Treatment response in childhood asthma

An interplay of genes and inflammatory signals

Susanne J.H. Vijverberg

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Thesis, Utrecht University – with a summary in Dutch

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Medicatierespons van kinderen met astma

Een samenspel van genen en inflammatoire signalen

(met een samenvatting in het Nederlands)

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Wisdom begins in wonder
(Socrates)

Voor dr. Willem R. Vijverberg

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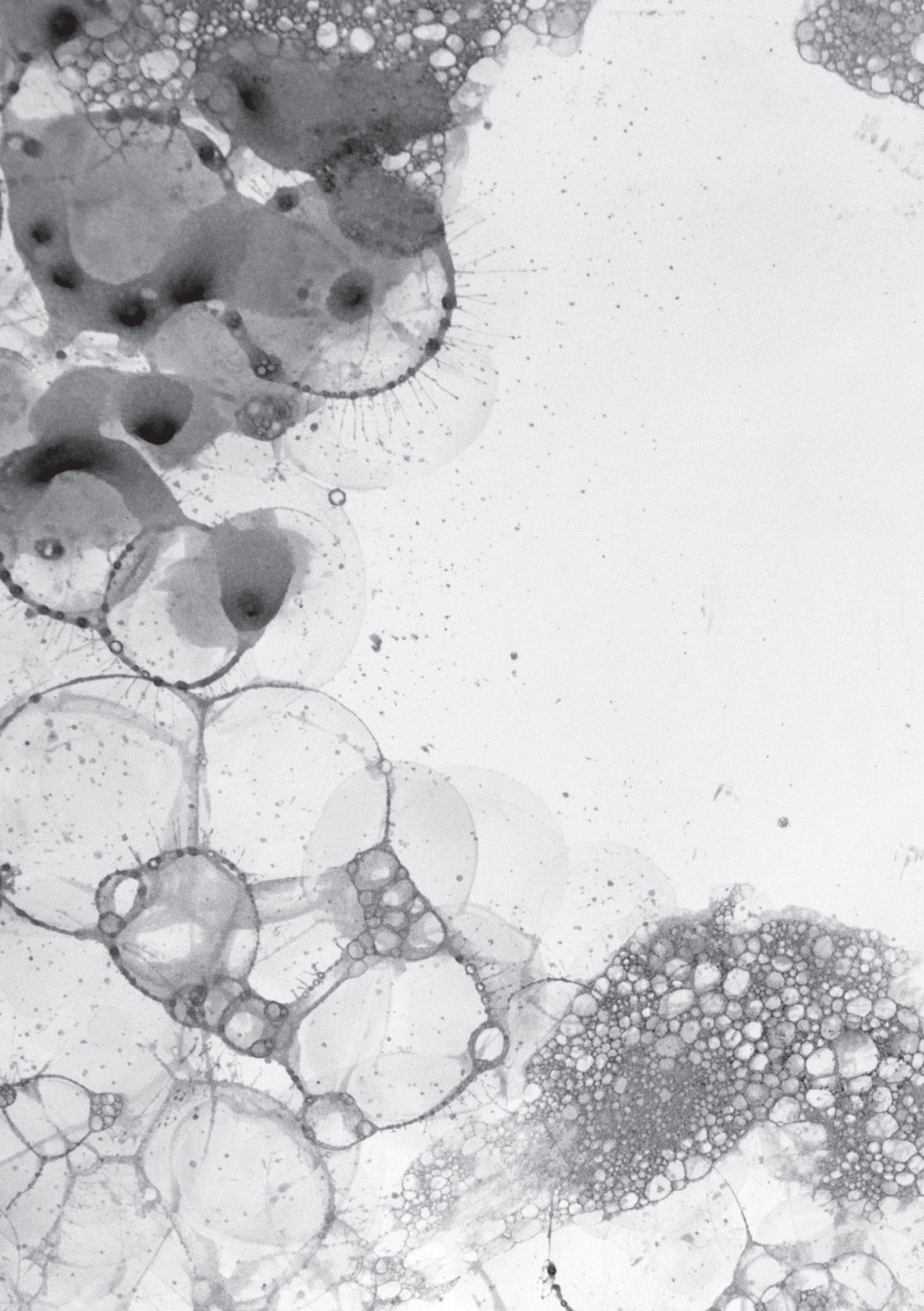
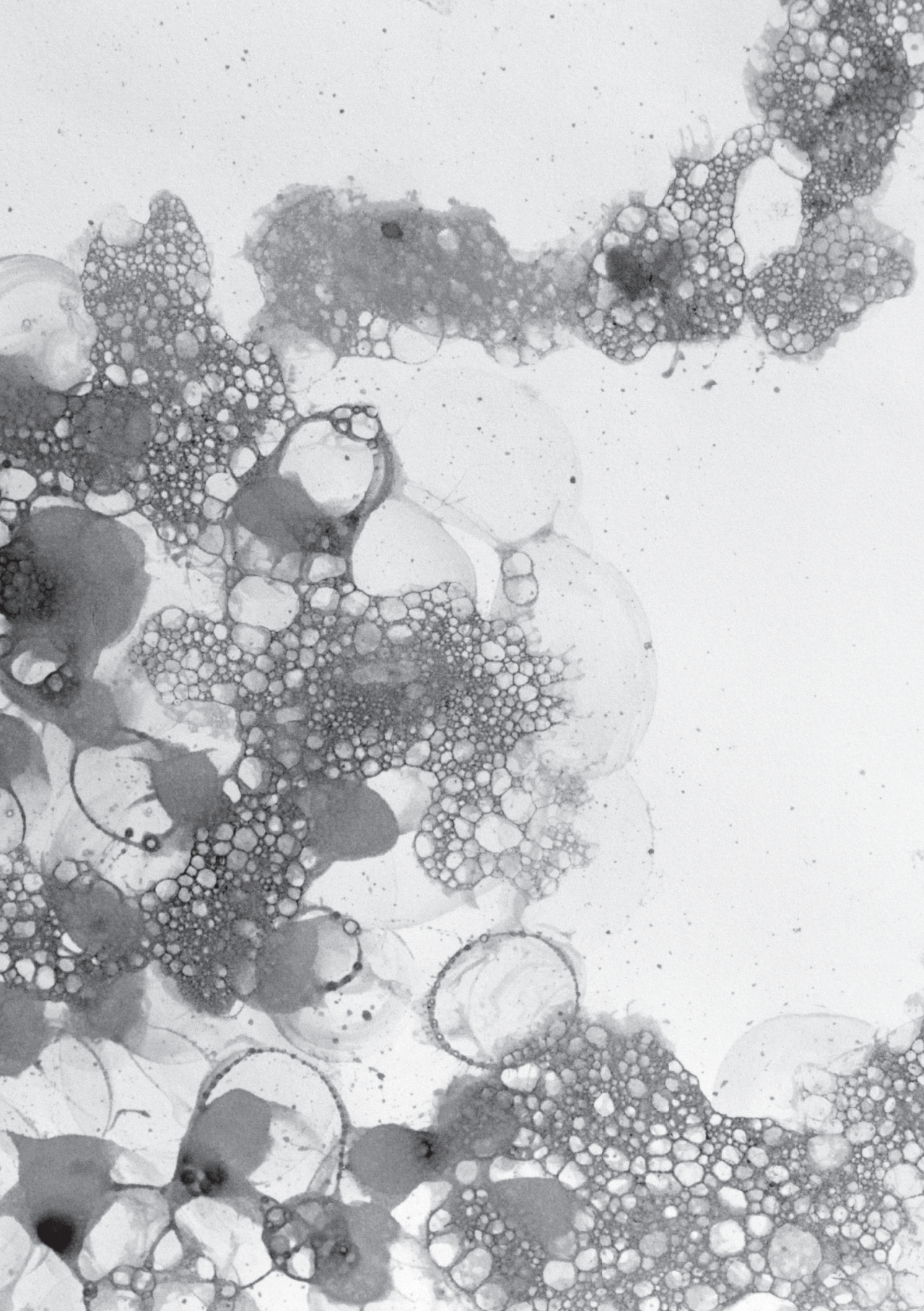


Chapter 1

Introduction



1.1

General Introduction

From black coffee and asthma cigarettes to inhaled corticosteroids

Asthma is a chronic disease affecting the airways, and is the most common chronic disease among children.[1] Patients suffering from asthma may experience recurrent periods of wheezing, coughing and breathlessness. Asthma-like diseases were already being recognised thousands of years ago with descriptions of the diseases being found in ancient Indian, Chinese and Egyptian texts.[2] The term 'asthma', as it is used nowadays, originates from the Greek verb *aazein*, meaning 'to exhale with open mouth, to pant',[3] and it appeared for the first time in the Greek epic poem the *Iliad*. In approximately 800BC, Homer described a warrior who dies at the end of a violent battle with asthma and perspiration. Centuries later physician John Floyer established the basis of the current commonly applied medical definition when he described asthma as an intermittent and episodic disease in his classic 1698 monograph 'A Treatise of Asthma'.[4] The term was further refined in 1860 in the work 'On Asthma: Its pathology and treatment' by Henry Hyde Salter, an international asthma expert and asthma patient himself. He introduced the role of the nervous system in asthma and described the disease as 'paroxysmal dyspnea of a peculiar character with intervals of healthy respiration between attacks.'[5] Salter advocated the use of depressants to suppress nervous irritation, sedatives to relieve airway irritability and stimulants to avert 'morbid activity' from the pulmonary system, with coffee being one of the proposed remedies. He wrote, 'One of the commonest and best reputed remedies of asthma, one that is almost sure to have been tried in any case that may come under our observation, and one that in some cases is more efficacious than any other, is strong coffee.'[5] We now know that caffeine is a weak bronchodilator, and that it also has the ability to decrease respiratory muscle fatigue.[6]

Using the concept of asthma as a disease of recurrent spasmodic constrictions of the airways (bronchi), initial asthma treatment strategies focused on bronchodilators, such as adrenalin and anticholinergic belladonna-related alkaloids, and other bronchodilators addressing the adrenergic system (e.g. ephedrine and theophylline).[7] Asthma powder and, later on, asthma cigarettes (Figure 1) containing the dried and ground leaves of *Datura stramonium* were sold commercially for asthma treatment from the end of the nineteenth century until late in the twentieth century as the therapeutic smoke could enter deeply into the lungs.[7,8] In the 1950s, the pressurised metered dose inhaler (pMDI) was introduced in combination with

isoproterenol (selective agonist to β-adrenoceptors), followed in the 1960s by salbutamol (selective agonist to β_2-adrenoceptors).[7, 9]

Figure 1. Asthma cigarettes containing the ground leaves of Datura stramonium.
Courtesy of prof. J.A.M. Raaijmakers

During the same period the concept of asthma as a chronic inflammatory disorder emerged.[10] In the early 1950s, case reports showed that treatment with adrenocorticotropic hormone or corticosteroids could decrease asthma symptoms,[11] however, initial enthusiasm was tempered due to the pronounced side effects of systemic corticosteroids.[12] In the 1960s, cromone medications were developed, and anti-inflammatory inhaler treatment with sodium cromoglycate became widely prescribed for childhood asthma.[7] Only a couple of years later, inhaled corticosteroids (ICS) entered the stage.[13] With the use of inhaler devices, low dosages of steroids could be delivered locally into the lungs, minimising the absorption in the bloodstream and the associated severe side effects. Results of clinical trials showed that ICS were more successful in achieving asthma control when compared to bronchodilator treatment alone.[14, 15] Furthermore, ICS were found to be superior to sodium cromoglycate in the treatment of childhood asthma.[16] Gradually ICS, in combination with short-acting β_2-agonists (SABA) as needed, became the cornerstone therapy of persistent asthma.

Other types of asthma treatment were introduced in the years that followed, such as long-acting β_2-receptor agonists (LABA), leukotriene receptor antagonists (LTRA), and more recently, treatment with anti-Immunoglobin E monoclonal antibody (omalizumab). Various other biologics, anti-interleukin 5 monoclonal antibody (mepolizumab), anti-interleukin 13 monoclonal antibody (lebrikizumab) and anti-interleukin 17 monoclonal antibody (brodalumab) for example, are currently under investigation.[17-20] Nevertheless, ICS hold their position as the first-line maintenance therapy for persistent asthma. 'Inhaled glucocorticosteroids are the most effective controller therapy, and are therefore the recommended treatment for asthma for children of all ages,' states the recently updated Global Initiative for Asthma (GINA) report.[1]

In the Netherlands, a stepwise approach to gain and maintain control over asthma is recommended in the clinical guidelines (Figure 2).[21] SABA as needed should be

prescribed initially to relieve symptoms of bronchoconstriction, according to the established guidelines. When adequate asthma control is not achieved, ICS are added to the treatment regime in order to reduce airway inflammation. A child should be referred to a paediatrician when symptoms remain uncontrolled on this regime. Subsequently, a LABA and/or LTRA can be added to the treatment regime. In cases where asthma symptoms remain uncontrolled and frequent asthma exacerbations occur, a course of OCS may be necessary.

Glucocorticoids: molecular multi-taskers

Glucocorticoids (GCs) are thought to exert their effects primarily by binding to a ubiquitously expressed glucocorticoid receptor (GR) in the cytoplasm. Two isoforms of GR have been described: GRα and GRβ,[22] both are expressed in various tissues and cells, including inflammatory cells and bronchial epithelial cells.[23-25] GRα is the predominant isoform and shows steroid-binding activity, while GRβ can interact with DNA, but not with GCs. GRβ is considered to be a dominant negative regulator of GRα, by competing for DNA binding sites. Furthermore, GRβ can bind to GRα and form a transcriptionally inactive heterodimer.[26] When GCs bind to the GRα, chaperone proteins dissociate, allowing the activated GR-glucocorticoid complex to translocate to the nucleus where it can bind to specific sites on the DNA called glucocorticoid response elements (GRE). Through this binding at promoter regions of responsive genes, GRα can stimulate or repress transcription of the gene.[27]
In addition to the direct regulation of genes through GRE-binding, GRα can also influence gene regulation indirectly, by binding to pro-inflammatory transcription factors such as nuclear factor κB (NF-κB) and activator protein 1 (AP1) for example, or by influencing mRNA stability.[28, 29] Furthermore, glucocorticoids are suspected of exerting non-genomic actions, for example through distinct membrane receptors linked to intracellular signalling pathways,[30] or through the activation of kinases, phosphatases and acetylases associated to the GR complex.[30, 31] These non-genomic processes may explain the very rapid effects of glucocorticoids, such as the change in bronchial blood flow within minutes after ICS treatment.[32]

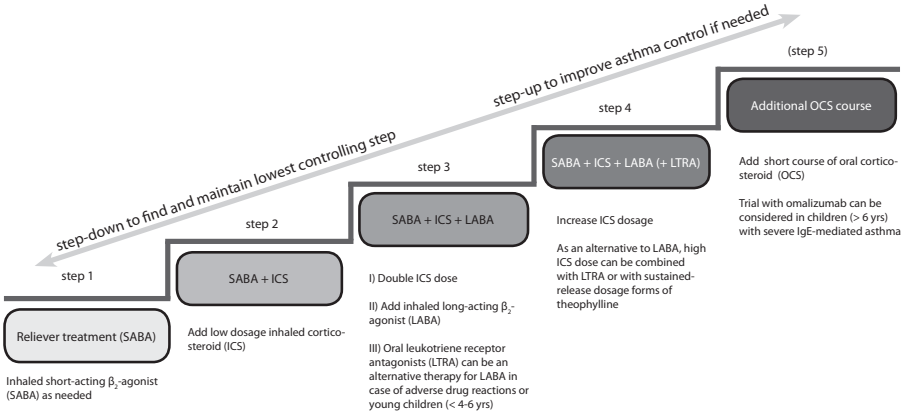


Figure 2. Summary of stepwise asthma treatment in children in the Netherlands. Based on the clinical guideline 'Treatment of Asthma in Children' (Dutch General Practitioners Society)[21] and 'Asthma and COPD Medications' of the Pharmacotherapeutic Compass (Health care Insurance Board).[33]

Heterogeneity in treatment outcome

The majority of asthmatic patients respond well to inhaled corticosteroid treatment; asthma symptoms decrease and lung function improves. However, there is a large interindividual variability in the extent to which symptoms improve upon treatment, and a small group of patients do not seem to respond to treatment at all. A study by the National Heart, Lung, and Blood Institute's Childhood Asthma Research and Education Network detailed the responses of 144 children with mild-to-moderate asthma to 8-weeks of ICS.[34, 35] A large variation in lung function improvement from baseline (see Figure 3) was found.[34] Change in asthma-controlled days showed a similarly wide distribution, varying between an increase of seven asthma-controlled days per week to a decrease of four asthma-controlled days per week.[35]

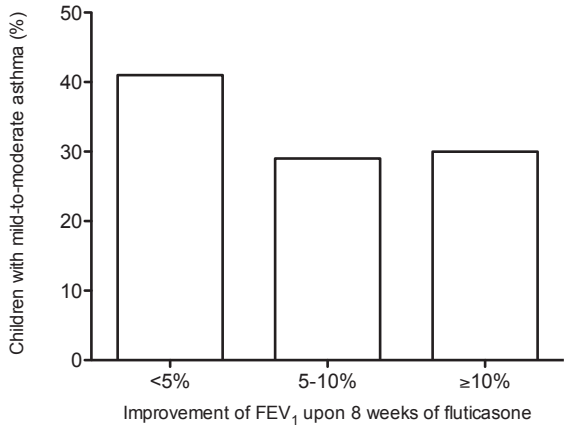


Figure 3. Variation in lung function improvement upon ICS treatment in a clinical trial population of asthmatic children. Percentage change of forced expiratory volume in 1 second (FEV_1) from baseline to 8 weeks of ICS treatment in 144 children with mild-to-moderate asthma. Based on data of Szefler et al.[34]

Heterogeneity in treatment response seem to be due in part to genetic variation.[36] Candidate gene approaches and, to a lesser extent, whole-genome association studies have identified several genetic loci associated with poor treatment response or severe asthma, including *FCER2* (coding for a low-affinity immunoglobin E (IgE) receptor, also known as CD23),[37,38] the 17q21 locus,[39] and *GLCCI1* (encoding glucocorticoid-induced transcript 1 protein).[40] However, overall the evidence is far from conclusive, and there is a definite need for additional work and replication of the identified loci in other study populations.

The many faces of asthma

Asthma is characterised clinically by recurrent episodes of reversible airway obstruction and airway hyperresponsiveness (AHR). Chronic airway inflammation, with innate immune cells such as eosinophils playing an important role, is one of the main pathological characteristics. However, asthma is very heterogeneous in its onset, course and treatment response and appears to encompass a broad collection of heterogeneous disease subtypes with different underlying pathophysiological mechanisms.[41, 42]

The growing consensus for the use of asthma as an umbrella term for various asthma subtypes is accompanied by the further need for clinical markers to distinguish distinct clinically relevant asthma phenotypes, to optimise diagnosis, and to

develop and guide (new) treatment. The measurement of inflammatory markers in bronchial lavage, bronchial biopsies and sputum is invasive and restricted to specialized medical centers. Inflammatory markers in exhaled air (i.e. fraction of exhaled nitric oxide [FeNO][43] or patterns of volatile organic compounds [VOCs]),[44] as well as inflammatory markers of the innate immune system in peripheral blood (e.g. activation of peripheral blood leukocytes)[45] may be promising asthma markers for general application in clinical practice.

The burden of uncontrolled asthma

Uncontrolled asthma exerts a substantial health, financial and societal burden. Almost half of the costs associated with asthma management arise from hospital admissions and unscheduled health care visits.[46] The number of asthma-related hospital admissions has been increasing over the years.[47] In 2005, the total costs of childhood asthma (calculated for children < 15 years of age) for the 25 countries of the EU were estimated at three billion euros, with the Netherlands contributing approximately 106 million euros to this total.[48] These costs included medical and non-medical direct costs (e.g. GP visits, hospitalisation and medication, diagnostics, transportation), as well as indirect costs (e.g. school days lost, caregiver productivity loss).

Furthermore, uncontrolled asthma has a significant impact on the physical and social functioning of children.[49] The disease can affect sport activities, school participation, social contacts, and uncontrolled disease has been associated with behaviour problems.[48, 49] Understanding the biological risk profiles underlying asthma treatment response is of great clinical value in order to improve and stratify treatment strategies and to identify new drug targets. This may also have a considerable social and economic impact.

The PACMAN cohort study

The Pharmacogenomics of Asthma medication in Children: Medication with ANti-inflammatory effects (PACMAN) cohort study was initiated to investigate the role of genetic variation in the treatment outcomes of children who regularly use asthma medication.[50] Patient inclusion started in the spring of 2009. Chronic inflammation and airway remodelling may modulate treatment outcome.[51] Therefore, studying the influence of genetic variation in treatment response might be more successful in asthmatic children than asthmatic adults, since the relationship between response and genetic factors is expected to be less biased in children. They have been exposed to chronic airway inflammation and/or other modulating environmental factors such as air pollution and smoking for a shorter period of time as compared to asthmatic adults.

In the PACMAN cohort study, children (aged 4-12 years) who were regular users of asthma medication (at least three asthma medication prescriptions in the past two years, including one or more in the preceding six months) were recruited with the help of pharmacists belonging to the Utrecht Pharmacy Practice Network for Education and Research (UPPER). During study visits in community pharmacies, an extensive set of data was collected, including data on asthma symptoms, medication use, adherence, environmental factors, inflammatory markers in exhaled breath, and saliva samples for DNA extraction. This resulted in a unique community pharmacy-based cohort of children with respiratory symptoms, representing a cross-section of children who use asthma medication on a regular basis.

Scope of this thesis

This thesis aims to study uncontrolled or exacerbation-prone childhood asthma despite ICS treatment at three different levels: I) genetics, II) inflammatory signals in peripheral blood and III) inflammatory signals in exhaled breath. Using this approach we hope to gain a better understanding of the underlying pathological mechanisms, identify novel asthma biomarkers associated with poor treatment outcomes, and evaluate the clinical value of previously identified asthma biomarkers. The main focus will lie on the treatment response to inhaled corticosteroids.

The following research questions will be addressed:

1. Can we identify new, or replicate previously identified, genetic loci associated with medication response in childhood asthma?
2. Are peripheral blood leukocytes distinctly pre-activated in children with uncontrolled asthma despite ICS treatment as compared to children treated with ICS and controlled asthma?
3. Are inflammatory markers in exhaled breath associated with uncontrolled childhood asthma despite treatment?

Thesis outline

This thesis is divided into three parts, which are preceded by an introductory chapter and concluded by a general discussion. The introductory chapter consists of a general introduction (**Chapter 1.1)**, and is followed by a more in-depth review of the current knowledge on asthma biomarkers for treatment response and asthma phenotypes (**Chapter 1.2**). The three subsequent chapters reflect the distinct dimensions studied.

The studies described in **Chapter 2**, **Genetics**, focus on the influence of genetic variation and treatment response. In **Chapter 2.1** the influence of genetic variation in the glucocorticoid signalling pathway on the risk of exacerbations is studied in four different study populations of children and young adults treated with ICS. In the following three sections (**Chapters 2.2 to 2.4**) replication studies of previously identified pharmacogenetic loci are described. **Chapter 2.2** addresses ICS response and genetic variation in the 17q21 locus and **Chapter 2.3** focuses on ICS response and *GLCCI1* genotype. **Chapter 2.4** investigates the association of the *ADRB2* Arg16Gly genotype and response to LABA as add-on treatment to ICS. In addition to these candidate gene approaches, **Chapter 2.5** describes the approach of using a targeted SNP array to study ICS response in a clinical trial population.

In **Chapter 3**, **Inflammation**, the view shifts towards detecting inflammatory patterns in peripheral blood underlying distinct asthma phenotypes. **Chapter 3.1** describes the design and rationale of the PACMAN2 study, a clinical follow-up to the PACMAN cohort. **Chapter 3.2** addresses the preliminary results of the PACMAN2

study with a focus on the expression of activation markers on peripheral blood leukocytes and the relationship with asthma control.

In **Chapter 4**, **Breath**, markers in exhaled air are investigated, as these markers are thought to reflect underlying inflammation. **Chapter 4.1** addresses the validity of FeNO as a marker of asthma control in children with reported use of asthma medication. **Chapter 4.2** describes initial work on patterns of VOCs in exhaled breath as markers of asthma control in paediatric patients.

In conclusion, a general discussion, as well as an outlook for future work, is presented in **Chapter 5**.

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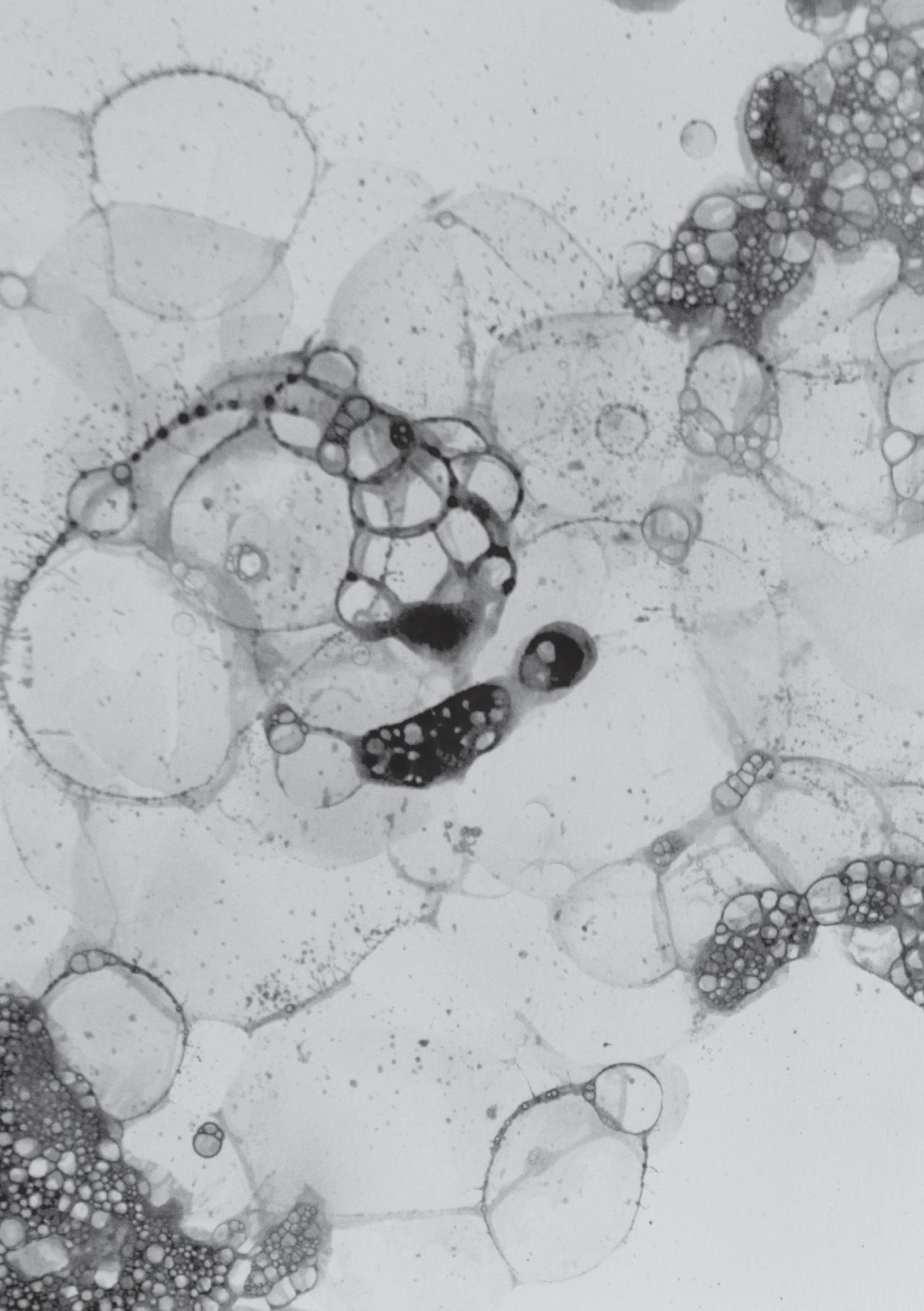

1.2

Asthma biomarkers for clinical phenotyping and treatment responsiveness

This chapter is adapted from:

Clinical utility of asthma biomarkers: from bench to bedside.
Susanne J.H. Vijverberg*, Bart Hilvering*, Jan A.M. Raaijmakers, Jan-Willem J. Lammers, Anke-Hilse Maitland-van der Zee* & Leo Koenderman*
* authors contributed equally

Biologics: Targets and Therapy 2013; 7(1):199-210

Biomarkers of therapy responsiveness in asthma: pitfalls and promises.
Susanne J.H. Vijverberg, Leo Koenderman, Ellen S. Koster, Cornelis K. van der Ent, Jan A.M. Raaijmakers & Anke-Hilse Maitland-van der Zee

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Abstract

Asthma is a chronic disease characterized by airway inflammation, bronchial hyperresponsiveness and recurrent episodes of reversible airway obstruction. The disease is very heterogeneous in onset, course and response to treatment and seems to encompass a broad collection of heterogeneous disease subtypes with different underlying pathophysiological mechanisms. There is a strong need for easily interpreted clinical biomarkers to assess the nature and severity of the disease and assess treatment responsiveness. Currently available biomarkers for clinical practice, for example markers in bronchial lavage, bronchial biopsies, sputum or Fraction of exhaled Nitric Oxide (FeNO), are limited due to invasiveness or lack of specificity. The assessment of markers in peripheral blood might be a good alternative to study airway inflammation more specifically, compared to FeNO, and at a less invasive manner, compared to BAL, biopsies or sputum induction. In addition, promising novel biomarkers are discovered in the field of breath metabolomics (e.g. volatile organic compounds) and (pharmaco)genomics. Biomarker research in asthma is increasingly shifting from the assessment of the value of single biomarkers to multidimensional approaches in which the clinical value of a combination of various markers is studied. This could eventually lead to the development of a clinical applicable algorithm composed of various markers and clinical features to phenotype asthma and improve diagnosis and asthma management.

Introduction to the pathophysiology of asthma

Asthma affects over 300 million individuals worldwide,[1] making it one of the most prevalent common chronic diseases. Although the respiratory disease is rarely fatal, the economic burden is extensive, due to direct and indirect medical expenses, including prescription drug costs, health care costs and productivity losses.[2]

The disease is characterized by airway inflammation, bronchial hyperresponsiveness and recurrent episodes of reversible airway obstruction. Asthma can be classified as "atopic" or "non-atopic" based on the presence (atopic) or absence (non-atopic) of specific IgE antibodies to common environmental allergens. Atopic asthma is the most common form of asthma. In allergen-sensitized patients with atopic asthma, re-exposure to an aeroallergen will lead to an IgE-mediated inflammatory cascade in the airways. Airway resident cells (i.e. macrophages and mast cells), newly mobilized immune cells (i.e. eosinophils and neutrophils) as well as epithelial cells play an important role in this inflammatory cascade.[3] In allergic inflammation, there seems to be a disturbed balance in Th1-type and Th2-type cytokines - with dominance towards Th2 cytokines.[4] Th2 cells produce cytokines such as Interleukin (IL)-4 and IL-13, which induce a class-switch in B-cells to the production of IgE. Th2 cells also produce IL-5, which recruits eosinophils to the lung and IL-9, which stimulates mast cell proliferation. Upon activation, mast cells start to produce histamine, cysteinyl-leukotrienes (Cys-LT's), and prostaglandin D2, which in its turn will lead to additional recruitment of eosinophils, Th2 cells and basophils to the tissue.[5]

Parallel to the allergic asthma model with airway epithelial cells and the adaptive immune response as important pillars, an additional non-allergic asthma paradigm has been proposed. In the non-allergic asthma model the innate immune system responds to constantly invading respiratory viruses and bacteria. This systemic innate response is driven by sentinel cells such as macrophages, dendritic cells, granulocytes and innate lymphoid cells. A recent review by Holtzman and colleagues provides a comprehensive overview of both the allergic and non-allergic immune response in asthma.[6]

A prolonged presence of activated inflammatory cells in the airways may lead to chronic inflammation and might induce tissue alterations in composition, content and organization of the airways ("airway remodelling"). Important cytokines released by epithelial cells and associated with remodelling are IL-25, thymic stromal lymphopoietin (TSLP), and IL-33. The remodelling response is characterized by subepithelial basement membrane thickening, epithelial cell disruption,

neoangiogenesis, globlet cell metaplasia, enlarged submucosal glands and airway smooth muscle hyperplasia[7] and has been observed in chronic asthmatics.[8] However, airway remodelling has also been observed in young asthma patients, suggesting that the process may even precede airway inflammation.[9]
Airway inflammation in asthma is often described as eosinophilic, based upon the presence of primed eosinophils in airways. In addition, various studies have shown that inflammation may also occur in the absence of increased levels of eosinophils.[10-13] As a consequence, a concept has evolved that more than one inflammatory pattern in asthma pathogenesis exists, and that the heterogeneity of the clinical expression of asthma may be associated with distinct underlying inflammatory patterns.[10]

Asthma biomarkers for clinical phenotyping and treatment efficacy

Asthma diagnosis and management is generally based on reported asthma symptoms often combined with lung function and bronchial provocation tests to assess reversible airway obstruction and airway hyperresponsiveness. However, symptoms and lung function measurements may not reflect underlying airway inflammation. Bronchoscopy with biopsies and bronchoalveolar lavage (BAL) are considered gold standard to assess airway inflammation, but are too invasive for general application in clinical practice.[14] In addition, asthma seems to encompass a broad collection of heterogeneous disease subtypes with different underlying pathophysiological mechanisms.[15] In order to identify clinical relevant asthma phenotypes, optimize diagnosis and guide treatment, there is significant interest in the development of asthma biomarkers. In this chapter we will provide an overview of asthma biomarkers already available for clinical practice and promising biomarkers currently under development (Figure 1).

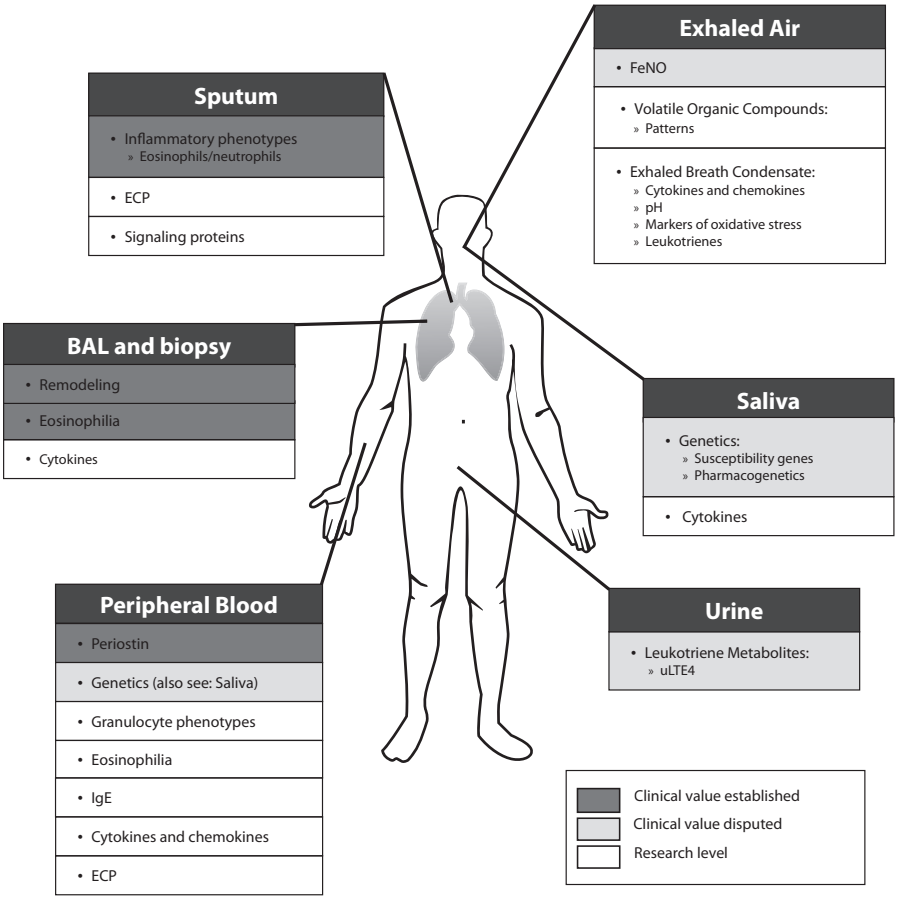

Figure 1. Asthma biomarkers. BAL, bronchoalveolar lavage; ECP, eosinophil cationic protein; FeNO, fraction of exhaled nitric oxide; IgE, immunoglobulin E; uLTE4, urinary leukotriene E4.

Lungs: the gold standard

The most tissue-specific and reliable method to assess airway inflammation in asthmatic patients is a combined procedure of bronchoscopy, biopsy and BAL. Airway remodeling has been observed in bronchial biopsies of both adults and children with asthma.[16] BAL fluid of asthmatic patients shows elevated levels of Th2 cytokines compared to healthy individuals.[17] In difficult-to-treat asthma, BAL and endobronchial biopsy should be considered to objectify the presence of airway eosinophilia and other typical pathological features of asthma.[18] However, the invasiveness and potential complications of these procedures preclude this

method in daily clinical routines. In recent years, several potential and less invasive diagnostic tools to assess airway inflammation have been investigated, but the validity and value of various methods and markers are subject to dispute.

Markers in induced sputum

Compared to bronchoscopy, biospy and BAL, sputum induction is less invasive. The aim of sputum induction is to obtain lower airway secretions, preferably without contamination of saliva and epithelial cells from the oral cavity. During the procedure, the patient inhales increasing concentrations of nebulised saline solution to liquefy sputum. There is a strong correlation between cellular components present in airway fluid obtained by bronchoalvealor lavage (BAL) and cells present in airway fluid obtained by sputum induction.[10, 19] Nevertheless, sputum induction is restricted to specialized medical centers due to the requirement of trained physicians to carry out the procedure in a safe and technically successful manner. Additionally, there is need for a skilled laboratory to perform the analysis.[20]
Currently, four distinct inflammatory phenotypes have been identified based on eosinophil and neutrophil percentages of total non-squamous cells in sputum (Figure 2):

1) Neutrophilic asthma: marked by an increased neutrophil proportion;
2) Eosinophilic asthma: marked by an increased eosinophil proportion;
3) Mixed granulocytic asthma: marked by increased neutrophils and increased eosinophils levels and;
4) Paucigranulocytic asthma: marked by normal levels of neutrophils and eosinophils.

Several studies have shown that higher levels of sputum eosinophils are associated with a better response to corticosteroids[21-23]; in contrast, others could not find, or only find a poor correlation[24, 25] (Supplementary Table 1). Additionally, the assessment of sputum eosinophils seems to be of value to guide treatment. Petsky et al.[20] evaluated three randomized-controlled trials that compared adjusting asthma therapies based on sputum eosinophils to adjustment based upon clinical symptoms. All three studies[26-28] included asthmatic adults and used asthma exacerbations, defined as the requirement for rescue oral corticosteroids, as the primary study

outcome. These studies showed a significant reduction in the frequency and the severity of asthma exacerbations in the group for whom sputum eosinophils counts were used to guide therapy, however clinical symptoms did not significantly differ between groups. This is in line with work by Haldar et al.[29] and Nair et al.[30] that showed that eosinophilic inflammation plays a role in asthma exacerbations, but is not necessarily involved in asthma symptoms or lung function. Both studies were randomized clinical trials that assessed the effect of Mepolizumab on adults with refractory asthma and sputum eosinophilia. Mepolizumab is a monoclonal antibody directed against IL-5, an important regulator of eosinophil production, activation and survival. By neutralizing IL-5, Mepolizumab inhibits eosinophilic inflammation. Treatment with Mepolizumab led to a reduction in the number of severe exacerbations in asthmatics with sputum eosinophilia despite high doses of corticosteroids. This may suggest that patients with high sputum eosinophils respond preferentially to Mepolizumab with fewer exacerbations; nevertheless, neither of the studies included asthmatic patients presenting with low sputum eosinophils. Moreover, Mepolizumab had no effect on asthma symptoms, FeNO or lung function. This implies that eosinophilic inflammation might not always underlie classical asthma symptoms (e.g. bronchial hyperresponsiveness).

In children, the interpretation and consistency of the results from analysis of induced sputum, BAL and tissue biopsies is also more variable and complex than one would expect. De Blic et al.[31] investigated bronchial inflammatory profiles in 28 children with persistent bronchial obstruction despite high dosages of ICS (age: 9–15 years). Bronchial biopsies showed that eosinophil and neutrophils counts in the epithelium were significantly higher in the children with persistent symptoms compared with children with few symptoms. In addition, BAL showed a trend for higher percentages of neutrophils in children with persistent symptoms. Wang et al.[32] assessed inflammatory phenotypes in sputum in 77 asthmatic children and 52 asthmatic adults and observed remarkable differences in the distributions between the adult and pediatric population. The asthma phenotype was predominantly eosinophilic in children with acute asthma and predominantly paucigranucolytic in children with stable asthma. The majority of adults with stable asthma also showed a paucigranulocytic phenotype, however, in adults with acute asthma, the predominant asthma phenotype was neutrophilic. This shows that one should be careful extrapolating results from paediatric studies to an adult population and vice versa. Furthermore, Fleming et al.[33] have shown that inflammatory phenotypes in children with asthma can change over time.

Corticosteroid treatment can also influence the inflammatory phenotype. Studies by Cowan et al.[34] and Luijk et al.[35] showed that corticosteroid treatment could lead to an increase of sputum neutrophils. This increase was observed after an allergen challenge,[35] as well as during regular treatment.[34] Cowan and colleagues analysed the sputum of 88 asthmatics after steroid withdrawal and subsequently after a period of ICS use. Upon steroid withdrawal, none of the asthmatics were classified as neutrophilic. However, after a (281 day) trial of fluticasone two of the 63 asthmatics originally classified as eosinophilic, and three of the 29 asthmatics originally classified as paucigranulocytic, were classified as neutrophilic. In total 32 asthmatics presented an altered inflammatory phenotype after steroid treatment. The effect of corticosteroids on inflammatory patterns might be explained by the differential effect of these drugs on the survival of distinct types of inflammatory cells. *In vitro* studies have shown that corticosteroids induce apoptosis in eosinophils while they inhibit apoptosis in neutrophils.[36, 37] Thus, the neutrophilic phenotype observed after corticosteroid treatment might be a consequence of the selective mechanism of action of the steroids. Nevertheless, the neutrophilic phenotype may also occur in the absence of steroid treatment, as sputum neutrophilia has also been observed in steroid-naïve patients.[38, 39]

Eosinophilic Cationic Protein (ECP) is another sputum marker that has been investigated as an asthma biomarker.[40] ECP is released during degranulation of eosinophils and can be measured in sputum, BAL fluid and in serum. It is considered to be a non-specific marker for inflammation and, therefore, lacks the specificity for diagnosing asthma. Meijer et al. showed that sputum ECP has no predictive value for clinical response to corticosteroids in asthmatic patients.[41] Its added value as a diagnostic tool would be in the measurement of the extent of inflammation and severity of asthma, e.g. moderate versus severe asthma.[42]

Peripheral blood

Peripheral blood is easy to obtain and the procedure itself is less invasive in comparison to sputum induction and bronchoscopy. Since inflamed tissue releases chemo-attractants and cytokines, which recruit activated immune cells from the peripheral blood, the dynamic process of immune cells entering and leaving the blood stream can be used as an indirect readout of the state of disease.

From a cellular point of view, peripheral blood eosinophilia has been described

extensively as a potential asthma biomarker.[43] Blood eosinophilia correlates with bronchial hyperresponsiveness and asthma-related inflammation.[44] The specificity of using peripheral blood eosinophilia to diagnose asthma is, however, rather low, as allergies, autoimmune disease and parasitic infections cause blood eosinophilia as well. Therefore, its role as a diagnostic biomarker remains limited. The same applies to total and allergen-specific IgE levels in serum.[45] However, there are some reports that these markers are (partly) associated with ICS response (Supplementary Table 2).

Szefler et al.[46] showed that asthmatic children with increased levels of serum eosinophil cationic protein, serum total IgE levels and blood eosinophil counts were more likely to respond to ICS. In addition, Meijer et al.[41] reported that the eosinophil percentage in the blood was a predictor of a therapeutic response in adults with asthma, yet the baseline values of clinical parameters used as outcome parameters (i.e. FEV_1, PC20Mch and QOL) were the major predictors of corticosteroid response and prediction of an individual response based on blood eosinophilia remained poor.

Several studies have evaluated whether the presence of inflammatory soluble mediators such as chemokines and cytokines were applicable as biomarkers for type and extent of asthma phenotypes.[47] Recent studies utilized multiplex analysis allowing the parallel analysis of multiple cytokines within one serum/plasma sample.[48, 49] Unfortunately, these studies have neither led to a clinically useful diagnostic tool to identify distinct disease phenotypes, nor to a tool to assess disease severity. A weakness of studies assessing inflammatory chemokine and cytokine profiles lies in the fact that the choice of mediators to be studied determines the (lack of) success of this approach, and that several inflammatory mediators may still be unidentified. Anti-inflammatory mediators (such as receptor antagonists) are often neglected. In addition, little consideration has been given to the complex interaction between inflammatory mediators.[50]

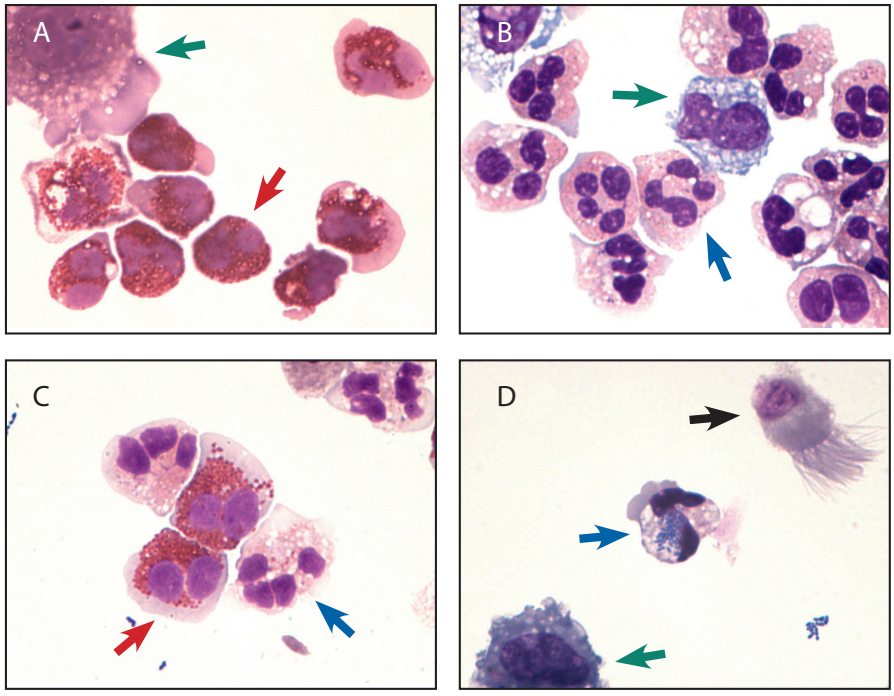

Figure 2. Inflammatory phenotypes of adult asthma patients obtained by sputum induction.

(A) Eosinophilic type; marked by the presence of eosinophils ≥3% (red arrow). The green arrow indicates and an alveolar macrophage, (B) Neutrophilic type; marked by the presence of neutrophils ≥61% (blue arrow). The green arrow indicates an alveolar macrophage, (C) Mixed type; marked by the presence of both eosinophils (red arrow) ≥3% and neutrophils (blue arrow) ≥61% (D) Paucigranulocytic type; marked by a lack of eosinophils (<3%) and neutrophils (<61%). The black arrow in this photo shows a ciliated pseudostrafied columnar airway epithelial cell, the blue arrow a neutrophil with phagocytosed bacteria inside and the green arrow an alveolar macrophage (green arrow). *May-Grünwald/Giemsa staining, photograph at 100x magnification, courtesy of dr. J.A.M. van der Linden (UMC Utrecht, The Netherlands).*

A different approach is to examine shifts in activation profiles of inflammatory cells in peripheral blood and attempt to link these shifts to clinical phenotypes. These inflammatory cells will integrate all pro- and anti-inflammatory signals and change their phenotypes accordingly. Studies on activation status of peripheral blood cells have provided some insights into the systemic innate immune response in allergic asthma. Various studies have shown that inflammatory cells such as monocytes and granulocytes respond with upregulation of several activation markers in response to inflammatory signals.[51-53] Many of these markers such as Mac-1 (CD11b), CD63, CD66b, CD69 are typically found in granules that fuse with the plasma membrane upon activation of the cells with inflammatory mediators.[54] Unfortunately most

studies[55, 56] compared the presence of the markers on blood cells and tissue cells obtained from sputum and BAL and did not take into account that cells homing to the tissue under homeostatic conditions exhibit the same phenotype.[57] The process of homing of the cells towards the tissue compartment is already sufficient to activate the cells both in homeostasis as well as disease. The expression of these markers in the peripheral blood has not lead to a clear link between expression profiles of granulocytes and type of asthma.

Elegant work by Johansson and colleagues has shown that eosinophils change their activation status of membrane bound integrins rather than overall expression of the integrins in response to inflammatory signals.[58] Application of antibodies specifically recognizing activated states of integrins provided solid data that shows that blood eosinophils in poorly controlled asthma are characterized by activated integrins. This situation is consistent with the hypothesis that these cells are primed and prepared to leave the peripheral blood for the tissues. Our group has obtained similar data by application of antibodies recognizing activated FcγR's.[51, 59] These data demonstrated that eosinophils first become activated in the peripheral blood and subsequently home for the tissue leaving behind unprimed cells.[60] More insight into the activation profiles of inflammatory cells in asthmatic patients, both in the presence and absence of stimuli, might be a promising strategy to identify novel asthma biomarkers.

Closer to clinical implementation is the biomarker-phenotype combination serum periostin and lebrikizumab response. Periostin is a recently discovered matricellular protein that is secreted by bronchial epithelial cells under the influence of IL-13. The presence of periostin in serum correlates strongly with sputum eosinophilia.[61] A study by Corren et al. showed that patients with high levels of serum periostin responded better to lebrikizumab (anti-IL-13 therapy) compared to patients with low levels of periostin.[62]

Fraction of exhaled Nitric oxide (FeNO)

Almost a decade ago the first reports emerged of elevated levels of nitric oxide in exhaled breath (FeNO) in patients with asthma.[63, 64] Since then a high number of studies have assessed the clinical value of exhaled nitric oxide in asthma management. Several FeNO analyzers became commercially available, and international guidelines on FeNO measurement were published.[65, 66]

NO is a highly reactive gaseous molecule that is produced in the airways when the amino acid L-arginine is oxidized to the amino acid L-citrulline. The synthesis of NO is catalysed by nitric oxide synthases (NOS), of which currently three isoforms are known: two forms of constitutive NOS and one form of inducible NOS (iNOS). Especially iNOS (inducible NOS) seems to play a role in the elevated levels of NO in exhaled breath of asthmatics. The expression of the enzyme is upregulated by a wide range of inflammatory cytokines. It remains unclear which cells are responsible for the increased NO production, but airway epithelial cells and eosinophils are considered to be the important candidates.[67] It is thought that the increased FeNO in asthmatics is caused by upregulated iNOS in the inflamed airways. Upon synthesis in the airways, NO diffuses into the airway lumen that is essentially NO free, thereby causing the rise in FeNO. High FeNO is regarded to be a surrogate marker of ongoing eosinophilic airway inflammation and may reflect uncontrolled asthma and predict asthma exacerbations.[68]

Despite the initial enthusiasm of FeNO as a new and non-invasive marker of airway inflammation, the clinical usefulness of FeNO to measure asthma control is still debated. Studies that have investigated the association between asthma control and FeNO provide inconsistent results, as well as studies assessing the relationship between FeNO and other airway inflammation markers, such as sputum eosinophilia or the presence of eosinophils in bronchial specimens.[69, 70] This may be partly caused by a non-overlap in asthma symptoms and airway inflammation. Furthermore, this relationship is complicated due to various other factors that seem to influence FeNO levels; including age, atopy, medication use, therapy adherence and airway infections.[68, 71, 72] In addition, tailoring asthma treatment based on FeNO measurements did not decrease asthma exacerbations or lead to better asthma control according to a meta-analysis performed by Petsky et al.[73] FeNO might, nevertheless, still be a valuable marker to predict ICS responsiveness (Supplementary Table 3). Zacharasiewicz et al. showed that the combination of increased levels of FeNO and the percentage of sputum eosinophils were significant predictors of exacerbation upon steroid reduction in children with stable asthma.[74] Still, the predictive value of FeNO alone was limited. More than 30% of the children with elevated FeNO levels remained stable upon ICS reduction. Studies by Szefler et al. and Knuffman et al. showed that paediatric asthma patients with elevated FeNO levels were more likely to respond to corticosteroids compared to montelukast.[46, 75] Although the clinical value of a single FeNO measurement is limited, combining this measure with other markers

of airway inflammation may lead to a more accurate assessment of underlying disease state.

Volatile organic compounds in exhaled breath

The measurement of volatile organic compounds (VOCs) in exhaled breath is a novel metabolomic approach to study molecular signatures of respiratory disease. Exhaled breath contains a complex mixture of up to thousands of VOCs. These compounds are produced due to metabolic processes in the airways and the presence and/or concentrations of the different compounds are likely influenced by the presence of airway inflammation. Different methods to assess VOCs exist; one can assess profiles of VOCs ('breathprints') present in exhaled breath using polymer-based gas sensor arrays ('electronic nose')[76] or identify individual molecular components using gas chromatography-mass spectrometry (GC-MS).[77] Asthma patients can be differentiated from healthy controls based on their breathprints,[78] as can asthmatic patients from COPD patients.[79] However, the method was less successful in distinguishing mild asthmatic from severe asthmatics.[78] Breathprints of COPD patients do correlate with the presence of eosinophil and neutrophils in induced sputum, as well as with levels of ECP and myeloperoxidase (MPO) in induced sputum, suggesting that the electronic nose might be capable of assessing distinct types of underlying airway inflammation.[80]

Using the other approach, GC-MS, Dallinga et al. showed that the measurement of a limited set of VOCs in exhaled air could differentiate asthmatic children from controls with high sensitivity (95%) and high specificity (89%).[77] A study by Ibrahim et al. showed that a set of 15 VOCs could accurately discriminate asthmatic patients from controls, and also could classify patients according to inflammatory sputum phenotype and asthma control (based on the ACQ).[81]

The assessment of VOCs in exhaled breath seems to be a very promising approach, especially when knowledge of clinical relevant VOCs is integrated in a user-friendly handheld device such as an electronic nose. However, validation of clinical relevant VOC patterns in a large population of asthmatic patients is necessary, as well as longitudinal assessment of these VOC patterns. Furthermore, the influence of asthma treatment should be assessed and international guidelines on VOC measurement should be developed. A large Europe-wide study to assess the clinical utility of VOCs in asthma in-depth is currently ongoing.[82]

Exhaled breath condensate

Biomarkers in breath can also be measured in exhaled breath condensate (EBC). When exhaled breath is cooled a liquid phase can be obtained, which contains condensed water vapour, as well as non-volatile substances. It is thought that changes in EBC content reflect biochemical changes in the airway surface fluid.
Various markers in EBC have been found to be elevated in asthmatics when compared to healthy individuals, including: adenosine concentration,[83] markers of oxidative stress (i.e. hydrogen peroxide),[84] cytokines and chemokines,[85] nitric oxide-related products,[86] isoprostanes and leukotrienes.[87] Furthermore, acute asthmatics and poorly controlled asthmatics show a decreased pH of EBC.[88, 89]
In spite of these results, the measurement of markers in EBC is still in its research phase and several important methodological problems complicate the clinical utility of EBC.[90] A standardized methodology for EBC collection is lacking, as are established reference values. Various factors such as the type of condenser equipment used, cooling temperature, condenser tube coating, cleaning procedures, breathing patterns, ambient air pollution or concentrations of relevant cytokines too low for reliable determination influence the measurement and compromise reproducibility. Furthermore, it remains uncertain from which compartments of the respiratory system specific markers originate and how the dilution of distinct markers should be assessed.[90]

Urine: leukotriene metabolites

Cysteinyl leukotrienes (LTs) C_4 and D_4 are lipid mediators, which are thought to play a role in asthma pathogenesis. They can be released from various cells, including eosinophils, neutrophils and mast cells. LTC_4 and LTD_4 in the plasma are rapidly converted into the less active LTE_4 metabolite. A fraction of LTE_4 is excreted in urine. The urinary LTE_4 (uLTE4) concentration is used as a marker of total body LT production.[91] Studies by Szefler et al. and Cai et al. showed that asthmatic patients with higher levels of $uLTE_4$ were more likely to respond to leukotriene antagonists (LTRA) when compared to asthmatic patients with lower $uLTE_4$ levels.[46, 92]

Pharmacogenetics

Twin studies have shown that asthma contains a considerable genetic component.[93] Genome-wide association studies (GWAS) have identified several loci to be associated with asthma risk, including: the 17q21 locus, *ADAM33* and various cytokines and cytokine receptor genes; *IL18R1, IL33, IL2RB, IL10, TGFB1* and *IL6R*.[94-97] A recent review by Dijk et al. provides a thorough overview of asthma susceptibility genes that have been found by genome-wide association studies.[98] Nevertheless, effect sizes are small and the identified genetic variants can only explain a small part of the asthma heritability. This could be due to the heterogeneity in asthma phenotypes and the underestimated influence of environmental factors. For example, recent work by Ierodiakonou and colleagues showed an interaction between variation in *TGFB1* and smoking on asthma severity.[99] Carrying a G-allele of rs6957 in *TGFB1* was associated with higher submucosal eosinophils and basement membrane thickness, but only in current or ex-smoking asthmatics.

A more promising genetic approach for clinical asthma practice might be pharmacogenomics: the association of genomic variations and medication response. More than a decade ago, Drazen et al.[100] suggested that up to 80% of the interindividual variance in treatment response of (Caucasian) asthmatics might be due to genetic variations. These variations could lead to an altered expression or function of therapeutic targets and pathways. Since then, several genetic variants have been described to be associated with treatment response.[101, 102]

Polymorphisms in the glucocorticoid receptor gene (*NR3C1*) are apparent pharmacogenetic candidates to be associated with altered corticosteroids response, however, results have been inconsistent and few studies have specifically focused on ICS-treated asthmatic patients.[101] More promising results have been found for other genes involved in the corticosteroid signaling pathway including *STIP1*[103] and *CRHR1*.[104, 105] A study by Hawkins et al. found a positive correlation with variations in *STIP1*, coding for an adaptor protein in the glucocorticoid receptor complex, and baseline lung function and improvement in lung function upon corticosteroid treatment in 382 adults with asthma.[103] Variation in the corticotrophin-releasing hormone receptor type 1 (*CRHR1*) gene has been associated with lung function improvement upon ICS treatment in asthmatic adults and children in three large clinical trial populations.[104, 105] Nevertheless, this association could not be replicated by Dijkstra et al. in 164 asthmatic patients.[106]

Other genes that have been associated with ICS response in asthmatic patients include *GLCCI1*,[107] *FCER2*[108, 109] and *TBX21*.[110] A study by Tantisira et al. showed that asthma patients with a variant in the *GLCCI1* have less improvement in lung function upon inhaled corticosteroids (ICS) treatment.[107] *GLCCI1* encodes Glucocorticoid Induced Transcript 1, a protein of unknown function. Furthermore, a SNP in the *FCER2* gene, coding for a low affinity IgE receptor, has been associated with an increased risk of asthma-related hospital visits, uncontrolled asthma and higher daily steroid dosages,[108, 109] and variation in *TBX21* (encoding transcription factor T-bet) has been related to improved airway responsiveness in childhood asthma upon treatment with ICS.[110] T-bet is thought to be an important regulator of the Th1/Th2 balance.[111]

Because of the complex mechanism of action of corticosteroids, it is likely that multiple genetic variations, rather than a single or just a few variations, will influence a therapeutic response. Hakonarson et al.[112] examined gene expression profiles in peripheral blood mononuclear cells from glucocorticoid-sensitive and glucocorticoid-resistant asthma patients. They showed that an expression pattern of 11 genes could predict the corticosteroid response in asthmatic patients with an accuracy of 84%. Notably, the expression of one single gene at baseline, *NFKB1*, encoding for the DNA-binding subunit of the transcription factor NF-kB, could already predict corticosteroid response with an accuracy of 81%. A study by Donn et al.[113] examined gene expression profiles in T lymphoblasts from glucocorticoid-sensitive and glucocorticoid-resistant healthy individuals but identified a different discriminatory gene set than Hakonarson and colleagues had done.

Pharmacogenomic studies on response to LTRA have found the strongest association with *ALOX5*,[114, 115] a 5-lipoxygenase, and *LTC4S*, a glutathione S-transferase.[116, 117] However, closer to clinical implementation might be assessment of the β_2-adrenergic receptor gene (*ADRB2*) Arg16Gly polymorphism in order to determine response to long-acting β_2-agonists for which randomized clinical trial (RCT) data are available.[118-120] The β_2-adrenergic receptor is a G-protein coupled receptor that is expressed in smooth muscle in the airways and activation induces bronchial relaxation. β_2-agonists are the most frequently prescribed drugs to relieve airway obstruction and act through the β-adrenergic receptor. Evidence suggests that genetic variations in the gene are associated with an altered response to long-acting β_2-agonists. Recently, a small RCT based on prospective testing of genetic variation in the *ADRB2* gene (alteration in amino acid at position 16; Arg16Gly) showed encouraging results in 62 children with persistent asthma.[118] Asthmatic

children homozygous for the variant genotype were randomized to a long-acting β_2-agonist (LABA) plus ICS or to LTRA plus ICS. The group treated with ICS and LTRA scored better on asthma symptoms and quality of life, used less rescue medication and were fewer days absent from school compared to the group children treated with LABA plus ICS, suggesting that asthmatic children homozygous for *ADRB2* Arg16Gly substitution (B16 Arg/Arg) benefit more from LTRA compared to LABA as add-on treatment to ICS. Yet, there was no difference in lung function improvement. On the other hand, RCTs performed in adults found no such differences in efficacy. A post-hoc pharmacogenetic analysis of two large RCTs in which asthmatic patients were treated with LABA or with a combination of LABA and ICS, found no differences in exacerbations, use of rescue medication, nights of awaking and lung function when patients were stratified according to *ADRB2* Arg16Gly genotype.[121] In a crossover RCT asthmatic patients with the B16 Arg/Arg (homozygote for the risk allele) or B16 Gly/Gly (homozygote for the wild type allele) were randomized to LABA plus ICS or placebo plus ICS. There was no difference in lung function improvement between the groups when ICS was added. Remarkably, airway responsiveness in the patients with B16 Gly/Gly did improve significantly when ICS was added to the treatment, while it did not in the B16 Arg/Arg group.[119] Airway responsiveness was measured as methacholine PC_{20} doubling dose: the dose of methacholine that provokes a 20 percent drop in the volume of exhaled air during the first second of a forced expiratory maneuver (FEV_1).

So far pharmacogenetic studies have been limited by small sample sizes, heterogeneous populations and lack of replication. However, the emergence of new sequencing technologies, innovative strategies of analyses and the upcoming of international research consortia may lead to the identification and replication of clinical relevant associations in the near future. In addition, the development of innovative – though expensive – targeted treatment strategies (such as omalizumab [anti-IgE], mepolizumab [anti-IL5] and lebrikizumab [anti-IL13]) may provide a novel clinical context for pharmacogenetics in order to identify subgroups of asthma patients that will benefit the most from these treatments.

Conclusions and future directions

Consensus is present in the asthma field that new and non-invasive biomarkers are necessary to better diagnose and stage the various asthma phenotypes in clinical practice and improve asthma treatment. In the clinical setting, asthma control is often used as a surrogate endpoint for treatment response and refers to the absence of clinical manifestations. Nevertheless, asthma may be characterized by recurrent periods of symptoms, and the absence of clinical symptoms does not necessarily correlate with the absence of underlying airway inflammation. Furthermore, not only biological factors (such as inflammatory patterns and variations in drug receptors) may play a role in a therapeutic response, but psychological, sociological and environmental factors may also be involved. More knowledge on the pathobiological mechanism underlying asthma aetiology may be of great importance to identify relevant biomarkers for asthma diagnosis and management. Currently, various surrogate markers for airway inflammation have been identified, though few studies have addressed whether these markers are also associated with response to corticosteroids. Studies that have been performed varied considerably in study design and definition of treatment response and are therefore difficult to compare.

Furthermore, single biomarker approaches to phenotype asthma are increasingly regarded to be inaccurate and outdated. To diagnose the presence of eosinophilic inflammation for example, FeNO is a very sensitive biomarker, but not very specific. Intuitively, combining FeNO with markers of eosinophilic inflammation (such as the percentage of eosinophils in peripheral blood or eosinophil receptor expression) or other biomarkers would increase specificity. To test this hypothesis, studies combining multiple known biomarkers, should be performed. Currently, research consortia like U-BIOPRED (Unbiased Biomarkers for Prediction of Respiratory Outcomes, http://www.ubiopred.european-lung-foundation.org/) and SARP (Severe Asthma Research Program, http://www.severeasthma.org) aim to integrate the process of data collection and multidimensional approaches to phenotype asthma.

Single biomarker approaches remain important in the process of biomarker discovery, as newly identified biomarkers can be integrated in a multidimensional approach to strengthen the diagnostic ability of a clinically applicable algorithm to phenotype asthma. Only then personalized asthma treatment will be in reach.

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Supplementary Table 1. Overview of studies assessing markers in induced sputum for steroid responsiveness in individuals with asthma

Study	Ref	Marker	Cut-off value	Study population	Design	Definition of response	Study outcome	Association/ Predictor?
Meijer et al.	41	ECP	120 µg/L	118 adults with unstable asthma	2-week trial of ICS	$\Delta FEV_1 \geq 9\%$ / ΔPC_{20} Mch ≥ 1 / ΔQOL≥ 0.5	Baseline values of the clinical parameters used as outcome were the major predictors of clinical response. Sputum ECP provided no additional information compared to other predictors.	-
Pavord et al.	21	Eosinophilia	3%	23 asthmatic adults	2-month trial of ICS	Improvement in PC_{20}Mch	Sputum eosinophilia was correlated with improvement in PC_{20}Mch (r: 0.41; p<0.05	+
Little et al.	122	Eosinophilia	4%	37 adult asthmatics	2-week trial of OC	ΔFEV_1 of ≥15%	There was no correlation between sputum eosinophil count and ΔFEV_1 (rho: 0.19, p: 0.36). Sputum eosinophilia had a NPV of 68% and a PPV of 64% for ΔFEV_1 ≥15%	+/-*
Godon et al.	25	Eosinophilia	1%	46 adults with mild uncontrolled asthma	1-month trial of ICS	Improvement in symptom score, QOL, FEV_1, PC_{20}Mch	There was no correlation between sputum eosinophilia and improvement in symptom score, PC_{20}Mch or QOL. Sputum eosinophilia did show a correlation with improvement of FEV_1 (rho: 0.52, p: 0.002)	+/-
Meijer et al.	41	Eosinophilia	3%	118 adults with unstable asthma	2-week trial of ICS	$\Delta FEV_1 \geq 9\%$/ ΔPC_{20} Mch ≥ 1 / ΔQOL≥ 0.5	Baseline values of the clinical parameters used as outcome were the major predictors of clinical response. Sputum eosinophilia provided additional information for ΔFEV_1 (β:0.32), ΔPC_{20}Mch (β:0.20) and for ΔQOL (β:0.22). Prediction of response of individual patients remained poor (correct classification: 57-65%)	+/-
Szefler et al.	22	Eosinophilia	-	26 adult asthmatics	24-week trial with ICS	ΔFEV_1≥15% (good),ΔFEV_1<5% (poor) / ΔPC_{20}Mch>3 (excellent), ΔPC_{20}Mch<1 (poor)	Patients with an excellent improvement in PC_{20}Mch had higher sputum eosinophil levels compared to patients with a poor improvement in PC_{20}Mch (p: 0.013)	+
Bacci et al.	123	Eosinophilia	3%	67 adult asthmatics	4-week trial of ICS	$\Delta FEV_1 \geq 12\%$, ΔPC_{20} Mch ≥ 1	Eosinophilic patients showed significant improvement in ΔFEV_1 and ΔPC_{20}Mch, while non-eosinophilic patients did not. Sputum eosinophils had a high NPV (100%) for $\Delta FEV_1 \geq 12\%$, but a low PPV (34%).	+
Berry et al.	23	Eosinophilia	1.9%	16 adult asthmatics	Cross-over design of 8 weeks of ICS and 8 weeks of placebo	Improvement in PC_{20}Mch, QOL	Eosinophilic patients showed a greater improvement in PC_{20}Mch (p: 0.018) and QoL (p: 0.008) upon treatment with ICS (compared to placebo) than non-eosinophilic patients	+

Study	Ref	Marker	Cut-off value	Study population	Design	Definition of response	Study outcome	Association/ Predictor?
Lex et al.	124	Eosinophilia	2.5%	17 children with difficult asthma	OC for 14 days or one single intramuscular dose of steroids	$\Delta FEV_1 \geq 9\%$ and/or overall subjective improvement	There was a similar clinical improvement in eosinophilic and non-eosinophilic patients.	-
Martin et al.	24	Eosinophilia	-	72 adult asthmatics	6-week trial of ICS	$\Delta FEV_1 > 5\%$	Levels of sputum eosinophils did not differ between responders ($\Delta FEV_1 > 5\%$) and non-responders ($\Delta FEV_1 \leq 5\%$) (p:0.09)	-
Cowan et al.	34	Eosinophilia	2%	88 asthmatics	28-day trial of ICS	$FEV_1 \geq 12\%$ and/ or $\Delta ACQ \geq 0.5$ point decrease and/or $\Delta PC_{20}AMP \geq 2$ and/or $\Delta FeNO \geq 40\%$ decrease	Patients with sputum eosinophilia showed more improvement in ACQ (p:0.001), FEV_1 (p<0.001), $PC_{20}AMP$ (p: 0.008) and FeNO (p<0.001), though a proportion of the non-eosinophilic patients also showed a response	+/-
Green et al.	27	Neutrophilia	65.3%	49 adult asthmatics	2-month trial with ICS	Improvement in symptom score, improvement in FEV_1, improvement in $PC_{20}MCh$	Patients with sputum neutrophilia showed less improvement in symptom score (p:0.04), FEV_1 (p:0.026) and $PC_{20}Mch$ (p:0.029)	+

*Little et al. showed that the combination of FeNO and sputum eosinophilia gave the highest predictive value; however, the predictive values for the combined measurements are not shown here.
ACQ, Asthma Control Questionnaire; ECP, Eosinophil cationic protein; FeNO, Fraction of nitric oxide in exhaled breath; FEV_1, Forced expiratory volume in 1s; ICS, Inhaled corticosteroids; NPV, Negative predictive vale; OC, Oral corticosteroids; $PC_{20}AMP$, Doubling provocative dose of adenosine monophosphate; $PC_{20}MCh$, Doubling provocative dose of metacholine; PPV, Positive predictive value; QOL, Quality of life; r, Pearson's correlation coefficient; β, Standardized regression coefficient; rho, Spearman's rank correlation coefficient.

Supplementary Table 2. Overview of studies assessing markers in peripheral blood for steroid responsiveness in individuals with asthma

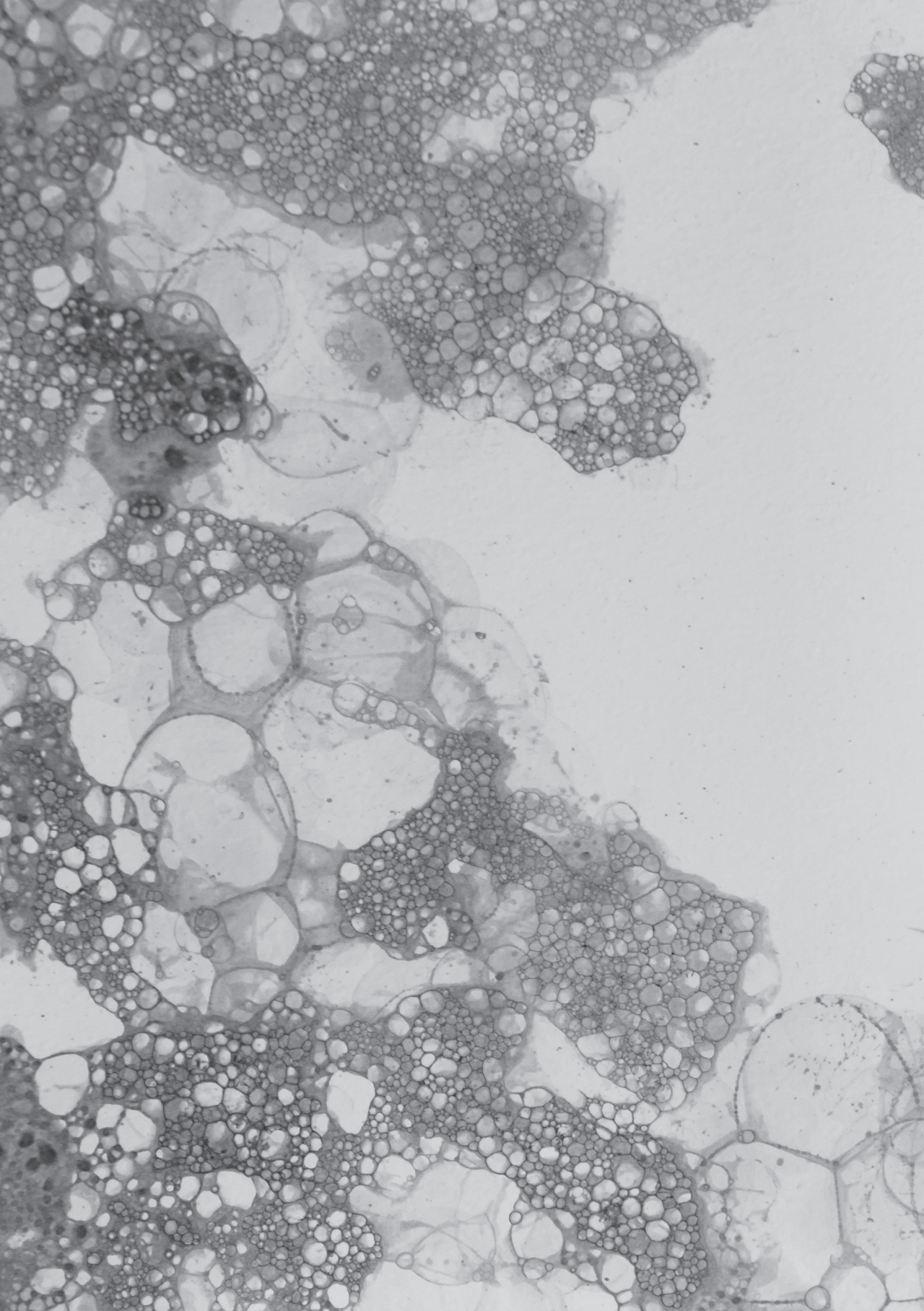
Study	Ref	Marker	Cut-off value marker	Study population	Design	Definition of response	Study outcome	Association/ Predictor?
Meijer et al.	41	ECP	15 µg/L	118 adults with unstable asthma	2-week trial of ICS	$\Delta FEV_1 \geq 9\%$ / $\Delta PC20$ Mch ≥ 1 / $\Delta QOL \geq 0.5$	Baseline values of the clinical parameters used as outcome were the major predictors of clinical response. Serum ECP provided no additional information compared to other predictors	-
Szefler et al.	46	ECP	15 µg/L	126 asthmatic children	Cross-over design of 8 weeks of ICS and 8 weeks of LTRA	$\Delta FEV_1 \geq 7.5\%$	Improvement of FEV_1 upon ICS was associated with higher serum ECP (OR:2.8; $p<0.01$)	+
Meijer et al.	41	Eosinophilia	3%	118 adults with unstable asthma	2-week trial of ICS	$\Delta FEV_1 \geq 9\%$ / $\Delta PC20$ Mch ≥ 1 / $\Delta QOL \geq 0.5$	Baseline values of the clinical parameters used as outcome were the major predictors of clinical response. Blood eosinophils provided some additional information for ΔFEV_1 (β: 0.35) and ΔQOL (β: 0.29), but not for ΔPC20 Mch. Prediction of response of individual patients remained poor (correct classification: 44-63%)	+/-
Szefler et al.	46	Eosinophilia	350 cells/ mm^3	126 asthmatic children	Cross-over design of 8 weeks of ICS and 8 weeks of LTRA.	$\Delta FEV_1 \geq 7.5\%$	Improvement of $FEV_1 \geq 7.5\%$ upon ICS was associated with higher blood eosinophil counts (OR:2.3, $p<0.05$)	+
Bacci et al.	123	Eosinophilia	5%	67 adult asthmatics	4-week trial of ICS	$\Delta FEV_1 \geq 12\%$, $\Delta PC20$ Mch ≥ 1	Both patients with high and low blood eosinophils showed a significant improvement in FEV_1 and symptom scores. Improvement in ΔPC20 Mch was only significant in the high blood eosinophil group. Predictive values were poor (NPV: 35%, PPV: 54%)	-
Meijer et al.	41	IgE	-	118 adults with unstable asthma	2-week trial of ICS	$\Delta FEV_1 \geq 9\%$ / $\Delta PC20$ Mch ≥ 1 / $\Delta QOL \geq 0.5$	Baseline values of the clinical parameters used as outcome were the major predictors of clinical response. Serum IgE provided some additional information for ΔQOL when combined with other predictors (β: 0.29; β: 0.22)	+/-
Szefler et al.	46	IgE	200 kU/L	126 asthmatic children	Cross-over design of 8 weeks of ICS and 8 weeks of LTRA	$\Delta FEV_1 \geq 7.5\%$	Improvement of $FEV_1 \geq 7.5\%$ upon ICS was associated with higher IgE levels (OR: 2.9, $p<0.01$)	+

ECP, Eosinophilic cationic protein; FEV_1, Forced expiratory volume in 1s; ICS, Inhaled corticosteroids; LTRA, Leukotriene receptor antagonist; OR, Odds ratio; PC_{20}Mch, Doubling provocative dose of methacholine causing 20% fall in FEV_1; QOL, Asthma quality of life; β, Standardized regression coefficient.

Supplementary Table 3. Overview of studies assessing FeNO for steroid responsiveness in individuals with asthma

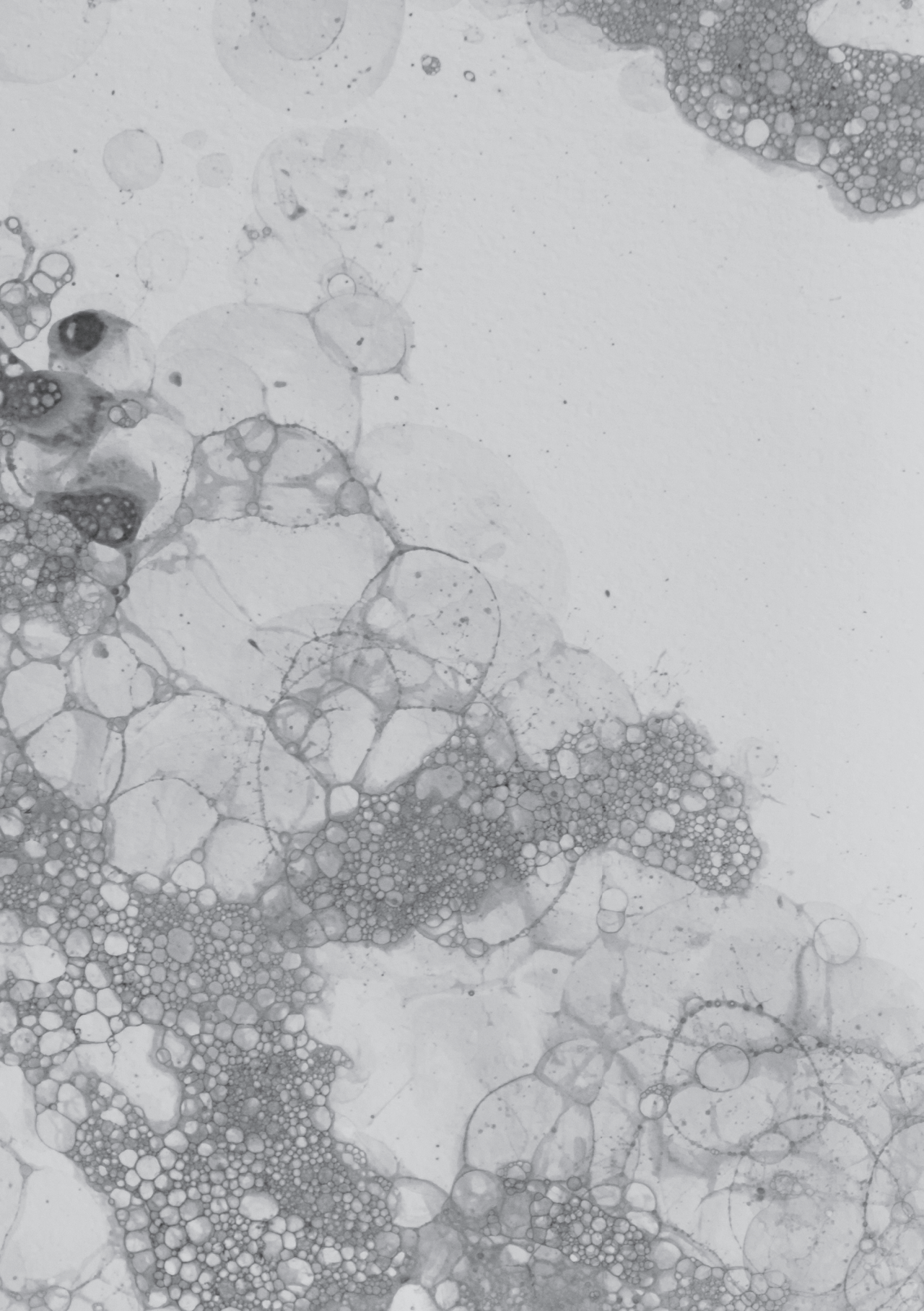
Study	Ref	Marker	Cut-off value marker	Study population	Design	Definition of response	Study outcome	Association/ Predictor?
Little et al.	122	FeNO	10 ppb	37 adults with stable asthma	2-week trial of OC	ΔFEV_1 ≥15%	There was a correlation between FeNO and improvement in FEV_1 (rho: 0.47, p: 0.003). FeNO had a NPV of 72% and a PPV of 83% for ΔFEV_1 ≥15%	+*
Meijer et al.	41	FeNO	n/a	118 adults with unstable asthma	2-week trial of ICS	ΔFEV_1 ≥ 9%, ΔPC20 Mch ≥ 1, ΔQOL≥ 0.5	Baseline values of the clinical parameters used as outcome were the major predictors of clinical response. FeNO provided no additional information	-
Szefler et al.	22	FeNO	n/a	26 adult asthmatics	24-week trial with ICS	ΔFEV_1≥15% (good), ΔFEV_1<5% (poor) / ΔPC_{20}Mch>3 (excellent), ΔPC_{20}Mch<1 (poor)	ΔFEV_1≥15% was associated with higher median FeNO (p: 0.002)	+
Smith et al.	125	FeNO	47 ppb	52 patients (children and adults) with undiagnosed respiratory symptoms	4-week trial of ICS	ΔFEV_1 ≥ 12% / mean morning peak flow ≥ 15% / ΔPC_{20}AMP ≥ 2 /composite symptom score reduction of ≥ 1	High FeNO levels were associated with a better steroid response on all of the assessed endpoints. AUC: 0.76 (PPV: 47%, NPV: 89%) for ΔFEV_1 ≥ 12%; AUC: 0.81(PPV: 53%, NPV: 94%) for increase in morning peak flow, AUC: 0.64 (PPV: 35%, NPV: 77%) for decrease in symptom score; AUC: 0.91 (PPV: 82%, NPV: 91%) for PC_{20}AMP	+
Szefler et al.	46	FeNO	25ppb	126 asthmatic children	Cross-over design of 8 weeks of ICS and 8 weeks of LTRA.	ΔFEV_1≥7.5%	Favorable response to fluticasone alone was associated with higher FeNO (OR: 2.8, p<0.05)	+
Martin et al.	24	FeNO	n/a	72 adult asthmatics	6-week trial of ICS	ΔFEV_1 > 5%	FeNO levels did not differ between responders (ΔFEV_1 > 5%) and non-responders (ΔFEV_1 ≤ 5%) (p:0.68)	-
Cowan et al.	34	FeNO	33 ppb	88 asthmatics	28-days trial of ICS	FEV_1≥12 and/ or ΔACQ≥ 0.5 point decrease and/or ΔPC_{20}AMP≥2 and/or ΔFeNO≥ 40% decrease	In non-eosinophilic patients FeNO was a predictor of improvement of ΔPC_{20}AMP (AUC: 0.810), but not for improvement of ACQ or FEV_1. In eosinophilic patients FeNO was a predictor of improvement of ΔPC_{20}AMP (AUC: 0.778) and of improvement in ACQ (AUC: 0.727)	+
Pérez-de-Llano et al.	126	FeNO	30 ppb	102 adults with difficult-to-treat asthma	Stepwise increase in ICS for 1 month. Patients who remained uncontrolled received OC for 1 month	ACT ≥ 20	FeNO was a good predictor of improvement in ACT (AUC: 0.925, PPV: 88%, NPV: 91%)	+

*Little et al. showed that the combination of FeNO and sputum eosinophilia gave the highest predictive value; however, the predictive values for the combined measurements are not shown here.
ACQ, Asthma Control Questionnaire; ACT, Asthma Control Test; AUC, Area under the curve; FeNO, Fraction of nitric oxide in exhaled breath; ICS, Inhaled corticosteroids; NPV, Negative Predictive Value; OC, Oral corticosteroids; OR, Odds ratio; PPV, Positive Predictive Value; PC_{20}AMP, Doubling provocative dose of adenosine monophosphate; PC_{20}MCh, Doubling provocative dose of metacholine; ppb, Parts per billion; rho, Spearman's rank correlation coefficient.



Chapter 2

Genetics



2.1

ST13 polymorphisms increase the risk of exacerbations in steroid-treated asthmatic children and young adults

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Abstract

Background: The clinical response to inhaled corticosteroids (ICS) is associated with single nucleotide polymorphisms (SNPs) in various genes. This study aimed to relate variations in genes in the steroid pathway and asthma susceptibility genes to exacerbations in children and young adults treated with ICS.

Methods: We performed a meta-analysis of three cohort studies: PACMAN (n=357, age: 4-12 years, the Netherlands), BREATHE (n=820, age: 3-22 years, UK) and PAGES (n=391, age: 2-16 years, UK). Genes were selected based on a role in the glucocorticoid signaling pathway or a reported association with asthma. Two outcome parameters were used to reflect exacerbations: hospital visits and oral corticosteroid (OCS) use in the previous year. A fourth study population (CAMP, clinical trial, n=172, age: 5-12 years, USA) was included to test the robustness of the findings.

Results: Two SNPs in *ST13* were associated with an increased risk of exacerbations despite ICS treatment in the three cohort studies. When CAMP was included in the meta-analysis the two SNPs remained associated with exacerbations. In a meta-analysis of the four studies *ST13* was associated with asthma-related hospital visits; OR=1.28 per G allele for rs138335 (p=0.02) and OR=1.31 per G allele for rs138337 (p=0.007) and OCS usage in the previous year, OR= 1.30 per G allele for rs138335 (p=0.003) and OR=1.18 per G-allele for rs138337 (p=0.03).

Conclusion and clinical relevance: A novel susceptibility gene, *ST13*, coding for a co-chaperone of the glucocorticoid receptor, is associated with exacerbations in asthmatic children and young adults. Genetic variation in the glucocorticoid signaling pathway seems to add to the interindividual variability in clinical response to ICS treatment in children and young adults.

Introduction

Inhaled corticosteroids (ICS) are considered first line therapy for reducing airway inflammation, improving lung function, and controlling asthma stability in patients with persistent asthma.[1,2] While most asthmatic patients have a beneficial response to inhaled corticosteroid therapy, approximately 10% of the patients suffer from severe symptoms despite regular use of ICS,[3] and almost half of the costs of asthma management arises from unscheduled health care visits due to exacerbations.[4] Heterogeneity in treatment response may partly be due to genetic variation.[5] An example of genetic variation in the *FCER2* gene contributing to exacerbations despite ICS treatment has been published previously.[6,7]

Corticosteroids are thought to exert their anti-inflammatory effects primarily by binding to a ubiquitously expressed glucocorticoid receptor (GR) in the cytoplasm.[8] In the absence of glucocorticoids the receptor is predominantly sequestered in the cytoplasm in a multi-protein chaperone complex. Various chaperones and co-chaperones have been described to be involved in the stabilization and maturation of the receptor.[9] Upon binding of glucocorticoids to receptor, the complex translocates to the nucleus where it can block gene expression of a wide range of pro-inflammatory genes and promote the expression of anti-inflammatory genes. To date, there have been few studies addressing variations in corticosteroid receptor complex genes and steroid treatment response in patients with asthma.[10,11]

We hypothesized that susceptibility genes might also be associated with an increased risk of exacerbations despite steroid treatment, due to a potential link with exacerbation-prone asthma phenotypes. In the present study we aim to relate genetic variations in genes in the steroid pathway and asthma susceptibility genes to asthma exacerbations despite ICS treatment.

Methods

Study population

Tag SNPs in 17 candidate genes were studied in three independent North-European cohorts of steroid-treated asthmatic children and adolescents: 1) the Pharmacogenetics of Asthma Medication in Children: Medication with Anti-inflammatory effects (PACMAN) cohort study, 2) the BREATHE study and, 3) the Paediatric Asthma Gene Environment Study (PAGES). For the current analyses we excluded participants of non-Northern European origin.

PACMAN

The PACMAN study is an observational cohort study of children (age: 4-12 years) with a reported (regular) use of asthma medication through community pharmacies in the Netherlands. Details of the study protocol have been described elsewhere.[12] We analyzed the PACMAN data obtained between 2009 and 2012. Data were collected with the help of pharmacists belonging to the Utrecht Pharmacy Practice Network for Education and Research (UPPER), and the work was conducted in compliance with the requirements of the IRB of the Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht University. A detailed history of the subjects is obtained, including information on asthma symptoms, exacerbations and medication use over the preceding 12 months during a study visit in the community pharmacies. Saliva samples are collected for DNA extraction (Oragene DNA Self Collection kit, DNA Genotek, Inc., Ontaria, Canada). The Medical Ethics Committee of the University Medical Centre Utrecht has approved the PACMAN study.

BREATHE

The BREATHE study includes children and young adults (age: 3-22 years) with physician-diagnosed asthma through primary or secondary clinics in either Tayside or Dumfries (Scotland, United Kingdom).[13,14] We analyzed the BREATHE data obtained between 2004 and 2006. At the asthma clinic a detailed history was obtained, including information on symptoms, treatment and asthma exacerbations over the preceding 6 months. Mouthwash samples were collected and DNA was isolated using Qiagen DNAeasy 96 kits (Qiagen GmbH, Hilden, Germany). The Tayside Committee on Medical Research Ethics has approved the BREATHE study.

PAGES

The PAGES study recruited children and adolescents (age: 2-16 years) with physician-diagnosed asthma through 15 secondary care asthma clinics across Scotland from 2008 to 2011. Details of the study protocol of the PAGES have been described elsewhere.[15] Briefly, a detailed history was obtained including information on symptoms, treatment and exacerbations over the preceding 6 months. Saliva samples were collected for DNA extraction (Oragene DNA Self Collection kit, DNA Genotek, Inc., Ontaria, Canada). The Plymouth and Cornwall Research Ethics Committee has approved the PAGES study.

First and second meta-analysis

We performed two meta-analyses; in the first we included PACMAN, BREATHE and PAGES, according to the similarity in design (cohort) and uniformity in genotyping strategy (Sequenom platform, performed at Dundee University). In order to test the robustness of our findings, we assessed the identified associations in a fourth population, the CAMP trial. This study differed in design (clinical trial) and genotyping strategy (imputation of GWAS data), and therefore was included in a second meta-analysis.

CAMP trial

We studied 172 non-Hispanic white ICS-treated children with asthma included in CAMP (USA). CAMP is a multi-center trial that randomized 1,041 children with mild-to-moderate asthma aged 5 to 12 years to budesonide (ICS), nedocromil, or placebo twice daily. The participants were followed for a mean of 4.3 years and follow-up visits took place at 2 and 4 months after randomization and every 4 months thereafter. The design of the study has been described previously.[16] We restricted our analysis to the non-Hispanic white subjects randomized to budesonide with available genotyping data (n=172).

Definition of ICS use

Pharmacological management of asthma was categorized based on the British Thoracic Society (BTS) guidelines[2]: step 0: no use of inhaled albuterol on demand in the past month, step 1: inhaled short-acting β_2- agonists (SABA) as needed, step 2: step 1 plus regular ICS, step 3: step 2 plus regular long-acting inhaled β_2-agonists (LABA) and, step 4: step 3 plus oral leukotriene receptor antagonists. For the present study we selected children and young adults on BTS treatment step 2, 3 and 4.

SNP selection and genotyping

Ten genes were selected based on their involvement in the glucocorticoid (GC) receptor complex (*NR3C1, HSPCA, HSPA4, FKBP4, ST13*), GC transport (*SERPINA6*) or GC-mediated signalling (*CREBBP, TBP, NCOA3, SMAD3*). In addition, seven genes were selected based on a previously reported association with asthma susceptibility, severity or asthma medication response (*ARG1*, 17q21 locus, *IL2RB IL18R1*, *PDE4D, HLA-DQ, BCL2*).[17-19] We selected 50 tag SNPs. SNPs were included if the MAF > 0.2. Tag SNPs were selected using Tagger (http://www.broadinstitute.org/mpg/tagger/server.html) with a gene coverage threshold of 90%. Previously described SNPs

in the genes of interest were also selected. Genotyping was performed using the Sequenom Mass Array platform (Sequenom, San Diego, California, USA). Genotype calls of all DNA samples and SNPs were examined for quality. Samples that consistently failed genotyping (≥ 20% of the SNPs) were excluded for further analyses. Subsequently, SNPs with a call rate < 95% were excluded, as well as SNPs not in Hardy-Weinberg equilibrium. A total of 38 SNPs (78%) in twelve genes passed this quality control. The following genes did not pass quality control and were excluded from further analyses: *HSPCA, HSPA4, IL18R1, HLA-DQ and BCL-2*. Selected genes and corresponding SNPs that passed quality control are listed in Supplementary Table 1. Illumina Infinium II 550 K SNP Chips and 610 Quad Chip (Illumina, Inc, San Diego, California) were used for genotyping in the CAMP study. SNPs of interest for replication were imputed based on 1000 Genomes.

Definition of outcome

As indicators for asthma exacerbations we studied: 1) asthma-related hospital visits and, 2) course(s) of oral corticosteroid (OCS) use reported by parent or child. The following outcome definitions as a measure for severe exacerbations were used:

1) asthma-related hospital visits reported by the parent of child:
    - BREATHE and PAGES: asthma-related hospitalization in the past 6 months
    - PACMAN: asthma-related ED visits in the past 12 months
    - CAMP: asthma-related ED visits and hospitalizations in first 12 months of the trial.
2) course(s) of OCS reported by the parent or child:
    - BREATHE and PAGES: in the past 6 months
    - PACMAN: in the past 12 months
    - CAMP: first 12 months of the trial

Statistical analysis

Logistic regression analysis was used to study the association between the SNPs and risk of exacerbations (OCS use or asthma-related hospital visits). Odds ratios (OR), 95% confidence intervals (CI) and p-values were calculated per study. The model was adjusted for age, gender and BTS treatment step. An additive genetic model was assumed. ORs were meta-analyzed assuming random effects with the inverse variance weighing method. I^2 was used to quantify between-study heterogeneity.[20] False Discovery Rates (FDR) were calculated to estimate the proportion of false positives due to multiple testing.[21] In addition, the risk estimates per genotype were

calculated. Statistical analysis was carried out using IBM SPSS 19.0 for Windows (SPSS, Inc, Chicago, Ill, USA) and PLINK.[22] Haplotype frequencies of the two *ST13* SNPs were estimated using the EM algorithm implemented in the 'haplo.stats' package in R, forest plots were made with R and the 'meta' package.[23]

Results

Characteristics of the study populations

Data were available for 820 children and young adults of the BREATHE cohort, 391 children and adolescents of PAGES, 357 children of the PACMAN cohort and 172 children of CAMP (Table 1). Most patients were on BTS treatment step 2 (as needed short-acting β_2-agonist use combined with regular low dose ICS). Compared to the other studies, the participants in the PACMAN cohort reported the lowest rates of asthma-related hospital visits (6.2%) and OCS usage (6.2%) in the past year.

Table 1. Baseline characteristics study population

	BREATHE (n=820)	PAGES (n=391)	PACMAN (n=357)	CAMP (n=172)
Child characteristics				
Age, mean (SD)	9.8 (4.0)	9.0 (3.8)	8.7 (2.3)	8.8 (2.1)
Male gender, %	61.2	55.8	61.1	55.2
Asthma exacerbations in preceding 12 months / 6 months				
Asthma-related ED visit/hospital admission*, %	19.0 (156/819)§	15.5	6.2 (22/356)§	13.4
Oral steroid use*, %	31.6 (259/819)§	43.2	6.2	47.1
BTS treatment step				
2, %	65.9	48.8	71.7	¶
3, %	18.3	42.2	23.0	-
4, %	15.9	9.0	5.3	-

* PACMAN cohort: preceding 12 months, BREATHE/PAGES: preceding 6 months.
§ data not available for all individuals; (number of individuals / number of individuals with data available). For BREATHE, the individual with missing hospital data is different from the individual with missing OCS data.
¶ CAMP is Randomized Clinical Trial of mild-to moderate asthmatics. All children were on 200 µg of budesonide (ICS) plus SABA as needed.
BTS, British Thoracic Society.

Associations with exacerbations in BREATHE, PAGES and PACMAN

In a meta-analysis of the three North-European cohorts BREATHE, PAGES and PACMAN, we found two out of the 38 SNPs to be associated with an altered risk of severe exacerbations as defined by asthma-related hospital visits. *ST13* SNP rs138335 increased the risk of asthma-related hospital visits (OR=1.35 per G allele; 95%CI: 1.07-1.70, p=0.01, FDR: 25%). Rs138337 in the same gene, had a similar effect on the risk of asthma-related hospital visits (OR: 1.36 per G allele, 95%CI: 1.11-1.67, p=0.003, FDR: 11%) (Table 2). In addition, rs138335 was also associated an increased risk of OCS use (OR: 1.33 per G allele; 95%CI: 1.11-1.59, p=0.002, FDR: 11%). In Supplementary tables 2 and 3 the summary effect estimates of all investigated SNPs are shown.

Table 2. Results of the first and the second meta-analysis

				First meta-analysis			Second meta-analysis		
Outcome: hospital visits									
SNP	**Chr**	**Gene**	**Risk allele**	**n**	**OR (95%CI)**	**p**	**n**	**OR (95%CI)**	**p**
Rs138335	22	ST13	G	1535	1.35 (1.07-1.70)	0.010	1707	1.28 (1.04-1.59)	0.024
Rs138337	22	ST13	G	1546	1.36 (1.11-1.67)	0.003	1718	1.31 (1.08-1.59)	0.007
Outcome: OCS use									
Rs138335	22	ST13	G	1541	1.33 (1.11-1.59)	0.002	1713	1.30 (1.09-1.54)	0.003
Rs138337	22	ST13	G	1552	1.19 (0.98-1.47)	0.099	1724	1.18 (1.01-1.38)	0.032

Random effects meta-analysis was used to calculate combined adjusted ORs. First meta-analysis included BREATHE, PAGES and PACMAN. Second meta-analysis included BREATHE, PAGES, PACMAN and CAMP.
Chr, chromosome; n, number of participants included in the analysis; ORadj, adjusted Odds Ratio per increase in G-allele; 95%CI, 95% Confidence Intervals.

ST13 in a fourth independent asthma population

In order to assess the robustness of findings we studied rs138337 and rs138335 in a fourth independent study population; the North-American CAMP study. Genotyping data for *ST13* were available for 172 asthmatic steroid-treated non-Hispanic white children. In this clinical trial population, the two SNPs in *ST13* were not significantly associated with the risk of severe exacerbation, but this might be due to a lack of power considering the small study population. In a second meta-analysis including all four studies (Table 2), both SNPS increased the risk of asthma related hospital visits. For rs138337 the OR per G-allele for asthma-related hospital

visits was 1.31 (95%CI: 1.08-1.59, p=0.007, FDR: 19%) and for rs138335 the OR per G-allele was 1.28 (95%CI: 1.04-1.59, p=0.02, FDR: 36%). Furthermore, both SNPs were associated with an increased risk of OCS usage; the OR per G-allele was 1.18 for rs138337 (95%CI: 1.01-1.38, p=0.03, FDR: 41%) and 1.30 per G allele for rs138335 (95%CI: 1.09-1.54, p=0.003, FDR: 10%). Forest plots are shown in Figure 1.

Table 3. Risk of severe exacerbations stratified per *ST13* genotype

Study	Genotype rs138335	OR hospital visits	p-value	Genotype rs138337	OR hospital visits	p-value
BREATHE	CC	1.00 (ref)	-	AA	1.00 (ref)	-
	CG	2.54	0.02	GA	1.79	0.02
	GG	2.61	0.02	GG	1.69	0.06
PAGES	CC	1.00 (ref)	-	AA	1.00 (ref)	-
	CG	1.52	0.47	GA	1.47	0.32
	GG	1.97	0.23	GG	1.98	0.09
PACMAN	CC	1.00 (ref)	-	AA	1.00 (ref)	-
	CG	2.40	0.42	GA	1.73	0.38
	GG	2.99	0.30	GG	3.37	0.06
CAMP	CC	1.00 (ref)	-	AA	1.00 (ref)	-
	CG	0.28	0.05	GA	0.823	0.72
	GG	0.46	0.23	GG	0.708	0.62
Study	**Genotype rs138335**	**OR OCS usage**	**p-value**	**Genotype rs138337**	**OR OCS usage**	**p-value**
BREATHE	CC	1.00 (ref)	-	AA	1.00 (ref)	-
	CG	2.08	0.02	GA	1.42	0.07
	GG	2.21	0.01	GG	1.51	0.06
PAGES	CC	1.00 (ref)	-	AA	1.00 (ref)	-
	CG	1.36	0.41	GA	1.21	0.46
	GG	2.18	0.04	GG	1.57	0.11
PACMAN	CC	1.00 (ref)	-	AA	1.00 (ref)	-
	CG	N/A	N/A	GA	0.71	0.49
	GG	N/A	N/A	GG	0.56	0.37
CAMP	CC	1.00 (ref)	-	AA	1.00 (ref)	-
	CG	0.99	0.98	GA	1.40	0.39
	GG	1.10	0.87	GG	1.23	0.67

A genotypic model was used to assess risk per genotype. ORs were adjusted for age, sex and BTS treatment step. N/A: could not be calculated due to low numbers of cases per genotype group.

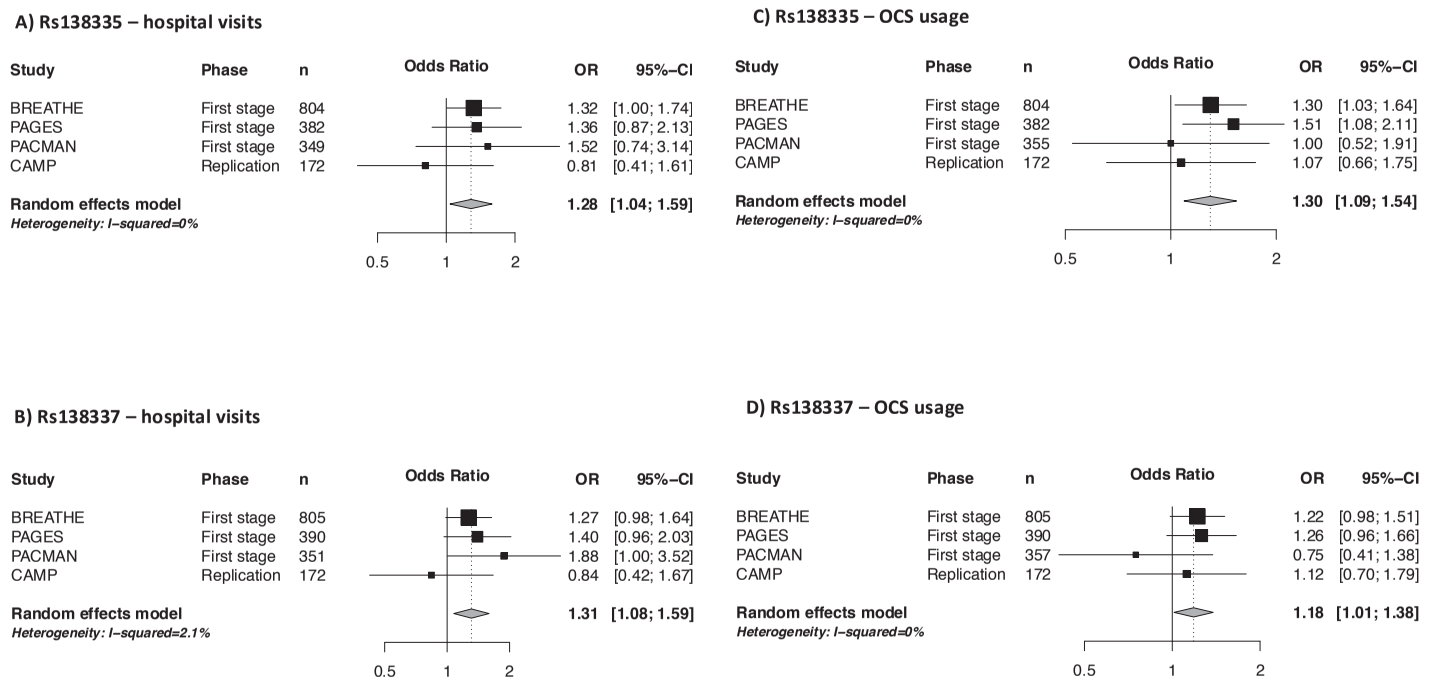

Figure 1. Forest plots for *ST13* and risk of severe exacerbations. Forest plots reflect the Odds Ratios for asthma-related hospital visits (A, B) and OCS usage (C, D) in BREATHE, PACMAN, PAGES and CAMP for *ST13* rs138335 and rs138337.

To assess the risk stratified per genotype we additionally performed a genotypic analysis whereby rs138335 CC and rs138337 AA were used as reference groups (Table 3). In a meta-analysis the highest risk estimates were found for carriers of rs138335 GG and risk of OCS usage (OR rs138335 GG compared to CC: 1.98, p=0.002) and carriers of rs138337 GG and risk of asthma-related hospital visits (OR rs138337 GG compared to rs138337 AA: 1.75, p=0.007).

ST13 haplotypes are associated with asthma-related hospital visits

Based on the available genotype information, three haplotypes were estimated to have a frequency >2% (Table 4). The most common haplotype was rs138335-G/rs138337-G. There was a significant association between *ST13* haplotype and asthma-related hospital visits, whereby compared to the most common haplotype, the haplotype without risk alleles (rs138335-C/rs138337-A) conferred the most protection (OR: 0.96, 95%CI: 0.94-0.99) (Table 5).

Table 4. Frequency of identified *ST13* haplotypes

	Rs138335 C/G	Rr138337 A/G	BREATHE	PAGES	PACMAN	CAMP
Haplotype			%	%	%	%
	G	G	50	52	46	48
	C	A	32	33	32	38
	G	A	18	15	22	14

Haplotype frequencies were estimated using the EM algorithm implemented in the 'haplo.stats' package in R. Three haplotypes were estimated to have a frequency >2%.

Table 5. The association between ST13 haplotypes and severe asthma exacerbations.

Hospital visits		
	OR	**p-value**
BREATHE		
CA vs. GG	0.95 (0.92-1.00)	0.03
GA vs. GG	0.98 (0.93-1.03)	0.39
PAGES		
CA vs. GG	0.96 (0.91-1.01)	0.15
GA vs. GG	0.96 (0.89-1.03)	0.24
PACMAN		
CA vs. GG	0.97 (0.93-1.01)	0.11
GA vs. GG	0.96 (0.92-1.01)	0.11
CAMP		
CA vs. GG	1.25 (0.60-2.63)	0.56
GA vs. GG	1.06 (0.39-2.89)	0.92
Summary		
CA vs. GG	0.96 (0.94-0.99)	0.003
GA vs. GG	0.97 (0.94-1.00)	0.04
OCS usage		
	OR	**p-value**
BREATHE		
CA vs. GG	0.94 (0.90-0.99)	0.02
GA vs. GG	0.99 (0.93-1.05)	0.72
PAGES		
CA vs. GG	0.91 (0.84-0.98)	0.02
GA vs. GG	1.02 (0.93-1.13)	0.67
PACMAN		
CA vs. GG	(0.97-1.05)	0.68
GA vs. GG	1.03 (0.98-1.08)	0.22
CAMP		
CA vs. GG	0.91 (0.54-1.51)	0.71
GA vs. GG	0.86 (0.43-1.72)	0.67
Summary		
CA vs. GG	0.96 (0.91-1.01)	0.13
GA vs. GG	1.01 (0.98-1.05)	0.41

The most common haplotype ('GG') was set as a reference. Models are adjusted for age, gender and BTS treatment step. The summary risk estimate was calculated using a random effect model.

Discussion

In a meta-analysis of three independent North-European cohorts we identified *ST13* as a novel risk gene for the occurrence of asthma exacerbations despite inhaled corticosteroid treatment in asthmatic children and young adults. For rs138335 the risk of exacerbations was increased with each substitution of the minor allele for

the major allele variant. For rs138337, oppositely, the minor allele variant was found to be associated with an increased risk of exacerbations. The two SNPs were in moderate LD (r^2=0.47) in our study. None of the other investigated genes could be linked to an increased risk of severe exacerbations.

SNPs rs138335 and rs138337 both lie in the non-coding intronic regions of the *ST13* gene, but may still affect gene expression, splicing or be in high LD with a variant that has functional consequences. However, there were no expression quantitative trait loci (eQTL) data available for both SNPs (http://www.ncbi.nlm.nih.gov/gap/PheGenI), nor could we identify a coding SNP in high LD with rs138335 and/or rs138337 (www.ensembl.org). *ST13* encodes a co-chaperone protein (Hsp70 interacting protein; hip) of the steroid-receptor complex and is involved in the functional maturation of the corticosteroid receptor, but the mechanism by which it does so remains to be elucidated.[24] *STIP1* (coding for another co-chaperone protein in the GR receptor complex, namely Hsp70/Hsp90-organizing protein: hop) has previously been associated with lung function and lung function improvement in 382 asthmatic patients treated with ICS.[10] At the time of SNP selection, *STIP1* was not included in our study. Hip (encoded by *ST13*) and hop (encoded by *STIP1*) are thought to function in a cooperative manner in GR maturation,[24] building evidence that alterations in the expression or folding of these co-chaperones may influence the binding of corticosteroids to the receptor or downstream signaling and therefore, ICS responsiveness. Functional studies are necessary to support our hypothesis.

A number of limitations need to be noted regarding the present study. Two SNPs in *ST13* were associated with both outcomes of exacerbations in the meta-analysis of all four patients populations, but the expected proportions of false discoveries (FDR rates) due to multiple testing ranged for both SNPs and the two outcomes in the second meta-analysis between 10-41%. We used FDR rates as a measure to correct for multiple testing, Bonferroni corrected p-values might be too conservative in candidate-gene approaches where SNP are in background LD. Although, the FDR rates are > 5% (which is often used as cut-of value), the biological function of *ST13* and the previous identified association of *STIP1* with ICS response in asthmatic patients,[10] strongly suggest that the identified association is not a false discovery. Furthermore, the SNPs were tested in four distinct study populations, and the effect estimates for both measures of severe exacerbations pointed in the same direction in the three largest study populations included in our study.

The populations we studied varied in age and severity of asthma symptoms. This

probably due to the design of the studies; the PACMAN population is recruited in community pharmacies, whereby most participants had well-controlled symptoms,[25] while patients in PAGES, BREATHE and CAMP were recruited through primary and secondary care. In addition, differences in health system and prescription behavior between the two countries might also play a role.[26] Notwithstanding these differences, heterogeneity was limited for *ST13* in the meta-analysis.

Our study was also limited due to the selection of tagging SNPs with a MAF ≥ 0.20. We could not study rare variants, which might have had larger effects. However, our total study population was small to study rare variants. Furthermore, the incorporation of common variants with smaller effects in clinical risk models might be valuable for a larger group of the asthma patient population.

In summary, variations in a novel risk gene *ST13* are associated with an increased risk of severe exacerbations in children and young adults despite ICS treatment. Although the effect sizes are modest, these results may provide insights into the biological mechanisms that underlie severe exacerbations in asthmatic patients treated with steroids. Heterogeneity in corticosteroid response is probably caused by complex interaction of genetic and environmental factors. Including *ST13* risk status in a multidimensional model with other genetic and non-genetic risk factors (e.g: exposure to tobacco smoke[27] or vitamin D levels[28]) might explain a larger part of the observed variability in treatment response.

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Supplementary Table 1. Selected genes with corresponding SNPs that passed quality control

Gene	SNPs	Description	Function
NR3C1	rs4607376 rs4912912 rs7701443 rs9324924 rs2963155 rs4912905 rs6865292 rs17209258 rs6196	Nuclear receptor subfamily 3 group C member 1	Glucocorticosteroid receptor (GR)[1]
KFBP4	rs1981655 rs11833878	FK506 binding protein 4	Chaperone, binds dynein upon ligand binding of GR[2]
ST13	rs138335 rs138337	suppression of tumorigenicity 13	Co-chaperone, mediates assembly chaperone GR complex[3]
CREBBP	rs130021 rs11076787 rs886528 rs2526689	cAMP-response element binding protein	Transcriptional co-activator[4]
TBP	rs2235506 rs3800235	TATA box binding protein	Transcriptional co-activator[5]
NCOA3	rs2425941 rs6066394 rs2143491 rs6018600 rs11700063	nuclear receptor coactivator 3	Transcriptional co-activator, acylates histones[6]
SMAD3	rs744910	Mothers against decapentaplegic homolog 3	Transcription factor, regulated by GR. Associated with asthma susceptibility[7]
SERPINA6	rs1956179 rs7158343 rs10498639 rs1998056 rs2281518 rs2281519 rs2281520 rs11629171	Corticosteroid-binding globulin	Protein involved in plasma corticosteroid-binding globulin activity[8]
ARG1	rs2781667	Arginase, liver	Arginase (enzyme), thought to be involved in asthma pathogenesis through effects on nitrosative stress. Associated with bronchodilator response[9]
17q21 locus	rs7216389	Involved in expression of orosomucoid - like protein 3	Locus thought to be associated with asthma susceptibility and therapy response[10-13]
PDE4D	rs1544791 rs1588265	phosphodiesterase 4D	Degrades cAMP. Thought to be associated with asthma susceptibility[14]
IL2RB	rs2284033	Interleukin 2 receptor, beta	Binds interleukin 2, involved in T cell mediated immune responses. Associated with asthma susceptibility[7]

Supplementary Table 2. Summary effect estimates of SNPs in candidate genes and asthma-related hospital visits in a meta-analysis of BREATHE, PAGES & PACMAN‡

SNP	CHR	BP	Gene	Effect allele	p-value	OR	I^2 (%)	q-value
rs138337	22	39560999	*ST13*	G	0.003	1.36	0	0.11
rs138335	22	39557032	*ST13*	C	0.010	0.74	0	0.25
rs6066394	20	45643560	*NCOA3*	T	0.099	1.21	0	0.92
rs6018600	20	45691984	*NCOA3*	A	0.116	1.18	0	0.92
rs1956179	14	93855495	*SERPINA6*	G	0.129	1.18	0	0.92
rs2963155	5	142736197	*NR3C1*	G	0.207	0.86	0	0.92
rs4607376	5	142776725	*NR3C1*	A	0.219	1.13	0	0.92
rs7216389	17	35323475	*17q21locus§*	C	0.266	0.81	59	0.92
rs2425941	20	45590796	*NCOA3*	T	0.269	0.89	0	0.92
rs17209258	5	142653590	*NR3C1*	G	0.273	1.25	55	0.92
rs2526689	16	3857884	*CREBBP*	G	0.337	0.90	0	0.92
rs2281518	14	93858870	*SERPINA6*	C	0.342	1.13	0	0.92
rs2281520	14	93846140	*SERPINA6*	C	0.358	0.88	10	0.92
rs10498639	14	93845279	*SERPINA6*	A	0.400	1.09	0	0.92
rs3800235	6	170718978	*TBP*	C	0.410	1.10	0	0.92
rs11700063	20	45586555	*NCOA3*	A	0.427	0.86	44	0.92
rs1588265	5	59405551	*PDE4D*	G	0.438	0.88	44	0.92
rs1998056	14	93859248	*SERPINA6*	G	0.448	0.92	0	0.92
rs2281519	14	93846385	*SERPINA6*	T	0.484	1.08	0	0.92
rs4912912	5	142787343	*NR3C1*	C	0.516	0.93	0	0.92
rs1544791	5	59474839	*PDE4D*	A	0.531	0.90	44	0.92

rs886528	16	3751557	*CREBBP*	C	0.566	1.14	70	0.92
rs130021	16	3772472	*CREBBP*	G	0.606	0.89	67	0.92
rs4912905	5	142710569	*NR3C1*	C	0.625	0.94	0	0.92
rs6865292	5	142773183	*NR3C1*	C	0.662	1.05	0	0.92
rs7701443	5	142772843	*NR3C1*	G	0.699	0.96	0	0.92
rs2235506	6	170720811	*TBP*	C	0.721	0.96	0	0.92
rs11833878	12	2780498	*KFBP4*	G	0.738	0.95	0	0.92
rs744910	15	65233839	*SMAD3*	A	0.741	0.96	28	0.92
rs2143491	20	45662074	*NCOA3*	A	0.747	0.97	0	0.92
rs6196	5	142641683	*NR3C1*	G	0.748	0.96	0	0.92
rs9324924	5	142772677	*NR3C1*	T	0.762	0.94	55	0.92
rs11629171	14	93843203	*SERPINA6*	T	0.867	1.02	21	0.96
rs2284033	22	35863980	*IL2RB*	A	0.875	1.02	0	0.96
rs11076787	16	3792777	*CREBBP*	T	0.901	0.98	0	0.96
rs1981655	12	2777987	*KFBP4*	A	0.926	1.03	0	0.96
rs2781667	6	131936837	*ARG1*	T	0.950	1.01	0	0.96
rs7158343	14	93852906	*SERPINA6*	G	0.963	1.01	0	0.96

Positions were based on NCBI Build 36. OR adjusted for age, gender and BTS treatment step.
‡PACMAN: asthma-related ER visits in the preceding 12 months, BREATHE/PAGES: asthma-related hospitalization in the preceding 6 months.
§ locus involved in the regulation of several genes.
OR, Odds Ratio assuming random effect model; I^2, heterogeneity (in %); BP, base pair.

Supplementary Table 3. Summary effect estimates of SNPs in candidate genes and oral corticosteroids use in the previous year in a meta-analysis of BREATHE, PAGES & PACMAN‡

SNP	CHR	BP	Gene	Effect allele	p-value	OR	I^2 (%)	q-value
rs138335	22	39557032	*ST13*	C	0.002	0.75	0	0.11
rs138337	22	39560999	*ST13*	G	0.099	1.18	20	0.92
rs7216389	17	35323475	*17q21 locus§*	C	0.102	0.80	49	0.92
rs6196	5	142641683	*NR3C1*	G	0.169	1.24	40	0.92
rs2963155	5	142736197	*NR3C1*	G	0.179	1.20	39	0.92
rs744910	15	65233839	*SMAD3*	A	0.188	0.89	6	0.92
rs6018600	20	45691984	*NCOA3*	A	0.215	1.11	0	0.92
rs4912912	5	142787343	*NR3C1*	C	0.224	0.90	0	0.92
rs7158343	14	93852906	*SERPINA6*	G	0.262	1.12	5	0.92
rs2284033	22	35863980	*IL2RB*	A	0.292	1.15	47	0.92
rs11629171	14	93843203	*SERPINA6*	T	0.308	0.89	17	0.92
rs1998056	14	93859248	*SERPINA6*	G	0.317	0.92	0	0.92
rs11833878	12	2780498	*KFBP4*	G	0.326	1.19	42	0.92
rs6865292	5	142773183	*NR3C1*	C	0.348	1.11	25	0.92
rs1981655	12	2777987	*KFBP4*	A	0.357	1.23	0	0.92
rs10498639	14	93845279	*SERPINA6*	A	0.410	0.89	56	0.92
rs2235506	6	170720811	*TBP*	C	0.458	1.07	0	0.92
rs9324924	5	142772677	*NR3C1*	T	0.463	1.07	0	0.92
rs7701443	5	142772843	*NR3C1*	G	0.466	0.94	0	0.92

rs4607376	5	142776725	*NR3C1*	A	0.543	1.05	0	0.92
rs130021	16	3772472	*CREBBP*	G	0.554	0.95	0	0.92
rs11700063	20	45586555	*NCOA3*	A	0.620	1.05	0	0.92
rs6066394	20	45643560	*NCOA3*	T	0.642	1.06	30	0.92
rs1544791	5	59474839	*PDE4D*	A	0.646	0.94	48	0.92
rs2143491	20	45662074	*NCOA3*	A	0.654	1.04	0	0.92
rs2281519	14	93846385	*SERPINA6*	T	0.686	0.96	0	0.92
rs2281520	14	93846140	*SERPINA6*	C	0.693	1.06	43	0.92
rs2526689	16	3857884	*CREBBP*	G	0.710	0.97	0	0.92
rs17209258	5	142653590	*NR3C1*	G	0.714	0.96	0	0.92
rs1588265	5	59405551	*PDE4D*	G	0.749	0.95	64	0.92
rs2281518	14	93858870	*SERPINA6*	C	0.750	1.04	23	0.92
rs11076787	16	3792777	*CREBBP*	T	0.809	1.03	0	0.96
rs2425941	20	45590796	*NCOA3*	T	0.823	0.98	0	0.96
rs2781667	6	131936837	*ARG1*	T	0.864	1.02	15	0.96
rs1956179	14	93855495	*SERPINA6*	G	0.872	0.98	31	0.96
rs3800235	6	170718978	*TBP*	C	0.912	1.01	0	0.96
rs886528	16	3751557	*CREBBP*	C	0.931	1.01	0	0.96
rs4912905	5	142710569	*NR3C1*	C	0.950	0.99	12	0.96

Positions were based on NCBI Build 36. OR adjusted for age, gender and BTS treatment step.
‡PACMAN: OCS use in the past 12 months, BREATHE/PAGES: OCS use in the past 6 months.
§ locus involved in the regulation of several genes.
OR, Odds Ratio assuming random effect model; I^2, heterogeneity (in %); BP, base pair.

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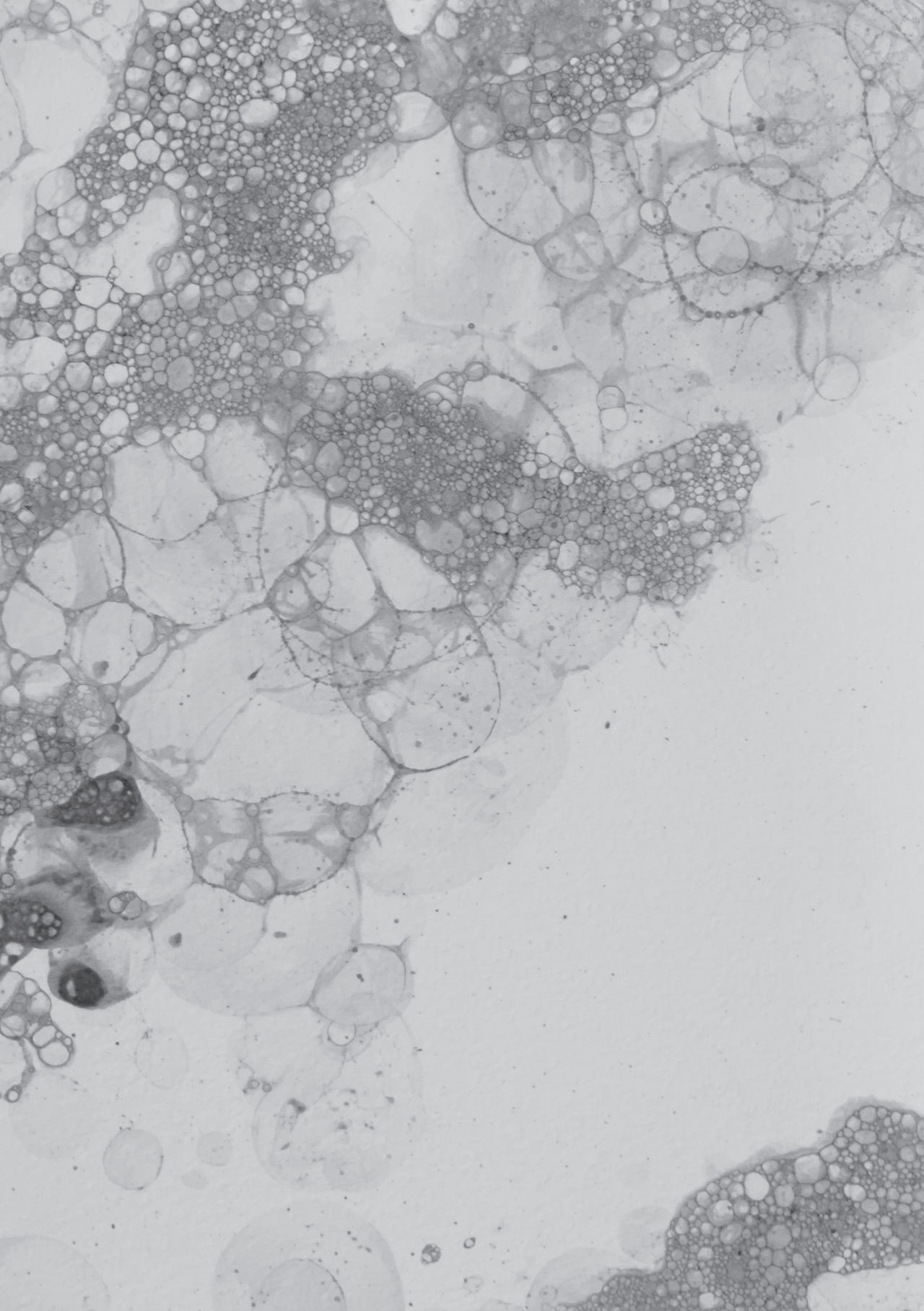

2.2

17q21 locus contributes to the risk of exacerbations in asthmatic children treated with inhaled corticosteroids: a meta-analysis

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Submitted for publication

Abstract

The 17q21 locus was initially identified as a susceptibility locus for early onset childhood asthma, but has also been associated with severe childhood-onset asthma. In this meta-analysis of three cohort studies and one clinical trial, we studied the relationship between the rs7216389 genotype and asthma exacerbations in children and young adults treated with inhaled corticosteroids (ICS). Rs7216389 was significantly associated with an increased risk of asthma-related hospital visits (summary Odds Ratio (OR) TT vs. CC: 1.93, 95% Confidence Intervals (CI): 1.29-2.89, p=0.001), as well as with an increased risk of prescribed course(s) of oral corticosteroids (OCS) (summary OR TT vs. CC: 2.00, 95%CI: 1.42-2.81, p=0.0007). The present study shows that variation in the 17q21 locus is associated with exacerbation-prone childhood-onset asthma which is more difficult to control by ICS.

Introduction

A polymorphism (rs7216389) at the 17q21 locus was initially identified as a risk factor for childhood onset asthma,[1] but was also found to be associated with uncontrolled asthma despite asthma treatment[2] and childhood onset severe asthma.[3-5] The SNP not only controls *ORMDL3* (orosomucoid 1-like 3) expression, which encodes a transmembrane protein localized in the endoplasmic reticulum, but also regulates lung mRNA expression of *GSDMA* (encoding gasdermin A), *GSDMB* (encoding gasdermin B) and *CRKRS* (encoding cell division cycle 2-related protein kinase 7).[6] In this study we aimed to assess the association between variation in the 17q21 locus and the risk of severe asthma exacerbations in four distinct study populations of children and young adults treated with inhaled corticosteroids (ICS).

Methods

Rs7216389 was genotyped in children and young adults using ICS from three cohort studies and one clinical trial population: the Pharmacogenetics of Asthma Medication in Children: Medication with Anti-inflammatory effects (PACMAN) cohort study (Netherlands, age: 4-12 years),[7] the BREATHE study (Scotland, United Kingdom, age: 3-22 years),[2] the Paediatric Asthma Gene Environment Study (PAGES) (Scotland, United Kingdom, age: 2-16 years)[8] and the Childhood Asthma Management Program (CAMP, age: 5-12 years at the start of the trial).[9] We restricted the analysis to children with a North-European background in PAGES, BREATHE and PACMAN and to non-Hispanic whites in CAMP. Genotyping in PACMAN, BREATHE and PAGES was performed using the Sequenom Mass Array platform (Sequenom, San Diego, California, USA). Genotyping in CAMP was performed using Illumina Infinium II 550 K SNP Chips and 610 Quad Chip (Illumina, Inc, San Diego, California). Rs7216389 was imputed based on 1000 Genomes. The following outcome definitions for severe exacerbations were used in order to meta-analyze the data from the different studies:

1) asthma-related hospital visits reported by the parent or child:
   - BREATHE: hospitalization in the past 6 months
   - PAGES: hospitalization in the past 12 months
   - PACMAN: emergency department (ED) visits in the past 12 months
   - CAMP: ED visits and/or hospitalizations in first 12 months of the trial.

2) Course(s) of OCS use reported by the parent or child:
   - BREATHE: in the past 6 months
   - PACMAN/PAGES: in the past 12 months
   - CAMP: in the first 12 months of the trial

For PAGES, data on hospital visits and OCS in the past 12 months, as well as in the past 6 months were available, and sensitivity analyses were performed with the 6 months outcome window. Furthermore, data on asthma control were available in PAGES and PACMAN. Uncontrolled asthma was defined as an Asthma Control Questionnaire score > 0.75 (PACMAN) or a Childhood Asthma Control Test score ≤ 19 (PAGES). In PACMAN, BREATHE and PAGES data on parental smoking data were available and we assessed whether there was an interaction between variation in 17q21 and parental smoking on the risk of severe exacerbations as defined above.

Statistical analysis

Logistic regression analysis was used to assess the risk of exacerbations when carrying the 17q21 variant. Odds ratios (OR) were calculated per study. The model was adjusted for age, gender and BTS treatment step (step 2: ICS plus short-acting β_2-agonist, step 3: step 2 plus long-acting β_2-agonist, step 4: step 3 plus leukotriene antagonist). The ORs were meta-analyzed assuming random effects with the inverse variance weighing method. I^2 was used to quantify between-study heterogeneity.[10] Statistical analysis was carried out using IBM SPSS 19.0 for Windows (SPSS, Inc., Chicago, Ill, USA) and PLINK.[11] Forest plots were made with R and the 'meta' package.[12] The Bonferroni corrected p-value for statistical significance was set at 0.05/3 (outcomes) = 0.017.

Results

Data from 1689 steroid-treated children and young adults were available. The characteristics of the study populations are listed in Table 1. The T-allele frequency of rs7216389 ranged between 0.56-0.58 in the different studies. In the meta-analysis of the four studies the rs7216389 TT genotype was significantly associated with an increased risk of severe exacerbations. Strongest effects where found when

the children homozygous for the TT genotype were compared with the children homozygous for the CC genotype, with similar effect estimates for asthma-related hospital visits and OCS use; summary OR for hospital visits: 1.93 (p=0.001), summary OR for OCS use: 2.00 (p=0.0007). Forest plots depicting the effect of the rs7216389 TT genotype compared to the CC genotype on severe exacerbations per study are depicted in Figure 1. When severe exacerbations in PAGES were assessed in the past 6 months instead of the past year, the assocations remained significant (summary OR for hospital visits: 1.87 [p=0.009], summary OR for OCS use: 1.79 [p=0.003]).
There was no significant interaction between parental smoking and rs7216389 for risk of severe exacerbations (parental smoking data available in PACMAN, BREATHE and PAGES). Furthermore, rs7216389 TT-genotype was not associated with an increased risk of not-well controlled asthma in PACMAN and PAGES (summary OR: 1.29, p=0.65).

Table 1. Characteristics of the study populations

	BREATHE (n=806)	PAGES (n=354)	PACMAN (n=357)	CAMP (n=172)
Child characteristics				
Age, mean (SD)	9.8 (4.0)	9.2 (3.8)	8.7 (2.3)	8.8 (2.1)
Male gender, %	60.8	56.5	61.1	55.2
Asthma exacerbations in past year				
Asthma-related ED visit/hospital admission, %#	19.0 (153/805)‡	31.9	6.0 (21/351)	13.4
Oral steroid use, %#	31.7 (255/805)‡	58.8	6.2	47.1
BTS treatment step				
2, %	65.6	46.0	71.7	§
3, %	18.4	44.4	23.0	-
4, %	16.0	9.6	5.3	-
Rs7216389 T-allele frequency	0.56	0.58	0.58	0.58

BREATHE, preceding 6 months; PACMAN/PAGES: preceding 12 months, CAMP: first 12 months of the trial.
§ CAMP is Randomized Clinical Trial of mild-to moderate asthmatics. All children were on 200 µg of budesonide (ICS) plus SABA as needed.
‡ individual with missing hospital data is different from the individual with missing OCS data.
BTS, British Thoracic Society; ED; Emergency Department; SD, standard deviation.

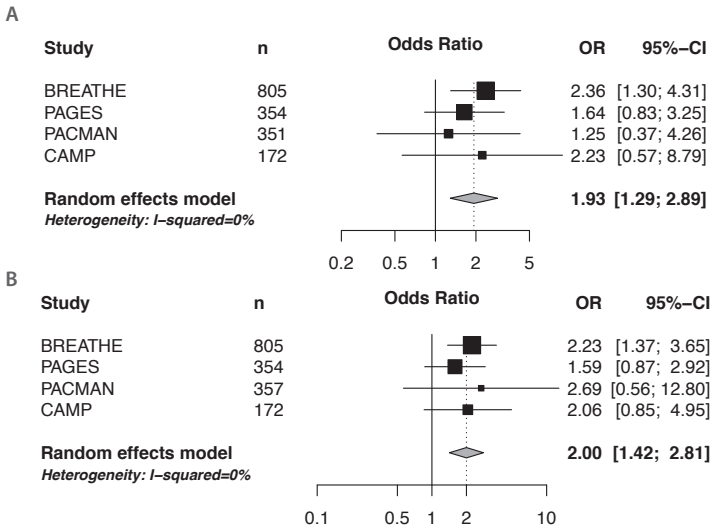

Figure 1. Forest Plots of rs7216389 for asthma related hospital visits (A) and OCS use (B). Odds Ratios (OR) and corresponding 95%CI for individuals with a rs7216389 TT genotype compared to individuals with the CC genotype, controlling for age, sex and BTS treatment step.

Discussion

In this study, we found rs7216389 TT genotype at the 17q21 locus to be associated with a higher risk on severe exacerbations in children and young adults with a reported use of ICS. Associations for both outcomes reflecting exacerbations remained significant after correction for multiple testing. These findings suggest that variation in the 17q21 locus contributes to a poor response to ICS. Other studies have reported associations with severe asthma exacerbations in pediatric patients, but did not restrict their analyses to steroid-treated patients.[2, 3]

Rs7216389 was also included in the panel of loci assessed in a previous study (Vijverberg et al., *submitted for publication*) but was not found to significantly associated with an increased risk of exacerbations in a meta-analysis of the three North-European studies PACMAN, PAGES and BREATHE, with p-values of 0.10 for the risk of OCS use and p: 0.27 for the risk of asthma-related hospitalizations in the previous year. This was probably due to a lack of power. When CAMP was included in the meta-analysis the associations were significant. This demonstrate the value of collaboration in the field of asthma pharmacogenetics to identify and validate genetic markers.

Asthma severity and treatment response are difficult to entangle in observational studies; patients with severe symptoms might be undertreated and may respond well to higher dosages of asthma medication. However, we restricted our analysis to children treated with ICS and adjusted the analysis for BTS treatment step, we therefore argue that the association we found reflects, at least partly, response to ICS.

This study is limited by the use of retrospective reporting of exacerbations in the three observational cohort studies. Nevertheless, in the clinical trial population CAMP, exacerbations were reported prospectively with the use of diaries. The effect estimates of all four studies pointed in the same direction.

The functional role of the 17q21 locus in asthma pathogenesis remains unclear. *ORMDL3* seems to be involved in sphingolipid metabolism,[13] which has been linked to the sensitivity of bronchial smooth muscle and airway hyperreactivity.[14] Miller et al. showed that in mice *ORMDL3* is mainly expressed in airway epithelial cells and can regulate the expression of various metalloproteases, chemokines and oligoadenylate synthetases antiviral genes which play a role in allergic inflammation, remodeling and antiviral responses.[15] Furthermore, a recent study by Ha et al. observed *ORMDL3* protein expression in lung tissue and eosinophils of allergen-challenged mice.[16] On a molecular level, *ORMDL3* was found to be involved in the regulation of eosinophil trafficking, recruitment and degranulation.[16] This suggests that altered expression of *ORMDL3* may increase susceptibility of the airway epithelial for airway remodeling and promote eosinophilic inflammation. Since the 17q21 locus also regulates other gene transcripts, such as *GSDMA* and *GSDMB*, expressed in the airway epithelium, other mechanisms may underlie asthma pathogenesis. The functions of the genes in the *GSDM* family remain largely unknown, though *GSDMA* has been associated with the regulation of apoptosis in gastric epithelium.[17]

In summary, in this study we show strong evidence that 17q21, a widely replicated asthma susceptibility locus, is also associated with an exacerbation-prone childhood asthma phenotype in Caucasian children. This phenotype seems to be less responsive to ICS. Although, the molecular mechanisms underlying this exacerbation-prone phenotype need to be elucidated, the incorporation of genetic information in clinical algorithms could eventually facilitate diagnosis of asthma phenotypes and guide treatment.

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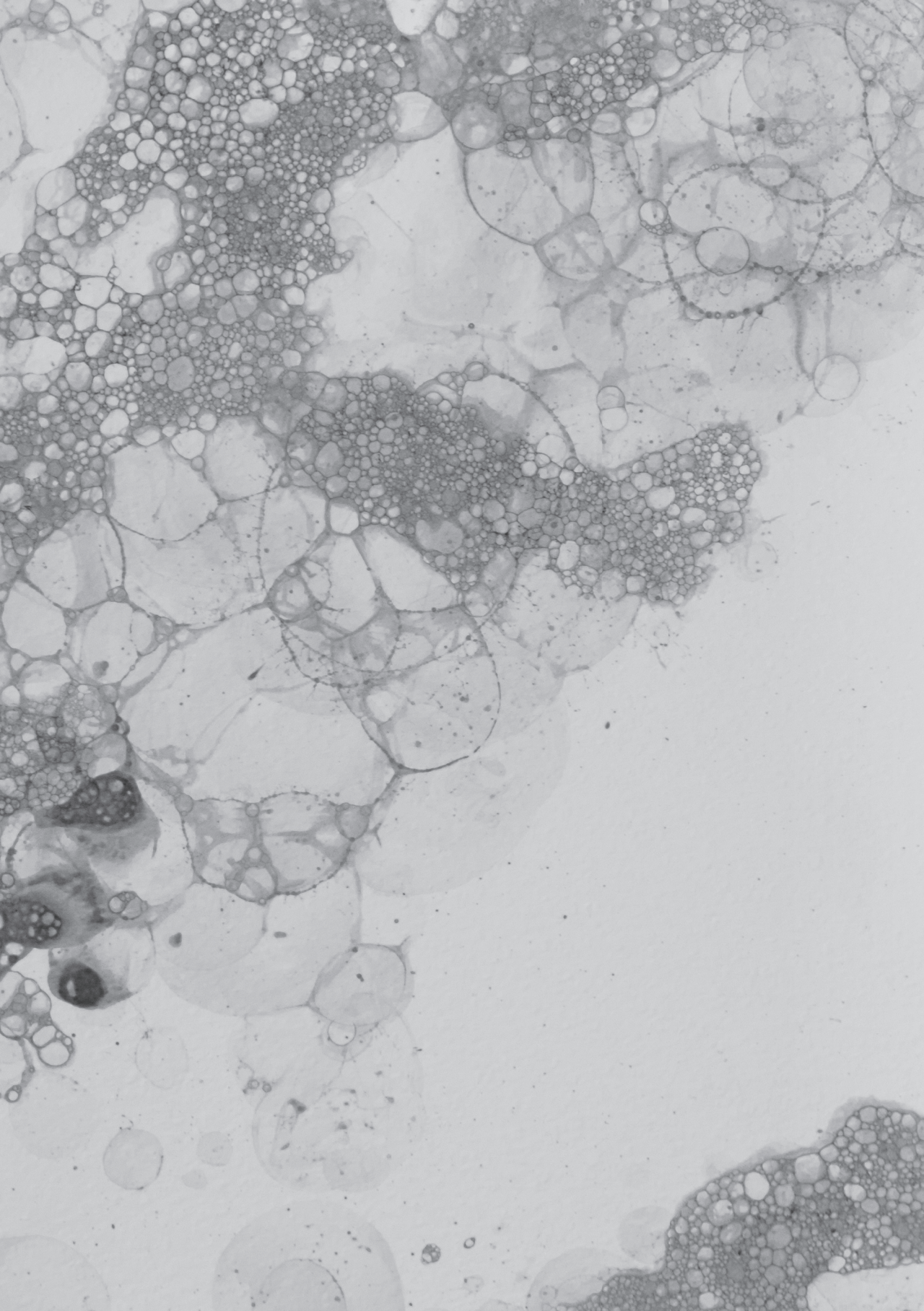

2.3

Pharmacogenetic analysis of *GLCCI1* in three North-European pediatric asthma populations with a reported use of inhaled corticosteroids

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Abstract

Background: *GLCCI1* rs37972 has previously been associated with decreased lung function improvement upon treatment with inhaled corticosteroids (ICS) in asthmatics.

Aim: To assess whether variation in rs37972 is associated with altered ICS efficacy in North-European asthmatic children and young adults with a reported use of ICS.

Patients & Methods: Rs37972 was genotyped in three cohort studies of asthmatic children with a reported use of ICS. As indicator for asthma exacerbations, asthma-related hospital visits and oral corticosteroid (OCS) use were studied. Asthma control was assessed using a questionnaire.

Results: Rs37972 T-allele was not significantly associated with an increased risk of OCS use (summary Odds ratio: 1.20; 95%CI: 0.99-1.45), an increased risk of asthma-related hospital visits (summary Odds ratio: 1.07; 95%CI: 0.89-1.29), uncontrolled symptoms (summary Odds ratio: 1.01; 95%CI: 0.75-1.36) or higher ICS dosages (summary β: 0.01; 95%CI: -0.06-0.08).

Conclusion: Variation in *GLCCI1* rs37972 genotype does not seem to affect ICS efficacy in North-European asthmatic children.

Introduction

Asthma is a complex chronic inflammatory disease that affects millions of individuals worldwide. Patients whose asthma remains uncontrolled by the use of short-acting bronchodilators alone, will be prescribed inhaled corticosteroids (ICS).[101] ICS are the main maintenance treatment in asthma. Although most patients with asthma show a beneficial response to ICS, intra-individual variability in the level of treatment response is large.[1] There is growing evidence that this variability is partly caused by intra-individual genetic differences.[2,3]

In 2011, Tantisira and co-workers identified a variation in the glucocorticoid induced transcript 1 gene (*GLCCI1*) on chromosome 7p21.3 to be associated with a decreased improvement of lung function upon treatment with corticosteroids.[4] The SNP, rs37972, was initially identified in a genomewide association analysis of 118 asthmatic child-parent trios, and subsequently evaluated in four additional clinical studies with adult asthmatics, studying a total of 935 subjects. Individuals homozygous for the mutant T-allele had a significantly higher risk of a poor response to inhaled corticosteroids, compared to heterozygotes or wild-type homozygotes. Functional analyses showed that rs37972 was in complete linkage disequilibrium (r^2=0.99) with a functional variation (rs37973) that affected the expression of *GLCCI1*. Furthermore, preliminary results by Thompson et al. showed that *GLCCI1* rs37973 variance was associated with higher dosages of inhaled and intranasal corticosteroid dosages and increased hospital admission in 402 asthmatic children.[5] Nevertheless, Hosking et al.[6] recently reported that they could not replicate the results of Tantisira et al. in post-hoc analyses of seven clinical trials including 1924 adolescents and adults with asthma.

In the current study, we aimed to assess whether variation in the *GLCCI1* rs37972 genotype was associated with an increased risk of severe exacerbations, uncontrolled asthma and higher daily ICS dosages in three North-European populations of asthmatic children and young adults.

Patients & methods

Study population

DNA samples were collected through saliva samples or mouthwash samples from children and young adults who participated in the BREATHE study (Scotland,

UK, age: 3-22 years), the Paediatric Asthma Gene Environment Study (PAGES) (Scotland, UK, age: 2-16 years)[7] and the Pharmacogenetics of Asthma Medication in Children (PACMAN) study (Netherlands, age: 4-12 years).[8] BREATHE and PAGES are observational cohort-studies of physician-diagnosed asthmatic children and young adults. Asthmatic children and young adults in BREATHE are recruited through primary or secondary clinics in either Tayside or Dumfries (Scotland, United Kingdom). At the asthma clinic, a clinical interview was performed to obtain information on symptoms, treatment and exacerbations in the 6 months preceding the study visit. Mouthwash samples were collected and DNA was isolated using Qiagen DNAeasy 96 kits (Qiagen GmbH, Hilden, Germany). Asthmatic children and adolescents in PAGES are recruited through secondary care asthma clinics across Scotland. A detailed clinical history was obtained from the parents and child including information on asthma symptoms, treatment and exacerbations over the preceding 6 months, as well as 12 months. A saliva sample was obtained to collect DNA (Oragene DNA Self Collection kit, DNA Genotek, Inc., Ontaria, Canada). The PACMAN cohort consists of children with a regular use of asthma, recruited with the help of pharmacists belonging to the Utrecht Pharmacy Practice Network for Education and Research (UPPER). Children were selected based on the use of regular asthma medication [≥ three prescriptions (Anatomical Therapeutic Chemical code R03) within the last two years, including ≥ one prescription in the past 6 months]. During a study visit in their own community pharmacy parents and children completed an extensive questionnaire including questions on respiratory symptoms, medication use and exacerbations in the past year. A saliva sample was obtained for DNA extraction (Oragene DNA Self Collection kit, DNA Genotek, Inc., Ontaria, Canada).

BREATHE has been approved by The Tayside Committee on Medical Research Ethics, PAGES by the Plymouth and Cornwall Research Ethics Committee. PACMAN was conducted in compliance with the requirements of the Institutional Review Board of the Department of Pharmacoepidemiology and Clinical Pharmacology (Utrecht University) and has been approved by the Medical Ethical Committee of the University Medical Centre Utrecht. We restricted the analyses to children with a North-European ancestry who reported the use of ICS.

Definition of outcomes

As indicators for acute severe asthma exacerbations we studied: 1) asthma-related ER visits in the past 12 months for PACMAN and asthma-related hospitalization

in the past 6 months for BREATHE and PAGES, and 2) courses of OCS in the past 12 months for PACMAN, and in the past 6 months for BREATHE and PAGES. For PAGES, data on hospital visits and OCS in the past 6 months, as well as in the past year were available, and sensitivity analyses were performed with the 12 months outcome window. In addition, we assessed daily ICS dosages to analyse whether children with the variant were treated with higher dosages of ICS compared to children without the variant. In PACMAN, daily ICS dosages were based on the last refill prescription recorded in the pharmacy system before the study visit, in PAGES and BREATHE parentally reported ICS dosages were recorded during the study visit. Furthermore, data on asthma control were available in PACMAN and PAGES. Asthma control in the PACMAN cohort was assessed with the use of the 6-item version of the Asthma Control Questionnaire (ACQ) (symptoms plus rescue medication use).[10] An ACQ-score ≥ 1.50 was considered 'not well-controlled asthma'. A sensitivity analysis was performed using a cut-off value of ≥ 0.75 for 'not well-controlled asthma'. In PAGES, asthma control was assessed using the 7-item childhood Asthma Control Test (c-ACT).[10] A c-ACT score of ≤ 19 was considered 'not well-controlled asthma'.

Treatment step

Treatment step was modified from British Thoracic Society (BTS) guidelines,[11] as follows: step 2: use of inhaled short-acting β_2-agonists (SABA) as needed plus regular ICS, step 3: step 2 plus regular long-acting inhaled β_2-agonists (LABA) and, step 4: step 3 plus oral leukotriene receptor antagonists (LTRA).

DNA extraction and genotyping

DNA was extracted from saliva samples according to the protocol of the manufacturer (Oragene DNA Self Collection kit, DNA Genotek, Inc., Ontaria, Canada). DNA was extracted from the mouthwash samples using Qiagen DNAeasy 96 kits (Qiagen GmbH, Hilden, Germany). Genotyping of rs37972 was performed using a TaqMan-based allelic discrimination assay with a 7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Genotype frequencies of rs37972 were consistent with Hardy-Weinberg equilibrium for PAGES (p=0.68) and PACMAN (p=0.57), but not for BREATHE (p=0.02).

Statistical analysis

Logistic regression analysis was used to study the association between rs37972 and the outcomes. Odds ratios (OR) and their corresponding 95% confidence

intervals (95%CI) and p-values were calculated per study, adjusting for age, gender and BTS treatment step. The ORs were meta-analyzed assuming random effects with the inverse variance weighing method. Linear regression was used to assess whether *GLCCI1* genotype was an independent predictor of daily ICS dosage. Log transformation was used to obtain a normal distribution of daily ICS dosage. Univariate linear regression analyses were performed to assess the influence of height, age, sex, weight, BMI on daily ICS dosage. Height, weight and age were significant predictors of ICS dosage and were included as covariates in the multivariate linear model. Beta's and 95%CI were back transformed for presentation. An additive genetic model was assumed, as was used by Tantisira et al.[4] The Kruskal Wallis test was applied to assess whether the symptom scores and daily ICS doses differed between rs37972 genotypes. Statistical analysis was carried out using IBM SPSS 19.0 for Windows (SPSS, Inc, Chicago, Ill, USA) and PLINK.[12] Forest plots were made with R and the 'meta' package.[13] A power analysis was performed to assess the minimal genetic risk for severe exacerbations that could be identified assuming an additive genetic model.[14] For both outcomes the study was powered to identify a minimal genetic risk effect of 1.2 per rs37972 T-allele (power = 80%, $\alpha = 0.05$).

Results

Characteristics of the study population

Genotyping data were available for 685 children of the PACMAN cohort, 1570 children and young adults of the BREATHE cohort and 523 children and adolescents of PAGES. We restricted our analysis to children and young adults from North-European descent who were treated with ICS; 431 children of the PACMAN cohort, 1037 participants of BREATHE and 323 children in PAGES met these criteria. Characteristics of the study population are shown in Table 1. The mean age ranged between 8.6-9.8 years between the three cohorts. The majority of the children in PACMAN and BREATHE were treated according BTS treatment step 2, in contrast to PAGES, where the majority of the children were treated according BTS treatment step 3.

GLCCI1 and severe exacerbations

In none of the cohort studies we could observe an effect of *GLCCI1* rs37972 genotype on the risk of asthma-related hospital visits; summary OR: 1.07 (95%CI: 0.89-1.29,

I^2=0%) (Figure 1a). When asthma-related hospitalization were assessed in the past year in PAGES, instead of in the past six months, the OR changed slightly from 1.01 per increase in T-allele (95%CI: 0.66-1.55) to 0.96 (95%CI: 0.69-1.77). Variation in *GLCCI1* rs37972 genotype was also not significantly associated with an increased risk of OCS use in the separate studies, nor in the meta-analysis, summary OR: 1.20 (95%CI 0.99-1.45, I^2=16.6%) (Figure 1b). When OCS use was assessed in the past year in PAGES, instead of in the past six months, the OR changed slightly; from 1.26 per increase in T-allele (95%CI: 0.90-1.76), to 1.03 (95%CI: 0.74-1.42).

Table 1. Characteristics of the study populations

	PACMAN cohort (n=431)	PAGES (n=323)	BREATHE (n=1037)
Child characteristics			
Age, mean (SD)	8.6 (2.3)	8.9 (3.8)	9.8 (4.0)
Male gender, %	59.9 (258/431)	57.7 (184/319)	60.2 (624/1037)
Asthma-related hospital visit[#], %			
Within past six months	-	16.8 (54/322)	17.5 (181/1037)
Within past year	6.4 (27/423)	31.4 (101/322)	-
Oral steroid use, %			
Within past six months	-	41.2 (133/323)	31.0 (322/1037)
Within past year	7.4 (32/431)	58.1 (187/322)	-
BTS treatment step			
2, %	71.6 (298/416)	29.4 (95/323)	67.6 (701/1037)
3, %	22.6 (94/416)	58.2 (188/323)	17.6 (182/1037)
4, %	5.8 (24/416)	12.4 (40/323)	14.9 (154/1037)
Uncontrolled asthma, %	15.7 (66/420)[‡]	65.3 (124/190)[§]	-
***GLCCI1* genotype, rs3797**			
CC	30.9 (133/431)	35.0 (113/323)	32.4 (336/1037)
CT	50.6 (218/431)	47.4 (153/323)	45.9 (476/1037)
TT	18.6 (80/431)	17.6 (57/323)	21.7 (225/1037)

BREATHE/ PAGES: asthma-related hospital admissions, PACMAN: asthma-related ER visits.
‡ Asthma Control Questionnaire Score ≥ 1.50.
§ Childhood Asthma Control Test ≤ 19.
BTS, British Thoracic Society; SD, standard deviation; ICS, Inhaled corticosteroids; IQR, Interquartile Range.

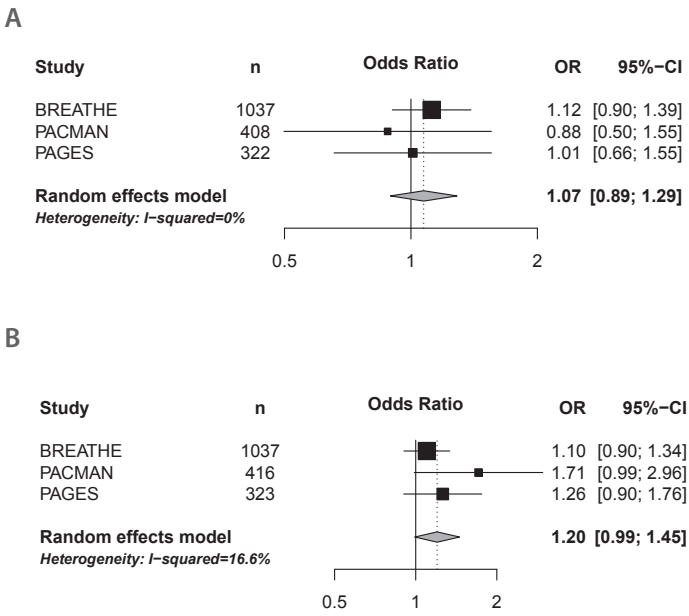

Figure 1. Forest plots of the Odds Ratios per increase in T allele for asthma-related hospital visits (A) and OCS usage (B) in BREATHE, PACMAN and PAGES for *GLCCI1* rs37972. An additive genetic model was assumed. In PACMAN ER visits for asthma were recorded, in BREATHE and PAGES asthma-related hospitalizations were recorded. For the summary effect estimate ORs were meta-analyzed assuming a random effects model. I^2 of the meta-analyses are shown to quantify between-study heterogeneity.

GLCCI1 and symptoms

In PACMAN and PAGES questionnaires on asthma control were included in the study protocols. In the PACMAN cohort; 15.7% of the children were uncontrolled (ACQ ≥ 1.50), compared to 65.3% of the children included in PAGES (c-ACT ≤ 19). In both studies, we could not identify a significant effect of *GLCCI1* rs37972 genotype on asthma control; in PACMAN the OR for uncontrolled symptoms was 0.95 per increase in T-allele (95%CI: 0.64-1.41), and it was 1.10 in PAGES (95%CI: 0.69-1.76). The effect summary was 1.01 (95%CI: 0.75-1.36, I^2=0%). The symptom scores did not significantly differ between rs37972 genotypes in PAGES and PACMAN (Table 2).

Table 2. Median symptom scores per *GLCCI1* rs37972 genotype

Study	Questionnaire	CC	CT	TT	p-value
		Median score (IQR)	Median score (IQR)	Median score (IQR)	
PAGES (n=190)	ACT	18 (12-22)	18 (14-21)	16 (13-22)	0.88
PACMAN (n=420)	ACQ	0.5 (0.3-1.2)	0.7 (0.0-1.0)	0.8 (0.17-1.17)	0.14

P-value of the Kruskal-Wallis test is shown. A lower ACT score indicates more asthma symptoms; in contrast, a higher ACQ score indicates more asthma symptoms.
ACT, Asthma Control Test; ACQ, Asthma Control Questionnaire; IQR, interquartile range.

GLCCI1 and ICS dosages

The median ICS dosages were lowest in PAGES and highest in PACMAN, there was no significant difference in ICS dosage between children with different *GLCCI1* genotypes (Table 3). Furthermore, in a linear regression model with age, height and weight as covariates, *GLCCI1* genotype was not an independent predictor of daily ICS dosage in PACMAN (β: 0.07, 95%CI: -0.06-0.22) BREATHE (β: 0.03, 95%CI: -0.03-0.09) or PAGES (β:-0.07, 95%CI: -0.16-0.04). In a meta-analysis assuming random effects the summary β was 0.01 (95%CI: -0.06-0.08, I^2=38.4).

Table 3. Daily ICS dose stratified per *GLCCI1* rs37972 genotype

	CC daily ICS dose, mcg (IQR)	CT daily ICS dose, mcg (IQR)	TT daily ICS dose, mcg (IQR)	p-value
PACMAN (n=323)	500 (400-500)	500 (400-500)	400 (394-813)	0.87
PAGES (n=317)	200 (200-400)	200 (100-300)	200 (200-300)	0.50
BREATHE (n=1011)	400 (200-400)	400 (200-500)	400 (200-500)	0.49

Median daily ICS dosage per genotype (budesonide equivalent). P-value of the Kruskal-Wallis test is shown.
IQR, Interquartile Range.

Discussion

In this replication study we assessed the effect of variation in the *GLCCI1* rs37972 genotype on ICS treatment outcome in three cohort studies and found no evidence that asthmatic children and young adults with a variant *GLCCI1* genotype require higher ICS dosages or have a higher risk of uncontrolled asthma or severe exacerbations.

To date little is known on the function of the *GLCCI1* gene. Functional experiments have shown that dexamethasone induces changes in the transcription of *GLCCI1* in lymphoblastoid cells.[4] In addition, gene knockdown experiments in zebrafish suggest *GLCCI1* might be involved in glomerular development and function.[15] In 2011, Tantisira and colleagues identified *GLCCI1* as a risk gene for poor ICS response. Genetic variation in *GLCCI1* was associated with a decreased improvement of forced expiratory volume in 1 second upon ICS administration in asthmatic children and adults.[4] Hosking et al.[6] could not replicate these findings in a large set of asthmatic adults from seven clinical studies (n=1924), though they did observe a non-significant trend toward poorer ICS response in rs37973 GG homozygotes compared to AA homozygotes, suggesting that the effect of *GLCCI1* on ICS treatment response might be more apparent in children. However, we could not find evidence for this hypothesis in our study. This could be due to differences in outcome definition. Variation in *GLCCI1* may influence lung function response upon steroid treatment in children, but not affect other dimensions of ICS response such as severe exacerbations or lack of asthma control. Improvement in lung function upon steroid treatment could not be tested in the current study due to the cross-sectional design of the three cohorts.

The hypothesis that *GLCCI1* has a stronger effect on ICS treatment response in children is in line with preliminary data by Thompson et al.[5] They studied 402 steroid-treated asthmatic children and found that children with the rs37973 variant were treated with higher corticosteroid dosages and had increased asthma-related hospital admissions.[5] There was no significant association with OCS use. Nevertheless, these findings need to be interpreted with caution, as all children had a clinically indicated low dose short Synatchen test, indicating a possible dysregulation of natural steroid (cortisol) production by the adrenal glands, complicating the extrapolation of these results to the general pediatric asthma population.

Our work shows that *GLCCI1* is not associated with an increased risk of asthma-related hospital visits, uncontrolled asthma or higher ICS dosage regimens in the general pediatric asthma population. We cannot exclude that the effect of *GLCCI1* might be associated with steroid responsiveness in specific patient groups, maybe due to environment-gene interactions. A prospective study in patients with bacterial meningitis, for example, found an association between *GLCCI1* genotype and a higher mortality upon dexamethasone treatment.[16]

Several potential limitations should be noted. OCS use and asthma-related hospital visits were based on parentally reported retrospective data and might be prone

to recall bias. This might be more pronounced for OCS use compared to asthma-related hospital visits, as the impact of an asthma-related ER visit or asthma-related hospitalization on a family is expected to be larger.
In addition, definitions of outcomes slightly differed between the cohorts, as well as the populations included into the cohorts. Children in the PACMAN cohort were recruited through community pharmacies based on regular asthma medication use, while participants of PAGES and BREATHE were recruited through secondary and tertiary asthma clinics, and therefore likely reflect a more severe asthmatic population compared to the PACMAN population. In order to take into account that the effect of *GLCCI1* might be different within the different cohort populations, we meta-analyzed the effect estimates assuming random effects. Heterogeneity between the studies (I^2) was generally low.
Tantisira et al.[2] reported an OR for poor lung function response upon ICS treatment in asthmatic patients of 1.52 per increase in risk allele at rs37973. Our study was powered to identify a significant OR >1.2 for severe exacerbations per increase in risk allele at rs37972, a SNP in complete LD with rs37973. We cannot exclude that *GLCCI1* rs37972 has a small effect on severe exacerbations, which we were unable to detect. However, this demonstrates the lack of clinical utility of this genetic marker in the general childhood asthma population.
Additionally, the genotype frequencies of BREATHE were not consistent with HWE. PAGES, BREATHE and PACMAN samples were all genotyped according to the same genotyping protocol, in the same center, using the same reagents. Analyses of BREATHE duplicate DNA samples did not alter genotype frequencies. We therefore assume that there might be a specific population effect in BREATHE for *GLCCI1* and the deviation from HWE is not caused by a technical error.

Conclusion and Future perspective

In this analysis of three pediatric asthma cohorts, including 1791 patients, we could not find evidence that *GLCCI1* genotype is associated with decreased ICS efficacy in North-European children and adolescents. *GLCCI1* genotype did not significantly influence daily ICS dosage, risk of exacerbations or risk of poor symptom control.
Asthma is a very heterogeneous disease composed of various subtypes with different underlying pathophysiological mechanisms. There is a strong need of markers that can be used in a clinical applicable algorithm to distinguish clinically relevant asthma phenotypes, optimize diagnosis and guide treatment. The risk effect of genetic markers might be different in specific patient populations and for

distinct definitions of treatment response. Advances in multi-dimensional unbiased cluster-analyses, combining a wide range of clinical parameters and biological markers, might provide more insights in the different asthma phenotypes.[17] An international consortium on childhood asthma can facilitate these types of analyses.[18] Furthermore, to profit optimally from identified pharmacogenetic asthma markers, future research should focus on exploring causative relationships and underlying biological pathways.

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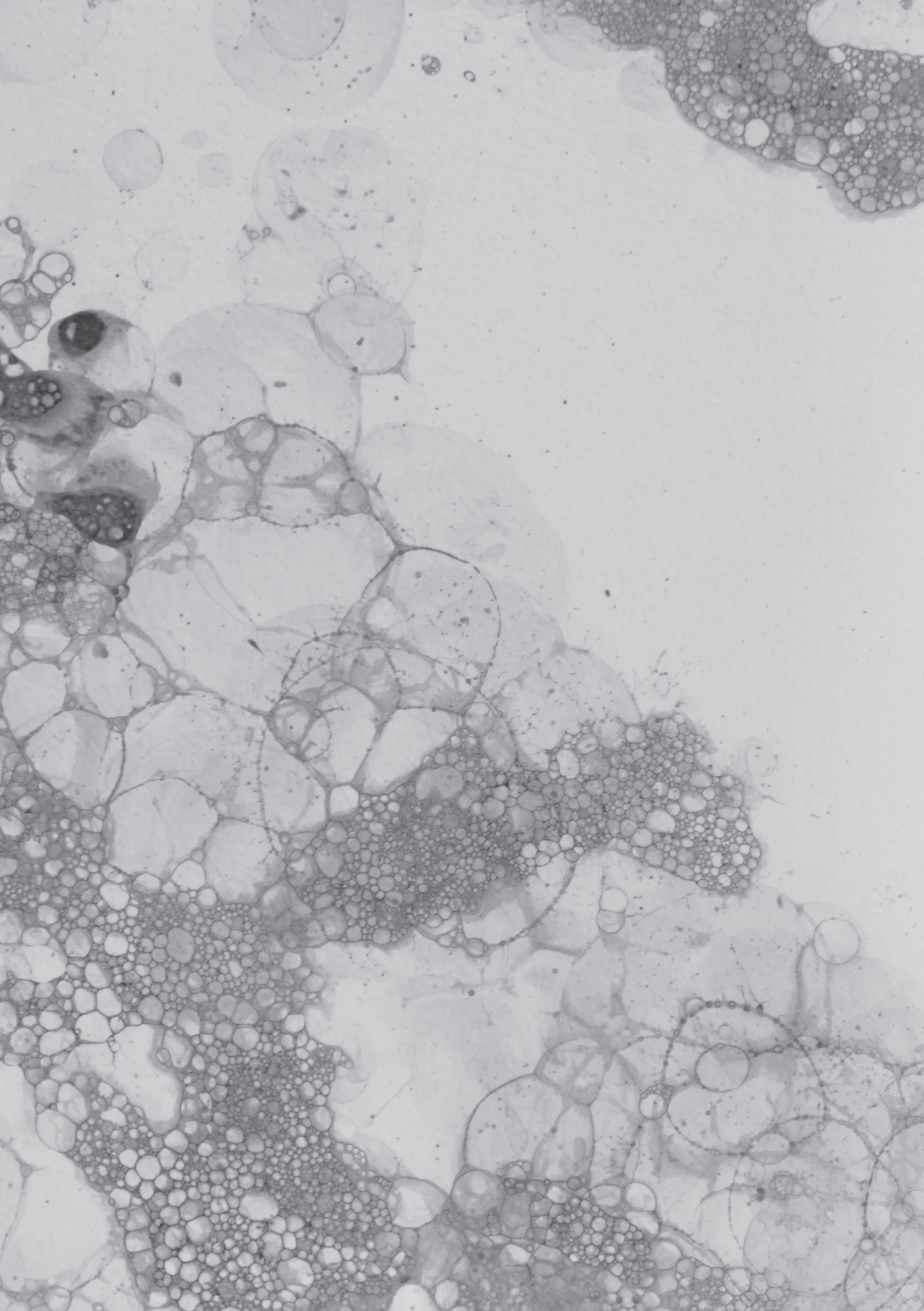

2.4

Arg16 *ADRB2* genotype increases the risk of asthma exacerbations in children with a reported use of long-acting β_2-agonists: results of the PACMAN cohort

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Abstract

Introduction: Current evidence suggests that asthma patients with the *ADRB2* Arg16 genotype have a poorer response to long-acting β_2-agonists (LABA), but the results remain inconsistent.

Aim: This study assessed the association between Arg16 variants and treatment outcome in children treated with inhaled corticosteroids (ICS) and LABA.

Methods: *ADRB2* Arg16 was genotyped in 597 children (4-12 years of age) participating in the PACMAN-cohort study. A questionnaire was used to assess asthma control, frequency of asthma-related emergency department visits and use of oral corticosteroids in the past year.

Results: Arg/Arg carriers with a reported use of ICS and LABA had an increased risk of oral corticosteroid use (OR 14.9; 95%CI: 1.59-140.1)) and emergency department visits in the past year (OR 11.9; 95%CI: 1.22-115.8)) compared with Gly/Gly carriers. This effect was not observed in Arg/Arg genotype carriers reporting ICS use only.

Conclusion: Children who are homozygous for *ADRB2* Arg16 have an increased risk of exacerbations when treated with combined LABA and ICS.

Introduction

Asthma is a major clinical health problem, with high prevalence and morbidity rates and large health care costs.[1] The variation in response to asthma maintenance treatment is large and approximately 10% of the children remain uncontrolled despite high levels of treatment. SNPs in the gene that codes for the β_2-adrenergic receptor (*ADRB2*) have been associated with asthma treatment outcome.[2] The effect of the Arg16 (Gly16>Arg16) polymorphism (rs1042713) on asthma treatment outcome has been studied in both adults and children. However, the results of these studies tend to be conflicting. Various studies suggest that the Arg16 variant allele negatively influences the response to long-acting β_2-agonists (LABA), resulting in an increased risk of exacerbations and a reduced lung function,[3-7] whereas other studies did not find a pharmacogenetic effect regarding this allele.[8-11] Recently, a clinical trial by Lipworth et al. randomized 62 asthmatic children who were homozygous for the Arg16 genotype to inhaled corticosteroids (ICS) combined with LABA, or to ICS combined with leukotriene receptor antagonists (LTRA). The children randomized to ICS combined with LTRA had fewer exacerbations and school absences compared with the group treated with ICS combined with LABA.[3] The two treatment arms did not differ in the level of lung function improvement upon treatment. Therefore, in our large observational study, we assessed whether there is an association between the Arg16 variant and severe exacerbations and uncontrolled asthma symptoms in a real-life population of children with a reported use of ICS and LABA compared with children that use only ICS.

Methods

This study is a nested case-control study within the PACMAN cohort study. Details of the PACMAN cohort study design have been published elsewhere.[12] Children between the ages 4 and 12 that used asthma medication on a regular basis (≥1 prescription in the last 6 months and ≥3 prescriptions within the last 2 years) were selected from Dutch community pharmacies affiliated with the Utrecht Pharmacy Practice Network for Education and Research (UPPER; The Netherlands). Data on asthma symptoms, recent exacerbations and current medication use were assessed using a questionnaire. Saliva samples were collected for DNA extraction using Oragene® saliva collection kits (DNA Genotek Inc., Kanata, Ontario,

Canada). Genotyping of rs1042713 was performed at LGC Genomics (UK). LGC Genomics provides outsourcing genotyping services utilizing the proprietary KASP™ chemistry. The Medical Ethics Committee of the University Medical Centre Utrecht (The Netherlands) and the Institutional Review Board of the Division of Pharmacoepidemiology and Clinical Pharmacology approved the PACMAN study.

Treatment steps

For this study, we only selected children with a reported use of maintenance treatment with ICS. We stratified the population into different treatment groups based on the type of maintenance medication use reported in the questionnaire. For the analysis, we selected two groups of patients: those who reported the use of ICS and those using a combination of ICS and LABA.

Definition of outcome

Two measures of asthma exacerbations were applied in this study; asthma-related visits to an emergency department (ED) in the past year, and prescribed courses of oral corticosteroids (OCS) in the past year. "Any exacerbation" was defined as the use of OCS and/or asthma-related ED visits. In addition, we assessed uncontrolled asthma with the use of the Asthma Control Questionnaire (ACQ-6), which assesses control of asthma symptoms in the past week.[13,14] A cut-off value of ACQ ≥ 1.50 was applied for uncontrolled asthma. A sensitivity analysis was performed using a cut-off value of ACQ ≥ 0.75 for uncontrolled asthma. Additionally, a sensitivity analysis was performed for Dutch ethnicity.

Statistical analysis

Binary logistic regression analysis was used to study the association between Arg16 genotype and the outcome measures. Odds Ratio (OR) and corresponding 95%CIs and p-values are reported. Crude ORs were adjusted for age and sex. A genotypic genetic model was assumed to assess the effect of the individual genotypes, with Gly16 homozygotes set as a reference. Additionally Hardy-Weinberg equilibria was calculated. Statistical analyses were performed with IBM SPSS® Statistics 20 (SPSS, IL, USA).

Results

Maintenance treatment use in the PACMAN population

Within the PACMAN population, Arg16 genotype and medication data were available for 765 children. In this study, rs1042713 was not consistent with Hardy-Weinberg equilibria (p=0.04). Of this group, 660 children (86%) were reported to use ICS singularly or concomitant with LABA. Table 1 presents the characteristics of the study population stratified for the different treatment groups. In our population, 468 (61%) children reported only ICS use as maintenance treatment, whereas 129 (17%) children reported ICS combined with LABA. Furthermore, 40 children (5%) reported LTRA use in addition to ICS and LABA use; 23 children (3%) used ICS in combination with LTRA and 11 children (1%) used LTRA only. Unfortunately, the numbers of children using LTRA were too small to include into the analyses.

Table 1. Characteristics of the study population

	ICS only (n=468)	ICS plus LABA (n=129)	p-value
Mean age ±SD (years)	8.52 ± 2.37	9.34 ± 2.15 (128/129)	0.037
Male gender (%)	60.9	65.1	0.38
Not well-controlled asthma‡ (%)	16.3 (75/459)	19.0 (24/126)	0.47
ED visit within the past year, %	6.2 (28/454)	4.8 (6/125)	0.57
Median FeNO in ppb (IQR)	14 (8-27), n=407	14 (7-36), n=120	0.84
SABA use within the past year (%)	88.0	77.5	0.002
Median ICS dosage (µg, budesonide equivalent; IQR)	400 (237-500), n=378	500 (400-1000), n=114	0.001
OCS use within the past year (%)	6.2	6.2	1.00
Arg16 genotype distribution Gly/Gly16 (%) Gly/Arg16 (%) Arg/Arg16 (%)	32.7 (n=153) 50.6 (n=237) 16.7 (n=78)	35.7 (n=46) 48.1 (n=62) 16.3 (n=21)	0.81

‡ ACQ-6 score ≥ 1.50.

ACQ, Asthma Control Questionnaire; ED, Emergeny department; FeNO, Fraction of exhaled Nitric Oxide; ICS, Inhaled corticosteroids; IQR, Interquartile range; LABA, Long-acting β_2-agonists; LTRA, Leukotriene receptor antagonist; ppb, Parts per billion; SABA, Short-acting β_2-agonists; SD, Standard deviation.

Arg16 genotype and treatment outcomes in children with a reported ICS and LABA use (N=129)

The children in the ICS plus LABA group were significantly older compared with the children in the ICS-only group (9.3 vs 8.5 years, p=0.04), reported using short-acting β_2-agonists (SABA) less frequently in the past (78% vs. 88%, p=0.002) and used higher ICS dosages (500 vs 400 µg median daily ICS dosage [budesonide equivalent]; p=0.001)). Children who were homozygous for the Arg16 allele with a reported use of ICS and LABA had a significantly higher risk of OCS in the past year (OR Arg16 homozygotes vs Gly16 homozygotes: 14.9 95%CI: 1.59-140.1, p=0.02) (Table 2). In the Arg/Arg group (n=21), there were five cases of OCS courses, compared to two cases within the Gly/Arg group (n=62) and one case within the Gly/Gly group (n=46). Furthermore, there were significantly more asthma-related ED visits (OR Arg16 homozygotes vs Gly16 homozygotes: 11.9, 95%CI: 1.22-115.8, p=0.03)). In the Arg/Arg group (n=19) there were four cases of ED visits, against one case in the Gly/Arg group (n=62) and one case in the Gly/Gly group (n=45). When these outcome were combined in 'any exacerbation' we found a similar significant association with the Arg/Arg genotype: (OR Arg16 homozygotes vs Gly16 homozygotes: 12.1, 95%CI: 2.18-67.6, p=0.004). In the Arg/Arg group (n=21) there were seven cases of severe exacerbations compared with three cases within the Gly/Arg group (n=62) and two cases within the Gly/Gly group (n=46) (Table 3). No significant association was found between the Arg16 genotype and uncontrolled asthma symptoms. A sensitivity analysis with a cut-off value of ACQ ≥ 0.75 did not alter our results.

Table 2. Effect of Arg16 genotype on exacerbations in children with a reported use of inhaled corticosteroids with or without long-acting β_2-agonists use

		ICS-only (n=468)		ICS plus LABA (n=129)	
		Adj. OR (95%CI)	p-value	Adj. OR (95%CI)	p-value
Not well-controlled asthma#	Gly/Gly	1 (ref)	-	1 (ref)	-
	Gly/Arg	1.36 (0.75-2.46)	0.32	0.63 (0.22-1.78)	0.38
	Arg/Arg	2.15 (1.05-4.42)	0.04	1.64 (0.49-5.55)	0.42
ED visit within the past year	Gly/Gly	1 (ref)	-	1 (ref)	-
	Gly/Arg	0.51 (0.23-1.15)	0.10	0.76 (0.05-12.68)	0.85
	Arg/Arg	0.29 (0.06-1.35)	0.12	11.87 (1.22-115.77)	0.03
OCS use within the past year	Gly/Gly	1 (ref)	-	1 (ref)	-
	Gly/Arg	0.62 (0.27-1.41)	0.25	1.47 (0.13-16.88)	0.76
	Arg/Arg	0.78 (0.26-2.32)	0.66	14.91 (1.59-140.06)	0.02
Any exacerbation‡	Gly/Gly	1 (ref)	-	1 (ref)	-
	Gly/Arg	0.62 (0.33-1.17)	0.14	1.11 (0.18-6.99)	0.92
	Arg/Arg	0.43 (0.15-1.19)	0.11	12.13 (2.18-67.60)	0.004

All data are p-values or ORs and 95%CI and were calculated by binary logistic regression analysis assuming a genotypic genetic model. Crude ORs were adjusted for age and gender.
ACQ-6 score ≥ 1.50.
‡ ED visits and/or OC use in the past year.
ACQ, Asthma Control Questionnaire; ED, Emergency department; ICS, Inhaled corticosteroids; LABA, Long-acting β_2-agonists; OCS, Oral corticosteroids; OR, Odds ratio.

Table 3. Arg16 genotype and exacerbations in children with a reported use of long-acting β_2-agonists combined with inhaled corticosteroids

Exacerbations in the past year	Gly/Gly16	Gly/Arg16	Arg/Arg16	Total
No	44	59	14	117
Yes	2	3	7	12
Total	46	62	21	129

'Exacerbation' was defined as oral corticosteroids use and/or emergency department visits in the past year.

Arg16 genotype and treatment outcomes in children treated with ICS, but without LABA (N=468)

In order to assess whether the observed association of the Arg16 allele with higher exacerbations rates was mainly caused by a reduced efficacy of the LABA treatment, we assessed the association of Arg16 allele in children who only used ICS (Table 2). Children who were homozygous for the Arg16 genotype did not differ significantly in their risk for OCS use or ED visits in the past year compared with children homozygous for Gly16. In contrast to the LABA treatment group, Arg16 genotype was associated with an increased risk of uncontrolled asthma symptoms in the past week in the ICS-only treatment group (OR Arg16 homozygotes vs Gly16 homozygotes: 2.15 (95%CI: 1.05-4.42) p=0.04). In the Arg/Arg group (n=77) there were 18 cases of not well-controlled asthma, 38 cases in the Gly/Arg group (n=232) and 19 cases in the Gly/Gly group (n=150). However, in a sensitivity analysis with ACQ ≥ 0.75 as cut-off value, this association was not found.

Dutch vs. non-Dutch decent

In order to assess the influence of a non-Dutch descent, we performed a sensitivity analysis in which we restricted the analyses to children of Dutch descent; this did not alter our results.

Table 4. Studies assessing the influence of Arg16 genotype on asthma exacerbations in children

Study	Design	Study population	Results	Ref.
Zuurhout et al., 2013	Observational; PACMAN cohort study.	468 children (4-12 yrs) with a reported use of ICS, and 129 children with a reported use of ICS and LABA	Increased risk of exacerbations in Arg16 homozygotes with a reported use of LABA and ICS compared to Gly16 homozygotes (OR: 12.13, 95%CI: 2.18-67.60)	[This Study]
Giubergia et al., 2013	Prospective clinical cohort	97 children with severe asthma receiving ICS and LABA regularly	The numbers of overall asthma exacerbations did not differ among Arg16 genotypes	[15]
Lipworth et al., 2013	RCT; children selected from the BREATHE study	62 asthmatic children who were homozygous for Arg16 genotype; children were randomized to ICS+LTRA or ICS+LABA treatment.	Exacerbations scores were reduced in children treated with LTRA+ICS compared to children treated with LABA+ICS (difference in score: -0.39, 95%CI: -0.15 to -0.64)	[3]
Basu et al., 2009 Palmer et al., 2006	Observational; BREATHE study	1182 young asthmatics (3-22 years), 401 of whom used β_2-agonists daily	Increased risk of exacerbations in patients receiving daily LABA or SABA with Arg16 variant, OR Arg16 homozygotes vs. Gly16 homozygotes: 2.70 (95%CI: 1.46-4.99)	[4,5]
Turner et al., 2004	Prospective community cohort	253 children enrolled at birth; 27 had asthma status at 11 years of age and Arg16 genotyping data available; medication data not available; LABA use was low‡	Increased risk of hospitalization for asthma in children who were homozygous for Gly16 genotype (OR of Gly16 homozygotes vs other genotypes: 3.2, 95%CI: 1.0-9.9). OR Arg16 homozygotes vs Gly16 homozygotes: 0.2, 95%CI: 0.02-1.42)‡	[16]

‡personal communication with the authors.
ICS, Inhaled corticosteroids; LABA, Long-acting β_2-agonists; LTRA, Leukotriene receptor antagonist; OR, Odds ratio; RCT, Randomized controlled trial; SABA, Short-acting β_2-agonists.

Discussion

This study provides additional evidence that the *ADRB2* Arg16 genotype is associated with an increased risk of severe exacerbations in children treated with LABA. This increased risk of OCS use and ED visits was restricted to children who were homozygous for the Arg16 genotype and treated with LABA, and was not observed in the ICS-only treatment group, suggesting that LABA are less effective specifically in children homozygous for *ADRB2* Arg16 genotype.

Few studies have assessed the effect of Arg16 genotype on the risk of exacerbations in asthmatic children (Table 4), and we were unable to perform a meta-analysis due to the heterogeneity of published data or the absence of ORs. Basu et al. and Palmer et al.[4, 5] have previously shown that the *ADRB2* Arg16 genotype is associated with an increased risk of exacerbations in Scottish children receiving daily SABA or LABA. In a RCT by the same group it was shown that children who were homozygous for *ADRB2* Arg16 respond better to LTRA than LABA as an add-on treatment to ICS when assessed on asthma control, quality of life, school absence and exacerbations.[3] However, a recently published study in Argentinean children with severe asthma treated with ICS and LABA did not find a difference in frequency of exacerbations when stratified per *ADRB2* Arg16 genotype.[15] The Argentinean study population (n=96) included only severe asthmatics, which might have influenced the results. Furthermore, differences in ethnic background might explain the discrepancy in results. Additionally, a prospective community cohort by Turner et al. found an increased risk of Gly16 homozygotes for asthma-related hospitalizations compared with other Arg16 genotypes, but this study only included 27 children with asthma (at 11 years of age) and use of LABA or SABA was not taken into account during the analysis.[16] These findings suggest that the Arg16 genotype is associated with an increased risk of exacerbations in Caucasian children with a reported regular use of inhaled β_2-agonists.

Clinical trials that mainly focused on adults could not demonstrate a modifying effect of *ADRB2* Arg16 genotype on LABA treatment outcome,[8, 9] suggesting that the effect of *ADRB2* might be restricted to LABA response in childhood-onset disease. In our study, LABA were used simultaneously with ICS, which means that the deleterious effect of having the Arg16 allele would exist even when children are treated with ICS therapy. However this is similar to previous studies.[3, 5, 6, 8, 10, 11] In children, the genetic effect might be stronger compared with adults, since the disease is less biased by long-term treatment use, airway remodeling and persistent

inflammation. Additionally, the genetic profile of childhood-onset disease may differ from adult-onset disease.[17]

The studies in adults that showed no effect of Arg16 genotype assessed lung function improvement as the main response outcome. Wu et al.[18] showed that clinical predictors of exacerbations in children differ from clinical predictors of uncontrolled asthma, suggesting that exacerbation-prone asthma is likely to be a different asthma phenotype compared with symptomatic asthma. This was observed in our own study as well - Arg16 was associated with severe exacerbations in LABA-treated children, but not with uncontrolled asthma symptoms.

Remarkably, we found an association between Arg16 genotype and poor asthma control (ACQ≥1.50) in the ICS-only treatment group. This might be explained by differences in the efficacy of SABA rescue medication, which is prescribed concomitantly with ICS.[2, 7, 20] In our study a higher percentage of the children who used only ICS as maintenance treatment reported the use of SABA in the past year compared with the children in the ICS plus LABA treatment group. A poor response to SABA might be reflected in less asthma control. Nevertheless, in our study we lacked detailed information about frequency of SABA use; therefore, we could not further test this hypothesis.

The Arg16 genotype frequencies in our population were not consistent with Hardy-Weinberg equilibria. This might be caused by the fact that our study population consisted of children that were treated with asthma medication, and therefore contained a high percentage of asthmatic children. The Arg16 variant allele is also associated with a higher risk of asthma symptoms.[5, 19]

Some limitations need to be noted. Although the cohort consists of a large study population of children with a reported use of asthma medication, the use of LABA and LTRA was limited, as well as the number of children with a homozygous Arg16 genotype. In addition, the incidence of severe exacerbations was relatively low in our population, as children were recruited through pharmacies and reflected a cross-section of the asthma population. Despite these limitations, we had enough power to detect significant associations. Nevertheless, our data have to be interpreted with caution due to multiple testing. However, Bonferroni correction, often applied to correct for multiple testing, was considered to be too conservative, as we only tested one previously identified SNP with related outcomes. We also have to bear in mind that the observed pharmacogenetic association might be biased by the risk conferred by other *ADRB2* SNPs. However, our results are in line with the results

of Lipworth et al.[3] and Basu et al.[4]; therefore, it can be assumed that the association we found is not a false positive.

Conclusion

Our data show that children who are homozygous for the *ADRB2* Arg16 genotype and who use LABA in addition to ICS have an increased risk of severe asthma exacerbations.

Future perspective

Our study strengthens the suggestion that children who are homozygous carriers of the Arg16 genotype could benefit from the use of personalized medicine. For example, as suggested in the trial of Lipworth et al. these patients might benefit more from LTRA compared with LABA treatment as an add-on therapy to ICS.[3] However, there is an ongoing need to further assess the value of Arg16 genotype testing in children in large, well-designed, prospective trials.

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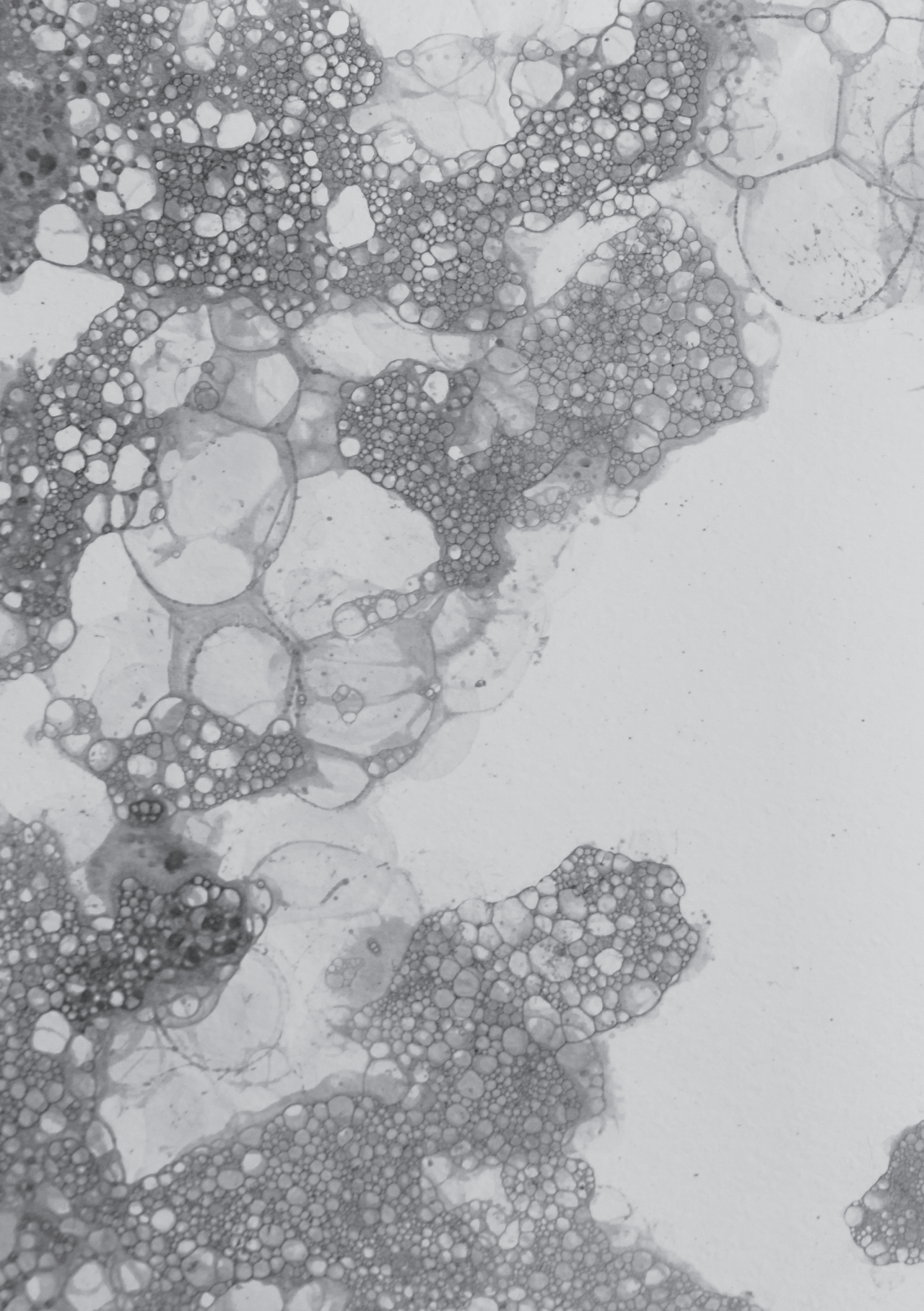

2.5

Genetic variation in uncontrolled childhood asthma despite inhaled corticosteroid treatment

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Abstract

Introduction: Genetic variation may partly explain the heterogeneity in asthma treatment response. We aimed to identify common and rare genetic variants associated with asthma that was not well controlled despite inhaled corticosteroid (ICS) treatment.

Methods: Data of 110 children was collected in the Children Asthma Therapy Optimal (CATO) trial. Measures of lung function (FEV_1%pred) and airway hyperresponsiveness to methacholine (Mch PD20) were analyzed longitudinally using mixed models. In addition, treatment response outcomes based on measures of FEV_1%pred or Mch PD20 and medication level were analyzed. Burden tests were used to analyze rare genetic variants (MAF<1%). The 17q12-21 locus previously associated with childhood asthma and treatment response was investigated separately.

Results: No SNPs or burden tests of rare variants were significantly associated with FEV_1%pred, Mch PD20 or treatment response. SNPs in the 17q12-21 locus were found nominally associated with the outcomes. The strongest association in this region was found for rs72821893 in *KRT25* with FEV_1%pred (p=3.75e-05), Mch PD20 (p=0.00095, and Mch PD20-based treatment outcome (p=0.006).

Conclusions: We replicated the association of the 17q12-21 region with asthma in this population. Using longitudinal data, this locus was additionally associated with FEV_1%pred and AHR, and additionally with ICS treatment response.

Introduction

Childhood asthma is a chronic inflammatory disease of the airways, which is associated with significant morbidity.[1, 2] Asthma is characterized by airway inflammation, airway hyperresponsiveness (AHR) and recurrent episodes of reversible airway obstruction.[3] Despite international guidelines on asthma treatment advising inhaled corticosteroids (ICS) as treatment to provide asthma control, some patients still have respiratory symptoms, exacerbations and get admitted to the hospital. There is increasing evidence that, in addition to environmental factors, genetic variation may partly explain heterogeneity in asthma treatment response.[4] Genome-wide association studies (GWAS) have mainly focused on (childhood) asthma susceptibility or severity[5] and have identified the 17q12-21 locus, containing *IKZF*, *GSDMB* and *ORMLD3*, which was consistently replicated in other studies.[6-8] One genome-wide study focusing on ICS treatment response has identified a SNP in the *GLCCI1* gene to be associated with change in forced expiratory volume in 1 second (FEV_1%pred) upon ICS treatment in asthma.[9] While providing cost-effective genome-wide coverage of common variation (SNPs >5%), GWAS yields little information about rare variation. To specifically investigate the role of functional variation in protein-coding genes, the exome-chip was developed which contains putatively functional and mostly rare (minor allele frequency (MAF) <1%) exonic variants, but also includes more common SNPs selected for a specific purpose (e.g. validated SNPs by GWAS,[10] ancestry informative SNPs, Human leukocyte antigen (HLA) SNPs).[11]

In the current study we have performed a post-hoc pharmacogenomic analysis using exome-chip data of participants of the Children Asthma Therapy Optimal (CATO) study.[12] This trial compared stepwise treatment of asthmatic children based on AHR and symptoms, or based on symptoms only.

We aimed to identify common and rare genetic variation associated with asthma that was not well controlled despite ICS treatment, focusing on lung function and AHR as main outcomes.

Material and Methods

CATO study

The CATO study is a two-year randomized clinical multi-center trial (ClinicalTrials.gov: NCT00158834) designed to compare treatment guidance of asthmatic children based on methacholine (Mch) bronchial provocation testing (provocative dose of methacholine that caused a fall in (FEV_1) of 20%, Mch PD20) and asthma symptoms, or based on asthma symptoms only. The design of the study has been published previously.[12] Briefly, atopic asthmatic children using inhaled corticosteroids, who had a positive radioallergosorbent test result (≥ 0.35 KU/L) for ≥ 1 airborne allergen were included based on current symptoms and/or AHR. Children were randomized to adjustment of treatment on symptom scores or to adjustment of treatment based on bronchial provocation testing and symptom scores. Participants were followed up for two years, with study visits every three months. During each visit the following parameters were measured: symptom-free days in the two weeks before each visit based on diary cards, lung function (FEV_1%pred)[13] and AHR (Mch PD20). AHR was tested by methacholine challenge using a dosimeter method.[14] Treatment dosage was when necessary adjusted upon each study visit according to the algorithm of the symptom (reference) or the AHR/symptom (intervention) strategy.

Medication levels

Study medication was divided into five increasing levels (Table 1). Level 1 and 2 consisted of ICS maintenance treatment only. In level 3-5 a long-acting β_2-agonist (LABA) was added to the ICS regime. All medication was administered via Diskus© dry powder inhalers.

Table 1. Levels of medication used in the CATO trial

Level of medication	Description
level 1	100 µg fluticasone per day
level 2	200 µg fluticasone per day
level 3	200 µg fluticasone and 100µg salmeterol per day
level 4	500 µg fluticasone and 100µg salmeterol per day
level 5	1000 µg fluticasone and 100µg salmeterol per day

Outcomes

Four outcomes were studied (specified below), two reflecting continuous measurements of phenotypes relevant to asthma, and two binary outcomes reflecting poor treatment response. As there was no significant effect of the intervention in the CATO study, we did not adjust for the treatment arm in these analyses.

Outcomes reflecting lung function and AHR

The measured outcomes FEV_1%pred and Mch PD20 were used to find SNPs affecting these parameters during treatment which was closely monitored (for further explanation see statistical methods). These outcomes are referred to as 'continuous outcomes'.

- **FEV_1%pred** expressed as a percentage of the average value for sex and height, corrected for medication level.
- **Mch PD20** as a continuous variable was log-transformed, and adjusted for age, sex and medication level.

Outcomes reflecting treatment response

To assess treatment response, we calculated a summary statistic to describe the increase or decrease of FEV_1%pred and AHR. We fitted a linear regression model for each subject and continuous outcome (FEV_1%pred and log PD20), with time as the independent variable. A positive or a negative value of the regression coefficient indicates an overall increase or decrease in the outcome (Figure 1). We selected children with worse than average lung function or AHR for these treatment outcomes, as these children are being treated with high doses of medication. The following outcomes are referred to as 'treatment response outcomes'.

- **Poor treatment response based on decrease in lung function despite high levels of treatment**

  Poor treatment response based on lung function was defined as a decreasing FEV_1%pred during the trial. Furthermore, children were required to have an FEV_1%pred < 100% at baseline, while medication administered was at level 4 or 5 for at least 5 out of 9 visits. Children not belonging to this group were considered to be responders to treatment. This outcome is referred to as "Lung function-based treatment outcome".

- **Poor treatment response based on persistent AHR (low PD20) despite high levels of treatment**
  Poor treatment response for AHR was defined as starting with a PD20 value lower than the median of the whole group, which decreased over time, while medication administered was at level 4 or 5 for at least 5 out of 9 visits. Children not belonging to this group were considered to be responders to treatment. This outcome is referred to as "AHR-based treatment outcome".

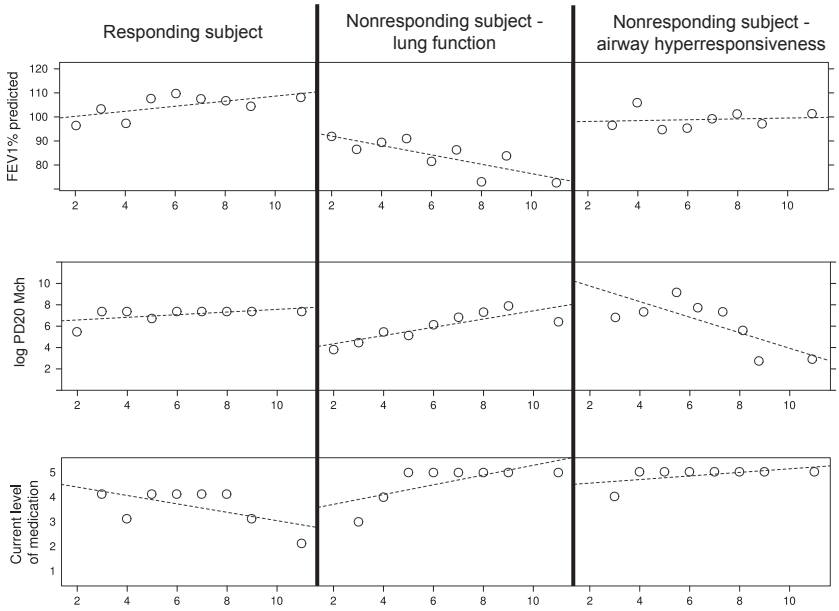

Figure 1. Progress over time of FEV_1%pred, PD20 and current level of medication for three subjects during the course of the study

Genotyping

DNA, isolated from buccal swabs, was available from 143 individuals and was extracted using a salt extraction method. Genotyping was performed using the Infinium HumanExome chip (Illumina, San Diego, CA), version 1.1, which contains 242,902 variants. The chip is designed to focus on nonsynonymous variation, but also features SNPs found associated to various phenotypes in previous GWAS, including: HLA tagging SNPs, ancestry informative markers.[11] Genotype calling was done using zCall[15] to facilitate calling of rare SNPs.

Quality control

The dataset was filtered on the basis of SNP genotyping call rates (≥95% completeness), only nonmonomorphic SNPs (MAF>0%), Hardy-Weinberg Equilibrium (HWE) p-value > 1e-6, and sample completion rate (≥95%) for further analysis. European ancestry was verified using EIGENSTRAT.[16] Identity-by-descent estimates from PLINK were used to identify siblings or otherwise related children (pi-hat > 0.2), one of which was randomly excluded.

Common SNP analysis

Using a cutoff of MAF ≥ 1%, 36,519 SNPs were selected for the common SNP analysis. The continuous outcomes FEV_1%pred and the log-transformed Mch PD20 were measured longitudinally. A linear mixed model approach was used to incorporate all data available in the trial, taking into account the correlated data within individuals. The results thus show the effect a SNP has on the outcome, adjusting for the fact that it has this effect at every time point.

Using the nlme R package,[17] a mixed model was fitted with age, sex (both only for AHR), and current level of medication as covariates. The random part of the model consisted of random slopes (allowing for systematic effects in the data) and random intercepts. Significance of each SNP was assessed using a likelihood ratio test comparing the model with and the model without the SNP.

Common SNP analyses for treatment response (a binary outcome) were performed using PLINK[18] for logistic regression analysis,[18] grouping children with a poor treatment response as 'cases', and the rest of the group as 'controls', adjusting for age and sex.

P-values were Bonferroni corrected for 36,519 tests to adjust for multiple testing, giving a cutoff value of 1.4e-6 for the common SNP analyses.

Rare SNP analyses

We used rare variant burden testing to group rare variants per gene, as these are unsuitable for single SNP testing. Gene and exon locations were based on RefSeq, including all nonsynonymous, splice and stop variants with a MAF < 1%. 24,944 SNPs were available for the rare SNP analyses in 10,157 genes, which resulted in a Bonferroni corrected p-value threshold of 2.75e-6. Calculations were performed using R version 2.15.2[19] and the nlme package for the continuous outcomes FEV_1%pred and Mch PD20. For these outcomes a T1 test was used, summing the number of variant alleles in a gene for each participant. This number is then used

as the independent variable in the linear mixed model analysis.
For the treatment response outcomes two tests were used in SCORE-SEQ version 5.2[20]: a simple sum of variants in a gene for each participant (T1 test) and the Sequence Kernel Association Test (SKAT).[21] SKAT allows for different directions of effect, possibly giving more power to detect effects.

Analysis of the 17q12-21 locus
We selected the whole 17q12-21 locus that is known to be associated with childhood-onset asthma[5, 7] and asthma treatment response,[22] to specifically investigate variants previously found to be associated with asthma, lung function and AHR. We compared the frequency of the rs7216389 T allele (the strongest previous association with asthma in this region) in the study population to the frequency in the Dutch population (samples of 500 parents from the GoNL project[23]) using a chi-square test.
For the association analyses the locus was defined as the region starting 100 kb upstream of the first SNP found associated with asthma in GWAS (rs907092 in *GSDMB*, base pair (BP) 37922259) and ending 100 kb downstream of the last SNP found associated with asthma (rs758632, upstream of *KRT25*, BP 38892689), the region of interest thus ranging from BP 37822259 to BP 38992689 on chromosome 17. This region contained 47 SNPs with a MAF ≥ 1%.

Results

Baseline statistics
110 children with European ancestry (80%) were included in the analyses, with age ranging from 6 to 16 years; 66 of which were boys (Table 2). During the trial, the mean FEV_1%pred remained stable, while the geometric mean PD20 rose from 88.7 to 333 µg. Fifteen out of 110 subjects were classified as nonresponders based on lung function, and 10 out of 110 subjects were nonresponders when considering AHR. There was little overlap between the two nonresponder phenotypes, 3 subjects being nonresponders for both outcomes. Nonresponders were on average a bit younger, and received more medication. Airway hyperresponsiveness decreased (higher Mch PD20) on average, and decreased for AHR nonresponders. Lung function did not change for the whole group, both nonresponder groups showed decreasing lung function over time.

Table 2. Characteristics of study subjects

	All subjects	Nonresponders FEV_1	Nonresponders Mch PD20
N (% of total)	110	15 (13%)	10 (8.7%)
Number of males (% male)	66 (59%)	7 (47%)	7 (70%)
Age, years	10.9 (2.47)	10.4 (1.84)	10.5 (2.42)
FEV_1 %pred at start of trial	97.6 (13.2)	91.9 (5.86)	100 (8.52)
FEV_1 %pred at end of trial	97.1 (15.0)	86.7 (9.08)	89.5 (14.7)
Mch PD20 (µg) at start of trial†	88.7 (4.86)	55.1 (3.72)	133 (5.76)
Mch PD20 (µg) at end of trial†	333 (5.01)	250 (4.94)	71.0 (7.36)
Mean level of medication‡	3.58 (1.24)	4.35 (0.729)	4.41 (0.688)

Values are mean (standard deviation) unless noted otherwise.
†Geometric mean and geometric standard deviation.
‡Mean level of all visits.

Genetic data quality control

After quality control, 64,581 variants were retained for analysis. 24,944 SNPs had a MAF < 1% and were included in the rare SNP analyses, leaving 39,637 SNPs for the common SNP analysis.

Lung function and AHR

Using FEV_1%pred as the outcome and the mixed model approach to account for the correlated data within individuals, we did not find chip-wide significant statistically results (Supplementary Figure 1). Lead SNPs from the loci with p < 1e-4 are shown in Table 3.

Using AHR as the outcome, the most significant finding was rs921561 at p=8.26e-06. Rs10484568 in the HLA region was associated with nominal statistical significance with both the FEV_1%pred outcome (at p=7.28e-05) and the AHR outcome (p=9.30e-05)(Supplementary Figure 2).

Table 3. Loci with p< 1e-4 for the continuous outcomes (FEV_1%pred and Mch PD20).

Chromosome pos.	SNP	Nearby gene(s)	MAF	Effect (SE)	P-value
		FEV_1%pred			
6p24.3	rs35742417	*RREB1*	0.1503	10.5 (2.13)	2.44E-06
16q24.3	rs117053233	*GAS8*	0.01748	-32.4 (7.41)	2.63E-05
17q21.2	rs72821893	*KRT25*	0.03497	-20.8 (5.00)	6.14E-05
6p21.32	rs10484568	*BRD2, HLA-DOA*	0.04545	-16.14 (3.9)	6.21E-05
7p12.2	rs1456896	*IKZF1*	0.3112	-7.62 (1.80)	7.42E-05
5q34	rs11953266	*RPS15P6*	0.3986	7.17 (1.76)	8.80E-05
		Mch PD20			
11q22.1	rs921561	*CNTN5, JRKL-AS1*	0.4792	-0.960 (0.212)	1.44e-05
12q21.31	rs1551120	*OTOGL*	0.3427	0.977 (0.224)	2.56e-05
13q22.2	rs716655	*TBC1D4*	0.4097	0.885 (0.204)	2.67e-05
6q21	rs847005	*SCML4*	0.3776	-0.861 (0.201)	5.56e-05
19q13.11	rs142299823	*ZNF30*	0.01389	-3.239 (0.766)	4.49e-05
10q11.23	rs2574951	*SGMS1*	0.1049	-1.97 (0.47)	4.60e-05
1p22.3	rs4655852	*LMO4*	0.4371	0.82 (0.2)	4.68E-05
19q13.41	rs12462608	*ZNF766*	0.03147	-3.3 (0.79)	5.18E-05
5p13.1	rs151191974	*C5orf51, OXCT1*	0.01399	-3.44 (0.84)	6.44E-05
19q13.43	rs34282745	*ZNF154*	0.03846	-2 (0.49)	8.13E-05
4p16.3	rs17768776	*ZFYVE28*	0.0979	-1.29 (0.32)	8.69E-05
6p21.32	rs10484568	*BRD2, HLA-DOA*	0.04545	-1.88 (0.47)	9.3E-05

Effect, SE and P-value from mixed model analysis, P-value calculated from likelihood ratio test.
MAF, minor allele frequency; SE, standard error.

Rare SNPs (burden tests)

Using the T1 burden test with 10,157 genes, we found 3 genes associated at p < 1e-4 (LAG3, ANK3, and NPBWR2; Table 4). *LAG3* and *ANK3* were associated with both FEV_1%pred and PD20.

Table 4. Burden test associations with continuous outcomes

Outcome	Gene	Beta	SE	P
		FEV_1%pred		
FEV	*LAG3*	-43.36	8.95	5.0E-06
FEV	*ANK3*	-24.93	5.89	4.2E-05
		Mch PD20		
PD20	*LAG3*	-4.85	1.09	1.7E-05
PD20	*ANK3*	-2.97	0.72	5.8E-05
PD20	*NPBWR2*	-4.44	1.10	8.9E-05

P-value calculated from likelihood ratio test.
SE, standard error.

17q21

The frequency of the T allele of rs7216389, the lead SNP in the 17q21 region incurring a higher risk for asthma,[5] was higher (61% vs. 50%, p=0.00165) in the CATO population compared to a representative sample of the Dutch population (GoNL[23]) .

Several common SNPs in the 17q12-21 region were found to be nominally statistically significantly associated with the outcomes of this study when investigating the 17q12-21 region. For the FEV_1%pred outcome (Supplementary Table 1), the most significant SNP was rs72821893 in the *KRT25* gene (p=3.97e-05), ranked third in the chip-wide analysis of common SNPs. One SNP reached nominal significance for PD20 Mch (Supplementary Table 2), rs72821893 (p=0.000954), which is the same SNP as the most significant SNP for the FEV_1%pred outcome.

Treatment response phenotypes

Common SNPs

Neither for treatment response based on FEV_1%pred, nor when based on AHR as the outcome, we found chip-wide significant results (Manhattan and QQ-plots in Supplemental Material). The five most significant loci for both outcomes are shown in Table 5.

Table 5. Five most significant loci for treatment response outcomes

Chromosome pos.	SNP	Nearby gene(s)	OR (SE)	P-value
		FEV_1%pred treatment response		
1q32.1	rs12748961	*NUCKS1, SLC45A3*	17.3 (0.710)	5.76e-05
8p11.23	rs4994	*ADRB3*	22.4 (0.781)	6.96e-05
16p12.1	rs113388806	*TNRC6A*	184 (1.33)	9.46e-05
6q25.3	rs894124	*SYTL3*	10.2 (0.622)	0.000229
16q24.1	rs72799568	*CRISPLD2*	10.9 (0.667)	0.000339
		Mch PD20 treatment response		
5p15.33	rs11745750	*IRX1*	33.6 (0.875)	6.42e-05
3p26.3	rs2727943	*CNTN4*	13.6 (0.741)	0.000426
11q12.3	rs1293035	*AHNAK*	35.6 (1.03)	0.000546
11p15.1	rs61733595	*MRGPRX3*	23.4(0.923)	0.000633
20q11.22	rs3746429	*EDEM2*	7.01 (0.577)	0.000732

OR, SE and P-value from logistic regression (Wald test).
MAF, Minor allele frequency; OR, Odds ratio; SE, Standard error.

Rare SNPs (burden tests)

Using the T1 and SKAT burden tests, we tested 10,157 genes on their association with treatment response. The most significant result for the burden tests of treatment response was the *DOCK2* gene, with a p-value of 7.10E-04 for the T1 test and 2.62E-04 for the SKAT test (Table 6).

For the treatment response based on AHR, the gene *GAB1* was ranked in the top 5 of both types of test (p=4.12E-04 for T1 and p=4.12E-04 for SKAT, Table 6).

Table 6. Burden test results for rare SNPs (MAF <1%) for both T1 and SKAT tests

Outcome	Gene	TEST	Rank T1	P T1	Rank SKAT	P SKAT
FEV_1%pred treatment response, top T1 results						
FEV_1 treat. resp.	*DOCK2*	T1	1	0.00071	4	0.000262
FEV_1 treat. resp.	*MTF1*	T1	2	0.00184	51	0.00346
FEV_1 treat. resp.	*DNAH5*	T1	3	0.00191	928	0.0883
FEV_1 treat. resp.	*BACH2*	T1	4	0.00198	17	0.00141
FEV_1 treat. resp.	*ZNF518B*	T1	5	0.00253	132	0.0105
FEV_1%pred treatment response, top SKAT results						
FEV_1 treat. resp.	*PWP2*	SKAT	3181	0.441	1	6.62E-05
FEV_1 treat. resp.	*RNASE10*	SKAT	8278	1	2	0.000118
FEV_1 treat. resp.	*C3orf15*	SKAT	2312	0.418	3	0.000148
FEV_1 treat. resp.	*DOCK2*	SKAT	1	0.00071	4	0.000262
FEV_1 treat. resp.	*CHD6*	SKAT	5422	0.571	5	0.000304
Mch PD20 treatment response, top T1 results						
PD20 treat. resp.	*ARSF*	T1	1	0.000355	58	0.00438
PD20 treat. resp.	*GAB1*	T1	2	0.000412	8	0.000412
PD20 treat. resp.	*CDH12*	T1	3	0.000449	82	0.00650
PD20 treat. resp.	*BICC1*	T1	4	0.000571	57	0.00428
PD20 treat. resp.	*ZNF518B*	T1	5	0.000675	131	0.0105
Mch PD20 treatment response, top SKAT results						
PD20 treat. resp.	*C20orf152*	SKAT	424	0.0427	1	0.000366
PD20 treat. resp.	*GAB1*	SKAT	2	0.000412	2	0.000412
PD20 treat. resp.	*ACTL8*	SKAT	479	0.0466	3	0.000562
PD20 treat. resp.	*NQO2*	SKAT	NA	NA	4	0.000765
PD20 treat. resp.	*ERRFI1*	SKAT	7059	0.762	5	0.000798

P-value calculated from likelihood ratio test.

17q21

Several SNPs in the 17q12-21 locus were found to be associated at $p < 0.05$ to FEV_1%pred based treatment response (Supplementary Table 3), such as rs907092 (p=0.0275) and rs9303277 (p=0.0271). The risk allele of the lead SNP at this locus

found in GWAS, rs7216389, is found at an allele frequency of 50% in a Dutch reference population,[23] at 61% in the CATO participants and 73% in the group not responding to treatment based on FEV1%pred (p=0.0551 in the association analysis).
For the AHR based-treatment outcome (Supplementary Table 4), the same SNP found associated to the continuous outcomes FEV_1%pred and PD20 MCh, rs72821893, was found as the strongest association in this region (p=0.00593).

Discussion

In an analysis of 36,519 common SNPs, no SNP was statistically significantly associated with lung function or AHR during ICS treatment. Several other findings seem relevant since analyses of the different phenotypes in this study pointed in the same direction. Our results show that in an asthmatic population treated with ICS, enriched for SNPs associated with asthma, these same SNPs may affect lung function and AHR. When investigating the 17q12-21 locus we found an enrichment of the risk allele of the lead SNP previously found in GWAS, rs7216389, in our population when compared with the general Dutch population. Furthermore, several SNPs in this locus were nominally associated with FEV_1%pred, including SNPs previously associated with asthma susceptibility. The most significant SNP, rs72821893 (in the keratin 25 (*KRT25)* gene), was associated with a reduction in FEV_1%pred by 20.8 percentage points per T-allele (95%CI -30.4; -11.3). Rs72821893 lowered PD20 Mch by 2.00 µg per T-allele (95%CI -3.16; -0.830, p=0.001), agreeing in direction with the effect found for FEV_1%pred. This same SNP was found to be associated with AHR treatment response. A 7-fold risk increase for being a poor responder was found per T-allele (OR 7.73, 95%CI 1.31; 45.5; p: 0.00593). *KRT25* is a keratin gene, an important protein in epithelial cells, but this family of proteins was not previously associated with asthma or pulmonary function. While rs72821893 is a nonsynonymous SNP, further investigation is necessary to explain this association.
The common SNP analysis for both treatment response outcomes showed no statistically significant results. However the first-ranking SNP, rs12748961 (close to *SLC45A3*) which was previously described in relation with the number of peripheral blood basophils (components of allergic inflammation),[24] is 2 kb away from a SNP associated with lung function decline in asthma.[25]
The burden tests for treatment response did not reveal statistically significant associations. *DOCK2* was found in the top-20 of both the T1 and SKAT test of

FEV_1%pred based treatment response, as was *BACH2*. The fact that we did not find rare variants with a large effect size might suggest that their importance for complex diseases such as asthma is small.

The greatest strength of our study is the longitudinal nature of the data, with follow-up for two years, and measurements of several clinically relevant phenotypes in a standardized way. Using a mixed model, we made optimal use of this data. In addition the cohort was well characterized, and treatment was standardized. With these features this study is a unique albeit small resource for asthma related investigations. The main weakness of our study is the low number of participants. This results in low power to find associations, although this is partially compensated by the longitudinal data. Our replication of associations at the 17q12-21 locus shows that a relatively low number of participants may be useful in (pharmaco)genetic research, if detailed phenotype data are available. The assessment of treatment responses in children with asthma is complex and response phenotypes are often simplified and do not take into account fluctuations of symptoms over time.[26] We were able to assess phenotypes over a longer time period, diminishing the impact of fluctuating phenotypes and measurement errors.

In conclusion, we used the exome-chip to find SNPs associated with lung function and AHR, and their treatment response based on the improvement of both variables during ICS treatment. We could not identify rare SNPs with major effects on these asthma phenotypes. We did show that the main asthma risk allele from GWAS (in the 17q12-21 locus) was more frequent in our population than in unaffected Dutch controls. This locus is associated with FEV_1%pred, AHR to methacholine and AHR based treatment response, with rs72821893 in *KRT25* as the most significant result for all three outcomes. Our study suggests that the 17q12-21 locus affects both asthma and treatment response to ICS in asthma.

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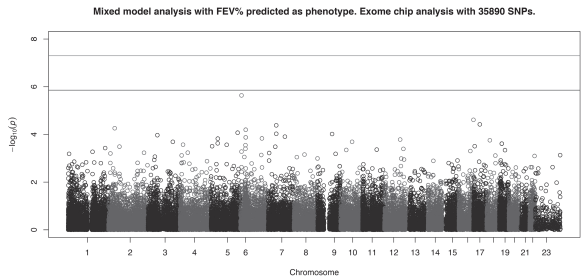
Supplementary figures and tables

A



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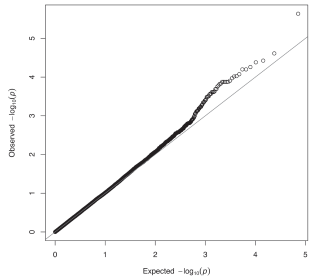
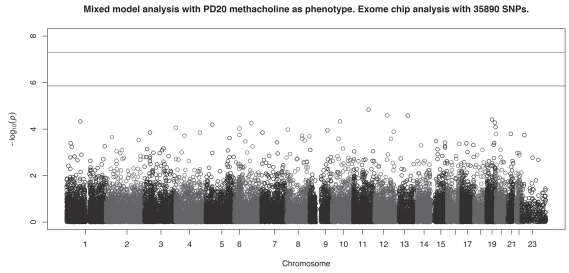


Supplementary Figure 1. Manhattan and QQ-plots for the chip-wide analysis of lung function. Manhattan plot (A) and QQ-plot (B).

A



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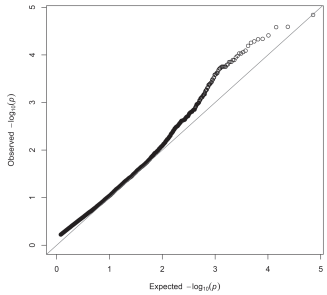


Supplementary Figure 2. Manhattan and QQ-plots for the chip-wide analysis of AHR. Manhattan plot (A) and QQ-plot (B).

Supplementary Table 1. Association of all SNPs in the 17q21 locus with FEV_1

SNP	CHR	BP	Allele	MAF	BETA	SE	P
rs35464006	17	37840860	C	0.01399	-488.441	697.427	0.4842
rs2643195	17	37853118	A	0.4266	2.119.985	1.940.872	0.278077
rs1136201	17	37879588	G	0.2238	-0.80572	2.082.727	0.704474
rs907092	17	37922259	A	0.3566	-319.367	2.006.653	0.113547
rs112301322	17	37944519	G	0.03846	0.889188	436.802	0.839136
rs9303277	17	37976469	T	0.4196	-283.604	196.021	0.150513
rs11557467	17	38028634	T	0.3951	-305.663	1.966.577	0.123536
rs2872507	17	38040763	A	0.3531	-320.134	1.983.409	0.107829
rs8067378	17	38051348	G	0.4126	-283.604	196.021	0.150513
rs2305480	17	38062196	A	0.3427	-341.387	1.951.795	0.081376
rs2305479	17	38062217	T	0.3811	-320.328	1.919.001	0.097455
rs35266519	17	38062390	T	0.01399	-598.443	6.863.059	0.390591
rs35104165	17	38062503	C	0.03846	0.579456	4.329.649	0.894044
rs11078928	17	38064469	C	0.3427	-341.387	1.951.795	0.081376
rs2290400	17	38066240	C	0.4056	-314.803	1.928.544	0.104281
rs7216389	17	38069949	C	0.3916	-335.297	1.931.838	0.084827
rs72832968	17	38100673	G	0.06294	5.383.617	3.392.832	0.113992
rs17609240	17	38110689	T	0.3077	-27.246	1.734.553	0.118892
rs7212944	17	38122686	A	0.2867	-344.119	1.862.406	0.066673
rs56030650	17	38131187	C	0.4441	-474.017	1.764.126	0.008476
rs4794822	17	38156712	T	0.4615	4.152.019	1.907.009	0.0351
rs4135012	17	38450248	A	0.03147	-286.608	50.143	0.568123
rs13706	17	38457151	A	0.1154	8.251.209	3.581.588	0.022488
rs202055764	17	38519545	A	0.03497	-299.535	4.735.996	0.531707
rs13695	17	38545193	A	0.2517	0.030473	2.017.959	0.987937
rs34300454	17	38547868	T	0.03497	-299.535	4.735.996	0.531707
rs2290207	17	38640744	T	0.2343	1.644.896	2.224.404	0.459874
rs3764424	17	38645125	G	0.2448	1.374.939	2.160.484	0.525001
rs1901187	17	38646147	C	0.3951	2.133.978	1.715.396	0.214143
rs2228015	17	38715186	C	0.01748	129.228	6.967	0.852257
rs7221109	17	38770286	T	0.3776	1.192.204	1.922.475	0.534106
rs2469825	17	38841662	A	0.4965	0.660706	1.922.074	0.731889
rs2462961	17	38855772	C	0.4965	0.73063	1.903.572	0.701751
rs874889	17	38857446	A	0.4825	0.207934	186.869	0.911358
rs9972941	17	38867922	A	0.1538	-312.822	2.584.286	0.227833
rs72821893	17	38907448	T	0.03497	-20.926	4.880.925	3.75E-05
rs9898164	17	38928014	G	0.1294	0.497476	2.748.353	0.856522
rs62622790	17	38933388	C	0.05944	-0.35566	3.616.243	0.921303
rs981684	17	38935812	A	0.4336	-292.101	1.866.225	0.119306
rs17558560	17	38936659	T	0.4301	2.490.202	1.879.211	0.184685
rs12453124	17	38938316	T	0.1294	0.497476	2.748.353	0.856522
rs7209228	17	38955991	T	0.1783	-0.90686	2.571.373	0.723299
rs142165420	17	38956007	G	0.01399	6.705.188	692.168	0.331974
rs77919366	17	38978462	T	0.2308	-33.702	2.122.197	0.112593
rs150048434	17	38978703	C	0.01049	-118.113	7.991.175	0.881974
rs17474506	17	38990780	G	0.03846	3.163.107	4.216.579	0.455136
rs1044806	17	38991052	G	0.2133	-154.475	2.413.386	0.52055

Base pair position is based on NCBI build 37. P-value (P) based on likelihood ratio test.
CHR, Chromosome; BP, Base pair; MAF, Minor allele frequency.

Supplementary Table 2. Association of all SNPs in 17q21 locus with AHR

SNP	CHR	BP	Allele	MAF	BETA	SE	P
rs35464006	17	37840860	C	0.01399	0.283398	0.817354	0.735195
rs2643195	17	37853118	A	0.4266	-0.04944	0.227732	0.827365
rs1136201	17	37879588	G	0.2238	0.026878	0.241115	0.910865
rs907092	17	37922259	A	0.3566	-0.23265	0.234058	0.319098
rs112301322	17	37944519	G	0.03846	0.168737	0.509023	0.739286
rs9303277	17	37976469	T	0.4196	-0.10543	0.23317	0.649848
rs11557467	17	38028634	T	0.3951	-0.16151	0.233928	0.488415
rs2872507	17	38040763	A	0.3531	-0.20052	0.235606	0.393324
rs8067378	17	38051348	G	0.4126	-0.10543	0.23317	0.649848
rs2305480	17	38062196	A	0.3427	-0.16786	0.232489	0.468838
rs2305479	17	38062217	T	0.3811	-0.12802	0.228737	0.574219
rs35266519	17	38062390	T	0.01399	0.936119	0.80237	0.24562
rs35104165	17	38062503	C	0.03846	0.168737	0.509023	0.739286
rs11078928	17	38064469	C	0.3427	-0.16786	0.232489	0.468838
rs2290400	17	38066240	C	0.4056	-0.05615	0.230119	0.806369
rs7216389	17	38069949	C	0.3916	-0.10996	0.230634	0.632118
rs72832968	17	38100673	G	0.06294	0.260997	0.401436	0.513909
rs17609240	17	38110689	T	0.3077	-0.18771	0.205531	0.361795
rs7212944	17	38122686	A	0.2867	-0.2062	0.221728	0.352228
rs56030650	17	38131187	C	0.4441	0.093105	0.214491	0.663782
rs4794822	17	38156712	T	0.4615	-0.13852	0.22878	0.545287
rs4135012	17	38450248	A	0.03147	-0.45062	0.593747	0.447511
rs13706	17	38457151	A	0.1154	0.180882	0.429797	0.672575
rs202055764	17	38519545	A	0.03497	-0.59749	0.558997	0.287219
rs13695	17	38545193	A	0.2517	0.206851	0.236321	0.379659
rs34300454	17	38547868	T	0.03497	-0.59749	0.558997	0.287219
rs2290207	17	38640744	T	0.2343	0.184921	0.261368	0.480107
rs3764424	17	38645125	G	0.2448	0.172921	0.253707	0.497336
rs1901187	17	38646147	C	0.3951	0.248433	0.20203	0.224508
rs2228015	17	38715186	C	0.01748	0.151125	0.818608	0.853781
rs7221109	17	38770286	T	0.3776	-0.24233	0.22662	0.289302
rs2469825	17	38841662	A	0.4965	0.032303	0.226417	0.886108
rs2462961	17	38855772	C	0.4965	0.020907	0.22433	0.925467
rs874889	17	38857446	A	0.4825	-0.04732	0.220087	0.829077
rs9972941	17	38867922	A	0.1538	-0.07249	0.30551	0.814402
rs72821893	17	38907448	T	0.03497	-200.683	0.594934	9.54E-04
rs9898164	17	38928014	G	0.1294	0.12883	0.322701	0.694732
rs62622790	17	38933388	C	0.05944	0.172348	0.424256	0.685196
rs981684	17	38935812	A	0.4336	-0.46891	0.218622	0.034162
rs17558560	17	38936659	T	0.4301	0.460999	0.218975	0.035696
rs12453124	17	38938316	T	0.1294	0.12883	0.322701	0.694732
rs7209228	17	38955991	T	0.1783	-0.32691	0.301765	0.283036
rs142165420	17	38956007	G	0.01399	0.138165	0.817832	0.868395
rs77919366	17	38978462	T	0.2308	-0.17665	0.252281	0.482521
rs150048434	17	38978703	C	0.01049	0.427082	0.935348	0.647651
rs17474506	17	38990780	G	0.03846	0.528498	0.495429	0.302257
rs1044806	17	38991052	G	0.2133	-0.30142	0.284018	0.289243

Base pair position is based on NCBI build 37. P-value based on likelihood ratio test.
CHR, Chromosome; BP, Base pair; MAF, Minor allele frequency.

Supplementary Table 3. Association of all SNPs in 17q12-21 locus with FEV_1 treatment response

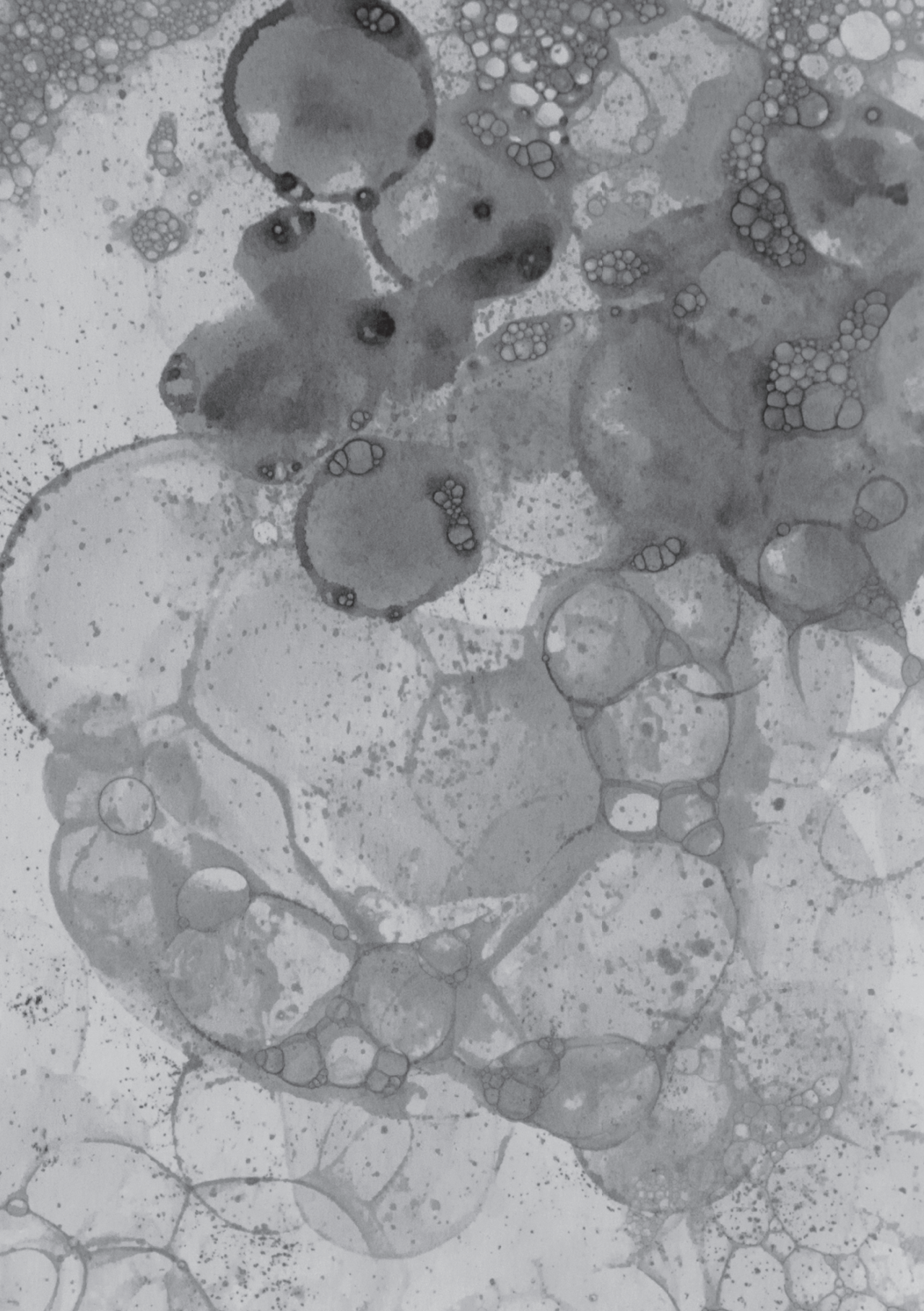
SNP	CHR	BP	Allele	MAF	OR	SE	P
rs35464006	17	37840860	C	0.01399	1.233	0.2744	0.3622
rs2643195	17	37853118	A	0.4266	0.4212	0.8302	0.129
rs1136201	17	37879588	G	0.2238	0.572	0.1221	0.086
rs907092	17	37922259	A	0.3566	0.518	0.1156	0.02748
rs112301322	17	37944519	G	0.03846	1.097	0.06044	0.5495
rs9303277	17	37976469	T	0.4196	0.491	0.1291	0.02713
rs11557467	17	38028634	T	0.3951	0.4899	0.1334	0.03141
rs2872507	17	38040763	A	0.3531	0.496	0.1472	0.05712
rs8067378	17	38051348	G	0.4126	0.491	0.1291	0.02713
rs2305480	17	38062196	A	0.3427	0.511	0.1301	0.04216
rs2305479	17	38062217	T	0.3811	0.4965	0.1237	0.02452
rs35266519	17	38062390	T	0.01399	1.233	0.1979	0.5183
rs35104165	17	38062503	C	0.03846	1.097	0.06044	0.5495
rs11078928	17	38064469	C	0.3427	0.511	0.1301	0.04216
rs2290400	17	38066240	C	0.4056	0.4786	0.152	0.04808
rs7216389	17	38069949	C	0.3916	0.4767	0.1574	0.0551
rs72832968	17	38100673	G	0.06294	0.6093	0.6005	0.2615
rs17609240	17	38110689	T	0.3077	0.4359	0.2595	0.2564
rs7212944	17	38122686	A	0.2867	0.4529	0.2656	0.3332
rs56030650	17	38131187	C	0.4441	0.4007	0.3397	0.4624
rs4794822	17	38156712	T	0.4615	0.4315	0.3313	0.5483
rs13706	17	38457151	A	0.1154	1.081	0.04791	0.3945
rs13695	17	38545193	A	0.2517	0.4216	0.62	0.4088
rs2290207	17	38640744	T	0.2343	0.5102	0.3466	0.9069
rs3764424	17	38645125	G	0.2448	0.4904	0.3414	0.8166
rs1901187	17	38646147	C	0.3951	0.3754	0.5018	0.9021
rs2228015	17	38715186	C	0.01748	1.238	0.2547	0.3923
rs7221109	17	38770286	T	0.3776	0.432	0.3929	0.8397
rs2469825	17	38841662	A	0.4965	0.4244	0.4839	0.803
rs2462961	17	38855772	C	0.4965	0.4223	0.4902	0.7857
rs874889	17	38857446	A	0.4825	0.4161	0.5102	0.7317
rs9972941	17	38867922	A	0.1538	0.5161	0.5841	0.3586
rs72821893	17	38907448	T	0.03497	1.131	0.1154	0.9599
rs9898164	17	38928014	G	0.1294	0.6034	0.2704	0.8356
rs62622790	17	38933388	C	0.05944	0.7621	0.2238	0.9964
rs981684	17	38935812	A	0.4336	0.4168	0.8772	0.09986
rs17558560	17	38936659	T	0.4301	0.4589	0.1999	0.1217
rs12453124	17	38938316	T	0.1294	0.6034	0.2704	0.8356
rs7209228	17	38955991	T	0.1783	0.5408	0.4378	0.6653
rs77919366	17	38978462	T	0.2308	0.4408	0.6712	0.2912
rs17474506	17	38990780	G	0.03846	1.053	0.1046	0.8534
rs1044806	17	38991052	G	0.2133	0.5186	0.4499	0.6748
rs142165420	17	38956007	G	0.01399	0.138165	0.817832	0.868395
rs77919366	17	38978462	T	0.2308	-0.17665	0.252281	0.482521
rs150048434	17	38978703	C	0.01049	0.427082	0.935348	0.647651
rs17474506	17	38990780	G	0.03846	0.528498	0.495429	0.302257
rs1044806	17	38991052	G	0.2133	-0.30142	0.284018	0.289243

Not all available SNPs are listed as for the rarer SNPs (MAF < 2%) the model could not always be fitted. 5 SNPs were removed for this reason. Base pair position is based on NCBI build 37. P-value (P) based on likelihood ratio test. Odds ratio (OR) per minor allele (additive model).
CHR, Chromosome; BP, Base pair; MAF, Minor allele frequency.

Supplementary Table 4. Association of all SNPs in 17q12-21 locus with AHR treatment response

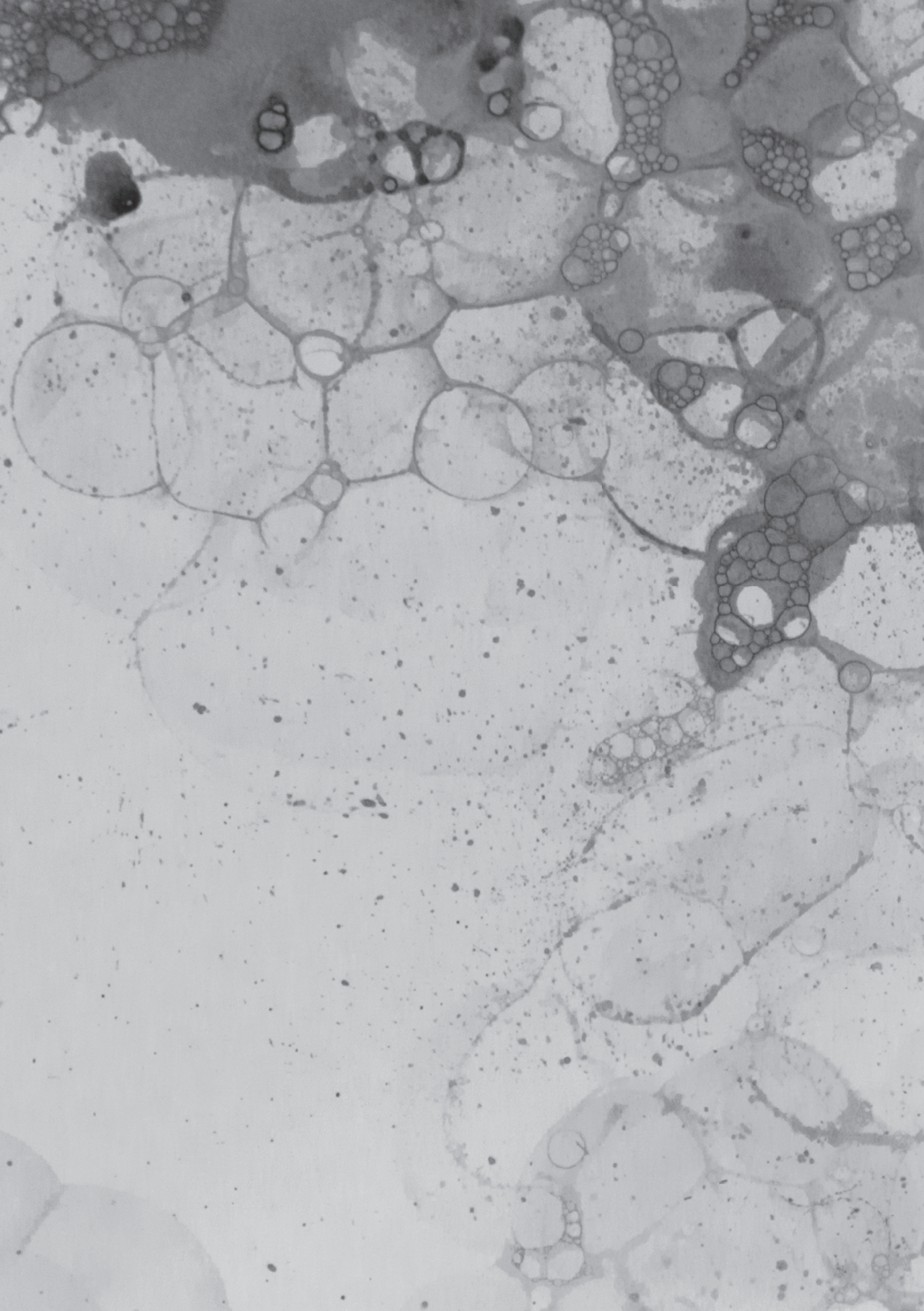
SNP	CHR	BP	Allele	MAF	OR	SE	P
rs2643195	17	37853118	A	0.4266	1.237	0.4962	0.668
rs1136201	17	37879588	G	0.2238	1.612	0.5002	0.3398
rs907092	17	37922259	A	0.3566	0.833	0.5313	0.7309
rs9303277	17	37976469	T	0.4196	0.5872	0.5442	0.3279
rs11557467	17	38028634	T	0.3951	0.6158	0.5439	0.3727
rs2872507	17	38040763	A	0.3531	0.8148	0.5339	0.7013
rs8067378	17	38051348	G	0.4126	0.5872	0.5442	0.3279
rs2305480	17	38062196	A	0.3427	0.8678	0.5266	0.7877
rs2305479	17	38062217	T	0.3811	0.6635	0.5348	0.4431
rs35266519	17	38062390	T	0.01399	3.278	1.212	0.3272
rs11078928	17	38064469	C	0.3427	0.8678	0.5266	0.7877
rs2290400	17	38066240	C	0.4056	0.6087	0.5427	0.3604
rs7216389	17	38069949	C	0.3916	0.6391	0.5412	0.4081
rs72832968	17	38100673	G	0.06294	0.6476	1.063	0.6828
rs17609240	17	38110689	T	0.3077	1.153	0.442	0.7471
rs7212944	17	38122686	A	0.2867	1.065	0.4846	0.897
rs56030650	17	38131187	C	0.4441	1.042	0.4791	0.9312
rs4794822	17	38156712	T	0.4615	0.9929	0.509	0.9889
rs13695	17	38545193	A	0.2517	0.4624	0.6462	0.2326
rs2290207	17	38640744	T	0.2343	0.16	1.049	0.08071
rs3764424	17	38645125	G	0.2448	0.1623	1.044	0.08167
rs1901187	17	38646147	C	0.3951	0.09721	1.029	0.02344
rs7221109	17	38770286	T	0.3776	0.631	0.5273	0.3825
rs2469825	17	38841662	A	0.4965	0.4766	0.5125	0.1481
rs2462961	17	38855772	C	0.4965	0.49	0.5088	0.1609
rs874889	17	38857446	A	0.4825	0.5263	0.4989	0.1983
rs9972941	17	38867922	A	0.1538	0.9665	0.6709	0.9594
rs72821893	17	38907448	T	0.03497	11.21	0.8784	0.005932
rs981684	17	38935812	A	0.4336	1.006	0.4903	0.99
rs17558560	17	38936659	T	0.4301	1.489	0.482	0.4091
rs7209228	17	38955991	T	0.1783	1.115	0.665	0.8701
rs77919366	17	38978462	T	0.2308	0.6473	0.5976	0.4668
rs17474506	17	38990780	G	0.03846	1.287	0.9921	0.7991
rs1044806	17	38991052	G	0.2133	0.7717	0.6484	0.6894

Not all available SNPs are listed as for the rarer SNPs (MAF < 2%) the model could not always be fitted. 12 SNPs were removed for this reason. Base pair position is based on NCBI build 37. Odds ratio (OR) per minor allele (additive model). P-value (P) based on likelihood ratio test.
CHR, Chromosome; BP, Base pair; MAF, Minor allele frequency.



Chapter 3

Inflammation



3.1

Inflammatory phenotypes underlying uncontrolled childhood asthma despite inhaled corticosteroid treatment: rationale and design of the PACMAN2 study

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Abstract

Background: The diagnosis of childhood asthma covers a broad spectrum of pathological mechanisms that can lead to similarly presenting clinical symptoms, but may nonetheless require different treatment approaches. Distinct underlying inflammatory patterns are thought to influence responsiveness to standard asthma medication.

Methods/design: The purpose of the PACMAN2 study is to identify inflammatory phenotypes that can discriminate uncontrolled childhood asthma from controlled childhood asthma by measures in peripheral blood and exhaled air. PACMAN2 is a nested, case–control follow-up study to the ongoing pharmacy-based "Pharmacogenetics of Asthma medication in Children: Medication with Anti-inflammatory effects" (PACMAN) study. The original PACMAN cohort consists of children aged 4–12 years with reported use of asthma medication. The PACMAN2 study will be conducted within the larger PACMAN cohort, and will focus on detailed phenotyping of a subset of the PACMAN children. The selected participants will be invited to a follow-up visit in a clinical setting at least six months after their baseline visit based on their adherence to usage of inhaled corticosteroids, their asthma symptoms in the past year, and their age (≥ 8 years). During the follow-up visit, current and long-term asthma symptoms, medication use, environmental factors, medication adherence and levels of exhaled nitric oxide will be reassessed. The following measures will also be examined: pulmonary function, exhaled volatile organic compounds, as well as inflammatory markers in peripheral blood and blood plasma. Comparative analysis and cluster-analyses will be used to identify markers that differentiate children with uncontrolled asthma despite their use of inhaled corticosteroids (ICS) (cases) from children whose asthma is controlled by the use of ICS (controls).

Discussion: Asthmatic children with distinct inflammatory phenotypes may respond differently to anti-inflammatory therapy. Therefore, by identifying inflammatory phenotypes in children with the PACMAN2 study, we may greatly impact future personalised treatment strategies, uncover new leads for therapeutic targets and improve the design of future clinical studies in the assessment of the efficacy of novel therapeutics.

Background

Asthma is one of the most common chronic diseases in childhood.[1] It is increasingly recognized that asthma is not a homogeneous disease and that different pathological mechanisms can lead to the clinical expression of asthma.[2] Inhaled corticosteroids (ICS) have become the first-line controller therapy for asthma, and the standard treatment of persistent asthma is generally guided by symptom control.[1] Most children with persistent asthma symptoms will have a beneficial response to ICS. Nevertheless, there is large inter-individual variability[3] and a portion of children with asthma will remain uncontrolled despite intensive treatment with high dosages of inhaled corticosteroids and/or oral corticosteroids. Uncontrolled asthma leads to a lower quality of life, may induce lung damage, can cause life-threatening exacerbations and results in increased health care resources utilization and expenditures.[4] Therefore, it is essential to identify asthmatic children with a high risk of poor response to standard asthma medication at an early stage.

A poor treatment response to ICS can be caused by various factors, including poor therapy adherence, misdiagnosis or continued exposure to allergens.[5] In addition, biological factors, including genetic variations, seem to play an important role in inter-individual ICS responsiveness. A recent study by Tantisira et al. showed that asthma patients with a single-nucleotide polymorphism (SNP) in the gene *GLCCI1* have a worse response in lung function upon ICS treatment.[6] Furthermore, a SNP in the *FCER2* receptor gene has been associated with an increased risk of asthma-related hospital visits, uncontrolled asthma and higher daily steroid dosages.[7,8] Nevertheless, despite the progress in asthma pharmacogenetic research, only a small percentage of the variability in treatment response can currently be explained by variations in SNPs.

In addition to genetic polymorphisms, other biological factors such as distinct inflammatory patterns may influence ICS responsiveness. Inflammation in asthma is often described as 'eosinophilic', based upon the presence of primed eosinophils in the airways. However, it has been shown that airway inflammation in asthmatic patients may also occur in the absence of increased levels of eosinophils and in the presence or absence of neutrophilia.[9,10] Corticosteroids induce cell death in eosinophils, but can induce survival in other immune cells such as neutrophils.[11] Therefore, it is likely that asthmatic patients with distinct inflammatory phenotypes may vary in their response to corticosteroids. This has been confirmed by various studies showing that asthmatic patients with non-eosinophilic inflammation

have a less beneficial response to corticosteroids when compared to those with eosinophilic inflammation.[12]

In addition, a RCT carried out by Green and colleagues showed that titrating ICS treatment based on sputum eosinophilia led to better asthma control compared to titrating treatment based on standard asthma guidelines in adults without a significant difference in corticosteroid usage.[13] A cluster analysis by Haldar et al. showed that titrating treatment based on sputum eosinophilia to prevent exacerbations was superior in two clusters of patients (specifically refractory asthma) where markers of eosinophilic inflammation were discordant with the presence of asthma symptoms.[2] A recent RCT in severe asthmatic children found no differences in exacerbations or improvement of asthma control when treatment was adjusted based on sputum eosinophilia.[14]

Various surrogate markers for airway inflammation have been described, including fraction of nitric oxide in exhaled breath (FeNO), volatile organic compounds (VOCs) in exhaled breath, sputum eosinophil counts and serum eosinophil cationic protein. Although these are, to a certain extent, applicable in clinical practice, few studies have assessed whether these markers are associated with ICS response in children.[12] In the PACMAN2 study we will focus on inflammatory phenotypes that may distinguish children who despite ICS use continue to suffer from asthma symptoms from children who are well controlled on ICS treatment. We aim to integrate clinical, proteomic, cellular and breath metabolomic data in order to more accurately define inflammatory mechanisms underlying asthma in children. PACMAN2 is an exploratory follow-up study of the ongoing Pharmacogenetics of Asthma medication in Children: Medication with Anti-inflammatory effects study (PACMAN).[15]

Methods/design

Study design

PACMAN2 is a nested case–control study within the observational pharmacy-based PACMAN cohort. PACMAN is an ongoing, cross-sectional study, including children aged 4–12 years with reported use of asthma medication. The inclusion criteria requires that they have had ≥3 prescriptions for Anatomical Therapeutic Chemical (ATC) code R03 medication in the past 2 years, including ≥1 prescription for R03 medication in the past 6 months. ATC code R03 medications are drugs prescribed

for obstructive airway diseases and are comprised of short-acting β_2-agonists, long-acting β2-agonists and inhaled corticosteroids (http://www.whocc.no/atc_ddd_index/?code=R03). Inclusion of children in the PACMAN cohort started in April 2009 and is still currently ongoing with over 990 children having been included thus far. Details of the study protocol of the PACMAN cohort study have been described elsewhere.[15]

For the PACMAN2 study, specific subsets of children included in the PACMAN cohort (PACMAN) will be selected for a follow-up visit in a clinical setting. This visit will be planned for at least six months after the original baseline visit and will include the reassessment of asthma symptoms, medication use, adherence to ICS and levels of FeNO. Furthermore, additional measurements will be made including pulmonary function testing and the measurement of exhaled volatile organic compounds in exhaled breath and inflammatory markers in peripheral blood by immunophenotyping and proteomics approaches. Comparative analyses and cluster-analyses will be used to identify markers that discriminate children with uncontrolled asthma despite ICS use (cases) from children with controlled asthma on ICS (controls). Cases and controls will be classified according to current and long-term asthma control at the time of the follow-up study visit, as this is most likely to reflect their current disease state. Current, uncontrolled childhood asthma despite ICS usage will be the primary study endpoint. Figure 1 presents a flowchart of the PACMAN study and the follow-up (PACMAN2).

Selection of study subjects

Children in PACMAN2 will be selected from the PACMAN cohort based on the inclusion criteria shown in Table 1. In order to increase the probability of including both children with long-term well controlled and long-term poorly controlled asthma, children will be selected from the PACMAN cohort based on the following:

- The child's asthma is classified as long-term controlled or long-term uncontrolled (see section below) at baseline, and
- The child is adhering to his/her inhaled corticosteroid regimen (Medication Adherence Rating Scale ≥ 21) [16] at baseline.

Current use of ICS will be checked when children are invited to participate in the follow-up study visit. Long-term and current asthma control will be reassessed during the follow-up study visit, as this may have changed over time. In the primary

analysis we will compare children that are currently uncontrolled despite ICS usage at the time of the follow-up study to children whose asthma is controlled by the use of ICS at the time of the follow-up study.

Table 1. Inclusion criteria PACMAN2 study

Long-term uncontrolled	Long-term controlled
• Parental consent to be approached for future research	• Parental consent to be approached for future research
• 8 years of age or older	• 8 years of age or older
• Current ICS user	• Current ICS user
• Adherent to corticosteroids (MARS≥21)‡	• Adherent to corticosteroids (MARS≥21)‡
• Long-term uncontrolled in the past year‡	• Long-term controlled asthma in the past year‡
≥ 3 seasons in the past year in which symptoms were uncontrolled:	≥ 3 seasons in the past year in which symptoms were controlled:
o ≥ 3 of the following symptoms (daily or weekly)	o Following symptoms are not present or occur less than weekly:
• Daytime asthma symptoms (cough, wheeze, shortness of breath)	• Daytime asthma symptoms (cough, wheeze, shortness of breath)
• Nighttime asthma symptoms	• Nighttime asthma symptoms
• Limitations in daily activities	• Limitations in daily activities
• Rescue medication use	• Rescue medication use
	• No asthma-related ER visit in the past year‡
	• No OCS use in the past year‡

Current ICS use was confirmed during telephone contact prior to the follow up study visit.
‡ Based on the retrospective questionnaire data obtained during the PACMAN pharmacy study visit (baseline).
ICS, Inhaled corticosteroids; MARS, Medication Adherence Report Scale; OCS, Oral corticosteroids; ER, Emergency room.

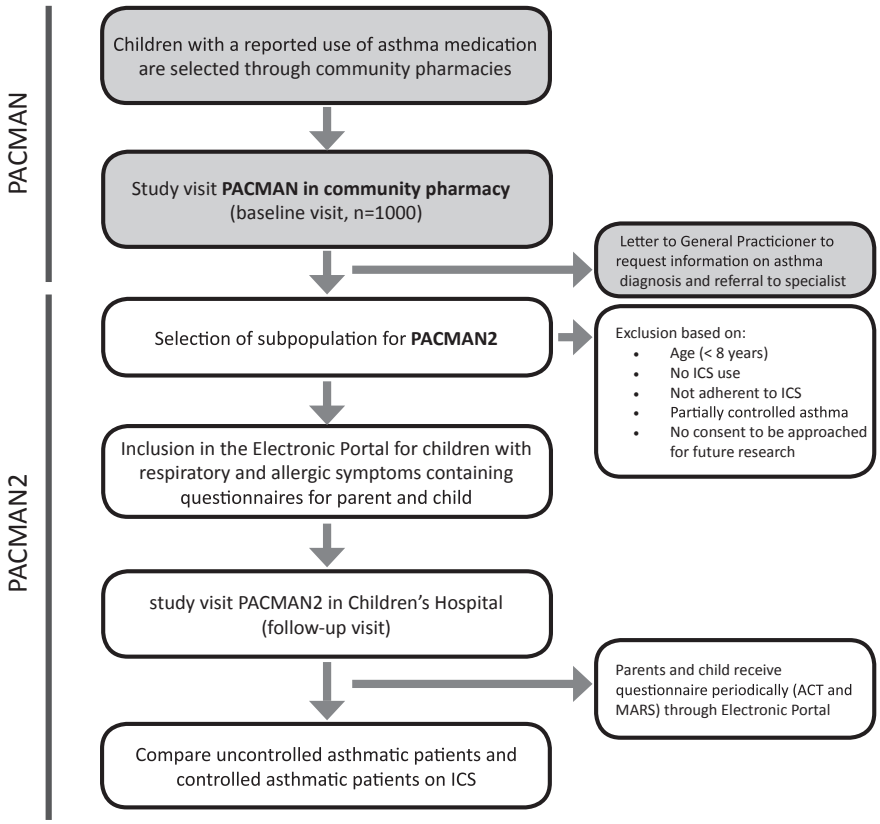

Figure 1. Flow chart data collection PACMAN cohort study and follow up

Current asthma control

Current asthma control will be assessed at both the baseline study visit and the follow-up study visit using the 6-item version of the Asthma Control Questionnaire (ACQ) (symptoms plus rescue medication use).[17] An ACQ-score of < 0.75 will be considered as 'well controlled asthma', a score ≥ 0.75 will be considered 'poorly controlled asthma'.

Long-term asthma control

The definition of long-term asthma control is based on the guidelines of the Global Initiative for Asthma.[1] During the baseline study visit and the follow-up study visit, parents will be asked to score the presence of and frequency of the following asthma symptoms: 1) daytime symptoms (wheezing, coughing, and shortness of breath), 2) nighttime symptoms, 3) limitations in daily activities, and 4) use of rescue medication during all four seasons of the previous year. Using this data, the children's asthma will be classified using the following definitions. Long-term, uncontrolled asthma is defined as ≥ 3 seasons of uncontrolled asthma in the past year with a season being considered uncontrolled when ≥ 3 asthma symptoms (daytime symptoms, daytime limitations, nighttime limitations or use of co-medication) occur on a daily or weekly basis.[18] Long-term controlled asthma is defined as ≥ 3 seasons of controlled asthma in the past year. A season is considered to be 'controlled' when asthma symptoms do not occur or occur less than weekly. In addition, children whose asthma is defined as long-term, controlled during the past year, but who reported the use of oral corticosteroids (OCS) or asthma-related ER visit(s) in the past year will be excluded.

Data collection PACMAN2

Children and their parents selected from the PACMAN cohort will be invited to a study visit at the Wilhelmina Children's Hospital. During this visit, data will be collected through the use of questionnaires and the measurements of lung function, exhaled breath, peripheral blood and blood plasma. Table 2 lists the instruments being used during the baseline visit (PACMAN) and the follow-up (PACMAN2).

Electronic portal

Before the scheduled follow-up visit, children and parents will be asked to complete an extensive online questionnaire regarding the child's general health, respiratory symptoms, respiratory infections, hay fever, food allergy, eczema, as well as environmental factors. The online questionnaire is located in a patient portal ('The Electronic Portal for children with respiratory and allergic symptoms') developed by the Wilhelmina Children's Hospital. The rationale and design of the Electronic Portal has been previously published.[33]

In order to screen for the presence of atopic diseases, parents and children will be asked to answer screening questions based on the core questions of the International Study on Asthma and Allergies in Childhood (ISAAC).[34] Based on their

initial answers, the participants will then be prompted by the system to complete additional disease-topic specific questionnaires including the Asthma Control Test (ACT),[35] the Medication Adherence Rating Scale (MARS)[16] and the Paediatric Asthma Quality of Life Questionnaire (PAQLQ).[23] The online questionnaire also contains questions on environmental factors such as tobacco smoke exposure, pet exposure and living environment. Additionally, after the follow-up study visit, parents and children will receive a short questionnaire (ACT and MARS) on the child's current asthma symptoms and use of medication during each season (every three months).

Additional questionnaire

In addition to the Electronic Portal questionnaires, parents and children will be asked to complete an additional, short questionnaire during their follow-up visit. This questionnaire will include the asthma control questionnaire (ACQ)[19] to assess asthma control in the previous week, questions regarding asthma symptoms during the previous seasons (to assess long-term asthma control), as well as questions about asthma-related health care utilization, recent severe exacerbations (OCS use, asthmarelated ER visits and hospitalisation) and current asthma medication use.

Lung function measurements and FeNO

Trained lung function technicians will perform spirometry and FeNO measurements. A single-breath, on-line measurement of FeNO will be carried out with a handheld electrochemical analyser (NIOX Mino, Aerocrine, Solna, Sweden). FeNO is measured during the baseline visit in a similar manner. Lung function measurements will include: forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), and FEV_1/FVC ratios before and after the inhalation of 800 µg salbutamol.

Volatile organic compounds (VOCs) in exhaled breath

VOCs will be measured according to a validated method described previously.[36] In short, while wearing a nose-clip, children will be asked to breathe normally for 5 minutes through a three-way, non-rebreathing valve with a VOC filter (A2, North Safety, Middelburg, the Netherlands) at the inspiration port and a silica filter at the expiration port. Then, after taking a maximal deep inspiration, the child will be asked to exhale a single, vital capacity volume into a Tedlar bag connected to the expiration port and a silica reservoir to dry the exhaled air. The VOCs present

in at least 500 ml of exhaled air in the Tedlar bag will be captured in Tenax GR Tubes (Interscience, Breda, The Netherlands) by using a peristaltic pump. The VOCs captured in the Tenax GR Tubes will be analysed with a validated panel of electronic noses (including carbon-poloymer, quartz microbalance metalloporphyrins, metal oxide sensors and ion mobility spectrometry) in the Department of Respiratory Medicine at the Academic Medical Centre in Amsterdam, The Netherlands.[37]

IgE levels, cytokines and chemokines in peripheral blood plasma

Levels of total and specific IgE against major allergens in plasma will be measured according the manufacturer's instructions using the Phadia ImmunoCAP system (UniCAP, Pharmacia, Sweden). These tests allow quantitative measurements (in kilo antibody units per litre; kU/l) of total IgE antibodies and specific IgE antibodies against common respiratory allergens. A concentration of specific IgE of 0.35 kU/l will be used as a cut-off value for a positive test result. In addition, cytokines and chemokines will be measured using multiplex immunoassay technology.

Immunophenotyping of peripheral blood cells

Expression of a wide range of surface markers on peripheral blood cells will be determined using multi-colour flow cytometry (Gallios, Beckman Coulter, Woerden, The Netherlands). Venous blood will be collected in sterile collection tubes containing sodium heparin as anticoagulant. Shifts in activation profiles of inflammatory cells will be assessed. The function of inflammatory cells is associated (in part) with the activation status of the cells' receptors, which can be modulated upon priming with inflammatory mediators, such as cytokines, chemokines and bacterial products. The expression of surface markers on distinct types of inflammatory cells will also be measured.

Proteomic profiling of peripheral blood granulocytes *in vitro* treated +/– dexamethasone

The *in vitro* effect of corticosteroids on the protein expression of peripheral blood granulocytes will be assessed using fluorescence 2-dimensional difference (2D) gel electrophoresis.[38] In brief, granulocytes will be isolated from whole blood anticoagulated with sodium-heparin using Ficoll-Paque. Granulocytes (5.10^6/mL) in incubation buffer will be treated *in vitro* with dexamethasone (10^{-6} M) or sham-treated with phosphate buffered saline for 15 minutes at 37°C. Subsequently, the cells will be stimulated with TNFα (100U/mL) for 3 hours. Cells will be lysed in lysis

buffer complemented with protease inhibitors, and proteins will be precipitated with 80% acetone and dissolved in 2D labelling buffer. 2D-DIGE technology will be used to analyse the proteomics samples and the differential protein expression after dexamethasone treatment. Spot detection will be performed with DeCyder 7.0 Difference in-gel Analysis software (GE Healthcare, Uppsala, Sweden) and gel images will be matched using DeCyder 7.0 Biological Variation Analysis software (GE Healthcare, Uppsala, Sweden).

Table 2. Instruments used during the baseline visit (PACMAN1) and follow-up study visit (PACMAN2)

	Baseline visit (PACMAN1)	Follow-up (PACMAN2)
Questionnaires		
	Questions on general health, allergies, asthma and respiratory symptoms	Questions on general health, allergies, asthma and respiratory symptoms
	Asthma control (ACQ-6)[19]	Asthma control (ACQ-6)[19]
	-	Childhood Asthma Control Test (c-ACT), Asthma Control Test (ACT)[20]§
	Questions on asthma control in the past 4 seasons[18]	Questions on asthma control in the past 4 seasons[18]
	Environmental factors (passive smoking, pets, living environment)	Environmental factors (passive smoking, pets, living environment) Active smoking is assessed in children > 12 years of age
	Believes about Medicines Questionnaire (BMQ)[21]	-
	Health care utilization for respiratory symptoms	Health care utilization for respiratory symptoms
	Exacerbations in the past year (ER visits/OCS usage)	Exacerbations in the past year (ER visits/OCS usage)
	Demographics	Demographics
	Current asthma medication use	Current asthma medication use
	Medication Adherence Rating Scale (MARS)[16]	Medication Adherence Rating Scale (MARS)[16]§
	-	General RAND questionnaire[22]
	-	Growth parameters, breast feeding and vaccination status
	-	Paediatric Asthma Quality of Life Questionnaire (PAQLQ)[23]§
	-	Paediatric and Adolescent Rhinoconjunctivitis Quality of Life Questionnaire (PRQLQ and AdolRQLQ)[23]§
	-	Allergic Rhinitis and its Impact on Asthma (ARIA)[26]§
	-	6-item Otitis Media Questionnaire (OM-6)[27] §

	-	Brouilette Score[28] §
	-	Food Allergy Quality of Life Questionnaire for children and teenagers (FAQLQ-CF and FAQLQ-TF)[29,30]§
	-	Self-Administered Eczema Area and Severity Index (SA-EASI)[31] §
	-	Children's Dermatology Life Quality Index (CDLQI) and Infant's Dermatitis Quality of Life Index Questionnaire[32] §
Inhalation technique		
	Inhalation technique (checklist)	-
Medication history		
	Medication history through pharmacy system	-
Lung function		
	Lung function testing and airway reversibility hand-held diagnostic spirometer	Lung function testing and airway reversibility in a clinical setting by a trained lung function technician
Exhaled breath		
	Exhaled Nitric Oxide (FeNO) (Niox Mino)	Exhaled Nitric Oxide (FeNO) (Niox Mino)
	-	Volatile Organic Compounds
Saliva		
	Saliva sample (Oragene) for DNA	Saliva sample (Oragene) for DNA
Peripheral blood and plasma		
	-	Peripheral blood and plasma sample

§ In the Electronic Portal, parents and children are asked to answer ISAAC screening questions which aim to screen on the presence of atopic diseases. Based on their initial answers, the participants will then be promted by the system to complete additional disease-topic specific questionnaires.[33]

Study endpoints

We will assess whether a (combination of) inflammatory marker(s) is (/are) associated with:

- current, uncontrolled childhood asthma despite ICS usage (primary study endpoint).
- long-term, uncontrolled childhood asthma despite ICS usage (secondary study endpoint).

Statistical analyses

Statistical analyses to compare controlled and uncontrolled asthma patients with respect to lung function, FeNO, total and specific IgE levels and flow cytometry data (surface and internal markers on immune cells) will be performed using independent sample t tests or one-way ANOVA with Dunnett's multiple comparison test for variables with a normal distribution, and Mann–Whitney and Kruskall Wallis tests for variables with non-normal distributions. In addition, unbiased cluster analysis will be used to assess patterns of inflammation. Statistical analysis of 2D-DIGE spot intensity will be performed using DeCyder 7.0 Extended data analysis software (GE Healthcare, Uppsala, Sweden) as described previously.[38] Since we aim to identify a fingerprint of markers that are differentially expressed, we will further explore the data using principal component analysis and other clustering analyses on the entire data set (including VOCs data) with adequate (cross-)validation according to recent recommendation in order to limit false-discovery.[39,40] Sensitivity analyses will be performed on the variables current adherence to corticosteroid treatment and continued exposure to environmental factors (pet exposure, passive/ active smoking).

Sample size calculation

Data concerning inflammatory markers in peripheral blood for long-term, uncontrolled asthma in paediatric asthma patients are lacking in the current literature; therefore, we are not able to perform a sample size calculation. However, it has been shown that proteomic approaches can distinguish protein expression profiles of peripheral blood cells in studies with small numbers of asthmatic patients and controls (n≥6).[41] Therefore, the following sampling approach has been selected. Based on a preliminary analysis of 744 children included in the PACMAN cohort, we found that 86.4% of the children use ICS and 60.2% are adherent to ICS treatment. When we assessed long-term asthma control at baseline, 33.4% of the children were long-term well controlled, 53.3% of the children were long-term partially controlled and 13.3% of the children were long-term poorly controlled. We expect that approximately 5.3% (n=53) of the children in the final PACMAN population (n=1000) will fulfil all the inclusion criteria for the uncontrolled (adherent) asthma patients and 12.5% (n=125) will fulfil all the inclusion criteria for the controlled (adherent) asthma patients. We will therefore invite all of the children that fulfil the inclusion criteria of 'uncontrolled asthma patients', as well as an equal number of controlled asthma patients.

Ethics

Only children whose parents consented to being approached for future research studies during their PACMAN study visit in the pharmacy will be invited to participate in the PACMAN2 study. A written informed consent will be obtained from the parents and from children who are ≥12 years. The Medical Ethics Committee of the University Medical Centre Utrecht approved this study.

Discussion

The PACMAN2 study represents an in-depth approach to the assessment of inflammatory phenotypes of steroid-treated, asthmatic children. It is our aim to integrate clinical data with inflammatory patterns in exhaled breath and peripheral blood in order to accurately assess paediatric asthma phenotypes related to asthma control, thereby gaining more insight into the underlying inflammatory mechanisms.

Many recent studies have focused on improving response to asthma medication using inflammatory or genetic markers; so far, success has been limited. Asthma is a heterogeneous disease and individual corticosteroid responsiveness is mostly likely to be the sum of various factors, including adherence to treatment, absence or presence of co-morbidities, exposure to allergens, genetic variations in therapeutic targets or pathways, as well as inflammatory patterns that may be intrinsically more or less sensitive to corticosteroid treatment.[5] All these different factors have to be taken into account for optimal guidance of individual treatment. Therefore, accurate phenotyping of children with asthma is paramount.

In 2009, the PACMAN cohort study was started in order to assess the effectiveness of asthma medication in children and the influence of genetic factors.[15] This resulted in a unique, pharmacy-based paediatric cohort representing a cross-section of children who use asthma medication on a regular basis. The asthma phenotypes ranged from controlled to uncontrolled asthma and from patients with mild disease primarily treated by general practitioners to patients with moderate to severe disease receiving specialized care from paediatricians or paediatric pulmonologists. The added value of the PACMAN cohort over that of other existing population-based asthma cohorts is its primary focus on medication use, in contrast to other paediatric asthma cohorts that have mainly concentrated on determinants of asthma susceptibility or respiratory symptoms.[42-44] Furthermore, large studies that

have assessed treatment effectiveness in asthmatic children, such as the Childhood Asthma Management Program (CAMP)[45] or the BREATHE study[46] have not taken underlying inflammatory patterns into account.

Defining appropriate therapy responses for asthma is a complex issue because of the heterogeneity of the disease. Various outcomes have been used to study the effectiveness of asthma therapies, for example improvement in lung function, symptoms scores or exacerbations (frequently defined by asthma-related hospital admissions, ER visits and/or oral corticosteroid use). Yet it is important to realize that predictors of treatment response depend upon the chosen definition of outcome variables.[47,48] Work by Haldar et al. showed that distinct clusters of adult asthmatics can be identified when studying two distinct dimensions of disease, i.e. asthma symptoms and eosinophilic inflammation.[2] These clusters can be concordant (asthmatic symptoms and measures of inflammation correlate) or discordant (asthmatic symptoms and measures of inflammation do not correlate). Others have described distinct inflammatory phenotypes based on sputum profiles of asthmatics,[9,10] indicating that defining outcomes solely on the presence or absence of symptoms or solely on markers of (eosinophilic) inflammation may only be informative for a subgroup of the total patient population. Therefore, in PACMAN2, we aim to collect data on symptoms as well as on inflammation markers.

During the first phase of the PACMAN study we obtained saliva samples for DNA extraction from our participants. To date, enough saliva has been collected for sufficient DNA isolation in 74% of the children (550/744). Recently, we replicated the genetic association identified by Tantisira et al. between the *FCER2* T22026 gene variant and treatment response in asthmatic children and showed that this SNP is associated with an increased risk of asthma-related hospital visits in our population.[7,8] The follow-up of specific subsets in PACMAN2 will give us the opportunity to identify new pharmacogenetic targets using proteomic and cellular profiling strategies and to validate these in the PACMAN cohort.

Markers in exhaled breath, FeNO and VOCs, will also be measured in an attempt to further elucidate their applicability in identifying asthma phenotypes in children; however the direct correlation between FeNO and airway inflammation remains unclear. FeNO is thought to be a marker of eosinophilic airway inflammation, but various other factors including steroid use and atopy seem to significantly influence FeNO levels.[49] Several studies have reported that high levels of FeNO in asthmatics are associated with a better response to ICS.[50-52]

Measuring patterns of VOCs in exhaled breath is a relatively novel metabolomic approach to study molecular signatures of respiratory disease. Exhaled breath contains a complex mixture of up to thousands of VOCs. These compounds are produced due to metabolic processes and the concentrations are likely to be influenced by the presence of airway inflammation. An electronic nose assesses the spectrum of volatiles present in exhaled breath without determining the individual molecular components.[53,54] Previous studies have shown that measurements of patterns of VOCs ('breathprints') using an electronic nose could discriminate adults with asthma from nonasthmatic controls[55] and asthmatic patients from COPD patients.[36,56] Furthermore, a recent study showed that breathprints of COPD patients with mild disease correlate well with the activation status of eosinophils and neutrophils in induced sputum samples of these patients,[57] suggesting that the electronic nose might be promising non-invasive diagnostic tool to assess ongoing airway inflammation.

An important strength of PACMAN2 is the extensive phenotyping of steroid-treated asthmatic children and the follow-up over time. We will reassess asthma symptoms, medication use, adherence and FeNO levels in children who were long-term, uncontrolled or controlled at the baseline visit. Nonetheless, due to the fact that the definition of long-term asthma control will be based upon retrospective questionnaire data, with parents and children being asked to answer questions about asthma symptoms over the past four seasons, recall bias may occur leading to an overor underestimation of the symptoms, constituting a potential limitation of our approach. Even so, long-term asthma control will likely provide additional information compared to current asthma control solely and findings from PACMAN2 will provide a better understanding of inflammatory phenotypes that may underlie uncontrolled asthma in inhaled steroid-treated children.

Childhood asthma affects millions of children worldwide and it is the leading cause of emergency room visits and hospitalizations in children, resulting in increased health care resources utilization and expenditures, and ultimately, costs to society. A substantial proportion of these asthma-related hospital visits occur despite high dosages of corticosteroid treatment. The identification of (inflammatory) phenotypes that reflect the pathobiological mechanisms underlying poor corticosteroid response may be of great importance in identifying high-risk patients at an early stage. Results from the PACMAN2 study might eventually lead to a more individualized treatment approach for asthmatic children, as well as to the discovery of new leads for innovative therapeutic strategies.

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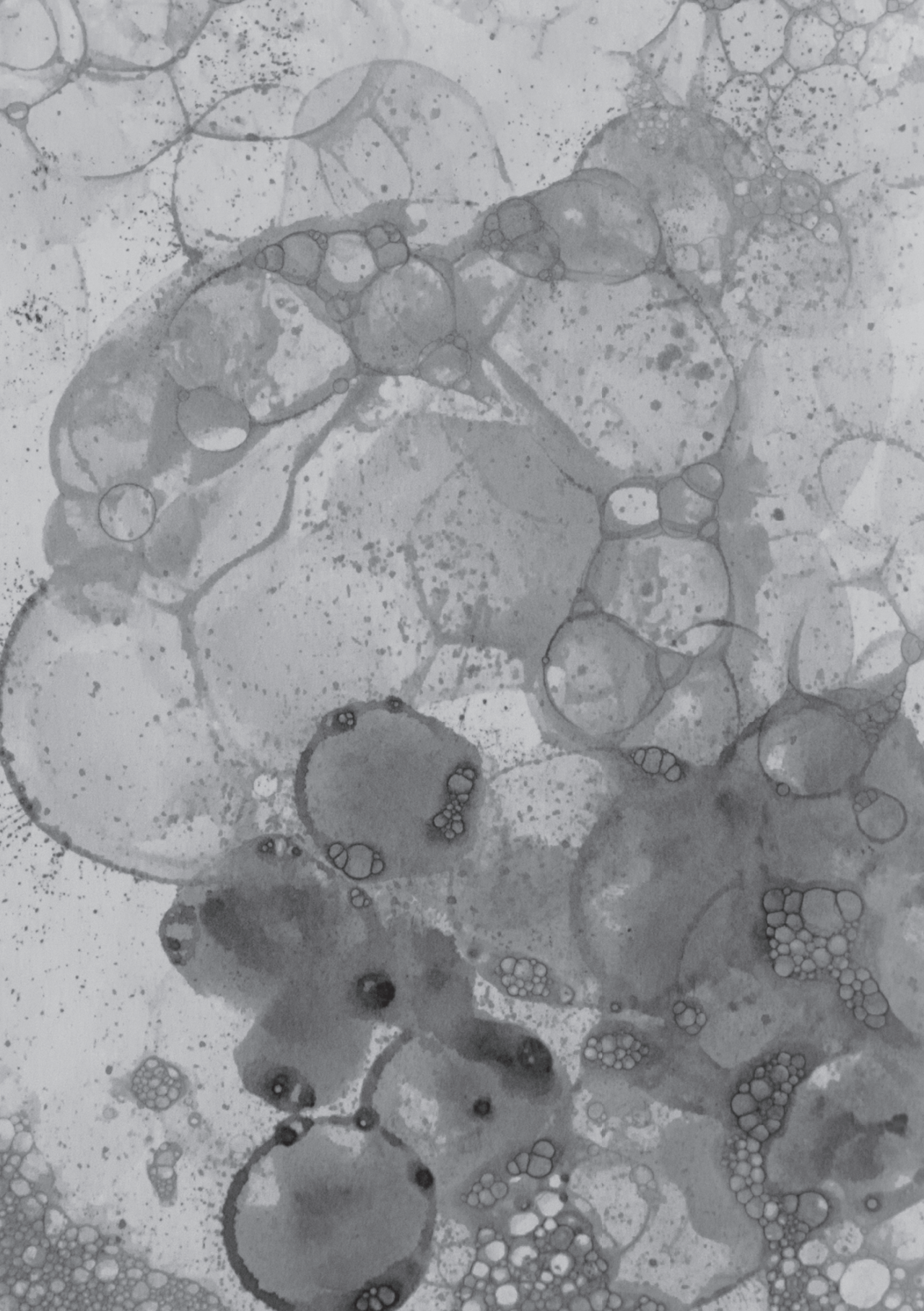

3.2

Priming phenotypes of peripheral blood granulocytes in asthmatic children treated with inhaled corticosteroids

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In preparation

Abstract

Background: The activation status of peripheral blood granulocytes has been associated with the state of disease in asthmatic adults. Distinct asthma phenotypes can be identified by determination of types of granulocytes in the tissue.

Objective: To investigate whether distinct priming phenotypes of granulocytes in blood of asthmatic children treated with inhaled corticosteroids (ICS) correlate with asthma control and clinical characteristics.

Methods: Blood samples were obtained from children who participated in the PACMAN2 study, a follow-up of the PACMAN cohort study. All children were current users of ICS. Children and parents completed a questionnaire on asthma control, medication use and exacerbations. Lung function was measured, as well as the fraction of exhaled nitric oxide (FeNO). Priming of eosinophils and neutrophils was determined by expression of Mac-1 (αm/CD11b) and expression of active FcγRII (CD32) both in an absence or presence of the innate immune mediator fMLF.

Results: Children with controlled asthma and uncontrolled asthma did not significantly differ in priming phenotypes of granulocytes in peripheral blood. The expression of active FcγRII on eosinophils (recognized by MoPhab A27) was associated with FeNO and eosinophil counts, but none of the other clinical features were associated with granulocyte priming. Eosinophils highly sensitive for stimulation ('pre-activated cells') were only observed in the context of low FeNO and low eosinophil counts in peripheral blood. Eosinophils with a low increase in activation epitopes upon stimulation ('refractory cells'), were observed in the context of high as well as low FeNO, and in the context of high as well as low eosinophil counts.

Conclusion: Based on the sensitivity of eosinophils for the priming stimulus, FeNO and blood eosinophil counts, three distinct phenotypes could be identified in asthmatic children with a reported use of ICS, yet these did not correlate with clinical features such as loss of asthma control. Different types of airway inflammation might not correlate directly with asthma symptoms.

Introduction

Asthma is characterized by airway inflammation. Inflamed tissue releases chemo-attractants and cytokines which recruit (pre)activated immune cells from the peripheral blood. This feed forward mechanism can lead to chronic inflammation in the tissue. The dynamic process of immune cells entering and leaving the blood stream could, therefore, be an indirect readout of the state of disease. Granulocyte functions *in vitro* and *in vivo* are tightly controlled by a process generally referred to as priming. This priming response does not necessarily lead to direct activation but rather leads to facilitation of responses evoked by heterologous agonists. Priming of these cells is associated with modulation of expression of several membrane receptors, both in number and in function. This activation status is typically modulated by interaction with environmental factors such as microbial products (e.g. N-formyl-methionyl-leucyl-phenylalanine; fMLF), as well as immunological factors such as cytokines and chemokines.[1]

Studies by Luijk et al.[2] and Johansson et al.[3] showed increased expression of active FcγRII, integrin receptor αd (CD49d) and β1 (CD29) on peripheral blood eosinophils of asthmatic patients upon allergen challenge. This priming process increases the responsiveness of cells to inflammatory signals such as opsonized targets and adhesion ligands. This responsiveness differs between asthmatic patients with and without a late asthmatic response.[4] In addition, priming can be used to discriminate between inflammatory responses that target neutrophils or eosinophils in peripheral blood.[4]

Unpublished work of our group shows that peripheral eosinophils of difficult-to-treat asthmatic adult patients are associated by blunted responsiveness to bacterial ligands. This hyporesponsiveness can be visualized by differential expression of activation markers on their cell surface upon *in vitro* treatment with a chemo-attractant. This is in marked contrast to mild asthmatics that are hyperresponsive to the same stimuli.[5] This data suggests that a low responsiveness of peripheral blood eosinophils to innate stimuli reflects a less favorable asthma phenotype.

In the current study we investigated whether distinct priming phenotypes could be observed in asthmatic children treated with inhaled corticosteroids and whether priming status of granulocytes correlated with clinical features in this patient group.

Material & Methods

Subjects and study design

Asthmatic children (age: 8-16 years) were recruited from the PACMAN cohort.[6] The rationale and overall design of the PACMAN2 study, a follow up of the PACMAN cohort, has been published previously.[7] All children were current users of inhaled corticosteroids. During a study visit in the Wilhelmina Children's Hospital, parents and children completed an asthma questionnaire,[8] spirometry was performed, FeNO was measured and venous blood was collected. The Medical Ethics Committee of the University Medical Centre Utrecht has approved the PACMAN2 study.

Asthma questionnaire

Asthma control was stratified into current and long-term asthma control. Current asthma control was assessed by the 6-item version of the Asthma Control Questionnaire (ACQ).[9] An ACQ-score ≥ 0.75 was considered 'not well-controlled asthma'.[10] Furthermore, parents and patients were asked to score the presence and frequency of asthma symptoms during all four seasons of the year preceding the PACMAN2 study visit. Long-term uncontrolled asthma was defined as ≥ 3 seasons of uncontrolled asthma in the past year. A season was considered to be uncontrolled when ≥3 asthma symptoms (daytime symptoms, daytime limitations, nighttime limitations or use of co-medication) occur on a daily or weekly basis (adapted from Koster et al.[11]). Treatment adherence was assessed using the Medication Adherence Report Scale.[12] The parents and children had also completed these questions during the initial study visit in the pharmacy (PACMAN cohort study).

Lung function and FeNO

Lung function and FeNO were measured by trained lung function technicians. A single breath measurement of fraction of exhaled nitric oxide was carried out with a hand-held electrochemical analyzer (NIOX Mino, Aerocrine, Solna, Sweden) with an expiration time of 6 seconds.[13] Lung function measurements included: forced expiratory volume in one second (FEV_1) and percentage change in FEV_1 predicted upon inhalation of 800µg salbutamol.

Isolation of granulocytes and assessing priming

Venous blood was collected in sterile collection tubes containing sodium heparin as anticoagulant. Blood was incubated at 37°C and in the presence or absence of the

bacterial formyl-peptide N-formyl-methionyl-leucyl-phenylalanine (fMLF, Sigma) (10^{-6}mol/L, 10 minutes at 37°C) to assess the expression of activation epitopes upon stimulation. After stimulation the cells were kept on ice. Samples were stained with fluorescein isothiocyanate (FITC) labeled antibodies directed against activation epitopes on FcγRII (CD32) (monoclonal phage antibody (MoPhab) A17 and A27), and against αm (CD11b) (clone 2LPM19c, PE-labelled, Dako, Glostrup, Denmark) as described previously.[4] CD11b is the specific α chain of the leukocyte integrin αMβ2, also known as Mac-1. Subsequently, erythrocytes were lysed in isotonic ice-cold NH_4Cl solution followed by centrifugation (1500 rpm for 7 minutes at 4°C). The pelleted samples were washed and resuspended in ice-cold PBS containing 1% human serum albumin (Sanquin, Amsterdam, the Netherlands). The samples were analyzed in a Gallios Flow Cytometer (Beckman Coulter). Eosinophils and neutrophils were identified according to their specific side scatter and forward scatter characteristics and CD16 fluorescence (clone 3G8, Alexa647-labelled). In order to assess the level of induction of the expression of the activation epitopes upon fMLF stimulation, 'fold induction' was calculated as: 1- (mean fluorescence units (MFU) after fMLF stimulation / mean MFU non-stimulated sample).

Eosinophil counts

Granulocyte counts in whole blood were measured by Cell-Dyn 1800 cell counter (Abbott Diagnostics). Using the percentages of CD16 negative granulocytes, the concentration of circulating eosinophils were calculated (in 10^9 cells per L).

Statistical analysis

To test the differences between clinical groups, non-parametric distributed variables were compared using Independent-Samples Median Test, and Pearson Chi-Square test was used to compare proportions. Differences between unstimulated and stimulated samples were compared using Related-Samples Wilcoxon signed rank tests. Correlations between activation markers were tested using Spearman Rank test, as were correlations between FeNO and eosinophil counts. Linear regression was used to assess whether clinical characteristics such as asthma control, lung function and exacerbations in the past year, were associated with the level of *in vitro* activation of peripheral blood eosinophils. Log transformation was used to obtain a normal distribution of priming induction.

Results

Characteristics of the study population

Of the 37 children included in the PACMAN2 study, data on priming status of granulocytes was available for 27 children (73%).Two children did not give consent for the venipuncture, and for 8 blood samples the CD16 staining was unsuccessful. Fifteen children were currently well-controlled, whereas 12 children were currently not-well controlled. Characteristics of the study population are shown in Table 1. Lung function measures (baseline FEV_1% predicted and bronchodilator response) were similar between well-controlled and not-well controlled children. Although children had been selected from the PACMAN cohort based on adherence to ICS at the time of the study visit in the community pharmacy, not all children were still adherent to treatment during the current follow up visit; 81.8% of the children who were uncontrolled were adherent, compared to 66.7% of the children with controlled symptoms (p=0.41).There was an incomplete overlap of distinct measures of poor asthma control (Figure 1). Four children who were currently uncontrolled had suffered from a severe exacerbation in the past year (33%) compared to none of the children who were currently well controlled (p=0.02). Nevertheless, only half of the children with current uncontrolled asthma were classified as long-term uncontrolled asthmatics according to reported symptoms in the past 4 seasons.

Table 1. Characteristics of the study population

	Not controlled asthmatics (ACQ ≥ 0.75)	Controlled asthmatics (ACQ <0.75)
	n=12	n=15
Age, mean ± SD	10.5 ± 1.6	12.9 (2.1)
Gender, male/female	6/12	7/15
FEV_1 baseline (% predicted), mean ± SD	88.2 ±11.9	86.3± 11.9
Change in FEV_1 from baseline	4.7 ± 5.5	5.2 ± 4.7
FeNO, median [IQR]	35 [11-42]	22 [13-79]
LABA use / no LABA use	50 (5/10)	58.3 (7/12)
LTRA use / no LTRA use	1/12	0/15
Adherent / not adherent	9/11	8/12
Severe exacerbations‡ / no exacerbations	4/12	0/15
Long-term uncontrolled / long-term controlled in the previous year	5/10	3/15

‡ER visits or OCS use.

ACQ, Asthma Control Questionnaire; BMI, Body Mass Index; FeNO, Fraction exhaled Nitric Oxide; FEV_1, Forced Expiratory Volume in 1 second.

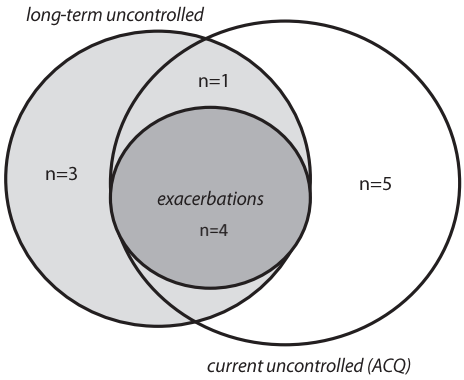

Figure 1. Distinct measures of poor asthma control. White circle reflects the groups of children that suffer from current uncontrolled asthma according to the Asthma Control Questionnaire (ACQ) (n=10). Light grey circle reflects the children with long-term uncontrolled symptoms according to reported symptoms in the previous 4 seasons (n=8) and the dark grey circle the children with severe exacerbations (ER-visits, hospitalizations or OCS use) in the previous year. Two children had missing questionnaire data on long-term asthma control.

Priming of granulocytes in asthmatic children treated with ICS

Children with uncontrolled asthma and controlled asthma did not significantly differ in lung function (Table 1). Baseline expression of activation epitopes or expression of activation epitopes upon *in vitro* fMLF stimulation on peripheral blood eosinophils and neutrophils did not differ between groups (Figure 2). Significant levels of upregulation of the expression of activation epitopes upon fMLF stimulation was observed in both groups (Figure 2). This did not significantly change when patients were grouped to the other measures of poor control, such as long-term control (at baseline and follow-up) or having suffered severe exacerbation in the past year (results not shown).

We studied whether priming of peripheral blood eosinophils was associated with clinical characteristics (Table 2). Univariate linear regression analysis showed a significant association between upregulation of the activation epitope recognized by MoPhab A27 upon stimulation and FeNO (log transformed β: -0.009, R^2=0.20, p=0.04) and between MoPhab A27 upon stimulation and blood eosinophil counts (log-transformed β: -0.87, R^2=0.27, p=0.02). Combining FeNO and eosinophil counts in a multivariate linear regression for eosinophil priming recognized by MoPhab A27 decreased the accuracy of the model (R^2=0.10) and the two predictors did not remain significant when combined. None of the other clinical characteristics were associated with granulocyte priming.

Table 2. Univariate linear regression analysis to study the association between clinical characteristics and priming window

	A17 induction		A27 induction		CD11 induction	
	β	p	β	p	β	p
Age	-0.008	0.95	-0.23	0.75	0.03	0.57
Gender	0.64	0.20	0.38	0.23	-0.04	0.83
FEV_1 baseline (% predicted)	0.01	0.63	0.005	0.69	-0.001	0.88
Change in FEV_1 from baseline	-0.074	0.33	-0.32	0.68	-0.12	0.57
FeNO in ppb	-0.01	0.21	-0.009	0.04	-0.004	0.21
Eosinophil counts	-0.62	0.42	-0.87	0.02	-0.36	0.13
ACQ score	-0.51	0.22	-0.42	0.83	-0.12	0.33
Long-term uncontrolled asthma	-0.28	0.82	-0.35	0.26	-0.02	0.86
Severe exacerbations in the past year	0.33	0.98	-0.19	0.41	0.003	0.99

Log-transformed Betas (β) are reported. Priming of eosinophils was measured by determination of expression of MoPhab A17, MoPhab A27 directed against activation epitopes on FcγRII and αm (CD11b) at baseline and upon *in vitro* fMLF stimulation.
FEV_1, forced expiratory volume in 1 second; ACQ, Asthma Control Questionnaire.

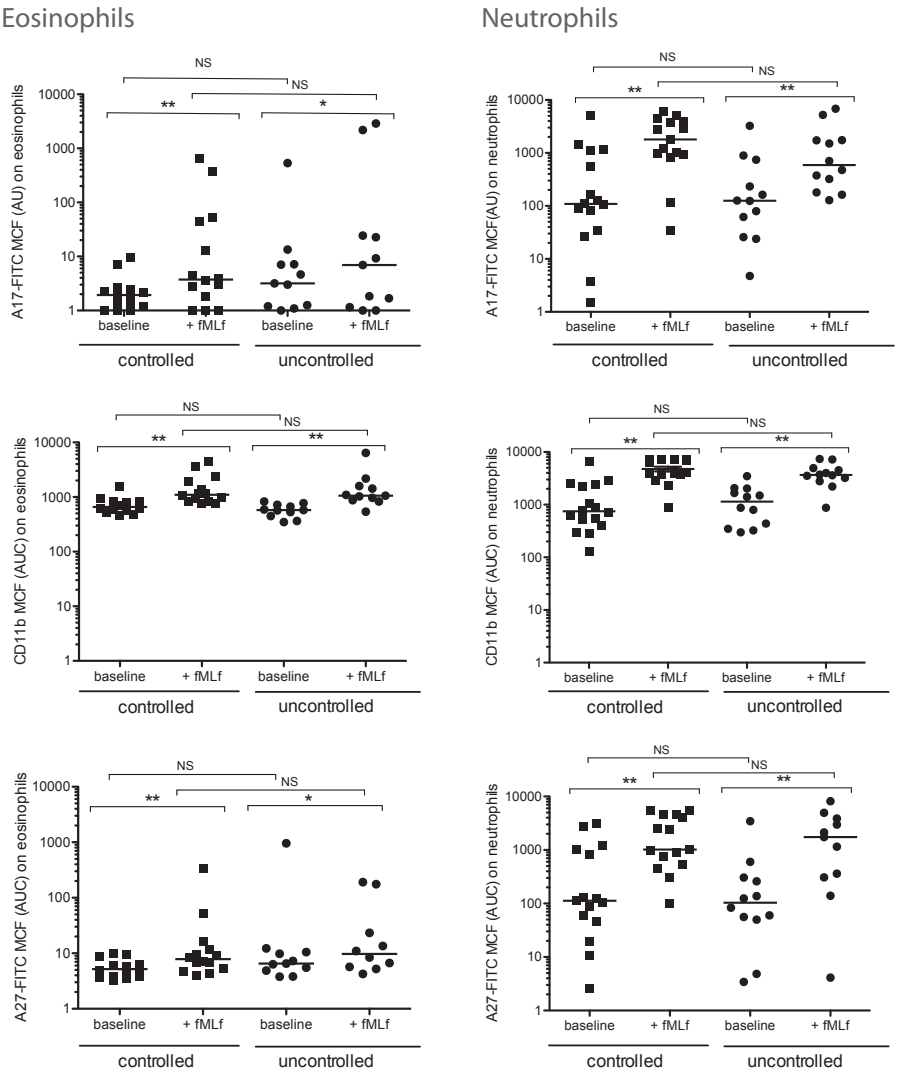

Figure 2. Priming of peripheral blood eosinophils and neutrophils in controlled and uncontrolled asthmatic children. MoPhab A27 and A17 are directed against activation epitopes on FcγRII. Mean channel fluorescence (MCF) is given in arbitrary units (AU). Asthma control was defined based on the Asthma Control Questionnaire. Independent-Samples Median Test was used to compare controlled asthmatics and uncontrolled asthmatics. Unstimulated and stimulated samples were compared using the Related-Samples Wilcoxon signed rank tests. *p<0.05, **p<0.005

Priming of eosinophils, eosinophil counts and FeNO

FeNO is thought to be a marker of ongoing eosinophil airway inflammation. There was a fairly strong positive correlation between FeNO and the number of blood eosinophils (Spearmans rho=0.61, p<0.001) (Figure 3).

Eosinophils highly sensitive for stimulation ('pre-activated cells') were only observed in the context of low FeNO and low eosinophil counts in peripheral blood (Figure 4). In contrast, eosinophils with a low increase in activation epitopes upon stimulation ('refractory cells'), were observed in the context of high as well as low FeNO, as well as in the context of high and low eosinophil counts.

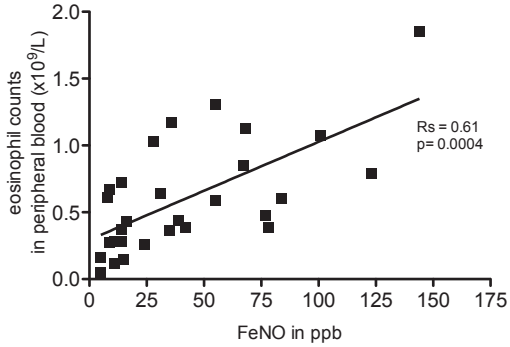

Figure 3. Correlation eosinophil counts in peripheral blood and FeNO. FeNO, fraction of exhaled nitric oxide; Rs: Spearman's rank correlation coefficient.

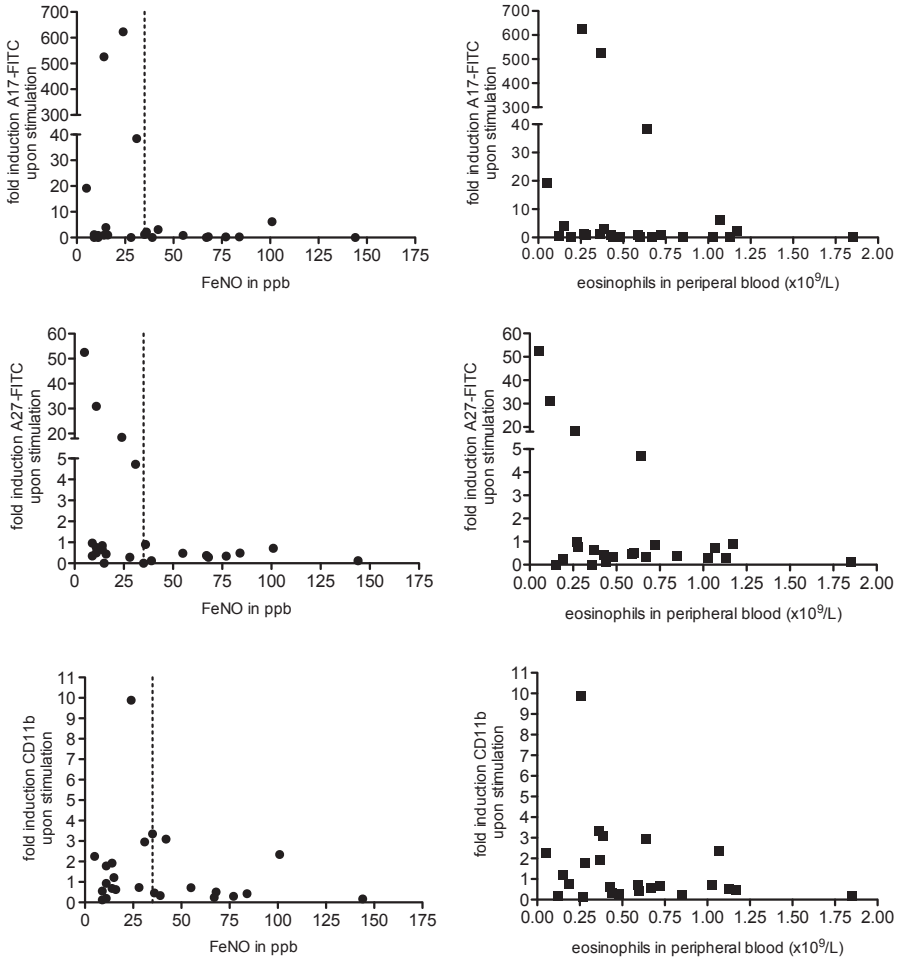

Figure 4. Sensitivity of peripheral blood eosinophils for stimulation and FeNO or eosinophil counts in asthmatic children. Dashed line depicts border of FeNO normal values (<35 ppb). Priming of eosinophils was measured by determination of expression of MoPhab A17, MoPhab A27 directed against activation epitopes on FcγRII and αm (CD11b) at baseline and upon in vitro fMLF stimulation. FeNO, fraction of exhaled nitric oxide.

Discussion

In this study, we found that ICS-treated children with uncontrolled asthma did not differ in priming phenotypes of peripheral blood granulocytes compared to their counterparts with controlled asthma. Priming of eosinophils recognized by MoPhab 27 was associated with eosinophil counts and FeNO, but not with clinical features such as FEV_1 or bronchoconstriction.

Peripheral blood eosinophilia has often been used as marker for asthma-related inflammation.[14] Studies have shown that blood eosinophilia correlates with bronchial hyperresponsiveness in asthmatic patients.[15] Furthermore, blood eosinophilia has been related to lung function improvement upon ICS treatment in asthmatic children.[16] The specificity of peripheral blood eosinophilia for asthma-related inflammation is, however, rather low, as allergies, autoimmune diseases and parasitic infections also cause blood eosinophilia. Furthermore, recent work by Ullmann et al. showed that peripheral eosinophil counts in children with severe asthma did not correlate with eosinophilia in BAL or airway biopsy samples.[17] Airway eosinophilia often occurred in the absence of blood eosinophils.

Peripheral blood eosinophil counts correlated well with FeNO levels in our study. It is thought that the enzyme inducible nitric oxide synthase (iNOS) is upregulated in inflamed airways, which leads to increased FeNO levels often observed in asthmatic patients.[18] FeNO cannot differentiate between severe and non-severe asthma,[19] but has been associated with eosinophils in endobronchial biopsies in children and adults with difficult asthma.[20,21] Nevertheless, various factors are known to influence FeNO including age, atopy, nutrition, medication use, therapy adherence and airway infections.[18] The interpretation of high FeNO levels is, therefore, complicated. An extra complication in the interpretation of FeNO and eosinophil count is the fact that corticosteroids can directly repress the transcription of the iNOS gene,[22] and can inhibit eosinophil survival[23] irrespective of disease. In parallel, corticosteroids are anti-inflammatory in part by repressing the expression of interleukins controlling eosinophil-mediated inflammation (such as IL-5).[24] It is, therefore, clear that associations between corticosteroid effects and clinical outcome do not need to be causal. FeNO has been reported to be a marker of ICS responsiveness, but this might have been a non-causal association. Corticosteroids may inhibit iNOS and inflammation independently.[16,25] Another puzzling finding is that children with severe therapy resistant asthma often express high levels of FeNO as well as airway eosinophilia despite the use of high doses of glucocorticoids.[26] Apparently, there is

a poor correlation between FeNO levels and disease severity in more severe asthma patients and/or parts of the immunological response might be ICS insensitive. Therefore, there is an urgent need for (a combination of) biomarkers that can easily identify children with a prognosis of poor treatment response. Importantly, these markers themselves should not be directly affected by the use of glucocorticoids.
Assessment of priming phenotypes of granulocytes could be a more predictive marker for the activity of inflammation. Johansson et al. and Luijk et al. focused on the mechanism of recruitment of eosinophils to the airways, which involves the expression of activated integrins.[2, 3] Their studies have shown that allergen challenge in asthmatics induced activation of integrins on blood eosinophils.[2, 3] Primed eosinophils with activated integrins might be prepared to exit the peripheral blood for the tissues, leaving behind eosinophils with a low response to inflammatory stimuli in the circulation. The observation that eosinophils with a refractory response to an *in vitro* stimulus are found in the peripheral blood of adults with difficult-to-treat asthma,[5] might reflect homing of activated cells to inflammatory loci in the airways.
In our study, blood eosinophils of asthmatic children with uncontrolled symptoms did not differ in sensitivity to an activating stimulus compared to children with controlled asthma symptoms. In contrast to the earlier studies mentioned above, our two groups of children did not differ in lung function. Maybe our analyses were performed in difficult-to-treat children earlier in their disease progression. This fits with the unpublished findings by Hilvering et al. that difficult-to-treat adult patients were characterized by eosinophils with a more pronounced insensitivity for fMLF compared to cells from controlled asthmatics.
Based on the sensitivity of eosinophils for the activating stimulus fMLF, FeNO and blood eosinophil counts; three phenotypes seemed present in asthmatic children with a reported use of ICS:

1) Sensitive blood eosinophils in the absence of systemic inflammation (low FeNO, low eosinophil counts)
2) Refractory blood eosinophils with no indication of systemic inflammation (low FeNO, low eosinophil counts)
3) Refractory blood eosinophils associated with systemic inflammation (high FeNO, high eosinophil counts).

We could not relate these subgroups to clinical characteristics, yet similar phenotypes have been observed in asthmatic adults (Hilvering et al., unpublished results). Airway inflammation and asthma symptoms may reflect different dimensions of disease and may not directly be related. Several lines of evidence support the view that inflammation rather than lung function is an important determining factor for the clinical picture of asthma. Johansson et al. found that expression of active β1 integrin on eosinophils was associated with loss of asthma control in 8 mild persistent asthmatics who participated in an ICS withdrawal study.[27] Furthermore, it was shown by the same group that the upregulation of active integrins on blood eosinophils upon allergen challenge is IL-5 mediated. Mepolizumab treatment (anti-IL5) attenuated the upregulation of active integrins on blood eosinophils of mild asthmatics upon allergen challenge, but not on BAL eosinophils.[28] Importantly, treatment with anti-IL-5 (Mepolizumab) has been shown to reduce asthma exacerbations in asthmatic patients with severe eosinophilic asthma[29, 30] without influencing asthma symptoms or lung function.[29]

Taken together our data suggest that the recruitment of active blood eosinophils to the tissue may influence asthma exacerbations, but this does not underlie other dimensions of disease such as symptoms or lung function. Multi-dimensional analyses including various biomarkers and clinical features,[31] rather than lung function measurements might identify clinical applicable asthma phenotypes in which different dimensions of disease (such as inflammation and asthma control) are successfully combined.

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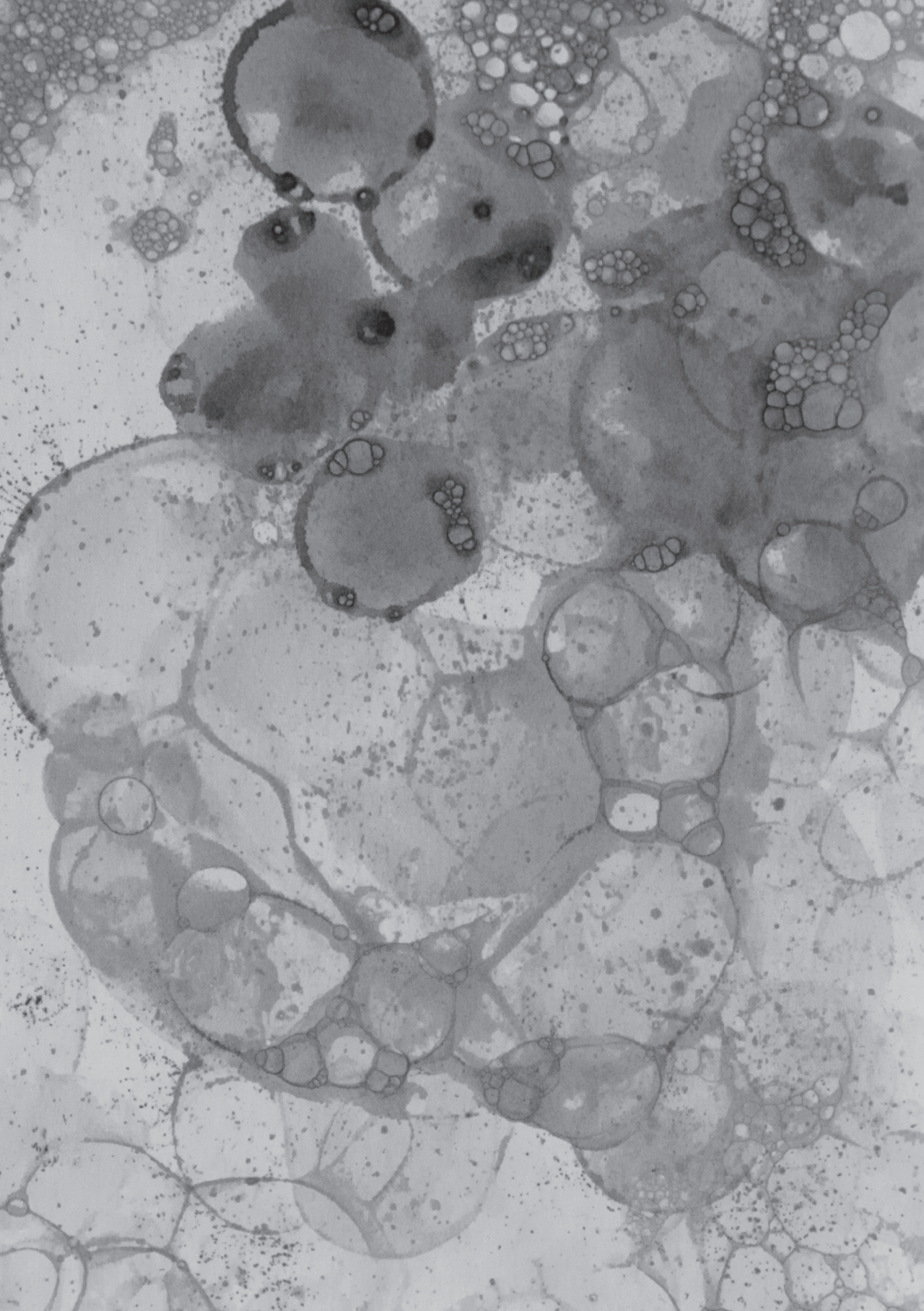
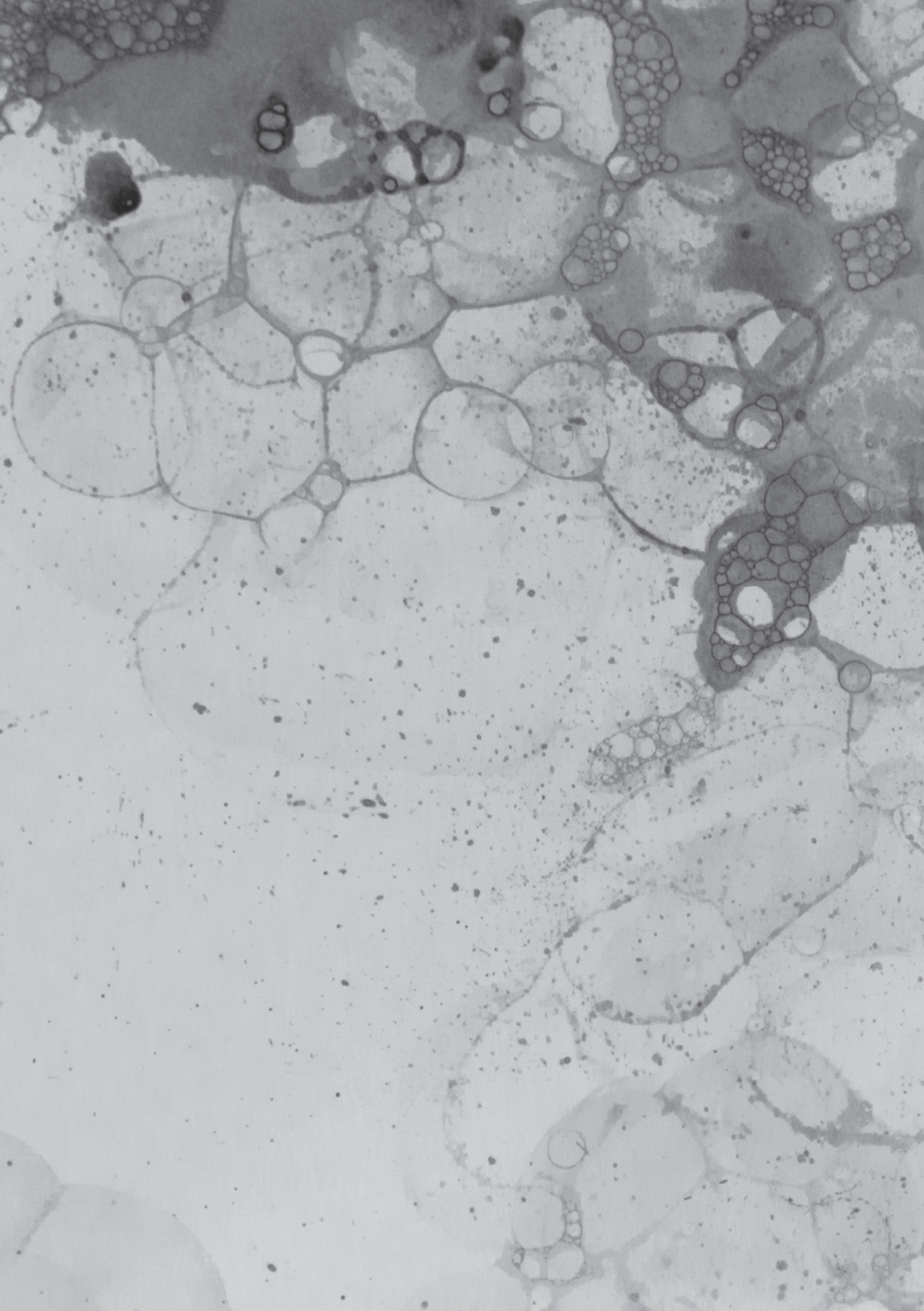


Chapter 4

Breath



4.1

Exhaled NO is a poor marker of asthma control in children with a reported use of asthma medication: a pharmacy-based study

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Abstract

Background: A high fraction of nitric oxide in exhaled breath (FeNO) has been suggested to be a marker of ongoing airway inflammation and poorly controlled disease in asthma. The usefulness of FeNO to monitor asthma control is still debated today.

Aim: To assess the validity of FeNO as a marker of asthma control in children with reported use of asthma medication.

Methods: Fraction of nitric oxide in exhaled breath was measured in 601 children (aged 4–12 yr) with reported use of asthma medication in the past 6 months and in 63 healthy non-asthmatic children (aged 5–12). Asthma control was assessed by the Asthma Control Questionnaire (ACQ). A receiver-operator characteristics (ROC) curve was generated to assess the accuracy of FeNO as a marker for asthma control. Logistic regression analysis was used to study whether clinical, health care, medication, and environmental factors are associated with high FeNO levels (>25 ppb).

Results: Fraction of nitric oxide in exhaled breath had a poor accuracy to discriminate well-controlled from not well-controlled asthma [area under the ROC curve: 0.56 (95%CI: 0.52–0.61, p=0.008)]. In addition, high FeNO (>25 ppb) was associated with lower medication adherence rates (OR: 0.4; 95%CI 0.3–0.6), fewer anti-biotic courses in the past year (OR: 0.6; 95%CI: 0.4–0.9), fewer leukotriene antagonists use in the past year (OR: 0.4; 95%CI: 0.2–0.9), and fewer visits to a (pulmonary) pediatrician (OR: 0.6; 95%CI: 0.4–0.9). Children living in a non-urban environment had more often high FeNO levels (OR: 1.7; 95%CI: 1.1–2.6).

Conclusion: High FeNO is a poor marker of asthma control in children with reported use of asthma medication. Various other factors, including medication adherence and medication use, are associated with increased FeNO levels.

Introduction

Over the last decade, a high fraction of nitric oxide in exhaled breath (FeNO) has emerged as a promising non-invasive marker of airway inflammation in asthma.[1, 2] A high FeNO has been reported as a surrogate marker for eosinophilic airway inflammation and may reflect uncontrolled asthma and predict asthma exacerbations.[1]

The production of nitric oxide (NO) from l-arginine is catalyzed by nitric oxide synthases (NOS). There are three known isoforms of NOS; two forms of constitutive NOS (neuronal NOS and endothelial NOS) and a form of inducible NOS. All three forms are expressed in airway epithelium. Inducible NOS can be upregulated by a wide range of inflammatory cytokines, and it is thought that this isoform is predominantly responsible for the elevated FeNO levels observed in atopic asthma.[2] Apart from inflammatory cytokines, various other factors like age, atopy, viruses, lipo-polysaccharide (LPS), oxidative stress, and corticosteroid use are thought to influence the production of NO.[1]

The clinical usefulness of FeNO to monitor asthma control is still debated. Studies assessing the relation between clinical symptoms, pulmonary function, and FeNO have provided inconsistent results.[3-7] Furthermore, a meta-analysis showed that the adjustment of asthma treatment based on FeNO levels does not improve asthma outcomes.[8]

Most studies focussing on FeNO in asthmatic children have recruited patients through secondary or tertiary health care services. Therefore, these patients may represent a subpopulation of patients with more severe disease compared with the total population of individuals with asthma symptoms that has generally mild-to-moderate severe disease. In this study, we assessed the specificity and sensitivity of high FeNO as a marker for not well-controlled asthma in a large population of children with a reported use of asthma medication, recruited through community pharmacies (primary care setting). In addition, we investigated whether other factors including medication adherence, health care usage, and living environment are associated with high FeNO levels in this population.

Methods

Study setting and study population

Our study population consists of children (aged 4–12 yr) who participate in the PACMAN (Pharmacogenetics of Asthma medication in Children: Medication with Anti-inflammatory effects) cohort study. The ongoing PACMAN cohort study has been initiated in April 2009 and has currently enrolled 744 children. Details of the study protocol have been described elsewhere.[9] Data were collected with the help of pharmacists belonging to the Utrecht Pharmacy Practice Network for Education and Research (UPPER), and the work was conducted in compliance with the requirements of the UPPER institutional review board of the Department of Pharmacoepidemiology and Clinical Pharmacology, Utrecht University. Briefly, children who are regular users of asthma medication [≥three prescriptions (Anatomical Therapeutic Chemical code R03) within the last 2 yr and ≥one prescription in the last 6 months] were recruited through community pharmacies in the Netherlands.[9] Children and their parents (or guardian) were invited for a visit to their own pharmacy. During this visit, the child's FeNO was measured and the caregivers filled in a questionnaire including questions on their child's respiratory symptoms, asthma diagnosis, medication use, adherence, caregivers' beliefs about medicines, environmental and socio-demographic factors. The questionnaire included the Asthma Control Questionnaire (ACQ)[10] and the Medication Adherence Report Scale (MARS).[11] Upon inclusion of a child in the PACMAN cohort study, a letter was sent to the child's treating general practitioner to inquire whether the participant has been diagnosed with asthma. Written informed consent was provided by the parents or guardian. For this study, we restricted our study population to children who were able to perform the FeNO expiration maneuver correctly and whose parents/guardian completed the questionnaire (n = 601). The PACMAN cohort study has been approved by the Medical Ethics Committee of the University Medical Centre Utrecht.

Fraction of nitric oxide in exhaled breath

A single-breath on-line measurement of FeNO was carried out with a hand-held electrochemical analyzer (NIOX Mino; Aerocrine, Solna, Sweden) with an expiration time of 6 s.[12] To investigate correlations between FeNO and other factors (e.g., asthma control, age, gender), FeNO values below 5 ppb (the detection limit of the NIOX Mino) were set on 2.5 ppb. Sensitivity analyses were performed whereby the FeNO values below 5 ppb were set on 0 and 5 ppb, respectively, which did not

influence our results. To assess factors related to high FeNO levels in children with reported use of asthma medication, FeNO was dichotomized using a cutoff value of 25 ppb based on the work of Buchvald et al.[13] who showed that FeNO values in healthy children and adolescents are below 25 ppb. In addition, sensitivity analyses were performed to test the robustness of our findings using cutoff values of 17.5 ppb, based on the results of our receiver-operator characteristics (ROC) analysis (Supplementary Table 1) and 35 ppb, the threshold value advised for children aged < 12 yr by the manufactory guidelines of NIOX Mino and the American Thoracic Society[14] (Supplementary Table 2).

Asthma control

Asthma control was assessed using the 6-item version of the ACQ (symptoms plus rescue medication use).[15] An ACQ score < 0.75 was considered 'well-controlled asthma', a score ≥0.75 was considered 'not well-controlled asthma'.[10]

Definition of main factors studied

Factors that could be associated with high FeNO (>25 ppb) were divided into five categories; (i) child characteristics, (ii) clinical characteristics, (iii) medication use in the past year, (iv) health care use in the past year, and (v) environmental factors.

1. Child characteristics: age and gender.
2. Clinical characteristics: atopy was defined as a parental-reported history of eczema, allergic rhinitis, and/or food allergy. Current use of inhaled corticosteroids (ICS) was defined as parental-reported use of ICS in the week previous to the study visit.
3. Medication use in the past year: ICS use, oral corticosteroids (OCS), short-acting β_2-agonists (SABA), long-acting β_2-agonists (LABA), leukotriene antagonists (LTRA), or antibiotic courses (AB) in the past year. Parental-reported compliance to medication was assessed using the MARS, comprising five questions on medication use behavior,[16] for example questions on forgetting to take a dose, altering the dose, deciding to miss a dose, taking less medication as instructed by the physician and stop taking medication for a while. The MARS was dichotomized using a cutoff point for the sum-score, ≥21 being considered as high adherence. A previous study by Menckeberg et al.[11] showed that the MARS is a satisfactory screening tool for non-adherence among adult ICS users irrespective of the chosen cutoff point, and that it correlates well with pharmacy dispensing data.

4. Health care use in the past year: general practitioner visit and specialist physician visit for respiratory complaints (pediatric pulmonologist or pediatrician visit).
5. Environmental factors: pet exposure, passive smoking, and current living environment (non-urban/urban).

Healthy controls

To compare FeNO values of our population of children with reported use of asthma medication with healthy controls, we studied healthy non-asthmatic children (aged 5–12 yr) who participated in the previous cross-sectional Utrecht Pulmonary Function Reference Data Study (The Utrecht dataset).[17] The Utrecht dataset is a cross-sectional study of the department of Paediatric Pulmonology of the University Medical Center Utrecht that was designed to generate new reference values for spirometry, lung volumes, airway resistance, CO diffusion, and interrupter resistance (Rint). Healthy non-asthmatic children were recruited at ten randomly selected primary and secondary schools between January 2004 and April 2009 by experienced pediatric pulmonary function technicians.[17] Data collection included FeNO measurement with NIOX Mino. For this study, we restricted our study population to children without a history of asthma, who did not show symptoms of wheezing in the last 12 months and did not use asthma medication. Written informed consent was obtained from the parents. The Utrecht dataset study has been approved by the Medical Ethics Committee of the University Medical Centre Utrecht.

Statistical analyses

Differences in characteristics between the population of children with reported use of asthma medication and the control group and between children with a high or normal FeNO were tested using chi-square test, independent sample t-test, or Mann–Whitney U-test when appropriate. Correlations between FeNO and other factors (e.g., age, asthma control) were tested using Spearman rank tests. A ROC curve was generated to assess the value of FeNO to discriminate well-controlled asthma (ACQ < 0.75) from not well-controlled asthma (ACQ ≥ 0.75). In a ROC curve, the true-positive rate is plotted against the false-positive rate for different cutoff values of a diagnostic test. The area under the ROC curve (AUC) is a measure for test accuracy; an AUC of 0.5 reflects poor predictive accuracy – no better than flipping a coin – while AUC of 1.00 implies excellent predictive accuracy. To study factors associated with high FeNO in children with reported use of asthma medication,

logistic regression analyses were used, resulting in the estimation of (adjusted) Odds ratios (OR) and 95 percent confidence intervals (95%CI). Only variables associated with high FeNO values ($p < 0.10$) in the univariate analyses were included in the multivariate analysis. Potential confounding factors were included in the multivariate model if they induced a 10% change or more in the crude regression coefficient for the determinant of interest.[18] Age, gender, current ICS use, OC use in the past year, SABA use in the past year, LABA use in the past year, LTRA use in the past year, asthma control, therapy adherence, pet exposure, passive smoking, parents' level of education and atopy were considered potential confounding factors. All analyses were performed using spss version 16 for Windows (SPSS, Chicago, IL, USA).

Results

Characteristics of children with reported use of asthma medication

From April 2009 until August 2011, 744 children were included in the PACMAN cohort study. Details on the response rate have been described elsewhere.[19] Of the included children, 137 (18.4%) were unable to perform the FeNO expiratory maneuver and 6 (0.8%) caregivers did not fill in the questionnaire. Therefore, 601 children (80.8%) were included for analyses. Additional data were made available by the general practitioners for 267 of these children (44.4%). General characteristics of the children with reported use of asthma medication are listed in Table 1. The majority of patients were boys (62.9%) and the mean age of the population was 9.0 yr. Of these children; 88.5% reported to have used ICS in the past year and 54.4% to have used ICS less than a week previous to the study visit. In addition, 56.2% could be classified as 'well-controlled' based on the ACQ score. Of the children from whom additional data were provided by the general practitioners, 76.0% had a doctor's diagnosis of asthma. FeNO was >25 ppb in 161 children (26.8%).

Table 1. General characteristics of the children with a reported use of asthma medication

	Children with a reported use of asthma medication (n = 601)
General characteristics	
Gender, male %	62.9
Age (yr), mean (SD)	9.0 (2.2)
Clinical characteristics	
Doctor-diagnosed asthma, %	76.0 (203/267)*
Well-controlled asthma†	58.0 (338/583)*
Atopy‡	80.1 (463/578)*
Current inhaled corticosteroids (ICS) users§	54.4
Medication use in past year	
Short-acting β_2-agonists, %	84.4
Long-acting β_2-agonists, %	25.0
ICS, %	88.5
Oral corticosteroids, %	6.3
Leukotriene antagonists, %	9.3
Antibiotic course, %	32.0 (191/596)*
Environmental factors	
Passive smoking	12.9 (77/597)*
Pet exposure	41.3 (247/598)*
Urban living environment	65.1 (388/596)*

* Data not available for the total population (number of children / total number of children with available data).
† Asthma Control Questionnaire score < 0.75.
‡ Parental-reported history of eczema, hay-fever or food allergy.
§ Use of ICS less than a week previous to the study visit.

Characteristics of children with reported use of asthma medication who were unable to perform the FeNO measurement

Children who were not able to perform the FeNO measurement were significantly younger than children who were able to perform the measurement (mean age 5.7, respectively 9.0 yr) ($p < 0.0001$) and were less likely to use ICS in the past year (81.0%, 87.6%, respectively) ($p=0.041$), whereas gender and SABA use did not significantly differ between both groups.

Fraction of nitric oxide in exhaled breath in children with reported use of asthma medication compared to a healthy population

A FeNO value was available for 63 healthy non-asthmatic children (age: 5–12). Compared to the children with reported use of asthma medication, the healthy population consisted of fewer males (46.0% vs. 62.9%, p=0.009) and was older (mean age: 11.1 yr vs. 9.0 yr, p<0.001). FeNO was significantly higher in the children with a reported use of asthma medication (median: 13.0 ppb, IQR: 7.0–27.0 pbb) compared with the healthy children (median: 9.0 ppb, IQR: 5.0–14.0 pbb) (p<0.0001) (Figure 1). Of the children with a reported use of asthma medication, 6.0% had a FeNO value below 5 ppb, compared with 12.7% of the healthy children.

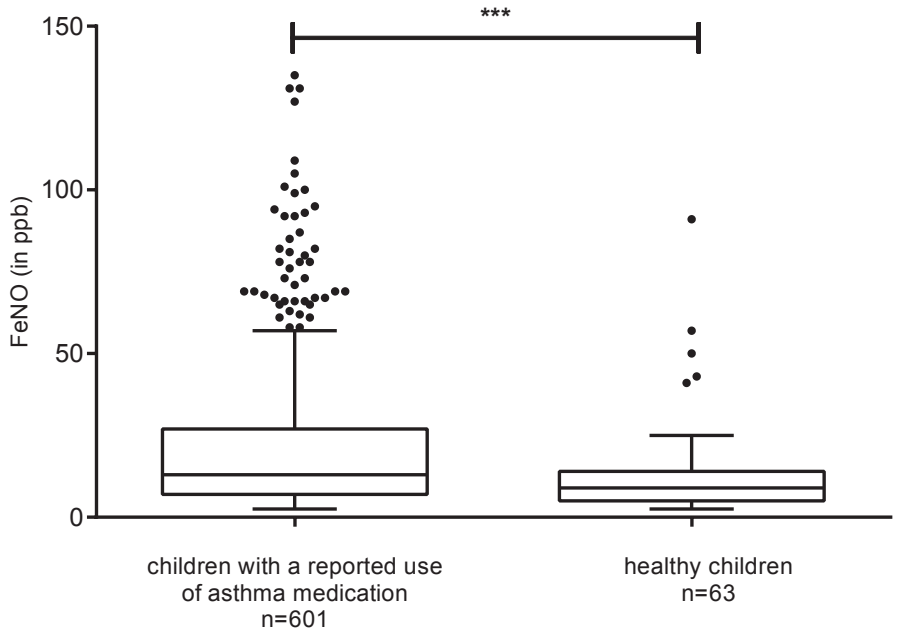

Figure 1. Fractional exhaled nitric oxide (FeNO) values in children with a reported use of asthma medication compared to healthy children (no history of asthma or asthma medication use). Box and whisker plots show median, 25th and 75th percentiles, minimum, maximum values and outliers. *** p<0.0001.

The influence of a doctor's diagnosis of asthma on FeNO in children with a reported use of asthma medication

Within the group of children with reported use of asthma medication, those with a doctor's diagnosis of asthma had a slightly higher FeNO level (median: 13.0 ppb, IQR: 8.0–27.0) compared with the children without a doctor's diagnosis of asthma (median: 9.0 ppb, IQR: 7.0–18.8), yet the difference was not statistically significant (p=0.10).

The influence of age and gender on FeNO in healthy children and children with a reported use of asthma medication

Fraction of nitric oxide in exhaled breath levels did not significantly differ between boys and girls with reported use of asthma medication (median: 13.0 ppb, IQR: 7.0–26.0 ppb vs. 12.5 ppb, IQR: 7.0–27.3, respectively, p=0.51), nor between healthy boys and girls (median: 9.0 ppb, IQR: 5.0–15.5 ppb vs. 8.0 ppb, IQR: 5.0–10.3, p=0.22). We did observe a weak correlation between FeNO and age in the group with reported asthma medication use (Rs=0.30, p<0.0001), but the correlation was borderline significant in the healthy population (Rs=0.25, p=0.05).

Fraction of nitric oxide in exhaled breath as a marker of asthma control

There was a very weak correlation between FeNO levels and total ACQ score (Rs=0.13, p=0.002) (Figure 2). In a sensitivity analysis in children with a doctor's diagnosis of asthma, the Rs value for the correlation between FeNO and ACQ score was comparable; however, it did not remain statistically significant in this smaller population (Rs=0.11, p=0.14). To assess the predictive accuracy of FeNO as a marker for not well-controlled asthma (ACQ≥0.75), a ROC curve was generated (Figure 3). FeNO showed to be a poor marker of not well-controlled asthma, the AUC being 0.56 (95%CI: 0.52-0.61, p=0.008). The highest sensitivity and specificity were observed with a cutoff value of 17.5 ppb (47.3%, 66.9%, respectively). A sensitivity analysis in children with confirmed doctor's diagnosis of asthma showed that the accuracy of FeNO as a marker of not well-controlled asthma did not improve when children without an asthma diagnosis were excluded from the analysis (AUC: 0.54; 95%CI: 0.46-0.62, p=0.33).

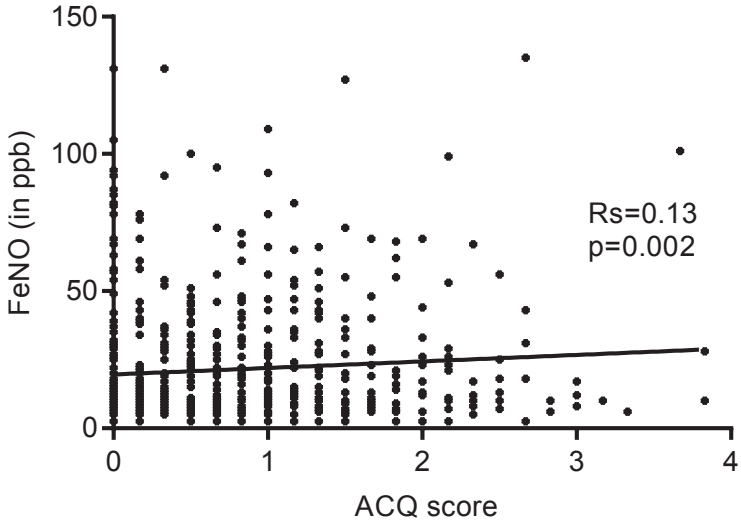

Figure 2. Correlation between fractional exhaled nitric oxide (FeNO) concentrations and Asthma Control Questionnaire score (ACQ) in children using asthma medication. FeNO levels are shown as parts per billion (ppb). Rs: Spearman's Rank correlation coefficient.

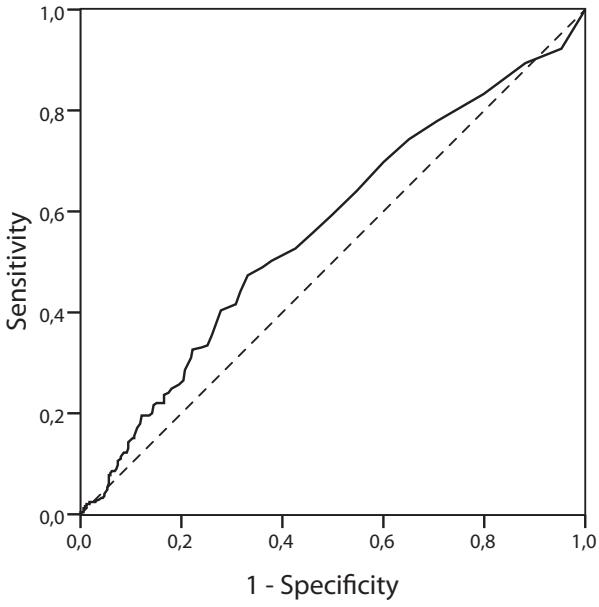

Figure 3. Receiver-operator characteristic (ROC) curve indicating the sensitivity and specificity of FeNO for predicting not well-controlled asthma (ACQ ≥ 0.75) in our population of children using asthma medication. Area under the curve (AUC):0.56, 95%CI: 0.52-0.61, p=0.008.

Factors associated with high FeNO in children with reported use of asthma medication

In the multivariate analysis, age, asthma control, therapy adherence, LTRA, and antibiotic use in the past year were significantly associated with high FeNO values (Table 2). Patients with high FeNO levels were less well controlled (OR: 0.6; 95%CI: 0.4–1.0), less adherent (OR: 0.4; 95%CI: 0.3–0.6) and used fewer courses of antibiotic (OR: 0.6; 95%CI: 0.4–0.9) in the past year. High FeNO levels were also associated with fewer LTRA use (OR: 0.4; 95%CI: 0.2–0.9) in the past year, but this association did not remain significant in the sensitivity analysis using a cutoff value of 17.5 ppb (Supplementary Table 1). In addition, a non-urban living environment was significantly associated with FeNO levels >25 ppb ($p < 0.05$), though the association between non-urban living environment and high FeNO (>35 ppb) was just above the threshold of significance in the sensitivity analysis ($p=0.05$) (Supplementary Table 2). Furthermore, we found a significant negative association between high FeNO levels (>25 ppb) and visits to a pediatrician or pediatric pulmonologist in the past year (OR: 0.6; 95%CI: 0.4–0.9); this association did not remain significant when we set the FeNO threshold to 35 ppb (Supplementary Table 2). There was no significant independent association between high FeNO levels (>25 ppb) and current use of ICS, though we did observe a significant negative association in the sensitivity analyses (Supplementary Tables 1 and 2).

Table 2. Factors associated with high FeNO values (>25 ppb)

	Univariate analysis OR (95%CI)	Multivariate analysis adjusted OR (95%CI)
General characteristics		
Male gender	1.1 (0.8-1.6)	
Age	1.3 (1.2-1.4)*	1.3 (1.2-1.4)*
Clinical characteristics		
Well-controlled asthma†	0.6 (0.4-0.9)*	0.6 (0.4-1.0)*
Atopy‡	1.1 (0.7-1.8)	
Current use of inhaled corticosteroids (ICS)§	0.7 (0.5-0.9)*	0.7 (0.4-1.1)
Good adherence¶	0.4 (0.2-0.5)*	0.4 (0.3-0.6)*
Medication use in the past year		
ICS use	0.7 (0.4-1.1)	
Oral corticosteroids use	0.8 (0.4-1.8)	
Short-acting β_2-agonists use	1.2 (0.7-1.9)	
Long-acting β_2-agonists use	0.8 (0.5-1.3)	
Leukotriene antagonists use	0.3 (0.1-0.7)*	0.4 (0.2-0.9)*
Antibiotic use	0.5 (0.3-0.8)*	0.6 (0.4-0.9)*
Health care use in the past year		
General practitioner visit	1.0 (0.7-1.5)	
Specialist physician visit**	0.5 (0.3-0.7)*	0.6 (0.4-0.9)*
Emergency Room (ER) visit	0.3 (0.1-1.1)	
Environmental factors		
Pet exposure	1.1 (0.8-1.6)	
Passive smoking	1.1 (0.7-1.9)	
Non-urban environment	1.8 (1.2-2.6)*	1.7 (1.1-2.6)*

Factors that caused a 10% change or more in the regression coefficient were included in the multivariable model.
* p <0.05. † Asthma Control Questionnaire-score < 0.75
‡ History of eczema, hay fever or food allergy
§ Use of inhaled corticosteroids less than a week previous to the study visit
¶ Medication Adherence Report Scale-score ≥ 21
** Pediatric pulmonologist or pediatrician visit

Discussion

To the best of our knowledge, this is the first study assessing the relationship between FeNO and asthma control in a large pharmacy-based cohort of children with reported use of asthma medication. We found a weak positive correlation between FeNO and not well-controlled disease. Our results are in line with previous (smaller) studies.[4, 5] Rosias et al.[4] found no correlation between FeNO and ACQ in 23 children with mild-to-moderate asthma (age: 6–16 yr). Mahut et al.[5] made similar observations in 200 asthmatic patients (children and adults) who were followed for 12 wks.

High levels of FeNO are thought to be a surrogate marker of ongoing eosinophilic inflammation. Clinical symptoms of asthma and underlying eosinophil airway inflammation seem to overlap only partially.[20] This is also supported by the poor accuracy of FeNO in our study to discriminate well-controlled children from not well-controlled children.

Nevertheless, Robroeks et al.[21] have reported that FeNO was a good marker of asthma control in 114 asthmatic children (age: 5–16 yr), especially when combined with levels of 8-iso-prostane, IFN-γ and IL-4 in exhaled breath condensate (EBC) (sensitivity = 82%; specificity = 80%). Specificity and sensitivity of FeNO solely for asthma control have not been reported previously, and the additional predictive value of the inflammatory markers in EBC remains unclear. Because asthma is a complex disease characterized by a wide variety of distinct phenotypes, measuring a combination of inflammatory markers, such as markers in EBC and FeNO, might give a more complete view of disease status than focusing on a single marker. However, the measurement of inflammatory markers in EBC is still in its experimental phase, and many methodological issues (e.g., standardization of EBC collection, the establishment of reference values for individual markers in EBC) need to be addressed before this technique can enter clinical practice.[22] More research in large populations is necessary to assess the clinical validity of such an approach.

We found that several factors were independently associated with high FeNO values, including age, poor therapy adherence, antibiotic use, and LTRA use. The (weak) positive correlation between age and FeNO has also been observed in healthy children[13] and is probably related to the developmental increase in total pulmonary surface and thereby increased production capacity of NO.

The other associations we identified are less clear, especially because the direct relationship between FeNO and airway inflammation remains to be elucidated.

Generally, the concept has evolved that high levels of FeNO are indicative of more uncontrolled eosinophilic airway inflammation and that high levels of FeNO in steroid-treated children might be caused by low medication compliance, ongoing allergen exposure and infections, imminent exacerbations, inadequate ICS dosing, or steroid resistance.[1] Our finding that children with high FeNO are more often non-compliant to therapy fits within this concept. A lack of compliance to corticosteroids may contribute to persistent eosinophilic airway inflammation and, thereby, increased FeNO levels. Children with asthmatic symptoms and high FeNO in our study used less LTRA, less AB and visited their pediatrician or pediatric pulmonologist less often than their counterparts with normal FeNO levels. This is less compatible with the hypothesis that high FeNO could be an indicator of a more severe asthma phenotype. However, it may be supporting the observation that tailoring treatment on FeNO is not effective in decreasing asthma exacerbations.[8]
An alternative view is that high FeNO might be a sign of a steroid-responsive asthma phenotype and that inter-individual differences in FeNO levels might represent distinct underlying inflammatory patterns. A recent study by Wang et al.[23] identified four different inflammatory patterns in stable asthmatic children based on sputum analyses: paucigranulocytic (49.0%), eosinophilic (28.6%), neutrophilic (20.4%), and mixed granulocytic (2.0%). Considering that eosinophils and neutrophils differ with regard to corticosteroids sensitivity,[24] patients with eosinophilic asthma might respond better to corticosteroids than patients with non-eosinophilic asthma. FeNO correlates positively with the presence of eosinophils in sputum[25] and may therefore reflect a more corticosteroid-responsive phenotype. This idea is supported by various prospective clinical studies that reported a better therapy response in asthmatic patients with elevated baseline FeNO levels.[26]
It is tempting to speculate that fewer LTRA and antibiotic use and fewer visits to a pediatrician or pediatric pulmonologist that were associated with high FeNO levels in our cohort might indirectly be a sign of a more steroid-responsive (eosinophilic) asthma phenotype. Clinical practice shows that an antibiotic course is regularly prescribed in the case of asthma exacerbations,[27] and therefore, fewer courses of antibiotics might indirectly reflect fewer asthma exacerbations. Furthermore, children with high FeNO were less likely to use LTRA. The pediatric asthma guidelines of the Dutch Pediatrician Society advice to prescribe LTRA when treatment with corticosteroids and SABA is not successful to obtain asthma control.[28] This also suggests that high FeNO might be indirectly associated with a better response to steroid treatment. LTRA have also been approved for the treatment of allergic rhinitis,

but the prevalence of parental-reported rhinitis did not significantly differ between the children with or without reported use of LTRA (51.8% vs. 43.9%, respectively, p=0.18). Lastly, there was a negative trend between FeNO and visits to pediatrician or pediatric pulmonologist in the past year, which also fits in the concept of high FeNO as a marker of a steroid-responsive inflammation. However, we could not assess therapy response in this cross-sectional study, and a prospective design is needed to further investigate the relationship between levels of exhaled NO, underlying inflammatory patterns and response to corticosteroids in children.

Another remarkable finding of our study was the positive trend between FeNO and a non-urban living environment. Epidemiological studies showed that children living on farms have a lower risk in developing asthma, atopic dermatitis, or allergic sensitization.[29] Because atopy has reported to be closely related to FeNO, one would expect lower FeNO values in children living in non-urban environments. However, in our study, we did not find an association between FeNO and parental-reported atopic disease. To the best of our knowledge, few studies addressed the influence of urbanization on FeNO. A study by Malmberg et al.[30] found a trend between growing up in the countryside and lower FeNO levels in Finnish and Russian populations, but the countryside in those countries might be incomparable to the countryside in the densely populated Netherlands. It is unlikely that ambient NO has contaminated our results because it was removed during the FeNO measurement through the use of a NO filter.[31] The socio-economic status of a child is often determined by assessing the parent's educational level and the household income. We did not have information on the latter parameter, but we did assess whether the parent's educational level confounded the association between living environment and high levels of FeNO, which was not the case. More research is needed to assess the influence of living environment on exhaled NO.

Potential limitations of our study include the relatively low response rate of the general practitioners: we are unaware of a possible doctor's diagnosis of asthma for 56% of our participants. Therefore, the percentage of asthma diagnosis might not be representative for our total study population. In addition, the ACQ used in this study has been validated and should therefore officially be assessed, in asthmatic children. Not all the children in our study with reported use of asthma medication have an official diagnosis of asthma. However, sensitivity analyses showed that the correlation between FeNO and ACQ, nor the accuracy of FeNO as a marker for not well-controlled asthma, improved when we excluded children without a confirmed doctor's diagnosis. Another limitation of our study is that we cannot

exclude that confounding by disease severity might have influenced our results. We did correct for OCS use in the past year, but lacked information on the number of hospital admissions or severity of previous exacerbations. Children with more severe disease might have been more compliant to therapy and have used health care more frequently, which may have confounded the identified associations.

Furthermore, symptoms and adherence were based on reporting by caregivers, which could also be a possible flaw. Parents and caregivers might over- or underestimate the health status and/or compliance of their child. But this resembles daily clinical practice, where asthma management in young children is mainly based on parental reporting. Atopy was also based on parental reporting in the questionnaire, and measurements of specific immunoglobulin E levels might have been more accurate to establish the atopic status of the child. Furthermore, other comorbidities such as chronic rhinosinusitis can also increase FeNO and might have confounded the association between FeNO and asthma control. In this study, we found that 6% of the children with a reported use of asthma medication and 12.7% of the healthy children had a FeNO below 5 ppb. Very low FeNO levels have also been observed in primary ciliary dyskinesia or cystic fibrosis patients, however; these diseases have a low prevalence and are unlikely to account for all the cases of low FeNO we found.

A major strength of our study was the availability of information on asthma symptoms and FeNO measurements in a large population of pediatric patients included in the primary care setting. This has the advantage that the PACMAN cohort focuses on a very broad range of asthmatics and not only on children in a specialized care group, as most asthmatic children are also treated within the primary care setting.

In summary, this study does not support a role for FeNO to assess current asthma control in every day practice. FeNO appears a poor marker of asthma control in children with reported use of asthma medication. Symptoms may overlap only partly with underlying airway inflammation, and our study shows that various other clinical health care and environmental factors influence FeNO.

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Supplementary Table 1. Factors associated a high fraction of nitric oxide in exhaled breath (>17.5 ppb)

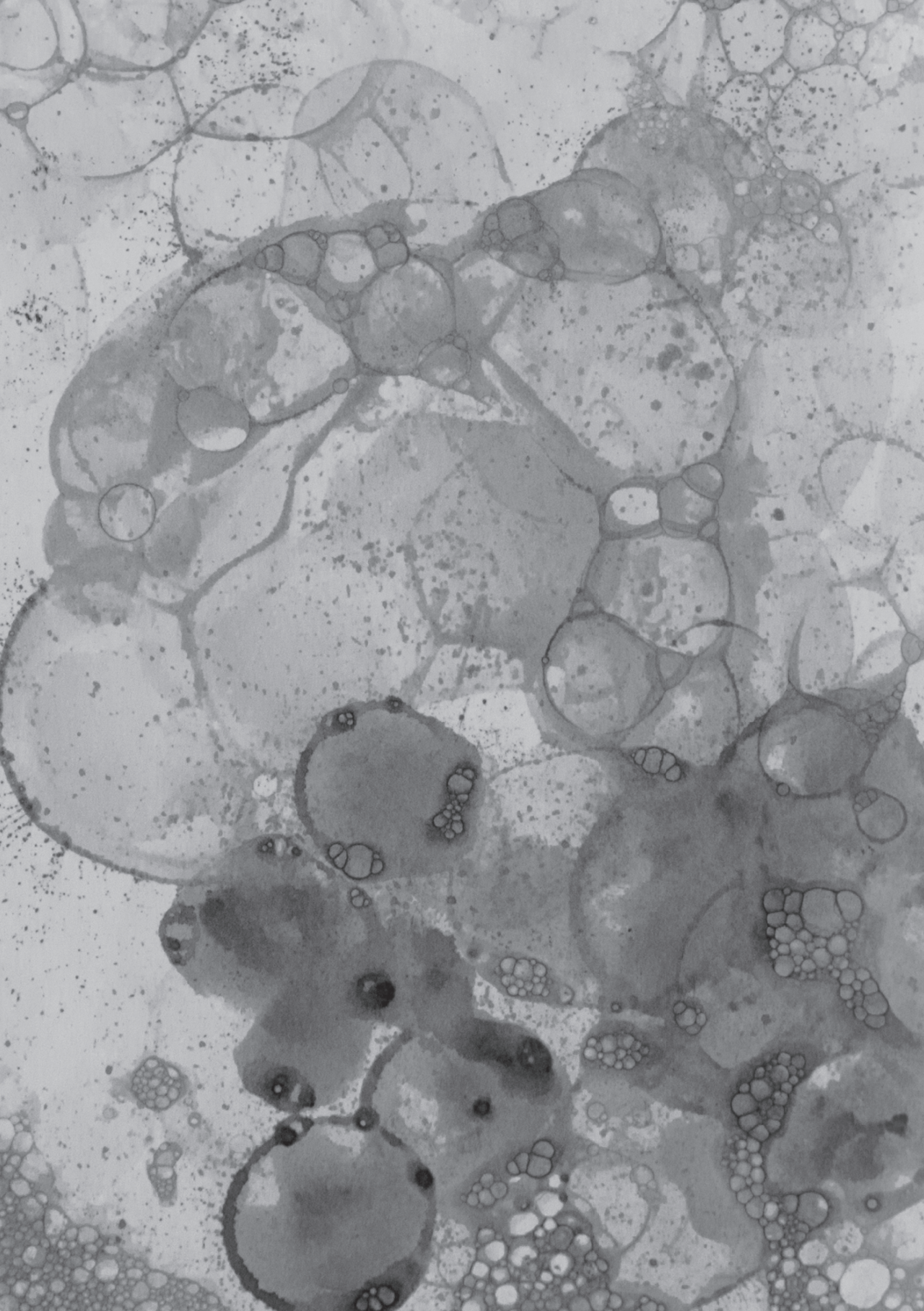
	Univariate analysis OR (95%CI)	Multivariate analysis Adjusted OR (95%CI)
General characteristics		
Male gender	1.1 (0.8-1.5)	
Age	1.3 (1.2-1.4)*	1.3 (1.2-1.4)*
Clinical characteristics		
Asthma control†	0.6 (0.4-0.8)*	0.6 (0.4-0.8)*
Atopy‡	1.1 (0.7-1.7)	
Current use of inhaled corticosteroids (ICS)§	0.6 (0.5-0.9)*	0.7 (0.4-1.0)*
Good adherence¶	0.4 (0.3-0.6)*	0.4 (0.3-0.6)*
Medication use in the past year		
ICS use	0.8 (0.5-1.4)	
Oral corticosteroids use	1.3 (0.7-2.5)	
Short-acting β_2-agonists use	1.4 (0.9-2.2)	
Long-acting β_2-agonists use	0.9 (0.6-1.3)	
Leukotriene antagonists use	0.5 (0.3-0.9)*	0.5 (0.3-1.1)
Antibiotic use	0.6 (0.4-0.8)*	0.6 (0.4-1.0)*
Health care use in the past year		
General practitioner visit	0.9 (0.7-1.3)	
Specialist physician visit**	0.5 (0.4-0.8)*	0.6 (0.4-0.9)*
ER visit	0.4 (0.2-1.0)*	0.4 (0.1-1.0)*
Environmental factors		
Pet exposure	1.0 (0.7-1.4)	
Passive smoking	1.4 (0.8-2.2)	
Non-urban environment	1.8 (1.3-2.5)*	1.8 (1.2-2.7)*

Factors that caused a 10% change or more in the regression coefficient were included in the multivariable model.
* p <0.05
† Asthma Control Questionnaire-score < 0.75.
‡ History of eczema, hay fever or food allergy.
§ Use of inhaled corticosteroids less than a week previous to the study visit.
¶ Medication Adherence Report Scale-score ≥ 21.
** Pediatric pulmonologist or pediatrician visit.

Supplementary Table 2. Factors associated with a high fraction of nitric oxide in exhaled breath (> 35 ppb)

	Univariate analysis OR (95%CI)	Multivariate analysis Adjusted OR (95%CI)
General characteristics		
Male gender	1.0 (0.7-1.5)	
Age	1.3 (1.2-1.4)*	1.3 (1.2-1.4)*
Clinical characteristics		
Asthma control†	0.6 (0.4-0.9)*	0.5 (0.4-0.8)*
Atopy‡	1.6 (0.9-2.9)	
Current use of inhaled corticosteroids (ICS)[c]	0.5 (0.4-0.8)*	0.6 (0.4-1.0)*
Good adherence[d]	0.4 (0.2-0.6)*	0.4 (0.2-0.6)*
Medication use in the past year		
ICS use	0.7 (0.4-1.3)	
Oral corticosteroids use	1.5 (0.7-3.2)	
Short-acting β_2-agonists use	1.4 (0.8-2.7)	
Long-acting β_2-agonists use	1.2 (0.7-1.9)	
Leukotriene antagonists use	0.2 (0.0-0.6)*	0.2 (0.0-0.8)*
Antibiotic use	0.5 (0.3-0.8)*	0.5 (0.3-0.9)*
Health care use in the past year		
General practitioner visit	1.0 (0.6-1.5)	
Specialist physician visit[e]	0.5 (0.3-0.8)*	0.6 (0.3-1.1)
ER visit	0.5 (0.2-1.7)	
Environmental factors		
Pet exposure	1.0 (0.7-1.5)	
Passive smoking	1.6 (0.9-2.9)	
Non-urban environment	1.6 (1.0-2.4)*	1.6 (1.0-2.5)

Factors that caused a 10% change or more in the regression coefficient were included in the multivariable model.
* $p < 0.05$
† Asthma Control Questionnaire-score < 0.75.
‡ History of eczema, hay fever or food allergy.
§ Use of inhaled corticosteroids less than a week previous to the study visit.
¶ Medication Adherence Report Scale-score ≥ 21.
** Pediatric pulmonologist or pediatrician visit.



4.2

eNose breathprints as a tool to assess asthma control in children treated with inhaled corticosteroids. A preliminary study

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In preparation

Abstract

Introduction: Measuring patterns of VOCs in exhaled breath ('breathprint') is a novel metabolomic approach to study molecular signatures of respiratory disease. These patterns are likely to be influenced by the presence of airway inflammation and can be non-invasively measured by gas multi-sensor arrays ('eNose').

Aim: to study whether controlled and uncontrolled children treated with inhaled corticosteroids (ICS) can be identified according to their breathprint.

Methods: VOCs were measured in thirty-three asthmatic children (age: 11.8±2.3 years) who participated in the PACMAN2 study, a follow-up of the PACMAN study. All children were current users of ICS. Current asthma control was assessed using the Asthma Control Questionnaire. Long-term asthma control was based on reported symptoms in the four seasons preceding the baseline visit (PACMAN) and follow-up visit (PACMAN2). A single vital capacity volume of exhaled air was collected upon 5 minutes of normal breathing through a three-way non-re-breathing valve with a VOC filter in a Tedlar bag. The VOCs in the breath sample were subsequently captured on Tenax GR Tubes and analyzed offline on a validated panel of four different eNoses. Breathprints were analysed per eNose using principal component analyses (PCA). ROC curves and cross-validated accuracy values were used to assess the accuracy of the devices to discriminate between controlled and uncontrolled patients. Furthermore, unbiased cluster analyses using Wards methods and similarity profile analysis was applied to study clusters of breath profiles based on the sensor signals. Clinical features of the distinct clusters were assessed.

Results: Two eNoses in the panel were able to discriminate current uncontrolled asthmatic children and controlled asthmatic children according to their breathprint (accuracy: 69.7-75.8%). Three eNoses were able to identify long-term uncontrolled asthmatic children and long-term controlled asthmatic children (accuracy: 66.9-87.5%). For both outcomes the metal oxide semiconductor sensor eNose was most accurate in separating both groups. Using unbiased cluster analysis four different phenotypic clusters of breathprints were identified.

Conclusion: Breathprint analyses can accurately identify current uncontrolled, as well as, long-term uncontrolled asthmatic children. Moreover, breathprint analyses may categorize distinct childhood asthma phenotypes. These results suggest that breathprint analyses might be a non-invasive tool to monitor asthma control and identify clinically distinct childhood asthma phenotypes.

Introduction

Asthma diagnosis and management is generally based on reported asthma symptoms often combined with spirometry and the assessment of airway responsiveness to aspecific stimuli such as histamine or methacholine.[1] Asthma is regarded as a condition that encompasses a collection of heterogeneous disease subtypes with different underlying pathophysiological mechanisms.[2, 3] There is a need for objective asthma biomarkers to identify clinical relevant asthma phenotypes, to optimise diagnosis and to guide treatment.

Measuring patterns of VOCs in exhaled breath is a novel metabolomic approach to study molecular signatures of respiratory disease.[4, 5] Exhaled breath contains a complex mixture of thousands of VOCs. These compounds are produced during metabolic processes and the concentrations are likely to be influenced by the presence of airway inflammation. A gas multi-sensor array ('electronic nose') can assess the spectrum of volatiles present in exhaled breath without determining the individual molecular components.[6, 7] Previous studies have shown that measurements of patterns of VOCs ('breathprints') using an electronic nose could discriminate adults with asthma from non-asthmatic controls,[7] asthmatic patients from COPD patients[5, 8] and steroid responsive asthmatics from steroid unresponsive asthmatics.[9] Furthermore, a recent study showed that breathprints of COPD patients with mild disease correlated well with with the presence of eosinophil and neutrophils in induced sputum, as well as with the levels of eosinophil cationic protein (ECP) and myeloperoxidase (MPO) in induced sputum. This suggests that the electronic nose might be capable of identifying distinct types of underlying airway inflammation in a non-invasive fashion.[8]

Uncontrolled asthma may reflect ongoing eosinophilic airway inflammation. Nevertheless, the fraction of exhaled NO (FeNO), which is considered to be a surrogate marker of eosinophilic inflammation, correlates poorly with asthma control.[10] In this study we aimed to investigate whether controlled and uncontrolled children treated with ICS could be identified accurately according to their breathprint, and whether breathprints were a more accurate marker of asthma control compared to a single FeNO measurement. Secondly, we aimed to assess whether unbiased cluster analysis of breathprints of children treated with ICS could identify childhood asthma phenotypes with distinct clinical or inflammatory features.

Methods

Subjects and study design

This study had a cross-sectional design. Asthmatic children taking regular ICS treatment (age: 8-16 years) participated in the PACMAN2 study, a follow-up study of the PACMAN cohort study. The rationale and overall design of the PACMAN2 study, as well as of the PACMAN cohort study have been published previously.[11, 12] Briefly, children were included in the pharmacy-based PACMAN cohort study based on the regular use of asthma medication. During a baseline study visit in community pharmacies (PACMAN cohort study) parents and children completed an extensive asthma questionnaire. For the PACMAN2 study children were selected from the PACMAN cohort based on (current) age (≥ 8 years), long-term asthma control (well-controlled or not well controlled) and adherence to inhaled corticosteroids (ICS) at the time of the baseline visit. Previous to the follow-up study visit (PACMAN2) it was checked whether the child was still using regular ICS. PACMAN2 consisted of a single follow-up study visit in the hospital. During this study visit, parents and children completed an asthma questionnaire, a VOC sample was obtained, spirometry was performed, FeNO was measured and venous blood was collected. The Medical Ethics Committee of the University Medical Centre Utrecht has approved the PACMAN2 study.

Asthma control

During the PACMAN2 study visit parents and child filled in a questionnaire on current and long-term asthma control and severe exacerbations in the past year. Current uncontrolled asthma was assessed with the 6-item version of the Asthma Control Questionnaire (ACQ) (symptoms plus rescue medication use).[13] An ACQ-score ≥ 0.75 was considered 'uncontrolled asthma'.

Long-term uncontrolled asthma was based on reported asthma symptoms of the preceding year. During the baseline study visit (PACMAN) as well as during the follow up study (PACMAN2) the parents and child completed questions on 1) day-time symptoms (wheeze, cough, shortness of breath), 2) night-time symptoms, 3) limitations in daily activities, and 4) the use of rescue medication in the preceding four seasons.

Long-term uncontrolled asthma was defined as: ≥ 3 seasons of uncontrolled asthma in the year preceding the baseline visit, as well as in the year preceding the follow up visit. A season was considered to be uncontrolled when ≥ 3 asthma

symptoms (day-time symptoms, day-time limitations, night-time limitations or the use of co-medication) occurred on a daily or weekly basis. Long-term controlled asthma was defined as: ≥ 3 seasons of controlled asthma in the year preceding the baseline visit, as well as in the year preceding the follow up visit. A season was considered to be 'controlled' when asthma symptoms did not occur or occurred less than weekly (adapted from Koster et al.[14]). In addition, children could only be classified as 'long-term controlled' if they had not used oral corticosteroids (OCS) or visited the ER for asthma-symptoms in the past year.

Breathprints

VOCs were measured in thirty-three PACMAN2 children according to a validated method.[5] In short, children were asked to breathe normally for 5 minutes through a three-way non-re-breathing valve with a VOC filter (A2, North Safety, Middelburg, the Netherlands) at the inspiration port, while wearing a nose-clip. Subsequently, upon a maximal deep inspiration, the child was asked to exhale a single vital capacity volume into a Tedlar bag connected to the expiration port and silica reservoir to dry the exhaled air. The VOCs present in at least 500 ml exhaled air in the Tedlar bag were captured on Tenax GR Tubes (Interscience, Breda, the Netherlands) with the use of a peristaltic pump. The VOCs captured on the Tenax GR Tubes were analyzed on a validated panel of four different electronic noses (Lonestar, Owlstone, Cambridge, United Kingdom; Cyranose 320; Sensigent, Baldwin Park, California, USA; Comon Invent eNose, Comon Invent, Delft, the Netherlands; TEN, University of Rome Tor Vergata, Rome, Italy) at the Department of Respiratory Medicine of the Academic Medical Centre in Amsterdam, the Netherlands. The four eNose brands are based on different measurement technologies; field asymmetric ion mobility spectrometry (Lonestar), carbon-polymer sensors (Cyranose), metal oxide semiconductor sensors (Comon Invent eNose) and quartz microbalance metalloporphyrins sensors (TEN).

Lung function and FeNO

Lung function and FeNO were measured by trained lung function technicians. The fraction of exhaled nitric oxide (FeNO) was measured with a hand-held electrochemical analyzer (NIOX Mino, Aerocrine, Solna, Sweden) with an expiration time of 6 seconds. Lung function measurements included: forced expiratory volume in one second (FEV_1), maximum mid-expiratory flow (MEF25-75%), and the percentage change in FEV_1 predicted upon inhalation of 800µg salbutamol.

Blood eosinophil counts

Venous blood was collected in sterile collection tubes containing sodium heparin as anticoagulant. Granulocyte counts in whole blood were measured by a Cell-Dyn 1800 cell counter (Abbott Diagnostics). Samples were stained with Alexa647-labelled antibodies directed against CD16 (clone 3G8) and analysed in a Gallios Flow Cytometer (Beckman Coulter). Eosinophils and neutrophils were identified according to their specific side scatter and forward scatter characteristics and CD16 fluorescence. Using the absolute granulocyte counts and the percentage of eosinophils, the concentration of circulating blood eosinophils was calculated (in 10^9 cells per L).

Statistical analyses

Statistical analysis was carried out using standardized approaches and applying internal validation procedures that minimize false discovery rate.[15] The raw eNose data (change in resistance) was normalised by expressing all values as percentage of the highest obtained resistance change per sensor. In case of non-normal distribution, the data was transformed through Box-Cox transformation. After preparation, data per electronic nose was restructured by principal component analysis (PCA) to a set of principal components (PCs) that capture the highest variance within the dataset. Student T-test was used to assess whether PCs were statistically different between controlled and uncontrolled asthmatic patients. ROC analyses were performed to assess the accuracy of eNose principal components to discriminate between controlled and uncontrolled asthma patients. Stepwise discriminant analysis and cross-validation was performed on all significant ($p<0.05$) associated PCs resulted from t-test. Furthermore, it was assessed whether combining the significant PCs of multiple eNoses resulted in increased accuracy of the technique to identify uncontrolled asthmatic patients. Additionally, clustering of the breathprints was performed using Wards method and similarity profile analysis[16] to assess whether different clusters of breathprints could be identified based on the raw eNose data solely. Subsequently, we assessed how clinical features related to the different clusters. Differences in patient characteristics were analysed using students t-test, chi-square test and independent samples-median test. Receiver operating characteristics (ROC) curves and corresponding Area Under the Curve (AUC) were used to assess the accuracy of FeNO to discriminate between controlled and uncontrolled asthma patients. All eNose data was analyzed in R version 3.01 [15] and the packages 'clustsig', 'Hmisc' and 'pgirmess'. Clinical data was analyzed using

IBM SPSS 19.0 for Windows (SPSS, Inc., Chicago, Ill, USA). The sample size of this preliminary study was based on a previous study in adult asthmatics that showed that breathprints of 7 to 11 subjects per group could distinguish steroid responsive subjects from steroid-unresponsive subjects with the use of Cyranose 320.[9] We estimated that similar group sizes would be sufficient to distinguish controlled from uncontrolled asthmatic children treated with ICS.

Results

Study participants

A VOC breath sample was obtained in 33 out of the 37 children included in the PACMAN2 study. Fifteen children were currently uncontrolled (ACQ score <0.75) and 18 were currently controlled (Table 1). The children with current uncontrolled symptoms were slightly younger compared to the children with current controlled symptoms (mean age 11.0 vs 12.6, p=0.04). Furthermore, when long-term asthma control was assessed; nine children were classified as long-term uncontrolled and seven were classified as long-term controlled. Six of the currently uncontrolled children were classified as long-term uncontrolled.

Measures of lung function or FeNO did not significantly differ between the groups. In this study, FeNO was a poor marker to distinguish current uncontrolled asthma from current controlled asthma (AUC: 0.56, 95%CI: 0.35-0.77), as well as long-term uncontrolled asthma from long-term controlled asthma (AUC: 0.61, 95%CI: 0.30-0.92).

Accuracy of the distinct eNoses to distinguish uncontrolled asthma from controlled asthma

Two eNoses in the panel were able to significantly discriminate current well controlled asthmatic children and current uncontrolled asthmatic children according to their breathprint (Table 2). The cross-validated accuracy of the model based on the output of the Cyranose 320 was 69.7%, whereas the accuracy of the Comon Invent was 75.8% to discriminate between current well and current not-well controlled asthmatic children. The combination of the significant PCs of the Cyranose 320 and the Comon Invent did not lead to additional accuracy of the model (accuracy; 75.8%).

Three eNoses were able to distinguish between long-term controlled and long-term uncontrolled patients (Table 3). The Cyranose 320 showed an accuracy of 81.2% to group the patients, and the Comon Invent had an accuracy of 87.5%. Additionally, the Owlstone Lonestar could discriminate the breathprints of long-term well controlled from the breathprints of long-term uncontrolled patients with an accuracy of 66.9%. The Comon Invent was the only eNose with two PCs significantly associated with long-term asthma control. Figure 1 shows the position of each child according to PC1 and PC2 of the PCA of the sensors of the Comon Invent. Combination of the significant PCs of the three eNoses led to an increased accuracy of the model (accuracy; 87.5%). Figure 2 depicts a spider chart of the average breathprint values from long-term controlled and long-term uncontrolled subjects based on the combined Principle Components of the combined eNoses.

Table 1. Patient characteristics

	Current uncontrolled ACQ < 0.75	Current controlled ACQ ≥ 0.75	p-value
n	15	18	
Age, mean±SD (years)	11.0±2.2	12.6±2.1	0.04
Gender, boys (%)	46.7	50.0	0.85
FEV_1 (% pred), mean±SD	89.5±11.3	86.9±11.2	0.52
Change in FEV_1 upon SABA, mean±SD	3.9±5.4	5.5±4.4	0.37
FeNO, median (in ppb) [IQR]	26 [11-40]	17 [13-81]	0.84
Severe exacerbations in the past year‡	4/15	0/17	0.02
	Long-term uncontrolled	**Long-term controlled**	**p-value**
n	9	7	
Age, mean±SD (years)	11.1±2.2	12.6±2.0	0.17
Gender, boys %	55.6	57.1	0.95
FEV_1 (% pred), mean±SD	88.9±9.8	87.3±13.9	0.78
Change in FEV_1 upon SABA (% pred), mean±SD	4.9±6.4	2.6±2.3	0.37
FeNO, median (in ppb) [IQR] baseline visit	8 [7-42]	10 [5-16]	0.62
FeNO, median (in ppb) [IQR] follow-up visit	42 [14-101]	28 [14-55]	1.00
Severe exacerbations in the past year‡	4/8	0/7	0.03
Current uncontrolled (ACQ<0.75)	6/9	0/7	0.006
Time between baseline and follow-up, mean±SD (months)	26.7±11.3	27.7±8.2	0.84

‡OCS use or asthma-related ER visits.
ACQ, Asthma Control Questionnaire; FeNO, Fraction exhaled Nitric Oxide; FEV_1, Forced expiratory volume in 1 second; OCS, Oral corticosteroids; SABA, Short-acting β_2-agonist.

Table 2. Accuracy of the distinct eNoses to separate the breathprints of controlled and uncontrolled asthmatic children

	Current uncontrolled asthma vs current well controlled asthma			
eNose	**Sign PC**	**p-value PC**	**Accuracy**	**AUC [95%CI]**
Owlstone Lonestar	-	NS	-	-
Cyranose 320	2	0.040	69.7	0.74 [0.56-0.92]
Comon Invent	2	0.044	75.8	0.71 [0.52-0.90]
Tor Vergata TEN	-	NS	-	-
	Long-term uncontrolled asthma vs long-term controlled asthma			
eNose	**Sign PC**	**p-value PC**	**Accuracy**	**AUC [95%CI]**
Owlstone Lonestar	4	0.037	66.8	0.83 [0.61-1.00]
Cyranose 320	1	0.038	81.2	0.81 [0.59-1.00]
Comon Invent	1,2	0.010, 0.030	87.5	0.97 [0.89-1.00]
Tor Vergata TEN	-	NS	-	-

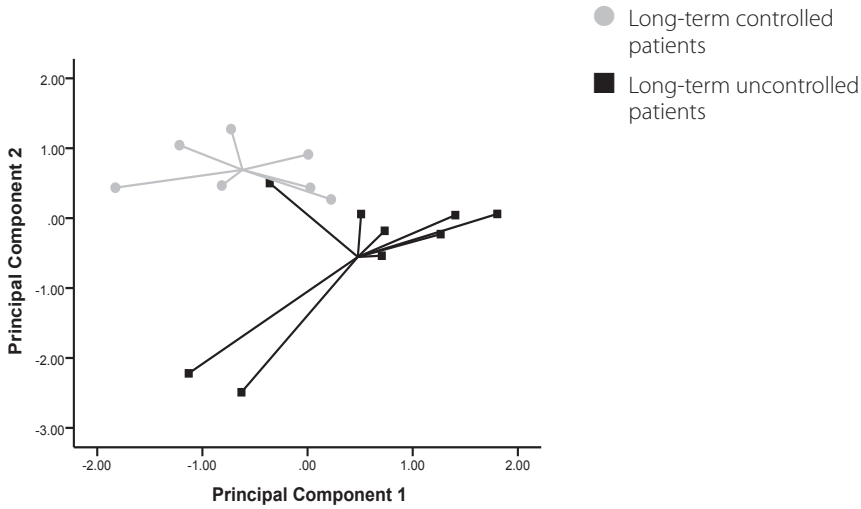

Figure 1. Scatter plot depicting the separation of long-term controlled and long-term uncontrolled children according to breathprint analysis by the Comon Invent

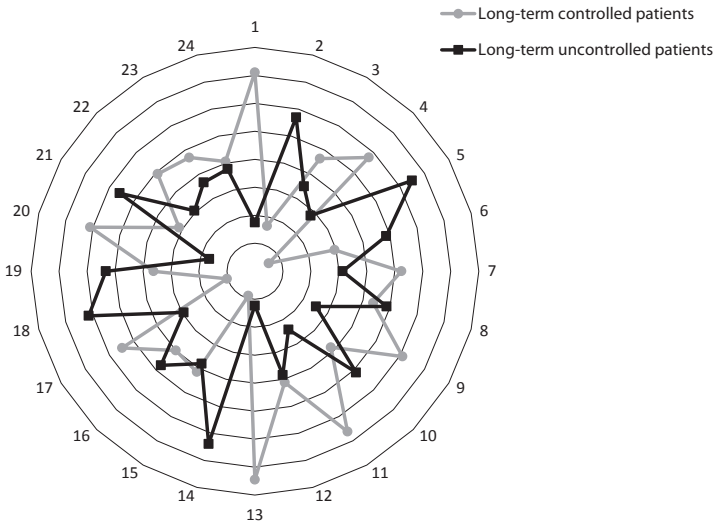

Figure 2. Spider chart of average breathprint values of long-term controlled and long-term uncontrolled subjects based on the combined Principle Components of the eNoses

Unbiased clustering of breathprints

In addition, an unbiased clustering analysis based on eNose data was performed using Wards methods and similarity profile analysis.[16] Using this approach four significantly different clusters of breathprints were identified (p=0.01) (Figure 3). Subsequently, we tried to relate clinical features to the different groups (Table 3). Although the study was too small to identify significant clinical differences between the clusters, interesting trends could be observed. Cluster 1 was characterized by the highest BMI scores. In cluster 2, three of the four children were non-adherent to maintenance treatment. This cluster was further characterized by the most favorable lung function measures and a high percentage of girls. Of the four children in our study with severe exacerbations, three clustered in cluster 3. Finally, cluster 4 was marked by the lowest baseline FEV_1 (% predicted) and highest levels of bronchoconstriction, but lowest mean ACQ score. When inflammatory markers were assessed, clusters 3 and 4 expressed higher blood eosinophil counts than cluster 1 and 2. This separation was not reflected in FeNO values.

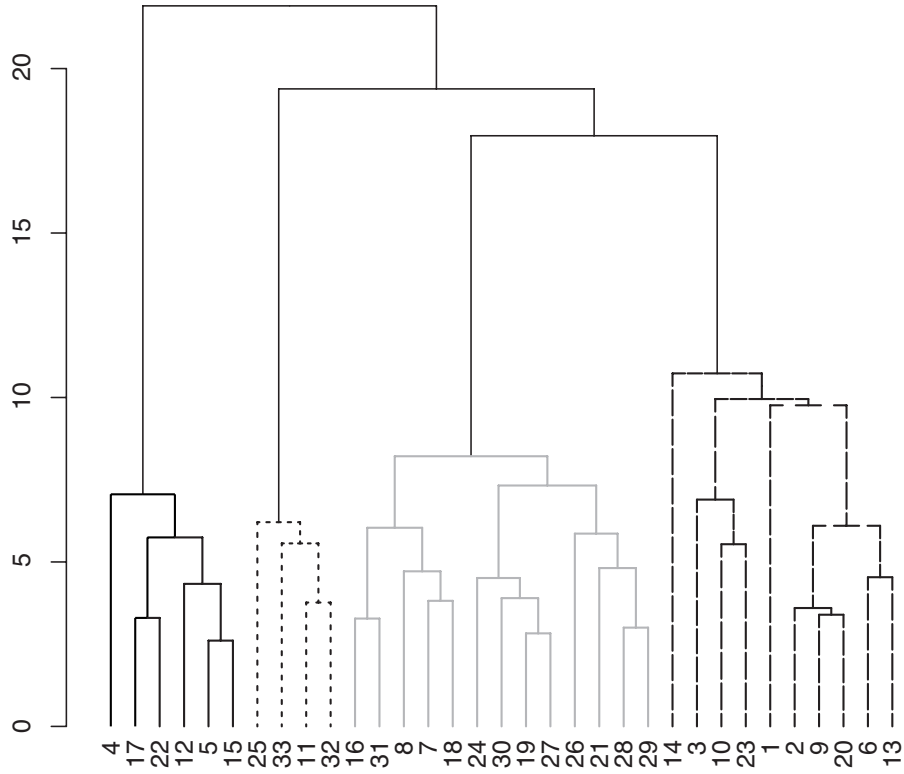

Figure 3. Unbiased clustering of breathprints using Wards methods and similarity profile analysis

Table 3. Clinical features of distinct breathprint clusters

	Cluster 1 N=6	Cluster 2 N=4	Cluster 3 N=13	Cluster 4 N=10	p-value
Patient characteristics					
Age, mean±SD (years)	13.0±2.4	11.4±2.4	11.4±2.5	11.0±1.9	0.57
Gender, boys/girls	3/3	1/3	9/4	3/7	0.21
BMI	20.5±3.6	18.5±1.1	18.4±3.9	18.7±1.3	0.55
Non adherent treatment, proportion	1/5	3/4	2/10	1/8	0.11
Spirometry					
FEV_1 (% pred), mean±SD	88±9.4	94±8.0	89.7±12.9	83.8±10.5	0.42
MEF 25-75% (% pred), mean±SD	74.5±19.4	93.8±21.3	81.1±33.7	79.3±30.3	0.78
Change in FEV_1 upon SABA (% pred), mean±SD	5.3±3.3	1.8±3.3	4.3±5.8	6.4±4.8	0.43
Symptoms and exacerbations					
Severe exacerbations in the past year, proportion	1/6	0/4	3/12	0/10	0.28
ACQ score, mean±SD	1.02±0.28	0.88±0.66	1.12±1.23	0.65±0.62	0.66
Uncontrolled asthma in the year preceding PACMAN2, proportion	2/6	2/4	6/12	1/9	0.28
Uncontrolled asthma at baseline (PACMAN) and follow-up (PACMAN2), proportion	1/6	2/4	6/12	0/9	0.10
Inflammatory markers					
Eosinophil counts (10^9/L), mean±SD	0.44±0.16	0.41±0.45	0.62±0.48	0.68±0.53	0.69
FeNO, median [IQR]	25 [11-40]	11 [#]	30 [14-83]	21 [11-41]	0.79

N/A: three valid measurements: 9 ppb, 11 ppb and 101 ppb. Proportions reflect number children / number children with available data.

FEV_1, Forced expiratory forced expiratory volume in one second; IQR, Interquartile range; MEF25-75%, Maximum mid-expiratory flow; SD, Standard deviation.

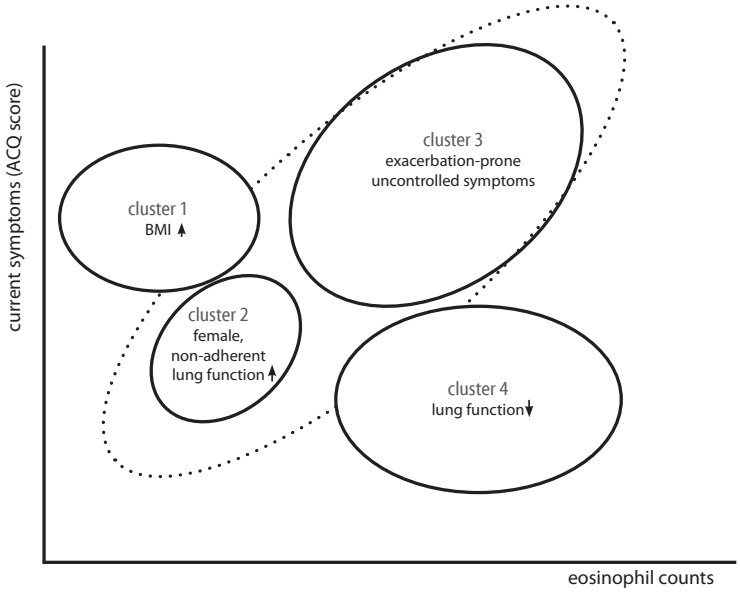

Figure 4. Proposed model of distinct childhood asthma phenotypes recognized by breathprints

Discussion

The present study showed that three out of the four eNoses included in the eNose panel could distinguish the breathprints of long-term uncontrolled asthmatic children from the breathprints of long-term controlled asthmatic children with moderate to high accuracy. Furthermore, two eNoses in our study could distinguish current uncontrolled childhood asthma from current controlled childhood asthma with moderate accuracy. For both outcomes one eNose, the metal oxide semiconductor sensors eNose (Comon Invent), gave the most accurate results. These preliminary results show that analyses of breathprints can objectively measure asthma control in children treated with ICS.

Compared to FeNO, breathprints were more accurate in identifying current asthma control. High FeNO levels are regarded to be a measure of ongoing eosinophilic airway inflammation.[17] FeNO correlates positively with the presence of eosinophils in bronchial specimens in children with difficult asthma.[18] Nevertheless, studies

that have investigated the association between asthma control and FeNO provided inconsistent results.[19,20] We have previously shown that a single FeNO measurement is a poor marker of asthma control in children using asthma medication participating in the PACMAN study.[10] Measuring a combination of markers might give a more complete view of disease status than focusing on a single marker. This is in line with work of van der Schee et al. [9] who showed that breathprints were more accurate in predicting steroid response in steroid-free asthmatic adults, compared to the percentage of sputum eosinophils or FeNO.

Our results also accord with earlier observations of Ibrahim et al. in asthmatic adults. With the use of gas chromatography-mass spectrometry, Ibrahim et al. identified a set of 15 VOCs which could accurately assess asthma control.[21] Uncontrolled asthma symptoms are thought to be influenced by inflammatory activity in the airways. Nevertheless, physiological or psychological factors might also influence asthma control.[22] Breathprints have been shown to correlate with sputum eosinophils and neutrophils in asthmatic patients[21] as well as COPD patients.[8] The study of Ibrahim et al., nonetheless, showed that the discriminating VOCs for asthma control were different compared to the discriminating VOCs for sputum profiles.[21]

There is a need of objective parameters to identify clinical relevant asthma phenotypes and guide asthma management. Unbiased clustering of the breathprints of the PACMAN2 children identified four different groups of breathprints. The children in our study were current users of inhaled corticosteroids, excluding a dominant influence of ICS use on breathprint clustering. Although the four identified clusters of breathprints were significantly different, the power of the study was likely too low to identify significant clinical differences between the four clusters. Nonetheless, interesting findings were observed, and it is tempting to speculate that the breathprints identified distinct childhood asthma phenotypes. When the clusters are compared based on eosinophil counts and current symptoms (ACQ symptom score) (Figure 4), the identified clusters show striking similarities with the clusters identified by Haldar et al. in adult asthmatics.[3] Haldar and colleagues identified four clinical asthma clusters in primary and secondary care asthma populations: two concordant phenotypes in which markers of eosinophilic inflammation (sputum eosinophilia and FeNO) corresponded with asthma symptom expression and two discordant phenotypes in which symptoms or inflammation were dominant. Using blood eosinophils as a marker of eosinophilic inflammation, we hypothesize that cluster 2 and 3 in our study reflect concordant asthma phenotypes, while cluster 1 reflects a symptom-dominant phenotype and cluster 4 reflects an inflammation-

dominant phenotype. Cluster 2 seems to be the most 'favorable' asthma group in our study reflecting current non-active inflammation; the children in this cluster have a good lung function, few current symptoms and low blood eosinophils. The lack of adherence in this group might be consequence of few current symptoms. Nonetheless, two out of the four children in this group reported uncontrolled symptoms in the year preceding the study visit. This suggests that during active disease their breathprint might change and be more similar to the breathprints in cluster 3. This latter cluster seems to reflect active eosinophilic inflammation, causing severe exacerbations in several of these patients. Cluster 1 is characterized by a higher BMI compared to the other clusters, moderate current symptoms and low blood eosinophil counts. Children in cluster 4 have a poorer lung function compared to the other clusters and the highest level of bronchoconstriction. In addition, this cluster has elevated blood eosinophil counts, but few current symptoms. These findings accord with the results of Haldar et al., who showed that the inflammation-dominant phenotype in adult asthmatics was characterized by few daily symptoms, but active eosinophilic inflammation.[3] However, in the study of Haldar et al. this cluster was restricted to the refractory asthma population, while the children in cluster 4 in our study reported few exacerbations in the past year. The poor lung function of the children in this cluster could indicate that these children are not yet suffering from refractory asthma, but might be at risk to develop refractory asthma later in life.

In the study of Haldar et al. levels of sputum eosinophils of the different asthma clusters correlated well with FeNO levels.[3] Furthermore, in the total PACMAN2 population FeNO correlated well with blood eosinophil counts (Chapter 3.2). In the current study, however, median FeNO levels did not mirror the mean blood eosinophils in the distinct breathprint clusters. This discordance might explain part of the controversy regarding the clinical utility of FeNO; while ongoing eosinophilic inflammation often correlates with high FeNO levels, some asthmatic children might be characterized by low FeNO levels despite ongoing eosinophilic inflammation.

The identified clusters of breathprints should be replicated and validated in a larger childhood asthma population. Despite the small study sample, our study demonstrates the potential of non-invasive breathprint analyses to monitor asthma control and identify clinically distinct childhood asthma phenotypes.

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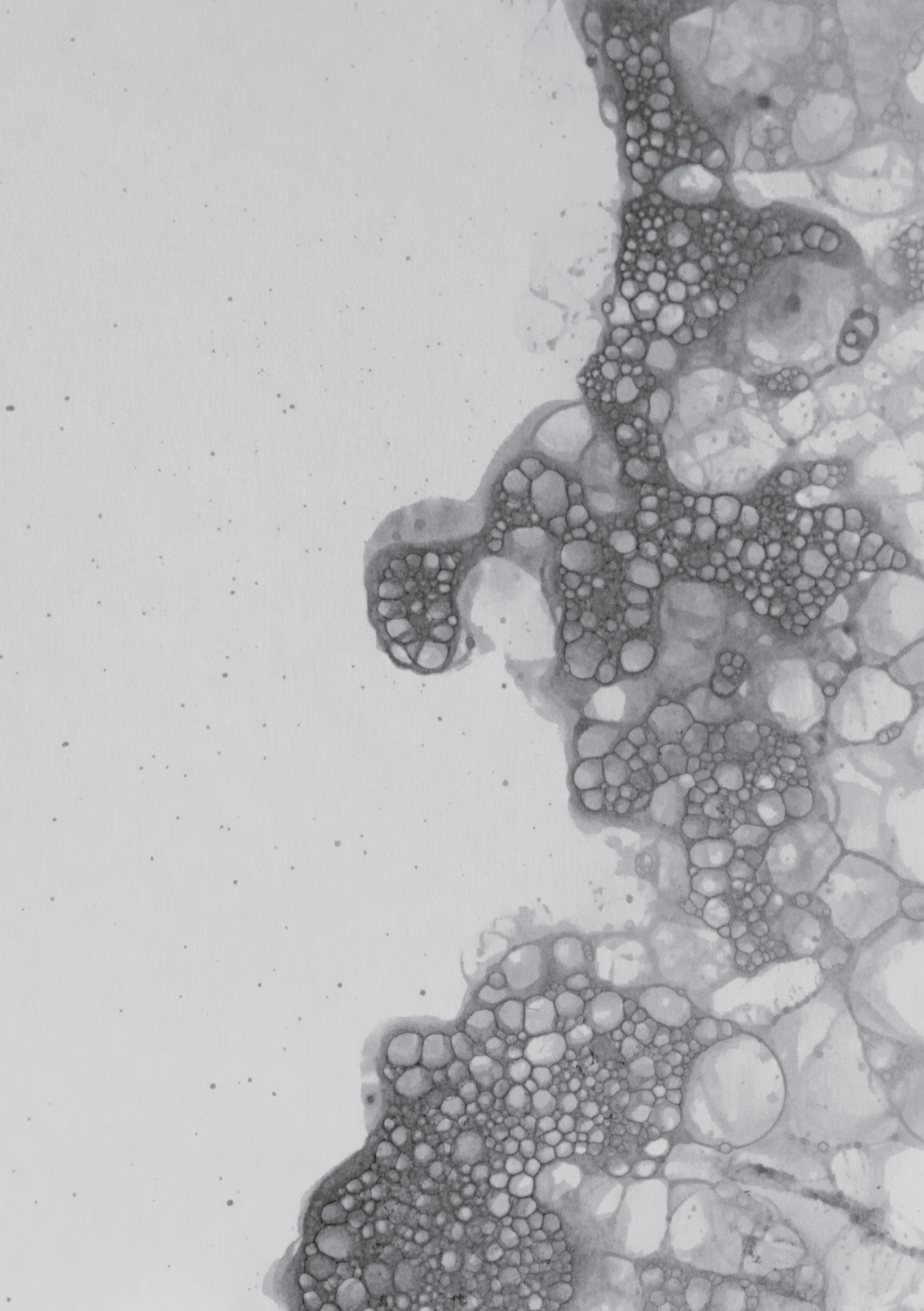

Chapter 5

General discussion

Scope of this thesis

The introduction of inhaled corticosteroids (ICS) in asthma management in the 1970s was an enormous step forward in controlling the disease in asthmatic patients. Currently, ICS are the mainstream treatment of choice for persistent asthma both in children and adults. However, although the majority of asthmatic patients can be treated satisfactorily, there is a significant intersubject variability in ICS responsiveness. Therefore, elucidating the mechanisms underlying impaired responsiveness is important in order to optimise the efficacy of asthma treatment and to identify novel therapeutic targets. For this thesis, asthma treatment response in children was studied, focussing predominantly on their response to inhaled corticosteroids. A multi-disciplinary approach was applied, combining epidemiology, pharmacogenomics and immunology. In this context, the influence of genetic variation on the measures of treatment outcomes in asthmatic children was assessed. Furthermore, priming phenotypes of peripheral blood granulocytes were investigated, as were markers in exhaled breath. This general discussion will elaborate on the results described in this thesis. First, the context in which most of the studies have been performed, the PACMAN cohort study, will be discussed. Secondly, the main findings will be addressed and placed in a broader perspective. Thirdly, methodological challenges will be addressed, and, lastly, an outlook for future research and clinical practice will be presented.

The PACMAN cohort: a multidisciplinary platform

The PACMAN cohort was initiated to assess the effects of genetic variation on asthma treatment outcomes in children. The inclusion of patients in the PACMAN cohort started in 2009. The PACMAN study closely collaborated with the Utrecht Pharmacy Practice Network for Education and Research (UPPER) and the Respiratory Research Group of the University Medical Center Utrecht. The focus on primary school children was chosen specifically in an attempt to avoid bias introduced by modulation of the disease due to environmental factors, such as smoking or long-term medication use. A major strength of the PACMAN cohort study is the inclusion of patients in a primary care setting. This gives it an advantage over other such studies because it focuses on a very broad range of asthmatics and not only on children in a specialised care group. Paediatric asthma studies often recruit patients

through secondary or tertiary health care services. Therefore, these patients may represent a subpopulation of patients with more severe disease when compared to the total population of individuals with asthma symptoms who generally have mild-to-moderately severe disease.

Due to the increasing numbers of children included in our cohort and the establishment of several (inter)national collaborations, the PACMAN cohort has now developed into more than just a tool to study the role of genetic variation in treatment response. It has become a research platform for a wide variety of research questions related to the treatment response of children with respiratory symptoms. Some of the research topics that have been addressed include: factors influencing adherence to ICS treatment,[1] seasonal differences in asthma symptoms[2] and the relationship between asthma control and inflammatory markers in exhaled breath (**Chapters 4.1 and 4.2**). Furthermore, the PACMAN study has become a foundation for additional studies. In 2012, PACMAN2, a clinical follow up of a selection of PACMAN participants, was initiated.

The aim of PACMAN2 was to study inflammatory markers in peripheral blood and exhaled breath in a small set of well-characterized children treated with ICS (**Chapter 3.1**). PACMAN children adherent to ICS, with long-term controlled symptoms or long-term uncontrolled symptoms were invited to participate. The in-depth analyses of peripheral blood, combined with spirometry and exhaled breath measurements restricted the study to a clinical setting.

During that same year, all the PACMAN children who had previously consented to being approached for future research were asked to participate in a web-based portal developed by colleagues at the Wilhelmina Children's Hospital; the Portal for Children with Respiratory and Allergic Symptoms.[3] This portal, which contains an extensive online baseline questionnaire, as well as additional short questionnaires that are made available every three months, makes it possible to follow-up on the symptoms and medication use of the PACMAN population over time, shifting the cross-sectional character of our cohort to a longitudinal one.

The main goal of the PACMAN cohort study is to connect scientific research with clinical practice and to study asthma treatment response in children at different levels. This might lead to the identification of clinical markers or therapeutic targets, and to a more personalized treatment approach of childhood asthma. The studies described in this thesis are all building blocks aiming to achieve that goal.

Main findings of this thesis

Genetic profile influences asthma treatment outcome in children

Various genetic loci influence the risk of severe exacerbations or poor symptom control in children with a reported use of asthma medication (**Chapter 2.1-2.5**). Analyses were performed using the PACMAN cohort, but other paediatric asthma study populations, such as the Scottish BREATHE and PAGES cohort studies, the North American CAMP trial and the Dutch CATO trial, were also studied.

With the use of a candidate gene approach, a novel risk gene, *ST13,* was identified. This gene is associated with severe exacerbations despite ICS treatment in children and young adults. *ST13* encodes a co-chaperone of the GR receptor complex: hsp70-interacting protein (hip) (**Chapter 2.1**). Although the genetic loci lie in non-coding regions of the gene, these loci might still influence gene expression or be in high linkage disequilibrium with other variants, which do have functional consequences. Hip is thought to be involved in the functional maturation of the corticosteroid receptor, but the mechanism by which it does so remains unclear. Hip cooperates with Hsp70/Hsp90-organising protein (hop), another co-chaperone of the GR receptor complex.[4] The gene encoding hop has been previously associated with lung function improvement in asthmatic patients treated with ICS.[5] This provides additional evidence that alterations in the expression or folding of these co-chaperones may influence the binding of corticosteroids to the receptor and, therefore, ICS efficacy.

Furthermore, variation in the *17q21* locus was found to be associated with an increased risk of exacerbations, poor symptom control (**Chapter 2.2**) and persistent airway hyperresponsiveness (**Chapter 2.5**) in children treated with ICS. One of the genes in the 17q21 locus, *ORMDL3,* has recently been reported to influence eosinophil activation.[6] Knockdown of *ORMDL3* in mouse eosinophils resulted in impaired cell adhesion, migration, degranulation and activation-dependent cell shapes required for cell motility. In contrast, overexpression of *ORMDL3* resulted in increased eosinophil rolling, activation of NF-κB and cytoskeleton changes.[6] *ORMDL3* seems to regulate eosinophil trafficking and degranulation. Overactive eosinophils might underlie an inflammatory asthma phenotype less responsive to the anti-inflammatory properties of ICS. Furthermore, it has been shown that *ORMDL3* can regulate the expression of various metalloproteases, chemokines and oligoadenylate synthetases (anti-viral regulated genes) in mice airway epithelial cells.[7] Therefore, *ORMDL3* might prime the airway epithelial for airway remodeling

and promote eosinophilic inflammation. The 17q21 locus also regulates the expression of other genes, such as *GSDMA, GSDMB* and *CRKRS*.[8] Yet it remains to be seen whether these genes might also be involved in asthma pathology.

Genetic variation influences ICS response in children with asthma, but the effect sizes are modest, and large study populations are generally needed in order to have enough power to identify these effects. In contrast, when the influence of *ADRB2* genotype was assessed on LABA response in the PACMAN population (**Chapter 2.4**) significant and relatively high effect estimates were found in a single study population. *ADRB2* encodes the β_2-adrenergic receptor. Receptor binding causes smooth muscle relaxation and bronchodilation.

Functional studies have reported that amino acid changes at position 16 (arginine vs glycine) influences down-regulation of the receptor upon prolonged β_2-agonist treatment, but results are inconsistent in which isoform is down-regulated faster.[9, 10] Down-regulation of the receptor corresponds to functional desensitisation of the receptor [9] and could underlie the clinical differences in LABA response. Prospective genotyping of *ADRB2* might have important clinical consequences for childhood asthma management[11] and should be studied in further detail in a well-designed clinical trial.

Different response phenotypes are associated with different genetic profiles

Poor response to treatment can be defined by various measurements: lack of improvement of lung function upon treatment, persistent airway hyperresponsiveness despite treatment, uncontrolled symptoms or severe exacerbations despite treatment, for example. The definition of response seems to influence the genetic profiles underlying that response phenotype. Variation in *GLCCI1* has previously been associated with impaired lung function improvement when treated with ICS; but no association between variation in this gene and the risk of exacerbations could be demonstrated in the children of the PACMAN cohort, the BREATHE cohort or the PAGES cohort (**Chapter 2.3**). It cannot be excluded that the population was too small to identify the effect, however, this might also demonstrate the lack of clinical utility of this genetic marker in the general childhood asthma population. Moreover, it might emphasise the heterogeneity in asthma phenotypes and the related complexity of asthma treatment response. In **Chapter 2.4** the *ADRB2* Arg16 genotype was associated with an increased risk of severe exacerbations in LABA-treated children, but not with an increased risk of uncontrolled asthma symptoms, suggesting that exacerbation-prone asthma

is likely to be a different asthma phenotype compared to symptomatic asthma.
In the CATO trial, lung function and AHR in children treated with ICS were measured over a 2 year period. The top 5 of genetic loci most significantly associated with poor lung function improvement was different from the top 5 of genetic loci most significantly associated with poor AHR response (**Chapter 2.5**). However, the clinical value of AHR in children might not be straightforward. AHR is the exaggerated narrowing of the airways in response to nonspecific stimuli.[12] AHR has been linked to disease severity, but has limited specificity. Asthmatic children and non-asthmatic wheezers do not differ in airway hyperresponsiveness,[13] and individuals without respiratory symptoms may also exhibit airway hyperreponsiveness.[14] Various factors seem to influence AHR, including age, gender and atopic status.[15] In the CATO trial, treatment was guided based upon symptom score or on symptom score and AHR. Lung function, AHR and symptom scores were measured over a period of 2 years. The trial showed that guiding treatment based on AHR and symptom score led to higher FEV_1 levels compared to guiding treatment on symptoms solely.[16] Nevertheless, there was a poor correlation between AHR and reported symptoms, as well as between AHR and measures of lung function in participants of the CATO trial. Furthermore, there were large interindividual fluctuations in AHR over time in a substantial group of the children, complicating the clinical interpretability of AHR.

Priming phenotypes of blood granulocytes do not correlate with asthma symptoms in children

Airway inflammation is one of the main pathological characteristics of asthma. Inflamed tissue releases chemo-attractants and cytokines which recruit more activated immune cells from the peripheral blood, leading to chronic inflammation in the tissue. The dynamic process of immune cells entering and leaving the blood stream could, therefore, be an indirect readout of the state of disease. Granulocyte functions *in vitro* and *in vivo* are tightly controlled by a process generally referred to as priming.[17] This priming response does not lead to direct activation but rather leads to facilitation of responses evoked by other stimuli. Priming can regulate *in vivo* granulocyte function and converts cells refractory to activation into cells which are easily activated. Under normal conditions, blood granulocytes are not primed. However, in the context of systemic inflammation such as in asthma and COPD, primed blood granulocytes have been observed.[18, 19]
The activation status of blood granulocytes ('priming phenotype') can be studied *in vitro* by measuring the expression of activation markers on their cells surface upon

in vitro treatment with a chemo-attractant. Ten Hove et al.,[20] as well as unpublished work of Hilvering et al. showed that blood eosinophils of adults with mild asthma are hyperresponsive to such stimuli, while blood eosinophils of difficult-to-treat asthmatics and controls are poorly responding. In **Chapter 3.2** distinct priming phenotypes of peripheral blood granulocytes were also observed in asthmatic children treated with inhaled corticosteroids, but these priming phenotypes were not related to clinical features. Upregulation of an activation epitope recognized by MohPhab A27 on eosinophils was associated with other inflammatory markers, such as blood eosinophil counts and FeNO, yet did not relate to asthma symptoms or lung function. Airway inflammation and asthma control may reflect different dimensions of disease and may not be directly related.

VOCs beat FeNO as a read-out for asthma control

Health care professionals have been searching for a clinically applicable non-invasive, read-out system to 1) confirm the diagnosis of asthma, 2) identify clinically distinct asthma phenotypes, and 3) guide treatment. The non-invasive character of such systems is especially important in paediatric patients. One such read-out system is the analysis of the fraction of nitric oxide in exhaled breath (FeNO). High FeNO levels are thought to reflect eosinophilic airway inflammation. NO is a highly reactive gaseous molecule that is produced in the airways when the amino acid L-arginine is oxidised into the amino acid L-citrulline. The synthesis of NO is catalysed by nitric oxide synthases (NOS). Expression of inducible NOS, an isoform of NOS, is influenced by a wide range of inflammatory cytokines.[21] Standardised measurements of FeNO are currently applied in asthma management.[22] However, its value for asthma treatment remains unclear. The association between a single FeNO measurement and asthma control was assessed in 601 PACMAN children, and there was a weak correlation between the value of FeNO and asthma control as assessed by the asthma control questionnaire (**Chapter 4.1**). Various other factors including age, living environment, health care visits and medication use were independently associated with high FeNO levels, complicating the interpretation of the high FeNO levels.

An additional read-out system is the analysis of volatile organic compounds (VOCs) in exhaled breath. Exhaled breath contains a complex mixture of thousands of VOCs. These compounds are produced as a result of metabolic processes, and VOC concentrations are likely to be influenced by the presence of airway inflammation. A gas multi-sensor array ('eNose') can assess patterns of VOCs present in exhaled

breath ('breathprints'). This approach seems to be more accurate than using FeNO to discriminate between controlled and uncontrolled asthmatic children (**Chapter 4.2**). The breathprints of 27 children participating in the PACMAN2 study were assessed on a panel of four eNoses, and the breathprints of children with long-term uncontrolled asthma could be separated from the breathprints of the children with long-term controlled asthma with accuracy up to 87.5%.

Uncontrolled asthma symptoms are thought to be influenced by inflammatory activity in the airways. Nevertheless, physiological factors (lung function, airway remodeling), as well as psychological factors (adherence, stress) might also play a role.[23] The measurement of a combination of exhaled markers seems to be more accurate in capturing the different aspects of asthma control than the measurement of one single exhaled inflammatory marker.

Potential causes of poor ICS responsiveness in childhood asthma

Inadequate inhalation technique, psychosocial problems and poor adherence

The studies described in this thesis have focused on the biological mechanisms underlying treatment response. However, non-biological factors, such as inadequate inhalation techniques, psychosocial problems and poor adherence, may also influence treatment outcomes.[24] The incorrect use of an inhaler device can lead to reduced deposition of asthma medication in the lungs, resulting in a decreased efficacy of the drug.[25] In the PACMAN population, 17% of the children demonstrated a poor inhalation technique (inhalation score <80% on inhalation device specific checklist[26]), with the most common errors being as follows: not shaking the aerosol inhaler before use, exhaling too quickly or not fully exhaling before inhalation, and not holding breath after inhalation (unpublished results).

Furthermore, childhood asthma is associated with an increased prevalence of psychosocial problems, such as anxiety disorders, depression and concentration problems.[27] These psychosocial problems may affect symptom recognition and adherence to treatment.[28] In addition, the work of Miller et al. showed that chronic stress in asthmatic children has been associated with a diminished expression of glucocorticoid and β_2-adrenergic receptor genes.[29]

Improving adherence to maintenance treatments has become an important target

to improve asthma control.[12] In the PACMAN cohort, 43% of the children were non-adherent to ICS.[1] Especially when children reach adolescence, adherence may become even more complicated.[30] In PACMAN2, a follow-up was done on a small subset of children who were included in the PACMAN cohort. At the time of the pharmacy study visit (baseline visit) they were all adherent to ICS (mean age baseline visit: 9.5 years). However, only 77% of the children were still adherent to ICS at the follow-up visit (mean age at follow-up: 11.8 years).

Improving adherence will most likely lead to better treatment outcomes.[24] Nevertheless, studies have shown that a subgroup of patients will remain symptomatic, despite being adherent to maintenance treatment with ICS.[31] For this group of patients particularly, non-adherence might become a consequence rather than a cause of impaired ICS responsiveness. Why should they be adherent to a drug that does not control their disease? This is supported by the results of a double blind randomized controlled trial (RCT) by Jonasson et al., who showed that adherence rates declined more rapidly in asthmatic children receiving a placebo compared to children receiving ICS.[32]

A defective drug pathway?

Genetic variation seems to predispose certain children for an asthma phenotype that is less responsive to ICS. The biological mechanisms linked to the genetic loci that have been associated with altered ICS responsiveness remain largely unknown. Loci in genes of co-chaperones of the GR complex such as hip (*ST13)* (**Chapter 2.1**) and hop (*STIP1)*[5] were found to be associated with ICS treatment outcomes. Nevertheless, the investigated loci were non-coding and the functional effect of alteration in these genes remains unclear. Hip and hop play a role in the assembly and maturation of the GR receptor complex,[4] and it has been hypothesised that they may indirectly influence efficient binding of corticosteroids to the GR receptor complex. A study in patients with acute lymphoid leukaemia, however, showed that mRNA levels of the co-chaperones were not significantly different in GC sensitive and GC resistant patients.[33]

There is little evidence that variation in the GR (encoded by *NR3C1*) itself plays an important role in explaining the heterogeneity in ICS response.[34, 35] This is in contrast to LABA responsiveness where genetic variation in the gene that codes for the β_2-adrenergic receptor (*ADRB2*) has been directly associated with treatment outcome in asthmatic children **(Chapter 2.4)**. Glucocorticoids play a pivotal role in internal basal and stress-related homeostasis. If poor ICS responsiveness

in asthmatic children is mainly caused by a defective GR, it would be likely that children responding poorly to ICS would also be more prone to symptoms of generalised glucocorticoid insensitivity. This situation has been described in a rare familial glucocorticoid resistance syndrome: inactivating polymorphisms in *NR3C1* lead to abnormalities of cortisol secretion and hypothalamic-pituitary-adrenal (HPA) axis sensitivity.[36] However, this does not seem to be the case in glucocorticoid-insensitive asthma.[37] Nevertheless, GC signalling could be disturbed more downstream, due to a decreased nuclear translocation, for example, or due to a reduced ability to bind to the DNA.[37] Other molecular mechanisms that have been thought to underlie (at least partly) corticosteroid insensitivity in asthmatic patients include: increased expression of GRβ (splice variant that cannot bind CS) acting as an negative inhibitor of GRα, altered cross talk between the GR receptor complex and transcription factors, and defective histone acetylation which might limit the GR-mediated inhibition of NF-κB on pro-inflammatory gene expression.[37, 38] However, these mechanisms cannot explain why it is the response to ICS that is specifically affected in this asthmatic population and not the intrinsic corticosteroid response.

A steroid resistant immune response?

Although pharmacogenomics studies often focus on variations in drug pathways, it appears that several genes that have been associated with ICS responsiveness are involved in immune regulation (Table 1). An alternative explanation for poor ICS response, therefore, could be that poor treatment response is caused by an immunologic response that is intrinsically less responsive to corticosteroids.

Table 1. Genes associated with ICS responsiveness and their biological function

Genes	Ref. PGx study	Biological function of the encoded protein
CRHR1	Tantisira et al., 2004 [39]	Mediates corticotrophin-releasing hormone expression in the hypothalamic–pituitary–adrenal (HPA) axis
GLCCI1	Tantisira et al., 2011 [40]; no association was found in **Chapter 2.3**	Unknown
FCER2	Koster et al., 2011 [41] Tantisira et al., 2007 [42]	Low-affinity receptor for IgE, involved in the regulation of IgE synthesis[43]
DUSP1	Jin et al., 2010 [44]	Inhibits mitogen-actived protein kinase (MAPK) by dephosphorylation, reducing the expression and production of proinflammatory cytokines[45]
TBX21	Ye et al., 2009 [46] Tantisira et al., 2004 [47]	Transcription factor, controls expression interferon-γ, IL-4 and IL-13[48]
T gene	Tantisira et al., 2012 [49]	Transcription factor crucial to notochord development[50]
NK2R	Ye et al., 2009 [46]	Receptor for neurokinin A. Involved in airway sensory nerve activation[51]
STIP1	Hawkins et al., 2009 [5]	Co-chaperonne of the GC receptor complex[4]
ST13	**Chapter 2.1**	Co-chaperonne of the GC receptor complex[4]
ORMDL3	Tavendale et al., 2008 [52] / **Chapter 2.2** and **2.5**	Linked to sphingolipid pathway,[53, 54] to eosinophil activation,[6] to the expression of metalloproteases and chemokines[7] and to IL-17 secretion early in life[55]

A neutrophilic asthma phenotype has been associated with poor treatment response.[56] Neutrophils, in contrast to eosinophils, appear to be insensitive to the anti-inflammatory properties of steroids.

In vitro studies have shown that steroids induce apoptosis in eosinophils, whereas they promote survival in neutrophils.[57] The same pro-survival effect was observed when peripheral blood neutrophils obtained from children with a history of RSV infection were treated with dexamethasone (Figure 1). This neutrophilic asthma phenotype could be steroid-induced, as ICS might selectively induce apoptosis in the eosinophils present in the airways, while promoting neutrophil survival in the airways. However, neutrophilic asthma has also been observed in steroid-naïve asthma patients.[58, 59] In adults, increased levels of sputum neutrophils have been linked to asthma severity [60, 61] and poor ICS responsiveness.[56] On the other hand, persistent eosinophilic asthma has also been linked to severe asthma.[62, 63] Moreover, the role of the neutrophil in asthma pathology is still unclear. It could play a causative role, but could also be a bystander induced by external factors such as tobacco smoke, viral infections, or tissue damage.[61, 64]

Children are less exposed to above mentioned factors, and the neutrophil seems

to play a less important role in severe or ICS responsive childhood asthma.[65] Work by Wang et al. showed that the most common inflammatory phenotype in adults with acute asthma is 'neutrophilic', but is 'eosinophilic' in children with acute asthma.[65] Furthermore, Bossley et al.[63] showed that children with severe therapy-resistant asthma were characterised by increased levels of eosinophils in BAL fluid, endobronchial biopsies and sputum samples, but not by neutrophilia. A small retrospective analysis of the pathological reports of nine children with a diagnosis of 'difficult-to-treat' asthma who underwent a bronchoscopy showed that, in the majority of these children, eosinophils were absent in the BAL, but present in lung biopsies (Table 2) (unpublished results). Furthermore, the children were characterised by airway remodeling and increased FeNO levels. In the children included in the study of Bossley et al.,[63] as well as in the children with difficult-to-treat asthma of the Wilhelmina Children's Hospital, eosinophils in the lung and high FeNO seemed to persist despite high dosages of ICS. This suggests that these children suffer from a type of eosinophilic asthma unresponsive to ICS.

It remains unknown what mechanism causes this apparently steroid-insensitive eosinophilia. Corticosteroids can inhibit *iNOS* expression[66]; coding for the enzyme thought to be responsible for the increased FeNO levels, as well as the transcription of *IL-5*; coding for interleukin-5, a key mediator of eosinophil activation.[67] However, in these difficult-to-treat children, these genes do not seem to respond to the inhibiting effect of corticosteroids. One might hypothesize that corticosteroids regulate a transcription factor that recognises a currently unknown binding site in these genes under normal 'steroid-responsive' circumstances. In steroid unresponsive asthmatic patients this pathway might be defective, leading to eosinophilia and increased FeNO levels.

Work of Saglani et al. suggested that IL-33 might be a mediator of severe disease in children with difficult-to-treat disease.[68] This steroid-insensitive cytokine is released rapidly upon epithelial damage, and is a chemoattractant for Th2 cells.[69] Saglani showed that IL-33 is upregulated in submucosal cells of biopsy samples of difficult-to-treat asthmatics and promotes airway remodeling. Combined with the observation that Th2 mediators were below detection levels in BAL fluid samples of these children, it could be speculated that IL-33 can orchestrate an alternate, less steroid-responsive immune mechanism in these patients.[68]

A cytokine produced by Th17 cells, IL-17, might also play a role in poor ICS reponse. This cytokine is involved in neutrophil recruitment to the airways.[70] Recent work by Nanzer et al. showed that PBMCs of adults with steroid resistant asthma produce

increased levels of this cytokine, and that this production is not inhibited by glucocorticoids.[71] Furthermore, variation in the 17q21 locus has been associated with elevated IL-17 secretion early in life.[55]

Overall, there is a strong suggestion that poor treatment outcomes are caused by genetic risk factors influencing the efficacy of drug pathways, as well as by immune responses that are intrinsically less sensitive to ICS. These steroid-insensitive immune responses may also be genetically determined.

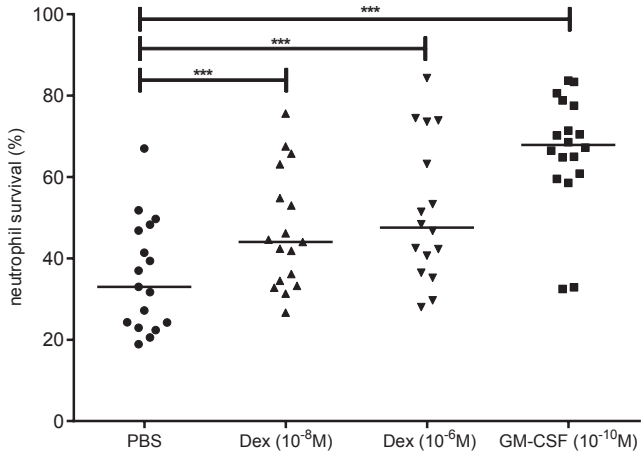

Figure 1. Dexamethasone-induced survival in peripheral blood neutrophils

Peripheral blood neutrophils were isolated from the peripheral blood of 18 children with a history of respiratory syncytial virus (RSV) infection early in life. Overnight survival assays were performed with phosphate-buffered saline (PBS; control), dexamethasone (10^{-8}M and 10^{-6}M) and granulocyte macrophage-colony stimulating factor (GM-CSF; 10^{-10}M, positive control). Cell viability was measured using 7-AAD/Annexin-V flow cytometry staining. *** $p < 0.001$

Table 2. Children who underwent a bronchoalveolar lavage (BAL) and bronchoscopic biopsy for difficult-to-treat asthma in the Wilhelmina Children's Hospital

	Children with difficult-to-treat asthma (n=9)
Age, mean ± SD	9.7 ± 5.4
Boys, %	8/9 (88.9%)
BAL eosinophils, %	1/9 (11.1%)
BAL neutrophils, %	6/9 (66.7%)
Eosinophils in biopsy, %	5/8 (62.5%)
Neutrophils in biopsy, %	0/8 (0.0%)
Basal membrane thickening	6/8 (75.0%)
FeNO, median [IQR] in ppb	55 [29-55]#

Bronchoscopies were performed between 2006-2009.
#FeNO data present for 5 of the 9 patients.
BAL, Bronchoalveolar lavage; FeNO, Fraction of exhaled Nitric Oxide; IQR, Interquartile Range.

Methodological challenges

Defining treatment response

Assessing treatment response in asthmatic children remains a challenging subject as illustrated by pharmacogenomics analysis of the CATO trial (**Chapter 2.5**): their symptoms vary over time and different dimensions of response (increasing AHR, decreasing FEV_1) are associated with different genetic risk profiles. Pharmacogenomics studies with a longitudinal character [5, 40, 72] often use the increase in lung function or the decrease of AHR assessed at the beginning and end of the trial as measures of good response. However, these assessments might fail to take into account the variations in response over time. Such fluctuations might be part of a distinct asthma phenotype. Using a fluctuation analysis of lung function, Frey et al. have previously shown that increased variability in peak expiratory flow (PEF) is associated with more severe disease.[73] In **Chapter 2.5** response definitions were proposed based upon lung function decrease over time and AHR increase over time, taking into account the correlation of determination (r^2), baseline values and the treatment levels. Although we did not specifically focus on fluctuations in response, these definitions do at least partially take these into account with the use of the r^2 and might, therefore, be a more accurate reflection of treatment response. In the observational pharmacogenomics studies described in this thesis (**Chapter 2.1-2.4**) exacerbations (OCS use / ED visits) or uncontrolled asthma symptoms

(based on ACQ) despite treatment have been used as surrogate markers of response. These definitions have been generally applied in pharmacogenomics asthma studies. However, it is important to emphasise that exacerbation-prone asthma seems to be a different asthma phenotype compared to poorly controlled asthma.[74] Children with limited symptoms can be prone to severe exacerbations.[75] In addition, cultural differences might further complicate the comparability of study results among countries when assessing OCS use or ED visits as markers for exacerbations. A study by Wahlström et al. presented case simulations from patients with an asthma exacerbation to physicians in five countries (40-100 physicians per country): the Netherlands, Germany, Sweden, Norway and the Slovak Republic.[76] The physicians were asked to give their treatment recommendations based on the information given in the cases. The study showed significant differences between the doctors in the five countries in their recommendations concerning OCS prescriptions despite having been given similar case descriptions. The Dutch GPs decided to prescribe an OCS to the simulated patients more often than the GPs from other countries. Yet, the mean proportion of decisions to prescribe an OCS by the GPs was still lower than the proportion of decisions to prescribe an OCS according to the 'gold standard' national guidelines.[76]
Observed national differences in prevalence of asthma-related hospital visits[77] might also be due to differences in the individual health care systems. Defining treatment response in observational studies is further complicated by the absence of a consensus regarding definitions for severe disease and treatment response. Consequently, there is need for consensus on these definitions in order to interpret treatment responses in longitudinal and cross sectional asthma pharmacogenomics studies.

'Difficult asthma' nomenclature

Terms such as 'difficult asthma',[78] 'difficult-to-treat asthma',[12] 'problematic asthma',[79] 'severe refractory asthma'[80] and 'severe therapy-resistant asthma'[31] are all used to designate asthma patients that respond poorly to treatment. However, these varying definitions all have their own specific criteria, and different scientific groups favour a different definition, which all adds to the confusion (Table 3). 'Severe asthma' should be regarded as a different entity than 'difficult-to-treat asthma', but these terms seem to be used interchangeably.[81] Severity reflects the baseline intensity of symptoms (in the absence of treatment), while treatment response focuses on how well treatment can control the symptoms. Patients with severe asthma can be well-controlled by treatment.[82]

Bush and colleagues have proposed an elegant solution to solve this confusion. They use the term 'problematic asthma' to describe all children with chronic symptoms and/or acute severe exacerbations despite the prescription of multiple drugs. Additionally, they apply the term 'severe therapy-resistant asthma' (STRA) for the children in that group that have a genuine intrinsically poor response to treatment.[31] Studying the pathology and biomarkers for treatment response in this well-phenotyped, and most likely more homogenous, STRA group might be more effective compared to a large heterogeneous population of difficult asthmatics. Identified markers should subsequently be validated in heterogeneous populations covering the whole spectrum of asthmatic patients to test clinical utility.

The children with persistent uncontrolled symptoms in the PACMAN2 study seem to reflect this heterogeneous population of difficult asthmatics. The group is likely a mixture of 'steroid-responsive' children that would benefit from higher dosages of treatment or improvement of adherence, as well as children in an early stage of 'difficult-to-treat' disease. This fits with the observation that children with persistent uncontrolled symptoms did not differ in their blood eosinophil priming phenotypes compared to long-term controlled children (**Chapter 3.2**), as well as with the observation that unbiased clustering of breathprints did not cluster all persistent uncontrolled children in the same group (**Chapter 4.2**). The unbiased cluster analysis also identified an inflammation-dominant cluster with a poorer lung function and fewer symptoms (cluster 4). When comparing these clusters with the clusters identified by Haldar et al. in an adult population,[62] it is tempting to speculate, that the children in cluster 4 might be in an early stage of their disease development and might be at risk to develop exacerbation-prone asthma later in life. It would be interesting to compare the breathprints and eosinophil blood priming phenotypes of persistent uncontrolled children in PACMAN2 with paediatrician diagnosed severe therapy-resistant asthmatic children and healthy controls.

Table 3. Definitions of difficult/severe asthma used by scientific organisations

Scientific organization or initiatives	Nomenclature	Criteria
Global Initiative for Asthma (GINA)[12]	Difficult-to-treat asthma	Patients that do not reach acceptable level of control at treatment step 4 (reliever medication plus two or more controllers).
Severe Asthma Research Program (SARP)[83]	Severe asthma	Patients who: · have a diagnosis of asthma · require high or continuous doses of ICS or OCS · have persistent asthma symptoms on a regular basis, · have a history of frequent or severe exacerbations of asthma (requiring prednisone, ER visits, hospitalisations), · are current non-smoker and <5 pack years total · are between 6 and 75 years of age
American Thoracic Society[84]	Refractory asthma	Definition of refractory asthma requires one or both major criteria and two minor criteria. Furthermore, other conditions should have been excluded, exacerbating factors should be treated, and patient should be generally adherent. Major characteristics: · treatment with continuous or near continuous (>50% of year) oral corticosteroids · requirement for treatment with high-dose inhaled corticosteroids Minor characteristics: · requirement for daily treatment with a controller medication in addition to inhaled corticosteroids · asthma symptoms requiring SABA use on a daily or near daily basis · persistent airway obstruction · one or more urgent care visits for asthma per year · three or more OCS courses per year · prompt deterioration with < 25% reduction in oral or inhaled corticosteroid dose · near fatal asthma event in the past
European Respiratory Society[78]	Difficult/ therapy-resistant asthma	Patients who: · have uncontrolled symptoms despite ≥ 800 µg budesonide equivalent (children) · have a continued requirement for SABA despite delivery of a reasonable dose of inhaled corticosteroids. · require OCS courses or a regular dose of OCS to maintain reasonable control of the disease. · rarely, control of asthma in these patients may be totally uninfluenced by corticosteroid therapy. · issues such as compliance with treatment, identification of exacerbating factors and exclusion of other diagnoses are dealt with.

Scientific organization or initiatives	Nomenclature	Criteria
Paediatric Pulmonology section of the Dutch Society of Paediatrics[85]	Difficult-to-treat asthma	Patients who: · are 6 years or older · have been treated for at least 6 months according treatment step 4 Fulfil ≥ 1 of the following criteria: · decreased exercise tolerance and/or; · night-time symptoms and/or; · ≥2 times per week SABA use; · frequent exacerbations requiring OCS; · history of exacerbations requiring ER visits; · persistent airway obstruction. · ≥ 6 months supervision by paediatrician · adherent to treatment · correct inhalation technique · confirmed asthma diagnosis by spirometry

'Missing' heritability

Family and twin studies have shown that asthma contains a considerable heritable component, with heritability estimates ranging between 50-90%.[86, 87] Genome-wide association studies (GWAS) have identified several loci to be associated with asthma risk.[88-90] Nevertheless, effect sizes are small and the identified genetic variants can only explain a small part of the asthma heritability. Missing heritability is also observed in asthma treatment responses. Few studies have assessed heritability of pharmacological responses in families or twins, since it requires family members to be users of medication.[91] However, based on the interindividual repeatability of asthma treatment responses, it was estimated that 60-80% of the observed variance in treatment responses might be due to genetic differences.[92] Current identified genetic variants comprise only a portion of the estimated heritability of asthma treatment responses. This could mean that the current applied methods of studying genomic variations are inefficient (i.e. studies are underpowered, searching methods are inadequate), but it could also mean that the influence of non-genomic factors has been underestimated.

In the field of genomics, there are currently two main approaches to performing DNA analysis: 1) candidate-gene studies, which are predominantly hypothesis-driven, and 2) genome-wide association studies or whole-genome/exome-sequencing studies, which are often data-driven. In a candidate gene approach, the association between variations in selected genes and an outcome (i.e. therapy response) is studied. Genes are selected based on biological knowledge. This type of study is not designed to identify novel genomic areas that might be associated with the outcome, but rather to confirm a hypothesised association between a gene, or

genes in a pathway, and the studied outcome. In **Chapter 2.1** this approach was applied to study variation in the genes involved in the glucocorticoid signalling pathway. In contrast, data-driven approaches including genome-wide association studies (GWAS) and whole genome/exome sequencing are often used to identify novel genomic variants. Since genes are not selected based on *a priori* knowledge in this approach, variation in the genome can be studied in an unbiased context. Both methods can be used, or combined, to study pharmacogenomics-related traits.

In a GWAS, single nucleotide polymorphisms (SNPs) are assessed across the entire genome. It studies if the investigated SNPs are associated with a specific outcome (i.e. therapy response). Genotype imputation, the estimating of missing genotypes that are not assayed on the chip, can be performed with greater precision using reference panels and knowledge about genetic linkage disequilibrium (the correlation of alleles at two or more loci). Large sample sizes are required to have enough power to detect loci with small to moderate effect. This might especially account for complex traits such as asthma and asthma treatment response. The establishment of asthma consortia has made it possible to perform GWAS of asthma in large patient samples. In 2010, Moffatt et al. published GWAS data of 10,365 asthmatic patients and 16,110 controls.[89] Subsequently, Ferreira et al. combined the previously-published GWAS data with their own samples and samples of replication cohorts. In total, they could study genetic loci of 15,797 asthmatic patients and 42,003 controls.[93] Compared to these numbers, sample size numbers of GWAS for asthma treatment responses lag behind considerably, with study populations of less than 3,000 patients.[40, 49, 94, 95] With increases in GWAS sample size; more genetic variants associated with asthma treatment response might be identified.

GWAS arrays are often designed to include mainly common genetic variants and are less suitable for capturing functional or rare variants. In addition, the investigated SNPs are often non-functional and/or in noncoding regions. Genotype imputation might partly solve these issues. Other approaches such as targeted, whole-exome or whole-genome sequencing might be more appropriate in finding the functional or rare variants underlying association, however, these remain expensive. An intermediate approach is using targeted SNP arrays with content focused on functional variation. In **Chapter 2.5,** an example of this approach, the exome-chip, is used. This chip assessed over two hundred thousand genetic variants at once (focusing on coding regions in the DNA). Samples from children participating in the CATO trial were investigated using this chip. During this trial, the lung function and

airway hyperresponsiveness in asthmatic patients treated with ICS were studied over a time period of two years. Response phenotypes were defined based on lung function or airway hyperresponsiveness improvement during the trial, taking into account medication use. Nevertheless, no loci on the exome chip were significantly associated with these response phenotypes when corrected for multiple testing. Correction for multiple testing is common practice in genomic research; however, it is complicated due to the existence of various methods that all have their own limitations.[96] Balancing the risk associated with finding false positives versus the risk of false negatives remains an important challenge.

Current exome strategies are limited because they do not (yet) cover all the coding regions present in the human genome, nor can they detect structural variants or chromosomal rearrangements,[97] which might also explain part of the missing heritability. Underestimation of gene-gene interactions might also play a role. System biology approaches are increasingly used in order to identify genetic networks associated with therapeutic response.[98] Furthermore, genotype data is progressively integrated with expression data through functional genomic screens, which will facilitate the identification of genetic variations contributing to altered gene expression and function, so-called expression quantitative trait loci (eQTL).[98] Epigenetic regulation might also play a role in treatment responses, further complicating the link between genotype and phenotype. It is thought that environmental factors, such as smoke exposure and vitamin D, can drive epigenetic changes. These are inheritable variations that do not occur in the DNA sequence, but rather in the DNA structure, which is highly dynamic and regulates gene expression.[99, 100] A study by Cohen et al. found that children who had been exposed to smoke *in utero* experienced fewer beneficial effects from ICS with regards to airway responsiveness, compared to children who had not been exposed to smoke *in utero*. [101] Furthermore, low levels of vitamin D have also been associated with decreased lung function, poor asthma control and increased steroid use.[102] Important epigenetic mechanisms include DNA methylation, post-translational modifications of histone proteins and modulation of gene expression by noncoding RNAs.[103] Epigenetics is a promising field, yet also a complex field to study due to the dynamic character of epigenetic modifications and the tissue-specificity of DNA methylation patterns.[104] Although various studies have related epigenetic variations to risk of asthma susceptibility, the direct link between epigenetic alterations and asthma susceptibility or treatment responses remains unclear.[105]

Several factors complicate the identification of genetic factors that may account for the heritability of asthma and asthma treatment responses. DNA analysis and data-mining technologies are rapidly evolving. In combination with larger sample sizes, this might resolve - at least part of - the missing heritability in the near future.

Future directions

Asthma phenotyping: from single- to multidimensional analyses

There is a definite need for asthma biomarkers to guide treatment and to discriminate clinically relevant asthma phenotypes. A single biomarker approach is increasingly being seen as an outdated concept, even when combined with clinical parameters such as lung function and symptom expression. As early as 2005, Wardlaw and colleagues proposed new approaches in the study of asthma using multi-dimensional, (semi)unbiased cluster analysis based on objectively measured clinical, pathobiological and physiological variables.[106] In 2008, Haldar et al. published a seminal paper on the analysis of the clinical data and the inflammatory markers of a UK cohort using cluster analysis.[62] They identified four different clusters: 1) early-onset atopic asthma, 2) obese non-eosinophilic asthma, 3) early-onset symptom predominant asthma and 4) late-onset, inflammation predominant asthma. Clusters 3 and 4 were characterised by a remarkable discordance between symptom expression and markers of eosinophilic airway inflammation. Cluster analyses have also been performed in childhood asthma (Table 4). A cluster analysis of children participating in the Severe Asthma Research Program (SARP) identified four clusters of patients.[107] The major discriminators for cluster assignment were asthma duration, number of used controller medication and baseline lung function. In contrast to the clusters identified in adults, health care use was not a strong discriminator in childhood asthma. Recently, these clusters have been validated in three clinical trial populations.[108] In this latter study, treatment response was also assessed. Overall, there was no clear pattern of treatment response across the different clusters, but there were differences between clusters within the different trials.[108]

Advances in mathematical techniques used to interpret multi-dimensional data are rapidly evolving. The statistical method used and the clinical parameters and biomarkers included in the dataset guide the analysis process. Ideally, cluster analysis should be unbiased or unsupervised. However, until now most cluster studies in

asthma[62, 107, 109, 110] have been performed with defined steps of supervision; by, for example, using hierarchical cluster analysis to obtain a number of clusters (*k*) and then use this *k* for a subsequent *k*-means cluster analysis. Although not optimal, at this time, the inclusion of data supervision steps seems essential for the creation of a clustering model.

In addition to clinical values, future research should include a multitude of biomarkers (e.g. genetic markers and inflammatory markers) that have demonstrated discriminative value in prior studies in order to assess which combination of factors have the highest predictive values. However, the promise of multi-dimensional analyses does not mean that there will no longer be a motivation for single biomarker discovery, as this will remain important to support/feed clustering algorithms. Eventually, these new approaches to respiratory care could contribute to the development of simple primary care algorithms to facilitate the diagnosis of asthma phenotypes, as well as leading to the implementation of complex tertiary care models to measure and optimise treatment response.

Table 4. Cluster analyses in childhood asthma

Study	Study population	Clusters	Description of clusters
Fitzpatrick et al., 2010 [81]	161 children (6-17 years)	4	Cluster 1: late-onset symptomatic asthma Cluster 2: early-onset atopic asthma with normal lung function Cluster 3: early onset atopic asthma with mild airflow limitation and comorbidities Cluster 4: early-onset atopic asthma with advanced airflow limitation Main predictors of cluster assignment: asthma duration, number of asthma controller medications, and baseline lung function
Chang et al., 2013 [108]	611 children (6-18 yrs)	4	Replication of the 4 clusters of Fitzpatrick et al. [81] in three Childhood Asthma Research and Education (CARE) Network clinical trials
Just et al., 2012 [109]	315 children (6-12 yrs)	3	Cluster 1, asthma with severe exacerbations and multiple allergies Cluster 2, severe asthma with bronchial obstruction Cluster 3, mild asthma (no clinical features)

International collaboration

Despite the value of single replication studies, the validation and clinical implementation of asthma biomarkers for treatment response requires a joint effort. Especially with regard to the field of pharmacogenomics, large patient numbers are required in order to have enough power to detect significant effects.

Collaborations are starting to emerge, but the field will only evolve when 1) experts are exchanging knowledge on a regular basis, 2) studies are performed in larger study populations or carried out as large meta-analyses, and 3) a consensus is reached on how to define treatment response. Given that there is a need for an official consortium to guide these issues in childhood asthma, a global consortium on Pharmacogenomics in Childhood Asthma (PiCA) was recently founded.[111] The consortium aims to collect data on at least 10,000 asthmatic children with well-defined phenotype and genotyping data to be made available by uniting birth cohorts and paediatric asthma studies. This consortium could facilitate replication and knowledge exchange, and when GWAS data of all these children would become available, PiCA could be a powerful platform to identify new pharmacogenomics markers. A recent inventory of studies and cohorts that have shown interest in participating in PiCA shows that the consortium will likely cover the whole spectrum of pediatric asthmatic patients. This will make it possible to stratify analyses, and assess genetic effects in the overall paediatric asthma population, as well as in specific subgroups, such as severe asthmatic children. PiCA could also be a platform to study other biomarkers, such as inflammatory markers in blood and exhaled air, and would be a suitable tool to perform unbiased cluster-analysis.

Randomised controlled trials for *ADRB2*-guided treatment

RCTs are the gold standard for evaluating the effectiveness of clinical interventions. RCTs involving pharmacogenomics, as an intervention to guide treatment or *ad hoc* pharmacogenomics analysis, are very rare in asthmatic children.[11] Pharmacogenomics of ICS is still in its discovery phase and has not yet let to translation into health applications.[112] The pleiotropic effects of corticosteroids on multiple signalling pathways, which are not fully understood, complicate the search for clinical useful markers. Further studies are necessary to identify and validate genetic markers associated with ICS response.

Pharmacogenomics of LABA, in contrast, has progressed further into clinical translation. A recent, RCT of Lipworth et al.,[11] assessed the clinical consequences of *ADRB2* genotyping in childhood asthma. Asthmatic children homozygous for the *ADRB2* variant genotype (Arg16) benefited more from a LTRA than from a LABA as an add-on treatment to inhaled corticosteroids. It is debatable as to if the choice of LTRA (an extra anti-inflammatory agent) in addition to ICS, instead of another type of bronchodilator, is the most scientifically sound. Nevertheless, the study showed that the group treated with LTRA scored better on asthma symptoms

and quality of life, used less rescue medication and were absent fewer days from school compared to the LABA group. The two groups did not differ in lung function improvement. These data suggests that prospective genotyping of *ADRB2* may have clinical consequences for childhood asthma management, at least when taking subjective, but still important, clinical outcomes such as asthma symptoms and quality of life into consideration.

Large randomised controlled trials are necessary to evaluate the clinical value of *ADRB2*-guided treatment in asthmatic children in comparison to current clinical practises. Pharmacoeconomic evaluations to assess the cost-benefit ratio of these interventions are needed as well.

In conclusion

The studies described in this thesis focused on the genetic and inflammatory mechanisms underlying ICS response in asthmatic children. Taken together, these studies provide more insight into decreased drug responsiveness in asthmatic children and propose potential valuable biomarkers for clinical practice. Poor treatment response in childhood asthma is likely to be caused by interplay of genetic risk factors influencing the efficacy of drug pathways and immune mechanisms that are intrinsically less sensitive to these drugs. In the near future, clinically applicable algorithms incorporating genetic and inflammatory markers may help to diagnose and guide treatment. The first steps towards this personalised treatment involve the identification and validation of potential biomarkers. Patient populations recruited in a secondary care setting and included in clinical trials, might be very suitable for the identification of biomarkers. Nevertheless, these markers should subsequently be validated in heterogeneous patient populations, covering the whole spectrum of asthmatic children. The association of a biomarker with a certain outcome or asthma phenotype might be stronger in pre-selected and well-monitored clinical trial populations, as compared to the 'real world' asthma population. The value of cohort studies recruited through primary care, such as the PACMAN cohort, should not be underestimated.

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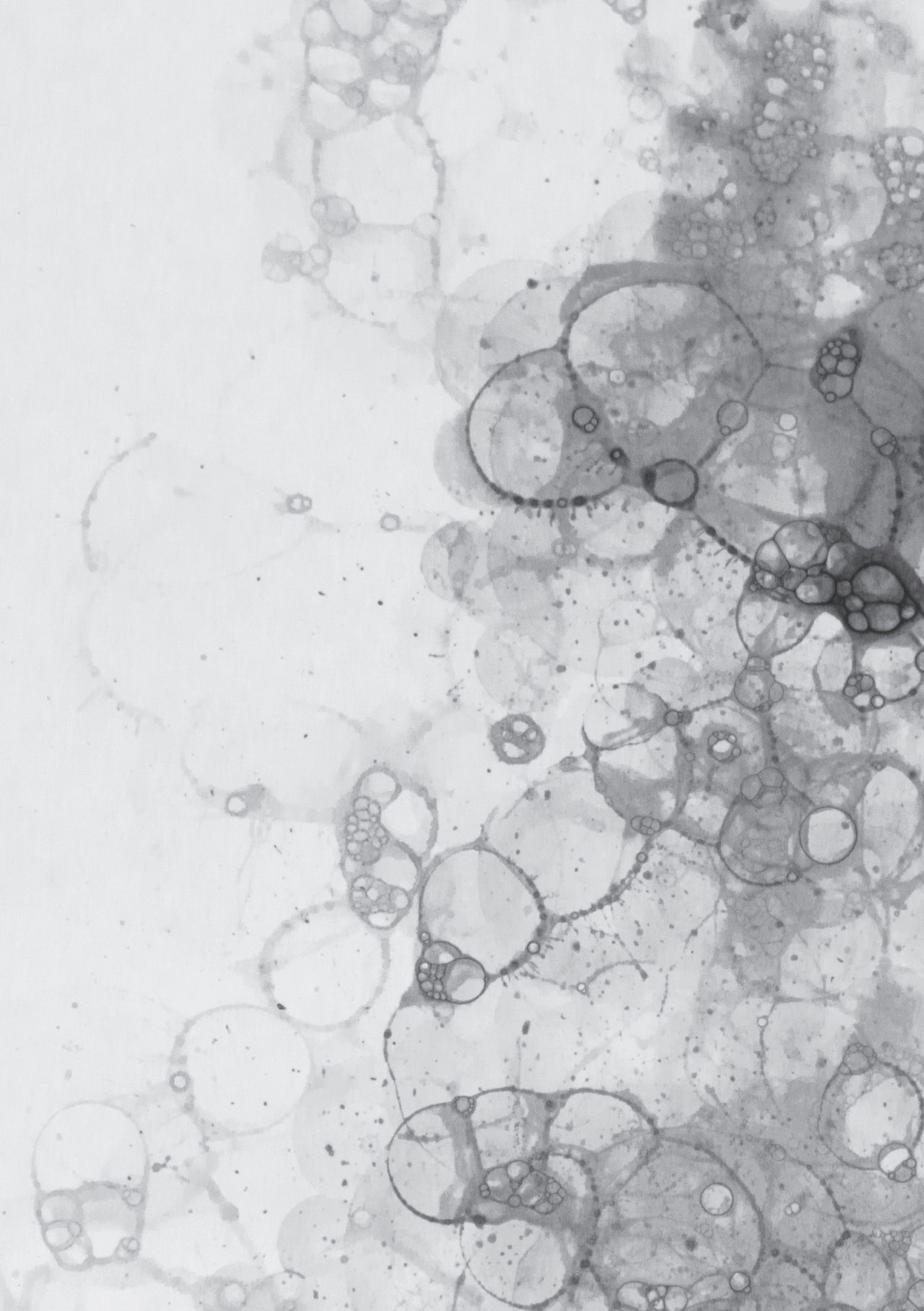
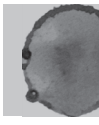


Appendices

Scientific summary

Asthma is a chronic disease of the airways and the most common chronic disease among children. Asthma patients often exhibit symptoms such as wheezing, coughing and breathlessness. Pathologically, the disease is characterised by airway inflammation, airway hyperresponsiveness (AHR) and recurrent episodes of reversible airway obstruction. Inhaled corticosteroids (ICS) are the cornerstone of persistent asthma treatment and are thought to function due to their anti-inflammatory properties. Additionally, β_2-adrenergic receptor agonists (short- or long-acting) are used in asthma treatment to relieve asthma symptoms. Although asthma treatment is effective in many patients, there is large variability in the level of symptom control and lung function improvement. Furthermore, a small group of patients continues to suffer from severe exacerbations, or long-term uncontrolled asthma symptoms, despite asthma maintenance treatment. Understanding biological profiles underlying treatment response is of great clinical value in order to improve and stratify treatment strategies and to identify new drug targets. In this thesis the biological profile underlying asthma treatment outcomes in children was studied on three different levels: I) genetics (**Chapter 2**), II) inflammatory signals in blood (**Chapter 3)** and III) inflammatory signals in exhaled breath (**Chapter 4**).

Chapter 1 'Introduction' describes the background of this thesis. In **Chapter 1.1** the scope and objective of the research described in this thesis are addressed. This chapter also provides a historical perspective on the rise of ICS as the leading treatment for persistent asthma. Furthermore, the heterogeneity of asthma is addressed, as well as the rationale behind the PACMAN cohort, a Dutch pharmacy-based cohort study of children with a reported use of asthma medication. **Chapter 1.2** provides an in-depth review of the current knowledge on asthma biomarkers for treatment response and asthma phenotypes. As underlying airway inflammation is considered to be the hallmark of asthma pathology, and different types of airway inflammation have been described in asthma patients, there is a significant interest in the development of asthma biomarkers in order to identify clinical relevant asthma phenotypes, optimise diagnosis and guide treatment. Currently, the most tissue-specific and reliable method to assess airway inflammation is a combined procedure of bronchoscopy, biopsy and bronchoalveolar lavage. However, these procedures are very invasive. In recent years several less invasive diagnostic tools using sputum, peripheral blood, exhaled breath, urine and saliva to assess



airway inflammation have been investigated. Nevertheless, very few studies have addressed whether or not these inflammatory markers are also associated with treatment response.

Chapter 2 'Genetics' focuses on the genetic profiles associated with asthma treatment responses in children. The field of pharmacogenetics/genomics studies the effect of variation in the DNA sequence on drug response. These genetic variations are present in all tissues and can be measured in the DNA, which is most often obtained from blood samples, but can also be obtained non-invasively from saliva samples. **Chapter 2.1** describes the details of our investigation into whether variations in genes involved in the glucocorticoid signalling pathway, as well as variations in asthma genes, could perhaps explain why some children suffer from severe asthma exacerbations despite using ICS. Using DNA samples obtained from asthmatic children who participated in our own PACMAN cohort, as well those who participated in two Scottish studies (BREATHE and PAGES), fifty genetic loci in seventeen genes were analysed. Two genetic loci in the gene *ST13* were found to be associated with an increased risk of severe exacerbations despite ICS treatment. The *ST13* gene codes for a co-chaperone of the glucocorticoid receptor complex and might be indirectly involved in the functionality of the glucocorticoid receptor. We then investigated this association in a fourth study population consisting of a North American clinical trial population of asthmatic children on ICS (CAMP). When the CAMP population was included in a meta-analysis with the PACMAN, PAGES and BREATHE population, *ST13* continued to be associated with severe exacerbations. However, the effect sizes were modest, indicating that variation in this gene can only explain a small part of the observed heterogeneity in treatment response. *ST13* had not previously been identified as a risk gene for poor ICS response.

Previous studies have reported that the 17q21 locus has an influence on the risk of childhood asthma, as well as with the risk of more severe asthma. The locus was also included in the panel of loci assessed in **Chapter 2.1**, but was not found to be significantly associated with an increased risk of exacerbations in a meta-analysis of the three North-European studies PACMAN, PAGES and BREATHE. However, in **Chapter 2.2** the locus was studied in more detail, and it was observed that the locus was associated with an increased risk of severe exacerbations despite ICS treatment when CAMP was added to the meta-analysis of the North-European studies. This suggests that the analysis described in **Chapter 2.1** lacked statistical

power to identify the association. It has been recently reported that one of the genes in the 17q21 locus, *ORMDL3,* influences eosinophil activation. Since increased levels of eosinophils are often observed in the airways of symptomatic asthmatic patients, altered eosinophil activation might reflect an inflammatory phenotype less responsive to the anti-inflammatory properties of ICS.

Another gene that has been implicated in ICS treatment response is *GLCCI1*. Previous studies reported that the lung function of asthma patients with a variant *GLCCI1* genotype improved less upon ICS treatment as compared to patients without the variant. However, there was no evidence that the children and young adults from the PACMAN, PAGES and BREATHE study, with a reported use of ICS and with a variant *GLCCI1* genotype, had an increased risk of severe asthma exacerbations or suffered from more asthma symptoms, nor were they receiving higher daily ICS dosages (**Chapter 2.3**).

Pharmacogenetic studies have not only addressed the role of genetic variation in response to ICS, but have also examined the role of genetic variation in the response to treatment with long-acting ß$_2$-adrenoceptor agonists (LABA). A LABA is added as needed to a treatment regime when an asthmatic patient is still symptomatic despite the use of ICS and short-acting ß$_2$-adrenoceptor agonists (SABA). Variations in the gene coding for the β_2-adrenergic receptor (*ADRB2*) have been associated with a poorer response to LABA. Recent clinical trial data from asthmatic children showed that children with the *ADRB2* Arg16Arg risk genotype have a poorer response to the addition of LABA to the treatment regime, in comparison to the addition of an alternative drug (leukotriene antagonists). In an attempt to replicate the association between the Arg16Arg genotype and a poor response to LABA, the *ADRB2* genotype and LABA outcome was studied in the PACMAN cohort (**Chapter 2.4).** It was observed that children in the PACMAN cohort carrying the Arg16Arg genotype who reported the use of ICS and LABA had an increased risk of severe exacerbations in comparison to their counterparts carrying the Gly16Gly genotype. This effect was not observed in Arg16Arg genotype carriers reporting ICS use only. These results suggest that LABA are indeed less effective in children homozygous for *ADRB2* Arg16 genotype.

Chapters 2.1-2.4 describe so-called candidate-gene studies, in which the association between variations in selected genes and an outcome (i.e. drug

response) was studied, and gene selection was based on biological knowledge or on previous reported associations. **Chapter 2.5** describes the use of a different approach in which a pharmacogenomic analysis of participants of the clinical trial CATO was performed using an exome-chip. This chip assessed over two hundred thousand genetic variants at one time (focusing on coding regions in the DNA) using a data-driven, rather than hypothesis-driven approach. In the CATO trial, the lung function and airway hyperresponsiveness of asthmatic children treated with ICS were studied over a period of two years. In this pharmacogenomic analysis, we defined response phenotypes based on lung function or airway hyperresponsiveness improvement during the trial, taking the level of medication into account. However, no loci on the exome chip were significantly associated with these response phenotypes when corrected for multiple testing. Despite the presence of extensive longitudinal measurements, the study was limited by a relative small sample size (110 children). Nevertheless, closer investigation of the 17q12-21 locus (also known as the 17q21 locus) resulted in interesting findings: an enrichment of risk alleles in the 17q12-21 region in the CATO population, as well as nominal significant associations with measures of ICS treatment response were observed.

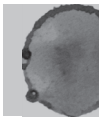
Chapter 3 'Inflammation' shifts from genetic markers to the expression of inflammatory patterns in childhood asthma. In **Chapter 3.1** the design and rationale of the PACMAN2 study are described. This explorative follow-up study of the PACMAN cohort was initiated in order to study inflammatory patterns in peripheral blood and exhaled air in well- and poorly controlled asthmatic children treated with ICS. Children from the PACMAN cohort were selected based on their age (≥ 8 years), long-term asthma control (poor or well-controlled symptoms in the year preceding the baseline visit to the pharmacy) and adherence to ICS. During a follow-up study visit (PACMAN2) to the Wilhelmina's Children Hospital, the children's asthma symptoms, medication use and adherence were assessed using a questionnaire. Furthermore, lung function and bronchodilator response, and markers in exhaled air were measured. Lastly, a venepuncture was performed in order to study inflammatory signals in peripheral blood.

Thirty-seven children were seen in PACMAN2, and in **Chapter 3.2** preliminary results of this study concerning inflammatory markers on peripheral blood granulocytes are described. Inflammatory cells, such as eosinophils and neutrophils,

in the peripheral blood of asthmatic adults have been reported to be increasingly sensitive to inflammatory stimuli compared to healthy controls. This process, called 'priming', seems to be an intermediate step to the activation of these cells. Fully primed cells will extravasate rapidly into the tissue, and studying the priming status of these cells in peripheral blood has been reported to be a read-out of the activity of current airway inflammation. The priming status of the peripheral blood eosinophils and neutrophils of 27 children participating in the PACMAN2 study has been investigated. Remarkably, in this study, children with well-controlled asthma did not differ in the priming status of their eosinophils and neutrophils, when compared to children with poorly controlled asthma. Yet, it was observed that high levels of exhaled nitric oxide (FeNO) were accompanied with low levels of priming.

Chapter 4 'Breath' investigates asthma biomarkers in breath. Almost a decade ago the first reports emerged of elevated levels of nitric oxide in exhaled breath (FeNO) in patients with asthma. High FeNO levels are regarded as a surrogate marker of ongoing eosinophilic airway inflammation and can easily be measured. Despite the initial enthusiasm about FeNO as a new and non-invasive marker of airway inflammation, the clinical usefulness of FeNO in the assessment of asthma control is still being debated. In the PACMAN study the correlation between a single measurement of FeNO and the asthma control questionnaire score was poor (**Chapter 4.1**). Furthermore, various other factors, including age, living environment and medication use, were independently associated with increased FeNO levels, complicating the clinical interpretability of a single FeNO measurement. In **Chapter 4.2,** a novel metabolomics approach used to study the molecular signatures of asthma in children participating in the PACMAN2 study is described. Profiles of volatile organic compounds ('breath prints') were measured in exhaled breath and analysed on a panel of gas multi-sensor arrays ('electronic noses'). Three of the four electronic noses could distinguish children with uncontrolled asthma symptoms from those with well-controlled symptoms with moderate to high accuracy.

Finally, the **General Discussion** in **Chapter 5** further elaborates on the studies described in this thesis. Their main findings are discussed and placed in a broader context. This chapter argues that poor treatment response in childhood asthma is caused by an interplay of genetic risk factors influencing the efficacy of drug pathways, and inflammatory patterns that are intrinsically less sensitive to these drugs. These inflammatory patterns may, in turn, be genetically determined. This



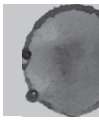
chapter also addresses related limitations such as the definition of treatment response and the nomenclature of difficult asthma. It concludes with concrete suggestions for future research: asthma phenotypes should be studied using a multi-dimensional biomarker-approach, rather than a single biomarker-approach. Secondly, there is a need for international collaboration to exchange knowledge, obtain larger study populations and validate biomarkers. Lastly, large RCTs are required to evaluate the clinical value of *ADRB2*-guided treatment in asthmatic children in comparison to current clinical practises.

Samenvatting

Astmatische kinderen en medicatierespons

Astma is de meest voorkomende chronische ziekte onder kinderen. Astmapatiënten hebben vaak last van terugkerende periodes van benauwdheid, hoesten en een piepende ademhaling. De longen van astmapatiënten zijn overgevoelig voor prikkels, bijvoorbeeld voor kou, pollen, sigarettenrook of huisstofmijt. Dit leidt tot het samentrekken van de spiertjes rondom de luchtwegen waardoor vernauwing optreedt. Tevens gaat het slijmvlies aan binnenkant van de luchtwegen meer slijm produceren en zwelt het op. Verschillende soorten ontstekingscellen kunnen uit het bloed naar de luchtwegen trekken en veroorzaken daar luchtwegontsteking. Een ontsteking is een reactie van het lichaam op schadelijke prikkels, maar kan dus ook ontstaan in de afwezigheid van bacteriën of virussen, als het afweersysteem 'denkt' dat een bepaalde stof toch schadelijk is.

Astma is nog niet te genezen en astmamedicijnen zijn voornamelijk gericht op het verminderen van de astmaklachten. Kinderen met milde astmaklachten krijgen vaak een kortwerkend inhalatiemiddel voorgeschreven om de vernauwing van de luchtwegen tegen te gaan, een zogenaamd kortwerkende luchtwegverwijder. Ze kunnen dit, indien nodig, gebruiken op het moment dat er aanvallen van benauwdheid optreden. Door het medicijn te inhaleren met behulp van een inhalator komt het direct in de luchtwegen terecht. Als de astmaklachten blijven aanhouden kan de huisarts daarnaast ontstekingsremmers zoals inhalatiecorticosteroïden (ICS) voorschrijven. Deze medicijnen onderdrukken de ontsteking in de luchtwegen, die op hun beurt de benauwdheidsklachten veroorzaken. Als de klachten niet onder controle blijven ondanks het gebruik van kortwerkende luchtwegverwijders en ICS, kan er eventueel een langwerkende luchtwegverwijder aan de behandeling worden toegevoegd. Ook kan er een alternatieve ontstekingsremmer, een leukotrieenantagonist, aan de behandeling worden toegevoegd. Bij een zeer ernstige astma-aanval kan een korte stootkuur van orale corticosteroïden worden voorgeschreven. Astmamedicijnen, zoals ICS en luchtwegverwijders, werken in het lichaam doordat ze op celniveau kunnen binden aan speciale eiwitten op de celwand, zogenaamde receptoren, en daardoor signaalroutes activeren. Door een serie van dergelijke signalen in de long teweeg te brengen, kunnen de processen verantwoordelijk voor de klachten worden beïnvloed.



De meeste kinderen reageren goed op astmamedicijnen; de astmaklachten verdwijnen of verminderen sterk en de functie van de longen verbetert. Niet elk kind reageert echter even goed op de medicijnen. Het ene kind blijft meer klachten houden dan het andere. Een kleine groep van de kinderen met astma (5-10%) houdt last van ernstige astmaklachten ondanks het gebruik van astmamedicijnen zoals ICS. Biologische factoren, zoals variaties in het genetische materiaal (DNA) en het type ontsteking in de luchtwegen, kunnen hierbij een rol spelen. Er lijken verschillende vormen van astma te bestaan, die beter of slechter reageren op astmabehandeling. Om astma beter te kunnen behandelen is het belangrijk om kinderen die slecht reageren op astmamedicatie vroegtijdig te herkennen. Dit geeft de mogelijkheid om deze kinderen tijdig door te verwijzen naar het ziekenhuis of om de behandeling op voorhand aan te passen. Daarvoor is echter meer kennis nodig over de biologische factoren die de verschillende soorten astma onderscheiden. Deze kennis zou ook nieuwe aangrijpingspunten kunnen bieden voor de ontwikkeling van innovatieve astmamedicijnen.

Dit proefschrift

Dit proefschrift beschrijft onderzoek naar de respons op astmamedicatie bij kinderen. De focus van het onderzoek ligt op het bestuderen van factoren in het lichaam die zouden kunnen voorspellen of een kind veel klachten zal houden ondanks het gebruik van astmamedicijnen. Van verschillende stoffen is onderzocht of ze gebruikt kunnen worden als biomarker, een biologische parameter die in het lichaam gemeten kan worden en waarmee een bepaalde conditie of ziektestaat kan worden bepaald of voorspeld. Bijvoorbeeld; een stof in het bloed die meet hoe actief de ontsteking in het lichaam is. Maar ook een stukje genetische informatie, een zogenaamde genetische marker, dat voorspelt hoe groot de kans is dat een bepaald type behandeling aan zal slaan, is een biomarker. In het onderzoek beschreven in dit proefschrift zijn onderzoeksmethoden uit de farmaco-epidemiologie gecombineerd met onderzoeksmethoden uit de genetica en de immunologie. De farmaco-epidemiologie houdt zich bezig met het bestuderen van medicijngebruik in grote groepen patiënten. De genetische en immunologische methoden beschreven in dit proefschrift, zijn vaak analyses van cellen en genetisch materiaal uitgevoerd in een laboratorium.

Hoofdstuk 1 is een inleidend hoofdstuk. In **hoofdstuk 1.1** wordt de achtergrond van het onderzoek beschreven. In dit hoofdstuk wordt onder meer kort de geschiedenis

van astmamedicijnen uiteengezet. Ook wordt de PACMAN cohortstudie toegelicht. In de PACMAN-studie worden gegevens verzameld van kinderen die regelmatig astmamedicatie gebruiken. Kinderen en hun ouders zijn benaderd via apotheken aangesloten bij het UPPER netwerk van de Universiteit Utrecht. Van de kinderen is onder meer speeksel verzameld voor DNA onderzoek. Ook is er bij deze kinderen een blaastest uitgevoerd om bepaalde stoffen in uitademingslucht te meten en hebben de ouders een uitgebreide astma-vragenlijst ingevuld om de aard en ernst van de luchtwegklachten en de reactie op medicijnen vast te leggen. Veel studies beschreven in dit proefschrift zijn gebaseerd op data verzameld in de PACMAN studie. In **hoofdstuk 1.2** wordt een overzicht gegeven van wat er in de wetenschappelijke literatuur momenteel al bekend is over astma-biomarkers. Biomarkers die al gebruikt worden bij de behandeling van astma worden beschreven, evenals biomarkers die momenteel onderzocht worden om de verschillende astmatypen beter te kunnen karakteristeren.

Genetische factoren en medicatierespons

De studies beschreven in **hoofdstuk 2** richten zich op de genetische verschillen tussen kinderen met een slechte en kinderen met een goede respons op bepaalde astmamedicijnen. In het onderzoek beschreven in **hoofdstuk 2.1** zijn variaties in 17 genen bestudeerd. Deze genen werden geselecteerd op basis van hun rol in de corticosteroïde-signaalroute in het lichaam, of hun associatie met het ontwikkelen van astma. Het onderzoek werd uitgevoerd bij kinderen en jongvolwassenen die ICS gebruikten voor luchtwegklachten en waarvan gegevens zijn verzameld in de eerder genoemde Nederlandse PACMAN-studie, en in de Schotse PAGES en BREATHE studies. De hypothese van het onderzoek was dat veranderingen in bepaalde genen het risico op ernstige astma-aanvallen zouden kunnen beïnvloeden. Uit een analyse van deze drie studies samen bleek dat twee variaties in het gen *ST13* geassocieerd waren met een verhoogd risico op ernstige astma-aanvallen. Kinderen die drager waren van één van deze genetische varianten, hadden een verhoogde kans op ernstige astma-aanvallen. Vervolgens werden gegevens van een vierde studie van astmatische kinderen aan de analyse toegevoegd; de Noord-Amerikaanse CAMP trial. Ook in een gecombineerde analyse van de vier studies samen bleef variatie in het *ST13* gen geassocieerd met ernstige astma-aanvallen ondanks het gebruik van ICS. Het *ST13* gen speelt een rol in de corticosteroïde-signaalroute in het lichaam en is nog niet eerder in verband gebracht met een verminderde respons op astmamedicatie.

Het onderzoek beschreven in **hoofdstuk 2.2** laat zien dat variatie op een andere specifieke plek (of 'locus') in het DNA, de 17q21 locus, ook geassocieerd is met een verhoogde kans op ernstige astma-aanvallen. Deze plek in het DNA werd onderzocht bij kinderen en jongvolwassenen uit de PACMAN, BREATHE, PAGES en CAMP studies. De genetische variant in de 17q21 locus reguleert verschillende genen, en het is nog onduidelijk welke van deze genen precies betrokken is/zijn bij astma. Eerder onderzoek heeft wel beschreven dat één van de genen in dit gebied betrokken is bij de activatie van ontstekingscellen, een proces dat bij astma-aanvallen van belang is.

In de studie beschreven in **hoofdstuk 2.3** is variatie in een ander gen, *GLCCI1*, onderzocht bij kinderen uit de PACMAN, BREATHE en PAGES studies. Het is nog onduidelijk welke rol het gen heeft in het lichaam. Eerder onderzoek liet zien dat de longfunctie van volwassenen met astma met een variant *GLCCI1* gen minder verbeterde tijdens ICS behandeling, dan bij astmapatiënten zonder een variant *GLCCI1* gen. De hypothese van het onderzoek beschreven in dit hoofdstuk was dat kinderen met het variante gen vaker last zouden hebben van ernstige astma-aanvallen en ook vaker een hogere dosis ICS zouden gebruiken, omdat het geneesmiddel bij deze kinderen wellicht minder goed werkt. In het onderzoek werd echter geen bewijs gevonden voor deze hypothese; er werden geen verschillen gevonden tussen de kinderen met, en kinderen zonder, de genetische variant.

In **hoofdstuk 2.4** wordt onderzoek beschreven naar het *ADRB2* gen bij kinderen van de PACMAN studie. Dit gen bevat informatie voor een receptor waar bepaalde typen veelgebruikte kort- en langwerkende luchtwegverwijders op aangrijpen; de β_2-adrenerge receptor. PACMAN kinderen, die een variant *ADRB2* gen droegen en behandeld werden met een combinatie van ICS en een langwerkende luchtwegverwijder (die zijn werking uitvoert door aan te grijpen op de β_2-adrenerge receptor), hadden een verhoogde kans op een ernstige astma-aanval. Dit verhoogde risico werd niet waargenomen bij kinderen die alleen ICS gebruikten. Deze resultaten suggereren dat de langwerkende luchtwegverwijders die aangrijpen op deze specifieke receptor minder goed werken bij kinderen met een variant *ADRB2* gen.

In **hoofdstuk 2.5** wordt een studie beschreven waarbij vele genetische verschillen tegelijkertijd werden bestudeerd bij 110 astmatische kinderen die deelnamen aan

de CATO studie. Met behulp van een DNA chip, een plaatje waarop duizenden minuscule DNA stukjes zijn gehecht, kon de aanwezigheid van meer dan tweehonderdduizend verschillende genetische varianten tegelijkertijd worden bepaald. In de CATO studie zijn astmatische kinderen twee jaar lang behandeld met ICS. De ICS dosering kon tussentijds aangepast worden op basis van klachten en/of de prikkelgevoeligheid van hun longen. Tevens werd er DNA verzameld van deze kinderen. Het doel van de studie beschreven in dit hoofdstuk was om te onderzoeken of bepaalde genetische verschillen vaker voorkwamen in de CATO kinderen die weinig verbetering lieten zien in hun longfunctie of in de prikkelgevoeligheid van hun longen, in vergelijking met de CATO kinderen die op die vlakken wel verbeterden. In deze studie werden geen significante verschillen gevonden, wat wellicht toe te schrijven is aan de beperkte patiëntenpopulatie in deze studie. De DNA chip bevatte ook informatie over de 17q21 locus en nadere bestudering van die locus gaf wel aanwijzingen dat deze DNA regio ook in de CATO patiëntenpopulatie gerelateerd is aan een verminderde ICS respons.

De pre-activatie van ontstekingscellen in het bloed

In **hoofdstuk 3** wordt het karakteriseren van ontstekingspatronen bij astmatische kinderen beschreven. Allereerst wordt de opzet van de PACMAN2 studie uiteengezet (**hoofdstuk 3.1**). PACMAN2 is een studie, uitgevoerd in het PACMAN cohort, waarbij een specifieke groep kinderen werd uitgenodigd voor aanvullend onderzoek in het ziekenhuis. Voor dit onderzoek werden PACMAN kinderen met veel of juist weinig astmaklachten geselecteerd. Om in aanmerking te komen voor het PACMAN2 onderzoek, moesten de kinderen ook ICS gebruikers zijn. Tijdens het studiebezoek werden de astmaklachten in kaart gebracht, evenals het medicatiegebruik en er werd gecontroleerd of het kind de medicatie gebruikte zoals voorgeschreven door de arts. Daarnaast werden er longfunctie testen gedaan, werden stoffen in uitgeademde lucht gemeten en werd er bloed geprikt bij de kinderen om de ontstekingscellen die een rol spelen bij astma te karakteriseren.

De eerste resultaten van de analyses van ontstekingscellen in het bloed van kinderen uit de PACMAN2 studie worden gepresenteerd in **hoofdstuk 3.2**. Ontstekingscellen, zoals eosinofielen en neutrofielen, bevinden zich ook in het bloed van gezonde mensen, maar zijn daar niet actief. Door bepaalde signalen kunnen de cellen aangezet worden om de bloedbaan te verlaten en zich naar specifieke weefsels te verplaatsen, zoals naar de geprikkelde luchtwegen. Daar

kunnen ze geactiveerd worden. Eerder onderzoek heeft aangetoond dat voordat de ontstekingscellen de bloedbaan verlaten, ze eerst worden gepreactiveerd, dit proces wordt 'priming' genoemd. Bij astmatische patiënten wordt vaak een toename gezien in het aantal gepreactiveerde ontstekingscellen. In deze studie werd onderzocht of de mate van pre-activatie van de ontstekingscellen in het bloed samenhing met het aantal astmaklachten bij de PACMAN2 kinderen, dit was echter niet het geval. Gepreactiveerde ontstekingscellen in het bloed werden zowel waargenomen bij kinderen met veel astmaklachten als bij kinderen met weinig astmaklachten.

Stoffen in uitademingslucht

Hoofdstuk 4 richt zich op astma-biomarkers in uitgeademde lucht. Allereerst werd onderzocht welk deel van de uitgeademde lucht bestond uit stikstofmonoxide bij een grote groep PACMAN kinderen (**hoofdstuk 4.1**). De hoeveelheid stikstofmonoxide in uitademingslucht wordt FeNO genoemd. Eerder onderzoek heeft laten zien dat de hoeveelheid FeNO vaak verhoogd is bij astmatische patiënten. Gedacht wordt dat de hoogte van FeNO aangeeft hoe actief de ontsteking in de luchtwegen is. FeNO wordt in de klinische praktijk regelmatig gemeten bij astmatische patiënten, maar de klinische relevantie staat nog ter discussie. Het onderzoek in dit hoofdstuk laat zien dat een hoge FeNO niet noodzakelijkerwijs gepaard gaat met veel astmaklachten. Verschillende andere factoren waren geassocieerd met verhoogde FeNO waarden bij kinderen in de PACMAN studie, waaronder; leeftijd, leefomgeving en het type medicatie dat gebruikt werd. Dit maakt het moeilijk om FeNO eenduidig te interpreteren.

In de studie beschreven in **hoofdstuk 4.2** werd, door gebruik te maken van een zogenaamde "electronic nose", niet één specifieke stof bestudeerd, maar patronen van vele verschillende stoffen die aanwezig zijn in uitademingslucht. De uitgeademde lucht van 33 PACMAN2 kinderen werd geanalyseerd door een panel van vier verschillende typen elektronische neuzen die stoffen in die lucht kunnen detecteren. De eerste resultaten laten zien dat drie van de vier onderzochte elektronische neuzen onderscheid kunnen maken tussen kinderen met veel klachten en kinderen met weinig astmaklachten aan de hand van hun uitademingslucht.

Discussie en toekomstig onderzoek

Het proefschrift wordt afgesloten met een algemene discussie gepresenteerd in **hoofdstuk 5**, waarin de belangrijkste resultaten uit de hiervoor beschreven studies besproken worden en in een breder kader worden geplaatst. Het onderzoek beschreven in dit proefschrift laat zien dat niet elke type astma hetzelfde is, en dat factoren in speeksel of uitgeademde lucht wellicht mede kunnen voorspellen welke kinderen klachten zullen blijven houden ondanks het gebruik van medicijnen. In dit hoofdstuk wordt beargumenteerd dat een slechte medicatierespons van kinderen met astma het resultaat is van een ingewikkeld samenspel van genetische factoren en ontstekingspatronen. De genetische varianten die iemand draagt kunnen de aangrijpingspunten van een bepaald medicijn en de werking van medicijn-signaalroutes in het lichaam beïnvloeden. Hierdoor kan een medicijn beter of slechter zijn werk doen. Tevens kunnen ontstekingspatronen in de luchtwegen meer of minder gevoelig zijn voor de medicijnen. Welk type ontstekingscellen geactiveerd wordt en betrokken is bij de ontstekingscascade in de luchtwegen kan weer genetisch vastgelegd zijn. Verder worden in dit hoofdstuk wetenschappelijke beperkingen besproken, zoals het gebrek aan uniforme definities van medicatierespons en 'moeilijk behandelbaar' astma. De discussie wordt afgesloten met aanbevelingen voor toekomstig onderzoek:

- Verdere bestudering van astmafenotypes bij kinderen door middel van analyses waarin klinische en biologische parameters gecombineerd kunnen worden zonder van te voren een selectie van deze parameters te maken. Deze geavanceerde analysemethoden ontwikkelen zich momenteel zeer snel en zouden uiteindelijk kunnen leiden tot klinisch toepasbare behandelalgoritmen en behandeling op maat.

- Internationale samenwerking om verschillende cohorten met patienten bij elkaar te brengen om geïdentificeerde biomarkers snel te kunnen valideren in grote en verschillende patiëntengroepen.

- Het opzetten van een grote gecontroleerde klinische studie om te bepalen of het klinisch relevant is om kinderen met een variant *ADRB2* gen te behandelen met een luchtwegverwijdend medicijn dat niet aangrijpt op de β_2-adrenerge receptor.

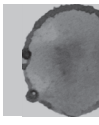
Dankwoord

Mijn promotietraject is een bijzondere reis geweest. Een tocht langs onbekende oorden. Er zijn stormen getrotseerd, monsters verslagen en er is nieuw land verkend. Velen hebben, ieder op hun eigen manier, bijgedragen aan dit prachtige avontuur. Dank hiervoor.

De voorbereidingen van de tocht waren al lange tijd in gang. Reeds in 2006 bereidden Mohsen en Tomas me in Stockholm voor op de reis, waarvan ik toen nog niet wist dat die er ook daadwerkelijk zou gaan komen. Ze leerden me te vertrouwen op mijn innerlijk kompas. In Amsterdam leerde ik vervolgens van Toine en Frans wijze lessen over het bepalen van routes die niet voor de hand lagen en het veilig manoeuvreren van een schip in woelige wateren.

Maar in 2009 ging de reis pas echt van start, in Utrecht. Gesteund door mijn co-promotor en mijn promotoren, met mijn bootje de wijde blauwe zee op. Anke-Hilse hield het roer nauwkeurig in de gaten. Lagen we nog op koers? Was er storm op komst? Jan en Leo keken vanaf het achterdek goedkeurend mee. Gebogen over de zeekaarten waren er genoeg momenten van discussie over de juiste koers. Moesten we niet iets meer afbuigen naar stuurboord, op zoek naar de wondere watervallen van de farmaco-epidemiologie? Of toch iets meer hellen naar bakboord, op zoek naar de eindeloze eilanden van de immunologie? Een kleine kapitein omringd door kapiteins. Mijn blik op de kim, en met steeds meer vertrouwen het avontuur tegemoet. Anke-Hilse, Jan en Leo, dank jullie wel voor al jullie betrokkenheid, steun en wijze woorden.

De wateren waren voor een deel al in kaart gebracht door Ellen, die twee jaar eerder van start was gegaan. Ze leerde me mijn boot snel te beheersen. De collega's van UPPER hielpen de vaart er in te houden, tientallen apothekers werden door hen benaderd. Marloes, een oud-studiegenootje en vriendin, creëerde een prachtig PACMAN logo, zodat er onder een heuse PACMAN vlag gevaren kon worden. Van tijd tot tijd kwamen er ook stagiaires aan boord. Ze hielpen om de zeilen te hijsen. Tientallen hebben een stukje van de reis meegemaakt. Zonder hun hulp was ik nooit zo ver gekomen. Maar ook alle kinderen en ouders die deelnamen aan het onderzoek, alle apothekers en alle artsen die mee hielpen, ben ik erg dankbaar.



Collega's bevoeren dezelfde zeeën, maar hadden andere bestemmingen. Sommigen hebben hun reisdoel al bereikt, anderen moeten nog een stukje. Lieve collega's, dank voor alle avonturen die we samen hebben beleefd, en alle bemoedigende woorden en kopjes thee als het even tegenzat. Maar vooral bedankt voor alle hulp om de knopen uit de lijnen te krijgen. De ondersteuning van Vera en Tamar in het lab behoedde me veelvuldig voor het raken van immunologische ijsschotsen. Maar ook mijn andere collega's in het lab wil ik bedanken voor al hun support en mooie taartlab-momenten. My pharmaco-epi colleagues, guided me through the waves of databases and statistical analyses. Special thanks go to my former 8th floor-roomies in 'het Went' for all the lovely Latte Macchiato moments, and to my former DDW 4th floor AIO colleagues for making me smile in times of high winds and rough seas. Ali; I wish you a safe trip with your own PACMAN boat. Furthermore, thanks post-doc roomies in 4.70 for showing me that there will be a life after this journey. Tevens wil ik de dames van het secretariaat bedanken voor alle hulp om mijn bootje bijeen te houden.

Twee eigenwijze, muitende collega-kapiteins, Bart en Maarten, varen als paranimfen ook het laatste stukje met me mee. Ik ben trots op die twee stoere mannen, afkomstig uit verschillende werelden, die me vaak hebben bijgestaan als mijn bootje weer eens dreigde te stranden.

De reizen werden exotischer door de diverse samenwerkingsverbanden die onderweg werden aangegaan. Graag wil ik alle co-auteurs bedanken die mijn boot ooit een zetje gaven in de juiste richting. Samen met de afdeling Kinderlongziekten van het UMCU ging ik op PACMAN2 expeditie. Kors, Kim, Francine, Myriam, Eltje, Lydia en de dames van de longfunctie; dank voor jullie hulp daarbij! Vanuit Groningen hebben Dirkje en Gerard mij veelvuldig kritisch laten nadenken over mijn koers, en hielp Nicole tijdelijk mee aan boord. Vanuit Amsterdam loodsten Paul en Peter mij naar nieuwe spannende 'eNOSE' gebieden. Avonturen die hopelijk nog niet ten einde zijn!

Mijn reis bracht me ook naar Schotse wateren. In the fall of 2012, Colin, Roger, Natalie and their colleagues taught me in Dundee how to sail the 'genotyping' waters and made me feel at home there. Thenk ye! My journey also would not have been the same without close collaboration with the studies BREATHE, PAGES and CAMP. Somnath, Steve, Kelan and Sze Man: I really appreciated all your scientific input.

En dan zijn er nog de vrienden en (schoon)familie die me langs de kade aanmoedigden. Ze dwongen me aan te meren als het nodig was en stuurden me chocolade in tijden van nood. Met sommige vrienden keek ik al uit over de Steinse haven in mijn tienerjaren dromend van het avontuur. Met anderen werd ik samen volwassen, studeerde ik, deelde ik studentenhuizen, stond ik vol adrenaline op de planken, filosofeerde ik over het leven, genoot ik van sushi, of danste ik salsa tot mijn voeten het begaven. Jullie zijn me zeer dierbaar.

Mijn ceremoniemeesters, Marlien en Marije, leerden me de continenten kennen toen mijn boot nog gebouwd moest worden. A nossa amizade sempre foi uma parte muito especial da minha vida.

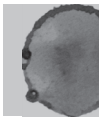
Grote broer, jij hebt me, wellicht onbewust, altijd aangespoord om niet zomaar de makkelijkste weg te kiezen. Dank daarvoor. En ook voor dat prachtige neefje dat je samen met Emma creëerde. Een avonturier in de dop?

Mijn reis zou niet hetzelfde zijn geweest zonder de steun van mijn ouders. Ze stonden er als ik aankwam en zwaaiden me uit als ik weer vertrok. Een veilige haven in het zuiden waar ik altijd voor anker mocht. Mama, ik kon altijd bij jou terecht. Wat ben ik trots op jouw kunstwerk dat dit boekje siert! Papa, dank voor al onze goede gesprekken. Als klein meisje stimuleerde je me al om net als de kleine kapitein van Paul Biegel niet bang te zijn voor het avontuur. Pas twee jaar geleden leerde je me op Santo Antão dat een zaklamp uiterst handig gereedschap is voor een avonturier. Papa en mama, dank jullie wel voor jullie onvoorwaardelijke liefde en support.

En dan is er nog Mattijs. Zelfs als de golven over het dek slaan, de mast van mijn boot dreigt te bezwijken, en ik het als kapitein echt niet meer weet, loods jij mij naar rustige wateren. Als een vuurtoren in de donkere nacht. Ik heb je zo ontzettend lief.

De kleine kapitein kiest altijd het ruime sop en ziet wel waar de wind hem brengt.

Opa, jij was de wind in mijn zeilen.



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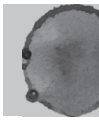

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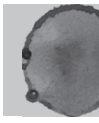

List of publications

ADRB2 Arg16 and the need for collaboration in childhood asthma pharmacogenomics. Vijverberg SJH, Raaijmakers JAM, Maitland-van der Zee AH. Pharmacogenomics. 2013; 14(16):1937-1939. [Editorial]

Arg16 *ADRB2* genotype increases the risk of exacerbations in children with a reported use of long-acting β_2-agonists: results of the PACMAN cohort. Zuurhout MJL, Vijverberg SJH, Raaijmakers JAM, Koenderman L, Postma DS, Koppelman GH, Maitland-van der Zee AH. Pharmacogenomics. 2013; 14(16):1965-1971.

Clinical utility of asthma biomarkers: from bench to bedside. Vijverberg SJH*, Hilvering B*, Raaijmakers JAM, Lammers JWJ, Maitland-van der Zee AH*, Koenderman L*. Biologics. 2013; 7:199-210. **Authors contributed equally*

Inflammatory phenotypes underlying uncontrolled childhood asthma despite inhaled corticosteroid treatment: rationale and design of the PACMAN2 study. Vijverberg SJH, Koenderman L, van Erp FC, van der Ent CK, Postma DS, Brinkman P, Sterk PJ, Raaijmakers JAM, Maitland van der Zee AH. BMC Pediatr. 2013; 13(1):94.

Exhaled NO is a poor marker of asthma control in children with a reported use of asthma medication: a pharmacy-based study. Vijverberg SJH, Koster ES, Koenderman L, Arets HG, van der Ent CK, Postma DS, Koppelman GH, Raaijmakers JAM, Maitland van der Zee AH. Pediatr Allergy Immunol. 2012; 23(6):529-536.

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R, Mukhopadhyay S, Vijverberg SJH, Raaijmakers JAM, Palmer CN. Allergy. 2011; 66(12):1546-1552.

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Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics. Arzenani MK, Zade AE, Ming Y, Vijverberg SJH, Zhang Z, Khan Z, Sadique S, Kallenbach L, Hu L, Vukojevic, Ekström TJ. Mol Cell Biol. 2011; 31(19):4119-4128.

Ethical and social issues in pharmacogenomics testing. Vijverberg SJH, Pieters T, Cornel MC. Curr Pharm Des. 2010; 16(2):245-254.

Heredity and predictive testing of alcoholism: an exploratory study of the views of Dutch alcoholics, at-risk drinkers and health care providers. Vijverberg SJH, van Berkel J, Pieters T, Snelders S, Braam R, Dupont H, Kaplan CD, Meijman FJ. J Addict Nurs. 2010; 21(4):174-182.

Conference scene: initiatives on future biobanking in pharmacogenomics. Vijverberg SJH, Daly AK, Maitland-van der Zee AH. Pharmacogenomics. 2009; 10(7):1135-1138.

Large interindividual differences in cellular sensitivity to calicheamicin may influence gemtubuzmab ozogamicin response in acute myeloid leukemia. Goemans BF, Zwaan CM, Vijverberg SJH, Loonen AH, Creutzig U, Hahlen K, Reinhardt D, Gibson BE, Cloos J, Kaspers GJ. Leukemia. 2008; 22(12):2284-2285.

About the author

Susanne was born on the 26th of October 1982 in Sittard, the Netherlands. In 2000, she obtained her athenaeum diploma at Scholengemeenschap Groenewald in Stein. In the same year she started studying Biology at Utrecht University. In 2001, she received her propaedeutic exam (*cum laude*). Two years later she participated in the Erasmus exchange programme at Coimbra University (Coimbra, Portugal). In 2004, she obtained her BSc degree in Biology and started with a Master's programme in Oncology at the VU University in Amsterdam. During her Master's programme, she performed two six-month research internships: the first at the department of Paediatric Oncology and Haematology of the VU Medical Center (VUmc) (Amsterdam, the Netherlands) studying drug sensitivity of childhood leukaemia cell lines. The second at the research group of prof. Tomas Ekström at the department of Clinical Neuroscience of the Karolinska Institute (Stockholm, Sweden) investigating cellular epigenetic regulation. Upon obtaining her MSc degree (*cum laude*) in 2006, she started working as a junior researcher at the department of Medical Humanities of the VUmc, under the supervision of prof. Toine Pieters, focussing on the societal impact of genomic knowledge. In 2007, she temporarily left science to work as a volunteer for 5 months at a Brazilian NGO with social programs for vulnerable groups within the local community (ONG IDEAIS, Volta Redonda, Brazil). After this adventure, Susanne returned to the department of Medical Humanities as a junior researcher / teacher. In 2009, she started her PhD project at the division of Pharmacoepidemiology and Clinical Pharmacology at Utrecht University and at the department of Respiratory Medicine of the UMC Utrecht, under the supervision of prof. Jan Raaijmakers, prof. Leo Koenderman and dr. Anke-Hilse Maitland-van der Zee. The focus of Susanne's research was to study biological mechanisms underlying treatment response to asthma medication in children, as described in this thesis. An important part of the project was the coordination of the PACMAN cohort. In the fall of 2012 and with financial support of the Ter Meulen Fund, she visited the group of prof. Colin Palmer at Dundee University as a guest researcher and gained experience with genotyping. In September 2013, Susanne started working as a junior lecturer at the division of Pharmacoepidemiology and Clinical Pharmacology, combining research and teaching.