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Nitric oxide within the concept of united airway disease

Exhaled and nasal nitric oxide in cystic fibrosis, asthma and upper airway inflammatory diseases

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Abstract

Krantz, C. 2021. Nitric oxide within the concept of united airway disease. Exhaled and nasal nitric oxide in cystic fibrosis, asthma and upper airway inflammatory diseases. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1721. 90 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1143-2.

Background: Within the concept of united airway disease, it is postulated that inflammatory disorders in the upper and lower airways are interrelated and influence each other. Fractional exhaled nitric oxide (FeNO) is an established biomarker of type-2 inflammation in the lower airways and is elevated in patients with asthma. However, the relation between nasal nitric oxide (nNO) and upper airway inflammation is less clear. Although cystic fibrosis (CF) is associated with increased airway inflammation, nitric oxide is not elevated in patients with CF.

Aims: To study nNO and FeNO as biomarkers of type-2 inflammation in the upper and lower airways, respectively, in relation to symptoms, disease control and treatment of both upper and lower airway diseases, and in relation to systemic inflammation.

Methods: This thesis is based on the MIDAS cohort of children and young adults with asthma (n=411) with a follow-up after 2-5 years (n=258), as well as one cohort of children and adults with CF (n=38) and one multicentre population-based cohort of middle-aged adults (n=5,824). Cross-sectional (Paper I-IV) and longitudinal (Paper III) analyses were performed. The main outcomes were nNO (Paper I-III) and FeNO (Paper II and IV) and their relations to IgE sensitisation, upper and lower airway symptoms and treatment, and systemic inflammation.

Results: In subjects with asthma, nNO was associated with FeNO and increased bronchial responsiveness and nNO was higher in subjects with perennial sensitisation. In non-asthmatic middle-aged subjects with perennial sensitisation, rhinitis and rhinoconjunctivitis were associated with higher FeNO. There was also a positive interaction with perennial sensitisation for the relation between upper airway inflammatory disorders and FeNO. Treatment with nasal or inhaled corticosteroids was associated with lower nNO levels in subjects with asthma. In middle-aged subjects with asthma and perennial sensitisation, use of nasal corticosteroids related to lower FeNO, whereas use of inhaled corticosteroids related to higher FeNO levels. Patients with CF had lower levels of nNO and FeNO than controls. Moreover, lower FeNO levels were associated with lower lung function and higher blood neutrophil counts in CF.

Conclusion: Within the concept of united airway disease, nNO is related to lower airway inflammation, responsiveness and treatment, and FeNO is related to upper airway inflammatory disorders, with a significant interaction with perennial sensitisation. In CF, lower FeNO is related to more severe disease with lower lung function and more systemic inflammation.

Keywords: nitric oxide, nasal NO, exhaled NO, asthma, cystic fibrosis, airway inflammation, upper airway inflammatory disorders, united airway disease, IgE sensitisation

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To Oliver, Anton and Sonja;
the pride and joy of my life

List of Papers

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

I Krantz, C., Janson C., Borres M. P., Nordvall L., Alving K., Malinovschi A., Nasal nitric oxide is associated with exhaled NO, bronchial responsiveness and poor asthma control. *Journal of Breath Research,* 2014 Jun;8(2):026002

II Krantz C., Janson C., Hollsing A., Alving K., Malinovschi A., Exhaled and nasal nitric oxide in relation to lung function, blood cell counts and disease characteristics in cystic fibrosis. *Journal of Breath Research,* 2017 Mar 20;11(2):026001

III Krantz C., Janson C., Alving K., Malinovschi A., Nasal nitric oxide in relation to asthma characteristics in a longitudinal asthma cohort study. *Nitric Oxide,* 2021 Jan 1; 106: 1-8

IV Krantz C., Accordini S., Alving K., Corsico A. G., Demoly P., Ferreira D. S., Forsberg B., Garcia-Aymerich J., Gislason T., Heinrich J., Jõgi R., Johannessen A., Leynaert B., Macron A., Martinez-Moratella Rovira J., Nerpin E., Nowak D., Olin A-C., Olivieri M., Pereira-Vega A., Raherison-Semjen C., Real F. G., Sigsgaard T., Squillacioti G., Janson C., Malinovschi A., (2021). Cross-sectional study on exhaled nitric oxide in relation to upper airway inflammatory disorders with regard to asthma and perennial sensitisation *Submitted for publication*

Contents

Abbreviations

ACT	Asthma Control Test
ADMA	Asymmetric dimethyl arginine
AR	Allergic rhinitis
ARIA	Allergic Rhinitis and its Impact on Asthma
ATS	American Thoracic Society
B-Eos	Blood eosinophil count
BudEq	Budesonide equivalent
$Calv_{NO}$	Concentration of alveolar NO
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator (chloride channel)
CI	Confidence interval
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
ECHRS	European Community Health Respiratory Survey
ERS	European Respiratory Society
FEF_X	Forced expiratory flow when x% of FVC remains
FeNO	Fraction of exhaled NO
FESS	Functional endoscopic sinus surgery
FEV_1	Forced expiratory volume at 1 second
FVC	Forced vital capacity
GINA	The Global Initiative for Asthma
GMP	Guanosine monophosphate
ICS	Inhaled corticosteroid
IgE	Immunoglobulin E
IL-1β	Interleukin 1β
INFɣ	Interferon ɣ
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
LLN	Lower limit of normal
LTRA	Leukotriene receptor antagonist
MIDAS	Minimally-Invasive Diagnostics in Asthma and allergic diseaseS
MF	Minimal function
NF_KB	Nuclear factor kappa B

nNO	Nasal nitric oxide
NO	Nitric oxide
NOS	Nitric oxide synthase
PCD	Primary ciliary dyskinesia
PD_{20}	Cumulative methacholine (provocative) dose resulting in a 20% drop in FEV_1
ppb	Parts per billion
RF	Residual function
STAT-1	Signal transducer and activator of transcription 1
STAT-6	Signal transducer and activator of transcription 6
Th2	T helper cell type 2
TNFα	Tumor necrosis factor α
UAID	Upper airway inflammatory disorders

Introduction

Nitric oxide

Biological effects

Nitric oxide (NO) is a gas connected to air pollution, but in the late 1970s it was discovered that NO is also an important signalling substance in human cells. The discovery of NO´s effect as signalling substance was awarded the Nobel Prize in Physiology and Medicine in 1998. Its effect on vascular smooth muscle cell relaxation through cyclic guanosine monophosphate (GMP) was discovered first [1]. Later, an effect on endothelial cells and blood coagulation was also seen. Nitric oxide has since been found to have many other effects, involving most of the human body, including the central and peripheral nervous system, the immune system, and the intestine, as well as the airways.

Nitric oxide causes smooth muscle relaxation also in the airways, and inhibition of nitric oxide synthase (NOS), which participate in synthesis of NO, causing increased bronchial responsiveness [2]. Nitric oxide has also been reported to stimulate ciliary beating frequency in vitro in human ciliated epithelial cells from healthy individuals [3].

Nitric oxide in high concentrations has an effect on infectious agents through directly interfering with the deoxyribonucleic acid (DNA) of target cells to cause breaks and fragmentations, as well as through inhibiting key iron-containing enzymes in cells [4]. In vitro studies have shown that *Pseudomonas aeruginosa* adhesion to, and survival in, infected cells is reduced after NO exposure [5]. NO also reduces biofilm production of bacteria (one of the most well-studied examples is *Pseudomonas aeruginosa*) through lowering cyclic dimeric GMP levels [6]. A phase I trial with inhaled gaseous NO in cystic fibrosis (CF) patients has shown a reduction in colony-forming units of bacteria and fungi in the sputum of patients [7].

In contrast to its beneficial effect on infectious agents, high levels of NO are also thought to play a part in the pathophysiology of many inflammatory diseases in humans including asthma [8] and nasal polyposis [9,10].

Nitric oxide production and regulation in healthy individuals

Nitric oxide is produced in cells through oxidation of L-arginine to L-citrulline via NOS. The process requires reduced nicotinamide adenine dinucleotide

phosphate (NADPH) and oxygen as co-substrates [11]. NO has a very short half-life and is quickly metabolised into either nitrite or nitrate or converted into peroxinitrite ($ONOO^-$) through reaction with superoxide anion O_2^-. Peroxinitrite and other reactive nitrogen species contribute to NO´s cytotoxic effects in both bacterial defence and inflammatory disorders [8].

There are three different forms of NOS. They were originally named after the cell lines in which they were first found, but they also have numerically based names. Endothelial NOS (eNOS or NOS3) is found at the cell surface in many types of cells, including endothelial cells. Neural NOS (nNOS or NOS1) is soluble in the cytoplasm and found mostly in neural cells near synapses. Both NOS1 and NOS3 are calcium-calmodulin-dependent and have a short peak of NO production. Inducible NOS (iNOS or NOS2) is not calcium-dependent and can produce high concentrations of NO. Expression of iNOS is low in unstimulated cells, but can be found in most cell lines upon stimulation, though it is mainly described in immune cells (macrophages) and epithelial cells [11]. The expression of iNOS and production of NO are triggered by cytokines (e.g. interferon ɣ (IFNɣ), interleukin (IL)-1β, IL-4 and IL-13) and transcription factors (signal transducer and activator of transcription (STAT) - 1 and – 6, and activator protein 1) [12,13]. The continuous expression of iNOS in epithelial cells of healthy individuals is believed to be triggered mainly by IFNɣ via STAT-1, while type-2 cytokines (e.g. IL-4, IL-13), via STAT-6, are involved in the increase in iNOS expression in inflammatory airway diseases [14,15]. As L-arginine is the substrate for NO production, L-arginine deficiency can lower NO production. NO production via NOS can be inhibited via naturally occurring NOS inhibitors, such as asymmetric dimethyl arginine (ADMA) [16].

NO is produced in much larger quantities in the upper airways (mainly in the paranasal sinuses, but also in nasal mucosa) [17] than in the lower airways and is thought to be part of the human innate immune defence against infectious organisms [18]. In human sinus mucosa, retrieved from patients without rhinitis or rhinosinusitis, there is a higher expression of eNOS than iNOS [3]. NO diffuses over cell membranes quickly and can be measured in gaseous form in exhaled and aspirated air from the airways. The amount of gaseous NO in air from the nose is determined by both the production of NO in the upper airway epithelium and the diffusion of NO from the paranasal sinuses into the nasal cavity. In exhaled air from the lower airways, NO derives mainly from iNOS expressed in the epithelial cells[19].

Measurements of exhaled and nasal nitric oxide

As nitric oxide diffuses rapidly over the cell membranes in the cells of the airway wall and follows the airflow towards the mouth and nose, it can be measured in gaseous form in exhaled and aspirated air in the airway. Detection and quantification of the levels of NO in exhaled or aspirated air is performed

using chemiluminescence or electrochemical sensors. The concentration of NO from the airways is flow-dependent, with lower fractions of exhaled NO at higher exhalation flow rates. As previously mentioned, the concentration of NO is much higher in upper than lower airways, making it important to obtain velum closure, to ensure no contamination from the upper airway when measuring lower airway NO and vice versa. Due to these factors, standardised procedures are necessary to compare NO levels within and between different studies. The American Thoracic Society and the European Respiratory Society (ATS/ERS) have constructed guidelines on how to perform exhaled and nasal NO measurements to ensure comparability between studies and in clinical settings [20].

For evaluating NO in the lower airways, the standard measurement is the fraction of NO in exhaled air (FeNO). This is performed at an exhalation rate of 50 mL/s, against a resistance resulting in a pressure of exhalation > 5 cm H_2O, during a single breath manoeuvre, where NO is analysed at a stable plateau phase. As NO is present in air, and the levels of NO in air are influenced by air pollution, inhalation of NO-free air through a scrubber is recommended before the exhalation manoeuvres.

As exhaled NO is flow-dependent, with a decrease of FeNO and an increase of NO output (fraction of NO multiplied by exhalation flow rate) with increasing flow rate, measurements of exhaled NO at different flow rates can be used to estimate the peripheral (alveolar = $Calv_{NO}$) and central (bronchial flux = $J'aw_{NO}$) contributions to the NO production [21].

NO production in the upper airways can be measured using a nasal suction or exhalation technique. The method least influenced by lower airway contamination is trans-nasal aspiration during velum closure, through breath-hold or oral exhalation against resistance. Both aspiration and exhalation must be done during a long enough period to reach a steady-state plateau phase.

Non-disease factors influencing exhaled and nasal nitric oxide

Nitric oxide levels in the airways are influenced by both constitutional and environmental factors and these factors can partially differ between FeNO and nasal NO (nNO).

FeNO relates to age and height in both children and adults [22-24]. In contrast, no relations between nNO and age or height were seen in a population-based study in adults [25], while in children under 12 years of age there was a positive relation between nNO and age, but no relation between nNO and height [26,27]. Sex has shown no relation to nNO[28], but a correlation to FeNO, with higher FeNO in males than females among both adults and children over 12 years of age [23].

Environmental factors that influence both nNO and FeNO are oral tobacco and smoking, which are associated with a moderate decrease in nNO [25,28]. Smoking is associated with a significant decrease in FeNO, by 40–60%, in

both healthy subjects and patients with asthma [29-31]. Intake of green-leafed vegetables, which are rich in nitrate, is associated with a transient increase in FeNO [32]. The major environmental factor influencing nNO is the NO level in ambient air [26,28], which is therefor important to take into account when evaluating nNO levels.

Cystic fibrosis

Genetic background

Cystic fibrosis is a rare, but severe, monogenic, autosomal recessive disease, linked to decrease in the expression of the protein cystic fibrosis transmembrane conductance regulator (CFTR) or dysfunction in CFTR [33]. CFTR acts as an anion channel in the cell membrane, primarily conducting chloride ions and bicarbonate [34,35] out of the cell. Dysfunction in CFTR is most prominent and well-known in epithelial cells, although CFTR is expressed in other cells as well. A dysfunction in CFTR leads to lower hydration and pH of the airway surface and ducts of the involved organs. The CFTR gene is located on chromosome 7 and there are over 2,000 known mutations of this gene [36]. The prevalence rates of different mutations vary greatly world-wide; the most common mutation in Western countries is F508del [37].

Not all of the mutations are pathogenic, but those that are have been divided into six different classes based on the effects they have on production, circulation and function of CFTR in the cell membrane[38]. In Class I mutations, there is insufficient translation of CFTR, and in Class II mutations, processing is impaired. Both of these mutation classes lead to no or insufficient CFTR on the cell membrane, and they are classified as minimal function (MF) mutations. In Class III mutations, the channel does not open properly; these are called gating mutations. In Class IV mutations, the conductance of CFTR is defective, in Class V mutations, there is a reduced stability or synthesis of CFTR, and in Class VI mutations, the turnover of CFTR in the cell membrane is increased. In these last three mutation groups, some functionality remains in the channel, and they are classified as residual function (RF) mutations. The more severe mutations (MF and gating mutations) are usually linked to pancreatic insufficiency and CF-related lung disease, whereas the RF mutations are more often related to pancreatic sufficiency and a milder CF lung disease, though the genotype is not the only predictor of phenotype in CF [33,39].

Diagnosing CF

Historically, and still to this day in countries without new-born screening for CF (such as Sweden), diagnosis of CF is most often based on symptoms in combination with sweat tests and genetic testing. CF is a multi-organ disease

with symptoms from upper and lower airways, pancreas, liver, sweat glands, and reproductive systems (Table 1).

Table 1. Typical symptoms of CF at diagnosis

Organ involved	Symptom	Typical age of presentation
Upper airways	CRS, nasal polyposis	Childhood, adolescence
Lower airways	Acute or recurrent infections	Childhood
	Increased sputum production	Childhood
	Increased cough	Childhood
	Chronic infections	Adolescence
	Obstructive lung disease	Adolescence
	Progressive lung disease	Adulthood
Gastrointestinal tract	Meconium ileus	Neonatal
	Pancreatic insufficiency and failure to thrive	Infancy
	Rectal prolapse	Infancy, childhood
	Hepatobiliary disease	Adolescence
	Recurrent pancreatitis	Adulthood (RF mutations)
Sweat glands	High Cl^- concentration in sweat	Infancy
Reproductive organs	Azoospermia	Adulthood (RF mutations)
Metabolism	Deficiency in fat soluble vitamins	Infancy
	Salt-loss syndrome	Childhood

CRS = chronic rhinosinusitis, RF = residual function,

In Sweden, the diagnosis of CF is based on the European terminology defined by diagnostic working group of the European Cystic Fibrosis Society [40]. Classic CF is when a patient has one or more typical symptoms of CF in addition to a sweat chloride concentration higher than 60 mmol/L in two measurements in a sweat test. Where there are two CF-causing mutation (located on different alleles), one measurement of sweat chloride concentration higher than 60 mmol/L in combination with one or more typical symptoms is sufficient for diagnosis. Patients with at least one RF mutation can have a borderline sweat test vid chloride concentrations between 40–60 mmol/L and are then diagnosed with atypical CF [40].

Disease manifestations and progression

The disease manifestations vary between subjects with MF or gating mutations and RF mutations. Patients with MF or gating mutations suffer from pancreatic insufficiency already at birth or shortly thereafter, due to blocking of the exocrine pancreatic ducts. This results in malabsorption of fat and proteins,

with a failure to thrive, steatorrhea and reduced levels of fat-soluble vitamins. Approximately 10–15% of patients with CF (all severe mutations) present with meconium ileus at birth, due to increased protein concentration in the meconium leading to plugging in the distal ileum or proximal colon. Patients with residual function mutations have normal pancreatic function at birth, but can develop pancreatic insufficiency or recurring pancreatitis in adulthood. The chronic obstructive pancreatitis in CF can cause a reduction in beta cells in the pancreas over time. This, in turn, in combination with a direct effect of CFTR dysfunction in the beta cells, results in insufficient insulin production and development of CF-related diabetes, which occurs in up to 50% of adult CF patients[41].

In the lungs, dysfunction of CFTR leads to dehydration of the airway surface liquid. In combination with altered pH, this results in decreased transportation of mucus from the distal parts of the airways and subsequent mucus stagnation. This is a risk factor for recurrent and chronic bacterial and fungal infections and inflammation [42]. The neutrophilic inflammation with increased neutrophil elastase often seen in patients with CF is related to bronchiectasis [43], which creates a suitable environment for gram-negative bacteria and fungi. There is evidence that the lung disease in CF starts early, with structural changes with bronchial dilation present as early as 3 months of age in over 15% of CF patients diagnosed through new-born screening[44]. During childhood, the most predominant infections are viral and bacterial infections with *Staphylococcus aureus* and *Haemophilus influenzae*, whereas infections with gram-negative bacteria, predominantly *Pseudomonas aeruginosa*, as well as fungal infection, predominantly *Aspergillus fumigatus*, increase with age and the presence of structural lung damage. Over time the chronic infections and inflammation in the lungs of CF patients lead to lung remodulation, increased structural changes of the lungs and lung function impairment. This may in turn lead to respiratory failure, lung transplantation or premature death.

The upper airways are also affected in patients with CF, with nasal blockage, nasal discharge and impaired olfactory function. Structural changes of the sinuses on CT scan are seen in over 80% of CF patients, with hypoplasia of paranasal sinuses and aplasia or hypoplasia of the frontal and sphenoidal sinuses being the most frequent changes [45]. Chronic rhinosinusitis with or without nasal polyposis is common and there are more neutrophil and fewer eosinophil infiltrations in the nasal polyps of patients with CF than in the nasal polyps of patients without CF [46].

Nitric oxide in cystic fibrosis

Patients with CF have a high degree of chronic infection and inflammation in the airways, but despite this, they have much lower levels of nNO [47,48] and lower or equal FeNO as compared with controls [49,50]. The lower levels of NO

in the upper airway are thought to be in part due to decreased diffusion of NO from the paranasal sinuses. CF patients with nasal polyps have lower nNO than those without, and the nNO levels rise after functional endoscopic sinus surgery (FESS), but fail to reach the same levels as in healthy controls [51]. Patients with CF also have thicker mucus and more neutrophil inflammation in the airways. The decreased diffusion of NO through the thicker mucus layer, as well as a reduction of NO levels by reaction with superoxide anions from neutrophils, can contribute to the lower NO levels in subjects with CF. In CF patients with malnutrition, L-arginine levels are low, as NO is produced from L-arginine, this can also be a factor contributing to low NO levels in subjects with CF [52]. Additional explanations are that CF patients have higher levels of ADMA (which is an inhibitor of NOS) in their sputum [53], and a lower expression of iNOS in their bronchial and nasal epithelium than controls [54,55]. A schematic picture of factors influencing nNO in CF is found in Figure 1.

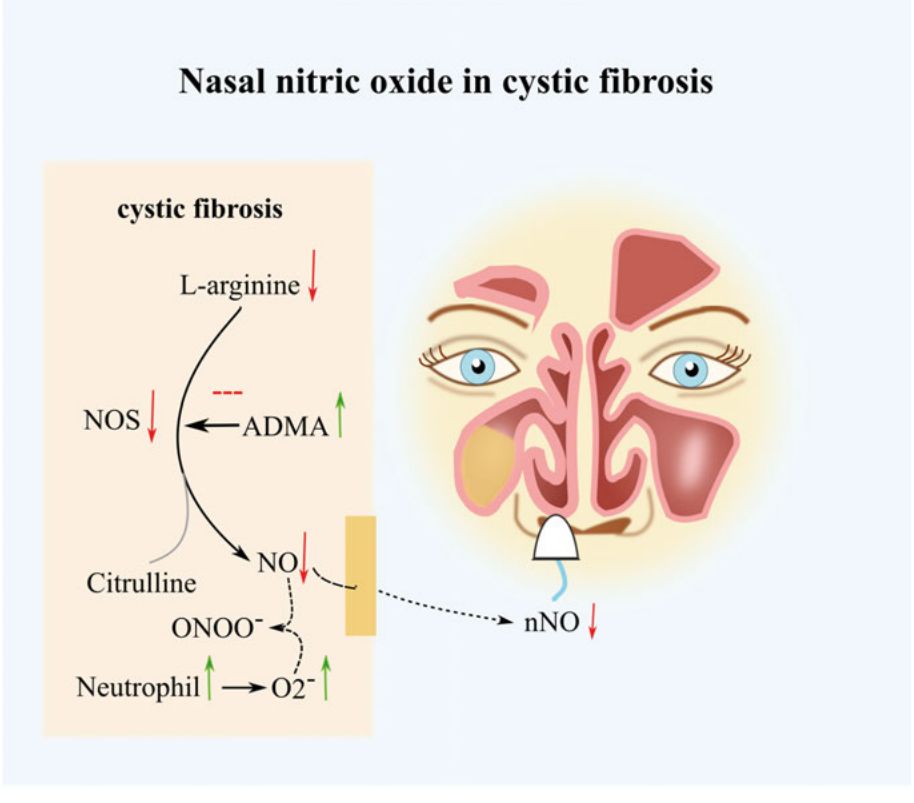

Figure 1. *Schematic picture illustrating factors potentially contributing to low nasal nitric oxide (nNO) in patients with cystic fibrosis. NOS = nitric oxide synthase, ADMA = asymmetric dimethyl arginine.*

Some previous studies have shown lower NO levels in CF patients with a more severe genotype, than in those with a mild genotype, and also in those with

pancreatic insufficiency compared with those without pancreatic insufficiency [56]. Recent studies have shown elevation in FeNO [57,58], as well as in nNO [59], after treatment with Ivacaftor in CF patients with gating-mutations, suggesting that a correction in CFTR function influences the NO production or availability in CF airways. A relation between NO levels and chronic colonisation with *Pseudomonas aeruginosa* has also been described [49,56]. One study has shown that treatment with antibiotics during exacerbation causes an increase in NO levels [60], but in another study, treatment with azithromycin, which is used as an inflammatory treatment in patients with chronic *Pseudomonas aeruginosa*, was cross-sectionally associated with lower NO levels [61].

Studies on the relations between nitric oxide levels and lung function in patients with CF have yielded diverging results, with some studies showing a positive association between FeNO and lung function in patients with CF [49,62], while other studies have shown no association [61,63,64]. The relation between blood leukocyte count, as a marker for systemic inflammation, and nitric oxide in CF is not well-studied.

IgE sensitisation and allergy

Allergy is an immune response reaction towards an exogenous substance (most often a protein) giving rise to a hypersensitivity reaction in the affected organ. The most common allergic inflammation is type-2 inflammation, in which IgE antibodies are formed towards the exogenous substance (the allergen). When the antibodies reencounter the allergen, an inflammatory process is initiated. The forming of IgE antibodies towards an allergen is called sensitisation, and atopy is the term for having a sensitisation, regardless of whether or not this sensitisation gives rise to symptoms [65,66].

The sensitisation process starts by the allergen entering through the mucosa or the skin, where it is taken up by an antigen presenting cell and transported to a lymph node, where naive (Th0) CD4+ cells are stimulated to transform into T helper cell type 2 (Th2 cells). The Th2 cells then release cytokines (mainly IL-4 and IL-13), which in turn stimulate B cells to produce allergen-specific IgE antibodies. The IgE antibodies attach to the cell surface of mast cells and basophils, which, in the event of a new encounter with the antigen, release preformed substances through degranulation. These substances – histamine, leukotrienes, heparin, proteases and tryptase – cause the inflammatory reaction through inducing vasodilation and increased permeability of the capillaries, causing swelling and secretion of mucosa, and through neural stimulation, causing itching and smooth muscle contraction [67]. During the allergic inflammatory reaction, IL-4 and IL-5, as well as chemokines, are produced by mast cells and Th2 cells and stimulate migration of additional inflammatory cells, e.g. eosinophils, to the inflammation. This further enhances the inflam-

matory process [67]. The allergic inflammation also stimulates increased expression of iNOS, with higher iNOS expression in nasal epithelial cells in patients with allergic rhinitis (AR) than in controls [68] and an increased expression of iNOS in bronchial epithelial cells and alveolar macrophages in allergic asthma [69,70]. As a proof of concept of the connection between sensitisation and iNOS expression, an ex vivo study on sensitised human mast cells showed that adding IgE increased the expression of iNOS, which was further upregulated upon cytokine stimulation with IL-1β, TNFα and INFɣ [71].

Nitric oxide and IgE sensitisation

Both FeNO and nNO have been reported to be higher in both symptomatic and asymptomatic subjects sensitised to aeroallergens than in controls in large population-based studies [25,72,73]. In patients with asthma, FeNO is positively associated with IgE sensitisation [73,74]. There is also a relation between degree and type of sensitisation and NO levels, with higher FeNO in subjects with perennial than seasonal sensitisation [75] and a positive relation between FeNO levels and multiple sensitisation [76]. However, the relation between degree and type of sensitisation and nNO is scarcely studied.

Asthma

In the latest GINA guidelines, asthma is defined as follows: "[A] is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation." [77] . It is the most common chronic disease that affects both children and adults. The prevalence of asthma symptoms in children in Sweden is around 10% [78], and the prevalence of asthma in adults worldwide is 4% with a large variation in prevalence between countries [79,80]. The cause of asthma can differ between individuals with allergic, non-allergic and intrinsic asthma, which have different underlying causes of the chronic airway inflammation. In allergic asthma, the underlying inflammation is mainly Th2-driven with increases in cytokines (including IL-4, IL-5 and IL-9), while non-allergic and intrinsic asthma have more diverse and partly unknown underlying mechanisms [81]. In recent years, the group 2 innate lymphoid cell (ILC2) has been discovered to play an important role in amplifying the inflammatory process in asthma and is thought to be involved in the inflammation in non-allergic hyper-eosinophilic asthma [82]. In children, allergic asthma is the most common, whereas the prevalence of non-allergic asthma is higher in adults, especially in late-onset asthma [83].

Asthma diagnoses in children are primarily based on respiratory symptoms collected from parents or guardians. When the children seek medical attention with symptoms, a medical examination can verify the bronchial obstruction through presence of wheeze or expiratory rhonchi that decrease upon treatment with short-acting bronchodilators. In older children (from approximately 7 years of age) and adults, the bronchial obstruction can by objectively verified through a lung function test with spirometry and a reversibility test or through repeated measurements of peak expiratory flow showing any variability in the obstruction. Studies on the use of oscillometry as a tool to assess bronchial obstruction and reversibility in children have proven technically feasible from younger ages (4–7 years) [84,85], but are not routinely used in clinical practice.

Treatment of asthma depends, in part, on the type of asthma. In allergic asthma, the main treatment objective is to decrease the underlying Th2-driven inflammation. This can be done through use of inhaled corticosteroids (ICS) or leukotriene receptor antagonists (LTRA), which both have a well-documented effect in treatment of allergic asthma. Both ICS and LTRA are sometimes used also in non-allergic asthma, though the effect is less well documented.

Nitric oxide in asthma

The relation between asthma and FeNO and the role of FeNO in diagnosing and monitoring asthma have been widely studied. Patients with asthma have higher levels of FeNO than controls [86] and FeNO is decreased in asthma after treatment with ICS [87] and/or LTRA [88,89]. FeNO is higher in allergic asthma than in non-allergic asthma [90]and correlates with increased sputum and blood eosinophil counts [91,92]. High FeNO in asthma patients also correlates with bronchial hyperreactivity and studies have shown a negative correlation between FeNO and FEV_1 in children with asthma [93] and that persistent high FeNO is related to decline in FEV_1 over time [94]. There has been a debate on the value of FeNO as a treatment guide for asthma, but a recent Cochrane review in adults with asthma has shown it to be a valuable tool in guidance of asthma treatment [95].

The role of nNO in patients with asthma, and if it has an additive effects in the monitoring of asthma control and treatment are less studied.

Upper airway inflammatory disorders

Rhinitis

Rhinitis is caused by inflammation in the respiratory epithelium of the nose and is characterised by symptoms from the nose, including nasal blockage, sneezing and rhinorrhoea and/or itching of the nose, during at least two consecutive days [96]. Rhinitis can have many causes, but AR is the most common non-infectious cause, with a prevalence of around 25% among adults in Europe, varying somewhat between countries [96]. According to Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines, AR can be classified as intermittent or persistent (rhinitis symptoms without a cold during more than 4 days a week and more than 4 weeks in a row during the preceding year), and mild, moderate or severe [96] based on the impact of rhinitis on everyday life, activities and sleep. Rhinitis is associated with impairment in quality of life and in school and/or work performance, this association is most pronounced in patients with moderate to severe rhinitis [96]. Persistent (chronic) rhinitis can be associated with chronic rhinosinusitis with or without nasal polyposis.

Chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is a chronic inflammation of the mucosa in the nose and sinuses causing symptoms due to swelling and purulent secretion. CRS has a high impact on quality of life and CRS is thought to be common, with a prevalence in the range 6.9–27.1% in Europe, based on population surveys assessing symptoms of CRS [97]. The prevalence of CRS increases with age and are rare in children.

Diagnostic criteria for CRS are: presence of two or more CRS symptom (nasal obstruction, nasal discharge, hyposmia, facial pain or pressure (in children cough), with at least one being nasal obstruction or discoloured nasal discharge) for 12 or more weeks, in addition to findings of inflammation through a CT scan or endoscopy, or objective findings of purulence originating from the sinuses [98,99]. CRS is further divided into CRS with nasal polyposis (CRSwNP) or CRS without nasal polyposis (CRSsNP) based on presence or abscence of nasal polyposis in endoscopy or CT scan. Though there is a considerable overlap between underlying inflammation in CRSwNP and CRSsNP, CRSwNP is more characterised by type-2 inflammation with elevated IL-5 and IgE concentrations than CRSsNP, which has a higher levels of INF-ɣ and TGF-β [100].

In most epidemiological studies, CRS is based on symptoms only, but the agreement between survey diagnosis of CRS and clinical findings and self-reported doctor's diagnosis of CRS has been validated, they are significantly positively interrelated [101].

Nitric oxide in upper airway inflammatory disorders

The use of nNO to evaluate allergic inflammation in rhinitis and rhinosinusitis is controversial. Some studies have shown higher levels of nNO in patients with AR than in controls [102,103], while others have not [104]. However, a recent meta-analysis on the relation between AR and nNO concluded that most studies have confirmed that nNO is higher in AR; however, further studies are needed to evaluate the clinical role of nNO in monitoring AR [105]. In non-allergic rhinitis, more severe symptoms and nasal polyposis are associated with lower nNO levels [106,107], and treatment with nasal corticosteroids in patients with nasal polyposis can result in elevation of nNO levels [107,108], as can medical or surgical treatment in patients with CRS [109]. A recent systematic review with meta-analysis, on the relation between CRS and nNO found lower nNO in patients with CRSwNP than in controls and patients with CRSsNP, but patients with CRSsNP did not differ in nNO levels compared with controls after adjustment for nasal steroid treatment [110]. The reason for the complexity in using nNO in rhinitis and rhinosinusitis is its dual origin, with NO both diffusing from the sinuses, where it is present in high levels, and arising through the activation of iNOS in the nasal mucosa upon exposure to allergens in AR. This is supported by the results from Palm *et al* reporting nNO levels similar in AR and controls, but a higher decrease in nNO in subjects with AR upon intranasal administration of a NOS inhibitor [111]. A schematic picture of factors influencing nNO in AR is found in Figure 2.

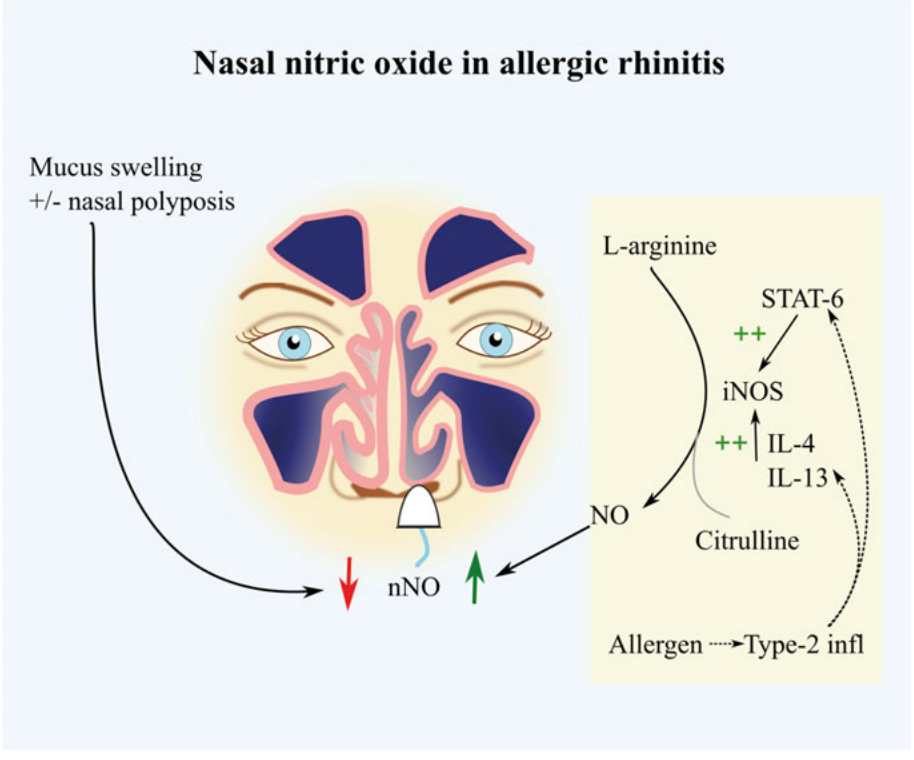

Figure 2. *Schematic drawing of how nNO is influenced by both type-2 inflammation, with activation of iNOS induced NO production, and by ostia obstruction leading to a reduced diffusion of NO from the sinuses, resulting in a reduced contribution to nNO.*

The united airway concept

The upper and lower respiratory tracts show many similar anatomical and histopathological features and are sometimes considered a unified entity called the united airway. One of the connections between the upper and lower airways in health and disease is morphological, with the nose acting as an air-conditioner for the lungs, providing humidification, temperature regulation, and filtering of particles in the passage of air from the nose to the lungs. In addition, the nose can contribute to the protection of the lungs from antigens and airborne pathogens by release of antibacterial proteins and IgA [112]. However, there are also other interrelations between upper airway inflammatory diseases, such as rhinitis and rhinosinusitis, and lower airway inflammatory diseases, such as asthma and hyperreactivity, suggesting that these diseases could be different manifestations of a united airway disease. It has been postulated that local inflammation in the respiratory mucosa triggering systemic

inflammation also contributes to the interrelation [113], as do neural reflexes, such as the nasobronchial reflex and the bronchonasal reflex[114].

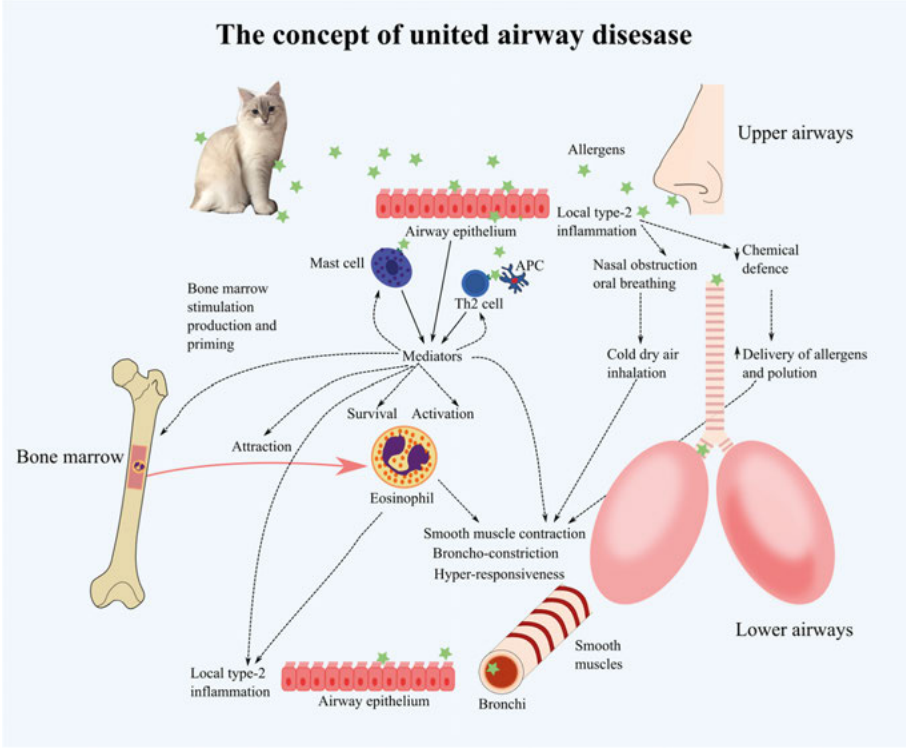

Figure 3. *Schematic figure of factors contributing to united airway disease and how they interact.*

Allergic rhinitis is a common co-morbidity in patients with asthma [96], with a high prevalence in patients with severe asthma, occurring in as many as 91% [115], and has an impact on both asthma control and asthma severity [116]. Furthermore, AR is considered a risk factor for development of asthma [96], and in patients without asthma, AR is related to increased bronchial hyper reactivity [117,118], small airway impairment [117,119] and increase in sputum eosinophil count [120]. Persistent rhinitis, regardless of whether it is allergic or non-allergic, is associated with more lung function impairment in patients with asthma [121], as well as with increased bronchial hyperreactivity in subjects without asthma [122]. Uncontrolled moderate to severe rhinitis is related to impaired asthma control [123].

Patients with asthma have an increased prevalence of CRS, with an even higher prevalence in the subjects with concomitant AR [99]. CRS comorbidity is associated with lower asthma-related quality of life and increased exacerbation rates in asthma patients [124]. In patients with severe asthma, concomitant

CRSwNP has an impact on health-related quality of life [125] and is related to structural lung changes and more exacerbations [126].

The effect of upper airway inflammation on asthma and bronchial responsiveness is relatively well-studied, but there are fewer studies addressing the relation of asthma to upper airway inflammation. However, in a study of non-atopic subjects with asthma and non-atopic controls, patients with asthma had higher numbers of $CD4^+$ cells and eosinophils in the nasal mucosa that controls, irrespective of presence of rhinitis [127]. In addition, bronchial allergen provocation in patients with AR, but without asthma, resulted in nasal symptoms and inflammation [128].

In CF, the term united airway disease can be used to describe infections in both the upper and lower airways. The same genotypic bacterial infection is often found in both upper (nose and sinus aspirate) and lower (sputum) airway samples in patients with CF, suggesting that the infections have a common origin [129]. In addition, studies have reported that upper airway culture positive for *Pseudomonas aeruginosa* often precedes positive sputum culture [130,131], indicating that the upper airway is the entry point for lower airway infections. In a Danish study, CF patients treated with FESS and consecutive local antibiotic treatment in the sinuses and nose had a lower incidence of chronic and intermittent *Pseudomonas* pulmonary infections during the following year than the control group [132].

Nitric oxide within the concept of united airway disease

FeNO, as a marker of type-2 inflammation in the lower airways, and nNO, as a marker of type-2 inflammation in the upper airways, could be used to strengthen the link between upper and lower airway inflammation within the concept of united airway disease.

In subjects without asthma, FeNO is higher in patients with AR than in controls [133,134], and higher in patients with CRSwNP than with CRSsNP [135]. Additionally, in non-asthmatic subjects, increased FeNO is related to an increased risk of developing new-onset wheeze [136], and in non-asthmatic subjects with AR, high FeNO is related to increased risk of asthma development[137-139].

The same associations are seen in patients with asthma, where concomitant AR is associated with higher FeNO [140,141]. A reduction in FeNO has been reported in a cohort study of patients with asthma and AR, when intranasal corticosteroids were added to the treatment[142]. This suggests that treatment of upper airway inflammation can have an effect on lower airway inflammation. In subjects with asthma, higher FeNO is also found in those with concomitant CRSwNP, than in those without nasal polyposis [126,143], and surgical treatment (FESS) of the nasal polyps relates to a decrease in FeNO [144].

However, it is not fully understood how much of the relation between upper airway inflammatory disorders and FeNO is due to IgE sensitisation and systemic type-2 inflammation, and how much is related to the local upper airway inflammation triggering lower airway inflammation.

The relation between nNO and lower airway disease and inflammation is even less studied.

Aims

The overall aim of this thesis was to study how nNO and FeNO, as markers of type 2-inflammation in the airways, related to symptoms, disease control and treatment, as well as to systemic inflammation.

Paper I: To analyse nNO in subjects with asthma in relation to FeNO, bronchial responsiveness, asthma symptoms and control, as well as to relate the findings to IgE sensitisation, upper airway symptoms and treatment in the upper and lower airways.

Paper II: To compare levels of nNO and FeNO in subjects with CF to those in subjects with asthma and healthy controls, as well as to investigate if nNO and/or FeNO related to lung function, systemic inflammation, evaluated based on blood cell count and total IgE levels, or disease related variables in subjects with CF.

Paper III: To investigate if longitudinal changes in nNO in subjects with asthma related to changes in asthma control or lung function, changes in type-2 inflammation, measured as FeNO and blood eosinophil count, changes in upper airway symptoms, or changes in treatment of asthma or rhinitis. A secondary aim was to investigate if the relations between nNO and FeNO, asthma control and upper airway symptoms found at baseline in Paper I could be found at follow-up 2–5 years later.

Paper IV: To study the associations between FeNO and upper airway inflammatory disorders (i.e., rhinitis, rhinoconjunctivitis, chronic rhinosinusitis or nasal polyposis), when asthma and IgE sensitisation were accounted for, in a large multi-centre population-based study.

Materials and methods

Study subjects and definition of asthma and cystic fibrosis

Paper I and III were based on subjects who participated in the Minimally-Invasive Diagnostics for Asthma and allergic diseaseS (MIDAS) asthma cohort. The healthy controls and asthma controls in Paper II were randomly selected from the baseline visit of the MIDAS study.

MIDAS

The MIDAS study was conducted within a framework of an industry-academy collaboration between the years 2010 and 2012. A total of 411 children and young adults with asthma were recruited to the study from primary or secondary care.

The inclusion criteria were physician-diagnosed asthma (identified through medical records) and self-reported use of daily anti-inflammatory treatment with ICS or LTRA during at least 3 months in the preceding year.

A total of 118 age- and gender-matched subjects without asthma or other respiratory diseases were randomly chosen from the population registry and recruited as healthy controls.

All subjects with asthma as well as the healthy controls were invited to participate in a follow-up that was performed during the years 2013–2015 (MIDASII). A total of 257 subjects with asthma and 63 healthy controls participated in this follow-up.

Analyses in Paper I were based on subjects in the asthma cohort at baseline visit who had technically acceptable nNO measurements and blood samples drawn (n=371).

Analyses in Paper III were based on subjects from the asthma cohort who performed both the baseline and follow-up visits, and had nNO measurements from both time-points (n=197). One subject was excluded due to self-reporting no asthma at follow-up.

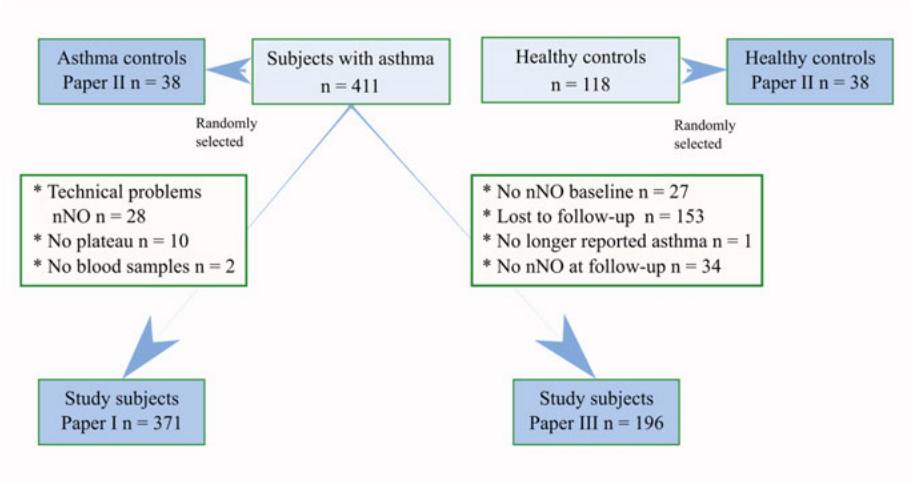

Figure 4. *Descriptive flow chart depicting inclusion and exclusion of study population in Papers I and III, as well as controls in Paper II.*

Paper II was based on 38 subjects with CF, aged 9-50 years, treated at the Uppsala Centre for Cystic Fibrosis, who performed measurements of nNO and FeNO within a study on "minimally-invasive methods for CF follow-up". These measurements were in addition to the regular examinations with lung function test, blood samples and sputum cultures at one of their regular annual check-ups during the period 2011–2015.

CF diagnosis was identified from medical records, and based on positive sweat-tests and, in most cases, two CF-causing mutation in CFTR. Information on current medication, chronic infections in the lungs, exocrine pancreatic status, and CF-related comorbidities such as CF-related diabetes, was also collected from medical records. Lung transplantation was an exclusion criterion.

For comparison of nNO and FeNO in relation to diagnosis, 38 subjects with asthma and 38 healthy controls, matched by gender and similar in age, were randomly chosen from the MIDAS I study.

Paper IV was based on subjects participating in the second follow-up of the European Community Respiratory Health Survey (ECRHSIII).

ECRHS

The European Community Respiratory Health Survey (ECRHS) is a population-based multicentre study involving mainly European countries, with the aim of mapping respiratory symptoms and diseases across Europe. The first survey was sent out to 200,000 randomly selected young adults (age 20–44 years) in the early 1990s. Among the responders, both a random sample and a sample of subjects reporting respiratory symptoms were invited to participate in a clinical visit at their respective study centre. A total of 26,000 subjects

participated in this clinical phase of the first study, and they were later invited to participate in follow-up studies, ECRHS II and ECRHS III, in 2000–2002 and 2010–2013, respectively.

The study population in Paper IV encompassed subjects with current asthma, as well as non-asthmatic subjects without treatment with ICS during the preceding year, who had participated in ECRHS III and had performed measurements of FeNO during their clinical visit.

Current asthma was defined as self-reported doctor-diagnosed asthma and at least one respiratory symptom (self-reported asthma attack, wheezing, nocturnal chest tightness, attacks of shortness of breath at night, after strenuous exercise or at rest) during the preceding 12 months and/or use of asthma medication during the same period. Subjects with self-reported chronic obstructive pulmonary disease (COPD) or emphysema were excluded from the study.

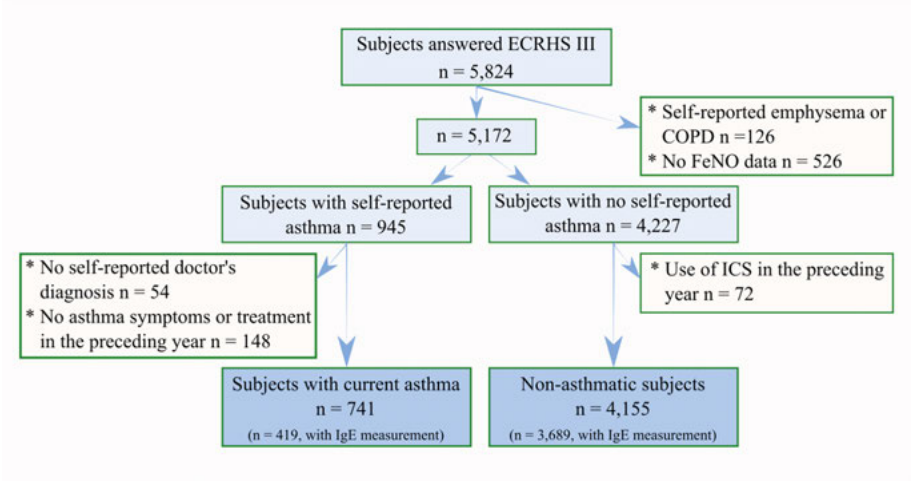

Figure 5. *Descriptive flow-chart, showing the selection of study population in Paper IV.*

Measurements of nitric oxide

Subjects were instructed not to eat or drink, smoke or use oral tobacco during 30 minutes prior to measurement of nitric oxide. They were also instructed to refrain from strenuous exercise and intake of large quantities of green-leafed vegetables on the day of the measurement.

Nasal nitric oxide

For Papers I–III, the same method, analyser and laboratory staff were used for measuring nNO. It was measured using the trans-nasal aspiration method in

ambient air with a suction pump generating a flow rate of 5 mL/s, in accordance with ATS/ERS guidelines [20]. Measurements were done using a chemiluminescence analyser (NIOX Flex, Aerocrine AB, Solna, Sweden) and the value was recorded from a stable plateau phase within a 40-second long breath-hold manoeuvre, to ensure velum closure. One measurement was taken from each nostril and a mean value was calculated. Only measurements where a stable plateau was reached were considered technically acceptable and included in the Papers I and II. As study subjects inhaled ambient air before the measurements, ambient NO was recorded in MIDAS I. In the MIDAS I cohort median ambient NO was 1.4 ppb (interquartile range (IQR) 0.8–2.5) and among the CF subjects it was 1.4 ppb (IQR 0.6-3.2). The analyser was calibrated every 14 days with certified calibration gas (2,000 ppb) in accordance with the manufacturer's recommendations.

Exhaled NO

For Papers I–III, the same method, analyser and laboratory staff were used for measuring FeNO as for the nNO measurements. The measurements were performed in accordance with ATS/ERS recommendations using a single breath technique after the subject inhaled NO-free air from a scrubber, with NO levels analysed using a chemiluminescence analyser (NIOX Flex, Aerocrine AB, Solna, Sweden). Exhalations were performed in duplicates (triplicate, if the values from the first two measurements were not within 10% of each other) at a flow rate of 50 mL/s, and a mean value was calculated. The analyser was calibrated every 14 days with certified calibration gas (200 ppb) in accordance with the manufacturer's recommendations.

In Paper IV, FeNO was measured using an electrochemical analyser (NIOX MINO; Aerocrine AB) at a flow rate of 50 mL/s. The measurements were performed in accordance with ATS/ERS recommendations, with the exception that only single measurements were made. NIOX MINO detects FeNO values from 5 to 300 ppb. No value above 207 ppb was measured. 15 subjects (10 subjects without asthma and 5 with current asthma), had levels "below 5 ppb" (no actual value) and those subjects were assigned an arbitrary FeNO value of 3.5 ppb (5 divided by √2).

Lung function tests

Spirometry was performed in Papers II–III and bronchial provocation test in Paper I.

Spirometry

For Papers II–III, spirometry was performed using a MasterScope spirometer (Erich Jaeger, Wurzburg, Germany) generating flow-volume curves. All subjects performed three technically acceptable trials, in accordance with ATS/ERS recommendations, and the highest values for FVC and FEV_1,, were recorded. For Paper II FEF_{25}, FEF_{50} and FEF_{75} were also recorded from the exhalation manoeuvre with the highest FVC and FEV_1 values. In Paper II, reference values from Hedenström [145,146] for adults and Solymar [147] for children (< 18 years of age) were used. Since some children in MIDAS I had become adults in MIDAS II, percent predicted lung volumes were calculated for Paper III using the Global Lung function Initiative (GLI2012) regression equation [148]. In Paper III, impaired lung function was defined as under the lower limit of normal (LLN) for FEV_1 or FEV_1/FVC.

Bronchial responsiveness test

For Paper I, bronchial responsiveness was recorded using a methacholine provocation with Aerosol Provocation System (Viasys Healthcare GmbH). A simplified protocol was followed, using a single concentration of methacholine with stepwise increase in methacholine doses up to a maximum cumulative dose of 3.63 mg [149]. The FEV_1 was measured two minutes after each inhalation with subsequent calculation of the cumulative methacholine dose resulting in a 20% drop in FEV_1 (PD20 FEV_1) through logarithmic interpolations using an integrated program.

Blood samples

Blood samples were drawn at the study visit for the MIDAS I and II cohort, as well as for the participants in the ECRHSIII study, and as part of the annual routine check-up at the CF center for subjects with CF included in Paper II.

Blood cell count

In the MIDAS I and II studies, as well as in subjects with CF included in Paper II, the blood samples were analysed for total leukocyte, neutrophil and eosinophil cell counts using routine methods (Cell-Dyn 4000, Abbott, IL, USA) at the Department of Clinical Chemistry and Pharmacology, Uppsala University Hospital, Uppsala, Sweden.

Total and specific IgE

Total IgE was measured in MIDAS I and in subjects with CF in Paper II using the UniCAP system (Immunodiagnostics Thermo Fisher Scientific, Uppsala, Sweden).

In the MIDAS I cohort, additional measurements of IgE against a mix of aeroallergens (Phadiatop, Immunodiagnostics Thermo Fisher Scientific, Uppsala, Sweden) were made. In addition, measurements of specific IgE against each individual allergen in the mix of aeroallergens (cat, dog, horse, house dust mite, mould, birch, timothy grass and mugwort), as well as towards one additional aeroallergen (the mould *Alternaria alternata*), were made using ImmunoCAP (Immunodiagnostics Thermo Fisher Scientific, Uppsala, Sweden).

In the ECHRS III study, measurements were made of specific IgE against *Dermatophagoides pteronyssinus* (house dust mite), timothy grass and cat, at a single central laboratory (AMC Amsterdam) using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden). Blood samples for analysis of specific IgE were obtained in 4,108 study participants (419 individuals with current asthma and 3,689 non-asthmatic subjects) in the examinations for Paper IV.

Definition of sensitisation

In Paper I, subjects with specific IgE antibodies ≥ 0.35 kU_A/L towards any of the tested allergens were considered to be sensitised to the relevant allergen(s) and to have atopy. If the subjects were sensitised towards perennial aeroallergens (cat, dog, horse, house dust mite or mould), they were defined as having a perennial sensitisation; if they were only sensitised towards birch, timothy grass or mugwort they were defined as having a seasonal sensitisation.

The measurement from MIDAS I were used for definition of IgE sensitisation in Paper III. The subjects who had IgE antibodies ≥ 0.35 kU_A/L towards the mix of aeroallergens (Phadiatop) were defined as sensitised towards airborne allergens. Subjects with airborne sensitisation who had specific IgE antibodies ≥ 0.35 kU_A/L towards a perennial allergen (cat, dog, horse, house dust mite or mould) were considered to have a perennial sensitisation.

In Paper IV, participants were defined as sensitised towards an individual allergen if the concentration of IgE against that specific allergen was ≥ 0.35 kU_A/L. As the number of participants with only grass sensitisation was low (only 35 in subjects with current asthma and 253 in non-asthmatic controls), two sensitisation groups were used: perennial sensitisation (cat- and/or house dust mite-sensitised, with or without simultaneous sensitisation towards timothy grass) or no perennial sensitisation (no sensitisation or sensitisation only towards timothy grass).

Definitions of upper airway disorders

In Papers I, III and IV, occurrence of upper airway disorders was based on subject responses to detailed interviewer-led questionnaires. The study subjects responded to questions regarding symptoms of rhinitis and rhinosinusitis, as well as frequency of symptoms and how the symptoms affected daily life. In Paper IV, the subjects also responded to if they had a doctor's diagnosis of CRS or nasal polyposis.

Rhinitis

Current rhinitis was defined as occurrence of one or more symptom of rhinitis (blocked nose, nasal discharge, itching of the nose, sneezing) without a cold during the preceding year. Subjects with current rhinitis were grouped into either persistent rhinitis, if they had current rhinitis with symptoms during more than 4 days in a week and more than 4 weeks in a row in the preceding year, or intermittent rhinitis, if the symptoms had been present less frequently, in accordance with ARIA guidelines [96]. In Paper I, the definition of persistent rhinitis was incorrectly described in the methods section in the publication (symptom during more than 12 weeks in the preceding year); the definition described above was used in the analysis.

In Papers I and III, severity of rhinitis was based on whether or not the nasal symptoms had an impact on daily activities and sleep. Moderate to severe rhinitis was defined as nasal symptoms having an impact on either sleep, daily activities, school or work during the preceding year. In Paper IV, the severity of rhinitis was based on the extent to which each symptom of rhinitis had affected daily activity and sleep (1. not a problem, 2. a slight problem, but not bothersome, 3. a bothersome problem, but without effect on daily activities or sleep, 4. a problem that affects some activities or sleep). Moderate to severe rhinitis was defined as having at least one of the nasal symptoms affecting some activities or sleep, while mild rhinitis was defined as current rhinitis not fulfilling the criteria for moderate to severe rhinitis.

In Paper IV, rhinoconjunctivitis was defined as current rhinitis with concomitant runny or itchy eyes.

CRS and nasal polyposis

In Paper I, CRS was defined as a combination of two of the symptoms (blocked nose without a cold, discoloured nasal discharge, pain or pressure in the forehead or eyes, or loss of smell) for at least 12 weeks in the preceding year. In Paper III, CRS was defined as nasal blockage in combination with at least one of the following symptoms: discoloured nasal discharge, pain or pressure in the forehead or eyes, or loss of smell, during at least 12 weeks in the preceding year. That same definition was used for symptoms of CRS in

Paper IV, in which self-reported doctor's diagnosis of CRS was used as the definition of CRS. The definition of nasal polyposis in Paper IV was based on self-reported doctor's diagnosis.

Definition of asthma control and severity

In Papers I and III, the association between asthma control and nNO levels was assessed. The Asthma Control Test (ACT) was used to assess asthma control; this is a validated measure based on responses to questions regarding obstructive symptoms, frequency of usage of short-acting bronchodilators, the subject's own opinion on asthma control, and impact of disease on everyday activities and sleep, within a 4-week recall [150]. An ACT score of ≥20 was defined as well-controlled asthma, a score below 15 as poor asthma control, and a score of 15–19 as uncontrolled asthma [150,151].

In Paper I, the subjects answered questions on asthma symptoms such as wheezing, shortness of breath at night, during rest or during exercise and asthma attacks, during the preceding year.

In Paper III, subjects were grouped at baseline as mild, moderate or severe asthma in accordance with GINA guidelines, based on frequency of asthma symptoms, asthma attacks and nocturnal symptoms, as well as lung function [152].

Definitions that differed between papers

Below is a summary of the variables where the definitions differed between Papers I, III and IV (Table 2).

Table 2. Variables for which the definition differed between the papers.

	Paper I	Paper III	Paper IV
Asthma	Diagnosis from medical records	Diagnosis from medical records	Self-reported doctor's diagnosis
Atopy (I)/ IgE sensitisation towards airborne allergens (III)	IgE ≥ 0.35 kU_A/L towards any individual aero allergen	IgE ≥ 0.35 kU_A/L towards Phadiatop	N/A
Perennial sensitisation	IgE ≥ 0.35 kU_A/L towards cat, dog, horse, house dust mite or mould	IgE ≥ 0.35 kU_A/L towards cat, dog, horse, house dust mite or mould	IgE ≥ 0.35 kU_A/L towards cat or house dust mite
Sensitisation towards furry animals	IgE ≥ 0.35 kU_A/L towards cat, dog, horse	N/A	N/A
CRS	≥Two symptoms of CRS*	Blocked nose in combination with ≥1 additional symptom of CRS*	Self-reported doctor's diagnosis of CRS
Symptoms of CRS	Refers to individual symptoms of CRS*	Refers to individual symptoms of CRS*	Blocked nose in combination with ≥1 additional symptom of CRS*

*CRS= chronic rhinosinusitis. * Blocked nose without a cold, discoloured nasal discharge, pain or pressure in the forehead or eyes, or loss of smell, for at least 12 weeks in the preceding year*.

Asthma and rhinitis medication

In Papers I, III and IV, the participants answered interviewer-led questions regarding their use of asthma and rhinitis medication in the form of ICS, LTRA or nasal steroid, how often they used medications and in what doses. In addition to this, in Paper I and III, the prescribed dose of ICS was collected from medical records and transformed into budesonide equivalents (BudEq) using these factors: budesonide dose × 1, fluticasone dose × 2, mometasone dose × 1 [153,154].

Statistical analyses

Statistical analyses were performed using STATA/IC 12.1 and STATA 15.1(StataCorp LP, Collage Station, Texas, USA) for Papers I–III and Paper IV, respectively.

The results were presented as numbers and percentages for categorical variables. In Papers I, III and IV, results were presented as means with standard deviations for continuous variables with normal distribution, medians with

IQRs for variables without normal distribution (i.e., blood eosinophil count (B-Eos) and ACT), and geometric means with 95% CI for variables with a right-skewed distribution (i.e., FeNO, total IgE). For variables with a right-skewed distribution, logarithmic transformation was performed before statistical testing. In Paper II, the study groups were small and normal distribution could not be assumed, hence, all results for continuous variables were presented as medians with IQRs.

In Paper II, the cc-match command was used in STATA to randomly choose healthy controls and asthma controls from MIDAS I, matched by gender and age group (≤ 11 years, 12–13 years, 14–15 years, 16–18 years, 19–21 years, 22–25 years, 26–29 years and ≥ 30 years). As the study groups were small and normal distribution could not be assumed, analyses of variance between groups were performed with the Kruskal-Wallis test, and pairwise differences between groups calculated with the Mann-Whitney test. Correlations between different NO parameters and lung function as well as with blood cell counts were calculated with Spearman's test.

In Papers I, III and IV, with larger study cohorts, statistical tests based on normal distribution theory were used, with analysis of variance (with Bonferroni correction) or t-tests used to evaluate univariate differences between groups and multiple linear regressions used for multivariate tests.

In Paper I, FeNO, blood eosinophil count, total IgE and bronchial responsiveness were divided into quartiles when analysing their individual relations to nNO. The relations were studied using both trend analyses and univariate tests between different quartiles. In the multivariate tests, formerly reported determinants of nNO as well as variables demonstrated to relate to nNO in univariate analyses were included. When factors were strongly related to each other (for example CRS and loss of smell), the one with the highest explanatory value (r^2) for in univariate analyses was chosen for multivariate tests.

In Paper III, multivariate tests were performed on baseline and follow-up values to study factors associating with nNO (as independent variables). The research group looked at differences between baseline and follow-up using paired t-tests for continuous variables. Pearson's test was used for studying correlations between changes in continuous parameters over time. Longitudinal changes in nNO levels were investigated as absolute changes (Δvalue defined as value at follow-up - value at baseline) and their correlations to various baseline characteristics and changing characteristics were investigated with t-tests and one-way analysis of variance. Multivariate tests were used for assessment of independent associations of the studied variables with changes in nNO levels over time, in relation to both absolute change and relative change (% change defined as 100 x (follow-up value – baseline value)/(baseline value)).

In Paper IV, differences in log FeNO levels in relation to categorical variables, such as sex, smoking habits, upper airway inflammatory disorders

(UAID) and perennial sensitisation, were initially tested separately for participants with current asthma and non-asthmatic subjects, and tests for interactions with asthma were performed. To assess differences in the relation between UAID and FeNO with regard to sensitisation, subjects were further stratified by perennial sensitisation to evaluate differences in FeNO levels in relation to UAID in the stratified groups. Interaction analyses between sensitisation and UAID with FeNO as outcome were also performed.

To further evaluate the independent association of UAID with FeNO in Paper IV, multiple linear regression analyses were performed on the groups stratified by current asthma and perennial sensitisation, as well as on the entire study population, with adjustments for sex, age, height, weight, smoking status, centre and treatment for rhinitis and asthma, with log-transformed FeNO as outcome. The UAID that had a relationship to FeNO in the univariate analysis (i.e., current rhinitis, rhinoconjunctivitis and nasal polyps) were tested individually in these multiple linear regression models, as they are related to each other. The results from the analyses, β coefficients with 95% confidence interval (CI), were then back-transformed and presented as % difference in FeNO (($(10^{\beta}$-1)X100) with 95% CI (($(10^{CI}$-1)X100)) in relation to the reference group. A test for interactions with perennial sensitisation was added individually to all models; the model including all study subjects also had a test for interactions with asthma added.

In all papers, a p-value of <0.05 was considered statistically significant.

Ethical approvals

In all studies, written informed consent was obtained from all participants, or from their legal guardian if they were under 18 years of age, before inclusion. The protocols for the Papers I–III were approved by Uppsala Regional Ethical Review Board; EPN number (2009/349) MIDAS I, (2011/097) Study II and (2012/420) MIDAS II. As ECRHS III was a multi-centre study, each participating centre obtained approval for the study from the regional committee for medical research ethics, in accordance with national legislation (EPN number for Uppsala (2010/068)).

Results

Levels of nitric oxide in cystic fibrosis, asthma and healthy controls

Nasal and exhaled NO in CF, asthma and healthy controls (Paper II)

Subjects with CF had much lower nNO levels than healthy controls or subjects with asthma. This was consistent in subjects both under and over 18 years of age (Figure 6). FeNO levels were also lower in subjects with CF than in healthy controls or subjects with asthma in the whole population and in subjects ≥ 18 years (Figure 7).

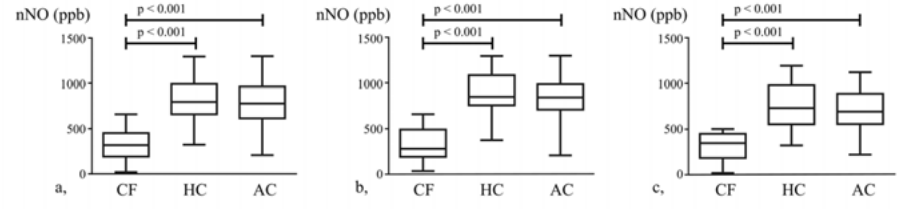

Figure 6. *Nasal NO (ppb) (presented as medians with IQRs and upper and lower limits) in the three investigated groups (CF = cystic fibrosis, HC = healthy controls, AC = asthma controls), a: all subjects, b: subjects under 18 years of age, c: subjects 18 years of age and older.*

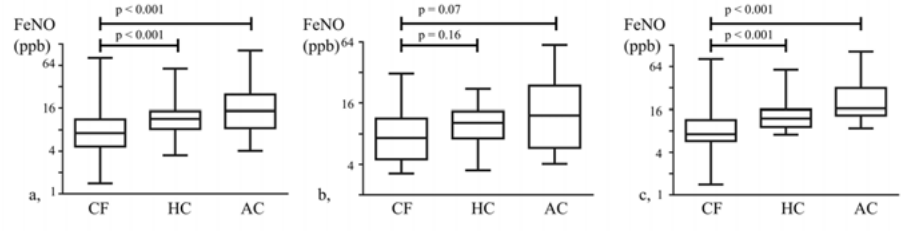

Figure 7. *FeNO (ppb) (presented as medians with IQRs and upper and lower limits) in the three investigated groups (CF = cystic fibrosis, HC = healthy controls, AC = asthma controls), a: all subjects, b: subjects under 18 years of age, c: subjects 18 years of age and older.*

Nasal NO in asthma (Papers I and III)

Subjects with asthma participating in both MIDAS I and II had an overall increase in nNO between the study visits. This was consistent for both those under and those 18 years of age and over at baseline, p-value for all < 0.01(Table 3).

Table 3. Nasal NO (ppb) expressed as means (SDs) at baseline and follow-up in the MIDAS study.

	Baseline	Follow-up
All subjects (n = 196)	764 (± 269)	855 (± 288)
Under 18 years of age at baseline (n = 95)	832 (± 244)	922 (± 278)
18 years and older at baseline (n = 101)	700 (± 278)	785 (± 282)

FeNO in asthma and healthy controls (Paper I, III and IV)

In subjects with asthma, there was an overall increase in FeNO between baseline and the follow-up visit in the MIDAS study (Paper III). The increase was seen in both those under 18 years of age at baseline and those 18 years of age or older at baseline, p-value for all < 0.05 (Table 4).

Table 4. FeNO (ppb) expressed as geometric means (95% CIs) at baseline and follow-up in the MIDAS study.

	Baseline	Follow-up
All subjects (n=196)	15.0 (13.5–16.6)	17.9 (16.2–19.9)
Under 18 years of age at baseline (n=95)	15.9 (13.3–18.9)	19.9 (16.8–23.6)
18 years and older at baseline	14.2 (12.5–16.1)	16.3 (14.3–18.5)

Paper IV, a large population-based study, revealed significantly higher FeNO in subjects with current asthma (21.2 (20.1–22.3) ppb) than in subjects with non-current asthma (18.7 (17.1–20.5) ppb) and non-asthmatic subjects (16.4 (16.1–16.7) ppb).

Nitric oxide in relation to non-disease factors

Nasal NO in relation to constitutional factors

In subjects with CF, no association was found between nNO and height, weight or BMI (Paper II).

In subjects with asthma, a negative association was found between nNO levels and age ($r = -0.17$, Paper I, $r = -0.23$ at baseline and $r = -0.24$ at follow-up, Paper III; $p < 0.01$ for all). There was no association between nNO and height, weight, BMI or sex (Paper I).

FeNO in relation to constitutional factors and smoking

In subjects with CF, no association was found between FeNO and height or age, but a positive association was found between FeNO and weight (rho = 0.41, $p = 0.01$) and BMI (rho = 0.48, $p = 0.002$) (Paper II).

In both subjects with current asthma and non-asthmatic subjects, a positive association was found between FeNO and height (($r = 0.12$, $p < 0.001$, for subjects with current asthma; ($r = 0.14$, $p < 0.001$) for non-asthmatic subjects) (Paper IV).

In non-asthmatic subjects, there was a positive association between FeNO and weight ($r = 0.08$, $p < 0.001$) and age ($r = 0.11$, $p < 0.001$), but not BMI. In subjects with current asthma, no association was found with age, weight or BMI (Paper IV).

Males had higher FeNO than females and current smokers had lower FeNO than never smokers and ex-smokers, among both subjects with current asthma and non-asthmatic subjects (Table 5), p-value for all < 0.01 (Paper IV).

Table 5. FeNO levels (ppb) in relation to smoking habits and sex in subjects with current asthma and non-asthmatic subjects. Results presented as geometric means (95% CI)

	Subjects with current asthma N = 741	Non-asthmatic subjects N = 4,155
Females	19.6 (18.3 – 20.9)	14.8 (14.5 – 15.2)
Males	23.7 (22.0 – 25.7)	18.1 (17.7 – 18.5)
Never smoker	23.4 (21.7 – 25.1)	11.8 (10.6 – 13.2)
Ex-smoker	22.8 (21.2 – 24.7)	17.5 (17.1 – 17.9)
Current smoker	12.0 (10.7 –13.4)	11.5 (11.1 – 11.9)

Nitric oxide in relation to disease characteristics

Nitric oxide in cystic fibrosis (Paper II)

Nitric oxide in relation to CF disease and treatment

No differences in FeNO or nNO levels were seen in relation to different genotypes, pancreatic insufficiency, chronic colonisation with *Pseudomonas aeruginosa*, CF-related diabetes, treatment with azithromycin or continuous antibiotic treatment with flucloxacillin.

Nitric oxide in relation to lung function

A positive association was found between FeNO and lung function parameters (Figure 8).

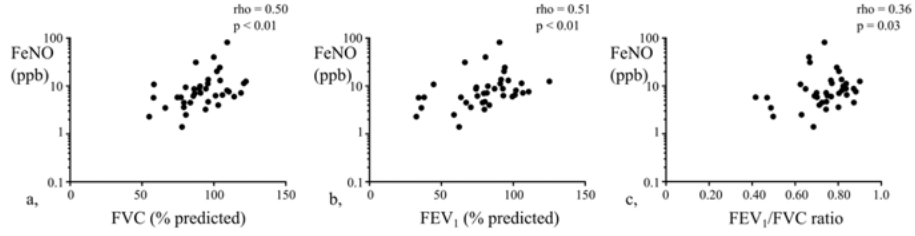

Figure 8. *Associations between FeNO (log-scale) and lung function parameters;; a, FeNO (ppb) and FVC = forced vital capacity (% predicted), b, FeNO (ppb) and FEV_1 = forced expiratory volume at 1 second (% predicted), c, FeNO (ppb) and FEV_1/FVC ratio in subjects with CF = cystic fibrosis (n = 38).*

The associations between FeNO and all lung function parameters were consistent for subjects ≥ 18 years, as were the associations between FeNO and FEV_1, FEF_{50} and FEF_{25} for subjects < 18 years (Table 6).

Table 6. Associations between FeNO levels and lung function parameters (% predicted) in subjects with CF, divided by age group. Associations calculated using Spearman's test.

	Under 18 years of age (n = 20)	18 years and older (n = 18)
FVC	rho = 0.43, p = 0.06	rho = 0.62, p < 0.01
FEV_1	rho = 0.47, p = 0.04	rho = 0.59, p = 0.01
FEV_1/FVC	rho = 0.32, p = 0.2	rho = 0.51, p = 0.03
FEF_{75}	rho = 0.28, p = 0.3	rho = 0.53, p = 0.02
FEF_{50}	rho = 0.48, p = 0.03	rho = 0.59, p = 0.01
FEF_{25}	rho = 0.49, p = 0.03	rho = 0.60, p < 0.01

No association between nNO and any of the lung function parameters was seen in patients with CF.

Nitric oxide in relation to blood cell count

In subjects with CF, a negative association was found between FeNO and blood leukocyte counts (rho = -0.33, p = 0.046) and blood neutrophil counts (rho = -0.37, p = 0.03) when assessing the whole population of subjects with CF. The association between FeNO and blood neutrophil count remained in subjects over 18 years of age (rho = -0.68, p = 0.004). No associations was found between FeNO and blood eosinophil count, nor between nNO and any of the blood cell counts, nor between total IgE levels and FeNO or nNO.

Nasal nitric oxide in asthma (Papers I and III)

Univariate associations

Associations between nNO and asthma control and symptoms

In Paper I (n = 371), subjects with an ACT score < 15 (n = 22) were found to have lower nNO levels than those with higher ACT score (619 ± 278 ppb vs. 807 ± 274 ppb, p = 0.002). No association was found between individual asthma symptoms and nNO levels.

In Paper III (n = 196), no relation between poor asthma control (ACT score < 15) and nNO was found at either baseline (n = 11) or follow-up (n = 7), nor any association between asthma severity and nNO, nor any association between change in nNO levels and change in asthma control between the visits.

Associations between nNO and bronchial responsiveness, B-Eos and FeNO

When FeNO, B-Eos and bronchial responsiveness were divided into quartiles, nNO was positively associated with higher FeNO, higher B-Eos and increased bronchial responsiveness (p-value for trend < 0.01 for all). There was also a significant difference in nNO levels between the highest and lowest quartiles of FeNO and bronchial responsiveness (Figure 9), as well as between the highest (> 0.3 x 10^9/L) and lowest (≤ 0.1 x 10^9/L) quartiles of B-Eos, p < 0.01 (Paper I).

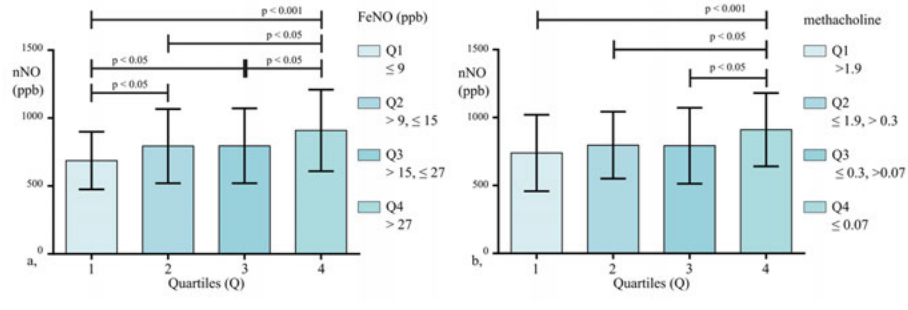

Figure 9. *Nasal NO levels expressed in parts per billion (ppb) and presented as mean and standard deviations, in relation to a, FeNO and b, bronchial responsiveness toward methacholine, divided into quartiles*.

In the follow-up study, there was a cross-sectional positive association between nNO levels and FeNO (Figure 10). Between the visits, there was a positive association between absolute change in nNO and absolute change in FeNO (r = 0.29, p < 0.001). In addition, a positive relation between absolute levels of B-Eos and FeNO was found at baseline (r = 0.17, p = 0.02) and follow-up (r = 0.20, p < 0.01) (Paper III).

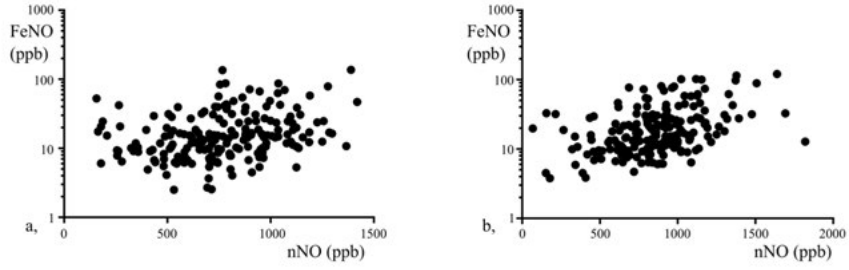

Figure 10. *Association between nNO levels (ppb) and log10 FeNO (ppb) at a, baseline, and b, follow-up.*

Associations between nNO and IgE sensitisation

There is a positive association between nNO and total IgE levels ($r = 0.22$, $p < 0.001$) (Paper I).

Subjects with atopic asthma (n = 290) had higher nNO levels than subjects with non-atopic asthma (n = 81) (822 ± 279 ppb vs. 703 ± 252 ppb, $p < 0.001$) (Paper I). These findings were consistent in Paper III, with higher nNO in atopic asthma compared with in non-atopic asthma at both baseline (791.7 ± 262.3 ppb vs. 685.2 ± 273.2 ppb, $p = 0.02$) and follow-up (887.7 ± 284.6 ppb vs. 737.2 ± 255.0 ppb, $p = 0.001$).

Subjects with sensitisation towards perennial allergens had higher nNO levels compared with non-atopic subjects or subjects with sensitisation only towards seasonal allergens (Figure 11), and a positive association was seen between nNO levels and the number of perennial allergens that a subject was sensitised to (p-value for trend < 0.001) (Paper I).

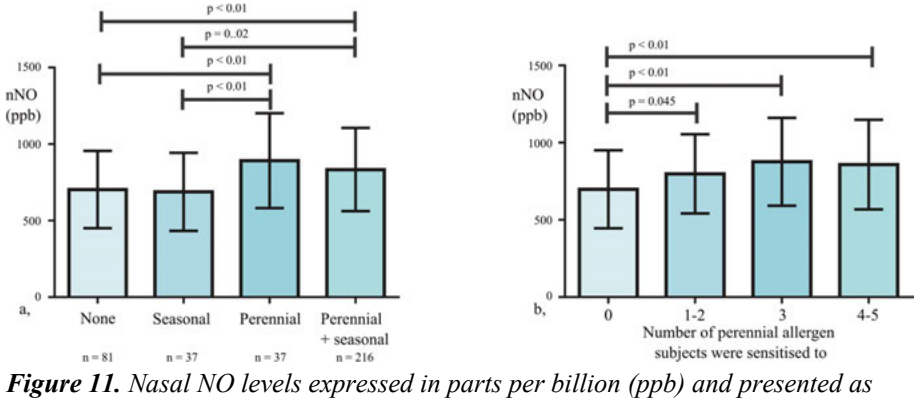

Figure 11. *Nasal NO levels expressed in parts per billion (ppb) and presented as means and SDs in relation to presence of IgE sensitisation towards aeroallergens and a, type of sensitisation (only seasonal, only perennial or combined seasonal and perennial), and b, number of perennial allergens a subject was sensitised to.*

IgE sensitisation towards any furry animal (cat, dog or horse) had the highest explanatory value for nNO levels among the perennial allergens and was associated with higher levels of nNO than no sensitisation towards furry animals (846 ± 280 vs. 707 ± 251, $p < 0.001$) (Paper I).

When dividing into groups of perennial (+/- seasonal) or non-perennial (no or only seasonal) sensitisation, nNO was higher in subjects with perennial sensitisation at both baseline and follow-up (Figure 12) (Paper III).

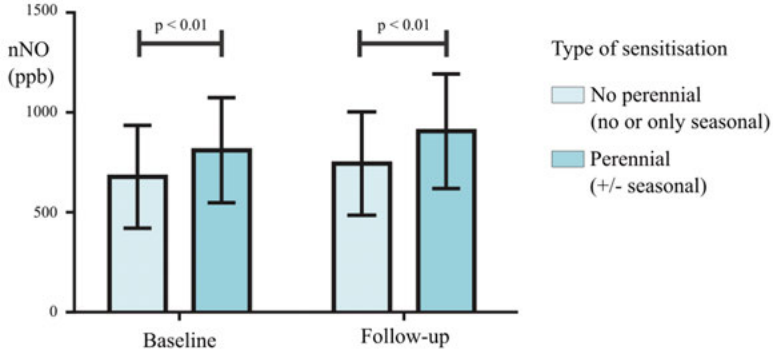

Figure 12. *Nasal NO (nNO) levels (ppb) at baseline and follow-up in relation to sensitisation towards perennial allergens.*

Associations between nNO and rhinitis and rhinitis persistency and severity

Subjects with non-atopic rhinitis had lower nNO levels than subjects with atopic rhinitis (Figure 13), and when pooling subjects without rhinitis, regardless of sensitisation, subjects with non-atopic rhinitis had lower levels of nNO than those with no rhinitis ($p = 0.01$) (Paper I).

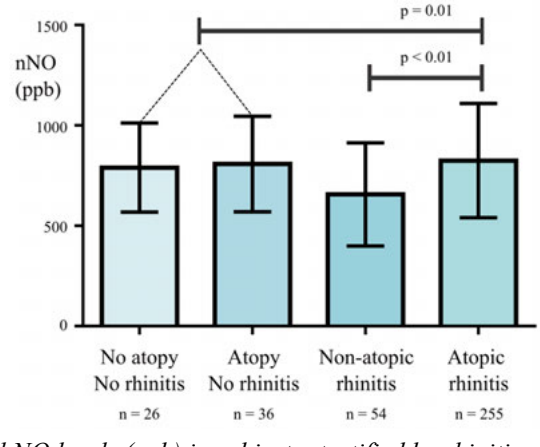

Figure 13*. Nasal NO levels (ppb) in subjects stratified by rhinitis and atopy.*

Subjects with persistent rhinitis (n = 186) had lower nNO than subjects with intermittent rhinitis (n = 123) (762 ± 279 ppb vs. 845 ± 291 ppb, p = 0.01) (Paper I). This relation remained in the follow-up study, with lower nNO in subjects with persistent rhinitis than in subjects with intermittent rhinitis or no rhinitis at both baseline (718 ± 266 ppb vs. 802 ± 267 ppb, p = 0.03) and follow-up (796 ± 314 ppb vs. 903 ± 252 ppb, p < 0.01) (Paper III).

No difference was found in nNO levels in relation to severity of rhinitis when looking at the whole study population in either Paper I or Paper III. In Paper III, the groups were stratified by atopy, which showed that, in the group without atopy, those with moderate to severe rhinitis had lower nNO than those with mild or no rhinitis. In contrast, in subjects with atopy nNO was higher in those with moderate to severe rhinitis than in those with mild rhinitis (Table 7).

Table 7. Nasal NO (ppb) in relation to severity of rhinitis at baseline and follow-up, in the whole population and divided by sensitisation or no sensitisation toward airborne allergens.

	Moderate to severe rhinitis	**Mild rhinitis**	**No rhinitis**
BASELINE			
All	764 (± 280) (n = 92)	766 (± 261) (n = 69)	757 (± 264) (n = 35)
With sensitisation	834 (± 253) (n = 69)	754 (± 272) (n = 58)	757 (± 256) (n = 20)
Without sensitisation	543 (± 254)* # (n = 22)	833 (± 187) (n = 11)	793 (± 260) (n = 14)
FOLLOW-UP			
All	869 (± 322) (n = 108)	796 (± 232) (n = 57)	860 (± 176) (n = 30)
With sensitisation	929 (± 290)* (n = 88)	799 (± 245) (n = 46)	846 (± 192) (n = 12)
Without sensitisation	575 (± 288)# (n = 18)	784 (± 167) (n = 11)	870 (± 170) (n = 18)

* is significantly different compared with mild rhinitis, # is significantly different compared with no rhinitis ($p<0.05$).

Association between nNO and chronic rhinosinusitis

Subjects with CRS or any of the individual symptoms of CRS, except discoloured nasal discharge, had lower nNO levels than subject with rhinitis but without CRS symptoms, p < 0.01 (Table 8) (Paper I).

Table 8. Nasal NO (ppb), expressed as means with SDs, in relation to individual symptoms of chronic rhinosinusitis and chronic rhinosinusitis in subjects with current rhinitis (n = 309).

Rhinosinusitis symptoms >12 weeks in the preceding year	Number of subjects with symptoms	Subjects without symptoms	Subjects with symptoms
Nasal blockage	117	829 ± 283	740 ± 285
Loss of smell	48	817 ± 280	680 ± 296
Pressure in the forehead	19	808 ± 284	609 ± 262
Discoloured nasal discharge	34	804 ± 280	728 ± 331
At least two of the above symptoms	63	818 ± 270	714 ± 321

Similar differences were found in Paper III, with lower nNO levels in subjects with CRS or symptoms of CRS than in those without CRS or symptoms of CRS, at both baseline and follow-up (Table 9).

Table 9. Difference in nNO levels between subjects with and without symptoms (β (95% CI)) at baseline and follow-up. n = number of subjects with that symptom. Significant association ($p < 0.05$) if the 95% CI does not include 0.

	Difference in nNO between subjects with or without symptoms	
	Baseline	Follow-up
Chronic rhinitis (> 12 weeks in the preceding year)	-105 (-191, -19), n = 58	-101 (-183, -19), n = 79
Pain or pressure in the forehead/nose/eyes	-213 (-360, -66), n = 14	-201 (-339, -64), n = 18
Discoloured nasal discharge	-143 (-276, -10), n = 18	-152 (-291, -12), n = 18
Loss of smell	-118 (-232, -4), n = 26	-166 (-305, -27), n = 18
CRS	-136 (-238, -34), n = 31	-103 (-224, 18), n = 25

No association between change in nNO and change in symptoms of CRS between the visits was found.

Associations between nNO and asthma or rhinitis medication

There was a negative association between nNO levels and treatment with inhaled or nasal corticosteroids. Subjects on ICS ≥ 500 μg/day in BudEq (n = 76) had lower levels of nNO than those receiving < 500 μg/day in BudEq (n = 295), and subjects on daily nasal steroid treatment (n = 71) had lower levels of nNO than those without nasal steroid treatment (n = 250) (Figure 14 a). A negative association was also found between prescribed daily doses of ICS ($r = -0.15$, $p = 0.004$) and nNO levels (Paper I). The relation to lower nNO was found in both subjects with daily nasal steroid treatment (n = 39 at baseline, n

= 32 at follow-up) compared with those without, and in subjects with ICS > 400 µg/day in BudEq (n = 45 at baseline, n = 46 at follow-up) compared with those without (Figure 14 b) (Paper III).

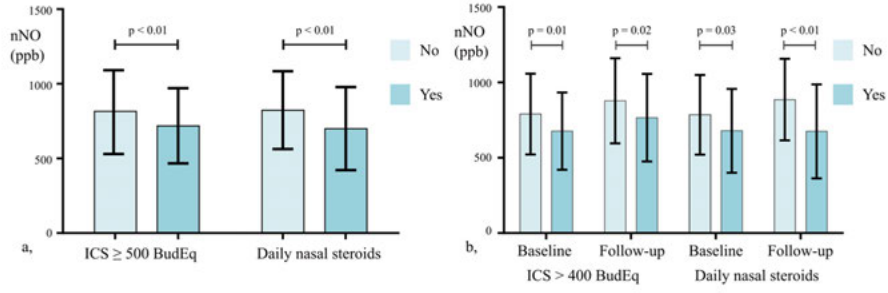

Figure 14. *Nasal nitric oxide (nNO) levels (ppb) in relation to use of daily nasal steroid treatment and treatment with ICS (inhaled corticosteroids) over or under 500* µg in BudEq (budesonide equivalents)/day *(Paper I) or 400* µg in BudEq/day *(Paper III), a, Paper I, b, Paper III.*

In Paper III, a greater increase in nNO was seen in the subjects who had had daily use of ICS at baseline but not at follow-up (n =40) than in those with daily ICS treatment at both time points (n = 120) (234 ± 274 ppb vs. 35 ± 276 ppb, $p < 0.01$). No differences in change in nNO levels was seen in relation to changes in treatment with nasal steroids.

Independent associations with nNO in asthma

Increasing age, daily treatment with nasal corticosteroids, reported loss of smell and ACT score < 15 were independently associated with lower nNO levels, while sensitisation to any furry animal was independently associated with higher levels of nNO in a multiple linear regression model (Table 10). Increased FeNO levels and high bronchial responsiveness were also independently associated with higher levels of nNO, when added into the model individually (Table 10). The relations between nNO and age and nasal steroid treatment were no longer significant in the model including bronchial responsiveness; the other associations remained.

Table 10. Effect sizes (regression coefficients (95% CIs) from multiple linear regression model) on nNO levels of different determinants. The model was additionally adjusted for ambient NO and current smoking. FeNO and bronchial responsiveness were added to the model individually.

	Regression coefficient (95% CI)
Age (/10 years)	-41.7 (-82.9, -0.5)
Loss of smell	-96.9 (-179, -15.2)
Daily nasal corticosteroid use	-77.8 (-149, -6.7)
Inhaled corticosteroids ≥500 µg/day BudEq	-34.7 (-104, 34.9)
IgE sensitisation to any furry animal	123 (58.5, 188)
IgE sensitisation to timothy	9.6 (-54.2, 73.3)
ACT score <15	-128 (-242, -14.7)
FeNO quartile 4 (≥ 26.75 ppb) vs. quartile 1 (≤ 8.8 ppb)	167 (85.1, 248)
Bronchial responsiveness quartile 4 (≤0.0672) vs. quartile 1 (> 1.948)	108 (24.8, 191)

In the follow-up study, no association between nNO and poor asthma control or CRS was found in the multivariate regression model at either baseline or follow-up. An independent positive association between perennial sensitisation at baseline and higher nNO levels at baseline and follow-up was found, as were independent negative associations between daily nasal steroid use, ICS > 400 µg BudEq/day and nNO levels at follow-up, but not at baseline (Table 11). When FeNO was added to the regression, an independent positive association between nNO and FeNO was seen at both baseline (beta coefficient (ppb per log unit increase of FeNO) (95 % CI) 213.7 (86.6, 340.9)) and follow-up (beta coefficient (95% CI) 351.1 (212.3, 489.9)). The association between nNO and perennial sensitisation was no longer significant in the model including FeNO.

Table 11. Independent determinants of nasal nitric oxide (nNO) at baseline and follow-up presented as regression coefficient (95% CI) Significant association ($p < 0.05$) if the 95% CI does not include 0.

	Baseline	Follow-up
Age/10 years	-52.7 (-107.8, 2.5)	-35.3 (-92.8, 21.2)
CRS	-89.4 (-194.5, 15.8)	-0.9 (-120.6, 118.9)
ACT score < 15	-96.1 (-255.5, 63.2)	72.9 (-133.8, 279.5)
B-Eos ≥ 0.3 (x 10^9/L)	11.1 (-76.7, 98.9)	27.2 (-59.7, 114.1)
Perennial sensitisation	101.3 (18.5, 184.1)	136.5 (50.5, 222.5)
Chronic nasal steroids	-65.4 (-160.6, 29.9)	-159.2 (-270.2, -48.3)
ICS > 400 µg BudEq/d	-42.1 (-138.6, 54.4)	-126.3 (-223.0, -29.7)

In Paper III, independent associations with change in nNO between baseline and follow-up visit were also studied and a negative association between

change in nNO levels and nNO levels at baseline was found. Discontinued daily use of ICS was associated with greater increase in nNO levels than continued daily use of ICS. Subjects on nasal steroid treatment at both timepoints had lower increase in nNO than subjects with no nasal steroid treatment. Those with sensitisation towards perennial allergens at baseline had higher absolute increases in nNO levels than those without sensitisation towards perennial allergens (Table 12). When change in FeNO was added to the model, an independent positive association between absolute change in FeNO and absolute change in nNO was found (3.0 [0.8, 5.2] (coefficient [95% CI])). The association between perennial sensitisation and nNO was no longer significant when FeNO was included in the model.

Table 12. Independent predictors of change in nasal nitric oxide (nNO) levels presented as regression coefficient (95% CI). The model is adjusted for all variables in the table as well as for time between visits, sex, age at start, change in blood eosinophil levels and change in CRS group. Significant association ($p < 0.05$) if the 95% CI does not include 0.

	Change [ΔnNO] (ppb)
nNO at start/100 ppb	-56.6 (-70.7, -42.5)
Change in ICS treatment	
discontinued vs. chronic	146.3 (51.0, 241.7)
never daily vs. chronic	49.9 (-56.6, 156.4)
start daily vs. chronic	129.8 (-49.2, 308.7)
Change in nasal steroid use	
discontinued vs. never	-17.8 (-135.3, 99.6)
start daily vs. never	-34.4 (-169.9, 101.1)
chronic vs. never	-184.5 (-321.0, -48.1)
IgE sensitisation to perennial allergens	92.4 (16.0, 168.8)

FeNO in relation to upper airway inflammatory disorders with regard to asthma and perennial sensitisation (Paper IV)

Univariate relations between FeNO and sensitisation and upper airway inflammatory disorders

Those with perennial sensitisation had higher FeNO levels than those without perennial sensitisation, in both subjects with current asthma and non-asthmatic subjects. Current rhinitis was also associated with higher FeNO in both subjects with current asthma and non-asthmatic subjects, while the association between FeNO and rhinoconjunctivitis was statistically significant only in non-asthmatic subjects. Nasal polyposis was associated with increased FeNO in asthmatics only (Table 13). A significant interaction with asthma was found

for the relation between FeNO and nasal polyposis (p = 0.04). No association was found between FeNO and severity or persistency of rhinitis, or with CRS.

Table 13. FeNO levels (ppb) in relation to sensitisation and upper airway inflammatory disorders in subjects with current asthma and non-asthmatic subjects. Results presented as geometric means (95% CIs).

	Current asthma (n = 741)	Non-asthmatic subjects (n = 4,155)
Perennial sensitisation		
No	18.1 (16.7–19.7) *	16.0 (15.7–16.3) *
Yes	24.4 (21.9–27.3)	19.2 (18.2–20.4)
Current rhinitis		
No	19.5 (17.9–21.2) #	16.1 (15.7–16.4) *
Yes	22.1 (20.7–23.5)	17.0 (16.6–17.5)
Rhinoconjunctivitis		
No	20.3 (18.9–21.9)	16.1 (15.8–16.4) *
Yes	22.0 (20.5–23.6)	17.8 (17.1–18.6)
Nasal polyposis		
No	20.7 (19.6–21.9) #	16.4 (16.1–16.7)
Yes	24.2 (21.2–27.5)	16.6 (15.5–17.7)

*p < 0.001, # p < 0.05.

When the groups were further stratified by perennial sensitisation, it was found that both current rhinitis and rhinoconjunctivitis related to higher FeNO only in non-asthmatic subjects with perennial sensitisation, with a positive interaction with perennial sensitisation for the relation between FeNO and current rhinitis (p = 0.04) as well as that between FeNO and rhinoconjunctivitis (p < 0.01).

Adjusted independent associations between FeNO, upper airway inflammatory disorder, perennial sensitisation and asthma

Current rhinitis was associated with 12.1% (95% CI 0.2–25.5%) higher levels of FeNO and rhinoconjunctivitis with 15.9% (95% CI 3.4–29.9%) higher levels of FeNO in non-asthmatics with perennial sensitisation, after adjusting for sex, age, height, weight, use of asthma or rhinitis medication, smoking status and centre (Table 14). Although both current rhinitis and nasal polyposis were numerically related to higher FeNO in subjects with current asthma, no statistically significant associations between UAID and FeNO were found in the other groups stratified by asthma and perennial sensitisation after adjustments. In subjects with current asthma and perennial sensitisation, use of nasal corticosteroids related to lower FeNO, whereas regular or intermittent use of ICS related to higher FeNO (Table 14).

Table 14. Independent associations of UAID and treatment with FeNO in subjects with current asthma and non-asthmatic subjects, stratified by perennial sensitisation, adjusted for age, weight, height, sex, treatment with LTRA, smoking status and centre. Results presented as % difference ($(10^{\beta\text{-coefficient}} - 1) \times 100$), with 95% confidence intervals (CIs), in relation to reference group (i.e., no rhinitis, no treatment, no nasal polyposis, no rhinoconjunctivitis). 95% CIs that do not include 0 are statistically significant ($p < 0.05$).

	Current asthma Perennial atopy (n = 168)	Current asthma No perennial atopy (n = 243)	Non-asthmatic Perennial atopy (n = 482)	Non-asthmatic No perennial atopy (n = 3,164)
Current rhinitis	21 (-10–61)	8 (-10–29)	12 (0.2–25)	2 (-2–6)
Rhinoconjunctivitis	7 (-16–35)	5 (-12–24)	16 (3–30)	2 (-23–7)
Nasal polyposis	16 (-21–69)	19 (-8–54)	1 (-18–26)	2 (-5–9)
Nasal steroids preceding year	-31 (-48–(-9))	9 (-10–32)	-7 (-21–10)	4 (-2–11)
Use of ICS				
Intermittent	40 (4–89)	-2 (-20–20)	Not applicable	Not applicable
Regular	67 (15–143)	5 (-15–30)		

Perennial = perennial +/- grass sensitisation, non-perennial = no sensitisation or sensitisation only towards grass, ICS = inhaled corticosteroids.

An analysis was performed of the whole study population, without stratification, in a regression model additionally adjusted for centre, smoking, age, weight, height, sex, and treatment with ICS, nasal steroids and LTRA. This showed that asthma, perennial sensitisation and current rhinitis, as well as rhinoconjunctivitis (added individually in exchange for current rhinitis), all related to higher FeNO (Figure 15). There was a significant interaction with perennial sensitisation for the relationship between FeNO and current rhinitis and rhinoconjunctivitis ($p = 0.03$ for both).There was also an association with 20% (95% CI 5–37%) higher FeNO among regular ICS users than among non-ICS users.

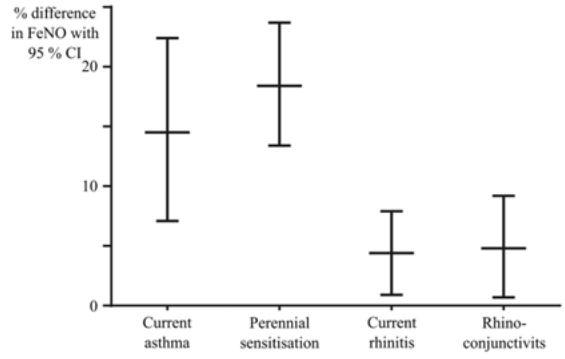

Figure 15. *Effect sizes of associations of asthma, perennial sensitisation, current rhinitis and rhinoconjunctivitis on FeNO. Additional adjustment were made for age, sex, height, weight, treatment for asthma and rhinitis, current smoking status and centre. Current rhinitis and rhinoconjunctivitis were added in the model individually.*

Discussion

Nitric oxide in cystic fibrosis

Nitric oxide in cystic fibrosis and it´s relation to disease characteristics

Patients with CF had lower levels of NO in their airways than subjects with asthma and healthy controls. This difference was most pronounced in the upper airways, with median nNO levels in the CF study population being about half of those in subjects with asthma and healthy controls, among both children and adults. FeNO was also lower in patients with CF than in subjects with asthma and healthy controls, but not to the same extent as nNO, and this difference was seen primarily in adults.

The findings of low nNO in patients with CF were in line with numerous previous studies showing that patients with CF have significantly lower levels of nNO than healthy controls[47,48,61,64] and subjects with asthma[155], but not as low levels as patients with primary ciliary dyskinesia (PCD) [156-158]. Nasal nitric oxide is distinctively lower in patients with PCD than in healthy controls [158-160] and nNO has been suggested as a screening tool and as part of the diagnostics of PCD[161,162]. Due to the similarity in respiratory symptoms in CF and PCD, as well as the fact that both diseases have low nNO, exclusion of CF is required in diagnosing PCD [162].

Part of the reason that nNO is low in patients with CF can be the high prevalence of concomitant upper airway disease, with CRS and nasal polyposis. In over 80% of patients with CF, structural changes in the upper airways are visible on CT scans, and a high proportion of CF patients develop nasal polyps with age [163]. Chronic rhinosinusitis, especially with nasal polyposis, is associated with lower nNO levels in non-CF patients [106,107,109] and increases upon medical or surgical treatment [108,109]. Similarly, among patients with CF, those with nasal polyposis have a further reduction in nNO levels than those without nasal polyposis. Though surgery of nasal polyposis raises nNO in these patients, nNO still fails to reach levels seen in healthy subjects [51]. This suggests that low nNO in patients with CF is not due solely to reduced diffusion of NO through the sinus ostia, but is more complex.

The finding of lower FeNO in patients with CF was also in line with some previous studies. However, the results from different studies are somewhat diverging, with some studies showing lower FeNO [49,50,64,164] and others showing similar FeNO levels as in healthy controls [155,165]. This indicates that even

though CF is associated with increased inflammation in the airways, FeNO is not increased, but rather decreased – raising the question why NO is decreased in CF. There are ample theories that address this question.

One explanation for low NO in patients with CF is the diffusion through the thick mucus in CF airways. Furthermore, NO can be reduced to nitrite and nitrate. This theory is supported by increased levels of NO metabolites in CF sputum, even though NO in exhaled air is not elevated[166,167]. However, treatment studies targeting the thick mucus in CF by inhalation with hypertonic saline or dornase alfa show no increase in FeNO upon these treatments [57], although Grasemann *et al.* reported a positive relation between change in FeNO and change in lung function in CF patients treated with dornase alfa[168].

Another possible explanation for reduced NO in the airways of CF patients can be that expression of NOS within the airways is reduced. This has mainly been studied in relation to iNOS, with a reduction of iNOS protein and mRNA expression in bronchial epithelial cells [55,169], tracheal cells [54], and in nasal mucosa biopsies [170]. The response in iNOS expression and activity upon stimulation with neutrophils in epithelial cells is also reduced in patients with CF compared with controls [171]. There are studies showing a reduction in nNOS in samples from upper airways in patients with CF[170], and polymorphisms in eNOS and nNOS in CF patients have been found to associate to both NO levels in the airways and infections with *Pseudomonas aeruginosa* [172,173].

These findings, with reduced NOS in CF, indicate that there might be a link between CFTR dysfunction and NOS. Some studies have shown a relation between NO levels and severity of genotype [56], although this author's research group could not find that association, in parallel with other study groups [64]. If there is a direct link between CFTR and NOS, one could assume that NO levels would be lower already in infancy in CF patients than in controls. However, the few studies looking at FeNO during infancy in CF show diverging results, with two studies showing lower FeNO in infants with CF [174,175], but a larger study failing to verify this result [176]. During the last decade, treatment of the underlying dysfunction of the CFTR protein has been developed, and CFTR modulator treatment has been made available, first for patients with gating mutations, and more recently for patients homozygous or heterozygous for the F508del mutation [177]. Two independent studies have looked at FeNO in relation to treatment with ivacaftor in patients with gating mutations and found that FeNO increased in patients treated with ivacaftor, and that the increase in FeNO related to increase in lung function [57,58]. A more recent study looked at long-term effects of treatment with both ivacaftor for gating mutations and ivacaftor/lumacaftor for patients homozygous for F508del. It found that the elevation of FeNO upon treatment with ivacaftor for gating mutations was maintained over time [178]. However, they found no significant effect on FeNO in relation to ivacaftor/lumacaftor treatment in patients homozygous for the F508del mutation [178]. These treatment studies suggest that there is a relation between CFTR function and FeNO; however, it is not possible to deduce

if this is a direct link between CFTR and NO or if it is mediated through underlying mechanisms of decreased mucus and inflammation by enhanced function of the CFTR protein. In the latest study by Grasemann *et al.*, the authors reported a decrease in myeloperoxidase (MPO) and the NO metabolite nitrite upon treatment with ivacaftor, strengthening the latter theory [178].

As arginine is the substrate used by NOS in NO production, low arginine levels in patients with CF have been proposed as an additional explanation for reduced NO in CF. Classic CF is accompanied by exocrine pancreas insufficiency leading to a malabsorption of fat and protein, which can cause arginine deficiency. CF patients have been shown to have lower arginine levels in blood than controls [179], and pancreatic insufficiency has in some studies been associated with lower FeNO in CF patients [56]. This relation could not be verified in Paper II. To test the relation between arginine deficiency and NO levels, treatment studies have been performed with injection, inhalation or oral supplementation with arginine. These studies have shown an increase in FeNO upon arginine supplementation, but no change in lung function [180-182]. A recent study has looked at arginine supplementation and inhibition of arginine breakdown in human epithelial cells from patients homozygous for F508del, to see if this amplified the effect of treatment with ivacaftor/lumacaftor. The researchers found an increase in cytoplasmic NO and an increased function of CFTR on the cell surface after increasing arginine availability in the cells [183].

Paper II could only confirm that patients with CF had reduced levels of both nasal and exhaled NO, but could add no information on the mechanism behind these findings.

FeNO in relation to inflammation in patients with cystic fibrosis

Paper II showed that, in CF patients, FeNO was negatively associated with the amount of leukocytes in blood and, in particular, blood neutrophil count. This is in contrast to in patients with asthma where FeNO has been associated with type-2 inflammation and increase in blood eosinophil count [184], indicating that the levels of FeNO in CF are dependent on other mechanisms. The relation between lower FeNO and higher leukocyte count has been reported in ex-smoking patients with chronic obstructive lung disease (COPD), but in these patients, there was also a positive relation between blood eosinophil count and FeNO [185]. In Paper II no association between blood eosinophil count and FeNO was found in patients with CF. The relation between systemic inflammation and NO in patients with CF is not as widely studied, though a relation between nNO, $Calv_{NO}$ and blood leukocyte count have been reported [49,56]. One plausible reason for the association between FeNO and blood neutrophil count found in Paper II is that the blood neutrophil count mirrored the neutrophil inflammation seen in CF lung disease [186]. The increase in neutrophil inflammation in CF lungs, with increase in MPO, might increase the conversion of NO to its metabolites, primarily peroxinitrite, resulting in lower levels of gaseous NO and thus lower FeNO [187,188].

As NO is believed to play a part in the human innate immune defence [4], an alternative explanation to this association could be that the reduced NO in patients with CF contributes to the bacterial and fungal infection in CF airway, which in turn leads to increased inflammation. In ex vivo studies, NO impairs growth and construction of biofilm in *Pseudomonas aeruginosa* [5,6], which is a common pathogen in CF lung disease. The prevalence of chronic infections with *Pseudomonas aeruginosa* in patients with CF increases with age and is related to decline in lung function [189]. Even though more aggressive treatment regimens are used in clinical practice to eradicate primary infections, which has led the prevalence of chronic *Pseudomonas* infections to decrease in the last decades, it is still the most common pathogen in chronic lung infection in CF patients [189]. Other pathogens, apart from *Pseudomonas aeruginosa*, associated with a more rapid decline in lung function in patients with CF include *Achromobacter Xylosoxidans*, *Burkholderia cepacia* complex and *Mycobacterium abscessus* complex [190]. A small Phase I trial has tried to use inhaled gaseous nitric oxide in high concentrations to treat these difficult to treat infections, resulting in a decrease in colony-forming units of the bacteria, as well as an increase in lung function [7]. Similar findings have also been reported in additional case studies in patients with CF and difficult-to-treat bacteria [191,192].

A relation between chronic *Pseudomonas* infection and lower levels of FeNO have been reported in previous studies [49,56,193], but causality in this relation is not clear. Paper II showed no relation between FeNO and chronic infection with *Pseudomonas aeruginosa*, but the frequency of chronic *Pseudomonas* infection was high in this study-population, making the chance to differentiate between those with and without *Pseudomonas* small.

FeNO in relation to lung function in patients with cystic fibrosis

Paper II showed a positive association between FeNO and lung function in both children and adults with CF. This is in line with a few other studies finding a relation between FeNO and lung function assessed through spirometry [49,62] and measurement of small airway involvement in children, using lung clearance index (LCI) measured with multiple breath washout [49]. However, other studies have reported no relation between FeNO and lung function assessed through spirometry [63,64,194].

Due to the cross-sectional design of Paper II, it was not possible to address any causality, but it is theoretically plausible both that the lung function decline proceeds or is parallel to the decline in FeNO and that the lower NO can contribute to decline in lung function.

The first theory is that as the CF lung disease progresses, with increased mucus stagnation and neutrophil inflammation within the lungs, it contributes to both a decrease in lung function and reduced gaseous NO in the airways. The latter, for the reasons discussed above, would lead to decreased diffusion of NO and increased reduction of NO to its metabolites within the thick mucus rich in inflammatory cells and cytokines. The second theory, discussed above

in regard to the relation of inflammation and FeNO, is that the low levels of NO in patients with CF contribute to susceptibility to infections, which in turn, through increased inflammation, lead to structural lung damage, thus exacerbating lung function impairment.

Nasal nitric oxide in asthma

Nasal nitric oxide in asthma and its relation to sensitisation

In patients with asthma, those with IgE sensitisation towards airborne perennial allergens had higher levels of nNO than those without IgE sensitisation (Papers I and III). In addition, perennial sensitisation at baseline was associated with a higher increase in nNO after 2–5 years (Paper III).

Whereas the relation between FeNO and sensitisation is well-known, with increased FeNO in non-asthmatic subjects with atopy [73,75,76], as well as higher FeNO in atopic vs. non-atopic asthma [73,74,76], the relation between nNO and IgE sensitisation is less clear-cut. In population-based studies the results have been diverging, with some studies showing a relation [25,72] and others showing no relation [195]. The same diversities are seen in studies on rhinitis, with some studies supporting an increase in nNO in relation to sensitisation [72,102], as well as an increase in nNO in seasonal AR upon allergen exposure [196], while others have found no association between sensitisation and nNO [197]. One reason for this inconsistency can be that the studies failing to show a relation have looked at sensitisation regardless of whether it is seasonal or perennial.

It is theoretically plausible that sensitisation towards airborne allergens would give rise to higher levels of nNO, as the upper airways are the first line of exposure for airborne allergens and IgE sensitisation with a type-2 inflammation stimulates iNOS expression and activation [71], with increase in NO production as a result. This is supported by a recent study showing that treatment with allergen-specific immunotherapy is related to a reduction in airway NO, with a reduction of nNO in patients with AR [198] and of FeNO in subjects with allergic asthma and concomitant rhinitis, and a simultaneous decrease in mucosal swelling in the airways [199].

Moreover, Paper I showed that multiple sensitisations was related to higher nNO levels, indicating a dose response in nNO in relation to the degree of sensitisation.

Nasal nitric oxide in asthma in relation to symptoms and treatment of upper airway inflammatory diseases

In patients with asthma, loss of smell, as a marker of more pronounced nasal mucosa swelling in CRS (Paper I) – and treatment of upper airway inflammation with nasal steroids (Papers I and III) are associated with reduced levels of nNO.

In contrast to FeNO, which is sometimes used as a diagnostic and monitoring tool in asthma, the use of nNO in rhinitis has been debated. Studies on patients with rhinitis show diverging results on its relation to nNO, with some showing increased nNO in AR [102,200] and increase upon allergen exposure, whereas others show similar levels as controls [197], or in the case of CRS, especially in the presence of nasal polyposis, decreased levels of nNO [106,110,201,202]. This is thought to be due to the dual contribution of nNO from both the nasal mucosa and the sinuses, where nitric oxide is present in much higher concentration than in other parts of the airways [17]. That nNO levels can be influenced by the passage from the sinuses is supported by the fact that patients with CRSwNP have lower nNO than those without nasal polyposis [106], and that nNO levels in patients with CRS or CRSwNP increase upon surgical treatment or treatment with intranasal steroids [108,109]. In the MIDAS study nNO levels were lower in subjects with persistent rhinitis than those with intermittent or no rhinitis, suggesting that a more persistent rhinitis is associated with more mucosal swelling and impaired passage of NO from the sinuses. However, the studies on nNO in relation to persistent rhinitis are diverging, with some results in line with those of the MIDAS study, i.e., lower nNO in persistent than intermittent rhinitis [134], while other studies have reported higher levels of nNO in patients with persistent rhinitis than controls [203], and a reduction of nNO upon treatment with nasal steroids [204]. The relation between nNO and severity of rhinitis was found to differ between subjects with atopy and those without atopy (Paper III). In atopic subjects, moderate to severe rhinitis was associated with increased nNO, whereas in non-atopic subjects, the relation was the opposite, with lower nNO in subjects with moderate to severe rhinitis. Previous reports on nNO in relation to severity of rhinitis are diverging. Wang *et al.*, looking at children with AR, reported higher levels of nNO in moderate to severe rhinitis [205], while Lee *et al.,* in a study of young adults, found that moderate to severe rhinitis was associated with numerically lower nNO [134]. These findings further highlight the difficulty in interpreting nNO in rhinitis and that sensitisation must be taken in to account when analysing nNO measurements.

Treatment of local type 2-inflammation with nasal corticosteroids related to lower nNO levels. Cross-sectionally, a relation could be seen between lower nNO and daily use of nasal steroids (Papers I and III); longitudinally, it was found that those with daily treatment with nasal steroids at both baseline and follow-up had a lower increase in nNO than those without treatment at both time-points (Paper III). This is in line with other studies showing a reduction in nNO in treatment of AR [206-208], though the results relating to nNO and treatment with nasal steroids are inconsistent [209]. Once again, the dual contribution of nNO can influence the outcome of treatment, as nasal corticosteroids can reduce local type-2 inflammation, leading to both reduced NO production via iNOS and reduced swelling of the mucosa, resulting in increased passage of NO from the sinuses.

A relation between lower nNO and symptoms of CRS in the cross-sectional univariate analyses of MIDAS I and II, as well as a relation between loss of smell, as a marker for increased severity of CRS, and lower nNO in regression model adjusted for confounders in MIDAS I. However, no relation was found between change in nNO and change in CRS symptoms over time. The relation between lower nNO and olfactory impairment has previously been reported in patients with CRS [202]. As nNO is lower in patients with CRSwNP than those with CRSsNP [106] and nasal polyposis is associated with loss of smell [210,211], it is plausible that low nNO in patients with loss of smell can be caused by nasal polyposis. However, a recent study on subjects with olfactory impairment with different aetiologies found that although nNO was lower in patients with loss of smell, it was not possible to differentiate between underlying reasons for loss of smell [212].

Nasal nitric oxide in asthma in relation to asthma control within the concept of united airway disease

Subjects with asthma and poor asthma control, defined as ACT < 15, had lower nNO levels than those without poor asthma control (Paper I). This relation remained after adjustment for sensitisation, loss of smell and treatment with inhaled or nasal corticosteroids.

Patients with CRS have been reported to have lower nNO levels than controls [106,201,202], and among patients with CRS, those with CRSwNP have lower nNO than those with CRSsNP [106]. As CRS with nasal polyposis is associated with impaired quality of life and lung function in patients with asthma [124,125], the finding in Paper I of lower nNO in subjects with poor asthma control could be a sign that upper airway disease in these patients causes both poor asthma control and low nNO. The relation between impaired asthma control and low nNO has also been reported by Asano *et al.* in asthmatic patients with concomitant CRS [213], as well as by Heffler *et al.* [214]. The latter study encompassed patients with moderate to severe asthma, with a high proportion of patients with a doctor's diagnosis of CRS (>50%). In addition to reporting a relation between poor asthma control and low nNO levels, Heffler *et al.* also reported lower levels of nNO in subjects with CRSwNP than in those with CRSsNP and in controls [214]. This suggests that nNO could be used in patients with asthma and impaired asthma control, to screen for upper airway comorbidity. On the other hand, in Paper III, with fewer study subjects and fewer patients with CRS symptoms and higher ACT scores, no relation could be found between asthma control and nNO or between change in nNO and change in asthma control.

Nasal nitric oxide in asthma within the concept of united airway disease

In subjects with asthma, nasal nitric oxide, as a marker of type-2 inflammation in the upper airways, was positively related to FeNO, as a marker of type-2 inflammation in the lower airways (Papers I and III), and was associated with

increased bronchial reactivity (Paper I). Levels of nNO in patients with asthma were also related to treatment of lower airway inflammation with ICS (Papers I and III).

The relation found between FeNO and nNO in asthma is in line with the concept of united airway disease, in which inflammations in the upper and lower airways relate to and influence each other. Both Paper I and III found independent relations between nNO and FeNO after adjustment for confounders, as well as a relation between change in nNO and change in FeNO between the two visits. It could be argued that the relation is due to cross-contamination of NO between the airway compartments, but as both FeNO and nNO measurements were performed during velum closure, minimising the passage of NO between the upper and lower airway compartments, this is unlikely. A more likely explanation is that systemic type-2 inflammation influences NO production in both upper and lower airways. It has been postulated, that local type-2 inflammation in one part of the airway, through stimulation of cytokines and chemokines, triggers a systemic cell response, which in turn activates inflammation in other parts of the airways [112]. This hypothesis is supported by findings of increased local nasal inflammation upon segmental bronchial allergen provocation [128], as well as by increase of bronchial reactivity upon nasal allergen provocation [215]. However, Pedroletti *et al.* found no increase in FeNO after nasal allergen provocation [216]. An association between nNO and FeNO has previously been described in population-based studies of asymptomatic subjects [72,104], as well as in patients with AR [104], but Paper I describes the first study of a large cohort of subjects with asthma addressing this relation. The relation between nNO and FeNO has recently been confirmed in patients with asthma and concomitant AR [213]

The findings that daily use of inhaled corticosteroids was associated with lower nNO levels (Paper I) and that discontinued use of inhaled corticosteroids > 400 µg/d BudEq was related to increase in nNO levels (Paper III) might be further support of the concept of united airway disease. Treatment of lower airway inflammation might theoretically reduce inflammation also in other parts of the airways. As CRS, especially with nasal polyposis is related to both more severe asthma [124,125], and lower nNO levels [106,107], an alternative explanation for the cross-sectional relation might be that subjects with severe upper airway comorbidities are prescribed more ICS. However, the relation between nNO and daily treatment of ICS in Paper I was consistent after adjustment for loss of smell, as a marker for CRS and CRS related symptoms.

The relation between nNO and bronchial responsiveness seen Paper I is also in line with the concept of united airway disease. There is a connection between AR and increased bronchial reactivity in patients without asthma [117,217,218], as well as increased bronchial reactivity in patients with asthma and concomitant AR [219]. It is not known if this relation is due to more blockage of the nasal mucosa leading to more oral breathing and less filtration of allergens

in the nose, making the lower airways more exposed to irritants from the environment, or if it is the inflammation in the upper airway that triggers lower airway inflammation. The relation between nNO and bronchial reactivity seen in Paper I can be an effect of either of these explanations, with higher nNO as a signal of more type-2 inflammation in the upper airway leading either to more systemic inflammation or to more symptomatic manifestations of inflammation with rhinitis. A relation between higher nNO and more bronchial responsiveness has previously been reported by Makris *et al.* in a population of patients with seasonal AR studied during pollen season [196].

Exhaled nitric oxide in upper airway disorders, in relation to asthma and IgE sensitisation

Current rhinitis and rhinoconjunctivitis are associated with higher FeNO in middle-aged adults after adjustment for known confounders including asthma and perennial sensitisation. The presence of either of these UAID are, on a population basis, associated with approximately 4–5% higher FeNO. Asthma and perennial sensitisation are associated with 15% respective 18% higher FeNO, respectively. There is a significant interaction with perennial sensitisation for the relation between FeNO and current rhinitis or rhinoconjunctivitis. When the population in Paper IV was stratified by current asthma and perennial sensitisation, the relation between FeNO and current rhinitis and rhinoconjunctivitis was significant only in non-asthmatic subjects with perennial sensitisation, where the presence of current rhinitis was associated with 12% higher FeNO and rhinoconjunctivitis with 16% higher FeNO.

IgE sensitisation is known to relate to higher FeNO in population-based studies of both children [76,220,221] and adults [72,73,222], as well as in asthma [73,74] and in allergic vs. non allergic rhinitis [222,223]. A dose response in the relation between FeNO and IgE sensitisation has also been reported, with higher FeNO in subjects with multiple sensitisations [224,225], as well as in those with perennial vs. seasonal sensitisation [75]. In addition, allergen exposure in subjects with IgE sensitisation is associated with increase an in FeNO in subjects with asthma and AR [223,226]. This gives rise to the question if it is only the IgE sensitisation that explains the higher FeNO in AR, or if the local nasal inflammation, independent of sensitisation, is related to or adds to the higher FeNO seen in AR. In a studt of 246 adults, Olin *et al.* reported that FeNO was elevated in subjects with atopy only in the presence of symptoms of inflammation in the airways with asthma or AR and when there had been recent allergen exposure [195]. Similar findings have been reported in population-based studies in children, where the difference in FeNO between atopic vs. non-atopic children was more pronounced in the presence of symptoms (wheeze) [227] and FeNO was higher in non-asthmatic atopic children with rhinitis than in those without rhinitis [76].

The findings in Paper IV of higher FeNO in subjects with perennial sensitisation than in those without perennial sensitisation, seen in both subjects with current asthma and non-asthmatic subjects, were in line with previous findings of a relation between sensitisation and higher FeNO.

Allergic rhinitis has previously been reported to be associated with higher FeNO in both patients with asthma [140,141,228] and non-asthmatic subjects[134,223,229,230], however, most studies have not been adjusted for sensitisation and type of sensitisation. Paper IV showed that the relation between current rhinitis as well as rhinoconjunctivitis and higher FeNO remained after adjusting for perennial sensitisation and asthma at a population level, and in non-asthmatic subjects with perennial sensitisation after stratification by asthma and sensitisation. It also revealed an interaction with perennial sensitisation on the relation between current rhinitis as well as rhinoconjunctivitis and FeNO. These findings suggest that perennial sensitisation and the presence of rhinitis have an additive effect on FeNO. If this is due to more inflammation in the upper airways, caused by allergen exposure, triggering lower airway inflammation within the concept of united airway disease, or simultaneous allergen exposure in the upper and lower airways independently triggering the inflammation in both compartments could not be determined or deduced from Paper IV.

After stratification, the relation between current rhinitis and FeNO was not significant in subjects with current asthma. This could be because the subjects with current asthma were fewer than the non-asthmatic subjects, making the study underpowered to assess this relationship in stratified groups. It could also be because in subjects with asthma, allergen exposure triggers a direct lower airway inflammation, yielding an increase in FeNO and diminishing the additive effect of rhinitis on FeNO in the presence of asthma-related inflammation.

Although a relation between nasal polyposis and higher FeNO was found in univariate analysis in subjects with asthma, no significant relation was found in the multivariate analyses between nasal polyposis and FeNO, either stratified by asthma and perennial sensitisation, or in the whole study population. The lack of significance on the relation between nasal polyposis and FeNO in the stratified analysis might be due to too few subjects in each strata, making the study underpowered for assessing the relation between nasal polyposis and FeNO in stratified groups. A relation between higher FeNO in subjects with asthma and concomitant nasal polyposis has been reported in previous studies [126,135,143,144,201]. In patients with asthma, concomitant nasal polyposis is related to more exacerbations, decreased quality of life and increased asthma severity [125,231,232]. In biopsies from nasal polyps, there is increased expression of iNOS, most prominent in patients with atopy [233] and higher iNOS expression in polyps than in nasal mucosa [234]. In addition, there is increased eosinophilia in biopsies from CRSwNP compared with CRSsNP [235]. Increased systemic typ-2 inflammation, with its increase in cytokines (e.g. IL-5 and IL-

13), trigger iNOS expression and recruitment of eosinophils in both the upper and lower airways.

The difference between subjects with asthma and non-asthmatic subjects in the relation between nasal polyposis and FeNO was further highlighted by the interaction of asthma on the relation between nasal polyposis and FeNO. On the other hand, no interaction with perennial sensitisation was found for relation between nasal polyposis and FeNO, suggesting that the relation between and nasal polyposis in asthma was not influenced by perennial sensitisation. This is in line with studies on CRSwNP, indicating that although CRSwNP is associated with increased type-2 inflammation and is often associated with high levels of polyclonal IgE antibodies; such inflammation can be present without atopy [236,237]. In recent years, there has been increasing evidence that group 2 innate lymphoid cells (ILC2) play an important role in the type-2 inflammation seen in eosinophilic nasal polyposis [238].

In subjects with current asthma and perennial sensitisation, those with regular use of nasal corticosteroids had lower FeNO compared with those without, after adjustment for asthma treatment, UAID and other known predictors of FeNO (Paper IV). These findings could theoretically be plausible within the concept of united airway disease; treatment of inflammation in the upper airway could lead to reduced inflammation in the lower airways, either through reduced systemic inflammation or through reduced swelling and mucosa impairment in the upper airway, leading to less oral breathing and exposure of the lower airways. However, the cross-sectional analyses could add no information on the causality of the relation between nasal steroids and FeNO. That nasal corticosteroids as add-on therapy in adult asthmatics are related to lower FeNO and improved asthma quality of life scores has previously been reported by Oka *et al.* [142], however, longitudinal studies in asthmatic children have reported no relation between add-on therapy with nasal steroids and change in FeNO [239,240].

In contrast to the relation between nasal steroid treatment and lower FeNO levels, regular, as well as intermittent, use of ICS was found to be related to higher levels of FeNO in subjects with current asthma and perennial sensitisation. This might seem counter-intuitive, as treatment with inhaled corticosteroid in patients with asthma has been reported to reduce FeNO [87,241]. However, as this is a cross-sectional analysis, it was not designed to assess whether regular use of ICS changed FeNO. The relation in Paper IV could be due to subjects with more severe asthma having both higher FeNO and more ICS treatment. Further, although ICS treatment can clinically improve asthma symptoms and asthma control, FeNO may still remain elevated [242]. An additional explanation could be a lack of adherence to prescribed ICS doses, resulting in maintained high FeNO despite reported regular ICS use [243].

Strength and limitations

A strength of the thesis was that simultaneous measurements of nNO and FeNO, using the same equipment, protocol and research staff, were performed in patients with CF, as well as in subjects with asthma and healthy controls in the studies for Papers I–III. Information on mutation, chronic infections, pancreatic insufficiency and CF-related comorbidities in patients with CF was collected from medical records in Paper II, minimising the risk for recollection bias and misclassification. To look at differences in NO between disease and healthy controls, age- and sex-matched controls were randomly selected from the MIDAS I cohort, as both age and sex are known predictors of FeNO.

The major limitation of Paper II was that the group of patients with CF was relatively small and very heterogeneous in regard to mutations, age, lung function and exocrine pancreatic status, and there was no objective measurement of IgE sensitisation, which is a known predictor of FeNO. Further, information on concomitant upper airway disorders and symptoms of CRS, which may influence nNO levels, was lacking. Another limitation was that, as a cross-sectional design was used no assumption could be made on the causality of the relation between FeNO and lung function or blood neutrophil count. Furthermore, there was no information on how FeNO or nNO changed over time in patients with CF, or if NO related to change in lung function or systemic inflammation. Originally, Study II was part of a longitudinal cohort in which part of the aim was to look at changes in FeNO and nNO over time, but due to technical problems and many subjects being lost to follow-up , the study population became too small for adequate statistical power in the longitudinal setting.

The main strengths of Papers I and III were that the study population was, to the best of the knowledge of the research group, the largest cohort of subjects with asthma with available nNO measurements that could be studied in relation to asthma control, bronchial reactivity and FeNO, with information on upper and lower airway symptoms and medication. Another strength with the study population was that it was based on children and young adults with physician-diagnosed asthma from primary and secondary care, making it generalisable to children and young adults with mild to moderate asthma in the region. All the measurements and questionnaire-based interviews were made by the same highly trained research nurses, and the same equipment and facilities were used at both baseline and follow-up visits.

The major limitation with Papers I and III was that assessment of upper airway disorders, especially CRS, was only questionnaire-based and no examinations were made of the upper airways to validate diagnoses or to assess the presence of nasal polyposis. However, the assessment of upper airway disorders were in accordance with ARIA guidelines, and validation studies have shown a high concordance between questionnaire-assessed CRS and self-reported doctor's diagnosis of CRS. Paper III lacked information on ambient

NO, as well as information on plateau during the nNO measurements. There were also a high number lost to follow-up between baseline and follow-up, although there were no significant differences between those included in Paper III and those lost to follow-up, apart from a higher number of males in the group lost to follow-up. Between the two visits, there was an overall improvement in asthma control and fewer subjects reported symptoms of CRS at follow-up. This, in combination with a high proportion being lost to follow-up, made the study potentially underpowered to look at nNO in relation to poor asthma control, CRS, or change in grouped variables. An additional limitation in Paper III was that IgE sensitisation was measured only at baseline, and there could have been a change in the number of allergens the study subjects were sensitised to or a conversion from non-perennial to perennial sensitisations between the visits.

The main strengths of Paper IV were that it was a large multi-centre study, making it potentially possible to investigate weaker associations and interactions of variables with adjustments made for known confounders. In addition, it could, in this setting, be possible to assess if the association between FeNO and upper airway inflammatory disorders varied between subjects with asthma and non-asthmatic subjects, accounting for perennial sensitisation. Another strength was that all IgE measurements were done at a single central laboratory, thereby excluding methodological and interpretation differences. Being a population-based study, the results may be generalisable for middle-aged adults.

A main limitation of Paper IV is the cross-sectional design of our analysis meaning that no assessment could be made of the causality of relations between FeNO and upper airway disorders, or of how FeNO related to changes in symptoms or diagnosis over time. Another major limitation was the high number of subjects for whom measurements of IgE sensitisation were missing (43% of subjects with current asthma and 19% of non-asthmatic subjects), making the groups smaller when stratification for perennial sensitisation and multivariate regressions was performed. In addition, a limited number of aeroallergens was assessed, making underestimation of the number of subjects with perennial or seasonal sensitisation possible. Further, there was no physician-based diagnosis of either asthma, CRS or nasal polyposis; instead, these diagnoses were based on self-reported doctor's diagnosis, which may have led to misclassification. To minimise this risk, only those with current asthma, defined as self-reported doctor's diagnosis and presence of asthma symptoms and/or treatment for asthma during the preceding year, were included; those with use of ICS were excluded from the group with no asthma. As regards CRS, both self-reported doctor's diagnosis and questionnaire-assessed symptoms of CRS were taken into account; while for nasal polyposis, only self-reported doctor's diagnosis was taken into account. This was a multi-centre study encompassing many countries, and both treatment for asthma, levels of

FeNO and degree and type of sensitisation varied between countries and centre, making the groups with current asthma and perennial sensitisation somewhat heterogeneous. To address this limitation, adjustment for centre was made in the multivariate analyses.

Clinical implications and future perspectives

Nitric oxide in cystic fibrosis

The finding that nitric oxide is lower in both upper and lower airways in patients with CF than in healthy controls and patients with asthma has been described previously and was confirmed in Paper II. FeNO was lower in CF than in control groups in adults, and the relation between lower FeNO and lower lung function and higher blood neutrophil count could be an indicator that FeNO relates to the disease progression in CF. However, Study II, being cross-sectional, added no information on the relation between change in FeNO and the progression in CF lung disease and inflammation. Recent studies looking at CFTR modulator therapy in relation to FeNO have suggested that a FeNO increase might be associated with an increase in lung function upon CFTR modulator therapy. Therefore, FeNO might be useful as an additional monitoring tool of CFTR modulator therapies. New CFTR modulator therapies with indication for more CF patients, including those heterozygous for F508del, have recently been approved for use in Europe.

It would be interesting to perform longitudinal studies of if treatment with new CFTR modulators is related to change in FeNO and nNO, and if any such changes relates to changes in inflammation, lung function, upper airway symptoms and/or infections in the airways.

Further, the low levels of NO seen in patients with CF and the fact that NO has a role in the human innate immune defence give rise to the question if the low NO levels in CF contribute to infections in the airways of patients with CF. Treatment trials with arginine as well as low-dose NO inhalations have shown an increase in FeNO, but no change in infections or lung function. However, recent studies with high-dose NO inhalation have shown promising results on both bacterial load and lung function. Randomised controlled trials are warranted to verify these results and the safety of high-dose inhalation treatment with NO in patients with CF.

Nitric oxide within the concept of united airway disease

The concept of united airway disease within inflammatory airway diseases is widely accepted. It is important with assessment and treatment of upper airway disorder in asthma, as well as continuous screening for lower airway involvement in AR. Asthma control and asthma-related quality of life are influenced by concomitant upper airway disorders and therefore it is important to

ask about symptoms of rhinitis and rhinosinusitis in patients with asthma, especially in the assessment of difficult-to-treat asthma. A marker for assessment of upper airway inflammation would facilitate finding and monitoring concomitant upper airway disease in asthma. Even though nNO could, in theory, be a useful marker of type-2 inflammation in the upper airways, its usefulness is complicated due to factors influencing nNO in different directions, most importantly allergic sensitisation, CRSwNP and treatment with corticosteroids. The importance of taking sensitisation in to account when assessing nNO is highlighted by the diverging results on the relation between nNO and severity of rhinitis in atopic and non-atopic subjects with asthma, respectively. Patients with poor asthma control and those with anosmia had lower nNO, suggesting that low nNO might be a potential marker for CRS or nasal polyposis in patients with poor asthma control. However, these findings were not consistent at follow-up of the asthma patients in the MIDAS study.

The usefulness of nNO as a marker of upper airway inflammatory disorders in uncontrolled asthma should be investigated in a prospective study in patients with impaired asthma control, with simultaneous measurements of IgE sensitisation, nNO and FeNO in combination with objective evaluation of CRS and nasal polyps.

The findings of a relation between nNO and FeNO in patients with asthma, in both cross-sectional and longitudinal analyses, as well as between nNO and bronchial responsiveness, further supported the concept of united airway disease. The same can be said about the findings of an independent relation between FeNO and current rhinitis and rhinoconjunctivitis in non-asthmatic patients with perennial sensitisation. The studies was not designed to look at incidence of asthma or rhinitis development in relation to FeNO or nNO. The results can rather be looked upon as a proof of concept of “one airway one disease” and, a reminder to look beyond the organ under treatment.

Conclusions

Patients with CF had lower FeNO and much lower levels of nNO than subjects with asthma and healthy controls. In patients with CF, FeNO was associated with lung function and blood neutrophil counts, in that lower FeNO was related to greater lung function impairment and higher blood neutrophil counts.

In adolescents and young adults with asthma, there was both a cross-sectional and a longitudinal association between FeNO and nasal NO levels, and higher levels of nasal NO were associated with bronchial hyper-responsiveness. These findings were in line with the concept of united airway disease, i.e., that there is a relation between upper and lower airway disease and inflammation.

Sensitisation towards perennial airborne allergens was a predictor of nNO in children and young adults with asthma, and nNO was related to the degree of sensitisation, with higher nNO in relation to multiple perennial allergen sensitisations. Furthermore, perennial sensitisation was associated with an increase in nNO over time in subjects with asthma.

No firm conclusions could be drawn on the relations between nNO and symptoms of CRS and poor asthma control. There were diverging results between the larger cross-sectional baseline analysis, showing lower nNO in subjects with poor asthma control and in subjects with loss of smell, and the analysis of the smaller study population that participated at follow-up, when these relations could not be confirmed. In addition, no relation was found between changes in nNO and changes in asthma control or CRS symptoms.

Current rhinitis and rhinoconjunctivitis were related to higher FeNO in middle-aged adults at a population level, with a significant interaction with perennial sensitisation on the relation between current rhinitis and rhinoconjunctivitis and FeNO. In non-asthmatic middle-aged subjects with perennial sensitisation, presence of current rhinitis or rhinoconjunctivitis was independently associated with 12% or 16% higher FeNO, respectively.

Sammanfattning på svenska

Bakgrund

Vid lungsjukdomar och sjukdomar i övre luftvägarna är inflammation en viktig bidragande komponent. Vid den vanligaste formen av allergisk inflammation (typ 2-inflammation) ökar mängden kväveoxid (NO) i luftvägarna pga. ökad produktion och aktivering av det enzym (inducerbart kvävesyntetas) som ansvarar för produktionen av NO i kroppen. Kväveoxid diffunderar snabbt över cellernas membran och kan enkelt mätas i utandningsluft och aspirerad luft från övre luftvägarna, med icke-invasiva metoder. Fraktionerad utandat NO (FeNO) är en väletablerad markör för typ-2 inflammation i nedre luftvägarna. Individer med astma har högre FeNO än friska kontroller och en sänkning av FeNO ses vid behandling av astma med inhalationssteroider. Kopplingen mellan FeNO och typ-2 inflammation visar sig även i att FeNO är högre hos individer med allergisk sensibilisering mot luftburna allergener och vid allergisk rinit (inflammation av nässlemhinnan), även utan astma.

Kväveoxid kan även mätas icke-invasivt i övre luftvägarna. Den mest etablerade metoden för att mäta nasalt NO (nNO) är via transnasal aspiration (man suger ut luft ur ena näsborren, medan den andra hålls öppen) under stängning av mjuka gommen (t ex genom att man håller andan). Mätning av nNO används kliniskt vid screening och diagnostik av sjukdomen primär ciliär dyskinesi (PCD), där nivåerna av nNO är betydligt lägre än hos friska individer, men värdet av att mäta nNO vid allergi och övre luftvägssjukdomar är mer debatterat. Detta eftersom nNO påverkas av både typ-2 inflammation och av hur luften kan diffundera till näsan från bihålorna där NO finns i betydligt högre nivåer än i övriga luftvägar hos friska individer. Vid rinit och kronisk bihåleinflammation, med mer uttalade och långvariga besvär från näsa och bihålor, kan således nNO öka till följd av inflammation, men även minska om svullnaden i slemhinnorna är så uttalad att passagen från bihålorna till näsan minskar.

Inom astma och allergi används begreppet ”united airway disease” om samsjuklighet mellan astma och övre luftvägssjukdomar. Allergisk rinit är vanligt vid astma och är kopplad till svårare sjukdomsförlopp och sämre astmakontroll. Hos personer med allergisk rinit, men utan astma, ses inflammation och ökad reaktivitet i nedre luftvägarna och studier talar för att personer med allergisk rinit med ökad reaktivitet eller förhöjd inflammation i nedre luftvägarna (mätt genom FeNO) har en ökad risk att utveckla astma. Även kronisk

bihåleinflammation är vanligare vid astma och är, speciellt vid samtidig förekomst av nasala polyper, kopplad till svårare sjukdomsförlopp och sämre astmakontroll. Hos patienter med svår astma är nasala polyper relaterade till högre FeNO, vilket talar för en ökad typ 2-inflammation även i nedre luftvägarna vid samsjuklighet med nasala polyper och astma.

Cystisk fibros (CF) är en recessivt ärftlig sjukdom med mutationer på en gen som kodar för en kloridkanal (CFTR). När kloridkanalen inte fungerar som den ska blir sekretet i kroppens slembildande organ segt, och i luftvägarna leder det till ansamling av segt slem med upprepade och i förlängningen ofta kroniska infektioner och inflammation som följd. Denna inflammation är ofta dominerad av neutrofiler (de vita blodkroppar som vanligen är förhöjda vid bakteriella infektioner). Vid CF är nNO lägre och FeNO på samma nivå som eller lägre än hos friska kontroller, men kopplingen mellan nNO och FeNO och inflammation och lungfunktion hos patienter med CF är studerad i mindre utsträckning.

Syfte

Målet med avhandlingen var att utvärdera hur nNO, som en markör för typ 2-inflammation i övre luftvägarna, relaterade till symtom, behandling, inflammation och reaktivitet i nedre luftvägarna, samt till symtom och behandling från övre luftvägarna hos patienter med astma (Arbete I och III).

Ett annat syfte var att utvärdera om och hur nivåer av FeNO och nNO skiljde sig hos personer med cystisk fibros jämfört med friska personer och personer med astma och hur NO relaterade till lungfunktion, systemisk inflammation och karaktär av sjukdom vid CF (Arbete II).

Ett ytterligare syfte var att se hur FeNO, som markör för typ-2 inflammation i nedre luftvägarna, relaterade till sjuklighet och symtom från övre luftvägarna, och hur denna relation påverkas av IgE-sensibilisering och astma (Arbete IV).

Metod

Arbete I baserades på personer med astma ur MIDAS-studiens (Minimally-Invasive Diagnostics in Asthma and allergic diseaseS) första besök (2010–2012) och Arbete III på de personer med astma ur MIDAS-kohorten som deltog vid både baslinje- och uppföljningsbesök (2013–2015). I Arbete II har studiepopulationen varit 38 patienter med CF som följs vid Uppsala Centrum för Cystisk Fibros (i samband med en årsuppföljningsstudie av minimal-invasiva metoder för uppföljning av CF), med två ålders- och könsmatchade kontrollgrupper (en med astma, en med friska kontroller) som valts slumpmässigt bland dem som genomförde MIDAS-studiens baslinje besök. Arbete IV baserades på den andra uppföljning av European Community Respiratory Health Survey (ECRHS III), som innefattade 25 centra i 11 länder.

Alla arbeten hade lokal inflammation i luftvägarna, definierat utifrån NO-halt, som huvudutfall. Nasalt NO (nNO) mättes via trans-nasal aspiration från

vardera näsborren (Arbete I, II och III). FeNO-mätningar gjordes genom utandning mot motstånd och analyserades med kemiluminiscens i Arbete I, II och III. I Arbete IV gjordes FeNO-mätningar med elektrokemisk analysator.

I Arbete I analyserades 377 barn och unga vuxna med astma avseende nNO i relation till systemisk allergisk inflammation (mätt i form av B-eosinofiler, samt totalt och specifikt IgE mot luftburna allergener), inflammation (FeNO) och reaktivitet i nedre luftvägarna (provokationstest), symtom från övre luftvägarna, astmakontroll och astma- och rinitbehandling. Variablernas oberoende relationer till nNO analyserades genom en justerad linjär regressionsmodell.

I Arbete II analyserade 38 barn och vuxna med CF och 76 kontroller (38 med astma och 38 friska kontroller) avseende nNO and FeNO. Jämförelser av FeNO- och nNO-nivåer mellan grupperna gjordes. I CF-populationen analyserades om nNO och/eller FeNO relaterade till lungfunktion, systemisk inflammation (B-eosinofiler, B-leukocyter, B-neutrofiler och total IgE), eller CF-relaterade variabler (mutationstyp, pankreasinsufficiens, kronisk bakteriell infektion i lungorna, eller CF-relaterad diabetes). Eftersom studiedeltagarna i detta arbete var få användes här icke-parametriska statistiska tester.

I Arbete III studerades de personer ur Arbete I som hade ett uppföljande besök inom MIDAS-studien. Totalt 196 personer med astma inkluderades i Arbete III, där analyser gjordes av förändringen i nNO över tid och hur den förändringen relaterade till förändringar i inflammation, symptom och behandling. Multivariabla linjära regressionsmodeller användes för att utvärdera oberoende relationer till förändring i nNO över tid.

I Arbete IV studerades medelålders vuxna med aktiv astma och personer utan astma eller annan lungsjukdom. Totalt inkluderades 741 personer med aktiv astma och 4 155 personer utan astma. I arbetet studerades om FeNO relaterade till övre luftvägsbesvär i form av rinit, rinokonjunktivit, kronisk bihåleinflammation eller nasala polyper, efter justering för faktorer som är kända för att påverka FeNO. Studiepopulationen studerades dels uppdelat för astma och sensibilisering för perenna allergener, dels som helhet, med linjära regressionsmodeller. Modellerna kompletterades även med test för analyser av om astma och perenn sensibilisering påverkade relationen mellan FeNO och övre luftvägsbesvär.

Resultat

Arbete I: Hos ungdomar och unga vuxna med astma är nNO (som markör för inflammation i övre luftvägarna) relaterad till närvaro av IgE-antikroppar mot pälsdjur, inflammation i nedre luftvägarna (FeNO) och ökad reaktivitet i nedre luftvägarna. Vidare sågs en koppling mellan lågt nNO och förlust av luktsinne och bristande astmakontroll. Daglig medicinering med kortisonnässpray eller inhalationskortison var också kopplad till lägre nivåer av nNO.

Arbete II: Personer med CF hade ungefär hälften så höga nivåer av nNO som personer med astma och friska kontroller. De hade även betydligt lägre nivåer av FeNO än personer med astma och friska kontroller. Ingen skillnad sågs i FeNO- eller nNO-nivåer relaterat till mutationstyp, kronisk infektion i lungorna, underfunktion av bukspottkörteln, eller närvaro av diabetes. Däremot var lågt FeNO relaterat till lägre lungfunktion och till ökad mängd neutrofila vita blodkroppar i blodet hos personer med CF.

Arbete III: Vid uppföljning av barn och unga vuxna med astma 2–5 år efter Arbete I sågs att nNO ökade över tid och att ökningen var relaterad till närvaro av IgE-antikroppar mot perenna allergener, samt till en ökning av FeNO. De som hade slutat med daglig behandling med inhalationskortison hade en större ökning av nNO än de som hade stått kvar på daglig behandling, medan de som hade haft daglig behandling med kortisonnässpray vid båda tillfällen hade en mindre ökning av nNO än de som inte hade haft kortisonnässpray vid något av tillfällena.

Arbete IV: Hos vuxna sågs en relation mellan förekomst av symptom på rinit eller rinokonjunktivit (hösnuva) och mer inflammation i nedre luftvägarna (FeNO). Denna relation påverkades av samtidig förekomst av IgE-antikroppar mot perenna allergener. Vidare sågs att förekomst av nuvarande astma och förekomst av IgE-antikroppar mot perenna allergener var för sig relaterade till nivåer av FeNO. Hos personer med astma och förekomst av IgE-antikroppar mot perenna allergener sågs lägre nivåer av FeNO hos dem som stod på daglig behandling med kortisonnässpray än dem utan samma behandling och högre nivåer av FeNO hos dem som stod på daglig behandling med inhalationskortison än dem utan samma behandling.

Sammanfattningsvis är de huvudsakliga fynden en koppling mellan inflammation och symtom i övre och nedre luftvägarna till stöd för ”united airway”-konceptet, med en oberoende koppling mellan nNO och ökad reaktivitet och inflammation i nedre luftvägarna, samt FeNO och övre luftvägssymtom. Vidare sågs vissa belägg för en koppling mellan förändring av behandling med inhalationssteroider i lungorna och förändring i nivåer av inflammation i övre luftvägarna (nNO), samt, hos astmatiker med perenn sensibilisering, en koppling mellan behandling med nasala steroider och lägre inflammation i nedre luftvägarna (FeNO). Avhandlingen visade även att vuxna och barn med CF hade lägre nivåer av både nNO och FeNO än friska kontroller och personer med astma, och att lägre FeNO var relaterat till lägre lungfunktion och mer systemisk inflammation i form av högre nivåer av neutrofila vita blodkroppar i blodet hos personer med CF.

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