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VIRAL WHEEZE AND RISK FACTORS FOR CHILDHOOD ASTHMA

- AN EVALUATION OF CLINICAL, IMMUNOLOGICAL AND GENETIC FACTORS

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Viral wheeze and risk factors for childhood asthma - an evaluation of clinical, immunological and genetic factors

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

It's not fully understood why some children wheeze with viral infections, and why some develop severe asthma. In this study we compared two study groups; children presenting with acute wheeze (AW) before the age of four and age-matched healthy controls (HC), and we investigated factors that might contribute to increased vulnerability for airway infections and risk of later asthma development.

In **Study I** we identified several hereditary and environmental risk factors in the AW group, including significantly lower vitamin D levels and recurrent episodes of viral wheeze compared with the HC group. Rhinovirus (RV) was the most common virus detected. Bacterial co-infections were also common at the acute visit in the AW group.

In **Study II** we investigated which subtypes of RV were detected during the acute phase, and the change in RV-specific IgG_1 between the acute visit and a follow-up three months later. It is currently debated whether or not RV-C is more pathogenic than RV-A and RV-B. RV-C was the most frequently detected subtype, but we found no correlation between RV subtypes and clinical symptoms, or RV-specific IgG_1 increase at follow-up. Children with an increase in specific IgG_1 against both RV-A and RV-C, reported the longest duration of respiratory symptoms, indicating a possible synergistic effect of two RV subtypes and possibly an increased risk of asthma.

Recently, *CDHR3* has been identified as an asthma susceptibility gene, and it encodes the RV-C receptor, cadherin-related family member 3. In **Study III** we investigated *CDHR3* rs6967330 G>A genotype in the AW and HC groups, and AG/AA was found to be overrepresented in the AW group. Furthermore, reduced mRNA levels for *CDHR3* were shown in children with acute wheeze.

The chitinase like protein YKL-40 has been associated with airway remodeling, and severe asthma in school-children. In **Study IV** we investigated blood YKL-40 at the acute, 3-month and 1-year follow-up visits. We studied the distribution of the genetic variant rs4950928 (-131C>G) in the gene encoding YKL-40, *CHI3L1*. The distribution was similar in the AW and HC groups, although rs4950928 variants were found to strongly affect circulating YKL-40 levels. The levels of YKL-40 were higher in the AW children during acute wheeze and at the 3-month follow-up, but did not differ between the groups at the one-year follow-up visit.

In conclusion, preschool children in the AW group had several environmental and hereditary risk factors for later asthma development, as well as lower levels of vitamin D. RV-C was detected in the majority of children in the AW group and co-infection with bacteria was common. The asthma susceptibility gene variant rs6967330 in *CDHR3* was associated with wheeze, and reduced mRNA levels of *CDHR3* were shown in children with acute wheeze. However, the proposed biomarker YKL-40 did not facilitate the identification of children with persistent airway inflammation.

Several of our findings indicate that children with wheeze may constitute a group of children with an increased vulnerability, both immunologically and genetically, placing them at greater risk of developing asthma compared to the healthy, age-matched HC group.

LIST OF PUBLICATIONS

The thesis is based on the following publications. The publications will be referred to by their Roman numerals (I-IV) * = shared first authorship, # = shared last authorship.

I. **K.Stenberg Hammar**, G.Hedlin, J.R.Konradsen, B.Nordlund, I.Kull, C.G.Giske, C.Pedroletti, C.Söderhäll, E.Melén
Subnormal levels of vitamin D are associated with acute wheeze in young children
Acta Paediatrica 2014 Aug;103(8):856-61. doi: 10.1111/apa.12666.

II. **K.Stenberg Hammar***, K.Niespodziana*, C.Söderhäll, A.James, C.Cabauatan, J.R.Konradsen, E.Melén, M.van Hage, R.Valenta#, G.Hedlin#
Rhinovirus-specific antibody responses in preschool children with acute wheeze reflect severity of respiratory symptoms
Allergy 2016 Jul 22. doi: 10.1111/all.12991 .

III. **K.Stenberg Hammar**, K.Niespodziana, M.van Hage, J.Kere, R.Valenta, G.Hedlin, C.Söderhäll
CDHR3 in preschool children with acute wheeze
Manuscript

IV. A.James*, **K.Stenberg Hammar*,** L.Reinius, J.R.Konradsen, S-E.Dahlén, C.Söderhäll, G.Hedlin
A longitudinal assessment of YKL-40 levels in preschool children with wheeze
Pediatr Allergy Immunol. 2016 Oct 12. doi:10.1111/pai.12669.

CONTENTS

LIST OF ABBREVIATIONS

25(OH)D	25-hydroxyvitamin D
APC	Antigen presenting cells
API	Asthma predictive index
CI	Confidence interval
CDHR3	Cadherin related family member 3
CHI3L1	Chitinase-3-like protein 1 (also known as YKL-40)
DBP	Vitamin D binding protein
HSA	Human serum albumin
ICAM-1	Intercellular adhesion molecule 1
ICS	Inhalations with corticosteroids
IQR	Interquartile range
LDL-R	Low-density lipoprotein receptor
LRI	Lower respiratory tract infections
OR	Odds ratio
PTH	Parathyroid hormone
RSV	Respiratory syncytial virus
RV	Rhinovirus
RT-PCR	Reverse transcription polymerase chain reaction
TCRS	The Tucson Children’s Respiratory Study
TRAP	Traffic-related air pollution
URI	Upper respiratory tract infection
VDR	Vitamin D receptor
WBC	White blood cells

1 INTRODUCTION

Asthma is the most common chronic disease in children, about 14% of the world's children had symptoms of asthma in the previous year, according to The Global Asthma Report of 2014. (1) The number of individuals with asthma has been increasing for a long time, but a decrease in prevalence has been reported in recent years. (2, 3)

Asthma has traditionally been considered an allergic disease, but a large proportion of children with early asthma/wheeze do not appear to be allergic. (1) In the majority of cases, wheeze in children is triggered by viral infection, (5) and it has been shown that children wheezing with rhinovirus (RV) infection in their first year of life have a 3-fold increased risk of asthma at six years of age, but children wheezing with RV in their third year of life, have a 32-fold increase in asthma at school age. (4) The Global Asthma Report of 2014 (1) highlights the recommendation that "recurrent wheeze in infancy, especially when frequent and/or severe episodes are present, should no longer be regarded as a benign condition but as part of the spectrum of asthma". (1)

Today preschool children with wheeze have no reliable classification to predict outcome over time (5), and clinicians have no easily attainable tools or biomarkers to measure lung function or airway inflammation, and to assess risk of chronic asthma.

The pathogenesis of asthma is heterogeneous with a complex interplay between the immune system, genetic factors and the environment. A large number of genetic studies have attempted to find genetic variants associated with the risk of childhood wheeze and asthma. In many cases, candidate chromosomal loci are discovered by genome-wide association studies (GWAS) using large subject cohorts, followed by further studies of genetic variants in candidate genes (single nucleotide polymorphisms (SNPs)).

It is important to combine clinical, immunological and genetic research to find new insights into the prevention and development of asthma. Early identification of preschool children with recurrent wheeze who are at risk of developing chronic asthma could make it possible to provide more specific and earlier therapeutic interventions, which could hopefully interfere with the disease trajectory.

2 BACKGROUND

2.1 WHEEZE

It has been difficult to find agreement among definitions of preschool wheeze, since there is a large overlap in phenotypes (phenotype being defined as a description of physical characteristics including disease history, see 2.2). To add further complication, patients may also move from one phenotype to another over time. The term episodic (viral) wheeze has been proposed to describe children who wheeze intermittently and are well between episodes (usually children less than three years of age), and the term multiple-trigger wheeze for children who wheeze both during and outside episodes of infection. (5) This classification has however been questioned, as many children change from one to the other if the classification is based on a retrospective questionnaire. (6)

The underlying process of wheeze includes inflammation of the airways, mucus production and reversible tightening of the smooth muscles in the airway walls. The whistling sound is produced by the vibration of opposing walls in the narrowed airways, causing dyspnea and cough (Figure 1). In the Tucson Children's Respiratory Study (TCRS), (7) one of the first longitudinal assessments of the natural history of asthma, 1246 children were followed from birth, and it was found that 1/3 of all children had an episode of wheeze before the age of three years, and 14% still had wheeze or asthma at school age.

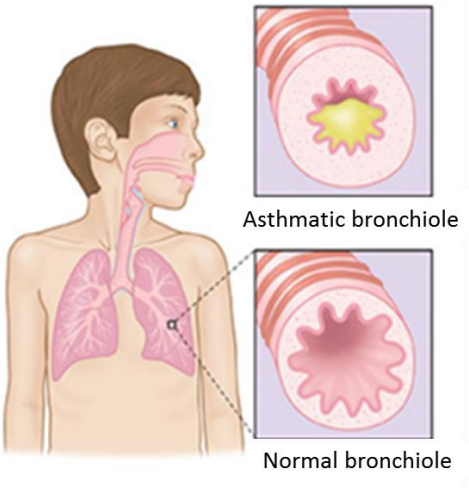

Figure 1. *The process of wheeze includes inflammation of the airways with contraction of smooth muscle, hypersecretion of mucus and mucosal edema, narrowing the caliber of the airways causing a whistling sound when exhaling.*

It is not currently known why respiratory infections trigger symptoms of bronchial obstruction in some children, but not others (50% had not experienced any episodes of wheeze at the age of six in the TCRS). (7) Underdeveloped airways are considered to be an important factor that may cause wheeze during the first year of life, but children wheezing during the second year of life are at greater risk of continuing to have reduced lung function. (8) The persistence of lung function abnormalities in those who wheeze beyond the first year of life is suggestive of a different disease process, possibly early airway inflammation. (8, 9) Other well known risk factors for the development of wheeze and later asthma are parental asthma/allergy, early sensitization, exposure to tobacco smoke and specific characteristics of the immune system. (9, 10) In a review article on risk factors for non-atopic asthma in children, 30 risk factors were evaluated in 43 different studies, and out of them only 3 risk factors showed consistent associations with non-atopic asthma/wheeze: family history of asthma/rhinitis/eczema, dampness/mold in the household, and lower respiratory tract infections during childhood. (11)

2.2 GENOTYPE, PHENOTYPE

A genotype is the sequence of the DNA code that determines a specific characteristic of an individual, such as eye color, blood group or various hereditary diseases. Genotypes interact with environmental factors (e.g. exposure to smoke, viruses or starvation) resulting in expression of a certain phenotype (e.g. asthma or allergies).

2.3 GENE EXPRESSION

Proteins are the link between genotype and phenotype. Protein formation starts with the transcription of genes in the DNA to form messenger RNA (mRNA). The mRNA is then translated into polypeptides/proteins (Figure 2).

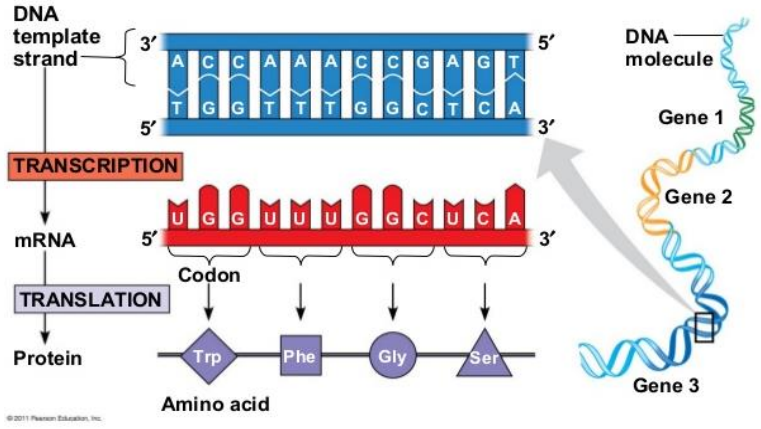

Figure 2. *Gene expression: a gene in the DNA is transcribed into messenger RNA (mRNA). This mRNA is then translated into a protein. (©2011 Pearson Education Inc.)*

2.4 VITAMIN D

Vitamin D is known to regulate calcium absorption and bone health, but recently the effect of vitamin D and its metabolites on other extra-skeletal functions and associations with chronic inflammatory diseases, including asthma, have come into focus.

2.4.1 Vitamin D metabolism

25(OH)D, is used to clinically assess vitamin D status. It can be formed in two ways in the human body; cholesterol in the skin is transformed by UVB sunrays (290-315 nm) to vitamin D3 (cholecalciferol), and in the gut vitamin D2 (ergocalciferol) is extracted from foods such as fish, mushrooms and fortified milk. Both of these inactive forms (vitamin D2 and D3) are transported in plasma to the liver to form the pre-hormone 25(OH)D (calcidiol).

The active hormone, 1,25 dihydroxyvitamin D_3 (calcitriol), is formed from 25(OH)D in the kidneys, stimulated by parathyroid hormone, and inhibited by high plasma levels of calcium and phosphate. (12) Calcitriol binds to the vitamin D receptor (VDR) in the nucleus of cells, and activates a nuclear transcription factor which regulates the gene transcription of many hundred genes (Figure 3). (12) VDR is expressed by many cell types throughout the body.

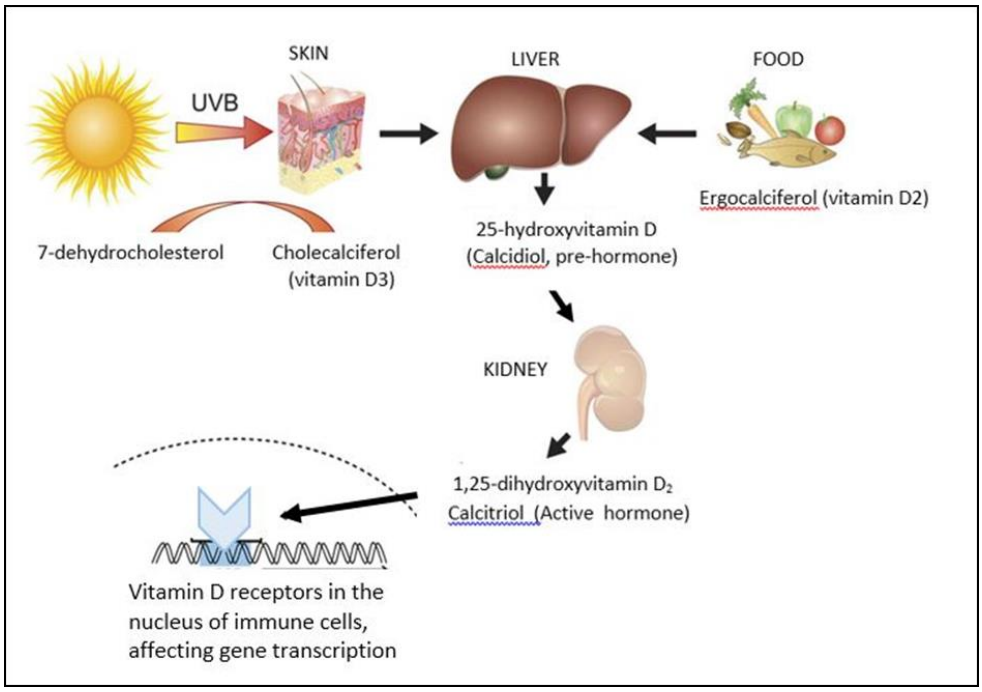

Figure 3. *The metabolism of vitamin D*

2.4.2 Optimal 25(OH)D levels

The association between 25(OH)D, calcium regulation and bone health has been known since the beginning of the last century, yet reference values are still being discussed. (13) The levels to define the lower limits of 25(OH)D are levels that maintain normal calcium homeostasis. The most common definition of a normal vitamin D range has been a concentration of 25(OH)D of 25-75 nmol/L (10 -30 ng/mL). It is known that children need to have >50 nmol/L (20 ng/mL) to avoid the risk of developing Rickets (14), and a 25(OH)D concentration of ≥50 nmol/L is also recognized as an optimal level for adults. (14, 15)

Vitamin D insufficiency is now commonly defined as 25(OH)D levels of 25-50 nmol/L, and deficiency is defined as levels of less than 25 nmol/L.

Levels of the active hormone 1,25-dihydroxyvitamin D_3 cannot be used for measuring vitamin D status, as it has a short half-life (hours) and levels are regulated by other factors such as serum parathyroid hormone (PTH). Therefore the level of 1,25-dihydroxyvitamin D_3 can be normal or elevated as a result of secondary hyperparathyroidism, even in the presence of 25-(OH)D deficiency. (12)

2.4.3 Risk factors for 25(OH)D deficiency

During the last decade, it has been shown exactly how prevalent 25(OH)D deficiency is worldwide, with high rates of 25(OH)D deficiency reported both in children and adults. (13, 16)

There is a clear relationship between latitude and levels of vitamin D detected. (16) For people living at latitudes higher than 35° (Stockholm is situated at 59° North), few UVB photons reach the earth's surface from September to April, leading to a higher risk of vitamin D deficiency or insufficiency during half of the year for this population (Figure 4). (16, 17)

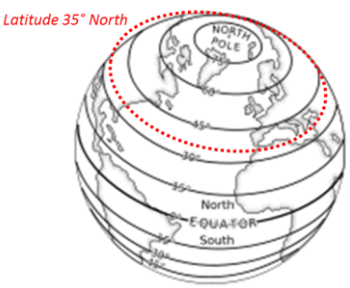

Figure 4*. For individuals living at latitudes above 35°, few UVB photons reach the earth's surface from September to April.*

Other factors associated with vitamin D deficiency/insufficiency include: a change in lifestyle (less outdoor activities), an awareness of skin cancer (using sunscreen with a sun protection factor of 15 blocks approximately 99% of cutaneous vitamin D production), increased BMI, increased age, having dark skin (which requires longer time with UVB exposure compared to people with lighter skin to synthesize equivalent amounts of vitamin D), being exposed to air pollution (which can filter out UVB radiation), and finally, having renal or liver disease. (18, 19)

2.4.4 Vitamin D and asthma

Epidemiological studies demonstrate that suboptimal level of 25(OH)D are associated with recurrent viral respiratory infections, (20) and asthma. (18, 21, 22)

The mechanisms whereby 25(OH)D is protective against asthma may be observed as early as the *in utero* period, when vitamin D plays a role in lung growth and maturation. (23) Vitamin D is also important for both innate and adaptive immune responses. The epithelium in the lung has the ability to locally convert inactive 25(OH)D to the active 1,25-dihydroxyvitamin D_3 leading to increased expression of genes important for innate immune defenses (24, 25) and activation of locally produced antimicrobial peptides (AMPs). (22) The vitamin D receptor is expressed on B cells, T cells and antigen presenting cells, all capable of synthesizing the active vitamin D metabolite, and thus vitamin D has the capability of acting locally, and independently of PTH levels. (25, 26)

2.5 RHINOVIRUS

Rhinovirus (RV), also known as the common cold virus, belongs to the Enterovirus genus, within the Picornaviridae family, and is one of the smallest viruses. It was discovered in the 1950s and was first classified into A and B species, (27) but in 2006 a third species, RV-C was discovered. (28) There are currently 74 known subtypes of RV-A, 26 subtypes of RV-B, and at least 50 subtypes of RV-C. (29, 30) Most RV grow best in temperatures between 33–35°C (as in the upper respiratory tract) and usually cause only mild symptoms, but some grow equally well at 37°C (as in the lower respiratory tract) and might be the cause of more severe respiratory symptoms involved in asthma exacerbations, as well as acute bronchiolitis and wheeze in young children. (29, 31) Despite 50 years of trying to develop a vaccine against RV the antigenic heterogeneity amongst the >150 RVs has been a major barrier resulting in little progress. (53)

2.5.1 Rhinovirus structure

Rhinoviruses have a genome composed of single-stranded positive RNA (the viral RNA functions as mRNA and can be directly translated into viral proteins within the host cell) and are non-enveloped viruses with capsids that express four viral proteins (VPs); VP1, VP2, VP3, and VP4. These proteins are arranged in overlapping fashion to form an icosahedral structure, with VP1, VP2, and VP3 expressed on the surface of the capsid while VP4 is located on the internal side of the capsid (Figure 5). (30, 32)

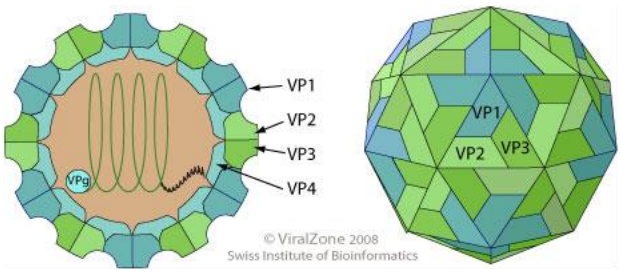

Figure 5. *Model of a rhinovirus with capsids that express four viral proteins VP1, VP2, VP3, and VP4 forming an icosahedral structure, with VP1, VP2, and VP3 expressed on the surface and VP4 located on the internal side of the capsid. (©Viral Zone 2008, Swiss Institute of BioInformatics)*

2.5.2 Rhinovirus receptors

RV-A and RV-B are also classified according to major or minor groups, depending on the receptor used to bind to human respiratory epithelial cells. Major group RVs (90% of all RV, both RV-A and RV-B) bind to the intercellular adhesion molecule 1 (ICAM-1) receptor and minor group RVs (all RV-A) bind to the low-density lipoprotein receptor (LDL-R).

Until recently, the RV-C receptor was unknown, but it has now been proposed that CDHR3, a cadherin-related family member protein, may function as the RV-C receptor. (30, 33, 34)

CDHR3 is a member of the cadherin family of transmembrane proteins with an, as yet, unknown biological function. Cadherins are involved in homologous cell adhesion processes that are important for epithelial cell-cell interactions and tissue differentiation (34-37).

2.5.3 Genetic regulation of the RV-C receptor

The gene coding for the RV-C receptor, CDHR3, is the asthma susceptibility gene *CDHR3*, located on chromosome 7q22. It was recently discovered to be associated with a specific asthma phenotype characterized by recurrent, severe exacerbations occurring between 2 and 6 years of age in a genome-wide association study. (34). It has been suggested that the gene variant rs6967330 A/G in *CDHR3* could be a risk factor for RV-C wheezing illnesses since it has been found that the risk rs6967330-A allele can increase RV-C binding and virus replication in HeLa cells that synthesize CDHR3. (33, 34)

2.5.4 Rhinovirus and asthma

The significance of early RV infections in the development of wheeze and subsequent asthma is under investigation. There could be a critical time period early in life, when RV infections may change the pattern of the immune reaction (38-40), or this might be true only in a subgroup of susceptible children with impaired lung physiology or antiviral responses. (41-43)

The pathophysiology of RV infection has some unique characteristics. The primary site of inoculation is the nasal mucosa and the infection involves only a small portion of the epithelium causing histamine release and nasal discharge. The virus does not cause any destruction of airway epithelial cells, but disrupts the tight junctions between cells destroying our first line of defense, the epithelial barrier. (44) Gaps within epithelial layers allow cytokines, immune cells and further viruses and bacteria to penetrate deeper into the airways, causing a massive upregulation of inflammatory mediators in the host. RV infections are often described as a “cytokine disease”. (45)

2.5.5 Immune response against rhinovirus

The clearance of a RV infection is dependent on both rapid innate immune responses (involving interferons, macrophages, antimicrobial peptides) as well as the humoral (B-cells with antibody formation) and cell-mediated immune responses (T-cells with cytokine regulation). (46)

Interferons (IFNs) are cytokine protein mediators, important for the early innate antiviral immune response. (47) In asthmatic individuals, impaired virus-induced IFN expression has been shown, and IFNs are important both during recovery from RV infections as well as in the prevention of virus-induced exacerbations. (42) Defective IFN secretion has also been seen in non-atopic children with viral-induced wheeze. (48)

The antiviral effector mechanism of the adaptive immune system involves the formation of neutralizing antibodies, whereby serotype-specific neutralizing serum antibodies (IgG) as well as secretory antibodies (IgA) develop. (49) Since they occur weeks after the infection, the humoral immune responses with antibody formation are thought to be most important for preventing RV infection. Antibody production in natural RV infections occurs on average in only 50% of patients. (49)

In contrast to the specific antibody response, T-cells show serotype cross-reactivity and can be activated either by serotype-specific or shared viral epitopes. (50, 51) Rhinovirus belonging to the major-group RV has been shown to directly infect and activate human T-cells without intervention of antigen presenting cells (APCs; monocytes, macrophages, or B-cells.). (52) This activation results in cytokine release from T-cells and activation of eosinophils, and the subsequent triggering of other inflammatory effector cells, ultimately causing exacerbations of airway disease. (52)

2.6 CHITINASE-LIKE PROTEIN YKL-40

Today, we do not have adequate biomarkers (defined as biological substances that can be detected and measured in for example blood or tissue, such as specific cells, gene or gene products) that are able to reflect chronic inflammation within the airways. However, one protein that has shown potential for this purpose is the chitinase-like protein YKL-40.

Chitin is the second most abundant polysaccharide on earth after cellulose, and a component of the cell walls of fungi and of the external skeleton of insects such as grasshoppers and cockroaches, and arthropods such as crabs and lobsters. Chitinases are hydrolytic enzymes that

break down the glycosidic bonds in chitin. (54) Humans do not produce chitin, but have two functional chitinases, Chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase), as well as the structurally-related chitinase-like protein, YKL-40. YKL-40 lacks chitinase activity and specific cell surface or soluble receptors have not yet been identified. The name of the protein YKL-40 derives from its molecular weight (40 kDa) and the one letter code for its three N-terminal amino acids, tyrosine, lysine, and leucine.

2.6.1 YKL-40 production

The main cell types able to secrete YKL-40 include a small group of highly differentiated tissue macrophages (type I) with pro-inflammatory properties, but with no production of anti-inflammatory cytokines and a low capacity for phagocytosis. (55, 56) Neutrophil granulocytes, which share a common progenitor cell with macrophages, store YKL-40 in specific granules, and release them after full activation at inflammatory sites. (57) Other cell types that express YKL-40 are, chondrocytes, synovial cells, vascular smooth muscle cells and hepatic stellate cells. (56)

2.6.2 YKL-40 function

YKL-40 levels are known to show a slow increase during adulthood and a steep increase after the age of 70, even in healthy elderly individuals. (57) Elevated levels of YKL-40 have been associated with multiple human inflammatory diseases including severe asthma in school age children (58) and adults. (59) The exact biological function of YKL-40 is not known, but it has been suggested that YKL-40 could act as an opsonin with a role in the human immune response (60) and be involved in airway remodeling due to its effect on smooth muscle proliferation and correlations with sub-epithelial fibrosis and bronchial wall thickness. (61)

2.6.3 Genetic regulation of YKL-40

The chitinase-3-like-1 gene (*CHI3L1)* located on chromosome 1q32.1 encodes the protein YKL-40. Several single nucleotide polymorphisms in this gene have been identified, and the genotype variant *CHI3L1*rs4950928 (-131C>G) has been associated with hyper responsiveness in the airways and reduced lung function (62) suggesting that genetic variations in *CHI3L1* may influence serum YKL-40 levels and the risk of asthma.

3 AIMS

We hypothesize that certain preschool children with wheeze have a specific genetic disposition that results in a vulnerability towards airway infections, and that certain viruses may trigger signs of remodeling at an early age.

The specific aims of this study were:

I. To identify risk factors for preschool wheeze, including specific sensitization patterns and vitamin D status.

II. To analyze subtypes of rhinovirus in relation to clinical symptoms and serological response after an acute episode of wheeze.

III. To investigate the gene variant *CDHR3* rs6967330 G>A and mRNA expression of *CDHR3* in relation to RV subtypes, RV-specific IgG_1 increase and clinical symptoms in preschool children with acute wheeze and at a follow-up visit.

IV. To investigate distribution of the gene variant *CHI3L1* rs4950928 (-131C>G), and its effect on the chitinase-like protein YKL-40, and whether YKL-40 is a possible biomarker of relevance to acute wheeze.

V. To identify possible markers of risk for chronic asthma in preschool children presenting with acute wheeze.

4 METHODS

4.1 STUDY DESIGN

The present thesis is based upon a longitudinal prospective study of a group of preschool children with acute symptoms of obstructive airways/wheeze and an age matched control group of healthy children, see Figure 6 for study design. The group of children with wheeze will be followed yearly until the age of seven, and this thesis covers the first year with one follow-up after approximately three months and one follow-up a year after inclusion.

The study was approved by the regional board of ethics at Karolinska Institutet (Dnr 2008/378-31/4 and 2014/399-31/3).

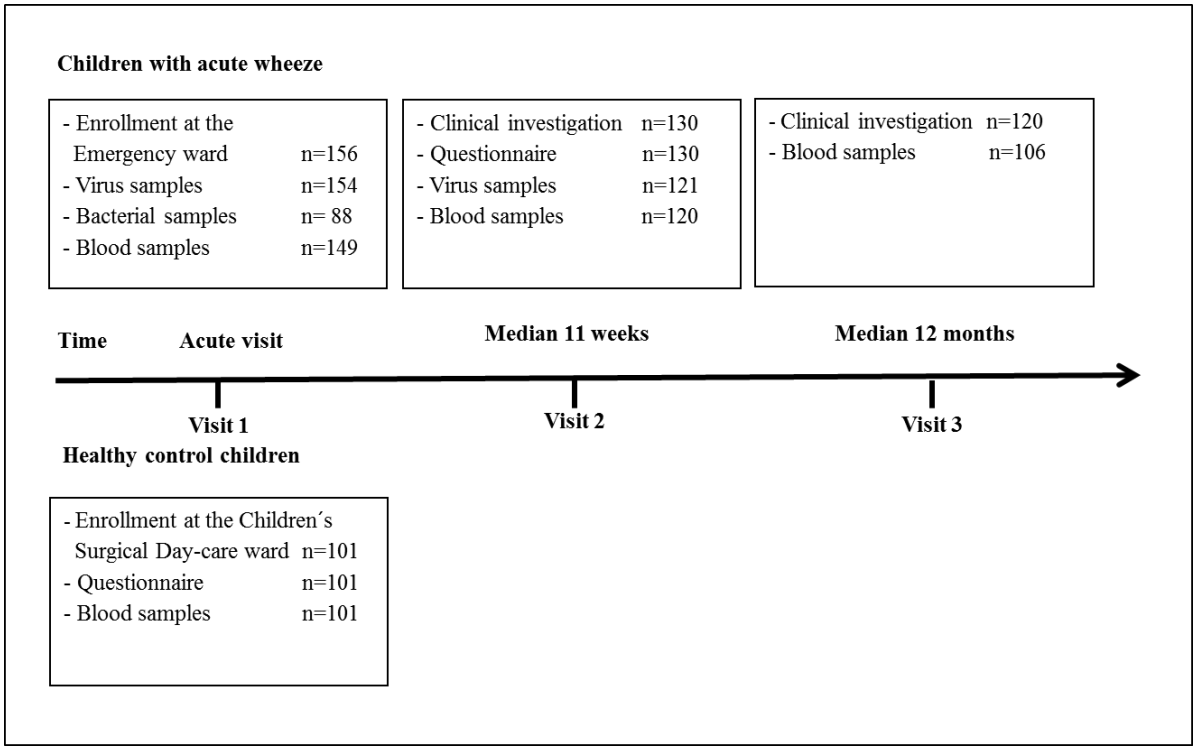

Figure 6. *Study design*

4.2 STUDY MATERIAL (I-IV)

The children with acute wheeze (AW) included in the study were recruited by the study doctor and the research nurse when attending the Pediatric Emergency Department or after admission to the Pediatric Emergency Ward as a result of their symptoms, at Astrid Lindgren Children's Hospital, Stockholm, Sweden, between October 2008 and September 2012.

Age-matched, healthy control children (HC) were recruited by the research nurse at the Children's Surgical Daycare Ward, Astrid Lindgren Children's Hospital during the same time period. The HC group had minor day surgery performed (retentio testis/hernia 44%, cystoscopy/micturating cystourethrogram 32%, hypospadias/circumscision/phimosis 9%, laparoscopy 12%, minor incisions 4%). For inclusion and exclusion criteria, see Table 1.

Table 1. *Inclusion and exclusion criteria*

	Inclusion criteria	**Exclusion criteria**
Children with wheeze/asthma	▪ Age 6-48 months ▪ Presenting at the emergency with acute symptoms of wheeze	▪ Prematurity (birth before 36 gestational w.) ▪ Any chronic disease ▪ Any simultaneous complications such as sepsis, bacterial pneumonia or diabetes at the time point of inclusion
Healthy children	▪ Age 6-48 months	▪ Prematurity (birth before 36 gestational w.) ▪ History of bronchial obstruction/asthma ▪ Known sensitization to airborne allergens

4.3 PROCEDURES

4.3.1 Inclusion of children with wheeze at the first acute visit (I-IV)

At the Pediatric Emergency Department or ward at Astrid Lindgren Children's Hospital, the diagnosis of wheeze was based on a clinical diagnosis made by the treating physician at the Pediatric Emergency Department and treatment was with salbutamol inhalation. After the enrolment criteria were confirmed by the study doctor or research nurse, the guardians of children with AW 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 nasal swab tests were obtained for virus detection. In addition, study children enrolled between September 2010 and September 2012 also had nasal swab tests for bacteria at the emergency visit. (Table 2)

4.3.2 Follow-up visit after 2-4 months and after 12 months (I-IV)

After approximately 2-4 months, the children with AW came back to the study doctor for a follow-up visit. The guardians were asked to fill out a standardized questionnaire concerning demographic factors, hereditary factors for asthma and allergy, birth weight, smoking during pregnancy, contact with furry animals, eczema, reported food intolerance, time with breast feeding and previous history of respiratory infections. The children underwent a clinical check-up and the study doctor carried out a structured interview with the guardians concerning the number of days the children had suffered respiratory symptoms, the number of acute visits and hospitalizations and medication since the acute visit. This was also confirmed using medical journals. The children had blood samples drawn after local anesthesia (EMLA cream, Astra Zeneca, Sweden) and again, nasal swab tests for virus detection were obtained**. (**Table 2)

Approximately 12 months after the acute visit, the children with AW had a second follow-up with the same clinical check-up, structured interview concerning time spent with symptoms and medication, and blood samples were drawn. (Table 2)

4.3.3 Inclusion of control children (I, III-IV)

The guardians of the healthy control children were informed about the study by the research nurse. After they had provided written informed consent, blood was drawn at one occasion from the children at the same time as an intravenous line was inserted prior to surgery and anesthesia. Guardians were asked to fill out the same standardized questionnaire about hereditary factors, lifestyle and environmental factors as the study children with AW. (Table 2)

Table 2*. Procedures at visits 1, 2 and 3 in children with acute obstructive airway disease (AW), and on one occasion in a group of healthy age-matched control children (HC).*

Procedures	**AW Visit 1 n=156**	**AW Visit 2 n=130**	**AW Visit 3 n=120**	**HC n=101**
Age in months, median (min-max)	18 (6-42)	20 (8-46)	30 (18-57)	18 (6-44)
Clinical investigation	156	130	120	
Standardized Questionnaires		130		101
Nasal swab samples for bacteria	88[1]			
Virus analysis on a 15 virus platform (I)	154	121		
Blood count; neutrophils/eosinophils (I-IV)	149	120	106	101
Total IgE in serum (I-IV)		124		81
Specific IgE in serum (Phadiatop/Fx5) (I-IV)		124		81
25(OH)D (I)		114		99
RV subtype analyze (II)	108	102		
Levels of RV specific IgG_1 and IgA antibodies (II)	120	120		
CDHR3 rs6967330 genotype (III)		122		94
Levels of YKL-40 (IV)	128	120	95	101
CHI3L1 rs4950928 genotype (IV)		123		95

[1]Of the 88 children tested for bacteria at the acute visit, 76 children came back for the first follow-up visit and were included in Study I.

4.4 LABORATORY ANALYSES

4.4.1 Blood count and IgE (I-IV)

All blood samples from both groups of children (drawn at the acute visit, the first and second follow-up visits for the AW group, as well as at the time of surgery for the HC group) were analyzed for total blood counts including hemoglobin, thrombocytes, leukocytes, and differential numbers of basophils, neutrophils and eosinophils according to standard procedures at the Karolinska University Hospital Laboratory, Stockholm, Sweden.

Levels of total IgE antibodies and allergen specific IgE antibodies against common airborne allergens (Phadiatop®; birch, timothy, mug worth, cat, dog, horse, moulds (Cladosporium herbarum), dust mites (Dermatophagoides pteronyssinus, Dermatophagoides farina) and food allergens (fx5®; cow's milk, egg white, soy bean, peanut, cod fish and wheat) (Thermo Fisher Scientific, Copenhagen) were analyzed in blood samples from the AW group (taken at first follow-up visit) and the HC group. Sensitization was defined as levels of specific IgE antibodies ≥0.35 kUA/L.

4.4.2 Vitamin D (I)

Levels of 25(OH)D were measured in serum samples from the AW group obtained at the first follow-up visit, approximately 3 months after the acute visit, and in samples from the HC group. The method for analysis used at the Karolinska University Hospital Laboratory was a standard procedure involving direct, competitive chemiluminescence analysis (CLIA, DiaSorin inc, USA). Levels of 25(OH)D <75 nmol/L (30 ng/mL) were used as cut off for suboptimal levels of vitamin D (insufficiency), and levels < 25 nmol/L (10 ng/mL) for deficiency.

4.4.3 Bacterial nasal swab tests (I)

The presence of bacteria was examined in 88 children at the acute visit, using a nylon nasal swab (Copan Eswab, Copan Diagnostics Ltd, Murrieta, California, USA) that was transported within two hours at room temperature to the Department of Clinical Microbiology, Karolinska University Hospital, for qualitative aerobic culture on solid media with *Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis* and beta-haemolytic *Streptococci* of groups A, C and G as target bacteria.

4.4.4 Virus analyses on a 15 virus platform (I)

The nasal swabs for virus detection were collected by the research nurse at the acute visit and at the first follow-up visit after 2-4 months on children in the AW group, and transported

within two hours after collection in a standardized virus medium (Sigma-Virocult, Medical Wire & Equipment Co Ltd, Corsham, Wiltshire, UK). The swabs 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) (63), and then stored in the biobank at the Department of Clinical Microbiology, Karolinska University Hospital.

4.4.5 Subtyping of rhinovirus species (II, III)

Nasopharyngeal swab samples from the AW group were obtained at the acute visit and at the follow-up 2-4 months later (see 4.4.4). After the first analysis (described above), these were stored in the biobank at the Department of Clinical Microbiology, Karolinska University Hospital until subsequent nested PCR analyses and sequencing were performed at the Immunology and Allergy Unit, Department of Medicine, Solna, Karolinska Institutet, Karolinska University Hospital Solna, in collaboration with the Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria. This procedure has been described previously (paper II).

4.4.6 Rhinovirus specific IgA and IgG_1 (II, III)

Levels of species-specific IgA and IgG_1 antibodies against the three known rhinovirus subtypes (RV-A, RV-B and RV-C) were measured in serum taken at the acute visit and the first follow-up visit in the AW group as described previously (paper II). In short, ELISA plates were coated with recombinant VP1 proteins from three representative strains of the RV-A subtype (RV-A2, RV-A16, RV-A89), one from the RV-B subtype (RV-B14) and one from the RV-C subtype (RV-YP) and human serum albumin (HSA). The reactivity to human serum albumin, as determined in serum from each patient was used as a negative control.

The optical density (OD) values corresponding to the levels of bound antigen-specific antibodies were measured using an ELISA reader (Dynatech, Denkendorf, Germany). The change in OD values were calculated as the OD value at the acute visit subtracted from the OD value at the follow-up visit (ΔOD). A change in OD value ≥ 0.1 in antibody levels between the acute visit and follow-up visit were considered to show an immunological response to rhinovirus.

4.4.7 Genotyping (III, IV)

In both groups of children, the gene variants rs6967330 G>A located in the *CDHR3* gene (III) and rs4950928 (-131C>G) located in the *CHI3L1* gene (IV) were analyzed using TaqMan allelic discrimination on the ABI Prism 7500 detection system according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Genotype calls were achieved from both children with wheeze (n=122 and n=123 respectively) and control children (n=95 and n=96 respectively).

4.4.8 *CDHR3* and levels of mRNA expression (III)

In a randomly selected subgroup of 50 children with wheeze, *CDHR3* expression was analyzed in blood leukocytes from blood samples taken at both the acute visit and the follow-up visit after 2-4 months. Likewise, *CDHR3* expression was analyzed in 17 healthy control children using a multiplexed TaqMan assay on the 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were multiplexed in order to analyze *CDHR3* (Hs00541677_m1) and the endogenous control (cyclophilin A [*PPIA*]: Hs99999904_m1) in the same well (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's recommendations.

4.4.9 Levels of YKL-40 (IV)

Levels of the chitinase-like protein YKL-40 were measured in plasma samples collected at the three visits in children with AW (the acute visit; n=95, 2-4 months follow-up; n=98, and one-year follow-up; n=83 children. 54 children came to all three visits), and also in plasma from the HC group (n=94) by ELISA (3) according to the manufacturer's instructions (Human Chitinase 3-like 1 DuoSet ELISA Development Kit, R&D Systems, Abingdon, United Kingdom). In short, two different dilutions were made for each sample from which an average was obtained, and all samples were analyzed in duplicate, in random order, and presented as ng/mL. The detection limit of this assay was 31 pg/mL, inter-assay variability was 3%, and intra-assay variability was 14%.

5 STATISTICS

Paper I-II: The χ^2-test was used to examine proportional differences between binary data. Variables with unadjusted p-values ≤ 0.05 were then analysed in multivariate logistic regression analyses for potential confounders. For normally distributed continuous variables (blood count), an unpaired t-test was used to compare between-group means. For data that did not show a normal distribution, the non-parametric independent samples Mann-Whitney U test (for two groups) and Kruskal-Wallis median test (several groups) were used to compare distribution and medians. SPSS software (version 19-22 IBM Corporation, Armonk, NY, USA) (I-4) was used for all statistical analyses.

Paper III: The χ^2 test was used to examine proportional differences for *CDHR3* gene variants in the AW and HC group. In addition, the χ^2 test was used when comparing the AW group according to variants of *CDHR3* rs6967330 genotype in relation to basic characteristics, medication, hospitalization, emergency visits until follow-up visit, PCR detection of RV and species-specific IgG_1 increase against RV. Variables with p-values ≤ 0.05 in the χ^2-test were considered significant. The independent samples Mann-Whitney U test was used to compare medians and distributions between the two groups. The Wilcoxon signed rank test was used to compare paired samples not following a normal distribution (the expression levels of mRNA acute and at revisit). Spearman Rank correlations were performed to analyze correlations between *CDHR3* expression at the acute visit and follow-up visit in children with different genotypes and clinical symptoms. SPSS software (version 22, IBM Corporation, Armonk, NY, USA) was used for statistical analyses. Allelic association was calculated in Haploview software version 4.2 (22). Expression levels of mRNA in each sample were determined by the comparative Ct method of relative quantification (64). (Relative quantification analyze changes in gene expression in a given sample relative to a control sample.) Data was presented as $2^{-\Delta Ct}$.

Paper IV: Basic comparisons were performed using GraphPad Prism statistical software (La Jolla, CA, USA). YKL-40 levels did not have a normal distribution and were presented as median with interquartile range and non-parametric tests were performed; the Wilcoxon Signed Rank test for paired datasets, the Mann Whitney test for un-paired datasets and the Spearman Rank test for univariate correlations. Regression analyses were performed using SPSS software following log transformation of YKL-40 levels (version 22, IBM Corporation, Armonk, NY, USA).

6 MAIN RESULTS

6.1 CLINICAL CHARACTERISTICS (I-IV)

In total, 156 children with acute wheeze (AW) were enrolled at the Pediatric Emergency Department or Ward at Astrid Lindgren Children's Hospital. An age matched group of 101 healthy children (HC) were recruited at the Children's Surgical Daycare Ward during the same time period. Of the children belonging to the AW group, 80% were admitted to the ward for in-house treatment and 82% received an oral steroid burst of 4 mg betamethasone within 24 hours of admission. The majority had experienced wheeze before, and 52% had previously been diagnosed with asthma, but 22% of the children were experiencing their first episode of wheeze. At the acute visit, 42% had ICS (17% continuously), at the first follow-up visit 75% had ICS (42% continuously) and at the one-year follow-up 90% had ICS (37% continuously).

The age distribution was similar in both groups at the acute visit (median 18 months (range 6-42 *vs* 6-44 months)). There was however a male dominance in the HC group compared with the AW group (79% *vs* 65%, p=0.02), this was due to the fact that the dominating diagnoses at the Children's Surgical Daycare Ward were related to the male genitalia (see Study material 4.2). (Table 3) The gender distribution remained within the AW group at the second follow-up visit (79% *vs* 68%).

6.2 HEREDITARY AND ENVIRONMENTAL FACTORS (I)

A standardized questionnaire regarding family history, environmental factors and prior illnesses was collected from 130 children that came back for the first follow-up visit in the AW group and for all 101 children in the HC group at inclusion.

The AW group had significantly more parents with pollen allergy and more mothers with asthma, more maternal smoking during pregnancy, less exclusive breastfeeding time (the children had infant formula from birth) and higher childcare center attendance ($p \leq 0.04$ for all, Table 3).

Children in the AW group had significantly more recurrent respiratory infections, earlier reported infections with respiratory syncytial virus (RSV) and bacterial pneumonias compared with the HC group ($p \leq 0.01$). At the clinical follow-up of 130 children after a median of 11 weeks, (range 6 to 31 weeks), 76% had had one or several episodes of respiratory infections, 38% had made an unplanned visit to a doctor because of acute wheeze and 15% had been re-admitted to the hospital because of respiratory problems since the initial emergency visit.

Table 3. *Clinical characteristics of 130 preschool children with acute wheeze (AW) compared with 101 healthy age matched controls (HC).*

	AW (n=130)	**HC** (n=101)	**p-value**
Male, %	68	79	**0.02**
Age 6-24 months, %	75	69	0.30
Median age in months (min-max)[1]	17 (6-42)	18 (6-44)	0.34
Mother caucasian, %	82	76	0.22
Father caucasian, %	85	76	0.1
Asthma mother, %	27	7	**<0.01**
" father, %	12	7	0.23
Pollen allergy, mother, %	36	13	**<0.001**
" " father, %	28	9	**<0.001**
Breastfeeding, total time ≥ 4 months	68	75	0.30
No exclusive breastfeeding time, %	28	16	**0.04**
Attend Childcare Center, %	73	53	**<0.01**
Smoking during pregnancy mother, %	11	3	**0.02**
Current smoking at home, %	22	20	0.62
Furred pets at home, %	25	22	0.5
Current eczema, %	18	7	**0.02**
Reported clinical food reaction, %	15	3	**<0.01**
>6 respiratory infections a year, %	66	19	**<0.01**
Reported RSV infections ever, %	31	5	**<0.01**
Bacterial pneumonia ever[2], %	8	0	**0.01**
Total IgE ≥100 kU/L (%)	19	13	0.30
Total IgE, kU/L (median (min-max))	26.6 (0.7-810.7)	13.5 (0.2-414.1)	**0.03**
Neutrophils, 10^9/L (median (min-max))[2]			
acute visit	7.1 (1.6-27.8)	2.6 (0.9-11.7)	**<0.001**
3-month follow-up	3.4 (0.5-12.9)		0.30
1-year follow-up	3.5 (0.1-9.4)		0.22
Eosinophils,10^9 /L (median (min-max))[3]			
acute visit	0.1 (0.0-4.6)	0.2 (0.1-2.1)	**<0.001**
3-month follow-up	0.3 (0.1-6.8)		**0.001**
1-year follow-up	0.3 (0.1-1.1)		**0.06**

[1] Median age at the inclusion of the study of the 130 children that came back for the second visit and healthy control children

[2]Definition of bacterial pneumonia: fever >38°C, CRP >50 mg/L and confluent infiltrates on X-rays and treated with antibiotics, or diagnosis made by the pediatrician at the Emergency Ward.

[3] Blood cell counts were analyzed in n=96 control children, and in children with acute wheeze; n =149 at visit 1, n=120 at visit 2 and n=106 at visit 3

6.3 SENSITIZATION (I, II, IV)

The percentage of children with total serum IgE>100 kUA/L was similar in the AW group and the HC group (19% *vs* 13%, p=0.30). No significant difference was found between the two study groups with regard to sensitization towards airborne allergens (phadiatop, 9 % *vs* 4%, p=0.14.) In the AW group, 4 children reported allergic rhinitis; one reacted towards birch, one against cat and two against grass. All children were asymptomatic in the HC group. There was no difference in the number of children sensitized to food allergens (fx5) between the groups

(24% *vs* 20%, p=0.49). Of children reporting clinical food reactions in the AW group (n=28), 30% (n=8) were positive in the fx5 panel (>0.35 kUA/L). Two of the children in the HC group reported clinical food reactions, and one of these had an fx5>0.35 kUA/L. Nine children in the AW group showed positive IgE against peanuts and one of the children in the HC group.

6.4 VITAMIN D (I)

Serum levels of 25(OH)D were analyzed in blood samples obtained at the first follow-up visit from 114 of children in the AW group, and from 99 children in the HC group. 25(OH)D insufficiency with values <75 nmol/L was found in 39% (n= 44) of children with acute wheeze and in 24% (n=24) of the control children (p=0.04). A significant difference between the two groups was also observed using 25(OH)D levels as a continuous variable (p=0.03) (Figure 7A). There was a significant correlation between 25(OH)D levels and age (p<0.001). When the age groups 6 to 24 months and 25 to 46 months were studied separately, the older children had a significantly lower median vitamin D level in both study groups (p<0.001 for both, Figure 7B). Atopy levels with specific sensitization, eczema or reported food allergies were not associated with 25(OH)D insufficiency. The degree of bacterial or viral colonization was no different in children with 25(OH)D insufficiency compared to 25(OH)D sufficient children.

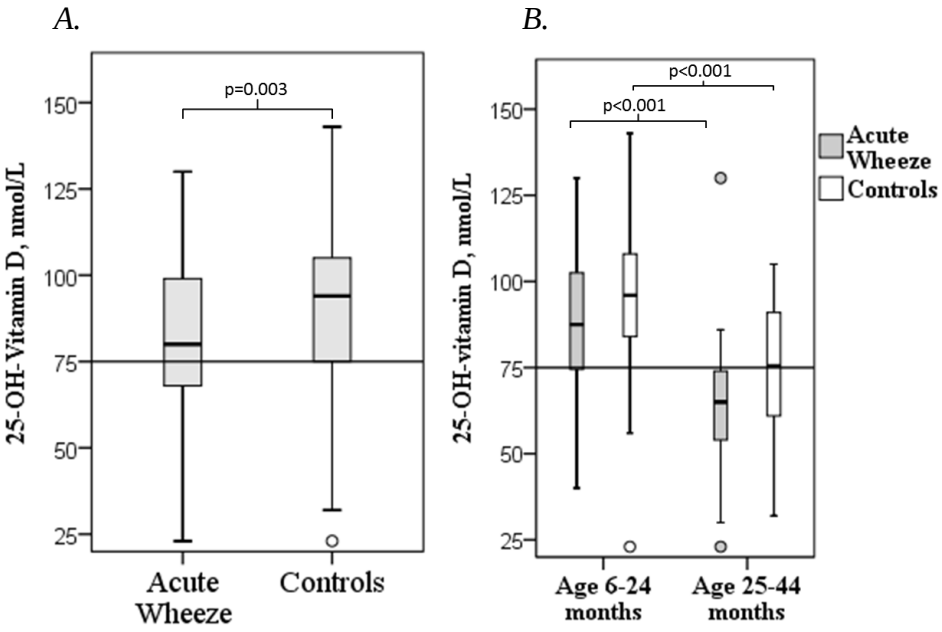

Figure 7. *Levels of 25(OH)D in a group of children with acute wheeze and a healthy control group (p=0.03) (A) and in two age groups (p<0.001 for both) (B).*

6.5 DETECTION OF VIRUS ON A 15 VIRUS PLATFORM AND BACTERIA (I)

Virus was detected in 69% of 154 children tested in the AW group at the acute visit. Rhinovirus was most common (36%, n= 56), followed by respiratory syncytial virus (17% n=26) (Table 4). Bacteria were found in nasopharyngeal cultures from 70% (n=52) of the 88 children tested. *Moraxella catarrhalis* was the most common bacterial finding, detected in 59% (n=52) of the children followed by *Streptococcus pneumoniae* (24%, n=21) and *Hemophilus influenzae* (19%, n=17). When detected, *M. catarrhalis* was the single pathogen in 40% (n=21) of cases. (In paper I, the results are reported on the 130 children that came back for a second follow-up visit, of which 76 children were tested for bacteria.)

Table 4. *Viruses detected in 154 children during the acute episode of wheeze, and in 121 children at the 3 month follow-up visit.*

Virus	**Acute visit** n=154		**Follow-up visit,** n=121	
	n	*%*	*n*	*%*
Adenovirus	*7*	4	*1*	1
Bocavirus	*17*	11	*6*	5
Coronaviruses	*9*	6	*6*	5
Enterovirus	*30*	19	*5*	4
Non typable Entero-/ Rhinovirus	*9*	6	*0*	0
Influenza A virus	*1*	1	*1*	1
Parainfluenzaviruses 1-3	*5*	3	*1*	1
Rhinovirus	*56*	36	*17*	14
RS virus	*26*	17	*4*	3
Metapneumovirus	*4*	2	*2*	2

6.6 RHINOVIRUS SPECIES (II, III)

Of 108 children in the AW group tested for different RV species (RV-A, RV-B or RV-C), 74% (n=80) had detectable RV (RV-A 20%, n=16, RV-B 5%, n=4 and RV-C 61%, n=49, Figure 8). A combination of two different RV species was detected in ten children (nine with RV-A and -C, one with RV-B and -C), (Figure 8). At the follow-up visit after approximately three months, RV species was reanalyzed in 102 of the children, and of these only 4 were positive for RV, all with a different species compared to the acute visit.

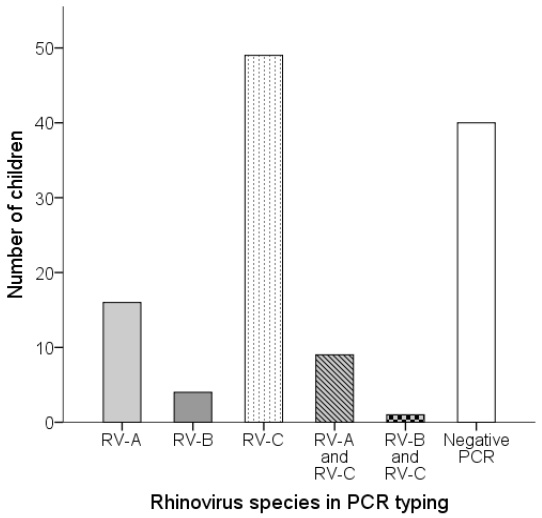

Figure 8. *Rhinovirus subtypes detected during an acute episode of wheeze in 108 preschool children.*

6.7 RV-SPECIFIC IgG_1 AND IgA ANTIBODIES (II, III)

Of the 120 children in the AW group with measurements of RV-specific IgG_1 and IgA levels at the acute visit and at the follow-up visit three months later, 92% (n=111) had prior IgG_1 antibodies against one or several RVs. An increase in RV-specific IgG_1 antibodies was seen in 61% (n=73) of the children at the follow-up visit (Table 5). The results of the IgA analyses were similar to IgG_1, but lower (Table 5). The initial levels of RV-IgG_1 measured at the acute visit did not correlate with reported time with respiratory symptoms until the follow-up visit. Irrespective of the RV species detected at the acute visit, an increase in IgG_1 against RV-A (RV89, 16 and/or 2), or against both RV-A (RV89, 16, 2) and RV-C (RV-YP) were significantly associated with more respiratory symptoms (p=0.03 for children with RV-A (RV89,16 and/or 2) and p=0.007 for children with both RV-A (RV89,16 and/or 2) and RV-C (RV-YP), Figure 9).

Table 5. *Presence and increase in RV-specific IgA and IgG$_1$ antibodies in 120 preschool children with acute wheeze and at follow-up (median 11 weeks later).*

RV subtypes	**IgA at acute visit** n=108 (90%)		**IgA increase at follow-up** n=68 (57%)		**IgG$_1$ at acute visit** n=111 (92%)		**IgG$_1$ increase at follow-up** n=73 (61%)	
	n	%	*n*	%	*n*	%	*n*	%
RV-A89	*104*	87	*45*	38	*102*	85	*62*	52
RV-A16	*97*	81	*42*	35	*103*	86	*39*	32
RV-A2	*104*	87	*50*	42	*106*	88	*47*	39
RV-A89,16,2	*96*	80	*38*	32	*100*	83	*42*	35
RV-B14	*65*	54	*23*	19	*73*	61	*20*	16
RV-C	*80*	67	*29*	24	*93*	78	*26*	22

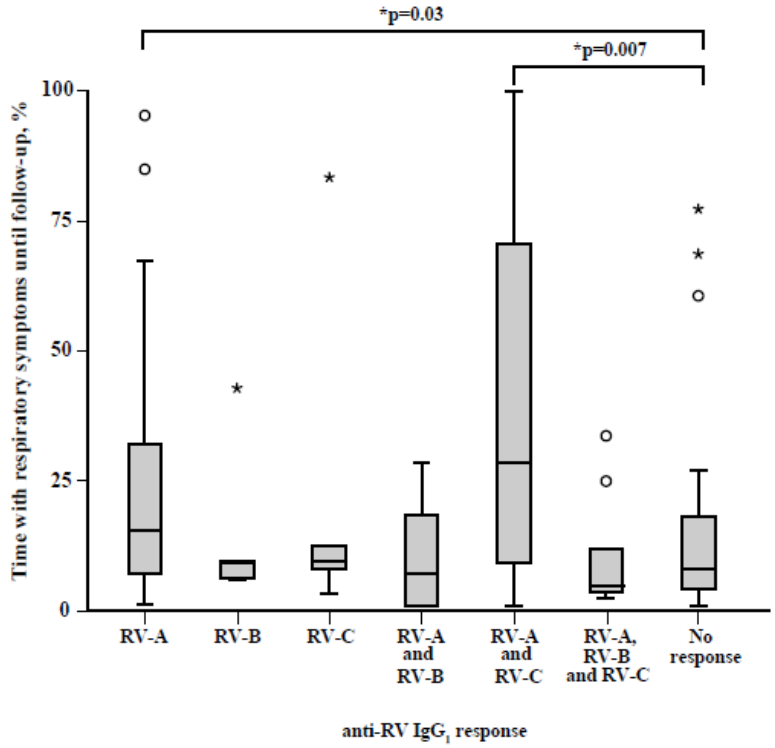

Figure 9. *Irrespective of RV species detected at the acute visit, children showing a RV-specific IgG$_1$ increase against RV-A and both RV-A and RV-C reported the longest time with respiratory symptoms until follow-up.*

6.8 *CDHR3* rs6967330 G>A AND mRNA EXPRESSION (III)

DNA was available from 122 preschool children with AW and 94 children from the HC group in whom the rs6967330 G>A polymorphism in the *CDHR3* gene was analyzed. Significant associations were found between the rs6967330-A allele and AW, and on a genotypic level AG/AA was associated with AW (p= 0.0006 and p=0.002, respectively).

We also analyzed the level of expression of *CDHR3* in a subgroup of 50 children in the AW group compared to 17 children in the HC group. *CDHR3* mRNA levels at the acute visit were 0.56 times the expression level in the HC group (p=0.001, Figure 10). At the follow-up visit, 2-3 months later, the same children in remission showed less of a reduction in *CDHR3* mRNA expression compared to that observed during the acute phase (p=0.001, Figure 10). Children in the AW group with the AA/AG genotype expressed less *CDHR3* mRNA at the acute visit than the follow-up visit (p=0.005).

At a clinical level, significantly more children with the AG/AA genotype (42%, n=31) compared to the GG genotype (20%, n=10) required emergency care due to respiratory symptoms between the acute visit and follow-up visit 2-3 months later (p=0.01).

No correlation between *CDHR3* mRNA and RV species detected at the acute visit could be found, but children with increased RV-specific IgG_1 levels against both RV-A and RV-C at the follow-up visit showed significantly reduced levels of *CDHR3* expression (p=0.006).

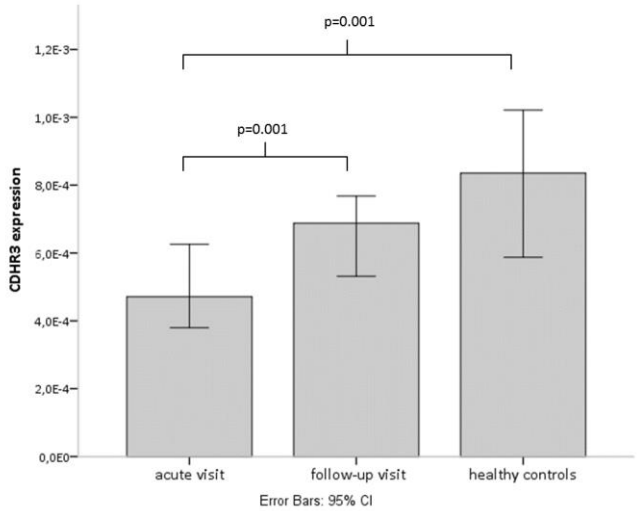

Figure 10. *Comparing levels of CDHR3 mRNA expression in children during an acute episode of wheeze and at follow-up after 2-3 months, as well as in a group of age-matched healthy controls. Data are presented as* $2^{-\Delta Ct}$.

6.9 *CHI3L1* rs4950928 C>G AND YKL-40 (IV)

DNA was available from 123 children in the AW group and 96 children from the HC group and the gene variant *CHI3L1* rs4950928-131C>G was analyzed. Genotype frequency was found to be similar in the two groups. (CC 63%, CG 31%, GG 6% respective CC 55%, CG 39%, GG 6%, p=0.51). *CHI3L1* rs4950928 affected YKL-40 in all subjects, with highest levels present in those with the CC genotype (p<0.001).

Serum levels of YKL-40 were examined in the AW group (n=128 from the acute visit, n=120 children from the second visit 3 months later and n=95 children after 1 year) and in 100 children from the HC group. The AW group had higher levels at the acute visit (median 14.7 ng/ml, p<0.001) and 3-month follow-up (median 15.9 ng/ml, p<0.001) compared to the 1-year follow-up (median 11.9 ng/ml).

YKL-40 levels in the HC group (median 13.6 ng/ml) tended to be lower than in the AW group during the first acute visit (p=0.07) and the second 3-month follow-up (p=0.04), but were no different at the 1-year follow-up.

7 DISCUSSION

7.1 STUDY DESIGN AND INCLUSION CRITERIA

This thesis is based on the first year of a longitudinal prospective study of a group of preschool children with wheeze (AW) and an age-matched group of healthy control children (HC). The children were aged 6 months ≤4 years at inclusion. They were approached in the emergency unit during an airway infection causing an acute episode of wheeze. The children in this study will have yearly follow-up visits until the age of seven, which offers a unique opportunity to follow clinical and immunological changes, and investigate genetic variants with the overall aim to find early biomarkers for the development of chronic asthma. The HC group will also have a second follow-up visit when they are seven. The inclusion criteria were based on the fact that most children with wheeze before 4 years of age have not yet developed allergies and are found to have a neutrophilic airway inflammation, (65, 66) making it difficult in this thesis to evaluate the prognosis for risk of persistent asthma.

The HC group was recruited at the Children's Surgical Day-Care Ward where they had minor day surgery performed, and selection bias for this group of children related to the variables and outcomes used in this study seems unlikely in view of the inclusion criteria. In comparison with the BAMSE cohort study, (designed to study risk factors for asthma, allergic diseases and lung function in childhood), which covers children living in the same inclusion area in Stockholm, the proportion of children with parental allergy, lifestyle factors such as the presence of furry pets, breastfeeding and ethnicity did not differ to any major extent with the HC group in our study. (67, 68)

The group of children in this study is small given that asthma as a disease is so heterogeneous, and the prevalence of severe asthma is low. The prevalence of asthma is reported to have increased continuously during primary school ages, with allergic sensitization and a family history of asthma being the most important risk factors. (69). In a study of 10 year old children in Norway, the prevalence of severe asthma was 0.5% in all 10 year olds, and 4.5% among current asthmatics. (70) Nevertheless, our group of wheezing children is at higher risk of developing asthma than the general population, and constitutes a valuable source of clinical and biological data, providing material for future studies on asthma development.

7.2 CLINICAL CHARACTERISTICS (I)

Environmental and hereditary risk factors for wheeze are well known, including maternal smoking during pregnancy, short breastfeeding time, high childcare attendance and allergy in mother and/or father (11), and these were also confirmed in our study as significant risk factors

in the AW group. Childcare attendance as a risk factor for asthma has however been debated. In earlier studies, it has been shown that children with greater exposure to other children at home or daycare were more likely to experience frequent wheeze at the age of 2 years, but less likely to have frequent wheeze from the age of 6 onwards. (71, 72) Childcare attendance before the age of 12 months seems to protect against later asthma development. (72)

The most striking difference between the AW group and the HC group was the number of reported respiratory infections during the past year: the average number of expected colds in a preschool child is approximately 6 infections/year and this was reported by 58% of the AW group but only 19% of the control children ($p<0.001$). This difference indicates a vulnerability of the immune system in the AW group, possibly reflecting an impaired ability to clear virus and/or bacterial infections. Recent studies have pointed out that the number of episodes of wheeze before one year of age, but not the particular viral trigger, was associated with later asthma development (73).

One may speculate about other environmental risk factors that could contribute to the wheezing phenotype of our AW group in the study. In the majority of cases, the children were living in areas north of Stockholm City. This area has several large, heavily trafficked roads as well as two airports. The control children lived in a more scattered area over the whole of Stockholm including the suburban areas on the south side without airports, since Astrid Lindgren Children's Hospital is the only hospital with a pediatric surgery department in Stockholm. There is an increasing awareness of early-life exposure to traffic-related air pollution (TRAP) and increased risk of persistent wheeze, and long-term exposure to TRAP with asthma development. (74, 75) Of the 120 children that came back for the third follow-up after a year, 90% had ICS medication and 37% took ICS medication continuously, 34% reported more than 100 days with inhaled β_2-agonist use.

7.3 ALLERGIC SENSITIZATION (I)

Rhinovirus infections have been associated with wheeze and allergic sensitization, (76) and Jackson et al reported that sensitization to aeroallergens leads to viral wheeze, but viral infection does not lead to sensitization. (77) In our study the AW group reported significantly more clinical symptoms of food allergy than the HC group, but the number of children positive for allergen-specific IgE antibodies did not differ significantly between the groups. In the AW group, 9% (n=11) were sensitized against aeroallergens, but 24% (n=22) were sensitized against food (6 children were sensitized against both), including 9 children with peanut allergy

(7%). Taken together these findings confirm a low prevalence of allergic sensitization in our group of children with viral wheeze.

Interestingly, one of the children with very high levels of antibodies against peanuts in the AW group, had never to the parents knowledge tasted peanuts, and had never suffered a reaction, indicating another route of sensitization, possibly through external exposure. An association between peanut allergy and the intake of soy milk, skin rash and the use of skin preparations containing peanut oil has been reported. (78) Recent studies have found an independent effect of IgE sensitization towards food allergens and local and systemic markers of inflammation in asthma, independent of allergy against aeroallergens (79, 80), but no association between asymptomatic sensitization to food allergens and asthma suggesting that there may be other links between food allergy and asthma. (80) Twenty-six (20%) of the children with AW had eczema, and of these 6 were sensitized to food, but only two displayed clinical symptoms. The initiation of sensitization via cutaneous exposures has received a great deal of current attention due the recent discovery that mutations in the filaggrin gene, which induce a defect in the skin barrier, have been linked to both atopic dermatitis and asthma, as well as peanut allergy. (81-83) One may speculated that recurrent RV infections could contribute to sensitization not only by affecting the airway epithelia, but also by having a systemic effect on the immune system with aggravated eczema symptoms as a result. Intervention studies have shown a reduction in the risk of developing childhood asthma in high risk children if both dietary and environmental allergen exposures are reduced, but not if only aeroallergens are avoided. (84)

7.4 VITAMIN D (I)

We found that 25(OH)D concentrations were inversely correlated with age, which is in agreement with previous studies on children and adults. (85, 86) There seems to be a drop in 25(OH)D levels after approximately 2 years of age in both the AW group and the HC group, which might be due to the fact that this is the time point/age at which free supplementation of vitamin D from childcare centers in Sweden comes to an end.

The children in the AW group had significantly lower serum levels of 25(OH)D than the HC group, but the majority of the children could still not be regarded as showing vitamin D-insufficiency, as only 4 of the children in the AW group and one in the HC group had levels below 50 nmol/L. The reference values for 25(OH)D are evaluated according to bone health, but evidence is still insufficient for other outcomes e.g. respiratory health. (15) It has been reported that other limits might be more correct, since the cell-regulatory actions of 1,25-dihydroxyvitamin D_3 are found at 25(OH)D concentrations higher than 75 nmol/L. (14)

One may speculate that children in the AW group with recurrent infections might have a dysfunction whereby vitamin D conversion to the active hormone 1,25-dihydroxyvitamin D_3 in tissue is impaired, or whereby regulation of the vitamin D receptor (VDR) and the vitamin D binding protein (DBP) is somehow altered compared to the HC group. VDR activation by the binding of 1,25-dihydroxyvitamin D_3 affects the expression of over 900 genes, and genome-wide studies have identified several genes that contribute to 25(OH)D variability. (87) Dysregulation of the VDR may lead to exaggerated inflammatory responses associated with immune-mediated diseases such as Crohn's disease and tuberculosis, characterized by an imbalance in helper T-cell development. Genetic variants in VDR have also been identified as risk factors for asthma. (88, 89) More detailed future investigations of DBG function may be important since the DBG has shown an association with circulating 25(OH)D concentration in healthy children. (90, 91) Tissue-specific production of 1,25-dihydroxyvitamin D_3 that is important both for the innate and the adaptive immune systems, has been identified. (92) 1,25-dihydroxyvitamin D_3 may be involved in direct modulation of the T-cell antigen receptor, important in T-cell activation. (93) Furthermore, macrophages can convert circulating 25(OH)D into 1,25 dihydroxyvitamin D_3 during a bacterial infection thus inducing the expression of genes encoding antimicrobial peptides which are central to the innate immune response. (94)

A difficulty in the diagnosis of vitamin D deficiency is the inconsistency between different measurement methods. In our study, direct, competitive chemiluminescence analysis (CLIA; DiaSorin Inc, Stillwater, MN, USA) was used as a routine procedure at the Karolinska University Hospital Laboratory and this method has been reported to show a higher proportion of low values. (95) Other studies have also shown considerable differences in measurements compared with those obtained at a certified laboratory, and differences have even been observed between laboratories using the same assays. (96)

Significant associations between lower vitamin D levels and a greater use of inhaled glucocorticoids have been shown, indicating that lower vitamin D levels could contribute to a more severe asthmatic condition. (97) In our study, we did not find a correlation between "time with steroid inhalation" and vitamin D levels in the AW group. 42% were treated with ICS at the acute visit (17% continuously), and at the first follow-up visit 75% had ICS (42% continuously). In this age group it is difficult to evaluate possible relationships between 25(OH)D levels and steroid inhalation, since vitamin D levels decrease with age, and usually the children with recurrent wheeze begin ICS treatment after an episode severe enough to require a visit to the emergency department and meet a pediatrician, which is usually also after

they began to attend childcare centers after one year of age. Steroid resistance might also be related to a more chronic airway inflammation, but it is known that even children with wheeze may not always respond to steroids. (98)

7.5 VIRUS AND BACTERIA (I-III)

7.5.1 Rhinovirus detection

In this study, two different PCR-based methods have been used to detect RV using the same patient material. In paper I, the laboratory at Karolinska University Hospital used real time PCR with an established 15 virus platform (63) (n=154 children, 36% positive for RV) and in paper II, our collaborators in the Allergy and Pathophysiology Department at the Medical University of Vienna, Austria, used nested PCR to detect RV strains and to subtype them (n=108, 74% (n=80) positive for RV). A discrepancy in the results could be observed between the two methods; of the 108 children tested using the nested PCR method in paper II, only 36% (39/108) were positive according to the PCR technique used in the first paper. It should be noted that children later subtyped with RV-C were often not positive in the first analysis with real time PCR, only 37% (n=22 of 59 children with RV-C in paper II) were found with this method, the percentages for detection of RV-A and RV-B found in paper II were 48% and 75% respectively of those in paper I. At follow-up the detection rate was the opposite, the real time PCR detected 17 children with RV and the nested PCR only 4 children. In a study comparing the relative sensitivity of a cross-section of published RV-specific PCR primers (most of which were first published before RV-C was reported), a high degree of variability in performance was found, and none of the pairs could detect all RVs in 2 panels of genotyped clinical specimens, suggesting that inefficient RV detection by PCR may be a problem both in the clinic as well as in research studies. (99)

7.5.2 Rhinovirus and bacterial co-infections

The contribution of bacterial colonization to the severity of respiratory symptoms is being discussed, and some studies have reported an aberrant immune response to bacteria in infancy in children with later asthma development. Both bacteria and viruses were independently shown to be associated with wheeze (100-102).

Our current investigations regarding bacteria as co-pathogens, were similar to earlier studies performed on children with wheeze in Denmark (100). The distribution of bacterial detection in the Danish study was approximately equal for *S. pneumoniae* (46%), *H. Influenzae* (44%) and *M.catarrhalis* (50%). (100) Interestingly, in our study the presence of *M.catarrhalis* was the same as the Danish study, but fewer cases were observed with

S. pneumonia and *H. Influenzae* (59%, 24% and 19% respectively). There is a discussion concerning whether the introduction of pneumococcal vaccines in the general vaccination program can alter colonization patterns in children, and cause a selection and increase in colonization and infection by *M. catarrhalis*. (103) The children in the Danish cohort study were born between 1998-2001, and this was before vaccination against *S.pneumoniae* was introduced into the Danish vaccination program in 2007. (100, 104) In our study 25% of the children enrolled were born before 2009 when Sweden introduced vaccinations against *S.pneumoniae*. When looking at the pattern of bacteria in our study in children born before or after 2009, we found no difference (p=0.75 for *M. catarrhalis*, p=0.80 for *H. Influenzae*, p=0.49 for *S. pneumoniae*), which suggests that the introduction of vaccination against *S.pneumoniae* did not alter colonization pattern in our study children.

In a study where children aged 4-12 years provided nasal samples over a period of 5 consecutive weeks in the autumn, detection of RV increased the risk of detecting bacteria within the same sample or that obtained the following week. In particular, detecting *M. catarrhalis* alongside RV increased the likelihood of experiencing a cold and/or asthma symptoms compared with isolated detection of RV. (105) In our study, children with RV had *M. catarrhalis* as a co-pathogen in 59% of cases, and *M. catarrhalis* was also found alone in nine of children negative for RV, indicating the ability of this bacteria alone to induce wheeze. It has recently been reported that epithelial cells infected with *M. catarrhalis* showed increased expression of RV compared with uninfected cells, and that *M. catarrhalis* can reduce antiviral defense functions of bronchial epithelial cells by diminished secretion of IFN-β and IFN-λ. (106) This may increase susceptibility to viral infections, and in our group also symptoms of wheeze.

7.5.3 Rhinovirus species

Differences in the pathogenicity of different RV-species is currently under debate. In general, RV-A and RV-C are a more common finding in children with wheeze, than RV-B, and RV-B is associated with milder disease, which might be a reason why RV-B is detected at a lower frequency in hospital-based studies (107). RV-C was discovered in respiratory samples of patients from New York with influenza-like illness in 2004, and in infants with bronchiolitis from Queensland hospitals in 2006 (108, 109) where it was reported to cause more severe lower respiratory tract infections than RV-A or RV-B. Recent reports however found that the clinical manifestations of infections appear to be similar between RV-A and RV-C. (110, 111) In our group of preschool children with acute wheeze, the reported time with symptoms until

follow-up visit was the same in groups with different species of RV, also in children with double virus species.

7.5.4 Serum levels of RV-specific IgA and IgG_1

The antiviral effector mechanisms of the adaptive immune system involve the formation of neutralizing antibodies whereby serotype-specific neutralizing serum antibodies (IgG) as well as secretory antibodies (IgA) are developed in the airways. (49) In our study, levels of RV-specific IgA and IgG_1 in plasma were measured, both at the acute visit and at the follow-up visit 2-3 months later. The reason IgG_1 and IgA were examined was based on earlier experimental studies of RV infections in adults, performed by our collaborators in the Allergy and Pathophysiology Department at the Medical University of Vienna, where RV-specific IgG_1 and IgA were found to show the strongest responses. (112) No children with wheeze included in our study had an IgA-deficiency, but given that innate immunity in the nasal mucosa most likely relies upon secretory IgA, and our measurements were of serum levels, the interpretation of IgA levels is difficult and we did not include these results in the paper (II). IgG_1 can remain elevated for years after a RV infection, making this easier to relate to reported illness. (113, 114) We found that children with increased RV-specific IgG_1 against RV-A and RV-C at the follow-up visit, reported the longest time spent with respiratory symptoms between the visits, which is similar to adults experimentally infected with RV where individuals with more severe manifestations of respiratory symptoms showed the highest increases in RV-specific IgG_1 antibodies. (112) In our study, older children had the highest levels of RV-specific IgG_1 antibodies at the acute visit, but their increase was not stronger compared with that of the younger children (paper II). It is not clear whether or not an increase in RV-specific antibodies is an indicator for risk of asthma later in life, but it is known that asthmatic individuals have higher levels of IgG_1 antibodies against RV than non-asthmatics. (115)

7.6 *CDHR3* AND mRNA EXPRESSION (III)

In our study (III) we genotyped AW and HC children for the rs6967330 A>G polymorphism in the *CDHR3* gene. The risk allele A was significantly overrepresented in the AW group, both at the allelic (A) and genotypic (AG/AA) level.

In addition, we analyzed *CDHR3* mRNA expression in a subgroup of 50 children from the AW group and 17 children from the HC group. Levels of mRNA expression were measured in white blood cells from both the first acute episode and the second follow-up visit 2-3 months later. Interestingly, in contrast to earlier findings on HeLa cells *in vitro* (33), the mRNA levels

for *CDHR3* in children with AW with the AA/AG genotype were significantly reduced at the acute visit compared to the HC group, but at the follow-up visit 2-3 months later the same children in remission showed a smaller reduction in *CDHR3* mRNA compared to HC. This is in agreement with a recent study of *CDHR3* mRNA expression in human epithelial cells cultured *ex vivo*, where cells from asthmatics showed lower expression than cells from non-asthmatics. (116) Interestingly, they also showed that expression was further reduced by RV infection with RV-A16, and that levels correlated with RV infection. (116)

We did not find an association between expression levels of *CDHR3* mRNA and the RV species detected at the acute visit in our study, but children with increased RV-specific IgG_1 levels against both RV-A and RV-C at follow-up did show significantly reduced levels of *CDHR3* at the acute visit compared to follow-up. This is in parallel with our earlier finding (paper II), where children with increased RV-specific IgG_1 against both RV-A and RV-C at the follow-up visit reported most symptoms between the acute and follow-up visits. Since these results are from a subgroup of a small study, larger studies are needed to confirm the finding.

Asthma has been associated with over a hundred different genes, but a major problem in many genetic studies is the lack of replication of results, only a subset of identified genes has been found to be associated with asthma in more than one study. (117) An exception however has been the finding that the *ORMDL3* gene is associated with childhood-onset asthma worldwide. (118, 119)

Again, our study highlights the difficulty in trying to investigate genetic findings, the degree of expression of an asthma susceptibility gene and the influence it has on clinical severity. It also points out the importance of cell-specific investigations when investigating expression levels. As acute wheeze primarily affects the airways, the most relevant samples would be, e.g. bronchial epithelial cells or bronchial biopsies. Blood is more accessible due to a less invasive sampling method, and changes in blood could be assumed to mimic the expression pattern in the airways. Our attempts to draw conclusions from genetic findings, expression levels and clinical data have to be interpreted cautiously, but hopefully may provide ideas for larger studies in the future.

7.7 *CHI3L1* GENE VARIANT AND YKL-40 (IV)

Levels of YKL-40 are associated with airway inflammation in both children and adults with asthma (58, 120), We therefore wanted to investigate whether this was also the case at an early age in a high-risk, preschool group of children with acute airway infection. The gene variant

CHI3L1 rs4950928 and protein levels of the chitinase-like protein YKL-40 were analyzed in the AW group and the HC group, and we found that rs4950928 variants strongly affected YKL-40 levels, but we did not find a difference in the distribution of rs4950928 genotypes among the AW and HC groups. Circulating YKL-40 levels were increased at the acute visit and the 3-month follow-up in children with AW compared to the HC group, but not at the one-year follow-up suggesting that the serum YKL-40 may be increased during acute infections.

Dividing children into those with and without more severe respiratory symptoms based on symptoms and treatment was generally not associated with higher YKL-40 levels. Interestingly, significantly higher YKL-40 levels were observed in children taking montelukast at the acute visit, which could indirectly suggest higher YKL-40 levels in a group of children with more severe airway disease requiring asthma medication, however, this difference was not observed at follow-up visits during remission. We therefore conclude that although YKL-40 may be increased during an acute episode of airway inflammation, it is unlikely to be a useful biomarker of persistent wheeze in this age group, possibly because the biological processes in which YKL-40 is involved in chronic adult asthma are not occurring at this early age.

In the present study, YKL-40 levels and circulating neutrophil numbers were found to correlate. This is in line with previous studies of schoolchildren with severe asthma. (56) Neutrophils are a known source of YKL-40, and are increased in the circulation during acute infections. (56) In studies of children with cystic fibrosis, a disease characterized by neutrophilic airway inflammation, the difficulty of trying to investigate local inflammation in the airways using a systemic blood test were highlighted as YKL-40 levels were high in the sputum but only moderately elevated in serum. (121) In adults, allergen challenge has been shown to cause local increases in YKL-40 as determined by increased sputum and BAL levels, but these changes were not mirrored in the circulation. (122) In addition, it has recently been shown that well differentiated human airway epithelial cells express *CHI3L1*, and mechanical stress during bronchoconstriction trigger epithelial cells and induce *CHI3L1* expression leading to local secretion of YKL-40 protein. (123)

Earlier studies have examined YKL-40 levels in very young children and found that levels are higher at birth and then plateau up to 5 years of age. (60) When we plotted serum YKL-40 concentrations for the different patient groups divided into 6 month intervals, it did not reveal higher levels in the younger children, or an increase with age, suggesting that over the age span of 6 months to 4 years, YKL-40 levels do not seem to be affected by age. Even though YKL-40 is not useful as a biomarker for risk of chronic asthma in this age group, our investigation has nevertheless contributed to knowledge of normal YKL-40 levels in preschool children.

8 MAIN RESULTS AND CONCLUSIONS

In the first study (I) we found that the preschool children attending the emergency department with acute wheeze shared known hereditary and environmental risk factors such as a family history of asthma and allergy, maternal smoking during pregnancy, less exclusive breastfeeding time and higher childcare center attendance. Children with wheeze had lower vitamin D levels, significantly more clinical food allergies and recurrent airway infections which may indicate an impairment in the immune system and reduced ability to combat viral infections, along with an increased risk of developing allergies. In addition, we detected both viruses and bacteria in the majority of children with wheeze, possibly suggesting that bacterial colonization could contribute to the severity of respiratory symptoms.

In the second study (II) RV was detected in nasopharyngeal samples in the majority of children with acute wheeze. RV-C was the most common finding, but symptoms and medication did not differ from children with RV-A infection. Children with an IgG_1 increase against both RV-A and RV-C reported most respiratory symptoms until revisit, irrespective of the RV species found at the acute visit, possibly indicating synergistic effects of RV-A and RV-C and a more severe inflammation.

In the third study (III) the asthma susceptibility gene variant *CDHR3* rs6967330: G>A encoding the RV-C receptor cadherin-related family member 3, was analyzed. Carriers of the risk allele-A were found to be overrepresented in the AW group. Children with the AG/AA genotype showed the lowest levels of *CDHR3* mRNA in the acute phase, as well as the largest differences in *CDHR3* mRNA expression between the acute visit and follow-up, possibly indicating a group with increased vulnerability towards RV infections.

Children with increased RV-specific IgG_1 levels against both RV-A and RV-C at remission showed significantly reduced levels of *CDHR3* expression during the acute phase compared to follow-up, possibly indicating synergistic effects of RV-A and RV-C and a more severe inflammation.

In the fourth study (IV) the chitinase-3-like-1 (*CHI3L1)* rs4950928 genotypes were investigated and found to show a similar distribution in the AW and HC groups. Circulating levels of the chitinase-like protein YKL-40, encoded by the *CHI3L1* gene, were elevated in the AW group compared with the HC group at the acute visit and at the follow-up visit three months later, but not at the one-year follow-up. These findings speak against YKL-40 as a useful biomarker of persistent wheeze in this young age group.

The levels of YKL-40 in serum in the present study were associated with the number of circulating neutrophils, indicating a reflection of a neutrophilic inflammation with an increase in the number of neutrophils, or possibly neutrophils producing more YKL-40.

In conclusion, preschool children in the AW group had several environmental and hereditary risk factors for the later development of asthma, as well as lower levels of vitamin D. We investigated whether specific RV-species could contribute to a more severe form of wheeze, but did not find any associations between reported symptoms and detected species. Bacteria were identified as co-pathogens in the majority of children with wheeze, possibly indicating a synergistic effect of virus and bacteria, creating an excessive immune reaction in the airways of children with wheeze. We found an association between increased RV-specific IgG_1 against both RV-A and RV-C, and longer time spent with reported respiratory symptoms at the first follow-up visit. When investigating the asthma susceptibility gene variant *CDHR3* rs6967330 and *CDHR3* mRNA expression, we found reduced mRNA levels in children with wheeze, especially in children with increased RV-specific IgG_1 levels against both RV-A and RV-C at remission. One may speculate that reduced mRNA levels indicate a reduced cadherin production, with increased permeability of the airways through destroyed epithelial cell tight junctions as a consequence, leading to exaggerated inflammation and increased vulnerability to infection. The proposed biomarker YKL-40 did not facilitate the identification of children with persistent airway inflammation in this young age group with acute wheeze, but was correlated with levels of neutrophils in acute inflammation.

Taken together, most of our findings indicate that children with wheeze constitute a group of children with an increased vulnerability, both immunologically as well as genetically, who are at high risk of developing asthma during later childhood.

9 FUTURE PERSPECTIVES

In this study we have shown that among children with wheeze, there is a subgroup with a possible aberrant immune system and a genetic vulnerability that could result in an increased risk of later asthma development. This group of children may require close attention from an early age, with optimal medication and the reduction of risk factors in the environment if possible. Future studies will hopefully identify preventable weaknesses in the innate and adaptive immunity, and make it possible to decrease the number of airway infections, and prevent later problems with airway hyper-responsiveness, and maybe also the development of an airborne allergy in high-risk children with wheeze. Further studies on the effect of vitamin D supplementation in children with suboptimal levels in combination with symptoms of wheeze are needed, as well as studies on genetic variants regulating the vitamin D receptor and the vitamin D-binding protein.

Research into the association of RV with asthma and allergy development will continue and hopefully give us tools to prevent and attenuate infections, by vaccine development, the use of antimicrobial peptides, or other ways to strengthen the innate immune system and eradicate co-infections by colonizing bacteria.

The pursuit of easily attainable biomarkers, that reflect the progression of inflammation in the airways, needs to be continued and will hopefully be successful since it is important with an individualized assessment of inflammation, even in young children with wheeze who are at risk of more chronic inflammation.

This study highlights the importance of combining genetic analyses with detailed clinical data from smaller selected groups of children, as a model for larger, more targeted studies.

All children in this study are being followed until school-age with yearly follow-ups, and with the combined collection of clinical data, lung function measurements, as well as yearly blood samples they offer a valuable research material for future studies, and will contribute to our understanding of the interplay between environmental and genetic factors in the development and progression of asthma and allergy.

10 SAMMANFATTNING PÅ SVENSKA

Astma är globalt sett den vanligaste kroniska sjukdomen hos barn. Antalet barn som har eller som insjuknar i astma har under lång tid ökat fram till 2008, nu verkar ökningen avstannat med sjunkande antal fall de senaste åren. (2) Många av de äldre barnen har en bakomliggande allergi som orsak till sin astma, men en stor andel av de yngre barnen har inte någon allergi men får ändå astma. (11)

Ett av huvudsymptomen vid astma är ett visslande ljud vid utandning (eng: wheeze) som beror på att slemhinnan i luftrören blir svullen och inflammerad, och musklerna i luftrören drar ihop sig, barnen får hosta och svårt att få luft. Små förskole barn med förkylningsutlöst astma har ökad risk att utveckla kronisk luftrörsinflammation i skolåldern. (4) Vissa virus verkar vara kopplat till astma, t ex vårt vanligaste förkylningsvirus, Rhinovirus (RV). Oftast orsakar rhinovirus bara en lindrig snuva, men de barn som får förkylningsastma av rhinovirus har högre risk att ha kvar astmabesvär vid skolstart. (4)

Ett stort antal genetiska studier har försökt att hitta genetiska varianter som ger en ökad risk att få astma. Det har i många fall gjorts genom att studera stora födelse grupper med många tusen individer för att hitta ett område eller gen på en kromosom som skiljer friska och sjuka individer åt. Detta har gjort att man sett hur miljöfaktorer ex cigarettrök, luftföroreningar, virusinfektioner kan påverka en individ som har en viss känslig genotyp att bli sjuk.

I vår studie ingår 156 förskolebarn i åldern 6 månader till 4 år, som sökt barnakuten på Astrid Lindgrens Barnsjukhus i Stockholm med akuta andningsbesvär pga virus infektion (AW). Även 101 åldersmatchade friska kontroller (HC) utan förkylningsastma eller luftburen allergi inkluderades som jämförande grupp i samband med att de genomgick en mindre operation på dagvården. Föräldrarna till barnen i båda grupperna har svarat på frågeformulär angående ärftlighet för astma och allergi, miljöfaktorer som rökning under graviditet och tidigare luftvägsinfektioner och allergiska symptom hos barnen. Barnen har lämnat blodprover, AW gruppen vid tre tillfällen (akut, vid återbesök efter 2-4 månader samt efter 1 år), kontrollerna i samband med operationen efter att de blivit inkluderade i studien. Barnen i AW gruppen har dessutom tagit näsprov med avseende på virus och bakterieförekomst akut och vid första återbesöket efter 2-3 månader.

Studie I: I den första studien ingick de 130 barn som kom tillbaka på det första återbesöket efter tre månader och 101 kontroller. Vi jämförde sedan tidigare kända riskfaktorer för astma mellan AW och HC grupperna, inklusive antikroppar mot luftburna allergen (björk, timotej, gråbo, katt, hund, häst, mögel och kvalster) och födoämnesallergen (komjölk, ägg,

soja, jordnöt, torsk, vete). Vitamin D nivåer i blodet analyserades också, då studier har visat att låga D-vitamin nivåer ger ökad risk för återkommande virusinfektioner, (20) och astma. (21, 22)

Vi fann att barn i AW gruppen hade flera riskfaktorer för astma jämfört med kontrollerna (ökad ärftlighet för astma/allergi, fler mammor som rökte under graviditeten, mindre tid med enbart amning utan samtidig komjölksbaserad ersättning, flera som gick på förskolan). AW gruppen hade också lägre nivåer av vitamin D, betydligt fler luftvägsinfektioner samt rapporterade mer födoämnesreaktioner än HC gruppen.

Studie II: Det finns tre typer av rhinovirus: RV-A, RV-B och RV-C. Det är framför allt RV-A och RV-C som man hittar hos barn som söker med andningssvårigheter på akutmottagningen. Vissa studier anser att RV-C är mer sjukdomsframkallande, andra studier visar att RV-A och RV-C ger lika mycket besvär. I den andra studien tittade vi på vilka typer av RV barnen hade vid akutbesöket samt om nivåerna av RV specifika antikroppar ökade vid återbesöket jämfört med nivåerna vid akutbesöket. RV var vanligt, och hittades hos 75 % av barnen, RV-C var vanligast (74% av barnen där vi hittade RV). Vi såg ingen koppling mellan RV typ vid det akuta besöket och hur länge barnen var sjuka eller använde astmamediciner. Barn med ökning av antikroppar mot både RV-A och RV-C vid återbesöket hade haft mest luftvägssymtom, vilket kanske beror på en förstärkt effekt av flera virus samtidigt och en svårare inflammation.

Studie III: RV-A och RV-B upptäcktes på 50-talet, RV-C upptäckte man runt 2006. Man har inte förrän nyligen vetat hur RV-C fäster till cellerna i luftvägarna och orsakar infektion, men nu har man upptäckt proteinet som fungerar som receptor, (cadherin-related family member 3). Genen som kodar för det proteinet heter *CDHR3*. I en stor studie på flera tusen barn (Genome Wide Association Study, GWAS) kopplade man nyligen en genetisk variant av genen (*CDHR3* rs6967330 G>A) till förkylningsastma hos förskolebarn. Vi analyserade DNA från blod på AW och HC gruppen och jämförde förekomsten av den genvarianten, och såg att barnen i AW gruppen hade betydligt fler med riskvarianten.

Vi jämförde också nivåerna av budbärar RNA (mRNA, av engelskans messenger-RNA) i två mindre grupper (50 barn från AW gruppen och 17 barn från HC gruppen) Budbärar-RNA för över information från DNA i cellkärnan och deltar sedan i framställning av protein-molekyler i cellen. Barnen som hade risk varianten av genen visade betydligt lägre nivåer av mRNA både akut och vid första återbesöket jämfört med kontrollerna. Den RV typ som barnen hade då de var på akutbesöket verkade inte påverka mRNA nivåerna, men barnen med

förhöjda RV-specifika antikroppar mot både RV-A och RV-C när de kom på återbesöket efter ett par månader hade lägst mRNA nivåer jämfört med barn med antikroppar mot bara ett RV.

Om lägre mRNA nivåer betyder att det bildas mindre protein, kan det innebära en ökad genomsläpplighet och känslighet i luftvägarnas slemhinnor vid RV infektion, med en svårare inflammation i luftvägarna som följd.

Studie IV: I den fjärde studien har vi tittat på nivåerna av ett visst protein som kallas för YKL-40. Höga nivåer av det proteinet har hittats i blodet hos många individer som har olika kroniska inflammationer, och också hos barn och vuxna med svår astma. (58-60) Protein produktionen styrs av en gen (*CHI3L1* (Chitinase-3-like-1)) Det finns flera genvarianten som påverkar YKL-40 nivåerna och genvarianten *CHI3L1* rs4950928 (C/G) har visat sig vara en sådan. Vi undersökte hur genvarianterna var fördelade i AW gruppen och HC gruppen, och fann inte någon skillnad. Vi jämförde också nivåerna av YKL-40 i plasma hos AW gruppen vid akut besök, återbesök efter tre månader och efter ett år och kontrollgruppen. YKL-40 nivåerna var högre i AW gruppen vid de två första besöken, men på samma nivå som kontrollerna vid årsbesöket då det inte var någon skillnad. Nivån av YKL-40 var kopplat till antalet vita blodkroppar (neutrofiler) i blodet, och kan vara en reflektion av en pågående icke allergisk inflammation. (60, 61)

Sammanfattningsvis har vi i denna studie följt en grupp barn med återkommande obstruktiva besvär vid infektioner, och de uppvisar flera riskfaktorer både ärftliga och miljö faktorer men också en immunologisk och genetisk känslighet jämfört med en grupp friska kontroller utan obstruktiva besvär.

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13 REFERENCES

1 Network GA. The Global Asthma Report 2014. Auckland, New Zealand 2014.

2 Akinbami LJ, Simon AE, Rossen LM. Changing Trends in Asthma Prevalence Among Children. *Pediatrics*. 2016; **137**.

3 Engelkes M, Janssens HM, de Ridder MA, de Jongste JC, Sturkenboom MC, Verhamme KM. Time trends in the incidence, prevalence and age at diagnosis of asthma in children. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2015; **26**: 367-74.

4 Jackson DJ, Gangnon RE, Evans MD, *et al.* Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *American journal of respiratory and critical care medicine*. 2008; **178**: 667-72.

5 Brand PL, Baraldi E, Bisgaard H, *et al.* Definition, assessment and treatment of wheezing disorders in preschool children: an evidence-based approach. *The European respiratory journal*. 2008; **32**: 1096-110.

6 Schultz A, Brand PL. Episodic viral wheeze and multiple trigger wheeze in preschool children: a useful distinction for clinicians? *Paediatric respiratory reviews*. 2011; **12**: 160-4.

7 Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *The New England journal of medicine*. 1995; **332**: 133-8.

8 Young S, Arnott J, O'Keeffe PT, Le Souef PN, Landau LI. The association between early life lung function and wheezing during the first 2 yrs of life. *The European respiratory journal*. 2000; **15**: 151-7.

9 Guerra S, Lohman IC, Halonen M, Martinez FD, Wright AL. Reduced interferon gamma production and soluble CD14 levels in early life predict recurrent wheezing by 1 year of age. *American journal of respiratory and critical care medicine*. 2004; **169**: 70-6.

10 Goksor E, Amark M, Alm B, Gustafsson PM, Wennergren G. Asthma symptoms in early childhood--what happens then? *Acta paediatrica*. 2006; **95**: 471-8.

11 Strina A, Barreto ML, Cooper PJ, Rodrigues LC. Risk factors for non-atopic asthma/wheeze in children and adolescents: a systematic review. *Emerging themes in epidemiology*. 2014; **11**: 5.

12 Holick MF. Vitamin D: evolutionary, physiological and health perspectives. *Curr Drug Targets*. 2011; **12**: 4-18.

13 Cashman KD, Dowling KG, Skrabakova Z, *et al.* Vitamin D deficiency in Europe: pandemic? *The American journal of clinical nutrition*. 2016; **103**: 1033-44.

14 Misra M, Pacaud D, Petryk A, *et al.* Vitamin D deficiency in children and its management: review of current knowledge and recommendations. *Pediatrics*. 2008; **122**: 398-417.

15 Ross AC, Manson JE, Abrams SA, *et al.* The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *The Journal of clinical endocrinology and metabolism*. 2011; **96**: 53-8.

16 Holick MF, Chen TC, Lu Z, Sauter E. Vitamin D and skin physiology: a D-lightful story. *J Bone Miner Res*. 2007; **22 Suppl 2**: V28-33.

17 Moan J, Dahlback A, Porojnicu AC. At what time should one go out in the sun? *Advances in experimental medicine and biology*. 2008; **624**: 86-8.

18 Kerley CP, Elnazir B, Faul J, Cormican L. Vitamin D as an adjunctive therapy in asthma. Part 2: A review of human studies. *Pulmonary pharmacology & therapeutics*. 2015; **32**: 75-92.

19 Lee JH, O'Keefe JH, Bell D, Hensrud DD, Holick MF. Vitamin D deficiency an important, common, and easily treatable cardiovascular risk factor? *J Am Coll Cardiol*. 2008; **52**: 1949-56.

20 Cannell JJ, Vieth R, Umhau JC, *et al.* Epidemic influenza and vitamin D. *Epidemiology and infection*. 2006; **134**: 1129-40.

21 Litonjua AA, Weiss ST. Is vitamin D deficiency to blame for the asthma epidemic? *The Journal of allergy and clinical immunology*. 2007; **120**: 1031-5.

22 Arikoglu T, Kuyucu S, Karaismailoglu E, Batmaz SB, Balci S. The association of vitamin D, cathelicidin, and vitamin D binding protein with acute asthma attacks in children. *Allergy and asthma proceedings : the official journal of regional and state allergy societies*. 2015; **36**: 51-8.

23 Kho AT, Sharma S, Qiu W, *et al.* Vitamin D related genes in lung development and asthma pathogenesis. *BMC Med Genomics*. 2013; **6**: 47.

24 Hansdottir S, Monick MM, Hinde SL, Lovan N, Look DC, Hunninghake GW. Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *Journal of immunology*. 2008; **181**: 7090-9.

25 Hansdottir S, Monick MM. Vitamin D effects on lung immunity and respiratory diseases. *Vitam Horm*. 2011; **86**: 217-37.

26 Aranow C. Vitamin D and the immune system. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research*. 2011; **59**: 881-6.

27 Savolainen C, Blomqvist S, Mulders MN, Hovi T. Genetic clustering of all 102 human rhinovirus prototype strains: serotype 87 is close to human enterovirus 70. *The Journal of general virology*. 2002; **83**: 333-40.

28 McErlean P, Shackelton LA, Andrews E, *et al.* Distinguishing molecular features and clinical characteristics of a putative new rhinovirus species, human rhinovirus C (HRV C). *PloS one*. 2008; **3**: e1847.

29 Stone CA, Jr., Miller EK. Understanding the Association of Human Rhinovirus with Asthma. *Clinical and vaccine immunology : CVI*. 2016; **23**: 6-10.

30 Jacobs SE, Lamson DM, St George K, Walsh TJ. Human rhinoviruses. *Clin Microbiol Rev*. 2013; **26**: 135-62.

31 Papadopoulos NG, Sanderson G, Hunter J, Johnston SL. Rhinoviruses replicate effectively at lower airway temperatures. *Journal of medical virology*. 1999; **58**: 100-4.

32 Rossmann MG, Arnold E, Erickson JW, *et al.* Structure of a human common cold virus and functional relationship to other picornaviruses. *Nature*. 1985; **317**: 145-53.

33 Bochkov YA, Watters K, Ashraf S, *et al.* Cadherin-related family member 3, a childhood asthma susceptibility gene product, mediates rhinovirus C binding and replication. *Proceedings of the National Academy of Sciences of the United States of America*. 2015; **112**: 5485-90.

34 Bonnelykke K, Sleiman P, Nielsen K, *et al.* A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. *Nature genetics*. 2014; **46**: 51-5.

35 Kowalczyk AP, Nanes BA. Adherens junction turnover: regulating adhesion through cadherin endocytosis, degradation, and recycling. *Sub-cellular biochemistry*. 2012; **60**: 197-222.

36 Leckband D, Sivasankar S. Cadherin recognition and adhesion. *Current opinion in cell biology*. 2012; **24**: 620-7.

37 Nelson WJ, Dickinson DJ, Weis WI. Roles of cadherins and catenins in cell-cell adhesion and epithelial cell polarity. *Progress in molecular biology and translational science*. 2013; **116**: 3-23.

38 Martinez FD. New insights into the natural history of asthma: primary prevention on the horizon. *The Journal of allergy and clinical immunology*. 2011; **128**: 939-45.

39 Papadopoulos NG, Christodoulou I, Rohde G, *et al.* Viruses and bacteria in acute asthma exacerbations--a GA(2) LEN-DARE systematic review. *Allergy*. 2011; **66**: 458-68.

40 Lukkarinen M, Jartti T. The first rhinovirus-wheeze acts as a marker for later asthma in high-risk children. *The Journal of allergy and clinical immunology*. 2016; **138**: 313.

41 Gern JE. Rhinovirus and the initiation of asthma. *Current opinion in allergy and clinical immunology*. 2009; **9**: 73-8.

42 Wark PA, Johnston SL, Bucchieri F, *et al.* Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *The Journal of experimental medicine*. 2005; **201**: 937-47.

43 Lemanske RF, Jr., Jackson DJ, Gangnon RE, *et al.* Rhinovirus illnesses during infancy predict subsequent childhood wheezing. *The Journal of allergy and clinical immunology*. 2005; **116**: 571-7.

44 Yeo NK, Jang YJ. Rhinovirus infection-induced alteration of tight junction and adherens junction components in human nasal epithelial cells. *The Laryngoscope*. 2010; **120**: 346-52.

45 Atkinson SK, Sadofsky LR, Morice AH. How does rhinovirus cause the common cold cough? *BMJ Open Respir Res*. 2016; **3**: e000118.

46 Kelly JT, Busse WW. Host immune responses to rhinovirus: mechanisms in asthma. *The Journal of allergy and clinical immunology*. 2008; **122**: 671-82; quiz 83-4.

47 Katze MG, He Y, Gale M, Jr. Viruses and interferon: a fight for supremacy. *Nat Rev Immunol*. 2002; **2**: 675-87.

48 Leech SC, Price JF, Holmes BJ, Kemeny DM. Nonatopic wheezy children have reduced interferon-gamma. *Allergy*. 2000; **55**: 74-8.

49 Message SD, Johnston SL. Host defense function of the airway epithelium in health and disease: clinical background. *Journal of leukocyte biology*. 2004; **75**: 5-17.

50 Gern JE, Brooks GD, Meyer P, *et al.* Bidirectional interactions between viral respiratory illnesses and cytokine responses in the first year of life. *The Journal of allergy and clinical immunology*. 2006; **117**: 72-8.

51 Gern JE, Dick EC, Kelly EA, Vrtis R, Klein B. Rhinovirus-specific T cells recognize both shared and serotype-restricted viral epitopes. *The Journal of infectious diseases*. 1997; **175**: 1108-14.

52 Ilarraza R, Wu Y, Skappak CD, Ajamian F, Proud D, Adamko DJ. Rhinovirus has the unique ability to directly activate human T cells in vitro. *The Journal of allergy and clinical immunology*. 2013; **131**: 395-404.

53 Glanville N, Johnston SL. Challenges in developing a cross-serotype rhinovirus vaccine. *Current opinion in virology*. 2015; **11**: 83-8.

54 Jolle\s P, Muzzarelli RAA. *Chitin and chitinases*. Basel: Birkha\0308user 1999.

55 Kirkpatrick RB, Emery JG, Connor JR, Dodds R, Lysko PG, Rosenberg M. Induction and expression of human cartilage glycoprotein 39 in rheumatoid inflammatory and peripheral blood monocyte-derived macrophages. *Exp Cell Res*. 1997; **237**: 46-54.

56 Volck B, Price PA, Johansen JS, *et al.* YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils. *Proceedings of the Association of American Physicians*. 1998; **110**: 351-60.

57 Johansen JS. Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. *Dan Med Bull*. 2006; **53**: 172-209.

58 Konradsen JR, James A, Nordlund B, *et al.* The chitinase-like protein YKL-40: a possible biomarker of inflammation and airway remodeling in severe pediatric asthma. *The Journal of allergy and clinical immunology*. 2013; **132**: 328-35 e5.

59 Specjalski K, Jassem E. YKL-40 protein is a marker of asthma. *The Journal of asthma : official journal of the Association for the Care of Asthma*. 2011; **48**: 767-72.

60 Renkema GH, Boot RG, Au FL, *et al.* Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur J Biochem*. 1998; **251**: 504-9.

61 Lee CG, Da Silva CA, Dela Cruz CS, *et al.* Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu Rev Physiol*. 2011; **73**: 479-501.

62 Rathcke CN, Holmkvist J, Husmoen LL, *et al.* Association of polymorphisms of the CHI3L1 gene with asthma and atopy: a populations-based study of 6514 Danish adults. *PloS one*. 2009; **4**: e6106.

63 Tiveljung-Lindell A, Rotzen-Ostlund M, Gupta S, *et al.* Development and implementation of a molecular diagnostic platform for daily rapid detection of 15 respiratory viruses. *Journal of medical virology*. 2009; **81**: 167-75.

64 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; **25**: 402-8.

65 McDougall CM, Helms PJ. Neutrophil airway inflammation in childhood asthma. *Thorax*. 2006; **61**: 739-41.

66 Stevenson EC, Turner G, Heaney LG, *et al.* Bronchoalveolar lavage findings suggest two different forms of childhood asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 1997; **27**: 1027-35.

67 Lannero E, Wickman M, van Hage M, Bergstrom A, Pershagen G, Nordvall L. Exposure to environmental tobacco smoke and sensitisation in children. *Thorax*. 2008; **63**: 172-6.

68 Kull I, Melen E, Alm J, *et al.* Breast-feeding in relation to asthma, lung function, and sensitization in young schoolchildren. *The Journal of allergy and clinical immunology*. 2010; **125**: 1013-9.

69 Bjerg-Backlund A, Perzanowski MS, Platts-Mills T, Sandstrom T, Lundback B, Ronmark E. Asthma during the primary school ages--prevalence, remission and the impact of allergic sensitization. *Allergy*. 2006; **61**: 549-55.

70 Lang A, Carlsen KH, Haaland G, *et al.* Severe asthma in childhood: assessed in 10 year olds in a birth cohort study. *Allergy*. 2008; **63**: 1054-60.

71 Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. *The New England journal of medicine*. 2000; **343**: 538-43.

72 Nicolaou NC, Simpson A, Lowe LA, Murray CS, Woodcock A, Custovic A. Day-care attendance, position in sibship, and early childhood wheezing: a population-based birth cohort study. *The Journal of allergy and clinical immunology*. 2008; **122**: 500-6 e5.

73 Bonnelykke K, Vissing NH, Sevelsted A, Johnston SL, Bisgaard H. Association between respiratory infections in early life and later asthma is independent of virus type. *The Journal of allergy and clinical immunology*. 2015; **136**: 81-86 e4.

74 Brunst KJ, Ryan PH, Brokamp C, *et al.* Timing and Duration of Traffic-related Air Pollution Exposure and the Risk for Childhood Wheeze and Asthma. *American journal of respiratory and critical care medicine*. 2015; **192**: 421-7.

75 Esposito S, Galeone C, Lelii M, *et al.* Impact of air pollution on respiratory diseases in children with recurrent wheezing or asthma. *BMC pulmonary medicine*. 2014; **14**: 130.

76 Jartti T, Kuusipalo H, Vuorinen T, *et al.* Allergic sensitization is associated with rhinovirus-, but not other virus-, induced wheezing in children. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2010; **21**: 1008-14.

77 Jackson DJ, Evans MD, Gangnon RE, *et al.* Evidence for a causal relationship between allergic sensitization and rhinovirus wheezing in early life. *American journal of respiratory and critical care medicine*. 2012; **185**: 281-5.

78 Lack G, Fox D, Northstone K, Golding J, Avon Longitudinal Study of P, Children Study T. Factors associated with the development of peanut allergy in childhood. *The New England journal of medicine*. 2003; **348**: 977-85.

79 Patelis A, Janson C, Borres MP, Nordvall L, Alving K, Malinovschi A. Aeroallergen and food IgE sensitization and local and systemic inflammation in asthma. *Allergy*. 2014; **69**: 380-7.

80 Schroeder A, Kumar R, Pongracic JA, *et al.* Food allergy is associated with an increased risk of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2009; **39**: 261-70.

81 McLean WH, Irvine AD. Heritable filaggrin disorders: the paradigm of atopic dermatitis. *J Invest Dermatol*. 2012; **132**: E20-1.

82 McLean WH. The allergy gene: how a mutation in a skin protein revealed a link between eczema and asthma. *F1000 Med Rep*. 2011; **3**: 2.

83 Brown SJ, Asai Y, Cordell HJ, *et al.* Loss-of-function variants in the filaggrin gene are a significant risk factor for peanut allergy. *The Journal of allergy and clinical immunology*. 2011; **127**: 661-7.

84 Maas T, Kaper J, Sheikh A, *et al.* Mono and multifaceted inhalant and/or food allergen reduction interventions for preventing asthma in children at high risk of developing asthma. *The Cochrane database of systematic reviews*. 2009: CD006480.

85 Jartti T, Ruuskanen O, Mansbach JM, Vuorinen T, Camargo CA, Jr. Low serum 25-hydroxyvitamin D levels are associated with increased risk of viral coinfections in wheezing children. *The Journal of allergy and clinical immunology*. 2010; **126**: 1074-6, 76 e1-4.

86 Alvarez-Rodriguez L, Lopez-Hoyos M, Garcia-Unzueta M, Amado JA, Cacho PM, Martinez-Taboada VM. Age and low levels of circulating vitamin D are associated with impaired innate immune function. *Journal of leukocyte biology*. 2012; **91**: 829-38.

87 Schlingmann KP, Kaufmann M, Weber S, *et al.* Mutations in CYP24A1 and idiopathic infantile hypercalcemia. *The New England journal of medicine*. 2011; **365**: 410-21.

88 Sun J. Vitamin D and mucosal immune function. *Curr Opin Gastroenterol*. 2010; **26**: 591-5.

89 Poon AH, Laprise C, Lemire M, *et al.* Association of vitamin D receptor genetic variants with susceptibility to asthma and atopy. *American journal of respiratory and critical care medicine*. 2004; **170**: 967-73.

90 Powe CE, Evans MK, Wenger J, *et al.* Vitamin D-binding protein and vitamin D status of black Americans and white Americans. *The New England journal of medicine*. 2013; **369**: 1991-2000.

91 Navas-Nazario A, Li FY, Shabanova V, *et al.* Effect of vitamin D-binding protein genotype on the development of asthma in children. *Ann Allergy Asthma Immunol*. 2014; **112**: 519-24.

92 Adams JS, Hewison M. Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. *Nat Clin Pract Endocrinol Metab*. 2008; **4**: 80-90.

93 von Essen MR, Kongsbak M, Schjerling P, Olgaard K, Odum N, Geisler C. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. *Nat Immunol*. 2010; **11**: 344-9.

94 White JH. Vitamin D as an inducer of cathelicidin antimicrobial peptide expression: past, present and future. *The Journal of steroid biochemistry and molecular biology*. 2010; **121**: 234-8.

95 Snellman G, Melhus H, Gedeborg R, *et al.* Determining vitamin D status: a comparison between commercially available assays. *PloS one*. 2010; **5**: e11555.

96 Black LJ, Anderson D, Clarke MW, Ponsonby AL, Lucas RM, Ausimmune Investigator G. Analytical Bias in the Measurement of Serum 25-Hydroxyvitamin D Concentrations Impairs Assessment of Vitamin D Status in Clinical and Research Settings. *PloS one*. 2015; **10**: e0135478.

97 Searing DA, Zhang Y, Murphy JR, Hauk PJ, Goleva E, Leung DY. Decreased serum vitamin D levels in children with asthma are associated with increased corticosteroid use. *The Journal of allergy and clinical immunology*. 2010; **125**: 995-1000.

98 Ducharme FM, Krajinovic M. Steroid responsiveness and wheezing phenotypes. *Paediatric respiratory reviews*. 2011; **12**: 170-6.

99 Faux CE, Arden KE, Lambert SB, *et al.* Usefulness of published PCR primers in detecting human rhinovirus infection. *Emerg Infect Dis*. 2011; **17**: 296-8.

100 Bisgaard H, Hermansen MN, Bonnelykke K, *et al.* Association of bacteria and viruses with wheezy episodes in young children: prospective birth cohort study. *BMJ*. 2010; **341**: c4978.

101 Larsen JM, Brix S, Thysen AH, Birch S, Rasmussen MA, Bisgaard H. Children with asthma by school age display aberrant immune responses to pathogenic airway bacteria as infants. *The Journal of allergy and clinical immunology*. 2014; **133**: 1008-13.

102 Liang Z, Zhang Q, Thomas CM, *et al.* Impaired macrophage phagocytosis of bacteria in severe asthma. *Respir Res*. 2014; **15**: 72.

103 Dunne EM, Manning J, Russell FM, Robins-Browne RM, Mulholland EK, Satzke C. Effect of pneumococcal vaccination on nasopharyngeal carriage of Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, and Staphylococcus aureus in Fijian children. *Journal of clinical microbiology*. 2012; **50**: 1034-8.

104 Ingels H, Rasmussen J, Andersen PH, *et al.* Impact of pneumococcal vaccination in Denmark during the first 3 years after PCV introduction in the childhood immunization programme. *Vaccine*. 2012; **30**: 3944-50.

105 Kloepfer KM, Lee WM, Pappas TE, *et al.* Detection of pathogenic bacteria during rhinovirus infection is associated with increased respiratory symptoms and asthma exacerbations. *The Journal of allergy and clinical immunology*. 2014; **133**: 1301-7, 07 e1-3.

106 Heinrich A, Haarmann H, Zahradnik S, *et al.* Moraxella catarrhalis decreases antiviral innate immune responses by down-regulation of TLR3 via inhibition of p53 in human bronchial epithelial cells. *FASEB J*. 2016; **30**: 2426-34.

107 Lauinger IL, Bible JM, Halligan EP, *et al.* Patient characteristics and severity of human rhinovirus infections in children. *J Clin Virol*. 2013; **58**: 216-20.

108 Lamson D, Renwick N, Kapoor V, *et al.* MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004-2005. *The Journal of infectious diseases*. 2006; **194**: 1398-402.

109 McErlean P, Shackelton LA, Lambert SB, Nissen MD, Sloots TP, Mackay IM. Characterisation of a newly identified human rhinovirus, HRV-QPM, discovered in infants with bronchiolitis. *J Clin Virol*. 2007; **39**: 67-75.

110 Lee WM, Lemanske RF, Jr., Evans MD, *et al.* Human rhinovirus species and season of infection determine illness severity. *American journal of respiratory and critical care medicine*. 2012; **186**: 886-91.

111 Iwane MK, Prill MM, Lu X, *et al.* Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. *The Journal of infectious diseases*. 2011; **204**: 1702-10.

112 Niespodziana K, Cabauatan CR, Jackson DJ, *et al.* Rhinovirus-induced VP1-specific Antibodies are Group-specific and Associated With Severity of Respiratory Symptoms. *EBioMedicine*. 2015; **2**: 64-70.

113 Message SD, Johnston SL. The immunology of virus infection in asthma. *The European respiratory journal*. 2001; **18**: 1013-25.

114 Barclay WS, al-Nakib W, Higgins PG, Tyrrell DA. The time course of the humoral immune response to rhinovirus infection. *Epidemiology and infection*. 1989; **103**: 659-69.

115 Iwasaki J, Smith WA, Khoo SK, *et al.* Comparison of rhinovirus antibody titers in children with asthma exacerbations and species-specific rhinovirus infection. *The Journal of allergy and clinical immunology*. 2014; **134**: 25-32.

116 Bai J, Smock SL, Jackson GR, Jr., *et al.* Phenotypic responses of differentiated asthmatic human airway epithelial cultures to rhinovirus. *PloS one*. 2015; **10**: e0118286.

117 Thomsen SF. Genetics of asthma: an introduction for the clinician. *Eur Clin Respir J*. 2015; **2**.

118 Moffatt MF, Gut IG, Demenais F, *et al.* A large-scale, consortium-based genomewide association study of asthma. *The New England journal of medicine*. 2010; **363**: 1211-21.

119 Moffatt MF, Kabesch M, Liang L, *et al.* Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature*. 2007; **448**: 470-3.

120 Ober C, Tan Z, Sun Y, *et al.* Effect of variation in CHI3L1 on serum YKL-40 level, risk of asthma, and lung function. *The New England journal of medicine*. 2008; **358**: 1682-91.

121 Fantino E, Gangell CL, Hartl D, Sly PD, Arest CF. Airway, but not serum or urinary, levels of YKL-40 reflect inflammation in early cystic fibrosis lung disease. *BMC pulmonary medicine*. 2014; **14**: 28.

122 Lee JH, Park KH, Park JW, Hong CS. YKL-40 in induced sputum after allergen bronchial provocation in atopic asthma. *J Investig Allergol Clin Immunol*. 2012; **22**: 501-7.

123 Park JA, Drazen JM, Tschumperlin DJ. The chitinase-like protein YKL-40 is secreted by airway epithelial cells at base line and in response to compressive mechanical stress. *The Journal of biological chemistry*. 2010; **285**: 29817-25.