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**EXHALED NITRIC OXIDE;  
VARIABILITY AND ASSOCIATION WITH  
BRONCHIAL HYPERRESPONSIVENESS AND  
ATOPY**

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ACADEMIC DISSERTATION

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# 1 Abstract

Airway inflammation is a key feature of bronchial asthma. In asthma management, according to international guidelines, the gold standard is anti-inflammatory treatment. Currently, only conventional procedures (i.e., symptoms, use of rescue medication, PEF-variability, and lung function tests) were used to both diagnose and evaluate the results of treatment with anti-inflammatory drugs. New methods for evaluation of degree of airway inflammation are required.

Nitric oxide (NO) is a gas which is produced in the airways of healthy subjects and especially produced in asthmatic airways. Measurement of NO from the airways is possible, and NO can be measured from exhaled air. Fractional exhaled NO (FENO) is increased in asthma, and the highest concentrations are measured in asthmatic patients not treated with inhaled corticosteroids (ICS). Steroid-treated patients with asthma had levels of FENO similar to those of healthy controls. Atopic asthmatics had higher levels of FENO than did nonatopic asthmatics, indicating that level of atopy affected FENO level. Associations between FENO and bronchial hyperresponsiveness (BHR) occur in asthma.

The present study demonstrated that measurement of FENO had good reproducibility, and the FENO variability was reasonable both short- and long-term in both healthy subjects and patients with respiratory symptoms or asthma. We demonstrated the upper normal limit for healthy subjects, which was 12 ppb calculated from two different healthy study populations. We showed that patients with respiratory symptoms who did not fulfil the diagnostic criteria of asthma had FENO values significantly higher than in healthy subjects, but significantly lower than in asthma patients.

These findings suggest that BHR to histamine is a sensitive indicator of the effect of ICS and a valuable tool for adjustment of corticosteroid treatment in mild asthma. The findings further suggest that intermittent treatment periods of a few weeks' duration are insufficient to provide long-term control of BHR in patients with mild persistent asthma. Moreover, during the treatment with ICS changes in BHR and changes in FENO were associated. FENO level was associated with BHR measured by a direct (histamine challenge) or

indirect method (exercise challenge) in steroid-naïve symptomatic, non-smoking asthmatics. Although these associations could be found only in atopics, FENO level in nonatopic asthma was also increased.

It can thus be concluded that assessment of airway inflammation by measuring FENO can be useful for clinical purposes. The methodology of FENO measurements is now validated. Especially in those patients with respiratory symptoms who did not fulfil the diagnostic criteria of asthma, FENO measurement can aid in treatment decisions. Serial measurement of FENO during treatment with ICS can be a complementary or an alternative method for evaluation in patients with asthma.

## 2 Abbreviations

AMP	Adenoside monophosphate
ATS	American Thoracic Society
BHR	Bronchial hyperresponsiveness
BMI	Body mass index
CI	Confidence interval
CoV	Coefficient of variation
COPD	Chronic obstructive pulmonary disease
DD	Doubling dose
ECP	Eosinophilic cationic protein
EIB	Exercise-induced bronchoconstriction
ERS	European Respiratory Society
FENO	Exhaled nitric oxide
FP	Fluticasone propionate
FEV1	Forced expiratory volume in one second
FVC	Forced vital capacity
HDM	House dust mite
HIB	Histamine-induced bronchoconstriction
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MEF <sub>50</sub>	Maximal expiratory flow at 50% of FVC
NO	Nitric oxide
PC <sub>20</sub>	Provocative concentration of histamine causing a 20% fall in FEV1
PD <sub>15</sub> FEV <sub>1</sub>	Provocative dose of histamine causing a 15% fall in FEV1
PEF	Peak expiratory flow
ROC	Receiver operating characteristic
SPT	Skin prick test

### 3 List of original publications

This thesis is based on the following original communications, referred to in the text by their Roman numerals (I - V). In addition, some unpublished data are presented.

- I. Ekroos H, Tuominen J, Sovijärvi ARA. Exhaled nitric oxide and its long-term variation in healthy non-smoking subjects. *Clin Physiol* 2000;20(6): 434-439.
- II. Ekroos H, Karjalainen J, Sarna S, Laitinen LA, Sovijärvi ARA. Short-term variability of exhaled nitric oxide in young male patients with mild asthma and in healthy subjects. *Respir Med* 2002; 96(11): 895-900.
- III. Sovijärvi ARA, Haahtela T, Ekroos H, Lindqvist A, Saarinen A, Poussa T, Laitinen LA. Sustained reduction in bronchial hyperresponsiveness with inhaled fluticasone propionate within three days in mild asthma: time course after onset and cessation of treatment. *Thorax* 2003;58: 500-504.
- IV. Rouhos A, Ekroos H, Karjalainen J, Sarna S, Sovijärvi ARA. Exhaled nitric oxide and exercise-induced bronchoconstriction: association only in atopics. *Allergy* 2005; 60: 1493-1498.
- V. Ekroos H, Rouhos A, Pallasaho P, Karjalainen J, Sarna S, Sovijärvi ARA. Equally elevated concentrations of exhaled nitric oxide in nonatopic and low-sensitised atopic asthmatics. *Respiratory Medicine* 2008  
doi:10.1016/j.rmed.2008.03.021.

The publications are referred to in the text by their roman numerals. The original publications are reprinted with permission of the copyright holders.

## 4 Introduction

The word "asthma" is derived from the Greek, meaning "to pant heavily" or "gasp for breath". The term originally did not define a disease as we understand it today, but was employed to connote respiratory symptoms of a host of pulmonary and cardiac conditions. Over time, the meaning contracted and by the beginning of the last century, most authorities thought asthma to be a unique illness characterized by "spasmodic afflictions of the bronchial tubes" (Osler 1901). In 1922, Huber and colleagues examined the microscopic features of 15 reported deaths and added 6 cases of their own. Their work described the classic features of patients dying from asthma, including mucous impaction in the bronchi; thickening of the airway walls; hypertrophy of the smooth muscle; edema of the submucosa; and eosinophilic, lymphoid, and neutrophilic infiltration (Huber et al. 1922). They also undertook the first attempt at quantifying the extent of abnormalities present and correlating them with the type and severity of the patient's asthma. Dunnill (1960) added mucosal denudation and thickening of the basement membranes of the airways to the list. These features have been repeatedly demonstrated in the inflammatory reactions in the airways of patients who die of asthma (Dunnill 1969).

Asthma is a chronic inflammatory disorder which tends to increase, affecting about 6% people in Finland (Kotaniemi et al. 2001) and over 100 million people worldwide (Global Initiative for Asthma, NHLBI, 1995). Asthma produces an economic burden on health care, and the cost of medical treatment of asthma was 280 million marks in Finland in 1994. Clinical evidence that airway inflammation plays the major role in the development of asthma was presented more than 20 years ago (Laitinen et al. 1985, Bousquet et al. 1990). Airway inflammation is central to the development of asthma and underlies the clinical features of asthma, which are bronchial hyperresponsiveness and variable airway obstruction (Djukanovic et al. 1990). Knowledge of inflammatory mechanisms in asthma has accumulated recently, and new treatment modes for asthma have been developed in the past decade.

In 1993 it was first reported that levels of exhaled nitric oxide (FENO) were increased in bronchial asthma compared to those of healthy controls (Alving et al. 1993), and was demonstrated that patients with asthma treated with oral or inhaled steroids had levels of exhaled nitric oxide similar to those of healthy controls (Kharitonov et al. 1994). Exhaled nitric oxide might therefore be a new marker of airway inflammation (Barnes and Kharitonov 1996).

In the present series of investigations, the aim was to examine with two methodological studies the FENO levels in healthy subjects and the long-term variation and also short-term variability of FENO in healthy subjects and patients with suspected asthma. Secondly, the aim was to evaluate the short-term effects of inhaled fluticasone (FP) on FENO and bronchial hyperresponsiveness (BHR) in mild asthma. Furthermore, we examined the association between exhaled nitric oxide, exercise-induced bronchoconstriction (EIB), and bronchial hyperresponsiveness in patients with suspected asthma. In addition, we studied levels of FENO in patients with nonatopic asthma compared with those of healthy subjects and low- and high-sensitised atopic asthmatics.

## 5 Review of the literature

### 5.1 Nitric oxide (NO)

#### 5.1.1 What is nitric oxide?

NO is a gas under atmospheric conditions but soluble within cells and tissues. Its solubility and diffusion properties resemble closely those of oxygen. NO is chemically reactive, but for a radical it is relatively stable and it does not react with itself, and it has a physiological half-life of seconds to minutes depending on its concentration and immediate chemical environment (Wink et al. 1996).

#### 5.1.2 Production of NO

NO is synthesised universally from L-arginine and molecular oxygen by an enzymatic process that utilises electrons donated by NADPH. The NO synthase (NOS) enzymes convert L-arginine to NO and L-citrulline via intermediate N-hydroxy-L-arginine.

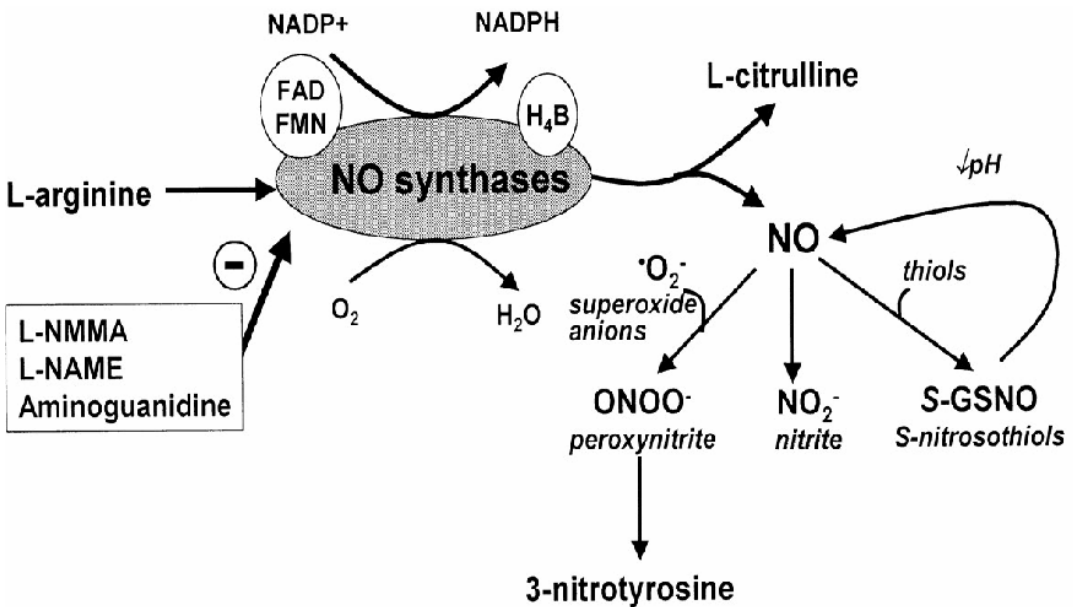


Figure 1. Synthesis of NO and NO-related products. Modified from Kharitonov and Barnes (2001).

There are three types of NOS. Two of these are constitutively expressed, while the other is expressed only in activated cells. One constitutive form was originally characterised in neurons and was therefore known as neuronal NOS (nNOS), while the other, originally characterised in endothelial cells, was known as endothelial NOS (eNOS). These two NOS isoforms have been renamed NOS-1 and NOS-3 (Nathan et al. 1994). The third type of

NOS is not expressed in resting cells, but is synthesised upon cell activation. This inducible form of NOS is known either iNOS or NOS-2 (Stuehr et al. 1997).

The constitutive forms of NOS (nNOS and eNOS) are activated in response to a calcium signal generated for example by the arrival of an action potential at a nerve ending, or activation of endothelial cell receptors by acetylcholine. Enzyme activation occurs rapidly and transiently, according to the kinetics of the calcium signal (Moncada et al. 1991). iNOS, on the other hand, is not activated by a calcium signal but is continuously active once expressed. Its expression is induced by several agents including cytokines such as interferon- $\gamma$ , interleukin-1 (IL-1), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Nathan et al. 1994).

At high concentrations, as produced by the inducible form of NOS and under aerobic conditions, NO is rapidly oxidised to reactive nitrogen oxide species (RNOS) with the generic formula  $\text{NO}_x$ . Under gaseous conditions, the RNOS formed are nitrogen dioxide ( $\text{NO}_2$ ), dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ), and dinitrogen tetroxide ( $\text{N}_2\text{O}_4$ ), but in aqueous solution, and in a biological system,  $\text{N}_2\text{O}_3$  is the major oxidative product (Wink et al. 1996). Under conditions of combined nitrosative and oxidative stress, when both NO and the superoxide anion ( $\text{O}_2^-$ ) are formed at high concentrations, these two radicals interact to generate the highly reactive oxidant peroxynitrite anion ( $\text{ONOO}^-$ ) (Figure 1). Peroxynitrite is thought to mediate many of the most severe toxic effects of NO (Wink et al. 1996).

### 5.1.3 NO in asthmatic inflammation

NO is generated at high levels during human inflammatory reactions such as asthma (Kharitonov et al. 1994) and, as in the immune response, the principal NOS isotype involved is iNOS (Figure 2) (Robbins et al. 1994). Higher iNOS expression has been reported in bronchial biopsies from patients with asthma than in healthy subjects (Hamid et al. 1993; Belvisi et al. 1995). Furthermore, iNOS expression can be reduced by corticosteroids (Saleh et al. 1998; Redington et al. 2001). Epithelial cells have been shown to express iNOS the most, but macrophages, eosinophils, and smooth muscle cells express iNOS as well (Saleh et al. 1998; Redington et al. 2001). Only iNOS expression was associated with FENO in respiratory epithelial cells obtained from children, suggesting that FENO variability is largely determined by epithelial iNOS expression with little contribution from other isoforms (Lane et al. 2004). Certainly the role of NO in inflammation considered to be uncertain. NO has toxic, regulatory, apoptotic and anti-apoptotic effects on different cell types at different stages of the inflammatory process.

In IgE-mediated inflammatory disease such as asthma, the activation of mast cells by antigens is the first event. Mast cells release mediators which cause classic inflammation but also cytokines, such as TNF- $\alpha$ , that may promote the later phases of inflammation by recruiting other inflammatory cell types. NO inhibits mast cell activation and mediates the inhibitory effects of IFN- $\gamma$  on mast cells in mixed cell populations. NO has been found to inhibit secretion of IL-2 and IFN- $\gamma$  in  $\text{Th}_1$ -lymphocytes. NO may also regulate the balance between  $\text{Th}_1$ - and  $\text{Th}_2$ -lymphocytes and may favour the  $\text{Th}_2$  response, which activates secretion of IL-4 and IL-5, causing more IgE production and eosinophil recruitment (Barnes and Liew 1995). Studies with knock-out mice and iNOS-inhibitors are so conflicting that clinical implications for human asthmatics are modest.

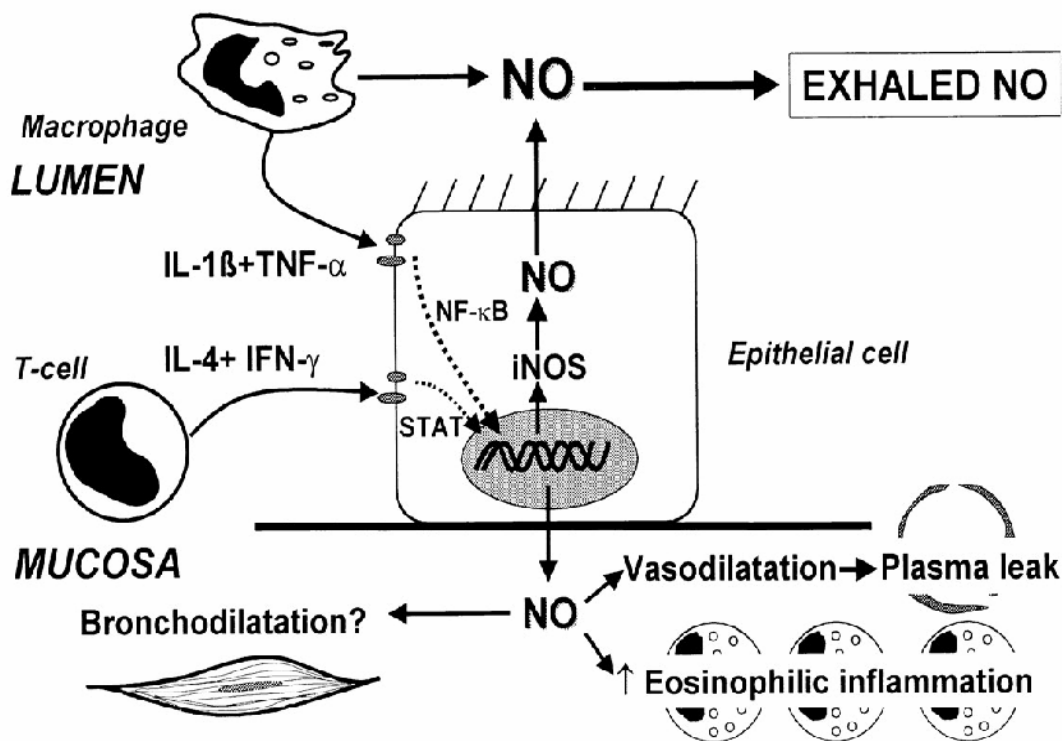


Figure 2. Sources of NO in exhaled air in asthma. Modified from Kharitonov and Barnes, 2001.

### 5.1.4 Measurement of exhaled nitric oxide

Gustaffsson and colleagues (1991) reported that exhaled NO is endogenously produced in rabbits, guinea-pigs, and humans. This pioneering study demonstrated that NO can be in the exhaled air of all three species, with no NO in inhaled air. They also demonstrated that when NOS inhibitors were used for test animals, the levels of NO were decreased and further administration of L-arginine raised NO levels back to normal.

In 1993 Alving and colleagues reported that exhaled NO is increased in asthma in humans. The fundamental studies, which were published simultaneously in *the Lancet* in 1994, finally demonstrated that measurement of exhaled NO can be used in diagnosis and treatment of asthma (Kharitonov et al. 1994; Persson et al. 1994). These studies confirmed that patients with steroid-naïve asthma had an increased level of exhaled NO compared to that of healthy controls. Moreover, patients with steroid-treated asthma had levels of exhaled NO similar to the levels of healthy subjects. Furthermore, smoking patients with asthma had lower levels of exhaled NO than did patients with steroid-naïve asthma. These findings showed that a non-invasive method to assess airways inflammation is possible, and for that reason methodological and clinical aspects of exhaled nitric oxide will be discussed.

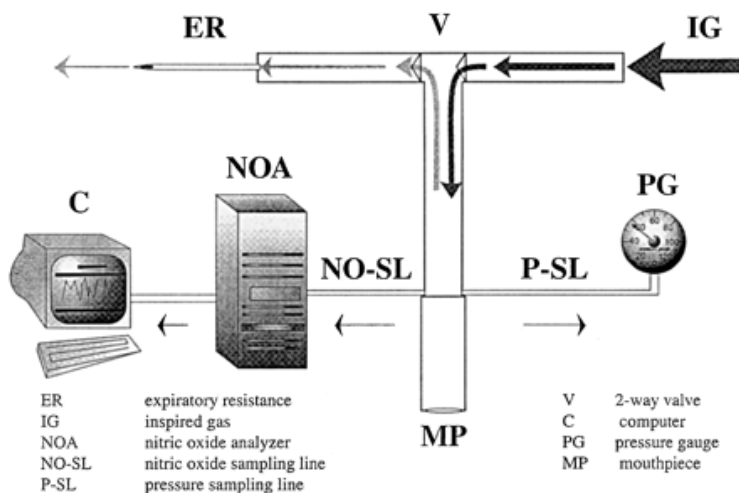


Figure 3. Diagram of one configuration for the breathing circuit employed in the restricted breath technique (Modified from ATS 1999).

#### 5.1.4.1 Chemiluminescence method

Exhaled NO is measured by the chemiluminescence method (Figure 3). It is based on the reaction between NO and the ozone ( $O_3$ ), which is generated from the ozone generator in the analyzer. NO and ozone form nitrogen dioxide ( $NO_2$ ), part of which is the excited form  $NO_2^*$ . When the excited form of  $NO_2$  resumes its stable form, light is emitted and can be quantified by a photomultiplier. The amount of light emitted is proportional to the amount of NO in gas collected from samples (Figure 4).

#### 5.1.4.2 Exhalation procedure

Earlier studies used tidal breathing or slow vital capacity manoeuvres. The method with tidal breathing is easy to perform, but the level of exhaled NO varies in the course of the breathing cycle; therefore, no constant level of FENO can be achieved. Breathholding was used to maximise the concentrations of FENO, which allowed nasal NO to mix with exhaled NO. Wearing a nose-clip during measurement keeps the soft palate from inadequately closing; nasal NO can contaminate the levels of FENO. The first studies used the peak-FENO measurement which starts with high peak-NO concentration (probably caused during breathholding) and after that, levels of FENO in the end-expiratory phase (= plateau) representing the endogenous level of FENO in the lower airways. Recently introduced standardised methods will be measured with a standardised expiratory flow rate without breathholding and without a nose-clip (Kharitonov et al. 1997; ATS 1999 and ATS/ERS Recommendation 2005).

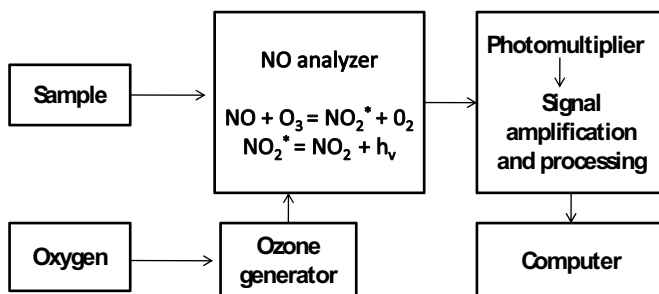


Figure 4 Schematic presentation of an ozone-chemiluminescence NO analyser

#### 5.1.4.3 Exhalation flow rate

Flow-dependency has the greatest effect on FENO. The level of FENO decreases with increasing flow rates and vice versa. This is partly explained by the fact that increased ventilation reduces the concentration of FENO in the bronchial tree. Moreover, the repeatability of FENO is dependent on expiratory flow, and low variation in expiratory flow reduces variation in FENO. With a controlled exhalation flow rate, the variability of FENO is low.

With a standardised expiratory flow rate there is no possibility of contamination of FENO from the nasal sinuses at slow exhalation flow rates. Therefore, during production of low positive mouth pressure with a resistor, the soft palate will be closed to prevent contamination from the nasal cavities (Figure 5). Devices to achieve mouth pressures from 5 to 20 cmH<sub>2</sub>O are recommended during FENO measurement see section 5.1.6.

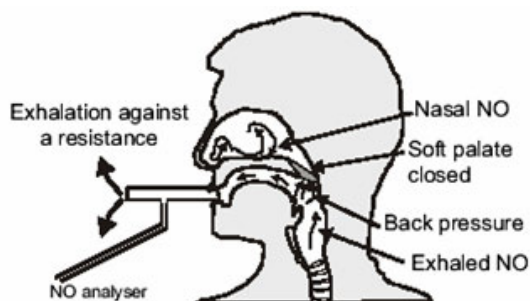


Figure 5. Schematic presentation of FENO procedure (exhalation against resistance produce positive mouth pressure which closes the soft palate). Modified from Kharitonov and Barnes (1998).

One application of FENO measurement is a two-compartment model of the lungs (Tsoukias and Georges 1998). Several clinically important findings were published with this method (Lehtimäki 2003).

#### 5.1.4.4 Peak exhaled nitric oxide

During the first years of FENO measurement, the procedure was not standardised. After breathholding with a noseclip, subjects exhaled with different flows. This procedure ensured that the maximum amount of NO is released from the airways, and this peak amount of NO was measured. Current knowledge confirms that previous methods of NO measurement were inadequate.

### 5.1.5 Factors affecting FENO level

Several factors affect the level of FENO in healthy subjects as well as in patients (Table 1).

#### 5.1.5.1 Diet

NO can be produced in the oral cavity by a non-enzymatic reduction from nitrite. The acid environment and bacteria in the oral cavity cause the formation of nitrate to nitrite and further to NO. Zetterquist and colleagues (1999) have shown that ingestion of a meal rich with nitrate prior to FENO measurement increases the level of FENO. When the mouth is rinsed with a basic solution or anti-bacterial solution, this increase in FENO can be partly eliminated. Caffeine and alcohol consumption have been shown to reduce the level of FENO (Yates et al. 1996; Persson et al. 1992), but a recent study by Taylor et al. (2004) showed that caffeine had no effect on FENO.

#### 5.1.5.2 Other factors

Repeated spirometry reduces the level of FENO (Silkoff et al. 1999; Deykin et al. 2000), and the short-acting beta-agonist after spirometry leads to an increased level of FENO (Yates et al. 1997). Physical exercise and sputum induction can reduce FENO level

(Phillips et al. 1996; Piacentini et al. 2000), if these procedures have been done before FENO measurement. Therefore, measurement of FENO should be done before lung function measurements (including measurement of bronchial hyperresponsiveness), and before any exercise test or sputum induction.

### 5.1.5.3 Smoking

Smoking reduces the level of FENO (Kharitonov et al. 1995; Robbins et al. 1996). High levels of NO occur in cigarette smoke and may affect endogenous NO production in smokers by downregulation of cNOS activity. Increased metabolic consumption of NO in smokers has also been postulated, which is a reaction of NO with superoxide, producing peroxynitrite. In any case, the level of FENO increases gradually during the 4 weeks after cessation of smoking (Högman et al. 2002).

**Table 1 Factors affecting exhaled nitric oxide (FENO) measurements in healthy subjects**

<b>Increased NO</b>	<b>Decreased NO</b>
<ul style="list-style-type: none"><li>• Upper respiratory tract infection</li><li>• Intake of L-arginine, ACE-inhibitors, papaverin, sodium nitroprusside</li><li>• Nitrite / Nitrate-enriched food</li><li>• Air pollution (ozone, NO)</li><li>• Occupational risks (chlorine dioxide, ozone, formaldehyde)</li></ul>	<ul style="list-style-type: none"><li>• NOS inhibitors</li><li>• Smoking (acute and passive)</li><li>• Alcohol ingestion</li><li>• Caffeine</li><li>• Mid-point of the menstrual cycle</li><li>• Repeated spirometry</li><li>• Physical exercise</li><li>• Sputum induction</li><li>• 100% inhaled oxygen</li><li>• Moderate altitude</li></ul>

### 5.1.6 International guidelines of FENO measurement

During the development of measurement of FENO, several factors affecting FENO have been found. The most important is flow-dependency (Silkoff et al. 1997), and an effect from exhalation manoeuvres and positive mouth pressure has also been detected. Because the validity of FENO must be evaluated, international guidelines have therefore been published to standardise measurement technique, the European Respiratory Society (ERS) published the first guidelines of FENO measurement in 1997 (Kharitonov et al. 1997). In these guidelines, several steps in FENO measurement have been standardised

to minimise the variation in FENO and bring closer the conflicting findings of different researchers. ERS guidelines suggest an exhalation flow range of between 0.167 mL/s and 250 mL/s (10–15 L/min).

Guidelines from the American Thoracic Society (ATS 1999) recommend that the exhalation flow should be a constant 0.050 mL/s. Variation in plateau should be less than or equal to 10% or 1 ppb and less than or equal to 5% of variation between the three measurements. Mouth rinsing with a basic solution is recommended in ATS guidelines. Recent guidelines from ATS/ERS combine the best elements from previous guidelines (ATS 2005).

A new hand-held device (NIOX MINO) for the measurement of FENO has been developed with a different assay for measuring FENO levels in exhaled air. A good correlation appeared between the NIOX MINO and standard FENO analysers (Khalili et al. 2007).

## **5.2 Bronchial hyperresponsiveness (BHR)**

### **5.2.1 What is BHR?**

BHR is currently defined as an increase in sensitivity to a wide variety of airway-narrowing stimuli. Most patients with asthma and chronic obstructive pulmonary disease (COPD) exhibit such an enhanced sensitivity. In asthma, in particular, this hypersensitivity is accompanied by excessive degrees of airway narrowing. Bronchial hyperresponsiveness is a composite functional disorder which requires treatment of each of its components (Sterk and Bel 1989). BHR is a fundamental abnormality in asthma, representing both structural and inflammatory changes in the airways due to the disease process.

### **5.2.2 BHR and genetics**

The association between BHR and genetics is a matter of conflict. The 5q region has been studied with respect to phenotypes such as asthma and BHR (Postma et al. 1995) in differing populations. Recent meta-analysis used a rank-based genome-scan meta-analysis (GSMA) to combine linkage data for asthma and related traits: BHR, allergen-positive skin prick test (SPT), and IgE in nine Caucasian asthma populations. They found significant evidence that susceptibility loci could be identified for quantitative traits including BHR 2p12-q22.1, 6p22.3-p21.1 and 11q24.1-qter; allergen SPT 3p22.1-q22.1 and 17p12-q24.3; and total IgE 5q11.2-q14.3 and 6pter-p22.3. Analysis of the asthma phenotype did not identify any region showing genome-wide significance. This study represents the first linkage meta-analysis to determine the relative contribution of chromosomal regions to risk for developing asthma and atopy. Several significant results were obtained for quantitative traits but not for asthma, confirming the greater numbers of phenotypes and greater genetic heterogeneity in asthma. These analyses support the contribution of regions that contain previously identified asthma susceptibility genes and provide the first evidence for susceptibility loci on 5q11.2-q14.3 and 11q24.1-qter (Denham et al. 2008).

### 5.2.3 Methods for evaluation of BHR

Both direct and indirect challenge tests can be used for evaluation of asthma, as in the standard definition: "The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli".

### 5.2.4 Direct challenges

There are several methods to evaluate BHR in direct challenges. Acetylcholine, metacholine, carbachol, histamine, prostaglandin D<sub>2</sub>, and leukotriene C<sub>4</sub> have been used for both clinical and research purposes. The most often used direct challenges (histamine, methacholine provocation tests) can cause a marked bronchoconstriction in asthmatics but not in healthy subjects. Both tests are pharmacological stimuli that cause bronchoconstriction by directly activating contraction of bronchial smooth muscle cells after binding to cholinergic receptors or histamine receptors. The physiological basis of this increased contractility remains fundamentally unresolved. Increased BHR to histamine is more prominent in chronic asthma when compared to newly detected asthma (Sovijärvi et al 1993). Recently, use of BHR as an additional guide to long-term treatment in clinical control of asthma has shown that more effective control of asthma can be achieved (Sont et al 1999).

### 5.2.5 Indirect challenges

During indirect challenges, stimuli cause airflow limitation by an action on cells other than the effector cells; these cells then interact a second time with the effector cells. Cells that act as an intermediary between the indirect stimuli and effector cells are inflammatory cells (such as mast cells) and neuronal cells. Different challenges have been used in both clinical and experimental studies: adenosine, tachykinins (substance A and neurokinin A), bradykinin, metabisulphite, mannitol, propranolol, cold air ventilation, exercise, hypertonic saline, and isocapnic hyperventilation (van Schoor et al. 2000).

Exercise testing is a basic measurement for study of children and young adults with suspicion of asthma. Exercise-induced asthma (EIB) is a phenomenon existing only in asthma. There is no evidence of EIB after exercise in healthy subjects; on the contrary healthy subjects and patients with COPD exhibit bronchodilation. The severity of EIB is clearly relevant to the severity of asthma in children and young adults. EIB is a short-term response to exercise, and spontaneous recovery is usually complete within an hour; no evidence for a late asthmatic reaction after exercise was found in 404 young men with asthma (Karjalainen 1991). In sum, evidence is sufficient to suggest that the release of constrictor mediators: histamine, prostaglandins, and leukotrienes is an important contributor to the bronchoconstriction induced by exercise and hyperventilation. The relative contribution of these mediators has not been determined, and it is likely that among individuals with EIB, the relative actions of these mediators vary. Presumably, airway cooling and drying during exercise constitutes the stimuli for mediator release. There may even be a direct role for exercise as a stimulus (Gilbert et al. 1993) for mediator release, although this has been little studied. In addition, bronchodilating mechanisms may play a more significant role in modifying the action of these constricting mediators than previously thought. Agents such as mast cell-produced heparin and PGE<sub>2</sub>, atrial natriuretic

peptide, kinins, substance P, vasoactive intestinal peptide, plus other vasoactive agents may also play a modifying role in EIB, although much more investigation is required before specific roles can be assigned.

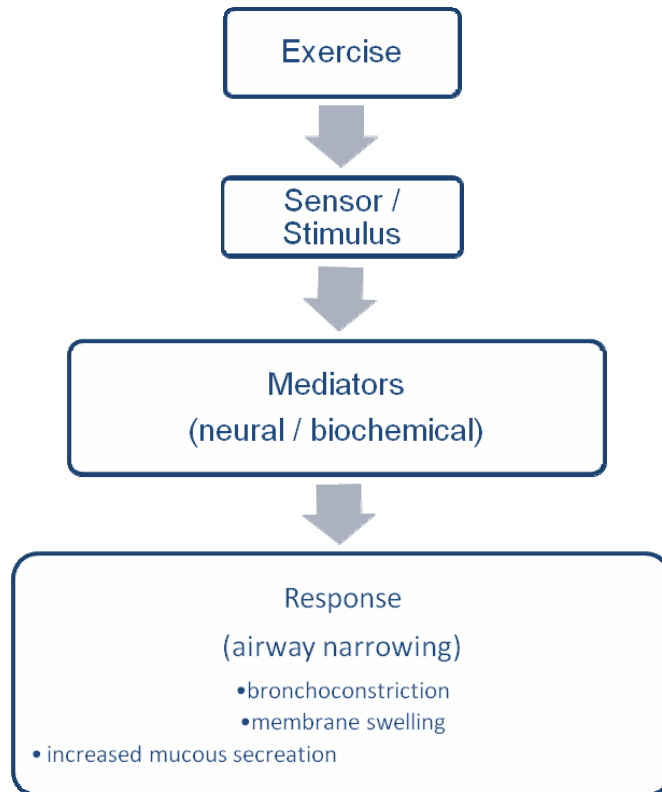


Figure 6. Schematic of potential sites for mechanisms of exercised-induced bronchospasm. Modified from Gotshall (2002).

Whereas exercise may provide multiple stimuli for EIB, hyperpnoea is the dominant stimulus inducing EIB (Figure 6). Cooling and drying, and possibly rewarming, affect airways, resulting in local multiple inflammatory mediator release of which prostaglandins and especially leukotrienes and histamine are important. Airway narrowing occurs post-exercise as mediators, possibly along with rewarming, cause bronchoconstriction, vascular engorgement and leakage, and increased mucus production (Figure 6). The airways narrow progressively post-exercise, peaking from 3 to 10 minutes typically. The obstruction dissipates over time, resolving in 30 to 60 minutes. This is followed by a refractory period of up to 3 hours that is dependent on prostaglandins that protect the airways from subsequent periods of exercise (Virant 1992).

Osmotic challenges with mannitol (Brannan et al. 1998) have been used as alternative surrogate tests to identify EIB in individuals with clinically recognized asthma. Relative to exercise and eucapnic voluntary hyperventilation, the osmotic challenges require less

complex and expensive equipment. The mannitol challenge, in particular, has the potential to be used in field, clinic, and laboratory environments to identify EIB in elite athletes. The response to mannitol fell within the normal range in asymptomatic subjects with BHR to methacholine and may be a more specific test for diagnosing asthma (Porsbjerg et al. 2007). BHR to mannitol also seems to be a more sensitive marker than BHR to metacholine in asthma patients not under treatment with steroids (Koskela et al. 2003), and in their study, BHR to mannitol reflected the degree of airway inflammation more closely than did BHR to methacholine. Furthermore, mannitol is more sensitive than cold air in demonstrating BHR in patients with mild or atypical asthma, and if specific cut-off values are used, sensitivity values of mannitol and histamine challenges were comparable (Koskela et al. 2003).

Mannitol challenge is both a sensitive and a valid test to demonstrate the effects of ICS in asthma. Histamine challenge is equally sensitive for this purpose, but its validity may be lower. Cold air challenge seems to be a valid test to demonstrate the effects of ICS, but its sensitivity may be lower than that of mannitol and histamine challenges (Koskela et al. 2003). Moreover, mannitol is a convenient challenge which is easy to administer and well-tolerated by children. It is a highly reproducible test of BHR in children with moderate to severe persistent asthma who are on inhaled corticosteroids for 7 days under laboratory conditions (Barber et al. 2003)

A study by Berkman and colleagues (2005) compared exercise, metacholine, and AMP as diagnostic tools for asthma and found that the ROC curves were comparable; furthermore, they concluded that measurement of FENO can be used as a safe, simple, and rapid test for the diagnosis of asthma and is as good as bronchial provocation tests.

## **5.3 Asthma**

### **5.3.1 Definition of asthma**

The International Consensus Report on the Diagnosis and Treatment of Asthma (1992) defined asthma as follows: "Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils and T-lymphocytes. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough particularly at night and / or in the early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneous or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli". Of course, asthma has more heterogeneity than in the definition of the Consensus Report. No single feature or group of features is common to all asthmatics (Howell 1995).

For research purposes, asthma is defined by the criteria of the Global Initiative for Asthma, NHLBI (2007): "An increased BHR to metacholine or histamine (specific cutoff values depend on method used), a PEF variability across 24 hours (amplitude percent mean) of 20 percent or more, and an increase in FEV1 of 15 percent or more from baseline with an inhaled short-acting beta<sub>2</sub>-agonist. In children and young adults the exercise test with a 15 percent fall in PEF or FEV1 from baseline has found to be diagnostic". For clinical purposes, asthma is defined by its components or a combination of these: obstruction

measured with FEV1 or PEF, bronchial hyperresponsiveness, increased level of eosinophils or ECP in induced sputum, atopy measured by total or specific serum IgE or a skin prick test.

### **5.3.2 Atopy**

Atopy is a personal or familial tendency to produce IgE antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis (Johansson et al. 2001). Furthermore, neither a positive skin prick test nor the presence of IgE antibody per se can be a criterion for atopy; such patients should be referred to as "skin prick test-positive" and "IgE sensitised", respectively. In this work, atopy is defined on the basis of a skin prick test.

### **5.3.3 Pathophysiology and pathogenesis of asthma**

#### **5.3.3.1 Methods for evaluation of inflammation**

One of the earliest studies of bronchial structure by bronchial biopsies was done by Laitinen et al. (1985). Performance of bronchoalveolar lavage (BAL) (Jarjour et al 1998), segmental allergen challenge (Makker et al. 1993) or bronchial brushing (Gibson et al 1993, Vignola 1998) will also reveal more about inflammatory processes in the airways. Recently, the use of induced sputum has been confirmed as a research and clinical tool for studying inflammation in asthma and COPD (Ryttilä 2003).

#### **5.3.3.2 Inflammatory cells**

An increased number of eosinophils has been found in asthma (Laitinen et al 1992; 1993). Eosinophils are the prominent cells in the airway epithelium of patients with symptomatic asthma (Bousquet 1990, Laitinen 1992; 1993). The clinical severity of asthma and the number of eosinophils in biopsies are correlated significantly (Bousquet et al 1990, Laitinen et al 1991). Activation of the eosinophils makes them produce their basic proteins such as ECP, eosinophil peroxidase (EPO), and major basic protein (MBP) (Carroll et al 1992). These proteins, especially ECP and EPO, are cytotoxic, and their levels in induced sputum correlate with the severity of asthma and the other markers of asthma (Ryttilä 2002). Eosinophils produce many cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3, -5 and -6 (IL-3, IL-5 and IL-6), and tumour necrosis factor- $\alpha$  and - $\beta$  (TNF- $\alpha$  and TNF- $\beta$ ) (Barnes 1994). Eosinophils also produce potent mediators such as cysteinyl leukotrienes (LTC4, LTD4 and LTE4) and platelet-activating factor (PAF) (Rodger et al 1997).

Higher numbers of metachromatic mast cells are present in the airways of patients with asthma than in healthy subjects (Laitinen et al. 1993). Mast cells produce active mediators such as histamine, tryptase, PAF, and prostaglandin D2 (PGD2) all of which are bronchospastic agents in asthma (Wasserman, 1994). Furthermore, an association between the severity of asthma and the level of histamine and tryptase in the BAL fluid has

been documented (Bousquet et al. 1991). Recently, evidence is increasing of the important role of mast cells in asthma. Activation of mast cells is one possible mechanism in EIB (O'Sullivan et al. 1998). An increased number of mast cells within the smooth muscle layer was evident in a group of mainly atopic asthmatics as compared to mast cells in those with eosinophilic bronchitis or in healthy controls (Brightling et al. 2002). Furthermore, mast cells accumulate in the smooth muscle compartment more in atopic than in nonatopic asthma (Amin et al. 2005).

T-lymphocytes are also involved in the airways of patients with asthma. Activated T-lymphocytes with increased expression of the cell surface markers occur in the airways. T-lymphocytes are usually of CD4 type, and the numbers of CD4-type T-lymphocytes are correlated with asthma severity (Corrigan et al 1995). Th2-lymphocytes are the major type of T-lymphocytes in asthma (Corrigan and Kay, 1992). Th2-lymphocytes produce mainly IL-3, IL-4, IL-5, and IL-10, and the products of cytokine by Th1-lymphocytes are interferon- $\gamma$  (IFN- $\gamma$ ), IL-2 and GM-CSF (Chang et al 1990).

The role of macrophages in the pathogenesis of asthma is still controversial. An increased number of macrophages expressed as cells/ml have appeared in induced sputum (Keatings et al 1997) and in the airway inflammatory infiltrate (Poston et al 1992, Laitinen et al 1993) from patients with asthma and COPD than in healthy subjects. In contrast, macrophages were not higher in patients with atopic asthma than in healthy subjects (Jeffery 1996). Alveolar macrophages capable of producing several cytokines, including IL-1, IL-6, IL-8, GM-CSF, TNF- $\alpha$  and IFN- $\gamma$  (Kelley, 1990). Potent bronchoconstrictors such as cysteinyl leukotrienes, prostaglandins, and leukotriene B4 (LTB4) are also produced by macrophages (Lee, 1992).

Prominent neutrophilic airway inflammation is related to severe persistent asthma (Jatakanon et al 1999). Fatal asthma of sudden onset has been demonstrated with prominent neutrophilic inflammation in the airway mucosa (Sur et al 1993) and in sputum (Fahy et al 1995). Neutrophils are the major cell type in patients with severe asthma who are receiving oral corticosteroids (Wenzel et al 1997). Neutrophil numbers and activation are also increased in the airways of subjects with noninfectious status asthmaticus (Lamblin et al 1998), and neutrophilic inflammation is also evident in COPD. The number of neutrophils and the neutrophil granule proteins myeloperoxidase (MPO) and human neutrophil lipochalin (HNL) are higher in COPD than in patients with asthma, numbers which differed significantly from those of healthy subjects (Keatings et al 1997). Jatakanon and colleagues (1999) found higher levels of neutrophils, IL-8, and MPO in patients with severe asthma receiving oral steroids than in healthy subjects and in patients with mild steroid-naïve asthma. Neutrophilic airway inflammation is one possible reason for steroid resistance in severe asthma and in COPD (Keatings et al 1997).

### 5.3.3.3 Pathogenesis

In atopic persons, genetic and environmental factors can launch the initiation of atopic inflammation, and then the major cell type will be Th-2 lymphocytes. Cytokines released by Th-2 lymphocytes activate several types of inflammatory cells and encourage inflammatory cells to migrate to the bronchus epithelium. Histopathological studies have shown that all structures of the bronchi are involved (Haley et al 1998). Bronchial inflammation is involved even in mild asthma (Laitinen et al 1993) and presents in both large and small airways. These inflammatory processes cause reversible airway limitation and symptoms.

The clinical severity of the disease correlates with bronchial inflammation (Bousquet et al 1990).

A major feature of asthma is epithelial shedding (Laitinen et al 1985, Jeffery et al 1989, Laitinen et al 1993). Damage to the epithelium may produce edema in the airways and further narrowing of the airway calibre. Airway smooth muscle hyperplasia and hypertrophy are regarded as classic histopathologic features of asthma. Remodelling processes of the airways are irreversible changes which are characteristics of activated inflammatory cells in the airways and changes in the bronchial extracellular matrix with thickening of the subepithelial basement membrane (Bousquet et al 1995).

#### **5.3.4 Treatment of asthma**

Inhaled corticosteroids are the "gold standard" in asthma treatment independent of asthma severity. Short-acting beta-agonists as needed should be prescribed for every patient with asthma. Combination therapy with ICS and long-acting beta-agonists should be considered in patients with moderate to severe persistent asthma (GINA 2007). A classification of asthma based on severity is of importance when decisions have to be made about asthma management (GINA, 2007). Assessment of asthma based on clinical or symptom indices of disease severity has been shown to relate to pathological indices of airway inflammation (Bousquet et al 1990). Both the level of airflow limitation and its variability enable asthma to be subdivided by severity into intermittent, mild persistent, moderate persistent, and severe persistent (GINA 2007). These descriptions of asthma severity have been useful because asthma therapy follows a stepwise approach in which the level of therapy is increased as the severity of the asthma increases, but recent guidelines recommend that asthma treatment is based on asthma control (Figure 7).

Step 1	Step 2	Step 3	Step 4	Step 5
Asthma education Environmental control				
As needed rapid-acting $\beta_2$ -agonist	As needed rapid-acting $\beta_2$ -agonist			
Controller options	Select one	Select one	Add one or more	Add one or both
	Low-dose inhaled ICS*	Low-dose ICS plus long-acting $\beta_2$ -agonist	Medium- or high-dose ICS plus long-acting $\beta_2$ -agonist	Oral glucocorticosteroid (lowest dose)
	Leukotriene modifier U	Medium- or high-dose ICS	Leukotriene modifier	Anti-IgE treatment
		Low-dose ICS plus leukotriene modifier	Sustained release theophylline	
		Low-dose ICS plus sustained release theophylline		

\* ICS=inhaled glucocorticosteroids  
U=Receptor antagonist or synthesis inhibitors

Figure 7. Stepwise treatment protocol of asthma according GINA guidelines

## 5.4 BHR and asthma therapy

Treatment with inhaled corticosteroids reduces BHR markedly, and in parallel, it improves asthma symptoms (Juniper et al 1990). Furthermore, treatment with inhaled corticosteroids improves the structure of the damaged airway epithelium (Laitinen et al 1992) and reduces the infiltration of inflammatory cells into the bronchial mucosa, a process which may be linked to the reduction in BHR (Boulet et al 2000). These findings support the view that BHR and inflammation may be associated.

Short-term effect (rapid effect) has been evident with budesonide; a single dose of 2400  $\mu\text{g}$  of budesonide has produced a 2.2-fold improvement in BHR to hypertonic saline with a simultaneous significant reduction in sputum eosinophils (Gibson et al 2001). A meta-analysis by van Grunsven and colleagues (1999) in steroid-naive asthmatics concluded that 1000  $\mu\text{g}$  budesonide or the equivalent reduced BHR on average by 1.16 doubling doses compared with placebo within 2 to 8 weeks of treatment, and no clear relationship was found between the dose of inhaled steroid and the decrease in BHR. Van Rensen and colleagues (1999) observed that a higher dose of FP (1000  $\mu\text{g}$ ) for 4 weeks reduced BHR by 1.82 doubling doses compared with placebo.

Lindqvist and colleagues (2003) examined the effects of 16 weeks of treatment with FP 250  $\mu\text{g}$  twice daily of BHR to histamine in 80 patients with newly diagnosed asthma and found that PD15FEV1 to histamine increased by 5.2 doubling dose units. A study by Hofstra and colleagues (2000) demonstrated that the protection afforded by inhaled FP against BHR to methacholine is time- and dose-dependent, whereas protection against

EIB is not, which suggests different modes of action of inhaled steroids in protecting against these pharmacological and physiological stimuli.

Results of the long-term effect of ICS on BHR are controversial. Ward and colleagues (2002) studied BHR to methacholine before and after treatment with high-dose inhaled FP 750 µg twice daily, and showed that BHR improved throughout the study year. Some adult patients with asthma whose BHR is normalised by ICS therapy can achieve remission from disease exacerbation after discontinuation of ICSs. However, patients with severe asthma or asthma of long duration may not achieve remission even if their BHR is normalised (Tsurikisawa et al. 2008).

## **5.5 FENO and asthma**

### **5.5.1 FENO and asthma diagnostics**

FENO has been shown to discriminate between patients with asthma and from patients with chronic cough. The sensitivity and specificity of FENO for detecting asthma were 75% and 87%, respectively. The positive and negative predictive values were 60% and 93%. The conclusion of that study was that FENO may play a role in the evaluation of chronic cough. In that group of patients, low FENO suggested little likelihood of asthma. The patients with chronic cough not attributable to asthma showed a low FENO value as compared with that of healthy volunteers and asthmatics (Chatkin et al. 1999). One study among children showed FENO to be superior to baseline respiratory function and bronchodilator responsiveness measured with impulse oscillometry in identifying preschool children with probable asthma (Malmberg et al. 2003).

Conventional tests (spirometry and PEF), FENO, and sputum eosinophils were compared in asthma diagnostics. Sensitivities for each of the conventional tests (0-47%) were lower than for FENO (88%) and sputum eosinophils (86%). Overall, the diagnostic accuracy with FENO and sputum eosinophils was significantly greater. FENO measurements and induced sputum analysis are superior to conventional approaches, with exhaled nitric oxide being most advantageous because the test is quick and easy to perform (Smith et al. 2004). Another study to identify the sensitivity and specificity of FENO in asthma diagnostics showed a specificity for the diagnosis of asthma of 90% and a positive predictive value of more than 90%. These findings suggest that the simple and absolutely non-invasive measurement of exhaled NO can be used as an additional diagnostic tool for the screening of patients with a suspected diagnosis of asthma (Dupont et al. 2003).

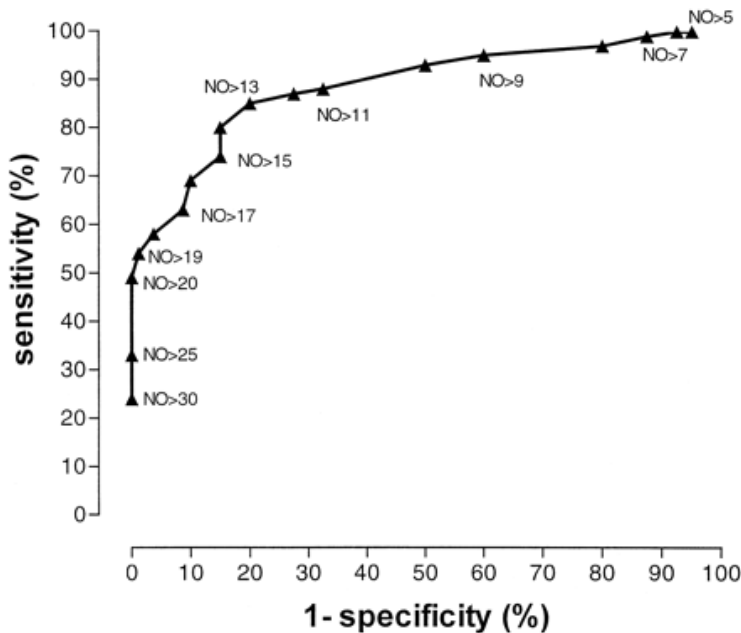


Figure 8. ROC curve for the measurement of exhaled NO in the diagnosis of asthma. Data labels feature different cutoff points of exhaled NO levels. Modified from Dupont et al. (2003)

Receiver operating characteristic (ROC) curves for the diagnosis of asthma indicate that FENO is a robust discriminator between individuals with asthma and healthy subjects and data of that study indicate that the choice of expiratory flow rate and collection method can be based on practicality and patient comfort without compromising the utility of this test for asthma (Deykin et al. 2002).

### 5.5.2 FENO and conventional assessment of asthma

In adult asthma there was no correlation between FENO and values from flow-volume spirometry. Only in children with stable asthma was a positive correlation found between FENO and percentage change in FEV1 and percentage change in FEF25-75%. A negative correlation appeared between prebronchodilator FEV1 and the level of FENO (Colon-Semidey et al. 2000). Exhaled NO correlated positively with PEF diurnal variability, but neither with symptom scores nor beta-agonist use; furthermore, the lack of correlation between symptom score and beta-agonist use, FEV1 % of predicted and FENO suggests that these measures are reflective of differing aspects of asthma (al-Ali et al. 1998). Furthermore, the level of FENO does not correlate with asthmatic symptoms, use of rescue medication (ie, short-acting beta-agonist), and variation in PEF (Lim et al. 2000). It seems that the association between FENO and conventional assessment is poor.

### 5.5.3 FENO and asthma and atopy

Patients with atopic asthma have higher levels of FENO than do patients with nonatopic asthma (Ludviksdottir et al. 1999), and patients with atopic asthma or rhinitis show higher levels of FENO than do nonatopic patients with asthma or rhinitis (Gratziou et al. 1999). In the same study, no difference emerged in FENO levels between atopic and nonatopic healthy subjects. When asymptomatic, non-smoking healthy subjects have been studied, the levels of FENO are higher in atopics than in nonatopics (Horvart et al. 1999). Moreover, the FENO level correlates with the number of positive prick tests and total IgE level (Ho et al. 2000). Moody and colleagues (2000) have found that the levels of FENO are increased in asymptomatic, non-smoking healthy subjects sensitised only to house dust mite (HDM) among Pacific islanders, indicating the subclinical allergic inflammation.

The type of sensitisation is important. Atopic subjects monosensitised to HDM, independent of having asthma or not, had increased levels of FENO (Barreto et al. 2001). Sensitisation to perennial allergens in asthma produced increased levels of FENO compared with that of asthmatic patients sensitised to seasonal allergens or with nonatopic asthma (Olin et al. 2004). Furthermore, levels of FENO are significantly higher in patients with asthma who are both sensitised and exposed to a relevant allergen than in those who are sensitised but not exposed, and FENO may be a marker of the airway inflammation induced by domestic exposure to an allergen in sensitised patients with asthma (Simpson et al. 1999). Sensitisation to perennial allergens (i.e., cat and house dust mite) has been shown to lead to increased levels of FENO in asthma (Langley et al. 2003). In atopic children, raised FENO levels are associated with sensitisation to perennial allergens, but not to seasonal allergens such as grass pollen. In this population, an increase in FENO is associated with BHR and current wheezing, suggesting that FENO is more than just a marker for atopy (Leuppi et al. 2002).

During an allergen challenge test, the levels of FENO have not increased during the early phase of the asthmatic reaction, but during the late phase both FEV<sub>1</sub> and the FENO level have changed significantly (Kharitonov et al. 1995). Moreover, an increase in FENO levels is correlated with decrease in FEV<sub>1</sub> during the late phase. The baseline level of FENO has been found to correlate with the magnitude of the late fall in FEV<sub>1</sub> following allergen challenge (Deyklin et al. 1998). The levels of FENO were increased during the low-dose allergen exposure in asthma when asymptomatic worsening of airway inflammation (i.e., increase of sputum eosinophils) occurred (de Kluijver et al. 2002). Gratziou and colleagues (2001) have shown that FENO is significantly elevated in patients with seasonal allergic rhinitis with and without symptoms, and the levels of FENO are dependent upon whether patient had bronchial hyperresponsiveness.

In a study by Lopuhää et al (2003) the increase in FENO after bronchial allergen challenge in non-asthmatic rhinitis, in particular in those patients with a dual asthmatic response, significantly exceeded the increase in asthma, resulting in similar levels of FENO after challenge. After allergen exposure the difference in FENO between non-asthmatic rhinitis and asthma at baseline is abolished, due to a significantly greater increase in FENO in non-asthmatic rhinitis. Recently, the levels of FENO were found to decrease significantly with specific immunotherapy for HDM after 4 months of therapy (Hung et al. 2004).

In summary, both level and type of sensitisation to allergens are important concerning atopy and FENO.

#### **5.5.4 FENO and asthmatic inflammation**

Blood eosinophils correlate with the level of FENO both in adults (Salome et al. 1999) and in children (Silvestri et al. 1999; Strunk et al. 2003; Franklin et al. 2003) with asthma. In children with asthma, only in atopic subjects did a significant correlation between the level of FENO and blood eosinophils appear (Franklin et al. 2003; Steerenberg et al. 2003). Finally, FENO level has been found to associate with blood eosinophils and the increase in FEV1 after a bronchodilatation test in atopic children with asthma (Silvestri et al. 2003).

The number of eosinophils in sputum and the level of FENO correlate both in adults (Jatakanon et al. 1998) and in children (Piacentini et al. 1999; Mattes et al. 1999) with asthma. Furthermore, the level of FENO correlates with sputum ECP in children with asthma (Mattes et al. 1999). FENO levels have correlated with blood eosinophils within both ICS-untreated and ICS-treated groups with asthma, but with sputum eosinophils only in ICS-untreated subjects (Reid et al. 2003).

Because FENO correlates closely with percentage of eosinophils in BAL fluid in asthmatic children, it is therefore likely to serve as a useful non-invasive marker of peripheral airway inflammation (Warke et al. 2002). The level of eosinophils is significantly correlated with FENO after treatment with inhaled budesonide in mild asthma (Lim et al. 1999). Blood eosinophil cell counts and FENO levels correlate significantly with the quantity of tissue eosinophils in patients with clinical remission of atopic asthma (van der Thoon et al. 2001). In contrast, levels of FENO did not correlate with eosinophils from bronchus biopsies in a study by Lim and colleagues (2000), involving both steroid-naïve and steroid-treated asthmatics. Otherwise, a strong and significant correlation between FENO and mucosal eosinophilic inflammation appeared in children with difficult asthma, following treatment with prednisolone (Payne et al. 2001).

It can thus be concluded that FENO and eosinophilic airway inflammation have a moderate association.

#### **5.5.5 FENO and BHR**

BHR measured by direct methods (i.e., histamine and methacholine) correlates with the level of FENO. In steroid-naïve patients with asthma, FENO level has correlated significantly with the PC20 to histamine (Dupont et al. 1998). In a study by de Gouw and colleagues (1998) rhinovirus infection has raised FENO levels in asthmatics, an increase inversely associated with worsening of airway hyperresponsiveness to histamine. The correlation between FENO and PC20 to methacholine suggests that FENO or the mechanisms leading to its increase may contribute to airway hyperresponsiveness in asthma (Jatakanon et al. 1998). In atopic subjects with asthma, the level of FENO has been significantly correlated with the dose-response slope for methacholine, while no such correlation appeared in the nonatopic group (Ludviksdottir et al. 1999). Similarly, BHR and the number of blood eosinophils both have been positively associated with FENO levels in atopic but not in non-atopic children (Leuppi et al. 2002). No correlation appeared between FENO and either of two provocative concentrations of methacholine or AMP causing a 20% fall in FEV1 in patient with allergic rhinitis without asthma (Prieto et al. 2002). These

findings suggest that BHR, which is a common phenomenon in asthma, associated with the level of FENO in asthma.

Indirect methods (i.e., exercise, AMP, and mannitol) to measure BHR were associated with the level of FENO. A positive correlation between the maximal percent decrease in FEV1 after exercise and the baseline FENO value has been found in asthmatic children (Terada et al. 2001). Baseline FENO values correlate with the magnitude of postexercise bronchoconstriction in children with asthma, suggesting that FENO may be a predictor of airway hyperresponsiveness to exercise (Scollo et al. 2000). None of the subjects with very low pre-exercise FENO levels (< 12 ppb) demonstrated bronchial hyperresponsiveness to exercise, and FENO measurement may obviate the need for bronchoprovocation testing in patients who complain of exertional dyspnea (El Halawani et al. 2003). However, in clinically well-controlled asthmatics taking inhaled corticosteroids, no relationship emerged between markers of airway inflammation (such as exhaled nitric oxide and sputum eosinophils) and airway responsiveness to either direct (histamine) or indirect (mannitol) challenge. Airway hyperresponsiveness in clinically well-controlled asthmatics thus appears to be independent of eosinophilic airway inflammation (Leuppi et al. 2001).

A significant correlation could be established between FENO and responsiveness to AMP, but not between FENO and responsiveness to methacholine in patients with clinical remission of atopic asthma (van den Toorn et al. 2000). Moreover, despite a significant correlation between FENO and PC20 AMP values, no correlation was detected between FENO and PC20 to methacholine in patients with asthma monosensitised to Parieta pollen (Prieto et al. 2002). Nonatopic subjects with nasal polyposis also have shown higher concentrations of FENO than do healthy control subjects, and inhaled AMP has caused airway narrowing in a significantly higher proportion of nonasthmatic subjects with nasal polyposis than in healthy controls (Prieto et al. 2004).

The most recent study by Porsbjerg et al. (2008) found that both BHR to mannitol as well as to methacholine was associated with elevated markers of airway inflammation: In over 80% of asthma patients with BHR to mannitol or to methacholine, the FENO level was >20 ppb. Furthermore, BHR to mannitol was more closely associated with the percentage of sputum eosinophils. In addition, there was a stronger correlation between BHR to mannitol and the level of FENO compared with BHR to methacholine, indicating that BHR to mannitol reflected the degree of airway inflammation more closely than does BHR to methacholine in steroid-naïve asthmatics.

## **5.5.6 FENO and asthma therapy**

### **5.5.6.1 Corticosteroids**

Early studies dealing with FENO have shown that the level of FENO in steroid-treated asthma is similar to that in healthy controls (Kharitonov et al. 1994). The level of FENO has also decreased when the dose of inhaled steroids is increased, and a phenomenon associated with a reduction in diurnal variability of PEF, and in nocturnal symptoms (Kharitonov et al. 1996). Dose-dependent speed of onset of action of budesonide and its cessation has occurred in FENO and asthma symptoms in mild asthma (Kharitonov et al. 2002).

FENO levels are higher in subjects with difficult asthma than in normal controls, but lower than were levels in steroid-naive mild asthmatics (Stirling et al. 1998). Prednisolone-treated patients have had higher FENO levels than did patients requiring only inhaled corticosteroids, suggesting greater disease severity in the former group. This indicates that FENO may serve as a useful complement to lung function and symptomatology in the assessment of patients with chronic severe asthma, and in the control and rationalisation of steroid therapy in these patients (Stirling et al. 1998).

Furthermore, FENO has been increased in a placebo-treated group after antigen exposure; in contrast, treatment with inhaled flunisolide has prevented such an increase in FENO in allergic asthmatic children re-exposed to allergens (Piacentini et al. 2000).

### 5.5.6.2 Other anti-inflammatory treatments

Montelukast, a leukotriene antagonist, has been shown to reduce FENO levels in mild asthma, an effect that is evident as early as 1 day following start of treatment and persisting for  $\approx$  1 week following treatment cessation (Sandrini et al. 2003). Moreover, montelukast reduces bronchial hyperreactivity in response to exercise and reduces exhaled nitric oxide levels but has little effect on bronchial responsiveness to methacholine and adenosine challenges (Berkman et al. 2003). Furthermore, after montelukast treatment there occurs a reduction in FENO in asthmatic children receiving maintenance therapy with inhaled corticosteroids. This suggests an anti-inflammatory effect of montelukast additive to that of inhaled corticosteroids (Ghiro et al. 2002). The combination of FP plus montelukast was superior to FP/SM for FENO and PC20 to AMP but was inferior for lung function. Thus, in patients taking FP/SM or FP, montelukast conferred complimentary effects on surrogate inflammatory markers, effects dissociated from lung function (Currie et al. 2003). However, some studies show no significant effect on FENO by montelukast therapy in asthmatic adults (Kanniess et al. 2002) or children (Strauch et al. 2003). Furthermore, both fexofenadine and montelukast significantly suppressed the levels of exhaled nitric oxide, while only montelukast significantly reduced the peripheral blood eosinophil count compared to placebo (Lee et al. 2004).

In a preliminary study based on FENO measurements, treatment with omalizumab, an IgE antibody, may have inhibited airway inflammation during steroid reduction in children with allergic asthma; the degree of inhibition of FENO was similar to that seen for inhaled corticosteroids alone, suggesting an anti-inflammatory action for this novel therapeutic agent in asthma (Silkoff et al. 2004).

In children with mild-to-moderate asthma no differences have been noted between nedocromil and placebo (Covar et al. 2003), and a study of stable asthmatic children showed that budesonide, but not nedocromil sodium, significantly reduces FENO even in the absence of changes in the lung function (Carra et al. 2001). Gratziou and colleagues have shown that FENO is significantly elevated in patients with seasonal allergic rhinitis with and without symptoms, and these increased FENO levels can be modulated only by inhaled steroids given as anti-inflammatory treatment without any effect on inhaled nedocromil (Gratziou et al. 2001).

### 5.5.6.3 Short- and long-acting beta<sub>2</sub>-agonists

Salbutamol may raise FENO levels in asthmatics taking inhaled glucocorticosteroids. Single high-dose salbutamol has not raised FENO levels in asthmatics not taking inhaled glucocorticosteroids, but regular use of salmeterol resulted in no change in FENO, either used alone or in combination with inhaled glucocorticosteroids (Yates et al. 1997). Nor was there any significant difference in another study between the levels of FENO before and after inhalation of salbutamol (Colon-Simedei et al. 2000). In asthmatic subjects, salbutamol has caused a significant increase in FENO for one hour as compared with placebo inhaler. These results suggest that a beta<sub>2</sub>-agonist may perturb FENO values and leads to the recommendation that studies control for that factor (Silkoff et al. 1999).

Healthy children show no statistically significant differences in FENO values before and after inhalation of albuterol, but in children with asthma, FENO values have increased significantly from pretreatment and postbronchodilator levels when the effect of spirometry and albuterol was studied, suggesting that FENO values should be obtained consistently either pre- and at a specific time post-albuterol treatment or spirometry (Kissoon et al. 2002). Neither montelukast nor salmeterol has affected FENO levels in asthma control when given as second-line therapy, but montelukast has produced significant effects on AMP challenge, suggesting anti-inflammatory activity (Wilson et al. 2001).

### 5.5.6.4 Combination therapy

Combination inhalers improve pulmonary function without potentiating anti-inflammatory effects on exhaled NO and serum ECP as compared with ICS alone (Lee et al. 2003). Double the dose of FP alone relative to FP+salmeterol has conferred superior effects on FENO but not on lung function (Currie et al. 2003). The levels of FENO and sputum ECP showed significant reductions, compared to those of placebo, with formoterol plus budesonide or budesonide alone but not with formoterol alone (Aziz et al. 2000).

## 5.5.7 FENO and asthma control

Assessment of FENO after an oral steroid course in patients receiving regular inhaled steroid have shown that the levels of FENO correlated with the percentage improvement in FEV<sub>1</sub> from baseline to the post-steroid, post-bronchodilator value with a FENO level of >10 ppb at baseline, having a positive predictive value of 83% for an improvement in FEV<sub>1</sub> (Little et al. 2000)

The usefulness of FENO for diagnosing and predicting loss of control (LOC) was demonstrated in asthma following steroid withdrawal. When comparisons were made against sputum eosinophils and BHR to hypertonic saline, correlations were highly significant between the changes in FENO and symptoms, FEV<sub>1</sub>, sputum eosinophils, and saline PD<sub>15</sub>. Both single measurements and changes in FENO had positive predictive values that ranged from 80 to 90% for predicting and diagnosing LOC. These values were similar to those obtained using sputum eosinophils and saline PD<sub>15</sub> measurements. Jones and colleagues (2001) conclude that FENO measurements are as useful as induced sputum analysis and BHR in assessing airway inflammation.

Recent results suggest that FENO measurements may be useful for monitoring asthma patients (Meyts et al. 2003). They found significantly different FENO levels between a group of paediatric asthma patients with insufficient and good/sufficient control, as defined by clinical assessment (the frequency of use of beta<sub>2</sub>-agonists, occurrence of day- and night-time asthma symptoms, and spirometry results). Recently, a similar result has been shown by Delgado-Corcocan and colleagues (2004), evaluating whether FENO levels are reflective of asthma severity in childhood asthma and determining the usefulness of FENO using the single-breath exhalation technique for monitoring asthma control and compliance with steroid treatment. FENO was significantly different in the mild, moderate, and severe asthma categories, and levels of FENO correlated significantly with asthma severity, compliance, and control.

The clinical utility of routine monitoring of FENO lies in determining its ability to predict future asthma exacerbations compared with other standard clinical measures of spirometry, peak flows, quality of life score, medication usage, and symptoms. Those with an exacerbation had a higher level of FENO than did those who did not. A nominal logistic regression model to determine those variables that predict asthma exacerbation found that FENO was the only significant predictor (Harkins et al. 2004).

A recent study in children with asthma showed that one year of steroid titration on FENO did not result in higher steroid doses and did improve airway hyperresponsiveness and inflammation (Pijnenburg et al. 2005). Furthermore, in asymptomatic asthmatic children, FENO may be an objective predictor of asthma relapse 2 and 4 weeks after discontinuation of steroids (Pijnenburg et al. 2005). More recently, a study by Smith and colleagues (2005) demonstrated that with the use of FENO measurements, maintenance doses of inhaled corticosteroids may be significantly reduced without compromising asthma control. These findings support the role of FENO as an additional tool in asthma monitoring.

## **6 Aims of the study**

The aims of the study were to evaluate:

1. exhaled nitric oxide levels in healthy subjects and their long-term variation.
2. short-term variability of exhaled nitric oxide in patients with suspected asthma compared to healthy subjects.
3. short-term effects of inhaled fluticasone on exhaled nitric oxide and bronchial hyperresponsiveness in mild asthma.
4. the association between exhaled nitric oxide, exercise-induced bronchoconstriction, and bronchial hyperresponsiveness in patients with suspected asthma.
5. levels of exhaled nitric oxide and bronchial hyperresponsiveness in patients with nonatopic asthma compared with healthy nonatopic subjects and low- and high-sensitised atopic asthmatics.

## 7 Methods

### 7.1 Study populations and designs

Three hundred and seventy-five subjects participated in the studies, the local ethics committee had approved the studies. The main characteristics of the study population are shown in Table 2.

Table 2 Subject characteristics in Studies I - V

Study	Group	N	Age	Males	FEV1	Atopy	PD15FEV1	EIB
			mean (range)	N	mean (% pred)	N	mean (SD)	% fall in PEF
I	Healthy	29	28.6 (21-40)	16	97	-	-	-
II	Healthy	13	27.4 (19-42)	8	95	0	-	-
II	Respiratory symptoms	21	19.7 (19-21)	21	95	13		4.7
II	Asthma	10	19.5 (19-20)	10	93	8		15.9
III	Asthma	26	36.5 (21-59)	11	81.8 (8.2)	19	0.18	-
IV	Asthmatic symptoms	181	20 (18-26)	181	94.8 (10)	128	1.5 (1.2)	10.2
V	Healthy	10	19.8 (18-22)	10	102.4 (13.8)	0	3.1 (0.28)	-
V	Asthma	85	20.3 (18-27)	85	93 (9.2)	71	1.00 (1.08)	16.2 (12.7)

#### 7.1.1 Long-term variability in FENO in healthy subjects (Study I)

The study population consisted of 29 healthy subjects from the Department of Medicine at Helsinki University Central Hospital where subjects participated in the phase I study with inhaled insulin. Only perfectly healthy subjects were included. Medical history, physical examination, clinical laboratory tests (including haematology, clinical chemistry, urinalysis; and human chorionic gonadotropin for female subjects), chest x-ray, and ECG were studied. The pulmonary function test included flow-volume spirometry and a diffusion capacity test. FENO was performed on the subjects to eliminate any inflammatory effect of inhaled insulin. The baseline levels of FENO before inhalation of insulin were used and time intervals between FENO measurements were selected according to the criteria from the phase I study.

The main inclusion criteria were BMI < 30, FEV1 > 80% predicted, normal chest x-ray. The main exclusion criteria were history of asthma, allergic rhinitis or atopic eczema, use of any medication < 7 days before entering the study, and blood donation less than 2 months before entering the study. None of the subjects had had a respiratory-tract infection within the 4 weeks before entering the study.

In baseline screening laboratory tests, FENO, spirometry, and diffusing capacity were measured on the same day in that order. If subjects met the criteria, the 7-day or 23-day variability or both were measured. After 7 days  $\pm$  1 day, the next FENO measurement was done. After 23 days  $\pm$  2 days the final FENO measurement was done. Baseline and all other FENO measurements were measured at the same time point (between 7-8 AM).

### **7.1.2 Short-term variability of FENO (Study II)**

Thirty-one non-smoking consecutive patients with asthmatic respiratory symptoms were recruited to the study; all had a high suspicion of asthma. Only short-acting beta-agonists were allowed, and the last dose was taken at least 12 h before the FENO and lung function measurements. Asthma was confirmed in ten patients. Diagnosis of asthma was assessed by the following criteria:  $\geq$  15% reversibility of peak expiratory flow (PEF) or of FEV1 in bronchodilatation test, or  $\geq$  20% spontaneous daily variation in PEF compared to the mean value of the day, or  $\geq$  13% fall in PEF or FEV1 after a standardised exercise challenge. The 13 healthy subjects were selected from among hospital employees or healthy conscripts. They had never smoked and had normal flow-volume spirometry. None had had any respiratory-tract infection within the 3 weeks before entering the study.

All patients were studied with flow-volume spirometry and a skin prick-test in the first study (day 1). On day 2 was an exercise challenge. The baseline determination of FENO was obtained at 7 to 8 a.m. on the third study day (day 3). FENO determinations were repeated at 10 min, 6 h and 24 h after the baseline. A histamine challenge test was performed immediately after the last FENO determination on day 4. The healthy subjects were studied on two different days. On the first day baseline determination of FENO was performed at 7 to 8 a.m., followed by repeated determination of FENO 10 min, 6 h and 24 h thereafter. Flow-volume spirometry was performed for all healthy subjects after the last FENO determination.

### **7.1.3 Short-term effect of inhaled fluticasone on BHR (Study III) and peak FENO**

The study population comprised of 26 adult patients with mild asthma. Diagnosis of asthma was based on ATS criteria (ATS 1991), and the main inclusion criteria were PD15FEV1 to histamine of 0.6 mg or less during the run-in period and FEV1 at baseline at least 65% of predicted.

The exclusion criteria were seasonal or unstable asthma, respiratory tract infection or exacerbation of asthma during the 4 weeks before entry into the study, current smoking or cessation of smoking within the year preceding the study, history of any pulmonary disease other than asthma, use of inhaled or oral steroids, inhaled chromones, or

leukotriene antagonists during the 2 months before the study, use of antihistamines within 2 weeks or long-acting beta<sub>2</sub> agonists within 4 weeks before entry into the study, pregnancy or breast feeding, any severe chronic disease, or alcohol or drug abuse.

The study design was randomised, placebo-controlled, double-blind, and parallel-group. During the run-in period of 1 to 2 weeks, the patients continued to take their usual asthma medication and recorded daily the asthma symptom score (ranging in daytime between 0 and 5 and at night between 0 and 4), use of salbutamol (Ventolin MDI, 100 µg, Glaxo Wellcome, Germany) and morning and evening PEF values (best of three) using a mini-Wright peak flow meter (Clement Clarke International, Essex, UK). During the 6-week treatment period, the patients inhaled either FP 250 µg MDI (Glaxo Wellcome) or placebo twice daily at 12-hour intervals by means of a spacer (Volumatic, Glaxo Wellcome). The patients were allowed to use inhaled salbutamol as needed but not within 12 hours before measurements of PD<sub>15</sub>FEV<sub>1</sub> and FEV<sub>1</sub>. No other asthma drugs were permitted. A spirometric test, a bronchial challenge with inhaled histamine, and peak FENO were performed at baseline, at 6, 12, 24, and 72 hours, and at 2, 4, and 6 weeks after the first dose of the treatment regimen. After cessation of treatment (after the last dose) the measurements were repeated at 48 hours and at 1 and 2 weeks (follow-up). The study drugs were administered immediately after the measurements of FEV<sub>1</sub>, BHR, and peak FENO at baseline and at 12, 24, and 72 hours during the treatment period. During the last treatment week and the second week after treatment cessation, the patients recorded daily their symptoms and PEF as during the run-in period.

#### **7.1.4 Association between FENO and BHR in asthma (Study IV)**

We studied 181 young male conscripts (18-26 years) referred to the Central Military Hospital during 1998 to 2002 because of suspected asthma. Their symptoms included dyspnea, wheezing, cough, sputum production, or symptoms related to exercise, all during the previous month. They were consecutively included in the study providing that they were nonsmokers, had used no inhaled or oral steroids, chromones, or leukotriene receptor antagonists during the preceding 2 months, and had no evidence of respiratory infections within the preceding 3 weeks. For safety reasons, all subjects had to have forced expiratory volume in one second (FEV<sub>1</sub>) > 70% of the predicted value. Use of inhaled short-acting beta-agonists was allowed not less than 12 hours before lung function and FENO measurements.

On the first study day, flow-volume spirometry, a bronchodilatation test, and skin prick tests were carried out. On the second day, the exercise challenge was performed. FENO was measured at 7 to 8 a.m. on the third study day. The histamine challenge was performed immediately after FENO measurement.

#### **7.1.5 Equally elevated concentrations of exhaled nitric oxide in nonatopic and low-sensitised atopic asthmatics (Study V)**

The 85 study subjects had been referred to the Central Military Hospital by military physicians from various areas in Finland because of respiratory symptoms including dyspnoea, wheezing, cough, sputum production, or symptoms related to exercise during the 1 month prior to the study. They were aged 18 to 27 years, with no history of childhood asthma, and were recently diagnosed as having asthma according to American Thoracic

Society (ATS) criteria: 12% reversibility in FEV1 in spirometry, 15% reversibility in PEF after bronchodilator, or 20% daily variability in PEF. In Finland, all conscripts are exposed to much outdoor exercise in the forest and thus have a similar exposure to allergens (same kind of accommodation, no animal exposure). Conscripts were consecutively included in the study if they were lifelong non-smokers, had used no inhaled or oral steroids or chromones during the preceding 2 months, and had no evidence of respiratory infections within the preceding 3 weeks. Inclusion criteria for FEV1 was >70% of predicted. Use of inhaled short-acting beta-agonists was allowed not less than 12 hours before the FENO and lung function measurements. In addition, 10 non-smoking, nonatopic healthy conscripts, who were hospitalised for non-respiratory reasons, volunteered to become our control group.

On the first study day, flow-volume spirometry, a bronchodilatation test, and skin prick tests were carried out, and on the second day, an exercise challenge was performed. FENO was measured at 7 to 8 a.m. on the third study day. The histamine challenge test was performed immediately after the FENO determination. No study measurements were performed during the pollen season.

## **7.2 Clinical methods**

### **7.2.1 Exhaled nitric oxide measurement**

FENO was measured with a chemiluminescence analyser (Sievers 270B, Boulder, CO, USA). Expiratory airflow and exhaled volume were measured with a pneumotachograph simultaneously with FENO in real time, an exhalation procedure performed according to European guidelines for exhaled NO measurement (18). Before the measurement, patients rinsed their mouths with sodium bicarbonate solution to eliminate any nitric oxide (NO) eventually produced in the mouth. For measurements of end-expiratory NO, subjects inhaled 100% oxygen (Aga Oy, Riihimäki, Finland) and then, without nose-clips, exhaled slowly from total lung capacity over a period of 15 seconds. They exhaled against a flow resistor (Hans Rudolph, Model #7100R, 100 cmH<sub>2</sub>O/L/s, flow range 0-0.5 L/s) to close the soft palate, thus avoiding any nasal NO contamination. Exhalation flow in the flow window was between 0.08 and 0.15 L/s, and the mean individual exhalation flow ranged between 0.09 and 0.12 L/s. The mean value was recorded for a 3-second period from the end-exhaled NO plateau. For at least three successive measurements, the mean value was recorded for analysis. The acceptable coefficient of variation of a single measurement was less than 0.15.

### **7.2.2 Peak-FENO measurement (Unpublished data)**

The concentration of nitric oxide in exhaled air was measured with a chemiluminescence analyser (Sievers 270B). Expiratory air flow and exhaled volume were measured in real-time with a pneumotachograph. The sampling rate through the reaction chamber of the analyser was 250ml/min for all measurements. The airflow and NO signals were fed

through AD and amplifier units to a microcomputer. A software programme for the purpose monitored in real time the signals in time domain.

The analyser was calibrated daily using NO-free oxygen to set absolute zero and then a certified concentration of nitric oxide in nitrogen of 184 ppb (AGA Edelgas, Germany). For the NO measurements, subjects inhaled pure oxygen and then exhaled from total lung capacity after breath holding of 10 seconds with exhalation flow 0.7 to 1.2 l/s. Peak FENO levels were analysed. Results of the analyses were computed and graphically displayed. At least three successive recordings were made. The mean values of measurements were used as the result.

### **7.2.3 Flow-volume spirometry**

FEV<sub>1</sub>, FVC, and the FEV<sub>1</sub>/FVC ratio were determined with a flow-volume spirometer (Medikro M904, Kuopio, Finland) according to the European Respiratory Society guidelines (ERS, 1993), with Finnish reference values for spirometry (Viljanen et al. 1982).

### **7.2.4 Pulmonary diffusing capacity**

The pulmonary carbon monoxide diffusing capacity (DLCO) was measured by the single breath technique according to the European Respiratory Society guidelines (Cotes et al. 1993). The DLCO was expressed as an absolute value in mmol/min per kPa and as a percentage of the predicted value. The ratio DLCO to alveolar volume (DL / VA) was also calculated. Measurement was done with the subject in a sitting position wearing a nose clip. Finnish reference values were adapted (Viljanen 1982).

### **7.2.5 Histamine challenge**

Bronchial challenge tests were performed by a dosimetric method with controlled tidal breathing. An automatic inhalation synchronised jet nebuliser (Spira Elektro 2, Respiratory Care Center, Hämeenlinna, Finland) was used for administration of buffered histamine diphosphate in a four-step non-cumulative dosage scheme (0.025, 0.1, 0.4, and 1.6 mg). In cases where FEV<sub>1</sub> did not decrease from baseline by 15% or more after the highest dose, an extra dose of 3.2 mg was given (Study III). If FEV<sub>1</sub> did not decrease at least 10% after the maximum dose of 1.6 mg of histamine, a PD<sub>15</sub>FEV<sub>1</sub> of 3.2 mg served as the final result (Studies II, IV-V). The histamine response was measured by use of FEV<sub>1</sub> determinations with a wedge spirometer (Vitalograph PF 2, Vitalograph, UK). When FEV<sub>1</sub> decreased from baseline by 15% or more after any dose, further administration of histamine was discontinued. After the last histamine dose, patients inhaled two puffs of a short-acting beta<sub>2</sub> agonist, salbutamol 200 µg (Ventoline, GlaxoSmithKline, UK) using a Volumatic spacer to resolve bronchoconstriction. PD<sub>15</sub>FEV<sub>1</sub> values were calculated from logarithmically transformed histamine doses by linear interpolation.

### **7.2.6 Exercise test**

A standardised 8-minute running test (Karjalainen et al. 1991) was performed outdoors on a 150-meter circular track between 9 and 11 AM. Running speed was adjusted by

monitoring each subject's heart rate with a Sport Tester™ PE 3000 heart rate meter (Polar Electro Ky, Kempele, Finland). Subjects raised their heart rate to 170 beats /min, 85% of the predicted maximal rate, during a 2-minute warm-up, and maintained this rate (165-175 beats /min) for the remaining 6 minutes of the exercise. PEF values were measured just before the exercise and immediately and 5, 10, 20, and 30 minutes after the exercise. Of the three successive PEF measurements obtained on each occasion, the highest value was recorded for analysis.

The response to exercise challenge was maximum percentage fall in PEF ( $\Delta$ PEF%) after exercise:

$$\% \text{ fall in PEF} = (\text{PEF (baseline)} - \text{PEF (after)}) / (\text{PEF (baseline)} \times 100)$$

Because the temperature of ventilated air can affect EIB, and the exercise challenge was conducted outdoors in temperatures ranging from  $-17.5$  to  $+21.4^{\circ}\text{C}$  (mean  $6.5^{\circ}\text{C}$ ), for all exercise challenges we adjusted the  $\Delta$ PEF% according to temperature ( $6.5^{\circ}\text{C}$ ). In an earlier study, the regression coefficient between air temperature and  $\Delta$ PEF% was  $-0.327$ , calculated from similar exercise challenges in 1809 conscripts with similar symptoms (Latvala et al. 2000).

### 7.2.7 Diary card

During the run-in period of 1 to 2 weeks, the patients continued to take their usual asthma medication and daily recorded the asthma symptom score (ranging daytime between 0 and 5 and at night between 0 and 4), use of salbutamol (Ventoline MDI, 100  $\mu\text{g}$ , Glaxo Wellcome) and morning and evening PEF values (best of three) using a mini-Wright peak flow meter (Clement Clarke International, Essex, UK).

### 7.2.8 Skin prick test

Skin tests for assessment of atopy were performed by the prick method with 13 common aeroallergens (ALK, Copenhagen, Denmark): alder, birch, cocksfoot, timothy, mugwort, horse, dog, cat, sheep's wool, house dust mite (*Dermatophagoides farinae* and *D. pteronyssinus*), alternaria, and cladosporium, and saline as the negative and histamine as positive controls. A wheal with a diameter of  $\geq 3$  mm was regarded as positive, and a patient with  $\geq 1$  positive reactions was regarded as atopic. In Study V, total prick wheal sum was calculated by adding the diameters of the wheals of each positive reaction excluding histamine (Miles et al. 1995). Subjects with a total prick wheal sum of 3 to 10 mm were regarded as low sensitised, and those with  $> 10$  mm were regarded as high sensitised.

## 7.3 Statistical analyses

There were several statistical points of view in each study, and statistical analyses were described separately for different studies. All tests were 2-tailed, with a p-value of less than 0.05 considered significant. All analyses were performed with SPSS version 11.0 (SPSS, Chicago, IL, USA).

### 7.3.1 Long-term variability in FENO in healthy subjects (Study I)

All data were expressed as means (SD). At baseline, Pearson correlation analysis was used to examine the relationship between FENO and age. A two-sided *t* test was used for comparisons between FENO and gender. Long-term variability in FENO was assessed by calculating the coefficient of variation ((mean / SD) x 100) intraindividually, and the mean CoV was the final result.

### 7.3.2 Short-term variability in FENO (Study II)

Because FENO was skewed, all subsequent analyses were carried out on log-transformed data. The difference between levels of FENO in different groups was analysed with one-way ANOVA and pairwise comparisons were used with the Bonferroni correction.

The reproducibility of FENO determinations was assessed with those measured after a 10-min interval by comparing the FENO data obtained at baseline. Intra-class correlation coefficients ( $r_i$ ) after 10 min as compared with baseline were calculated for all measurements and for each group. The coefficients of variability (CoV) compared with baseline at 10 min, 6 h, and 24 h were also calculated. Variability in FENO was assessed at 6 h and 24 h after baseline by calculating the difference of means compared with baseline. This evaluation was made for each group. A two-sided *t*-test was used to analyse the difference of variability in FENO between groups.

### 7.3.3 Short-term effect of inhaled fluticasone on BHR (Study III) and peak-FENO

Sample-size calculations were based on the primary variable PD<sub>15</sub>FEV<sub>1</sub>. In the groups of 13 patients a mean difference of 1.0 dose step (SD 0.84) was estimated to have an 80% probability of being detected with a *t* test at a significance level of 5%. The changes in PD<sub>15</sub>FEV<sub>1</sub> from baseline were calculated by the doubling dose (DD) technique. The within-group changes in doubling dose units and the comparisons between the groups were given as means with 95% confidence intervals at each time-point. The repeated DD measurements were summed by the area under the curve (AUC) method with linear trapezoidal application. AUC analysis was split into three successive time segments. The measurements from 6 to 72 hours were summed to assess the short-term effect of FP treatment, and the subsequent measurements from 2 to 6 weeks to assess the long-term effect. In addition, after cessation of treatment the changes were summed by the AUC method; comparisons were made both with baseline and with the last dose of FP or placebo.

All AUC values were divided by the total time of each AUC segment to get an average level of doubling doses over the time segment (standardised AUC). The standardised AUC values in the treatment groups and the differences between the groups were calculated as means with 95% confidence interval. A *t* test for independent samples was used to compare the standardised AUC values between the treatment groups.

Changes in FEV<sub>1</sub> (percentage predicted) from baseline during the trial were also calculated using standardised AUC values over the same time segments as for DD units. Changes in morning and evening PEF (percentage predicted) from baseline to the last week of treatment were analysed by a *t* test for independent samples. The changes in

symptom scores and the use of rescue medication were analysed by the Mann-Whitney U-test.

#### **7.3.4 Association between FENO and BHR in asthma (Study IV)**

FENO, PD15FEV1 were skewed and were  $\log_{10}$  transformed before analysis to achieve a near normal distribution. Exhaled NO levels are reported as medians and 25 to 75%-quartile range and the other parameters as mean (SD). Differences between anthropometric data, FENO, PD15FEV1, and  $\Delta$ PEF% in the atopic and nonatopic patients were analysed with the Mann-Whitney U-test. Mutual correlations between FENO, PD15FEV1, and  $\Delta$ PEF% were analysed separately in the atopic and nonatopic groups with the Spearman rank test, with 95% confidence intervals calculated. Multiple linear regression analyses were undertaken using  $\log$  (FENO) values as the outcome variable. In the first model, the effect of atopy and asthma were evaluated. In the second model,  $\Delta$ PEF% and  $\log$  (PD15FEV1) were introduced by a stepwise method, separately. Variables in all models were excluded in a stepwise fashion if they did not reach significance at the 5% level.

#### **7.3.5 Equally elevated concentrations of exhaled nitric oxide in nonatopic and low-sensitised atopic asthmatics (Study V)**

FENO and PD15FEV1 were skewed and  $\log$  transformed before analysis to achieve a near-normal distribution. FENO and PD15FEV1 levels were reported as medians and 25 to 75% quartiles, and the other parameters as means (SD). Differences between anthropometric data, FENO, PD15FEV1, and severity of EIB in the high-sensitised and low-sensitised atopic and nonatopic patients and in healthy controls were analysed with ANOVA. ANOVA tests were continued with Dunnett's test based on two hypotheses: In the first, healthy subjects served as the reference group. In the second hypothesis, the high-sensitised atopic asthma group served as the reference group. A separate comparison was made between those with nonatopic asthma and low-sensitised atopic asthma.

Multiple linear regression analysis was undertaken using the  $\log$  (FENO) value as the outcome variable. The model evaluated the severity of EIB ( $\Delta$ PEF%),  $\log$  (PD15FEV1), B-eosinophils, atopy score, seasonal vs perennial sensitisation, number of positive prick reactions, and size of each prick reaction with a stepwise method for atopic patients. Variables in the model were excluded in a stepwise fashion if they did not reach significance at the 5% level.

## 8 Results

### 8.1 Long-term variability in FENO in healthy subjects (Study I)

The mean (SD) value of FENO in all subjects (n = 26) was 6.9 ppb (2.5 ppb). The 95% confidence interval was 6.0 to 7.9 ppb. The upper limit of intraindividual variation (+2 SD) was 11.9 ppb, and the lower limit (-2 SD) was 1.9 ppb. The mean (SD) value of NO output was 39.1 pmol/s (20 pmol/s). We found no correlation between FENO and age ( $r = -0.06$ ,  $p = 0.78$ ). Nor any association between FENO and gender ( $p = 0.40$ ).

The mean intraindividual coefficient of variation (CoV) within the interval of 7 days was 15.8% of FENO and 20.7% of NO output. Within an interval of 23 days the mean CoV was 16.8% of FENO and 18% of NO output. Within an interval of 7 days standard deviation was 1.96 ppb, and was 2.4 ppb within an interval of 23 days.

### 8.2 Short-term variability in FENO (Study II)

For variability in FENO, 44 subjects showed a mean (SD) FENO at baseline for all of 16.7 (23.5) ppb. In healthy subjects (n=13), mean (SD) FENO baseline values were 6.6 (2.3) ppb; in patients with respiratory symptoms who did not fulfill the diagnostic criteria of asthma (n=21), FENO values were significantly higher 14.6 (11.1) ppb than in healthy subjects ( $p = 0.0076$ ). Asthma patients (n=10) had the highest mean (SD) FENO levels, at 34.2 (43) ppb, as compared with healthy subjects ( $p < 0.001$ ) or patients with respiratory symptoms ( $p = 0.02$ ) (Table 3).

Table 3 Exhaled nitric oxide (ppb) and its short-term variability in different groups during the study

	Baseline mean (ppb)	SD	10 min mean	CoV %	6 h mean	CoV %	24 h mean	CoV %
Healthy subjects	6.6	2.3	6.7	5.1	6.5	10.8	5.8	11.7
Respiratory symptoms	14.6	11.1	15	7.1	14.7	16.4	14.5	22.2
Asthma	34.2	43	32.7	13.5	33.3	19.4	32.8	26.4

ppb = parts per billion, SD = standard deviation, CoV = Coefficient of variation

The intra-class correlation coefficient ( $r_1$ ) was 0.955 for all measurements between baseline and after 10 min. The  $r_1$  was 0.959 in healthy controls, 0.986 in the respiratory

symptoms-group, and 0.936 in asthmatics. The mean difference in FENO after 10 min compared with baseline was 0.18 ppb (Fig. 1). The CoV of FENO after 10 min, 6 h, and 24 h was good (Table 3).

### **8.3 Short-term effect of inhaled fluticasone on BHR (Study III) and on peak FENO**

#### **8.3.1 Short-term effect of inhaled fluticasone on BHR**

The increase in PD<sub>15</sub>FEV<sub>1</sub> from baseline was greater in the FP group than in the placebo group. The short-term difference between the treatment groups in 6 to 72 hours was significant ( $p=0.048$ ), and the effect was sustained until the end of the treatment period (2–6 weeks, Table 4 and Figure 9). In the FP group, the PD<sub>15</sub>FEV<sub>1</sub> between 72 hours and 6 weeks after the start of treatment was 1.85 to 2.07 doubling doses above baseline level, and the difference between the treatments during that period was 1.19 to 1.98 doubling doses. At the end of the 6-week treatment period the geometric mean PD<sub>15</sub>FEV<sub>1</sub> in the FP group was 0.510 mg (range 0.061–2.369) and in the placebo group 0.248 mg (range 0.003–1.912).

After cessation of treatment, the effect of FP on BHR diminished significantly within 2 weeks (Figure 9). At the end of the 2-week follow-up PD<sub>15</sub>FEV<sub>1</sub> had returned to near pretreatment levels in the FP group (0.85 doubling doses above baseline). The geometric mean PD<sub>15</sub>FEV<sub>1</sub> at the end of the 2-week follow-up period was 0.294 mg (range 0.042–1.925) in the FP group and 0.280 mg (range 0.014–1.827) in the placebo group.

FEV<sub>1</sub> (percentage predicted) did not increase significantly from baseline in the FP group compared with the placebo group within 72 hours (mean change 2.9% v 0.3%,  $p=0.084$ ), but the long-term effect was significant (mean difference from 2 to 6 weeks 3.8% v -0.3%,  $p=0.028$ ). No statistically significant differences were seen between treatment groups in the PEF values recorded at home. Morning PEF values increased 2.4% and evening values 2.5%, in the FP group, with a corresponding decrease of 0.4% and 0.5% in the placebo group. Symptom scores or the use of rescue medication between the groups did not significantly differ during or after treatment.

**Table 4** Mean (95% CI) changes in PD<sub>15</sub> FEV<sub>1</sub> values in doubling doses (DD) compared with baseline in fluticasone propionate (FP) and placebo groups and mean (95% CI) differences between groups

Time from treatment onset	Fluticasone (n=13)	Placebo (n=13)	Difference (FP v placebo)
<b>Treatment v baseline</b>			
6 hours	0.58 (-0.07 to 1.22)	0.63 (-0.17 to 1.43)	-0.06 (-1.03 to 0.92)
12 hours	1.26 (0.70 to 1.82)	0.43 (-0.26 to 1.12)	0.83 (-0.01 to 1.67)
24 hours	1.75 (1.08 to 2.43)	1.03 (0.47 to 1.60)	0.72 (-0.12 to 1.56)
72 hours	1.98 (1.33 to 2.63)	0.79 (-0.11 to 1.70)	1.19 (0.13 to 2.25)
2 weeks	1.92 (1.07 to 2.77)	0.59 (-0.16 to 1.34)	1.33 (0.26 to 2.40)
4 weeks	1.85 (0.88 to 2.82)	0.58 (-0.15 to 1.31)	1.27 (0.12 to 2.42)
6 weeks	2.07 (1.30 to 2.83)	0.09 (-0.64 to 0.81)	1.98 (0.98 to 2.98)
<b>Post-treatment v baseline</b>			
48 hours	1.47 (0.32 to 2.61)	0.66 (-0.12 to 1.45)	0.81 (-0.48 to 2.09)
1 week	1.15 (0.01 to 2.29)	0.11 (-0.74 to 0.96)	1.04 (-0.29 to 2.3)
2 weeks	0.85 (-0.57 to 2.27)	0.26 (-0.37 to 0.89)	0.59 (-0.82 to 2.00)
<b>Post-treatment v 6 weeks</b>			
48 hours	-0.40 (-1.00 to 0.20)	0.43 (-0.04 to 0.90)	(-0.83 (-1.54 to -0.12)
1 week	-0.72 (-1.34 to -0.09)	-0.12 (-0.72 to 0.48)	-0.60 (-1.41 to 0.22)
2 weeks	-1.02 (-2.19 to 0.16)	0.03 (-0.58 to 0.63)	-1.04 (-2.25 to 0.17)
Post-treatment values are compared with those at baseline and at 6 weeks (last dose). n =11 in the FP group and n =12 in the placebo group in the post-treatment period at 48 hours, 1 week and 2 weeks.			

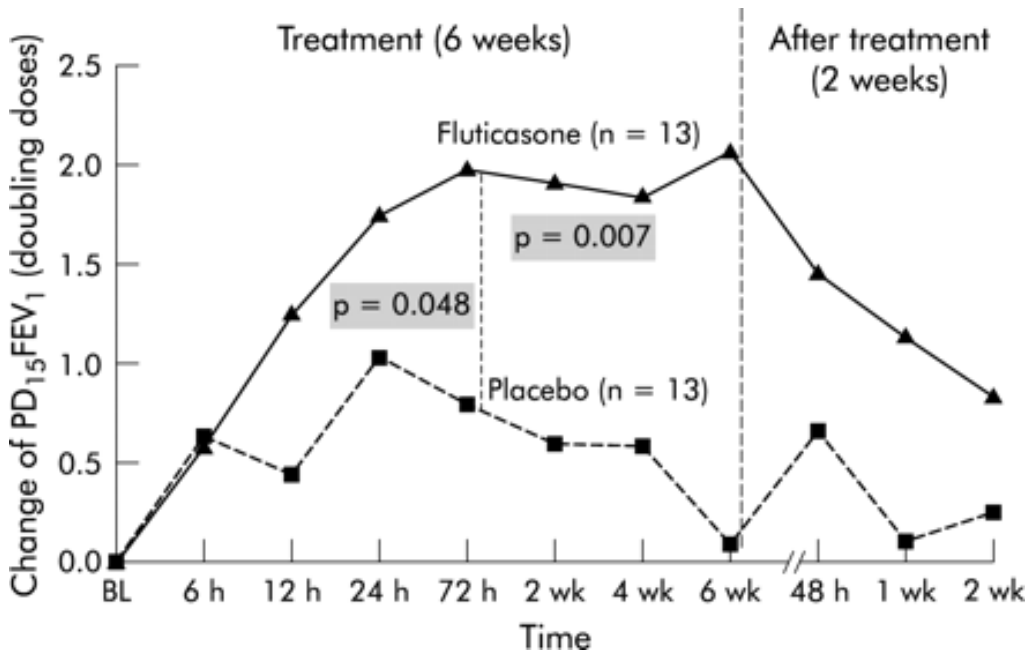


Figure 9. Time-course of the effect of inhaled fluticasone propionate (FP) 250 µg twice daily (n=13) or placebo (n=13) on bronchial hyperresponsiveness (BHR) in patients with mild asthma. Changes in BHR are presented as mean changes in PD<sub>15</sub>FEV<sub>1</sub> for histamine expressed as doubling dose units compared with baseline PD<sub>15</sub>FEV<sub>1</sub> values. Patients were treated with FP for 6 weeks and followed for 2 weeks after cessation of treatment; p values indicate significant differences between the FP and placebo groups from 6 to 72 hours (p=0.048) and from 2 to 6 weeks (p=0.007) (area under the curve analysis). BL=baseline.

### 8.3.2 Short-term effect of inhaled fluticasone on peak FENO (unpublished data)

At baseline, the geometric mean of peak FENO was 59.9 ppb in the FP group and 90.7 ppb in the PL group. After 2-week treatment, peak FENO decreased from baseline significantly more in the FP group than in the PL group (-54.7% vs. -5.9%, p=0.01). Peak-FENO levels remained stable during the rest of the treatment period. The difference between groups (FP minus PL) was -15.9 at week 4 (p=0.10) and -14.1 at week 6 (p=0.07). At follow-up peak-FENO levels were slowly increased in the FP group, with no change in the PL group. After 1 week of cessation of treatment peak-FENO levels were still significantly lower the FP group compared to the PL group (p=0.04).

After 2-week treatment, a positive correlation appeared between the within-subject changes in PD<sub>15</sub> (as doubling doses) and peak FENO (as% changes,  $r = -0.52$  (p=0.008) (Figure 10). Including all the repeated observations of PD<sub>15</sub> and peak FENO during the treatment period, the correlation was  $r = -0.44$  (p=0.0007).

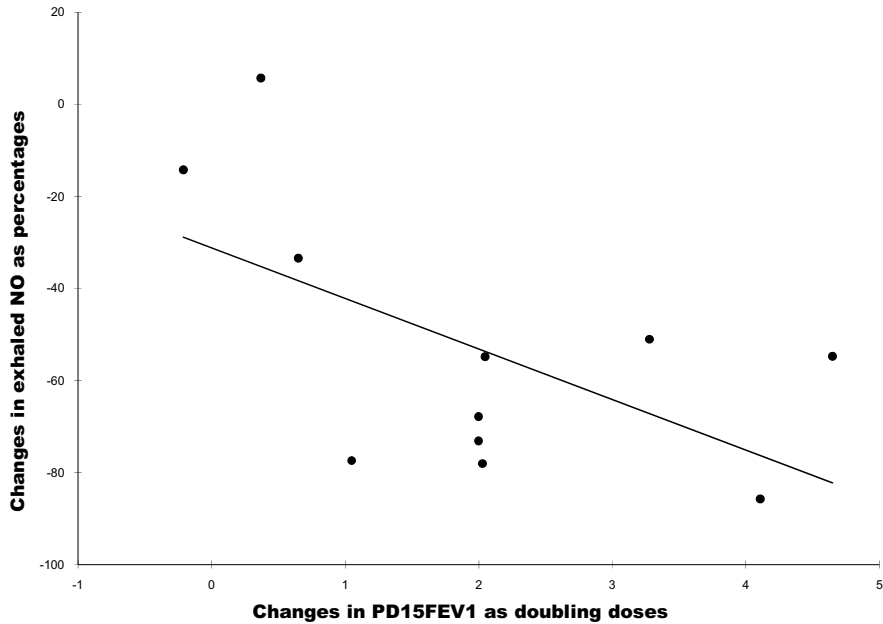


Figure 10. Correlation between changes in PD15FEV1 and changes in peak FENO after 2 weeks of treatment with fluticasone propionate (unpublished data)

#### 8.4 Association between FENO and BHR in asthma (Study IV)

In atopic patients, level of FENO was on average over twice that of nonatopic patients (Table 5). In atopic patients, EIB was slightly but significantly more severe, but their BHR in histamine challenge did not differ significantly from that of nonatopics (Table 5). When atopic and nonatopic patients with a confirmed diagnosis of asthma were analysed, there was no difference in either EIB or HIB between the groups (Table 5).

**Table 5** Exhaled nitric oxide, exercise-induced bronchoconstriction and histamine-induced bronchoconstriction in young male patients

	All atopic patients n = 128	Atopic asthmatics n = 68	All nonatopic patients n = 53	Nonatopic asthmatics n = 19
FENO, ppb #	21.2 (13.2 - 44.6)***	29.6 (18.4 - 52.0)▣▣▣	10.2 (8.4 - 14.8)	12.4 (8.5 - 20.0)
ΔPEF% ##	11.2 (10.8)**	16.6 (12)	7.1 (7.4)	12.3 (9.3)
PD15FEV1, mg #	1.0 (0.4-2.5)	0.4 (0.2-1.0)	1.5 (0.5-3.2)	0.4 (0.2-0.7)

NOexp: exhaled nitric oxide; PD15FEV1: provocative dose of histamine causing a 15% fall in FEV1; ΔPEF%: fall in PEF after exercise challenge; # : median (25 -75% quartiles), ## : mean (SD) atopics vs nonatopics: \*\* p < 0.01; \*\*\* p < 0.001 atopic asthma vs nonatopic asthma: ▣▣▣ p < 0.001

In atopic patients, FENO correlated significantly with both EIB and HIB (Table 6 and Figure 11). In contrast, in nonatopic patients, FENO did not correlate with BHR measured by either an indirect or direct method. A multiple linear regression model showed that atopy (p < 0.001), severity of EIB (p <0.001), and severity of HIB (expressed as log PD15FEV1) (p=0.006) all significantly associated with FENO (Table 6). A significant interaction occurred between atopy and severity of EIB, as well as atopy and HIB; therefore, separate regression models were constructed for atopic and nonatopic patients. In these models, the severity of EIB and HIB was associated with FENO in atopic but not in nonatopic patients. These results did not change even when only patients with confirmed diagnosis of asthma were analysed.

**Table 6** Variables included in multiple linear regression model with log (FENO) as the dependent variable in all patients (n = 181)

Variable	B	95% CI	p value	partial correlation
Atopy	0.517	0.293 to 0.742	< 0.001	0.323
ΔPEF%	0.028	0.016 to 0.039	< 0.001	0.335
log (PD15FEV1)	-0.128	-0.219 to -0.037	0.006	-0.204

B = regression coefficient; CI = confidence interval; ΔPEF%: maximum percentage fall in PEF after exercise challenge; PD15FEV1: provocative dose of histamine causing a 15% fall in FEV1

Partial correlation = correlation between dependent and each independent variable taking into account the other variables in the model

Adjusted coefficient of determination (R<sup>2</sup>) for all variables is 0.32

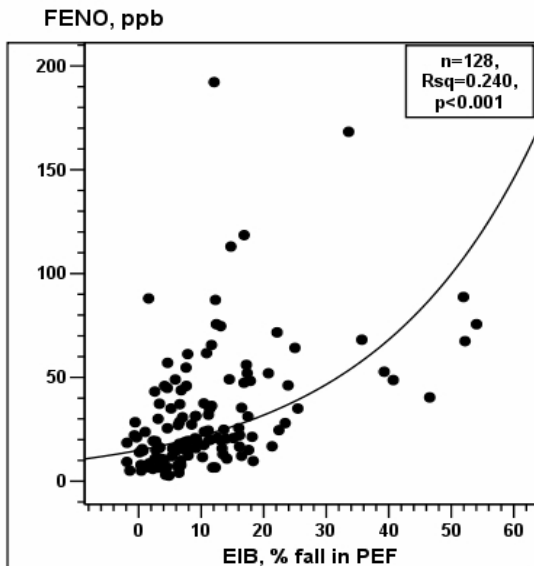


Figure 11 Association between exercise-induced bronchoconstriction (EIB,% fall in PEF) and exhaled nitric oxide (FENO, ppb) in atopic patients. The curve is exponentially fitted.  $Rsq = r^2$  (coefficient of determination)

### 8.5 Equally elevated concentrations of exhaled nitric oxide in nonatopic and low-sensitised atopic asthmatics (Study V)

Based on the skin prick test, 71 (84%) of the subjects were atopic and 14 (16%) nonatopic. Of those atopic, 58 (82%) were high-sensitised (total prick wheal sum >10 mm) and 13 (18%) low-sensitised (total prick wheal sum 3 to 10 mm). FENO levels and degree of BHR in the study groups are shown in Table 7 and Figures 12 and 13. The median FENO of the high-sensitised atopic patients was more than double that of the nonatopic asthmatics. Low-sensitised and nonatopic asthmatics had similar FENO levels, with FENO level regarded as elevated if it exceeded 12 ppb. Elevated levels appeared in 57% of subjects with nonatopic asthma, while this level was exceeded by 71% of subjects with low- and 91% with high-sensitisation atopic asthma.

FEV<sub>1</sub> of predicted was slightly higher in subjects with low-sensitisation atopic asthma than in nonatopic asthma. BHR to histamine and to exercise between subjects with nonatopic asthma and with low-sensitisation atopic asthma was similar, but high-sensitised atopic asthmatics had significantly more severe BHR to both histamine and exercise than did nonatopic asthmatics ( $p < 0.05$ , both comparisons) (Table 7).

Table 7 Exhaled nitric oxide and bronchial responsiveness to histamine and to exercise in study groups

		Healthy subjects n = 10	Nonatopic asthma n = 14	Low-sensitized atopic asthma n = 13	High-sensitized atopic asthma n = 58
FENO, ppb	median (25 -75% quartiles)	6.5 (5.1 - 8.0)§§§	15.2 (9.7 - 24.7)***§§§	16.7 (10.8 - 25.6)***§§	34.9 (21.3 - 53.8)***
PD15FEV1, mg	median (25 -75% quartiles)	- <sup>a</sup>	0.82 (0.38 - 3.2)§	1.1 (0.59 - 1.1)§§	0.37 (0.16 - 0.96)
PD15FEV1 <1.6 mg	% of subjects	0	57	43	86
PD15FEV1 <0.4 mg	% of subjects	0	29	14	53
EIB,% fall in PEF	mean (SD)	ND	10.3 (7.7)§	12.3 (6.8)	18.6 (14.1)
EIB,% fall in PEF > 10%	% of subjects	ND	36	69	78

FENO: exhaled nitric oxide; PD15FEV1: provocative dose of histamine causing a 15% fall in FEV1;

EIB: exercise-induced bronchoconstriction; and ND: not done.

\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001 compared to healthy subjects.

§ p < 0.05, §§ p < 0.01, §§§ p < 0.001 compared to high-sensitized atopic asthma.

<sup>a</sup> all values over 1.6 mg (no bronchial hyperreactivity).

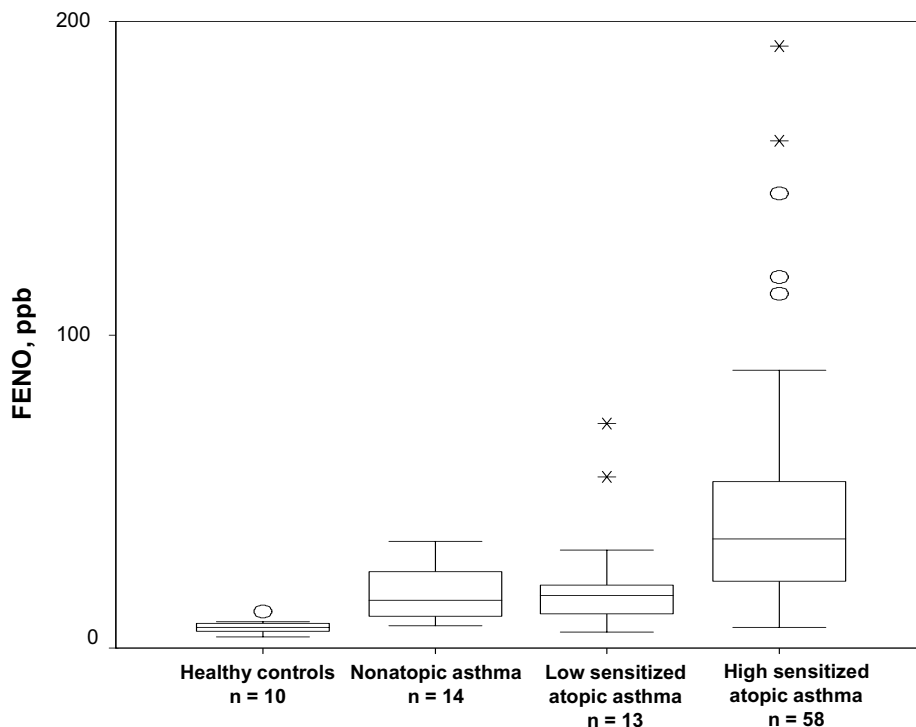


Figure 12. Boxplot presentation of the distribution of exhaled nitric oxide (FENO) in the groups (circles are outliers and asterisks are extreme values).

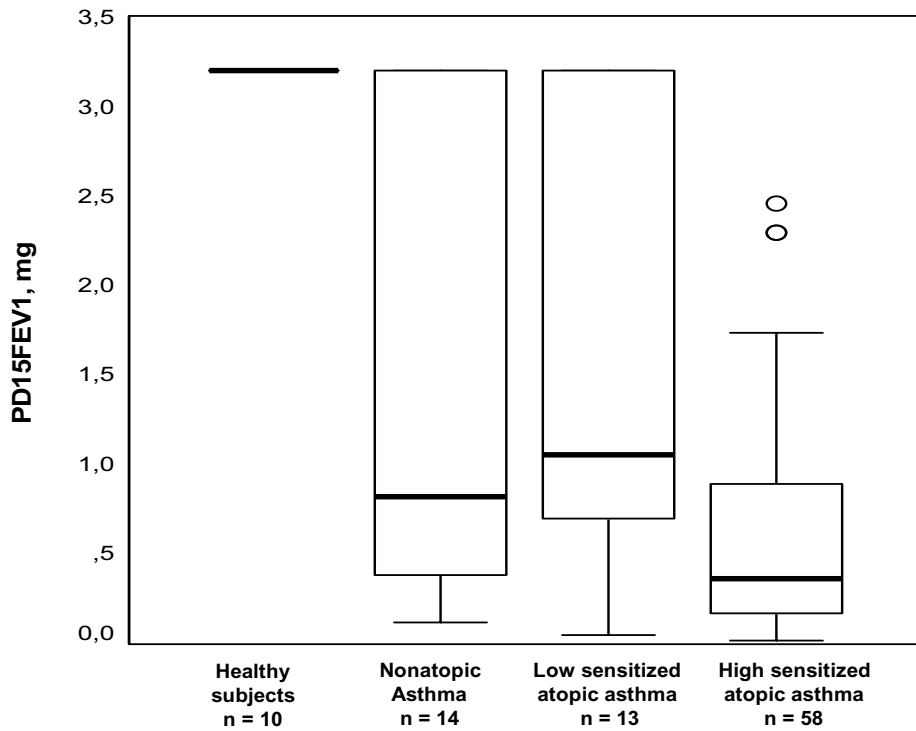


Figure 13. Boxplot presentation of the distribution of PD15FEV1 in the groups (outliers are circles) (unpublished data).

## **9 Discussion**

### **9.1 Study population and methods**

#### **9.1.1 Study population**

In Study I, healthy subjects were carefully evaluated from the phase I study with inhaled insulin (see Methods). Atopy was evaluated regarding their medical history. We did not perform the skin prick tests or measurements for specific IgE to exclude possible hidden allergy. Measurements of FENO were performed during winter time and timing of measurement eliminated the possible effect of seasonal allergic rhinitis. However, study subjects in Study I were healthy and examined carefully.

Study III comprised 26 adult patients with mild asthma diagnosed based on ATS criteria. In short, we studied steroid-naïve, non-smoking asthma patients with mild to moderate BHR to histamine. The mean PD15FEV1 was 0.18mg, which indicated room for PD15FEV1 to increase during the treatment with FP. However, the duration of the disease in most of the patients had been more than 2 years. Although the disease was persistent, its severity was mild based on low symptom scores, only slightly (if any) reduced lung function, and limited use of rescue medication.

Our study population of non-selected male conscripts (II, IV-V) was very homogenous: They were of the same age, were nonsmoking and steroid-naïve, and all had respiratory symptoms suggesting asthma, but had no other interfering diseases. Such a population is most suitable for evaluation of airway inflammation and airway reactivity. As all the patients had symptoms suggesting asthma, we also included those patients who did not fulfill the standard criteria of asthma, in order to obtain a wider range of variety in airway inflammation and bronchial hyperresponsiveness (IV). The main results did not change when only those patients with an unequivocal diagnosis of asthma underwent analysis. The study population of 181 patients was sufficiently large for assessment of the differences between atopic and nonatopic groups. Study V included only patients with asthma followed by ATS criteria.

#### **9.1.2 Methodological considerations**

##### **9.1.2.1 FENO measurement**

In the present series of investigations (Studies I-II and IV-V) the measurement of FENO was made according to the ERS guidelines. Furthermore, the exhalation flow was restricted to the range in the flow window between 0.08 and 0.15 L/s. Actual individual exhalation flow was maintained between 0.09 and 0.12 L/s. Limitation of exhalation flow rate minimises variation in FENO (Silkoff et al. 1997). Our measurements of FENO started

in 1998, and ATS guidelines for FENO measurements were published in 1999; for that reason we did not change methodology during the study. The preliminary study by Zetterquist and colleagues (1999) showed that ingestion of a meal rich in nitrate prior to FENO measurement raised the level of FENO. When the mouth is rinsed with a basic solution or anti-bacterial solution, the increase in FENO can be eliminated. Our method included mouth rinsing before measurement, so this possible contamination could be eliminated. We also did measure FENO before lung function tests (including histamine challenge), because repeated spirometry and measurement of BHR could influence FENO (Silkoff et al. 1999). We measured FENO the day after the exercise challenge for practical reasons, besides the effect of exercise on airway inflammation or HIB has been considered negligible (Gauvreau et al. 2000).

Unfortunately, we did measurements of FENO according to ERS guidelines, not according to ATS, and this makes comparison of the results with ATS guidelines from other investigators challenging. Moreover, the criteria of reproducibility were stricter than with our method. Repeated reproducible exhalations should be performed, resulting in three NO plateau values that agree within 10% of the mean value. Exhaled NO is then calculated as the mean of these three values.

Peak FENO measurements were done in Study III. The first patients were randomised to the study in the beginning of 1996. After that, the measurement of FENO has been developed in different ways. At the present time, the knowledge of FENO measurement has increased logarithmically compared to that in 1996. Many weaknesses emerged in peak FENO methodology: using the nose clip, breathholding, no standardised exhalation flow, and no use of the resistor. Because of high variability in peak FENO, the results of the peak FENO in Study III were not included in the final manuscript.

#### 9.1.2.2 BHR measurement

Histamine challenge tests were performed by a dosimetric method with controlled tidal breathing (Sovijärvi et al. 1993). PD<sub>15</sub>FEV<sub>1</sub> values were calculated from logarithmically transformed histamine doses by linear interpolation. The method was carefully validated and had good reproducibility (Sovijärvi et al. 1993). In cases where FEV<sub>1</sub> did not decrease from baseline by 15% or more after the highest dose, an extra dose of 3.2 mg was given (Study III). If FEV<sub>1</sub> did not decrease at least 10% after the maximum dose of 1.6 mg of histamine, PD<sub>15</sub>FEV<sub>1</sub> of 3.2 mg served as the final result (Studies II, IV-V). If FEV<sub>1</sub> did decrease at least 10% but less than 15% after the maximum dose of 1.6 mg of histamine, the PD<sub>15</sub>FEV<sub>1</sub> were calculated by linear interpolation (Cockcroft et al. 1983). In this way, we were unable to calculate the exact PD<sub>15</sub>FEV<sub>1</sub>, and this therefore had a possible effect on the results. However, the number of patients with PD<sub>15</sub>FEV<sub>1</sub> more than 1.6 mg were quite few.

#### 9.1.2.3 Exercise test

We used a standardised 8-minute running test outdoors with adjusted heart rate monitoring (Karjalainen et al. 1991). PEF values were measured just before the exercise and immediately and 5, 10, 20, and 30 minutes after the exercise. An 8-minute running test has been shown to be adequate long for evaluation of EIB and using the shorter test

time, the severity of EIB might be lower. We used PEF measurements for evaluation of the severity of EIB; recent guidelines, however, recommended FEV<sub>1</sub> (ATS 1999) measurement. PEF measurements in the exercise test served as the standard method for more than 20 years at the Central Military Hospital. We did not measure ventilation during the exercise test but did measure heart rate. There were studies showing that intensity, duration, mode, and environmental conditions during exercise must all be considered. While historically the exercise challenge has been prescribed at an intensity of 85% maximum heart rate (Andersson et al. 1971), recent studies suggest that a significantly higher exercise intensity should be used (up to 95 to 100% of maximum effort), especially in the athlete population (Rundell et al. 2000). The duration of 6 to 8 minutes of exercise is consistent with a mathematical model of airway drying (Anderson and Daviskas 1997). However, a successful diagnosis has been obtained using short-duration; high-intensity exercise. The 2-minute challenge described by Rundell and colleagues (2000) for speed skaters is a high-intensity, high-ventilation exercise that sufficiently dries the airways to provoke a response in susceptible individuals within 2 minutes.

We were advised about the seasonal variability in EIB outdoors (Karjalainen et al. 1989), and therefore did not include patients in the study during the high pollen season. Temperature and air humidity affect EIB severity (Rundell et al. 2000). We adjusted the severity of EIB according to temperature in Studies IV and V. For adjustments, the data for temperature and relative humidity during the study hours were collected from the database of the Finnish Meteorological Institute. Absolute air humidity was computed according to a study (Latvala et al, 2000) using the same exercise challenge method in the same place and with similar subjects: 1809 army conscripts with suspected asthma. This method has shown a significant linear dependence of % PEF on temperature (°C) and water content of the air (H<sub>2</sub>O / m<sup>3</sup>). The regression coefficient used with respect to absolute air humidity was -1.026, and that with respect to air temperature was -0.327.

#### 9.1.2.4 Determination of atopy

Skin prick tests for assessment of atopy were performed with 13 common aeroallergens: alder, birch, cocksfoot, timothy, mugwort, horse, dog, cat, sheep's wool, house dust mites (*Dermatophagoides farinae* and *D. pteronyssinus*), and the fungi *Alternaria*, and *Cladosporium*, with the solvent as the negative and histamine as the positive control. A wheal with a diameter of at least 3 mm in the presence of expected reactions to the control solutions was regarded as a positive reaction, and subjects with at least one positive reaction were regarded as atopic. Total prick wheal sum was calculated by addition of the diameters of the wheals of each positive reaction excluding histamine. Subjects with a total prick wheal sum 3 to 10 mm were regarded as low sensitised, and those with >10 mm were regarded as high sensitised (Study V).

These are the major allergens in Finland, and according to the results from the Skin and Allergy Hospital, more than 90% of atopics can be found with these allergens (Leena Petman and Tari Haahtela, oral communication). We did not measure specific IgE levels, and there was only a low possibility for hidden allergy in nonatopic patients.

## 9.2 Primary findings and their relation to Studies I – V

### 9.2.1 Long-term variation in FENO in healthy subjects

FENO was unrelated to age or sex in healthy subjects, and we found no significant long-term variability in FENO. Age-related changes in FENO have been reported in healthy and in asthmatic children (Franklin et al. 1999; Malmberg et al. 2003), but we found no significant correlation between age and FENO in adults. An association between FENO and height has been found in several studies (Olin et al. 2006, Bohadana et al. 2008). Obesity can change the levels of FENO (Busia et al. 1999), but in the present study all subjects had BMI less than 30. Menstrual cycle has affected the long-term variability at 7 days in female subjects (Kharitonov et al. 1994; Morris et al. 1996), but long-term variability at 23 days was measured at almost the same cycle phase, and changes in hormone levels between measurements were not very large.

In several studies, atopic asymptomatic subjects appeared to have higher FENO than did nonatopic asymptomatic subjects (Horváth and Barnes 1999, Franklin et al. 2004 and Olin et al. 2007). After critical evaluation, all these studies had one or more weakness in their patient selection: Patients with BHR to methacholine had previous asthma, or atopy was defined by increased IgE level only. The most recent finding showed that an atopic constitution defined as positive skin prick test results does not elevate FENO in healthy nonsmoking adults with no signs or symptoms of airway disorders, meaning that the same reference ranges for FENO can be applied to both skin prick test-positive and -negative subjects (Rouhos et al. 2008).

These results suggest that FENO measurement was reproducible, and the levels of FENO were less than 12 ppb in healthy subjects. We found no significant long-term variability in FENO in healthy subjects.

### 9.2.2 Short-term variation in FENO in healthy and asthmatic subjects

This study showed that in young men with asthma or asthma-like symptoms as well as in young healthy adults, determination of exhaled NO is reproducible. Patients with respiratory symptoms who did not fulfil the diagnostic criteria of asthma had FENO values significantly higher than in healthy subjects, but significantly lower than in asthma patients. This finding support results showing that patients with respiratory symptoms suggesting asthma but with normal lung function had a higher number of sputum eosinophils than did healthy subjects (Rytilä et al. 2000), but the degree of eosinophilic inflammation was less pronounced than in asthmatics. Furthermore, Lehtimäki and colleagues (2005) have shown that both the bronchial and alveolar components of FENO were increased in patients who did not fulfil the diagnostic criteria of asthma compared with those of healthy subjects.

In all groups studied, short-term reproducibility after the 10-min interval was good. The intra-class correlation coefficient was 0.95, which was similar to previous findings of  $r_1 > 0.92$  (van Rensen et al. 1999) and 0.98 in one population study (Salome et al 1999).

Knowledge of short-term reproducibility of FENO in asthma was scant. An  $r_1$  of 0.81 in mild to moderate asthmatics was used by de Gouw and colleagues (1998). A mean difference of 0.094 ppb between the two closest FENO values has been reported (Gabbay et al. 1998). In our study, the mean difference in FENO after 10 min compared with baseline was 0.08 ppb in healthy subjects and 0.18 ppb overall, including in patients with asthma. Coefficients of variation (CoV) have been 19% in healthy subjects and 10% in asthmatics after a 10-min interval (ten Hacken *et al* 1998).

Previously, FENO measured with a flow of 250 mL/s at different airway pressures, found CoV to be 6% (Högman et al. 1997). In our study, the variation in FENO in healthy subjects was 5.1%, lower than in previous reports. In our study, variation in FENO in asthmatics was similar to that previously reported (ten Hacken *et al* 1998). The most recent study showed FENO levels to be stable during repeated measurements (intraclass correlation coefficient, 0.81) in healthy, nonsmoking African-Americans (Levescuc et al. 2008).

CoV values for FENO within the interval of 6 h and 24 h in healthy subjects or in patients with asthma-like symptoms or with asthma did not mutually differ. An intra-day variation (CoV) of 8.6 to 11.1% and inter-day variation of 19.0 to 23.2% at different expiratory flows have been shown in healthy subjects (Silkoff et al. 1998). We found no circadian variation in FENO in any group; however, we did not measure FENO during the early morning (i.e., 4 a.m.). Previously, a circadian variation in FENO showed that FENO was significantly decreased at 4 a.m. as compared with 4 p.m. in nocturnal asthma (ten Hacken et al. 1998). No circadian rhythm occurred in the study with healthy children measured on 6 consecutive days, but their FENO showed an intra-individual coefficient of variation of 25.9%. In a two-compartment model of the lung, the alveolar NO concentration was constant with age, whereas the airway part of NO steadily increased with age (Latzin et al. 2002)

In a recent study, FENO, according to ATS guidelines, was measured on 5 consecutive days (four measurements on the same day) for adults and twice on the same day for children. In both children and adults the coefficient of reproducibility was 2.11 parts per billion (ppb), and the intraclass correlation coefficient was 0.99. No diurnal or day-to-day variation occurred, nor any learning effect, and the results of FENO measurements were identical at the beginning and at the end of the study (Kharitonov et al. 2003). The most recent study demonstrated that the reproducibility of FENO is not influenced by gender or smoking status in subjects refraining from smoking before FENO assessment, and it provided evidence that FENO is potentially useful in the survey of populations, only fairly small samples being necessary to assess significant changes in FENO levels (Bohadana et al. 2008).

According to the results of our study and from other studies, measurement of exhaled nitric oxide by use of a standardised method based on ERS suggestions was well reproducible over a short period in the short term in healthy subjects, in patients with asthma-like symptoms, and in mild asthma.

### **9.2.3 Short-term effects of FP on BHR and peak FENO in mild asthma**

The hourly or daily changes in BHR after the start or cessation of treatment with ICS have not been studied. No data are available on how rapidly a sustained reduction in BHR can

be obtained after starting ICS treatment. In the present study, the BHR in terms of PD<sub>15</sub>FEV<sub>1</sub> for histamine was reduced and reached a plateau within 72 hours of starting treatment. The maximal effect occurred within 6 weeks. A significant improvement was also found in FEV<sub>1</sub> within 6 weeks of commencing FP treatment. Most of the treatment effect on BHR disappeared within 2 weeks of cessation of treatment.

These results are in line with previous findings, as Van Rensen and colleagues (1999) observed that a higher dose of FP (1000 µg) for 4 weeks reduced BHR by 1.82 doubling doses compared with placebo, the effect being similar to that found in our study. Another study showed the effect of a high dose of FP (2000 µg), at 2-week intervals for testing BHR; the maximum reduction of 1.9 doubling doses of methacholine was achieved after 6 weeks of treatment (Convery et al. 2000). The maximum effect in the present study at 6 weeks was similar, but obtained with a much smaller dose. Furthermore, Vathenen et al. (1991) found a significant effect on BHR in adults with a single dose of 800 µg budesonide after 6 hours, and Sherrington and Mallol (1999) reported a significant effect in children with a single dose of 2000 µg budesonide or 400 µg fluticasone after 8 hours. The effect of the single dose disappeared after 12 hours. Furthermore, Gibson and colleagues (2001) demonstrated a significant reduction in BHR to hypertonic saline in 6 hours with a large (2400 µg) single dose of budesonide. These results, together with ours, suggest that a higher dose of ICS can relieve BHR faster than a low dose (i.e., in a dose-dependent manner).

A short-term effect from ICS on BHR is linked to a reduction in airway inflammation. A single dose of budesonide reduced bronchial responsiveness to hypertonic saline concomitantly with a reduction in sputum eosinophils but not in mast cells (Gibson et al. 2001). Interestingly, a reduction in FENO occurred with lower doses of ICS than the decrease in BHR to methacholine (Jatakanon et al. 1999). FENO has been shown to be correlated with BHR in many studies of steroid-naïve asthmatics, reflecting the role of BHR as an indirect marker of airway inflammation (Dupont et al. 1998; Jatakanon et al. 1998). ICS reduce FENO in a dose-dependent manner, the effect starting within 3 to 5 days after start of treatment (Kharitonov et al. 1996). In our study, a significant correlation between reduction of BHR and fall in peak FENO emerged after 2 weeks of treatment with FP (unpublished data).

The reversal of BHR after cessation of treatment may be dose-dependent, but the evidence for this is scanty. Gershman and colleagues (2000) found that, after a 6-week treatment period with FP, BHR reversed within 3 days when the daily dose was small (100 µg) but within 2 weeks after treatment with a higher dose (1000 µg). We observed a significant reversal of BHR in 2 weeks after cessation of 6 weeks of treatment with FP 500 µg daily; at that time BHR returned to near pretreatment levels.

Our study was designed to evaluate short-term effects of ICS on BHR, but evidence that monitoring BHR during long-term treatment for adjustment of the corticosteroid dose in asthma has been clinically useful. When corticosteroid treatment was adapted by assessing BHR to methacholine, patients suffered about half the exacerbations observed in patients treated according to PEF measurements and symptoms only (Sont et al. 1999). Even a reduction in the thickness of the subepithelial reticular layer was evident in bronchial biopsy specimens of patients whose treatment was guided by BHR measurements (Sont et al. 1999).

#### **9.2.4 Association between FENO, BHR, and EIB only in atopics**

Our study showed a significant association between FENO and severity of EIB in the atopic group only. To the best of our knowledge, no previous reports are available comparing the association between FENO and bronchial responsiveness to exercise between atopic and nonatopic patients. The results of our study are in accordance with the findings of Scollo and colleagues (2000), who reported a significant association between baseline FENO and EIB in asthmatic children, most of whom were atopic. Our study found no association between FENO and EIB in the nonatopic group. El Halawani and colleagues (2003) demonstrated the role of FENO as a predictor of exercise-induced bronchoconstriction in subjects with suspected diagnosis of EIB with no history of allergic rhinitis or eczema. In their study, however, atopic status was not verified by objective tests. Measurement of FENO can possibly be of use to screen for EIB testing and therefore optimise the resources for exercise testing in paediatric asthma monitoring (Buchwald et al. 2005).

We also found EIB to be slightly but significantly more pronounced in atopic than in nonatopic patients despite similar reactivity in these groups in histamine challenge. The presence of atopy seems to facilitate the development of exercise-induced airway hyperresponsiveness. In a group of mainly atopic asthmatics, Koh and colleagues (2002) reported a significant correlation between atopy score and severity of EIB, which correlated with markers of eosinophilic airway inflammation in sputum and blood. Atopic patients have been more reactive also to AMP than are nonatopic patients (Ludviksdottir et al. 2000). FENO, blood eosinophils, and responsiveness to AMP correlate with eosinophils in bronchial biopsies from atopic asthmatics (van der Thoorn et al. 2000), suggesting that both level of FENO and response to AMP challenge depend on the degree of allergic airway inflammation.

The findings of this study indicate that concentration of exhaled nitric oxide correlates significantly with bronchial hyperresponsiveness, assessed either directly (histamine) or indirectly (exercise), in atopic but not in nonatopic young male patients with asthmatic symptoms. The findings also indicate that EIB correlates with level of histamine-induced bronchoconstriction in atopics but not in nonatopics. Whether these results in part depend on differences in the inflammatory profile or cytokine production, or depend on possible differences in duration of the inflammatory process between atopic and nonatopic patients, demands further study.

#### **9.2.5 Equally elevated concentrations of exhaled nitric oxide in nonatopic and low-sensitised atopic asthmatics**

Higher levels of FENO appeared in our nonatopic asthmatics than in healthy controls. The elevated FENO in 57% of our nonatopic asthmatics is consistent with earlier findings on the association of FENO level with asthma, irrespective of atopy (Malmberg et al. 2005), and with a decrease in FENO in nonatopic asthmatics after treatment with inhaled steroids (Dal Negro et al. 2003). In contrast, Ludviksdottir and colleagues (1999) found similar FENO levels in nonatopic and atopic asthma, but the majority of their patients were on inhaled steroids. In contrast with our results, FENO levels have been similar in steroid-

naive nonatopic asthma, nonatopic rhinitis, and in nonatopic healthy subjects, which can in part explain the very stable or mild asthma during that study (Gratziou et al. 1999).

Our study confirms earlier findings that the FENO level is higher in atopic asthma than in nonatopic asthma (Olin et al. 2004). The mechanisms for this are still obscure. The similar FENO in nonatopic and low-sensitised atopic asthma probably reflects a similar low level of eosinophilic airway inflammation between them, even though their levels of blood eosinophils are not similar, whereas the high eosinophilic bronchial inflammation may be more marked in the high-sensitised group (Amin et al. 2000 and Ryttilä 2002).

Linear regression analysis showed, moreover, that in atopic asthmatic patients, the severity of EIB and number of blood eosinophils, and also sensitisation to cats were the most important determinants of FENO. Our results are consistent with those of Olin and colleagues (2004) showing that being sensitised to perennial allergens in asthma raised the level of FENO compared to asthmatic patients' sensitisation to seasonal allergens and to that of nonatopic asthmatics. Moreover, atopic subjects sensitised only to house dust mite (HDM), independently of having asthma or not, had an increased level of FENO (Barreto et al. 2001). Furthermore, levels of FENO have been significantly higher in patients with asthma who were both sensitised and exposed to relevant allergens than in those who were sensitised but not exposed. FENO may thus be a marker of the airway inflammation induced by domestic exposure to allergens in sensitised asthmatics (Simpson et al. 1999). Sensitisation to perennial allergens (e.g., cat and HDM) has also led to increased levels of FENO in asthma (Langley et al. 2003). In atopic children, elevated FENO was associated with sensitisation to perennial allergens, but not to seasonal allergens such as grass pollen. Furthermore, an increase in FENO was associated with BHR and current wheezing, suggesting that FENO was more than just a marker for atopy (Leuppi et al. 2002). In Finland HDM is not a major allergen because of central heating and dry indoor air; cats produce the most important perennial allergen, especially for younger adults (Pallasaho et al. 2006).

To the best of our knowledge, this is the first study showing that steroid-naive nonatopic asthmatics and low-sensitised atopic asthmatics have equal levels of FENO as well as BHR both to histamine and to exercise. Similar exposure to relevant allergens during military service (the same kind of accommodations, no animal exposure) may contribute. All study subjects had asthmatic symptoms during the previous month, which in atopic patients was associated with FENO in one population study (Olin et al. 2006). In atopic patients, sensitisation to cats was strongly associated with FENO.

Our finding that the level of BHR to histamine and exercise is lower in nonatopic asthma than in high-sensitised atopic asthma is consistent with previous findings (Obase et al. 1999). Furthermore, asthmatics sensitised and then exposed to high levels of sensitizing allergens had lower baseline FEV1 and more severe BHR than did asthmatics not sensitised and exposed to relevant allergens (Langley et al. 2003). Schwartz and colleagues (2002) showed in a population study that BHR to methacholine increased with number of positive skin-prick tests. In one retrospective study in asthmatics, skin prick reactivity to aeroallergens was also associated with BHR to methacholine (Fowler et al. 2003). In our study, the degree of atopy was significant where BHR to an indirect stimulus was concerned: The magnitude of EIB was higher in high-sensitised atopic asthmatics than in nonatopic asthmatics. Similarly, the reactivity to AMP has been higher in atopic than in nonatopic asthmatics (Ludviksdottir et al. 2000). Differences in BHR between these

groups may be explained by the recent finding that accumulation of mast cells in the smooth muscle compartment is more prominent in atopic than in nonatopic asthma (Amin et al. 2005).

Our study indicates that FENO is equally elevated in nonatopic asthma and in low-sensitised atopic asthma but is lower than in high-sensitised atopic asthma among young adult conscripts. These differences in FENO between the asthma groups parallel the differences in airway function disturbance in terms of responsiveness to histamine or exercise in steroid-naive non-smoking young men.

### **9.3 Clinical implications**

Asthma has been traditionally diagnosed with lung function and PEF follow-up. These conventional measurements can only detect the functional disturbance of asthma. Based upon recent knowledge of the pathophysiology of asthma, the functional disturbance was the end result of inflammation processes. Inflammation in the airways can be measured by exhaled nitric oxide measurements; the level of FENO is directly associated with the degree of airway inflammation, particularly eosinophils. The present study showed that patients with respiratory symptoms who did not fulfil the diagnostic criteria of asthma had FENO values significantly higher than in healthy subjects but significantly lower than in asthma patients. This is in line with other studies in such patients, showing that elevated levels of FENO have been found in both the bronchial and alveolar components of FENO, and the alveolar component of FENO correlated with blood eosinophils and urinary leukotriene E4 (Lehtimäki 2003). Furthermore, sputum eosinophils have been increased in patients with symptoms suggesting asthma but with normal lung function and with their level of sputum eosinophils lower than in asthma (Ryttilä et al. 2000). When these patients were followed up for 1 year, 13% of patients developed asthma during the follow-up. Similarly, among children with asthmatic symptoms but normal lung function, 33% developed asthma during a 2-year follow-up (Remes et al. 1998). The present and other studies have demonstrated the important of early detection of bronchial inflammation before lung function disturbance caused by remodelling of asthma.

The level of FENO was associated with bronchial hyperresponsiveness measured by a direct method (histamine challenge) or an indirect method (exercise challenge) in steroid-naive symptomatic, non-smoking asthmatics. Although these associations can be found only in atopics, the level of FENO is also increased in nonatopic asthma. These findings demonstrate that FENO can be used in the diagnosis of asthma in both atopic and nonatopic patients with asthma; moreover, knowledge of the atopic status of each patient is relevant, before measurement of FENO.

The present study demonstrated that measurement of FENO had good reproducibility, and the variability of FENO was reasonable in the short- and long-term in both healthy subjects and patients with respiratory symptoms or asthma. We demonstrated the upper normal limit for healthy subjects, which was 12 ppb calculated from two different study populations (II, V). From our experience, this level was the best for distinguishing between healthy subjects and patients with asthmatic symptoms or asthma. Based on our results, the limit for a significant change after 6 hours and 24 hours was also calculated for possible future intervention studies.

In short, measurement of FENO is easy to perform and is a noninvasive method to assess inflammation in the airways. The present series of investigations thus has several important clinical indications.

These findings (Study III) suggest that BHR to histamine is a sensitive indicator of the effect of ICS and a valuable tool for adjustment of corticosteroid treatment in mild asthma. The positive correlation found between the within-subject changes in PD15FEV1 and the peak FENO suggests that both methods can serve in evaluation of treatment with ICS in asthma. The findings further suggest that intermittent treatment periods of a few weeks' duration are insufficient to provide long-term control of BHR in patients with mild persistent asthma. Furthermore, the results of the present study suggest that use of inhaled corticosteroids should be stopped 4 weeks before a diagnostic procedure for BHR to histamine.

## 10 Conclusions

1. FENO was not related to age or sex in healthy subjects. We found no significant long-term variability in FENO. These results suggest that FENO measurement can be reproducible. We found that FENO levels in healthy subjects were less than 12 ppb.
2. Patients with suspected asthma who did not fulfil the diagnostic criteria of asthma had FENO values significantly higher than in healthy subjects, but significantly lower than asthma patients. Measurement of exhaled nitric oxide by using a standardised method based on ERS guidelines was well reproducible over a short period in the short term in healthy subjects, in patients with suspected asthma, and in mild asthma.
3. A daily dose of 500 µg FP significantly reduced BHR to histamine by inducing a sustained effect within 3 days and a maximal effect within 6 weeks after the start of treatment in patients with mild asthma. After cessation of FP treatment, BHR seems to return to near pretreatment levels within 2 weeks. These findings suggest that BHR to histamine may be a sensitive indicator of the effect of ICS and a valuable tool for adjustment of corticosteroid treatment in mild asthma. The findings further suggest that intermittent treatment periods of a few weeks' duration are insufficient to provide long-term control of BHR in patients with mild, persistent asthma.
4. FENO was associated with bronchial hyperresponsiveness measured by a direct method (histamine challenge) or an indirect method (exercise challenge) only in atopics when steroid-naive symptomatic, non-smoking asthmatics were studied. These associations were non-significant in nonatopics.
5. FENO was equally elevated in nonatopic asthma and in low-sensitised atopic asthma but was lower than in high-sensitised atopic asthma among young adult conscripts. These differences in FENO between the asthma groups parallel the differences in airway function disturbance in terms of responsiveness to histamine or exercise in steroid-naive non-smoking young men.

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