INVESTIGATING PROBLEMATIC SEVERE ASTHMA IN CHILDREN – A TRANSLATIONAL APPROACH

Jon R Konradsen
When you can measure what you are speaking about and express it in numbers, you know something about it; but when you can not measure it, when you cannot express it in numbers, your knowledge is of a meager and unsatisfactory kind’

Lord Kelvin, 1891
Abstract

Children with problematic severe asthma (PA) have persistent symptoms and/or severe exacerbations despite treatment with high doses of currently available asthma medications. The term PA includes children who are difficult to treat due to unidentified exacerbating factors (e.g. allergens or environmental hazards, comorbidities, psychological and social issues, and/or poor adherence) and those lacking identifiable aggravating factors but, nonetheless, do not respond well to asthma therapy. Children with PA are a heterogeneous group of patients with varying clinical presentations, pulmonary function and patterns of inflammation.

This thesis is based on the results of a Swedish nationwide cross-sectional study in which school aged children with PA (n=57) were compared to age matched peers with persistent, but controlled asthma (CA), (n=39). The major objectives were to identify distinguishing features of children suffering from PA, to differentiate between children who were difficult to treat and those who were severely resistant to therapy and to investigate novel and potentially clinical relevant biomarkers of PA. PA was defined by insufficient asthma control despite high doses of inhaled corticosteroids.

The protocol included a detailed characterization of: history and clinical presentation; pulmonary function; bronchial hyperresponsiveness; inflammatory biomarkers in blood (including white blood cells, interleukin-5 and chitinases (chitotriosidase and the chitinase-like protein YKL-40)), urine (EPX) and exhaled air (FeNO); allergy (IgE antibodies, component resolved allergy diagnostics, basophil allergen threshold sensitivity (CD-sens)); morphology (computerized tomography of sinuses and lungs (in the PA group only)).

The major distinguishing features of children with PA involve familial background (heredity, socioeconomic status), clinical presentation (comorbidities and triggering factors) and pathophysiological differences including degree of airway obstruction, bronchial hyperresponsiveness and inflammatory profile (IL-5, number of eosinophilic and neutrophilic cells in blood). Sixty percent of children with PA had therapy-resistant asthma, with the remainder being difficult to treat due to identified aggravating factors.

Individual IgE-responses were similar between children with PA and CA. Children with PA were more often multi-sensitized to > 3 single lipocalin (nMus m 1, rEqu c 1, Fel d 4, rCan f 1, 2), kallikrein (rCan f 5) and secretoglobin (rFel d 1) allergens compared to children with CA. Cat-allergic children with PA had higher allergen sensitivity, as measured by CD-sens, compared to cat-allergic peers with CA. Furthermore, CD-sens correlated with clinical markers of asthmatic disease, including asthma control and biomarkers of eosinophilic inflammation.

YKL-40 levels and chitotriosidase activity were increased in the serum of children with PA, and YKL-40 specifically correlated with airway remodelling (as assessed by computerized tomography) and blood neutrophils in children severely resistant to asthma therapy.

By employing a comprehensive and standardized clinical assessment we have discerned specific features of children with PA and identified children who are severely resistant to therapy. We have applied two novel methods of allergy diagnostics (Component resolved diagnostics and CD-sens) and found that these two methods provide relevant information when investigating children with PA. Finally, our findings confirm that YKL-40 is a potential biomarker of asthma severity and airway remodeling. A translational research approach is necessary when investigating associations between disease mechanisms and clinical presentation in complex diseases.
LIST OF PUBLICATIONS

The thesis is based on the following papers. The papers will be referred to by their Roman numerals (I-IV).

Problematic severe asthma: A proposed approach to identifying children who are severely resistant to therapy.

IgE antibodies to animal-derived lipocalin, kallikrein and secretoglobin are markers of bronchial inflammation in severe childhood asthma.
PMID: 22339365 [Epub ahead of print].

High basophil allergen sensitivity is associated with severe allergic asthma in children.
PMID: 22432913 [Epub ahead of print].

* Have contributed equally to the preparation of this manuscript.
Chitinases are markers of airway remodeling in children with therapy resistant asthma.
Submitted.
Other publications/manuscripts related to this project:

Identifying problematic severe asthma in the individual child - does lung function matter?

Crystal structure of the dog lipocalin allergen Can f 2: implications for cross-reactivity to the cat allergen Fel d 4.

Nordlund B, Konradsen JR, Pedroletti C, Kull I, Hedlin G.
The clinical benefit of evaluating health-related quality-of-life in children with problematic severe asthma.

Christina Orsmark Pietras, Anna James, Jon R Konradsen, Björn Nordlund, Cilla Söderhäll, Christophe Pedroletti, Juha Kere, Sven Erik Dahlen, Gunilla Hedlin, Erik Melén.
Genome Wide Transcriptome Analysis Suggests Novel Mechanisms in Severe Childhood Asthma.
Manuscript in preparation.

Jon R Konradsen, Björn Nordlund, Åsa Wheelock, Hans Grönlund, Joachim Lundahl, Gunilla Hedlin.
Inflammatory cytokines in serum from children with severe asthma compared to controlled asthmatics.
Manuscript in preparation.

Jon R Konradsen, Björn Nordlund, Marika Lidegran, Christophe Pedroletti, Kjell Alving, Gunilla Hedlin.
High levels of exhaled Nitric oxide are associated with increased morbidity in children with persistent asthma.
Manuscript in preparation.
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<tbody>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BHR</td>
<td>Bronchial hyperresponsiveness</td>
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<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BWT</td>
<td>Bronchial wall thickening</td>
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<tr>
<td>CA</td>
<td>Controlled persistent asthma</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CD-sens</td>
<td>Basophil allergen threshold sensitivity</td>
</tr>
<tr>
<td>CI</td>
<td>Confidential interval</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DRS&lt;sub&gt;methacholine&lt;/sub&gt;</td>
<td>Slope of the dose-response curve for provocation with methacholine</td>
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<tr>
<td>ECP</td>
<td>Eosinophilic cationic protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPX</td>
<td>Eosinophilic protein x</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (and others)</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (for example)</td>
</tr>
<tr>
<td>FceRI</td>
<td>The high affinity IgE receptor</td>
</tr>
<tr>
<td>FcεRII</td>
<td>The low affinity IgE receptor</td>
</tr>
<tr>
<td>FEF&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Forced expiratory flow at 50% of forced vital capacity</td>
</tr>
<tr>
<td>FeNO</td>
<td>Fraction of nitric oxide in exhaled air</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume during one second</td>
</tr>
<tr>
<td>FRC</td>
<td>Functional residual capacity</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GA&lt;sup&gt;2&lt;/sup&gt;LEN</td>
<td>Global Allergy and Asthma European Network</td>
</tr>
<tr>
<td>GERD</td>
<td>Gastro esophageal reflux disease</td>
</tr>
<tr>
<td>GINA</td>
<td>Global initiative for asthma</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HRCT</td>
<td>High resolution computerized tomography</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>i.e.</td>
<td>Id est (that is)</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>ISAC</td>
<td>Immunosolid-phase allergen chip</td>
</tr>
<tr>
<td>kU/l</td>
<td>Kilounits per liter</td>
</tr>
<tr>
<td>kU&lt;sub&gt;A&lt;/sub&gt;/l</td>
<td>Kilounits allergen per liter</td>
</tr>
<tr>
<td>LABA</td>
<td>Long-acting β-2 agonist</td>
</tr>
<tr>
<td>LN</td>
<td>The natural logarithm</td>
</tr>
<tr>
<td>LTRA</td>
<td>Leukotriene receptor antagonist</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nNO</td>
<td>Fraction of nitric oxide in nasal air</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PA</td>
<td>Problematic severe asthma</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD20</td>
<td>The dose of methacholine producing a 20% reduction in FEV₁</td>
</tr>
<tr>
<td>p.p.b.</td>
<td>Parts per billion</td>
</tr>
<tr>
<td>PSACI</td>
<td>The Problematic severe asthma in childhood initiative</td>
</tr>
<tr>
<td>RBM</td>
<td>Reticular basement membrane</td>
</tr>
<tr>
<td>RV</td>
<td>Residual volume</td>
</tr>
<tr>
<td>SABA</td>
<td>Short acting β-2 agonist</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>YKL-40</td>
<td>A chitinase-like protein</td>
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<tr>
<td>WHO</td>
<td>World health organization</td>
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1 INTRODUCTION

Asthma is defined by airway inflammation, completely or partly reversible airway obstruction and bronchial hyperresponsiveness which collectively induce cough, dyspnoea and wheeze in the affected patient. Figure 1. Symptoms of asthma may be caused by allergen exposure, viral infections, physical exercise and airway irritants such as tobacco smoke.

![Diagram of asthma symptoms, airway inflammation, and abnormalities of airway function](image)

Figure 1. Relationships between symptoms, airway inflammation, and abnormalities of airway function, with permission from (2).

The prevalence of asthma increased during the second half of the 20\textsuperscript{th} century, (4-5). However the worldwide prevalence of childhood asthma seems to have reached a plateau during the two last decades (6) and in Swedish children (age 7-8), the prevalence of current wheeze is 13.0% whereas the prevalence of physician diagnosed asthma is 5.7% at age 7-8 and 7.7% at age 11-12 (7). Deaths from asthma were increasing until the end of the 20\textsuperscript{th} century (8) but this trend has also been broken (9). Nonetheless, 37 Swedish children and young adults died because of asthma from 1994 to 2008 (10), demonstrating that there are still children with uncontrolled disease who need special attention.

In an ideal situation, asthma should be suspected in a child with symptoms of airflow obstruction, and a spirometry should be performed to verify the diagnosis (11). Alternative diagnoses should be excluded, and whenever spirometry fails to confirm obstruction, a bronchial provocation test should be performed to demonstrate bronchial hyperresponsiveness. However, in clinical pediatric practice, the diagnosis of asthma is often based on clinical history and the response of the child to a trial of asthma medications, since both spirometry and assessment of bronchial hyperresponsiveness are troublesome, particularly in preschool children.

The goal of pediatric asthma treatment is to enable the children to control their symptoms, to be able to lead a normal active life, to have a normal lung function as well as prevent asthma exacerbations (12). A stepwise therapeutic approach is
applied to adjust medications according to symptoms and several guidelines have been published to support the physician in these treatment decisions (e.g. the Global initiative for asthma, National Asthma Education and Prevention Program).

Caring for children with asthma does not only include the prescription of asthma medications. The children and their families need to be convinced and educated so that the child actually takes the medication as prescribed, in a proper manner. Furthermore, health care providers must teach the families how to avoid or handle triggering factors, such as allergens, to recognize signs of asthma worsening and seek medical advice when needed.

The majority of children with asthma have mild or moderate disease and can obtain adequate control of symptoms through the avoidance of triggering factors and/or with the help of medications such as short acting inhaled β2-receptor agonists (SABA), inhaled corticosteroids (ICS) and when required, the addition of long acting β2-receptor agonists (LABA) and leukotriene receptor antagonists (LTRA) (13). However, approximately 5% of all children with asthma have chronic symptoms and/or recurrent exacerbations despite maximum treatment with conventional medications (14). These children are referred to as severe asthmatics, and due to the lack of specific biomarkers for this disease category, severe asthma is currently being defined on the basis of the intensity of the treatment required to improve asthma control, and the level of control achieved (15).

Children with such severe symptoms are heterogeneous with respect to triggering factors, pulmonary function (16) pattern of inflammation (17) and clinical symptoms (18-20). These children have a reduced quality of life (21), account for a large proportion of the health care costs related to asthma (22-23) and represent a continuous clinical challenge for the paediatrician (24). Previous investigations of patients with severe asthma, both adults (25-26) and children (20, 27), have involved varying definitions, geographical and ethnic backgrounds, inclusion criteria and study designs and the results are not necessarily applicable to Swedish children with severe asthma.

A GA2LEN task force, the Problematic Severe Asthma initiative has proposed the term Problematic Severe Asthma (PA) to define all children who suffer from chronic symptoms and/or severe exacerbations despite the prescription of several drugs (18, 28) and this concept has been incorporated in the recent WHO definition of severe asthma (29). The advantage of the newly introduced term is that it encompasses two groups of children; 1) children who after a thorough work up can be classified as difficult to treat because of identified exacerbating factors, and 2) children who following a thorough work up do not have any identifiable aggravating factors but are severely resistant to therapy. Figure 2.
Figure 2. Clinical classification of children presenting with Problematic severe asthma, from (18).
2 BACKGROUND

2.1 CLINICAL PRESENTATION
School-age children with PA either present with chronic symptoms on most days, or with acute exacerbations with or without symptoms between exacerbations. They might also present with persistent airflow obstruction which is unresponsive to a trial with steroids (18). Allergic comorbidity, including, rhinitis eczema and food allergies is common in this age group and severe anaphylactic asthma exacerbations after allergen exposure pose a particular risk to these children. Asthma symptoms are most commonly caused by exercise and infections. In these ages, adherence to prescribed treatment is still dependent upon carers but pulmonary function measurements and indirect assessments of inflammation can be reliably performed.

2.2 LONGITUDINAL STUDIES
There are only a few follow-up studies on children with PA; Fitzpatrick et al. found no changes in clinical presentation and severity in a 3 year follow-up of school age children (30) and Gupta et al. found that 34 of 44 school age children with PA still were treated with ICS and LABA, although one in six were well controlled on therapy in a 5 year follow up (31).

In the Melbourne study, 83 subjects with severe childhood asthma were followed from 10 years of age. At age 42, 90 % still had symptomatic asthma, and 45% had persistent asthma (32). The mechanism of the transition from predominantly atopic males in severe childhood asthma to non-atopic females (25) in severe adult asthma is largely unknown.

2.3 PULMONARY FUNCTION
Reversible obstruction of the airways is one of the pathophysiological hallmarks of asthma and is assessed objectively with spirometry. Forced expiratory volume in one second (FEV₁), has been considered the most helpful marker. FEV₁ has been shown to correlate with asthma symptoms, (33-34), exacerbations (35) and asthma control (36). However, some studies have failed to demonstrate significant correlation between FEV₁, daytime symptoms, quality of life and the need for rescue medication (37-39) and furthermore, pulmonary function might be normal in many children with asthma (11).

A decreased FEV₁ has also been used to define severe asthma in children in clinical studies (27, 40) but this criterion is an adaption from adult medicine and is not validated in a childhood population. Specific studies on severe asthma in childhood have yielded inconsistent results regarding pulmonary function, some studies found that FEV₁ was on average lower in children presenting with PA although the magnitude of airflow limitation was significantly less in children, compared to adults with severe asthma (27, 41). Other studies found no difference in FEV₁ on average (14, 16, 42-43), but there were large individual variations (44). Taken together, the results from these studies implicate that the usefulness of FEV₁ as indicator of severity in childhood asthma remains unclear.

2.4 BRONCHIAL HYPERRESPONSIVENESS
Bronchial hyperresponsiveness (BHR) is another characteristic feature of
asthma (45) and means that the lower airways constrict in response to a stimulus (i.e. allergen, tobacco smoke) which is harmless to a healthy person. Methacholine provocation is classified as a direct airway challenge since methacholine induces airflow limitation via a direct stimulatory effect on cholinergic receptors on the smooth muscle. In indirect challenges (such as inhaled Mannitol), airflow limitation is induced as a consequence of changed airway osmolarity, which leads to the release of mediators or cytokines from inflammatory cells, mast cells in particular, thus inducing a secondary bronchoconstriction.

Methacholine provocation is highly sensitive to detect BHR but it is not specific to asthma (46) as a positive test can also be found in other diseases such as rhinitis and chronic obstructive pulmonary disease. However, a negative test has a greater value in ruling out asthma, at least in the treatment naïve patient (11). One disadvantage of methacholine provocation is that it requires a baseline FEV$_1$ > 70% to be performed safely.

BHR to methacholine has been shown to correlate with asthma severity in a large cohort of children with different manifestations of persistent asthma (47) and this finding has been verified in specific studies on children with PA (14, 27). Furthermore, BHR to methacholine is reduced by anti-inflammatory treatment and has been shown to correlate with the number of inflammatory cells in biopsies (48) and markers of airway inflammation, including the fraction of nitric oxide in exhaled air (FeNO) in severe asthmatics (49-50).

### 2.5 INFLAMMATION

Airway inflammation is a pathophysiological characteristic of asthma mediated by the infiltration of inflammatory cells including mast cells, eosinophilic and neutrophilic granulocytes, plasma cells and structural cells in the airway wall. This cell infiltration subsequently leads to damage of the airway epithelium, neuroadrenergic imbalance, mucus secretion, unpredictable smooth muscle contractions, bronchial hyperresponsiveness and in the case of chronic inflammation, persistent morphological changes to the airways, i.e. airway remodelling (51).

The level of symptoms and markers of inflammation do not always correlate, some children might have persistent symptoms, without any histologically detectable inflammatory cells (52), whereas other children might have no symptoms between exacerbations but still have increased markers of airway inflammation (53). Nevertheless, the number of inflammatory cells present in the airway epithelium are higher in symptomatic compared to non-symptomatic children (40).

The aetiology of airway inflammation in asthmatic children varies according to age. Whereas viral infections, including rhinovirus and respiratory syncytial virus are linked to obstructive bronchitis in infancy and early childhood, sensitization and exposure to allergens is a major cause of airway inflammation in older children. Interestingly, recent evidence points to a synergistic effect between viral infections and aeroallergen exposure and the subsequent sensitization in genetically predisposed children (54). Furthermore, viral infections have been shown to be the most important cause of asthma exacerbations in all age-groups (55). Rhinovirus and respiratory syncytial virus are shown to damage the respiratory epithelium, making it less resistant to inhaled allergens subsequently leading to enhanced Th2 responses in liable children and the development of allergic inflammation.
The process of allergic sensitization is described in Figure 3 and the early phase allergic reaction is described in Figure 4. In addition, it should be noted that mast cells can be activated directly by pathogens such as bacteria and physical stimuli including osmolarity changes during exercise.

**Figure 3.** Sensitization to allergens in the airway of an atopic individual i.e. a person with a tendency to produce IgE antibodies in response to low doses of allergens, with permission from (3). The first time an atopic individual is exposed to an allergen, dendritic cells located in the airway mucosa recognize the allergen as body-foreign material, ingest the antigen and move towards the nearest lymph node. Fragments of the ingested antigen are presented on the major histocompatibility complex (MHC) which together with other co-stimulatory molecules bind to the respective receptor on a T-helper cell. The T-cell response varies depending on the individual’s genetic constitution and the nature of the presented antigen. In the atopic individual, naive T-helper (Th) cells will differentiate into Th2 cells producing IL-4, IL-5, IL-9, IL-13 GM-CSF and eotaxin. These cytokines stimulate B-cells to produce specific IgE antibodies, to recruit eosinophilic cells from the bone marrow and homing of mast cells. Mast cells are differentiated from CD34 precursor cells in the bone marrow, they are normally not identified in blood, but migrate, guided by chemotaxins, to tissues throughout the body, although the highest numbers are found in the skin and in the airway and gastrointestinal mucosa. The IgE produced by B-lymphocytes will circulate in serum with a half life of 2-4 days and the FC region of the IgE antibodies will bind to high affinity receptors (FccR1) on tissue mast cells with a half life of 2-4 weeks and the person is now sensitized.
The late phase allergic reaction includes the recruitment and migration of T-cells and other inflammatory cells including eosinophils and neutrophils to the site of allergen exposure within 4-6 hours. The granulocytes are recruited and developed from a pluripotent stem cell in the bone marrow via the circulation to the airways. Interleukin 3 (IL-3), IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) secreted by Th2 cells and mast cells are the most important interleukins for eosinophilic development and recruitment, although only IL-5 is specifically targeted towards the eosinophils. Once matured and recruited to the circulation, eosinophils adhere to the endothelial wall, connecting with adhesion molecules expressed on the eosinophil (Very late antigen-4) and endothelial cells (Vascular cell adhesion molecule-1).

After transmigration through the endothelial wall, the eosinophils are guided to the site of inflammation by chemokines, and release several stored toxic mediators, including free acid radicals, eosinophil cationic protein (ECP), eosinophil peroxidase, eosinophil protein x (EPX) and leukotrienes.

The physiological effects of the mediators excreted from eosinophils in the bronchial wall include smooth muscle contraction, increased vascular permeability,
increased BHR and production of toxic intermediate products and lipid peroxidation ultimately leading to injury and shedding of the surface epithelium. Collectively these mechanisms contribute to the establishment of chronic airway inflammation.

Markers of lipid peroxidation, nitrite and oxidized glutathione (the most important extracellular antioxidant) are increased in children with severe asthma and persistent airflow limitation compared to mild-to-moderate asthmatic children (56-57). In addition, oxygen free radicals in severe asthmatic children are associated with impaired alveolar macrophage phagocytic function (58), which could explain the increased susceptibility to infections with capsular polysaccharide bacteria in severe asthmatic children (59).

Neutrophils are attracted by cytokines such as GM-CSF and IFNγ. Neutrophilic pathogenic excretions include elastase which causes hypersecretion and hyperreactivity; matrix metalloproteinase 9 (MMP-9) which breaks down collagen and causes hypersecretion and free oxygen radicals which also induce bronchial hyperreactivity (60).

The relative contribution of each of these cell types to asthma severity is not clear and a sustained expression of pro-inflammatory cytokines and chemokines that are not Th2 dominant has been found in BAL fluid from severe asthmatic children (61). Nevertheless, airway eosinophilia has been shown to persist in children with PA, despite treatment with corticosteroids (62) although in a recent published study on children with therapy resistant asthma there was a huge variability in eosinophil counts in BAL fluid and biopsy material (63). In addition, correlations between asthma severity, eosinophil activation (64) and bronchial hyperresponsiveness (65) have been demonstrated. The number of neutrophils in bronchoalveolar lavage fluid has been shown to correlate with asthma severity in children (59), but in a recent study of children with therapy resistant asthma no signs of neutrophilic airway inflammation were found in BAL fluid and biopsy material (63).

2.5.1 Airway remodelling and computerized tomography

Airway remodelling refers to structural changes in the bronchial wall (66) causing reduced lung function (67) in asthmatic patients. These structural changes are confirmed by histological analysis and are characterized by sustained tissue eosinophilia, epithelial damage, thickening of reticular basement membrane (RBM), subepithelial fibrosis as well as mucus gland and airway smooth muscle hypertrophy and hyperplasia (68-69). Increased hypertrophy of the smooth muscle has been associated to reduced lung function in severe asthma (70). Remodelling is found in the majority of school age children with PA (43) and the progressive loss of lung function found in these patients (30) is probably caused by remodelling.

Bronchial wall thickening (BWT) can be identified by high resolution computerized tomography (HRCT), and has been proposed to be a surrogate marker of airway remodelling (71). BWT can be assessed quantitatively based on the number of visible bronchi at different levels of the lung by HRCT, and increased bronchial wall thickening has been found in children with moderate to severe asthma compared to healthy controls (72-73). In adults, BWT correlates with asthma severity, airflow obstruction (74) and RBM thickening (75-76).

In children, BWT has been shown to correlate with the thickness of the RBM (r=0.34) and ECP levels (r=0.45). However, these results have not been
confirmed in other studies (77) and no correlations have been found with lung function measurements or levels of IFN-γ, IL-4, IL-5 in BAL fluid (78).

2.6 BIOMARKERS IN PEDIATRIC ASTHMA

A biomarker reveals information about the investigated disease and can be used in one or more of the following settings; to assess risk for morbidity, identify disease or disease phenotypes and to monitor disease activity as well treatment effects (79). The ideal biomarker is attained non-invasively, is reproducible and easily measured and provides information about essential pathophysiological processes with high performance characteristics including sensitivity, specificity, negative and positive predictive values.

Using biomarkers in the context of childhood airway disease is particularly challenging. Imaging and spirometry, although providing relevant information as described previously, also have significant limitations. Biological events that occur episodically (bronchoconstriction) might not be captured and in early or mild disease these methods might show normal results despite the patient having symptoms (79).

Invasive methods such as those used to obtain biopsy and BAL fluid are possible in specialized centres, but in most circumstances they are not considered feasible in children. Induced sputum, a semi-invasive method, has been found to be safe in children but does require specially trained staff (80). In adults treatment guided by the presence or absence of sputum eosinophils has been shown to reduce asthma exacerbations (81), but this has not been proven effective in severe asthmatic children (82).

2.6.1 Exhaled Nitric oxide

Nitric oxide (NO) is produced by NO-synthase in airway epithelial cells and the resulting NO can easily diffuse into the airway lumen. The fraction of NO in exhaled air (FeNO) is currently being established as a non-invasive biomarker in asthma. FeNO has been shown to be elevated in patients with atopic asthma (83), to correlate with the degree of IgE sensitization (84), elevated in connection with allergen exposure (85) reduced by treatment with inhaled corticosteroids (86) and correlates with the number of eosinophils in blood (87), induced sputum (50) and biopsy specimens (62). Thus, FeNO seems to be a marker of the physiological consequences of atopy, i.e. the inflammation caused by allergen exposure. Nevertheless, the clinical situation in which FeNO provides the most useful information is currently debated (11), and the relationship between FeNO and asthma severity is unclear (49).

Height has also been shown to be an independent variable for FeNO, probably because of a larger airway mucosa in taller subjects and an increase in height from 120 to 180 cm is associated with a doubling of FeNO levels (88-89).

2.6.2 Chitinases

A group of proteins recently discovered to be potential biomarkers of asthma are the chitinases. Chitin is a tough structural polysaccharide, the second most abundant biopolymer in nature after cellulose, used by a variety of organisms including insects, crustaceans, parasites, fungi and bacteria to protect against harsh environmental conditions (90). Although chitin is not present in the human body, we express enzymes capable of its degradation. Two members of this family, the enzymatically active
chitotriosidase and the enzymatically inactive chitinase-like protein YKL-40, are increased in the serum of asthmatic patients (91-92). Furthermore, levels of YKL-40 have been shown to correlate with markers asthma severity (92) and it has been suggested that the chitinases may be involved in the development of fibrosis and remodelling of the airway (93).

A promoter single nucleotide polymorphism (SNP) in the gene encoding YKL-40, CHI3L1 rs4950928 (-131C→G) has also been associated with increased circulating levels in serum and features of asthma (94). Chitotriosidase activity is also subject to genetic regulation, with individuals homozygous for a 24bp duplication in exon 10 of CHIT1, the gene coding for chitotriosidase, displaying complete enzyme inactivity (95).

### 2.7 ALLERGY AND ALLERGENS

IgE mediated allergy leading to allergic inflammation, as described above, is common among children with persistent asthma. The presence of allergy predict both long-lasting (32) and troublesome asthma (96-97) in adult life and ongoing allergen exposure affects the frequency of asthma symptoms (98). Finally, comorbidities between different allergic diseases, including food allergies, atopic eczema and rhinitis are common, and the latter has been shown to be a risk factor for developing asthma (99) and contribute to reduced symptom control in asthmatic children (100).

#### 2.7.1 Allergens

Antigens which cause an IgE mediated reaction are defined as allergens. The most common sources of inhaled allergens that affect Swedish children are tree and grass pollens and furred animal dander (cat, dog) whereas peanut, egg and milk are the most important food allergen sources. Every allergen source consists of several allergens, each of which might have the capacity to bind to IgE (101). The allergens are named after the three first letters in the Latin family name followed by the fist letter of the species name and a number, e.g. Betula verucosa 1 (Bet v 1) refers to birch allergen 1.

In addition, every allergen has several epitopes, and similar epitopes might occur in allergens from different allergen sources, which is the biological explanation underlying cross sensitization (102).

Some allergens are unique markers of a specific allergen source whereas other allergens with similar structure and functions are found in different species, for example lipocalins is a group of proteins arising from cat, dog, horse and mice and cross-reactivity within these groups of proteins is common (103-104).

#### 2.7.2 Diagnosing allergy

A thorough clinical history and assessment of allergic sensitization are the cornerstones of an allergy diagnosis. Sensitization is usually determined by using allergen extracts to identify the disease eliciting allergen source, either by in vivo (skin prick testing) or in vitro testing, i.e. measurement of specific IgE in serum. However, one disadvantage with these extract based methods is that they only provide information about the possible sensitizing allergen source, but no information about sensitization towards specific allergens is provided. Thus, it is not possible to differentiate between the primary source of sensitization and cross-reactivity between allergens from different allergen sources (105), and it is not possible to determine whether the patient is sensitized to an allergen causing severe reactions or allergens causing mild reactions.
2.7.3 IgE component resolved diagnostics
Component-resolved allergy diagnostics is a new concept for measuring specific IgE towards single allergens in vitro e.g. by using an allergen microarray chip (106). The only commercially available component-based microarray platform for allergy is the immunosolid-phase allergen chip (ISAC, Phadia, Sweden). Although the ISAC chip lacks some minor allergens, the most common species-specific and cross-reactive allergens are represented. Some allergens are specific markers of an allergen source (107), and by identifying these allergens it is possible to determine which are the sensitizing allergen sources and which are the cross-reactive allergen sources (108). Furthermore, it is sometimes possible to predict whether the patient is at risk for a severe or mild reaction, based upon which allergen the patient is sensitized to. For instance, children allergic to peanut and sensitized to the allergens Ara h 1, Ara h 2 and Ara h 3 are at risk for significantly more severe symptoms upon peanut exposure compared to children sensitized to Ara h 8 (109), which is a homologue of the major Birch allergen, Bet v 1.

2.7.4 Basophils
Basophils share important features with mast cells as they are both developed from CD34 positive stem cells in the bone marrow, express FcεRI on their surface and store histamine containing granulae which are released upon crossbinding of allergens to IgE bound to FcεRI. Circulating basophils are more accessible than tissue resident mast cells, and this is the reason for using basophils as a marker of mast cell activity (110).

2.7.5 Basophil allergen threshold sensitivity (CD-sens)
Assessment of allergen specific IgE antibodies does not necessarily predict the degree of sensitivity to a particular allergen, i.e. the effector cell response and the subsequent clinical symptoms upon allergenic stimulation. For this purpose allergen provocation challenge tests are needed, but as they are potentially harmful, especially in severe asthmatic children, few studies on in vivo allergen challenge tests have been feasible and published in children with severe asthma.

An attractive in vitro alternative to the clinical allergen provocation test has been established (111). Basophil allergen threshold sensitivity (CD-sens) is a method for quantification of allergen sensitivity which correlates well with in vivo allergen provocation (112-113). Basophils express CD203c on their surface, which makes it possible to reliably identify these cells in a blood sample (114). Upon stimulation, basophils release their histamine containing granulae and CD63, which is situated in the vesicles of the granulae, subsequently becomes exposed on the cell surface (115). CD-sens is based on the flow cytometric detection of CD63 on basophils following an in vitro allergen titration. The allergen concentration causing 50% of maximum basophil activation (measured by CD63 up-regulation) is used to calculate CD-sens, Figure 5 and Figure 6. Therefore CD-sens is a measurement of the sensitivity of basophils to a particular allergen.
3 RATIONALE AND AIMS

Clinical characterization (I)
The clinical presentation of children with Problematic severe asthma (PA) in Sweden has not previously been described and the applicability of the recently introduced definition of PA, with the subgroups of difficult to treat asthma and severe therapy resistant asthma, has never been addressed. In addition, identification of novel biomarkers of asthma severity is needed.

The aims were to provide a detailed characterization of Swedish children suffering from PA and through a standardized and non-invasive protocol, investigate whether it would be possible to identify children who are severely resistant to therapy. A further aim was to compare children with PA to age-matched peers with controlled persistent asthma (CA) and to identify distinguishing features between these two patient groups. Finally, we also aimed to provide baseline data of these two patient groups and generate hypothesis for exploring biomarkers of asthma severity and follow up studies.

Component resolved allergy diagnostics (II)
Assessment of allergic sensitization by the established extract based methods have diagnostic limitations, both with respect to concordance (116), cross reactivity and prediction of severe allergic reactions. Component resolved diagnostics is a new concept for measuring allergens in vitro and the main advantage of this method is that it enables identification of cross-reactive or species-specific allergens, and allergens associated with mild or severe reactions. The added value of performing this analysis in children with persistent asthma has not been investigated previously. The aim was to assess the usefulness of component resolved allergen diagnostics in the assessment of children with different manifestations of persistent asthma.

Basophil allergen threshold sensitiviy (CD-sens) (III)
A possible mechanism for the development of PA in allergic children is increased sensitivity to allergens, which would imply that the allergic inflammation is triggered by lower allergen concentrations in children with PA compared to children with CA. This hypothesis can be tested using CD-sens.

The aim of this study was to examine whether CD-sens was increased in children with PA compared to CA and investigate correlations between CD-sens and other biomarkers of asthma.

Biomarkers of inflammation (IV)
Specific biomarkers of PA are needed to improve the characterization of this heterogeneous patient group, to increase the understanding of the underlying pathophysiology and to select patients for the most appropriate “beyond-the-guidelines” treatment. Chitotriosidase and the chitinase-like protein YKL-40 have been shown to be increased in the serum of asthmatic patients, to be involved in the development of remodelling of the airway and levels of YKL-40 have also been shown to correlate with markers of disease severity. Little is known about the expression and regulation of YKL-40 and chitotriosidase in relation to asthma in children.
The aim of this study was to examine serum YKL-40 levels and chitotriosidase activity in children with different manifestations of persistent asthma and further, to investigate potential correlations between YKL-40, bronchial wall thickening assessed by HRCT, pulmonary function measurements, and other biomarkers of inflammation.
4 METHODS
The present dissertation is based upon the Swedish severe asthma study. The study was approved by the regional board of ethics at Karolinska Institutet (Dnr 2006/1324-31/1).

4.1 STUDY DESIGN AND INCLUSION CRITERIA (I-IV)
The Swedish severe asthma study was designed as a nationwide observational study in which school aged children (6-18 years) with problematic severe asthma (PA) were compared to age-matched peers with controlled persistent asthma (CA). These two conditions were defined in accordance with the GINA guidelines (12), which take into account the severity of the underlying disease, as indicated by the need for treatment and degree of symptom control achieved. The inclusion criteria are presented in Table 1.

4.2 RECRUITMENT AND SUBJECTS (I-IV)
Allergists working at 27 pediatric clinics throughout Sweden were invited to refer children with PA and age-matched peers (±12 months) with CA for this investigation. Sixteen of these clinics (6 at university hospitals and 10 at general hospitals) from all regions of the country responded. In addition, journal charts from 4 clinics were systematically reviewed to identify children with CA. The children identified and referred in this manner were then invited by mail, telephone or verbally in connection with a visit to their doctors to participate. Following reassessment of their medical histories, medication and adherence to prescribed treatment, final selection of the participants was performed according to the criteria documented in Table 1. The children who admitted that they could miss more than 3 doses of prescribed medication per week were considered ineligible for inclusion, and likewise, children with vocal cord dysfunction, cystic fibrosis, immunodeficiencies, serious neurological disease or who had undergone major lung surgery or been born before 36 weeks of gestational age were excluded.

In paper I, 54 children with PA and 39 children with CA from 15 clinics were investigated. The analysis in Paper II and IV comprised 57 children (56 in Paper II) with PA and 39 children with CA, recruited from 16 clinics. Paper III included 11 cat allergic children with PA and 11 cat allergic children with CA from three clinics. In addition, in paper IV, 27 school-aged healthy controls were included for comparison.

4.3 PROCEDURES
Information was collected at each child’s regular clinic or at the research clinic at Astrid Lindgren Children´s Hospital in connection with two or three visits. A team consisting of myself and a research nurse (Björn Nordlund) visited the participating clinics and conducted the interviews and the majority of the procedures on site, utilizing brought equipment, to ensure standardized collection of biological samples and data. The children were required to have been free from airway infection or exacerbation of their asthma during the 2-week period prior to examination.
<table>
<thead>
<tr>
<th>Problematic severe asthma (PA)</th>
<th>Controlled persistent asthma (CA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major criteria</strong> (all required)</td>
<td><strong>Major criteria</strong> (all required)</td>
</tr>
<tr>
<td>- A diagnosis of asthma by a pediatric allergist</td>
<td>- A diagnosis of asthma by a pediatric allergist</td>
</tr>
<tr>
<td>- Daily high-dose administration of ICS (≥800 μg budesonide or ≥400 μg fluticasone/momethasone per day) in combination with LABA and/or LTRA*</td>
<td>- Daily low- to medium-dose administration of ICS (≥100 - ≤400 μg budesonide or ≥50 - ≤200 μg fluticasone per day). Use of either LABA or LTRA was acceptable.</td>
</tr>
<tr>
<td><strong>Minor criteria</strong> observed within the preceding 12-month period (minimum of at least one required)</td>
<td><strong>Minor criteria</strong> observed within the preceding 12 month period (all required)</td>
</tr>
<tr>
<td>- at least one emergency hospitalisation</td>
<td>- no hospitalisation</td>
</tr>
<tr>
<td>- at least two emergency out-patient visits</td>
<td>- no emergency out-patient visits</td>
</tr>
<tr>
<td>- at least one oral treatment with corticosteroid</td>
<td>- no oral corticosteroid treatment</td>
</tr>
<tr>
<td>- at least twelve exacerbations of asthmatic symptoms per year or symptoms present continuously for at least 3 months</td>
<td>- less than five exacerbations of symptoms**</td>
</tr>
<tr>
<td>- symptoms that limited daily activities (including sport or leisure activities) more than twice a week for at least three 3 consecutive months</td>
<td>- occasional symptoms related to strenuous exercise only, otherwise no symptoms</td>
</tr>
<tr>
<td>- nocturnal symptoms more than twice a week for at least three consecutive months</td>
<td>- no nocturnal symptoms</td>
</tr>
</tbody>
</table>

Table 1. Inclusion criteria for the children recruited to the study. ICS Inhaled corticosteroid; LABA long-acting β-2 agonist; LTRA leukotriene receptor antagonist, *High-dose administration of ICS for at least 6 months during the preceding year; previous use of LABA or LTRA only was considered acceptable if this treatment was discontinued due to inefficacy or the occurrence of unacceptable side-effects. **An increase in the ICS dosage for a maximum of 2 weeks in connection with asthma exacerbations was considered acceptable.

4.3.1 Interviews (I-IV)

The children and their parents were interviewed using a standardized questionnaire, which was a modified version of the one employed in the Environmental and Childhood Asthma Study (117). The questionnaire includes enquiries regarding demographic data; family history; indoor environment; exposure to tobacco smoke, pets and outdoor pollution; level of physical activity; symptoms or previous diagnosis of rhinoconjunctivitis (sneezing, congestion, secretions, itchiness, seasonal variation) and/or gastroesophageal reflux disease (GERD) (heartburn, regurgitation, upper
abdominal pain); occurrence of atopic disease; anaphylactic reactions; use of asthma medication; adherence to medication (evaluated with a three-point scale ranging from missing on the average < 1 to missing >3 doses of prescribed medications per week); asthmatic symptoms and their exacerbations (118); triggering factors; and utilisation of health care services. The percentiles of age- and sex-adjusted body mass indices (BMI) were calculated utilizing published algorithms (119). The research nurse observed the ability of each child to use the inhaler properly.

4.3.2 Asthma control test (II, III, IV)
Asthma control was estimated according to the Asthma control test (120). A total of 25 points can be achieved with optimal asthma control, and a score of 19 or less suggests poorly controlled asthma.

4.3.3 Spirometry and methacholine provocation (I-IV)
Treatment with SABA and/or LABA and/or LTRA was withheld for 8, 24 and 72 hours, respectively, prior to measurement of baseline pulmonary function and provocation with methacholine.

   Spirometry was performed using a Vitalograph® 2120 (Vitalograph®, Ennis, Ireland), in accordance with published recommendations (121) and FEV₁, FVC, FEF₅₀ were reported using the reference values reported by Polgar (122).

   Static lung volumes were determined at each local hospital and presented as TLC, RV and FRC, expressed as percentages of the locally applied reference values.

   Bronchial hyperresponsiveness (BHR) to a challenge with methacholine was assessed utilizing a Spira nebuliser (Spira Respiratory Care Centre, Hämeenlinna, Finland) (123). The dose of methacholine causing a 20% reduction in FEV₁ (PD20) (124) and the slope of the dose-response curve (DRS₅₀ methacholine) were calculated (125). Nebulised salbutamol (0.2mg/kg, maximally 5 mg) was administered following methacholine provocation and FEV₁ recorded 15 minutes later. Children with a baseline FEV₁ <70% were not exposed to the methacholine challenge, however, these children were also given salbutamol followed by spirometry.

4.3.4 Analyses in blood and urine (I-IV)
Following application of local anaesthesia (EMLA cream, Astra Zeneca, Sweden), samples of venous blood were obtained and the white blood cells and C-reactive protein (CRP) examined. The serum concentration of Immunoglobulin E (Total IgE, kU/L) and the levels of IgE antibodies directed specifically against the Phadiatop® mixture of common inhaled allergens, (birch, timothy, mugwort, cat, dog, horse and moulds (Cladosporium herbarum)); against house dust mite (Dermatophagoides pteronyssinus)); and against food allergens (fx5® = cow’s milk, egg white, soy bean, peanut, cod fish and wheat) (ImmunoCAP System™, Phadia AB, Uppsala, Sweden) were determined. Sera which demonstrated a positive reaction to Phadiatop® or fx5® (defined as a level of IgE antibodies ≥ 0.35 kU/l) were analysed further for IgE antibodies towards the individual allergens present in these mixtures. IgE antibodies to rFel d 1 were analysed by ELISA (103). Atopy was defined as level of antibodies towards either Phadiatop® and/or fx5® ≥0.7 kU/l (Paper I). Interleukin 5 was analysed by ELISA as part of a Multiplex cytokine assay. Eosinophilic protein X (EPX) in morning urine was analyzed with the EDN ELISA kit (Medical & Biological
Laboratories Co. Naka-Ku Nagoya, Japan), for which the lowest level of detection is 0.62 ng/ml, and expressed as nanogram EPX per millimol urine creatinine.

4.3.5 Component resolved diagnostics (II)
All serum samples were analysed using an experimental research ISAC prototype (Phadia AB, Sweden) containing 111 allergen components (all components of the ImmunoCAP ISAC 112 chip version except Ara h 6). The chip contained allergens derived from 51 sources, and required 30 µl of serum per test. Details of the tested components are listed in Figure 9. Positive sensitization was defined by a level of ≥ 0.30 ISAC independent units (ISU).

4.3.6 CD-sens (III)
CD-sens analyses were performed at one laboratory, as described previously (111), 18-24h after blood sampling. The basophils were exposed to decreasing concentrations of cat allergen extract (Aquagen) (ALK Laboratories, Copenhagen, Denmark) followed by staining with CD203c (Immunotech, Marseille, France) (basophil identification) and CD63 (Immunotech) (basophil activation), Figure 5.

![Figure 5](image.png)

Figure 5. Example of test report from CD-sens analysis, demonstrating the increased expression of CD63 with increasing allergen concentrations, with permission from Anna Nopp.

CD-sens was defined as the inverted lowest allergen concentration causing 50% of maximum CD63 up-regulation. This value was multiplied by 100 and used to describe a patient’s allergen-specific sensitivity. A high CD-sens indicates high basophil allergen sensitivity. Basophil reactivity was expressed as the maximum upregulation of CD63 shown as percentages. The number of IgE molecules and FceRI receptors per basophil was calculated using a FITC-conjugated antibody to IgE and FceRI and compared to a standard calibration curve (111).
4.3.7 Exhaled Nitric oxide (I-IV)
Employing a NIOX™ (Aerocrine AB, Solna, Sweden), FeNO was measured prior to spirometry, at an exhalation flow rate of 50 ml/s, in accordance with international guidelines (126) and presented as parts per billion (p.p.b). In the extended FeNO analysis, the FeNO -values obtained were adjusted for height; the difference between measured and expected height adjusted FeNO (Ln(FeNO) = 0.0112 x height (cm) + 0.641) was given as a percentage (%FeNO) (88).

4.3.8 nasal Nitric Oxide (nNO) (I)
Using nasal air samples obtained during breath-holding following maximal inhalation nNo was determined with NIOX™ and a nNO value greater than 105 ppb was considered indicative of the absence of primary ciliary dyskinesia (127).

4.3.9 YKL-40 assay (IV)
Serum YKL-40 levels were measured by according to the manufacturer’s instructions (Human Chitinase 3-like 1 DuoSet ELISA Development Kit, RnD Systems, UK). Two different dilutions were made for each sample from which an average was obtained, and all samples were analysed in duplicate.

4.3.10 Chitotriosidase assay (IV)
Chitotriosidase activity in the serum was determined using the fluorogenic substrate 4MU-deoxychitobiose as described and presented as nmol/ml/hour(128). Briefly: 25 μl serum, diluted with BSA/PBS (bovine serum albumin/phosphate buffered saline, 1 mg/ml) and 100 μl substrate mixture, 0.111 mM 4MU-deoxychitobiose and 1 mg/ml BSA in McIlvain buffer (100 mM citric acid, 200 mM sodium phosphate) pH 5.2, were incubated for 20 min at 37 °C. Reactions were stopped with 2.0 ml 0.3 M glycine NaOH buffer pH 10.6 and the formed 4MU was detected fluorometrically (excitation at 445 nm; emission at 366 nm). In order to discriminate between chitotriosidase activity and that of acidic mammalian chitinase (AMCase) in serum samples, a neutralizing antibody against AMCase was used.
4.3.11 Genotyping for chitinases (IV)
The CHIT1 24bp duplication (rs3831317) was genotyped using melting curve analysis to discriminate the different PCR products based on the presence or absence of the duplication. The following forward (5'-GGAGAAGCCGCAAGATC-3') and reverse primers (5'-AGCTATCTGAAGCAGAAG-3') were used. PCR reactions were performed with 10 µM of each primer, in a final volume of 10 µl, using SYBR Green PCR Master Mix (Applied Biosystem) following the manufacturer’s recommended protocol. Melting curve analysis was performed as a post-PCR procedure with the SDS v1.4 software using the ABI Prism 7500 detection system (Applied Biosystems). The -131 C/G polymorphism in the CHI3L1 gene (rs4950928) was analyzed using TaqMan allelic discrimination on the ABI Prism 7500 detection system according to manufacturer’s protocol (C__27832042_10, Applied Biosystems). Reruns of 10% of the samples for both genotypes showed 100% concordance.

4.3.12 CT scanning (I, IV)
Children suffering from PA underwent HRCT of their lungs at their local hospitals, within 3 months after inclusion in the study, to identify possible differential diagnosis and assess bronchial wall thickening. A standardized protocol was used and all images were evaluated by the same radiologist. HRCT scans of the lungs were obtained at full inspiration with 1 to 1.5 mm thick sections at 10 mm intervals through the thorax and images were reconstructed using a high spatial resolution reconstruction algorithm. The images were viewed at lung window settings optimized for paediatric lungs; window level -500 Hounsfield units (HU) and window width 1500 HU. The number of children with a significant differential diagnosis detected by HRCT is indicated and the degree of bronchial wall thickening (BWT) is assessed as described by Marchac et al. (72) using a semi-quantitative score based on the number of clearly identifiable segmental and sub-segmental airways at three pre-defined levels in the right lung. Sinus CT scans were scored and presented according to the Lund-MacKay system (129) with a score >5 indicating rhinosinusitis (130).

4.3.13 Definition of patient populations
Problematic severe asthma (PA): Children with chronic symptoms and/or recurrent exacerbations despite treatment with high doses of inhaled corticosteroids and other anti-asthmatic controller medications, according to the inclusion criteria presented in Table 1.

Difficult to treat asthma: A sub-group of children with problematic severe asthma. In these children a possible explanation to their poor asthma control has been identified, see Figure 2.

Therapy resistant asthma: A sub-group of children with problematic severe asthma. In these children no explanations for the severe symptoms have been identified, see Figure 2.

Controlled asthma (CA): Children with persistent asthma who achieve satisfactory control of symptoms with a low or moderate dose of inhaled corticosteroids, according to the inclusion criteria presented in Table 1.
Exacerbation phenotype: Children with Problematic severe asthma who had received more than 1 course of oral corticosteroids in the preceding 12 months, without experiencing daytime or night-time symptoms between exacerbations.

Chronic symptoms phenotype: Children with Problematic severe asthma that had experienced daytime and/or night-time symptoms more than twice a week for the preceding three months.

4.4 STATISTICS
All information obtained was recorded on a specially constructed, computerized form for subsequent analysis with the Statistical Package for Social Sciences Version 16.0-20.0. The values are presented as means and standard deviations (SD) for normally distributed data and as medians and inter-quartile ranges (IQR) for data not demonstrating a normal distribution. Categorical data are presented as percentages. For all comparisons a two-tailed probability of ≤ 0.05 was considered statistically significant. Groups of variables that exhibited a normal distribution were compared utilizing Student’s t-test, while for skewed variables the Mann-Whitney’s test was used.

In paper IV, normality was tested with the Kolmogorov-Smirnov test and groups of variables that exhibited a normal distribution were compared utilizing Student’s t-test in the case of equal variance, and by Welch’s t-test in the case of significantly unequal variance between groups as assessed by Levene’s test.

In all papers, associations between two categorical variables were tested with a Chi square test or Fisher’s exact test. Correlations were assessed employing the Pearson or the Spearman test.

Binary logistic regression analyses (Paper I) were conducted to further analyze applicable categorical variables which demonstrated significant differences between PA and CA in the univariate analysis. CA or PA was used as the dichotomous dependent variable, CA was assigned a value of 0 and PA was assigned a value of 1, and odds ratios (OR) were estimated along with the 95% confidence intervals (95% CI). Adjustments were made for possible confounding factors; age gender and ethnicity (model A) and in model B, the variables that came out with a significant result in model A were analyzed together. The history and trigger score (the sum of scores of the individual variables included in model A, each of which was assigned a value of 0 = no or 1=yes) were calculated to integrate information provided by the different variables concerning each patient’s history. Regression analyses of history score and trigger score were performed to assess the OR associated to PA or CA with every stepwise increase in each of these scores.
5  MAIN RESULTS

In total, 57 children with PA and 39 children with CA were included and investigated, Figure 7.

Figure 7. Flow of patients from referral to final classification.

5.1  CLINICAL CHARACTERISTICS (I)

54 children with PA were compared to 39 age matched children with CA in this analysis. Children with PA and CA were similar with regards to age (13.1 (3) vs.13.8 (2.9) years, p=0.28), gender distribution (39% vs. 41% females, p=0.84) and BMI percentile (63 (28) vs.55 (29), p=0.15) and the majority of children in both groups were Caucasians (83% vs. 92%, p=0.20)

5.1.1  History

In univariate analyses, children with PA were more likely to have parents with asthma (p=0.003), less income (p=0.03) and less formal education (p=0.01) compared to children with CA. Furthermore, children with PA were less physically active (p=0.04),
needed more health care services (p<0.001) and had a trend towards more smoking in their family (p=0.05). Finally, children with PA had more comorbidity with rhinoconjunctivitis, whereas eczema, food allergy and a history of anaphylactic reactions were equally as common in these two groups of patients (presented in 5.3.1.). Results from the binary logistic regression analysis are presented in Table 2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Model A (adjusted for age, gender and ethnicity)</th>
<th>Model B (Multivariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>OR</td>
</tr>
<tr>
<td>Parents without education beyond high school</td>
<td>85</td>
<td>3.4</td>
</tr>
<tr>
<td>At least one parent with asthma</td>
<td>92</td>
<td>3.9</td>
</tr>
<tr>
<td>At least one family member who smokes</td>
<td>92</td>
<td>2.7</td>
</tr>
<tr>
<td>Rhinoconjunctivitis</td>
<td>93</td>
<td>3.7</td>
</tr>
<tr>
<td>Parental income of 3 or less on a 4-point scale</td>
<td>82</td>
<td>3.0</td>
</tr>
<tr>
<td>Physically active ≤ 5 hours/week</td>
<td>93</td>
<td>2.2</td>
</tr>
<tr>
<td>History score*</td>
<td>93</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 2. Regression analysis of factors in the patient history associated with PA or CA. PA (1) or CA (0) was used as a dichotomous dependent variable. Model A: OR was estimated for each variable, adjusted for possible confounding factors. Model B: The four variables that came out with a significant result in Model A were analysed together. *History score: The sum of the scores of all the individual variables included in Model A, each of which was assigned a value of 0 = no or 1=yes.

5.1.2 Clinical presentation
Children with PA had a reduced score on the Asthma control test compared to the children with CA (23 (3) vs. 17 (2), p<0.001). The clinical phenotype of children with PA was either chronic symptoms or recurrent exacerbations with or without chronic symptoms between exacerbations, Figure 8. Children with chronic symptoms only had a reduced Asthma control test score (16 (3) vs. 20 (2), p=0.001) compared to the children with exacerbations only. There were no differences in age or gender distribution, prescribed medications, FEV₁, FeNO, response to methacholine provocation, blood eosinophils or neutrophils or total IgE between these two groups of children with PA, results from analysis not shown.
5.1.3 Symptom triggering factors
Children with PA reported more external symptom triggering factors (median 6/9 vs. 4/9, p<0.001). In univariate analysis, the following triggers were more common among children with PA: viral infection (p=0.03), birch and grass pollen (p=0.02), humid air (p=0.001), cold air (p=0.001) and tobacco smoke (p=0.04). Results from the binary logistic regression analysis of symptom triggering factors are presented in Table 3.
Table 3. Regression analysis of factors triggering asthma symptoms in children with PA and CA. PA (=1) or CA (=0) was used as a dichotomous dependent variable. Model A: OR was estimated for each variable, adjusted for possible confounding factors. Model B: The four variables that came out with a significant result in Model A were analysed together. *Trigger score: the sum of the scores of all the individual triggering factors included in Model A, each of which was assigned a value of 0 = no or 1=yes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Model A (adjusted for age, gender and ethnicity)</th>
<th>Model B (Multivariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>OR</td>
</tr>
<tr>
<td>Viral airway infection</td>
<td>93</td>
<td>3.6</td>
</tr>
<tr>
<td>Birch – grass pollen</td>
<td>92</td>
<td>2.8</td>
</tr>
<tr>
<td>Fog / humid air</td>
<td>90</td>
<td>4.9</td>
</tr>
<tr>
<td>Cold air</td>
<td>93</td>
<td>4.2</td>
</tr>
<tr>
<td>Tobacco smoke</td>
<td>89</td>
<td>2.4</td>
</tr>
<tr>
<td>Sum of all triggers (trigger score)*</td>
<td>93</td>
<td>2.6</td>
</tr>
</tbody>
</table>

5.1.4 Treatment and adherence
In line with the inclusion criteria, children with PA were prescribed more inhaled corticosteroids (800 (800-800) vs. 320 (200-400) µg Budesonide equivalents daily) and more courses with oral corticosteroids, (2 vs. 0 courses per year). Furthermore, 80% of children with PA were taking three controller medications (ICS, LABA, LTRA) and 79% were inhaling SABA more than twice weekly. By comparison, 65% of the children with CA were taking two controller medications. With respect to adherence, 59% and 44% of those with PA and CA, respectively, reported that they missed on the average less than one dose of controller medication per week (p=0.14) and 39% vs. 51% reported that they missed 1-3 doses per week (p=0.24).

5.1.5 Differential diagnosis
The detailed assessment of each child, including nNO sampling (PA: n=49) and computerized tomography (n=50) did not reveal any differential diagnosis. For the 48 children who underwent a sinus CT, the median Lund-MacKay score was 2, with 9 children demonstrating a score > 5.

5.1.6 Severely resistant to therapy vs. difficult to treat
In attempt to identify children with PA resistant to therapy, we excluded those patients subjected to identified possible aggravating factors, including untreated rhinoconjunctivitis (n=4), current exposure to tobacco smoke (n=12), untreated symptoms of GERD (n=5), the presence of pets in the home despite sensitization to such pets (n=3), sensitization to moulds and reported exposure to a problematic and/or humid indoor climate (n=4). After these exclusions, 33 (61%) of the children with PA were defined as suffering from severe therapy-resistant asthma. These children had
parents with a higher income (p=0.002) and education (p=0.004) and exhibited a non-significant trend towards higher FEV₁ (86 (19) vs. 76 (18), p=0.06) and lower FeNO (15 (10-38) vs. 32 (16-42), p=0.06) compared to those who were difficult to treat due to exacerbating factors.

5.2 SPIROMETRY AND METHACHOLINE PROVOCATION (I)

<table>
<thead>
<tr>
<th></th>
<th>PA</th>
<th>CA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dynamic spirometry (n)</strong></td>
<td>54</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>FEV₁, % of predicted</td>
<td>82 (20)</td>
<td>90 (11)</td>
<td>0.02</td>
</tr>
<tr>
<td>FVC, % of predicted</td>
<td>101 (18)</td>
<td>103 (16)</td>
<td>0.60</td>
</tr>
<tr>
<td>FEF₅₀, % of predicted</td>
<td>69 (28)</td>
<td>85 (18)</td>
<td>0.002</td>
</tr>
<tr>
<td>FEV₁/FVC x 100</td>
<td>78 (10)</td>
<td>85 (7)</td>
<td></td>
</tr>
<tr>
<td><strong>Static lung volumes (n)</strong></td>
<td>45</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>TLC, % of predicted</td>
<td>106 (15)</td>
<td>104 (15)</td>
<td>0.45</td>
</tr>
<tr>
<td>RV, % of predicted</td>
<td>133 (60)</td>
<td>125 (55)</td>
<td>0.60</td>
</tr>
<tr>
<td>RV/TLC x 100</td>
<td>23 (8)</td>
<td>20 (8)</td>
<td>0.14</td>
</tr>
<tr>
<td>FRC, % of predicted</td>
<td>120 (24)</td>
<td>111 (28)</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Methacholine provocation (n)</strong></td>
<td>32</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>DRS₅₀ methacholine median (IQR)</td>
<td>18 (1.5-65)</td>
<td>3 (0.4-27)</td>
<td>0.01</td>
</tr>
<tr>
<td>PD20 &lt; 2 µmol, %</td>
<td>69</td>
<td>33</td>
<td>0.003</td>
</tr>
<tr>
<td>PD20 negative, %</td>
<td>16</td>
<td>31</td>
<td>0.14</td>
</tr>
<tr>
<td>FEV₁ post-salbutamol, % of predicted</td>
<td>89 (16)</td>
<td>92 (11)</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Table 4. Measurements of pulmonary function and response to provocation with methacholine in children with PA and CA. The values presented are means (SD), unless otherwise indicated.

*Reported values are obtained before salbutamol was given.

# Fifteen children with PA were not subjected to provocation with methacholine because their baseline FEV₁ was <70% of predicted. Another 7 children with PA did not undergo methacholine provocation because they had taken β-2 agonist on the same day (n=3), because of technical problems with the equipment (n=2) or in accordance with the recommendation of their physician based on the severity of their asthma (n=2).

5.3 ASSESSMENT OF ALLERGY

5.3.1 Symptoms, allergic comorbidity and atopy (I)

Symptoms after exposure to animal dander (65% vs. 63%, p=0.88) and food allergens (33% vs. 23%, p=0.28) were equally common between children with PA and CA whereas symptom related to birch or grass pollen exposure were more common in children with PA, (74% vs. 49%, p=0.02). There were no statistically significant differences in comorbidity with reported atopic eczema (82% vs. 69%, p=0.17), food allergy (70% vs. 62%, p=0.37) or anaphylactic reactions (28% vs. 39%, p=0.28), but comorbidity with rhinoconjunctivitis was more common in children with PA (89% vs. 67%, p=0.01). The two groups of children also had similar levels of Total IgE (283
(118-853) vs. 280 (66-700), p=0.51). Most of the children with PA or CA were atopic (82% and 80%, respectively; p = 0.81), as assessed by Phadiatop® and fx5®, data not shown.

5.3.2 Component resolved allergy diagnostics (II)
Assessment of allergic sensitization by component resolved analysis revealed similar results, with eighty percent of children in both groups sensitized to at least one allergen and children in the two groups were similarly sensitized to food allergens, pollen and perennial allergens, Figure 9. Sensitization towards individual allergen sources and specific allergens were no different between children with PA and CA (results not shown). The most common allergen sources in both groups of children were cat (76% (rFel d 1 and 4)), timothy grass (64% (rPhil 1,2,5,6 and 11)) and birch (64% (nBet v 1)). The most common food allergy source was peanut; 42% (Ara h 1, 2, 3 and 9).

Figure 9. Sensitization patterns among children with PA and CA. Analysis includes allergen components of perennial aeroallergens, pollen allergens and food allergens: Perennial aeroallergens: Dog (rCan f 1, 2, 3, 5), horse (rEqu c 1, 3), cat (rFel d 1, 2, 4), mouse (nMus m 1) house dust mites (rBlo t 5, nDer f 1, rDer f 2, nDer p 1, rDer p 2, 10, rLep d 2, rEur m 2), cockroach (rBla g 1, 2, 5, 7), Alternaria (rAln a 1, 6), Aspergillus (rAsp f 1, 3, 6) and Cladosporium (rCla h 8). Pollen allergens: Bermuda grass (nCyn d 1), Timothy (rPhl p 1, 2, 4, 5, 6, 11), Alder (rAln g 1), Birch (nBet v 1), Hazel (rCor a 1.0101), Japanese cedar (nCry j 1), Cypress (nCup a 1), Olive (nOle e 1, 7, 9), Plane (rPla a 1, 2, 3), Ragweed (nAmb a 1), Mugwort (nArt v 1, 3), Goosefoot (rChe a 1), Wall pellitory (rPar j 2), Plantain (rPla l 1), Saltwort (nSal k 1) and cross-reactive markers of polcalcin (rBet v 4 and Phl p 7) and profilin (Latex rHev b 8, Birch rBet v 2, Timothy rPhl p 12 and Annual mercury rMer a 1). Food allergens: Kiwi (nAct d 1, 2, 5, 8), Celery (rApi g 1), Apple (rMal d 1), Peach (rPru p 1, 3), Cashew nut (rAna o 2), Brazil nut (rBer e 1), Hazel nut (rCor a 1.0.0401, 8, 9), Walnut (nJug r 1, 2, 3), Sesame seed (nSes i 1), Peanut (rAra h 1, 2, 3, 8, 9), Soy (rGly m 4, 5, 6), Buckwheat (nFag e 2), Wheat (rTri a 14, a 19, nTri aA _TI), Cow’s milk (nBos d 4, 5, 6, 8, lactoferrin), Cod (rGad c 1), Egg (nGal d 1, 2, 3, 5), Shrimp (nPen m 2, 4), and cross-reactive components of tropomyosin (Anisakis rAni s 3, Shrimp nPen m 1).
A specific IgE-response to >3 single animal-derived allergens from the lipocalin (nMus m 1, rEqu c 1, Fel d 4, rCan f 1, 2), kallikrein (rCan f 5) and secretoglobin (rFel d 1) protein families was more common among children with PA compared to children with CA (n=14 vs. n=3, p=0.03). Among children with PA, those who were multi-sensitized to more than 3 single animal derived lipocalin, kallikrein or secretoglobin allergens demonstrated increased levels of FeNO (p=0.02), blood eosinophils (p=0.02) and BHR to methacholine (p=0.002) compared to children with PA, but positive to fewer of these components. Moreover, the multi-sensitized group had higher total IgE levels (p=0.006) and demonstrated a non-significant trend towards requiring more treatment courses with oral corticosteroids (p=0.051).

5.3.3 CD-sens (III)
We compared CD-sens in 22 cat-allergic children, 11 with PA and 11 with CA, and found that children with PA had higher CD-sens compared to those with CA (7.1 (3.2-14) vs. 1.3 (0.2-4.8), p=0.02). No significant differences were observed when comparing basophil reactivity expressed as Max % Cat activated basophils (p=0.12), IgE molecules per basophil (p=0.33), FcεR1 on basophils (p=0.15) and number of basophils in the blood (p=0.69). There were no significant differences in IgE antibodies and specific IgE antibodies to cat between these two groups of children, Figure 10.

![Figure 10](image_url)

**Figure 10. Comparisons of CD-sens, Cat IgE and IgE between the two groups of cat allergic children with PA and CA.**

Furthermore, we found significant correlations between CD-sens and asthma control, markers of allergic inflammation and markers of sensitization when investigating all the children that were included in this analysis (n=22), Table 5.
<table>
<thead>
<tr>
<th>Variabel 1</th>
<th>Variabel 2</th>
<th>r-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma control test</td>
<td>CD-sens</td>
<td>-0.63</td>
<td>0.002 *</td>
</tr>
<tr>
<td>FeNO</td>
<td>CD-sens</td>
<td>0.55</td>
<td>0.01 *</td>
</tr>
<tr>
<td>Eosinophils in blood</td>
<td>CD-sens</td>
<td>0.49</td>
<td>0.02 *</td>
</tr>
<tr>
<td>IgE antibodies to cat</td>
<td>CD-sens</td>
<td>0.63</td>
<td>0.002 *</td>
</tr>
<tr>
<td>IgE antibodies to cat/ IgE</td>
<td>CD-sens</td>
<td>0.57</td>
<td>0.005 *</td>
</tr>
<tr>
<td>IgE to rFel d 1</td>
<td>CD-sens</td>
<td>0.54</td>
<td>0.01 *</td>
</tr>
<tr>
<td>ICS</td>
<td>CD-sens</td>
<td>0.30</td>
<td>0.17</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>CD-sens</td>
<td>0.31</td>
<td>0.17</td>
</tr>
<tr>
<td>DRS&lt;sub&gt;methacholine&lt;/sub&gt;</td>
<td>CD-sens</td>
<td>0.20</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Table 5. Correlations between CD-sens and various markers of allergic sensitization and asthma morbidity.

5.4 ASSESSMENT OF INFLAMMATION (IV)

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>PA (n=57)</th>
<th>CA (n=39)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total white blood cells (10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>6.7 (2.1)</td>
<td>5.9 (1.4)</td>
<td>0.04</td>
</tr>
<tr>
<td>Neutrophils (10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>3.2 (1.4)</td>
<td>2.7 (0.7)</td>
<td>0.02</td>
</tr>
<tr>
<td>Eosinophils (10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>0.5 (0.4)</td>
<td>0.3 (0.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>Interleukin 5 (pg/ml), median (IQR)</td>
<td>0.32 (0-2)</td>
<td>0 (0-0)</td>
<td>0.003</td>
</tr>
<tr>
<td>CRP (mg/L) median (IQR)</td>
<td>0 (0-2)</td>
<td>0 (0-0)</td>
<td>0.14</td>
</tr>
<tr>
<td>EPX* median (IQR)</td>
<td>136 (91-246)</td>
<td>133 (84-189)</td>
<td>0.32</td>
</tr>
<tr>
<td>FeNO, p.p.b. median (IQR)</td>
<td>22 (10-40)</td>
<td>17 (9-26)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 6. Biomarkers of inflammation from blood, urine (EPX) and exhaled air (FeNO) in children with PA and CA. * ng /mmol creatinine. All values given as means (SD) unless otherwise stated.

FeNO correlated with DRS<sub>methacholine</sub> (r=0.40, p<0.001), Asthma control test (r=−0.27, p=0.007) and blood eosinophils (r=0.42, p<0.001). In addition, blood eosinophils correlated with DRS<sub>methacholine</sub> (r=0.45, p<0.001) and urinary EPX (r=0.50, p=0.01).

5.4.1 Extended FeNO analysis

When adjusting for height, a difference in FeNO between children with PA and CA became noticeable. Children with PA had trend towards higher levels % FeNO compared to children with CA; 210% (101-367) vs. 139% (85-216), p=0.07, respectively. When analysing all children (n=96), those with % FeNO >200 (n=43, PA: n=30, CA: n=13) had reduced asthma control (18.5 (4) vs. 20.4 (4), p=0.04) and FEV<sub>1</sub>/FVC (77 (10) vs. 83 (8), p=0.004) and increased bronchial hyperresponsiveness (54 (5-67) vs. 2 (0.4-36), p=0.001), bronchial wall thickening on CT (25 (9) vs. 17 (6), p=0.004), blood eosinophils (0.5 (0.4) vs. 0.3 (0.3), p<0.001) and total IgE (539 (253-1525) vs. 140 (43-425), p<0.001) compared to those with % FeNO <200.
5.4.2 Serum levels of YKL-40 and chitotriosidase activity

YKL-40 levels and chitotriosidase activity were significantly higher in serum from children with PA compared to those with CA (19.1 (6.8) vs. 16.4 (3.9), p=0.01 and 67.1 (37) vs. 51.2 (24), p=0.01, respectively). Figure 11. YKL-40 levels were higher in children with PA compared to healthy controls (19.1 (6.8) vs. 15.4 (8.9), p=0.04), whereas there was no statistical significant difference in chitotriosidase activity between these two groups (67.1 (37) vs. 57.9 (28), p=0.25).

Figure 11. Comparison of serum levels of YKL-40 (A) and Chitotriosidase activity (B) in children with problematic severe and controlled persistent asthma.

Among all children with asthma, significant correlations between YKL-40 and asthma control (r=-0.26, p=0.01), FeNO (r=0.21, p=0.04) and blood neutrophils (r=0.49, p < 0.001) were observed. These correlations were more evident in the group of children with severe therapy resistant asthma; asthma control (r=-0.38, p=0.03), FeNO (r=0.48, p=0.004), and blood neutrophils (r=0.63, p=<0.001), Figure 12.

5.4.3 Bronchial wall thickening

BWT was assessed on 44 children with PA (30 children with severe therapy resistant asthma). In the group of children with severe therapy resistant asthma, there were significant correlations between YKL-40 and BWT diagnosed by HRCT (r=0.45, p=0.01). Figure 12.
5.4.4 Variations in the CHI3L1 gene and YKL-40 levels
Children homozygous for the C allele had higher levels of YKL-40 (18.8 (16.6-22.8)), compared to heterozygous carriers (14.0 (12.5-17.4), p<0.001), Figure 13A. Among the CC carriers, children with PA had higher levels of YKL-40 compared to those with CA (20.9 (18.6-24.9) vs. 16.7 (15.2-18.3), p<0.001), Figure 13A. Fifty-seven percent of children with PA were homozygous for the C allele, compared to 72% of children with CA, p=0.04.

5.4.5 Variations in the CHIT1 gene and chitotriosidase activity
Children lacking the 24 bp duplication in exon 10 (75/75 bp homozygous) had significantly higher chitotriosidase activity compared to those heterozygous for the deletion (75/99 heterozygous), (72.9 (55.3-86.2) vs. 35.9 (27.7-42.5)), p<0.001). Among those lacking the 24 bp duplication, children with PA had higher levels of chitotriosidase compared to those with CA (76 (66-107) vs. 58 (52-73), p=0.001), Figure 13B. Sixty-three percent of children with PA were lacking the 24 bp duplication in exon 10 (75/75 bp homozygous) compared to 68.4 % of children with CA, p=0.77.
Figure 13. The effect of genetic variability on chitinase levels in all asthmatic subjects. A) Serum YKL-40 levels according to \textit{CHI3L1} rs4950928 genotype. B) Serum chitotriosidase activity according to \textit{CHIT1} rs3831317 genotype. Grouped results from children with CA (○) and PA (■). Results are displayed as individual data points with median bars.
DISCUSSION

6.1 GENERAL COMMENTS REGARDING METHODS

6.1.1 Study design

In this observational study, the main objective was to provide a detailed characterization of Swedish children with Problematic severe asthma (PA), and investigate how children with PA differ from age matched peers with persistent, but controlled asthma (CA). PA is uncommon in children and a multi-centre approach was needed to acquire a sufficiently large group of children. No power calculations were performed beforehand, since our intention was to identify as many children as possible by a survey of 27 pediatric clinics throughout Sweden. Our cohort of 57 children with PA is larger than most (14, 27, 40, 131), but not all (43) studies with a similar design investigating severe childhood asthma. This might appear to be a low number compared to population based studies with several hundreds or thousands of children with asthma. However, a major advantage of this study is the vast amount of information gathered from each individual child, providing a meticulous characterization and making it possible to explore associations and disease mechanisms in relation to the presenting phenotype.

The different stages of this project were carried out in cooperation with several clinicians and researchers within this field. Paediatric allergists from paediatric centres throughout Sweden were involved in the development of the study protocol. The actual data collection, on the other hand, was performed by a mobile team consisting of two individuals only (myself and Björn Nordlund). We visited all the participating clinics and conducted the interviews and the majority of the procedures on site, utilizing brought equipment, which ensured the standardization of patient inclusion and collection of biological samples. An extensive network of basic researchers at Karolinska Institutet has been engaged in conducting analyses of the samples obtained within the areas of allergy, inflammation and genetics.

This study is cross-sectional in the sense that the children only were investigated once, but not in the sense that the included children were extracted from a population based study; accordingly this project was not designed to estimate the prevalence of severe childhood asthma in Sweden. Nevertheless, the children were broadly recruited, allergists at 27 hospital based pediatric clinics were invited to refer children to this study, and although 11 of these clinics are not represented, it is our interpretation that the children referred, represent the most challenging asthmatic children throughout Sweden.

Furthermore, this project could be classified as a comparative study with a case-control like design as we were comparing asthmatic subjects with problematic severe disease (the 'cases') with patients who did not have problematic severe disease, but were otherwise similar (the controls) (132). One advantage of this study design is the possibility to study multiple exposures associated with rare diseases simultaneously, whereas the major drawbacks are that only associations can be studied and recall bias might be a problem.
6.1.2 Criteria of inclusion and definitions

Detailed inclusion criteria are a prerequisite in case-control studies to ensure that the subjects studied are representative of all subjects with the investigated disease. In this project, all subjects were included on the basis of strictly defined criteria, with myself and the principal investigator taking sole responsibility for the final inclusion. Of the 70 children with PA referred for participation in the study, 13 were not included. Eight of these children did not fulfill the minor criteria regarding medications and symptoms / asthma control. The remaining 5 children had other diagnosis (born prematurely, immunodeficiency, major pulmonary surgery), diagnosis which could contribute to their symptoms.

Moreover, our observations concerning heredity (27), socioeconomic factors (133), health care (20), triggering factors (25), lung function (27, 134-135) and BHR (14) are comparable to those of previous studies. The frequency of atopic sensitization observed here is similar to that in the Childhood asthma management program (CAMP) study (134), as well as with preliminary findings in children with persistent asthma recruited from a large Swedish birth cohort (136) upon follow-up at 8 years of age (Inger Kull, PhD, personal communication, April 2010). Finally, the characteristics of our children with PA and CA agree well with the findings from a population-based study in Norway (14). Taken together, these considerations indicate that our subjects were representative of children with varying manifestations of persistent asthma.

The current definition of PA aims at including children who have poor asthma control despite high doses of inhaled corticosteroids. Asthma control is defined as “to which extent the manifestations of asthma have been reduced or removed by treatment” (15), a concept that not only includes the assessment of daytime and nocturnal symptoms, and the need for reliever medication, but also an estimation of future risk of severe exacerbations. Hence, the inclusion criteria for children with PA in this study combine an evaluation of medication, chronic symptoms and exacerbations. These inclusion criteria were developed from the GINA guidelines (12), in consensus with the Allergy section of the Swedish Pediatric Society and are similar, but not identical, to the inclusion criteria in other studies on severe childhood asthma (27, 40, 43).

It is noteworthy that specific biomarker criteria are not included in the current definition of PA, and the reason for this is the pathophysiological heterogeneity of asthma, as described in the introduction. This heterogeneity was confirmed in a cluster analysis to identify phenotypes in 161 asthmatic children with different manifestations of asthma (89 children with severe asthma) (19). Four clusters were identified based on 12 variables including demographic data, duration of asthma, FEV1 pre and post salbutamol, atopy, exhaled nitric oxide and medication. Children classified as severe asthmatics were present in all clusters, which confirms the heterogeneity of the disease.

In the Swedish severe asthma study, inclusion criteria based upon biomarkers would have provided different results, as exemplified with the extended FeNO analysis (section 5.4.1). The current definition is a reflection of how children with PA present to the pediatric allergist, i.e. having troublesome symptoms, despite prescription of maximum doses of conventional medications, and is in agreement with the recent WHO definition of severe asthma (29).
6.1.3 Control group

The selection of controls is particularly crucial for the validity of a case-control study, and in this project two aspects regarding the controls deserve comment. First of all, it must be emphasized that children in the control group are not mild asthmatics; they all have persistent asthma but achieve acceptable symptom control with continuous treatment with a low to moderate dose of inhaled corticosteroids. By making continuous treatment with inhaled corticosteroids inclusion criteria for the control group, we avoided investigating differences caused by steroid treatment per se.

Secondly, and rather surprisingly, children with CA were more difficult to identify and recruit to this study than the children with severe asthma, and as a consequence, less children with CA were included, and the majority were recruited from the Stockholm region. There are several explanations for this; children with controlled asthma do not need to visit a paediatric allergist at a hospital based clinic, therefore the allergists involved in this study had few patients of this category on their listings. Once identified, children with controlled asthma are also less motivated to undergo the extensive study protocol. Besides, many asthmatic children with adequate control of their symptoms discontinue their treatment with ICS and were therefore not even considered for inclusion.

6.1.4 Adherence to treatment

Reliable assessment of adherence to prescribed treatment is a challenge in clinical practice as well as in research on patients with asthma. In this study, adherence was determined by self-assessment and this information might be somewhat unreliable. The reliability might have been improved by, for example, examining pharmacy records. Nonetheless, the approach employed in this study represents current clinical practice in Sweden and the lack of any differences in adherence between the groups (PA vs. CA or children who were severely resistant to therapy vs. those who were difficult to treat due to aggravating factors) suggest that this shortcoming did not exert any major impact on our findings.

6.2 CLINICAL CHARACTERISTICS (I)

6.2.1 Family history and comorbidity

The current dataset indicates that a child with PA is more likely to have parents who smoke and have asthma, come from underprivileged circumstances and have untreated rhinoconjunctivitis, compared to a child with controlled asthma compared to a child with CA. Although this study was not designed to establish causality, there are plausible biological explanations to support that some of these observations are involved in the development of PA.

Regarding heredity, there is good evidence that inherited factors influence the risk of developing childhood asthma, and numerous candidate genes have been identified (137). In addition, a few genes have been associated with asthma severity, one example is mutations in the interleukin-4 receptor alpha gene, which regulates Th2 responses (138). The search for other genetic variants, possibly related to PA, is currently being conducted based on samples from the Swedish severe asthma study.

Furthermore, with respect to parental smoking, prenatal exposure to tobacco smoke influences lung function at birth (139), smoking is associated with steroid resistance (140) and passive smoke exposure, measured by urinary cotinine
levels, has been associated with more exacerbations, increased airflow obstruction and increased BHR (141).

The comorbidity between childhood asthma and rhinoconjunctivitis is well established (142). However, the observation that patients with PA report clinical symptoms of rhinoconjunctivitis more often than those with CA has only been reported in adults (143), which is in concordance with the concept of the united airways (144).

6.2.2 Clinical presentation
Three main clinical phenotypes were identified among the children with PA; children with exacerbations only, children with chronic symptoms only and children with both exacerbations and symptoms. As expected, those with chronic symptoms had a reduced score on the asthma control test compared to those with exacerbations only, but the fact that no differences were found with respect to prescribed medication, pulmonary measurements, bronchial hyperresponsiveness and biomarkers of inflammation indicates that these two groups of children have equally severe asthma, although the clinical presentation differs, and this finding is in line with the proposals from the PSACI group (18).

6.2.3 Difficult to treat asthma vs. therapy resistant asthma
Of all included children with PA, forty percent were exposed to exacerbating factors that could be treated, or at least partially eliminated. This finding provides support for the clinical utility of the recently formulated definition of PA. Moreover, it shows that the fundamental effort of taking a detailed history is helpful in identifying children with the most severe asthma. However, home visits (145) and assessment of steroid response with for example Triamcinolone injection (43) could have further improved the classification of children with PA.

The results indicate that children with PA, a low socioeconomical background and high FeNO have an increased probability of suffering from asthma which is difficult to treat because of identifiable aggravating factors. This suggests a shortcoming of the profession to educate these families on how to avoid or handle triggering factors, and furthermore, a high FeNO is a useful hint of increased risk of exacerbating factors in these children. However, it should be kept in mind that these children might still have severe symptoms after the exacerbating factors are dealt with.

Children classified as having severe therapy-resistant asthma might benefit from more invasive procedures, such as bronchoscopy, that provide a more detailed characterization of their disease, e.g. identification of bacterial infections which would justify treatment with macrolides (146). In addition, they could be candidates for novel therapeutic options, such as anti-IgE treatment and to participate in studies on other treatment alternatives currently in the pipeline, including Pitirakinra (IL4-/IL-13 antagonist) (147), Lebrikizumab (IL13 antagonist) (148) and Reslizumab (antibody against IL-5) (149). Finally, future studies on the underlying mechanisms of severe childhood asthma should only include children diagnosed with therapy resistant asthma. In Paper IV we provide an example of the usefulness of this classification in the analysis of correlations between chitinases and other features of asthma; correlations were most evident among children classified as being therapy resistant.
6.2.4 Statistical considerations
It should be noted that no correction for multiple testing has been performed in paper I. Such correction would have resulted in fewer statistically significant differences between these two groups of patients. Furthermore, this study was not designed for powerful regression analysis, but the results from the analysis conducted, are useful to generate hypothesis of which factors could be most important.

6.3 ASSESSMENT OF PULMONARY FUNCTION (I)
The most noteworthy finding regarding pulmonary function is the modest difference in $\text{FEV}_1$ between children with PA and CA, a finding which is in line with findings in other studies (14, 16, 27). There are several possible explanations for this finding; $\text{FEV}_1$ is a measurement of airflow in the larger airways, but it is insensitive to changes in the smaller airways, and symptoms in patients with severe asthma might be caused by small airway obstruction (150). Furthermore, measurements of pulmonary function correlate poorly with the distribution of inflammation in the airways (151). Finally, since we have investigated children, the decline in lung function is still not as pronounced as that observed in the adult population (25-26). However, it should also be noted that the children with CA do not have an entirely normal pulmonary function, due to the fact that the control group does not consist of mild asthmatics.

Looking at FEF$_{50}$ and $\text{FEV}_1$/FVC, the difference between the two groups of children appears to be more robust. The reasons for this could be that FEF$_{50}$ is a better indicator of obstruction in smaller airways and that $\text{FEV}_1$/FVC reflects exaggerated dysanapsis, i.e. the disproportionate growth of airways and lung parenchyma, in severe asthmatics (16). All observations regarding pulmonary function are in line with the findings from a more thorough analysis of pulmonary function in severe asthmatic children, partly based on patients from the Swedish Severe asthma study (135).

6.4 BRONCHIAL HYPERRESPONSIVENESS (I)
Children with PA had increased BHR compared to children with CA which support findings from previous studies (27) can could provide an explanation to why children with PA are reporting more symptom triggering factors. Somewhat surprisingly, there were 5 children with PA who did not respond to a provocation with methacholine, 4 of these children were not sensitized to any allergen (results not shown), which support findings in a similar study (14). The explanations could be that the high doses of inhaled corticosteroids suppress the methacholine response or that these particular individuals constitute a specific phenotype. In addition, it should be noted that sensitivity to methacholine could be reduced in non-atopic compared to atopic children (152).

6.5 ASSESSMENT OF ALLERGY (I, II)
The majority of children in both groups were atopic and there were no differences between the two groups when comparing allergic sensitization to allergen sources or individual specific allergens. Previous studies have found that being allergic increases the risk of having persistent asthma in childhood (134), but studies on allergic sensitization in relation to asthma severity have provided conflicting results. Siroux et al. found that the type and intensity of the allergic sensitization were not associated with any criteria of asthma severity in 216 asthmatic children (153). On the other hand,
Carroll et al. found significant associations between asthma severity score and Total IgE and in 400 asthmatic children (154). Ponsonby et al. also found that there are more atopic children with severe asthma (155). Furthermore, in the Fitzpatrick study from 2006 (27), which is similar to the Swedish severe asthma study in power and design, atopy was more prevalent in the severe asthma group. However, it should be noted that the major allergens in the Fitzpatrick study were house dust mites and weed mix, allergens which are rarely found in Sweden. Regarding sensitization to animal dander, tree and moulds, there were no differences between severe and mild asthmatics in the Fitzpatrick study either.

With the help of component resolved diagnostics, we were able to demonstrate that sensitization to more than 3 single allergens from the lipocalin, kallikrein and secretoglobulin protein families (allergens from cat, dog, horse and mouse) was more common among children with PA compared to those with CA. Multi-sensitization to these allergens was also associated with evidence of increased eosinophilic bronchial inflammation and a trend towards a greater number of oral corticosteroid courses and thus making this appear to be a clinically relevant finding.

From a clinical point of view, we interpret the association between increased bronchial inflammation and multi-sensitization to lipocalin, kallikrein and secretoglobulin allergens as a consequence of ubiquitous exposure to furred animals or an increased disposition for allergic disease. We have previously shown that cat dander is easily transferred from cat owner’s into school environments where it significantly affects asthmatic children (98). It is crucial to control the inhalation of animal-derived lipocalin, kallikrein and secretoglobulin allergens in dander particles as they are small in size and easily deposited in the respiratory tract. To date, only interventions involving a high-altitude environment (156) or temperature-controlled laminar airflow during sleep (Protexo) (157), have proven effective in this respect.

### 6.5.1 CD-sens (III)

In Paper III we explored the relationship between basophil allergen threshold sensitivity (CD-sens) and clinical parameters in 22 cat allergic children, of whom 11 had PA. We used CD-sens as a measure of allergen sensitivity and found that basophils from children with PA were significantly more sensitive to cat-allergens, compared to basophils from children with CA. Furthermore, CD-sens correlated with several other characteristics of asthmatic disease, including asthma control, IgE antibodies and biomarkers of allergic inflammation.

A high CD-sens implies that lower allergen concentrations are needed to activate basophils, and presumably the mast cells, which subsequently leads to the initiation of allergic inflammation. However, recent evidence indicates that basophils are not only markers of mast cell function but could also function as initiators of allergic inflammation and contribute to the development of chronic allergic inflammation (110). It may therefore be speculated that the higher allergen sensitivity in children with PA could be due to skewing of a basophil phenotype since basophils are a heterogeneous cell population with varying degranulation properties depending on the genetic background and surrounding cytokine environment at the stage of basophil precursor development (158).

Once matured, basophils require on average a cross-linking of 2000 IgE-FceR1 complexed molecules before intracellular signalling is initiated, but there are considerable variations from this mean value (159). Thus, another hypothesis is that
basophils in children with PA need fewer cross-bindings to be activated and start intracellular signalling. A third possible explanation to the observed difference in basophil activation involves a variation in intracellular regulatory mechanisms. The enzyme PI3-kinase is involved in the fusion of basophil granules to the plasma membrane (160) and individual variation in the activity of this enzyme could also be an explanation for the observed differences in CD-sens.

Once activated the basophils release their histamine containing granulae, containing IL-4 and IL-13 (160). These pro-allergenic cytokines induce IgE synthesis in B-cells and this could explain the observed correlations between a high CD-sens and IgE antibodies to cat and Fel d 1.

This is the first report of the potential clinical usefulness of analysing CD-sens in children with severe allergic asthma. The findings indicate that children with PA are more sensitive to cat allergen compared to children with CA and this finding is of particular interest since there were no significant differences in markers of cat sensitization between these two groups of children. CD-sens represents the in-vivo pathway leading to symptoms after allergen exposure and therefore provides additional information compared to the assessment of sensitization alone. As it is well known that most children, regardless of whether they own a cat, are exposed to cat-allergens at school and/or at home (161), a high allergen sensitivity may therefore explain why it is more difficult to achieve symptom control in some cat-allergic children with persistent asthma. Collectively, our findings suggest that CD-sens is more closely related to the clinical presentation of allergic asthma than the atopic status and therefore has the potential to be a useful marker of disease severity.

However, it should be noted that CD-sens is still a staff-intensive analysis and only can be performed in specialized laboratories. Furthermore, CD-sens varies between different allergens which could be because of lack of standardization in the allergen extracts used and finally, no reference values have been established. The results should probably be interpreted in a categorical manner because CD-sens>0 signifies activation of the allergic inflammation (162), although in the current study, we have demonstrated that cat allergic children with PA, at a group level, have higher CD-sens compared to those with CA.

6.5.2 Potential applications of CD-sens (III)
Assessment of allergen sensitivity is not only useful for investigating the severity of a child’s asthma, it could also be useful for selecting those with the highest allergen sensitivity who are likely to benefit most from more extensive treatment alternatives such as anti-IgE treatment (Omalizumab). Omalizumab binds to the Fc portion of circulating IgE and prevents IgE binding to the FcεRI and FcεRII on mast cells and basophils, thereby inhibiting activation of these cells (163). The clinical effect of this treatment in patients with severe allergic asthma has been demonstrated in several studies (164). The selection of patients, and the timing and dosing of Omalizumab injections are currently only based on the presence of severe allergic asthma and by measurement of total IgE and weight.

This is the first biomarker guided treatment option in asthma, but still, only one third of patients treated with Omalizumab experience a considerable improvement following this treatment (165). Biomarkers to improve the selection of responder from non-responders have not yet been established (163), although CD-sens has been shown to be useful in the monitoring of anti-IgE treatment efficiency (112).
and to correlate with the response to a bronchial allergen inhalation challenge (113). Thus, studies examining the capability of CD-sens to identify Omalizumab responders are warranted as CD-sens is a biomarker of the key pathological process related to Omalizumab treatment. Furthermore, CD-sens could be evaluated as a biomarker to select candidates for other established (subcutaneous immunotherapy) and novel immunomodulatory treatment alternatives.

6.6 ASSESSMENT OF INFLAMMATION (IV)
We found increased levels of blood neutrophils, blood eosinophils and serum IL-5 in children with PA which is in line with findings from adult severe asthmatics (166). The assessment of inflammatory biomarkers in blood will be extended with analysis of a broad assay of specific interleukins from these patients.

With respect to FeNO and EPX, we found no difference between the groups, which questions the value of these parameters as markers of the severity of asthma. In light of the high degree of self-reported adherence to medication, it is possible that the high doses of inhaled corticosteroids used by children with PA may have suppressed differences in the FeNO and EPX values that might have been seen without such treatment.

However, when normalizing FeNO values against the individual child’s height, the difference between PA and CA was more apparent. Comparing asthmatic children with % FeNO >200 to children % FeNO <200, irrespective of pre-determined severity classification, the former group had increased morbidity. This finding point to a clinical usefulness of FeNO measurement across all ranges of asthma severities and supports similar analyses performed in adults (49).

One limitation regarding the assessment of inflammation is that the presented inflammatory biomarkers were assessed in blood, urine and exhaled air only and not more invasively by the analysis of sputum, BAL fluid or biopsies. Sputum induction was, in fact, part of the protocol in this project. Unfortunately, no reliable cell counts could be performed due to contamination of the samples with saliva and epithelial cells, which was verified by analysis of amylases in the sputum supernatant (results not shown). This shortcoming confirms the challenging nature of more invasive assessments of inflammation in asthmatic children and underlines the need for minimally invasive biomarkers.

From this perspective, the finding of increased levels of YKL-40 in children with PA and the correlation between YKL-40 and bronchial wall thickening is particularly interesting. The current findings of increased serum YKL-40 levels in children with PA and the inverse correlation between YKL-40 and asthma control confirm and extend previous observations made in Chinese, French and North American cohorts of adult patients with asthma (92, 167).

Our findings strengthen the view that YKL-40 is a marker of remodelling or even somehow involved in the remodelling processes. Chupp et al. found a correlation between serum and BAL levels of YKL-40 and RBM thickness (92) and interestingly, YKL-40 binds to collagen I and can regulate the formation of collagen fibrils (168). YKL-40 has also recently been shown to increase the proliferation and migration of human bronchial smooth muscle cells (169). In relation to other diseases also characterized by chronic inflammation and tissue remodelling, YKL-40 has been shown to play a role in fibrosis and angiogenesis by regulating the survival, adhesion, migration and proliferation of various structural cell types (93). Thus, the relation
between YKL-40 and asthma severity could, in part, be mediated by the effects of YKL-40 on fibrosis and smooth muscle cells (170).

The role of BWT assessment by HRCT as a surrogate marker of chronic inflammation or airway remodelling deserves further comment. The correlation between RBM and BWT in children is weak (78) and has not been confirmed in all studies (77). However, it should be emphasized that the image of thickened bronchial walls represents not only the increased thickness of the reticular membrane, but probably also other features of remodelling; hypertrophy and hyperplasia of the bronchial smooth muscle, myofibroblastic proliferation, increased numbers and size of the mucus glands and angiogenesis (71).

Another noteworthy finding regarding the chitinases is the close correlation between YKL-40 and blood neutrophils. Previous studies have indicated that YKL-40 is produced by a number of cells, including epithelial cells and alveolar macrophages (171). It is also known that YKL-40 can be stored in the specific granules of neutrophils and released upon activation of these cells (172). A recent study of sputum YKL-40 levels in adults with asthma also demonstrated a strong correlation with sputum neutrophil count (173). The results from our study support and extend these findings, by indicating that neutrophils are major producers of YKL-40 particularly in severe asthma.

Variation in the CHI3L1 gene was strongly associated with serum YKL-40 levels. Children homozygous for the C-allele had higher levels of YKL-40 despite the fact that there were more children with CA with this genotype. However, the highest YKL-40 levels among CC-carriers were predominantly found in children with PA, demonstrating that YKL-40 levels are associated with asthma severity, irrespective of variations in CHI3L1. Genetic variants in the CHI3L1 gene have been associated with several phenotypes of asthma (174-176) although our sample size limits the possibility to draw firm conclusions about risk estimates related to PA and CA. In 2008, Ober et al. found a relationship between the CHI3L1 polymorphism -131 C/G, circulating YKL-40 levels, prevalence of asthma and pulmonary function (94). Furthermore, a large population based study by Rathcke et al., including over 6500 subjects demonstrated that the rs4905928 G allele, and not the C allele was associated with asthma. Taken together, there is a need for further studies investigating the complex genetic associations between CHI3L1 and asthma (175).

Variations in the CHIT1 genotype affected chitotriosidase activity. Children lacking the 24 bp duplication in exon 10 (75/75 bp homozygous) had the highest chitotriosidase activity compared to the 75/99 bp heterozygous and the 99/99 homozygous genotype. Within the 75/75 bp homozygous genotype, children with PA had the highest chitotriosidase activity, confirming that chitotriosidase activity is associated with asthma severity, independently of variations in the CHIT1 gene.
7 MAIN RESULTS AND CONCLUSIONS

A comprehensive and standardized clinical assessment discerns specific features of children with Problematic severe asthma (PA) and allows the identification of those who are severely resistant to therapy.

The major distinguishing features of children with PA involve familial background (heredity, socioeconomic status), clinical presentation (comorbidities and triggering factors) and pathophysiological differences including degree of airway obstruction, bronchial hyperresponsiveness and inflammatory profile (IL-5, number of eosinophilic and neutrophilic cells in blood).

Multi-sensitization towards animal-derived lipocalin, kallikrein and secretogoglobin allergens is more common in children with PA, compared to those with controlled asthma (CA) and is associated with increased bronchial inflammation.

Cat-allergic children with PA have a higher allergen sensitivity, as measured by CD-sens, compared to cat-allergic peers with CA. Furthermore, CD-sens correlates with clinical markers of asthmatic disease, including asthma control and biomarkers of eosinophilic inflammation.

YKL-40 levels and chitotriosidase activity are increased in the serum of children with PA, and YKL-40 specifically correlates with airway remodelling (assessed by HRCT) and blood neutrophils in children severely resistant to asthma therapy. The associations between YKL-40, chitotriosidase activity and asthma severity persisted after adjusting for variations in the $CHI3L1$ and $CHIT1$ genes, respectively.

In summary, we have provided a detailed characterization of children with PA in Sweden, and identified potential explanatory factors for their severe disease. Furthermore, we examined the usefulness of two novel diagnostical methods for allergy and found that these methods both provide relevant information when investigating children with PA. Our findings support that YKL-40 is a potential biomarker of asthma severity and airway remodeling in children with PA. Finally, a translational research approach is necessary when investigating associations between etiologies, mechanisms of disease and clinical presentation in complex diseases.
8 FUTURE PERSPECTIVES

The scientific community seems to have agreed upon the current definition of Problematic severe asthma and new studies using this definition are continuously being published. International collaborations are being established; one example of which is the European U-Biopred (Unbiased BIOmarkers for the Prediction of Respiratory Disease) study which our research group is participating in. The American SARP study (Severe asthma research program) is being continued and extended to include more children (177). Future reports from these projects will hopefully provide additional insights into the stability of the severe asthma phenotype, the mechanisms involved in the development of severe asthma, and novel therapeutic approaches in these children.

One particular point of interest is characterization of the inflammatory pattern in the peripheral parenchyma, as inflammation in severe asthma may predominantly be associated with peripheral airways. Sputum samples, BAL fluid and transbronchial biopsies do not necessarily reflect inflammation in the lung parenchyma. Several methods are being developed and introduced which can help to elucidate the role and distribution of peripheral airway inflammation, including the measurement of FeNO at different exhalation flow rates. Novel imaging techniques, for instance magnetic resonance imaging to identify ventilation defects and molecular imaging techniques that enables visualisation of areas of inflammation (178) will probably also enhance our understanding of this disease.

The pursuit for specific biomarkers to characterize asthma will continue, the goal being to find easily attainable biomarkers that will help us to understand disease mechanisms, select appropriate treatments and monitor the effect of these treatments. During the past few years, several new therapeutic alternatives have been developed which are increasingly being used in clinical practice (Anti IgE, temperature-controlled laminar airflow). Several new treatment alternatives are in the pipeline, including Pitrakinra (IL4-/IL-13 antagonist) (147), Lebrikizumab (IL13 antagonist) (148) and Reslizumab (antibody against IL-5) (149) all of which predominantly target allergic asthma. As these are, or are likely to be, expensive treatment alternatives, detailed characterization of patients in order to identify those most likely to benefit from these interventions will be increasingly important. As mentioned in the discussion CD-sens could turn out to be a valuable tool in this regard, and the possible availability of CD-sens as a chip based method in the foreseeable future would make this method more easily applicable.

Another novel marker of potential clinical relevance is periostin, a protein suggested to be a marker of excessive Th2-type inflammation in asthma (179) and the expression of periostin in airway epithelial cells is regulated by interleukin 13 (180). The effect of Lebrikizumab treatment has been shown to be greater in severe asthmatics with high periostin levels compared to those with low periostin levels, as demonstrated by an improvement in FEV$_1$ and reduction in FeNO) (148) thus providing an entirely new example of biomarker guided treatment in severe asthma. Periostin might also turn out to be a useful biomarker for other treatment alternatives.

However, the most likely scenario regarding biomarkers is that it is no single biomarker will characterise asthma but rather a set or a cluster of different
biomarkers (61), and the most useful of all will probably be clusters of markers combining genomics, transcriptomics and proteomics.

New treatment alternatives for asthma should probably not only focus on suppressing the inflammatory response. The idea of primary prevention of asthma is becoming an option (54) and in this regard combining vaccines for allergy and rhinovirus infections are a novel and interesting approach (181). Another novel approach is intralymphatic immunotherapy which might turn out to be a “fast-track”, effective alternative to subcutaneous immunotherapy (182). In addition, Vitamin D supplementation could be a straightforward treatment alternative to strengthen the immune response in children with therapy resistant asthma (183), although more trials are needed.

I denna avhandling redovisas resultat från en svensk multicenter studie i vilken skolbarn med problematisk svår astma har jämförts med jämnåldra barn som har kronisk astma och god effekt av sin astmabehandling (kontrollerad astma). Syftet med studien var att göra en detaljbeskrivning av barn med problematisk svår astma, identifiera eventuella undergrupper och studera skillnader mellan barn med svår astma och barn med kontrollerad astma avseende årtidighet, sjukdomsorsak, sjukdomsmekanismer och den kliniska presentationen av sjukdomen. Vidare ville vi utvärdera nyttan av nya biomarkörer för svår astma.

Barnen, som rekryterades från 16 barnkliniker i Sverige, fick svara på frågeformulär, göra lungfunktionsmätningar, test bronkial hyperreaktivitet, lämna urin prov och blodprov för analys av inflammationsmarkörer (däribland vita blodkroppar och chitinaser i serum), genetisk kartläggning och allergitester (allergenkomponentanalys och basofil känslighetstest (CD-sens)). Barnen med svår astma fick dessutom genomgå datortomografi av lungor och bikhålor.

I det första delarbetet fann vi att av 57 inkluderade barn med problematisk svår astma kunde 23 klassificeras som svårbehandlade, och övriga 34 var terapi resistenta. Barnen med problematisk svår astma skilde sig signifikant från de med kontrollerad astma avseende familjebakgrund (årtidighet för astma, socioekonomi), klinisk presentation (astmakontroll, samsjuklighet med rhinokonjunktivit och symptomutlösande faktorer) och patofysiologiska mekanismer (lungfunktion, hyperreaktiva luftrörs och inflammatorisk profil i blodet (vita blodkroppar, IL-5, chitinaser)). Således har vi med ett icke-invasivt protokoll som inkluderade en standardiserad och detaljerad klinisk karakterisering hittat särskilda kännetecken hos barnen med problematisk svår astma som möjliggjorde identifiering av barn med terapi resistent astma.

I det andra delarbetet analyserade vi förekomsten av antikroppar mot 111 specifika allergen hos alla barn i studien. Det var ingen skillnad i sensibilisering mot enskilda allergenkällor. Barnen med problematisk svår astma var ofta
multisensibiliserat mot flera än 3 specifika allergen från pälsdjur jämfört med barn med kontrollerad astma. Vi fann också att barnen med problematisk svår astma som var sensibiliserade mot fler än 3 av dessa allergen, hade tecken på ökad inflammatorisk aktivitet i blod och utandningsluft och mer hyperreaktiva luftrörs jämfört med svåra astmatiker utan denna multisensibilisering. Detta arbete visar att det kan ha ett mervärde att analysera specifika allergener hos barn med svår allergisk astma jämfört med traditionell allergidiagnostik som mer är riktad mot allergenkällor. Vidare, att multisensibilisering mot fler än 3 specifika pälsdjurallergen kan vara drivande för den allergiska inflammationen i en undergrupp av barn med svår astma.

I det tredje delarbetet undersökte vi om katt-allergiska barn med svår astma hade ökad känslighet för katt, utvärderat med allergenstimulering på isolerade basoefiler (basofil känslighetstest, CD-sens). I denna analys inkluderades 11 katt-allergiska barn med problematisk svår astma som jämfördes med 11 katt-allergiska barn med kontrollerad astma. Vi fann att barnen med problematisk svår astma hade signifikant högre CD-sens jämfört med kontrollbarnen och att värden av CD-sens korrelerade med graden av astmasymptom, specifika IgE antikroppar mot katt och kväveoxid i utandningsluften, en markör för eosinofil luftvägsinflammation. Vi konkluderade att kallt allergiska barn med svår astma har ökad känslighet för katt, och att detta kan vara en bidragande orsak till dessa barns svåra och kroniska symtom. Vi föreslår också att CD-sens skulle kunna användas som en markör för att identifiera barn med den svåraste allergiska astman som har störst nytta av nya, intensiva behandlingsinsatser så som anti-IgE behandling.

Syftet med det fjärde arbetet var att jämföra nivåerna av ett chitinase likt protein YKL-40 och chitotriosidase hos patienter med problematisk svår och lättbehandlad astma, samt att identifiera eventuella samband mellan YKL-40, kroniska luftrörsförändringar (utvärderat med skiktrotn) och andra markörer för astmasjukdomen.

I nuläget saknas biomarker för att tillförlitligt identifiera och karakterisera barn med svår astma. Chitinase YKL-40 har nyligen visat sig vara associerad till luftvägsinflammation, inflammationens svårighetsgrad och dessutom visat sig ha ett samband med kroniska förändringar i luftvägarna som utvecklas efter längre tids inflammation. Skiktrotnen kan också ge information graden av kroniska luftrörsförändringar genom att man värderar de synliga bronkväggsförjockningarna.

Resultaten visar att barnen med problematisk svår astma hade högre värden av YKL-40 och chitotriosidase jämfört med barnen med kontrollerad astma. Bland barnen med terapi resistant astma var det signifikanta korrelationer mellan YKL-40 och astmakontroll, kväveoxid i utandningsluften, neutrofiler i blod och graden av kroniska luftrörsförändringar.

Detta arbete stödjer att nivåerna av YKL-40 har samband med astmans svårighetsgrad även hos barn, och våra fynd indikerar dessutom att YKL-40 har en central roll i luftvägsinflammationen eftersom chitinase är associerad till neutrofil och eosinofil luftvägsinflammation, så väl som till kroniska förändringar i luftroten.

Sammanfattningsvis har vi detaljbeskrivit barn med Problematisk svår astma i Sverige och identifierat faktorer som kan vara avgörande för utvecklandet av deras svåra sjukdom. Vidare, har vi visat på mervärden av att använda nya metoder inom allergidiagnostik. Slutligen är detta avhandlingsarbete ett exempel på nyttan av att ha en noggrant karakteriserat patientkohort när man skall utvärdera nya biomarker samt att
en translationell forskning är nödvändig när man skall undersöka samband mellan orsaker, mekanismer och klinisk presentation av komplexa sjukdomar.
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