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Fractional exhaled nitric oxide and its relation to exercise, asthma and allergic rhinoconjunctivitis in a subarctic childhood population

A study of asthma and allergy among schoolchildren in Nordland County

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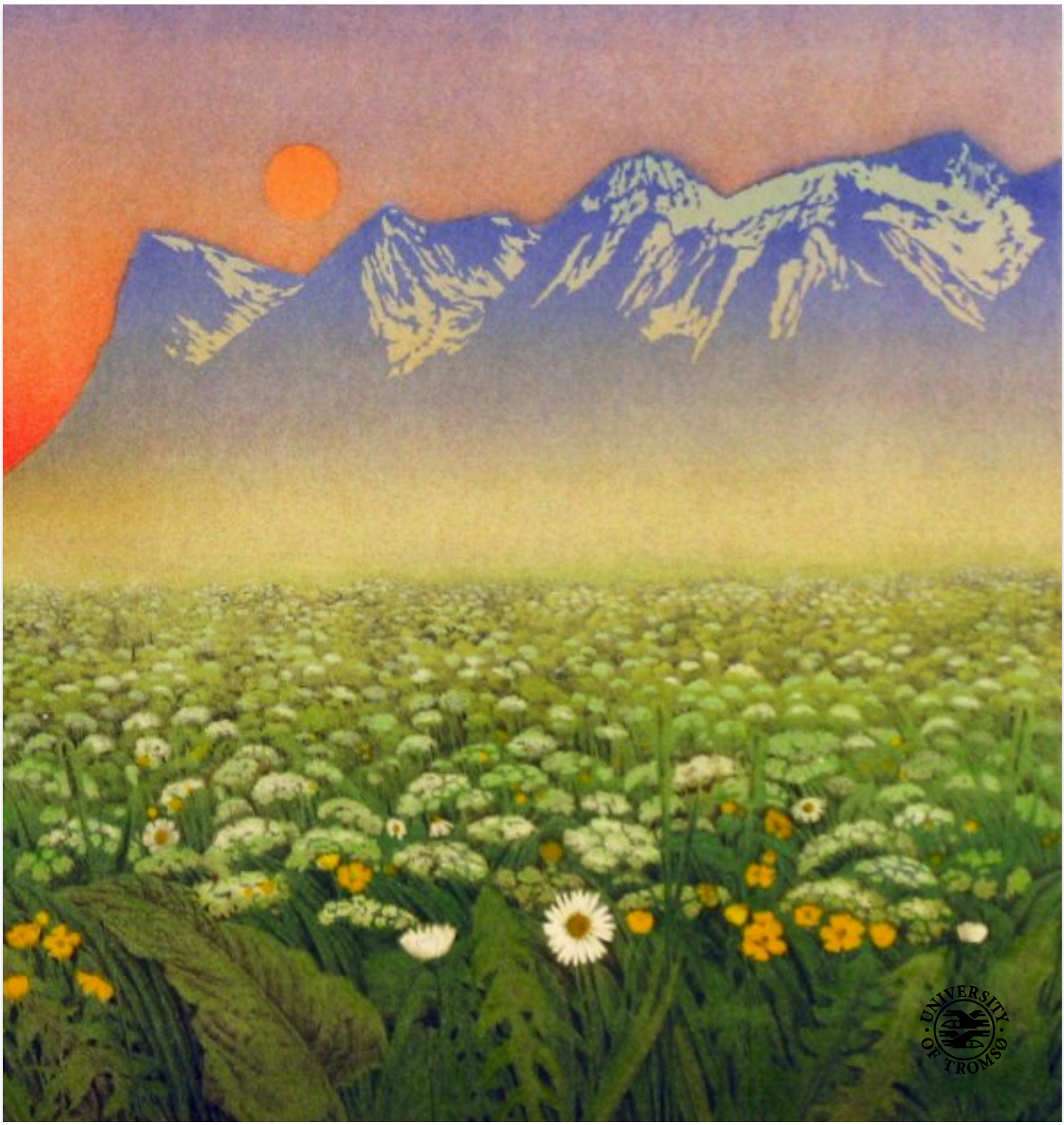
Bjørg Evjenth

A dissertation for the degree of Philosophiae Doctor – 2014



Fractional exhaled nitric oxide and its relation to exercise, asthma and allergic rhinoconjunctivitis in a subarctic childhood population

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Bodø, Norway
2014

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Acknowledgements

The studies included in this thesis were part of the research project ´Asthma and allergy among schoolchildren in Nordland´. They were carried out during the years 2008-2014 in collaboration with The Faculty of Health Science, Department of Community Medicine, University of Tromsø and The Department of Pediatrics, Division of Pediatrics, Obstetrics and Womens`s Health, Nordland Hospital, Bodø. The study was financed by grants from the Northern Norway Regional Health Authority, the Norwegian Respiratory Society and the Morten Jensens Foundation.

Many people have contributed to the work undertaken in this thesis. In particular I would like to thank the following persons for the support indicated.

First and foremost I gratefully acknowledge the enthusiasm and cooperation of all participating children and parents who have made this work possible.

My supervisor, Jan Holt MD PhD, for inviting me to be a PhD student in this project and for introducing me to the field of paediatric research. Thank you for always being accessible, and for your constructive discussions and outstanding supervision. I am ever grateful for your encouragement and patient support through all parts of the study.

Co-supervisor Professor Jon Øyvind Odland for initiating the study, contributing with language supervision, providing statistical advice, and for guidance with the PhD thesis.

My research partner Tonje Elisabeth Hansen for your hard work and `no problem` attitude. I thank you for generously letting me take part in the writing of Paper I, and your help and support during the writing of Paper II-IV. We travelled all over Nordland County. We performed all the examinations and procedures and shared valuable experiences.

Co-author Professor Ole-Lars Brekke for your sharp-minded and encouraging support during the writing of Paper IV.

Aud Sundsfjord and Birgit Andersen for performing the serum IgE analyses.

Professor Tom Wilsgaard and PhD Tonje Braathen for important contributions with statistical advice in Paper III.

Sandy Goldbeck-Wood for critical revising Paper III and IV, and Professor emeritus Evert Nieboer who has read through the thesis to improve my English.

Terje Tollåli for helpful discussions during the preparation of Paper III. I am also grateful to Olaf Alexandersen and Terje Tollåli for the opportunity I have been given to learn clinical lung medicine in their section.

Colleagues in the research group `Inflammation in human disease`, Institute of Clinical Medicine, University of Tromsø for helpful discussions and collaborations and Knut Dybwik for `Mac support`.

My gratitude is extended to Head of the Pediatric Deparment, Ingebjørg Fagerli who has been supportive of the project. I am also grateful for the support and flexibility received from all my enthusiastic colleges at the Pediatric Department.

Finally, I would like to express my sincere thanks to my family and friends and above all my husband Frank for your patience and enthusiasm. Thanks to Trine, Håvard and Torbjørn for your laughter and smiles.

Bodø, July 2014
Bjørg Evjenth

Sammendrag

Astma og allergisk øye- og nesekatarr (rhinokonjunktivitt, AR) er de vanligste kroniske sykdommene blant barn i den vestlige verden. I de siste tiårene har prevalensen (forekomsten) av sykdommene økt betydelig, men i enkelte Europeiske land rapporteres det om en utflating i prevalensen av astma. I klinisk praksis brukes laboratorietester til å understøtte diagnosene astma og AR. Analyser av biologiske markører i utåndingsluften kan gi verdifull informasjon om betennelsesmekanismer i luftveiene. Fraksjonen av ekshalert nitrogenoksid (FE_{NO}) er den eneste av disse markørene som er standardisert for bruk innen barnemedisin. FE_{NO} er en markør på eosinofil betennelse i nedre luftveier. FE_{NO} er omfattende studert, men hvilken effekt anstrengelsestester har på FE_{NO} hos barn er ikke fullstendig belyst. Diagnostikk av allergisk astma og AR inkluderer påvisning av allergen-spesifikt immunglobulin E (sIgE). Lite data er publisert om sammenhengen mellom nivåer av serum sIgE målt med Siemens IMMULITE® 2000 system (IMMULITE®) og hud prikk test (SPT) resultater hos barn. Det er ikke etablert kliniske grenseverdier for serum sIgE målt med IMMULITE® for å diagnostisere AR hos barn.

Formålene med studien var å undersøke prevalensen av astma, AR og eksem blant barn i en subarktisk befolkning, å kartlegge FE_{NO} nivåer i relasjon til astma og AR samt og undersøke effekten av anstrengelse på FE_{NO}. Likeledes ønsket vi å etablere kliniske grensenivåer for serum sIgE for å diagnostisere AR hos barn samt å utforske relasjonen mellom serum sIgE, total IgE og FE_{NO}.

Avhandlingen er basert på data fra fase I og fase II i studien `Astma og allergi blant skolebarn i Nordland`. Fase I var en tverrsnittstudie basert på et spørreskjema. Skolebarn (n=4150) i alderen 7-14 år fra 65 tilfeldige utvalgte skoler i Nordland fylke ble inkludert i denne undersøkelsen. Prevalensrater fra 2008 ble sammenlignet med data fra 1985 og 1995. Fase II var en klinisk undersøkelse av 801 skolebarn, rekruttert fra fase I. Foreldene besvarte et spørreskjema og et strukturert intervju. Videre ble det utført en klinisk undersøkelse, FE_{NO} målinger, spirometri, anstrengelsestest samt SPT og blodprøver.

Resultater fra fase I viste at prevalensen av astma, AR og eksem det siste året var 2-3 doblet fra 1995 til 2008. Livstidsprevalensen av astma og AR økte mens prevalensen av eksem, etter en økning mellom 1985 og 1995, var uendret i siste periode.

Resultater fra fase II viste at FE_{NO} nivåene var signifikant økt blant astmatiske barn sammenlignet med ikke-astmatiske barn, og signifikant høyere blant astmatiske og ikke-astmatiske barn med AR sammenlignet med barn uten AR. Barn med allergisk astma hadde de høyeste FE_{NO} verdiene. Ett minutt etter en submaksimal anstrengelsestest var FE_{NO} redusert hos både astmatiske og ikke astmatiske barn. FE_{NO} var ikke tilbake til utgangsnivået etter 30 min. Barn med AR viste større reduksjon i absolutt FE_{NO} verdi (parts per billion) enn barn uten AR, uavhengig av astma. Imidlertid var effekten av anstrengelse, målt som % endring i Ln (naturlig log) FE_{NO} størst hos barn uten AR.

Analyser av `Receiver operating characteristic` (ROC) kurver viste at IMMULITE® har generelt god nøyaktighet. Serum sIgE predikerte AR til allergenene pollen, dyr og husstøvmidd. For disse allergenene var sIgE cut-off nivåer med den beste kombinasjon av sensitivitet og spesifisitet høyere enn deteksjonsgrensen for IMMULITE® (0.23-1.1 kU/L). Serum sIgE for *Alternaria tenius*, *Cladosporium herbarium* og kakerlakk kunne imidlertid ikke predikere AR. Blant barn med AR, fant vi en positiv korrelasjon mellom FE_{NO} og serum total IgE samt sIgE mot katt og hund, men ikke til de andre testede allergenene.

Vi konkluderer med at prevalensen av astma, AR og eksem siste året økte betydelig mellom 1995 og 2008. Livstidsprevalensen for astma og AR økte fra 1985 til 2008 mens livstids-prevalensen for eksem nådde et platå.

Videre har astmatiske og ikke-astmatiske barn med AR høyere FE_{NO} enn barn uten AR. FE_{NO} reduseres signifikant etter en standardisert anstrengelsestest og er ikke tilbake til utgangsverdi etter 30 min. Derfor kan FE_{NO} verdier bli underestimert hvis barn er fysisk aktiv før FE_{NO} målinger. Dette er mest uttalt blant barn med AR som har de høyeste utgangsverdiene og det største fallet i FE_{NO} verdier etter anstrengelse.

Serum sIgE cut-off verdier for å diagnostisere AR er avhengig av den allergiske fenotypen. Blant sju av de ti testede allergenene var sIgE cut-off verdiene over IMMULITE® sin deteksjonsgrense. Dersom man bruker deteksjonsgrensen for sIgE som beslutningspunkt for å diagnostisere AR så vil dette bidra til å over-diagnostisere AR.

Summary

Asthma and allergic rhinoconjunctivitis (AR) are the commonest chronic diseases in children in the Western world. During the past decades, the prevalences of these diseases have increased: those of asthma and AR vary greatly, and recent reports indicate a levelling off for asthma in some European countries. In clinical practice, the diagnosis of asthma and AR are supported by laboratory tests. Analyses of exhaled breath biomarkers have been assessed to uncover pathological mechanisms of airway inflammation. Fractional exhaled nitric oxide (FE_{NO}) is the only exhaled biomarker that has been standardized for clinical paediatric application. FE_{NO} is a marker of eosinophilic airway inflammation and is extensively studied, although the impact of exercise on its release is not fully elucidated. Furthermore, the diagnosis of allergic airway diseases involves confirming sensitization by detecting allergen-specific immunoglobulin E (sIgE). Little comparative data have been available for sIgE testing using the Siemens IMMULITE® 2000 system (IMMULITE®) and skin prick test (SPT) results in children. Paediatric cut-off values for serum sIgE using IMMULITE® to diagnose AR have not been determined.

The aims of the study were to investigate the following: the prevalences and time trends of asthma, AR and eczema in a subarctic childhood population, the FE_{NO} levels in relation to asthma and AR, and the impact of exercise on FE_{NO}. Likewise, it was an aim to establish paediatric serum sIgE cut-off values for diagnosing AR and to explore the relationship between serum sIgE, total IgE and FE_{NO}.

This thesis is based on data from Phase I and Phase II of the study `Asthma and allergy among schoolchildren in Nordland`. Phase I was a cross-sectional questionnaire-based survey and included 4150 schoolchildren aged 7-14 years from 65 randomly selected schools in Nordland County. Prevalence rates of asthma, AR and eczema in 2008 were compared with results from 1985 and 1995. Phase II was a clinical investigation of 801 schoolchildren recruited during Phase I. The parents completed a questionnaire and a structured interview. FE_{NO} measurements, spirometry, an exercise challenge test, SPT and blood sampling were performed.

The Phase I survey revealed that the prevalence of current asthma, AR and eczema doubled and trebled between 1995 and 2008. The prevalence of asthma and AR ever increased

between 1985 and 2008, while the prevalence of eczema ever, after an increase between 1985 and 1995, remained unchanged in the last period.

In Phase II of the study, we found that the FE_{NO} level was significantly increased in asthmatics compared to non-asthmatics, and was significantly elevated in asthmatics and non-asthmatics with AR compared to individuals without AR. The highest FE_{NO} values were found in children with current allergic asthma. FE_{NO} decreased significantly in non-asthmatic and asthmatic children after a submaximal exercise test, and did not return to baseline value within 30 min. Children with AR demonstrated a significantly greater reduction in FE_{NO} value (parts per billion) than children without AR, irrespective of asthma. Although, the effect of heavy exercise (% change in natural log FE_{NO}) was more pronounced in subjects without AR.

Receiver operating characteristic (ROC) analysis demonstrated that the overall accuracy of IMMULITE® was good. Serum sIgE predicted AR to the tested pollen, animal and house dust mite allergens. sIgE cut-off values with the best combined sensitivity and specificity were above the detection limit of IMMULITE® for these allergens (0.23-1.1 kU/L). The sIgEs for *Alternaria tenius*, *Cladosporium herbarium* and German cockroach were not significant predictors of AR. In children with AR, positive correlations were found between FE_{NO} and serum total IgE, sIgE to cat and dog but not to the other tested allergens.

In conclusion, the prevalence of current asthma, AR and eczema in schoolchildren increased considerably between 1995 and 2008. The prevalence of asthma and AR ever increased between 1985 and 2008, while the prevalence of eczema ever reached a plateau.

Non-asthmatic and asthmatic children with AR expressed higher FE_{NO} values than children without AR. FE_{NO} decreased in all children after a submaximal exercise challenge and did not return to baseline level within 30 min. Hence, if children are physically active before FE_{NO} measurements, FE_{NO} values could be underestimated. This is especially pronounced in children with AR who have the highest baseline FE_{NO} and the largest decline in FE_{NO} value.

Cut-off values for diagnosing AR using serum sIgE were dependent on the allergic phenotype and were above the IMMULITE® detection limit for seven of ten inhalant allergens. Consequently, using the detection limit for serum sIgE as the decision point would result in over-diagnosing AR.

List of Papers

This thesis is based on the four papers listed below. The papers are referred to in the text by their Roman numerals (I-IV).

Paper I

Hansen TE, Evjenth B, Holt J. Increasing prevalence of asthma, allergic rhinoconjunctivitis and eczema among schoolchildren: Three surveys during the period 1985-2008. Acta Paediatr 2013;102:47-52.

Paper II

Evjenth B, Hansen TE, Holt J. Exhaled nitric oxide decreases during exercise in non-asthmatic children. Clin Respir J 2013;7:121-127.

Paper III

Evjenth B, Hansen TE, Holt J. The effect of exercise on exhaled nitric oxide depends on allergic rhinoconjunctivitis in children. Submitted.

Paper IV

Evjenth B, Hansen TE, Brekke OL, Holt J. Establishing IMMULITE® 2000 cut-off values for serum allergen-specific immunoglobulin and exploring their relationship to exhaled nitric oxide
Acta Paediatr 2014;103:759-65.

Abbreviations

AR	Allergic rhinoconjunctivitis
ARIA	Allergic Rhinitis and its Impact on Asthma
ATS	American Thoracic Society
AUC	Area under the curve
BHR	Bronchial hyperresponsiveness
CI	Confidence interval
EIB	Exercise-induced bronchoconstriction
ERS	European Respiratory Society
FEF_{50}	Forced expiratory flow in 50% of FVC
FE_{NO}	Fractional exhaled nitric oxide
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GINA	Global Initiative of Asthma
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL	Interleukin
IMMULITE®	IMMULITE® 2000
iNOS	Inducible NOS
Ln	Natural logarithm
LR+	Likelihood ratio positive
LR-	Likelihood ratio negative
nNO	Nasal NO
NO	Nitric oxide
NOS	Nitric oxide synthases
OR	Odds ratio
ppb	Parts per billion
rho	Spearman`s rank correlation coefficient
ROC	Receiver operating characteristic
SD	Standard deviation
sIgE	Allergen-specific IgE
SPT	Skin prick test
Th	T-helper

1 BACKGROUND

1.1 Asthma and allergic rhinoconjunctivitis in children

1.1.1 Prevalence of asthma and allergic rhinoconjunctivitis

Asthma and allergic rhinoconjunctivitis (AR) represent global health problems in children (1, 2). Asthma and AR are the commonest chronic diseases in childhood in developed countries today (2, 3). The burdens of the diseases have major impacts on the patients, families and the health care systems (4). Over the last decades, the prevalence of bronchial asthma and AR have increased substantially (5, 6). In Northern Norway the lifetime prevalence of childhood asthma increased from 5.1% in 1985 to 8.6% in 1995, while the lifetime prevalence of AR increased from 16.4% to 22.1% (7). In the mid-1990s, higher prevalences of asthma and AR were found in children of Sami ethnicity than Norse ethnicity, and Russian children had lower prevalence of asthma and AR than Norwegian children (7, 8).

The prevalence of asthma varies greatly in Europe, with higher prevalence reported in English speaking countries than in other Northern European countries (9). In 10-year old children in Oslo, the lifetime prevalence of asthma was 20.2% in year 2004 (10). However, recent reports indicate a levelling off in childhood asthma in some European countries (11, 12).

1.1.2 Asthma

Asthma history

The word ´asthma´ is derived from the Greek root άσθμα (aazein) meaning to pant heavily or gasp for breath (13). Asthma was probably first used as a medical term by Hippocrates, `the father of medicine` (460-370 B.C) (13). In 1860 Henry Hyde Salter described asthma as an inflammatory disorder triggered by external stimuli involving both neural and vascular mechanism, and William Osler stated in 1892 that asthma was a special form of inflammation of the smaller bronchi (14). Although asthma was for decades regarded largely as a neurotic disorder (14), it was not until the 1960s that airway inflammation was recognized as an underlying substrate (15). From the 1970s many pathognomonic elements of stimuli such as allergens, exercise, viral infections and airway pollutants, were uncovered (14). Likewise, much attention has been devoted to the hygiene hypothesis that scarcity of microorganism exposure in early life increases the risk of atopic diseases in later life (12). In the last decade,

researchers have attempted to understand the relation between genes and environmental factors that promotes the development of asthma and allergic diseases (16, 17). Increasing evidence points to that both intrauterine and early-life factors play an important role in the pathogenesis of asthma and AR (18, 19).

Asthma definition

Guidelines relating to the diagnosis and management of asthma have been made worldwide. Among them, the Global Initiative of Asthma (GINA) guidelines are probably the most internationally recognized framework. GINA was founded in 1993, and the first report was published in 1995 based upon expert opinion (20). Since the 2002 update, the GINA guidelines have been based on evidence-based methodology. In the definition of asthma, the role of chronic inflammation and the functional consequences of airway hyperresponsiveness are stressed. The definition of asthma remains descriptive since its pathogenesis is not fully understood. In the 2012 updated GINA guidelines, the operational description of asthma is:

´Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment.´ (4)

Asthma has been recognized as a heterogeneous disease with a complex pathogenesis. A wide range of features have been proposed to sub-classify asthma to support diagnosis and guide treatment decisions (21). Different asthma phenotypes have been suggested based on time-presentation of wheeze (22, 23), allergic sensitization (24), response to treatment (25, 26), inflammatory markers (27), pathophysiological mechanism including exercise-induced bronchial hyperresponsiveness (BHR) (28, 29), and disease severity (30). Lately new statistical approaches, specifically cluster analyses, have been applied to identify sub-phenotypes of asthma (31). Research on genetics linked to environmental factors (epigenetics) has also provided new pathways that may be important in the future understanding, classification and treatment of different asthma phenotypes.

1.1.3 Atopy and allergic diseases

Common allergic diseases in children include allergic asthma, AR, atopic eczema, food allergy, allergic urticaria and anaphylaxis. Allergic diseases are hypersensitivity reactions initiated by immunological mechanism usually mediated by immunoglobulin E (IgE) as identified in 1968 (32).

Atopy is defined as personal and/or family tendency to become sensitized and produce specific immunoglobulin E (IgE) antibodies in response to ordinary exposures to allergens. By contrast, allergic sensitization refers to the production of allergen specific IgE (sIgE) (33). Such sIgE antibodies can by determined in serum or by skin prick testing (SPT). Individuals are considered to have an allergic disease when they develop symptoms upon exposure to an allergen and sensitization to the allergen is confirmed. However, not all allergic hypersensitivity reactions are IgE-mediated, and IgE-mediated conditions may be atopic or non-atopic (34), Figure 1.

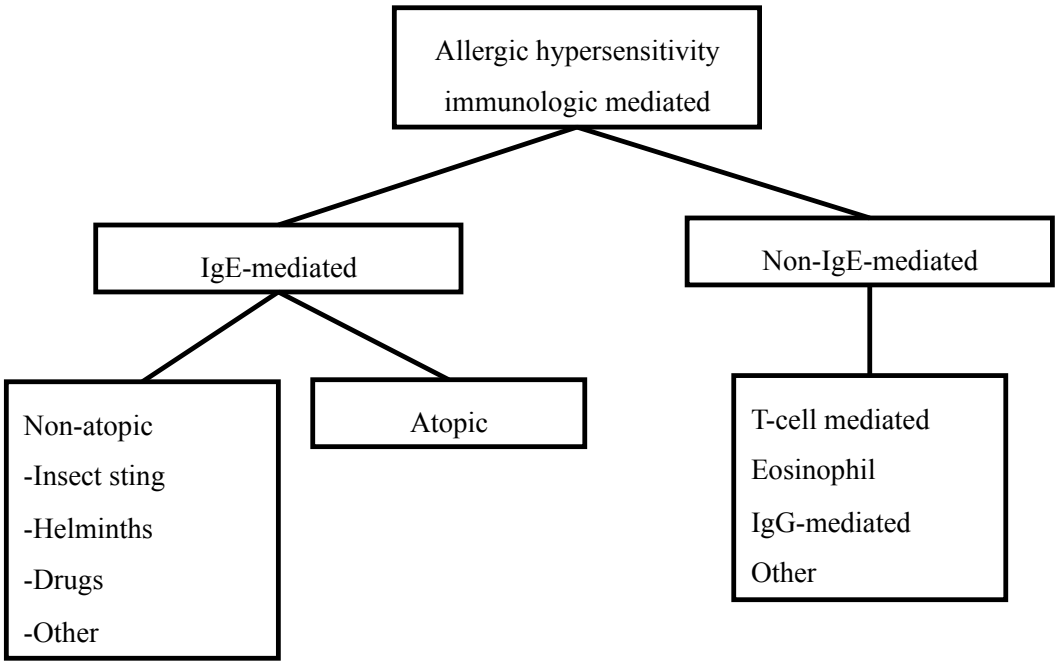

Figure 1. *Allergic pathways*

Adapted from (34). Reprinted by permission © 2008 John Wiley and Sons. All rights reserved.

1.1.4 Allergic rhinoconjunctivitis

In 1819, ´hay fever´ was described for the first time as a rare and unusual disease (35). Allergic rhinitis was defined in the medical literature in 1929, and its cause was at that time ascribed to pollens (36). In 1999 `The Allergic Rhinitis and its Impact on Asthma (ARIA)` Expert Panel published evidence-based guidelines on diagnosis and treatment of allergic rhinitis and concomitant conjunctivitis (37). The ARIA guidelines were last updated in 2010 (38).

Rhinitis is defined as an inflammation of the lining of the nose and is characterized by nasal symptoms including rhinorrhea, sneezing, nasal blockage and /or itching of the nose (39). By contrast, allergic rhinitis is defined as a symptomatic disorder of the nose induced after allergen exposure by an IgE-mediated inflammation (36). Allergic rhinitis is often accompanied by allergic conjunctivitis. For clinical application, the ARIA guidelines suggest clinical allergic rhinitis when watery running nose is accompanied by one of the following symptoms: sneeze, nasal obstruction, nasal itching or conjunctivitis. Allergic rhinoconjunctivitis (AR) is either classified as intermittent or persistent, or according to the causative allergen as either seasonal or perennial. Most studies refer to the latter classification (38).

1.1.5 Allergic *versus* non-allergic asthma

Asthma, AR, food allergies and atopic eczema are often concomitant diseases, and it is generally accepted that the majority of asthmatic children are allergic (40). Allergic asthma is not uniformly defined. In most studies ´allergic asthma´ is defined in the presence of asthma and at least one positive SPT or elevated serum sIgE. The risk of developing asthma symptoms and the severity of symptoms following allergen exposure may relate to the type of allergen, route of exposure, level of exposure and host genotype (16, 41). It has been shown that 80% of children with asthma have allergic rhinitis (42), and an association has been found between allergic rhinitis and asthma severity (43). Identifying and treating asthmatics with concomitant rhinitis is essential since it improves the control of asthma and reduces the risk of severe asthma exacerbations (42, 44).

A hallmark of allergic asthma is the T-helper 2 (Th2) driven eosinophilic inflammation (45). Eosinophilic cells are found in the airway wall, bronchoalveolar lavage fluid and sputum in subjects with allergic asthma (46). Both eosinophilic and neutrophilic cells play a role in the

pathogenesis of asthma. In general, eosinophilic inflammation is associated with atopy and persistent asthma symptoms, while neutrophilic inflammation is associated with viral triggered wheeze and increased asthma severity (17).

Markers of inflammation may be assessed in blood, exhaled breath and histological biopsies. The level of symptoms and markers of inflammation do not always correlate (21, 47). To some degree markers of inflammation aid diagnosis and the monitoring of asthma and allergy, since phenotypes demonstrate different inflammatory profiles. The most commonly used methods for assessing eosinophilic inflammation are measurements of the following: fractional exhaled nitric oxide (FE_{NO}), serum total and allergen-specific IgE (sIgE), serum eosinophilic cation product (s-ECP), and leukotrienes (LTs).

1.2 Airway inflammation

Aetiology of airway inflammation

Airway inflammation is a pathophysiological characteristic of asthma and rhinitis. The aetiology of airway inflammation is age dependent. In early childhood, airway inflammation is predominately triggered by viral infections, especially rhinovirus (48). In older children, airway hyperresponsiveness is mainly determined by allergic airway inflammation (49). Altogether, virus infections are involved in >80% of asthma exacerbations in childhood, and recent studies have suggested a synergistic effect between viruses and allergens on airway hyperresponsiveness (48). Respiratory viruses have been shown to damage the respiratory epithelium making it less resistant to inhaled allergens (17). Likewise, exposure to air pollution is associated with airway inflammation and asthma worsening (17, 50).

The immune responses and airway inflammation

The immune system is a complex system of interdependent cells and multiple mediators that collectively protect the host from various antigens and related diseases. The immune system is composed of two major parts. The innate and the adaptive immune system serve as the first and second line of defence, respectively. The innate immune system constitutes a non-specific defence and is composed of mechanical, physical and chemical barriers that act against invading microorganism. The highly specific adaptive immune system is activated by different cellular processes if the innate defence is not sufficient. The immune system can have both protective and harmful effects on the host.

The airway epithelium plays an important role in the first-line immune defence and in the pathogenesis of asthma and AR. In allergic airway diseases, the respiratory epithelium has reduced antioxidant defence and cytokine generation capabilities, which are essential for virus elimination (17). Increased permeability of the respiratory epithelium has also been shown to increase the access of inhalant allergens, pollutants and other agents to the underlying airway tissue (17). These factors may subsequently enhance the immune response in vulnerable airways. In addition, NO (nitric oxide) and other oxygen radicals are produced by macrophages and neutrophils to kill the invading organisms. In inflammatory airways, high concentrations of these agents are produced under oxidative stress, and these factors may injure the tissue and exaggerate the primary inflammatory response (51).

In allergic airway diseases, the immune response is a multicellular process involving mainly eosinophils, neutrophils, T lymphocytes (dominantly Th2) and mast cells. The most characteristic feature is the eosinophilic infiltration (16, 17, 46). The allergic inflammatory response consists of multiple steps. First and foremost an atopic individual must be sensitized to the allergen (Figure 2). The likelihood to develop a clinically significant sensitization is dependent on the type of allergen, and factors like the host genotype and the impact of environmental pollutants (52, 53). When a sensitized subject is re-exposed to the specific allergen an early-phase reaction also known as a Type I immediate hypersensitivity reaction may occur within minutes (min) of allergen exposure (Figure 3).

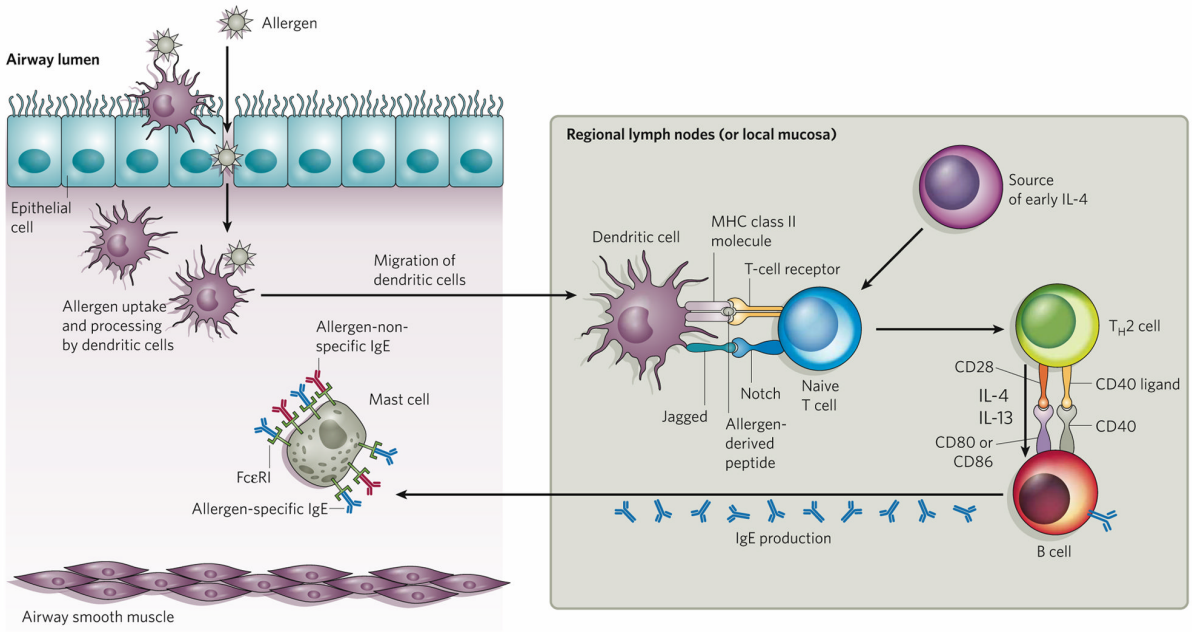

Figure 2. ***Sensitization to allergens in the airways***

Dendritic cells located in the airway epithelium and submucosa of an atopic individual may recognize an allergen as body-foreign material. These cells sample the allergen and receive signals to migrate to regional lymph nodes. The proceeded allergen is then presented on the major histocompatibility complex (MHC) and binds to receptors on naive T cells. In the presence of interleukin (IL)-4, naive T cells acquire the characteristics of T-helper 2 (Th2) cells. Th2 cells subsequently produce IL-4, IL-5, IL-9, IL-13, other cytokines and granulocyte-macrophage colony-stimulating factor (GM-CSF). These mediators stimulate B cells to undergo immunoglobulin class-switch that initiates the production of allergen-specific IgE (sIgE) and stimulates the recruitment of eosinophilic cells and mast cells from the bone marrow. sIgE is distributed systematically and binds to high affinity receptors for IgE (FcεRI) on tissue mast cells. The mast cells are now sensitized and capable to respond when the host is re-exposed to the allergen (16).

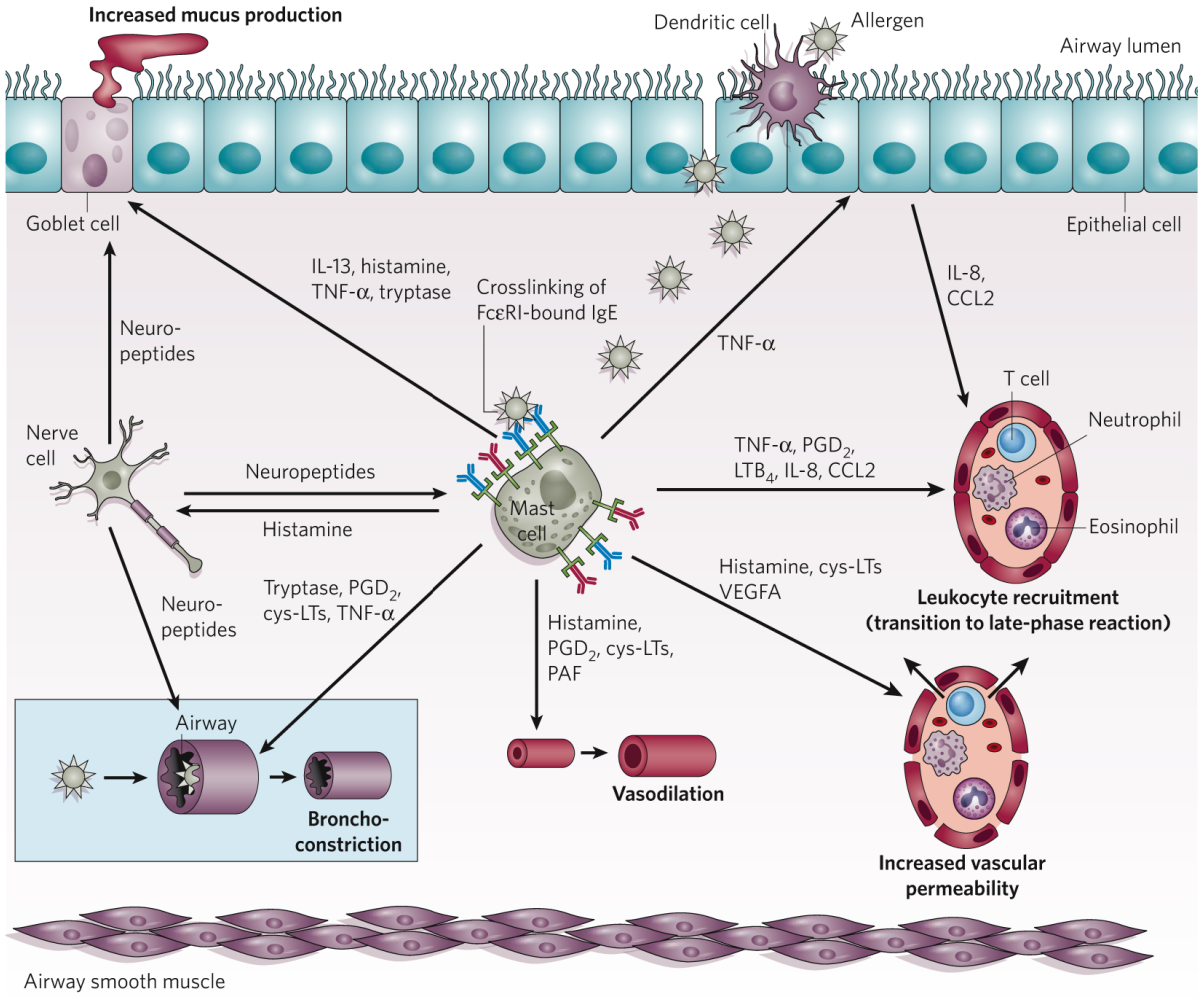

Figure 3. ***Early phase of allergen induced airway inflammation***

When a sensitized individual is re-exposed to the allergen, sIgE bound to FcεRI on mast cells are cross-linked by the allergen. This activates mast cells to release preformed mediators and increase the synthesis of cytokines, chemokines and growth factors. These mediators induce vasodilation, increased vascular permeability and oedema in affected organs. In asthmatic airways, bronchoconstriction and mucus hypersecretion occur. Some of the mediators released may promote local recruitment and activation of eosinophilic and other inflammatory leukocytes, initiating development of the late-phase reaction (16).

The late phase of the allergic reaction occurs hours after allergen exposure. It reflects the action of both tissue resident cells and immune cells recruited from the bone marrow (i.e., eosinophilic and neutrophilic cells and the effect of numerous pro-inflammatory mediators). The inflammation is particularly driven by Th2 cells that produce a range of cytokines, i.e., interleukin (IL)-4, IL-5, IL-13 and granulocyte-macrophage colony stimulating factor (GM-

CSF). IL-4 and IL-13 are able to induce iNOS (inducible NO synthase) expression, while IL-5 is involved in the differentiation and activation of eosinophilic granulocytes. Eosinophilic cells release potentially tissue-damaging basic proteins and oxygen free radicals and a wide range of cytokines and chemokines.

Continuous or repetitive allergen exposure may lead to a chronic allergic inflammation. In this phase, Th1 cells capable of secreting tumor necrosis factor (TNF)-α and interferon (INF)-γ are also recruited. The airway wall enters into `a chronic wound scenario` with enhanced cell infiltration and increased production of cytokines and growth factors. This airway remodelling process may contribute to processes such as sustained mucus production, altered barrier function; and in asthmatics bronchoconstriction and non-specific airway hyperreactivity (16, 17). In severe asthma bronchial biopsies have revealed wide airway damage such as epithelial metaplasia and injury, thickening of sub-epithelial basal lamina, increased number of myofibroblasts and other evidence of airway remodelling (54). Recently, similar findings (including eosinophilia) have been found at the onset of childhood asthma episodes (55).

1.3 Diagnosing asthma

Ideally, the diagnosis of asthma should be based on the presence of characteristic clinical symptoms and objective measurements of reversible airway obstruction. The latter may be obtained by lung function measurements with demonstration of reversible airway obstruction and by measurements of BHR. In addition, measuring exhaled markers of airway inflammation may support the asthma diagnosis.

1.3.1 Lung function and asthma

Forced expiratory flow volume measurement (spirometry) is the commonest lung function test used in schoolchildren. Forced expiratory volume in one second (FEV_1) has been proposed as the most useful variable. Current asthma symptoms have been associated with reduced FEV_1 (56, 57), and the magnitude of FEV_1 decrease has been associated with the risk of asthma attacks (58). However, normal FEV_1 has been reported in asthmatic patients (42, 59), and it is found to be an insensitive marker of severe persistent asthma (60, 61).

1.3.2 Bronchial hyperresponsiveness

BHR is considered to be a characteristic pathophysiological feature of paediatric asthma, although it is not specific for asthma, as it may also exist in non-asthmatics and in individuals with other lung disorders (10, 62-64). BHR is defined as an abnormal sensitivity of the airways to narrow following stimuli of chemical or physical origin (direct or indirect stimuli) (65). Direct stimuli (i.e., inhalation of methacholine or histamine) induce airflow limitation, predominantly *via* a direct effect on receptors on airway smooth muscles (66). This mechanism is in contrast to indirect stimuli, including exercise challenge, inhalation of cold dry air or non-isotopic aerosols, that enhance the release of endogenous mediators and neurotransmitters from airway cells causing airway smooth muscles contractions (66). Hence indirect tests mimic the natural pathophysiology of asthma, whereas direct stimuli are more closely related to structural changes in the airways (66). Furthermore, markers of airway inflammation have been shown to correlate with the extent of BHR, while anti-inflammatory treatment may reduce BHR (67, 68).

The exercise challenge test

During heavy exercise, tidal volume and respiratory frequency are increased due to increased demand of oxygen. Increased ventilation is accompanied by heat and water loss from the airways that lead to cooling and dehydration of the airway mucosa (69). Intracellular hyperosmolarity induction of mediator release has been proposed as the main mechanism of exercise-induced bronchoconstriction (EIB) (70, 71). In addition, airway cooling, mediator release and increased osmolarity may stimulate bronchoconstriction *via* parasympathic reflex pathways (69). On cessation of hyperventilation, reactive hyperaemia and oedema of the airways may occur that reduces the size of the airway lumen (72, 73). EIB has been shown to reflect ongoing airway inflammation (74). In asthmatics, FE_{NO} has been proposed to be a predictive marker of EIB (75).

In most children with current asthma EIB is triggered by exercise, although children without asthma symptoms may demonstrate it (10, 76). Therefore, the criterion for a positive EIB test is controversial (77). A reduction in FEV_1 ≥10% after a standardized exercise test is generally accepted as a positive test (71, 77). However, a fall in FEV_1 of 15% appears to be more diagnostic of EIB (77).

The latest American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines recommend an exercise load of 80-90% of predicted maximal heart rate (calculated as 220 minus age in years) (77). It has been demonstrated that a higher exercise load is more sensitive to reveal EIB and is better related to inflammatory activity (71).

1.3.3 Fractional exhaled nitric oxide

Exhaled breath biomarkers

Exhaled biomarkers have been explored to understand pathological mechanisms and to guide diagnosis and treatment decisions. The most studied exhaled biomarkers are NO, carbon monoxide, volatile organic compounds (VOC) and various biomarkers in exhaled breath condensate (EBC). FE_{NO} is the only exhaled biomarker that has been standardized and validated for clinical paediatric application (78, 79). FE_{NO} is a non-invasive surrogate measurement of eosinophilic airway inflammation that is easy to perform, provides immediate results and is well suited for children (51, 80).

Nitric oxide

NO is a free radical gas with one unpaired electron that avidly reacts with other molecules. In 1987, NO was recognized as the endothelium derived relaxing factor (ERDF) (81). In 1991 Gustafsson et al. measured endogenous NO in exhaled air of humans, and thereby started a new area in respiratory research (82). NO is known as a messenger molecule involved in multiple biological systems, including neurotransmission, platelet inhibition, inflammation and immunomodulation (83).

NO is generated *via* oxidation of L-arginine, a process catalysed by the enzyme system NO synthases (NOS) (84). Three isoforms of NOS have been described: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). The latter two are calcium and calmodium-dependent enzymes, which are released within seconds upon receptor stimulation. By contrast, iNOS is slowly regulated at the transcriptional level and releases large quantities of pro-inflammatory NO (83). The signal transducer and activator of the transcription (STAT) pathway is the main regulatory mechanism of iNOS gene transcription (85). iNOS is activated by endogenous mediators, namely chemokines and cytokines as well as exogenous factors such as viruses, allergens and pollutants (83). Current knowledge indicates that the induction of iNOS in asthmatics is primarily dependent on the activity of IL-4 and IL-13 in the

bronchial wall (86, 87). Besides NOS-catalysed formation, NO may be formed in high concentrations from peroxynitrite and tyrosine nitration (83, 88).

All of the three NOS isoforms are expressed in the respiratory system (83). In children, a highly significant correlation between epithelial iNOS mRNA expression and orally exhaled NO levels has been found (89). Nasally exhaled air contains higher NO concentrations than orally exhaled air (90). This has been attributed primarily to higher expression of iNOS in the paranasal sinuses than in the lower respiratory tract (91).

In the respiratory system, low NO concentrations have protective effects that promote bronchial dilatation, mediate ciliary beat frequency and stimulate mucus secretion (83, 92, 93). On the other hand, high NO concentrations have deleterious effects and promote inflammation *via* Th2-mediated mechanism and oxidizing agents. Pro-inflammatory effects of NO include vasodilatation, plasma extravasation, mucus hypersecretion, impaired ciliary motility and cytotoxicity (83).

FE_{NO} sampling technique

The chemiluminescence method was the first established technique to measure NO in exhaled breath of humans, and it became the gold standard (82). This sensitive technique uses ozone to react with NO and produces NO_2 in an excited state. The reaction emits light that correlates with the amount of NO present (94).

FE_{NO} is influenced by many factors of which the most crucial is exhaled flow. FE_{NO} is flow dependent and increases with reduced exhalation (95). According to the 2005 ATS/ERS guidelines, FE_{NO} should be measured at an exhalation flow of 50 mL/s (±10%) (78). The subject should inhale NO free air to avoid contamination of ambient NO (78). Exhalation is recommended to start immediately after inhalation to total lung capacity (TLC) to avoid accumulation of NO in the oro-pharynx (78, 96). Nasal NO (nNO) is present in higher concentrations relative to the lower respiratory tract (97). Therefore, it is recommended to exhale with an oral pressure of 5-20 cmH_2O to ensure closure of the soft palate (78).

Factors affecting FE_{NO} measurements

Height, age and gender have been shown to influence FE_{NO} measurements. FE_{NO} increases with age (80). Height has been found to correlate with FE_{NO} (98). The increased FE_{NO} in taller

individuals probably reflects the greater airway mucosal area available for NO exchange (99). Studies report conflicting data as to whether FE_{NO} is influenced by gender in children (80, 100-102).

Treatment with inhaled corticosteroids (ICS) reduces FE_{NO} (26, 51) as may exposure to tobacco smoke (103), whereas exposure to air pollution (50) and intake of nitrate-rich food may increase it (104). Kharnitov et al. found no diurnal variation in FE_{NO} in healthy and asthmatic children (105). Population-based studies have reported either no association (100, 101) or weak association between FEV_1 and FE_{NO} (106). Rhinovirus infections may induce iNOS leading to increased FE_{NO} levels (107, 108), while FE_{NO} is slightly decreased in the symptomatic phase of respiratory syncytial virus (RSV) and influenza virus infections (109, 110).

FE_{NO} and the relation to allergic sensitization, asthma and AR

It is well documented that FE_{NO} is increased in children with asthma compared to healthy controls (100, 111). FE_{NO} is found to correlate with measurements of eosinophilic activity in the airway mucosa (51). Therefore, FE_{NO} is often referred to as a surrogate marker of eosinophilic inflammation. FE_{NO} has also been shown to correlate with the degree of IgE sensitization, both in terms of number of SPTs (111, 112) and the sIgE levels to some allergens (113).

The FE_{NO} level is increased in children with AR, and the highest values have been found in children with allergic asthma (100, 101). In some studies, atopic individuals without asthma and/or AR have equal FE_{NO} concentrations relative to non-atopics (114, 115). In other studies, increased FE_{NO} levels have been observed in atopic individuals regardless of the respiratory tract symptoms (51, 100, 111) . It has been suggested that this might reflect subclinical airway inflammation (51, 111). The heterogeneity in the exhaled NO levels reported might be explained by unlike allergen exposure, different definitions of allergic sensitization, and whether subgroups are labelled by allergic sensitization alone or by allergic sensitization and allergy symptoms.

The effects of common laboratory procedures on FE_{NO} measurements

Bronchodilator administration, spirometric manoeuvres and EIB tests have been proposed to affect FE_{NO} measurements (116-118). The ATS/ERS guidelines recommend refraining from

exercise 1 hour before performing the FE_{NO} test because forced breathing have been shown in most studies to reduce FE_{NO} in healthy and asthmatic adults (78). It has been argued that increased NO elimination and reduced airway surface area during EIB are the main mechanisms of FE_{NO} decline post exercise (117-120). In children, few reports concerning the effects of exercise on FE_{NO} have been published and with conflicting results (117, 120, 121). Different conclusions may partly be explained by different NO sampling techniques and EIB tests performed (i.e., different activities and thresholds; 117, 120, 121). FE_{NO} levels have been found to correlate with the degree of eosinophilic airway inflammation (51). Although, the impact of allergic airway inflammation on FE_{NO} in relation to exercise has not been fully elucidated in asthmatic and non-asthmatic children.

1.4 Diagnosing inhalant allergy

The diagnosis of allergic diseases involves both the presence of allergy symptoms and confirmation of relevant allergic sensitization (33). Allergic sensitization is commonly determined either by *in vivo* skin prick testing or by *in vitro* measurement of sIgE in serum (122). Serum sIgE can be analysed for single allergens, allergenic molecules (components) of single allergens, a mix of allergens, and by multi-allergen tests for screening purposes. These tests identifies allergic sensitization and do not necessarily demonstrate clinical relevant allergies (123, 124). Serum sIgE cut-off points for clinically relevant allergies may be determined by plotting the sensitivity against 1-specificity using receiver operating characteristic (ROC) curves.

1.4.1 Skin prick test

The core diagnostic test for Type-1 hypersensitivity is the SPT test (125, 126). The SPT test utilizes the presence and degree of cutaneous reactivity to an allergen as a surrogate marker of sensitization. When an allergen is introduced into the skin, sIgE bound to surface receptors on mast cells may cross-link and induce mast cell degranulation thereby releasing histamine and other mediators (126). This may produce a wheal that can be quantified. A positive SPT is considered in the presence of a wheal diameter ≥3 mm larger than the negative saline control (125). A false negative result can be seen if the individual has ongoing antihistamine therapy, current eczema, or if topical steroids have been applied to the skin. Dermographism may lead to a false positive result (125). SPT results have been found to correlate with those of nasal allergen challenge (127), and very good correlations have been found between SPT results and clinical allergy symptoms (125, 128).

1.4.2 Serum IgE and *in vitro* immunoassays

Serum sIgE antibodies can be determined by a variety of *in vitro* immunoassays (122). There exist no absolute serum sIgE antibody reference standards against which to judge accuracy. However, ImmunoCAP® (Phadia) was the first established assay and has been accepted and validated as a quasi-standard (129-131). Allergen reagents produced by different manufactures vary in its protein composition and have been shown to detect dissimilar sIgE populations (130, 132). Thus, sIgE cut-off levels reported for one *in vitro* assay as defining positive allergic reactivity cannot be used with sIgE results generated employing test kits from a different manufacturer. In addition, allergens may have different cut-off values when employing the same immunoassay (41). The analyses of serum sIgE are feasible when patients are taking anti-histamines. However, therapeutic levels of omalizumab in sera will interfere in several of the clinically used immunoassays (132).

The Siemens IMMULITE® 2000 system (IMMULITE®) is a four-step chemiluminescent assay using biotinylated allergens in a liquid phase coupled to ligand-coated beads (41). Cut-off levels for IMMULITE® to some common inhalant allergens have been reported for adults (131), but not for children. Although IMMULITE® assays and SPT are used in some clinics, little comparative data are available for results in children; neither have paediatric cut-off values for sIgE using IMMULITE® to diagnose AR been established.

2 AIMS OF THE STUDY

The aims of the study were to investigate the prevalence and time trends of atopic diseases in a subarctic childhood population and to quantify FE_{NO} levels in relation to asthma, AR and exercise testing. Likewise, another object was to establish paediatric serum sIgE cut-off values for the diagnosis of AR and to explore the relationships between serum sIgE, total IgE and FE_{NO}.

The specific aims were:
Paper I: To explore whether or not the prevalence of asthma, AR and eczema continues to increase in Nordland County, Norway.

Paper II: To investigate FE_{NO} levels in non-asthmatic children, and to explore whether exercise testing affect FE_{NO} levels in non-asthmatic children with and without AR symptoms.

Paper III: To determine the effects of AR on FE_{NO} in response to a standardized treadmill exercise test in asthmatic and non-asthmatic children.

Paper IV: To establish paediatric cut-off values for serum sIgE using the Siemens IMMULITE® 2000 to diagnose AR, and to explore the relationships between serum sIgE, total IgE and FE_{NO}.

3 METHODS

3.1 Study design and subjects

This thesis is based on data from Phase I and Phase II of the study `Asthma and allergy among schoolchildren in Nordland´ (Figure 4).

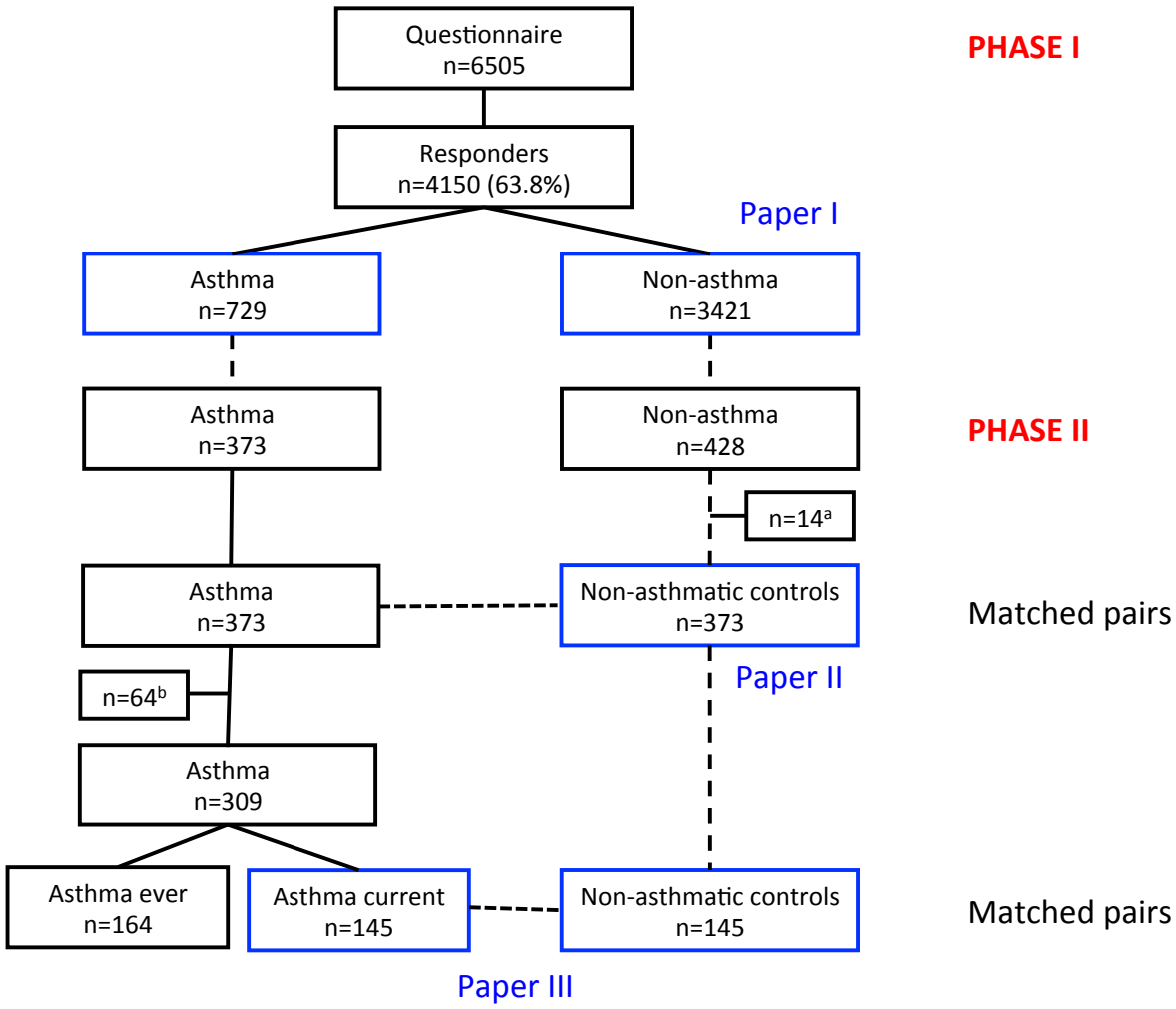

Figure 4. ***Subject flow chart in Phase I and Phase II of the study.***

[a]Subjects misclassified as non-asthmatics (n=14); subject who became asthmatic from Phase I to Phase II (n=8), subjects categorized as asthmatic in the structured interview (n=6).

[b]Subjects categorized as non-asthmatic in the structured interview (n=64).

Phase I of the study was a cross-sectional questionnaire based survey. Schoolchildren aged 7-14 years from 65 randomly selected schools of a total of 244 schools in Nordland County

were invited to participate. Parents received a questionnaire (Appendix 1) regarding asthma, AR and eczema between February and May 2008. All participants received one reminder. The study closed four weeks after the reminder was distributed. Based on the questionnaire responses, pupils were categorized as asthmatic or non-asthmatic (Paper I).

In Phase II of the study, pupils who reported having asthma in Phase I and lived nearby the study locations along with two age and gender matched non-asthmatic controls were invited to participate. Of the 1144 pupils invited, 801 children (373 of them reporting asthma in Phase I) accepted to participate. The parents completed a questionnaire and a structured interview. A clinical examination, spirometry, exercise treadmill testing, SPT and measurements of FE_{NO}, serum sIgE and total IgE were obtained. Based on information given in the structured interview and the clinical examination, the pupils were finally categorized as asthmatic or non-asthmatic (Figure 4). The participants were examined at least two weeks after any suspected respiratory tract infection during the school season from March 2009 to June 2010. The examinations took place at Nordland Hospital, Bodø, and at three other locations in Nordland County (Fauske, Mo i Rana and Sortland). PhD student Tonje E. Hansen and the author conducted all the interviews and procedures, and the same medical instruments were used throughout to secure standardized measurement conditions.

The study population of Paper II included 373 non-asthmatic pupils (non-asthmatic controls to the original asthma group). These children were similar with respect to demographic data to the non-asthmatic children who were not included in Paper II. In Paper III, the assessments of 145 pupils with current asthma and 145 non-asthmatic age- and gender-matched controls were compared. Of the 801 children enrolled in Phase II, 303 had measurements of serum sIgE, total IgE, SPT and FE_{NO} and constituted the study subjects of Paper IV.

Both Phase I and Phase II studies were approved by the Regional Committee for Medical and Health Research Ethics, and were conducted in accordance with the ethical standards of the 2000 Helsinki Declaration. In Phase I, the parents/guardians signed a written consent for their children's participation. In Phase II, written informed consent was obtained from all children and their parents.

3.2 Definitions

Phase I, Paper I

'Asthma ever' was considered if the parent answered 'yes' to the question `Has the pupil ever had asthma?`, and/or to the question `Does the pupil experience wheeze, periods of coughing or acute shortness of breath (asthma) due to external factors?`

´AR ever´ was estimated on the basis of a positive answer to the question `Has the pupil ever had hay fever (runny or blocked nose, sneezing, itching of the nose and/or eyes, or swollen or red eyes)?`

´Eczema ever´ was recorded if the pupils reported an itchy rash lasting at least four weeks, combined with lesions on the face, elbows or knee flexures, or a high degree of itching and lesions elsewhere.

´Current disease´ was considered among those answering yes to the main questions about asthma, AR or eczema and reporting symptoms the last 12 months.

Phase II, Paper II-IV

Asthma

Asthma (Paper II-IV): at least two of the following three criteria fulfilled at any time in life: 1) recurrent dyspnoea, chest tightness and/or wheeze; 2) a doctor´s diagnosis of asthma; and 3) use of asthma medication including β-2 agonist, sodium chromoglycate, corticosteroids, leukotriene antagonists and/or aminophylline.

Current asthma (Paper III): asthma as defined above plus symptoms and/or medication within the last year.

Current asthma (Paper IV): asthma as defined above plus symptoms and/or medication within the last year, and/or a positive exercise test.

Asthma in remission (Paper IV): asthma not defined as current asthma.

Allergic rhinoconjunctivitis (AR)

AR symptoms (Paper II-IV): a history of watery rhinorrhea, blocked nose, sneezing, nasal itching accompanied by itchy watery eyes in absence of airway infection.

AR (Paper III): AR symptoms in combination with allergic sensitization.

Allergic sensitization (Paper III): a positive serum sIgE and/or a positive SPT to at least one of the ten inhalant allergens.

Non-AR (Paper III): no AR symptoms or sensitization to inhalant allergens.

AR (Paper IV): a positive SPT and a history of related AR symptoms as evaluated by a doctor.

Food allergy

Food allergy (Paper IV): a positive SPT and a history of related food allergy symptoms as evaluated by a doctor.

3.3 Questionnaires, structured interview and clinical examination

Questionnaire Phase I (Appendix I): A questionnaire that focused on diagnosis and symptoms of asthma, AR and eczema was created in 1985 to assess disease among schoolchildren in northern Norway. The questions covered gender, age, family history of atopy, socio-economic conditions, passive smoke exposure and household animals. In 2008, we used the identical questions indicated but added some about physical activity, medical diagnosis of asthma and asthma medication. The additional questions did not change the definition of the diseases.

Questionnaire and structured interview Phase II: The parents completed a detailed questionnaire and a structured interview relating to asthma, AR, food allergy, urticaria, anaphylaxis and eczema symptoms and diagnosis, the use of medications, exposure to allergens and exposure to tobacco smoke. Additional questions regarding diet, infections, physical activity and demographic factors were answered and recorded.

Clinical examination, Phase II: A clinical examination was performed including height and weight measurements and assessment of the skin, the upper airways, lungs and the heart.

Inhaled corticosteroids (ICS) and short acting β-2 agonists were withheld for 12 hours (h) prior to testing; inhaled long acting β-2 agonists for the last 48 h; leukotriene modifiers for the last 24 h; and antihistamines in the last 5 days. No children were using oral steroids.

3.4 Allergic sensitization

Serum total IgE and sIgE: Blood samples were obtained using standard venepuncture using Vacutainer® tubes (Becton Dickinson, Plymouth, UK). Serum was collected and stored at -80°C until assayed. Total IgE and sIgE levels were analysed employing the IMMULITE® 2000 (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) using 3gAllergy® kits. The

detection range for sIgE was ≥0.10-100 kU/L. The following were tested: sIgE to timothy, birch and mugwort pollens; dog dander, cat and rabbit epithelial dander; house dust mite *Dermatophagoides pteronyssinus*; moulds *Alternaria tenius* and *Cladosporium herbarium* and German cockroach. Seroatopy was defined by a sIgE test ≥0.35 kU/L (132) to at least one of the listed allergens (Paper III). Blood samples were requested for all children.

Skin prick test: SPT was performed for the above listed inhalant allergens and egg white, milk, peanut and codfish with Soluprick® allergens (ALK Abello, Denmark). Histamine was used as positive control and saline as negative control. SPT was considered positive in the presence of a wheal diameter ≥3 mm larger than the negative control (125). During the initial study period, SPT was requested for all children. Thereafter, SPT was requested for children with asthma and/or allergy symptoms.

Allergic sensitization was not evaluated in 12 individuals without AR symptoms (Paper III). Of a total of 2673 serum analyses, 23 measurements of sIgE were missing due to low sample volume (Paper IV).

3.5 Fractional exhaled nitric oxide

FE_{NO}: was measured online by the single breath method with a chemiluminescence analyser, EcoMedics Exhalyzer® CLD 88sp with Denox 88 (Eco Medics AG, Duernten, Switzerland), (detection range 0.1-5000 ppb, accuracy ±2%). The procedure was performed in accordance with published guidelines (78). The participants inhaled NO free air (<5 ppb) to near total lung capacity to avoid contamination from ambient NO. The expiratory pressure was 5-20 cmH_2O to close the soft palate. Mean exhaled flow rate was 50 mL/s ±10% during the NO plateau. The manoeuvre was repeated until two exhalations agreed to within 5% coefficient of variation (CV) or three exhalations agreed to within 10% CV. The NO concentration, FE_{NO}, was defined as the mean of these values expressed in parts per billion (ppb). The analyser was calibrated daily using a standard NO calibration gas (Air Liquide Deutschland GmbH, Krefeld, Germany) and was corrected for ambient temperature and humidity. FE_{NO} was measured at baseline, prior to spirometry, and immediately after exercise (1 min) and 30 min later.

3.6 Lung function and exercise test

Spirometry: was performed in accordance with international guidelines (133) with an ambulant electronic spirometer, Spiro USB with Spida 5 software (Micro Medical, Rochester, UK). Forced vital capacity (FVC), FEV_1, and forced expiratory flow at 50% of FVC (FEF_{50}) were reported using the reference values of Zapletal (134) (Paper II) and the global lung function 2012 equation (135) (Paper III).

Standardized exercise test: An exercise challenge test was performed by running for 6-8 min on a motor-driven treadmill (Woodway PPS Med, Woodway GmbH, Weil am Rhein, Germany) following the ATS/ERS guidelines (77). The mean target heart rate during the last 4 min was 95% of maximum heart rate (calculated as 220 minus age in years), though a minimum heart rate of 180 beats per minute (85-88%) was accepted. In accordance with the study protocol, the EIB test was considered positive with a decrease in FEV_1 ≥10% (Paper II and IV) of baseline FEV_1 measured at 3, 6, 10, 15 and 20 min after the exercise. In Paper III, the threshold of a positive EIB test was a decrease in FEV_1 ≥15%, as recommended by reviewers of Paper III. Exclusion criteria were: strenuous exercise 4 hours prior to testing and pre-exercise FEV_1 lower than 75% of predicted value.

3.7 Statistical analyses

Normally distributed values were presented as means and standard deviations (SD) or 95% confidence intervals (CIs). Categorical data were presented as percentages. All tests were two-sided using a significance level of 0.05.

Phase I: The main outcome were differences in prevalence between the periods 1985-95 and 1995-2008. The analyses were performed using chi-square statistics, and the differences in secular prevalence were quantified with odds ratios (OR). For values measured three times, the chi-square test for trend (linear-by-linear associations) was carried out.

Phase II: The distribution of FE_{NO} values was right skewed, and hence the statistical analyses were executed with natural log (Ln)-transformed data. The results were presented as back-transformed values and expressed as geometric means with 95% CIs. Inter-group comparisons were analysed with an independent t-test for continuous variables and Pearson´s chi-square test for categorical variables. Differences in FE_{NO} concentrations measured before

the exercise challenge and at 1 min and at 30 min after it were analysed by paired sample t-test: the Wilcoxon signed rank test was used for comparison of untransformed FE_{NO} data (Paper II). Linear mixed models were used to assess differences in time trends between the groups (Paper III). The response variable in each model was $LnFE_{NO}$. Dependence between the three repeated time points was controlled for by including an unstructured covariance matrix to the model. `Matched pairs` were included as a random effect in the model. Bonferroni`s *post hoc* test was used for multiple comparisons for continuous variables. ROC curves were constructed, presenting sensitivity, specificity, positive and negative likelihood ratios (LR+ and LR-, respectively) in order to find the best cut-off values for serum sIgE for a diagnosis of AR (Paper IV). Spearman`s rho test was used for correlations. Correlations were assessed with sIgE values ≤100 kU/L (Paper IV).

Statistical analyses were performed using Graph Pad Prism version 5 (Graphical Software, San Diego Ca, USA) (Paper I), Statistical Package for Social Science (SPSS) software version 18.0, 19.0 and 21.0 (Paper I-IV) (SPSS Inc. IBM, Chicago, IL, USA) and MedCalc version 12.5.0 (MedCalc software, Ostend, Belgium) (Paper IV).

4 RESULTS

4.1 Prevalence of asthma, AR and eczema 1985-2008 (Paper I)

Of 6505 pupils invited to participate, 4150 (63.8%) answered the questionnaire and were enrolled in the study (49.1% boys). The main findings were: an increasing prevalence of asthma ever (7.3% in 1985 to 17.6% in 2008, p for trend <0.001), and AR ever (15.9% in 1985 to 24.5% in 2008, p for trend <0.001); and the prevalence of eczema ever, after an increase between 1985 and 1995, remained unchanged in the last time period. The prevalence of current disease doubled and trebled between 1995 and 2008 for all three diseases (Table 1). The proportion of children reporting at least one disease (asthma, AR or eczema) increased from 26.2% in 1985 to 43.3% in 2008 (p for trend <0.001).

Table 1. The prevalence of current asthma, allergic rhinoconjunctivitis and eczema in children aged 7-14 years from the 1995 and 2008 questionnaire-based surveys in Nordland.

	Prevalence (%)			
	Surveys		2008/1995	
	1995	2008	OR	95 % CI
All				
Current asthma	4.8	9.9	2.21	1.86-2.62
Current rhinoconjunctivitis	6.7	21.5	3.83	3.33-4.40
Current eczema	6.4	13.5	2.27	1.96-2.64
Boys				
Current asthma	5.6	12.0	2.29	1.83-2.87
Current rhinoconjunctivitis	7.5	24.4	3.80	2.15-4.58
Current eczema	6.2	12.3	2.11	1.70-2.62
Girls				
Current asthma	3.9	8.0	2.13	1.63-2.78
Current rhinoconjunctivitis	5.8	18.7	3.70	3.01-4.56
Current eczema	6.6	14.6	2.43	1.97-2.99

The difference in prevalence between 2008/1995 is quantified with odds ratio (OR). Corresponding 95 % confidence intervals (95% CI) are presented.

Adapted from Hansen et al. Acta Paediatr 2012;102:47-52.

4.2 The impact of exercise on FE_{NO} in non-asthmatic children (Paper II)

Of the 373 non-asthmatic children enrolled in this part of the study, 22 children were unable to comply with the study protocol and 21 children had a positive EIB test and were excluded. Three hundred and thirty children were included in the statistical calculations. Children reporting AR symptoms (n=71) were similar to children without AR symptoms (n=259) with respect to gender, age, height, weight and spirometric indices (all $p > 0.05$).

Geometric mean FE_{NO} values at baseline, at 1 min and at 30 min after the treadmill exercise test are given in Table 2. Baseline FE_{NO} was significantly increased in children reporting AR symptoms *versus* no AR symptoms: 15.1 (12.6-18.1) ppb *versus* 9.6 (9.0-10.3) ppb ($p < 0.001$). Subjects with AR symptoms had a significantly higher decline in geometric mean FE_{NO} value at 1 min post-exercise compared to children without AR symptoms: 4.2 ppb *versus* 2.6 ppb ($p < 0.001$). FE_{NO} did not return to baseline level in either of the groups at 30 min post-exercise (Table 2). Subjects with baseline $FE_{NO} \geq 20$ ppb demonstrated a higher decline in FE_{NO} value than subjects with baseline $FE_{NO} < 20$ ppb at 1 min post-exercise: 9.9 (8.7-11.4) ppb *versus* 2.4 (2.3-2.5) ppb ($p < 0.001$).

Table 2. Levels of FE_{NO} at baseline compared to levels of FE_{NO} at 1 min and at 30 minutes after a standardized exercise induced bronchoconstriction (EIB) test on a treadmill in non-asthmatic children with and without allergic rhinoconjunctivitis symptoms.

		Baseline FE_{NO}* †	FE_{NO} 1 minute post exercise*	*P* value vs. baseline	FE_{NO} 30 minutes post exercise*	*P* value vs. baseline
All children	(n=330)	10.6 (9.9-11.3)	7.7 (7.2-8.2)	<0.001	8.9 (8.3-9.5)	<0.001
No AR‡ symptoms	(n=259)	9.6 (9.0-10.3)	7.0 (6.5-7.5)	<0.001	8.0 (7.5-8.6)	<0.001
AR symptoms	(n=71)	15.1 (12.6-18.1)	10.9 (9.2-12.9)	<0.001	13.0 (10.9-15.5)	<0.001

*Results are given as geometric means (95% confidence intervals).
†Fractional nitric oxide (FE_{NO}) is expressed as parts per billion (ppb).
‡Self-reported allergic rhinoconjunctivitis symptoms.

Adapted from Evjenth et al. Clin Respir J 2013;7:121-127.

4.3 The effects of AR on FE_{NO} in response to a standardized exercise treadmill test in asthmatic and non-asthmatic children (Paper III)

In this part of the study, matched pairs of 145 pupils with current asthma (cases) and 145 non-asthmatic pupils (controls) were enrolled. Twenty pairs included pupils (n=23) who were unable to comply with the study protocol, and one pair included a control with a positive EIB test. These 21 pairs were excluded. Children who did not comply were younger than the included children (p =0.006). The included children with current asthma (n=124) had more frequent AR, and they had significantly lower FEV_1 and FEF_{50} than the non-asthmatic controls (n=124), (all p <0.05).

Baseline FE_{NO} was significantly higher in asthmatics compared to non-asthmatics, 21.0 (17.6-24.9) ppb *versus* 11.1 (9.9-12.4) ppb (p <0.001) and significantly elevated in asthmatics and non-asthmatics with AR compared to individuals without AR (Figure 5). Baseline FE_{NO} was not significantly influenced by ICS use in asthmatics or in the subgroup of allergic asthmatics (data not presented). Comparison of FE_{NO} levels (ppb) at each time point demonstrated parallel time trends between asthmatics and non-asthmatics (p =0.866). Adjustment for baseline FE_{NO} yielded no significant difference in time trends between the groups (p=0.848). However, the time trends depicted in Figure 5 were significantly different in children with AR compared to children without AR (p =0.039), irrespective of asthma (p =0.876). In children with AR, FE_{NO} declined by a mean of 6.1 ppb (5.1-7.5) at 1 min post exercise. At 30 min, FE_{NO} was reduced by a mean of 2.8 ppb (2.5-3.3). In children without AR, FE_{NO} declined by a mean of 2.7 ppb (2.1-3.5) at 1 min post exercise, while at 30 min FE_{NO} was reduced by a mean of 1.6 ppb (1.3-2.0) compared to baseline FE_{NO}.

The effect of exercise on FE_{NO} was evaluated by comparing the % change in $LnFE_{NO}$ from baseline to 1 min and 30 min post exercise (Figure 6). The time trend was dependent on AR (p <0.001), irrespective of asthma status (p =0.795). The effect of exercise was more pronounced in children without AR than in children with AR. In asthmatics the effect of exercise on FE_{NO} was independent of ICS treatment (p =0.583) and a positive EIB test (p=0.230).

Based on $LnFE_{NO}$, the % reduction at 1 and 30 min post exercise was less pronounced with increasing number of positive SPT/or sIgE tests. Significant differences were observed

between children without AR (non-sensitized, n=83) and those with AR and 1-3 (p=0.002, n=45) and 4-9 (p <0.001, n=78) positive tests. However, the differences between the latter two groups were not statistically significant (p=0.633).

Baseline FE_{NO} correlated positively with maximal post exercise FEV_1 decline in asthmatics (rho=0.331, p<0.001). In asthmatics with AR a positive correlation was found (rho=0.360, p<0.001) but not in asthmatics without AR.

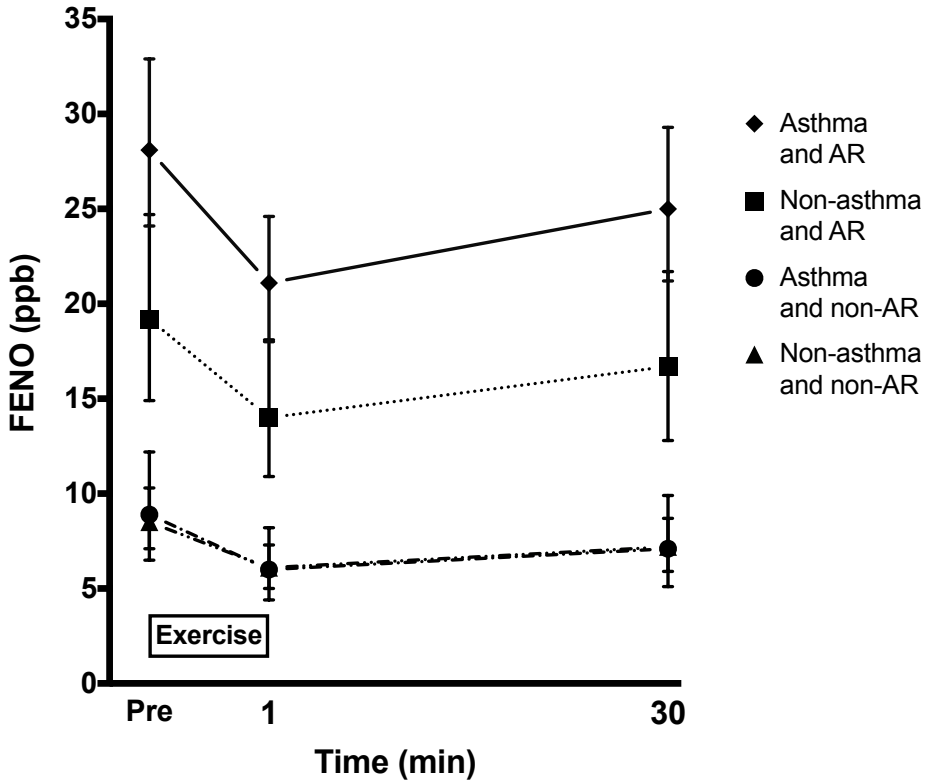

Figure 5. *Geometric mean FE_{NO} levels in asthmatics with allergic rhinoconjunctivitis (AR) (n=89), non-asthmatics with AR (n=34), asthmatics without AR (n=22) and non-asthmatics without AR (n=61). FE_{NO} was measured at baseline (pre) and at 1 min and 30 min after a standardized exercise induced-bronchoconstriction test on a treadmill. FE_{NO} is expressed in parts per billion (ppb). Error bars represents 95% confidence intervals.*

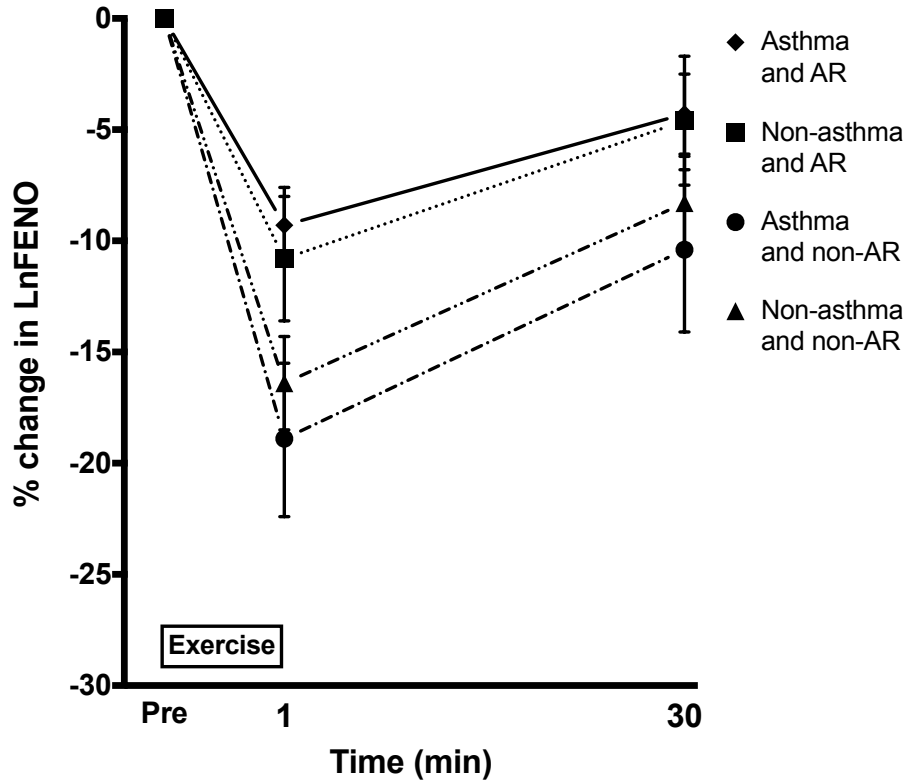

Figure 6. *Changes in LnFENO (%) after a standardized exercise-induced bronchoconstriction test on a treadmill in asthmatic and non-asthmatic children. Data are presented for four subgroups: asthmatics with allergic rhinoconjunctivitis (AR) (n=89), non-asthmatics with AR (n=34), asthmatics without AR (n=22) and non-asthmatics without AR (n=61). FE_{NO} was measured at baseline (pre) and at 1 min and 30 min after the exercise test. Error bars represents 95% confidence intervals.*

4.4 Paediatric cut-off values for serum sIgE to diagnose AR and its relation to FE_{NO} (Paper IV)

Of the 303 children enrolled, 223 had AR symptoms and 80 did not. In the group with AR symptoms, children with a reaction to the negative control (n=5), food allergy (n=23) and individuals who did not fulfil the AR definition (n=31) were excluded. In the group without AR symptoms, one child had food allergy and was also excluded. Children with AR (n=164) were similar to children without AR (n=79) with respect to age, height, weight, current eczema and urticaria (data not presented). Children with AR had more often current asthma than children without AR (p =0.044).

Diagnostic value of serum sIgE

Cut-off values for serum sIgE for a general optimal test with the best combined sensitivity and specificity were above the detection limit of the assay for seven of the ten allergens (0.23-1.1 kU/L). ROC curve analysis showed that the overall accuracy of the IMMULITE® in detecting AR was moderate to excellent, with areas under the curves (AUCs) at 0.852-0.954 (Table 3). However, the sIgEs for *Alternaria tenius*, *Cladosporium herbarium* and German cockroach were not significant predictors of AR (data not presented). Serum sIgE cut-off values differed according to the purpose of the test. Cut-off values for a diagnostic test at 90% specificity and for a screening test at 90% sensitivity are presented in Table 4.

FE_{NO} levels and the correlation with serum sIgE

FE_{NO} was elevated in children with AR, irrespective of asthma (Figure 7). In children with AR, FE_{NO} correlated moderately with total IgE (Spearmans´s rank correlation coefficient (rho)= 0.28, p <0.001), sIgE to cat (rho= 0.38, p =0.002) and dog (rho=0.59, p <0.001). FE_{NO} did not correlate positively with sIgE to other tested allergens (data not presented).

Pairwise comparisons of ROC curves

Serum sIgE was superior to total IgE and FE_{NO} in predicting AR to timothy, birch, mugwort, cat, dog and house dust mite. Total IgE predicted AR to timothy, birch and rabbit, while FE_{NO} did not. FE_{NO} and total IgE had equal power to predict AR in children sensitized to dog and *Dermatophagoides pteronyssinus* (Figure 8).

Table 3. ROC curve statistics for serum specific IgE to inhalant allergens in children with allergic rhinoconjunctivitis

Allergen	N*/ Positive†	AUC	95% CI	p-value	Cut-off value‡	Sensitivity	Specificity	LR+	LR-
Timothy	241/96	0.954	0.920-0.977	<0.001	1.1	94.8	84.1	6.0	0.06
Birch	241/73	0.905	0.861-0.939	<0.001	0.93	91.8	85.1	6.2	0.09
Mugwort	240/17	0.937	0.899-0.964	<0.001	0.59	82.4	94.2	14.1	0.19
Cat dander	240/89	0.924	0.882-0.954	<0.001	0.91	95.5	83.4	5.8	0.05
Dog dander	242/77	0.852	0.801-0.894	<0.001	0.27	83.1	78.2	3.8	0.22
Rabbit dander	242/23	0.856	0.805-0.897	<0.001	0.23	78.3	93.6	12.2	0.23
D.pteronyssinus	242/31	0.917	0.875-0.949	<0.001	1.00	87.1	97.2	30.6	0.13

AUC, area under the curve; CI, confidence interval; LR+, likelihood ratio positive; LR-, likelihood ratio negative; *D.pteronyssinus, Dermatophagoides pteronyssinus.*

*Complete result sets of serum specific IgE, skin prick test (SPT) and allergic rhinoconjunctivitis (AR) symptoms.

†Positive SPT and related AR symptoms as evaluated by a doctor.

‡Serum specific IgE cut-off values (kU/L) with the best combined sensitivity and specificity.

Adapted from Evjenth et al. Acta Paediatr 2014;103:759-65.

Table 4. Cut-off values and diagnostic utility of allergen-specific IgE for identifying children with allergic rhinoconjunctivitis

Allergen	Purpose	Cut-off value*	Sensitivity (95% CI)	Specificity (95% CI)	LR+	LR-
Timothy	Diagnostic†	4.1	78.1 (68.5-85.9)	90.3 (84.3-94.6)	8.1	0.24
	Screening‡	1.7	90.6 (82.9-95.6)	87.6 (81.1-92.5)	7.3	0.11
Birch	Diagnostic	2.8	80.2 (69.1-88.6)	90.2 (84.6-94.3)	8.2	0.22
	Screening	1.0	90.4 (81.2-96.1)	85.1 (78.8-90.1)	6.1	0.11
Mugwort	Diagnostic	0.35	82.4 (56.6-96.2)	91.0 (86.5-94.4)	9.2	0.19
	Screening	0.16	94.1 (71.3-99.9)	82.1 (76.4-86.9)	5.3	0.07
Cat dander	Diagnostic	7.4	69.7 (59.0-79.0)	90.1 (84.1-94.3)	7.0	0.34
	Screening	1.3	91.0 (83.1-96.0)	85.4 (78.8-90.6)	6.3	0.11
Dog dander	Diagnostic	1.7	52.0 (40.3-63.5)	90.3 (84.7-94.4)	5.4	0.53
	Screening	0.1	88.3 (79.0-94.5)	67.3 (59.5-74.4)	2.7	0.17
Rabbit dander	Diagnostic	0.11	78.3 (56.3-93.5)	90.4 (85.7-94.0)	8.2	0.24
	Screening	0.1	78.3 (56.3-93.5)	88.6 (83.6-92.5)	6.9	0.25
D.pteronyssinus	Diagnostic	0.36	87.1 (70.2-96.4)	90.5 (85.7-94.1)	9.2	0.14
	Screening	0.1	87.1 (70.2-96.4)	82.9 (77.2-87.8)	5.1	0.16

CI, confidence interval; LR+, likelihood ratio positive; LR-, likelihood ratio negative; *D.pteronyssinus, Dermatophagoides pteronyssinus*.

*Serum specific IgE cut-off values (kU/L)

†Diagnostic test; specificity at 90% or the closest specificity identified with the best combined sensitivity

‡Screening test; sensitivity at 90% or the closest sensitivity identified with the best combined specificity

Adapted from Evjenth et al. Acta Paediatr 2014;103:759-65.

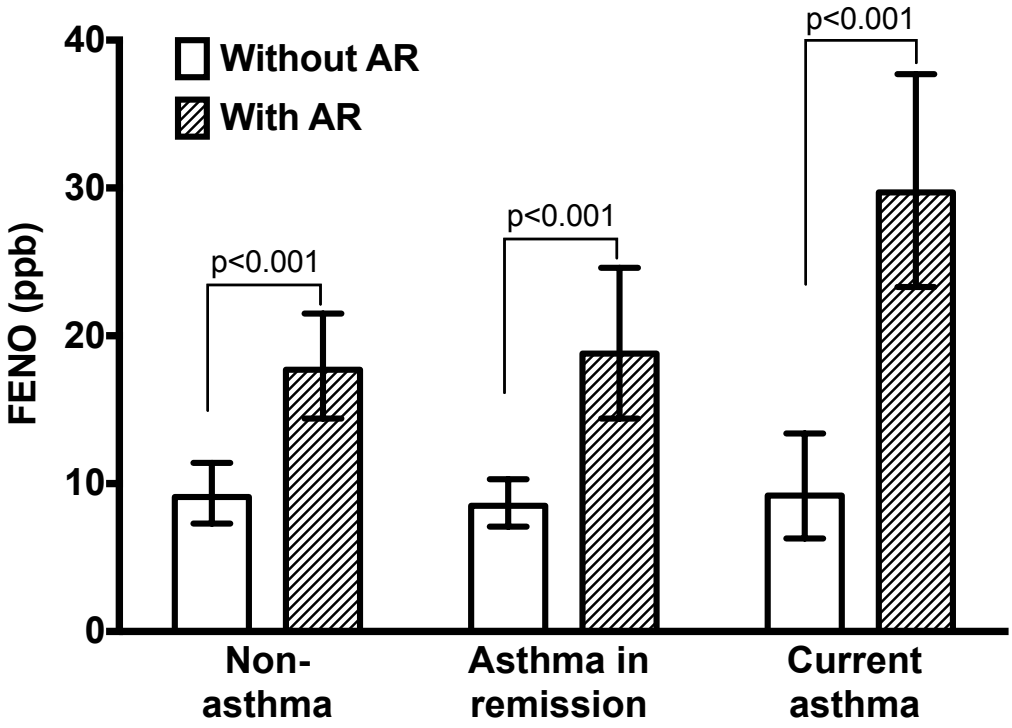

Figure 7. *Comparison of fractional exhaled nitric oxide (FE_{NO}) levels in children without asthma (non-asthma, n=110), asthma in remission (n=60) and current asthma (n=73) with (shaded bars) or without (white bars) allergic rhinoconjunctivitis (AR). AR was defined by a positive skin prick test and related AR symptoms. FE_{NO} was measured using the single breath technique and was expressed as parts per billion (ppb). Group comparisons were analysed by independent t-test with natural logarithm transformed data. Data are given as geometric means with 95% confidence intervals.*

Adapted from Evjenth et al. Acta Paediatr 2014;103:759-65.

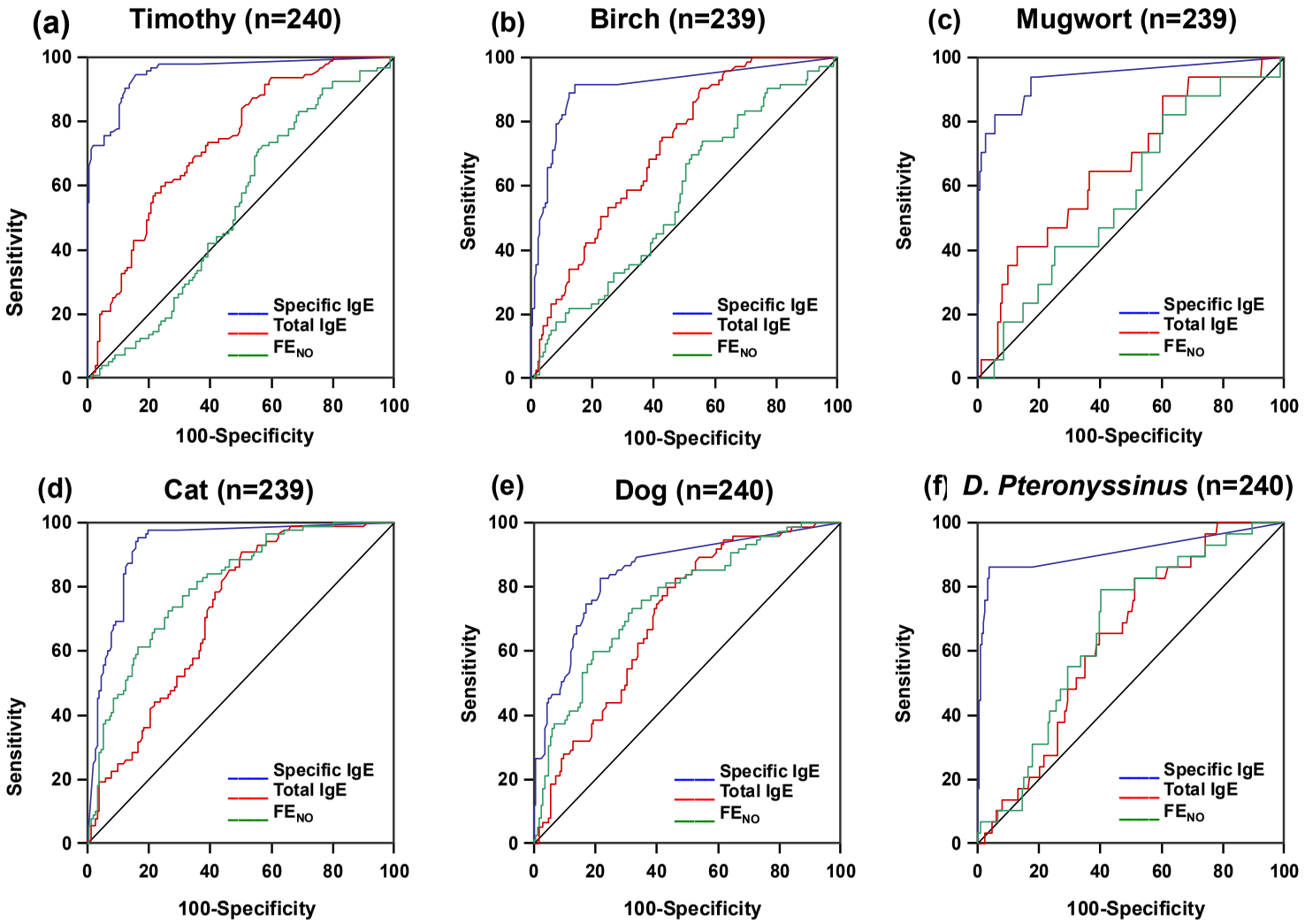

Figure 8. *Receiver operating characteristic (ROC) curves for specific IgE, total IgE and fractional exhaled nitric oxide (FE_{NO}) to predict allergic rhinoconjunctivitis (AR). AR was defined by a positive skin prick test and related AR symptoms. Specific IgE for (a) timothy-, (b) birch-, and (c) mugwort pollens, (d) cat, (e) dog and (f) D. pteronyssinus were analysed in serum using IMMULITE® 2000, and the results were expressed as kU/L.* Adapted from Evjenth et al. Acta Paediatr 2014;103:759-65.

5 DISCUSSION

5.1 Main findings

5.1.1 Prevalence of asthma, AR and eczema

The main findings were an increasing prevalence of asthma ever and AR ever between 1985 and 2008, while the prevalence of eczema ever reached a plateau. The prevalence rates found in 2008 were similar to those reported from the Environmental and Childhood Asthma study conducted in Oslo (10), but somewhat higher compared to results in the OLIN (Obstructive Lung Disease in northern Sweden) study (136). In contrast to prevalence studies in comparable populations (10, 137, 138), we found a substantial increase in the proportion of children reporting current diseases in the last time period.

Asthma, AR and eczema are closely related diseases (10, 139). Still the comorbidity of asthma and AR levelled off, while the comorbidity of asthma and eczema increased. These trends are in line with reports from the ISAAC study (6). We found a male predominance in asthma and AR. Male gender has been proposed to be a risk factor of asthma and AR (11, 140).

The increased prevalence of current asthma and AR in this subarctic childhood population may be related to changing environmental conditions (137). Global warming might increase the length and severity of the pollen season (141). It is widely recognized that air pollutants such as nitrogen dioxide (NO_2) and sulphur dioxide (SO_2) can damage the respiratory epithelium and also modify the allergenic potential of pollens (142). Information on the effect of environmental factors on respiratory allergic diseases in subarctic children is lacking, and future studies are needed on this issue.

5.1.2 FE_{NO} levels and the relation to asthma and AR

The aims of Paper II and III were to investigate the effects of exercise on FE_{NO}, whereas the primary aim of Paper IV was to establish serum sIgE cut-off values to diagnose AR and to examine the relationship between serum sIgE and FE_{NO}. Nevertheless, it is appropriate to compare FE_{NO} levels in the subgroups with other studies.

FE_{NO} levels in non-asthmatic children

We found that FE_{NO} was 10.6 (9.9-11.3) ppb in non-asthmatic children. FE_{NO} was significantly increased in non-asthmatic children reporting AR. FE_{NO} levels were not affected by gender or exposure to tobacco smoke (Paper II). In a multicentre study by Buchvald et al., non-asthmatic children with and without AR symptoms aged 4-17 years had geometric mean concentrations of FE_{NO} of 9.7 ppb (80). FE_{NO} increased significantly with self-reported rhinitis/conjunctivitis and age. FE_{NO} was not affected by passive smoke exposure or gender. Our FE_{NO} measurements in non-asthmatic children are in line with this multi-center study (80). However, in other investigations diverging FE_{NO} reference limits are reported, although the correlation with AR is consistent (98, 100, 111, 143, 144). Conflicting results are reported as to whether FE_{NO} is associated with gender (98, 100, 101, 145). In a Norwegian birth-cohort study, FE_{NO} was similar in boys and girls (101), while a Swedish childhood population study reported higher FE_{NO} levels in males than in females (100). They argued that larger lung volumes and higher degree of self-reported atopy could explain elevated FE_{NO} values in males.

FE_{NO} levels in relation to asthma and AR

In the case-control study (Paper III), we found that FE_{NO} was significantly increased in children with current asthma compared to non-asthmatic controls. The highest FE_{NO} levels (28.1 (23.0-34.3) ppb) were found in children with current allergic asthma. These findings are in line with other studies (76, 101, 111, 146). However, Nordvall et al. found that FE_{NO} was independently related in a multiple linear regression model to AR symptoms, although they reported considerably lower FE_{NO} values compared to our results (100). This may be explained by the use of an exhaled flow rate of 0.1 L/s rather than the recommended 0.05 L/s (78). We found that asthmatic and non-asthmatic children without AR had similar FE_{NO} concentrations (Paper III and IV), and that FE_{NO} levels were similar in asthmatics and non-asthmatics in remission (Paper IV). These findings are in line with the Environmental and Childhood Asthma study in Oslo (101), but contrasts to those of Norvall et. al who found increased FE_{NO} levels in children with ´ever asthma´, but allergic sensitization was not taken into account (100).

Interpreting FE_{NO} levels, the AR definition should be taken into account. In non-asthmatic with AR, we reported higher FE_{NO} values in Paper III and IV than in Paper II. This could be rationalized by different definitions of AR; AR was merely defined by self-reported

symptoms in Paper II, while it was defined by allergic symptoms and allergic sensitization in Paper III and IV.

It is well known that ICS treatment reduces FE_{NO} values (26, 51). In our study, FE_{NO} was not influenced by the use of ICS in asthmatics (Paper III). However, ICS treatment has been reported to be a marker of more severe disease (146).

In conclusion, our findings are in accordance with current literature namely that FE_{NO} is a marker of AR and allergic asthma (51, 111, 145). In clinical practice, AR and allergic asthma should be suspected when elevated FE_{NO} levels are measured. However, FE_{NO} has been reported to have a low positive predictive value (PPV) and a high negative predictive value (NPV) to diagnose current asthma (101). Our findings supports that FE_{NO} cannot be used to rule out asthma, as children with non-allergic asthma express FE_{NO} values similar to non-asthmatics. The lower FE_{NO} production found in non-atopic asthmatics than in atopic asthmatics supports the theory of different pathophysiological mechanism of airway inflammation in these groups (17, 30).

FE_{NO} and the correlation with serum total IgE and sIgE

In children with AR, total IgE correlated significantly with FE_{NO}. High total IgE is a well-known predictive marker of FE_{NO} increase in children (147, 148). However, different correlations with FE_{NO} have been demonstrated in different phenotypes of AR and allergic asthma (149-151). In children with AR, serum sIgE to cat and dog correlated significantly with FE_{NO}. This may partly be explained by allergen size. Sensitization to small molecules is associated with BHR, whereas sensitization to larger molecules such as pollen allergens is associated with allergic inflammation in the upper airways (45, 152). Allergens inhaled to the lower respiratory tract may induce FE_{NO} production by increased expression of iNOS (83). Serum sIgE to pollen allergens did not positively correlate with FE_{NO}, in line with other studies (146, 149) . On the other hand, FE_{NO} has been shown to increase substantially in the pollen season in children with seasonal AR and asthma (144, 153). A limitation of our study was that it was performed mainly out of the pollen season and pollen exposure is time-limited in cold climates. In contrast to other studies, we did not find a positive correlation between sensitization to *Dermatophagoides pteronyssinus* and FE_{NO} (151, 154). This may partly be explained by the few children with AR to *Dermatophagoides pteronyssinus.*

In conclusion, the correlation between FE_{NO} and serum sIgE is dependent on the allergic phenotype.

5.1.3 The impact of exercise on FE_{NO}

The main results were that FE_{NO} decreased in non-asthmatic and asthmatic children immediately after a submaximal exercise challenge and did not return to baseline value within 30 min. Children with AR expressed higher baseline FE_{NO} levels and demonstrated a significantly greater reduction in FE_{NO} value (ppb) than children without AR, irrespective of asthma. However, the effect of heavy exercise (% change in $LnFE_{NO}$) was more pronounced in subjects without AR.

The increased baseline FE_{NO} level found in children with AR and allergic asthma suggest that AR might be linked to both the upper and the lower respiratory tract, in concordance with the united airways disease concept (45, 155). This is supported by histochemical studies that have demonstrated eosinophilic inflammation from the nasal mucous membrane to the bronchial lining in subjects with AR (45). Therefore, the increased FE_{NO} at baseline in non-asthmatics with AR is likely to reflect subclinical lower eosinophilic airway inflammation.

Eosinophilic cells are known to provoke airway-epithelium injury *via* oxidative damage of proteins (156) and thereby promote the release of cytokines and other pro-inflammatory mediators. The expressions of iNOS and cNOS are enhanced by pro-inflammatory mediators, and the NO production is aggravated by oxidative stress (83, 88). During exercise, airway inflammation is triggered by cooling and dehydration of the airway mucosa, and inflammatory mediators are released in response to a hyperosmolar stimulus (69, 70). In addition, in children with AR and allergic asthma the nose can be blocked and mouth breathing is favoured. The reduced air-conditioning may enhance the inflammatory process (71). Based on our results we hypothesise that airway inflammation and oxidative stress during heavy exercise aggravate NO production in asthmatics and non-asthmatics with AR leading to a less % reduction in $LnFE_{NO}$ post exercise compared to children without AR.

The differences in the effect of exercise on FE_{NO} were more significant with increasing number of positive SPT/or sIgE tests in children with AR compared to children without AR. In children with AR, the effect was not significantly different with increasing numbers of positive allergy tests. The few children in each of these subgroups may partly explain the non-

significant difference. However, AR is an index of greater atopy. It is likely that the allergic inflammation drives both AR and FE_{NO} production in the lower airways.

Few reports regarding the effects of exercise on FE_{NO} in children have been published, and the results are conflicting (117, 120, 121, 157). In these studies differences in response were not reported according to AR or allergic sensitization in asthmatics and controls, and few subjects were included compared to the present study. Likewise, unlike NO sampling techniques may affect the results (158, 159).

The main mechanism of FE_{NO} decline post exercise has been explained by a washout of tissue NO store due to hyperventilation (160, 161). The EIB test has been reported with different activity and intensity (117, 120, 121). We used a high intensity load, which can explain a marked decline in FE_{NO} post exercise. High exercise intensity entails increased pulmonary blood flow. However, Borland et al. found that NO diffusion towards the pulmonary circulation did not increase during exercise (162).

The overall decreased FE_{NO} after exercise may partially be explained by a lower contribution of nNO during exercise. During exercise nNO falls rapidly, and oral breathing may contribute to lower contamination by nNO of the lower respiratory tract (163). Studies are conflicting as to whether nNO is altered in AR (97).

The positive relationship between baseline FE_{NO} and the severity of BHR has also been demonstrated in other studies (75, 76, 164). Bronchoconstriction could conceivably decrease the airway surface area, and thus decrease the diffusing capacity of NO. We found that the time trend was not associated with EIB in asthmatics. The reduced FE_{NO} after exercise has been found to be independent of changes in airway caliber in other studies (120, 165).

What are the clinical implications of these findings? In clinical practice, FE_{NO} is used to guide diagnosis and treatment decisions. If children are physically active before FE_{NO} measurements, FE_{NO} values could be underestimated. This is especially pronounced in children with AR who have the greatest reduction in FE_{NO} value post exercise. Therefore, FE_{NO} measurements should be performed before EIB tests, and children should be recommended to rest at least 30 min before FE_{NO} measurements.

5.1.4 IMMULITE® 2000 cut-off values for serum sIgE to diagnose AR

Previous studies using different immunoassays have shown wide disparity among serum sIgE levels (130, 166). In a proficiency survey by Hamilton et. al excellent agreement was demonstrated for total IgE measurements between the most commonly used assays including IMMULITE® (130). They reported a trend towards higher estimates of sIgE levels to common inhalant allergens for the IMMULITE® compared to those of ImmunoCAP® at sIgE levels above 1 kU/L (130). Likewise, IMMULITE® has been found to overestimate sIgE levels to cat, dog and *Dermatophagoides farina* (166). Thus sIgE cut-off values reported for one *in vitro* assay defining clinical allergy cannot be used with sIgE results from a different assay. To our knowledge, no previous studies have determined paediatric cut-off values for serum sIgE to diagnose AR using IMMULITE® 2000.

We found that serum sIgE was a powerful predictor of AR to the tested pollen, animal and mite allergens. Cut-off values with the best combined sensitivity and specificity were above the detection limit of IMMULITE® for seven of the ten allergens (0.23-1.1 kU/L) tested. At these levels the sIgEs were good predictors of AR to pollens, cat and rabbit; and sIgE was a very good predictor of AR to house dust mite. However, sIgE to dog had a low LR+ and was therefore poor in predicting AR to dog. Cut-off values for a diagnostic test were determined at 90% specificity. At these cut-off points most individuals with AR to pollens, rabbit and house dust mite were diagnosed. However, AR to dog and cat were under-diagnosed. Using sIgE as a screening test with sensitivity at approximately 90% resulted in lower sIgE cut-off points. The sIgE cut-off level for dog could not be used to rule out AR to dog. The sIgEs for *Alternaria tenius*, *Cladosporium herbarium* and German cockroach were not significant predictors of AR to these allergens. This may partly be explained by the few children with AR to these allergens.

It could be argued that over-sampling of children with AR affected the results. However, ROC curves are theoretically independent of disease prevalence (167). On the other hand, cut-off values may be affected by the severity of AR in the schoolchildren (167).

In conclusion, labelling serum sIgE as a dichotomous variable (positive or negative) based on the detection limit of IMMULITE® would result in over-diagnosing AR. The cut-off values were dependent on the allergic phenotype and the purpose of the test. Consequently, the cut-

off values used in the clinic should be chosen according to the allergen and the purpose of the test.

5.2 Methodological considerations

5.2.1 Phase I (Paper I)

A large representative fraction of schoolchildren in Nordland County were enrolled in Phase I of the study. The Nordland population is mainly of Caucasian ethnicity. Therefore, the external validity is restricted by ethnicity. The response rate of 63.8% might entail a selection bias. However, in a Swedish study the prevalence of airway symptoms and diseases did not differ between responders and non-responders (168). Thus, we find the study population to be representative for the Nordland childhood population. The questionnaire has been used in other Norwegian studies (169, 170), and its validity has been evaluated thereby proving to be a method with high sensitivity and specificity (169). However, self-reporting is affected by recall bias. Nevertheless, questions concerning current symptoms and diseases are expected to be the most reliable.

5.2.2 Phase II (Paper II-IV)

Study design

No power calculations were performed beforehand, since our intention was to include as many as possible within the scope of study. For each of the children who reported asthma in Phase I and was invited to Phase II, two non-asthmatic controls were invited as less attendance was expected in the control group. A relatively high attendance rate of 70% in Phase II was an important factor to control for selection bias. However, people attending surveys may tend to be more health-interested (171). This may be a cause of information bias (171). As a consequence of the population-based design most asthmatics had mild or moderate disease.

Detailed inclusion criteria are prerequisite in a case-control study (Paper III). Based on the structured interview and the clinical examination the participants were categorized as asthmatic or non-asthmatic. The pupils were matched according to age and gender as these features influence FE_{NO} and spirometric values and represent potential confounding factors (Paper III) (80, 135). One advantage of the case-control study design is the possibility to

study exposures associated with the diseases, whereas conclusions on causality cannot be drawn.

Definitions

One limitation in interpreting the results is the lack of a ´gold standard´ in defining asthma. However, asthma was defined more strictly than doctor´s diagnosis alone, or by questionnaire reported wheeze. Thus, a detailed structured interview and the requirement of at least two of three commonly used criteria reduced the risk of both over- and under-diagnosing, thereby increasing the validity of the data. AR symptoms were defined according to the ARIA guidelines (38). The reliability of the AR diagnosis may be affected by the definition of allergic sensitization (Paper III and IV) (132). In Paper III, seroatopy was defined by a sIgE ≥0.35 kU/L to reduce over-diagnosing AR.

Procedures

A major advantage of this study was the comprehensive clinical characterization of the children, including clinical examination, measurements of FE_{NO}, lung function, BHR and allergic sensitization as well as the detailed questionnaire and interview data. Although an obvious limitation was that the Phase II study was not blinded to the investigators.

Two paediatric doctors conducted all the interviews, clinical examinations and procedures and the same medical instruments were used to secure standardized measurement conditions. Further, the procedures were performed in accordance with validated published guidelines (77, 78, 125, 133). Thus, the clinical assessments can be regarded as consistently reported and reliable and thereby strengthen the statistical power and the internal validity of the results.

FE_{NO} measurements were performed with a chemiluminescence analyser that has demonstrated to exhibit good reproducibility and accuracy (78, 172). The participating children were examined at least two weeks after any respiratory tract infection. Hence, it was not likely that current viral infections influenced FE_{NO} values. Physical activity and food intake were restricted one hour prior to FE_{NO} measurements. None of the pupils smoked. However, FE_{NO} measurements may be confounded by the asthma status, asthma severity and use of ICS. Ideally, after the exercise FE_{NO} measurements should have been repeated until normalization.

Blood samples were requested for all children. During the initial study period SPT were requested for all children. Thereafter, SPT were requested for children with asthma and/or allergy symptoms. This approach resulted in an oversampling of children with AR (Paper IV). The participants went through a comprehensive program. Due to time limitations, we prioritized SPT testing in children with asthma and/or allergy symptoms. Ideally, SPT should have been requested for all children.

Statistical considerations

It should be noted that, in the published version of Paper II, the % change in FE_{NO} was calculated from geometric mean FE_{NO} values, and these percentages were not included in the statistical calculations.

5.3 Future perspectives

Asthma and AR are complex diseases and future research is needed in the areas of epidemiology, genetics and inflammatory markers.

The present study revealed an increasing prevalence of asthma and AR in Nordland County over the last three decades. This points to the need of repeated regional studies. Life-style and environmental factors may contribute to the development and the severity of asthma and allergic diseases (173, 174). Similarly gene-environmental interactions (epigenetics) influence airway disease susceptibility (173). Future epidemiological studies may help to identify primary preventive strategies to decrease the burden of asthma and allergic diseases. So far, most primary preventive programs based on allergy avoidance have failed to reduce asthma and allergic diseases (175). Interesting new concepts of primary prevention have emerged which propose that early exposure to allergens may induce tolerance (175).

Today, FE_{NO} is the only exhaled biomarker that has been standardized and validated for clinical paediatric application (78, 79). However, the main limitation is that FE_{NO} is a marker of eosinophilic inflammation. In this study, we have elucidated some clinical aspects of FE_{NO} measurements of importance in clinical care. To our knowledge, this is the first study reporting that the effect of exercise on FE_{NO} is dependent on AR in asthmatic and non-asthmatic children. The results are novel, and further studies are needed to confirm these findings.

The syndrome of asthma and also AR are frequently divided into clinical phenotypes that are heterogeneous, overlap and change over time. Comprehensive efforts are being made in identifying disease endotypes based on cellular and molecular disease mechanisms (176). Recent reports indicate that no single biomarker will characterize asthma subtypes, but rather a combination of biomarkers is required (176-178). Future studies on biomarkers will hopefully provide additional insight into the underlying disease endotypes, and may thus be helpful in tailoring individual treatment approaches. In addition, these studies will eventually reveal why some atopic individuals develop AR and allergic asthma while others do not.

6 CONCLUSIONS

In Nordland County, repeated cross-sectional surveys between 1985 and 2008 revealed an increase in the prevalence of asthma and AR ever among schoolchildren (7-14 years), while the prevalence of eczema ever reached a plateau. The prevalence of current asthma, AR and eczema doubled and trebled between 1995 and 2008.

The FE_{NO} level was significantly increased in asthmatic compared to non-asthmatic children, and significantly elevated in asthmatic and non-asthmatic children with AR compared to individuals without AR. The highest FE_{NO} values were found in children with current allergic asthma. The correlation between FE_{NO} and serum sIgE was dependent on the allergic phenotype.

FE_{NO} decreased in non-asthmatic and asthmatic children immediately after a standardized exercise treadmill test, and FE_{NO} did not return to baseline value within 30 min. Children with AR demonstrated a significantly greater reduction in FE_{NO} value (ppb) than children without AR, irrespective of asthma. However, the effect of heavy exercise (% change in $LnFE_{NO}$) was more pronounced in subjects without AR. Hence, if children are physically active before FE_{NO} measurements, FE_{NO} values could be underestimated. This is especially pronounced in children with AR who have the greatest reduction in FE_{NO} value post exercise.

The overall accuracy of IMMULITE® 2000 in detecting AR was good. The cut-off values with the best combined sensitivity and specificity were above the detection limit of IMMULITE® for seven of ten inhalant allergens (0.23-1.1 kU/L) tested. Consequently, using the detection limit for serum sIgE as the decision point would result in over-diagnosing AR.

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ERRATA

Published paper II: The correct name of the chemiluminescence device is ECO MEDICS Exhalyzer®.

Paper I

Paper II

Paper III

Paper IV

Appendix I

NORDLANDSSYKEHUSET
NORDLÁNDA SKIHPPIJVIESSO

Skjema nr. ☐

INNLEDNING

Dette er spørreskjemaet som vi ber dere fylle ut hvis dere vil delta i forskningsprosjektet. Spørreskjemaet inneholder 49 spørsmål. Undersøkelsen baserer seg på frivillig deltakelse, men for det beste resultatet, er det viktig at så mange som mulig deltar.

Vi ønsker å delta i forskningsprosjektet: **Ja** ☐

Sted/dato	**Underskrift foreldre/foresatte**

PERSONOPPLYSNINGER

Gutt ☐ Jente ☐ Alder i år ☐ Fødselsdato ☐

Skole:..... Klasse ☐

Hvor bodde eleven det første leveåret(poststed)?.....

Hvor lenge har eleven bodd i nåværende område (antall år)? ☐

Spørreskjemaet er fylt ut av:

Eleven selv		Mor		Far		Andre	

FAMILIE

1. Har noen i familien til eleven (foreldre, søsken) hatt astma, "høysnue", eksem, elveblest eller andre sykdommer som dere tror kan skyldes allergi? Ja ☐ Nei ☐
2. Hvis JA: kryss av:

	Mor	Far	Søstere	Brødre
Astma				
Høysnue				
Elveblest				
Eksem				
Andre allergiske sykdommer				

3. Hvor mange søsken har eleven? ☐

LUNGESYKDOMMER

4. Har eleven hatt astma? Ja ☐ Nei ☐
5. Hvis JA: har eleven hatt slike plager siste 12 måneder? Ja ☐ Nei ☐

6. Har eleven brukt astmamedisiner? Ja ☐ Nei ☐
7. Hvis JA: har eleven brukt slike medisiner siste 12 måneder? Ja ☐ Nei ☐
8. Har lege diagnostisert astma hos eleven? Ja ☐ Nei ☐
9. Har eleven hatt perioder med tetthet og piping i brystet, og/eller anfall med tung pust uten at dette har vært oppfattet som astma? Ja ☐ Nei ☐
10. Har eleven hatt perioder med hoste uten å være forkjølet? Ja ☐ Nei ☐
11. Har eleven hatt anfall med tung pust? Ja ☐ Nei ☐
12. Får eleven piping i brysteteller blir han/hun mer tungpustet enn jevnaldrende ved anstrengelser eller i rå, kald luft? Ja ☐ Nei ☐

13. Får eleven piping i brystet, perioder med hoste eller anfall med tung pust (astma) på grunn av ytre faktorer? Ja ☐ Nei ☐

14. Hvis JA: kryss av:

Dyr		Gress		Matvarer	
Værforandringer		Infeksjoner		Andre	

15. Har eleven noen gang vært behandlet av lege eller innlagt i i sykehus for annen sykdom enn ovenfor nevnt i bronkier eller lunger, f. eks bronkitt eller lungebetennelse? Ja ☐ Nei ☐

HØYSNUE

16. Har eleven hatt "høysnue"(Perioder med plager fra nese og/eller øynene som f. eks renning fra nesen, nesetetthet, nysing, kløe i nese/øyne, hovne øyne, "røde øyne")? Ja ☐ Nei ☐

17. Hvis JA: har eleven hatt slike plager siste 12 måneder? Ja ☐ Nei ☐

Hvis NEI: fortsett til spørsmål nr. 27.

18. Hvis JA: kryss av:

Nesetetthet		Renning fra nesen		Kløe i nesen	
Kløe i øynene		Hovne øyne		Nysing	
Hevelse rundt øynene		Rødhet i øynene		Andre	

19. Vet dere om forhold som utløser høysnueplagene? Ja ☐ Nei ☐

20. Hvis JA: kryss av:

Dyrekontakt		Gress		Trær	
Matvarer		Andre			

21. Er det noen årstid hvor høysnueplagene er verst? Ja ☐ Nei ☐

22. Hvis JA: kryss av:

Sommer		Høst	
Vinter		Vår	

23. Elevens alder (år) da høysnueplagene begynte? ☐

24. Dersom eleven tidligere har hatt høysnue, men nå er kvitt disse plagene: Hvor gammel var eleven da plagene forsvant? ☐

25. Bruker eleven medisiner for sine høysnue plager? Ja ☐ Nei ☐

26. Hvis JA: hvilke medisner bruker han/hun?

……

HUDSYKDOMMER

27. Har eleven hatt utslett som har vart i mer enn 4 uker? Ja ☐ Nei ☐

Hvis NEI: fortsett til spørsmål nr. 32.

28. Hvis JA: har eleven hatt slikt utslett siste 12 måneder? Ja ☐ Nei ☐

29. Hvis JA: med:

Mye kløe		Lite kløe		Ingen kløe	

30. Hvis JA: hvor var utslettet lokalisert?

Ansikt		Mage		Albuebøyer	
Rygg		Knehaser		Andre steder	

31. Hvis JA: hvor gammel var eleven da utslettet begynte ☐

32. Dersom eleven tidligere har hatt utslett som ovenfor nevnt, men nå er kvitt plagene: Hvor gammel var han/hun da utslettet forsvant? ☐

33. Har eleven hatt elveblest (kløe og hevelse i huden – utslettet flytter seg fra sted til sted ila minutter/timer og forsvinner etter timer eller dager)? Ja ☐ Nei ☐

Hvis NEI: fortsett til spørsmål nr. 36.

34. Hvis JA: hvor mange slike perioder har eleven hatt?

Mindre enn 5		Flere enn 5	

35. Hvis JA: hvor gammel var han/hun da plagene begynte? ☐

36. Har eleven reagert på matvarer? Ja ☐ Nei ☐

Hvis NEI: fortsett til spørsmål nr. 40.

37. Hvis JA:

Bare en gang		Flere ganger	

38. Hvis JA: hvordan reagerte han/hun?

Kløe i halsen		Tungpust	
Utslett/elveblest		Allergisjokk	

39. Hvis JA: hva reagerte han/hun på?

……

BOLIG

40. Hvor mange i familien bor nå sammen? ☐
41. I hvilket år ble boligen bygget? ☐
42. Hvor stor er boligen (ca boligareal i kvadratmeter)? ☐

43. Ligger boligen i et tettbebygget område med gater? Ja ☐ Nei ☐
44. Ligger skolen så langt unna hjemstedet at eleven må ha skyss til skolen? Ja ☐ Nei ☐
45. Røyker noen i familien daglig? Ja ☐ Nei ☐
46. Røyker noen i familien innendørs? Ja ☐ Nei ☐
47. Har familien selv dyr? Ja ☐ Nei ☐

48. Hvis JA: hvilke:

Hund		Katt		Hest	
Ku		Geit		Reinsdyr	
Sau		Kanin		Fugl (er)	
Marsvin		Hamster		Andre	

49. Hvis NEI: har eleven omtrent daglig kontakt med dyr? Ja ☐ Nei ☐

Nå er spørreskjemaet ferdig. Vi ber dere om å se over at alle spørsmål som dere ønsker å besvare, er besvart. Spesielt viktig er det at spørsmålene uthevet med gult er besvar.

I fase to av denne undersøkelsen ønsker vi å gjøre klinisk undersøkelse og testing av de barna som vi ut fra spørreskjemaet tenker har astma, samt kontrollbarn til disse. Dette vil bli et tilbud til disse elvene og det er frivillig om man vil delta. Vi kontakter de aktuelle elvene når spørreskjemaundersøkelsen er gjennomført.
Fase to planlegges gjennomført ila av høsten -08. Vi vil da reise rundt å undersøke barna, alternativt ta dem inn til undersøkelse her hos oss ved barneavdelingen.

TAKK FOR HJELPEN!