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Airway Responsiveness and Inflammation in Young Children with Respiratory Symptoms

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ACADEMIC DISSERTATION

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To Ella and Anna

Abstract

Background: The diagnosis of asthma in young children is based mostly on symptoms. The need for objective methods for diagnosing asthma in this age group is therefore obvious. Lung function in preschool children can be assessed with impulse oscillometry (IOS), which involves no voluntary breathing manoeuvers. Because most children with asthma have normal baseline lung function, the use of bronchoprovocative tests may improve diagnostics. Fractional concentration of nitric oxide (FeNO) is suggested to be a good measure for airway inflammation, and the method is also available for preschoolers.

Aims: To examine new methods for evaluating airway hyperresresponsiveness and inflammation in young children. Further aims were to study the effect of parental smoking on lung function and airway inflammation in wheezy children and whether children with severe exercise induced bronchoconstriction exhibit small airways dysfunction.

Methods: A total of 272 children (3 to 8 years old), 231 with obstructive syndromes and 41 healthy controls, were examined. Children with various clinical characteristics were recruited: troublesome lung symptoms, a history of bronchopulmonary dysplasia (BPD), early wheezing symptoms and multiple-trigger wheezing. Airway hyperresponsiveness was evaluated with exercise test, methacholine and mannitol challenge tests using IOS. FeNO measurements with two different analyzers were examined. Parental reports and children's urinary cotinine measurements served to monitor exposure to environmental tobacco smoke.

Results: Exercise test with IOS succesfully identified children with probable asthma, and the methacholine challenge test was able to differentiate children with probable asthma, BPD and early wheezing from the controls. The mannitol challenge test, however, was unable to distinguish between the study groups. Furthermore, children with severe exercise-induced bronchoconstriction (EIB) exhibited small airways dysfunction. A portable FeNO analyzer proved to be more difficult than a stationary device to use in young children. In addition, its poorer accuracy in low FeNO levels diminishes its feasibility in this age group. However, a portable analyzer differentiated children with asthma from the controls. Children with smoking mothers had poorer lung function and higher FeNO than children with non-smoking mothers. Urinary cotinine concentrations closely reflected reported smoking in the family. A father's smoking had no effect on children's FeNO or lung function.

Conclusions: The exercise test with IOS succesfully identified children with probable asthma. The methacholine challenge test aids in evaluating airway hyperresponsiveness in young children, although its cut-off value for this age group requires re-evaluation. A portable FeNO analyzer can also serve as a screening tool in young children, because it differentiates asthmatics from the controls with reasonable accuracy. Children with severe exercise induced bronchoconstriction exhibited small airways dysfunction, which suggests that peripheral airways are involved even in young asthmatic children. Maternal smoking clearly deteriorates lung function and increases bronchial inflammation in young children with wheeze. This objective finding with cotinine measurements emphasizes current knowledge; young children should not be exposed to environmental tobacco smoke.

Tiivistelmä

Tausta: Pienillä lapsilla on paljon hengitystieoireita. Hengitysoireiden selvittely ja astmadiagnostiikka tässä ikäryhmässä perustuu enimmäkseen oireiden tulkintaan ja riskitekijöiden kartoitukseen. Leikki-ikäisen lapsen keuhkojen toimintaa voidaan tutkia oskillometrialla, joka ei vaadi lapselta suurta yhteistyökykyä. Valtaosalla astmaa sairastavista lapsista on normaali keuhkojen toiminta perustilassa, jolloin diagnostiikka voi täsmentyä keuhkoputkien supistumistaipumuksen määrityksellä. Uloshengityksen typpioksidipitoisuutta mittaamalla voidaan arvioida keuhkoputkien astmaattista tulehdusta. Erilaiset ärsykkeet, kuten tupakansavulle altistuminen, voivat vaikuttaa lapsen hengitysteiden astmaattiseen tulehdukseen ja keuhkojen toimintaan.

Tavoitteet: Selvittää uusien menetelmien toimivuutta leikki-ikäisen lapsen keuhkoputkien supistumisherkkyyden ja astmaattisen tulehduksen arvioinnissa. Lisäksi tavoitteena oli tutkia vanhempien tupakoinnin vaikutusta lapsen keuhkojen toimintaan.

Menetelmät: Tutkimukseen osallistui 272 (3-8-vuotiasta) lasta, joista 231 oli hengitystieoireisia ja 41 terveitä verrokkeja. Tutkimukseen osallistui astmaepäilyn vuoksi selvityksiin lähetettyjä, vastasyntyneenä bronkopulmonaalisen dysplasian (keskosen keuhkosairaus) sairastaneita sekä alle 2-vuotiaana toistuvista hengitysvaikeuksista kärsineitä lapsia. Keuhkoputkien supistumistaipumusta tutkittiin ulkojuoksukokeella sekä metakoliini- ja mannitolialtistustestillä. Keuhkojen toiminta määritettiin oskillometrialla. Keuhkoputkien astmaattista tulehdusta arvioitiin uloshengityksen tyypioksidipitoisuutta mittaamalla kahdella eri laitteella. Tupakansavulle altistumista monitoroitiin vanhempien täyttämällä kyselykaavakkeella sekä lasten virtsan kotiniinimäärityksellä.

Tulokset: Ulkojuoksukoe oskillometriaseurannassa erotteli hyvin astmaoireista kärsineet lapset muista ryhmistä. Metakoliinialtistustesti osoitti keuhkoputkien supistumista kahdella kolmasosalla kaikista tutkituista lapsista, mutta hengitysoireista kärsineet lapset olivat sille herkempiä kuin kontrollilapset. Mannitolialtistustesti ei erotellut ryhmiä toisistaan. Lapsilla, joilla oli vaikein rasituksen aiheuttama keuhkoputkien supistumistaipumus, oli myös poikkeavuutta pienten ilmateiden toiminnassa. Uloshengityksen typpioksidin mittaaminen on vaikempaa kannettavalla mittarilla kuin isommalla laitteella, ja myös laitteen huonompi tarkkuus matalilla pitoisuuksilla rajoittaa sen käyttöä pienillä lapsilla. Tupakoivien äitien lapsilla oli huonompi keuhkojen toiminta ja enemmän keuhkoputkien astmaattista tulehdusta kuin tupakoimattomien äitien lapsilla. Isien tupakoinnilla ei ollut samanlaista vaikutusta lasten keuhkojen toimintaan. Virtsan kotiniinipitoisuudet kuvasivat hyvin vanhempien ilmoittamaa tupakointia kotona.

Johtopäätökset: Ulkojuoksukoe yhdistetty oskillometriatutkimukseen on hyvä tutkimus leikki-ikäisen astmaoireiden selvittelyssä. Metakoliinialtistus on tämän tutkimuksen mukaan toimiva menetelmä keuhkoputkien supistumistaipumuksen selvittelyssä, mutta pienten lasten viitearvojen puuttuminen rajoittaa sen käyttöä tässä ikäryhmässä. Kannettavaa uloshengityksen typpioksidimittaria voidaan käyttää seulontamielessä myös pienillä lapsilla. Äitien tupakointi huonontaa astmaoireista kärsivien lasten keuhkojen toimintaa ja lisää keuhkoputkien astmaattista tulehdusta. Tämä objektiivinen löydös vahvistaa käsitystä, että pienen lapsen ei tulisi altistua tupakansavulle.

Contents

List of original publications

This thesis is based on the following publications:

I   Kalliola S, Malmberg P, Rito T, Pelkonen AS, Mäkelä MJ. Can we use portable nitric oxide analyzer in young children? Pediatr Pulmonol 2011; 46:627-31.

II   Kalliola S, Pelkonen AS, Malmberg LP, Sarna S, Hämäläinen M, Mononen I, Mäkelä MJ. Maternal smoking affects lung function and airway inflammation in young children with multiple-trigger wheeze. J Allergy Clin Immun 2013: 131(3):730-5.

III   Kalliola S, Malmberg LP, Kajosaari M, Mattila PS, Pelkonen AS, Mäkelä MJ. Assessing direct and indirect airway hyperresponsiveness children using impulse oscillometry. Ann Allergy Asthma Immunol 2014: 113(2):166-72.

IV   Kalliola S, Malmberg LP, Pelkonen AS, Mäkelä MJ. Small airways dysfunction during induced bronchoconstriction in young children. Submitted.

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

Abbreviations

AHR	airway hyperresponsiveness
AMP	adenosine monophosphate
API	asthma predictive index
ATS	American Thoracic Society
AUC	area under curve
AX	reactance area
BAL	bronchoalveolar lavage
BPD	bronchopulmonary dysplasia
CI	confidence interval
COPD	chronic obstructive pulmonary disease
CV	coefficient of variability
ECP	eosinophilic cationic protein
EIB	exercise-induced bronchoconstriction
ELISA	enzyme-linked immunosorbent assay
ERS	European Respiratory Society
ETS	environmental tobacco smoke
EVH	eucapnic voluntary hyperpnea challenge
EW	early wheezer
FeNO	fractional concentration of nitric oxide
FEV_1	forced expiratory volume in one second
FOT	forced oscillation technique
Fres	resonance frequency
FVC	forced vital capacity
GWAS	genome-wide association study
HRCT	high-resolution computer tomography
HRV	human rhinovirus
Hz	Hertz
ICON	International consensus on pediatric asthma
IgG	immunoglobulin G
IgE	immunoglobulin E
IL	interleukin
INFγ	interferon gamma
IOS	impulse oscillometry
MBW	multiple-breath washout
MBNW	multiple-breath nitrogen washout
MEF_{25-75}	mean expiratory flow between 25% and 75% of vital capacity
MEF_{50}	mean expiratory flow at 50% of vital capacity
NO	nitric oxide
NOS	nitric oxide synthase
cNOS	constitutive nitric oxide synthase
iNOS	inducible nitric oxide synthase
OR	odds ratio

PCR	polymerase chain reaction
$PD_{20}FEV_1$	provocative dose to induce a 20% decrease in FEV_1
$PD_{40}R5$	provocative dose to induce a 40% increase in R5
PEF	peak expiratory flow
PIF	peak inspiratory flow
PNEC	pulmonary neuroendocrine cells
ppb	parts per billion
R5	resistance at 5 Hz
R20	resistance at 20 Hz
R5-20	difference between R5 and R20
RIA	radioimmunoassay
Rint	interrupter resistance
ROC	receiver-operator characteristics
Rrs	resistance
RSV	respiratory syncytial virus
SPT	skin prick test
sRaw	specific airway resistance
TNF-β	tumor necrosis factor beeta
TLC	total lung capacity
TLS	troublesome lung symptoms
X5	reactance at 5 Hz
X10	reactance at 10 Hz
Xrs	reactance
Zrs	impedance

1 Introduction

Wheezing in early childhood is common, but the underlying pathologies are heterogeneous. A viral infection is the most frequent cause of preschool wheezing, and up to 50% of all children wheeze at least once before their sixth birthday (1). Considering the high prevalence of wheezing, the symptom is an important health care issue worldwide. Researchers have proposed several wheezing phenotypes for preschool wheezing based on the large cohort studies. Transient, late-onset and persistent phenotypes are characterized according to the patient's age at onset and the duration of the symptoms (2). The temporal pattern of the symptom indicates the wheeze type: episodic (viral) and multiple-trigger (3). Episodic wheezers wheeze only during the viral infections, whereas multiple-trigger wheezers wheeze both during and outside descrete episodes. Both infections and other triggers such as allergens, tobacco smoke exposure and exercise can cause symptoms for multiple-trigger wheezers. However, wheezing phenotypes offer only modest help in the evaluating of preschool children, because the analysis is based on the retrospective studies. In addition, children frequently move from one phenotype to another during the preschool years and more than half of these children stop wheezing before they start school (1). Many children who had suffered bronchopulmonary dysplasia (BPD) also experience respiratory symptoms during their childhood, symptoms that may mimic asthma (4). Consequently, finding children who benefit from asthma medication and require follow-up in this large group of wheezers is challenging.

Some of the wheezers develop asthma, which is characterized by bronchial inflammation and airway hyperresponsiveness. Asthma diagnosis is based on assessment of lung function in school-age children and adults, but assessing lung function in preschoolers may be challenging. Impulse oscillometry (IOS) however, requires no voluntary breathing manoeuvers and over 80% of preschool-age children can perform it successfully (5). Assessing of airway hyperresponsiveness (AHR) with IOS may aid in evaluation of respiratory symptoms, because most of the mild and moderate asthmatic children exhibit normal baseline lung function. AHR can be assessed with direct and indirect challenge tests. Direct tests are highly sensitive but less specific for asthma. Methacholine is thought to act directly on smooth muscle cells in the bronchi and is widely used to exclude asthma (6). Indirect challenge tests, such as exercise and mannitol challenges, act through mediators, which are released by eosinophils, mast cells and neuronal cells (7). Based on their mechanism of action these tests are associated with bronchial inflammation, unlike direct tests, which are considered to be rather independent from inflammation. Indirect tests are therefore thought to be more specific than direct tests in diagnosing asthma.

The fractional concentration of nitric oxide (FeNO) is suggested to reflect airway inflammation (8). The method is both noninvasive and available to young children. A conventional, stationary device is available mostly in specialized clinics, but a portable, hand-held device can also be used ouside of these centers. Some have suggested that the

hand-held device is more difficult to use than the older device, whether this device is suitable for young children remains unknown.

Recently, research has linked small airways dysfunction to severe asthma. Researchers have proposed the IOS parameter R5-20 as a measure for small airways function (9). Others have also suggested that, when it comes to their peripheral airways, asthmatics respond in distinct ways to bronchoprovocative tests. Small airways dysfunction during methacholine challenge has been associated with severe asthma in adults (10). Knowledge of the peripheral airways function in young children is scarce.

Exposure to environmental tobacco smoke (ETS) is a well-known risk factor for childhood respiratory symptoms. ETS is also known to decrease lung function (11), but little is known about its effects on bronchial inflammation. Parental reports often monitore smoking at home, but measuring of cotinine levels in body fluids and hair offers a more objective method for estimating ETS (12).

Diagnosing asthma in young children is usually based on symptoms. The aim of this study was to evaluate new methods for determing objective airway hyperresponsiveness and assessing bronchial inflammation in young children. We used impulse oscillometry to study direct and indirect airway hyperresponsiveness tests; we specially inquired whether these tests could differentiate various patient groups from one another. We used IOS parameters reflecting peripheral airways function to examine small airways function in relation to asthma severity. In addition, we investigated the feasibility of a portable FeNO analyzer in young children. Finally, we studied the effect of parental smoking on children's lung function and airway inflammation.

2 Review of the literature

2.1 Obstructive respiratory symptoms in early childhood

Respiratory symptoms in the early years of life are extremely common. Typical obstructive symptoms in preschool children include recurrent cough, difficulty in breathing, chest tightness and wheezing (13). Large population studies have revealed that approximately one third of all children have at least one wheezing episode before the age three (2,14,15). Cohort studies indicate that the cumulative prevalence of wheezing during the first six years of life is 16-49% (2,16-24). Wheezing may result from number of conditions, but most wheezing episodes are associated with viral infections, which occur frequently in young children. Considering the high prevalence of wheezing in preschool children, the problem is a serious health care issue worldwide (14). Although the understanding of preschool wheezing has improved, our management of wheezing disorders remains challenging.

2.1.1 Phenotypes of preschool wheezing

Several large population-based studies have deepened our understanding of early obstructive symptoms and classification of wheezy children into distinct phenotypes. Table 1 shows basic data and the prevalence of preschool wheezing phenotypes in ten cohort studies.

The landmark study of wheezing disorders in young children, the Tucson Children's Respiratory Study in Arizona, followed 826 children from birth to the age of six years (2). The participitants in this cohort study were enrolled between 1980 and 1984. The children were investigated in their infancy, as well as at ages three and six. Assessment included IgE measurement, lung function testing, skin prick tests and questionnaires completed by the children's parents. The children in this study were divided into never wheezers (51.5%), transient early wheezers (19.9%, stopped wheezing before the age of 3), late-onset wheezers (15%, bagan wheezing after 3 years of age) and persistant wheezers (13.7%, wheezed both before and after 3 years of age) based on their age at onset and the duration of their symptoms (2) (Table 1).

The children of the Tucson cohort underwent further analysis at 11 years of age (25), which included peak expiratory flow (PEF), the methacholine challenge test, skin prick testing and serum IgE. Transient early wheezing was independent of airway hyperresponsiveness, and non-atopic wheezing in toddlers and young schoolers was associated with PEF variability, but not with a positive methacholine test result. IgE-associated wheezing/asthma was associated with persistent wheezing at any age, male sex, airway hyperresponsiveness to methacholine, PEF variability and positive atopy markers. The children of the Tucson cohort are still being followed up. Their follow-up data at 22

years of age indicated that asthma onset at 6 years, persistent wheezing in early life, sensitization to Alternaria alternata, reduced lung function and airway hyperresponsivenss at 6 years were independent risk factors for chronic asthma at the age of 22 (26).

The German Multicentre Allergy Study (MAS) followed 1314 children annually from birth to seven years of age (16). The overall wheeze prevalence was 35% (Table 1). The researchers found that children with wheezing episodes during only the first three years of life showed slight impairment in lung function at seven years of age. Children with persistent or late-onset wheezing, however, showed a significant decrease in expiratory flow volumes at seven years of age. This study indentifies several risk factors for lung function impairment in persistent wheezers: a history of parental atopy, time in years since the first wheeze episode, sensitization to indoor allergens, elevated cord IgE and a low ponderal index at birth (16). Maternal smoking was more common in all groups of wheezers than in the group in which children never wheezed (16).

A British cohort study (Isle of Wight) investigated 1456 children at 1, 2, 4 and 10 years of age to determine the natural history of childhood wheezing (21). Of these children 40% wheezed at least once before the age of 10, half of the wheezers were early transient wheezers, 30% were persistent wheezers and 19% late-onset wheezers (Table 1). In this study, 37% of early wheezers (onset before 4 years of age) continued to wheeze at the age of 10. These persistent wheezers had more physician-dignosed asthma before two years of age, hospital admissions, special referrals and use of inhaled corticosteroids than did transient early wheezers. In addition, they were more reactive to methacholine than were transient wheezers. Both groups had lower lung function at the age of ten years than did never-wheezers. Late-onset wheezers had a similar airway hyperresponsiveness pattern to that of early wheezers (21).

The ALSPAC study, another British cohort, used annual questionnaires to follow 6265 children from birth to age seven (18). The study assessed the children's lung function, AHR and atopy at seven to nine years of age. This large cohort revealed six different wheezing phenotypes (Table 1) and an overall wheezing prevalence of 41% (18). Repeated symptom-based evaluation yielded a novel intermediate-onset phenotype with onset after 18 months of age. The study also found a strong association between intermediate phenotype and atopy and AHR (18). The PIAMA study from the Netherlands also gathered annual data (22,27,28). This latter study reported similar findings to the ALSPAC study regarding phenotypes of preschool wheezing. However, the prevalence of wheezing was lower than in ALSPAC study (25%) (22). The PIAMA study had complete follow-up data on 2180 neonates until eight years of age (22) (Table 1). The MAAS cohort from the UK examined preschool wheezing in 1085 children (17,29-31). Of these children, 840 attended the five-year follow-up visit. The children were evaluated at 3, 5, 8 and 11 years of age. The overall prevalence of wheezing at preschool age was 44% and around half of all wheezers were categorized into the transient phenotype (17) (Table1).

Three large Scandinavian investigations have explored these questions. The ECA study from Norway recruited 3754 newborns and distributed questionnaires to their parents every six months until the newborn reached the age of two in order to evaluate their symptoms (20,32,33). Lung function was assessed at infancy, at 10 and 16 years of age. The researchers assessed high prevalence of never wheezers (69%) (20) (Table 1). The BAMSE study from Sweden used more stringent criteria for asthma/wheezing than did other studies with other cohorts, reporting the lowest prevalence of these cohorts (16%) (23) (Table 1). The COPSAC study from Denmark focused on troublesome lung symptoms rather than wheezing and found a symptom prevalence of 46% (34) (Table 1). These children were born to atopic mothers and were considered to be at high risk for atopic diseases. The study suggested that a quantitative measure of troublesome lung symptoms during the first three years of life is a better predictor of asthma than the observation of wheeze (35). In the final analysis the total number of acute clinic visits for asthma symptom was associated with later asthma, but the wheezing was not(35).

The PASTURE study evaluated effects of living on a farm on allergic diseases among a large birth cohort drawn from five European countries: Austria, France, Germany, Switzerland and Finland (24). The study investigated wheezing by distributing annual questionnaires to the caregivers of 953 children until they reached the age of six. The prevalence of wheezing in this cohort was 30%, the most common phenotype of which was transient wheezing (16.8%) (Table 1) (24). Unremitting wheeze was suggested as promising alternative asthma definition for epidemiologic studies.

Further attempts to clarify heterogeneous wheezing phenotypes in young children have resulted in recently published reports (36). A French study analyzed 20 variables among 551 wheezy children younger than 36 months (37) and identified three clusters of wheezy young children: mild episodic viral wheezers (59%), nonatopic uncontrolled wheezers (despite of high doses of inhaled corticosteroids) (29%) and multiple-trigger wheezers with eczema and increased spesific IgE levels (12%). Atopic multiple-trigger wheezing was associated with allergenic factors, male sex, and uncontrolled nonatopic wheezing with infectious factors such as day care attendance and female sex (37). A British birth cohort study of 1184 children revealed a novel group of persistent troublesome wheezers (3.2% of the cohort children) (38) that suffered substantially more exacerbations, hospitalizations and unscheduled health care visits than did all other groups.

The European Respiratory Society (ERS) Task Force proposed somewhat different phenotyping system in 2008 by suggesting the definitions 'episodic' (viral) and 'multiple-trigger wheezing' to describe different phenotypes of preschool wheezing (3). Episodic (viral) wheezing refers to those children who wheeze intermittently during the viral infections but are well between the episodes. Multiple-trigger wheezers wheeze both during and outside discrete episodes. An episodic pattern is usually caused by viral infections, declines over the time and disappears by the age of six, but may continue into school age as episodic wheezing, change to multiple-trigger wheezing or disappear later in life (3). If other than viral triggers, such as allergen exposure or environmental tobacco

smoke, cause wheezing in a preschooler, the ERS Task Force suggests the definition of 'multiple-trigger wheezing' and recommended caution with the term 'asthma' in preschool-age children (3) based on the heterogeneity of the symptom entity.

Many of the cohort studies have suffered from substantial loss of the children's participation in follow-up (39). The largest cohorts (ASLPAC and PIAMA) saw the highest numbers of phenotypes (six and five, respectively) (22). These two studies also evaluated symptoms on an annual basis. The Tucson study examined children at infancy, as well as at three and six years of age (2). Most of these studies used population-based birth cohorts, but an exception is the COPSAC study, which enrolled children with atopic mothers (40). Different diagnostic methods in the BAMSE study resulted in a low prevalence of wheezing (23).

Table 1. Preschool wheezing phenotypes in ten cohort studies.

Cohort	Patients	Methods	Wheezing phenotypes	Other outcomes
Tucson study, Tucson, Arizona,USA (2) Recruitment 1980-84	1246 newborns population based cohort 826 followed up to 6 years	Cord serum IgE (n=750) Lung function at infancy (176) IgE at 9 months (672) At 6 years: IgE (460), lung function (526), skin prick test (629)	Never wheezers: 51.5 % Transient early wheezers: 19.9% (at least one wheezing episode before 3 years, but not afterwards) Late-onset wheezers: 15% (wheezing only after 3 yrs) Persistent wheezers:13.7% (wheezing both before and after 3 years of age)	Transient wheeze associated with diminished lung function in infancy Persistent wheezing associated with elevated IgE Maternal asthma associated with decreased lung function at 6 years of age Maternal smoking a risk factor for wheezing
Isle of Wight, UK (21) Recruitment 1989-1990	1456 newborns, population based 1034 attended all study visits	Follow-ups at 1, 2 and 10 years, questionnaires Lung function (859) and AHR at 10 yr, skin prick test, IgE	Never wheezers: 60% Transient: 20% Late-onset: 8% Persistent: 12%	Persistent wheezing associated with atopy, increased AHR and impaired lung function
MAS Multiple cities, Germany (16) Recruitment 1990	1314 newborns, birth cohort, 815 unselected and 499 high-risk, 939 followed up to 7 years	Annual check-ups: interview, specific IgE Lung function in 800 children at 7 years	Never wheezers: 65% Transient: 25% Late-onset: 5.3% Persistent: 4.9%	Transient wheezers had normal/subnormal lung function at 7 years of age Persistent and late-onset wheezers had impaired lung function at 7 years of age
ALSPAC Avon, UK (18) Recruitment 1991-92	14 541 newborns, population based, 6265 followed up to 7 years of age	Questionnaires at 7 time points from birth to 7 years Assessment of atopy, lung function, AHR at 7-9 years	Never/infrequent wheezers: 59% Transient early: 16% Prolonged early: 9% (wheezing from age 6-54 months, low prevalence at 69 months and later) Intermediate: 3% (onset 18 – 42 months) Late-onset: 16% Persistent: 7%	Atopy and AHR associated most strongly associated with intermediate onset wheezing

ECA Oslo, Norway (20,32) Recruitment 1992-93	3754 newborns, birth cohort 803 investigated at 10 years	Questionnaires every 6 months until 2 years of age, lung function at birth (n=803), at 10 years (n=616) and 16 years (550), skin prick test	Never wheezers: 69% Transient: 9% Late-onset: 9% Persistent: 13%	At 10-16 years, 34% recurrent wheezers < 2 years were free of asthma symptoms, medication or increased AHR at 16 years. All symptomatics had lower lung function than never wheezers.
BAMSE Stockmann, Sweden (23) Recruitment 1994-96	4089 birth cohort, population based 2965 at 4 years 2630 at 8 years	Questionnaires at 1, 2, 4, and 8 years, physical and lung function examination at 4 (n=2965) and 8 (n=2630) years of age	Never asthma: 84% Transient: 9% Late-onset: 3% Persistent: 4%	More stringent criteria for asthma/wheezing than in other cohorts Transient and persistent symptoms associated with reduced lung function at 8 years
MAAS Manchester, UK (17,29,30) Recruitment 1995-97	1085 children, population based 840 attended 5 year follow up	Interview, lung function at 3, 5, 8 and 11 years AHR at 5 years Skin prick test	Never wheezers: 56% Transient: 23% Late-onset: 6% Persistent: 15%	Poor lung function at age 3 predicted persistent symptoms in children who had wheezed before the age of 3
PIAMA Multicenter, Netherlands (22,27,28) Recruitment 1996-97	4146 newborns 1327 high risk (maternal atopy) 2819 low risk 3164 follow-up at 8 years	Yearly questionnaires Feno at 4 (1808) and 8 (1554) years, spirometry at 8 years, total and specific IgE, eosinophil count	Never/infrequent: 75% Transient early: 16.7% Intermediate-onset: 3.1% Late-onset: 1.7% Persistent: 3.5%	All wheezing phenotypes had a lower predicted FEV1% predicted than did never-wheezers, with the greatest deficit in persistent wheezers
COPSAC Copenhagen, Denmark (34,41) Recruitment 1998-2001	411 newborns with atopic mothers 307 had all data available at 6 years	Troublesome lung symptoms assessed daily, recorded at home lung function in infancy (403) and at 7 years (317), skin prick tests, IgE	No symptoms: 54% Transient: 18% Late-onset: 11% Persistent: 17%	Children with asthma at age of 7 suffered lung function deficit and increased AHR as newborns(41) Analysis based on troublesome lung symptoms rather than wheezing
PASTURE Austria, Finland, France, Germany, Switzerland (24) Recruitment 2002-05	1133 birth cohort in rural areas 953 complete data on wheezing	Yearly questionnaires Specific IgE FeNO Spirometry at 6 years	No/infrequent: 70% Transient: 16.8% Intermediate: 5.9% Late-onset: 4% Persistent wheeze 3.3%	International study Used both latent class analysis and clinical phenotyping In utero smoke exposure was related to reduced FEV1 in children with unremitting wheeze

2.1.2 Bronchopulmonary dysplasia and lung function in early childhood

Improvements in perinatal care have substantially increased the survival of extremely preterm infants (4). Bronchopulmonary dysplasia (BPD) usually affects infants born very preterm and is characterized by dysregulated lung growth and prolonged oxygen dependency (42). The definition of BPD is based on the need for supplemental oxygen for at least 28 days after birth, and the severity of the disorder is graded according to need for respiratory support at 36 postmenstrual weeks (4). BPD most often affects infants born before 30 weeks of gestational age and weighing less than 1500 g at birth (4). Estimates indicate that BPD devolopes in 12% to 32% of these infants (4,43) and may also affect term infants with severe perinatal disorders (4).

Before the substantial advances in perinatal care the "old BPD" occured in slightly preterm infants receiving aggressive mechanical ventilation and high concentrations of supplemental oxygen (44). More effective methods of prevention with antenatal corticosteroids, surfactant replacement after birth and a more conservative approach to respiratory care have reduced the prevalence of this old BPD (4). However, higher survival rates for infants with earlier gestational ages have meant that the overall prevalence of BPD remains unchanged. These very premature infants are born before the alveolarization has begun (4). The loss of normal structures of the lung now characterize a "new BPD" pattern: the development of fewer, larger alveoli with dysmorphic vasculature, which reduce the overall surface for gas exchange (43,45).

Wheezing in infants born prematurely is markedly higher than in children born at term (4). Hospital readmission rates due to respiratory infections are high in children born before the gestational age of 29 weeks (approximately half of the infants in their first year of life) (46). The infants with BPD are the most vulnerable and suffer from more severe symptoms during the respiratory infections (4). Several studies have revealed that children who were born preterm experience more wheezing and chronic coughing at the preschool and school age, especially those with a history of BPD (47,48). Asthma-like symptoms and the use of inhaled corticosteroids are significantly more prevalent among young children born prematurely than among those born at term (49-52).

Weaning from supplemental oxygen in BPD children usually occurs before 2 years of age (43), presumably due to postnatal alveolar growth during the first two years of life (53). Studies have found that children and adults with a history of BPD show abnormal diffusion capacity and impaired small airway expiratory flow (54,55). A Finnish study examined 34 very low birth weight children at seven to eight years of age (56). One third of these children experienced respiratory symptoms during the exercise or in contact with cold air. They had lower forced expiratory volume in one second (FEV_1), higher airway resistance and higher rates of airway hyperreactivity than did term children (56). An earlier Finnish study showed that children born very preterm exhibited increased bronchial hyperresponsiveness, bronchial obstruction and bronchial lability at school age (57). A

recent study from Norway found lower FEV_1 values in children born at a gestational age $\leq$ 28 weeks or with a birth weight $\leq$ 1000 g than in term-born controls (58); their lung functions were assessed at the ages of 10, 18 and 25 years. Researchers used methacholine and adenosine 5'-monophosphate (AMP) challenge tests with a modified auscultation technique to evaluate preschool-age BPD survivors and asthmatics. This study found that 89% of children with a BPD history responded positively to methacholine, but only 21% responded positively to AMP. All asthmatics responded positively to methacholine and 92% had a positive AMP result (59). A recent report also raised concerns about reduced exercise capacity in persons born very preterm (43).

Despite substantial advances in perinatal care for premature births, the BPD survivors continue to experience increased respiratory morbidity until adulthood. Although the long term effects of the BPD remain unknown, persons who have suffered from BPD in the neonatal period may be at great risk for developing the COPD phenotype as they age (43).

2.2 Risk factors for childhood asthma

The prelavence of asthma has risen in recent decades in industrialized countries (15). According to surveys based on questionnaire data, asthma affects 5% to 16% of people worldwide (15). Knowledge of the risk factors associated with asthma has advanced considerable in recent years, mainly due to large cohort studies focused on this issue. Heredity is widely considered important in asthma, but the rapid change in the prevalence of asthma cannot result purely from genetic changes. Environmental and infectious factors are known to be relevant risk factors for childhood asthma. Genome-wide association studies (GWAS) have studied thousands of asthma cases and controls to identify associations between genes and the disease (15,60).

For most asthmatics, asthma symptoms present early in life during their preschool years. The strongest predictor for severe disease and persistent symptoms seems to be a persistent bronchial obstruction (61,62). In mild asthmatics the lung function is not substantially different from that of subjects without asthma; more severe asthmatics, however, seem to show deterioration in lung function already in infancy, though the most significant loss of lung capacity is thought to occur during the preschool years (15). The preschool years therefore constitute an important period of life, when it may be possible to influence the outcome of asthma later in life by modulating the risk factors and making accurate diagnoses and treatment decisions.

2.2.1 Family history / genetics

A Finnish twin study in the 1990s investigated the heredity of asthma and atopic diseases (63) by mailing a questionnaire to 2483 twin families. At the time of the study,

the twins were 16 years old. According to this report, families in which at least one of the parents had asthma, hereditary factors explained as much as 87% of their susceptibility to asthma (63). The cumulative incidence of asthma in twins with asthmatic parents was four-fold the incidence of twins without parental asthma.

In a Spanish population study of 2646 individuals, the risk for asthma in young adulthood was 4.5 times higher in subjects with parental asthma than in subjects without parental asthma (64). In a birth cohort from New Zealand, 18-year-olds with parental asthma or hay fever had a 1.5-fold higher incidence of asthma than did subjects with no parental asthma or hay fever (65).

Genome-wide association studies (GWAS) have reported several asthma-related gene loci in at least chromosomes 1, 2, 5, 6, 9, 15, 17 and 22 (15). In most cases, other studies have failed to replicate the genes reported in one GWAS with the exception of the 17q12-21 gene (encoding ORMDL3-GSDMB), which appears to be associated with childhood asthma but not with adult asthma (15,66). The PASTURE birth cohort study, which also included Finnish children, found an association between the persistent wheezing and the locus 17q21 (24). Nevertheless, associations of these loci with asthma are weak and probably account for only a small part of the disease's heritability. Multiple genes seem to affect the pathogenesis of asthma, but the current genetic knowledge offers little prognostic utility (15).

2.2.2 Infections

Viral infections are the most important causes of preschool wheezing. Bronchiolitis refers to a lower respiratory tract infection with bronchus obstruction, edema, cellular debris and mucus, and resulting symptoms of wheezing(67). Based on the population studies, the prevalence of bronciolitis is 18-32% in the first, 9-17% in the second, and 4-12% in the third year of life (68,69). The need for hospital admission with bronchiolitis among all infants under one year of age is 2-3% (70). The major viruses that cause wheezing are respiratory syncytial virus (RSV), human rhinovirus (HRV), human metapneumovirus and influenza viruses (71). A recent multicenter study of 2207 children under two years of age with hospitalization due to bronchiolitis, found RSV in 72%, HRV in 25.6%, other viruses or bacteria in 7.8% and multiple pathogens in 29.8% of children (72).

RSV is the most common cause of viral bronchiolitis in infants during the epidemic winter months, particularly in the first six months of life (72). The histopathological findings in cases of fatal RSV bronchiolitis have revealed extensive airway epithelium damage and small airway obstruction (73). A Swedish study followed 46 subjects who required hospitalization due to RSV bronchiolitis in their first year of life up to the age of 18 years (74). They found a higher prevalence of asthma or recurrent wheezing (39% vs

9%), sensitization to perennial allergens (41% vs 14%) and clinical allergy (43% vs 17%) in children with a history of RSV than in control subjects at the age of 18 (74). Lung function, as measured with spirometry, was reduced in individuals with past RSV bronchiolitis. Another Swedish study found a ten-fold higher risk for adult asthma after severe early wheezing over that of age-matched control subjects (75). Earlier reports have shown a higher prevalence of asthma and allergic sensitization at the ages of 3, 7 and 13 years after brochiolitis (76-78). However, the Tucson birth cohort found RSV bronchiolitis to be a significant risk factor for wheezing up to 11 years of age, but not at the age of 13 years (68). The severity of RSV bronchiolitis seems to be the strongest determinant of the future asthma risk related to RSV in infancy (79). The question of whether RSV bronchiolitis is a causal or genetic predisposing factor for asthma remains unanswered. Some have argued that atopic diseases and viral infections may interact in multiple ways to promote asthma in childhood. A genetic association study of RSV bronchiolitis in infancy found that the haplotype at the IL13-1L4 locus raises the risk for severe primary RSV bronchiolitis in infancy (80). This haplotype is associated with increased IL-13 production and atopic sensitization (80).

Human rhinovirus (HRV) is the second most common cause of wheezing disorders during the first half year of life and becomes the most common pathogen after the first birthday (81). After the development of PCR techniques, some have recognized that HRV causes not only common colds, but also lower respiratory tract infections (71). Reseachers have identified at least 100 HRV serotypes and classified them into three groups (A, B, C) (82). Rhinovirus bronchiolitis in infancy and asthma later in childhood are strongly associated (83,84). A prospective follow-up study of 259 children with wheezy bronchiolitis before three years of age found a ten-fold increase in asthma risk in children with wheezy HRV and a 2.6-times higher asthma risk in children with RSV at the age of six (84).

The mechanisms by which HRV increases the risk for asthma remains unclear, but immunological factors are suggested to play a role. Researchers have proposed that HRV damages the airway epithelium and consequently causes asthma (85). Another theory postulates that some children are genetically predisposed to wheezing. In these individuals HRV may act synergistically in a process, which leads to asthma (85). Low interferon responses and atopy have been associated with HRV bronchiolitis (86). A low interferon-gamma (INF-γ) level in cord blood has proved to be a risk factor for wheezing illnesses (87), and is also associated with atopy in childhood (71,88). Others have also suggested that a minor group of wheezy infants have aeroallergen sensitization, which predisposes them to severe wheezing during HRV infection (88). In addition, experimental studies on HRV infections have shown increased pro-inflammatory mediator production in the bronchial epithelium (89). Interestingly, reduced Th1 and increased Th2 cytokine production have been reported during a HRV infection (90).

The current understanding is that viruses other than RSV and HRV play only a minor role in the pathogenesis of early wheezing and later asthma. Viral bronchiolitis in early

life seems to be a major risk factor for wheezing and asthma in later life. A recent Finnish study showed that asthma was currently present in 20% of subjects with bronchiolitis in infancy, whereas the prevalence in controls at the age of 27 years was 5% (91). Whether the viral bronchiolitis in infancy is a causal factor or a reflection of a predisposition remains unclear.

2.2.3 Environmental factors

The main origin of the air pollutants is the combustion of fossil fuels (92). In urban areas, a major source of air pollution is transportation-related emission. Chronic exposure to diesel exhaust particles in childhood has been associated with increased risk for persistent wheezing and asthma (93). Several studies have found associations between the traffic pollution and childhood respiratory symptoms and sensitization to allergens (92). The mechanisms, for how air pollutants cause asthma or airway inflammation, are not fully understood, but evidence suggests that oxidative stress, the induction of persistent inflammation and epigenetic mechanisms are involved in the pathogenesis (92). Others have also suggested that exposure to indoor moulds is positively associated with the asthma incidence in children (94). The proposed mechanisms may not be associated with allergies, but the induction of inflammation due to fungal chitin may play an important role (95).

Several published reports have explored the effects of living on a farm in childhood on the development of atopic diseases. The original hygiene hypothesis refers to the assumption that unhygienic conditions, large families and overcrowding reduce the incidence of atopic diseases and asthma (96). Exposure to microbial diversity reportedly suppresses Th2 immunity, but this may be an oversimplification (96). Nevertheless, studies repeatedly show that living on a farm in early childhood or while in utero protects against asthma and atopy (97,98). A Finnish birth cohort study of 410 children showed that the quantity of microbial exposure in early childhood predicted asthma at the age of six years (99). Those at highest risk for asthma had a median level of microbial exposure (99).

Interestingly, the large multicenter study CABRIEL, found that children living on farms are protected from wheezing independently of atopy (100). The study analyzed 8023 children stratified according farming exposure. The prevalence of current wheezing among nonatopic children exposed to a farm environment was lower than in nonexposed children (100). This finding supports the concept that an imbalance of Th1/Th2 immunity does not exclusively explain the hygiene hypothesis (96). Research suggests that a farm environment may influence airway inflammation through antiviral properties and alter the microbiome of the airways (100).

2.2.3.1 Environmental tobacco smoke

Exposure to tobacco smoke products is a global health risk factor, especially for respiratory health, but also lung cancer, cardiac disease, chronic obstructive lung disease, sudden infant death syndrome, middle ear infections and asthma (101). Estimated worldwide mortality due to smoking is 5.7 million people annually (102). Nor are the harmful effects of smoking restricted to the active smoker. Exposure to environmental tobacco smoke (ETS) influences the unborn baby and child living with smoking parents. Research has suggested that the exposure level of a fetus of a smoking pregnant woman is 20 times higher than exposure level in ETS (103). Finnish legislation has diminished exposures to ETS in public and working places since 1985 (104), but a child can still be exposed to ETS at home. However, studies have shown smoke-free legislation to be to be associated with reduced pre-term births and hospital attendances for asthma (102).

According to the latest national statistics, 21% of Finnish men and 14% of Finnish women smoked in 2012 (105). The same statistics show that 16.6% of the pregnant women smoke. Children can be exposed to tobacco smoke not only at home but also in public places. ETS contains thousands of harmful chemicals, including both nitric oxide and superoxide (106), and consists of sidestream smoke from burning cigarettes and exhaled mainstream smoke (11). At least 50 ETS chemicals are known to cause cancer, heart diseases and respiratory illnesses such as asthma and COPD (11). Because sidestream smoke is produced at a lower temperature and under more reducing conditions than mainstream smoke, many toxicants are generated in higher concentrations in sidestream smoke than in mainstream smoke (107). Moreover, many chemicals are present in different phases in various constituents; nicotine, for example, is present in the particulate phase in mainstream smoke and in the vapour phase in sidestream smoke (11). Particle sizes in sidestream smoke are smaller than in mainstream smoke, and are therefore more easily penetrated deeper into the lungs and smaller airways of children (11).

Studies have shown that exposure to maternal prenatal smoking reduces newborn lung function. Some studies have measured lung function several weeks after birth, making it difficult to distinguish the effects of pre- and postnatal ETS (101). A Norwegian study which assessed the lung function of infants with smoking mothers on their second to fifth day of life showed a dose-response association between reduced lung function and exposure to tobacco smoke (108). British (109) and Australian (110) studies have reported similar findings and even in prematurely born infants with smoking mothers (111). Swedish researchers showed that pre- and postnatal ETS increased the risk for asthma in early adulthood (112). They also found that a connection between in utero smoke exposure and asthma appears to be mediated via the development of airway hyperresponsiveness. Furthermore, smoke exposure in infancy increased smoking in adulthood, which is linked to adult asthma (112).

Knowledge of the exact mechanisms for how ETS reduces lung function in newborns is lacking. The placenta offers no protection against nicotine or other pollutants (11). Nicotine affects placental function by inducing epinephrine release into the maternal blood and thereby reduces circulation in placenta (11). Consequently, some researchers have speculated that the smoking during pregnancy generally inhibits growth leading to smaller infants and lungs (101). However, several studies also show consistent findings of reduced lung compliance in infants, suggesting structural changes in the lungs (113). Studies have also found a significant increase in thickness of the inner airway wall in children with sudden infant death and those exposed to tobacco smoke (114). Another study of fetal tobacco smoke exposure found fewer and larger saccules in exposed rats than in non-exposed rats (115). Evidence suggests the involvement of pulmonary neuroendocrine cells (PNEC) in lung defects caused by ETS. PNECs are present in large numbers during the fetal period, but decrease rapidly during the first year of life (101). Several respiratory conditions, such as asthma, sudden infant death and BPD, are associated with high numbers of PNECs (116). Animal models have shown an association between altered fibrillar collagen expression and nicotine receptors in lungs (117).

ETS and an increased prevalence of asthma are associated (11). In a Finnish cohort study of 60 000 children found a dose-response fashion with maternal smoking during pregnancy and asthma in seven year old children was found: if a mother smoked < 10 cigarettes/day the risk for asthma was 1.2 fold; for > 10 cigarettes, it was 1.4-fold (118). Exposure to tobacco smoke is also associated with wheezing, upper and lower respiratory tract infections, otitis media and coughing in childhood (11). Recent reviews and meta-analyses revealed an association between the maternal smoking and higher risk for both wheezing and asthma. A meta-analysis of 79 prospective studies found a 30-70% higher risk for childhood wheezing and 21-85% for asthma if a mother smoked (119). Distinguishing between pre- and postnatal exposure is difficult, but studies suggest that postnatal maternal smoking raises the risk for wheezing in children two years of age or younger (119). Another recently published meta-analysis reported that postnatal maternal smoking was associated with a 1.2-fold higher risk for wheezing in children under six years old (120).

ETS also influences lung function, the deterioration of which is not restricted only to asthmatic children exposed to ETS, although the negative effect on lung function seems to be greater in asthmatics (121). A meta-analysis of childhood lung function in relation to ETS found that 18 of the 21 studies examined identified a relationship between a reduction in FEV1 and ETS (122). The authors concluded that maternal smoking is associated with a small but yet significant deterioration in lung function at school age but most of the effects appears to stem from maternal smoking during pregnancy (122). A large, international cohort study found that prenatal ETS reduced mean expiratory flow at 25% of vital capacity (MEF_{25}) by 6% and forced expiratory volume in one second ($FEV_{1)}$) by 1%. The association with current ETS was lower, but still significant: -0.5% for FEV_1 and -2% for MEF_{50} (123). Cultural differences may explain contradictory findings in

studies from Turkey (124) and China (125), where the reduced lung function in children was associated with paternal smoking.

A growing body of data indicate that exposure to tobacco smoke affects the immune system and may interact with genes in epigenetic or epistatic ways. Some researchers have postulated that tobacco smoke enhances Th2 immunity, induces oxidative stress and alters the histone acetylase balance (126). A murine model reported exaggerated immunoglobulin E (IgE), immunoglobulin G1 (IgG1), eosinophil, interleukine 4 (IL4) and IL-10 productions in the presence of ETS in sensitized mice (127). One study of asthmatic school-age children found an association between ETS and leukotriene E4 in urine (128). Another study reported an interaction between a CHRNA3/5 polymorphism and ETS for bronchial hyperresponsiveness in children. The locus of this gene encodes the subunits of the acetylcholine reseptor, which mediates airway cholinenergic activity (129). Studies have shown that the locus of gene 17q21, which is associated with childhood asthma, affects the development of asthma more strongly if the mother is smoking (130).

Cotinine and ETS

Most of the studies that have investigated children's exposure to parental tobacco smoke are based on questonnaires in which parents report their smoking habits. Because people often underestimate their bad habbits, this method may produce biased results. A more realiable method to monitor exposure to second-hand smoke is to measure the cotinine level in body fluids and hair (131). Cotinine is a major nicotine metabolite and can be measured in serum, urine, hair or saliva (131). Most of the nicotine metabolism occurs in liver, which metabolizes 70-80% of nicotine to cotinine (132). Cotinine is more stable and has a longer half-time (20-30 h) than nicotine (2 h) (131). Cotinine measurements in body fluids are estimated to reflect exposure to tobacco smoke in previous three to four days (12). Studies have found a positive association with children's urinary cotinine and parental smoking and in particular maternal smoking seems associate strongly with children's exposure (131,133,134). Some reports have shown an association between children's urinary cotinine levels and reduced lung function (133,135,136). Some individual variability in nicotine metabolism due to gene polymorphism in CYP2A6 may influence interpretation of the cotinine results (131). Urinary cotinine can be measured with chromatographic techniques, radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) (137). The lower detection limit of the methods is as follows: chromatographic techniques 0.1-1.0 μg/l, RIA 0.1-2.0 μg/l and ELISA 0.1-0.2 μg/l(137). The recommended cut-off value for urinary cotinine from exposure to tobacco smoke is 0.5 μg/l (131,137). Few studies have investigated the association between children's cotinine levels and FeNO or lung functions indices. Table 2 summarizes these studies.

Table 2. Studies reporting cotinine levels in relation to FeNO or lung function in asthmatic children.

Study	Subjects, n (age)	Cotinine level	Associations with cotinine
Chilmonczyk et al 1993(133)	199 (4-10 years) asthmatics (145 with lung function)	5.6 ng/ml (urine) in non-exposed 23.3 ng/ml in exposed	Reduced FEV1, FEF25-75% and FEV1/FVC Increased number of exacerbations
Oddozze et al 1999 (134)	90 (4-14 years) asthmatics	Urinary cotinine: No ETS: median 0.0 µg/l Mother only: 20 µg/l Father only: 3.5 µg/l Both: 7.5 µg/l	No association with baseline spirometry Association with AHR to carbachol
Spanier et al 2006(138)	170 (6-12 years) asthmatics	All exposed 2.2 ± 8.8 ng/ml (serum)	No association with FeNO
De la Riva-Velasco et al 2012 (139)	33 (8-18 years) asthmatics	urinary cotinine $\geq$ 1ng/ml (n = 10) < 1ng7ml (n = 23)	Poorer asthma control Lower FeNO level
Leung et al 2013 (136)	2763 (mean age 4.5 years) general population	High cotinine-to-creatinine ratio ($\geq$ 30 ng/mg) in 10.6% and measurable in 62.4% of children with cotinine assessments (n = 893)	High urinary cotinine and lower $FEV_{0.5}$, FEF_{25-75} and PEF associated
Hernández-Alvirez et al 2013(128)	49 asthmatics 41 controls 5-13 years	Urinary cotinine: Asthmatics 1.98, non-asthmatics 1.23 ng/mg creatinine	Urinary cotinine and FEV_1 and FVC inversely correlated
Howrylak et al 2014 (140)	619 (1-16 years) asthmatics	56.1% had detectable cotinine in serum and 79.6 % in saliva	Detectable cotinine levels in serum and saliva were associated with readmission to hospital within a year
Gill et al 2014 (141)	40 (8-18 years) asthmatics	70 % urinary cotinine $\geq$ 1ng/ml 30 % < 1ng/ml	No association with FeNO or FEV_1 Higher urinary LTE4 in ETS exposed with ICS, but not with montelukast
Valsamis et al 2014(135)	41 preschool asthmatics	15 had > 1ng/ml	FEV_1 decreased in children with > 5ng/ml cotinine, no effect on IOS indices

2.3 Diagnosing asthma in preschool children

Asthma is defined as a chronic disorder with airway inflammation and hyperresponsiveness, which lead to recurrent episodes of obstructive respiratory symptoms (wheezing, coughing, dyspnea or chest tightness). Taking into account the heterogeneous features of preschool wheezing or asthma-like symptoms and the difficulty in measuring objective lung function in this age group, no simple and clear definition for asthma in preschoolers exists.

Several guidelines for the diagnostics issues associated with preschool asthma are available (13,142,143). Current international guidelines recommend taking a history of recurrent respiratory symptoms as a key method for diagnosing asthma in children (13) (Table 3). Wheezing, coughing, dyspnea, and chest tightness are typical of asthma and the symptoms are often worse at night or early in the morning. Exposure to various stimuli such as cold, exercise, tobacco smoke, allergens, respiratory infections, laughter or crying may trigger episodes of respiratory symptoms (13). The guidelines do not specify the number of symptomatic episodes needed for a diagnosis of asthma in preschool children but do recommend an arbitrary number of three or more. A personal history of atopy (allergic rhinitis, eczema, food/aeroallergen sensitization) as well as a family history of asthma strengthens the diagnosis (13). Physical examination should include chest inspection and auscultation for wheezing or any other abnormalities in breathing sounds. A chest X-ray is recommended for a differential diagnosis, and a positive outcome in therapeutic trial may also support a diagnosis of asthma. Because symptoms of asthma may stem from various conditions, a differential diagnosis is crucial. A list of important differential diagnoses appears in Table 4.

An Asthma Predictive Index (API) was developed based on the Tucson cohort to help in assessing the risk for persistent asthma in children younger than three years of age (144). The API is based on both major (parental asthma and atopic eczema) and minor criteria (allergic rhinitis, wheezing without colds, eosinophilia) (145). A positive API score is defined as having recurrent wheezing episodes before the age of three (< 3 / year in loose and ≥ 3 in stringent index) and one of two major criteria or two of three minor criteria (144). Several studies have sought to validate the use of API or to develop new predictive indices (21,36,146-148). Positive likelihood ratio of API regarding the risk for asthma ranges from 2.5 to 7.9 among 6- to 13-year-old children with early wheezing (149). The negative likelihood ratio ranges from 0.53 to 0.91 (149). A modified API includes three major criteria (parental asthma, atopic dermatitis and aeroallergen positivity) and three minor criteria (allergic sensitization to milk, egg or peanuts, wheezing unrelated to colds and eosinophilia) (150).

The evaluation of lung function is essential in both diagnosis and monitoring of asthma control in school-age children and adults. In preschool-age children asthma diagnoses are mostly based on clinical symptoms. The International Consensus on pediatric asthma

(ICON) (13) recommends spirometry for children old enough to perform it properly (proposed range of minimum age is 5 to 7 years of age). ICON states that in children under five years of age, newer techniques such as oscillometry or spesific airway resistance can be used. Finnish guidelines recommend the use of oscillometry from the age of three years to assess lung function and to diagnose asthma (151). Normal lung function in mild to moderate asthmatics does not exclude asthma (152). In such cases the AHR tests may offer an additional aid to achieve the correct diagnosis. Given the heterogeneity of the asthmatic symptoms in young children, objective methods for the assessment of lung function, airway inflammation and airway hyperresponsiveness are needed to enhance proper asthma diagnoses and to avoid unnecessary medications.

Table 3. Diagnosis of preschool asthma (13,143)

History of respiratory symptoms
Recurrent obstructive respiratory symptoms (wheeze, cough, dyspnea, chest tightness)
Symptoms worse at night or in early morning
Exacerbations by exercise, viral infections, allergens, environmental factors
Personal history of atopy (eczema, food allergy, allergic rhinitis)
Family history of atopy or asthma
Physical examination
Inspection of the chest
Auscultation for wheezing
Inspection of the skin for eczema
Signs of rhinitis
Lung function (in Finland, oscillometry is recommended from the age of 3)
Evaluation of atopy (skin prick tests or specific IgE)
Chest X-ray for differential diagnosis
Therapeutic trial

Table 4. Diffrential diagnosis for respiratory symptoms in small children (13,143)

Immunological and infectious disorders	Mechanical obstructions
Recurrent respiratory tract infections	Congenital malformations
Bronchiolitis	Foreign body aspiration
Allergic rhinitis and sinusitis	Laryngotracheamalacia
Anaphylaxis	Tracheal stenosis, bronchostenosis
Tuberculosis	Enlarged lymph nodes or tumor
Sarcoidosis	Vascular rings or laryngeal webs
Immune deficiency	
Pertussis	
Bronchial pathologies	Other systems
Primary ciliary dyskinesia	Heart disease
Cystic fibrosis	Gastroesophageal reflux disease
Bronchiectasis	Neuromuscular disorder
Bronchopulmonary dysplasia	Psychogenic cough

2.4 Measuring lung function in young children

Lung function testing plays a key role in assessing of diagnosis and managing chronic pulmonary conditions in school-age children and adults. However, the objective measurement of pulmonary function in children under six years of age is uncommon, mostly due to the cooperational challenges in this age group. Manoeuvers needed in spirometry may be difficult for many preschoolers to perform. Consequently, significant efforts have aimed to develop techniques that noninvasively measure lung function in young children during tidal breathing.

A number of methods to measure preschool lung function have been introduced, many of which have proved safe, feasible and easy for a young child to perform (153). Spirometry has been suggested in preschoolers using the modified acceptability criteria, but there is limited data regarding the intepretation of the bronchodilatation response, usage with the exercise test and bronchoprovocative challenges in this age group (154,155). However, the ATS/ERS statement provides reference values for baseline spirometry values (153), and more recent publications present additional data (156,157). For preschool children who may be unable to produce exhalation for one second, researchers report the forced expiratory flow at 0.5 or 0.75 second (158).

One can use a mouthpiece in a body pletysmograph to assess specific airway resistance (sRaw) during the tidal breathing (159). Reports have shown sRaw to be higher in asthmatic children than in healthy controls (160). sRaw can also serve to assess bronchial hyperresponsiveness and the bronchodilator effect (161), but standardized guidelines for sRaw are necessary (161).

The interrupter resistance (Rint) method offers a noninvasive and quick assessment of respiratory resistance during tidal breathing. This method involves briefly occluding the airway opening, and the ratio between resistance difference and airflow is the interrupter resistance (Rint) (153,162). Rint has shown 70-92% specificity and 24-76% sensitivity in distinguishing between children with current wheezing and healthy controls (161). However, bronchoprovocative tests have revealed a large inter-individual distribution of normal values and discrepancies between Rint and other pulmonary function tests (158).

The MBW (multiple breath inert gas washout) technique is based on the measurements of gas distribution and mixing within the lungs (161). The method provides measurements of lung volume and ventilation inhomogeneity during tidal breathing (161). MBW has proved to be a good indicator of lung function deterioration in preschool wheezers (160). However, clinically meaningful thresholds for the method are unavailable and the lack of commercial equipment limits the use of this technique in everyday practice (161).

2.4.1 Impulse oscillometry

2.4.1.1 Technique

In 1956, DuBois first introduced the forced oscillation technique (FOT) (163). FOT is a general impression for the airway mechanistical measurements, which utilize non-invasive pressure fluctuations over the subject's normal, tidal breathing. Impulse oscillometry (IOS) is a modification of the FOT, which uses rectangular pulse signals during the tidal breathing instead of sine-curved waves in FOT (5,164).

IOS delivers a large sample of different frequencies of pressure waves, which are thought to provide a detailed characterization of the lung function (165). IOS can serve to measure lung function in both adults and children (165,166). IOS uses a methodology involving a loudspeaker that generates oscillation pressure waves. These regular square waves are transmitted to the airway system, usually at the mouth (166). The pressure oscillations act via movement of the air column in the airways and cause the elastic components of respiratory tract tissues to distend and recoil and create backpressure (165). Impedance (Zrs) is a term that refers to a pressure-flow relationship produced by the oscillation technique (166). Impedance is composed of the resistance (Rrs) and reactance (Xrs) of the respiratory system.

Rrs describes the mechanical properties of the respiratory system and is calculated from the pressure and airflow signals, where pressure is in phase with the flow (165). Rrs measures both central and peripheral resistance from the oropharynx to the distal lungs and the thoracic wall tissue (166). Reactance (Xrs) is related to the energy storage capability of the airway system and is determined by its elastic properties (165). It is calculated from the relationship of the pressure and volume (167). Elastic properties are prominent at low frequencies and the inert properties become more relevant with increasing oscillation frequencies. Resonance frequency (Fres) refers to the point at which the transition from the passive distension to active stress in the lungs occurs (166).

The oscillations in IOS are applied in a fixed square wave frequency at 5 Hertz (Hz), from which all other frequencies derive (165). Resistance and reactance at 5 Hz are termed as R5 and X5, respectively. Low frequencies of 5 Hz or less are considered descriptive of both peripheral and proximal lung mechanics and are thought to provide an overview of the lungs. Instead, high-frequency oscillations such as 20 Hz are proposed mainly to reflect the large airways function (165). The term frequency dependence refers to this distinct capability of low and high frequency oscillations to reflect conditions of the peripheral and proximal parts of the lungs (5,167). R5-R20 is calculated from the difference between R5 and R20 and is proposed to be a measure of the peripheral airways (168,169). The terms total resistance (R5), central resistance (R20), small airways resistance (R5-R20), distal reactance (X5) and reactance area (AX) are conventionally used in IOS measurements (166).

Coherence, a measure of the correlation between airflow and pressure, is an estimate of the reliability of the IOS (165). Low coherence may reflect the inability of the patient to relax, leakage from the mouthpiece, irregular breathing, improper bracing of the cheeks, an inadequate seal with the nose clip, swallowing, vocalization, coughing, closing of the glottis or closure of the airflow by the tongue (165). Three to five acceptable measurements should be performed (153). The coefficient of variability (CV) is calculated and serves as an indicator of trial-to-trial variability. Reports show that the CV in children ranges from < 5 to 17% (167). This range suggests that achieving similar results in consecutive measurements is easy (165). During the measurement, the sublect's or examiner's hands must support the cheeks in order to compensate for the compliance of the cheeks (5). A nose clip prevents the oscillations to escaping from the nares (5).

2.4.1.2 Clinical applications in young children

ATS/ETS Statement proposes the IOS technique as one of the techniques for measuring lung function in preschool children (153). The statement concludes that the oscillometric technique is a promising method for diagnosing airway obstruction, reversibility and hyperresponsiveness (153,165). Most reports have defined R5 as a specific primary outcome variable in IOS measurements (158). The Finnish guidelines

recommend measuring lung function with IOS in children from three years of age. Most children under six years of age are incapable of performing maneuvers needed in spirometry, which is widely used in school-aged children and adults. IOS requires only passive cooperation and can be performed during quiet, tidal breathing (165,166). More than 80% of young children are able to perform oscillometry successfully (5,158). Studies have reported success rates of 40% in four-year-olds and 83% in 5-year-olds (166). In a Finnish report 89% of two- to seven-year-olds were able to perform IOS successfully (5).

The normal reference values for children are based in their standing height (170), which appears to be the strongest dependent factor. The children's height and the R5 value are inversely associated (5,171,172). The IOS reference values for Finnish preschool-age children are provided based on a study of 109 healthy two- to seven-year-olds (5,173).

IOS can serve to determine lung function in both asthma and other lung disorders (167,174,175). The clinical diagnostic capacity of respiratory impedance of IOS is comparable to that of spirometry (167). In school-age children IOS has shown consistent results with spirometry (176).

Children with stable chronic lung diseases often have normal baseline resistance values (158). To determine positivity to a bronchodilator, researchers have suggested a decrease of 15-40% from baseline IOS resistance, and recently proposed a cut-off value of 40% (158,177,178). In healthy Finnish children the mean percentage decrease in R5 after inhalation of salbutamol was 19.2% (5). An American study examined 73 four-year-olds and found that bronchodilator response with IOS discriminated between the children with and without asthma (179). In a Finnish study of 96 preschool children, baseline R5 and post-bronchodilatation R5 were able to distinguish between the wheezy and healthy children (180).

No established cut-off values have been published for the bronchoprovocation tests using IOS. However, studies have shown an association between the asthma symptoms and positivity in the methacholine challenge with IOS in adult asthmatics (181). The exercise test with IOS can succesfully distinguish between the pediatric asthmatics and controls (182). IOS (increase in R5) and spirometry (decrease in FEV_1) showed agreement in the exercise test in young adults (183). The bronchoprovocative tests with IOS are addressed in more detailed later in this thesis.

The main limitation of the IOS technique is that it fails to differentiate between the obstructive and restrictive conditions (167). The technique is also sensitive to the artifacts, which should be carefully monitored during the measurement (167). Upper airway artifacts can be controlled for holding the cheeks during the measurement, and by avoiding movements during the performance (166,167). The strength of the IOS technique is its need of minimal cooperation, which is why it is recommended when cooperation difficulties preclude the use of spirometry (167).

2.4.2 Airway hyperresponsiveness assessment

Airway hyperresponsiveness (AHR) is one of the key features in asthma. AHR refers to an abnormal increase in airflow limitation following various natural or pharmacological stimuli (184). The first reports of the bronchoprovocative tests are by Tiffeneau in the 1940s (6). Bronchial challenge tests can serve to assess AHR in patients with symptoms and signs suggesting asthma. A large number of provocating agents have been developed since the first reports of AHR (185). These agonists are divided into direct and indirect agents to emphasize the heterogeneity of the reactions resulting from various stimuli (186,187). An overview of such direct and indirect stimuli appears in Table 5.

Direct stimuli act directly on airway smooth muscle cell reseptors, and indirect stimuli cause airway constriction through mediators. A schematic illustration of the mechanisms of direct and indirect bronchoconstrictive stimuli appear in Figure 1.

Two semi-independent components of AHR have been identified; variable and fixed components (188,189). The variable component is transient and inducible, and may be result of, for example, allergens. It improves rapidly with medication or environmental control (188) and is associated with airway inflammation, current asthma activity and exposures (188). The fixed component is relatively persistent AHR, which occurs in many but not in all chronic asthmatics. This phenomenon is also called airway remodelling, which refers to the structural changes that take plase in the airway walls (189).

Table 5. Direct and indirect challenge tests.

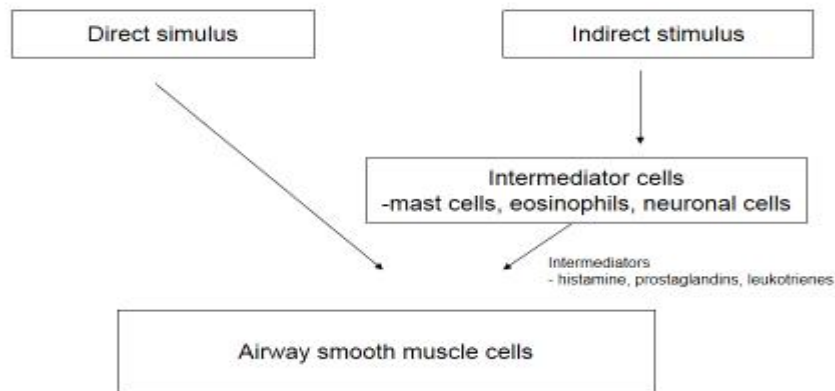| Direct stimuli	Indirect stimuli
Methacholine	**Exercise**
Histamine	**Eucapnic voluntory hyperpnea**
Asetylcholine	Hypertonic saline
Carbachol	**Mannitol**
Prostaglandin D_2	Adenosine
Leukotriene $C_4/D_4/E_4$	Metabisulfite
	Sulfur dioxide

Figure 1. Schematic illustration of the mechanisms of direct and indirect bronchial stimuli.

2.4.2.1 Direct challenges

Direct bronchoprovocative tests acts directly on the airway smooth muscles to cause airway contraction. The most widely used direct tests for clinical and research purposes are methacholine and histamine challenges (190). Histamine acts on histamine (H_1) receptors and methacholine in muscarinic (M_3) receptors on the bronchial airway smooth muscle cells (Figure 2). These activations cause the cells to depolarize and contract, thereby constricting the bronchi (191). Direct hyperresponsiveness is only weakly related to airway inflammation of the airways (192), but attenuates after the use of inhaled corticosteroid. Direct tests most often serve to rule out asthma, since they have high sensitivity, but low specificity (6). The list of direct AHR tests appears in Table 5, with the most frequently used tests appearing in bold.

Methacholine challenge

Methacholine chloride (acetyl-β-methacholine) is a parasympathomimetic analog of acetylcholine, and causes bronchial smooth muscle constriction by stimulating the

38

muscarinic, parasympathetic receptor (193). American Thoracic Society recommends two alternative protocols for the methacholine challenge: 1) the 2-min tidal breathing method and 2) the five-breath dosimeter method (194). The dosimeter method may have a lower sensitivity especially in mild asthmatics, because it includes deep breaths, which are considered to be bronchoprotective and bronchodilative (189). In addition, the delivered dose is lower in the dosimeter method than in the tidal breathing method (189).

A 20% fall in forced expiratory flow (FEV_1) is considered as a positive test result. The dose of the agonist ($PD_{20}FEV_1$) needed to cause this fall is calculated by interpolation from the dose response curve (190). Methacholine is considered to have a high sensitivity to current asthma but low specificity (6). Patients with allergic rhinitis, atopy, and other lung diseases, as well as patients who smoke may have AHR to methacholine (190). Therefore, the methacholine test often serves to rule out current asthma (6).

Studies have shown some genetic linkages between the direct AHR and asthma-associated genes (184). Association studies between the inflammatory markers and direct AHR have shown somewhat contradictory results (195). The number of mast cells and eosinophils has shown a weak association with AHR (184,196). Patchy desquamation of the epithelium, airway smooth muscle hyperplasia and hypertrophy as well as vascular hyperplasia may explain part of the AHR in asthmatics (184). Therefore, it is postulated that the direct AHR in asthmatics results from both inflammatory and structural changes in the airways (184).

Only few studies have examined the methacholine provocation test and other AHR tests in preschool-age children, mostly due to the challenges in measuring the lung function in this age group. Many of the children under six years of age are incapable of performing the manoeuvers needed in spirometry. They can, however, perform the impulse oscillometry, which demands minimal cooperation. Table 6 summarizes studies that have used AHR tests to distinguish various pediatric patient groups in children and studies that have examined AHR in preschoolers.

In the 1990's investigators used methacholine, adesonine and exercise challenges with spirometry to study a group of 6- to 25-year-olds. The investigators reported that methacholine distinguished children with asthma and pediatric COPD from controls, but not asthma and COPD patients from each others (197). Exercise and adenosine did differentiate asthmatics and COPD children from controls and from each other (197). A study of five- to six-year-old wheezy children found that neither the methacholine nor hypertonic saline tests with IOS successfully distinguished between the past and present wheeze or the degree of the clinical severity of the symptoms (198). A subsequent study reported higher adenosine, but not methacholine responsiveness in preschool age children with atopic asthma than in children with non-atopic asthma (199). AHR was assessed with a modified auscultation method.

A Danish study examined sRaw, Rint, IOS and the transcutaneous measurement of oxygen with methacholine challenge in two- to four-year-olds (200). They found that IOS was suitable for young children for measuring AHR. The most sensitive of these tests was sRaw, the second most sensitive was Xrs5 with IOS. IOS reportedly detected 70-80% of five-year-olds who responded to the methacholine test with spirometry (201).

Large cohort studies have used methacholine challenge test results as a sign of a childhood AHR and a possible trajectory for later asthma. A Danish study found an association of neonatal AHR to methacholine and asthma at seven years of age (41). A Norwegian report showed that AHR to methacholine at the age of ten years was a more accurate predictor than the exercise test for asthma six years later (202). A report from the Tucson cohort found that AHR to methacholine at the age of seven was associated with persistent wheezing and atopic markers in early life (25).

The exact role of the methacholine test in diagnosing childhood asthma remains unclear. Canadian researchers studied 215 children with doctor-diagnosed asthma and 197 healthy controls with methacholine challenge test (203). They found in these seven- to nine-year-old children that methacholine had a positive likelihood ratio of 2 for doctor-diagnosed asthma in atopic girls and 2.6 in atopic boys. In nonatopic children the methacholine test offered no help in diagnosing asthma (203).

2.4.2.2 Indirect challenges

Indirect challenges cause the airways to narrow by releasing endogenous mediators, which cause the airway smooth muscles to contract (7). Inhaled corticosteroids deteriorate or complete inhibit responses to indirect challenges, and these test are thought to reflect more closely airway inflammation than direct challenges (186). Indirect tests are considered to be highly specific but less sensitive for asthma (7). Most natural stimuli that provoke asthmatic attacks, such as exercise of allergens, in everyday life act indirectly (7). The list of indirect challenges appears in Table 1. Challenges that are commonly available in pulmonary laboratories appear in bold.

Exercise test

Exercise induced bronchoconstriction (EIB) is defined as an acute airway narrowing that results from vigorous exercise (204). The first reports of the exercise test in children were published in the 1960's (205-207). The exercise test is the first standardized indirect challenge test in children (208). Exercise is a natural way to induce airway symptoms in asthmatic children (209), and estimates indicate that 40–90% of asthmatics experience respiratory symptoms during exercise (210,211). EIB has been found also in subjects with no known diagnosis of asthma (204).

EIB results from changes in lung function evoked by exercise, and the FEV_1 is the recommended lung function measure to assess EIB (204) in school-age children and adults. Symptoms of EIB are nonspecific and variable and have poor predictive value for objectively confirmed EIB (212,213). The severity of EIB can be graded by the magnitude of change in lung function in the exercise test. According to the American Thoracic Society (ATS) guidelines, a 10-25% drop in FEV_1 from the pre-exercise level is considered mild EIB, a 25-50% drop indicates moderate EIB and a $\geq$ 50 % drop indicates severe EIB (204).

Two competing hypotheses describe the mechanism of EIB. The first is based on the heat loss and the subsequent vascular engorgement that ensues as the airways rewarm after exercise and initiate the constriction of the airways (214). This theory is unrelated to mediator release. The second proposed mechanism is grounded on the water loss from the airways during exercise as the lower airways must condition large volumes of air in a short time (210). This leads to a change in osmolarity, which is thought to activate mast cells, sensory nerves and epithelial cells and to release inflammatory mediators, such as leukotriens, prostaglandins and histamine, thereby constricting the bronchi (210,215).

The osmotic mechanism of EIB is the most accepted theory today. Investigation of 25 asthmatics with EIB found the realease of epithelial cells, mast cell mediators and eicosanoids into the airways during EIB (216). The eosinophil count in induced sputum and the severity of EIB shows a correlation (217). Sensory airway nerves may also be involved in mediator release into airways during EIB by releasing neurokinins, which cause airway constriction and mucous secretion (218).

The exercise test with IOS can serve to identify EIB in young children who are incapable of performing spirometry. A 35-40% increase in resistance at 5 Hz (R5) after exercise is considered as EIB (153,167,182). A study of three- to seven-year-old wheezy children and non-atopic controls revealed that wheezy children showed significantly larger responses in IOS indices than did nonatopic controls (182). The exercise test was performed as a free running test. Physical activity is natural for a child, so the exercise test is a good way to induce symptoms of asthma in children. IOS and spirometry parameters after the exercise test have correlated well in young adult asthmatics (183).

The exercise test defies standardization, in that controlling exercise intensity and environmental factors is challenging. This difficulty has lead to the search for surrogate tests, such as the eucapnic voluntary hyperpnea (EVH) challenge, and the hypertonic saline and mannitol tests (210). Direct challenges act directly on smooth muscle receptors and depend less on airway inflammation (219), so any association with indirect challenge tests is only modest (186).

Mannitol challenge test

Mannitol is a sugar alcohol, and has served in clinical use as a bronchoprovocative agent since 1997 (191,220). Mannitol is commercially available as dry powder, which is inhaled from gelatin capsules in increasing doses; up to a maximum of 18 mannitol doses are inhaled during the challenge test. Lung function is assessed one minute after each inhaled dose of mannitol (191,220). The challenge is completed with a documented decrease of 15% in FEV1 or when the total dose reaches 635 mg (220). The mechanism whereby mannitol causes airway narrowing is thought to involve an increase in airway osmolarity, which leads to the release of mediators from mast cells and neuronal cells (220). Mannitol reportedly stimulates release of histamine from human lung mast cells and basophils in vitro (191). Histamine initiates the bronchoconstriction following inhalation of mannitol and the leukotriens sustain the effect. In vitro experiments have also shown that mannitol releases protaglandin D2 and leukotriene E4 from human mast cells and leukotriens from eosinophils (221). Studies have shown mannitol inhalation to release prostaglandin D2 and leukotriene E4 in asthmatic patients and to minor extent in healthy persons (221,222). Inhalation of mannitol induces airway refractoriness for several hours (191), which may be do to tachyphylaxis at the level of airway smooth muscles rather than to lower mediator production (191).

The original study on safety and efficacy found a good condordance between responsiveness to hypertonic saline and mannitol in asthmatic and healthy subjects (223). In a study of subjects with asthmatic symptoms but no confirmed diagnosis of asthma, mannitol showed similar sensitivity and specificity to that of methacholine in identifying doctor-diagnosed asthma or EIB (224). Nevertheless, the concordance between the test results in these study subjects was not as good as between the persons with a known diagnosis of asthma and healthy controls (223).

The mannitol test has proved useful in optimizing inhaled doses of corticosteroid (225,226). In a study of 9- to 16-year-old asthmatic children with moderate to severe disease, mannitol test showed good reproducility and tolerance (227). Another study of asthmatic children (6-16 years of age) found positive and negative predictive values of 68% and 89% for the mannitol test in identifying exercise-induced bronchoconstriction (228). Furthermore, the mannitol test identified methacholine-positive 9- to 16-year-olds children with asthma (229). All these pediatric studies utilized spirometry to assess lung function.

Researchers investigating the mannitol challenge test with oscillometry in 8- to 21-year-olds found a significant correlation between a decrease in FEV_1 and an increase in R5 by IOS after the mannitol test (230). Use of forced oscillation technique to measure the mannitol response in adult patients showed high sensitivity and repeatability (231). No published studies have investigated the mannitol bronchoprovocation test with IOS in preschool-age children.

Table 6. Studies that have investigated the abilities of AHR tests to differentiate between various pediatric patient groups and studies among preschool-age children.

Study	Subjects	Age, mean (years)	AHR tests	Results
Godfrey et al 1991 (209)	52 with asthma 22 other chronic lung diseases 19 controls	11.7 11.4 14.4	Methacholine and exercise spirometry	Asthmatics more reactive in exercise test Both groups responded to methacholine
Avital et al 1995 (197)	51 asthmatics 21 pediatric COPD 19 controls	13.0 11.1-13.7 14.0	Adenosine Methacholine Exercise spirometry	Methacholine distinguishes asthma and COPD from controls, but not from one another Exercise and adenosine distinguished asthma from other groups
Wilson et al, 1995 (198)	40 asthmatics	4.6 - 6.0	Methacholine Hypertonic saline IOS	No differences between present or previous wheezing or the clinical severity
Malmberg et al 2008 (182)	130 wheezy children 79 controls	5.6 5.1	Exercise with IOS	Exercise test with IOS distinguished wheezy children from controls
Liem et al 2008	215 asthmatics 197 controls	8.3-8.7	Methacholine spirometry	Sensitivity for asthma 66%, specificity 64%
Suh et al 2011 (199)	122 asthmatics (97 atopic and 25 nonatopic)	5.3	Methacholine Adenosine with modified auscultation technique	Atopic asthmatics had greater AHR-to-adenosine than did nonatopics, no difference in methacholine responses
Schulze et al 2012 (201)	48 asthmatic children	5.3	Methacholine spirometry and IOS	R5 by IOS detected 70-80% of patients who responded in spirometry

2.4.3 Measuring small airway function

Small airways are defined as distal airways with a diameter of less than 2 mm (232) and start around generation eight in the bronchial three (169,233). This distal airway area is traditionally referred as a silent zone, but in recent years more evidence has accumulated to support the importance of the small airways in the pathogenenesis and clinical picture of asthma (233). The mucosal cross-sectional area is much larger in the distal than proximal airways, which is also reflected in overall airway resistance (234). Small airways dysfunction has been associated with noctural asthma, severe asthma, exercise-induced obstruction, recurrent exacerbations, symptoms and responses to inhaled corticosteroids (9,235,236).

Inflammation, hyperresponsiveness and remodeling can affect both the large and small airways. Functional features of small airway dysfunction include the peripheral heterogeneity of the ventilation, air entrapment and premature airway closure (237). Some have also proposed that the peripheral airways response to bronchoconstrictors differs among asthmatics (10,238). Studies have shown methacholine-induced changes in forced vital capacity, which are thought to reflect airway closure due to small airways, to be associated with asthma severity, steroid requirements and symptoms in adults(10).

Measuring the small airways is challenging, because the distal part of the lungs are relatively unaccessible. The conventional and most widely used methods for assessing lung function, such as spirometry, are not optimal for investigating the distal airways. Forced expiratory flow at 25% to 75% of forced vital capacity (FEF_{25-75}) is considered as a measure of the distal airways, but suffers from poor reproducibility (239). A retrograde catheter of 2 mm in diameter was wedged into the bronchus to measure airflow resistance in the distal airways in a landmark study of the small airways (232). High resolution computed tomography (HRCT), used to visualize regional air-trapping in the distal airways, and helium-oxygen breathing tests are also invasive and technically complicated methods for investigating the peripheral airways (240,241). Multiple-breath nitrogen wash-out (MBNW) and oscillation techniques are less invasive, yet still potentially sensitive methods to measure the distal airways and can also serve in young children (236).

The impulse oscillometry (IOS) indices R5-20, X5 and AX are suggested to reflect the distal airways function (166). Resistance at low frequency oscillations, such as 5 Hz (R5), is considered to be a measure of both the proximal and distal airways. Proximal obstruction increases both R20 and R5, and isolated distal obstruction increases R5 more than R20 (165). This phenomenon is called frequency-dependent change and is calculated as the difference between R5 and R20 (R5-20) (165).

2.4.3.1 Small airways and IOS in children

Although remodelling and peripheral disease were thought to affect only adults and only after a long asthma history, these structural changes already seem to be present in small children (242).

Only a few published studies have investigated measurement of the small airways in children with IOS. Children who lost their asthma control had a higher baseline R5-20 and reactance area (AX) than did children who maintained control during the control visit after 8-12 weeks (168). The same study group also showed an association between uncontrolled asthma and small airways dysfunction (R5-20, X5, Fres and AX) (243). Studies used IOS to examine the effect of inhaled corticosteroid/long-acting beta-agonist combinations on small-airway dysfunction in 12- to 45-year-olds (244). The investigators found that inhalation of the combination of medicines initially improved the peripheral lung function, as measured with R5-20 and AX in about five minutes. After four weeks' regular use of the combination, the initial effect diminished due to the improved pre-dose status of the peripheral IOS parameters (244).

2.5 Airway inflammation

Airway inflammation is one of the key features of asthma and is characterized by the presence of inflammatory cells and the release of inflammatory mediators into the airways (8). Mast cells, eosinophils, T-cells (Th2, Th17 and Treg cells), neutrophils, dendritic cells and endothelial cells can be activated in the asthmatic airways (245,246). Children with asthma show higher eosinophilic counts in induced sputum than do healthy subjects (247). Important mediators in asthma may involve interleukins IL-4, IL-5 and IL-13 (15,246). The need to control inflammation in the airways has driven to a development of devices that measure biological markers of airway inflammation. Because directly evaluating airway inflammation with brochoalveolar lavage (BAL) and bronchoscopic techniques is invasive, innovators have developed less-invasive techniques to evaluate asthmatic inflammation.

2.5.1 Fractional concentration of nitric oxide (FeNO)

2.5.1.1 Biology of FeNO in the airways

In the late 1980s the small molecule nitric oxide (NO) proved to be an important endogeneous mediator of various functions in both health and disease (248). Nitric oxide is a free-radical gas, which forms in the airways when L-arginine oxidates to L-citrulline (Figure 2) (249). The reaction is then catalyzed by NO-synthases (NOS), which exist in at least three different isotypes (8). The constitutive NO-synthase (cNOS) is activated by intracellular rises of calcium after cellular activation (8). Mediators such as histamine, leukotrienes, bradykinin, acetylcholine and platelet-activating factor can also activate cNOS (8). cNOS is further divided into neuronal NOS and endothelial NOS. Inducible NOS (iNOS) is induced by TNF-β, IL-1 and INF-γ and higher iNOS levels occur in inflammatory states (250). Subjects with asthma have shown an increased iNOS in airway epithelial cells (251), which may be due to increased transcription mediated by STAT-1 and nuclear factor-κB (252-254); iNOS is independent of calcium concentration (255).

NOSs are present in several various cell types. Endothelial NOS exists in the endothelial cells of lung vessels, the epithelium of the bronchi, alveolar epithelial cells, nasal mucosa and ciliary microtubules (8). Neuronal NOS occurs in the nerves that supply vessels and smooth muscle in the airways (8). iNOS has been found at least in type II alveolar epithelial cells, macrophages, fibroblasts, smooth muscle cells, neutrophils, endothelial cells and mast cells (249,255). Some cells may express two or more NOSs (255).

After forming in the airways, NO is exhaled, oxidized, reduced or complexed with other molecules in the airways (255). NO is highly unstable and reacts easily with other molecules. Combining with oxygen converts NO into nitrite (NO_2) and nitrate (NO_3). Peroxynitrite consist of NO and super oxide (O_2). NO can react with cysteine and glutathione to form S-nitrosoproteins and S-nitrosothiols, which as along with nitrite can release NO and carry or store NO (249).

The levels of NO in the nose and nasopharynx are much higher than those expired from the mouth suggesting that, at least in healthy subjects, the upper airways are a major contributor to exhaled NO (256). The level of nasal NO is lower in subjects with primary ciliary dyskinesia (257).

Nitrogen oxides can serve distinct functions in the respiratory tract and vascular system, such as smooth muscle relaxation and vasodilatation, platelet inhibition, ciliary function, viral inhibition and neurotransmission (258,259). Although understanding of the link between airway inflammation and NO remains to be incomplete, increasing evidence supports the conception that measuring NO in the breath may serve as a measure of airway inflammation at least in atopic asthma (260).
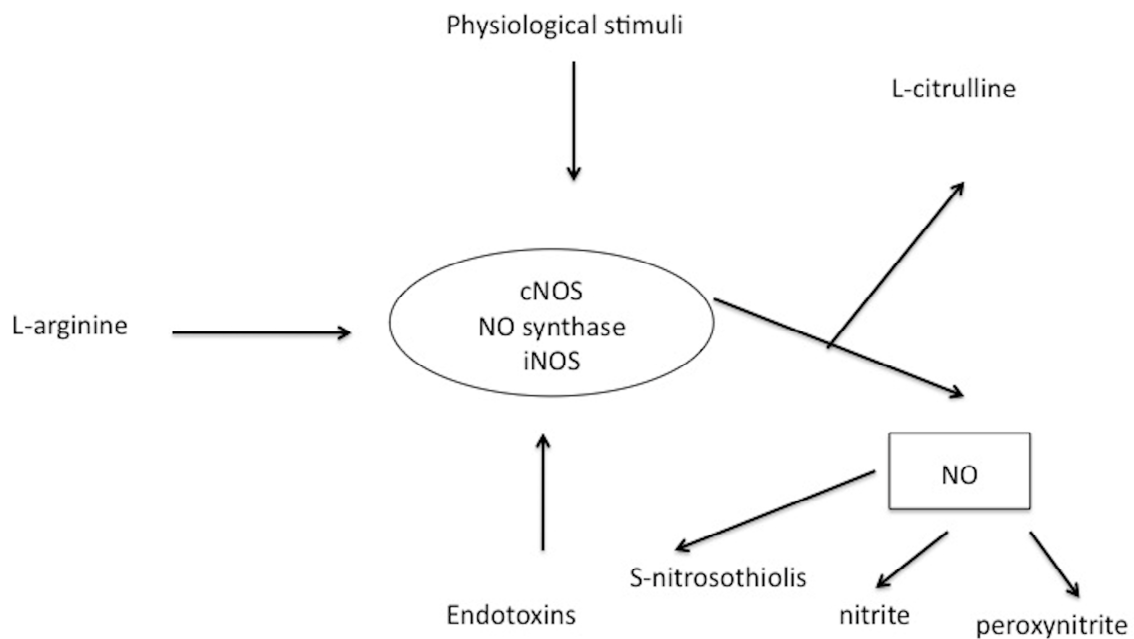
Figure 2. Nitric oxide (NO) synthesis from L-arginine (255).

2.5.1.2 FeNO measurement

The first descriptions of endogeneous NO in exhaled human breath are from 1991 (261). Direct measurement of NO is difficult, because NO is labile in the presence of oxygen. Moreover, NO is present only in small amounts (259).

The ERS/ATS statement on measuring exhaled nitric oxide in children was published in 2001 (260), and recommendations for standardized procedures for the online and offline measurements appeared in 2005 (262). Online and offline measurement techniques are available and thanks to their noninvasive, repeatable, safe and instantaneous characteristics, are also quite suitable for young children. Online methods refer to techniques, which directly analyze exhaled gas and offline methods analyze exhaled gas first collected in a reservoir. Both methods use single breath or tidal breathing, although the single breath technique is recommended (8). The single breath method, however, is difficult for young children under four years of age, because they are incapable of performing the manoeuvers needed to complete the measurements (260,262). The feasible options for the youngest children are the tidal-breathing techniques, which do not require controlled breathing manoeuvers.

FeNO is commonly assessed with the chemiluminescence method, which is based on the detection of photons when NO reacts with ozone (8). The Aerocrine NO Monitoring System NIOX was approved for clinical application in patients in Europe in 2000 and by the US Food and Drug Administration in 2003 (263). A photomultiplier tube detects the photons released by the reaction of NO and ozone, and generates a voltage linearly proportional to the amount of NO in the sample (263). The result is then expressed as a fractional concentration of exhaled NO (FeNO), in parts per billion (ppb). The lower detection limit of the chemiluminescence analyzers is < 1 ppb. A reliable measurement requires a constant exhalation flow, which is why the system incorporates a dynamic flow controller (264). ATS/ERS recommends an exhalation flow rate of 50 ml/s (262), which is monitored by the pneumotachograph.

Another method for measuring NO is based on electrochemical detection (8). This technology is incorporated in hand-held devices, which are easily available outside the specialized laboratories. These hand-held devices (e.g. NIOX MINO by Aerocrine) yield similar results to the simultaneously measurements of stationary chemiluminescence analyzers (265,266). The technique is also available for young children, but the lack of visual motivation in the NIOX MINO analyzer makes it more difficult to perform with young child (267). The lower detection limit in this device is 5 ppb (www.aerocrine.com).

For FeNO assessment (260), a child should be seated and breath quietly for about 5 min in order to acclimatize. The inspired gas should contain less than 5 ppb NO. Subjects should exhale against a resistance of 5-20 cmH$_2$O to close the velum in order to avoid contamination of expired air by nasal FeNO. The subject inhales to near-total lung capacity (TLC) and then exhales at a constant flow of 50 ml/s until reaching a plateau of $\geq$ 2 s during an exhalation of $\geq$ 4-6 s.

The Finnish height-adjusted reference values have been published for preschool-age (180) and school-age children (268). FeNO values should always be interpreted in consideration of the symptoms and factors that may affect the FeNO. Several algorithms for the use of FeNO in children have been published and the FeNO value of 5-25 (-35) ppb is considered normal for children (8,269).

2.5.1.3 Factors affecting FeNO

Several factors can influence the results of a FeNO measurement (Table 7). Height is the best determinant of FeNO in healthy children (268). According to a Finnish study, the FeNO increased from 7 to 14 ppb in the height range 120-180 cm(268). FeNO is also significantly associated with atopy, age, height, weight and body surface area, but not with gender in Caucasian children (8,268). The most important factors that increase the FeNO

level are atopy, infections and age/height (8,270). Very low FeNO levels can be seen in cystic fibrosis, HIV infection and primary ciliary dyskinesia (260).

Several studies have shown that smoking and ETS affect FeNO levels. FeNO levels are lower in active smokers than in non-smokers (271,272). Moreover, acute exposure to tobacco smoke reduces FeNO levels (271,272). The mechanisms by which active smoking decreases FeNO level remain unclear. Tobacco contains high concentrations of NO and may reduce FeNO via negative feedback on inducible NO synthase (272). Some investigators have also speculated that active smoking downregulates IL-10 production from macrophages and, consequently, attenuates Th2 and eosinophil responses (126). Reports of the effect of ETS on FeNO level in healthy children have found no associations (273,274). Studies of children with asthmatic symptoms have shown conflicting results, however. A study of infants with respiratory symptoms found an association between parental smoking and elevated FeNO levels (275). In contrast, a study of school-age asthmatic children reported an association between a lower FeNO level and ETS exposure (139,276). Several studies report no associations between ETS and FeNO levels in children with respiratory symptoms (138,141,273,277).

Table 7. Factors influencing FeNO.

Confounding factor	Effect on FeNO
Atopy	Increase(268,278)
Height	Increase with increasing height (268)
Age	Increase(268,279)
Spirometry	Transient decrease(280)
Exercise	Transient decrease(280)
Bronchoprovocation tests	Transient decrease (281)
Alcohol	Transient decrease (282)
Caffeine	Conflicting results(283,284)
Nitrate containing food	Increase(285)
Active smoking	Decrease(271,272)
Passive smoking	Conflicting results(275,276,286)
Infection	Increase (252,271)

2.5.1.4 FeNO and young children

The first report on increased FeNO levels in adult patients with allergic asthma was published in 1993 (287). Since then several studies have been conducted in both children and adults. Some of these studies found a correlation between exhaled NO and eosinophils in the induced sputum of adult asthmatics (288,289). FeNO was positively associated with sputum eosinophils and eosinophilic cationic protein in children with corticosteroid-treated asthma (290). In a study of difficult pediatric asthmatics, FeNO levels correlated with bronchoscopic biopsy eosinophils in children who adhered to systemic prednisolone treatment (291).

Studies on FeNO in relation to lung function, hyperreactivity, asthma diagnosis and disease severity have yelded conflicting results. Atopic asthmatic school-age children (median age 8.1 yrs) with previous symptoms had higher FeNO levels than did symptom-free asthmatics or healthy controls (292). In 6- to 17-year-old asthmatics, FeNO was correlated with blood eosinophils, IgE and ECP and methacholine challenge results, but weakly with FEV1/FVC and bronchodilatation response and not at all with clinical symptoms or PEF values (293). Most studies aiming to assess the relationship between FeNO and lung function has failed to show such (8,278). Atopic status seems to be a fundamental feature to be accounted for when interpreting FeNO results, and investigators have suggested that FeNO is not necessarily directly linked to respiratory symptoms or lung function (278).

Studies have explored FeNO in preschool-aged children. Because respiratory symptoms are extremely common in early childhood, a non-invasive FeNO measurement emerged as an attractive method for diagnosing and monitoring asthma in preschoolers. However, FeNO values should always be interpreted with the knowledge of the patients' current symptoms and possible influencing factors (Table 2).

A study from Israel succesfully differentiated two- to seven-year-old steroid-naive asthmatic children from healthy and steroid-treated children with the off-line tidal breathing method (294). Dutch investigators reported FeNO reference values for one- to five-year-old healthy children with off-line FeNO assessment. This population showed a mean FeNO of 7.1 ppb (295). A report of 4- to 27-month-old infants with recurrent lower respiratory track symptoms found an association of FeNO with a maternal history of asthma and increased airway hyperresponsiveness (296). Atopy, blood eosinophilia and lung function showed no association with increased FeNO level. In 3.8- to 7.5-year-old Finnish children, the FeNO level served to distinguish children with probable asthma from controls and asthmatics receiving inhaled corticosteroids (180). A recent study of 75 children of four- to eight-year-olds found that increasing age, severe asthma and atopic dermatitis were associated with higher FeNO levels (297). A follow-up study examined 391 preschool-age children to determine whether higher FeNO at an early age could predict asthma at school age (298). The investigators found higher FeNO values in children with later asthma and proposed a new index for predicting asthma that includes

FeNO. According this predictive index, children with higher scores ran a 58 % risk for later asthma: the negative predictive value of this index was 78 %. A recent study reported higher FeNO levels in 3- to 47-month-olds with wheezing episodes than in children with cough only (299).

A Danish study warns against diagnosing asthma based on a dichotomized approach with FeNO or AHR. They studied both asthmatic and symptom-free seven-year-olds and found AHR and FeNO to be associated in both groups regardless of symptoms (300). In addition, two published meta-analyses focused on FeNO-tailored asthma treatment (270,301). Both reports concluded against recommending FeNO-guided asthma treatment, because it fails to improve asthma control and may lead to increased daily doses of inhaled corticosteroids with no meaningful improvement in clinical symptoms and lung function (270,301). Several factors influence FeNO values, a fact that researchers must bear in mind when interpreting the results.

3. Aims of the study

Diagnosing asthma in preschool children is based mostly on clinical symptoms. New objective methods for assessing asthma diagnosis in this age group are needed. This study was designed to clarify the role of lung function, airway hyperresresponsiveness and bronchial inflammation in young children with respiratory symptoms. The specific aims were:

1. To determine whether young children were able to perform FeNO measurement with a portable nitric oxide analyzer (Study I).

2. To examine the effect of risk factors such as parental smoking on lung function and bronchial inflammation in young children (Study II).

3. To study airway hyperresponsiveness with the exercise, methacholine and mannitol challenge tests in different patient groups using impulse oscillometry (Study III).

4. To evaluate small airways function during the methacholine challenge using impulse oscillometry (Study IV).

4. Materials and methods

4.1 Study subjects

All the children were three to eight years old at the time of the studies and were evaluated at the Skin and Allergy Hospital in Helsinki University Central Hospital. A schematic illustration of the patients groups appears in Figure 3.
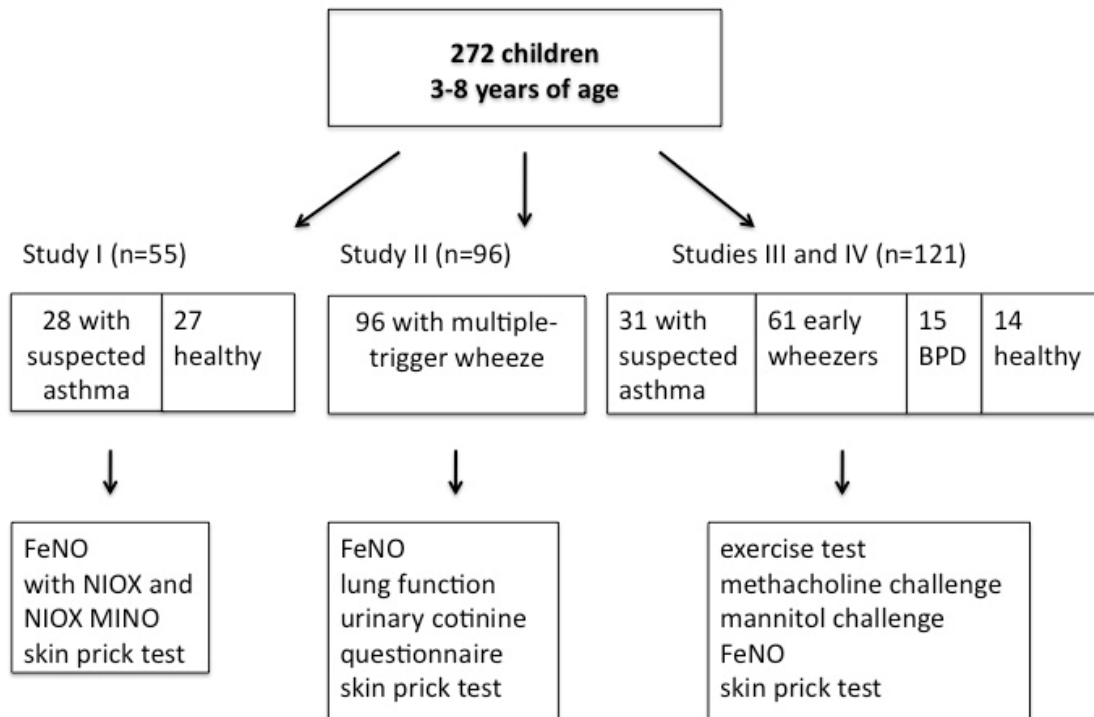

Figure 3. Schematic illustration of the study groups and main methods used.

Study I

A total of 55 children participated in the study from October 2004 to February 2005. Half of these children (n = 28) were referred to the hospital due to asthmatic symptoms. They took part in a study that evaluated lung function in wheezy atopic young children and non-atopic controls (182). Two of these children were using inhaled corticosteroids at the time of the evaluation; all other children were free from asthma-control medication. The other half of the children (n = 27) were age-matched children in good health with no symptoms or signs from the respiratory tract. They had participated in a study that examined the effect of adenoidectomy with tympanostomy tubes in children with recurrent or persistent otitis media at the age of 12 to 48 months (302). At the time of the present study, they were healthy.

Study II

Children with suspected asthma (n = 105) were referred to the hospital during August 2002 and June 2005. The study children participated in a trial investigating the efficacy of combining of salmeterol and fluticasone propionate over that of fluticasone propionate or salmeterol alone in reducing airway inflammation in preschool children with multiple-trigger wheezing (303). The children had a history of breathlessness or wheezing both during and outside viral infections (multiple-trigger wheezing) and showed bronchodilator responsiveness and/or exercise-induced bronchoconstriction as measured with IOS. Patients were excluded if they presented with seasonal symptoms only, had experienced signs of respiratory infection during the previous two weeks or had received inhaled or systemic corticosteroids in the previous six months.

Their lung function was measured with IOS in the first visit to the study clinic. If they fulfilled the inclusion criteria, the patients were invited to complete the study investigations. At enrollment, the parents of the children completed a questionnaire, which inquired the number of cigarettes smoked in the family per day and other background information.

Studies III and IV

A total of 121 children participated in the study during the period from October 2009 to February 2013. Any of the study children who were using regular asthma-control medication stopped it four weeks before the lung function tests.

Of these children, 31 were referred to our hospital because for probable asthma due to troublesome lung symptoms (denoted as TLS). The children suffered from chronic coughing, exercise-related symptoms or recurrent wheezing. The median duration of

symptoms was 11 months (range 2-36 months). Of the TLS children 13 (42 %) had suffered from troublesome coughing, 12 (39 %) from wheezing and 6 (19 %) presented with respiratory symptoms during exercise. The inclusion criteria for the study were: 1. age four to six years 2. asthmatic symptoms (wheezing, coughing, dyspnea) 3. satisfactorily completed exercise test by free running with IOS. The exlusion criteria were: 1. previous diagnosis of asthma 2. previous use of asthma control medication for over six months. 3. jnhaled corticosteroids during the previous two months.

A total of 61 study children had suffered from wheezing in early childhood. They were recruited for a follow-up study from a previous study that evaluated the effect of montelukast on respiratory symptoms and lung function in wheezy infants (304). These children had suffered from persistent or recurrent wheezing and/or dyspnea at the age of six to 24 months and a doctor had diagnosed at least one of their wheezing episodes. This group was called as early wheezers (EW). In total, 83 % of the EW children wheezed and/or had dyspnea both during and outside viral infections (multiple-trigger wheezing) at the time of the previous study.

We recruited 15 age-matched children with a history of bronchopulmonary dysplasia (BPD) during the neonatal period from the Helsinki University Central Hospital's Children's Hospital. The diagnosis of BPD was assessed according to the current guidelines during the newborn period (4). The mean gestational age of these children was 27 (range 25-39) weeks, their mean birth weight was 940 (475-1350) g, the mean number of surfactant boluses was 2 (1-6), the mean length of ventilator therapy was 26 (1-77) days and the mean length of oxygen therapy was 98 (30-240) days.

Finally, 14 healthy age-matched subjects were recruited as controls: they had participated in a study that evaluated the effect of adenoidectomy on respiratory symptoms (302) and had no signs or symptoms of atopic disease or asthma and succesfully performed the free running test and the methacholine challenge test.

4.2 Lung function and airway hyperresponsiveness assessments

4.2.1 Lung function

The children of the study groups II, III, and IV underwent lung function measurement with IOS. Respiratory system resistance at 5 (R5) and 20 (R20) Hz as well as reactance at 5 (X5), 10 (X10) Hz were assessed as described previously (5,182,305). The frequency dependence of resistance was calculated as the difference between the respiratory resistance at 5 Hz and 20 Hz (R5-20) in Study IV. Responsiveness to the bronchodilator

was assessed after an inhalation of 300 μg of salbutamol. A positive response was defined as an increase of 35% or more in R5 (5).

4.2.2 Airway hyperresponsiveness assessment

The children in studies II, III and IV performed exercise test. In addition, the methacholine challenge was performed in studies III and IV and the mannitol challenge test in study III.

4.2.2.1 Exercise test

An outdoor free running test served as an exercise test (182). The children ran for six to eight minutes at such exercise level that raised their heart rate (HR) to approximately 85-90% of their estimated maximum HR, as assessed with a hearth rate monitor (Vantage NV, Polar Ltd, Kempele, Finland). IOS measurements for lung function were taken at baseline and repeated at 1, 5, and 10 minutes after the exercise. A positive test result was defined as an increase of 35% (in study II) and 40% (in studies III and IV) in R5. Finally, the children inhaled 300 μg salbutamol, administered via a Babyhaler, and the lung function measurement was repeated 15 minutes after the inhalation.

4.2.2.2 Methacholine challenge test

The methacholine challenge test was applied as a dosimetric bronchial provocation test modified for preschool children (306). First, we assessed the baseline R5 with IOS. After that, we used an automatic, inhalation-synchronized dosimeter (Spira Electro 2, Spira Respiratory Care Centre Ltd, Hämeenlinna, Finland) connected to a calibrated nebulizer (Salter Labs 8900, Arvin, CA) to administer increasing doses of methacholine chloride. The R5 was remeasured 90 seconds after each inhalation dose. A rapid dosage scheme with five cumulative dose steps was applied by calculating the number of breaths with nebulized methacholine. The procedure was continued until R5 decreased by 40% or the subject received the maximum dose of methacholine. The dose of methacholine that provoked the 40% decrease in R5 was determined from the dose-response curves (175,307). The test result was considered positive, if the $PD_{40}R5$ was < 400 μg. This cut-off is based on data from a study in which this same cut-off corresponded approximately to the provocative dose of methacholine < 1 mg that decreased the forced expiratory flow in one second ($PD_{20}FEV_1$) by 20% (201). This result indicates significantly increased AHR (308) and is predictive of active asthma in the follow-up into adolescence (202).

4.2.2.3 Mannitol challenge test

The mannitol we used in the mannitol challenge test was dry powder (Aridol, Pharmaxis Ltd, NSW Australia). A mannitol challenge test kit consists of one empty capsule, two 5-mg capsules, two 10-mg capsules and eighteen 40-mg capsules. The subject inhales the encapsulated mannitol through the Osmohaler in cumulative doses and then holds his or her breath for five seconds. We checked the inhalation technique on every child and measured peak inspiratory flow (PIF). IOS served to assess lung function at baseline and 60 seconds after each mannitol dose (placebo, 5, 10, 20, 40, 80, 160, 160, 160 mg). We performed three consecutive IOS measurements at every step. The challenge was continued until R5 increased by 40% or the subject received cumulative dose of 635 mg (230,231,307). A $PD_{40}R5 \leq 635$ mg was considered a positive test result based on the established interpretation in adults (220).

4.3 FeNO measurements

The FeNO level was assessed in studies I, II and III. FeNO level was measured with the stationary chemiluminescence-based analyzer NIOX (Aerocrine AB, Sona, Sweden). In study I, we used NIOX and a portable electrochemical NIOX MINO (Aerocrine AB) to compare measurements between the two techniques.

We measured FeNO, according to ATS/ERS recommendations (262) and calibrated the analyzers according to the manufacturer's specifications. The test was performed while the children were seated and without a nose clip. The children filled their lungs completely with NO-free air; they then exhaled with a mean and instantaneous flow of 50 ± 5 ml/s for at least six seconds. Both devises automatically rejected the measurements that not fulfill the criteria. Consecutive, acceptable paired measurements were recorded, followed by calculation of the mean results and repeatability.

In study I, we first measured NIOX followed NIOX MINO measurements with an interval 10 to 15 minutes between the measurements. The lowest detection limit with NIOX is 2 ppb and in NIOX MINO 5 ppb. The analytical accuracy in NIOX is ± 2.5 ppb for measured values < 50 ppb and ± 5% of the measured values > 50 ppb (263). With NIOX MINO the analytical accuracy is ± 5 ppb up to a maximum of 15% of the measured value (www.aerocrine.com).

4.4 Urinary cotinine measurements

The urinary samples were collected from the children in study II during their second visit to the study clinic. The samples (3 X 10 ml) were stored at -26°C until analyzed. We used gas chromatographic method of Feyerabend and Russell to extract cotinine from urine samples (309). As an internal standard, 200 μg of 5-methylcotinine (100 μg/l in dichloromethane) was added and mixed with 200 μg of urine. We added 100 μg of 1,2 dichloroethane, 40 μl of antifoam/phenol red mixture (20 mg phenol red/100 μl 5 % antifoam) and 600 μl of sodium hydroxide solution (5 mol/l) and then centrifuged the mixture. We then injected 2 μl of this concentrated extract into a Hewlett-Packard FFAP silica capillary column of an Agilent 7890A gas chromatograph equipped with an Agilent blos bead nitrogen-phosphorus detector. Air and hydrogen served as detector gases, and helium as a carrier gas. The current was 30 pA, and the detector temperature was 330°C. The temperature programme in the oven was as follows: from 80°C to 120°C at 40°C/min, from 120°C to 220°C at 12°C/min, and 220°C for 10 minutes. The lower detection limit of the measurement was 0.7 μg/l (309).

4.5 Atopy assessment

We used skin prick tests (SPT) to screen for atopy in all the study children (SQ, ALK, Horsholm, Denmark). The following aeroallergens were tested: birch, timothy grass, meadow fescue, mugworth, cladosporium herbarum, cat, dog, horse, cow and house dust mite. The following foods were tested: egg, milk, fish, wheat, schrimp and peanut. SPT was considered positive if at least one of the tested allergens showed a wheal with a diameter of ≥ 3 mm and the control solution gave a negative result.

4.6 Eosinophilic activity

In study III, the blood samples were also taken during the first or second visit to the study clinic to assess eosinophilic cationic protein and eosinophilic count. The samples were examined under routine laboratory methods in the laboratory of the Helsinki University Central Hospital.

4.7 Statistical analysis

Sample size

We calculated sample size for study III by using the intraclass correlation coefficient (ICC). This method measures agreement between paired observations; in this study, we evaluated the agreement between the methacholine and mannitol challenge tests and the exercise test. Thus, the calculated sample size was 57.

The sample size in studies I, II and IV was based on the number of available measurements of lung function and FeNO level.

Study I

We analyzed agreement between the two FeNO devices with Bland-Altman plot and included 95% confidence intervals for limits of agreement. The coefficient of the repeatability (CoR) and the mean difference between the paired measurements served to estimate the repeatability. We compared the mean FeNO values between the devices with the student t-test and analyzed the results between the asthmatics and controls with the unpaired student t-test. For the correlation analyses the FeNO values were log transformed because the values were not normally distributed. Pearson correlation test was used for the correlation analyses.

Study II

The normality of the value distribution was tested with the Shapiro-Wilks test. Non-parametric tests were used, because the FeNO, cotinine and R5 values were not normally distributed. Parental smoking was categorized in two different ways. First four different categories were defined according to the smoking habits at home: no smoking, only mother, only father, and both smoking. Second, the children were categorized according to maternal smoking: yes/no. The Kruskall-Wallis test, the Jonckheere-Terpstra test or the Mann-Whitney U-test were used to compare the categorical data. Correlation analyses were made by Spearman correlation coefficient. A correlation coefficient of 0.1 was considered small, 0.3 moderate, and 0.5 large (310). We used Chi-square test to compare the two categorical datasets.

Study III

Because the data were not normally distributed, we used non-parametric tests. We used the Kruskal-Wallis test and Mann-Whitney test to compare continuos data and used Spearman's correlation test to the correlation analysis. To study whether the methacholine and mannitol challenge tests could identify EIB, we applied receiver-operator characteristics (ROC) curves. An area under the curve (AUC) of 0.9-1 was considered excellent, 0.8-0.9 good, 0.7-0.8 fair, 0.6-0.7 poor and 0.5-0.6 fail. BPD children were

excluded from the analysis, because their underlying lung function disorder was presumably different from that of the asthmatic children. Possible explanatory factors were analyzed with binary logistic regression. The mannitol and methacholine challenge tests and exercise test were included as dependent variables and skin prick test positivity, parental smoking and the FeNO level served as covariates.

Study IV
We used nonparametric tests and the Kruskal-Wallis test and Mann-Whitney tests to compare IOS indices R5, R20, R5-20, X5 and X10 at baseline and after the methacholine challenge.

The statistical siginificance was set at a $p < 0.05$. We analyzed the data with SPSS, version 19.0 (SPSS, Inc, Chigaco, IL).

4.8 Ethics

The Ethics Committee of the Helsinki University Central Hospital (81/E7/02 and 337/13/03/03/2008) approved this series of studies. Finnish Medical Agency approved the study (KLnro 246/2008 EudraCT 2008-007264-41).
Each parent provided his or her written, informed consent before their child took part in the study.

5. Results

The basic characteristics of the study populations appear in Table 8.

Table 8. Basic characteristics of the children in studies I, II, III and IV.

	Study I	Study II	Study III and IV
Number of subjects	55	96	121
Female, n (%)	24 (44)	31 (32)	47 (39)
Age (y), mean (range)	5.7 (3.9-8.5)	5.6 (3.9-7.9)	5.9 (3.7-8.1)
Height (cm), mean (range)	115 (99-134)	115 (99-113)	116 (97-153)
Skin prick test positivity, n (%)	30 (55)	72 (75)	46 (38)
Parental asthma, %		34	39
Symptoms	28 with asthmatic symptoms 27 healthy controls	multiple-trigger wheezing	31 with suspected asthma 61 early wheezers 15 BPD survivors 14 healthy controls

5.1 FeNO measurements (Study I)

5.1.1 FeNO measurements with stationary and portable analyzers

Of the children in the study, 40 (mean age 6.1 years, range 4.1-8.5 years) were capable of performing measurements with both FeNO analyzers (NIOX and NIOX MINO). Measurements were more often successful with NIOX than with NIOX MINO (93% vs. 73%, p = 0.004), and the children who were unable to produce reliable FeNO values with

MINO were younger than children with successful measurements. The success rates in different age groups appear in Table 9.

The NIOX analyzer produced slightly higher FeNO values than did the NIOX MINO analyzer (mean FeNO values 9.9. and 7.8 ppb, respectively, p = 0.002). However, the measurements with the two devices correlated significantly (r = 0.972). The difference between the devices according to the Bland-Altman plot was 1.1 ppb with limits of the agreement between -4.4 and 6.7 ppb.

The mean FeNO with NIOX MINO was higher (14.3 ppb, median 9.5, range 2-48 ppb) in children with asthmatic symptoms than in control children (median 6.0, range 1.0-15.0 ppb, p = 0.028, Figure 4). With NIOX, the difference between the groups did not reach the statistical significance. In children with asthmatic symptoms the mean FeNO with NIOX was 15 ppb (median 8.6, range 3.9-46.9 ppp) and in the control children 8.6 ppb (median 7.5, range 4.2-16.5 ppb, p = 0.071).

Table 9. Success rates in different age groups.

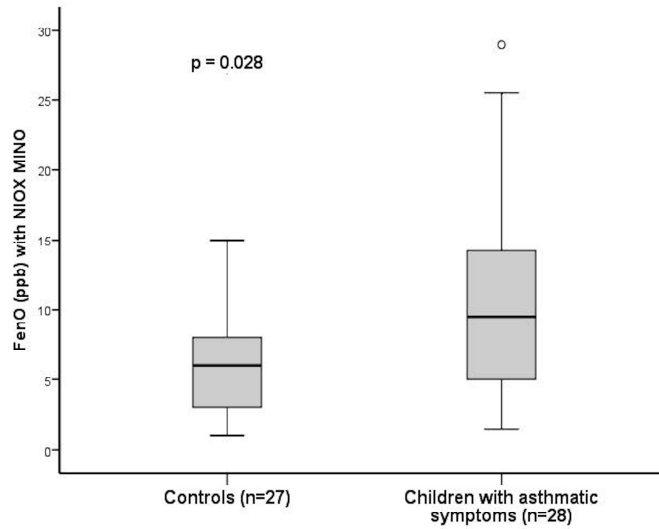| Age (years)	Number of children	NIOX	NIOX MINO
3 - 4	15	11/15 (73%)	7/15 (47%)
5 - 6	31	31/31 (100%)	24/31 (77%)
7 - 8	9	9/9 (100%)	9/9 (100%)
All	55	51/55 (93%)	40/55 (73%)

Figure 4. The median FeNO value measured with NIOX MINO in children with asthmatic symptoms and controls. The box and whisker plot describes the first and third quartiles (bottom and top of the boxes, 50% interquartile range, IQR). The band inside the box is the median and the whiskers show the top and bottom 25% of scores. The circle represents an outlier.

5.2 Environmental tobacco smoke (Study II)

5.2.1 Exposure to parental tobacco smoke

According to the questionnaires that the parents completed at the enrollment in the study, 43% of the children were exposed to ETS from parental smoking. The schematic illustration of the parental smoking appears in Figure 5.
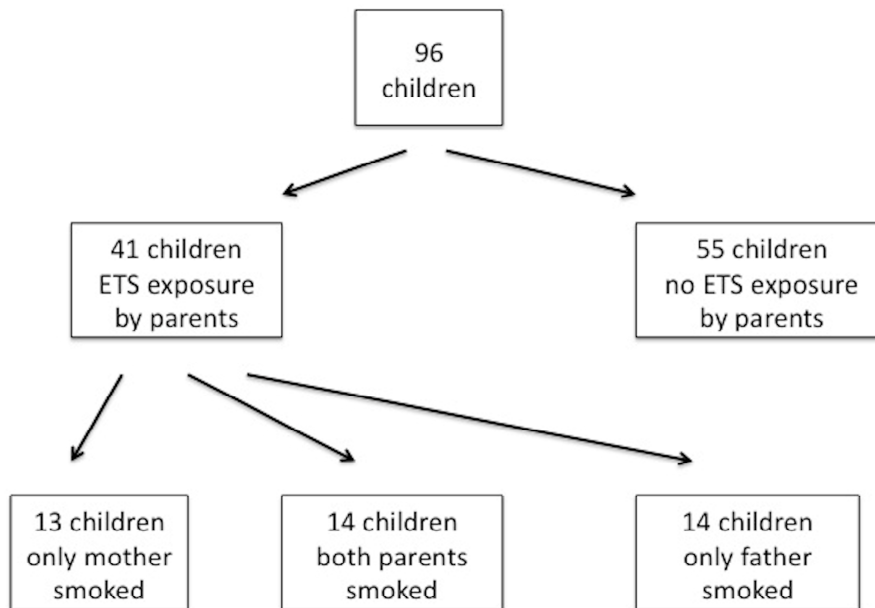
Figure 5. Schematic illustration of the smoking status in families according to the parental reports.

5.2.2 Parental reports and cotinine concentrations

The majority of the children (58%) had undetectable urinary cotinine concentrations. Therefore, the median cotinine concentration was < 0.01 µg/l (range 0-17.1 µg/l). The children with ETS exposure from parental smoking (median 2.0 µg/ml; range 0-17.1 µg/ml) had higher cotinine concentrations than did children with no parental smoking (median < 0.01 µg/l; range 0-13.9 µg/l, $p = 0.001$) (Figure 6). The number of cigarettes smoked by the parents and the urinary cotinine concentration correlated moderately ($r = 0.404$, $p < 0.001$). Urinary cotinine concentrations were highest in children with only the mother smoking (n=13; median 4.1 µg/l; range 0-9.9 µg/l) and in children with both parents smoking (n=14; median 4.6 µg/l; range 0-17.1 µg/l). We found a significant difference between these two groups and children with non-smoking parents (n=55; median < 0.01 µg/l; range 0-13.9 µg/l) or with only the father smoking (n=14; median < 0.01 µg/l; range 0-4.4 µg/l, $p < 0.001$).
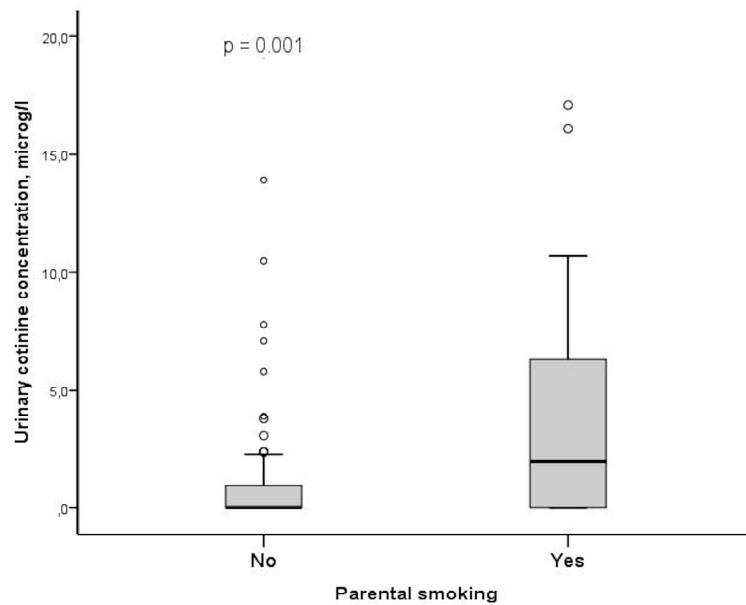
Figure 6. Urinary cotinine concentration grouped according to parental smoking. The box and whisker plot describes the first and third quartiles (bottom and top of the boxes, 50% interquartile range, IQR). The band inside the box is the median and the whiskers show the top and bottom 25% of scores. The circles represent outliers.

5.2.3 FeNO level and exposure to tobacco smoke

The median FeNO z-score was significantly higher in children with a smoking mother (median 3.3 SD; range 0.07-6.7 SD) than in children with a nonsmoking mother (1.9 SD; range -0.62 - 6.53 SD, Figure 7). The FeNO level was lower in children with only the father smoking (n = 14, median 11.2 ppb) and in children with nonsmoking parents (n = 55, median 12.9 ppb) than in children with both parents smoking (n = 14, median 25.4 ppb) or in children with only the mother smoking (median 23.3 ppb, p 0 0.46). The FeNO z-score and the urinary cotinine concentration correlated (r = 0.22, p = 0.03). In addition, the FeNO z-score and the number of cigarettes smoked in the family correlated (r = 0.22, p = 0.034).
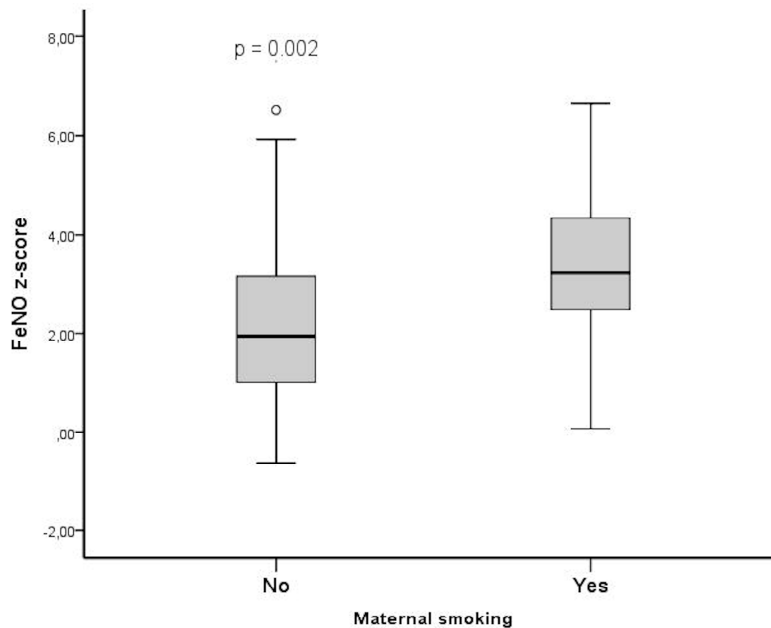
Figure 7. FeNO z-score in children grouped by maternal smoking. The box and whisker plot describes the first and third quartiles (bottom and top of the boxes, 50% interquartile range, IQR). The band inside the box is the median and the whiskers show the top and bottom 25% of scores. The circle represents an outlier.

5.2.4 Exposure to tobacco smoke and lung function

Lung function was assessed with IOS. The R5 z-score was higher in children with only the mother smoking (median 0.61, range -1.4 - 3.7) than in children with a non-smoking mother (median -0.008, range -2.1-4.8, p = 0.05, Figure 8). However, we found no difference in R5 between the children with a smoking father and children with a nonsmoking father, nor did we find correlation between the R5 and the cotinine concentration. We also found no correlation between the cotinine concentrations or reported ETS and X5, X10, bronchodilatation effect or the extent of the exercise-induced bronchoconstriction.
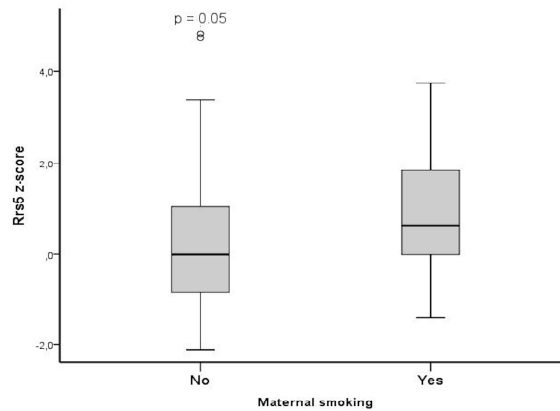
Figure 8. R5 z-score according to maternal smoking. The box and whisker plot describes the first and third quartiles (bottom and top of the boxes, 50% interquartile range, IQR). The band inside the box is the median and the whiskers show the top and bottom 25% of scores. The circles represent outliers.

5.2.5. Skin prick test positivity in relation to tobacco smoke exposure

Of all the study children 75% tested positive on the skin prick test (SPT). At least one positive reaction on the SPT was more common in children with ETS than in children with no tobacco smoke exposure (85% vs. 68%, p = 0.043). The urinary cotinine concentration showd no association with SPT positivity, eosinophilic count or eosinophilic cationic protein.

5.3 Airway hyperresponsivess (Study III)

Of all the 121 children, 23 (19%) were using regular asthma control medication at the time of the study (none of the children in the TLS group, 34% of the children in the EW group, 13% of the children in the BPD group and none of the controls). All of the children took the exercise test and methacholine challenge tests, and 97 children took the mannitol challenge test. The FeNO level was assessed for 114 children.

5.3.1 Baseline lung function

The children in BPD group had a higher baseline R5 (median R5 z-score 1.5 SD, range -1.4-6.2 SD) than did the children in other groups (TLS 0.04 SD; range -1.8-2.8 SD, EW 0.33 SD; range -1.8-3.5 SD, controls 0.02 SD; range -1.6-2.4 SD) (p = 0.023). We found no significant differences between the other study groups.

5.3.2 Exercise test

Altogether 27 (22%) children tested positive on the exercise test. The positivity rates in different patient groups appear in Table 10. The children in the TLS group showed a higher R5 increase after exercise (median 62%; range -13-159%) than children in the EW (median 19%; -16-111%), BPD (23%; -12-53%) or control groups (9%; 6-20%) (p < 0.001).

Table 10. Positivity rates for different airway hyperresponsiveness tests by patient groups.

	All (n = 121)	TLS (n = 31)	EW (n = 61)	BPD (n = 15)	Controls (n = 14)
Exercise test positive, n (%)	27 (22)	19 (61)	6 (10)	2 (13)	0
Methacholine test positive, n (%)	84 (69)	24 (77)	47 (77)	10 (67)	3 (21)
Mannitol test positive, n (%)	10 (11)	3 (10)	4 (7)	3 (20)	

TLS = troublesome lung symptoms, EW = early wheezers, BPD = bronchopulmonary dysplasia

5.3.3 Methacholine challenge test

Altogether 86 (69%) of the study children tested positive on the methacholine challenge test. The positivity rates in different patient groups appear in Table 10. The children with symptoms were more reactive to methacholine than were the children with no symptoms. However, the test was unable to differentiate the TLS, EW and BPD groups from each others. The median $PD_{40}R5$ in the TLS group was 90 µg, in the EW group 190 µg, in the BPD group 210 µg and in the controls 590 µg. The methacholine challenge succesfully distinguished between the TLS and control groups (p < 0.001), but not between the TLS and EW groups (p = 0.263) or the TLS and BPD groups (p = 0.348). We found a significant difference in $PD_{40}R5$ between the EW and control groups (p < 0.001), but the difference between the BPD children and controls did not reach statistical significance. The children with positive exercise test results displayed more reactivity in the methacholine challenge than did children with negative exercise test result (p = 0.004). The receiver operator curve for methacholine challenge to identify EIB appears in Figure 9.
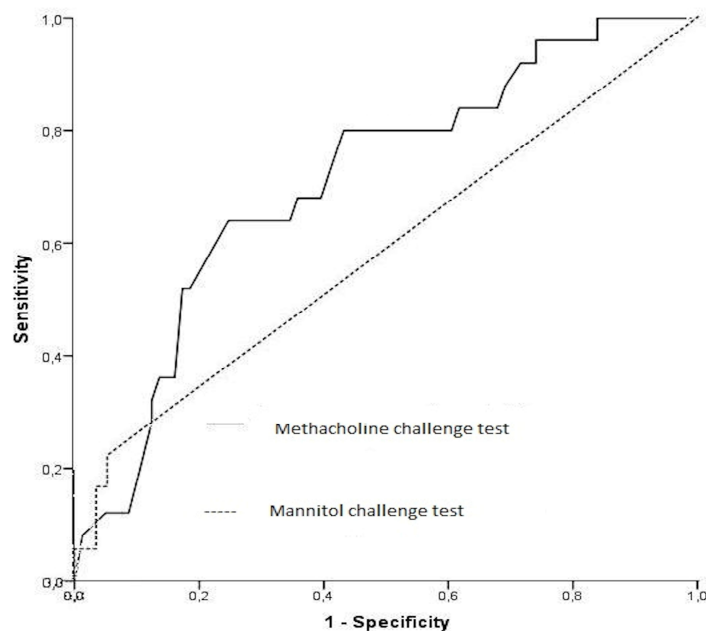

Figure 9. Receiver-operator curves (ROC) for the methacholine and mannitol challenge tests to identify exercise-induced bronchoconstriction. The area under the curve (AUC) for methacholine was 0.70 (CI 0.58-0.82, p = 0.02) and for mannitol 0.58 (CI 0.43-0.73, p = 0.32). Modified from Kalliola et al, the original for study III.

5.3.4 Mannitol challenge test

Of the 88 children who took the mannitol test 10 (11%) tested positive. The positivity rates for different patient groups appear in Table 10. The $PD_{40}R5$ was unable to distinguish between the study groups. The AUC for mannitol to identify EIB in the ROC analysis was 0.58 (Figure 9) indicating poor ability to identify children with a positive exercise test result.

The children who performed the mannitol challenge were also tested for peak inspiratory flow (PIF) before the challenge. Their median PIF was within the optimal range for the inhaler. Poor cooperation resulted in the failure of the mannitol challenge test in 9 cases. Coughing was the most common side effect during the challenge, and 65 children (73%) experienced coughing during the mannitol challenge. The median duration of the mannitol challenge test was 46 minutes (range 25-66 min).

5.3.5 FeNO, atopy and AHR tests

A total of 114 children were able to perform the FeNO measurement successfully. The FeNO z-score and the R5 increase during the exercise test correlated significantly (r = 0.43, $p < 0.001$, Figure 10). FeNO and the methacholine or mannitol challenge test results, however, did not correlate significantly.

Children with current TLS had higher FeNO z-scores (median 2.5 SD) than did children in the EW (1.0; -1.2-3.8), BPD (0.6; -0.9-2.1) or control (0.6; -1.2-2.3) groups (p < 0.001).

Skin prick test positivity was found in 46 (38%) of the children. The extend of the EIB was greater in atopic (median increase in R5 43%) than in nonatopic children (14%, p < 0.001, Figure 11). This increase was even more pronounced in children with current TLS. In this group, the atopic children saw a median 69% increase in R5 after exercise test compared to 17% increase among nonatopic children (p = 0.006). In addition, children with atopy showed more reactivity in the methacholine challenge test than did nonatopic children (median 130 µg vs. 250 µg, p = 0.02), but not in the mannitol challenge test.
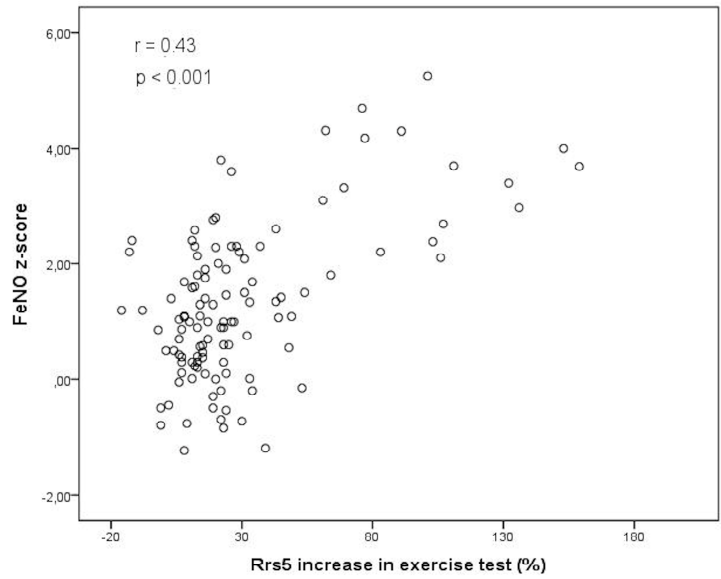
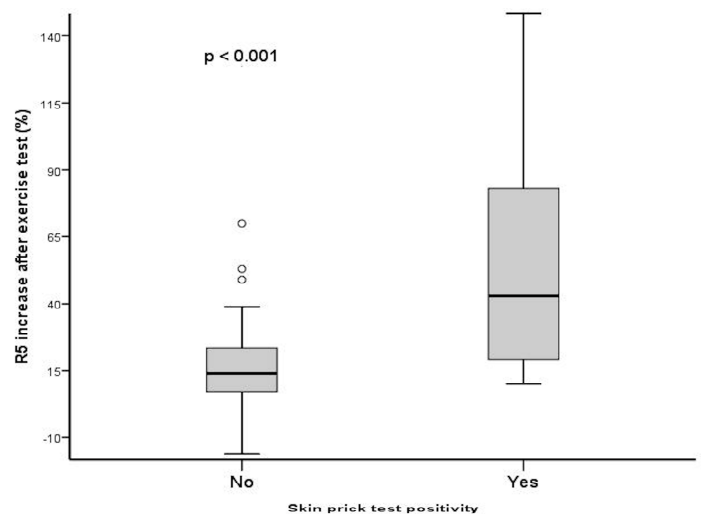*Figure 10. The correlation between the exercise-induced increase in R5 and the FeNO z-score.*

Figure 11. R5 increase after the exercise test among atopic and nonatopic children. The box and whisker plot describes the first and the third quartiles (bottom and top of the boxes, 50% interquartile range, IQR). The band inside the box is the median and the whiskers show the top and bottom 25% of scores. The circles represent outliers.

71

5.3.6 Multivariate analysis

A positive result in skin prick test (OR 10.2, 95% CI 2.9-36.4, p < 0.001) and a higher FeNO level (OR 1.1, 95% CI 1.0-1.2, p = 0.012) were associated with a positive result on the exercise test. We found no such associations with the methacholine or mannitol challenge tests.

5.4 Small airway function (Study IV)

All the selected oscillometry parameters were available for 109 of the 121 children. These children were divided into three groups according to their result on the exercise test. The EIB negative group was comprised children with R5 increase less than 40% on the exercise test (n = 84), 13 children had moderate EIB (R5 increase from 40% to 80%) and 12 children showed severe EIB (R5 increase over 80%).

Children with severe EIB had more skin prick test positivity (100%) than did children in the moderate EIB (77 %) or no-EIB groups (20%) (p < 0.001). These children were also using more short-acting beta-agonists (92%) than were children with moderate (31%) or no EIB (11%) (p < 0.001).

5.4.1 Baseline lung function

The children with moderate EIB had a higher R5 at baseline (median 0.92; range 0.72-1.66) than did children with severe (0.78; 0.54-1.02) or no EIB (0.82; 0.52-1.84) (p = 0.048, Table 11). We found no significant differences between the groups in R5-20, R20, X5 or X10.

5.4.2 Methacholine challenge test

Of all 109 children 68% had reactivity to methacholine indicated by an R5 increase 40% or more. The children with severe or moderate EIB were more reactive to methacholine than children with no EIB (Figure 12).
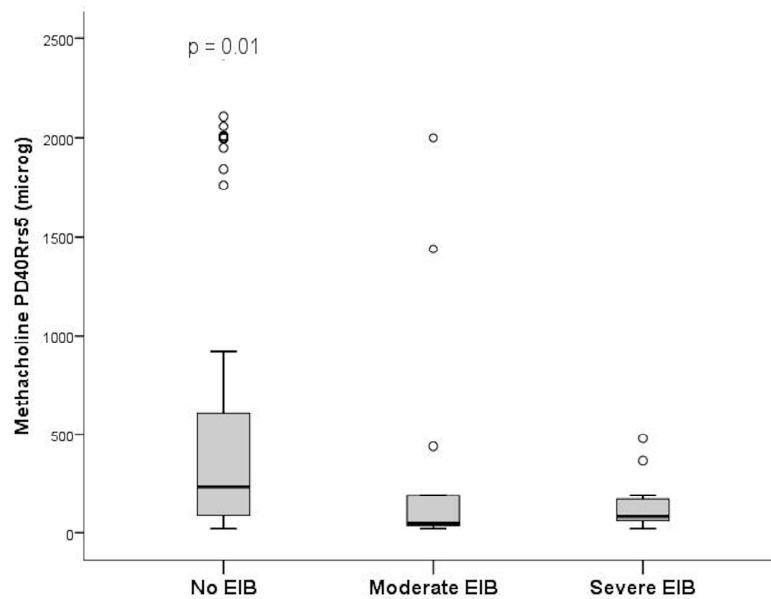
Figure 12. Reactivity to methacholine in three different patient groups. No EIB = R5 increase < 40 %, moderate EIB = R5 increase 40–80 %, severe EIB = R5 increase ≥80 %. The box and whisker plot describes the first and third quartiles (bottom and top of the boxes, 50% interquartile range, IQR). The band inside the box is the median and the whiskers show the top and bottom 25% of scores. The circles represent outliers.

5.4.3 IOS parameters after the methacholine challenge test

The median R5-20 increase after the methacholine challenge was higher in children with severe EIB (median increase 2.61-fold; range 1.13-4.00) than in children with moderate EIB (1.48; 0.7-2.84) or no EIB (1.74; -4.52-11.5, Table 11) (p = 0.036). In pairwise comparison the difference between the severe EIB group and the non-EIB group reached the statistical significance (p = 0.019, Figure 13).

Table 11. IOS parameters before and after the methacholine challenge in three different groups.

	All	ET-negative	ET 40-80 %	ET > 80 %	p-value
Baseline R5 z-score, median	0.16 (-3.0-5.53)	0.18 (-3.0-5.53)	0.41 (-1.14-3.64)	-0.63 (-2.47-0.62)	**0.028**
Baseline R20 (kPa/l/s), median (range)	0.62 (0.44-1.05)	0.63 (0.44-1.05)	0.67 (0.55-0.89)	0.59 (0.46-0.81)	0.182
Baseline R5-R20 (kPa/l/s), median (range)	0.19 (0.04-0.89)	0.19 (0.04-0.86)	0.25 (0.12-0.89)	0.18 (0.05-0.30)	0.064
Baseline X5 (kPa/l/s), median (range)	-0.23 (-0.82-0.42)	-0.23 (-0.82-0.42)	-0.23 (-0.52-0.21)	-0.20 (-0.3-0.25)	0.221
Baseline X10 (kPa/l/s), median (range)	-0.12 (-0.61-0.0)	-0.12 (-0.59- -0.01)	-0.14 (-0.61- -0.05)	-0.12 (-0.18-0.0)	0.151
R5 (fold increase), median (range)	0.52 (-0.13 -1.09)	0.50 (-0.13-0.90)	0.49 (0.28-0.94)	0.46 (0.35-1.09)	0.88
R5-20 (fold increase), median (range)	1.74 (-4.52 -11.5)	1.74 (-4.52-11.5)	1.48 (0.70-2.84)	2.61 (1.13-4.00)	**0.036**
R20 (fold increase), median (range)	0.12 (-0.22 -0.58)	0.12 (-0.22-0.47)	0.12 (-0.14-0.28)	0.12 (-0.10-0.58)	0.79
X5 (fold increase), median (range)	0.96 (-4.58 -3.91)	1.05 (-4.58-3.91)	0.67 (-3.48-2.62)	0.79 (-2.80-2.82)	0.58
X10 (fold increase), median (range)	2.13 (-0.50 -20.0)	2.17 (-0.50-20.0)	2.07 (0.38-3.71)	2.25 (0.92-5.60)	0.34
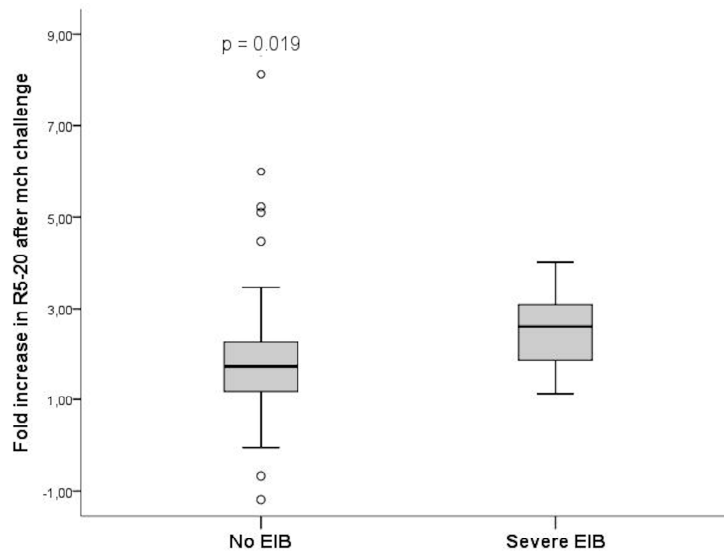
Figure 13. R5-20 increase after the methacholine challenge in children with severe EIB and children with no EIB. Mann-Whitney test is used for pairwise unbound comparison. No Bonferroni correction is used. The box and whisker plot describes the first and third quartiles (bottom and top of the boxes, 50% interquartile range, IQR). The band inside the box is the median and the whiskers show the top and bottom 25% of scores. The circles represent outliers.

6. Discussion

In this study, we examined airway hyperresponsiveness, bronchial inflammation, small airways function and the effects of parental smoking in young children with respiratory symptoms. According to present results, the exercise test with impulse oscillometry is a feasible method for evaluating preschool respiratory symptoms and can successfully identify children with probable asthma. The methacholine challenge test is less specific, but showed higher positivity rates in children with probable asthma, BPD history and early wheezing symptoms than in controls. The mannitol challenge test was unable to differentiate the patient groups. Children with severe exercise-induced bronchoconstriction seem to have small airways dysfunction despite their young age. To evaluate bronchial inflammation, a portable FeNO analyzer can serve to distinguish asthmatics from the controls, but its success rate in young children is lower than with the stationary device. Exposure to maternal smoking is a well-known risk factor for childhood asthma. This study was able to show a connection between the children's urinary cotinine concentration and bronchial inflammation; furthermore, children with a smoking mother had lower lung function than did children with a non-smoking mother.

6.1 Methodological considerations

6.1.1 Study populations

Of the 272 children (aged 3-8 years) examined in this study, 240 had obstructive lung symptoms and 41 were healthy controls. The study children were homogeneous: the mean ages in three study groups (Table 8) were 5.7, 5.6 and 5.9 years. The major strengths of this study are that the children were well characterized and the lung function of every child was assessed objectively with impulse oscillometry in studies II, III and IV. Children using asthma-control medication stopped doing so for four weeks before the lung function tests in order to exclude the masking effect of the inhaled corticosteroids.

The children in study II were multiple-trigger wheezers with normal basic lung function but had bronchodilator responsiveness or exercise-induced bronchoconstriction, which means that ascertaining the detrimental effect of the environmental tobacco smoke cannot be directly generalized to the normal population. The groups of 15 BPD children and 14 controls in study III were small which lowered the power of the study.

As noted earlier, several preschool wheezing phenotyping systems are provided mainly based on the retrospective analysis, but none of them is optimal when evaluating children with current respiratory symptoms. Regarding the cross-sectional study design, the various

76

patient groups presented in this study do not completely follow the current definitions of the preschool wheezing (3).

6.1.2 Lung function, airway hyperresponsiveness and FeNO measument

Lung function measurement with impulse oscillometry in young children is in routine use in the laboratory of Physiology at the Helsinki University Central Hospital's Allergy Department. Finnish reference values for the interpretation of the results are available and the measurements adhere to international guidelines (5,153,182).

No validated guidelines for the AHR tests with IOS exist, but the ATS/ERS statement provides recommendations for the AHR assessments in preschool children (153). The exercise test with IOS has shown the ability to distinguish wheezy children from controls in the Finnish population (182) and the method is in routine use in our laboratory. An increase of 35-40 % in R5 is widely accepted as a suitable endpoint in challenge tests with IOS measurements (153,167,182).

We used a modified dosimetric method to carry out a methacholine challenge that would be appropriate for preschool children (306). The method was easy to perform and all the study children were able to complete it successfully. A cut-of value of 400 μg for a positive $PD_{40}R5$ was chosen based on the data from Schulze et al (201). The value of 400 μg would correspond to a $PD_{20}FEV1$ of < 1 mg, which indicates significant AHR (308). The lower cut-off value for young children could more accurately distinguish between asthmatic and healthy children.

The mannitol challenge was adhered to the published protocol (220). The mannitol challenge test with IOS in school-age children has yielded comparable results to those of the spirometric technique (230). A positive test result was defined as $PD_{40}R5$ based on the established interpretation in adults (220). The inhalation technique was verified prior to the challenge, and all the study children passed the test. However, inhalation of up to 19 powder doses seemed demanding for children under school age. The control children did not perform the mannitol challenge test, which is one of the limitations of the study. However, given the low positivity rate on the mannitol challenge test in the present study, the result would probably be no different even if we have tested control children as well.

We used the characteristics of the receiver operator characteristics curves (ROC) to examine the ability of methacholine and mannitol tests to identify exercise-induced bronchoconstriction. Neither of the tests showed good ability to identify EIB. The assessment of asthma diagnosis in preschool-age children has no gold standard. However, previous studies have shown that the exercise test with IOS can distinguish children with current wheeze from healthy controls (182). According to the present study, the examined AHR tests cannot serve to test surrogates for each other.

FeNO was measured according to ATS recommendations (262); a well-experienced nurse assisted us with the measurements. The devices automatically reject non-acceptable exhalations. ATS/ERS recommends at least a 4 s exhalation for a child over four to five years old (262); the present study used an exhalation flow of at least 6 s. The shorter exhalation may improve the FeNO measurements in young children, and could have improved the success rate of measurements taken with a portable device. Measurement taken with the stationary NIOX analyzer was performed first, followed by the assessment of FeNO with MINO after a 10 to 15 min interval. This may have affected the study result through the learning effect, and could have lowered the success rate with MINO without first performing the NIOX.

6.2.3 ETS monitoring

Exposure to tobacco smoke was monitored with the measurement of urinary cotinine concentrations. The cotinine level measurement in body fluids offers a more objective method for monitoring ETS exposure than the parental reports. The used gas chromatographic method for the cotinine analysis has a lower detection limit of 0.7 µg/l (309). The recommended cut-off value for the second-hand tobacco smoke exposure is 0.5 µg/l, which means that some of the low cotinine levels may have gone undetected. However, the difference between the 0.7 µg/l and 0.5 µg/l is minor and may have had no significant influence on the study results.

6.2 Discussion of the main results

6.2.1 FeNO measurement with two analyzers in young children

Stationary FeNO analyzers are relatively expensive, require special expertise and are used mostly in specialized clinics. The present study, which compared the feasibility, accuracy and repeatability of using a portable, small and hand-held device (NIOX MINO) to a stationary device (NIOX) in young children showed that the portable analyzer is a practical tool for screening FeNO levels in young children. However, its use in this age group is limited due to relatively poor accuracy, a higher lower detection limit and a lower success rate than the stationary device. Still, both devices were able to distinguish between higher FeNO levels in asthmatics and lower levels in healthy children.

The overall success rate with the stationary NIOX analyzer was 93% and with the portable NIOX MINO 73%. These findings are in agreement with a report from Great Britain in which investigators saw a 71% success rate with NIOX MINO in median nine-year-old children (311). A study in Switzerland, which used a conventional analyzer and

hand-held device to examine children of a mean age of 11.8, found that all of the study children were able to perform the measurement successfully (265). In addition, they found a between-method agreement within a clinically acceptable range. Children in the present study were younger than those in earlier studies. The lack of visual motivation may be a factor that influenced the lower success rate with the hand-held device compared to the stationary device. With the stationary device the children are able to follow the balloon animation on the computer screen, which helps with constant exhalation. In the portable device, the pitch of the flow marker sound guides the exhalation. A retrospective analysis that recently evaluated a portable MINO device in four- to seven-year-olds (297) found a correlation between the FeNO and asthma severity but not with lung function. The study did not report success rates with the portable device. Therefore, the success rates in the present study are in line with those of the earlier reports. The younger the child, the more difficult the performance of the hand held device seems to be.

The limits of agreement were wide in NIOX MINO in relation to the low FeNO levels. The lower detection limit in NIOX MINO is 5 ppb compared to 1.5 ppb in NIOX (www.aerocrine.com). This latter detection limit may be acceptable in adults and asthmatic children, but in young healthy children it may be too high. In addition, the manufacturer specifies a wider accuracy in MINO (values < 50 ppb, ± 5ppb) than in NIOX (values < 50 ppb, ± 2.5 ppb). This may be important in lower FeNO levels (around 5 ppb), which frequently occur in young healthy children or in monitored pediatric asthmatics. On the other hand, the clinical relevance of the FeNO values at these low levels may be only modest. In conclusion, the study suggests that NIOX MINO can be used as a screening tool in young children, but its feasibility at low FeNO levels is limited.

6.2.2 Exposure to tobacco smoke and respiratory health in preschool-age multiple-trigger wheezers

This study, which investigated exposure to tobacco smoke with urinary cotinine measurements, found a correlation between the FeNO z-score and urinary cotinine levels in preschool-age children with multiple-trigger wheezing. In addition, a dose-response relationship was shown between the urinary cotinine level and the number of cigarettes smoked in the family. Children with a smoking mother had lower lung function and a higher FeNO level than did children with a non-smoking mother.

Most studies have used parental reports to monitor ETS. However, people tend to underestimate their bad habbits. Fortunately, ETS exposure can also be monitored by measuring of cotinine in bodily fluids. Cotinine is a major nicotine metabolite with anelimination half-time of approximately 20 hours, compared to two hours of nicotine, which renders it useful for monitoring ETS over the previous three to four days (12). Nicotine and cotinine metabolism may differ across individuals, which may influence the cotinine levels (312). Moreover, the timing of the spot measurements in relation to

tobacco smoke exposure can affect the outcome. However, measuring cotinine in bodily fluids offers an objective and accurate tool for estimating tobacco smoke exposure.

In the present study, the children with a smoking mother or two parents smoking had the highest urinary cotinine levels. This result is in agreement with the results of Japanese researchers who showed higher urinary cotinine concentrations in children with smoking mothers or two parents smoking than in children with only the father smoking or two non-smoking parents (131). The overall median urinary cotinine concentration of the children was low (median cotinine < 0.01 µg/l). However, some of the non-exposed children showed detectable cotinine concentrations possibly due to unreliable parental reports or exposure to ETS outside the home. An American study examined cotinine levels in the serum and saliva of 619 children who were hospitalized for asthma or wheezing (140). A detectable cotinine concentration was associated with readmission to hospital. Interestingly, 39% and 70% of those children whose caregivers reported no smoking had detectable serum or saliva levels, respectively (140). Some of the exposure may come from the third-hand smoking, which refers to the particulates that remain on the surfaces and in the dust after cigarettes finished burning (313). These pollutants can be re-emitted into the gas-phase or they can react with other environmental compounds to form harmful products (313). Overall, the measurement of urinary cotinine concentrations seems to be a realiable method for monitoring ETS exposure and offers additional information on parental reports.

In the current study, 43% of the children were exposed to parental tobacco smoke. According to the latest tobacco statistics, 14% of Finnish working-age women and 21% of men smoke (105) and during the study years from 2002 to 2005 21% of women and 26% of men were smokers (105). Consequently, exposure to ETS in the present study children was rather high. All of these study children had lower respiratory tract symptoms, and their exposure to parental tobacco smoke may have caused some of the symptoms and deteriorated lung function among this group.

The detrimental effects of exposure to tobacco smoke on children's health are well established (11,122,314). Children are more sensitive to the negative effects of ETS than are adults; children have higher respiratory volumes in relation to their body size than adults, their metabolism, respiratory and immune systems are still developing and their life-time expectancy is higher (313). Maternal smoking seems to influence children's health and lung function more than paternal smoking. This is suggested to be due to detrimental effects of maternal smoking during pregnancy (110,122). One of the limitations of the present study is that we had no data on the maternal smoking status at the time of the pregnancy. Separating pre- and postnatal ETS exposure is difficult, but some evidence suggests that postnatal exposure increases respiratory symptoms in children (119). Even if these children already had dimished lung function at birth, the permanent postnatal exposure to tobacco smoke surely has negative effects on lung function also. Moreover, children with a smoking mother had higher FeNO levels than did children with no ETS exposure or with only the father smoking, indicating that the

bronchial epithelium had an active inflammation at the time of the present study. Small children seem to be close to their mothers even if their mothers report smoking outside. A study that evaluated the effects of parental smoking on infants found eight times higher urinary cotinine levels in infants with parents who reported smoking outside than in children with non-smoking parents (315). Fathers probably smoke frequently at work or in other places outside the home. Thus, maternal smoking, especially during pregnancy, is more detrimental to a young child than is paternal smoking.

The patients in this study suffered from multiple-trigger wheezing and most of them were atopic (75%). These features may be the predisposing factors behind the detrimental effects of tobacco smoke exposure. Multiple-trigger wheezers are vulnerable to triggers such as allergens and ETS (3). R5 was higher in children with a smoking mother than in children with a non-smoking mother. A study of 41 preschool-age asthmatic children with decreased lung function in the presence of ETS reported similar findings (135). A recent report have showed that to locus of gene 17q21, which is associated with childhood asthma, has a sronger effect on the development of asthma in the presence of maternal smoking, thus suggesting interaction between the environment and genes via epistatic or epigenetic mechanisms (1,130). Moreover, one of the studies from the Tucson cohort found that parental smoking in infancy acts synergistically with the current smoking to reduce lung function in early adulthood (316). Therefore, ETS is especially harmful for subjects with a predisposition to asthma.

The negative influence of the maternal smoking on lung function was further supported by the finding that the FeNO level was higher in children with a smoking mother than in those with a non-smoking mother. In addition, the FeNO z-score and urinary cotinine level correlated positively. Earlier studies of FeNO and ETS have shown conflicting results, however. A study of young children with respiratory symptoms showed an association between the reported parental smoking and increased FeNO levels (275). In contrast, another study reported lower FeNO levels in ETS-exposed 8- to 18-year-olds with inhaled corticosteroids than in those children with no ETS exposure (139). A study of school-aged children with untreated allergic asthma found an association between decreased FeNO levels and parental smoking (276). A recently published report showed no association between ETS exposure and FeNO or lung function in school-age asthmatics (141). A study of school-age children found no association between the cotinine levels and FeNO (138). However, our study children were younger than in most of the other studies. To conclude, the contradictory results in studies of FeNO and ETS may be due to heterogeneous and small study populations and age-dependent differences in inflammatory responses.

The mechanism by which ETS raises the FeNO levels is unclear, but ETS may be an inflammation-causing irritant in the airway mucosa, especially in children with untreated multiple-trigger wheezing. Regular exposure to tobacco smoke is thought to change inflammatory responses and enhance Th2 reactions (126). FeNO levels in active smokers are lower than in non-smokers (271,272), possibly due to NO in tobacco smoke via the

negative feed-back system on inducible NO synthase (317). In a study of Scottish asthmatic bar workers, their FeNO levels decreased by 20% following smoke free legislation (318). A study of infants reported that maternal atopy and prenatal ETS exposure were associated with eleveated FeNO levels (319). Althought the current study could not assess the association, several studies have shown that atopy increases FeNO levels (8), which may be explained by the homogeneous study population with a high prevalence of atopy (75% of the children), and rather high FeNO levels in all the children. One of the strengths of the present study is that the population was relatively homogeneous and that the children were not using asthma-control medication, which can mask the inflammatory responses to various irritants. We studied confounding factors such as parental atopy and animals at home and found no associations with increased FeNO or decreased lung function. To summarize, ETS is suggested to alter inflammatory responses on airways and enhance atopic reactions.

6.2.3 Airway hyperresponsiveness

The current study used impulse oscillometry (IOS) to investigate airway hyperresponsiveness in preschool children. AHR was assessed with the exercise test, as well as the methacholine and mannitol challenge tests. The tested children represented various clinical patient groups. The study showed that the exercise test successfully identified children with current troublesome lung symptoms (probable asthma), whereas children with probable asthma, previous BPD and early wheezing symptoms tested positive on the methacholine test, but not children without respiratory symptoms. The mannitol test was unable to discern between the study groups.

Only a few studies have examined airway hyperresponsiveness in preschool-age children, partly due to the difficulties in lung function measurements in this age group. In addition, no standardized protocols for challenge tests are available for preschool children. In the 1990s a couple of papers were published on these issues. One study reported that the exercise test but not the methacholine challenge could differentiate asthmatic children from children with other chronic lung diseases (209). Another study showed that methacholine distinguished asthma and pediatric COPD from the controls but not from one another (197). Rather, the exercise test was able to differentiate asthmatics from subjects with other chronic lung diaseases. These results are in agreement with the present findings.

In the present study, we chose IOS parameter R5 as the end-point parameter as the ATS/ERS statement recommends (153). IOS is easy for a young child to perform, and success rates as high as 80 to 90% are common in this age group (5,166). In this study all the children were able to perform the challenge tests with IOS. IOS can also serve to assess AHR (320,321). In addition, research has shown that an increase in R5 and a decrease in FEV_1 in challenge tests correlate significantly (183,201). A German study that

used both spirometry and IOS to examine hyperresponsiveness to methacholine in 48 preschool children (201) found that IOS also detected roughly 70-80% of those subjects who responded to spirometry (201). Thus, IOS is a feasible method for assessing lung function and AHR in young children.

The current study showed that 69% of all the study children were reactive to methacholine, compared to 22% positivity in the exercise test. Children with probable asthma showed more often positivity in the exercise test than did children with early wheezing, BPD and controls. Instead, the methacholine challenge test result was often positive in children with probable asthma, BPD and early wheezing but not in controls. Methacholine is known to be highly sensitive, but less specific to asthma (189). The mechanisms by which direct and indirect challenge tests act in the bronchus differ. Methacholine acts directly on muscarinic receptors on the bronchial smooth muscle and does not directly depend on airway inflammation (322). Instead inflammatory cells such as eosinophils and mast cells, which release a variety of mediators under hypersosmotic conditions, may mediate exercise-induced bronchoconstriction (210). Furthermore, some some age-related and inherited features, may affect the results of airway hyperresponsiveness tests in young children (229). FeNO levels and exercise test result were associated, which may indicate the presence of eosinophilic inflammation, a conclusion that is in line with earlier reports (186,323). FeNO was also able to distinguish children with probable asthma from the other groups. To conclude, the exercise challenge test indentifies active asthma, but the presence of AHR for methacholine seems to be a less specific feature, at least in young children.

The children in the present study represented three groups with various clinical characteristics. However, grouping of these children with lower respiratory symptoms is difficult and some overlap between groups surely exists. Children with current troublesome lung symptoms were referred to a hospital because of suspected asthma. These children had no previous asthma diagnosis and their symptoms were started during their third to sixth years of life; according to widely used wheezing phenotypes could be called late-onset wheezers (2). The majority of these children (81%) were atopic, and 61% had a positive exercise test result. Children with positive exercise test results were diagnosed as asthmatics. This finding agrees with the concept that the exercise test is highly specific for pediatric asthma (209).

Of the children with early wheezing disorder who had wheezed before two years of age, 83% had multiple-trigger wheezing type. At the time of the present study, however, only 34% of them were using daily asthma control medication. This observation is in line with the knowledge that early wheezers often recover and cease to wheeze before school age. Early wheezing is associated with diminished lung function in infancy and maternal smoking but not to atopy (2). Wheezing phenotypes during the preschool years commonly change (2), the estimated prevalence of which is around 55% (1). In the present study, children with EW were less reactive in the exercise test than were children with under current suspicion of asthma. In contrast both groups showed high positivity rates in the

methacholine challenge test (77%), which agrees with the knowledge that the methacholine challenge test is sensitive, but less specific for current asthma (6), and even some (3 of 14) of the control children in this study exhibited reactivity in the methacholine challenge test.

Thus, the clinical implication of positive methacholine test results in preschool children is somewhat unclear. In Canada, 215 asthmatics and 197 healthy school-age children were examined with the methacholine challenge test. The study showed that methacholine may prove helpful when diagnosing asthma in atopic children, but is less useful in non-atopics (203). Several cohort studies have used AHR to methacholine to predict asthma or assess associations between different phenotypes and characteristics. A British study examined over 6000 children at seven time points during their first seven years of life to assess associations between the wheezing phenotypes and atopy, lung function and airway hyperresponsiveness (18). The strongest associations were found between the intermediate-onset wheezing phenotype and atopy (OR 8.36) and airway hyperresponsiveness to methacholine (OR 1.76) (18). Late-onset wheezing also associated with atopy and AHR, although more weakly than did intermediate-onset phenotype. Therefore, positivity in the methacholine challenge may indicate a tendency for airway hyperreactivity rather than be a marker of active asthma in young children.

Children with a history of neonatal bronchopulmonary dysplasia often exhibit diminished lung function and increased airway hyperresponsiveness in later childhood and in adulthood (55,57,174). The knowledge of AHR in BPD survivors at preschool age is scarce. Researchers in Korea used the methacholine and adenosine challenge tests with modified auscultation technique to examine prechool-age children with a history of BPD. Their results agree with those of the present study, in that the BPD survivors showed a high positivity rate in the methacholine test, but low responsiveness to the indirect adenosine test (59). The baseline R5 was higher in the BDP children in the present study than in the children of other groups indicating poorer lung function. Furthermore, the BPD survivors showed more reactivity in the methacholine challenge test than did the controls, although the difference did not reach the statistical significance. This finding supports the knowledge of the poor specificity of the methacholine challenge. Instead, the exercise test result failed to distinguish between the children with earlier BPD and controls or early wheezing symptoms but did differentiate them from children with current asthma suspicion. This result may be due to the presence of bronchial inflammation in children with probable asthma and the lack of it in BPD survivors; lower FeNO level in BPD survivors than in children with probable asthma supports this concept. To conclude, BPD children showed hyperresponsiveness to methacholine but not to the exercise challenge.

Mannitol challenge test

The mannitol challenge test is an indirect challenge test recommended as a surrogate for the exercise challenge (220,324), as it has shown results consistent with those of the

exercise test in school-age children (228), good power to diagnose asthma (185) and linear results with other bronchoprovocative tests (229). No previous studies have investigated the mannitol challenge test in preschool-age children with IOS.

The present study entailed 97 mannitol provocation tests. The mannitol challenge test was unable to differentiate patient groups from one another. Nine of these challenge tests were incompleted, because of cooperative problems. The mannitol challenge test involves inhaling up to a maximum of 19 doses of encapsulated mannitol powder if the entire protocol is completed. Although all of the study children were able to surpass the peak inhalation test before taking the challenge test, inhalating 19 powder doses seemed to be excessive for children of this age as repeated inhalations may cause impairment in inspiratory effort. As a result the powder may not have reached the peripheral airways. In addition, 75% of the children experienced coughing during the mannitol challenge test, which most of the children considered rather discomforting. Therefore, the results of the present study do not favour the use of the mannitol challenge test in young children.

6.2.4 Small airways function in relation to asthma severity

In the present study, we evaluated small airways function during the induced bronchoconstriction using impulse oscillometry. The study children were divided into three groups according their test results in the exercise challenge. The main finding was that severe exercise-induced bronchoconstriction was associated with small airways dysfunction and more frequent use of short-acting beta-agonists. We are aware of no earlier studies that have examined small airways function during a bronchoprovocative test in young children.

Exercise-induced asthma is a specific feature of childhood asthma (209), and free running is a natural way to induce asthmatic symptoms in preschool-age children. Assessment of asthma severity based on lung function assessment is challenging in young children. In the present study, asthma severity was defined by the extent of exercise-induced change in IOS R5 during the free running test. This change has been shown to correlate with the decrease in FEV_1 in school-age children (325,326) and adults (183,327). An increase of 35-40% in R5 in the challenge test is widely considered an abnormal response (167,182,237). The categorization was made analogously to common guidelines of EIB as assessed with spirometry (204). Therefore, the R5 increase from 40% to 80% was considered moderate EIB and an increase over 80% as severe EIB.

Although small airways dysfunction is considered important underlying feature of various asthma symptoms and severity, the assessment of perfipheral airways function has no gold standard (169). Airway inflammation, hyperresponsiveness and remodeling can affect the whole bronchial tree (169). The peripheral heterogeneity of the ventilation, air trapping and premature airway closure are considered as functional features in small airways dysfunction (237). Forced expiratory flow at 25% and 75% of forced capacity, as

measured with spirometry, is considered to be a marker of the peripheral airways, but has poor reproducibility (239,328,329). Some of the techniques for measuring small airways are invasive and complex to perform (240,241). Impulse oscillometry and multiple-breath nitrogen wash-out methods are non-invasive, and IOS is easily available also to young children. The effect of large airways cannot be exluded in most of the small airway measurements, so one should always interpret the results with caution (169).

Therefore, impulse oscillometry is proposed as a useful technique for assessing peripheral airways function (168,330) due to its ability to measure the frequency characteristics of respiratory mechanics (331). With increasing frequency and with heterogenous time constants in the peripheral airways due to redistribution of flows, studies have shown effective resistance and compliance in the lungs to decrease (331). Adult patients show this pattern, but mostly at frequencies below 5 Hz (332). The present study could assess the association between the increased R5-20 and severity of asthma. Although the frequency dependence of resistance above 5 Hz by the forced oscillation technique may reflect artifactual airway shunting (333), it cannot alone explain the finding of the present study. Thus, the present results suggest that children with severe EIB have small airways dysfunction as measured by frequency-dependent resistance in IOS.

Oscillometric parameters X5 (reactance at 5 Hz) and AX (reactance area) are also considered markers of small airways function, as explained by the changes in the elastic properties of the respiratory system during ventilation heterogeneities with airway closure or near closure (334). The current study could assess no association between the reactance parameters and asthma severity, possibly due to challenges in impedance assessment at low frequencies in young children with possible artifacts in the higher harmonics of the fundamental breathing frequency (335). An Australian study found changes in reactance after methacholine challenge to be a more sensitive marker for a small airways obstruction than changes in resistance parameters in adults (236). Therefore, the present study could assess no association between the reactance indices and asthma severity.

Recent pediatric studies have shown that the baseline IOS indices R5 and AX predicted loss of asthma control more accurately than did spirometry (168,243). Another investigation found that asthma patients with peripheral airway dysfunction, as measured by FEF25-75 had more severe AHR independently of the FEV1 level (336). An Italian study was able to assess an association between the R5, R5-20 and X5 and the magnitude of the hyperresponsiveness to methacholine in school-age children and adults (337). However, studies of small airways function in children are rare.

We chose to assess small airways function during the methacholine challenge because similar investigations in adults have found connections between the signs of peripheral airway dysfunction and asthma severity (10,238). The present findings suggest a similar phenomenon occuring in young children with obstructive symptoms. The present results suggest that the baseline lung function measurement in young children seems to be a less sensitive marker for peripheral airway dysfunction. Two bronhcoscopic studies aimed to

examine small airways function during bronchoprovocation test (338,339). The first study found that higher small airway resistance at baseline was associated with greater airway hyperresponsiveness to methacholine (339). The second study reported a faster increase in peripheral airway resistance in asthmatics than in healthy persons after histamine challenge (338). Furthermore, investigators from the UK reported an association between small airways obstruction with IOS during methacholine challenge and dyspnea perception in adult asthmatics (181). In addition, Dutch researchers found less peripheral obstruction as measured with IOS during the methacholine challenge in asymptomatic adults with AHR than in symptomatic subjects (330). According to the present results, the small airways dysfunction may present even in young asthmatic children.

7. Future considerations

According to the present study, exercise test with IOS is a feasible method for examining respiratory symptoms in young children. Methacholine challenge with IOS is a promising option for the assessment of bronchial hyperresponsiveness also in young children. However, the cut-off value of this test may need to be further evaluated in larger studies and may be lower than in older children and adults. The clinical implication of a positive response in methacholine challenge in this age group remains unanswered, thus warranting further research. Earlier studies have shown associations between the childhood bronchial hyperresponsiveness to methacholine and later asthma (18), but the evidence of the clinical relevance of this AHR to the present symptoms at preschool age is lacking. A further challenge in interpreting the direct AHR test results is the lack of a gold standard for asthma diagnosis in preschool children.

The findings of the mannitol challenge test in the present study discourage the use of this test in the assessment of bronchial hyperresponsiveness in preschool children. A shorter protocol may enhance the feasibility of the test in this age group, thereby making it easier to carry out.

The FeNO measurement can also serve to estimate airway inflammation in preschool children. The development of new hand-held FeNO analyzers could facilitate their usage in young children. The FeNO level seems to reflect eosinophilic inflammation in atopic asthmatics. However, the FeNO level is not recommended for use in tailoring asthma medication. In selected individuals, its assessment may help to monitor adherence to treatment and to characterize the asthma phenotype. For research purposes the FeNO assessment seems to be a feasible method for monitoring airway inflammation even in young children.

Tobacco smoke and exposure to tobacco smoke are known to harm health in many ways. Interestingly, the elevated risk for asthma conferred by known childhood asthma gene loci is further pronounced by environmental tobacco smoke in the early years of life (130). The upcoming years will likely see an increase in our knowledge of the gene-environmental interactions and the enhancement of our tools to modify early wheezing outcomes.

8. Conclusions

Estimating bronchial inflammation with a portable FeNO analyzer is a feasible option in children over four years old, especially when it comes to distinguishing between asthmatics and the healthy. However, it is more difficult to use with preschool-age children than the older, stationary device. Moreover, the hand-held analyzer has a higher lower detection limit and poorer accuracy at low FeNO levels, which limits its use in young children.

We used parental reports and children's urinary cotinine concentrations to examine exposure to parental tobacco smoke. Urinary cotinine concentrations closely reflected parental reports of their smoking habits. Furthermore, the FeNO z-score and urinary cotinine concentrations correlated. Exposure to tobacco smoke by a smoking mother was connected to reduced lung function and increased bronchial inflammation in young children with multiple-trigger wheezing, yet paternal smoking showed less impact.

The results of this study showed that bronchial challenge tests with impulse oscillometry can serve to examine airway hyperresponsiveness in young children. The exercise test performed as a free running test succesfully identified children with probable asthma. The methacholine challenge test showed lower specificity than did the exercise test, but yielded higher positivity rates in children with probable asthma, BPD and early wheezing symptoms than in healthy controls. Therefore, the finding suggests that the methacholine challenge may serve in assessing airway hyperresponsiveness even in young children, but the cut-off value for this age group may need to be re-evaluated. In addition, the clinical value of the positive methacholine challenge test in this age group remains unclear. The results of the mannitol challenge test discourage its use in preschool-age children.

We found small airways dysfunction in children with severe exercise-induced bronchoconstriction. The result was further supported with the finding that children with severe EIB were using short-acting beta-agonists more frequently than were children with no EIB or moderate EIB. The results postulate that the small airways may play an important role even in young children with severe asthma.

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Satu Kalliola

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