From The Department of Women's and Children's Health
Karolinska Institutet, Stockholm, Sweden

# FACTORS ASSOCIATED WITH PRESCHOOL WHEEZE DEVELOPING INTO SCHOOL-AGE ASTHMA

Idun Holmdahl


Karolinska Institutet

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# Factors associated with preschool wheeze developing into school-age asthma

Thesis for Doctoral Degree (Ph.D.)

By

**Idun Holmdahl**

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**Principal Supervisor:**
Associate professor Jon Konradsen
Karolinska Institutet
Department of Women's and Children's Health
Division of Clinical Pediatrics

**Co-supervisors:**
Associate professor Cilla Söderhäll
Karolinska Institutet
Department of Women's and Children's Health
Division of Clinical Pediatrics

Associate professor Anna Asarnoj
Karolinska Institutet
Department of Women's and Children's Health
Division of Clinical Pediatrics

Professor Marianne van Hage
Karolinska Institutet
Department of Medicine Solna
Division of Immunology and Allergy

Professor Magnus P. Borres
Uppsala University
Department of Women's and Children's Health
Division of Clinical Pediatrics

**Opponent:**
Professor James E. Gern
University of Wisconsin-Madison
Department of Pediatrics
Division of Allergy, Immunology and Rheumatology

**Examination Board:**
Adjunct professor Björn Fischler
Karolinska Institutet
Department of CLINTEC
Division of Pediatrics

Adjunct professor Lennart Nilsson
Linköping University Hospital
Department of Biomedical and Clinical Sciences
Division of Inflammation and Infection

Associate professor Ellen Tufvesson
Lund University
Department of Clinical Sciences
Division of Respiratory Medicine, Allergology and Palliative Medicine

This thesis is dedicated to my children, Ted and Loa.

*"It is fairer to listen to the string that broke than to never strain a bow"*

- Verner von Heidenstam

# Popular science summary of the thesis

Wheeze is defined as a whistling sound during expiration due to narrowed airways, accompanied by cough and troublesome breathing. Wheezing in preschool children is usually caused by respiratory viral infections, and most children are well between episodes. Preschool wheeze affects half of all preschool children below 6 years and is considered a risk factor for later asthma development since more than one third of all preschool wheezers later develop asthma in school age.

Risk factors associated with preschool wheeze developing into school-age asthma include eczema, allergic sensitization (presence of Immunoglobulin E antibodies), heredity for asthma and allergies, mode of delivery, wheezing onset, prematurity, vitamin D and environmental factors. Despite the many proposed risk factors, it is not possible to reliably predict which preschool wheezers will develop school-age asthma.

The aim of this doctoral project was to find associating factors with preschool wheeze developing into school-age asthma. Further, to examine the importance of allergic sensitization, the impact of different viruses and its combined effect on asthma development. Finally, to explore the inflammatory profile in blood during the acute wheezing episode.

All four studies in this doctoral project are based on the Gene Expression in Wheezing and Asthmatic Children (GEWAC) study, a longitudinal case-control study including 156 children with preschool wheeze (cases) and 102 healthy controls, ages 6-48 months, followed until age 11 years.

In study I we found that children with preschool wheeze with asthma at age 7 more frequently had a wheezing episode triggered by, the common cold virus, rhinovirus at inclusion. Further, preschool wheezers with asthma at age 7 experienced respiratory symptoms the year after inclusion in the study that demanded hospitalization in a higher extent than preschool wheezers without asthma at age 7.

In study II we found rhinovirus to be the most frequent trigger of wheeze at inclusion among preschool wheezers with asthma at 11 years of age. Rhinovirus-induced wheeze is most likely acting as a revealing factor in those children already at risk of asthma development due to hereditary factors and the presence of early allergic sensitization. We noted that children with both allergic sensitization and rhinovirus-induced wheeze at inclusion had a higher asthma prevalence at age 11.

In study III we explored inflammatory-related plasma proteins in children with acute preschool wheeze and compared those to healthy controls. Among 92 measured plasma proteins, we found that the ten most differentially expressed proteins could almost entirely separate children with acute preschool wheeze and healthy controls. These ten proteins, with their different functions, might contribute to a better understanding of preschool wheeze as a risk factor for asthma.

In study IV we examined sensitization to multiple allergen molecules at preschool age and at 7 years in relation to asthma at 7 years. We noted that preschool wheezers with asthma at age 7 had a significant increase of sensitizing allergen molecules from early preschool age to age 7 not seen in preschool wheezers without asthma at age 7. Furthermore, the number of sensitizing allergen molecules at age 7 was associated with asthma.

In summary, we found rhinovirus-induced wheeze to be associated with asthma both at age 7 and 11. Wheezing caused by rhinovirus is probably a revealing factor in children already at a higher risk of developing asthma. Both the increasing number of sensitizing allergen molecules from preschool age to 7 years of age as well as polysensitization at age 7 were associated with asthma, highlighting the importance of allergic sensitization in asthma development. Finally, among 92 measured inflammatory-related plasma proteins we found ten that could almost entirely separate children with acute wheeze and healthy controls. These proteins might contribute to a deeper understanding of why preschool wheeze is a risk factor for asthma.

# Abstract

Preschool wheeze affects one third of all toddlers up to the age of three years and half of the children before six years of age. Approximately one third of these children will develop asthma in school age, and several risk factors have been proposed. However, as of today, it is not possible to reliably predict which children, with preschool wheeze, will develop asthma.

All four studies in this thesis are based on the Gene Expression in Wheezing and Asthmatic Children (GEWAC) study, a longitudinal case-control study in which 156 cases and 102 healthy controls, ages 6-48 months, were included. The cases were recruited from the pediatric emergency department at Astrid Lindgren's Children's Hospital in Stockholm, when seeking care for an episode of acute wheeze. They came to a revisit after approximately 3 months and were followed annually up to the age of 7 years and a follow-up at age 11. The age-matched healthy controls were recruited from the same hospital at the surgical day-care ward and came to a follow-up at ages 7 and 11 years.

The study protocol included nasopharyngeal swabs for viral detection at inclusion, blood sampling, questionnaires, physical examination, measurements of lung function and fractional exhaled nitric oxide (FeNO).

Study I consisted of 113 children with an episode of preschool wheeze (cases) and 52 healthy controls who came to the 7-year follow-up. The prevalence of asthma at age 7 was 70.8 % among cases and 1.9 % in healthy controls. Rhinovirus-induced preschool wheeze was more common among cases with asthma in comparison to cases without asthma at age 7 years (48.1 % vs. 21.9 %, p = 0.011; OR 3.3, 95 % CI 1.3-8.5) and this association remained after adjustment for infection with other viruses (OR 3.8, 95 % CI 1.4-10.5). Cases with asthma at age 7 years were admitted to hospital more often because of respiratory difficulties (p = 0.024) and spent more time hospitalized (p = 0.01) during the year after inclusion in the study.

In study II we evaluated 107 cases and 46 healthy controls at the 11-year follow-up. We found that 62.6 % of cases and 13.0 % of healthy controls had asthma at age 11 years. Early-life factors associated with asthma at age 11 years, among cases, were rhinovirus-induced wheeze (OR 2.4, 95 % CI 1.02-5.6) and allergic sensitization at 2 years of age (OR 2.9, 95 % CI 1.05-8.1). However, in multivariate logistic regression only allergic sensitization at age 2 years (adjusted OR 3.0, 95 % CI 1.02-8.7) and parental heredity for asthma and/or allergy (adjusted OR 3.4, 95 % CI 1.1-9.9) were associated with asthma at age 11 years. Cases with both rhinovirus-induced wheeze at inclusion and allergic sensitization at age 7 years had a higher prevalence of asthma at age 11 years, in comparison to cases with rhinovirus-induced wheeze at inclusion but without allergic sensitization at age 7 years (92.9 % vs. 57.1 %, p = 0.03).

In study III we measured 92 inflammatory-related plasma proteins during an episode of acute preschool wheeze in 145 cases and compared them to 101 healthy controls. With

unsupervised clustering we found that the ten most differentially expressed inflammatory-related proteins could almost entirely separate cases from healthy controls. Seven proteins exhibited a higher expression in cases (OSM, IL-10, IL-6, CXCL10, FGF21, AXIN1 and SIRT2) and three proteins had a lower expression (TNFSF11, TNF-β and CASP8), in comparison to healthy controls. These proteins are implicated to be involved in airway epithelial dysfunction, airway remodelling, viral defence, and type 2 inflammation. Among the ten proteins, three (FGF21, SIRT2 and IL-10) were still differentially expressed between cases and controls at the revisit 3 month later.

Finally, in study IV we investigated sensitization to multiple allergen molecules longitudinally and its relation to asthma development at 7 years using the multiplex ImmunoCAP ISAC measuring 112 allergen molecules. In this study 72 cases were included and 43 healthy controls. Sensitization to each additional allergen molecule from preschool age to 7 years was associated with asthma at 7 years (OR 1.2; 95 % CI 1.01-1.5). The median number of sensitizing molecules increased from 3 (1-14) at inclusion to 10.5 (1-21) at 7 years of age among sensitized cases with asthma at 7 years of age ($p = 0.038$). No significant increase was seen in cases without asthma ($p = 0.26$). Lastly, the number of sensitizing allergen molecules at 7 years was associated with asthma at the same age (OR 1.2; 95 % CI 1.02-1.42).

In summary, we found rhinovirus-induced wheeze to be associated with asthma at both 7 and 11 years of age, although probably acting as an unveiling factor in children already predisposed to asthma development. We highlighted the importance of allergic sensitization in asthma development with molecular spreading and polysensitization being involved in disease development. Finally, we found ten inflammatory-related plasma proteins that could contribute to the understanding of why preschool wheeze is a risk factor for asthma development.

# LIST OF SCIENTIFIC PAPERS

I. **Holmdahl I**, Filiou A, Stenberg Hammar K, Asarnoj A, Borres MP, van Hage M, Hedlin G, Söderhäll C*, Konradsen JR*. **Early life wheeze and risk factors for asthma – A revisit at age 7 in the GEWAC-cohort**. *Equal contribution. Children (Basel). 2021 Jun 8;8(6):488. doi: 10.3390/children8060488. PMID: 34201058.

II. **Holmdahl I**, Lüning S, Wärnberg Gerdin S, Asarnoj A, Hoyer A, Filiou A, Sjölander A, James A, Borres MP, Hedlin G, van Hage M, Söderhäll C, Konradsen JR. **Rhinovirus induced wheeze is a potential unveiling factor for asthma development in predisposed children** *[Under review]*

III. **Holmdahl I**, Chakraborty S, Hoyer A, Filiou A, Asarnoj A, Sjölander A, Borres MP, van Hage M, Hedlin G, Konradsen JR, Söderhäll C. **Inflammatory related plasma proteins involved in acute preschool wheeze.** Clin Transl Allergy. 2023;e12308. doi: 10.1002/clt2.12308

IV. Filiou A, **Holmdahl I**, Asarnoj A, van Hage M, Ekenkrantz T, Rydell N, Sjölander A, Stenberg-Hammar K, Hedlin G, Konradsen JR, Söderhäll C. **Development of sensitization to multiple allergen molecules from preschool to school age is related to asthma**. Int Arch Allergy Immunol. 2022;183(6):628-639. doi: 10.1159/000521324. Epub 2022 Jan 18. PMID: 35042215.

# Scientific papers not included in the thesis

Chakraborty S, Hammar KS, Filiou AE, **Holmdahl I**, Hoyer A, Ekoff H, Sjölander A, Rydell N, Hedlin G, Konradsen JR, Söderhäll C. **Longitudinal eosinophil-derived neurotoxin measurements and asthma development in preschool wheezers.** Clin Exp Allergy. 2022 Nov;52(11):1338-1342. doi: 10.1111/cea.14210. Epub 2022 Aug 13. PMID: 35906849

Filiou A, Hoyer A, **Holmdahl I**, Chakraborty S, van Hage M, Nordlund B, Hedlin G, Konradsen JR, Söderhäll C. **Vitamin D receptor genetic variant associated with asthma in Swedish school-children.** Clin Exp Allergy. 2023 Oct;53(10):1045-1049. doi: 10.1111/cea.14349. Epub 2023 May 29. PMID: 37246605.

# CONTENTS

# List of abbreviations

| | |
|---|---|
| AD | Atopic dermatitis |
| Ara h 1, Ara h 2, Ara h 3, Ara h 6, Ara h 8, Ara h 9 | Arachis hypogea, peanut allergen molecules |
| AXIN1 | Axin inhibitor protein 1 |
| BDR | Bronchodilator reversibility |
| Bet v 1 | Betula verrucosa 1, birch allergen molecule |
| CASP8 | Caspase 8 |
| CDHR3 | Cadherin related family member 3 |
| CXCL10 | C-X-C chemochine ligand 10 |
| EDN | Eosinophil derived neurotoxin |
| EPA | Early preschool age, cases and controls at inclusion/revisit (study IV) |
| Fel d 1 | Felis domesticus 1, cat allergen molecule |
| FeNO | Fractional exhaled nitric oxide |
| FEV1/FVC | Forced expiratory volume in 1 sec / Forced vital capacity |
| FGF21 | Fibroblast growth factor 21 |
| GSDMB | Gasdermin B |
| GWAS | Genome wide association studies |
| HC | Healthy controls |
| ICAM-1 | Intracellular adhesion molecule |
| ICS | Inhaled corticosteroids |
| IFN | Interferon |
| IL | Interleukin |
| ILC | Innate lymphoid cells |
| LABA | Long acting β-2 agonist |
| LDL-R | Low density lipoprotein receptor |

| | |
|---|---|
| LTRA | Leukotriene receptor antagonists |
| Mal d 1 | Malus domestica 1, apple allergen molecule |
| OCS | Oral corticosteroids |
| ORMDL3 | Orosmucoid like-3 |
| OSM | Oncostatin M |
| Phl p 1 | Phleum pratense 1, timothy grass allergen molecule |
| PR-10 | Pathogenesis-related class 10 |
| Pru p 1 | Prunus persica 1, peach allergen molecule |
| PW | Children with preschool wheeze (cases) |
| PW-R | Children with preschool wheeze at the revisit after 3 months |
| RSV | Respiratory syncytial virus |
| RTI | Respiratory tract infection |
| RV | Rhinovirus |
| SABA | Short acting β-2 agonists |
| SIRT2 | Sirtuin 2 |
| SNP | Single nucleotide polymorphism |
| SPT | Skin prick test |
| T2 inflammation | Type 2 inflammation |
| Th1, Th 2 | T helper type 1 and type 2 |
| TNFβ | Tumor necrosis factor beta |
| TNFSF11 | TNF super family memeber 11 |
| TRAP | Traffic-realted air pollution |
| TSLP | Thymic stromal lymphopoietin |

# INTRODUCTION

Preschool wheeze is a common reason for attending the pediatric emergency department, affecting one third of all children before age three years. Approximately 40 % of children with an episode of wheeze will have recurrent wheeze and develop asthma at school age (1).

The global asthma prevalence in children and adolescents is approximately 10 % (2), with some studies indicating an increasing prevalence and incidence (3, 4). The increasing prevalence is thought to be a consequence of environmental factors rather than genetics (5). Asthma in childhood is an important public-health issue and causes a major burden on families, health care and the society (4).

Asthma is characterized by symptoms that are paroxysmal or persistent, variable airflow limitations, airway inflammation, bronchial hyperresponsiveness, and sometimes airway remodelling (6). Symptoms include recurrent episodes of wheeze, breathlessness and coughing (7).

Asthma is a widely heterogeneous and complex disease caused by the interplay between genes and environmental factors. Multiple risk factors have been proposed such as early onset and severity of wheeze, atopic diseases, parental smoking, vitamin D deficiency, obesity, male gender during childhood and female in adulthood, urbanisation, less commensal microbe exposure and heredity of asthma (8-11).

Preschool wheeze is mainly triggered by viral infections and a recognized risk factor for asthma development. However, it is currently unknown whether it is the viral agent that triggers the development of asthma or if viral wheeze only unveils the already genetically predisposed individuals (12).

Despite the many proposed risk factors for asthma, it is not possible to reliably predict which children with an episode of acute wheeze, as a toddler, will have asthma at school age. Early recognition of toddlers at risk for persistent asthma could lead to early prophylactic or therapeutic interventions that may help improve the prognosis.

# 1 LITERATURE REVIEW

## 1.1 Preschool wheeze – definition and prevalence

Acute wheeze (whistling sound during expiration accompanied by dyspnoea) is a symptom of an obstructive airway and asthma. Mucus secretion, airway inflammation and reversible tightening of the smooth muscles of the airways causes dyspnea and cough. The two major causes of wheeze in toddlers are bronchiolitis and asthma. Bronchiolitis is a virus-induced inflammation characterized by expiratory breathing difficulties and sometimes wheeze in children up to 2 years of age (13). Other, more uncommon, causes of wheeze include foreign body aspirations, cystic fibrosis, cardiac-immune- and gastrointestinal disorders and congenital anatomical abnormalities, these causes should be taken under consideration in atypical cases or in therapy resistant patients (14).

Approximately 30 % - 50 % of all preschool children suffer from episodes of viral induced wheeze which is one of the most common reasons for attendance to the pediatric emergency department (15-17). These children are at risk of later asthma since more than one third of preschool wheezers will continue to wheeze or develop asthma at school age (18). In high-risk cohorts, where the included children have severe recurrent wheeze, the prevalence of asthma at school age is around 70% (19).

Various genetic and environmental factors influence the clinical manifestation of preschool wheeze. At present, early viral infections, bacterial colonisation, and allergic sensitization, especially early allergic sensitization and polysensitization, are factors important in contributing to recurrent wheeze and asthma (20). Exposure to these early factors in combination with a genetic predisposition could influence the immune system that in turn could have a major effect on disease development (21).

**Knowledge gap:** Despite the many proposed risk factors, it is not possible to reliably predict which preschool wheezers will develop school-age asthma.

## 1.2 Genetic risk factors for wheeze and asthma

In birth cohorts, maternal asthma has been noted to be associated with persistent wheeze in the offspring (22). One possible explanation is that the combined effect of genetics and the in-utero environment could influence wheeze development. This could be due to pro-asthmatic mediators that are transferred through the placenta to the unborn child and affect the fetal immune system (22).

Genome-wide association studies (GWAS) have identified the 17q12-21 asthma locus including single nucleotide polymorphisms (SNPs) associated with pediatric asthma

development. Nonetheless, solely genetic factors are not the reason for the rapid increase in the prevalence of asthma (21).

Locus 17q21 has been recognised as the strongest asthma locus. SNPs at the 17q21 locus is associated with up-regulation of two, at the same locus, co-regulatory genes: *ORMDL3* (orosomucoid like-3) and *GSDMB* (gasdermin B). *ORMDL3* and *GSDMB* are linked to early life wheeze. Children with early life wheeze in combination with risk variants of 17q21 also have a higher risk of developing asthma (23, 24).

Exposure of Rhinovirus (RV) up-regulates *GSDMB* and *ORMDL3* expression (23). This overexpression of *ORMDL3* may increase the viral replication or the efficiency of the virus and cause damage to the respiratory epithelial cells, which limits the epithelial cell's ability to repair themselves after a RV-infection. These findings suggest a gene-pathogen interaction in the development of asthma (23, 25).

Another asthma-related gene, Cadherin-related family member 3 (*CDHR3*) is associated with early childhood asthma and recurrent wheeze in children between ages 2 and 6 years (26). However, CDHR3 is also identified to function as a receptor for the RV-C strain and *CDHR3* risk variants are associated with wheeze caused by RV-C in early life (27-29). Children carrying a *CDHR3* risk allele and with concurrent atopic disease have been shown to be more susceptible to RV-C infections (30).

## 1.3 Early-life exposures

Increasing evidence highlights the importance of the microbiome during early life. The microbiome affects immune maturation, and disruption during this critical period could cause allergic diseases and asthma (31). Exposure to a diverse group of microorganisms in early life leads to activation of the innate immune system, increased regulatory T cells with subsequent promotion of immunologic tolerance (32). Multiple early environmental exposures influence the microbial colonization and subsequently the immune responses to viruses and allergens (33). In populations, such as farm children and Amish populations, where they are extensively exposed to a microbial environment, the asthma prevalence is low, highlighting the importance of a diverse microbiome for immune maturation (34).

Both intrinsic factors (immune maturation) and extrinsic factors (delivery method, breastfeeding, hospital stay, gestational age, antibiotic use etc.) affect the intestinal microbiota in newborns. Studies have shown that the gut microbiota is no different in wheezing children developing asthma than those who do not, suggesting a window of opportunity before the wheezing episode (35).

Cesarian section reduces the diversity of the microbial composition compared to spontaneous delivery (36). In addition, antibiotics given during the neonatal period is associated with an elevated asthma risk, thought to be because of changes in the gut microbiome (37).

Short duration and non-exclusive breastfeeding are connected to an elevated risk of wheeze in preschool children, however it is not associated with asthma-like symptoms in older ages (38). Breastmilk contains various factors such as oligosaccharides, immune factors, hormones, growth factors, nutrients, and maternal microbiota, that is thought to influence immune development by promoting a healthy and diverse microbiome (39, 40).

Prenatal and early postnatal tobacco smoke exposure is associated with aberrant lung development with subsequent elevated risk of early-life wheeze and asthma (41). Traffic-related air pollutants (TRAP) also contribute to an elevated risk of recurrent wheeze in preschool children and asthma among children, although the duration of the exposure is of importance (42, 43).

Day-care attendance and older siblings who attend day-care also increase the risk of frequent wheeze during the preschool years. However, later in childhood day-care attendance is associated with a reduced risk for asthma and allergies (44).

## 1.4 Early-life immune responses

Newborns still have an immature adaptive immune response and to protect themselves against pathogens they need to rely on antibodies from their mothers, from breastmilk and their innate immunity (45). The unborn child's, as well as the infant's immune system, is skewed towards type 2 immune responses. Exposure to microbes is essential for immune maturation as well as adaptive immune system development. The immune system needs to learn how to adapt to invading pathogens as well as remain silent to commensal bacteria. A poorer microbial exposure early in life might lead to prolonged type 2 immunity, subsequent risk of allergic diseases and a defective response to viruses (46).

IFNs are crucial in our defense against viruses as they mediate innate immune responses to respiratory viruses. Low levels of IFN type II in cord blood and peripheral blood in infancy as well as low levels of IFN type I and III during infancy have been associated with wheeze during early life (47, 48).

Respiratory viruses infect the airway epithelium and trigger an inflammatory response, that in some cases, causes wheeze. One theory is that this innate immune response might impact the adaptive immune response to microbes and allergens that could lead to type 2 inflammation and further to recurrent wheeze. On the other hand some studies suggest that early contact with microbes could already have altered the immune response in the child, prior to exposure to viruses that triggers the first wheezing episode (33).

Others have shown that the path to childhood asthma already begins in utero. Maternal prenatal immune status with decreased interferon (IFN) -γ/IL-13 and IFN-γ/IL-4 ratio can predict childhood asthma (49) and the immune status among non-asthmatic mothers influence epigenetics and immune response to microbes in the offspring (50). Furthermore, increasing evidence suggests that maternal inflammation, infection and the microbiome influence fetal immune development in utero (51).

## 1.5 Vitamin D

Vitamin D deficiency have been hypothesised to contribute to asthma development as it has been shown to affect fetal lung development, lung maturation as well as immune cell function (52, 53). Vitamin D receptors are found in many tissues and cells, including the immune system. The active vitamin D metabolite 1,25-dihydroxyvitamin D (1,25 (OH)2D) affects both the adaptive and the innate immune system (10).

Vitamin D has protective effects against respiratory tract infections and also affects the expression of many genes in airway smooth muscle cells (54). Vitamin D deficiency has been associated with an elevated risk for severe asthma outcomes (55). A meta-analysis, restricted to RCTs involving children and adults given vitamin D supplements to improve asthma control and prevent asthma exacerbations, noted that vitamin D supplements reduced the risk of asthma exacerbations. However, the study was unable to comment on whether this was associated with factors like asthma severity and baseline vitamin D status (56).

## 1.6 Respiratory tract pathogens

Early infectious diseases in the lower airways and the number of respiratory infections have been implicated in both wheeze and later asthma development (12). Viral respiratory infections are important triggers of wheeze in preschool children, potentially due to a dysregulation of the innate immune response causing them to be more prone to severe viral infections (47). The interaction between viruses, the innate immune system and the respiratory microbiota has been suggested to contribute to infection severity with subsequent increased risk of wheeze progressing to asthma (47). Asthma is widely thought to be a result of airway inflammation that leads to bronchial hyperreactivity and lung function deficits. Increase of airway smooth muscle, airway eosinophilia, and reticular membrane thickening have been noted among recurrent preschool wheezers (57) suggesting that airway remodeling and inflammation are present even before asthma inception. Further, evidence of airway epithelial dysfunction with epithelial damage and barrier dysfunction due to insults have been associated with recurrent wheeze (58).

Virus-induced wheeze is a common presentation of asthma in early life, the episodes are intermittent, and the children are often well between the episodes (59). Respiratory virus infections play a major part in all wheezing illnesses from bronchiolitis to asthma and are identified in 90 % of all wheezing episodes in children below age 3 years (60).

Many different viruses can cause wheeze among preschool children; RV, Respiratory Syncytial Virus (RSV), Metapneumovirus, Influenza, Parainfluenza virus, Coronavirus and Bocavirus. RSV is the most important triggering virus during infancy (before 12 months of age) and RSV is over-representative among hospitalized cases (61). RV is the most common trigger after approximately 12 months (13). RV dominates as a trigger of wheeze among children with recurrent wheezing and asthma exacerbations (62).

The airway epithelial barrier is important in maintaining a proper defence against respiratory viral infections. Airway epithelial cells has been noted as an important source of type I (IFN-α/β) and III (IFN- λ) IFNs. They are involved in the control of virus replication as well suppression of airway inflammation. Disrupted epithelial signalling pathways make the airway epithelium more susceptible to environmental triggers which leads to epithelial injury, damaged repair, and airway remodelling. Further, disruption of the tight junctions in the epithelial cells contributes to an impaired barrier function allowing viruses into the airways where they interact with immune and inflammatory cells (63).

Studies have highlighted the importance of IL-33, thymic stromal lymphopoietin (TSLP), and type 2 inflammation in asthma exacerbations. Exhibiting its effect by being secreted from airway epithelial cells as a response to epithelial damage caused by respiratory viruses, leading to increased innate immune pathways and type 2 inflammation (64, 65).

**Knowledge gap**: Airway samples are not easily collected from children. The immunopathology of preschool wheeze using a wide range of systemic inflammatory markers might contribute to the understanding of why preschool wheeze is a risk factor for asthma development.

### 1.6.1 Respiratory syncytial virus

An association between RSV- induced wheeze and recurrent wheeze and asthma has been noted in pre-school children but at age 13 most children had outgrown their symptoms (66, 67). The subsequent risk of asthma is related to the severity of the acute illness (68). The highest risk of RSV-induced wheeze and asthma is among the youngest children with low lung function and is not associated with atopic characteristics. This indicates that the timing of RSV infection is of importance for later risk of asthma (69). RSV-induced wheeze is associated with non-atopic asthma (67, 70, 71).

RSV directly target airway epithelial cells and attach to the cellular receptor CX3CR1. RSV cause more harm to the airway epithelium, in comparison to RV, by induction of epithelial cell apoptosis and necrosis (69, 72). The induction of antiviral interferons and interferon-induced genes in the mucosa, as a response to RSV infection, is strong but the antiviral interferon responses are weaker and the inflammatory and adaptive immune responses are ineffective in severe RSV bronchiolitis (13). RSV infection is shown to decrease type 1 memory T-cell responses (antiviral) but does not influence the later type 2 responses, suggesting that it has a greater effect on the inception of non-atopic rather than atopic wheezing in children (73).

### 1.6.2 Rhinovirus

RV is within the Enterovirus genus in the Picornaviridae family, and compromise three different subtypes; RV-A, RV-B and RV-C (69). RV-A and RV-C are noted to trigger

respiratory illnesses of higher severity and have a stronger association with wheezing in early childhood in comparison to RV-B (74). RV-A and RV-B use the intercellular adhesion molecule (ICAM-1) receptor, expressed on, among others, epithelial cells, and the low-density lipoprotein receptor (LDL-R), expressed in almost all tissues. Cadherin-related family member 3 (CDHR3) is a unique receptor for RV-C (75). RV-C causes more severe respiratory infections and wheeze (76, 77) and one explanation for this could be the fact that CDHR3 is specifically expressed in the airway epithelium (26).

RV is a frequent trigger of asthma exacerbations and is the triggering agent in up to 80 % of exacerbations among children (78). As of today, it is among the most important risk factors for school-age asthma in children with wheeze, with an odds ratio (OR) of 45 dependent on aeroallergen sensitization as a cofactor (66).

RV has a well-known peak in September (79) whereas RSV is more common during winter (80). A population-based cohort study from the UK demonstrated that one of the strongest factors associated with asthma development in preschool wheezers is if the first event of wheeze occurs in September. This was a stronger risk factor than the other known risk factors atopic diseases and maternal family history of asthma (81). RV-induced wheeze has been accompanied by a 4 times higher risk of school-age asthma in comparison to those without RV-induced wheeze (82, 83). Increasing evidence point to that presence or predisposition of allergic sensitization in combination with RV-induced wheeze increases the risk of subsequent asthma, at least in genetically susceptible individuals (60, 84, 85).

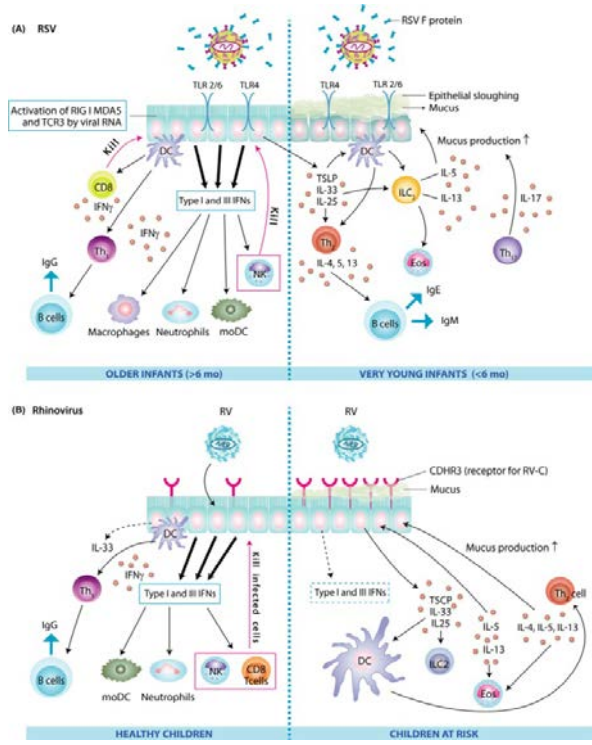



**Figure 1. Inflammatory response in RSV (a) and RV (b) wheeze, with permission from (13)**

RV directly targets airway epithelial cells and triggers release of the alarmins IL-25, IL-33 and TSLP (innate cytokines) from airway epithelial cells. RV infections trigger a stronger induction of innate cytokines in airway epithelial cells of asthmatics in comparison to non-asthmatics (13, 86) Alarmins trigger the release of IL-13, IL-5 and IL-4 due to activation of dendritic cells, T-cells and innate lymphoid cells (ILCs). These T-helper (Th)2 cytokines are capable of increasing ICAM-1 expression on the bronchial epithelial cell surface, making them more susceptible to infection (87).

Airway epithelial cells from allergic asthmatic children produce less cytokines involved in limiting viral replication (IFN-α, IFN-λ, INF-γ), causing a more severe RV infection since it impairs apoptosis of infected airway epithelial cells (87). In addition, other studies have noted that children with atopic characteristics have a higher risk of severe RV infection due to a defect in antiviral innate type I and III IFN responses in the airway mucosa, which is considered to be an effect of the cross-linking of high-affinity IgE receptors with IgE causing a significantly reduced RV-induced IFN response in atopic asthmatic children compared to non-asthmatics without atopy (13, 88-91).

The effect of the reduced type I interferon expression and the Th2 immune response leads to an increase of proinflammatory cytokines for example IL-6, IL-8, IL-16, CXCL10 and leukotrienes. This cause disrupted tight junctions and later airway damage, neutrophilic inflammation, bronchospasm, oedema, mucus production that causes airway obstruction and wheeze (87). In addition, RV triggers the induction of vascular growth factor TGF-β and chemoattractants, which contribute to airway remodelling (92).

Compared to RSV, RV-induced wheeze is associated with early decreased lung function, more severe and earlier wheezing illness (93).

**Knowledge gap**: It is debated whether RV is a causative agent for asthma development or solely a triggering factor of wheeze in children already predisposed to asthma.

### 1.6.3 Bacterial airway colonization

Asymptomatic one-month old babies colonized with *S. pneumoniae*, *M. catarrhalis* or *H. influenzae* in the airways have been associated with a 5 times higher asthma risk at 5 years compared to non-colonized infants (94). At 6 months of age the children that eventually developed asthma had increased systemic levels of IL-5, IL-13, IL-17 and IL-10, suggesting an aberrant immune response, which could result in chronic type 2 inflammation and asthma (95).

The duration of symptoms during an acute wheezing episode was shortened when antibiotic treatment was given, suggesting that there might also be a microbial effect (96).

## 1.7 Allergic sensitization

Allergen-specific immunoglobulin E (IgE) antibodies are developed throughout childhood and are a prerequisite for allergic reactions and the development of allergic diseases for example asthma, rhinitis and atopic dermatitis (AD) (97, 98). Specific IgE is crucial in the development of allergic reactions by binding to receptors on mast cells and basophils. Subsequent allergen exposure causes cross-linking of IgE antibodies that cause allergic symptoms by the release of cell mediators (99). Recent evidence suggests the defective skin barrier in AD is the trigger of development of allergic sensitization (100). The development of allergic diseases with AD and food allergy as most common during early life, followed by rhinoconjunctivitis and asthma in later childhood, is often referred to as the atopic march (101, 102).

Early-life allergic sensitization has been associated with recurrent wheezing and later asthma (18, 60, 103-105). There is a greater risk of asthma development in children with both early-life wheeze and aeroallergen sensitization than the two factors separately (66, 106). A greater risk of asthma development is also seen among children with early sensitization to multiple allergens (107). Polysensitized individuals have a higher risk of persistent asthma than monosensitized individuals measured at any time-points up to age 13 years, although the highest risk of asthma is in the youngest children with polysensitization (108).

Increased specific IgE levels to perennial allergens (mold, dust mites, pet dander) in childhood have been associated with a higher asthma risk. This is supported by results from the Manchester Asthma and Allergy Study (MAAS) which demonstrated that the presence of IgE antibodies to dust mites was strongly associated with asthma (107) and by Copenhagen Prospective Studies of Asthma in Childhood (COPSAC) where they noted an association between asthma and specific IgE to pet dander (109). In contrast, specific IgE to seasonal allergens (grass pollen, tree pollen) have been associated with a higher risk of rhinitis (110).

Even though the association between allergic sensitization and asthma is strong there are still many sensitized children who never develop asthma and many non-sensitized children with asthma. A comparison of sensitized children with and without atopic disease showed that those without symptoms had less heredity of allergies (111). This suggests that genetic factors that affect the expression of IgE matter in the development of allergic disease in childhood (98).

### 1.7.1 Molecular allergy diagnostics

Allergic sensitization is often diagnosed using skin prick test (SPT) or serum analysis measuring specific IgE to whole allergen extracts. Whole allergen extracts are composed of several proteins (components), some classified as allergen molecules. An allergen is defined as any molecule binding IgE antibodies. Some allergen molecules have similar structures and properties that could cause cross-reactivity between different foods and between food and inhalant allergen sources. Cross-reactivity normally causes mild symptoms from the oral

mucosa but, dependent on the cross-reactive allergen, for example lipid transfer proteins (Ara h 9, Cor a 8), could also cause severe reactions (112, 113).

One technique for molecular allergy diagnostics is the multiplex ImmunoCAP ISAC platform which enables the simultaneous measurement of IgE antibody levels against 112 allergen molecules from more than 40 allergen sources and utilizes only minute amounts of serum. Molecular allergy diagnostics makes it possible to determine sensitization at the molecular allergen level and the method provides additional information, particularly e.g., polysensitized patients to differentiate between genuine sensitization and cross-reactivity (112).

The most well-known example of IgE-cross-reactivity between inhalant and food allergen sources in Northern Europe is between Bet v 1 (birch) and the structurally homologous allergens in foods Mal d 1 (apple), Pru p 1 (peach) and Ara h 8 (peanut) (Pathogenesis-related 10 (PR-10 proteins)). Sensitization to these proteins is usually associated with mild symptoms from the oral cavity (114).

Storage proteins include, among others, Ara h 1, Ara h 2, Ara h 3, Ara h 6 (peanut), Cor a 9 and Cor a 14 (hazelnut). These proteins induce genuine peanut and hazelnut sensitization, respectively, and have been associated with atopic heredity and asthma (115). A recent Swedish study noted that genuine peanut sensitization most commonly developed before the age of 8 years and often persisted to adulthood. Also, sensitization to storage proteins was associated with respiratory and systemic T2 inflammation (116).

| Allergen | Storage protein | Beta expansin | Uteroglobin | PR-10, Bet v 1 family member |
|---|---|---|---|---|
| Apple | | | | Mal d 1 |
| Birch | | | | Bet v 1 |
| Cat | | | Fel d 1 | |
| Peach | | | | Pru p 1 |
| Peanut | Ara h 1<br>Ara h 2<br>Ara h 3<br>Ara h 6 | | | Ara h 8 |
| Timothy grass | | Phl p 1 | | |

**Table 1. Allergen molecules mentioned in this thesis, divided into groups with similar structures.**

**Knowledge gap**: Allergic sensitization is strongly associated with asthma, yet there are sensitized children who never develop asthma. Is there a role for molecular allergology in asthma prediction among preschool wheezers?

## 1.8 Lung function

The frequency and severity of wheeze have been associated with reduced lung function in school age (117), so have low birth weight, early-life wheeze, early allergic sensitization and tobacco smoke exposure (118). Infancy, a period of rapid lung growth and remodelling, could be a particularly sensitive period for viral- and allergen-induced inflammation (106).

Studies from a birth cohort only including children to mothers with asthma, demonstrated reduced lung function present already during infancy in children who later developed asthma. The lung function deficit, present in infancy, remained through adolescence even though symptoms of asthma ceased (119). These results suggest that lung function traits are established before the onset of airway inflammation and asthma.

Children with wheeze that persist from childhood to adulthood or relapse of wheeze in adulthood have been noted to be associated with a lower predicted FEV1 in adulthood in comparison to children who never wheeze (120). Others have shown that not only persistent wheeze, but also transient early wheeze is associated with subsequent persistently low FEV1, if the wheezing episode was severe (118). According to data from Tucson Children Respiratory Study (TCRS) (9) the airway dysfunction probably develops sometime between birth and age 6. Preschool children with persistent wheeze have reticular basement membrane thickening and eosinophilic inflammation (57). This suggests that preschool children with wheezing onset before 6 years have permanent lung function deficits that may continue into adolescence and adulthood (14). Further, birth cohort studies have noted an association between asthma-like symptoms during childhood and chronic pulmonary obstructive disease (COPD) 50 years later (121, 122).

## 1.9 Childhood asthma

Common triggers of asthma symptoms include allergens, respiratory viral infections, and exercise. Symptoms of asthma are the same in asthmatics unrelated to their underlying pathology: impaired or normal lung function, the presence or absence of atopy or intermittent or persistent symptoms (46).

The cardinal symptoms of asthma are caused by airway obstruction, which is a consequence of airway inflammation that contributes to narrowing of the airways. The inflammation is due to infiltration and activation of dendritic cells, eosinophils, neutrophils, lymphocytes, ILCs and mast cells, the communication between these immune cells, together with their effect on epithelial cells lead to airway hyperresponsiveness (123).

Epithelial cells have a central role in asthma through orchestrating inflammatory responses (by producing chemokines and cytokines) to external triggers and, in asthma, their function is altered by the loss of tight junctions, leading to increased permeability (124). Deficient epithelial cells are present in all asthma phenotypes and functional changes in epithelial cells are seen from an early age (125). The most studied epithelial-derived cytokines are the alarmins: TSLP, IL-33 and IL-25. External triggers such as allergens and virus induce the

release of alarmins. The alarmins contribute to activation of dendritic cells that promote Th2 development in draining nodes. They are also involved in activation and recruitment of innate immune cells, contributing to airway inflammation by release of mediators (124).

Type 2-high endotype is characterized by airway eosinophilia. Th2- and ILC2 cells are activated by alarmins and release IL-4, IL-5 and IL-13, contributing to airway inflammation. IL-4 and IL-13 bind to IL-4Rα and induce IgE immunoglobulin class switching by B cells. IL-5 has an important role in recruiting and activating eosinophils (126).

After generation of Th2 cells in draining nodes, some will connect with B cells to develop into plasma cells and antibody-producing cells. IL-4 and IL-13 promotes IgE production by B cells. IgE bind to high-affinity IgE receptors, expressed on various cell types for example basophils, mast cells, eosinophils, dendritic cells, airway smooth muscle cells as well as epithelial cells. Crosslinking of two IgE molecules by an allergen on the high affinity IgE receptor activates release of histamine, tryptase, chymase and produce lipid mediators as well as IL-4, IL-5, IL-9 and IL-13 from mast cells and basophils. This causes reinforcement of the pro-inflammatory Th2 environment, with vascular permeability, trigger mucus hyperproduction and contributes to smooth muscle hypertrophy and hyperplasia (124). IgE receptors expressed on airway smooth muscle cells can respond instantly to IgE through the production of cytokines and chemokines and contribute to airway hyperresponsiveness (127). IgE can bind to high-affinity IgE receptors on plasmacytoid dendritic cells, which produce type I IFN, and reduce the type I IFN signaling that is crucial for viral clearance (89).

The release of IL-5 from Th2 cells and ILC2s activates eosinophils. Eosinophils exert its effect in the airways through various factors including eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), type 2 cytokines, pro-inflammatory cytokines, chemokines, and lipid mediators. Eosinophilic airway inflammation leads to lung damage, airway remodelling and promotes airway hyperresponsiveness (124, 128).

The release of IL-13 and IL-4 from Th2 cells as well as from ILC2s promotes upregulation of the epithelial inducible NO synthase with subsequent elevated NO levels. Thus, fractional exhaled nitric oxide (FeNO) function as a T2 inflammation marker (129).

The definition of non- or low-type 2 phenotypes is the lack of Th2 signatures, such as blood eosinophils and elevated FeNO as well as non-responders to treatment with inhaled corticosteroids (ICS). In some patients with this phenotype, an increased IL-6 pathway has been noticed (130) and neutrophilic asthma has been proposed as an asthma phenotype. The theory behind is production of IL-1β, IL-6 and IL-8 from airway epithelial cells recruit neutrophils as well as induce Th1 and Th17 cells, which also contribute to neutrophil recruitment. Neutrophils release factors contributing to epithelial cell damage and mucus production. Although attempts to target these immune pathways have been ineffective. It is

debated now whether neutrophilic airway inflammation is a non-specific marker of severe asthma rather than a causative factor (131).

## 1.10 Diagnosing asthma from preschool to school age

No objective reliable test is available to diagnose asthma and it is difficult to establish an early diagnosis since preschool wheeze is a heterogeneous condition with different phenotypes that overlap in symptoms (16, 132, 133). Asthma diagnosis in young age is often based on clinical criteria such as symptom pattern, heredity, and physical examination along with the absence of an alternative diagnosis (14).

According to Swedish national guidelines (134) the preschool child receives a diagnosis of asthma after:

- Three wheezing episodes if the child is <3 years
- One episode of wheeze if the child is <3 years AND have other signs of atopic disease
- One episode of wheeze if the child is >3 years of age

The most recognized guidelines for asthma management are the Global Initiative for Asthma (GINA), the National Institute for Health and Care Excellence (NICE; United Kingdom) and the Expert Panel Report of the National Asthma Education and Prevention Program (NAEPP). According to GINA (135) the diagnosis of asthma from the age 6 years is based on "variable respiratory symptoms (wheeze, shortness of breath, chest tightness and cough) and variable expiratory airflow limitation" since expiratory lung function among asthmatics has a greater variability than the healthy population.

According to European Respiratory Society, an asthma diagnosis in children 6-15 years should be based on at least 2 objective criteria with the first-line test to diagnose asthma. The test criteria are spirometry (FEV1/FVC <80 % and/or FEV1 <80 % of predicted), FeNO >25 ppb or bronchodilator reversibility (BDR) with FEV1 ≥12 % or >200 ml increase. Since spirometry nor FeNO excludes asthma, they recommend repeated testing if asthma is strongly suspected (136).

## 1.11 Asthma prediction

Efforts have been made to find predictors of school-age asthma among children with early life wheeze and to find different phenotypes of wheeze to be able to find management strategies and maybe offer early therapy (14).

The most commonly used wheezing phenotypes among children are; non-wheeze, early transient wheezing, persistent wheeze and late-onset wheeze established by Martinez et al with data from TCRS (18). Studies have shown that the phenotypes associated with atopy are persistent and late-onset wheeze and that early transient wheeze is linked to reduced lung function (103).

Other wheezing phenotypes are derived from other birth cohort studies. One of them is the Avon Longitudinal Study of Parents and Children (ALSPAC) (17) involving six different phenotypes of wheeze which are identified based on atopy and lung function at mid-childhood and the Dutch Prevention and Incidence of Asthma and Mite Allergy (PIAMA) (137) which identified five phenotypes of wheeze.

These wheezing phenotypes may not be useful in clinical practice for early recognition of children who will develop asthma. Partly because there are no good biomarkers or predictors available but also due to the fact that there is an overlap between phenotypes and children can switch phenotype over time (138).

To be able to identify children at higher risk of developing asthma the asthma predictive index (API) was developed. API is based on TCRS data. Recurrent wheeze (≥3 episodes/year) up to the age of three years and either 1 major risk factor (asthma heredity or eczema) or 2/3 minor risk factors (eosinophilia, wheeze in the absence of a cold or allergic rhinitis) (139). The API has later been modified and the subsequent modified asthma predictive index (mAPI) entails recurrent wheeze (minimum 4 episodes of wheeze, among those ≥1 diagnosed by a physician) accompanied by 1 major criterion (asthma heredity, history of eczema or allergic sensitization to ≥1 airborne allergen) or 2 minor criteria (allergic sensitization to milk, egg, or peanuts, wheezing without colds or eosinophilia) (140). Even though the API and mAPI could be useful in clinical practice and have a high specificity it has low sensitivity (141).

A newly developed index: Pediatric Asthma Risk Score (PARS) (142) has shown a higher sensitivity (sensitivity=0.68, specificity=0.77) than API (sensitivity=0.28, specificity=0.96) and identifies children at mild to moderate risk of asthma including demographic and clinical data without need for blood sampling. PARS, validated based on the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS, n=762), includes parental asthma, eczema before age three, and wheeze without respiratory tract infections, wheeze before age three, sensitization to ≥ 2 food and/or airborne allergens and African-American race (142).

## 1.12 Asthma diagnosis in cohorts

There are many definitions of childhood asthma, both in guidelines, follow-up studies and clinical trials. In 122 papers, 60 different definitions were used, with the most common definitions: 1) doctor's diagnosis of asthma; 2) doctor's diagnosis of asthma together with asthma symptoms the previous year; 3) doctor's diagnosis of asthma together with asthma symptoms or asthma medication use the preceding year. Different definitions also yield different asthma prevalence, from 15.1 % to 51.1 % (143).

Asthma definitions in well-known follow-up studies (9, 23, 66, 71, 144, 145) are shown in Table 2.

| | BAMSE<br><br>(Children Allergy, Environment, Stockholm, Epidemiology) | COAST<br><br>(The Childhood Origins of Asthma Study) | $COPSAC_{2000}$ $COPSAC_{2010}$<br><br>(Copenhagen Prospective Study on Asthma in Childhood) | Vinku & Vinku2 | MAAS<br><br>(Manchester Asthma and Allergy Study) | TCRS<br><br>(Tucson Children's Respiratory Study) |
|---|---|---|---|---|---|---|
| **Study design** | Population based cohort study | High-risk birth cohorts, at least one parent with allergy/asthma | High-risk birth cohort. Mothers with a history of physician diagnosed asthma /population-based birth cohort | Originally RCT | Population-based birth cohort | Birth cohort |
| **Setting** | Stockholm, Sweden | Madison, Wisconsin, United States | Copenhagen, Denmark | Turku, Finland | Manchester, England | Tucson, Arizona, United States |
| **Study population** | n = 4089 | n = 287 | $COPSAC_{2000}$ n = 411<br>$COPSAC_{2010}$ n = 700<br>population-based pregnant women and their children | Vinku n = 293 children 3 months – 16 years hospitalized for acute wheeze<br>Vinku2 n = 124 children, 3-23 months, with first episode of wheeze | n = 995 | n = 1246 |
| **Time of asthma evaluation** | 1, 2, 4,8, 12, 16 and 24 years | 6,8,11 and 13 years | $COPSAC_{2000}$ : 7, 13 and 18 years<br>$COPSAC_{2010}$: 6, 8 and 10 years | 8 years | 3, 8 and 11 years | 6, 11 and 16 years |
| **Asthma definition** | ≥4 episodes of wheeze the past year or one episode of wheeze AND intermittent or regular use of ICS | ≥1 the previous year: a doctor's diagnosis of asthma, use of SABA for coughing or wheezing, use of daily controller medicine, step-up plan (SABA or ICS or OCS) | 5 episodes of symptoms the previous 6 months (lasting 3 consecutive days), symptoms typical of asthma, intermittent SABA and response to a 3 months course of ICS and relapse upon ended treatment. | Based on information from the preceding year:<br>Doctor's diagnosis and use of ICS, use of OCS for exacerbation, asthma attack relieved by SABA and/or positive BDR | 2/3 of the following: has the doctor ever told you that your child has asthma, has your child had wheezing the past 12 months or har your child had asthma treatment in the last 12 months. | Physician diagnosis of asthma with episodes or attacks of asthma and/or wheeze during the previous year. |

**Table 2. Design and criteria for asthma diagnosis in different follow-up studies.**

## 1.13 Markers of asthma

Blood eosinophilia is an easy to determine clinical biomarker of T2 inflammation, incorporated in the API, and a well-known asthma feature mainly driven by IL-5 (146). Peripheral blood eosinophilia, is noted to be related to FEV1, asthma symptom scores and bronchial hyperresponsiveness (147).

EDN is a basic protein contained in the granule of eosinophils and is released following stimulation, as well as involved in airway epithelium damage, mucus secretion, airway remodelling and inflammation. EDN has been noted to be associated with fixed airway obstruction in a cross-sectional study (148). EDN has been suggested to better reflect eosinophilic inflammatory response and activation (149).

Another marker for T2 inflammation is FeNO, induced by IL-13 and IL-4 (150). Levels of FeNO have been shown to be elevated among preschool children with wheeze and probable asthma in comparison to children at the same age with transient symptoms or without any symptoms of wheeze (151). The levels of FeNO correlate with the extent of airway inflammation and could serve as a marker for uncontrolled asthma or bad treatment compliance (152).

The metabolites prostaglandin D2 (PGD2) and leukotriene E4 (LTE4), measured in urine, are indicators of the total levels of cysteinyl leukotrienes (CysLTs). PGD2 and LTE4 are suggested to reflect mast cell and eosinophil activation and could be a biomarker for Th2 inflammation (153).

Low levels of type 2 biomarkers can be seen in a patient on inhaled corticosteroids (ICS) since these biomarkers are suppressed by anti-inflammatory treatment (131).

The non-type 2 phenotype with inflammation caused by Th1 and Th17 cells which contribute to an activation of neutrophils and macrophages. A potential biomarker is blood neutrophils although less studied as a biomarker than blood eosinophils (154). High levels of blood neutrophils have been associated with a worse prognosis and airway obstruction (155). However, a non-invasive biomarker that will help to identify this phenotype is still lacking.

From age 6, most children can perform a dynamic spirometry. In mild asthma, without on-going symptoms, lung function is usually normal, and the spirometry shows no sign of obstruction. Reduced FEV1 can be due to other lung diseases, not only asthma, or weak spirometric technique. Reduced FEV1/FVC ratio indicates expiratory airflow limitation (7).

Impulse oscillometry (IOS) uses pressure fluctuation during tidal breathing and is an alternative method to evaluate lung function in children unable to perform spirometry (156).

A characteristic feature of asthma is bronchial hyperresponsiveness (BHR), which can be evaluated with a methacholine challenge. Executing a bronchial challenge test includes escalating the dose of the trigger while monitoring lung-function and a decrease in FEV1 by 20 % from the baseline level is considered positive. In asthmatics the lower airways tighten in response to a stimulus, for example methacholine, which has been noted to be correlated with markers of airway inflammation (157).

**Knowledge gap**: Some markers have been noted to be useful in both predicting and diagnosing asthma. However, longitudinal analyses are needed to evaluate changes over time in relation to asthma development.

## 1.14 Prevention strategies

Through the identification of environmental exposures in epidemiological studies, some exposures have been translated into prevention strategies. The Finnish Asthma Programme has had some success with a smoking ban to reduce the burden of the disease (158). In COPSAC (159) supplementation of fish oil during pregnancy as well as Vitamin D supplementation in both COPSAC (160) and Vitamin D Antenatal Asthma Reduction Trial (VDAART) (161) have yielded some success regarding wheeze frequency but not consistent into school age.

A preventive measure taken for virus triggers is vaccination. Vaccination against RSV has successfully reduced recurrent wheezing, but not asthma (13), suggesting that RSV may not be causative for asthma development.

Commensals and commensal-derived components might have an important role in asthma inception and probiotics have been investigated in this aspect. This has resulted in less atopic

dermatitis in oral supplementation of various commensal strains given to infants, than those given placebo in the Trial of Infant Probiotic Supplementation (TIPS) study (162). Although, in the same study an evaluation at 7 years did not support the use of infant probiotics for prevention of AD, asthma or rhinitis (163). Other studies have shown that probiotics given during pregnancy result in less incident of wheezing in the offspring up to 2 years of age (164).

Some promising preclinical studies could result in novel prevention strategies, for example: synthetic toll-like receptor (TLR)2 agonist that has been noted to increase innate immunity in the airway mucosa and prevent respiratory infections (165). Other examples are cow's milk-derived β-lactoglobulin (lipocalin molecule) (166) and products of various helminths species that drive immunoregulatory responses or block pro-allergic responses (167).

## 1.15 Treatment

Because of the difficulties diagnosing asthma in preschool children, many children do not receive daily anti-inflammatory drugs for recurrent or persistent asthma symptoms (168). The purpose of asthma treatment is asthma control, meaning no exacerbations, symptom control and the ability to live an active life. To describe asthma control it is important to assess pulmonary function and symptom-burden using validated questionnaires such as Asthma Control Test (ACT) (169). Asthma control can be achieved by avoiding triggering factors and with asthma medication: short-acting inhaled β2-receptor agonists (SABA), ICS, long-acting β2-receptor agonists (LABA) and leukotriene receptor agonists (LTRA) (157).

### 1.15.1 Treatment of preschool asthma

Systemic corticosteroids as treatment for an episode of acute wheeze have shown conflicting results (170) but a post hoc analysis of an RCT found OCS, given at the first wheezing episode when RV was the trigger, to reduce the risk of recurrent wheeze. This effect persisted over a 7-year follow-up period (171, 172).

A medical algorithm for diagnosing and treating preschool asthma suggests ICS treatment only to those preschool children with wheeze and concomitant atopy and/or eosinophilia (≥300 cells/mcL) (173). Treatment dependent on the entity of bronchiolitis is supported by a newly published post hoc analysis, which suggests that high-dose SABA and OCS may be beneficial for some children with RV induced wheeze. The theory behind this is that some of the children with RV induced wheeze have an early asthma like airway inflammation (174).

### 1.15.2 Treatment of school-age asthma

ICS is the first choice of maintenance treatment for children of all ages with severe symptoms. ICS has a good effect on reducing exacerbations, improve symptoms as well as lung function in recurrent preschool wheezers at risk of developing asthma (146) but does not prevent asthma development among high-risk children (175). Treatment with steroids reduces T2 inflammation, therefore type 2 biomarkers should be evaluated over time and in the

context of current treatment (176). Since levels of eosinophils in blood correlate well with those in the airway, blood eosinophils can be helpful to guide ICS treatment (177).

There are several biologics approved for children with severe uncontrolled asthma. Biologics are considered in uncontrolled asthma when use of medium-high doses of ICS together with LABA and potentially along with muscarinic antagonist, LTRA, azithromycin and/or OCS have been insufficient. When considering biologic treatment, the patient should be tested for eosinophils in blood, total IgE and specific IgE levels, FeNO as well as lung function assessment, to be able to choose the appropriate treatment. The approved biologics for children > 6 years are Omalizumab (anti-IgE), Mepolizumab (anti-IL-5) and Dupilumab (anti-IL-4Rα). From age 12 years Benralizumab (anti IL-5Rα) and Tezepelumab (anti-TSLP) are also available. Tezepelumab is the only biologic available for patients who do not demonstrate an eosinophilic or T2-high phenotype (178).

# 2 RESEARCH AIMS

The overall aim of this doctoral project was to explore factors associated with preschool wheeze developing into school-age asthma in a high-risk preschool wheeze cohort.

All studies are based on data from the GEWAC (Gene Expression in Wheezing and Asthmatic Children) study. GEWAC is a longitudinal case-control study of children included at the pediatric emergency department when needing care for an acute episode of preschool wheeze (cases) and age-matched healthy controls.

The specific aims were:

**Study I**: To assess the prevalence, characteristics, and risk factors for asthma at age 7 through a detailed assessment of the clinical presentation, etiology of viral wheeze, inflammatory markers in blood and exhaled air, allergic sensitization, and measurement of pulmonary function.

**Study II**: To study early life factors associated with asthma at 11 years of age. Further, to explore the significance of RV-induced wheeze at inclusion and allergic sensitization at different timepoints during childhood. Finally, to longitudinally investigate potential markers of asthma such as lung function measurements, FeNO, blood eosinophil count and EDN.

**Study III**: To investigate the inflammatory profile, using the Olink platform with 92 inflammatory-related proteins in plasma, in children during an acute wheezing episode and at remission and to compare the inflammatory profiles to a group of healthy controls.

**Study IV**: To explore the relation between sensitization to multiple allergen molecules longitudinally and persistent asthma at age 7 years, using a comprehensive panel of 112 allergen molecules measured at early preschool age and at 7 years of age among cases and healthy controls.

# 3 MATERIAL AND METHODS

## 3.1 Study design and study population

GEWAC is a prospective case-control study in which 156 children with an episode of acute wheeze (cases) were recruited from the Pediatric Emergency Department or after admission to the Pediatric Emergency Ward at Astrid Lindgren's Children's Hospital between October 2008 and September 2012 (179). Inclusion and exclusion criteria for enrollment are presented in Table 3.

| | **Inclusion criteria** | **Exclusion criteria** |
|---|---|---|
| **Children with preschool wheeze (cases)** | • Age 6-48 months<br>• Presenting at the emergency with acute symptoms of wheeze | • Prematurity (birth before 36 gestational weeks)<br>• Any chronic disease<br>• Any simultaneous complication such as bacterial pneumonia, sepsis, diabetes at the time of inclusion. |
| **Control group** | • Age 6-48 months | • Prematurity (birth before 36 gestational weeks)<br>• A history of bronchial obstruction/asthma<br>• Known sensitization to airborne allergens |

**Table 3. Inclusion and exclusion criteria for enrollment in the GEWAC study.**

During the same time of inclusion of cases, 102 age-matched healthy controls were recruited by the research nurse from the Children's Surgical Daycare Ward at Astrid Lindgren's Children's Hospital. The control group had minor surgery performed (retention testis/hernia 44%, cystoscopy/micturating cystourethrogram 32%, hypospadias/circumcision/phimosis 9%, laparoscopy 12%, minor incisions 4%). Inclusion and exclusion criteria for controls are presented in Table 3.

Flowchart describing the inclusion and follow-up visits for cases and controls in GEWAC and the study population of each study (I-IV) are presented in Figures 2 and 3, respectively.

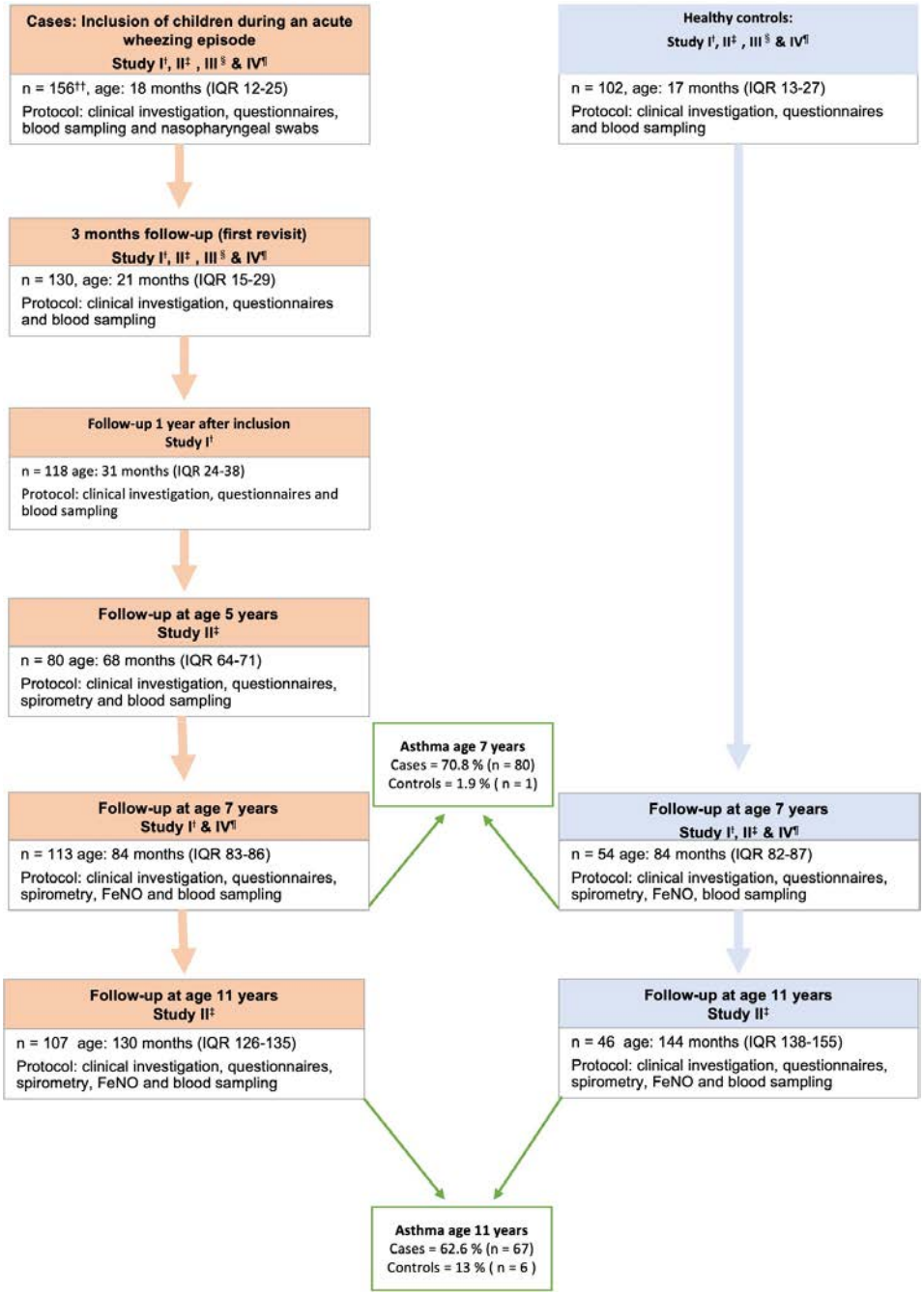



**Figure 2. Flowchart of GEWAC. †Study I: 113 cases attended the 7-year follow-up, of these 100 attended the first revisit and 97 at the first annual follow-up. ‡Study II: 107 cases attended the 11-year follow-up, of these 100 attended the first revisit, 80 attended the 5-year follow-up and 92 attended the 7-year follow-up. §Study III: 145 cases at inclusion and 113 cases at the first revisit with available plasma, along with 101 healthy controls. ¶Study IV: 72 cases with available sera at inclusion (n = 7) or first revisit (n = 65) and at the 7-year follow-up along with 43 healthy controls at inclusion. ††2 cases were excluded after inclusion due to prematurity and pseudo-croup.**

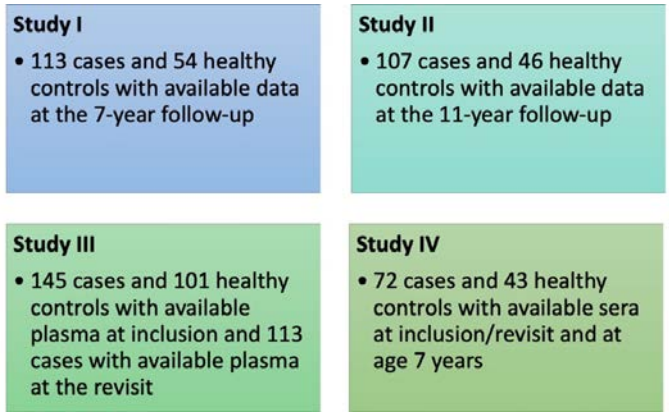



**Figure 3. Overview of the study population in each of the studies (I-IV) in the present thesis.**

## 3.2 Data collection and definition

At inclusion, the diagnosis of wheeze was based on a clinical diagnosis made by the treating physician at the Pediatric Emergency Department. After the enrolment criteria were confirmed by the study doctor or research nurse, the guardians of cases were informed about the study and after written informed consent was provided, samples of venous blood were drawn following local anesthesia (EMLA cream, Astra Zeneca, Sweden) and nasopharyngeal swabs were obtained. Data was collected from cases at inclusion, at the first revisit after approximately 3 months and then annually until age 7 years. A final revisit was made at our research department at age 11 years.

The guardians of the healthy controls were informed about the study by the research nurse. After they had provided written informed consent, blood was drawn at the same time as an intravenous line was inserted prior to surgery. Guardians of healthy controls filled out a standardized questionnaire at inclusion and controls were assessed at age 7 years and finally at age 11 years.

At the follow-ups, the children were required to be infection-free and not been using short-acting beta-2 agonists (SABA), leukotriene receptor antagonists (LTRA) or inhaled corticosteroids (ICS) for at least 24 h prior to examination. At age 7 and 11, all study participants were evaluated for an asthma diagnosis by the study doctor. The study protocol included a physical examination, standardized questionnaires, measurements of lung function, FeNO and blood sampling.

### 3.2.1 Physical examination

At each follow-up, a physical examination was performed including measurement of weight and length, auscultation of lungs and heart and examination of throat and skin.

### 3.2.2 Interviews

A standardized questionnaire was filled out at the first revisit for cases and at inclusion for healthy controls regarding demographic factors, hereditary factors for asthma and allergy, birth weight, smoking during pregnancy, contact with furry animals, eczema, reported food intolerance, time with breastfeeding and previous history of respiratory infections. At each follow-up the study doctor carried out a structured interview, based on a standardized questionnaire, with the guardians and participants concerning the number of days the children had suffered respiratory symptoms, number of acute visits and hospitalization and medication the previous year as well as reported intolerance to food or airborne allergens.

### 3.2.3 Nasopharyngeal samples

Nasopharyngeal samples for viral detection were obtained from cases at inclusion by the research nurse. The samples were transported within two hours after collection in a standardized medium (Sigma-Virocult, Medical Wire & Equipment Co Ltd, Corsham, Wiltshire, UK). The samples were analyzed according to standard procedures by real-time PCR using a 15-virus platform developed in 2007, including Adenovirus, Bocavirus, Coronavirus (229E, HKU1, NL63), Influenza A, Influenza A/H1N1 and Influenza B, Parainfluenza virus (1,2,3) Metapneumovirus, Enterovirus, Rhinovirus and Respiratory Syncytial virus). The samples were stored in the biobank at the Department of Clinical Microbiology, Karolinska University Hospital.

Between September 2010 and September 2012, 88 cases were tested for the presence of bacteria at inclusion by using a nylon nasal swab (Copan Eswab, Copan Diagnostics Ltd, Murrieta, Califonia, USA). The test was transported within two hours to the Department of Clinical Microbiology, Karolinska University Hospital, for a qualitative aerobic culture on solid media. The test was analyzed for Haemophilus influenza, Streptococcus pneumonia, Moraxella catarrhalis and beta-haemolytic Streptococci of groups A, C and G.

### 3.2.4 Assessment of allergic sensitization

Allergen-specific IgE antibodies against common airborne allergens (Phadiatop®; birch, timothy, mug worth, cat, dog, horse, molds (*Cladosporum herbarum*), dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*) and food allergens (fx5®; cow's milk, egg white, soy bean, peanut, codfish and wheat) (Thermo Fisher Scientific, Uppsala, Sweden) were analyzed in serum samples from cases at first revisit, 7 years- and 11 years follow-up and the control group at inclusion, 7 years- and 11 years follow-up at the Department of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital. Allergic sensitization was defined as allergen-specific IgE $\geq$0.35 $kU_A/L$.

Serum analysis for 112 allergen molecules using ImmunoCAP ISAC (Thermo Fisher Scientific, Uppsala, Sweden) was performed at Thermo Fisher Scientific, and measured at inclusion/revisit and 7 years. The allergen-specific IgE level is expressed semi quantitatively in ISU (ISAC Standardized Units). The cut-off IgE $\geq$0.3 ISU was considered positive.

### 3.2.5 Inflammation

All blood samples from both cases and controls were analyzed for total blood counts including hemoglobin, thrombocytes, leukocytes and differential number of basophils, neutrophils, and eosinophils. Blood samples were analyzed according to standard procedures at the Department of Clinical Chemistry, Karolinska University Hospital, Stockholm, Sweden, and are presented in $10^9$/L.

EDN levels were measured as arbitrary fluorescence intensity units (AU) at Thermo Fisher Scientific, Uppsala, Sweden, using microarray-based semi-quantitative research assay.

Fractional exhaled nitric oxide (FeNO) was measured using the non-invasive apparatus NIOX VERO (Circassia AB) and presented in parts per billion (ppb) at the 7- and 11-years follow-ups.

EDTA plasma was collected in a standardized way and stored at -80°C until 92 immune-related proteins were measured at inclusion and the first revisit with the Olink Target 96 Inflammation panel, using the antibody-mediated proximity extension technology (180) (Olink Proteomics, Uppsala, Sweden). Data from the Olink analysis was obtained as Normalized Protein eXpression (NPX) values, which are $\log_2$ transformed normalized values.

### 3.2.6 Lung function

Spirometry and airway reversibility were assessed at ages 5, 7 and 11 years using the Medikro Pro spirometer (Medikro Oy, Kuopio, Finland). The ratio FEV1 (L) /FVC (L) was reported using the reference values reported by Solymar (181) and Zapletal (182) depending on age. Positive bronchodilator test (BDR) was defined as an increase in FEV1 >12 %, evaluated 15 minutes after inhalation with 400 micrograms of salbutamol using a spacer. The patient's technique and quality were evaluated by the research nurse performing the test and a pediatric pulmonologist according to international guidelines (183).

### 3.2.7 Asthma diagnosis

The definition of asthma at 7 and 11 years, based on GINA guidelines, was defined by one mandatory criterion; a doctor's diagnosis of asthma, together with one of the following alternative criteria: symptoms typical of asthma (recurrent wheeze, shortness of breath, chest tightness, exercise induced bronchoconstriction, prolonged cough without ongoing cold) for 5 days or longer the past 12 months, use of asthma medication (ICS and/or LTRA) for 5 days or longer the past 12 months or airway reversibility >12 % after the use of bronchodilation with salbutamol, Table 4.

| CRITERIA: | |
|---|---|
| **DOCTOR'S DIAGNOSIS OF ASTHMA AND AT LEAST ONE OF FOLLOWING:** | |
| **1. SYMPTOMS** | Self-reported asthma symptoms for 5 days or longer during the previous 12 months |
| **AND/OR** | |
| **2. MEDICATION** | ICS or leukotriene receptor antagonist for 5 days or longer the previous 12 months |
| **AND/OR** | |
| **3. AIRWAY REVERSIBILITY** | Reversibility >12% in FEV1 after bronchodilation |

**Table 4. Criteria for asthma diagnosis in the GEWAC study.**

## 3.3 Data analysis

Statistical analyses were made using SPSS (version 25 or later). In study III some of the analyses were made using R (https://www.r-project.org/).

### 3.3.1 Study I

The results were expressed as numbers and proportions as well as median values with ranges. The chi-square test was used to examine dichotomous variables between two groups and Fisher's Exact test was used on small sample sizes. An unpaired t-test was used for continuous variables with normal distribution and a non-parametric test, Mann-Whitney, was used for skewed continuous variables. Odds ratios (OR) with 95 % confidence intervals (95% CI) were calculated with univariate and multivariate logistic regression with adjustment for different viruses. P-values less than 0.05 were considered significant.

### 3.3.2 Study II

The results were expressed as numbers and proportions as well as median numbers with ranges. The Chi-squared test was used to compare proportional differences between two groups and Fisher's exact test was used on small sample sizes. An unpaired t-test was used to analyze continuous variables with normal distribution and the non-parametric Mann-Whitney U test for skewed continuous variables. OR with 95% CI were calculated with logistic regression for the association with asthma at age 11 years in relation to early life factors. All estimates with a p-value <0.1 in a univariate logistic regression were chosen for further multivariate logistic regression analysis. A multivariate regression analysis was used for the

association with asthma at age 11 years in relation to viruses examined at inclusion. Differences in FEV1/FVC were examined using Wilcoxon signed rank test for evaluating paired differences between two time points. P-values <0.05 were considered significant.

### 3.3.3 Study III

Data from the Olink analysis was obtained as Normalized Protein eXpression (NPX) values; these are $\log_2$ transformed normalized values. NPX values were converted into a linear scale ($2^{(NPX)}$) and generalized linear model design =~ Sample-type (AW and HC) was used to calculate differential protein expression by the edgeR package (184). $\text{Log}_2$ transformed fold change and False Discovery rate (FDR) by the Benjamini-Hochberg method (185) were calculated for each protein. The top ten significant differentially expressed proteins between PW and HC with abs($\log_2$ fold change) > 1, FDR < 0.05 were selected for further analysis. A heatmap with unsupervised clustering of the top ten differentially expressed proteins in the 246 samples was plotted using their NPX value with the scaled row.

Descriptive statistics were performed using SPSS version 27 (IBM). The results were expressed as numbers and proportions as well as median number with ranges. The Chi-square test was used to examine proportional differences between two groups and Fisher's exact test was used on small sample sizes. Continuous variables with a normal distribution were examined using an unpaired t-test and skewed variables were examined with the non-parametric test, Mann-Whitney U test. A two-tailed probability of <0.05 was considered statistically significant.

### 3.3.4 Study IV

The results were expressed in numbers and proportions as well as the median number of positive IgE responses with ranges. The median values of specific IgE were calculated in groups of individuals sensitized to the respective molecule. Allergen molecules were ranked by prevalence and median value in the cases at two different time points: at early preschool age and at age 7 years. Combined ranking was then applied by ranking the sum of ranks for prevalence and median value for each allergen molecule. The chi-square test was used to analyze proportional differences between two groups and Fischer's exact test was used on small sample sizes. An unpaired t-test was used for continuous variables and a nonparametric test, Mann-Whitney, was used for skewed data. P-value <0.05 was considered significant. OR with 95% CI were calculated using univariate logistic regression. Probability curves for asthma at age 7 years based on the number of sensitizing allergen molecules were derived from the results of a univariate logistic regression.

## 3.4 Ethical considerations

Careful ethical consideration is important in all research, especially when including children. However, research is also important for understanding underlying mechanisms and could lead to better health care. It is important weighing the benefits of research against the harm that is done. We obtained informed consent from both legal guardians. Nothing was done against

the child's will. Participation in the study was voluntary and families were informed that participation in the study can be withdrawn at any time. The participants were informed to apply a local anesthesia (EMLA cream) before blood sampling to reduce the feeling of discomfort and pain. The research in this thesis conforms to the guidelines in the Declaration of Helsinki.

All papers included in this thesis have ethical approval obtained from the Swedish Ethical Review Authority, Sweden. Written informed consent were collected from guardians entering each follow-up.

Study I: Dnr: 2008/378-31/4 and supplements: 2010/2017-32 2014/399-31

Study II: Dnr: 2008/378-31 and supplements: 2010/2017-32 2014/399-31 Dnr: 2017/2527-32 and supplements: 2018/1404-32 2018/1456-32 2018/2522-32

Study III: Dnr: 2008/378-31/4 and supplements: 2010/2017-32 2014/399-31

Study IV: Dnr: 2008/378-31/4 and supplements: 2010/2017-32 2014/399-31

# 4 RESULTS

**Main findings:**

## 4.1 Study I

In study I we explored the associations between early-life factors and asthma development at age 7. Those included in this study were children with acute preschool wheeze (cases) (n=113) and healthy controls (n=54) who attended the follow-up at 7 years of age.

Asthma prevalence at 7 years was 70.8 % in preschool wheezers and 1.9 % in healthy controls. Cases had more first-grade heredity for asthma and/or allergies (73.8 % vs. 49.1 %, p = 0.002), attended childcare more frequently (76.9 % vs. 48.1 %, p<0.001) and had lower levels of Vitamin D at inclusion (83.0 vs. 96.0, p = 0.015) compared to healthy controls. Eighty-one percent of cases were hospitalized at inclusion and only 21 % of cases were first time wheezers at the time of inclusion in the study.

RV was the most frequent trigger of wheeze at inclusion, and more common in cases with asthma at 7 years in comparison to cases without (48.1 % vs. 21.9 %, p = 0.011). When adjusting for infection with other viruses, this association was still significant (adjusted (a)OR 3.8, 95 % CI 1.4-10.5), Figure 4. No association was found between RSV-induced wheeze and asthma (aOR 2.9, 95 % CI 0.8-10.7). In addition, cases with wheeze at inclusion caused by other viruses (Coronavirus (229E, HKU1, NL63), Adenovirus, Influenza A, Influenza A/H1N1, Influenza B, Bocavirus, Parainfluenza virus (1-3) or Metapneumovirus) was inversely associated with asthma at 7 years (aOR 0.3, 95 % CI 0.1-0.8).

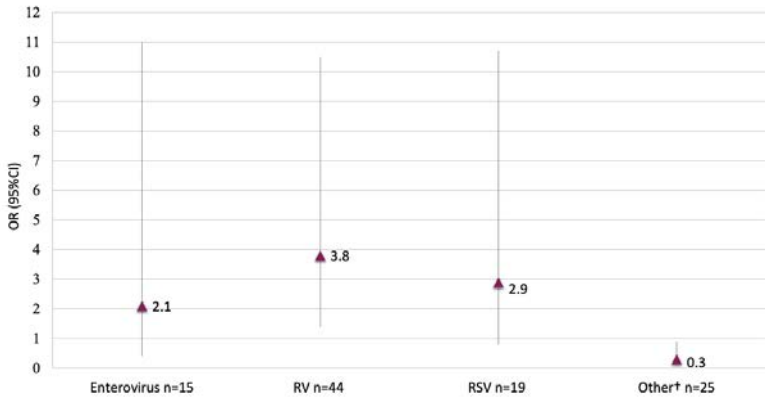



**Figure 4. The associations between viruses at inclusion and asthma at 7 years.**

Children with asthma at age 7 were hospitalized more frequently because of symptoms from the airways in the year following inclusion (p = 0.024) and these children were also hospitalized for more days during the same period (p = 0.01), Figure 5.

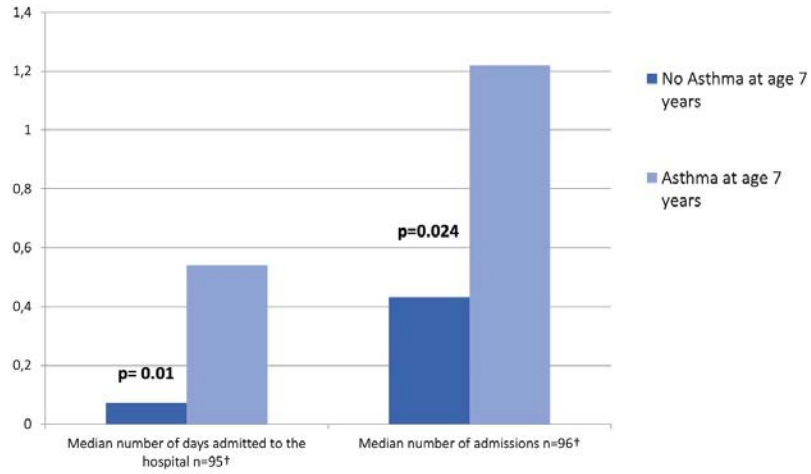



**Figure 5. Days admitted and number of hospital admissions the year after inclusion in GEWAC.**

Further, children with asthma at age 7 had lower FEV1/FVC ratio (87.3 vs. 91.2, p = 0.043), in higher extent aeroallergen sensitization (34.8 % vs. 12.5 %, p = 0.039) and had higher prevalence of eosinophils > 0.3 $10^9$/L (54.2 % vs. 29.2 %, p = 0.038) when evaluated at 7 years, in comparison to those without asthma. A total of 27 % of cases had a significant BDR.

## 4.2 Study II

In study II we investigated associations between early-life factors and asthma development at age 11, with focus on allergic sensitization and RV-induced wheeze. Those included in the analyses were 107 cases (preschool wheezers) and 46 healthy controls that attended the 11-year follow-up. We found an asthma prevalence of 62.6 % in cases and 13.0 % in healthy controls. We noted parental asthma and/or allergies (84.8 % vs. 68.4 %, p = 0.048), RV-induced wheeze at inclusion (49.3 % vs. 28.9 %, p = 0.043) and aeroallergen sensitization at the first revisit (18.0 % vs. 0 %, p = 0.006) to be more common in asthmatic cases at age 11 in comparison to non-asthmatic cases, Table 5.

| Variables | Number included | Cases with asthma n = 67 | Cases without asthma n = 40 | p value |
|---|---|---|---|---|
| ***Early life factors*** | | | | |
| Age in months at inclusion, median (IQR) | 107 (67/40) | 18 (11-27) | 15 (12-22.7) | 0.41 |
| Age in months at the first revisit, median (IQR) | 100 (61/39) | 22 (14-30.5) | 19 (15-26) | 0.51 |
| Male sex, n (%) | 107 (67/40) | 45 (67.2) | 28 (70.0) | 0.76 |
| Caucasian mother and/or father, n (%) | 105 (67/38) | 60 (89.6) | 36 (94.7) | 0.36 |
| Parental asthma/allergy, n (%) | 104 (66/38) | 56 (84.8) | 26 (68.4) | **0.048** |
| First time wheeze at inclusion, n (%) | 100 (61/39) | 11 (18.0) | 9 (23.1) | 0.54 |
| Hospitalized at inclusion, n (%) | 100 (61/39) | 48 (78.7) | 34 (87.2) | 0.28 |
| Doctor's diagnosis of asthma at the revisit, n (%) | 100 (61/39) | 32 (52.5) | 22 (56.4) | 0.70 |
| History of AD† at inclusion, n (%) | 105 (67/38) | 20 (29.9) | 5 (13.2) | 0.054 |
| RV‡ induced wheeze at inclusion, n (%) | 105 (67/38) | 33 (49.3) | 11 (28.9) | **0.043** |
| RSV§ induced wheeze at inclusion, n (%) | 104 (67/37) | 16 (23.9) | 5 (13.5) | 0.21 |
| Food allergen positive at the first revisit, n (%) | 98 (61/37) | 17 (27.9) | 6 (16.2) | 0.19 |
| Aeroallergen positive at the first revisit, n (%) | 98 (61/37) | 11 (18.0) | 0 (0) | **0.006** |
| EDN¶ at inclusion, median (IQR) | 56 (33/23) | 2771.2 (1646.5-4233.7) | 1878.4 (1485.2-3222.2) | 0.28 |
| EDN¶ revisit, median (IQR) | 79 (50/29) | 4377.2 (2725.5-6953.9) | 3256.4 (2225.4-6902.3) | 0.30 |
| Blood eosinophil count at the revisit $10^9$/L, median (IQR) | 96 (60/36) | 0.4 (0.2-0.57) | 0.3 (0.1-0.47) | 0.12 |
| ***5 and 7 year follow up*** | | | | |
| Age in months at the 7-year follow-up, median (IQR) | 92 (58/34) | 84 (83-85) | 84 (83-86) | 0.78 |
| Asthma at 7-year follow-up, n (%) | 92 (58/34) | 50 (86.2) | 18 (52.9) | **<0.001** |
| FEV1/FVC†† at age 5 years, median (IQR) | 80 (51/29) | 92.8 (84.6-99.1) | 96.4 (90.6-100) | 0.11 |
| FEV1/FVC†† at age 7 years, median (IQR) | 91 (57/34) | 87.3 (80.6-91.9) | 90.3 (83.4-96.9) | **0.035** |
| Food allergen positive 7-year follow-up, n (%) | 83 (53/30) | 13 (24.5) | 3 (10.0) | 0.11 |
| Aeroallergen positive 7-year follow up, n (%) | 83 (53/30) | 22 (41.5) | 3 (10.0) | **0.003** |
| EDN¶ 7-year follow-up, median (IQR) | 83 (54/29) | 4660.5 (3201.4-7465.9) | 3090.1 (2072.6-4524.7) | **0.004** |
| Blood eosinophil count 7-year follow up $10^9$/L, median (IQR) | 78 (49/29) | 0.4 (0.25-0.7) | 0.2 (0.15-0.45) | **0.014** |
| FeNO‡‡ 7-year follow up, median (IQR) | 33 (22/11) | 10.5 (6-14.5) | 8 (7-12) | 0.61 |
| ***11-year follow up*** | | | | |
| Age in months at 11-year follow-up, median (IQR) | 107 (67/40) | 130 (126-135) | 129 (126-135) | 0.74 |
| ACT¶¶ in %, median (IQR) | 107 (67/40) | 89 (78-93) | 100 (96-100) | **<0.001** |
| AQLQ†††, median (IQR) | 95 (62/33) | 6.6 (5.9-7) | 7 (6.9-7) | **<0.001** |
| FEV1/FVC†† at 11-year follow-up, median (IQR) | 97 (63/34) | 83.1 (77.7-88.9) | 87.0 (81.3-90.6) | **0.018** |
| Food allergen positive 11-year follow up, n (%) | 83 (54/29) | 23 (42.6) | 5 (17.2) | **0.02** |
| Aeroallergen positive 11-year follow up, n (%) | 82 (54/28) | 29 (53.7) | 7 (25.0) | **0.013** |
| Blood eosinophil count 11-year follow-up $10^9$/L, median (IQR) | 76 (52/24) | 0.3 (0.2-0.5) | 0.2 (0.05-0.2) | **0.001** |
| FeNO‡‡ 11-year follow-up, median (IQR) | 93 (59/34) | 8 (5-18) | 6 (5-8.3) | **0.037** |

**Table 5. Baseline characteristics of cases with and without asthma at 11 years.**

Among all common viruses measured at inclusion, RV was the only virus associated with asthma at 11 years, Table 6.

| Virus etiology of wheeze at inclusion | Crude Odds Ratios OR (95% CI) | Adjusted Odds ratios‡ OR (95% CI) |
|---|---|---|
| Rhinovirus | 2.4 (1.02-5.6) | 2.9 (1.2-7.2) |
| Respiratory syncytial virus | 2.0 (0.7-6.0) | 2.9 (0.9-9.3) |
| Enterovirus | 2.3 (0.6-8.8) | 2.1 (0.5-8.7) |
| Other† | 0.9 (0.3-2.4) | 0.8 (0.3-2.3) |

**Table 6. Viral etiology of wheeze and asthma risk at age 11.**

RV-induced wheeze and allergic sensitization at age 2 were associated with asthma at age 11 in an unadjusted analysis. However, in an adjusted model only allergic sensitization and parental asthma/allergy were associated with asthma, Table 7.

| Early-life factors | Crude Odds Ratios OR (95% CI) | Adjusted Odds ratios† OR (95% CI) |
|---|---|---|
| Parental asthma and/or allergy | 2.6 (0.99-6.7) | 3.4 (1.1-9.9) |
| Sensitisation at 2 years of age | 2.9 (1.05-8.1) | 3.0 (1.02-8.7) |
| Rhinovirus induced wheeze | 2.4 (1.02-5.6) | 1.9 (0.8-4.8) |

**Table 7. Early-life factors and their association with asthma at 11 years.**

Aeroallergen sensitization was more common at the first revisit (18 % vs. 0 %, p = 0.006), at age 7 years (41.4 % vs. 10 %, p = 0.003) and 11 years (53.7 % vs. 25 %, p = 0.013), and IgE antibodies to food was more common at age 11 years (42.6 % vs. 17.2 %, p = 0.02) in cases with asthma in comparison to cases without, Table 5.

We noted a statistically significant higher asthma prevalence at age 11 in cases with both RV at inclusion and allergic sensitization at age 7 compared to cases with RV but without allergic sensitization at 7 years of age (92.9 % vs. 57.1 %, p = 0.03). No differences were seen when comparing wheeze not caused by RV at inclusion with and without sensitization, Figure 6.

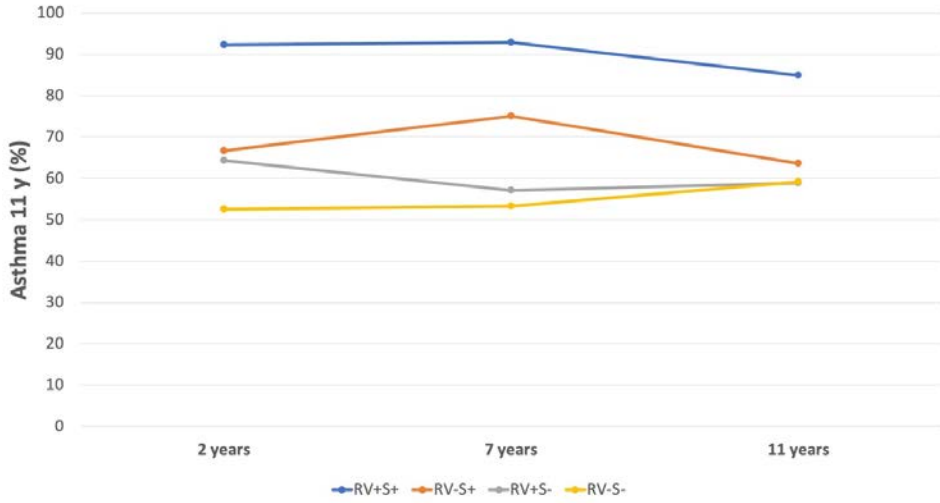


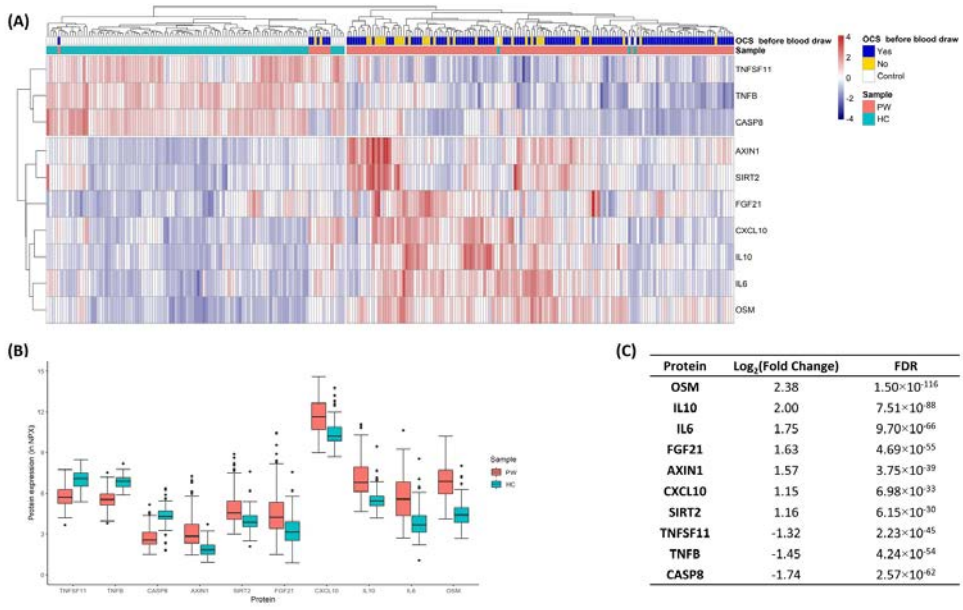

**Figure 6. Asthma prevalence at age 11 (y-axis) stratified on rhinovirus (RV+/-) in combination with and without allergic sensitization (S+/-) measured at different time points (x-axis)**

In addition, we noted that cases with asthma at age 11 had lower FEV1/FVC at both age 7 and 11, higher blood-eosinophil count at age 7 and 11, higher FeNO at age 11 and higher EDN levels at age 7, Table 5.

## 4.3 Study III

In study III we explored markers of inflammation in plasma obtained from 145 cases in GEWAC during the acute wheezing episode and at remission (n = 113). The results were compared to plasma obtained from healthy controls at inclusion (n = 101). The inflammatory response was studied by measuring a panel of 92 inflammatory-related plasma proteins.

Unsupervised clustering noted that the 10 most differentially expressed inflammatory-related proteins could almost entirely separate cases from healthy controls. Seven proteins had an increased expression in cases in comparison to healthy controls: Oncostatin M (OSM), Interleukin-6 (IL-6), Interleukin-10 (IL-10), C-X-C motif chemokine ligand 10 (CXCL10), Fibroblast growth factor 21 (FGF21), Sirtuin 2 (SIRT2) and Axis inhibitor protein 1 (AXIN1). Three proteins had a decreased expression in cases in comparison to healthy controls: TNF superfamily member 11 (TNFSF11), Tumor necrosis factor β (TNF-β) and Caspase 8 (CASP8), Figure 7.




| Protein | $Log_2$(Fold Change) | FDR |
|---|---|---|
| OSM | 2.38 | $1.50\times10^{-116}$ |
| IL10 | 2.00 | $7.51\times10^{-88}$ |
| IL6 | 1.75 | $9.70\times10^{-66}$ |
| FGF21 | 1.63 | $4.69\times10^{-55}$ |
| AXIN1 | 1.57 | $3.75\times10^{-39}$ |
| CXCL10 | 1.15 | $6.98\times10^{-33}$ |
| SIRT2 | 1.16 | $6.15\times10^{-30}$ |
| TNFSF11 | -1.32 | $2.23\times10^{-45}$ |
| TNFB | -1.45 | $4.24\times10^{-54}$ |
| CASP8 | -1.74 | $2.57\times10^{-62}$ |

**Figure 7. 10 proteins differentially expressed between cases (PW) and healthy controls (HC). A) Heat map showing protein expression, created with unsupervised clustering. B) Box-plot showing differences in protein expression. C) $Log_2$ fold change and false discovery rate (FDR) between PW and HC.**

The expression levels of 5 (TNF-β, CASP8, CXCL10, IL-10 and OSM) of the 10 most differentially expressed proteins were affected by the use of OCS <24 hours before blood draw. In cases with OCS treatment those 5 proteins were still differentially expressed in comparison to healthy controls, Figure 8.

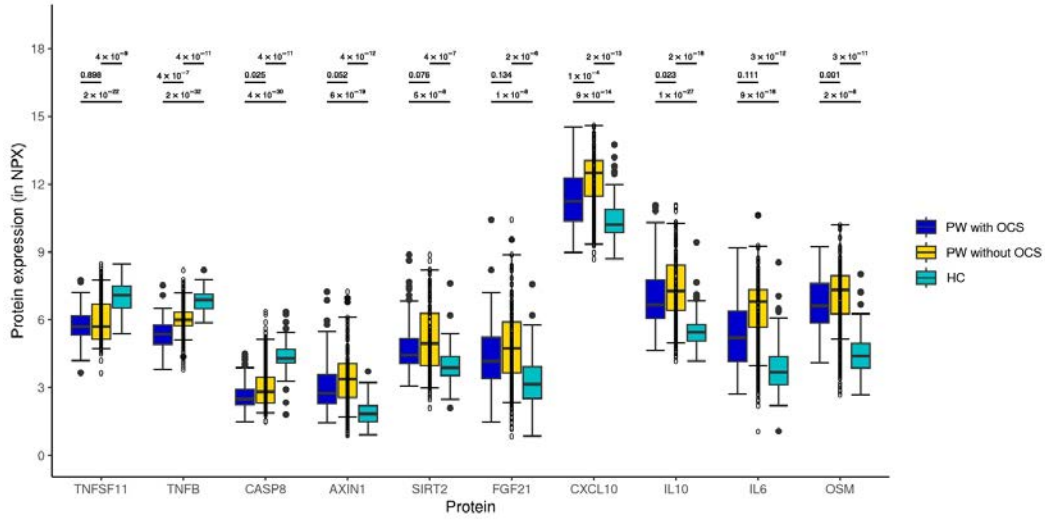
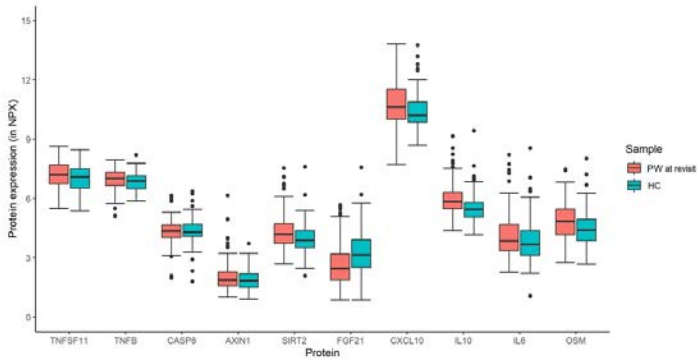



**Figure 8. Box-plot showing protein expression in cases (PW) with and without OCS at inclusion and healthy controls (HC).**

Among the 10 proteins, three (FGF21, SIRT2 and IL-10) were still differentially expressed between cases at the revisit 3 months later, in comparison to healthy controls at inclusion, Figure 9.

(A)




(B)

| Protein | $log_2$(Fold Change) | FDR |
|---|---|---|
| FGF21 | -0.79 | $7.78\times10^{-13}$ |
| SIRT2 | 0.41 | $3.07\times10^{-4}$ |
| IL10 | 0.25 | 0.034 |
| AXIN1 | 0.29 | 0.085 |
| OSM | 0.18 | 0.174 |
| CXCL10 | 0.15 | 0.246 |
| CASP8 | -0.13 | 0.454 |
| TNFSF11 | 0.07 | 0.811 |
| TNFB | -0.03 | 0.926 |
| IL6 | 0.04 | 0.926 |

**Figure 9. A) Box plot showing protein expression in cases (PW) at the revisit compared to healthy controls (HC). B) $Log_2$ fold change and false discovery rate (FDR) between PW and HC.**

## 4.4 Study IV

In study IV we explored sensitization to allergen molecules at inclusion/revisit and at 7 years and in relation to asthma development at 7 years. We used ImmunoCAP ISAC and measured 112 allergen molecules in serum. In this study 72 cases were included with available sera at inclusion/revisit (EPA) (n = 72) and at age 7 years, along with 43 healthy controls.

It was more common with sensitization to ≥2 allergen molecules at inclusion/revisit in preschool wheezers than in healthy controls (18.1% vs 4.6%, p = 0.039). An increase of sensitizing allergen molecules was seen from 3 (1-4) at inclusion/revisit to 6.5 (1-21) at 7 years in cases (p = 0.024). Figure 10.

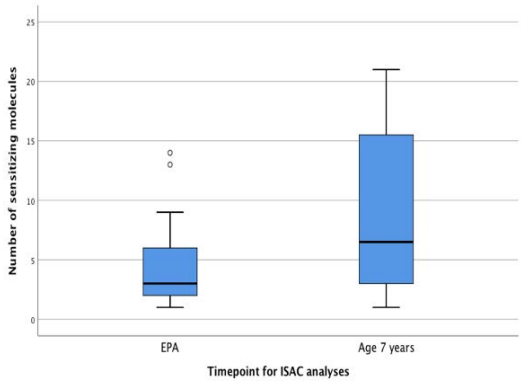



**Figure 10. Box plot of median number and range of number of sensitizing molecules at inclusion/revisit (EPA) and at age 7 years in cases.**

In cases, Mal d 1 was the highest ranked (ranked by prevalence + median value ISU) allergen molecule at inclusion/revisit and Bet v 1 was the highest ranked allergen molecule at 7 years of age. Among the three highest ranked molecules at both timepoints were Ara h 2 and Bet v 1.

An increase in prevalence of sensitization to the most common airborne allergen molecules was seen from inclusion/revisit to age 7 years in cases (Bet v 1; 4.2 % to 20.8 %, p = 0.002, Fel d 1; 2.8 % to 13.9 %, p = 0.016, Phl p 1; 0 % to 19.4 %, p <0.001). No increase was seen in Ara h 1, Ara h 2, Ara h 3 and Ara h 6.

Among the 72 cases, 68.1 % had asthma at age 7 years. An association with asthma was found for sensitization to every additional molecule with IgE reactivity from inclusion/revisit to age 7 (OR 1.2; 95 % CI 1.01-1.5), Figure 11.

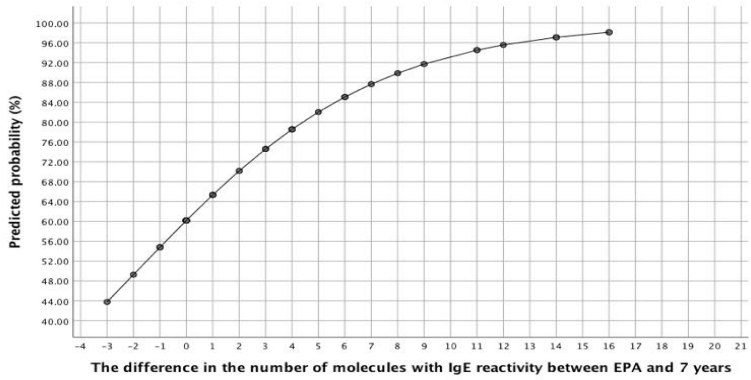



**Figure 11. Difference in number of allergen molecules from inclusion/revisit (EPA) to age 7 (x-axis) and predicted probability of asthma (y-axis)**

There was an increase in sensitizing molecules from 3 (1-14) at inclusion/revisit to 10.5 (1-21) at 7 years among sensitized asthmatic cases at age 7 (p = 0.038), no significant increase was seen in non-asthmatic cases (p = 0.26), Figure 12.

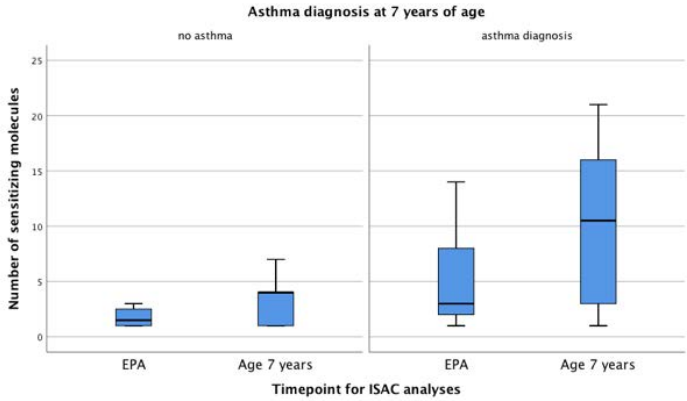



**Figure 12. Median number of sensitizing molecules at inclusion/revisit (EPA) and age 7 in cases with and without asthma.**

In asthmatic cases at 7 years, it was more common with sensitization to Bet v 1 in comparison to non-asthmatic cases (28.1 % (n = 14) vs. 4.3 % (n = 1), p = 0.027). Finally, the number of sensitizing allergen molecules at age 7 was associated with asthma (OR 1.2; 95 % CI 1.02-1.42), Figure 13.

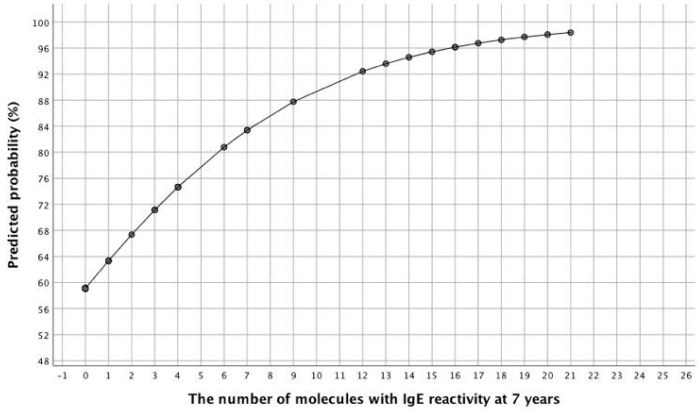



**Figure 13. The number allergen molecules in each case at age 7 (x-axis) and probability of asthma at age 7 (y-axis).**

# 5 DISCUSSION

## 5.1 Study design – strengths and weaknesses

GEWAC is a longitudinal case-control study where cases were included during an episode of acute preschool wheeze, along with healthy controls from the surgical day-care ward from one hospital, Astrid Lindgren's Children's Hospital in Stockholm, Sweden. The major strength is the thorough clinical characterization at inclusion as well as during follow-up with regular visits to our research department with clinical investigation, interviews, nasopharyngeal swabs for viral detection, blood sampling, spirometry and FeNO testing.

The prevalence of children with asthma at 7 (70.8 %) and 11 years (62.6 %) of age was higher in GEWAC in comparison to other prospective cohort studies of early wheezers (30-40 %) (9, 71). The high prevalence of asthma noted at school age could be because most children were recurrent and persistent wheezers at inclusion and that most children had a severe wheezing episode (81 % were admitted to hospital). However, we cannot exclude that the children who attended the 7- and 11-year follow-up had more respiratory difficulties in school age, potentially implying selection bias. Drop-out analysis showed that children with preschool wheeze who participated at the follow up at age 11 had more heredity for asthma/allergy and more commonly AD before inclusion in GEWAC.

One of the exclusion criteria for healthy controls was "Known sensitization to airborne allergens" which has caused an overestimation of the observed differences regarding allergic sensitization between cases and healthy controls.

Our outcome, asthma, was defined based on a doctor's diagnosis of asthma and one out of three of either: asthma symptoms, medication, and/or significant bronchodilator test. In the initial study design, we planned to perform a methacholine provocation, but due to logistical problems, this was not feasible. This would have strengthened our definition of the outcome since spirometry can be normal even in children with asthma.

Another limitation is the sample size, which caused limited statistical power to perform sub-analyses and to adjust for all potential confounders. The limited statistical power is demonstrated in the width of the confidence intervals. Not all the initially recruited cases and controls came to the follow-ups and in some of the studies we were limited by the availability of blood samples, nasopharyngeal swabs, and spirometry measurements.

Since GEWAC is a high-risk cohort of preschool wheezers with a severe wheezing episode at inclusion and mainly recurrent wheezers, the findings are valid in similar hospital-based settings.

## 5.2 Main findings

We aimed to identify early-life factors associated with school-age asthma in a high-risk cohort of preschool wheezers.

We found RV-induced wheeze at inclusion to be associated with asthma at both 7 and 11 years. There was a decreased risk of asthma at age 7 in cases with wheeze caused by common respiratory viruses, other than RSV, RV and Enterovirus. In addition, the severity of wheeze the year after inclusion was associated with asthma at 7 years. Even though an association was found between RV-induced wheeze at inclusion and asthma at age 11, the association was no longer significant after adjusting for parental heredity for asthma and/or allergic disease and allergic sensitization at age 2 years, suggesting RV-induced wheeze to be a revealing, rather than a causal, factor for asthma development in predisposed children. Further, an interaction between RV and allergic sensitization is possible since the combination of RV-induced wheeze and allergic sensitization contributed to a higher prevalence of asthma.

There was a significant increase in sensitizing allergen molecules from early life to age 7 among cases with asthma development at age 7. The increase in sensitizing allergen molecules from preschool age until 7 years was associated with asthma development at 7 years. Furthermore, polysensitization at age 7 was associated with asthma at the same age.

Finally, we found 10 inflammatory-related plasma proteins that could almost entirely separate children with an acute wheezing episode from healthy controls. Among these 10 proteins (IL-6, IL-10, OSM, CXCL10, SIRT2, FGF21, AXIN1, TNFSF11, TNF-β and CASP8) three were still differentially expressed at the revisit 3 months later (FGF21, IL-10 and SIRT2). These proteins are suggested to be involved in airway remodelling, airway epithelial dysfunction, T2 inflammation and antiviral response.

## 5.3 Asthma diagnosis

A review article identified multiple definitions of asthma in school age in follow-up studies, with varying prevalence, due to the lack of homogeneity in the definition (143). Most follow-up studies have a similar definition as ours, based on symptoms and medication. Our definition is based on GINA guidelines (186), and on symptoms and variable airflow obstruction. ERS recently published new guidelines for diagnosing asthma in children 5-16 years (136). They suggest that an asthma diagnosis should be based on at least 2 objective measurements, spirometry, BDR and FeNO, together with symptoms of asthma. If there is a high suspicion of asthma but no obtained objective measurements, the examination should be repeated. This approach is achievable in clinical practice but less feasible in clinical research due to scarcity of resources and its time-consuming nature for participants. Our mandatory criterion for a doctor's diagnosis of asthma was a diagnosis of asthma at the time of the follow-up visit or sometime during the last 12 months before the follow-up. For some participants the interpretation of the objective measurements was not reliable due to continuous ICS treatment. In participants without continuous ICS, at least one objective measurement was needed for an asthma diagnosis, in line with diagnosing asthma in a clinical setting.

## 5.4 Asthma prevalence

We found a high prevalence of asthma at both 7 years (70.8 %) and 11 years (62.6 %). Most children with preschool wheeze are asymptomatic later in life and cohort studies have reported an asthma prevalence between 30-40 % (9, 71, 187). A study of 100 children hospitalized for wheeze in Kuopio University Hospital, Finland, were evaluated for asthma in early adulthood together with healthy controls. Asthma was noted in 53 % of all cases and in 64 % in cases with RV-induced wheeze (188). Data from TCRS show that 63.6 % of persistent wheezers continued to wheeze at age 11 years (189). Combined results on asthma prevalence among preschool wheezers from the Isle of Wight Cohort (IOWBC) and the Food Allergy and Intolerance Research (FAIR) birth cohort demonstrate differences in asthma prevalence based on different wheezing phenotypes (190). Infantile-onset-non-atopic wheeze has an asthma prevalence of 67.3 % and an infantile-onset-persistent-atopic-wheeze has an asthma prevalence of 60.6 % at 10 years. The cluster early-childhood-onset-persistent-atopic-wheeze has an asthma prevalence of 78.6 % at age 10 years. In contrast, the prevalence is only 21 % in the cluster early-childhood-onset-transient-non-atopic wheeze at the same age. These findings suggest that the persistence, onset, and frequency of wheeze as well as atopic characteristics are associated with a higher asthma prevalence at 10 years. The Study Team for Early Life Asthma Research (STELAR) found similar results: asthma prevalence among intermittent and persistent wheezers was around 60 % in adolescence (191). In COAST, wheezing children with allergic sensitization at age 1 year had an asthma prevalence of 65 % and 40 % had asthma if sensitized at age 5 years (66). Given the high-risk nature of the GEWAC cohort with the majority being recurrent wheezers, the severity of the wheezing episode during inclusion and with many being persistent wheezers, our high prevalence of asthma at age 7 and 11 is in line with previous studies.

## 5.5 Markers of asthma

We found a lower ratio of FEV1/FVC at both 7 years and 11 years in cases with asthma in comparison to cases without, as well to healthy controls. It has been shown that wheezing in early life have a negative impact on lung function later in life (189) independent of asthma development. This has also been demonstrated in a Swedish cohort of preschool wheezers (192) and an even lower lung function in those with asthma in early adulthood. One possible mechanism behind lower lung function in children with previous preschool wheeze is that repeated early lower respiratory infections, with acute and acute on chronic inflammation, eventually cause airway remodeling (193).

Persistent childhood asthma is mostly dominated by type 2 inflammation, characterized by aeroallergen sensitization, increased FeNO, blood-eosinophilia, AD, and parental atopic asthma (194). Both FeNO and elevated blood-eosinophils are signs of type 2 inflammation and are well established markers of Th2-high asthma. We found elevated FeNO at 11 years and elevated blood-eosinophils at 7 years and 11 years in cases with asthma at age 11 years in comparison to non-asthmatic cases at the same age. The levels of FeNO were not as high as could be suspected in asthmatics, which may be due to ICS treatment in cases with asthma

since corticosteroids are known to suppress type 2 inflammation (175). FeNO measurements are suggested to be applied when evaluating asthma management in asthmatic children with symptoms despite treatment with ICS, and might be more helpful in confirming rather than excluding asthma (150). High levels of blood-eosinophils are associated with an increased risk of asthma exacerbations and can help to guide biologic treatment for T2 inflammation in children (195). The findings of elevated FeNO and blood-eosinophils and lower ratio of FEV1/FVC in cases with asthma are not surprising and further support their asthma diagnosis.

EDN levels were elevated when measured at 7 years among cases with asthma at age 11, but not when measured at inclusion or the first revisit. EDN has been suggested to be a good and stable marker for eosinophilic inflammation since it reflects the activation level of eosinophils and has no circadian rhythm (196). Previous results from GEWAC have shown that medication has an impact on blood-eosinophils but not on EDN levels and elevated EDN levels from 4 years were associated with asthma at 7 years (197). Other studies have found increased EDN levels among children in early life with wheezing illness (198).

## 5.6 Rhinovirus induced wheeze

RV is one of the most frequent triggers of wheeze among preschool children and the most common cause of exacerbation once asthma is established (69). Children with RV-induced wheeze have more frequently been shown to have AD and blood eosinophilia (84). We found RV induced wheeze to be associated with asthma at both 7 and 11 years, although most likely acting as a revealing factor for asthma development. As also reported in other studies (66, 199, 200), the highest asthma prevalence was present in children with both wheeze caused by RV and allergic sensitization, suggesting an interaction between the two. It is discussed whether RV is a causative factor for asthma development or whether it primarily causes wheeze in already predisposed children. In the high-risk cohort COPSAC, the number of wheezing episodes during early life, independent of the triggering virus, was associated with asthma development (12), arguing against causation. Results from our studies support that wheeze caused by RV is mainly an unveiling factor in predisposed children and there are several potential underlying mechanisms explaining this interaction.

Atopic asthma is associated with a reduced IFN response and a decreased viral clearance upon viral infection. This could be due to the inverse relationship between high affinity IgE receptors and type I and III IFN production by peripheral blood cells and the fact that the antiviral responses in airway epithelial cells can be inhibited by type 2 inflammatory cytokines (87, 201). Children with atopic asthma treated with omalizumab showed improved IFN responses and less exacerbations triggered by virus, suggesting that inflammation mediated by IgE increases the risk of exacerbations by reducing antiviral responses (202). Further, RV causes a stronger induction of innate and pro-inflammatory cytokines in children biased towards a type 2 inflammation, causing further damage and inflammation in the airways (86).

RV-induced first-time wheezing has been noted to increase the risk of atopic asthma, independent on atopic status during the wheezing episode (71). This finding suggests RV to be a first sign of allergic disease and that early type 2 inflammation increases susceptibility to RV- induced wheeze. Further, that downregulation of type 2 inflammation at an early stage could have long-term effect. Treatment with OCS at the time of the first RV-induced wheezing episode has been implicated to decrease the asthma risk among children hospitalized due to first-time RV-induced wheeze with or without sensitization and/or eczema (200). Further studies are needed to investigate this more thoroughly.

In addition, we found that parental heredity for asthma and/or allergies is associated with asthma development. Genetic susceptibility is important in determining wheezing outcomes. Being a carrier of genetic risk variants in the 17q21 locus together with RV wheezing have the highest risk for early-onset asthma in a high-risk cohort. Further, stimulation with RV caused an upregulation of *ORMDL3,* suggesting this to contribute to wheeze severity (23). Other genetic factors increasing the risk of children with RV-induced wheeze developing asthma involve a genetic risk variant in *CDHR3*, a gene coding the RV-C receptor CDHR3, that has been identified to be linked to severe wheezing episode (28). Previous studies from the GEWAC cohort have found RV to be the most identified trigger of wheeze, the RV-C subtype was the most detected and co-infection with bacteria was common in children with wheeze at inclusion (179, 203). Specific $IgG_1$ increase to RV-A and RV-C was associated with longer time with respiratory symptoms (203). Further, a genetic risk variant of *CDHR3* have been noted to be more common in cases in GEWAC in comparison to healthy controls (29).

## 5.7 Allergic sensitization

We noted that cases with asthma at age 11 had allergic sensitization to airborne allergens at the first revisit, 7 years, and 11 years to a higher extent than cases without. All cases with sensitization to airborne allergens at the first revisit (n = 11) had asthma at age 11 years. Early sensitization to both food and airborne allergens has been found to be associated with asthma development (66, 204). Sensitization to food allergens seems to dominate during preschool years in our cohort, whereas sensitization to airborne allergens increases over time and dominates at school age, results in line with other studies (102, 111).

We noted that molecular spreading, the increase in sensitization to allergen molecules over time, from early life to school age was associated with asthma at 7 years. Further, polysensitization at age 7 was associated with asthma at the same age, supported by results from other cohorts (205). Cases sensitized to Bet v 1, Ara h 2, Ara h 6 and Fel d 1 at preschool age had a larger increase in sensitization to allergen molecules from early life to school age. This finding suggest that these allergen molecules are initiators of molecular spreading. Other studies have found sensitization to specific allergen molecules (Der p 2, Der p 1, Der p 23 and Phl p 1) during childhood to be initiators of a further increase in sensitization to allergen molecules over time (206-208). Regarding timothy grass, early-onset

allergic sensitization has been shown to be related to asthma, whereas late-onset is related to rhinitis (207), suggesting that the timing is crucial for disease development.

In addition, we found that the highest ranked (ranked by prevalence and median ISU) allergen molecules were Mal d 1 at inclusion/revisit and Bet v 1 at age 7. Ara h 2 and Bet v 1 were in the top three highest ranked allergen molecules at both inclusion/revisit and age 7. There was a significant increase of the most prevalent airborne allergen molecules in cases (Bet v 1, Phl p 1 and Fel d 1) from inclusion/revisit to age 7. No increase was seen in sensitization to the peanut molecules Ara h 2, Ara h 3 and Ara h 6. Cases with asthma at 7 years were Bet v 1 sensitized, at age 7, in a higher extent than cases without asthma. Our findings of the PR-10 allergen molecules Mal d 1 and Bet v 1 being among the highest ranked during preschool years (Mal d 1 and Bet v 1) and at 7 years (Bet v 1), probably reflect the degree of cross-reactivity between the two molecules. We did not find an association between the highest ranked molecules and asthma at age 7, potentially due to the sample size. In the population-based cohort BAMSE, persistent asthma at age 16 years could be predicted by sensitization during preschool years to Ara h 1, Bet v 1, Fel d 1 and Phl p1 (97). We found Fel d 1 and Bet v 1 to be potential markers of molecular spreading that could affect the course of asthma, but we could not identify specific allergen molecules that were directly associated with asthma development.

In Sweden, Bet v 1 has shown to be a risk allergen molecule (97) while identified risk allergens in studies from Germany and the UK are Der p 1 and Der p 2 (dust mites) (209). Storage proteins (Ara h 1, Ara h 2, Ara h 3 and Ara h 6) have been associated with heredity for atopic diseases and asthma (115) and development of sensitization to storage protein in early life has been shown to persist through adulthood and is associated with airway and systemic type 2 inflammation (116). These findings are in line with our results, where all children sensitized to Ara h 1, Ara h 2, Ara h 3 or Ara h 6 (peanut molecules) at preschool age had asthma at age 7 years.

Polysensitization has been associated with a more severe disease outcome than solely monosensitization (210). Both polysensitization and early-onset allergic sensitization have been linked to a low lung function in early adulthood, a finding that is hypothesized to be due to a more atopic constitution in these children that in turn contribute to asthma severity (211).

To sum up, using molecular allergy diagnostics we were able to show the importance of polysensitization as well as molecular spreading in asthma development. This approach adds important pieces to the possibility of asthma prediction, which is not feasible using extract-based allergens.

## 5.8 Systemic inflammation in the acute wheezing episode

We used Olink proteomics when analyzing the inflammatory response during an episode of preschool wheeze and compared the results to healthy controls. To our knowledge, this extensive platform of inflammatory proteins has never before been used to study the acute

episode of wheeze. Out of 92 measured inflammatory-related plasma proteins we found 10 that could almost entirely separate cases from controls. Seven had a higher expression (IL-6, IL-10, OSM, CXCL10, AXIN1, SIRT2 and FGF21) and three had a lower expression (TNF-β, TNFSF11 and CASP8) in cases compared to controls.

OSM are part of the IL-6 family of cytokines and is suggested to be involved in asthma and asthma exacerbations by promoting airway inflammation and mucus secretion from epithelial cells (212). Elevated levels of OSM have been noted in atopic diseases and can stimulate inflammatory responses of airway epithelial cells, fibroblasts, and airway smooth muscle cells, contributing to epithelial dysfunction (213, 214). OSM induce both IL-6, associated with severe asthma (215), and IL-33 which plays a role in type 2 inflammation (216).

IL-6 is involved in both regulation and induction of chronic inflammation and guides the direction of CD4+ T cell differentiation, suppress regulatory T cells and promotes fibrosis (217). High plasma levels of IL-6 are associated with metabolic dysfunction and risk of asthma exacerbations (218). Two large birth cohorts identified plasma levels of CXCL10 and IL-6 to be associated with low lung function (219), suggesting that the elevated IL-6 and CXCL10 leading to frequent exacerbations in some children will cause lung function limitations. CXCL10 is a proinflammatory chemokine, secreted in response to IFN- γ, involved in the Th1 immune response and has been found elevated during asthma exacerbation and also in severe asthma (220). Previous studies have also found that high levels of IFN during exacerbations increase the risk of asthma development (221). In our study IL-6 and CXCL10 were elevated during acute wheeze and not at remission, it would have been interesting to measure plasma levels of these proteins before the wheezing episode.

IL-10 is an anti-inflammatory cytokine with the ability to regulate both Th1 and Th2 immune responses. Allergic inflammation can develop if IL-10 is not properly controlled. Low levels of IL-10 have been associated with asthma and atopic diseases (222). Others have found that higher plasma concentration of IL-10 after bronchiolitis is associated with recurrent wheeze (223).

AXIN1 is a negative regulator of the Wnt/β-catenin pathway and noted to be associated with a Th2-high phenotype (224). SIRT2 mediates Th2 immune responses and promotes airway inflammation. The selective SIRT2 inhibitor AGK2 causes a decrease in allergic inflammation (225). FGF21 has been found to be increased in sera of asthmatics, in comparison to non-asthmatics, and also to be able to distinguish controlled asthmatics from un-controlled (226).

The three down-regulated proteins, in comparison to healthy controls, were: TNF-β, TNFSF11 and CASP8. TNF-β and TNFSF11exhibit proinflammatory functions partially due to their activation of NF-κB signaling pathways, and they can also trigger apoptosis (227). CASP8 is in the same pathway and triggers apoptosis, low levels of CASP8 have been associated with exaggerated viral replication (228).

In summary, we found signs of activation of both type 1 and type 2 inflammation, suggested exaggerated viral replication and proteins that are both involved in epithelial dysfunction and in airway remodeling, with signs of type 2 inflammation that persists through remission. Interestingly, in line with the observed protective effects of OCS (200), we found that OCS suppresses CXCL10, which has been implicated in risk of exacerbations and low lung function (219).

It would have been interesting to have had a second control group, consisting of children with a regular cold, to distinguish features that are typical of wheeze.

# 6 CONCLUSIONS

In our high-risk cohort of preschool wheezers, in which a majority were recurrent wheezers already at inclusion, we found a high prevalence of asthma at both 7 and 11 years. At the acute wheezing episode, we found elevated levels of inflammatory plasma proteins, distinguishing children with acute wheeze from healthy controls, and signs of an already ongoing T2 inflammation, remodeling, affected antiviral defense, and epithelial dysfunction. This suggests that there are common immunopathological pathways between wheeze and asthma.

There was a significant increase of sensitizing allergen molecules from inclusion to 7 years among cases with asthma, an increase was not seen in cases without asthma. Furthermore, there was an increased risk of asthma with each additional sensitizing molecule from inclusion to 7 years and the risk was also associated with the number of sensitizing molecules at age 7.

We found RV to be associated with asthma at both 7 and 11 years and that the severity of wheeze after inclusion in the study was associated with asthma at 7 years. In an adjusted model, heredity for asthma/allergy and allergic sensitization at age 2 were associated to asthma in early adolescence, suggesting that RV could be a revealing factor for asthma development among children predisposed to asthma. In support of this hypothesis, we found a higher asthma prevalence at age 11 in children with both RV at inclusion and allergic sensitization at 7 years compared to children with RV at inclusion without allergic sensitization at age 7.

In summary, we found evidence of RV as a revealing factor for asthma development among children predisposed to asthma. This finding was strengthened by the fact that already at the acute wheezing episode there were signs of type 2 inflammation that persisted through remission.

# 7 POINTS OF PERSPECTIVE

## 7.1 Clinical implications

Our study adds to the evidence that mainly children predisposed of allergic diseases affected with RV-induced wheeze, have an increased risk of school-age asthma. We were able to show that allergic sensitization, RV-induced wheeze and signs of T2 inflammation already at the acute wheezing episode at inclusion are found in a group of children with more heredity for asthma and allergies.

RV could very well be an unveiling factor in predisposed individuals, and our results suggest that it may be of clinical value to collect nasopharyngeal samples for virus detection and further to make a regular assessment of allergic sensitization as well as to explore the heredity for allergic diseases. This could result in better risk assessment of preschool children at higher risk of asthma in adolescence.

## 7.2 Future research

Our results could help identify children on the course of asthma development but if we want to be able to change the course of disease development, we will probably need to look at factors before the first wheezing episode.

Systems immunology in large cohort studies could yield a greater understanding about the involved mechanisms and show unexplored immune pathways and define the best targets for protection against asthma, studies that have shown great promise in this regard (229).

Preventive strategies are carried out both with Vitamin D (230), fish oil (159) and probiotics with results that will be interesting to follow.

Implications that OCS treatment during RV-induced wheeze may be protective of recurrent wheeze (172), an RCT is ongoing to further address this question. RV-species have been found to have different risk profiles related to severity of wheeze. A newly developed high-resolution antibody assay can discriminate between different RV species (231). Identification of which RV-species (A-C) is the trigger of the wheezing episode could aid in future development of RV-specific therapeutic and prophylactic treatment.

Another study that will be interesting to follow is the Breathing Together, a birth cohort of 1000 babies, recruited from different centers in the UK. The aim is to relate the microbiome, transcriptomics and systems immunology measured longitudinally from birth, to wheeze and asthma outcomes (232). The study is unique and has the potential to give more answers to asthma development with the longitudinal approach to study the immune development.

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