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Banić, Ivana

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FACULTY OF SCIENCE

DEPARTMENT OF BIOLOGY

Ivana Banić

GENETIC POLYMORPHISMS ASSOCIATED WITH THE RESPONSE TO ASTHMA TREATMENT IN CHILDREN

DOCTORAL THESIS

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PRIRODOSLOVNO-MATEMATIČKI FAKULTET

BIOLOŠKI ODSJEK

Ivana Banić

POLIMORFIZMI GENA POVEZANIH S ODGOVOROM NA TERAPIJU DJECE S ASTMOM

DOKTORSKI RAD

Zagreb, 2019. g.

Ovaj je doktorski rad izrađen u Dječjoj bolnici Srebrnjak u Zagrebu, pod vodstvom izv.prof.dr.sc. Mirjane Turkalj, dr.med., u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu.

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Posvećeno mom savršenom savršenstvu, mom najvećem postignuću, Maksimu i Mališanu, sve ovo dosad i sve ono odsad- sve je to za VAS…

Sveučilište u Zagrebu Doktorski rad

Prirodoslovno-matematički fakultet

Biološki odsjek

POLIMORFIZMI GENA POVEZANIH S ODGOVOROM NA TERAPIJU DJECE S ASTMOM

IVANA BANIĆ

Dječja bolnica Srebrnjak, Zagreb

Astma je najčešća kronična bolest u djece s trendom porasta prevalencije u budućnosti koju karakterizira izrazita heterogenost s obzirom na moguću etiologiju, stupanj upale i oštećenja bronha, poremećaj funkcije pluća i tijek bolesti. Astma se danas više ne smatra jedinstvenom bolešću nego sindromom s više različitih fenotipova definiranih novokoncipiranim imunopatozihološkim mehanizmima u kojem genska predispozicija igra važnu ulogu. Svrha ovog istraživanja je identificirati genske varijante povezane s učinkovitošću uobičajene terapije u astmi (inhalacijski kortikosteroidi, β-agonisti i antagonisti leukotrienskih receptora) i specifične obrasce u odgovora na terapiju u astmi u djece (N= 365) te dati bolji uvid u patogenezu pojedinih podtipova bolesti. Rezultati ovog istraživanja pokazuju kako su određeni polimorfizmi u genima *GLCCI1*, *TBX21*, *CRHR1*, *ADRB2* i *MMP9* povezani s varijabilnim terapijskim ishodima te kako postoje diskretni obrasci terapijskih ishoda (pozitivni/ lošiji) povezani sa specifičnim fenotipskim karakteristikama poput razine i tipa upale, komorbiditeta i nekih genskih varijanti u djece s astmom.

(149 stranica, 17 slika, 31 tablica, 266 literaturnih navoda, jezik izvornika: engleski)

Ključne riječi: astma, terapija, genski polimorfizmi, djeca, uspješnost terapije, inhalacijski kortikosteroidi, fenotipovi u astmi

Mentor: izv.prof.dr.sc. Mirjana Turkalj, dr.med.

Ocjenjivači: prof.dr.sc. Kristian Vlahoviček

Izv.prof.dr.sc. Maja Matulić

Prof.dr.sc. John W. Holloway, PGCert

University of Zagreb

Faculty of Science

Department of Biology

Doctoral thesis

GENETIC POLYMORPHISMS ASSOCIATED WITH THE RESPONSE TO ASTHMA TREATMENT IN CHILDREN

IVANA BANIĆ

Srebrnjak Children`s Hospital, Zagreb

Asthma is the most common chronic condition in children. It is characterized by high levels of heterogeneity in disease etiology, inflammation, bronchial and lung function impairment and natural course of the disease. Asthma is no longer considered a single disease but rather an „umbrella“ term encompassing several different phenotypes defined by newly conceived pathophysiologic mechanisms and genetic predisposition. The purpose of this research is to identify genetic variants associated with treatment success in asthma (inhaled corticosteroids, β-agonists, leukotriene receptor antagonists) and specific treatment response patterns in children, providing better insight into the pathogenesis of specific disease subtypes. The results of this study indicate that certain polymorphisms in the *GLCCI1, TBX21, CRHR1, ADRB2* and *MMP9* genes are associated with variable treatment outcomes in asthmatic children and that there may be discrete treatment outcome patterns (good/poor) associated with specific phenotype characteristics such as inflammation level and type, comorbidity and certain genetic traits.

(149 pages, 17 figures, 31 tables, 266 references, original in: English)

Keywords: asthma, treatment, genetic polymorphisms, children, treatment success, inhaled corticosteroids, asthma phenotypes

Supervisor: Assoc.prof. Mirjana Turkalj, MD, PhD

Reviewers: Prof. Kristian Vlahoviček, PhD

Assoc.prof. Maja Matulić, PhD

Prof. John W. Holloway, PhD, PGCert

Mentor: Izv.prof.dr.sc. Mirjana Turkalj, dr.med.

Datum i mjesto rođenja: 28.10.1963., Slavonski Brod, RH

Adresa: Dječja bolnica Srebrnjak (DBS), Srebrnjak 100, 10000 Zagreb

Trenutno zaposlenje (od 2003.): pedijatar- subspecijalist alergologije i kliničke imunologije, voditelj Odjela za alergologiju i pulmologiju predškolske i školske dobi, zamjenik Ravnatelja, voditelj Referentnog centra za kliničku alergologiju djece Ministarstva zdravlja RH, DBS, Zagreb

Akademski rad: predavač na više od 50 stručnih i sveučilišnih kolegija i tečajeva u RH i inozemstvu u posljednijih 10 godina

Edukacija

1982. – 1987. **dr.med.** Medicinski fakultet Sveučilišta Zagrebu
1987. – 1989. Poslijediplomski studij alergologije i kliničke imunologije, Medicinski fakultet Sveučilišta Zagrebu
1994. **mr.sc.** u području Biomedicine, Medicinski fakultet Sveučilišta Zagrebu
1997. – 1999. Poslijediplomski studij Njege majke i djeteta, Medicinski fakultet Sveučilišta Zagrebu
1998. **dr.sc.** Biomedicinske znanosti, Medicinski fakultet Sveučilišta Zagrebu
2013. – 2015. Poslijediplomki studij Sportske medicine, Medicinski fakultet Sveučilišta Zagrebu

Znanstveno- istraživački projekti: 3 međunarodna (EU FP7 i EU FP6, uloga: PI, suradnik), 9 nacionalnih (uloga: voditelj, glavni istraživač, istraživač, suradnik), >5 kliničkih studija (uloga: voditelj)

Mentorstva: mr.sc.(1), dr.sc. (3), trenutna dr.sc. (6), diplomski radovi (1)

Istraživački fokus: razumijevanje kliničkih osobitosti i patogeneze alergija, osobito astme, ali i drugih respiratornih i imunoloških bolesti. Trenutni istraživački focus izv.prof.dr.sc. Mirjane Turkalj, dr.med. je u području personalizirane medicine i translacijskog istraživanja patofizioloških mehanizama u alergijama i respiratornim bolestima (uključujući astmu, alergije na hranu i primarne imunodeficijencije), i to osobito u: (i) interakcijama okoliša i ljudskog organizma u zdravlju i bolesti, (ii) glikozilacijskim promjenama u alergijama, (iii) funkcionalnoj genetici (farmakogenetici) astme, (iv) epigenetskoj regulaciji u razvoju alergijskih bolesti, (v) ulozi gornjih i donjih dišnih putova u alergijskim i drugim respiratornim bolestima, uključujući istraživanje mikrobioma i metaboloma gornjih i donjih dišnih putova korištenjem neinvazivnih dijagnostičkih metoda poput kondenzata izdaha, i (vi) prevencija razvoja alergija i respiratornih bolesti.

Publikacije (CROSBI): Radovi u časopisima (76), sažeci sa skupova (78), radovi u zbornicima radova (2), poglavlja u knjigama (13), obrazovni materijali (3)

Supervisor: Assoc. prof. Mirjana Turkalj, MD,PhD

Date and place of birth: 28th October 1963, Slavonski Brod, Croatia

Affiliation (address): Srebrnjak Children`s Hospital (SCH), Zagreb, Croatia

Current post (since 2003): Pediatrician- Allergy and Clinical Immunology subspecialist, Head of the Department of Allergology and Pulmonology, Deputy Director, Head of Referral Center for Clinical Allergology in Children, Republic of Croatia Ministry of Health, SCH, Zagreb, Croatia

Academic work: more than 50 courses and lectures in Croatia and abroad in the past 10 years

Education

1982 - 1987	**MD** School of Medicine, University of Zagreb, Croatia
1987 - 1989	Postgraduate studies of Allergy and Clinical Immunology, School of Medicine, University of Zagreb, Croatia
1994	**MSc** Master of Science in Biomedical research, School of Medicine, University of Zagreb, Croatia
1997 -1999	Mother and child care Postgraduate study, School of Medicine, University of Zagreb, Croatia
1998	**PhD** Biomedical research, School of Medicine, University of Zagreb, Croatia
2013 - 2015	Postgraduate study in Sports Medicine, School of Medicine, University of Zagreb, Croatia

Research projects: 3 international (EU FP7, role: PI, collaborator), 4 national (role: PI and Co-PI), 5 national (role: researcher), >5 clinical studies (PI)

Mentorships and supervision: MSc (1), PhD (3), current PhD mentorships (6)

Research focus: understanding the clinical features and pathogenesis of allergy, especially asthma, as well as other respiratory and immune disorders. Her current research program focuses on personalized medicine and translational research in pathophysiological mechanisms of allergy and respiratory diseases such as asthma and food allergy, more specifically: (i) the environment and health and their interactions, (ii) glycosylation in allergy, (iii) functional genetics (pharmacogenomics) of allergy and respiratory diseases, (iv) the epigenetic regulation of asthma/allergic diseases, (v) the role of upper and lower airways in respiratory diseases, including analysis of microbiome and metabolome in upper and lower airways using non-invasive diagnostic procedures such as the exhaled breath condensate (EBC) and (vi) prevention of allergy and respiratory diseases development.

Publications (CROSBI): Original research papers and reviews (76), congress abstracts (80), book chapters (13), books and scripts (3)

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1. INTRODUCTION

Although it is one the most common chronic conditions globally and the most common chronic disease in the pediatric population (up to 30% of children in the UK), the causes and pathophysiologic mecahnisms in asthma still remain poorly understood. Asthma is a major burden on healthcare systems worldwide and society in general, with significant financial costs both in terms of direct medical costs (such as those of medication, diagnostics and hospital admissions), as well as indirect costs (such as time lost from work or school and premature mortality). These costs are even more significant when accounted for under- or over- diagnosing and under- or over- medication of the disease in a large number of patients, with this effect being most prominent in people with uncontrolled or (more) severe asthma (GINA 2018, WHO, Masoli et al. 2004).

Asthma is a syndrome of very complex and largely unknown etiology characterized by reversible airway obstruction, airway hyperresponsiveness to specific and non-specific stimuli, and a chronic inflammatory process of the airways in which mast cells, eosinophils, T lymphocytes, epithelial cells, and airway smooth muscle cells play a prominent role (Elias et al. 2003).

Although asthma cannot be cured, with appropriate management adequate control and good quality of life can be achieved (GINA 2018, WHO 2013). Earlier common asthma classification by symptoms and lung function measurements enables only treatment options and selection at the initial disease presentation, but not adequate disease control monitoring. Even the latest GINA guidelines and recommendations, involving symptom control (daily and nocturnal symptoms, rescue treatment requirements, especially need for bronchodilatators, effect on daily activity, mainly on physical activity) do not offer insight into disease aetiology and true level of asthma control. Also, there are no recommendations as to treatment failure identification and changes recommended towards the treatment of choice (different drug classes or their combinations) or only general choice recommendations are made (the physician can choose between several treatment options with the generally preferred option recommended).

The lack of benefit to patients from this inadequate and ultimately arbitrary treatment selection process is probably due to the overgeneralized approach to asthma as a disease, disregarding very specific (individual) disease forms. Hence, in order to optimize medication

selection and maximize treatment response, further classification and characterization of specific asthma subtypes (phenotypes and endotypes) is more than necessary. Currently, a series of asthma subtypes have been reported and described, based on inflammation level and type (specific biomarkers), clinical features and natural course of disease, reversibility of airway obstruction, disease severity, response to treatment (resistance to inhaled corticosteroids, sensitivity to leukotriene receptor antagonists etc.), level of tissue remodeling and allergic sensitization (Bush and Menzies-Gow 2009).

There is mounting evidence that despite the availability of several classes of asthma medications and their overall satisfactory effectiveness, a significant portion of patients fail to respond adequately to these therapeutic agents. Available data, according to numerous pharmacogenetic studies, suggest that genetics may contribute for as much as 60-80% to the interindividual variability in treatment response for all asthma medications (Duong-Thi-Ly et al. 2017). Although many studies are limited by small sample sizes and replication of the findings is needed, several candidate genes have consitently been identified. These include polymorphisms in the *GLCCI1*, *CRHR1*, *TBX21* and *FCER2* genes associated with the response to treatment with inhaled corticosteroids; polymorphisms in the *ADRB2* gene associated with the response to treatment with β-agonists and polymorphisms in the *ALOX5* and *MRP1* genes associated with the response to treatment with leukotriene modifiers (Vijverberg et al. 2018). Pharmacogenetic research, such as that presented in this doctoral thesis, is a step towards more personalized treatment of asthma, which will improve therapeutic outcomes, minimize side effects and lead to a more cost-effective care.

The purpose of this translational research approach is to ultimately improve the health and wellbeing of asthmatic children. Today, asthma is diagnosed only after clinical symptoms arise, primarily because current technologies and guidelines do not enable earlier detection. Preventive measures and treatments are designed in a “one size fits all” approach, frequently leading to over- or under-medication and undesirable or possibly dangerous side effects. Gaining better insight into asthma pathophysiology and factors predisponing for asthma is important and in recent years advances in the pharmacogenetics of asthma have indicated that a number of genes associated with susceptibility to asthma or its intermediate phenotypes and disease characterization are also involved in an altered treatment response. The main focus of this doctoral research is to identify major factors underlying the huge interindividual variability in the response to common asthma medications, as well as genetic variants predisponing for the level of treatment success in children with asthma and specific disease

phenotypes. This might ensure more precise, individually tailored and personalized treatment options in this common chronic condition in the pediatric population, enabling them to be more effective, cause fewer side effects and be more cost-effective due to stratification of specific patient risk and even prediction of response to treatment.

The main objectives in this doctoral thesis are:

- To determine the differences in clinical presentation, genetic predisposition and response to treatment in children with asthma and specific disease subtypes (phenotypes);
- More specifically, to determine the differences in response to treatment with common medication classes (inhaled corticosteroids, leukotriene receptor antagonists and β-agonists) in children with asthma (and certain disease phenotpyes) in regard with specific genetic polymorphisms in the *GLCCI, TBX21, CRHR1, ADRB2* and *MMP9* genes.

The main hypothesis is that there is marked variability in clinical parameters, the level of response to treatment and genetic predisposition in children with specific asthma phenotypes and that this variability is associated with certain genetic variants in the *GLCCI, TBX21, CRHR1, ADRB2* and *MMP9* genes.

In order to address the issues in treatment success/failure in children with asthma, 365 patients (aged 2-22 years) with physician diagnosed asthma of the outpatient clinic at Srebrnjak Children`s Hospital in Zagreb, Croatia were recruited to the study. At their first visit patients underwent physical examination, skin prick tests and other allergy assays, lung function tests and blood sampling for routine laboratory diagnostics and subsequent genetic analysis. After they were diagnosed with asthma, patients started treatment with inhaled corticosteroids (alone or in combination with β-agonists) and/or leukotriene receptor antagonists, according to disease severity and previously assessed disease control (according to GINA guidelines, GINA 2018). Follow-up visits with lung function and other testing, physical examination as well as clinical assessment of treatment outcomes were made on average every 6 months over the period of 2.5 years. Patients were genotyped for the following genetic polymorphisms: rs37973 (*GLCCI1*), rs9910408 (*TBX21*), rs242941 and rs1876828 (*CRHR1*), rs1042713 (*ADRB2*) and rs17576 (*MMP9*). The level of response to treatment ("good", "moderate" and "bad") was analyzed in association with certain clinical

parameters and specific genotypes, and additionally, patients were stratified by cluster analysis (using the hierarchical clustering and Ward`s method) into several subgroups based on specific biomarkers, clinical features, plausible pathophysiological mechanisms, response to treatment and genetic predisponing factors (analyzed genetic polymorphisms).

2. A LITERATURE OVERVIEW IN ASTHMA AND PHARMACOGENETICS

Asthma is a heterogeneous disorder characterized by chronic airway inflammation. It is also associated with airway hyperresponsiveness and airway remodeling (to a more or less extent), and is classically considered to be a reversible airways disorder. Common symptoms of asthma include wheeze, shortness of breath, chest tightness and/or cough, particularly at night or early in the morning. Patients with asthma can also experience exacerbations (commonly known as asthma attacks or flare-ups), which are triggered by a number of endgenous and exogenous factors such as exercise, exposure to allergens or irritants (eg. air pollutants), changes in weather (particularly cold weather) or viral respiratory infections (GINA 2018).

Asthma is one of the most common chronic diseases in general, with up to 300 million people currently suffering from the disorder and up to 250 000 people estimated to now be dying from asthma annually. It is also the most common chronic disease in children (WHO 2013). Today, one child in three has some form of an allergic disorder (including asthma), and it is estimated that in 2015 half of the European population may have been suffering from one or more allergic disorders. One in four Europeans has some form of respiratory allergy and experts estimate that one in five infants develop asthma during childhood or later in life. Asthma symptoms vary from mild to life threatening and can have a devastating impact on patients` day to day life, their families and children's school activity. According to the World Health Organization, asthma kills one person in Europe each hour.

2.1. Global burden of asthma

Asthma typically begins much earlier in life than other chronic disorders, and consequently imposes a significant lifetime burden on individuals, their caregivers and society in general. The financial costs of asthma in Europe are estimated to be up to 18 billion € per year. These only include direct costs of asthma management (diagnostics, medication, management of exacerbations) and, along with other indirect costs, including diminished quality of life and social impact (eg. reduced professional capacity), the total costs of asthma management range from €55 - €151 billion per annum (Zuberbier et al. 2014, ERS 2003), and can be avoided to a large extent. The WHO has estimated that 15 million disability-adjusted life years (DALYs)

are lost annually due to asthma, representing 1% of the total global disease burden (WHO). In the case of childhood asthma, the repercussions of the disease affect not only the asthmatic child, but also the parents and other members of the family. Parental fears of a serious attack create anxiety, and even with mild forms of the disease, family activities may be limited. Children miss days at school and abstain from sports and other recreational activities. Breathing problems and other accompanying conditions can also harm the self-image of young children, adults and especially teenagers.

The European Commission (EC) is recognizing the growing health issue of asthma, particularly in children and is developing strategies to adequately address it. There is a strong link between poor health and environmental problems. A recent report from the European Environmental Agency (EEA) shows that as many as 60 000 deaths per year in large European cities are caused by long-term exposure to air pollution, including those caused by asthma (EEA 2009). Children are more sensitive to environmental risks than adults. In order to reverse this alarming trend the European Commission has launched a European Environment and Health Strategy. With this new strategy the Commission expects to achieve a better understanding of the complex relationship between environment and health and to identify and reduce diseases caused by environmental factors, including asthma. Also, this issue is of great importance for the Croatian Ministry of Health, as they included asthma as an important focus in the National Health Strategy (Croatian National Health Strategy 2012-2020).

2.2. The prevalence of asthma

Based on numerous epidemiological evidences it appears that there are marked variations in the prevalence of asthma in different countries. The prevalence, causes and clinical presentation of asthma all vary significantly with age. Many children first develop symptoms during infancy, but many cease wheezing in early childhood. Asthma can appear *de novo* throughout life, but it most commonly starts in early childhood.

Like in other western (developed) countries of the world, there has been a three- to four-fold increase in the prevalence of childhood asthma in Europe in the last three to four decades. According to the ISAAC Phase I study, the highest prevalence of childhood asthma in Europe was found in the British Isles, with lifetime prevalence rates of asthma ranging from 1.6% in

Albania to 20.7% in the UK for 13-14-year-old children, and from 1.4% in Estonia to 22.9% in the UK among 6-7-year-olds, with markedly increasing rates across Europe from East to West (Figure 1). This East-to-West difference has changed over recent years with a relative increase in lifetime prevalence rates in eastern Europe compared with the western countries. This may be related to simultaneous changes in lifestyle in eastern Europe- a shift from traditional lifestyle to „westernized" habits (sedentary lifestyle, changes in diet etc.). Currently, Croatia belongs to the countries with moderate prevalence of asthma in children with up to 10% of the paediatric population suffering from this (Figure 1), but further increase can be expected (GINA 2018).

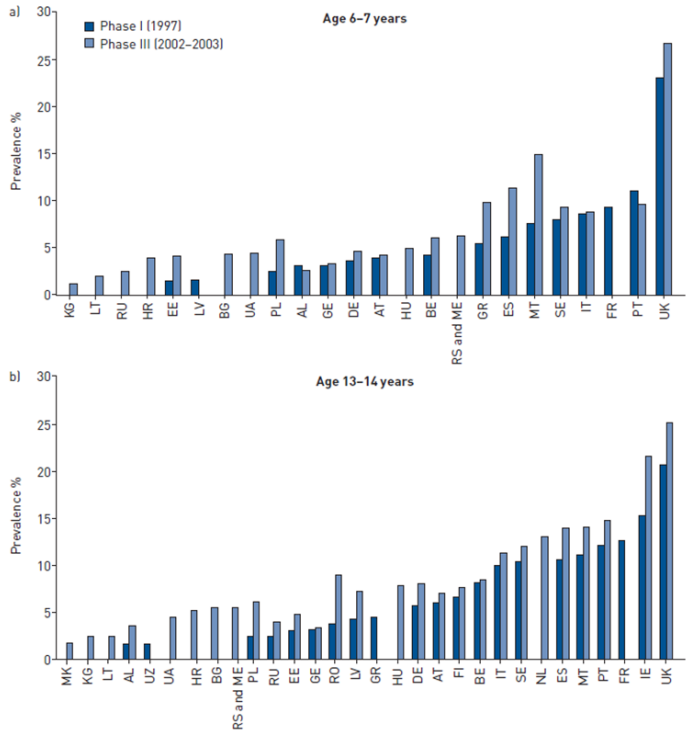


Figure 1. Lifetime asthma prevalence rates in European school children. a) Asthma prevalence rates in European countries in younger children (aged 6-7 years), b) asthma prevalence rates in European countries in older children (aged 13-14 years). Prevalence rates in Croatia (HR) range from 5 to 8 % (higher in older children). Source: ISAAC 1998 and Lai et al. 2009.

2.3. Pathophysiology of asthma

Asthma is characterized by reversible airway obstruction, bronchial hyper-responsiveness to specific and non-specific stimuli (such as allergens, exercise or cold air), persistent

inflammation, mucus hyper-production, airway tissue remodeling (primarily airway narrowing), sub-epithelial fibrosis, smooth muscle tissue hypertrophy and hyperplasia, epithelial cell metaplasia, vasodilatation, angiogenesis and increased vascular permeability which lead to oedema and changes in the extracellular matrix, due to protein leakage to the extracellular space. The level of these structural changes correlates with disease severity and progressive lung function deterioration (Ribatti et al. 2009, Towns and van Asperen 2009, Lemanske and Busse 2010, Harkness et al. 2014).

Characteristic pathophysiologic features of asthma are shown in Figure 2. Genetics, in combination with early life events as well as the environment, modulate the development of CD4+ (cluster of differentiation 4 or T) lymphocytes towards a type 2 helper (Th2) immunophenotype. These cells then produce cytokines, such as interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 13 (IL-13), and granulocyte–macrophage colony-stimulating factor (GM-CSF) and thereby promote the synthesis of immunoglobulin E (IgE), an important allergic effector molecule, creating an inflammatory airway milieu. Chemokines, such as eotaxin, Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)/Chemokine (C-C motif) ligand 5 (CCL5) and interleukin 8 (IL-8) produced by epithelial and inflammatory cells, serve to amplify and perpetuate the inflammatory events. Several bronchoactive mediators, such as histamine, leukotrienes, and neuropeptides are released into the airways and precipitate an asthma attack by causing airway smooth muscle constriction, mucus secretion and oedema. In time, smooth muscle tissue proliferates and the deposition of subepithelial connective tissue occurs- a process that is commonly referred to as airway remodeling. As a result, patients with asthma have difficulty exhaling air because of an increase in airway resistance that is a consequence of smooth muscle contraction, inflammation and remodeling (Barnes 1996, Weiss et al. 2006).

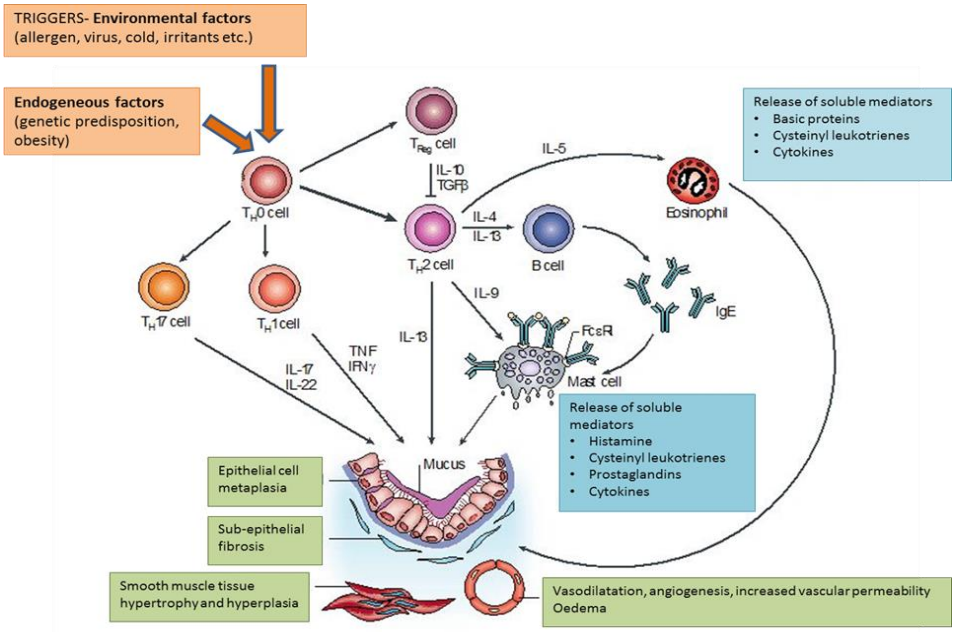


Figure 2. A schematic representation of major contributors to asthma pathophysiology. Asthma is an inflammatory disorder of the airways induced by various environmental and endogeneous factors, including genetic predisposition. Chronic inflammation leads to a number of structural and functional changes in the airways which in turn lead to airway narrowing and the rise of characteristic asthma symptoms. T_H17- type 17 T helper cells, T_H0- naive T cells, T_H1- type 1 T helper cells, T_{Reg}- regulatory T cells, IL-10- interleukin 10, IL-9- interleukin 9, IL-17- interleukin 17, IL-22- interleukin 22, TGF-β- transforming growth factor beta, TNF- tumor necrosis factor, IFNγ- interferron gamma, FcεR- FC fragment of IgE receptor. Modified from (Source): Holgate and Polosa (2008).

2.3.1. Airway inflammation in asthma

Chronic airway inflammation is a fundamental feature in asthma which involves different cell types: inflammatory cells such as mast cells, eosinophils and T lymphocytes, structural cells such as epithelial cells and numerous infammatory mediators (Holgate 2008, Olin and Wechsler 2014).

Since allergic asthma is the most common asthma type in children, the inflammation is initiated by infiltration of allergens into the lower airway, which are taken up by dendritic cells (DCs). DCs process allergens to peptides and present the peptides to naive T (Th0) cells, and in a suitable environment the naive T cells develop into type 2 helper T (Th2) cells (Vijverberg et al. 2013, Brugha et al. 2015). Th2 cells produce cytokines such as IL-4 and IL-13 which stimulates B lymphocytes to produce IgE as well as IL-3 and IL-5 which attracts eosinophils to the lungs; and IL-4 and interleukin 9I (IL-9), which stimulate mast cell hyperplasia. With repeated exposure to allergens that an individual is sensitized to, mast cells,

secondary to binding of allergens to IgE, release histamine and start to produce prostaglandin D2 (PGD2) and cysteinylleukotrienes (leukotriene C4- LTC4, leukotriene D4- LTD4, and leukotriene E4- LTE4), which attract inflammatory cells to the lungs. The early phase of asthma is a consequence of the effects of histamine and other mediators released from mast cells, while the delayed effect is a consequence of other inflammatory cells and the release of inflammatory mediators. The molecular mechanism of inflammation in asthma is characterized by increasing various inflammatory genes controlled by proinflammatory transcription factors, such as nuclear factor kappa beta (NF-kB) and activator protein-1 (AP1). Both NF-kB and AP1 are activated by mediators, including cytokines, tumour necrosis factor alpha (TNFα), IL-1β and other factors. A number of coactivators also participate in the activation and repression of inflammatory genes through acetylating core histones by recruiting histone acetyltransferases (HATs). As a result, inflammatory proteins or enzymes and other proteins are synthesized and their production perpetuates airway inflammation (Duong-Thi-Ly et al. 2017). Characteristic inflamamtory and molecular mechanisms involved in allergic asthma are shown in Figure 3.

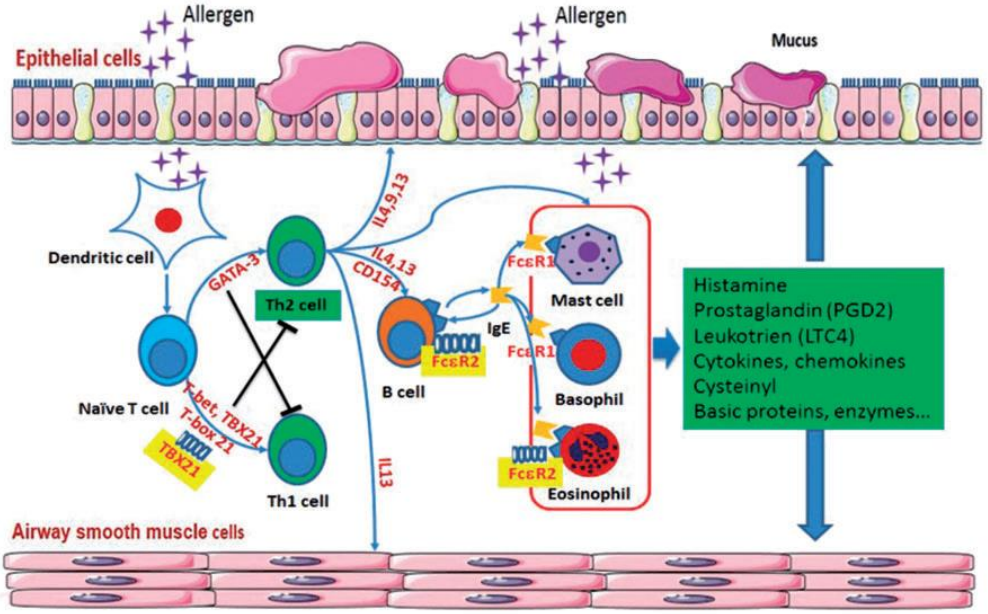


Figure 3. A schematic representation of mechanisms of airway inflammation in allergic asthma. GATA-3- GATA binding protein 3, CD154- cluster of differentiation 154; TBX21/Tbet- T-box 21, FcεR1- Fc fragment of IgE receptor I, FcεR2- Fc fragment of IgE receptor II. Source: Duong-Thi-Ly et al. 2017.

An overview of the roles of inflammatory cells and molecules involved in asthma pathophysiology is presented in Tables 1 and 2.

Table 1. Different cell types (inflammatory and structural) involved in asthma pathogenesis (Barnes 2016, Cosmi et al. 2011, McDougall and Helms 2006, Peters-Golden 2004, Ngoc et al. 2005, Loxham et al. 2014).

Cell type	**Role in the pathogenesis of asthma**
Mast cells	Activated by various triggers (allergens, exercise, hyperventilation etc.) Involved in releasing pro-inflammatory mediators such as histamine, cytokines and chemokines, which leads to bronchoconstriction Involved in promoting chronic inflammatory responses
Eosinophils	Most prominent cell type involved in allergic inflammation in asthma Secrete cytokines and chemokines that promote inflammation via the Th2 cell-mediated pathway Secrete lipid mediators (such as cysteinyl-leukotrienes) that cause airway epithelial damage and obstruct the airflow Associated with clinical responsiveness to glucocorticosteroids
Neutrophils	Predominatly found in the airways of patients with severe persistent disease, those with poor response to treatment with corticosteroids, smokers (with asthma) and during disease exacerbations Recruited by Th17 cell-mediated pathways and mediated by interleukin 8 (IL8) Exact mechanisms and roles in asthma not fully understood- corticosteroid medication supresses eosinophilia, which then in some patients results in neutrophilia
Macrophages	One of the most prominent and abundant immune cells in asthma pathophysiology Derived from monocytes Release inflammatory mediators and cytokines thet serve to amplify the inflammatory reponse in asthma
Dendritic cells	One of the major antigen-presenting cells (APC) in the airways Induce T-cell mediated immune responses Promote Th2 cell differentiation
Lymphocytes	**B lymphocytes** involved in the production of the major effector molecule-immunoglobulin E (IgE) **T lymphocytes**: role in coordinating the inflammatory response in asthma Release specific cytokines (Th2 subpopulation) that drive the inflammation Th1 cytokines also play a role in asthma inflammation (eg. IFNγ, TNF)
Structural cells (epithelial cells, fibroblasts, airway smooth muscle cells)	Important sources of inflammatory mediators Epithelial cells are central to hostt tissue response to environmental factors Epithelial signaling is crucial for the recruitment and localisation of inflammatory cells and for informing APCs about the local environment

Table 2. Inflammatory mediators involved in asthma pathogenesis (Barnes 2016, Chung and Barnes 1999, Bisset and Schmid-Grendelmeier 2005, Prado et al. 2011).

Inflammatory mediator	**Role in the pathogenesis of asthma**
Cytokines	Orchestrate and perpetuate the chronic inflammatory response in asthma Th2 cytokines mediate allergic inflammation (interleukines IL4, IL5, IL9, IL13 etc.) Pro-inflammatory cytokines (eg. IL-1β and TNF-α) serve to amplify the inlammatory response GM-CSF prolongs eosinophil survival in asthmatic airways
Chemokines	Essential in directing the migration of various inflammatory cells into the affected (asthmatic) airways Contribute to allergic inflammatory response Interact with eosinophils and mast cells to maintain local balance in favour of Th2 cells
Cysteinyl leukotrienes	Potent bronchoconstrictors Successfully targeted by treatment with leukotriene receptor antagonists (LTRA)
Markers of oxidative stress	Activated inflammatory cells (eg. eosinophils) produce reactive oxygen species (ROS) Levels of oxidative stress correlate with disease severity Amplify the inflammatory response May contribute to reduced responsiveness to corticosteroid treatment
Nitric oxide (NO)	Produced mainly by inflammatory and epithelial cells Potent vasodilatator with a range of effects (neurotransmission, vascular and non-vascular smooth muscle relaxation) Levels of exhaled nitric oxide (eNO) are increased in asthma- concentrations of NO in exhaled breath (fractional nitric oxide, FENO) is a useful biomarker of airway inflammation (predominantly of eosinophilia)

Inflammatory cells direct a complex network of mediators in the affected airways which results in a general inflammatory environment- the major contributor to the pathophysiology of asthma. Chronic inflammation then leads to other characteristic features of the disease: bronchoconstriction, mucus hypersecretion and other structural changes, including airway remodeling.

2.3.2. Effects of airway inflammation in asthma

Airway remodeling is defined as an increased thickness of the airway wall and increased airway narrowing, characterized by strucural changes such as subepithelial fibrosis, increased

smooth muscle mass, mucus gland and goblet cell hyperplasia, decreased cartilage integrity, angiogenesis, vascular proliferation and epithelial alterations- eg. subepithelial layer thickening and loss of epithelial integrity (Bergeron et al. 2009). The level of airway remodeling results from various factors, including genetic influences, early life exposure events, duration of the disease and long-term uncontrolled inflammation. These structural changes arise as a response to, for example, environmental exposure- to inhaled allergens, viral infections or air pollution, which can work synergistically to enhance them (eg. infections may exaggerate these changes in allergen-sensitized individuals). In most patients with asthma, airway obstruction and and remodeling (to a certain degree) is reversible and complete reversibility of long-standing impaired lung function parametres (such as forced expiratory volume in 1 second- FEV_1) may be achieved with adequate treatment. However, in some patients these changes are more permanent and airway obstruction is not fully reversible leading to a progressive loss of lung function, despite substantial anti-inflammtory medication use (including inhaled and systemic corticosteroids and other).

Airway epithelium can be damaged in asthma as a result of proteases released fro inflammatory cells and by inflammatory mediators. This contributes to airway hyperresponsiveness (another major feaure of asthma, AHR), defined as increased bronchoconstrictive reponse to various both specific (eg. allergens) and non-specific (cold air) stimuli. Several mechanisms can lead to AHR, such as loss of (epithelial) barrier function which enables the penetration of allergens, loss of enzymes that degrade inflammatory mediators and exposure of sensory nerves in the airway (Barnes 2016). The relationship between AHR and chronic inflammation remains unclear, but there is evidence that that anti-inflammatory treatment is usually effective at reducing AHR and improving asthma control (Busse 2010, Janssen-Heininger et al. 2012).

Subepithelial fibrosis in asthma leads to the thicknening of the basement membrane (Brewster et al. 1990). This is associated with eosinophil infiltration and presumably by the release of numerous cytokines, such as transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF) and certain Th2 cytokines (Bhakta and Woodruff 2011). This can lead to irreversible airway narrowing, especially in more severe asthma subtypes.

Airway smooth muscle tissue plays a key role in asthma by releasing several cytokines, chemokines and lipid mediators that contribute to bronchoconstriction. The thickness of the airway smooth muscle layer (consisting of smooth muscle tissue, matrix, inflammatory cells,

mast cells and blood vessels) increases in asthma due to hypertrophy and hyperplasia. This is caused by the stimulation of airway smooth muscle cells by factors such as PDGF and endothelin-1 and is also associated with an increase in extracellular matrix. Smooth muscle hypertrophy occurs in the large airways in both non-fatal and fatal types of asthma, but hyperplasia is usually present in fatal (more severe) asthma only, and affects both the large and small airways (James et al. 2012, Berair et al. 2013).

Inflammation in asthma also causes changes to blood vessels: vasodilatation, angiogenesis, vascular permeability etc. Vasodilatation leads to increased airway mucosal blood flow which then contributes to airway narrowing as well, but at the same time it also may be important for removing inflammatory mediators from the airways. Angiogenesis occurs in response to factors such as vascular endothelial growth factor (VEGF) as well as inflammatory mediators (IL-4, IL-5, IL-13 etc.) and is one of the key features of airway remodeling in asthma. Moreover, airway oedema is present in the airway mucosa of patients with asthma, resulting from increased microvascular permeability during the inflammatory process, which may then further increase airway wall thickening and lead to more severe airway obstruction (Zanini et al. 2010). Although oedema is a common asthma feature, it is very difficult to directly evaluate and quantify oedema in the airways of asthmatic patients.

Chronic inflammation in asthma also leads to increased mucus secretion. This then contributes to the formation of viscid mucus plugs that obstruct or even plug the airways, especially in fatal asthma. Also, in asthma hyperplasia of submucosal glands in the large airways occurs and the number of epithelial goblet cells (that secrete gel-forming mucins, the major component of mucus) increases. Airway mucus hypersecretion may be indicative of poor disease control and it certainly contributes to the morbidity (and mortality) in asthma (Rogers 2004, Shale and Ionescu 2004, Evans et al. 2009).

Remodeling in allergic diseases (including asthma) is not restricted solely to the airways, but it also affects the upper and lower airways and the skin. Moreover, the same profile of inflammation, mediators and adhesion molecules (eosinophil, mast cell and CD4+ T cell influx; histamine, cysteinyl-leukotrienes, cytokines etc.) is common for all allergic diseases, although long-term structural changes differ. As remodeling is observed in all atopic (allergic) diseases, this reinforces the hypothesis that it is a mainly inflammation- driven process (Bergeron et al. 2009).

2.4. Diagnosis and assessment of asthma

Establising and confirming a diagnosis of asthma can be difficult and challenging, as there is an absence of a gold standard for defining it (or diagnosing) and it is commonly defined simply as reversible airway obstruction. The initial diagnosis of asthma is often based on identifying the presence of symptoms such as (recurrent) wheezing, cough, shortness of breath (dyspnea), chest tightness and variable expiratory airflow limitation (Figure 4). However, these symptoms are not specific to asthma only (similar ones are characteristic to bronchitis, especially chronic bronchitis, pneumonia and a number of other respiratory diseases) and establishing a diagnosis requires rather extensive clinical experience and judgement. Moreover, signs and symptoms of asthma vary significantly between patients and may even fluctuate in the same patient at different times and under different circumstances (depending on, for example, exposure to allergens). Therefore, it is important to establish whether the symptoms are recurrent (and how often they occur) as well as to identify if the symptoms are provoked by specific triggers, such as exposure to allergens or exercise. Additionally, a detailed personal and family medical history, such as commencement and existence of respiratory symptomy in childhood and details on atopy (allergy) as well as any other clinically relevant data should be recorded- this significantly increases the certainty in diagnosis of asthma (GINA 2018).

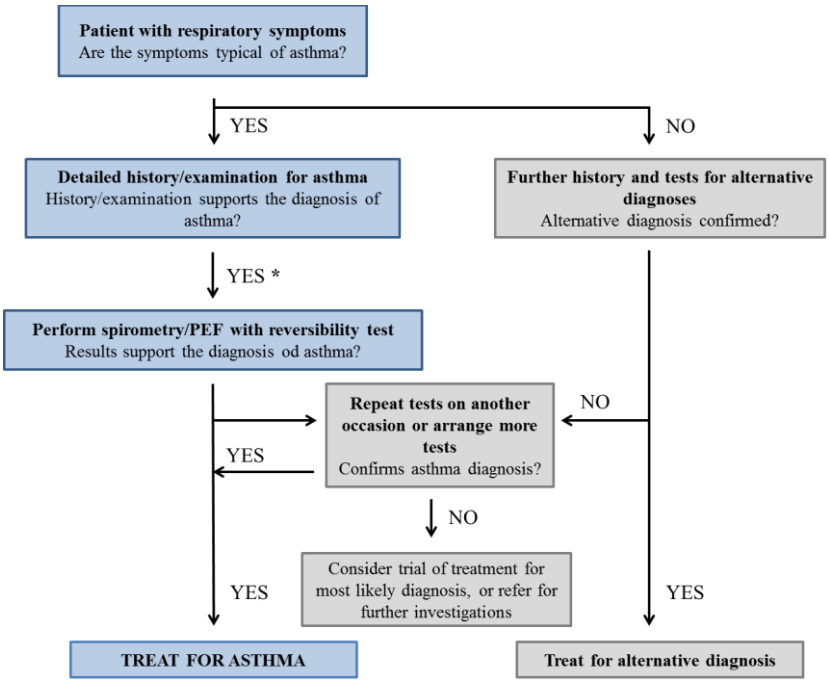


Figure 4. Diagnostic flow-chart for asthma in clinical practice. The diagnosis of asthma should be confirmed and evidence documented in the patient`s medical notes. Depending on clinical urgency and access to resources,

this should preferably be done before initiating controller treatment, as confirming a diagnosis of asthma is more difficult after treatment was started. ***** In case of clinical urgency (eg. current asthma attack), empiric treatment (eg. inhaled corticosteroids and bronchodilatator) should be administered and response to treatment assessed. Diagnostic tests should be performed again within 1 to 3 months after the event. Modified from (source): GINA 2018.

2.4.1. Objective measurements and tests for diagnosing and monitoring asthma

Of course, besides obtaining the clinical history, objective measurements are required in order to make a confident diagnosis of asthma. This is done by measuring lung function parametres by spirometry or peak expiratory flow measurement. Spirometry measures airflow obstruction, which is usually defined as a ratio of forced expiratory volume in 1 s (the volume of air exhaled in the first second of a forced exhalation from a position of maximum inhalation, FEV_1) to forced vital capacity (the total volume of air exhaled forcibly from maximum inhalation to maximum exhalation, FVC). Volumes (%) of FEV_1 and FVC below 80% of the predicted value for age, sex, height and ethnicity and FEV_1/FVC ratios below 0.7 (or 0.75) in adults and below 0.9 in children suggest airway obstruction. The lower the FEV_1/FVC ratio is, the more severe the obstruction. Peak expiratory flow (PEF) measures the maximum flow of air achievable from forced expiration, starting from maximum lung inflation. To establish a diagnosis of asthma, average daily diurnal PEF variability (calculated from 2 or 3 daily measurements as: PEF_{max} minus PEF_{min} for that day, divided by the mean of PEF_{max} and PEF_{min}, averaged during 1-2 weeks) should be greater than 10% (in children greater than 13%), or diurnal variability should be greater that 20% for at least 3 days in a week for a period of two weeks. Measures of gas trapping (residual volume- RV and the ratio of residual volume to total lung capacity- RV/TLC) as well as specific airway resistance (Raw), the so-called body plethysmography, may be superior to measurements of expiratory flow in detecting airway obstruction, especially in asymptomatic individuals and children (GINA 2018, BTS 2014, Criee et al. 2011).

When spirometry or PEF exhibit an obstructive pattern, in order to properly diagnose asthma, it is important to establish whether this obstruction is reversible. This is usually achieved by testing lung function with short-acting bronchodilatators and, when FEV_1 is less than 60% of the predicted value (for age, sex, ethnicity etc.), also with corticosteroids (oral or inhaled). A significant increase in FEV_1 (more than 12% from baseline value or more than 200 ml, in children more than 12% of the predicted value) after bronchodilatator indicates reversible airflow obstruction and supports the diagnosis of asthma. Increases in FEV_1 by more than

12% and 200 ml from baseline value (in children, by more than 12% of predicted value) after (at least) 4 weeks of anti-inflammatory treatment also strongly suggests a diagnosis of asthma. However, an absent response to bronchodilatators or corticosteroids does not exclude asthma. It is also worthy to note that response to bronchodilatators and corticosteroids is altered during and immediately after an exacerbation of the disease or viral infections, which is why it is recommended to perform diagnostic reversibility tests when the patient is clinically stable (BTS 2014).

2.4.2. Peripheral airways in asthma and methods of assessing peripheral airway dysfunction

The small, peripheral or distal airways, defined as airways with an internal diameter of less thna 2 mm, account for the majority of the luminal surface area within the airways, representing 98% of the total lung volume (James 2002). Due to a lack of accurate small airway dysfunction tests, direct evaluation of small airway tissue changes and obstruction has lead to the peripheral airways being termed the „lung`s quiet zone“ and to assessments in asthma being limited to the large central airways only. With the introduction of new techniques such as fiberoptic bronchoscopy, there has been a renewed interest in peripheral airways, which are now becoming increasingly appreciated for their significance to the clinical manifestations (expression) in asthma.

Assessments of the peripheral airway have traditionally been challenging due to their small internal diameter and deep location in the thoracic cavity. New and more specialized methods and techniques have been developed to better assess peripheral airway dysfunctions in asthma, such as forced expiratory flow at 50% ($FEF_{50\%}$) and at 25-75% ($FEF_{25-75\%}$) of forced vital capacity, measuring airway resistance with impulse oscilometry (IOS), single/multiple breath nitrogen washout, alveloar nitric oxide, late-phase sputum induction, imaging techniques etc (van der Wiel et al. 2013, Usmani 2014, Downie et al. 2007). Other methods such as transbronchial biopsy and bronchoalveolar lavage (BAL) are available, but far more invasive and as such not recommended to be performed in children, except in specific indications. A list of most commonly used small airways assessment methods are summarized in Table 3.

Table 3. Common methods of assessment of peripheral airways in asthma. $FEF_{25-75\%}$ and $FEF_{50\%}$- forced expiratory flow through 25-75% and at 50% of the forced vital capacity, R5- airway resistance at 5 HZ (IOS), R20- airway resistance at 20 Hz, R55-R20 – difference of R5 and R20, X5- reactance of the airways at 5 HZ,

AX- reactance area, Fres- resonant frequency of reactnace, FRC- functional residual capacity, CV- closing volume, CC- closing capacity, S_{acin} and S_{cond}- ventilation heterogenetiy indices in the acinar and conductive lung zones, respectively, HRCT- high-resolution computed tomography, H3HeMRI- magnetic resonance imaging with inhaed hyperpolarized helium-3 gas.

Method	**What it measures**	**Parameter**	
		Peripheral airways	**Central airways**
Spirometry	Airway obstruction	$FEF_{25-75\%}$, $FEF_{50\%}$, FVC/SVC	FEV_1, FEV_1/FVC, PEF
Impulse oscilometry	Airway resistance	R5-R20, X5, AX, Fres	R20
Body plethysmography	Air trapping	RV, RV/TLC, FRC	
Single breath nitrogen washout	Ventilation heterogeneity	CV, CC, Slope phase III	
Multiple breath nitrogen washout		S_{acin}, S_{cond}	
Exhaled nitric oxide (NO)	Airway inflammation	Alveolar NO	Bronchial NO
Induced sputum	Airway inflammation	Late-phase sputum	Early-phase sputum
HRCT/H3HeMRI	Imaging techniques	Lung attenuation	

Recognizing asthma as a disease of the entire respiratory tract is of great clinical significance and highlights the need to target the distal airways in therapeutic strategies for effective asthma management.

2.4.3. Other tests and investigations used in asthma diagnosing

Additionally, constrictor response tests can also be performed to assess the presence and severity of airway hyperresponsiveness. This is achieved by measuring the fall in FEV_1 after bronchoconstrictor stimuli, such as certain pharmacological agents in metacholine or histamine challenge tests, exercise in spiroergometry tests or allergens in allergen challenge (provocation) tests.

Above the age of 5 years, conventional lung function testing (measurements of airway obstruction, response to bronchodilatators and bronchoconstrictors and AHR) is usually possible in most children and most settings, although cooperation and compliance to the procedure is always an issue with children. In children between the age of 2 and 5 years, spirometry is commonly not performed, but most can perform other tests that do not rely so heavily on their ability to perform a forced expiratory manoeuvre. In general, these tests have not been evaluated as diagnostic tests for asthma specifically, as there is often substantial

overlap between the values measured in children with and without asthma. Of those tests, specific airways resistance (sRaw), impulse oscilometry (IOS) and measurements of residual volume (RV) appear most promising. In children under the age of 2 or even 3 years, lung function tests are almost impossible to perform and establishing a diagnosis of asthma is therefore extremely difficult, especially because episodic respiratory symptoms such as wheezing and cough are quite common in this population (associated with respiratory tract infections which occur 6-8 times per year in young children). This is why a confirmation of a diagnosis of asthma is often postponed for a certain period of time in which the child is carefully monitored (BTS 2014, GINA Pediatric 2015, Beydon et al. 2007, NHLBI 2007). Recently, novel objective respiratory function methods based on an impedance pneumography (IP) and flow-interruption (FI) technologies have been developed. These methods enable pulmonary function testing during tidal spontaneous breathing at home, even during sleep, which makes them suitable for use in very young children (Benoist et al. 1994, Lødrup et al. 1994, Dames et al. 2014., Gugten et al. 2013).

Other non-invasive tests are often performed to assess certain features of asthma, such as tests of eosinophilic airway inflammation. These include induced sputum differential eosinophil count and exhaled nitric oxide concentrations (FENO). Higher sputum eosinophil counts are associated with a higher degree of airway obstruction and reversibility, greater disease severity and atopy in general. In children with newly diagnosed mild asthma forms, sputum eosinophilia is present and usually declines with anti-inflammatory treatment (eg. with inhaled corticosteroids). Although sputum induction is easily obtainable in school-aged children, it is technically demanding as well as time consuming and therefore at present it remains mostly a research rather than a routine diagnostic tool (Lex et al. 2005, Rytila et al. 2004, Covar et al. 2004). Fractional exhaled nitric oxide (FENO) is feasible to measure in children from the age of 3 to 4 years. Increased levels of FENO are not a specfic marker of asthma and often ovelap with children who do not have asthma, because FENO is closely linked with atopy, age and height, but with underlying lung function. Still, measurement of FENO in exhaled breath is a quantitative, noninvasive, simple, and safe method of measuring the level of airway inflammation that provides a complementary tool to other ways of assessing asthma (Malmberg et al. 2005, Brussee et al 2005, Malmberg et al. 2006).

Atopy status is also often assessed in individuals with suspected asthma, especially in children. Positive skin tests (skin-prick tests- SPT), blood eosinophilia (more than 4% of relative count) or increased levels of total and especially allergen-specific immunoglobulin E

in serum increase the probablity of asthma in children with wheeze (Castro-Rodriguez et al. 2000, Chan et al. 2005, Simpson et al. 2005).

2.5. Asthma management and monitoring

Asthma management is defined as:

1. the control of symptoms such as wheeze, chest tightness, shortness of breath and cough,
2. reduction in the risk of severe and/or life-threatening exacerbations and long-term morbidity (including disease progression to more severe forms and other comorbidities) by therapeutic intervention (eg. with anti-inflammatory treatment), and
3. reducing adverse events and side-effects of treatment.

Recently, the main focus in the management of asthma has shifted from preventing (and sanation) of acute asthma attacks to achieving overall disease control, including improvements in symptoms score and overall quality of life (GINA 2018, BTS 2014). A list of tools commonly used to assess and monitor asthma and disease control are listed in Table 4.

Table 4. Summary of common tools in asthma management (monitoring and assessment of disease control), recommended to be clinically reviewed on at least an annual basis or more frequently, according to physician`s judgement (BTS 2014, Simon and Simon 2007, Hoffman et al. 2012).

Measurement/tool	**Description/comment**
Spirometry	Widely available, robust and enables clear demonstration of airflow obstruction. Adequate for children from the age of 5 years on.
Peak expiratory flow (PEF)	Widely available, simple (adequate for home use), results less reproducible than spirometry measurements. Changes in PEF are more meaningful that absolute values measured.
Asthma Control Test (ACT), Supplement 1	5 questions: 3 related to symptoms, 1 related to medication use and 1 to overall control. Score of ≥25 indicates good asthma control, 20-24 indicates moderate control and ≤19 indicates that asthma is poorly controlled. Validated in adults and children 4 years and older.
Airway hyperresponsiveness	Challenge tests (metacholine, indirect tests with inhaled mannitol, allergen challenge tests). Not applicable in patients with substantially impaired lung function ($FEV_1/FVC \leq 0.7$

Table 4. continued

	and FEV_1 ≤70% predicted).
Exhaled nitric oxide (FENO)	Normal range is <25 ppb at exhaled flow of 50 ml per second. Values >50 ppb in adults and >35 ppb in children highly predictive of eosinophilic airway inflammation and predicted positive response to corticosteroid treatment. Values <25 ppb highly predictive of absence of airway eosinophilia and of predicted poor response to corticosteroids or indicative for step down treatment with corticosteroids.
Eosinophil differential count (blood or induced sputum)	Feasible even in children, but time consuming and not available except in specialised centres. Normal range in sputum is <2% (or <3% in blood for adults and children older than 2 weeks), and <300 or <350 cells per microliter of blood- absolute values. Increased sputum eosinophil count closely related to corticosteroid responsiveness in adults and together with FENO in children.

Asthma management is adjusted in a continuous cycle to assess disease control, adjust treatment and review response (Figure 5).

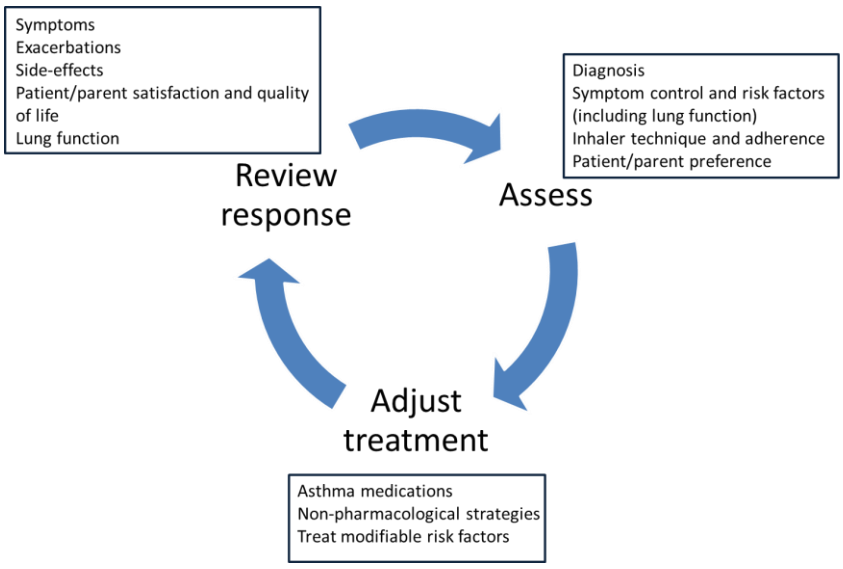


Figure 5. The control-based asthma management cycle and its main components. In children, especially those under the age of 5 years, parents or caregivers have a very important role in asthma management, which is why their preferences and satisfaction is cruicial in the management proccess. Modified from (source): GINA 2018, GINA Pediatric 2015.

A list of currently available and most commonly used treatment options for asthma is presented in Table 5.

Table 5. Most commonly used pharmacological treatment options for asthma today (Bonini and Usmani 2016).

Reliever medication (bronchodilatators)	**Controller medication (anti-inflammatory treatment)**
β2-agonists: Short-acting (SABA) Long-acting (LABA)	Corticosteroids: inhaled corticosteroids (ICS) oral corticosteroids (OCS) parenteral corticosteroids
Anticholinergics	Anti-leukotrienes (leukotriene receptor antagonists, LTRA)
Theophyline	Theophyline (slow-releasing)
	Cromones
	Macrolides
	Monoclonal anti-IgE antibody (omalizumab)

For the effective managament of asthma, experts recommend employing a stepwise approach. This stepwise approach is aimed at achieving early and adequate disease control by eliminating symptoms as soon as possible and to optimise lung function (eliminate airflow obstruction) by initiating treatment at the level most likely to achieve this. Based on this approach, asthma management in adults and children can be divided into five steps, as shown in Figure 6 (GINA 2018, GINA Pediatric 2015, BTS 2014).

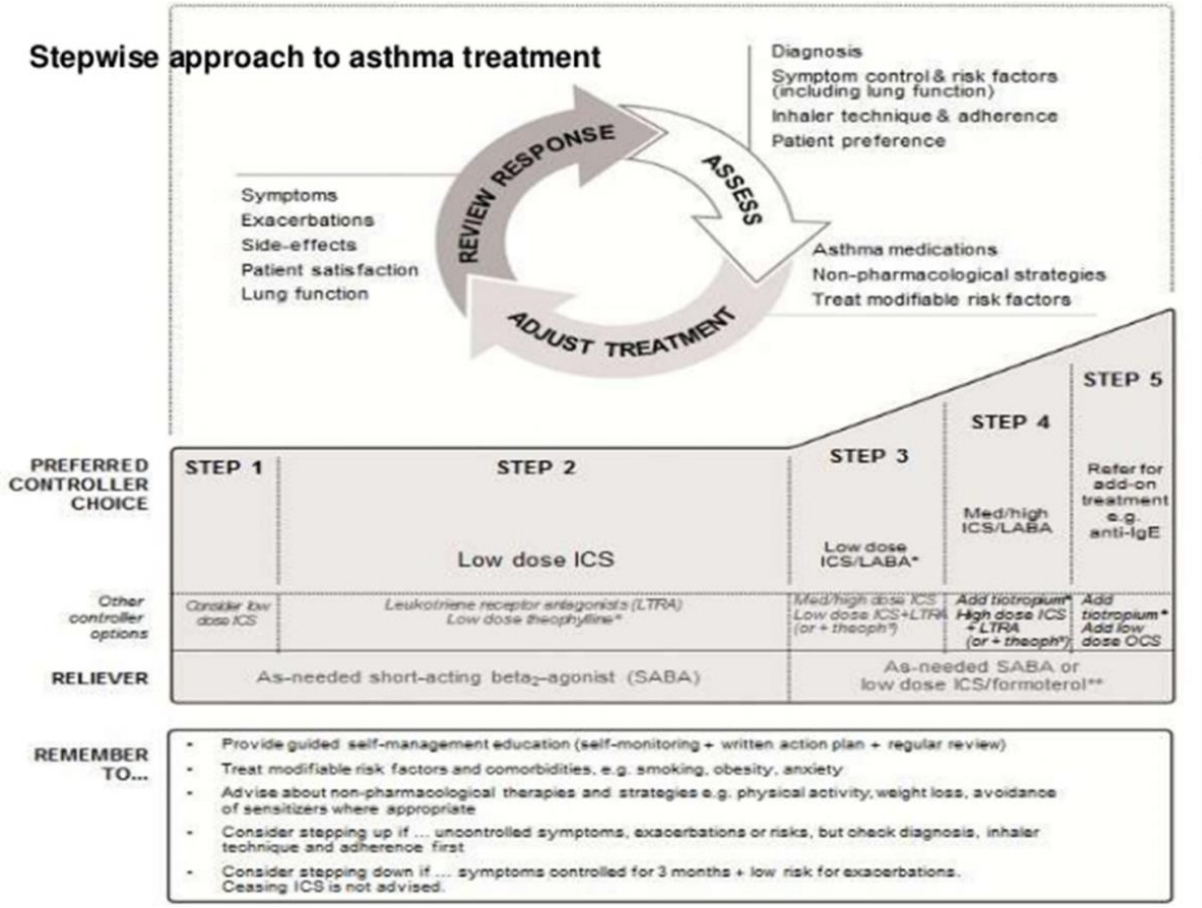


Figure 6. A schematic representation of the stepwise approach in asthma management. *For children aged 4 to 11 years, the use of theophyline is not recommended as controller treatment and the preferred option in Step 3 is medium dose ICS. In step 5, some patients may benefit from low dose OCS, but long-term systemic side-effects may occur. For children under the age of 5 years, step 1 treatment is as needed SABA, step 2 is daily or intermittent low dose ICS or LTRA, step 3 is „double low dose ICS“ and add-on option is LTRA, while step 4 is increasing the dose of ICS and add-on regular LTRA, with further expert advice, investigation and reconsidering diagnosis highly recommended. Source: GINA 2018, GINA Pediatric 2015.

Patients should be carefully monitored every 1 to 3 months after treatment initiation, and every 3 to 12 months after that, if adeqate control is achieved or 1 week after an exacerbation for response review. Since asthma is often a variable condition, periodic adjustment of controller treatment may be needed. If adequate control is not reached, treatment step-up is recommended in a:

1. sustained step-up manner for at least 2 to 3 months and if symptoms/exacerbations persist, other common causes such as incorrect inhaler technique and poor adherence (especially in young children), modifiable risk factors (like smoking) or comorbidities need to be assessed,
2. short-term step-up manner for 1-2 weeks due to viral infection or allergen exposure, or
3. day-to-day adjustment manner for patients on treatment steps 3-5.

If asthma control has been reached and maintained for at least 3 months, treatment step-down should be considered by reducing ICS dose by 25-50% at 2 to 3-month-long intervals, during which the patient should be carefully monitored and disease control reassessed. A summary of the current guidelines in the management and treatment of asthma is presented in Supplement 2.

2.6. Heterogeneity in asthma

Asthma and its key mechanisms remain poorly understood despite the fact that much progress has been made in understanding the underlying pathogenesis in recent years. This has largely been due to the failure in successfully identifying the distinct disease subtypes in asthma.

While asthma is generally considered an inflammatory disorder of the conducting airways, it is evident that the disease is extremely heterogeneous with respect to immunopathology, clinically observed phenotypes, response to treatment, and natural history. Although it was once considered purely an allergic disease dominated by Th2-type lymphocytes, IgE, mast cells, eosinophils, macrophages, and cytokines, it is becoming increasingly apparent that the pathogenesis of asthma also involves local epithelial, mesenchymal, vascular and neurologic events that are involved in directing the Th2 phenotype to the lung and through aberrant injury-repair mechanisms to remodeling of the airway wall (Holgate 2008). This demonstrates the urgent need for identifying additional immunologic and inflammatory pathways involved in asthma, in order to reveal new ways of intervening in the prevention and treatment of the disease.

The most common asthma form is allergic asthma (especially in children), induced by allergic sensitization (usually by inhaled allergens) and immunoglobulin E (IgE) as well as Th2-mediated immune response play a crucial role in the pathogenesis of allergic asthma. A common form of asthma is non-specific asthma, caused by respiratory infections (viral), air pollutants (smoke, ash and other large particles), probably due to physical (mechanical) damage to the airways, physical activity and emotional stress. Asthma is often accompanied by comorbidity- allergic rhinitis/rhino-conjunctivitis (AR), atopic dermatitis (AD), gastro-oesophageal reflux disease (GERD), obstructive sleep apnoea syndrome (OSAS), obesity etc. This, along with the variability in disease aetiology, level of inflammation, bronchial damage and lung function impairment, specific clinical features and natural course of the disease (persisting to adulthood or remission in adolescence), reflect the vast heterogeneity and complexity of asthma. Current knowledge of asthma pathophysiological mechanisms as a Th2 cell mediated allergic reaction does not suffice in explaining and dealing with a large portion of this heterogeneity, which is why in the past few years asthma has been revised and considered as a complex syndrome of several different subtypes (phenotypes) defined by newly conceived immuno-pathophysiological mechanisms called endotypes (Gagro 2011). This is also reflected in the multiplicity of asthma risk factors, both endogenous (sex, hormonal status, genetic predisposition and epigenetic status) as well as environmental factors (allergens, viral infections, air pollution etc.).

2.6.1. Phenotypes in asthma

Phenotypes are described as the observable characteristic of an organism produced by interactions of the genotype and the environment (Henderson 2014). The presence of distinct asthma phenotypes has been described for a number of years: in the mid 20th century there were two clinically distinct phenotypes of asthma defined- extrinsic (allergic) or early-onset asthma and intrisic (non-allergic) or late-onset asthma, with the age of onset emerging as one of the main clinical characteristics to define disease heterogeneity (Rackermann 1947). Subsequently, asthma phenotypes which identified the presence of eosinophilic inflammation and its link to corticosteroid responsiveness emerged (Brown 1961, Berry et al. 2002). Further studies confirmed the heterogeneity in asthma leading to the conclusion that not all patients respond to anti-inflammatory (corticosteroid) treatment and that treatment reponse is largely dependent on the presence and type of airway inflammation (Pavord et al. 1999, Gibson et al. 2001, Wenzel et al. 1999, Green et al. 2002), as well as that patients with poor response to treatment tend to develop severe, uncontrolled disease. The group of patients with non-eosinophilic type of inflammation were then further stratified into neutrophilic asthma, mixed granulocytic asthma with increased eosinophils and neutrophils and paucigranulocytic asthma with normal levels of both eosinophils and neutrophils (Simpson et al. 2006). Recently, there have been several attempts to use novel computing and machine learning techniques (eg. cluster analysis) to identify additional phenotypes in asthma (Haldar et al. 2008, Siroux et al. 2011, Wu et al. 2014). Although these studies have performed unbiased statistically based analyses on large cohorts of patients involving a wide range of clinical variables, they have been limited in the terms of clinical characteristics they have used to identify different phenotypes in asthma and still do not provide much insight into the underlying disease mechanisms (Gauthier et al. 2015, Ray et al 2015).

2.6.2. Endotypes in asthma

Although different phenotypic clusters of asthma have emerged, these have been primarily clinically oriented and while they are useful for classifying patients, they provide little reference to the underlying pathophysiological processes. This has been one of the major hindrances in the development of targeted therapies in asthma so far (Chung and Adcock 2015). Therefore, in order to capture disease mechanisms, molecular phenotypes or „endotypes“ are now being defined by integrating and correlating molecular markers with clinical phenotypes to describe a specific disease subtype based on distinct

pathophysiological mechanisms. For example, a clinical phenotype of asthma may be underpinned by several discrete endotypes, each of which leads to a final common pathway of disease manifestations that are characteristic to that particular phenotype. Conversely, a discrete endotype (for example, a particular inflammatory pathway such as eosinophilic airway inflammation) could be exant in a number of different clinical phenotypes, as shown in Figures 7A and 7B (Henderson 2014, Lötval et al. 2011, Wenzel 2012, Ray et al. 2015, Anderson 2008).

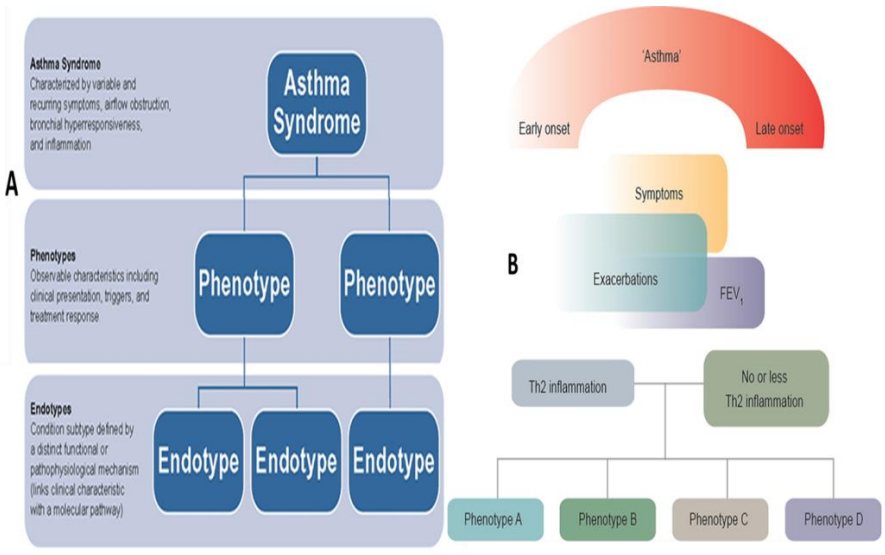


Figure 7. A schematic representation of asthma subtypes: phenotypes and endotypes. **A** hiearchy of asthma phenotypes and endotypes. **B** Schematic representation of potential asthma phenotypes. For example, Th2-associated asthma and non-Th2-associated asthma may share key features of symptoms, lung function and exacerbations but can be further subdivided according to other characteristics (Phenotypes A, B, C or D), including some molecular mechanisms. Source: Lötvall et al. 2011, Wenzel 2012.

Most recent studies utilizing genetic, epigenetic and transcriptomic data together with extensive clinical data have been even more successful in characterizing the underlying predisponing genetic, epigenetic and gene expression patterns associated with specific asthma endotypes. Such approaches further advance the possibility of stratifying asthma at the molecular level in order to achieve personalized treatments and individually tailored management strategies (Chung and Adcock 2015, Wesolowska-Andersen and Seibold 2015).

2.6.3. Asthma and obesity

Obesity, that is, overweightness is one the most common asthma comorbidities. Approximately 38% of current adult asthmatics are also obese in the US, and obesity has been shown to be an indepedent risk factor for developing asthma. Obese asthmatics have an

increased risk for asthma exacerbations, more frequent and worse respiratory symptoms as well as poor disease control and poor quality of life, despite often using high-dose inhaled corticosteroids (Baffi et al. 2015). A meta-analysis of 7 longitudinal cohort studies involving over 300,000 adults indicated a dose response effect between increasing BMI and the odds ratio (OR) of incident asthma (Beuther and Sutherland 2007). Since obesity is a risk factor for asthma and a potential culprit for the rise in prevalence of asthma in both children and adults and due to the fact that asthma and obesity have common pathophysiological mechanisms, it is now thought that being obese and having asthma constitutes a unique clinical phenotype; in other words, the interaction of obesity (as an environmental risk factor) with underlying genetic traits (or other host susceptibility factors) leads to a set of defined observable traits- one or, more likely, multiple specific phenotypes. Although the clinical manifestations have been well documented, the aetiologies of obese asthma still remain unclear (Scott et al. 2017).

The mechanisms by which obesity exacerbates asthma are both mechanistic and physiological. Increased adiposity around the chest wall and abdomen may lead to lung restriction, resulting in reduced total lung capacity and most notably, low expiratory reserve volume, from upward diaphragmatic displacement due to increased abdominal fat. Consequently, airway closure occurs at or above functional residual capacity in the dependent lung zones, which can lead to significant ventilation/perfusion mismatching (Salome et al. 2010). Although obesity is not associated with more airway obstruction, studies indicate that it is a risk factor for increased bronchial hyperresponsiveness (Dixon et al. 2010). Moreover, obesity promotes systemic inflammation, potentially exerting effects in the airways.

Sputum samples of patients with asthma reveal that the obese have the largest proportion of non-eosinophilic airway inflammation. This is probably not due to an absolute reduction of airway eosinophils in obese patients, but rather, due to reduced migration into the airway lumen. A study of severe asthmatics demonstrates that obesity is associated with increased sub-mucosal eosinophils (but not in the airway lumen) and with greater IL-5 sputum levels (Desai et al. 2013). In contrast, obesity has been associated with increased airway neutrophilia (Telenga et al. 2012). All this suggests appear that the obesity-mediated changes in airway inflammation are more consistent with a non-predominant Th-2 phenotype, and potentially a more Th-1 polarized immune response (Rastogi et al. 2012). IL-17 producing innate immune cells detected in human BAL may constitute an additional non-Th2 pathway in obese asthma (Celedon and Kolls 2014). Increase in adipose tissue correlates with an

increase in the levels of leptin as well as a reduction in the levels of adiponectin and both of these changes have been implicated in the obese asthma pathogenesis (Dixon 2009). The levels of leptin are higher among asthmatics, and increase in relation to plasma leptin levels and BMI (Holguin et al. 2011, Lugogo et al. 2012). The magnitude of leptin receptor expression in visceral fat has been related to BHR (Sideleva et al. 2012) and moreover, leptin has been shown to increase the oxidative and inflammatory response of alveolar macrophages derived from overweight and obese asthmatics *ex vivo* (Lugogo et al. 2012). Adiponectin, on the other hand, probably has protective effects. In females, higher plasma adiponectin levels are associated with decreased asthma risk (Sood et al. 2008), but whether it has anti-inflammatory or immunomodulatory effects in the human airway is not clear. Both asthma and obesity are characterized by greater oxidative stress and may act synergistically to further increase it, especially in the NO metabolism. Exhaled NO (eNO) and BMI are inversely correlated in late-onset compared to early onset (childhood) asthma. This may be due to an imbalance between L-arginine, the precursor of NO and substrate for inducible nitric oxide synthase (iNOS), and asymmetric di-methyl arginine (ADMA), which is an endogenous inhibitor of all NOS synthases (Holguin et al. 2013). Lower L-arginine might result from increased arginase activity, which is associated with asthma severity, and increased ADMA has been related to obesity and metabolic syndrome (Morris et al. 2004, Palomo et al. 2011). Having lower airway NO bioavailability at baseline may impair the degree of physiological bronchial dilation, leading to increased respiratory symptoms, poorer disese control and imparied lung function (Ricciardolo et al. 2004). Compared to those with normal BMI obese asthmatics have poorer response to treatment with ICS according to changes in lung function parametres and disease control. This reduced steroid response in obese asthmatics could be due to increased steroid resistance. Studies have shown that obesity was associated with an *in vitro* blunted response to dexamethasone-induced mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) and baseline tumor necrosis factor (TNF)-alpha in peripheral blood mononuclear cells (PBMCs) and BAL cells (Peters-Golden 2006, Sutherland et al. 2010). Obesity in asthma also increases the risk for other chronic diseases (such as OSA, GERD and metabolic syndrome), which are associated with worsened respiratory symptoms and poorer disease control (Teodorescu et al. 2013, Samson and Garber 2014). A summary of pathophysiological mechanisms involved in obese asthma are shown in Figure 8.

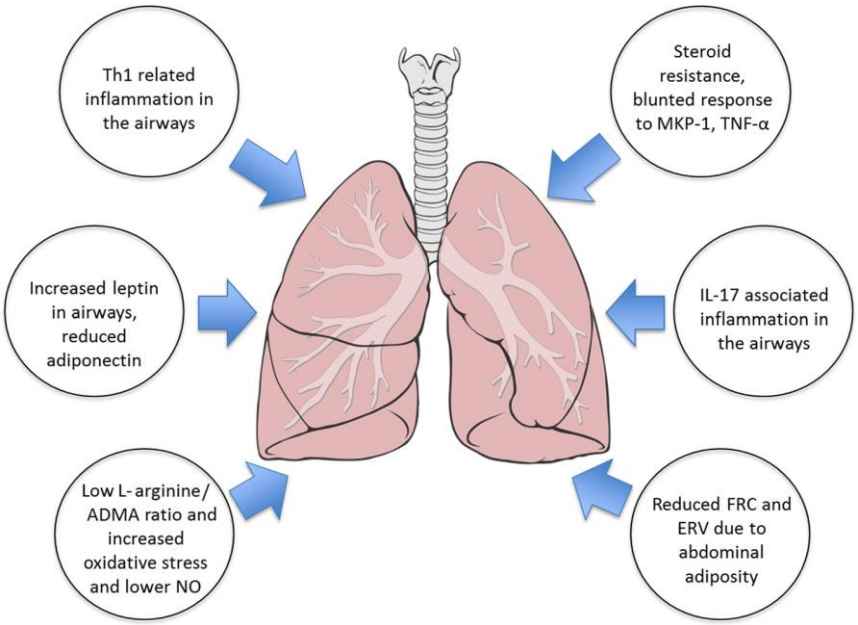


Figure 8. Pathophysiological mechanisms of obese asthma. A variety of mechanisms have been proposed as drivers of the physiologic and clinical observations in obese asthmatics, including changes in adipokines; Th-1 skewed airway inflammation, lower ADMA to L-arginine ratio resulting in increased oxidative stress and decreased physiologic NO- a mediator in smooth muscle dilatation, reduced FRC and ERV due to excess abdominal adiposity, IL-17 associated airway inflammation, steroid resistance and dampened response to MKP-1. Th-1- T-helper 1 cells, ADMA- asymmetric dimethylarginine, NO- nitric oxide, FRC- functional residual capacity, ERV- expiratory reserved volume, IL-17- interleukin 17, MKP-1- mitogen-activated protein (MAP) kinase phosphatase-1. Source: Baffi et al. 2015.

Because the origins of obesity and asthma are multifactorial, it is now believed there are multiple obese-asthma phenotypes, with varied aetiologies and clinical consequences, and with childhood or early-onset obese asthma being an independent one (Baffi et al. 2015).

2.7. Genetics of asthma

Genetic predisposition, that is, heritability, undoubtely plays a crucial role in the onset and development of asthma, as this disorder, as well as other allergic disorders, is more common in individuals with a positive atopic (allergic) and asthmatic family background. Twin studies have indicated that certain parameters, such as bronchial hyperresponsivness (BHR), are inherited independently (Los et al. 2001). Numerous genetic loci (SNPs, Single Nucleotide Polymorphisms) have been associated with the onset, progression and clinical features of asthma through genome-wide association studies (GWAS), and have recently been re-assessed through a large meta-analysis involving more than 10,000 asthma patients (Moffatt

et al. 2010). These studies indicated that certain chromosome regions are associated with susceptibility to asthma, such as: 6p- containing genes encoding the major histocompatibility complex (MHC), 11q- containing genes encoding the high-affinity IgE receptor and glutathione S-transferase, 20p- containing the gene encoding ADAM33, which plays an important role in cellular fusion, adhesion, signalling and proteolysis processes and certain regions on chromosome 17- containing the gene *ORMDL3* encoding an epithelial protein strongly associated with asthma development (Ober and Yao 2011). A GWAS meta-analysis has been recently conducted across different ethnicities highlighting the role of genes at the the 17q21 region (including genes encoding for interleukin 1 receptor-like 1- IL1RL1, thymic stromal lymphopoietin- TSLP, interleukin 33- IL33 etc.) across different ethnic groups (Torgerson et al 2011). Genetic risk of childhood-onset asthma has been reviewed recently emphasizing the role of the 17q21 region and more specifically, the *GSDMB-ORMDL3* (encoding for gasdermin B- GSDMB and oromucosoid 1 like-3- ORMDL3) locus in childhood asthma (Cookson et al. 2011). Novel technologies such as whole exome sequencing are only beginning to be utilized in the field. Recently variants in the *CBLB* (an E3 ubiquitin-protein ligase), *KALRN* (Kalirin RhoGEF kinase) and *PDE4DIP* (Phosphodiesterase 4D interacting protein) genes were foun d to segregate with patients in a family affected by asthma (DeWan et al. 2012). Certain cytokine and chemokine encoding genes, involved in the Th2 mediated immunological response, genes encoding proteins involved in oxidative stress processes and the keratin-binding protein filaggrin (FLG) have also been associated with asthma and other allergic diseases (Michel et al. 2010).

Genes implicated by genome-wide association studies (GWAS), genome-wide linkage studies, and candidate gene studies can broadly be divided into 4 groups (according to their function and role in the development of asthma and allergic diseases):

1. genes involved in epithelial barrier function,
2. genes involved in environmental sensing and immune detection,
3. genes ivolved in tissue response to allergic inflammation, and
4. genes involved in TH2 cell polarization and response (presented in Figure 9).

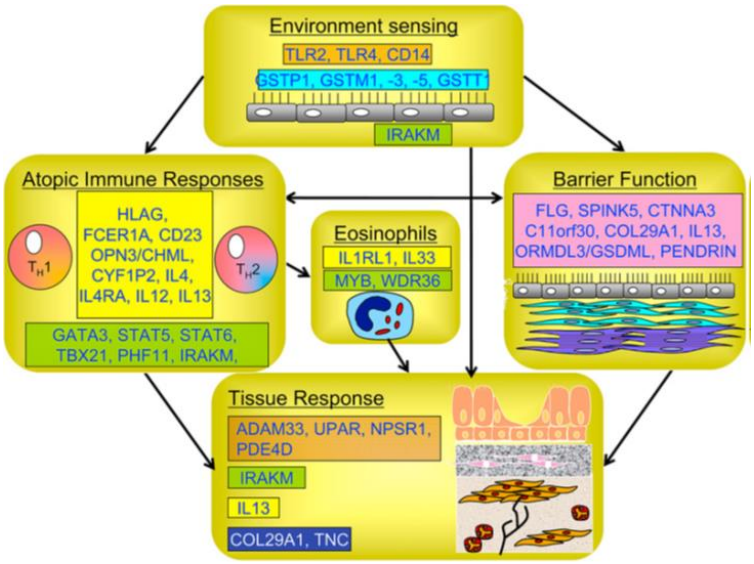


Figure 9. Susceptibility genes for asthma and allergic diseases. Source: Holloway et al. 2010.

2.7.1. Genes involved in epithelial barrier function

A high proportion of the novel genes identified for susceptibility to allergic disease through genome-wide linkage and association approaches have been shown to be expressed in the epithelium. The RAD50 homolog is important for DNA double-strand break repair, cell-cycle checkpoint activation, telomere maintenance, and meiotic recombination. This gene is also adjacent to the interleukin 4/13 (*IL4/IL13*) locus and asoociated with increased total IgE levels, eczema, and atopic dermatitis. The *FLG* (filaggrin) gene, as well as *RAD50* and *IL-13* have already been associated with allergic diseases (atopic dermatitis) and recent studies have implicated an overlap of certain loci within these genes with asthma and allergic rhinitis pathogenesis. Genes encoding filaggrin (a protein involved in keratin aggregation), defensin beta-1 and the *RAD50/IL-13/IL4* loci may be also associated with asthma susceptibility and level of response to treatment in patients with loss-of-function FLG variants or defensin and RAD50/IL-13, which may be due to allergen sensitisation that occurs after the breakdown of the epithelial barrier (Weidinger et al. 2013). Other susceptibility genes, such as *ORMDL3/GSDML* (gasdermin-like or gasdermin B), *PCDH1* (encoding protcadherin 1), and *C11orf30* (or *EMSY*, BRCA2 Interacting Transcriptional Repressor) are also expressed in the airway epithelium and might have a role in regulating epithelial barrier function (Koppleman et al. 2009, Moffat et al. 2007, Holloway et al. 2010).

2.7.2. Genes involved in environmental sensing and immune detection

Genes involved in sensing of environmental influences and immune detection include genes of the MHC class, genes encoding Toll-like receptors (TLR1, TLR6 and TLR10) an others. This group of genes encodes molecules that directly modulate the effect of environmental risk factors for allergic disease. For example, genes such as *TLR2*, *TLR4*, and *CD14*, encoding components of the innate immune system, interact with levels of microbial exposure to alter the risk of allergic immune responses (Yang et al. 2007). Additionally, polymorphisms in genes encoding glutathione-S-transferase (*GSTM1*, *GSTM2*, *GSTM3*, *GSTM5*, *GSTT1*, and *GSTP1*) have been shown to modulate the effect of environmental exposures involving oxidant stress, including tobacco smoke and air pollution, on susceptibility for asthma (Romieu et al 2009, Breton et al. 2009).

2.7.3. Tissue response to allergic inflammation

A variety of genes involved in mediating the response to allergic inflammation and oxidative stress on tissue level appear to be important contributors to asthma and allergy susceptibility as well as disease progression and treatment effectiveness. Examples include genes encoding ADAM33, a disintegrin and metalloprotease expressed in lung fibroblasts and smooth muscle cells, the alpha-1 chain of type 29 collagen (COL29A1), phosphodiesterase 4D (PDE4D), leukotriene C4 synthase (LTC4S), glutathione-S-transferase (GSTP1, GSTM1), arachidonate 5-lipoxygenase (ALOX-5), nitric oxide synthase 1 (NOS1), metallopeptidase 9 (MMP9), which are expressed in lung fibroblasts and smooth muscle cells, as well as the β2 adrenergic receptor (ADRB2) etc. (Lotvall et al. 2011, Himes et al. 2009). The latter two genes (*MMP9* and *ADRB2*) have also been significantly associated with remodeling events that occur in asthma and related conditions (such as chronic obstructive pulmonary disease, COPD) as well as with response to certain classses of common asthma treatment, with *MMP9* being pivotal in remodeling in asthma for its role in extracellular matrix degradation and *ADRB2* being crucial for bronchodilatation (Ohbayashi and Shimokata 2005, Litonjua et al. 2010). Polymorphisms in the *VEGFA* gene (vascular endothelial growth factor A, involved in tissue remodeling processes, mainly angiogenesis) have been associated with asthma, especially with phenotypes involving extensive airway tissue remodeling. The *TBXA2R* gene encodes the thromboxane receptor (TP), also known as the prostanoid TP receptor. Variations in this gene have been associated with asthma and level of response to therapy. *TBX21* encodes the T-box transcription factor TBX21, a member of a phylogenetically conserved family of genes

that share a common DNA-binding domain, the T-box. TBX21 protein is a Th1 cell-specific transcription factor that controls the expression of the hallmark Th1 cytokine, interferon-gamma (IFNγ). Variations in this gene have also been assoicated with asthma as well a to response to treatment (Van Eerdewegh al. 2002, Söderhall et al. 2007).

2.7.4. Th2 cell polarization and response

Th2 cell-mediated adaptive immune responses have been widely recognized as a crucial component of allergic disease. Genes involved in Th2 cell differentiation and function have been extensively studied in asthma candidate-gene association studies, and as one might expect, SNPs in many of these genes have been associated with asthma and other allergic phenotypes. Genes important for Th1 versus Th2 T cell polarization, such as those encoding transcription factors and certain cytokines (*GATA3, TBX21, IL4, IL4RA, STAT6*, and *IL12B*), have been implicated with asthma and specific disease phenotypes, as well as with response to treatment (Agache et al. 2012). The genes encoding IL-13 and the beta-chain of the IgE receptor FcεR1 are well replicated contributors to asthma susceptibility. This group includes genes that regulate Th1/Th2 differentiation and effector function (eg. *IL13, IL4RA, STAT6, TBX21* and *GATA3*), as well as genes such as *IRAKM, PHF11* and *UPAR*, that potentially regulate both allergic sensitisation and the level of inflammation that occurs at the end-organ location for allergic disease. This group also includes genes shown to regulate the level of blood eosinophilia: *IL1RL1, IL33, MYB*, and *WDR36* (Kabesch et al. 2006, Suttner et al. 2009, Pykäläinen et al. 2005, Barton et al. 2009).

2.7.5. Corticosteroid transport and signaling genes

Genes involved in corticosteroid transport have been associated with specific asthma endotype pathogenesis and response to treatment (ICS) in patients with different asthma phenotypes. These include: the gene encoding the corticosteroid-binding globulin SERPIN1, a major glucocorticoid transporter, that has already been associated with certain asthma subtypes and with the level of response to inhaled corticosteroids (Dijkstra et al. 2011), the gene encoding the solute carrier family 22, member 2 (SLC22A2), also known as organic cation transporter 2 (OCT2), which is predominantly expressed in the luminal membrane of airway epithelial cells and is involved in the release of acetylcholine from bronchial epithelium, and has previously been associated with certain asthma endotypes and response to medication (Park et al. 2011, Lips 2005) and other. Corticosteroid-induced genes, that is, corticosteroid-responsive genes, namely the gene *GLCCI1*, encoding the glucocorticoid

induced 1 protein of unknown function (although it may be an early marker for glucocorticoid-induced apoptosis), has already been associated with asthma pathogenesis and the level of response to asthma treatment, along with *CRHR1*, encoding the corticotropin releasing hormone receptor 1, essential for the activation of signal transduction pathways that regulate diverse physiological processes including stress, reproduction, immune response and obesity (Tantisira et al. 2011, McGeachie et al. 2013).

As all of these genes were identified in screens for asthma susceptibility genes, most of them are listed as risk factors. Protective factors for asthma are less well characterized, and are often more descriptive of general genetic or lifestyle factors, such as a negative family history, history of breastfeeding, and exposure to pets and/or livestock and farm animals (Sandini et al. 2011). Exposure to endotoxin at young ages has been shown to be protective against the development of asthma. There is also a correlation between higher levels of the endotoxin binding molecule CD14 (soluble form) and lower asthma prevalence, and in animal models this is dependent on the Toll-like receptor 4 (TLR4). The proteins encoded by the *TBX21* and *IL12B* genes promote the development of Th1 cells, which contribute to inflammation and generally suppress atopic phenotypes. It is likely that future studies of the genes identified as susceptibility loci will show that some have roles more consistent with protection from rather than susceptibility to asthma, at which point those genes would be recategorized.

2.8. Response to asthma treatment

As there is still no real cure to asthma (due to the overwhelming complexity of this disease), today, common asthma treatment is actually symptomatic treatment, with short-term medications that are mostly used to relieve current symptoms (reliever medication) and long-term medication used in case of persistent symptoms to control the underlying inflammation and prevent exacerbations (controller medication). These short term treatment options involve:

1. bronchodilators, such as short acting β-adrenoreceptor agonists (SABA), used for example in case of asthma attacks or shortness of breath,

2. both oral and parenteral corticosteroids, such as medrol (methylprednisolone), prednisone, pronisone, dexamethasone and solu-medrol (methylprednisolone sodium succinate),
3. and other drugs, such as theophylline, which are mostly used in the management of asthma exacerbations.

Long-term medication involves:

1. inhaled glucocorticoids (inhaled corticosteroids, ICS), the most commonly used treatment option in asthma,
2. leukotriene receptor modifiers (LTM)- most commonly leukotriene receptor antagonists (LTRA) and, less frequentely, leukotriene inhibitors (LTI), and
3. long acting β-adrenoreceptor agonists (LABA), which are commonly used in combination with ICS by millions of patients with asthma, mostly those with greater disease/symptom severity as maintenance treatment.

Corticosteroids are synthesized and secreted by the cortex of the adrenal gland as a result of stimulation by the hypothalamus-pituitar-adrenal (HPA) axis. The HPA axis is responsible for the adaptation to stress and inflammatory stimuli. This response is characterized by a hypothalamic release of corticotropin-releasing hormone (CRH), which acts by combining with the CRH receptor (CRHR), predominantly CRH receptor 1 (CRHR1). Both endogenous and exogenous corticosteroids act by binding intracellularly to glucocorticoid receptors (GR). A protein complex including heat shock proteins HSP70 and HSP90 binds the inactive GR. While HSP70 inactivates GR through partial unfolding, HSP90 reverses this inactivation, and is required for activation of GR. GR homodimers bind to glucocorticoid response elements (GREs) in the promoter region of steroid-sensitive genes, by which the anti-inflammatory genes such as annexin-1, secretary leukocyte protease inhibitor (*SLPI*), MAPK phosphatase-1 (*MKP-1/DUSP1/MAPK1*), *NF-kB*, inhibitor of NF-kB alpha (*NFKBIA*), *IL-10* and glucocorticoid-induced leucine zipper (*GILZ*) are activated. Certain transcription factors (NF-kB and AP1) activate coactivator molecules, like CBP (cAMP-response-element-binding-protein (CREB) binding protein) which bind to nuclear GRs, which inactivates a number of pro-inflamamtory genes, such as IL-8 (Barnes 2006, Duong-Thi-Ly et al. 2017). Molecular mechanisms involved in the anti-inflammatory action of corticosteroids are shown in Figure 10. Corticosteroids also exert their anti-inflammatory action by inducing histone acetylation (and subsequent activation) of anti-inflammatory genes (eg. *MKP-1*), and by recruiting

histone deacetylases (HDAC2) and inducing deacetylation (and subsequent silencing) of proinflammatory genes, for example *IL-8, NF-κB, AP-1* (Barnes 2009).

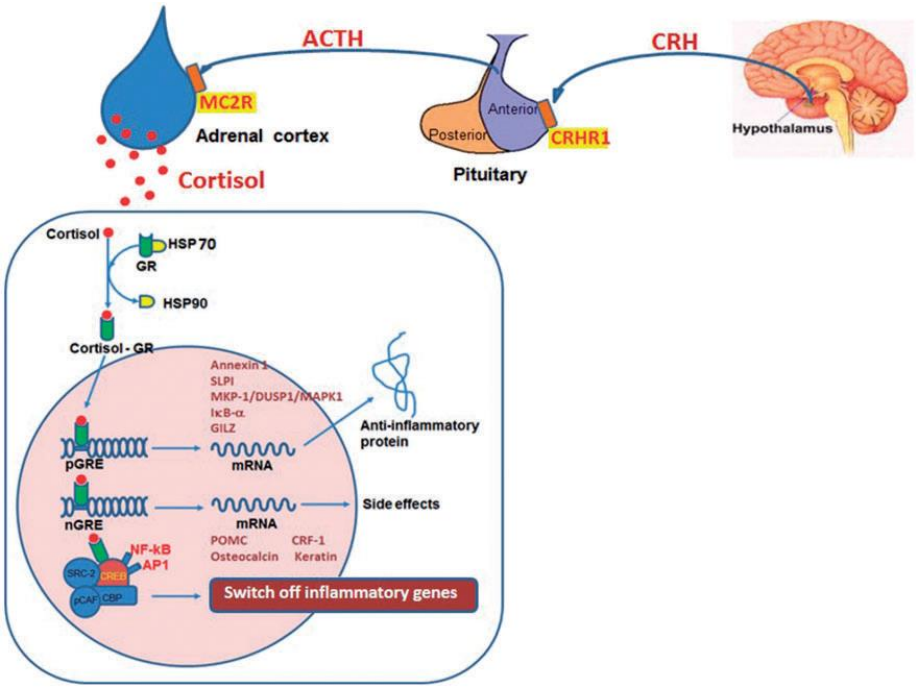


Figure 10. A schematic representation of anti-inflammatory mechanism of corticosteroids. ACTH- adrenocorticotropic hormone, MC2R- melanocortin 2 receptors, STIP1- stress induced phosphoprotein 1, NR3C1- nuclear receptor subfamily 3 group C member 1, DUSP1- dual specificity phosphatase 1, ORMDL3, ORMDL sphingolipid biosynthesis regulator 3, pCAF- p300/CBP-associated factor, SRC- steroid receptor co-activator, POMC- proopiomelanocortin, CRF- corticotrophin releasing factor. Source: Duong-Thi-Ly et al. 2017.

The leukotriene pathway begins with the conversion of arachidonic acid to leukotriene A4 (LTA4), a reaction catalyzed by the enzyme 5-lipoxygenase (5-LO). LTA4 is subsequently converted to LTC4 under the influence of leukotriene C4 synthase (LTC4S), which is transported extracellularly. Sequential cleavage of glutamate and glycine residues results in the formation of leukotriene E4 and D4. Leukotrienes bind to receptors present on leukocytes and lung smooth muscle cells, such as cysteinyl leukotriene receptor 1 (CysLTR1), to cause smooth muscle contraction and mucus secretion. Current antileukotriene treatment (LTM) include CysLTR blockers or LTRA and inhibitors of 5-LO or LTI (Tse et al. 2011). The leukotriene pathway and mechanisms of LTM action are shown in Figure 11.

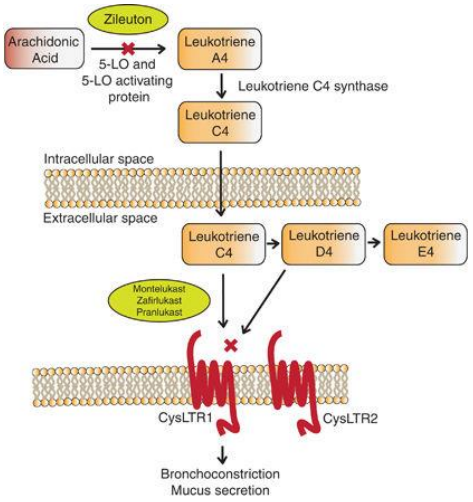


Figure 11. A schematic representation of the leukotriene pathway, with sites of action of antileukotriene treatment indicated in red x. Zileuton- an LTI, a 5-LO inhibitor montelukast, zafirlukast and pranlukast- LTRA, blockers of CysLTRs. CysLTR1- cysteinyl leukotriene receptor 1, CysLTR2- cysteinyl leukotriene receptor 2. Source: Tse et al. 2011.

Combination treatment (ICS+LABA) is frequently used in the control of asthma and it is now recognized that there are important molecular interactions between these two classes of drug. Corticosteroids increase β2-receptor gene transcription, resulting in increased expression of cell surface receptors, thus protecting against the down-regulation of β2-receptors after long-term administration. This is important for both bronchodilation and other β2-agonist effects, such as mast cell stabilization. Corticosteroids may also enhance the coupling of β2-receptors to G-proteins, which enhances β2-agonist effects and reverses the uncoupling of β2-receptors that may occur in response to inflammatory mediators, such as IL-1β through a stimulatory effect on a G-protein coupled receptor kinase (Figure 12). β2-agonists also act on glucocorticoid receptors to increase the anti-inflammatory effects of corticosteroids (Barnes 2010).

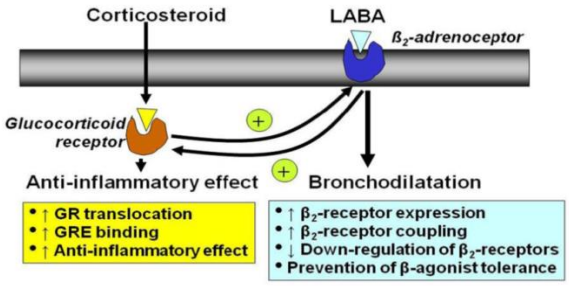


Figure 12. Biological actions of combination treatment (ICS+LABA). Corticosteroids have anti-inflammatory effects but also increase the number of β2-receptors, whereas β2-agonists, act on glucocorticoid receptors (GRs) to increase the anti-inflammatory effects of corticosteroids. Source: Barnes 2010.

Addtionally, low concentrations of theophylline, another drug commonly used for asthma treatment (usually in case of management of exacerbations), have been shown to reverse the effects of corticosteroid resistance by restoring HDAC2 activity, possibly via selective inhibition of phosphoinositide-3-kinase (PI3K)-δ and the phosphorylation of downstream kinases (Barnes, P. 2009).

When it comes to asthma controller treatment options, there is marked patient-to-patient variability in the therapeutic response. For example, about one in three patients with asthma who use inhaled glucocorticoids may not benefit adequately from this treatment (Szefler 2005). As asthma seems to be a complex genetic syndrome, the response to asthma treatment is also genetically complex and is characterized by high intra-individual repeatability and high inter-individual variability, with up to 50% of asthmatic patients having poor or even no response to treatment (Drazen et al. 2000, Szefler et al. 2002). Inhaled glucocorticoids are the most widely prescribed medications for controlling asthma. Levels of endogenous glucocorticoids are heritable and vary significantly, both at baseline and in response to environmental perturbation (Inglis et al. 1999, Ober et al. 2002, Steptoe et al. 2009). Moreover, studies in families with conditions other than asthma have shown both familial segregation and heritability in responses to glucocorticoid medications (Armaly 1967, Schwartz et al. 1972).

2.9. Pharmacogenetics of asthma

Given the heritability within response to therapeutic classes of common asthma as well as the high degrees of between-patient variability and within-patient repeatability in the response to treatment of asthma, it is likely that this response has a strong genetic basis. Previous studies have suggested that up to 80% of asthma patients have different responses due to genetic factors (Baye et al. 2011). In fact, a number of single nucleotide polymorphisms in candidate genes have been identified by genome-wide association studies (GWAS), linkage and candidate gene studies that might influence the clinical response to treatment in patients with asthma. Different genetic variants associate with the response to commonly used asthma drug classes (bronchodilators, inhaled corticosteroids and leukotriene modifiers) and their direct or indirect effects depend on their role in the inflammatory immune response in asthma or the anti-inflammatory action of the medication, respectively (Duong-Thi-Ly et al. 2017). Certain genetic loci associated with the response to asthma treatment are shown in Table 7.

Table 6. Genetic variants (loci) associated with the response to different classes of asthma medication (ICS, LABA, SABA, LTM, theophylline) identified by GWAS and candidate gene studies. Bronchodilatator response (BDR)- reversibility after bronchodilatator, change in FEV_1 after administration of SABA (Duong-Thi-Ly et al. 2017, Vijverberg et al. 2018, Morrow 2007, Lima et al. 2009, Fal and Rosiek-Biegus 2012, Tse et al. 2011).

Gene/genetic locus	Relevant medication	Outcome (assessed in study)
FGF14, ASB3, SOCS, PRKCQ, IL15RA, IL2RA, COL22A1, CLOCK, SPATA13-AS1, SLC22A15, SPATS2L; ADRB2 Gly16Arg, ADRB2 Gln271Glu	SABA	Bronchodilatator response (BDR)
CMTR1, ALLC, FBXL7, T gene, GLCCI1, CRHR1, FCER2, GR/NR3C1, TBX21, STIP1, DUSP1, HDAC, ORMDL3, VEGF	ICS	Lung function changes, Symptom frequency and severity, Exacerbation frequency and severity, AHR
CYP1A2, HNMT T314 allele	Theophylline	Drug metabolism and clearance, Toxicity
ADRB2 Gly16Arg, CRHR1, ARG1	LABA	Risk of exacerbations
LTC4S, ALOX5, LTA4H, CysLTR1, CYPBA4, CYP2C9, SLCO2B1, MRP1/ABCC1, MLLT3, GLT1D1, MRPP3	LTM	Lung function changes, Symptom and exacerbation frequency and severity

For example, certain loci in the gene encoding T-box 21 (TBX21) and Fc fragment of IgE receptor II (FCER2) contribute indirectly to the variability in the response to ICS by altering the inflammatory mechanisms involved in asthma pathogenesis, while other genetic loci such as those in the gene encoding corticotropin releasing hormone receptor 1 (CRHR1), nuclear receptor subfamily 3 group C member 1 (NR3C1), stress induced phosphoprotein 1 (STIP1), dual specificity phosphatase 1 (DUSP1), glucocorticoid induced 1 (GLCCI1), histone deacetylase 1 (HDAC), ORMDL sphingolipid biosynthesis regulator 3 (ORMDL3), and vascular endothelial growth factors (VEGF) directly affect treatment response variability through the anti-inflammatory mechanisms of ICS (Vijverberg et al. 2018).

TBX21 (T bet or T-box 21) is a transcription factor that acts as a regulator of Th1 cell development by inducing IFN-γ production and by inhibiting Th2 cytokines, such as interleukin IL-4, IL-5 and IL-13 (Lopert et al. 2013). Gene knockout mice lacking TBX21 spontaneously develop histological and physiological features of asthma, including bronchial hyperresponsiveness (BHR), peribronchial inflammation and collagen deposit in the lung

basement membrane. Among others, ICS also affect BHR in asthma patients, so it is more than likely that treatment efficacy may be altered by variants in the *TBX21* gene. Certain genetic polymorphisms, such as rs9910408 (c.-7947) and rs2240017 (H33Q C>G) have been associated with a decrease in BHR in both children and adults (Lopert et al. 2013, Raby et al. 2006, Tantisira et al. 2004).

CRHR1 is the key CRH (corticotropin releasing hormone) receptor in the pituitary gland, mediating the release of adrenocorticotropic hormone (ACTH, Figure 10) and the catecholaminergic response to CRH (Duong-Thi-Ly et al. 2017, Tantisira et al. 2004). Peripherally, CRH may bind to mast cells via CRHR1 (Theoharides et al. 1998). Alterations of any of these CRH effects, mediated by the *CRHR1* gene, have the potential to influence the pathogenesis of asthma. For example, the absence of CRHR1 leads to enhanced airway inflammation and dysfunction (Maitland-van der Zee and Daly 2012). Moreover, decreased expression or function of CRHR1, due to genetic variations, could diminish the capacity to secrete cortisol in response to inflammation, as a consequence of decreased ACTH release. Therefore, asthmatic patients with certain *CRHR1* variations would probably respond better following the administration of an exogenous corticosteroid. Certain genetic polymorphisms, such as rs242941 and rs1876828 have been associated with a positive treatment response in both children and adults with asthma (Tantisira et al. 2004, McGeachie et al. 2013).

The *GLCCI1* gene encodes encodes the glucocorticoid induced element 1, a protein of a still unknown function. The expression of *GLCCI1* is induced by glucocorticoids and may be an early marker for glucocorticoid-induced apoptosis. Certain genetic polymorphisms, such as rs37972 and rs37973, which are in complete linkage disequilibrium (i.e., perfectly correlated), are associated with decreases in *GLCCI1* expression and poorer response to treatment with ICS in asthmatic patients, that is with reduced lung function in response to ICS (Tantisira et al. 2011).

The non-intronic *ADRB2* gene encodes the beta-2 adrenergic receptor (β2 adrenoreceptor) that binds epinephrine (adrenaline) whose signaling, via a downstream L-type calcium channel interaction, mediates physiologic responses such as smooth muscle relaxation and bronchodilation. This receptor-channel complex also contains a G protein, a cAMP-dependent kinase, and the counterbalancing phosphatase, PP2A. Different polymorphic forms, point mutations, and/or changes in the expression of ADRB2 have been associated with asthma (especially severe asthma and exacerbations), obesity and type 2 diabetes

(Szczepankiewicz et al. 2009, Jocken et al. 2007, Puranik et al. 2017). For example, certain genetic polymorphisms such as rs1042713 (Arg→Gly16, 46A→G) and rs1042714 (Gln→Glu27, 79C→G) are associated with higher agonist promoted receptor down-regulation, rs1042714 with stronger desensitization of the β2 receptor and an additonal polymorphism rs1800888 (Thr→Ile164, 491C→T) with diminished affinity of β2-agonist to the receptor, decreased adenylate cyclase binding and 50% shorter lasting SABA. Consequently, patients with these polymorphisms had poorer response to SABA and LABA as well as more frequent and more severe asthma exacerbations (Fal and Rosiek-Biegus 2012, Green et al. 2001).

The *MMP9* gene encodes a matrix metalloproteinase (family of Zn^{2+}-dependent endoproteinases) or gelatinase B produced mainly by macrophages and neutrophils (but also epithelial cells, mast cells, fibroblasts nad smooth muscle cells). MMP9 has several functions, displaying gelatinolytic, elastolytic and collagenolytic activity, which is why it plays a key role in physiologic extracellular matrix turnover as well as tissue remodeling in certain diseases, such as asthma and chronic obstructive pulmonary disease (COPD). Additionally, MMP9 may also modulate the activity of various biological factors, including other proteinases (MMP13), their inhibitors (α1-antitrypsin) and cytokines such as IL-1 (Grzela et al. 2016). Moreover, MMP9 seems to have a regulatory role in neutrophil migration across the basement membrane (Delclaux et al. 1996). Certain genetic polymorphisms such as rs17576 (Gln279Arg) are associated with non-atopic asthma in children, obesity and increased levels of systemic inflammation in children (Grzela et al. 2016, Belo et al. 2012) and may be involved in the pathophysiology of non-eosinophilic asthma and moreover, response to treatment in certain asthma phenotypes (Goleva et al. 2007, Grzela et al. 2016, Naik et al. 2017).

3. MATERIALS AND METHODS

In order to study treatment success in asthmatic children, 365 pediatric patients (355 children aged 2-17 years and 10 adolescents aged 18-22 years) with atopic and non-atopic, intermitent to severe persistent asthma (according to GINA guidelines; GINA 2018, GINA Pediatric 2015), patients of the outpatient clinic at the Srebrnjak Children`s Hospital, were recruited in a prospective, non-interventional type of clinical study. Informed consent was obtained from the childrens` parents/legal guardians following a presentation of the study by the physician and distribution of written material regarding the study. Parents/guardians were also asked to provide clinically relevant information about the child (personal and family medical history). This study protocol was compliant with all national, EU and international ethics related rules and professional codes of conduct. The study was approved by the Ethics Committees of the Srebrnjak Children`s Hospital and School of Medicine at the University of Zagreb, and written parental consent was obtained.

3.1. Establishing a diagnosis of asthma

At their first visit patients underwent physical examination, anthropometric measurements (height and weight), along with a standard battery of diagnostic procedures and measurements to establish a diagnosis of asthma. These included skin prick tests for common allergens, lung function tests and blood sampling for routine laboratory tests (hematology, biochemistry and allergy assays) to establish a diagnosis of asthma. Peripheral whole blood samples were collected by venepuncture into EDTA coated vacutainers (for hematology analyses) and into vacutainers with clot activator and gel for serum separation (for biochemistry and certain allergy assays). Serum was separated by centrifugation at 3000 g for 10 min (Eppendorf centrifuge 5702R, Eppendorf AG, Germany). During this study a total of 10,5 ml peripheral blood samples per participant maximum was collected at the baseline visit (recruitment point). The remainder of blood samples (in EDTA coated vacutainers) and sera left over after diagnostic tests was stored at -20°C for subsequent analyses, including genotyping.

3.1.1. Assessment of allergy

To assess their atopy status, all participants underwent skin prick testing (SPT) to a standard battery of inhaled allergens (Alyostal, Stallergenes Greer, France), including house dust mite, grass pollen, animal dander, weed pollen, tree pollen and molds, as well as additional inhaled (eg. Mediterranean species) and food allergens, if indicated. A full list of allergens tested is presented in Supplement 3 (Table 30). SPT was performed on the volar surface of the non-dominant forearm with the listed allergen extracts, using plastic lancets and 0.9% phenolated glycero-saline solution as the negative and 10 mg/mL histamine hydrochloride solution as the positive control (Stallergenes Greer, France). The size of each urtica reaction wheal (in millimeters) was documented as the mean of the longest diameter and the diameter perpendicular to it at its mid-point, according to the International Study of Asthma and Allergies in Childhood (ISAAC) skin prick test (SPT) protocol (Asher et al. 1995).

In addition to SPT, other standard assays of allergy status assessment was performed. More specifically, concentrations of total immunoglobulin E (IgE) and allergen-specific IgE (sIgE) in serum was determined in all participants, using a standardized sandwich fluorescent enzyme immunoassay- ImmunoCAP® (Phadia AB, Sweden) on a Phadia 100 Laboratory system (Phadia AB, Sweden). In house established age dependent cut-off values were used to determinate elevated serum total IgE level (Dodig et al. 2006).

3.1.2. Lung function assessment

In order to evaluate the level of airflow obstruction, all participants underwent routine lung function measurements, according to age and indication (where applicable according to relevant clinical judgement). These included:

1. spirometry (FEV_1, FVC, PEF, $MEF_{50\%}$ and other relevant parameters), SpiroScout, Ganshorn Medizin Electronic, Germany
2. FEV_1 reversibility (changes in FEV_1 % predicted) 20 minutes after inhalation of 100-400 μg bronchodilatator (salbutamol), depending on body weight, SpiroScout, Ganshorn Medizin Electronic, Germany
3. impulse oscillometry (in younger children, as indicated), MasterScreen-IOS, Erich JÄEGER GmbH & CoKG, Germany
4. body plethysmography, Q-box, COSMED, Italy, and

5. bronichial/airway challenge tests to assess airway hyperresponsiveness (metacholine challenge test- APS Pro System, Erich JÄEGER GmbH & CoKG, Germany and spiroergometry- MTM-1500 med ergometer, Schiller AG, Switzerland).

Forced expiratory volume in one second (FEV_1) was measured with SpiroScout, Ganshorn Medizin Electronic, Germany. The predicted value (% of predicted) was calculated for each subject, according to Stanojevic et al. 2008.

As for the metacholine challenge test the decline in FEV_1 was calculated from the control value, i.e. the value of FEV_1 at baseline and after several inhalations of 3.2% metacholine solution (with the initial inhalation being with normal saline) using an automatic inhalation-synchronized dosimeter jet nebulizer. Methacholine chloride was nebulized automatically by the measuring device in cumulative doses of 22.5 μg, 45 μg, 90 μg, 180 μg, 360 μg, 720 μg, 1440 μg and 2000 μg. FEV_1 was measured, before the challenge and 2 minutes after inhalation of saline and each dose of methacholine (APS Pro System, Erich JÄEGER GmbH & CoKG, Germany). Changes in post-metacholine FEV_1 of 20% were considered a positive reaction (with a grading scale of: severe bronchial hyperresponsiveness- positive reaction to metacholine doses <24.5 μg, intermediate bronchial hyperresponsiveness- positive reaction to doses 24.5-389.3 μg and mild bronchial hyperresponsiveness- positive reaction to doses 389.3-1291.3 μg). The test was not performed if the post-saline FEV_1 was reduced by more than 10% compared with the pre-saline FEV_1. Methacholine challenges were terminated after a fall of FEV_1 greater than 20% from the control value or after the last dose inhaled (Brannan and Lougheed 2012).

Spiroergometry testing was performed according to the Bruce treadmill protocol for children (Van Der Cammen-van Zijp et al. 2009) on a MTM-1500 med ergometer, Schiller AG, Switzerland. Briefly, the participants had their heart rate, ECG and lung function monitored (oxygen uptake- V_{O2}), as well as carbon dioxide production- V_{CO2}, minute ventilation- V_E, and respiratory exchange ratio at 10-second intervals). The treadmill protocol consisted of a 60-second warm-up period (pacing at 2.74 km/h on a flat treadmill) followed by the initiation of the test at 2.74 km/h and a 10% gradient for 3 minutes then by incremental increases in speed and incline every 2 minutes until volountary exhaustion. After the test, participants were monitored for 2 min to ensure a normal recovery of heart rate (2 km/h with a flat treadmill). The test was terminated if at any point the participants felt diziness, exhaustion, shortness of breath etc.

Impulse oscillometry (IOS) was performed in younger children (preschool, under the age of 5 years) using sound waves to detect airway changes, requiring only normal tidal breathing from the patient. Pulmonary mechanics are determined by superimposing small external pressure signals on the spontaneous breaths of the participants. When analyzed, these pressure signals separately quantify the degree of obstruction in the central and peripheral airways. Briefly, in IOS a loudspeaker generates harmonic sound waves of single or multiple frequencies (2 and 4 Hz to between 30 and 35 Hz) that flow through a conduit tube and mouthpiece into the participant`s respiratory tract. The sound impulses travel superimposed on normal tidal breathing through the large and small airways, with higher frequencies reflecting back from the large airways to the mouth and lower frequencies traveling deeper into the lung before returning. A pressure and flow transducer measures inspiratory and expiratory flow and pressure. Respiratory impedance is the sum of all the forces (resistance and reactance) opposing the pressure impulses (oscillations) and is calculated from the ratio of pressure and flow at each frequency (Bickel et al. 2014).

Body plethysmography provides measures of the lung that reflect a multitude of functional and structural aspects (such as lung residual volume- RV, total lung capacity- TLC etc.) that cannot be assessed with other tecniques (eg. spirometry). Briefly, it was performed in certain participants by detecting changes in pressure in combination with either changes of mouth pressure or with flow rate under defined breathing conditions in a sealed box with rigid walls (chamber), according to the law of Boyle-Mariotte (for a fixed amount of gas in a closed compartment, such as the sealed box used, the relative changes in the compartment's volume are always equal in magnitude but opposite in sign to the relative changes in pressure, inferring relative volume changes from pressure changes). These signals are evaluated in order to determine static lung volumes and airflow resistance (Criee et al. 2011).

3.1.3. Assessment of levels of inflammation

In order to assess the levels and type of both systemic and local inflammation, certain inflammatory biomarkers were measured at baseline in all participants, including fractional exhaled nitric oxide (FENO), high-sensitive C-reactive protein (hsCRP) as well as certain inflammatory cell counts, such as eosinophils and neutrophils.

FENO is used to detect the level of airway inflammation as well as likeliness to respond to treatment with inhaled corticosteroids. It can also help to predict the onset of asthma symptoms or loss of asthma control, and to monitor compliance with corticosteroid therapy

and the effectiveness of such treatment. Briefly, the FENO small and portable device (Medisoft, Belgium) was used to determine exhaled nitric oxide concentration in the participant`s breath sample, requiring 10-second exhalation of breath at a pressure of 10–20 cm H_2O to maintain a fixed flow rate of 50 ± 5 ml/s. The last 3 seconds of the 10-second exhalation were analyzed by a calibrated electrochemical sensor to give a definitive result in parts per billion (ppb). Clinical cut-off values can be applied to the exhaled nitric oxide values to categorise readings as low, intermediate or high according to the reference ranges for children and adults (Dweik et al. 2011).

Serum C-reactive protein levels measured by high-sensitivity assays (hs-CRP) are known to be a marker of low-grade systemic inflammation, present in many chronic conditions, including cardiovascular diseases and asthma. It may also be associated with airflow obstruction and even serve as a surrogate marker of airway inflammation in asthma and moreover, it may indicate the level of disease control (Takemura et al. 2006, Navratil et al. 2009). Brielfy, a highly sensitive CRP assay was performed with an Olympus AU680 automated system (Beckman Coulter Inc., USA) using the latex agglutination method to quantitate CRP in serum samples of all participants at baseline (Rifai et al. 1999).

In order to assess both the type and level of inflammation, total eosinophil and neutrophil counts were measured at baseline in peripheral blood samples of all participants, absolute and/or relative (to total white blood cell- WBC count) counts, using a Sysmex cell counter (Sysmex XT-1800i Automated Hematology Analyzer, Sysmex Canada Inc., Canada).

3.1.4. Other assessments and diagnostic tests

In order to assess their health status, other diagnostic measurements and tests were performed in all participants at baseline, including complete blood count (absolute and/or relative)- white blood cells (basophils, monocytes, lymphocytes etc.) and total number of platelets using the same cell counter (Sysmex XT-1800i Automated Hematology Analyzer, Sysmex Canada Inc., Canada), as well as other relevant biochemistry assays, such as immunoglobulin levels (immunoglobulin A- IgA, immunoglobulin G- IgG and immunoglobulin M- IgM), using a commercial turbidimetry assay and an Olympus AU680 automated system (Beckman Coulter Inc., USA).

In order to identify additional conditions that might affect and aggravate the underlying disease (and asthma control), the participants were tested for common asthma comorbidities,

such as gastroesophageal reflux disease (GERD), obstructive sleep apnoea syndrome (OSAS), allergic rhinitis and atopic dermatitis.

All participants (and/or their parents/legal guardians) were asked about the child`s medical history on allergic rhinitis/rhinoconjuctivitis (AR) and atopic dermatitis (AD). Additionally, participants who were suspected to have AR, underwent measurements of nasal fractional exhaled nitric oxide (nasal FENO) for diagnostic purposes (to assess the degree of local upper airway allergic inflammation as well as subjective symptoms), using the same FENO analyzer as with bronchial FENO with a nose adaptor (Medisoft, Belgium).

Participants with unresolved persistent cough etiology or otherwise suspected reflux disease underwent 24-hour pH monitoring with a esophageal probe positioned approximately 5 cm above the lower esophageal sphincter barrier using a Ohmega pH and impedance monitoring system (Medical Measurement Systems B.V., The Netherlands), recording the number, type and duration of acidic, weakly acidic and non-acidic reflux episodes (Streets and DeMeester 2003). This data was then used to calculate certain indices (such as the Boix-Ochoa and Johnson-DeMeester score) and association to reflux symptoms in order to diagnose or exclude GERD.

Obstructive sleep apnoea syndrome (OSAS), characterized by episodes of complete or partial upper airway obstruction during sleep, is a common comorbidity in asthma and moreover, has been associated with disease severity (Salles et al. 2013). In order to diagnose or exclude any obstructive sleep disorder (including OSAS), certain participants (those with adequate indication- nocturnal symptoms, AR, extensive fatigue or affected cognitive and learning abilities due to sleep deprivation etc.) were subjected to all-night polysomnography (a multi-parametric sleep study). Briefly, the participants spent one night sleeping in the Sleep lab at the Srebrnjak Children`s Hospital, at least 8 hours in duration. They were put to bed at their usual bedtimes, without the use of any hypnotic agents. During this procedure, the particpants had multiple body functions monitored using Nihon Kohden (Japan) digital system and PolyT software as well as standardized proceudre (Gjergja Juraški et al. 2013). These included monitoring of brain functions (electroencephalography, EEG), eye movements (electrooculography, EOG), chin and leg muscle activity (electromyography, EMG) and heart rhythm (electrocardiography, ECG), along with breathing functions such as nasal pressure, nasal airflow, respiratory effort (thoracic and abdominal plethysmography), pulse oxymetry, snore detection, body position sensoring etc. These recorded sleep onset latency, sleep

efficiency, stages of sleep (1, 2, 3 and REM- Rapid eye movement), breathing irregularities, arousals, cardiac rhythm abnormalities, leg movements and body positions during sleep and oxygen saturation, required for the assessment of sleep disorders, according to standard criteria of Rechtschaffen and Kales, including the apnoea-hypopnoea index, AHI (Lerman et al. 2012, Gjergja Juraški et al. 2013).

3.1.5. Asthma severity assessment

After the participants were diagnosed with asthma, the level of disease control and disease severity (grade) was assessed according to GINA guidelines (GINA 2018, GINA Pediatric 2015), taking into account symptom severity and occurance (including nocturnal symptoms), need for reliever medications (namely SABA), number of asthma exacerbations, lung function and scoring based on certain health and quality-of-life questionnaires such as the Asthma Control Test (ACT). According to these severity criteria, participants were classified as either having: intermittent or persistent asthma, with persistent asthma being mild, moderate or severe. A summary of the GINA classification of asthma severity is presented in Tables 7 and 8.

Table 7. Classification (grading system) of asthma severity in adults and children older than 5 years of age, according to GINA guidelines, involving daytime and nocturnal symptom severity and frequency, as well as certain lung function parameters. *The presence of one of the features of severity is sufficient to place a patient in that category (grade). May be applicable to children 5 years of age and younger. Source: GINA 2018, GINA Pediatric 2015.

	Symptoms (daytime)	**Symptoms (nocturnal)**	**PEF or FEV_1 (% of predicted)**	**PEF variability**
STEP/GRADE 1 (Intermittent asthma)	< 1 time a week; Asymptomatic and normal PEF between attacks	≤ 2 times a month	≥ 80%	< 20%
STEP/GRADE 2 (Mild persistent asthma)	> 1 time a week but < 1 time a day; Attacks may affect daily functioning	>2 times a month	≥ 80%	20-30%
STEP/GRADE 3 (Moderate persistent asthma)	Daily; Attacks affect activity and daily functioning	>1 time a week	60-80%	>30%
STEP/GRADE 4 (Severe persistent asthma)	Continuous; Limited physical activity	Frequent	≤ 60%	>30%

Table 8. Classification (grading system) of asthma severity in children under 5 years of age, according to GINA guidelines. Source: GINA Pediatric 2015. For persistent asthma, consider severity and interval since last exacerbation. Frequency and severity may fluctuate over time.* Exacerbations of any severity may occur in patients in any severity category (grade).

Components of severity (criteria)		**Classification of asthma severity (children aged 0-4 yrs)**			
		Intermittent asthma (grade 1)	**Persistent asthma**		
			Mild (grade 2)	**Moderate (grade 3)**	**Severe (grade 4)**
Impairment	**Symptoms**	≤ 2 days per week	>2 days per week, but not daily	Daily	Daily, throughout the day
	Night-time awakenings	0	1-2 times per month	3-4 times per month	>1 times per week
	Use of reliever medication (SABA)	≤ 2 days per week	>2 days per week, but not daily	Daily	Severeal times per day
	Intereference with normal activity	None	Minor limitation	Some limitation	Extremely limited
Risk	**Exacerbations requiring OCS**	0-1 per annum	≥ 2 exacerbations in the last 6 months requiring OCS use, or ≥ 4 episodes per year lasting for > 1 day AND risk factors for persistent asthma		

3.1.6. Assessment of treatment response

After they were diagnosed with asthma, patients started treatment with inhaled corticosteroids, ICS (alone or in combination with LABA) and/or LTRA, according to disease severity and previously assessed disease control. Follow-up visits with lung function and airway inflammation testing as well as physical examination were made on average every 6 months over the period of 2 years. Additionally, treatment success (response) and the level of disease control (according to GINA guidelines) was assessed at each visit. A summary of GINA recommendations for asthma control monitoring is presented in Table 9.

Table 9. Grading system for the assessment of asthma control between clinical visits, according to GINA guidelines. Source: GINA 2018, GINA Pediatric 2015.

	Controlled	**Partly controlled**	**Uncontrolled**
Characteristic	Controlled if all of the following	Any of the features present in one week	
Daytime symptoms (wheezing, cough, difficulty in breathing)	None (≤ 2 times per week)	>2 times per week short periods-	

Table 9. continued

		minutes)	Three or more features of partly controlled present in one week
Limitation of activities (cough, wheezing or difficulty in breathing during exercise, playing or laughing)	None	Any	
Nocturnal symptoms/awakenings (cough, wheezing or difficulty in breathing)	None	Any	
Need for reliever/rescue treatment	None (≤ 2 times per week)	>2 times per week	
Lung function (PEF or FEV_1)	Normal	<80% predicted or personal best	
Exacerbations	None	One or more per year	One or more per week

Acute exacerbation severity and frequency was assessed at each follow-up visit as: none, mild, moderate and severe.

3.2. Genetic analysis

3.2.1. DNA isolation

Genomic DNA was extracted from EDTA-containing peripheral whole blood samples, previously stored at -20°C, using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions, by an automated (Qiacube, Qiagen GmbH, Germany) or manual spin protocol. DNA was eluted in a total volume of 100 μl. The quality and concentration (A_{260}/A_{280} ratio, concentration in ng/μl, A_{260}/A_{230} ratio) of each DNA isolate was checked using a NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific Inc., USA).

3.2.2. Genotyping

The genotypes of the SNPs analyzed were determined using a 5`-nuclease allelic discrimination assay in a 96-well format and Taqman technology. Primers and probes were purchased from Applied Biosystems (Life Technologies, USA) for SNP genotyping assays rs37973 in *GLCCI1*, rs9910408 in *TBX21* and rs242941 and rs1876828 in *CRHR1*, as well as rs1042713 in *ADRB2*. Allelic discrimination assays were performed in 5 μL reaction

volumes, using approximately 5 ng of DNA as a template, 2x TaqMan Fast Advanced Master Mix, and predesigned SNP genotyping assays provided by Applied Biosystems for rs37973, rs9910408, rs242941 and rs1876828. Temperature conditions for qPCR were set at 50°C for 2 minutes and 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds and at 60°C for 30 seconds.

For rs17576 SNP (*MMP9*) genotyping a primer and probe set were designed using a free online software qPCR primer & probe design tool and design service (Eurofins Genomics, Germany). The forward and reverse primer as well as probe sequences are presented in Table 10.

Table 10. Primers (forward and reverse) and probes (allele 1 and allele 2) design for the rs17576 genotyping assay (*MMP9* Gln279Arg, A/G transition detection). FWD- forward primer, REV- reverse primer, Allele 1 PR- probe for SNP allele 1, Allele 2 PR- probe for SNP allele 2, bp- base pairs (length of sequence in base paris), reporter- fluorescent dye, quencher- fluorescent dye quencher pair. For probe sequences nucleotides highlighted in red denote the ambiguity position (transition).

Oligonucleotide type	**Length (bp)**	**Sequence**	**Reporter/quencher**
MMP9 Gln279Arg FWD primer	19	TCCCCCTTTCCCACATCCT	
MMP9 Gln279Arg REV primer	21	CAGGGTTTCCCATCAGCATTG	
MMP9 Gln279Arg Allele 1 PR	17	CTCTACACCC**A**GGACGG	VIC-BHQ1
MMP9 Gln279Arg Allele 2 PR	17	TCTACACCC**G**GGACGG	FAM-BHQ1

Allelic discrimination assays for rs1042713 and rs17576 was performed in 12.5 µl reaction volume, using approximately 5 ng of DNA template, 100 µM of primer set (forward and reverse) and 10 µM of probes, with qPCR conditions as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute.

Genotyping of the amplified PCR products was determined by differences in VIC and FAM fluorescent levels, using the ABI Prism 7500 Fast Real-Time PCR system (system instrument equipped with SDS v2.0.5 software, Applied Biosystems, ThermoFisher Scientific Inc., USA) for rs37973, rs9910408, rs242941 and rs1876828 and using the Agilent AriaMX Real-Time PCR system (system instrument equipped with AriaMx software v1.0, Agilent Technologies, USA) for rs1042713 and rs17576.

3.3. Statistical analysis

The Hardy-Weinberg equilibrium was tested using the chi-squared (χ^2) test for the goodness-of-fit (one degree of freedom) model and Michael H. Court's (2005-2008) online calculator (Court 2012). Data distribution was evaluated by the Kolmogorov-Smirnov test and the Shapiro-Wilk's W test. Parametric statistics (one-way ANOVA) were used on normally distributed data, and non-parametric statistics (the Kruskal-Wallis test) was used if the distribution deviated from normal. Genotypic distribution and allelic frequencies in „good'', „moderate" and „bad'' responders (with regard to change from baseline in FEV_1, MEF_{50}, FENO and level of asthma control after 6 months, 12 months, 18 months and 2 years of treatment) were compared using the χ^2 test calculated on contingency tables. A Spearman's rank-order correlation was run to determine the relationship between the response to treatment (according to the above mentioned changes in lung function parameters- FEV_1 and MEF_{50}, changes in exhaled nitric oxide and level of asthma control) and other baseline parameters (demographic and clinical parameters, including cell count, inflammation biomarkers, comorbidity etc.). A multivariate general linear model (or a multi-way ANCOVA) was conducted to determine a statistically significant association between the level of response to treatment (according to changes in FEV_1, MEF_{50}, FENO and asthma control) and specific genotypes for rs37973, rs9910408, rs242941, rs1876828, rs1042713 and rs17576 controlling for certain confouding variables. IBM® SPSS® Statistics software was used for these analyses (version 21, release 21.0.0.0 for Windows; International Business Machines Corp., USA). A p-value of less than 0.05 was accepted as statistically significant.

3.4. Cluster analysis

Following the 2-year clinical assessment period, all participants were stratified into specific subgroups (asthma phenotypes/endotypes) by a cluster analysis, according to specific biomarkers- clinical features, treatment success, possible pathophysiological mechanisms and genetic predisposition by hierachical clustering analysis (HCA). HCA is considered an unsupervised machine learning method. It is used to reveal structures within data based on certain distance or similarity between objects and/or variables. All operations on data were performed either in R (https://www.r-project.org/) or Python (https://www.python.org/). Data taken for hierarchical clustering were the data gathered at baseline (demographic and clinical

parameters, including cell count, lung function, inflammation biomarkers, comorbidity etc.), follow up visits (lung function, airway inflammation- FENO and level of disease control according to GINA guidelines) and response to treatment data.

Hierachical clustering analysis was performed using the Ward method (Moore et al 2010, Kim et al. 2013, Qiu et al. 2018) with the use of Pythons' SciPy library. Ward's method minimizes the total within-cluster variance. This method is implemented in a way that each step finds the pair of clusters that leads to minimum increase in total within-cluster variance after merging. The clusters were subsequently tested on stability using bootstrapping, i.e. the data was reshuffled and separated on train-test splits. Jaccard similarity (Irani et al. 2016) was used to calculate similarities between the patients` association to the same cluster, i.e. the clusters are more stable the higher the Jaccard similarity. To increase cluster stability the entry data for HCA was performed on PCA (principal component analysis) transformed data (Deliu et al. 2018). PCA is a dimensionality reduction technique based on orthogonal transformation. The reduced data was represented as principal components which are mutually uncorrelated, each being a linear combination of original variables, and ordered by the amount of variance they cary.

4. RESULTS

Basic participant demographic and clinical data at baseline (age, gender and BMI distribution, common comorbidities, and disease severity assessment according to GINA guidelines, GINA 2018 as well as disease duration) are shown in Table 11.

Table 11. Basic participant data (clinical and demographic) at baseline (recruitment point). M- male, F- female participants; AR- allergic rhinitis; AD ever- atopic dermatitis ever; AD curr- atopic dermatitis in the last 12 months; GERD- gastroesophageal reflux disease; RI score- reflux index; OSA- obstructive sleep apnea, AHI- apnea/hipopnea index; Y- yes, N- no, SD- standard deviation, y- years. Underweight- ≤5 centile, normal- 5-85 centile, overweight- 85-95 centile, obese- ≥95 centile.

Age (years)- Mean (SD)	**Gender (male/female, M/F)- N (%)**	**Percentile of height and weight- N (%)**	**Disease severity (GINA)- N (%)**	**Duration of disease (y)- mean (SD)**	**Comorbidity- N (%), mean (SD)**
9.97 (3.97) M 9.68 (3.93) F 10.44 (4.01)	M 223 (61.10) F 142 (38.90)	Underweight- 11 (3.01) Normal- 253 (69.32) Overweight- 50 (13.70) Obese- 51 (13.97)	Grade 1- 221 (60.55) Grade 2- 119 (32.60) Grade 3- 19 (5.21) Grade 4- 6 (1.64)	3.27 (2.83) (N=302)	AR 312 (85.48) AD ever 101 (27.82) AD curr 23 (6.33) GERD 101 (27.67) RI score 9.10 ± 11.19 OSA 14 (3.84), AHI 0.57 ± 2.84

Participant atopy status as well as key allergy and inflammation features (total serum IgE, peripheral blood eosinophil count, peripheral blood neutrophil count, hsCRP, lung FENO) at baseline (recruitment point) are shown in Table 12.

Table 12. Participant atopy status and certain allergy and inflammation features. IgE- immunoglobulin, hsCRP- high-sensitive C-reactive protein, FENO- fraction of exhaled nitric oxide, WBC- white blood cells (leukocytes).

Atopy (Y/N)- N(%)	**Total IgE (kIU/l)- mean (SD)**	**Eosinophil count (Dunger)- median (SD)**	**Neutrophil count (% of total WBC)- mean (SD)**	**hsCRP (mg/l)- mean (SD)**	**Lung FENO (ppb)- mean (SD)**
Y 319 (87.40)	614.54 (1145.62)	416.35 (341.27) (N=355)	49.76 (12.86) (N=364)	2.23 (9.30) (N=311)	20.49 (20.07) (N=350)

Table 12. continued

N 46 (12.60)	(N=351)				

Participant lung function data (% of FEV_1 and MEF_{50} predicted for age, gender and posture) at baseline is shown in Table 13.

Table 13. Participant lung function parameters at recruitment point (baseline). FEV_1- forced expiratory volume in 1 second, MEF_{50}- maximum expiratory flow at 50%, N=365.

% of FEV1 predicted (at baseline)- mean (SD)	**% of MEF_{50} predicted (at baseline)- mean (SD)**
87 (17.14)	88 (23.11)

Genotype distribution (frequency) for each genetic polymorphism is shown in Figure 13. All participants were successfully genotyped for rs37973, rs9910408, rs242941 and rs1876828. For rs1042713 and rs17576 genotype data was missing for 1 and 19 participants, respectively, due to insufficient DNA extract material or degraded DNA samples in subsequent/repeated analysis.

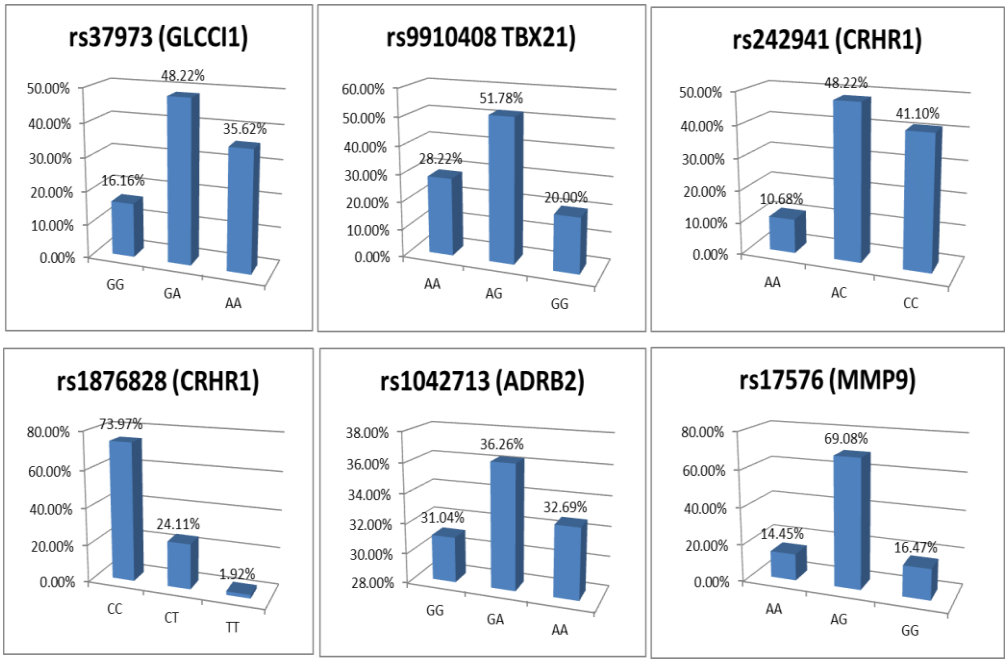


Figure 13. Genotype frequency (%) for respective genetic polymorphisms: rs37973, rs9910408, rs242941, rs1876828, rs1042713 and rs17576. *GLCCI1*- glucocorticoid induced 1; *TBX21*- T-box 21, T-bet; *CRHR1*- corticotropin releasing hormone receptor 1, *ADRB2*- beta-2-adrenergic receptor; *MMP9*- matrix metallopeptidase 9. For rs37973, rs9910408, rs242941 and rs1876828 N=365, for rs1042713 N=364 and for rs17576 N=346.

Consistency with the Hardy- Weinberg equilibrium (HWE) for each genetic polymorphism, along with global and population-specific minor allele frequency (MAF) is presented in Table 14.

Table 14. HWE consistency for genotype frequencies for rs37973, rs9910408, rs242941, rs1876828, rs1042713 and rs17576. χ^2– chi-squared value, $p < 0.05$ consistent with HWE. Global and population specific (Central European) MAF according to NCBI dbSNP (https://www.ncbi.nlm.nih.gov/SNP/). HWE- Hardy-Weinberg equilibrium, MAF- minor allele frequency, CEU- Central European, reference population. Calculated using Michael H. Court's (2005-2008) online calculator (Court 2012).

Genotype	rs37973		rs9910408		rs242941		rs1876828		rs1042713		rs17576	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Homozygote reference	130	130.2	103	106.9	150	155.2	270	270.1	113	88.0	50	83.0
Heterozygote	176	175.6	189	181.3	176	165.6	88	87.7	132	182.0	239	172.9
Homozygote variant	59	59.2	73	76.9	39	44.2	7	7.1	119	94.0	57	90.0
Variant allele frequency	0.40		0.46		0.35		0.14		0.51		0.51	
χ^2	0.002		0.664		1.433		0.003		27.433		50.508	
p (1 degree of freedom)	0.965		0.415		0.231		0.956		0.000		0.000	
Global MAF	0.396		0.384		0.323		0.086		0.476		0.456	
CEU MAF	0.442		0.456		0.282		0.240		0.358		0.381	

4.1. Definition of response

According to their response to specific classes of treatment (ICS alone, LTRA alone, combination treatment- ICS+LABA/LTRA), patients were divided into ‘‘poor’’ or „inadequate“ („moderate“ and „bad“) and ‘‘good’’ responders in accordance with the American Thoracic Society (ATS) and European Respiratory Society (ERS) task forces` interpretation of changes in lung function (FEV_1 and MEF_{50}) as well as data from other studies evaluating treatment response in asthma- by taking into account changes in the level of disease control (according to GINA), as well as changes in the level of airway inflammation, i.e. FENO values (GINA 2018, Pellegrino et al. 2005, Reddel et al. 2009, Oei et al. 2011, Dweik et al. 2011, Buchvald et al. 2003, Smith et al. 2005, de Jongste 2005, Smith et al. 2005).

4.1.1. Definition of response according to changes in lung function

Bad response to treatment according to changes in FEV_1 was defined as a decrease in FEV_1 predicted (for children of certain age, gender and posture- height, weight) by 10% or more between clinical visits/follow-ups (≤10%). Moderate response to treatment according to changes in FEV_1 was defined as a relative change in FEV_1 predicted by ±10% and good response to treatment was defined as an increase in FEV_1 predicted by 10% or more (≥10%) between clinical assessments/follow-ups.

Bad response to treatment according to changes in MEF_{50} was defined as a decrease in MEF_{50} predicted (for children of certain age, gender and posture) by 15% or more (≤15%); moderate response was defined as a relative change in MEF_{50} predicted by ±15% and good response to treatment was defined as an increase in MEF_{50} predicted by 15% or more (≥15%) between clinical visits/follow-ups (Telenga et al. 2013, Boskabady et al. 2008).

4.1.2. Airway inflammation

According to ATS recommendations, cut points rather than reference values were used when interpreting FENO levels (Dweik et al. 2011): low FENO (25 ppb in adults; 20 ppb in children), high FENO (50 ppb in adults, 35 ppb in children), intermediate FENO (between 25 ppb and 50 ppb in adults; 20- 35 ppb in children). Bad response to treatment was defined as an increase in FENO greater than 20% for values over 35 (50 for patients older than 18 years) ppb or more than 10 ppb for values lower than 35 (50) ppb between clinical visits. A reduction of at least 20% in FENO for values over 35 (50) ppb or more than 10 ppb for

values lower than 35 (50) ppb was defined as the cut point to indicate a significant (good) response to anti-inflammatory treatment. Moderate response to treatment was defined as changes in FENO values ranging from a reduction of ≤20% and increase ≤20% for FENO values over 35 (50) ppb and ±10 ppb for values lower than 35 (50) ppb from one visit to the next.

4.1.3. Asthma control

The level of asthma control between clinical visits was assessed according to GINA guidelines (GINA 2018): symptom occurance (including nocturnal symptoms), need for reliever medications (namely SABA), number and severity of asthma exacerbations, lung function and Asthma Control Test (ACT), where applicable. The level of control was defined as either controlled, partly controlled or uncontrolled, whereas bad response to treatment was defined as a deterrioration in asthma control between visits (controlled to uncontrolled or partly controlled), good response was defined as an improvement in asthma control (partly- or uncontrolled to controlled) and moderate response was defined as no changes in partial asthma control between clinical visits, with the exception of the patient having uncontrolled asthma from visit to visit, which was considered a bad response or good response to treatment, when the patient had controlled asthma symptoms between visits.

4.2. Association of response to treatment with genetic and other parameters

Significant correlations in response to treatment (in general, including all 3 major classes of treatment- ICS, LTRA and combination treatment- ICS+LABA/LTRA) according to relative changes in lung function parameters (FEV_1 and MEF_{50}), changes in markers of local (airway) inflammation (FENO) and changes in the level of asthma control according to GINA guidelines, between respective visits over the period of (on average) 2 years with specific genetic polymorphisms (rs37973, rs9910408, rs242941, rs1876828, rs1042713 and rs 17576) are presented in Table 15.

Table 15. Correlation of response to treatment after 6, 12, 18 and 24 months (on average), according to changes in FEV_1, MEF_{50}, FENO and asthma control, with the analyzed genetic polymorphisms: rs37973 (*GLCCI1*), rs9910408 (*TBX21*), rs242941 (*CRHR1*), rs1876828 (*CRHR1*), rs1042713 (*ADRB2*) and rs17576 (*MMP9*).

Spearman`s correlation test, p< 0.05. Abbreviations for respective responses to treatment are defined in Supplement 4. R- Spearman`s Rho (correlation coefficient).

	rs37973	rs9910408	rs242941	rs1876828	rs1042713	rs17576
Resp_MEF_{50}_diagn_1^{st} control				R= -0.116 p= 0.027 N= 365		
Resp_FENO_1^{st}_2^{nd} control		R= -0.141 p= 0.008 N= 350				
Resp_FENO_3^{rd}_4^{th} control						R= -0.175 p= 0.009 N= 223
Resp_MEF_{50}_3^{rd}_4^{th} control	R= 0.180 p= 0.006 N= 234					
Resp_FEV_1_diagn_4^{th} control			R= 0.158 p= 0.016 N= 231			
Resp_CRTL_diagn_4^{th} control					R= 0.147 p= 0.024 N= 234	

Significant correlations in response to treatment (in general, including all 3 major classes of treatment- ICS, LTRA and combination treatment- ICS+LABA/LTRA) according to relative changes in lung function parameters (FEV_1 and MEF_{50}), changes in markers of local (airway) inflammation (FENO) and changes in the level of asthma control according to GINA guidelines, between respective visits over the period of (on average) 2 years with other clinical and physiological parameters are presented in Table 16.

Table 16. Correlation of response to treatment after 6, 12, 18 and 24 months (on average), according to changes in FEV1, MEF50, FENO and asthma control, with all other physiological and clinical variables (medical history, biochemical, lung function, demographic and other parameters). Spearman`s correlation test, p< 0.05. Abbreviations for respective responses to treatment are defined in Supplement 4. R- Spearman`s Rho (correlation coefficient). Diagnosis- recruitment point; AD- atopic dermatitis, AR- allergic rhinitis, OSA- obstructive sleep apnea.

Response to treatment	Association with physiologic and clinical parameters
Resp_FEV_1_diagn_1^{st} control (N=365)	MEF_{50} at diagnosis (R= 0.236, p= 0.000), total IgE (R= 0.108, p= 0.040), FVC at 1^{st} control (R= -0.364, p=0.000), PEF at 1^{st} control (R= -0.303, p= 0.000), MEF_{50} at 1^{st} control (R= -0.376, p= 0.000), Atopy (R=0.120, p= 0.021), Asthma severity (R= 0.114, p= 0.029), Exacerbation severity at 1^{st} control (R= 0.111, p= 0.034), FVC at 2^{nd} control (R= -0.175, p= 0.001), PEF at 2^{nd} control (R= -0.112, p= 0.033), MEF_{50} at 2^{nd} control (R= -0.180, p=0.001), FVC at 3^{rd} control (R= -0.184, p= 0.000), PEF at 3^{rd} control (R= -0.190, p= 0.000), MEF_{50} at 3^{rd} control (R= -0.159, p= 0.002), FENO at 4^{th} control (R= 0.134, p= 0.010), Asthma control at 4^{th} control (R= 0.109, p= 0.038)
Resp_FENO_diagn_1^{st} control (N= 352)	Asthma control at diagnosis (R= -0.121, p=0.023), Age (R= 0.199, p= 0.000), Disease duration (R= 0.166, p= 0.002), total IgE (R= 0.113, p= 0.034), Eosinophil count (R= 0.114, p= 0.033), AD (R= -0.105, p= 0.049), Night symptoms at 3^{rd} control (R= -0.110, p= 0.039)
Resp_CTRL_diagn_1^{st} control (N= 365)	MEF_{50} at diagnosis (R= -0.118, p= 0.024), Age (R= 0.153, p= 0.003), total IgE (R= 0.204, p= 0.000), Basophil count (R= 0.125, p= 0.017), FEV_1 at 1^{st} control (R= -0.109, p= 0.037), MEF_{50} at 1^{st} control (R= -0.142, p= 0.007), FENO at 1^{st} control (R= 0.149, p= 0.004), AR (R= 0.121, p= 0.021), AD (R= 0.147, p= 0.005), Atopy (R= 0.134, p= 0.010), FEV1 at 2^{nd} control (R= -0.137, p= 0.009), FVC at 2^{nd} control (R= -0.115, p= 0.028), FENO at 2^{nd} control (R= 0.174, p= 0.001), FENO at 3^{rd} control (R= 0.169, p= 0.001)
Resp_MEF_{50}_diagn_1^{st} control (N= 365)	FEV_1 at diagnosis (R= 0.236, p= 0.000), Eosinophil count (R= -0.112, p= 0.033), FEV_1 at 1^{st} control (R= -0.375, p= 0.000), FVC at 1^{st} control (R= -0.168, p= 0.001), PEF at 1^{st} control (R= -0.294, p= 0.000), FEV_1 at 2^{nd} control (R= -0.227, p= 0.000), FVC at 2^{nd} control (R= -0.132, p= 0.012), PEF at 2^{nd} control (R= -0.156, p= 0.003), FEV_1 at 3^{rd} control (R= -0.141, p= 0.007), PEF at 3^{rd} control (R= -0.194, p= 0.000)
Resp_FEV_1_1^{st}_2^{nd} control (N= 365)	Disease duration (R= 0.130, p= 0.013), OSA (R= 0.122, p= 0.019), total IgE (R= -0.118, p= 0.024), Neutrophil count (R= -0.107, p= 0.040), Basophil count (R= 0.107, p= 0.041), FVC at 1^{st} control (R= 0.284, p= 0.000), PEF at 1^{st} control (R= 0.256, p= 0.000), MEF_{50} at 1^{st} control (R= 0.306, p= 0.000), Atopy (R= -0.111, p= 0.034), FVC at 2^{nd} control (R= -0.194, p= 0.000), PEF at 2^{nd} control (R= -0.221, p= 0.000), MEF_{50} at 2^{nd} control (R= -0.119, p= 0.023), FVC at 4^{th} control (R= -0.105, p= 0.046), Asthma control at 4^{th} control (R= -0.127, p= 0.015)
Resp_FENO_1^{st}_2^{nd} control (N= 350)	BMI percentile (R= -0.113, p= 0.035), Neutrophil count (R= -0.133, p= 0.013), PEF at at 2^{nd} control (R= -0.145, p= 0.007), FEV_1 at 4^{th} control (R= -0.151, p= 0.005), FVC at 4^{th} control (R= -0.182, p= 0.001), PEF at 4^{th} control (R= -0.188, p= 0.000), MEF_{50} at 4^{th} control (R= -0.135, p= 0.01), Asthma control at 4^{th} control (R= -0.154, p= 0.004)
Resp_CTRL_1^{st}_2^{nd} control (N=365)	Neutrophil count (R= 0.125, p= 0.016), AD (R= 0.116, p= 0.027), FEV_1 at 2^{nd} control (R= -0.231, p= 0.000), FVC at 2^{nd} control (R= -0.116, p= 0.027), MEF_{50} at 2^{nd} control (R= -0.212, p= 0.000), FEV_1 at 3^{rd} control (R= -0.131, p= 0.012), MEF_{50} at 3^{rd} control (R= -0.119,

Table 16. continued

	p= 0.023)
Resp_MEF_{50}_1^{st}_2^{nd} control (N=365)	Disease duration (R= 0.198, p= 0.000), Eosinophil count (R= 0.121, p= 0.020), Neutrophil count (R= -0.117, p= 0.025), Basophil count (R= 0.119, p= 0.024), FEV_1 at 1^{st} control (R= 0.354, p= 0.000), FVC at 1^{st} control (R= 0.243, p= 0.000), PEF at 1^{st} control (R= 0.337, p= 0.000), Exacerbation severity at 1^{st} control (R= -0.110, p= 0.035), FEV_1 at 2^{nd} control (R= -0.190, p= 0.000), FENO at 2^{nd} control (R= 0.110, p= 0.035), Asthma control at 4^{th} control (R= -0.123, p= 0.019)
Resp_FEV_1_2^{nd}_3^{rd} control (N= 365)	Age (R= 0.109, p= 0.037), BMI percentile (R= 0.112, p= 0.032), FVC at 2^{nd} control (R= 0.166, p= 0.001), PEF at 2^{nd} control (R= 0.175, p= 0.001), MEF_{50} at 2^{nd} control (0.229, p= 0.000), Asthma control at 2^{nd} control (R= -0.109, p= 0.037), FVC at 3^{rd} control (R= -0.112, p= 0.032), PEF at 3^{rd} control (R= -0.136, p= 0.009), MEF^{50} at 3^{rd} control (R= -0.167, p=0.001)
Resp_FENO_2^{nd}_3^{rd} control (N= 350)	Atopy (R= 0.167, p= 0.002), PEF at 2^{nd} control (R= 0.164, p= 0.002), FEV_1 at 4^{th} control (R= 0.214, p= 0.000), FVC at 4^{th} control (R= 0.254, p= 0.000), PEF at 4^{th} control (R= 0.243, p= 0.000), MEF_{50} at 4^{th} control (R= 0.198, p= 0.000), Asthma control at 4^{th} control (R= 0.228, p= 0.000), Exacerbation severity at 4^{th} control (R= 0.106, p= 0.049)
Resp_CTRL_2^{nd}_3^{rd} control (N= 365)	MEF_{50} at diagnosis (R= -0.152, p= 0.004), Age (R= -0.187, p= 0.000), Monocyte count (R= -0.112, p= 0,032), FEV_1 at 1^{st} control (R= -0.172, p= 0.001), FVC at 1^{st} control (R= -0.155, p= 0.003), PEF at 1^{st} control (R= -0.142, p= 0.006), MEF_{50} at 1st control (R= -0.127, p= 0.015), FENO at 1^{st} control (R= -0.225, p= 0.000), AD (R= 0.140, p= 0.007), FVC at 2^{nd} control (R= -0.105, p= 0.046), PEF at 2^{nd} control (R= -0.151, p= 0.004), MEF_{50} (R= -0.141, p= 0.007), FENO at 2^{nd} control (R= -0.132, p= 0.012), FEV_1 at 3^{rd} control (R= -0.314, p= 0.000), FVC at 3^{rd} control (R= -0.162, p= 0.002), PEF at 3^{rd} control (R= -0.230, p= 0.000), MEF_{50} at 3^{rd} control (R= -0.299, p= 0.000), FEV_1 at 4^{th} control (R= -0.139, p= 0.008), FVC at 4^{th} control (R= -0.134, p= 0.010)
Resp_MEF_{50}_2^{nd}_3^{rd} control (N= 365)	OSA (R= -0.115, p= 0.028), Platelet count (R= 0.122, p= 0.020), FEV_1 at 2^{nd} control (R= 0.137, p= 0.009), FVC at 2^{nd} control (R= 0.108, p= 0.039), PEF at 2^{nd} control (R= 0.120, p= 0.022), FEV_1 at 3^{rd} control (R= -0.201, p= 0.000), Asthma control at 3^{rd} control (R= 0.240, p= 0.000), Exacerbation severity (R= 0.222, p= 0.000)
Resp_FEV_1_3^{rd}_4^{th} control (N=234)	Age (R= -0.149, p= 0.023), Disease duration (R= -0.129, p= 0.049), FVC at 2^{nd} control (R= -0.136, p= 0.038), MEF_{50} at 3^{rd} control (R= 0.139, p= 0.034), FVC at 4^{th} control (R= -0.209, p= 0.001), PEF at 4^{th} control (R= -0.129, p= 0.049)
Resp_FENO_3^{rd}_4^{th} control (N= 223)	Exacerbation severity at 1^{st} control (R= -0.141, p= 0.035)
Resp_CTRL_3^{rd}_4^{th} control	hsCRP (R= 0.134, p= 0.040), Neutrophil count (R= 0.151, p= 0.021), MEF_{50} at 1^{st} control (R= -0.147, p= 0.023), PEF at 2^{nd} control (R=

Table 16. continued

Resp_MEF_{50}_3^{rd}_4^{th} control (N= 234)	FEV_1 at 3^{rd} control (R= 0.132, p= 0.044), FEV_1 at 4^{th} control (R= -0.181, p= 0.006), PEF at 4^{th} control (R= -0.143, p= 0.029)
Resp_FEV_1_diagn_2^{nd} control (N= 365)	MEF_{50} at diagnosis (R= 0.387, p= 0.000), Asthma control (R= -0.104, p= 0.047), Disease duration (R= 0.108, p= 0.040), Neutrophil count (R= -0.135, p= 0.010), FVC at 2^{nd} control (R= -0.240, p= 0.000), PEF at 2^{nd} control (R= -0.191, p= 0.000), MEF_{50} at 2^{nd} control (R= -0.306, p= 0.000), FVC at 3^{rd} control (R= -0.113, p= 0.032), PEF at 3^{rd} control (R= -0.114, p= 0.029), MEF_{50} at 3^{rd} control (R= -0.179, p= 0.001)
Resp_FENO_diagn_2^{nd} control (N= 349)	Disease duration (R= 0.145, p= 0.007), GERD (0.155, p= 0.004), Asthma control at 3^{rd} control (R= -0.133, p= 0.013), Exacerbation severity at 3rd control (R= -0.111, p= 0.039)
Resp_CTRL_diagn_2^{nd} control (N= 365)	Disease duration (R= 0.117, p= 0.025), Neutrophil count (R= 0.109, p= 0.037), Monocyte count (R= -0.104, p= 0.046), AR (R= 0.120, p= 0.022), FEV_1 at 2^{nd} control (R= -0.256, p= 0.000), FVC at 2^{nd} control (R= -0.129, p= 0.013), MEF_{50} at 2^{nd} control (R= -0.200, p= 0.000), FEV_1 at 3^{rd} control (R= -0.120, p= 0.022)
Resp_MEF_{50}_diagn_2^{nd} control (N= 365)	FEV_1 at diagnosis (R= 0.400, p= 0.000), Asthma control at diagnosis (R= -0.119, p= 0.023), MEF_{50} at 1^{st} control (R= -0.167, p= 0.001), FEV_1 at 2^{nd} control (R= -0.332, p= 0.000), PEF at 2^{nd} control (R= -0.217, p= 0.000), FEV_1 at 3^{rd} control (R= -0.170, p= 0.001), PEF at 3^{rd} control (R= -0.149, p= 0.004)
Resp_FEV_1_diagn_3^{rd} control (N= 365)	MEF_{50} at diagnosis (R= 0.457, p= 0.000), Asthma control at diagnosis (R= -0.122, p= 0.020), total IgE (R= 0.110, p= 0.036), Neutrophil count (R= -0.105, p= 0.044), AR (R= 0.104, p= 0.047), FVC at 2^{nd} control (R= -0.142, p= 0.007), PEF at 2^{nd} control (R= -0.124, p= 0.018), MEF_{50} at 2^{nd} control (R= -0.108, p= 0.039), FVC at 3^{rd} control (R= -0.188, p= 0.000), PEF at 3^{rd} control (R= -0.261, p= 0.000), MEF_{50} at 3rd control (R= -0.290, p= 0.000), Asthma control at 3^{rd} control (R= 0.149, p= 0.004), Exacerbation severity at 3^{rd} control (R= 0.173, p= 0.001)
Resp_CTRL_diagn_3^{rd} control (N= 365)	Age (R= -0.129, p= 0.014), Monocyte count (R= -0.162, p= 0.002), FEV_1 at 1^{st} control (R= -0.162, p= 0.002), FVC at 1^{st} control (R= -0.152, p= 0.004), PEF at 1^{st} control (R= -0.134, p= 0.010), MEF_{50} at 1^{st} control (R= -0.120, p= 0.022), FENO at 1^{st} control (R= -0.161, p= 0.002), AD (R= 0.127, p= 0.015), FEV_1 at 2^{nd} control (R= -0.166, p= 0.001), FVC at 2^{nd} control (R= -0.113, p= 0.032), PEF at 2^{nd} control (R= -0.120, p= 0.021), MEF_{50} at 2^{nd} control (R= -0.135, p= 0.010), FEV_1 at 3^{rd} control (R= -0.313, p= 0.000), FVC at 3^{rd} control (R= -0.181, p= 0.001), PEF at 3^{rd} control (R= -0.240, p= 0.000), MEF_{50} at 3rd control (R= -0.295, p= 0.000)

Table 16. continued

Resp_MEF_{50}_diagn_3rd control (N= 365)	FEV_1 at diagnosis (R= 0.435, p= 0.000), Asthma control at diagnosis (R= -0.123, p= 0.019), Age (R= 0.114, p= 0.029), FEV_1 at 2nd control (R= -0.119, p= 0.023), PEF at 2nd control (R= -0.124, p= 0.018), FEV_1 at 3rd control (R= -0.275, p= 0.000), PEF at 3rd control (R= -0.189, p= 0.000), FENO at 3rd control (R= 0.115, p= 0.028), Asthma control at 3rd control (R= 0.142, p= 0.007), Exacerbation severity at 3rd control (R= 0.141, p= 0.007)
Resp_FEV_1_diagn_4th control (N= 231)	MEF_{50} at diagnosis (R= 0.413, p= 0.000), hsCRP (R= -0.147, p= 0.025), AR (R= 0.137, p= 0.037), FVC at 2nd control (R= -0.143, p= 0.030), MEF_{50} at 3rd control (R= -0.194, p= 0.003), FVC at 4th control (R= -0.196, p= 0.003), PEF at 4th control (R= -0.184, p= 0.005), MEF_{50} at 4th control (R= -0.274, p= 0.000)
Resp_FENO_diagn_4th control	Disease duration (R= 0.152, p= 0.023), Basophil count (R= -0.144, p= 0.032), Atopy (R= 0.156, p= 0.020), FVC at 2nd control (R= -0.134, p= 0.046)
Resp_CTRL_diagn_4th control	FEV_1 at 3rd control (R= -0.155, p= 0.017), FEV_1 at 4th control (R= -0.143, p= 0.028)
Resp_MEF_{50}_diagn_4th control	FEV_1 at diagnosis (R= 0.417, p= 0.000), PEF at 2nd control (R= -0.137, p= 0.037), PEF at 4th control (R= -0.176, p= 0.007)

4.3. Association of treatment response with genetic and other parameters in specific treatment class groups

Significant associations in treatment response according to relative changes in FEV_1, MEF_{50}, FENO and level of asthma control between respective visits over the period of 2 years (recruitment to 4th control visit) with specific genetic polymorphisms (rs37973, rs9910408, rs242941, rs1876828, rs1042713 and rs17576), as well as significant associations adjusted for certain confouding variables- covariates (demographic and clinical parameters, previously identified by a Spearman`s correlation test- see Table 16) in specific subgroups of participants on different classes of asthma treatment (ICS alone, LTRA alone and combination treatment- ICS+LABA/LTRA) are presented in Tables 17 (use of ICS alone), 18 (use of LTRA alone) and 19 (use of combination treatment).

Table 17. Association of response to treatment after 6, 12, 18 and 24 months (on average), according to changes in FEV_1, MEF_{50}, FENO and asthma control with the analyzed genetic polymorphisms: rs37973 (*GLCCI1*), rs9910408 (*TBX21*), rs242941 (*CRHR1*), rs1876828 (*CRHR1*) and rs17576 (*MMP9*) in patients using inhaled corticosteroids (ICS) alone. Kruskal-Wallis test, $p < 0.05$, $\chi 2$- chi square, N= 158. Adjusted model (F adjusted) corrected for possible confounding variables: age, disease duration, atopy status, total IgE level, eosinophil count, neutrophil count, basophil count, hsCRP level, monocyte count, platelet count, BMI percentile category, comorbidity (AR, AD, OSA, GERD). General linear model test, $p < 0.05$, Partial $\eta 2$- partial eta squared (effect size statistics), N= 158. BMI percentile category: underweight (0-3 percentile), normal (5-85 percentile), overweight (86-95 percentile), obese (>95 percentile). Abbreviations for respective responses to treatment are defined in Supplement 4.

rs37973 (*GLCCI1*)								
	Resp_CTRL_ diagn to 1st control	**Resp_FEV_1_ 1st to 2nd control**	**Resp_FENO_ 1st to 2nd control**	**Resp_CTRL_ 1st to 2nd control**	**Resp_MEF_{50}_ 1st to 2nd control**	**Resp_CTRL_3rd to 4th control**	**Resp_CTRL_diagn to 2nd control**	**Resp_MEF_{50} _diagn to 3rd control**
χ^2	10.736	9.353	11.111	16.525	6.492	9.122	14.543	8.600
p value (two-tailed)	0.005	0.009	0.004	0.000	0.039	0.010	0.001	0.014
	Resp_FENO_1st to 2nd control				**Resp_FENO_2nd to 3rd control**			
F (adjusted)	2.397				2.139			
p value	0.005				0.012			
Partial η^2	0.519				0.491			

rs9910408 (*TBX21*)								
	Resp_MEF_{50}_diagn to 1st control	**Resp_FEV_1_1st to 2nd control**	**Resp_FENO_1st to 2nd control**	**Resp_FEV_1_ 2nd to 3rd control**	**Resp_FENO_ 2nd to 3rd control**	**Resp_MEF_{50}_ 2nd to 3rd control**	**Resp_CTRL_ 3rd to 4th control**	**Resp_MEF_{50} _diagn to 2nd control**
χ^2	6.768	6.545	25.672	17.028	20.671	7.337	15.960	7.314
p value (two-tailed)	0.034	0.038	0.000	0.000	0.000	0.026	0.000	0.026
	Resp_FEV_1_1st to 2nd control		**Resp_FENO_1st to 2nd control**			**Resp_CTRL_3rd to 4th control**		
F (adjusted)	1.769		2.685			2.143		

Table 17. continued

p value	0.047			0.002			0.013	
Partial η^2	0.428			0.532			0.476	
rs242941 (*CRHR1*)								
	Resp_FENO_ 1st to 2nd control	**Resp_MEF50_ 1st to 2nd control**	**Resp_FEV1_diagn to 2nd control**	**Resp_FENO_ diagn to 2nd control**	**Resp_FEV1_diagn to 3rd control**	**Resp_FEV1 _diagn to 4th control**	**Resp_CTRL_ diagn to 4th control**	**Resp_MEF50 _diagn to 4th control**
χ^2	12.335	11.023	17.452	15.395	10.556	17.303	12.966	11.617
p value (two-tailed)	0.002	0.004	0.000	0.000	0.005	0.000	0.002	0.003
	Resp_FENO_ 1st to 2nd control			**Resp_FENO_ diagn to 2nd control**				
F (adjusted)	2.550			2.077				
p value	0.003			0.015				
Partial η^2	0.535			0.484				
rs1876828 (*CRHR1*)								
	Resp_CTRL_ diagn to 1st control	**Resp_CTRL_ 1st to 2nd control**	**Resp_FENO_3rd to 4th control**	**RespFEV1_diagn to 2nd control**	**Resp_MEF50_diagn to 2nd control**	**Resp_FEV1_diagn to 3rd control**	**Resp_CTRL_diagn to 3rd control**	
χ^2	7.017	6.542	6.980	13.423	6.891	6.511	11.313	
p value (two-tailed)	0.030	0.038	0.031	0.001	0.032	0.039	0.003	
rs17576 (*MMP9*)								
	Resp_CTRL_diagn to 1st control			**Resp_FEV1_3rd to 4th control**			**Resp_FENO_diagn to 2nd control**	
χ^2	11.327			14.268			6.035	
p value (two-tailed)	0.003			0.001			0.049	

Table 18. Association of response to treatment after 6, 12, 18 and 24 months (on average), according to changes in FEV_1, MEF_{50}, FENO and asthma control with the analyzed genetic polymorphism rs17576 *(MMP9*) in patients using leukotriene antagonists (LTRA) alone. Kruskal-Wallis test, $p< 0.05$, χ2- chi square, N= 38. Adjusted model (F adjusted) corrected for possible confounding variables: age, disease duration, atopy status, total IgE level, eosinophil count, neutrophil count, basophil count, hsCRP level, BMI percentile category, comorbidity (AR, AD, OSA, GERD). General linear model test, $p< 0.05$, Partial η^2- partial eta squared (effect size statistics), N= 38. BMI percentile category: underweight (0-3 percentile), normal (5-85 percentile), overweight (86-95 percentile), obese (>95 percentile).Abbreviations for respective responses to treatment are defined in Supplement 4.

rs17576 (*MMP9*)	
	Resp_FENO_3rd to 4th control
χ^2	7.119
p value (two-tailed)	0.028
	Resp_CTRL_1st to 2nd control
F (adjusted)	8.488
p value	0.026
Partial η^2	0.971

Table 19. Association of response to treatment after 6, 12, 18 and 24 months (on average), according to changes in FEV_1, MEF_{50}, FENO and asthma control with the analyzed genetic polymorphisms: rs37973 (GLCCI1), rs9910408 (*TBX21*), rs242941 (*CRHR1*) and rs1042713 (*ADRB2*) in patients using combination treatment (ICS + LABA and/or LTRA). Kruskal-Wallis test, $p< 0.05$, χ2- chi square, N= 106. Adjusted model (F adjusted) corrected for possible confounding variables: age, disease duration, atopy status, total IgE level, eosinophil count, neutrophil count, basophil count, monocyte count, platelet count, hsCRP level, BMI percentile category, comorbidity (AR, AD, OSA, GERD). General linear model test, $p< 0.05$, Partial η2- partial eta squared (effect size statistics), N= 106. BMI percentile category: underweight (0-3 percentile), normal (5-85 percentile), overweight (86-95 percentile), obese (>95 percentile). Abbreviations for respective responses to treatment are defined in Supplement 4.

rs37973 (*GLCCI1*)					
	Resp_FENO_diagn to 1st	**Resp_CTRL_1st to 2nd**	**Resp_MEF_{50}_3rd to 4th**	**Resp_FENO_diagn to 2nd**	**Resp_CTRL_diagn to**

Table 19. continued

	control	control	control	control	2nd control
χ^2	9.076	13.275	11.903	13.822	14.722
p value (two-tailed)	0.011	0.001	0.003	0.001	0.001
rs9910408 (*TBX21*)					
	Resp_FENO_1st to 2nd control	**Resp_FEV_1_2nd to 3rd control**	**Resp_MEF_{50}_3rd to 4th control**	**Resp_FEV_1_diagn to 3rd control**	
χ^2	20.335	6.927	8.165	6.152	
p value (two-tailed)	0.000	0.031	0.017	0.046	
	Resp_FENO_1st to 2nd control				
F (adjusted)	2.060				
p value	0.031				
Partial η^2	0.586				
rs242941 (*CRHR1*)					
	Resp_MEF_{50}_diagn to 1st control	**Resp_FEV_1_1st to 2nd control**	**Resp_FEV_1_2nd to 3rd control**	**Resp_MEF_{50}_2nd to 3rd control**	**Resp_FEV_1_3rd to 4th control**
χ^2	8.844	14.822	23.482	18.020	8.162
p value (two-tailed)	0.012	0.001	0.000	0.000	0.017
	Resp_FENO_diagn to 4th control				
F (adjusted)	2.463				
p value	0.010				
Partial η^2	0.629				
rs1042713 (*ADRB2*)					
	Resp_FENO_diagn to 1st control	**Resp_CTRL_2nd to 3rd control**	**Resp_FENO_diagn to 2nd control**	**Resp_CTRL_diagn to 3rd control**	

	Resp_FENO_3rd to 4th control	**Resp_FENO_diagn to 3rd control**	**Resp_FENO_diagn to 4th control**	**Resp_CTRL_diagn to 4th control**	**Resp_MEF_{50}_diagn to 4th control**
χ^2	6.342	10.527	11.913	6.150	7.273
p value (two-tailed)	0.042	0.005	0.003	0.046	0.026

Table 19. continued

χ^2	16.814	7.926	12.803	12.942
p value (two-tailed)	0.000	0.019	0.002	0.002
	Resp_FENO_diagn to 1st control			
F (adjusted)	2.321			
p value	0.015			
Partial η^2	0.615			

Genotype distribution (frequencies) for respective genetic polymorphisms (rs37973, rs9910408, rs242941, rs1876828, rs1042713 and rs17576) in „good", „moderate" and „bad" responders according to relative changes in FEV_1, MEF_{50}, FENO and level of asthma control between each visit over the period of 2 years (recruitment to 4th control visit) in specific subgroups of participants on different classes of asthma treatment (ICS alone and combination treatment- ICS+LABA/LTRA) are presented in Tables 20 (use of ICS alone) and 21 (use of combination treatment).

Table 20. Genotype distribution for analyzed genetic polymorphisms: rs37973 (GLCCI1), rs9910408 (*TBX21*) and rs242941 (*CRHR1*) in patients with „good", „moderate" and „bad" response to treatment with ICS alone according to changes in FEV_1, MEF_{50}, FENO and asthma control after (on average) 6, 12, 18 and 24 months of medication use. Abbreviations for respective responses to treatment are defined in Supplement 4. Pearson`s χ^2 test, $p < 0.05$. χ^2- chi square, N= 158.

rs37973 (*GLCCI1*)							
		GG	**GA**	**AA**	**Total**	**χ^2**	**p value**
Resp_FENO_ 1st to 2nd control	**Good response**	10	64	38	112	13.980	0.007
	Moderate response	7	6	9	22		
	Bad response	3	5	9	17		
Total		20	75	56	151		
Resp_FENO_ 2nd to 3rd control	**Good response**	10	16	12	38	10.210	0.037
	Moderate response	6	47	38	91		
	Bad response	4	11	6	21		
Total		20	74	56	150		
rs9910408 (*TBX21*)							
		AA	**AG**	**GG**	**Total**	**χ^2**	**p value**
Resp_FEV_1_1st to 2nd control	**Good response**	2	24	5	31	19.340	0.000
	Moderate response	28	54	12	94		
	Bad response	15	9	9	33		
Total		45	87	26	158		
Resp_FENO_1st to 2nd control	**Good response**	40	63	9	112	27.663	0.000
	Moderate response	0	14	8	22		
	Bad response	2	8	7	17		
Total		42	85	24	151		
Resp_CTRL_3rd to 4th control	**Good response**	7	26	9	42	22.710	0.000
	Moderate response	16	36	7	59		
	Bad response	13	4	0	17		

Table 20. continued

Total		36	66	16	118		
rs242941 (*CRHR1*)							
		AA	**AC**	**CC**	**Total**	**χ^2**	**p value**
Resp_FENO_diagn to 2nd control	**Good response**	7	6	4	17	19.650	0.000
	Moderate response	11	38	48	97		
	Bad response	0	18	19	37		
Total		18	62	71	151		
Resp_FENO_1st to 2nd control	**Good response**	8	45	59	112	14.210	0.007
	Moderate response	4	11	7	22		
	Bad response	6	6	5	17		
Total		18	62	71	151		

Table 21. Genotype distribution for analyzed genetic polymorphisms: rs9910408 (*TBX21*), rs242941 (*CRHR1*) and rs1042713 (*ADRB2*) in patients with „good", „moderate" and „bad" response to treatment with combination therapy (ICS+LABA/LTRA) according to changes in FEV_1, MEF_{50}, FENO and asthma control after (on average) 6, 12, 18 and 24 months of medication use. Abbreviations for respective responses to treatment are defined in Supplement 4. Pearson`s χ^2 test, $p < 0.05$. χ^2- chi square, N= 106.

rs9910408 (*TBX21*)							
		AA	**AG**	**GG**	**Total**	**χ^2**	**p value (χ^2)**
Resp_FENO_1st to 2nd control	**Good response**	29	37	2	68	28.745	0.000
	Moderate response	8	6	11	25		
	Bad response	1	7	3	11		
Total		38	50	16	104		
rs242941 (*CRHR1*)							
		AA	**AC**	**CC**	**Total**	**χ^2**	**p value (χ^2)**
Resp_FENO_diagn to 4th control	**Good response**	0	7	8	15	13.522	0.007
	Moderate response	4	23	21	48		
	Bad response	7	7	4	18		
Total		11	37	33	81		
rs1042713 (*ADRB2*)							
		GG	**GA**	**AA**	**Total**	**χ^2**	**p value (χ^2)**
Resp_FENO_diagn to 1st control	**Good response**	13	30	30	73	17.460	0.001
	Moderate response	2	0	3	5		
	Bad response	12	1	13	26		
Total		27	31	46	104		

Significant allelic frequencies (distribution) for respective genetic polymorphisms (rs37973, rs9910408, rs242941, rs1876828, rs1042713 and rs17576) in „good", „moderate" and „bad" responders according to relative changes in FEV_1, MEF_{50}, FENO and level of asthma control between each visit over the period of 2 years (recruitment to 4th control visit) in specific subgroups of participants on different classes of asthma treatment (ICS alone, LTRA alone and combination treatment- ICS+LABA/LTRA) are presented in Tables 22 (use of ICS alone), 23 (use of LTRA alone) and 24 (use of combination treatment).

Table 22. Allelic distribution for analyzed genetic polymorphisms: rs37973 (*GLCCI1*), rs9910408 (*TBX21*) and rs242941 (*CRHR1*) in patients with „good", „moderate" and „bad" response to treatment with ICS alone according to changes in FEV_1, MEF_{50}, FENO and asthma control after (on average) 6, 12, 18 and 24 months of medication use. Abbreviations for respective responses to treatment are defined in Supplement 4. χ^2 test, $p < 0.05$. χ^2- chi square, N= 158.

rs37973					
Response	**GG**	**GA+AA**	**χ^2**	**p value**	
Good	10	102	8.707	0.0129	**Resp_FENO_1st to 2nd control**
Moderate	7	15			
Bad	3	14			
Good	10	28	9.713	0.008	**Resp_FENO_2nd to 3rd control**
Moderate	6	85			
Bad	4	17			
rs9910408					
Response	**AA**	**AG+GG**	**χ^2**	**p value**	
Good	2	29	12.130	0.0023	**Resp_FEV$_1$_1st to 2nd control**
Moderate	28	66			
Bad	15	18			
Good	7	35	21.05	<0.0001	**Resp_CTRL_3rd to 4th control**
Moderate	16	43			
Bad	13	4			
Good	40	72	14.14	0.0009	**Resp_FENO_1st to 2nd control**
Moderate	0	22			
Bad	2	15			
rs242941					
Response	**AA**	**AC+CC**	**χ^2**	**p value**	
Good	7	10	18.90	<0.0001	**Resp_FENO_diagn to 2nd control**
Moderate	11	86			

Table 22. continued

Bad	0	37			
Good	8	104	12.10	0.0024	**Resp_FENO_1st to 2nd control**
Moderate	4	18			
Bad	6	11			

Table 23. Allelic distribution for analyzed genetic polymorphisms: rs17576 (MMP9) in patients with „good", „moderate" and „bad" response to treatment with LTRA alone according to changes in FEV_1, MEF_{50}, FENO and asthma control after (on average) 6, 12, 18 and 24 months of medication use. Abbreviations for respective responses to treatment are defined in Supplement 4. $\chi 2$ test, $p < 0.05$. $\chi 2$- chi square, N= 38.

rs17576					
Response	**AA+AG**	**GG**	**χ^2**	**p value**	
Good	29	4	6.221	0.0446	**Resp_CTRL_1st to 2nd control**
Moderate	0	1			
Bad	2	1			

Table 24. Allelic distribution for analyzed genetic polymorphisms: rs9910408 (*TBX21*), rs242941 (*CRHR1*) and rs1042713 (*ADRB2*) in patients with „good", „moderate" and „bad" response to treatment with combination therapy (ICS+LABA/LTRA) according to changes in FEV_1, MEF_{50}, FENO and asthma control after (on average) 6, 12, 18 and 24 months of medication use. Abbreviations for respective responses to treatment are defined in Supplement 4. χ^2 test, $p < 0.05$. χ^2- chi square, N= 106.

rs9910408					
Response	**AA+AG**	**GG**	**χ^2**	**p value**	
Good	66	2	25.01	<0.0001	**Resp_FENO_1st to 2nd control**
Moderate	14	11			
Bad	8	3			
rs242941					
Response	**AA**	**AC+CC**	**χ^2**	**p value**	
Good	0	15	13.31	0.0013	**Resp_FENO_diagn to 4th control**
Moderate	4	44			
Bad	7	11			
rs1042713					
Response	**GG**	**GA+AA**	**χ^2**	**p value**	
Good	13	60	8.552	0.0139	**Resp_FENO_diagn to 1st control**
Moderate	2	3			
Bad	12	14			

4.4. Clustering analysis

Prior to cluster analysis, the data had to be preprocessed. The data consisted of 365 patients and 280 variables in total. The dataset did not include any incomplete cases up to the 3rd control visit, which corresponds to the roughly 1.5 year- follow-up period. At the 4th control visit, a significant proportion of the dataset had missing values due to the fact that not all patients were followed up to the 4th control visit (ca. 2 years after recruitment) or simply dropped out of the study. Some features had missing values due to patients` lack of cooperation (eg. patient was too young to cooperate for lung function or FENO measurement), there was insufficient blood/serum sample to perform certain biochemical tests etc.

The variables used in this study can be broadly separated into:

- baseline demographics (such as gender, age);
- subjective clinical data obtained from the parents/patient at the patient`s first research contact (personal and family medical history, such as atopy status, allergic rhinitis, atopic dermatitis);
- objective clinical data collected at the patient`s first research contact and other follow-up appointments. These include personal anamnesis in the period since the last visit, such as symptom control and frequency and severity of exacerbations, lung function and airway inflammation (FENO) measurement;
- biologically (and clinically) relevant data collected at the patient`s first research contact (such as skin prick and total and specific IgE allergy test results, hematologic and biochemical blood test results, comorbidity status- pH probing and reflux episodes monitoring for diagnostics of GERD, polysomnography for diagnostics of OSAS, anthropometric measurements- height and weight and calculation of BMI and BMI percentiles);
- genetic data- genotypes for rs37973 (*GLCCI1*), rs9910408 (*TBX21*), rs242941 (*CRHR1*), rs1876828 (*CRHR1*), rs1042713 (*ADRB2*) and rs17576 (*MMP9*).

The data was divided into treatment periods, i.e. diagnosis (baseline), first, second, third and fourth control visit. Feature selection in clustering was conducted through filtering based on the variance threshold, which was set to 5%, meaning variables with less than 5% variance were excluded from further analysis (Belgrave et al. 2018).

Features describing allergic sensitization were converted to binary or ordinal features, and grouped into 4 clinically relevant categories: seasonal allergens (i.e. grass, weed and tree pollen), perennial allergens (house dust mite and molds), insect venom (bee, wasp or hornet) and food allergens. Both SPT results and specific IgE results were into account for each respective sensitization category. Additionally, certain sensitization features were assigned separate variables, due to their possible clinical relevance- strong sensitization to house dust mite (*D. pteronyssinus, D. farinae*), cat dander and ragweed (*Ambrosia*). These sensitizations have previously been associated with disease severity and more severe outcomes (Sheehan and Phipatanakul 2016, Li et al. 2013, Lombardi et al. 2017). Strong sensitization was defined as sIgE class R4-R6 to the respective allergen.

Features with string notation (e.g. “medication (treatment) prescribed”) were numerically coded and converted to categorical features. Features with a large portion of missing values (ca. 20%) were imputed by median for continuous variables and most frequent value for categorical variables.

Data clustering was performed with data regarding treatment outcomes (level of response to treatment according to changes in lung function, airway inflammation and asthma control, as defined in Section 4.1) to identify patterns of response to treatment with common classes of asthma treatment ("response" clusters). These clusters were transformed to classes and relevant data (at recruitment point, baseline) was used to establish plausible underlying disease phenotypes (clusters) predisponing for certain treatment outcomes (classes). To understand feature importance for classification the decision tree algorhytm was used. For this, a Gini index (Gini coefficient) which represents a measure of statistical dispersion (Wittebole et al. 2009) was set to 0.2 as the lowest limit. A Gini coefficient of zero expresses perfect equality, but to avoid potential noise and overfitting the clustering data, an empirical cutoff of 0.2 was implemented.

Response clusters are presented in Figure 14. Relevant features (main discriminants) distinguishing each cluster according to the decision tree algorhytm for the response clusters are shown in Figure 15. The relevant features corresponding to clinical, demographic and genetic data from recruitment point (baseline) characterizing each response cluster/class (main discriminants) are shown in Figure 16. Cluster statistics related to response to treatment patterns and data from recruitment point (baseline) are shown in Tables 25 and 26, respectively.

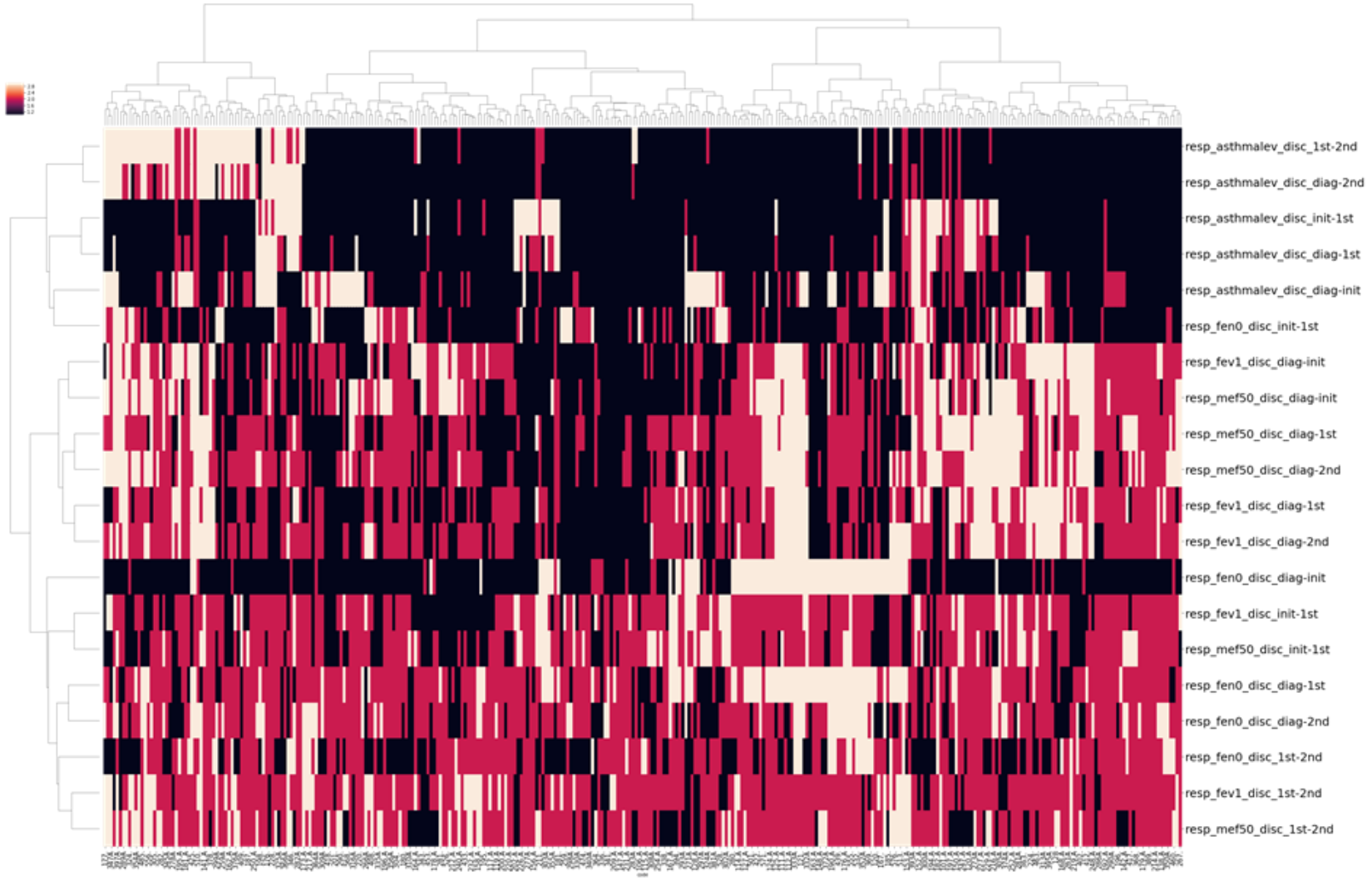


Figure 14. Heatmap of response clusters indicating patterns of response to treatment with common classes of asthma treatment (ICS alone, LTRA alone, ICS+LABA/LTRA). Ward`s Euclidean method, $p < 0.05$.

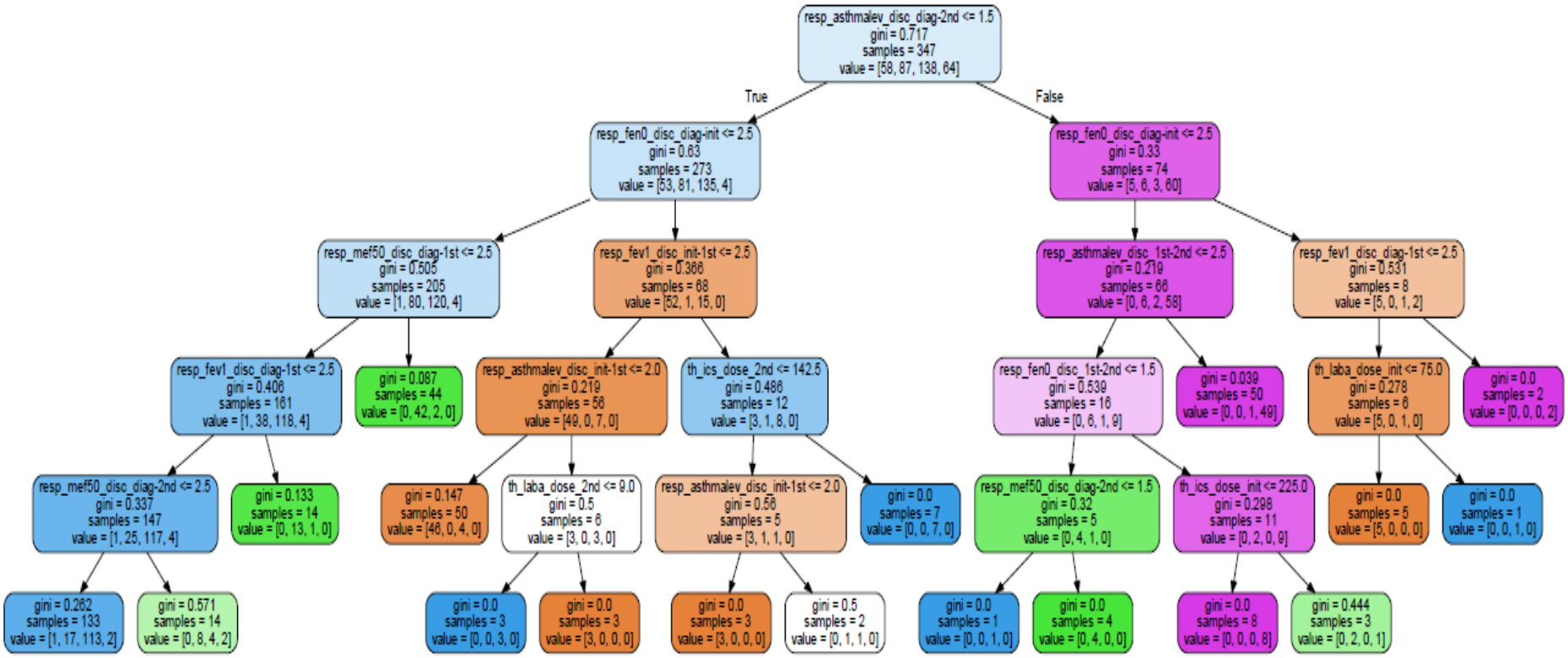


Figure 15. Main discriminants distinguishing between each response cluster (N= 4) according to the decision tree algorhytm. Ward`s Euclidean method, $p < 0.05$, Gini < 0.2. Disc- discrete variable, asthmalev- level of asthma control, fen0- FENO, th- treatment (therapy), init- 1st control visit (after 6 months of treatment use), 1st- 2nd control visit (after 12 months of medication use), 2nd- 3rd control visit (after 1.5 years of treatment use), resp- response.

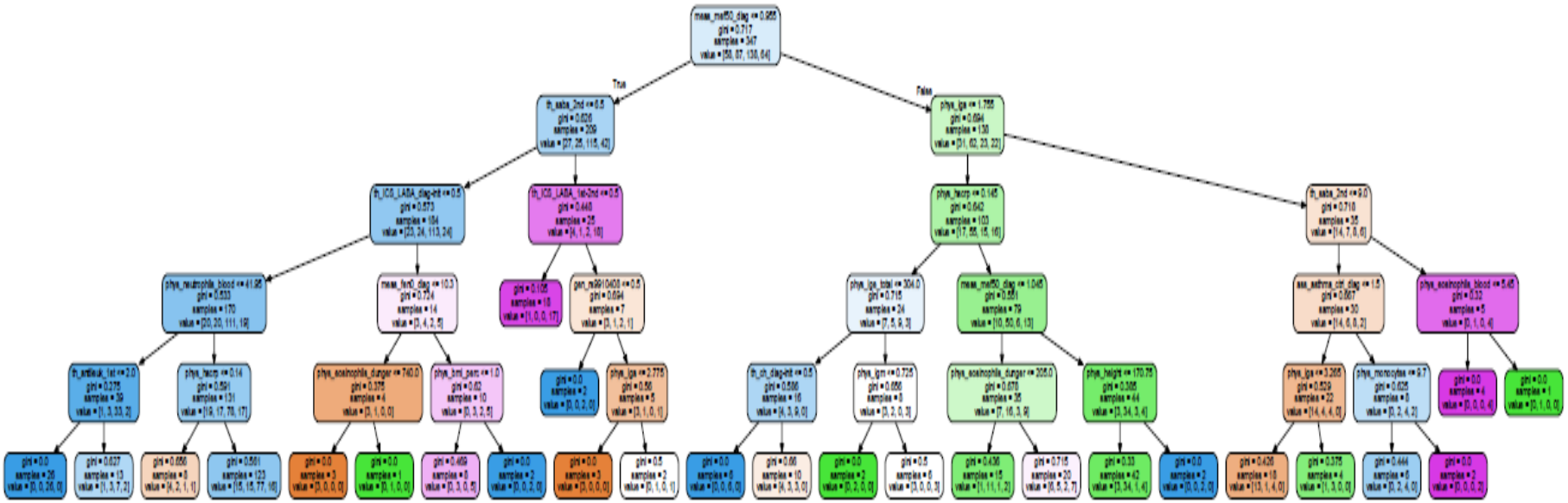


Figure 16. Main discriminants (relevant features) characterizing each response clusters/class corresponding to clinical, demographical and genetic data from recruitment point, according to the decision tree algorhytm. Ward`s Euclidean method, $p < 0.05$, Gini < 0.2. Disc- discrete variable, asthmalev- level of asthma control, fen0- FENO, th- treatment (therapy), init- 1st control visit (after 6 months of treatment use), 1st- 2nd control visit (after 12 months of medication use), 2nd- 3rd control visit (after 1.5 years of treatment use), antileuk- LTRA treatment, ICS_Laba- combination treatment, esoinophils_dunger- Eosinophil count (Dunger), eosinophils_blood_ relative eosinophil blood count (%), neutrophils_blood- relative neutrophil blood count, asthma_ctrl- asthma control.

Table 25. Response to treatment- related cluster statistics. Ward`s Euclidean method, χ^2 test, $p < 0.05$. Abbreviations for respective responses to treatment are defined in Supplement 4.

Feature	**Cluster 1**			**Cluster 2**			**Cluster 3**			**Cluster 4**			**Significance**
No of patients	N= 58			N= 87			N= 138			N= 64			**p value**
	Good N (%)	**Moderate N (%)**	**Bad N (%)**	**Good N (%)**	**Moderate N (%)**	**Bad N (%)**	**Good N (%)**	**Moderate N (%)**	**Bad N (%)**	**Good N (%)**	**Moderate N (%)**	**Bad N (%)**	
Resp_FEV_1_diagn to 1st control	17 (29.31)	27 (46.55)	14 (24.14)	8 (9.2)	45 (51.72)	34 (39.08)	75 (54.35)	49 (35.51)	14 (10.14)	19 (29.69)	24 (37.5)	21 (32.81)	< 0.001
Resp_FENO_diagn to 1st control	1 (1.72)	57 (98.28)	0 (0)	79 (90.8)	7 (8.05)	1 (1.15)	108 (78.26)	14 (10.14)	16 (11.59)	58 (90.62)	4 (6.25)	3 (3.12)	< 0.001
Resp_CTRL_diag to 1st control	34 (58.62)	12 (20.69)	12 (20.69)	55 (63.22)	22 (25.29)	10 (11.49)	94 (68.12)	16 (11.59)	28 (20.29)	34 (53.12)	11 (17.19)	19 (29.69)	< 0.05
Resp_MEF_{50}_diag to 1st control	18 (31.03)	22 (37.93)	18 (31.03)	5 (5.75)	43 (49.43)	39 (44.83)	75 (54.35)	43 (31.16)	20 (14.49)	20 (31.25)	22 (34.38)	22 (34.38)	< 0.001
Resp_FEV_1_1st to 2nd control	9 (15.52)	46 (79.31)	3 (5.17)	10 (11.49)	64 (73.56)	13 (14.94)	44 (31.88)	70 (50.72)	24 (17.39)	18 (28.12)	40 (62.5)	6 (9.38)	< 0.001
Resp_FENO_1st to 2nd control	48 (82.76)	10 (17.24)	0 (0)	55 (63.22)	21 (24.14)	11 (12.64)	84 (60.87)	30 (21.74)	24 (17.39)	39 (60.94)	14 (21.88)	11 (17.19)	< 0.05
Resp_CTRL_1st to 2nd control	52 (89.66)	2 (3.45)	4 (6.9)	59 (67.82)	11 (12.64)	17 (19.54)	118 (85.51)	4 (2.9)	16 (11.59)	46 (71.88)	6 (9.38)	12 (18.75)	< 0.01
Resp_FEV_1_2nd to 3rd control	3 (5.17)	45 (77.59)	10 (17.24)	11 (12.64)	73 (83.91)	3 (3.45)	21 (15.22)	89 (64.49)	28 (20.29)	4 (6.25)	42 (65.62)	18 (28.12)	< 0.001
Resp_FENO_2nd to 3rd control	16 (27.59)	26 (44.83)	16 (27.59)	29 (33.33)	48 (55.17)	10 (11.49)	54 (39.13)	75 (54.35)	9 (6.52)	23 (35.94)	29 (45.31)	12 (18.75)	< 0.01

Table 25. continued

Res_CTRL_2nd to 3rd control	53 (91.38)	3 (5.17)	2 (3.45)	82 (94.25)	5 (5.75)	0 (0)	128 (92.75)	6 (4.35)	4 (2.9)	2 (3.12)	9 (14.06)	53 (82.81)	< 0.001
Resp_MEF$_{50}$_2nd to 3rd control	3 (5.17)	41 (70.69)	14 (24.14)	20 (22.99)	57 (65.52)	10 (11.49)	38 (27.54)	76 (55.07)	24 (17.39)	4 (6.25)	36 (56.25)	24 (37.5)	< 0.001
Resp_FEV$_1$_diagn to 2nd control	22 (37.93)	23 (39.66)	13 (22.41)	6 (6.9)	42 (48.28)	39 (44.83)	79 (57.25)	54 (39.13)	5 (3.62)	19 (29.69)	30 (46.88)	15 (23.44)	< 0.001
Resp_FENO_diagn to 2nd control	7 (12.07)	0 (0)	51 (87.93)	13 (14.94)	65 (74.71)	9 (10.34)	25 (18.12)	90 (65.22)	23 (16.67)	14 (21.88)	42 (65.62)	8 (12.5)	< 0.001
Resp_CTRL_diagn to 2nd control	50 (86.21)	6 (10.34)	2 (3.45)	61 (70.11)	16 (18.39)	10 (11.49)	120 (86.96)	10 (7.25)	8 (5.8)	42 (65.62)	10 (15.62)	12 (18.75)	< 0.01
Resp_MEF$_{50}$_diagn to 2nd control	17 (29.31)	27 (46.55)	14 (24.14)	3 (3.45)	36 (41.38)	48 (55.17)	74 (53.62)	60 (43.48)	4 (2.9)	14 (21.88)	35 (54.69)	15 (23.44)	< 0.001
Resp_FEV$_1$_diagn to 3rd control	19 (32.76)	20 (34.48)	19 (32.76)	8 (9.2)	49 (56.32)	30 (34.48)	71 (51.45)	62 (44.93)	5 (3.62)	8 (12.5)	39 (60.94)	17 (26.56)	< 0.001
Resp_FENO_diagn to 3rd control	12 (20.69)	25 (43.1)	21 (36.21)	15 (17.24)	56 (64.37)	16 (18.39)	34 (24.64)	86 (62.32)	18 (13.04)	10 (15.62)	37 (57.81)	17 (26.56)	< 0.01
Resp_CTRL_diagn to 3rd control	53 (91.38)	5 (8.62)	0 (0)	81 (93.1)	6 (6.9)	0 (0)	135 (97.83)	3 (2.17)	0 (0)	4 (6.25)	20 (31.25)	40 (62.5)	< 0.001
Resp_MEF$_{50}$_diagn to 3rd control	11 (18.97)	30 (51.72)	17 (29.31)	5 (5.75)	37 (42.53)	45 (51.72)	71 (51.45)	61 (44.2)	6 (4.35)	4 (6.25)	36 (56.25)	24 (37.5)	< 0.001

Table 26. Cluster statistics related to relevant clinical, demographic and genetic data from recruitment point (diagnosis). Ward`s Euclidean method, Kruskal- Wallis and $\chi 2$ test, $p < 0.05$. Strong sensitization defined as sIgE to respective allergen of >17.51 kU/L (classes 4-6), food allergy defined as any sIgE to food allergens > 0.35 kU/L (> class 1). Abbreviations for respective responses to treatment are defined in Supplement 4.

Feature	**Cluster 1**		**Cluster 2**		**Cluster 3**		**Cluster 4**		**Significance**
No of patients	N= 58		N= 87		N= 138		N= 64		**p value**
	No	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	
Strong sensitization to dust mite (sIgE d1)	18 (31.03%)	40 (68.97%)	40 (45.98%)	47 (54.02%)	73 (52.90%)	65 (47.10%)	31 (48.44%)	33 (51.56%)	< 0.05
Food allergy	51 (87.93%)	7 (12.07%)	70 (80.46%)	17 (19.54%)	122 (88.41%)	16 (11.59%)	54 (84.38%)	10 (15.63%)	
AD comorbidity	44 (75.86%)	14 (24.14%)	60 (68.96%)	27 (31.03%)	94 (68.12%)	44 (31.88%)	34 (53.12%)	30 (46.88%)	< 0.05
Gene_rs37973									
	GG	**GA**	**AA**	**GG**	**GA**	**AA**	**GG**	**GA**	**AA**	**GG**	**GA**	**AA**	< 0.05
	13 (22.41%)	18 (31.03%)	27 (46.55 %)	13 (14.94 %)	48 (55.17%)	26 (29.89%)	15 (10.87 %)	70 (50.72%)	53 (38.41%)	12 (18.75%)	34 (53.12%)	18 (28.12 %)	
	Mean (STD)								
% FEV_1 predicted at baseline	0.89 (0.20)		0.96 (0.13)		0.82 (0.17)		0.88 (0.15)		< 0.001
% MEF_{50} predicted at baseline	0.92 (0.22)		1.03 (0.19)		0.79 (0.22)		0.84 (0.24)		< 0.001
FENO at baseline (ppb)	21.5 (23.61)		17.23 (16.15)		23.07 (23.14)		18.59 (13.38)		
Age (yrs)	11.79 (3.39)		10.16 (3.82)		9.98 (3.84)		9.61 (3.64)		< 0.01

Table 26. continued

Height (cm)	153.05 (17.78)			143.9 (19.11)			143.86 (21.12)			142.23 (21.24)			< 0.05
Disease duration (yrs)	5.72 (3.43)			5.08 (3.84)			4.12 (3.8)			3.45 (3.11)			< 0.001
Total IgE (kIU/L)	686.34 (744.36)			421.95 (578.5)			653.9 (1164.19)			484.79 (516.04)			
Eosinophil absolute count (Dunger)	419.99 (284.96)			418.71 (347.17)			380.16 (305.29)			377.22 (349.65)			
Neutrophil blood count (%)	49.72 (11.26)			50.79 (13.18)			49.57 (12.82)			51.99 (12.96)			
hsCRP (mg/L)	2.58 (6.31)			4.02 (14.45)			3.11 (7.52)			2.57 (3.72)			
Platelets (x 10^9/L)	291.09 (86.54)			278.68 (108.14)			283.72 (105.47)			257.7 (105.31)			
Asthma severity	**Grade 1-2**		**Grade 3-4**	**Grade 1-2**		**Grade 3-4**	**Grade 1-2**		**Grade 3-4**	**Grade 1-2**		**Grade 3-4**	
	55 (94.83%)		3 (5.17%)	84 (96.55%)		3 (3.45%)	131 (94.93%)		7 (5.07%)	55 (85.93%)		9 (14.06%)	< 0.05
BMI percentile	**0-5, N(%)**	**5-85, N(%)**	**>85, N(%)**	**0-5, N(%)**	**5-85, N(%)**	**>85, N(%)**	**0-5, N(%)**	**5-85, N(%)**	**>85, N(%)**	**0-5, N(%)**	**5-85, N(%)**	**>85, N(%)**	
	0 (0)	47 (81.03)	11 (18.87)	3 (3.45)	52 (59.77)	32 (36.78)	4 (2.9)	94 (68.11)	40 (28.99)	3 (4.69)	47 (73.43)	14 (21.88)	< 0.05

5. DISSCUSSION

A number of studies have demonstrated that there is a high degree of variability in the magnitude of response to common asthma medications and that this is largely due to genetic predisposition, i.e. specific genetic variants. Moreover, several genetic variants have reproducibly been identified by large genomic (GWAS) studies as well as candidate gene studies for each class of medications commonly used in asthma management (Vijverberg et al. 2018, Duong-Thi-Ly et al. 2017). These genetic variants (including those in the *TBX21*, *GLCCI1*, *CRHR1* and *ADRB2* genes) have been associated with the heterogeneity in bronchodilator reversibility and asthma worsening (exacerbations) in patients continuously using SABA and LABA (*ADRB2* polymorphisms), as well as with the variability in response regarding lung function improvement, airway hyperresponsiveness, and exacerbations in patients on ICS- *GLCCI1*, *CRHR1*, *TBX21* polymorphisms (Lima et al. 2009). Despite the urging need for standardization in reporting measures of clinical validity, there still no consensus in defining primary outcomes/endpoints in pharmacogenetic studies, although most of them focus on lung function parameters, BHR and exacerbation risk (Vijverberg et al. 2018).

5.1. Selection of outcomes (study endpoints)

In clinical trials, including pharmacogenetic studies such as this one, the degree of resistance and physiological lung function impairment is quantitated most commonly by the forced vital capacity in 1 s, FEV_1 (ATS 1987, Fal and Rosiek-Biegus 2012). FEV_1 is a commonly used endpoint in respiratory disease trials, because the measurement is reproducible, standardized and easy to measure. On the other hand, there is no consensus as to what constitutes a clinically meaningful change. In general, FEV_1 abnormalities tend to parallel clinical measures of respiratory health and the European Medicines Agency (EMA) definition of clinical endpoints and pulmonary exacerbations in lung disease list FEV_1 as a primary endpoint (EMEA/CHMP/EWP/9147 2008). However, certain studies have shown relatively poor correlations of FEV_1 with episodes of dyspnea or exercise tolerance in patients with stable forms of lung diseases (Niewoehner et al. 2000). Although EMA lists other lung function parameters (FVC, FEF_{25-75}) as surrogate endpoints, they are rarely assessed as outcomes in clinical studies investigating treatment success.

Recently, there has been a renewed interest in the peripheral airways, which are now becoming increasingly appreciated for their significance to the clinical manifestations (expression) in asthma. The level of inflammation present in the peripheral airways of asthmatics may be more intense than the one observed in the large airways (Corren 2008). Moreover, peripheral airways resistance in patients with asthma contributed to up to 50% of the total airways resistance and the distal parts of the lung have been recognized as a predominant site of airflow obstruction in asthmatics (Hamid and Tulic 2007). Small airways dysfunction has been associated with nocturnal asthma, exercise-induced asthma and more severe and difficult-to-treat disease forms (Yanai et al. 1992, Kraft et al. 1999, Anderson 2006, Veen et al. 2000). Also, the small (peripheral) airways seem to contribute significantly to the severity of bronchial hyperresponsiveness (BHR), independently of the level of FEV_1 measured (Telenga et al. 2013). Although it is not commonly used as a clinical endpoint, the level of reponse to treatment according to parameters of the distal (small or peripheral) airway function (MEF_{50}) was assessed as one of 4 primary study endpoints, due to the mounting evidence of the distal airway involvement being a crucial factor in asthma in children (Torrego Fernandez and Munoz Cano 2011).

The level of response to treatment according to the measurement of fractional nitric oxide (FENO) in exhaled breath was also assessed, because it is a quantitative, noninvasive (which is imperative in pediatric asthma management), simple, and safe method of measuring airway inflammation that provides a complementary tool to other ways of assessing airways disease, including asthma (Dweik et al. 2011). The latest GINA guidelines even suggest that FENO-guided treatment adjustment significantly reduces exacerbation rates compared with guideline-based treatment in children (GINA 2018).

According to GINA gudielines, the long-term goals in asthma management are achieving adequate symptom control, maintaining normal activity levels as much as possible (minimum impairment of quality of life), minimizing future risk of exacerbations, fixed airflow limitation and medication side-effects (GINA 2018). Although asthma control assessment is a multifactorial parameter that involves symptom occurance (including nocturnal symptoms) and severity, need for reliever medications (namely SABA), number and severity of asthma exacerbations (especially those that require the use of oral corticosteroids and hospitalization), as well as patient self-assessment (ACT) and provides good insight into the current disease status, it is rarely used as an endpoint in clinical studies probably because it encompasses certain subjective variables and there is always the risk of patient-originated

bias. On the other hand, objective measurements (such as lung function parameters) are not always in concordance with symptom control nor predictive of exacerbation episodes, and patient self- assessment is an essential part of the "shared- care approach" in asthma management (implying a high level of patient involvement in tailoring their own treatment care) associated with improved outcomes (Wilson et al. 2010), which is why the parameter of asthma control was used in assessment of the level of response to treatment in this doctoral research.

5.2. Selection of genetic variants

rs37973 is a polymorphism in the promoter region of the gene encoding the glucocorticoid induced transcript 1 (GLCCI1) located on chromosome 7. In patients with asthma, treatment with ICS increases the expression levels of the GLCCI1 protein in epithelial lung cells. This increase is suggested to be followed by inflammatory cell apoptosis and diminished airway inflammation levels. Studies report that the level of response to treatment with ICS as well as the amount of GLCCI1 protein induced by glucocorticoids in the bronchial epithelial cells of patients with the GG genotype is significantly lower/poorer as compared to wild type homozygotes- AA (Chiba et al. 2018, Tantisira et al. 2011, McGeachie et al. 2013), which is why it was chosen as a candidate genetic variant in this doctoral research.

rs9910408 (c.-7947) is a polymorphism in the 3`UTR region of the gene encoding the Th1 transcription factor T-bet (TBX21, T-box 21) located on chromosome 17. TBX21 influences naive T lymphocyte development and has been implicated in asthma pathogenesis by GWAS (Tantisira et al. 2004). It serves as a regulator of Th1 cell differentiation by inducing IFN-γ production and it may play a critical role in the supression of the Th2-mediated immune response by inhibiting interleukins IL-4, IL-5, and IL-13 (Lopert et al. 2013, Zhu et al. 2012) Moreover, TBX21 knockout mice spontaneously develop airway hyperresponsiveness and other asthma related features (Finotto et al. 2002). Studies have established the association of rs9910408 with BHR in both children and adults, as well the association of the AA genotype with good (adequate) levels of response to treatment with ICS in non-smoking and non-atopic adult patients with asthma (Raby et al. 2006, Lopert et al. 2013), which is why it was chosen as a candidate SNP in this doctoral research.

rs242941 and rs1876828 are polymorphisms in the intronic region of the gene encoding the corticotropin-releasing hormone receptor 1 (CRHR1) located on chromosome 17. CRHR1 is the key corticotropin releasing hormone receptor, mediating the release of adrenocorticotropic hormone and the catecholaminergic response to CRH (Duong-Thi-Ly et al. 2017). The absence of CRHR1 leads to enhanced airway inflammation and dysfunction (Maitland-van der Zee and Daly 2012). Certain genetic variants may cause decreased expression of CRHR1 and diminished cortisol secreting capacity in response to inflammation. Moreover, in both children and adult patients with asthma (and other respiratory conditions) certain CRHR1 genotypes (including rs242941 and rs1876828) have been shown to be associated with either better or poor response to treament in terms of lung function improvement (FEV_1), bronchodilatator reponse and risk of exacerbations following the administration of an exogenous corticosteroid, such as ICS (Tantisira et al. 2004, McGeachie et al. 2013, Rogers et al. 2009, Kim et al. 2009), which is why they were chosen as candidate genetic variants in this study.

rs1042713 is a non-synonymous variation in the intronless gene encoding the beta-2-adrenergic receptor located on chromosome 5. ADRB2 is a member of the G protein-coupled receptor superfamily, mediating a plethora of physiologic responses such as smooth muscle relaxation, bronchodilation, glucose and lipid metabolism, inhibition of histamine release fom mast cells etc. Genetic variants (including rs1042713) and changes in the expression of this gene have been associated with a number of disorders, including asthma (nocturnal asthma, severe forms and exacerbations), obesity and type 2 diabetes mellitus (Saliba et al. 2014, Rebordosa et al. 2011, Thomsen et al. 2012). The G allele encodes the G (glycine) form at position 16 in ADRB2, while the A allele encodes the R (arginine) residue. A Scottish study involving pediatric patients with asthma showed that the rs1042713 A allele is associated with disease exacerbations, regardless of treatment regime. The risk of exacerbations in patients with the A allele was higher in patients using both short- and long-acting β-agonists, odds ratio- OD ca 1.6 for AA homozygotes and 1.2 for hetergozygotes (Basu et al. 2009, Vijverberg et al. 2018), which is why it was chosen as a candidate genetic variant in this doctoral research, primarily regarding treatment success in patients using combination therapy.

rs17576 is a non-synonymous variation in exon 6 of the gene encoding the matrix metelloproteinase 9 (MMP9) located on chromosome 20. MMP9 plays an essential role in remodeling in asthma (and other conditions such as COPD) including airway wall thickening

and inflammation due to its role in extracellular matrix degradation and genetic variants in the *MMP9* gene (including rs17576) have been associated with different forms of childhood asthma (non-atopic phenotypes) as well as wheezing (Pinto et al. 2010). The rs17576 G allele encodes the R (arginine) residue at position 279 of MMP9 and has been associated with higer morbidity risk, while the A allele encodes the glutamine residue (Q). Additionally, rs17576 has been associated with childhood obesity causing lower plasma MMP9 levels which may modify relevant pathogenetic mechanisms involved in the development of a number of conditions (including asthma) associated with obesity in childhood (Belo et al. 2012). Although rs17576 has not been reviewed in the context of treatment outcomes in asthma, a recent study involving 127 pediatric patients with asthma indicated that other genetic variants in the MMP9 gene are associated with better asthma control and with better response to treatment (Dragicevic et al. 2018). It is therefore highly likely that other poylmorphisms in this gene, including rs17576 are also involved in modulating treatment success in asthmatic patients, which is why it was chosen as a candidate genetic variant in this study.

5.3. Association of genetic variants with treatment outcomes

Treatment outcomes were assessed at 4 timepoints (follow-up visits) over the period of 2 years with folow-up appointments on average 6 months apart (6-8 months), according to relative changes in lung function parameters (FEV_1 and MEF_{50} predicted for age, gender and posture), changes in FENO levels and changes in the level of disease (symptom) control as well as exacerbation rate and severity according to GINA guidelines (as discussed in section 4.1). These were then associated with genotype and allele frequencies for polymorpisms in the *GLCCI1* gene (rs37973), *TBX21* gene (rs9910408), *CRHR1* gene (rs242941 and rs1876828), *ADRB2* gene (rs1042713) and *MMP9* gene (rs17576) in patients using different classes of treatment (ICS alone, LTRA alone and combination treatment-ICS+LABA/LTRA).

5.3.1. Association of genetic variants with treatment outcomes in patients using ICS only

158 patients were treated with inhaled corticosteroids continuously over the period of 2 years. When success of treatment with ICS alone was assessed by changes in lung function parameters, the polymorphisms rs37973 in the *GLCCI1* gene, rs9910408 in the *TBX21* gene

as well as rs242941 and rs1876828 in the *CRHR1* gene were associated with the level of response to treatment according to % change in FEV_1 after 6 months (for rs37973 and rs9910408) as well as after 12 months and 1.5 years (for rs242941 and rs1876828) and 2 years of medication use (rs242941)- see Table 17, which was in concordance with previous studies (Chiba et al. 2018, Lopert et al. 2013, McGeachie et al. 2013). Additionally, the polymorphism rs37973 was significantly associated with the level of response to treatment according to relative changes in MEF_{50} (see Table 17) after 6 months and 1.5 years of ICS use, which was previously reported in a small pediatric asthma cohort (Ding et al. 2017). The rs9910408 polymorphism in the *TBX21* gene, rs242941 and rs1876828 in the *CRHR1* gene were also associated with the level of response to treatment according to relative changes in MEF_{50} after 6 and 12 months (for rs9910408), after 6 months and 2 years for rs242941 and after 12 months of medication use for rs1876828 (see Table 17), which was not previously reported. Interestingly, the rs17576 SNP in the gene encoding MMP9 was significantly associated with the level of response to treatment assessed by % change in FEV_1 after 6 months of ICS use, which has not been reported so far.

Moreover, when outcomes in treatment with ICS alone were assessed by relative changes in the level of airway inflammation (FENO level), the rs37973 polymorphism (*GLCCI1* gene), rs9910408 in the *TBX21* gene, rs242941 and rs1876828 in the C*RHR1* gene as well as rs17576 (*MMP9*) were significantly associated with the level of reponse to treatment after 6 months. The rs242941 and rs17576 polymorphisms were also associated associated with the level of reponse to treatment according to changes in FENO after 12 months of medication use (see Table 17). None of these associations (of these polymorphisms and changes in FENO) have been reported so far.

When treatment outcomes were assess by changes in the level of disease control (symptom control and exacerbation frequency and severity, as discussed in section 4.1), the rs37973 (*GLCCI1*), rs9910408 (*TBX21*), rs1876828 (*CRHR1*) and rs17576 (*MMP9*) polymorphisms were significantly associated with the level of response to treatment after 6 months of ICS use, while rs37973 was also associated with the level of response after 12 months. Additionally, the rs242941(*CRHR1*) polymorphism was associated with the level of response to treatment according to changes in disease control after 2 years of medication use (see Table 17). No such results have been reported previously.

In order to correct for potential confounding variables influencing these associations an adjusted model was applied according to parameters significantly correlating with response to treatment. The variables chosen as potential confounders (covariates) included: age, disease duration, atopy status, total IgE level, eosinophil count, neutrophil count, basophil count, hsCRP level, monocyte count, platelet count, BMI percentile category, comorbidities- AR, AD, OSA, GERD (Table 16). Other lung function parameters (such as FVC, PEF or FEV_1 for assessment of treatment response according to % change in MEF_{50} or FVC, PEF or MEF_{50} for assessment of treatment response according to relative changes in FEV_1) were not taken into account for correction although they significantly correlated with the level of response to treatment since all these variables are functions of the same exhaled breath (lung air) volume and this correlation provides little information on biologically relevant associations (plausible underlying pathophysiologic mechanisms). Additionally, since the level of response to treatment according to changes in FENO and asthma control were also selected as outcomes (study endpoints), these correlations involving FENO and disease control variables were not taken into consideration for the adjusted model, especially because the level of disease control is a multifactorial parameters that partially encompasses measures of lung function and local (airway) inflammation.

After the adjustment for potential confounders, the rs37973 polymorphism in the *GLCCI1* gene was significantly associated only with the level of response to treatment according to changes in FENO after 6 months (p= 0.005 and p= 0.012). Similarly, after correction for covariates, the rs242941 polymorphism in the *CRHR1* gene was associated only with the level of response to treatment according to changes in FENO after 6 and 12 months of ICS use (p= 0.003 and p= 0.015, respectively). In the adjusted model, only rs9910408 in the *TBX21* gene was associated with treatment outcomes assessed by changes in all parameters (lung function, airway inflammation level and asthma control): with the level of response according to % change in FEV_1 after 6 months (p= 0.047), with the level of response to treatment according to changes in FENO after 6 months (p= 0.002) as well as with the level of response to treatment with ICS according to changes in asthma control after 6 months (p= 0.013), see Table 17. This is the first such association of the analyzed polymorphisms with response to treatment according to changes in FENO and asthma control reported. rs9910408 in *TBX21* has previously been associated with response to treatment with ICS assessed by % change in FEV_1 in an adult asthma cohort (Lopert et al. 2013).

No significant associations of treatment outcomes assessed by changes in lung function (% change in FEV_1 and MEF_{50}), changes in FENO and asthma control were identified in the adjusted model for rs1876828 (*CRHR1*) and rs17576 (*MMP9*).

When treatment success was assessed by changes in FENO after 6 months, the frequency of the AA genotype in rs37973 (*GLCCI1*) was significantly higher in good responders (p= 0.007), as well as moderate responders (p= 0.037) compared to the GG genotype (see Table 20). Moreover, the frequency of the A allele was significantly higher in good responders (p= 0.0129) and patients with moderate levels of response ot treatment (p= 0.008) compared to bad responders (see Table 22). Although this association of FENO related treatment success and the rs37973 polymorphism has not been previously reported, a large study involving more than 900 pediatric patients with asthma has demonstrated a similar genotype related effect on response to ICS according to % change in FEV_1 predicted (Tantisira et al. 2011).

For the rs9910408 polymorphism (*TBX21* gene) the frequency of the G allele was significantly higher in good responders compared to moderate and bad responders, when treatment outcomes were assessed by changes in FEV_1 and asthma control after 6 months of medication use (p= 0.0023 and p<0.0001, respectively). When treatment success was assessed by changes in FENO levels after 6 months, the frequency of the G allele was significantly higher in moderate responders compared to patients with bad and good response (p= 0.0009), see Table 22. Although these associations were not previously reported, a study involving patients from the Childhood Asthma Management Program (CAMP) observed a similar allelic effect on the level of response to treatment according to changes in airway hyperresponsiveness (Raby et al. 2006). Conversly, a study involving a Slovenian adult asthma cohort reported that the AA genotype was overrepresented in good responders according to changes in FEV_1 and AHR (Lopert et al. 2013), but these were adult non-atopic participants, whereas our cohort involves children the majority of which had allergic asthma.

For rs242941 in the *CRHR1* gene the frequency of the C allele was significantly higher in good and moderate responders compared to patients with inadequate (bad) levels of response to treatment according to changes in FENO after 12 and 6 months of ICS use (p= 0.0024 and p< 0.0001, respectively), see Table 22. Although this association of response according to relative changes in FENO has not been reported so far, similar effects (association of the C allele) with positive treatment outcomes (improved lung function, % FEV_1 change) was reported in a Korean adult COPD cohort (Kim et al. 2009). Interestingly, a study involving

CAMP participants demonstrated that the minor allele (A) was associated with poor reposnse to treatment with ICS assessed by % change in bronchodilatation reversibility- % FEV_1 change after administration of bronchodilatator (Rogers et al. 2009).

There was no significant association of response to treatment with ICS alone with rs1042713 (*ADRB2*), neither prior nor post adjustment for confounding variables, which was expected, since this polymorphism was previously associated with response to β-agonists (Basu et al. 2009).

5.3.2. Association of genetic variants with treatment outcomes in patients using LTRA only

38 patients were treated with leukotriene receptor antagonists continuously over the period of 2 years. When success of treatment with LTRA alone was assessed by changes in FENO levels, only the rs17576 polymorphism (*MMP9* gene) was significantly associated with the level of response to treatment after 6 months of medication use. This association, however, was not reproduced in the adjusted model (after correction for confounding variables). Instead, treatment outcome assessed by changes in asthma control after 6 months of LTRA use was significantly associated with rs17576 (p= 0.026), see Table 18. The frequency of the A allele was significantly higher in good responders compared to patients with moderate and bad response to treatment with LTRA assessed by changes in disease control after 6 months (p= 0.0446), see Table 23. This is the first such association of rs17576 with the level treatment response in asthma reported so far, but similar effects of disease control improvement after treatment were reported for other genetic variants in the *MMP9* gene (Dragicevic et al. 2018). However, since the association with the level of response to treatment with LTRA accroding to changes in asthma control was rather weak and identified only post adjustment for confounders and, moreover, due to the small sample size (N= 38), there is a risk that this effect is random. Despite that, genetic variants in the MMP9 gene (including the rs17576 polymorphism) may be promising new biomarkers in asthma pharmacogenetics and deserve further research attention.

5.3.3. Association of genetic variants with treatment outcomes in patients using combination treatment

106 patients were treated with combination treatment (ICS+LABA and/or LTRA) continuously over the period of 2 years. When success of treatment with ICS+LABA/LTRA

was assessed by changes in lung function parameters, the rs37973 polymorphism in the *GLCCI1* gene was significantly associated with the level of response to treatment according to % change in MEF_{50} predicted for certain age, gender and posture after 6 months. Additionally, rs37973 was associated with treatment outcomes according to changes in FENO levels and asthma control after 6 and 12 months of medication use. The rs9910408 polymorphism (*TBX21*) was significantly associated with the level of response to combination treatment according to changes in lung function after 6 months and 1.5 years (% change in FEV_1- after 6 months and 1.5 years and MEF_{50}- after 6 months), as well as with treatment outcomes assessed by changes in FENO after 6 months of medication use. rs242941 in the *CRHR1* gene was significantly associated with treatment success assessed by changes in lung function parameters after 6 months and 2 years (% change in MEF50- after 6 months and FEV1- after 6 months and 2 years) of ICS+LABA/LTRA use. Additionally, rs242941 was associated with treatment outcomes according to changes in FENO levels after 6 months, 1.5 years and 2 years of medication use, as well as with the level of response to treatment assessed by changes in asthma control after 2 years of medication use (see Table 19). After adjustment for confounding variables, only treatment outcomes assessed by changes in FENO and asthma control were significantly associated with rs9910408 in *TBX21* (according to changes in FENO after 6 months) and rs242941 in the *CRHR1* gene (according to changes in FENO level after 2 years of treatment use), see Table 19. No significant associations of treatment outcomes were identified in the adjusted model for rs37973. Although the afore mentioned associations have not been reported for combination treatment use so far, they are likely associated with the ICS component in combination regimes, and certain studies have shown an association of all of these genetic variants with response to treatment with inhaled corticosteroids, although mainly according to changes in FEV_1 and airway hyperresponsiveness but not with changes in FENO or asthma control (see Section 5.3.1).

The rs1042713 polymorphism in the *ADRB2* gene was significantly associated with treatment outcomes assessed by changes in FENO after 6 and 12 months as well as with response to treatment according to changes in asthma control after 6 months and 1.5 years of ICS+LABA/LTRA use (see Table 19). After adjustment for confounders, rs1042713 was only associated with treatment success assessed by changes in FENO levels after 6 months of medication use (see Table 19). This association has not been previously reported.

When treament success was assessed by changes in FENO levels after 6 months of ICS+LABA/LTRA use, the frequency of the AA genotype in rs9910408 (*TBX21*) was significantly higher in good responders compared to the GG genotype (p= 0.000), see Table 21. Moreover, the frequency of the A allele was significantly higher in good responders compared to patients with moderate and bad response to treatment (p< 0.0001), see Table 24. Although this association with response to treatment according to changes in FENO was not previously reported for ICS+LABA/LTRA use, a study involving pediatric patients with asthma on ICS demonstrated a similar allelic effect on the level of response to treatment with assessed by changes in AHR (Raby et al. 2006), which is why this association is probably due to the ICS component of combination treatment.

For rs242941 in the *CRHR1* gene, when the level of response to treatment was assessed by changes in FENO after 2 years of treatment, the frequency of the CC genotype was significantly higher in good and moderate responders compared to the AA genotype (p= 0.007), see Table 21. Additionally, the frequency of the C allele was significantly higher in good and moderate responders compared to children with poor (bad) response to treatment (p= 0.0013), see Table 24. Although this association of response to treatment according to changes in FENO has not been previously reported for rs242941 and the C allele with combination treatment, a study involving adult patients with asthma using ICS reported similar results (allelic effect) and an association of positive treatment outcomes (improved lung function) with the C allele (Kim et al. 2009). Additionally, a study involving pediatric patients with asthma on treatment regimes with ICS reported an association of poor treatment response (according to changes in FEV_1 reversibility after bronchodilatator administration) with the A allele (Rogers et al.). This indicates that this association of rs242941 and treatment success is likely due to the ICS component in combination treatment.

When treatment outcomes were assessed by changes in FENO levels after 6 months of ICS+LABA/LTRA use, the frequency of the AA genotype in the rs1042713 polymorphism (*ADRB2*) was significantly higher in good responders compared to the GG genotype (p= 0.001). Moreover, the frequency of the A allele was significantly higher in good responders compared to patients with moderate and bad response to treatment (p= 0.0139). This association of FENO- related treatment outcomes with rs1042713 has not been reported so far. Conversly, the A allele (encoding the Arg16 residue) has been associated with diminished response to treatment with β-agonists (both SABA and LABA)- reduced PEF compared to baseline and higher asthma exacerbation rates (Cho 2010).

There was no significant association of response to treatment with ICS+LABA/LTRA with rs17576 (*MMP9*), neither prior nor post adjustment for confounding variables.

5.4. Identifying clusters in response to treatment

The results of this study indicate that there might be specific patterns in the level of response to treatment with ICS, LTRA or combination therapy (ICS+LABA/LTRA) assessed by changes in lung function parameters (% change in FEV_1 and MEF_{50}), FENO levels and asthma control, see Figure 14 and Table 25. By employing the Ward`s HCA on original response data (not PCA transformed, as using PCA did not affect cluster stability), 4 distinct response clusters were identified. Since the method itself is always prone to forming 2 separate classes by splitting the decision tree until it reaches a perfect fit, by employing a limit of the Gini index of 0.2 and based on cluster stability (Average proportion of non-overlap, APN= 0.4; APN is a measure of the average number of observations not placed in the same cluster by clustering based on the full data and clustering based on the data with a single variable removed iteratively, Brock et al. 2008) as well as empirical clinical and biological knowledge we have successfully identified 4 specific response clusters:

- Cluster 1- patients with overall good response to treatment according to level of asthma control, moderate response to treatment according to lung function parameters (relative changes in FEV_1 and MEF_{50}) and moderate or bad response to treatment according to changes in FENO (N= 58);
- Cluster 2- patients with overall good response to treatment according to level of asthma control, good or moderate levels of response to treatment according to changes in FENO, moderate response to treatment according % change in FEV_1 and bad or moderate levels of response to treatment according to changes in MEF_{50} (N= 87);
- Cluster 3- patients with overall good response to treatment according to changes in disease control, good levels of response to treatment according to changes in both FEV_1 and MEF_{50}, as well as good response to treatment according to changes in FENO levels (N= 138);
- Cluster 4- patients with overall poor (moderate and bad) response to treatment according to changes in lung function, moderate levels of response according to

FENO changes and long-term poor (bad) response to treatment according to level of asthma control (N= 64).

The main discriminant variable in the response clustering according to the decision tree algorhytm was the level of response to treatment according to changes in asthma control after 1.5 years of treatment (long-term control), followed by response to treatment according to changes in FENO, FEV_1 and MEF_{50} in shorter assessment periods (6 and 12 months), see Figure 15.

To date, only one study has focused on long-term treatment outcomes in 3 independent cohorts (including pediatric patients) and have successfully replicated SARP (Severe Asthma Research Program) clusters identified by Moore and colleagues (Moore et al. 2010) in distinguishing each cluster according to age of onset of asthma (early onset vs late onset disease), lung function (normal vs impaired lung function) and comorbidity, such as obesity and eczema (Chang et al. 2014). However, certain clusters had small sample size, especially those refering to more severe disease forms which may affect cluster stability and reproducibility of these results in other cohorts. In general, very few studies have focused on disease control and severity as outcomes. A study involving a cohort of almost 2000 adult asthmatics has successfully replicated 4 clusters in female participants and 3 clusters in men of variable severity (mild to severe persistent asthma) and symptom control (controlled, partly controlled, uncontrolled), although these findings were based on questionnaire data (use of medication, other healthcare use and self-assessment) and there were no objective parameters (such as lung function) measured (Mäkikyrö et al. 2017). Additionally, these were adult patients with asthma and these results may not be applicable in children.

5.4.1. Identifying phenotypes underlying specific response to treatment outcomes

The results of our study suggest specific patterns in the response to treatment assessed by changes in different parameters, including lung function, airway infammation (FENO) and asthma control. In order to identify possible disease features and phenotypes underlying such response patterns/outcomes, we have employed the same clustering method (Ward`s HCA on original data, not PCA transformed) on all relevant data from baseline (diagnosis, recruitment point), including: baseline demographics (gender, age, duration of disease), atopy status, lung function (FEV_1 and MEF_{50} predicted), FENO, sensitization and allergy data (distributed in categories, as discussed in Section 4.4), hematologic and biochemical blood test results,

comorbidity- GERD, OSAS, obesity/overweightness, AR, AD; treatment use and genetic data (rs37973, rs9910408, rs242941, rs1876828, rs1042713 and rs17576). These phenotyping results have identified 4 clusters (according to response ot treatment patterns):

- Cluster 1- the smallest cluster (N= 58), older children (mean age ca. 12 years), with age of onset at about 6 years of age, lung function still in physiologic range (mean FEV_1= 89% predicted and mean MEF_{50}= 92% predicted for age, gender and posture), predominantly sensitized to house dust mite (strong sensitization, 68.97%) and with a dominant genotype (AA) and allelic effect (A allele) for rs37973 in the *GLCCI1* gene;
- Cluster 2 (N=87)- mean age ca. 10 years, with age of onset at about 5 years of age, normal lung function (mean FEV_1= 96 % predicted and mean MEF_{50}= 103% predicted), with ca 54% of participants exhibiting strong sensitization to house dust mite and predominantly heterozygotic for rs37973;
- Cluster 3- the largest cluster (N= 138), mean age ca. 10 years with age of onset at about 6 years of age, impaired lung function (mean FEV_1= 82% and mean MEF_{50}= 79% predicted), no significant association with strong sensitization to house dust mite (53% with strong sensitization, 47% negative) and predominantly heterozygotic for rs37973;
- Cluster 4 (N= 64)- mostly younger children (mean age ca. 9.6 years) with age of onset at about 6 years of age, relatively normal FEV_1 (mean= 88% predicted) but lower MEF_{50} (mean= 84% predicted), no significant association with strong sensitization to house dust mite (51.5% with strong sensitization, 48.5% negative) and predominantly heterozygotic for rs37973, see Table 26.

The main discriminant variables in the phenotype clustering according to the decision tree algorhytm were MEF_{50} predicted at baseline, followed by use of reliever medication (SABA) which a parameter incorporated in asthma control assessment, use of combination treatment, hsCRP and neutrophil blood count (see Figure 16) which reflect type and level of inflammation, although some of these variables (ie. neutrophil count) were not significantly different between clusters in the cluster statistics. This may very well be an effect of different types of statistics applied in the decision tree algorhytm and cluster statistics (Kruskal- Wallis and χ^2 tests)- these two cannot be compared on the same scale of linearity.

Our results indicate that clusters 1-3 have overall good long-term treatment outcomes assessed by changes in asthma control. Cluster 1 had moderate levels of response to

treatment according to lung function parameters (both FEV_1 and MEF_{50}), which may explained by the fact that these patients didn`t have significantly impaired lung function at baseline. These patients also had relatively poor FENO-related response to treatment, which may be a consequence of strong sensitization to house dust mite, as the majority of these patients (almost 70%) had strong sensitization to house dust mite (class 4, sIgE > 17.51 kU/L), see Table 25 and 26. A study involving a pediatic cohort in Korea has demonstrated that the levels of sIgE to house dust mite correlate with increases in FENO (Lee et al. 2015) and, moreover, a study involving asthmatic children from the Southern Mediterranean region suggests that in atopic children, sensitization to indoor inhaled allergens, including house dust mite, may increase airway inflammation levels (FENO) which leads to worsening of the pulmonary function (Ruggieri et al. 2017). Also, these patients were older and and had later onset of the disease (at about 6 years of age), which may also contribute to poorer response to treatment according to FENO and lung function parameters, as most studies indicate that late-onset disease phenotypes imply more severe outcomes (Haldar et al. 2008, Siroux et al. 2011, Wu et al. 2014, Moore et al. 2010, Kim et al. 2013). House dust mite is an allergen with high allergenic potential (Calderon et al. 2016, D`Amato et al. 2007) and, moreover, sensitization to dust mite has been associated with poorer disease outcomes in children possibly indicating early remodeling as a consequence of inflammation secondary to exposure to these allergens (Gaffin and Phipatanakul 2009). Cluster 1 also had the highest eosinophil count and the highest serum total IgE levels (see Table 26), although these associations were not statistically significant, but were major discriminants in the decision tree (see Figure 16). This may indicate a higher level of Th2 inflammation, but not significantly increased FENO levels which may be the reason for their poor FENO-related treatment effectiveness (Robinson et al. 2016).

Cluster 2 was similar to cluster 1 in terms of response to treatment according to disease control and FEV_1 parameters (moderate levels of response according to FEV_1 and good according to asthma control), but they differed in FENO-related treatment outcomes. Cluster 2 patients had good or moderate levels of response to treatment according to changes in FENO which is probably due to the fact that this cluster was not significantly associated with sensitization to house dust mite. These children had relatively earlier age of onset of disease (ca. 5 years of age). Additionally, cluster 2 differed from cluster 1 in the level of response to treatment according to changes in MEF_{50}- these patients had poor (bad and moderate) MEF_{50}-related response, although their baseline MEF_{50} measurements were not impaired, see Tables

25 and 26. This suggests that lung function in the distal airways deterriorates with time in these patients despite regular medication use which contributes to the importance of the small (distal airways) in children with asthma (Torrego Fernandez and Munoz Cano 2011). The peripheral (distal) airways are the predominant site of airway inflammation (Corren 2008) and may very well be a predominant site of airflow obstruction in asthmatics (Hamid and Tulic 2007). Additionally, there is evidence that the obstruction in the small (distal) airways may be involved in the pathophysiology and resistance to treatment with ICS in children, especially those with increased BMI (Ye et al. 2013) and that the impairment of the small airways disease may be present despite rare and mild asthma symptoms and normal spirometry in children (Singer et al. 2014). Also, cluster 2 had the highest levels of serum hsCRP (see Table 26), although this association was not statistically significant, but was a major discriminant in the decision tree algorythm (see Figure 16). This may indicate that these patients have higher levels of systemic inflammation and hence, poorer disease and treatment outcomes. Several studies have demonstrated that increased levels of hsCRP are associated with more severe asthma outcomes and may be an interesting novel biomarker in predicting asthma control and treatment response (Kilic et al. 2012, Monadi et al. 2016). Moreover, cluster 2 patients had a higher proportion of overweight and obese patients (36.78 %) compared to other clusters, which is in concordance with other findings indicating that obesity in asthma is associated with poorer disease outcomes and poorer response to treatment with ICS according to changes in lung function parametres (Monahan et al. 2014) and moreover, that obese asthma could be a distinct and more severe asthma phenotype (Dixon et al. 2010, Teodorescu et al. 2013, Samson and Garber 2014). Additionally, since these patients had higher levels of serum hsCRP, this suggests that obesity in asthma promotes systemic inflammation which may reflect in the airways as well, which is in concordance with previous findings (Monahan et al. 2014). These patients also have higher levels of eosinophilic inflammation (eosinophil count) than clusters 3 and 4 but also higher neutrophil counts than clusters 1 and 3- although this was not statistically significant but major discriminants in the decision tree algorythm (see Table 26 and Figure 16), which supports recent findings that obesity in mice is associated with a mixed granulocytic inflammation and may contribute to a refractory therapeutic response as well as exacerbation of disease severity (Silva et al. 2017).

Cluster 1 was also different from cluster 2 in exhibiting a dominant genotype (AA) and allelic (A allele) affect for the rs37973 polymorphism in the *GLCCI1* gene, which has been

associated with positive treatment outcomes according to % change in lung function in patients using ICS (Tantisira et al. 2011) and according to similar findings in this study.

Cluster 3 was the largest of the four clusters and had overall good response to treatment according to all analyzed parameters (% change in FEV_1, MEF_{50}, FENO and asthma control). These patients were somewhat younger than patients in clusters 1 and 2 (mean age just under 10 years) but still had a relatively later onset of disease (at about 6 years of age). Interestingly, these patients had the lowest values of FEV_1 and MEF_{50} at baseline measured (see Tables 25 and 26), which indicates that they had the highest improvement in lung function in response to treatment. These patients also had a higher frequency of the A allele for rs37973, which may contribute to better responsiveness to the ICS component of their treatment regimes (Tantisira et al 2011) and is also in concordance with the findings of this study. Cluster 3 was also characterized by higher serum total IgE levels (as well as cluster 1), but not with significantly higher eosinophil or neutrophil count, which may indicate lower levels of airway inflammation in these patients contributing to positive treatment outcomes. Additionally, these patients also has the highest levels of FENO, although this association was not statistically significant but a major discriminant in the decision tree algorythm (see Table 26 and Figure 16), which may explain their better responsiveness to treatment with the ICS component (Price et al. 2018).

Cluster 4 was the only one characterized by poor long-term control-related response. Additionally, these patients had poor (moderate and bad) levels of response to treatment according to lung function parameters (both FEV_1 and MEF_{50}), but positive treatment outcomes assessed by FENO changes. These patients were the youngest (mean age 9.6 years) but also had later onset of disease (ca. 6 years of age). They had somewhat lower FEV_1 and MEF_{50} measurements at baseline, but still within acceptable physiologic range (see Tables 25 and 26). Cluster 4 patients had a significantly higher proportion of patients with atopic dermatitis (AD), see Table 26, which is more common in severe asthma (Lee and Han 2018). This may be associated with an impairment of the epithelial barrier (skin) causing immune dysregulation, and lead to the atopic march- progression from AD to allergic rhinitis and asthma. Asthma and AD are known to aggravate each other (Celakovská and Bukac 2016). Additionally, cluster 4 patients had a higher proportion of more severe asthma forms (moderate and severe asthma) compared to other clusters, which contributes to their poorer responsiveness to treatment (GINA 2018), see Table 26. Although this association was not statistically significant, but a major discriminant in the decision tree algorythm, cluster 4

patients had the highest neutrophil count, which has been associated with more severe asthma outcomes and, moreover, with non-responsiveness to corticosteroids (Ray and Kolls 2017). Additionally, although this association was also not statistically significant, but a trend may be present, cluster 4 had lower platelet counts compared to other clusters (see Table 26). Platelets may actively be involved in the pathogenesis of allergic asthma via the regulation of Th2 inflammation mediated by active platelet-derived IL-33 protein activation and lower platelet count due to systemic inflammation is more prominent in children (Kowal et al. 2006, Ellaurie and Wang 2004). Platelets may also be involved in more extensive airway remodeling, as well as in the development of steroid-refractory asthma, since ICS do not affect platelet function (Takeda et al. 2017).

Although this association was not statistically significant, clusters 2 and 4 had higher proportions of patients with food allergy, which is also likely why these patients had poorer response patterns since food allergy and asthma are known to aggravate each other and increase the risk for morbidity in each condition. Children with food allergy and asthma are more likely to have near-fatal or fatal allergic reactions to food allergens and are also more likely to have more severe forms of asthma (Wang and Liu 2011).

In the past few years a number of studies have attempted to perform asthma phenotyping by employing novel bioinformatic tools (eg. cluster analysis and various techniques). Most of them have identified age of onset- early onset vs late onset disease presentation, usually in adults (Haldar et al. 2008, Siroux et al. 2011, Wu et al. 2014, Moore et al. 2010, Kim et al. 2013); gender (Moore et al. 2010, Qiu et al. 2018); atopy status (Siroux et al. 2011, Howrylak et al. 2014), obesity (Haldar et al. 2008, Moore et al. 2010) and type of inflammation-eosinophil, neutrophil, mixed type, Th2 high/low (Wu et al. 2014, Loza et al. 2016, Qiu et al. 2018, Su et al. 2018) as main discriminants distinguishing specific clusters (phenotypes)-usually 3 to 5 clusters. Certain studies have focused on disease control and severity (Mäkikyrö et al. 2017), airway obstruction and exacerbations (Howrylak et al. 2014), recurrent chest infections (Belgrave et al. 2017) and type and level of sensitization (Schoos et al. 2017). A literature overview of clustering studies focusing on identifying distinct asthma phenotypes and their main findings as well as limitations is presented in Supplement 5.

Although phenotyping studies such as this one have performed unbiased statistically based analyses on larger cohorts of patients involving a wide range of clinical variables, they have been limited in the terms of clinical characteristics they have used to identify different

phenotypes in asthma and still do not provide much insight into the underlying disease mechanisms (Gauthier et al. 2015, Ray et al 2015). Additionally, different methods employed in these studies have been shown to yield different results in cluster assignments, especially in different populations (Prosperi et al. 2013). Our population, however, was very homogenous (all children, mostly milder disease forms, mostly atopic, ethnically homogenous), which was an advantage in identifying genetic traits associated with treatment response patterns, but a disadvantage in identifying clear disease phenotpyes. From all of this is is evident that further research and further classification involving larger numbers of patients, multi-centric, longitudinal and prospective studies and even more clinically relevant parameters are needed to adequately address the issues of the high levels of inter-patient variability in the level of response to treatment with common asthma medication.

6. CONCLUSIONS AND FUTURE PROSPECTS

The results of this study have confirmed that almost all of the selected genes and polymorphisms are likely candidate genes (genetic variants) in the pharmacogenetics of asthma. Although associations of treatment outcomes assessed by changes in FENO levels and asthma control have not been reported so far for the genetic variants analyzed in this study, all of the polymorphisms with the exception of rs1876828 in the *CRHR1* gene have been associated with either ICS, LTRA or combination treatment regimes, but this is most likely due to the fact that the frequency of the T allele and the TT genotype was very low (N_{TT}= 7 in total, see Figure 13). The rs37973 polymorphism (*GLCCI1*) was significantly associated with positive treatment outcomes in children using inhaled corticosteroids (ICS) alone (the A alelle), while the rs9910408 genetic variant in the *TBX21* gene (the A allele) as well as the rs242941 polymorphism in the *CRHR1* gene (the C allele) were associated with positive treatment outcomes in patients using both ICS alone and combination treatment regimes, which is in concordance with previous studies. This association of rs9910408 and rs242941 with good response to treatment with ICS+LABA/LTRA is most likely related to the ICS component of combination treatment regimes, since no such association with β-agonists or LTRA has been reported previously.

The rs17576 polymorphism (*MMP9*) was associated with positive treatment outcomes in patients using leukotriene receptor antagonists alone (A allele). This is the first such association of rs17576 and treatment success in pediatric patients with asthma (and asthmatics in general) ever reported, but similar effects have been shown in a recent study focusing on other genetic variants in the *MMP9* gene (Dragicevic et al. 2018). However, since the association with the level of response to treatment with LTRA and rs17576 was rather weak and identified only post adjustment for confounders and, moreover, due to the small sample size (N= 38), there is a risk that this effect is random. Despite that, genetic variants in the *MMP9* gene (including the rs17576 polymorphism) may be promising new biomarkers in asthma pharmacogenetics and definitely deserve further research attention.

The rs1042713 polymorphism in the *ADRB2* gene was significantly associated with positive treatment outcomes in children using ICS+LABA/LTRA (A allele, encoding the Arg16 residue). Although this association of FENO- related success of treatment with β-agonists

with rs1042713 has not been reported so far, several studies have demonstrated an association of the A allele with poor response to treatment with SABA and LABA- reduced PEF compared to baseline and higher asthma exacerbation rates (Cho 2010, Basu et al. 2009). This may indicate that our results are random and the sample size is too small (N= 106) or the assessment period is too short (6 months in this study and in Basu et al. 2009), although candidate gene studies are not rare to give contradictory studies. For example, an association of the A allele in rs37973 in the GLCCI1 gene with good response to treatment with ICS has been reported by Tantisira and colleagues (Tantisira et al. 2011), while a recent study involving a Slovenian cohort of patients with asthma showed an association of positive treatment outcomes assessed by % change in FEV_1 with the GG genotype (Rijavec et al. 2018).

In general, the results of this study have replicated genotype and allelic effects reported in other studies (larger and those involving adult and pediatric patients with asthma), with the exception of rs1042713 (*ADRB2*), although the sample size in specific treatment regime subsets (ICS alone, LTRA alone and ICS+LABA/LTRA) may be small (N= 158, N= 38 and N= 106, respectively). Although the assessment period was 2 years in total, the majority of genotype and allele-related effects were associated with shorter periods of time (6 months). This may reflect an actual and relatively immediate biological effect- genes being involved in the inflammatory response and asthma pathogenesis and their role in corticosteroid and β-agonist responsiveness, although there is a risk of the effect being confounded by other unrelated events such as poor response to treatment (impaired lung function and diminished disease control) due to a current respiratory infection or recent exposure to an allergen or elevated FENO levels due to poor adherence to treatment (Price et al. 2013).

Additionally, the results of this study have failed to replicate the association of treatment outcomes assessed by changes in lung function (except for rs9910408 and ICS use) as most studies focused on % change in FEV_1 as a primary endpoint. Instead, the majority of the associations in this study with specific genetic variants are related to treatment success according to changes in disease control and FENO levels. These results suggest that lung function may not be a preferred tool to be used to guide treatment in pediatric (as well as adult) patients with asthma, which is in absolute concordance with the latest GINA guidelines and the control-based management approach which focuses on achieving adequate control of symptoms and maintaining normal activity levels, as well as minimizing future risks of exacerbations (GINA 2018), see Supplement 2. Additionally, no previous studies have

investigated FENO as an outcome in pharmacogenetic research, although evidence suggests that FENO can also be used as a predictor of steroid responsiveness even more consistently than other parameters (Smith et al. 2005). FENO is a good biomarker of Th2- related allergic inflammatory response, as IL-13 promotes NO-synthase activity and NO production (Bagnasco et al. 2016). Hence, FENO may reflect corticosteroid effectiveness in reducing inflammation (which is the primary effect of ICS use) much better than other parameters such as lung function that encompasses a number of pathophysiologic mechanisms as well as structural changes in the airways. Moreover, the latest GINA guidelines suggest that treatment guided by FENO in children and young adults, is associated with a significant reduction in exacerbation rates and that it may be a good complementary approach compatible with control-based asthma management (GINA 2018).

In conclusion, the results of this study indicate that all of the selected genes (*GLCCI1, TBX21, CRHR1, ADRB2* and *MMP9*) are most likely candidate genetic variants in pharmacogenetics of asthma and may significantly contribute to the inter-individual variability in the level of response to all classes of common asthma treatment. The results of this study also indicate that other biomarkers and approaches (other than lung function parameters and airway hyperresponsiveness) may be more suitable in assessing treatment outcomes in pediatric patients and pharmacogenetic research, such as FENO and asthma control (symptom control and risk for exacerbations as well as exacerbation severity). Surely additional studies involving a larger number of patients, several (shorter and longer) treatment success assessment periods as well as functional studies to underpin possible pathophysiologic mechanisms underlying this treatment outcome heterogeneity are needed to confirm any of these results.

We have also successfully identified 4 distinct response clusters varying in the level of response to treatment according to the afore mentioned parameters and duration of treatment (short-term vs long-term). Clusters 1 and 3 seemed to have a more positive pattern of treatment outcomes and were characterized by more prominent atopic markers (higher sensitization levels and strong sensitization to house dust mite for cluster 1, higher total IgE levels, higher eosinophilia for cluster 1, higher FENO, low neutrophilia) and a predominant allelic (A allele) effect for rs37973 in the *GLCCI1* gene. Conversly, they had a relatively later onset of disease (6 years of age or more). Clusters 2 and 4 had poorer treatment success patterns and were characterized by higher levels of airway and systemic inflammation (higher hsCRP for cluster 4), multiple comorbidities (food allergy, atopic dermatitis for cluster 4 and

obesity for cluster 2, which is in concordance with the obese asthma phenotype), but varied in the type of inflammation (predominantly neutrophilic for cluster 4 and mixed-type for cluster 2). Cluster 2 was the only one with relatively earlier onset of asthma (5 years of age), while cluster 4 had more severe forms of asthma and lower platelet counts associated with more extensive airway remodeling and poorer treatment effectiveness.

The results of this study emphasize the issues in asthma treatment and management due to the overgeneralized approach to the disease, not taking into account specific disease phenotypes. This is the first study to identify treatment resposne patterns in children with asthma and plausible pathophysiologic and molecular mechanisms underlying such treatment outcomes. From the results of this study it is more than evident that in order to optimize medication selection and maximize treatment response in patients with asthma, especially children (since prevention and acting in a timely manner early in the disease presentation may prevent asthma progression later in life), further characterization of specific disease phenotypes is more than necessary. This may aid in developing complex prediction models which will stratify patients according to their specific disease traits and risk for treatment failure which will help in revisiting and recategorizing current treatment selection guidelines, aiming at establishing novel and better therapeutic options but surely further research and clinically based evidence is needed. A better understanding of individual variations in response to treatment, especially in children, will help clinicians in optimising asthma treatment and enable each patient to have full quality of life with minimal or no impediment from their asthma.

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8. CURRICULUM VITAE

Ivana Banić was born on 15th March 1987 in Banja Luka, BIH. She obtained her BSc degree in 2008 and MSc degree in 2010 at the Faculty of Science, University of Zagreb.

She worked as a Junior CRA at NovoNordisk Croatia Ltd., Zagreb, as a researcher/marketing associate at the Glycobiology Laboratory at Genos Ltd, Zagreb where she studied the changes in IgG glycosilation in the pathogenesis of allergy. She has been working at the Srebrnjak Children`s Hospital, Zagreb since 2012.

Her research focus is in molecular and pathophysiological mechanisms of asthma, allergy and other chronic conditions in children, such as primary immunodeficiencies (PID). She is involved in several research projects studying differential methylation in allergy, pharmacogenetics of asthma, small non-coding RNAs in severe asthma, changes in microbiome in specific asthma phenotypes, genetics and immunophenotyping in PID etc. She has been involved in several EU and national research projects. She has co-authored 3 scientific publications and 20+ conference abstracts. She did a part of her doctoral research at the University Clinic for Respiratory Diseases and Allergy Golnik, Slovenia and performed her ERS STRTF research project in severe asthma at the University of Southampton and University Hospital of Southampton, UK.

She has received several honors/prizes: student of the generation at both elementary and high school, fellowship of the Croatian MSE for exceptional students, ranked in the top 5% of students graduated from the study of Molecular biology at the Faculty of Science in 2010, travel grant and best presentation award at the EAACI PAAM Congress in Berlin in 2015, best poster award at the CYTO Congress in Glasgow in 2015, recipient of the ERS Short-term research and training fellowship in 2016.

9. SUPPLEMENTS

9.1. Supplement 1. Asthma Control Test for children between the age of 4 and 11 years

Childhood Asthma Control Test for children 4 to 11 years old.

Know the score.

This test will provide a score that may help your doctor determine if your child's asthma treatment plan is working or if it might be time for a change.

How to take the Childhood Asthma Control Test

Step 1 Let your child respond to **the first four questions (1 to 4)**. If your child needs help reading or understanding the question, you may help, but let your child select the response. Complete the remaining **three questions (5 to 7)** on your own and without letting your child's response influence your answers. There are no right or wrong answers.

Step 2 Write the number of each answer in the score box provided.

Step 3 Add up each score box for the total.

Step 4 Take the test to the doctor to talk about your child's total score.

19 or less If your child's score is 19 or less, it may be a sign that your child's asthma is not controlled as well as it could be. No matter what the score, bring this test to your doctor to talk about your child's results.

Have your child complete these questions.

1. How is your asthma today?



SCORE ☐

2. How much of a problem is your asthma when you run, exercise or play sports?



☐

3. Do you cough because of your asthma?



☐

4. Do you wake up during the night because of your asthma?



☐

Please complete the following questions on your own.

5. During the last 4 weeks, on average, how many days per month did your child have any daytime asthma symptoms?

5	4	3	2	1	0
Not at all	1-3 days/mo	4-10 days/mo	11-18 days/mo	19-24 days/mo	Everyday

☐

6. During the last 4 weeks, on average, how many days per month did your child wheeze during the day because of asthma?

5	4	3	2	1	0
Not at all	1-3 days/mo	4-10 days/mo	11-18 days/mo	19-24 days/mo	Everyday

☐

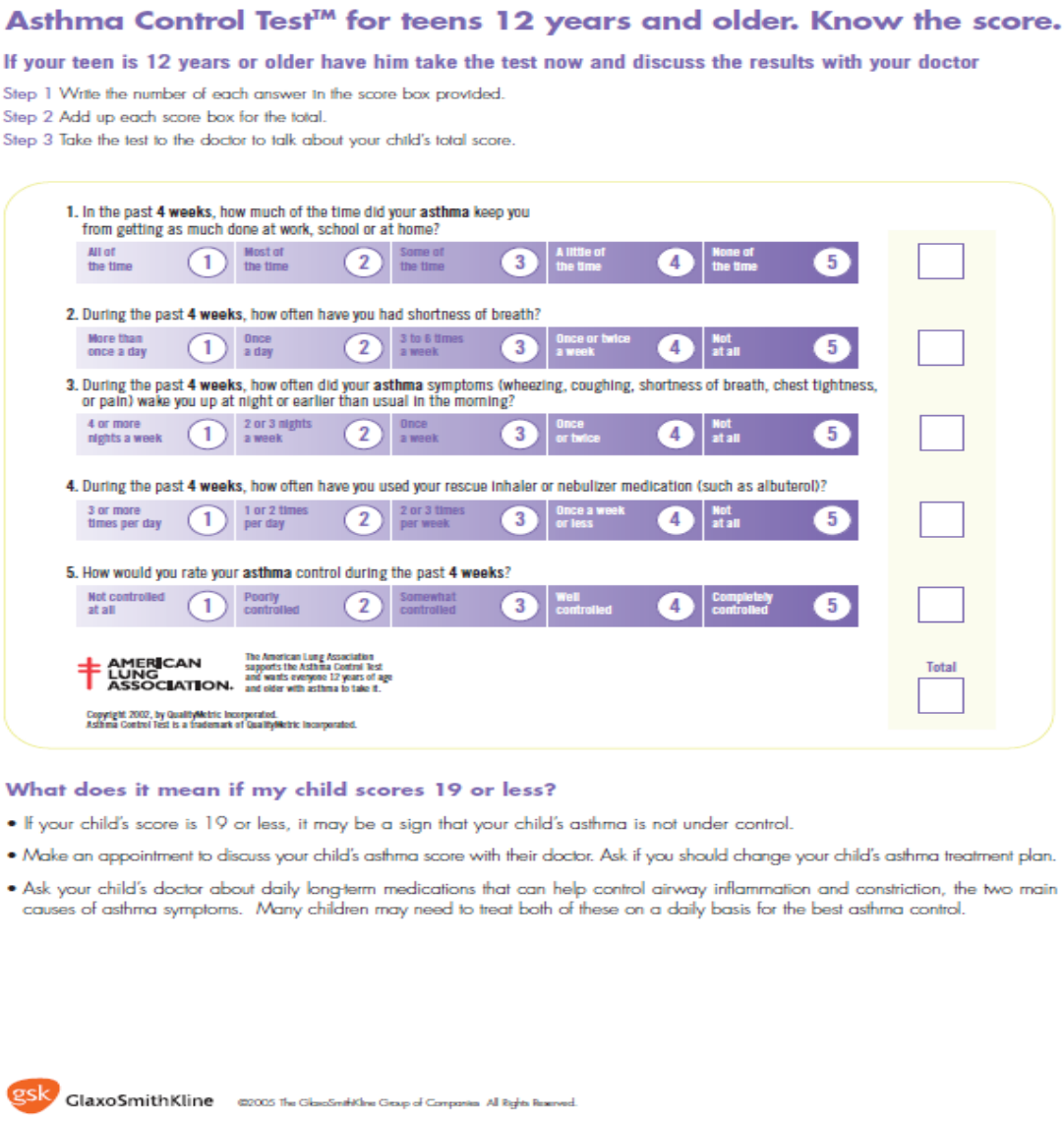
7. During the last 4 weeks, on average, how many days per month did your child wake up during the night because of asthma?

5	4	3	2	1	0
Not at all	1-3 days/mo	4-10 days/mo	11-18 days/mo	19-24 days/mo	Everyday

☐

TOTAL ☐

Please turn this page over to see what your child's total score means.



Asthma Control Test™ for teens 12 years and older. Know the score.

If your teen is 12 years or older have him take the test now and discuss the results with your doctor

Step 1 Write the number of each answer in the score box provided.
Step 2 Add up each score box for the total.
Step 3 Take the test to the doctor to talk about your child's total score.

1. In the past **4 weeks**, how much of the time did your **asthma** keep you from getting as much done at work, school or at home?

All of the time	Most of the time	Some of the time	A little of the time	None of the time
1	2	3	4	5

2. During the past **4 weeks**, how often have you had shortness of breath?

More than once a day	Once a day	3 to 6 times a week	Once or twice a week	Not at all
1	2	3	4	5

3. During the past **4 weeks**, how often did your **asthma** symptoms (wheezing, coughing, shortness of breath, chest tightness, or pain) wake you up at night or earlier than usual in the morning?

4 or more nights a week	2 or 3 nights a week	Once a week	Once or twice	Not at all
1	2	3	4	5

4. During the past **4 weeks**, how often have you used your rescue inhaler or nebulizer medication (such as albuterol)?

3 or more times per day	1 or 2 times per day	2 or 3 times per week	Once a week or less	Not at all
1	2	3	4	5

5. How would you rate your **asthma** control during the past **4 weeks**?

Not controlled at all	Poorly controlled	Somewhat controlled	Well controlled	Completely controlled
1	2	3	4	5

Total

AMERICAN LUNG ASSOCIATION. The American Lung Association supports the Asthma Control Test and wants everyone 12 years of age and older with asthma to take it.

Copyright 2002, by QualityMetric Incorporated.
Asthma Control Test is a trademark of QualityMetric Incorporated.

What does it mean if my child scores 19 or less?

- If your child's score is 19 or less, it may be a sign that your child's asthma is not under control.
- Make an appointment to discuss your child's asthma score with their doctor. Ask if you should change your child's asthma treatment plan.
- Ask your child's doctor about daily long-term medications that can help control airway inflammation and constriction, the two main causes of asthma symptoms. Many children may need to treat both of these on a daily basis for the best asthma control.

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Figure 17. Asthma Control Test (ACT) for children (ages 4 to 11 years).

9.2. Supplement 2. Summary of the current guidelines in the management and treatment of asthma

The long-term goals of asthma management are:

- To achieve good control of symptoms and maintain normal activity levels,
- To minimize future risks of exacerbations, fixed airflow limitation and side-effects.

In control-based asthma management, pharmacological and non-pharmacological treatment is adjusted in a continuous cycle that involves assessment, treatment and review (Figure 5). For many patients, symptom control is a good guide to a reduced risk of exacerbations. However, with add-on asthma therapies (including ICS/LABA) or different treatment regimes (eg. ICS/LABA maintenance and reliever therapy) and in patients with severe asthma or difficult-to-treat or brittle asthma, they may be a discordance in responses for symptom control and exacerbations. In these cases, stepping up ICS doses may raise issues on side-effects of long-term use.

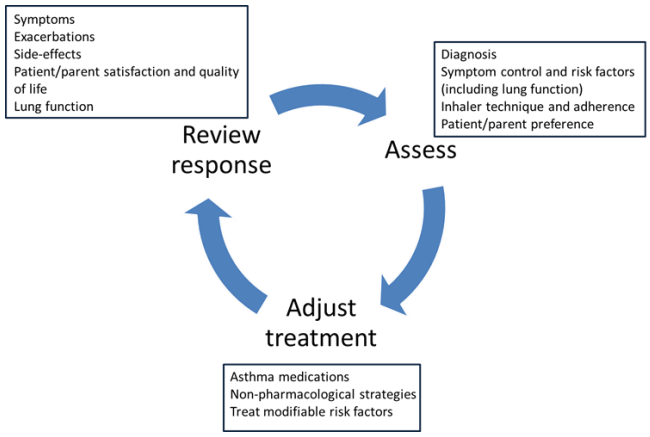


Figure 5. The control-based asthma management cycle and its main components. In children, especially those under the age of 5 years, parents or caregivers have a very important role in asthma management, which is wy their preferences and satisfaction is cruicial in the management process. Modified from (source): GINA 2018, GINA Pediatric 2015.

Some alternative strategies have been evaluated for adjusting asthma treatment, such as:

- Treatment guided by sputum eosinophil count: this approach is associated with a reduced risk of exacerbations and similar levels of symptom control and lung function;
- Treatment guided by fractional concentration of exhaled nitric oxide (FENO): in children anad young adults, this approach is associated with a significant reduction in the number of patients with 1 or more exacerbations and in exacerbation rate, there are no differences in symptom control or ICS medication use in this approach in comparison with others.

For each treatment step, a „preferred“ controller medication is recommended with the optimal benefit to risk ratio for both symptom control and exacerbation risk reduction, based on efficacy studies, safety dana and cost-effectiveness, on a population level. Any patient

characteristics that may predict a clinically important difference in their response to treatment compared to the „generalized model“, should be taken into account.

As there is still no real cure to asthma (due to the overwhelming complexity of this disease), today, common asthma treatment is actually symptomatic treatment, with short-term medications that are mostly used to relieve current symptoms (**reliever medication**) and long-term medication is used in case of persistent symptoms to control the underlying inflammation and prevent exacerbations (**controller medication**).

In general, the goal of asthma management is establishing good disease control with a tendency to stepping down treatment regimes if adequate control is achieved.

At present, Step 1 is no controller medication and as-needed SABA (reliever medication). However, there is more and more evidence that chronic inflammation is present even in patients infrequetn and recent-onset symptoms and that regulat daily low dose ICS is highly effective in reducing symptoms, risk of exacerbations, hospitalization and asthma-related death.

In case of persistent symptoms and/or exacerbations despite low dose ICS, consider stepping up but first check for common problems (inhaler technique, adherence, comorbidity etc.).

Consider stepping down once good asthma control has been achieved and maintained for about 3 months, to find the patient`s lowest treatment that controls both symptoms and exacerbations (Figure 6).

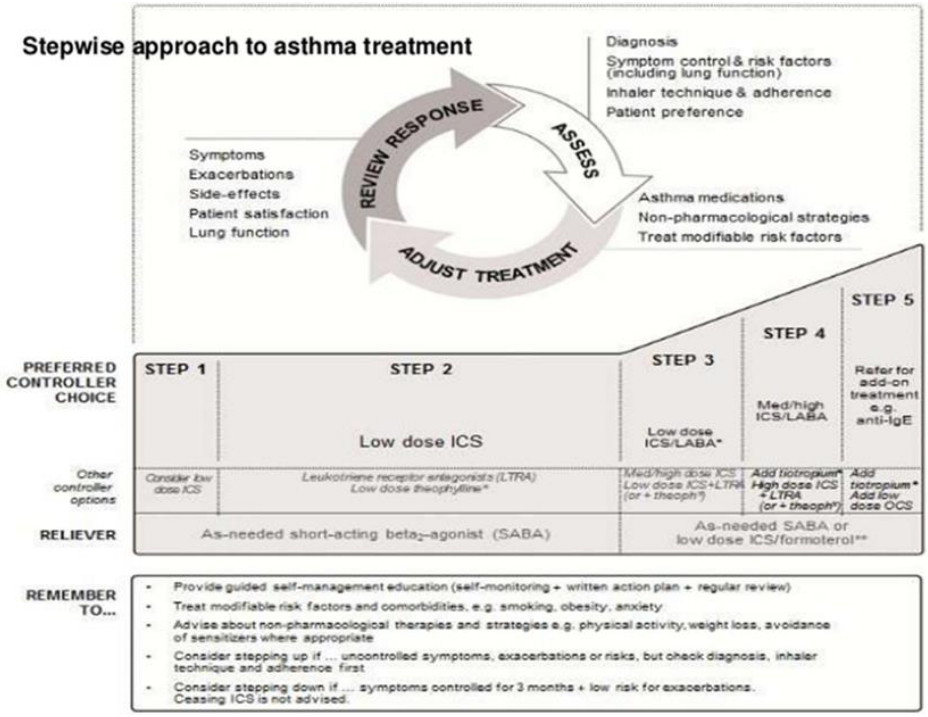


Figure 6. A schematic representation of the stepwise approach in asthma management. *For children aged 4 to 11 years, the use of theophyline is not recommended as controller treatment and the preferred option in Step 3 is medium dose ICS, whereas for adults and adolescents the preferred option in step 3 is ICS/LABA. In step 5, some patients may benefit from low dose OCS, but long-term systemic side-effects may occur. **For children under the age of 5 years, step 1 treatment is as needed SABA, step 2 is daily or intermittent low dose ICS or LTRA, step 3 is „double low dose ICS" and add-on option is LTRA, while step 4 is increasing the dose of ICS and add-on regular LTRA, with further expert advice, investigation and reconsidering diagnosis highly recommended.** Source: GINA 2018, GINA Pediatric 2015.

Recommended options for **INITIAL controller treatment** in adults and adolescents are presented in Table 27.

Table 27. Recommended options for initial controlled treamtent according to presenting symptoms, with levels of evidence.

Presenting symptoms	**Preferred initial controller**
Asthma symptoms or need for bronchodilatator (SABA) < 2 times a month; **no nighttime waking during the past month and no risk for exacerbations** (including no exacerbations in the last year)	No controller (Evidence D)
Infrequent symptoms, but the patient has one or more risk factors for exacerbations- low lung function, history of exacerbations requiring OCS (oral corticosteroids) in the last year, or lifetime history of	Low dose ICS (Evidence D)

Table 27. continued

hospitalization in intensive care for asthma	
Symptoms or need for SABA ≥ 2 times a month and ≤ 2 times a week or **nighttime awakening due to asthma ≥ 1 time a month**	Low dose ICS (Evidence B)
Symptoms or need for SABA > 2 times a week	Low dose ICS (Evidence A); other (but less effective) options include LTRA and theophyline
Troublesome symptoms most days or nighttime awakenings ≥ 1 time a week, especially if risk factors for exacerbations exist	Medium/high dose ICS (Evidence A) or low dose ICS/LABA (Evidence A)
Initial asthma presentation with severly uncontrolled asthma or with an acute exacerbation	Short course of oral corticosteroids (OCS) AND regular controller medication (high dose ICS- Evidence A or moderate dose iCS/LABA- Evidence D)
Review patient`s response after 2-3 months, or earlier depending on clinical urgency Step down treatment once good control has been achieved and maintained for 3 months	

The stepwise approach to control symptoms and minimize future risk for exacerbations is presented in Table 28.

Table 28. Stepwise approach to asthma management with levels of evidence.

	Medication	**Symptoms/exacerbations**
Step 1	As-needed reliever inhaler (SABA)	Occasional daytime symptoms (< 2 times a month) of short duration (few hours), NO nighttime awakening, normal lung function (≥ 80% of predicted or personal best)
	Other options- low dose ICS (recommended- Evidence B) or inhaled anticholinergics, oral SABA or short-acting theophyline- only in adults, slower onset of action and higher risk for side-effects (Evidence A)	Presence of any risk for exacerbation, such as FEV1 < 80% predicted or personal best, a history of exacerbation in the past 12 months (Evidence B)
Step 2	Low dose controller plus as-needed reliever (preferred option- low dose ICS plus as-needed SABA- Evidence A)	Step up if symptoms are not controlled well, if there is a history of exacerbations or risk for exacerbation (including lung function)
	Other options- LTRA (less effective than ICS), appropriate for patients with AR (Evidence B),for adults and adolescents, low dose ICS/LABA may be more effective in reducing symptoms and improving lung function, but does not further reduce risk for exacerbation compared to ICS alone (Evidence A)	
Step	One or two controller medications plus as-needed reliever	

Table 28. continued

3	(preferred option for adults and adolescents- low dose ICS/LABA plus as-needed SABA or low dose ICS/LABA as both maintenance and reliever; preferred option for children 6-11 yrs is moderate dose ICS plus as-needed SABA; for children ≤5 yrs preferred option is double low dose ICS and consider LTRA as add-on)- Evidence A	
	Other options- medium dose ICS, low dose ICS plus LTRA or sustained-release theophyline (less effective)- Evidence A/B	
Step 4	Two or more controllers plus as-needed reliever- preferred option for adults and adolescnets is low dose ICS/LABA as both maintenance and reliever or medium ICS/LABA plus asneeded SABA; for children 6-11 yrs if moderate dose ICS plus as-needed SABA does not achieve adequate control, refer to expert advice; for children ≤5 yrs, increase iCS dose and add-on LTRA- Evidence A	
	Other options- tiotropium or theophyline or high dose ICS/LABA-only in adults and in case good control is not achieved with medium dose ICS/LABA plus a third controller (LTRA, theophyline) on a trial basis- Evidence A/B	
Step 5	High level care and add-on treatment, such as tiotropium, anti-IgE (omalizumab), anti-IL5 (reslizumab), sputum-guided treatment approach, low dose oral corticosteroids (OCS)	Refer to expert advice

Categories of ICS treatment according to ICS dose are presented in Table 29.

Table 29. Low, medium and high doses of inhaled corticosteroids.

Adults and adolescents (12 yrs and older)			
Drug	**Low daily dose (mcg)**	**Medium daily dose (mcg)**	**High daily dose (mcg)**
Budesonide (dry powder inhaler, DPI)	200-400	>400-800	>800
Ciclesonide (hydrofluoroalkane propellant, HFA)	80-160	>160-320	>320
Fluticasone propionate	100-250	>250-500	>500

Table 29. continued

(DPI)			
Children 6-11 yrs			
Drug	**Low daily dose (mcg)**	**Medium daily dose (mcg)**	**High daily dose (mcg)**
Budesonide (dry powder inhaler, DPI)	100-200	>200-400	>400
Ciclesonide (hydrofluoroalkane propellant, HFA)	80	>80-160	>160
Fluticasone propionate (DPI)	100-200	>200-400	>400

9.3. Supplement 3. List of all inahled, food and other allergens used in standard SPT in participants.

Table 30. List of all inahled, food and other allergens used in standard SPT in participants.

Type of allergen	**Allergen species**	**Binomial nomenclature**
House dust	House dust mite	*Dermatophagoides farinae*
		Dermatophagoides pteronyssinus
	House dust mix- mite, cockroach and animal dander	
Animal dander	Cat dander	*Felis domesticus*
	Dog dander	*Canis familiaris*
	Rabbit epithelium	*Oryctolagus cuniculus*
Grass pollen	Cocksfoot	*Dactylis glomerata*
	Timothy grass	*Phleum pratense*
	Cultivated rye	*Secale cereale*
	5 grasses mix- cocksfoot, sweet vernal-grass, rye-grass, meadow grass, and timothy	*Dactylis glomerata, Anthoxanthum odoratu*m, *Lolium perenne, Poa pratensis* and *Phleum pratense*
Weed pollen	Common ragweed	*Ambrosia elatior*
	Mugwort	*Artemisia vulgaris*
Tree pollen	Common silver birch	*Betula verrucosa*
	Hazel	*Corylus avellana*
	Olive	*Olea europaea*
	Pine	*Pinus radiata*
	Trees mix- maple, horse-chestnut,	*Acer pseudoplatanus, Aesculus*

Table 30. continued

	plane tree and lime tree	*hippocastanum, Platanus acerifolia* and *Tilia x europaea*
Shrub pollen	Mimosa	*Mimosa pudica*
Molds	Alternaria	*Alternaria alternata*
	Cladosporium	*Cladosporium herbarum* or *spp.*
Food allergens	Egg white	*Gallus spp.*
	Egg yolk	*Gallus spp.*
	Cow`s milk	*Boss spp.*
	Wheat	*Triticum aestivum*
	Corn/maize	*Zea mays*
	Soy	*Glycine max (Soja hispida)*
	Rye	*Secale cereale*
	Peanut	*Arachis hypogaea*
	Sesame	*Sesamum indicum*
	Tuna (yellowfin)	*Thunnus albacares*
	Hake	*Merluccius merluccius*
	Trout	*Oncorhynchus mykiss*
	Kiwi	*Actinidia deliciosa*
	Hazelnut	*Corylus avellana*
	Walnut	*Juglans spp.*
	Almond	*Amygdalus communis*
	Rice	*Oryza sativa*
	Strawberry	*Fragaria vesca*
	Apple	*Malus x domestica*
	Orange	*Citrus sinensis*
	Peach	*Prunus persica*
	Potato	*Solanum tuberosum*
	Tomato	*Lycopersicon esculatum*
	Parsley	*Petroselinum crispum*
Drugs	Penicilloyl G	
	Amoxicilloyl	
Insect venom	Common wasp (Yellow jacket)	*Vespula spp.*
	European hornet	*Vespa crabro*

9.4. Supplement 4. Definition of response to treatment abbreviations

Resp_FEV_1_diagn to 1st control- response to treatment according to changes in FEV_1 after 6 months (recruitment to 1st control)

Resp_FENO_diagn to 1st control- response to treatment according to changes in FENO after 6 months (recruitment to 1st control)

Resp_CTRL_diagn to 1st control- response to treatment according to changes in asthma control after 6 months (recruitment to 1st control)

Resp_MEF_{50}_diagn to 1st control- response to treatment according to changes in MEF_{50} after 6 months (recruitment to 1st control)

Resp_FEV_1_1st to 2nd control- response to treatment according to changes in FEV_1 after 6 months (1st to 2nd control)

Resp_FENO_1st to 2nd control- response to treatment according to changes in FENO after 6 months (1st to 2nd control)

Resp_CTRL_ 1st to 2nd control- response to treatment according to changes in asthma control after 6 months (1st to 2nd control)

Resp_MEF_{50}_1st to 2nd control- response to treatment according to changes in MEF_{50} after 6 months (1st to 2nd control)

Resp_FEV_1_2nd to 3rd control- response to treatment according to changes in FEV_1 after 6 months (2nd to 3rd control)

Resp_FENO_2nd to 3rd control- response to treatment according to changes in FENO after 6 months (2nd to 3rd control)

Resp_CTRL_ 2nd to 3rd control- response to treatment according to changes in asthma control after 6 months (2nd to 3rd control)

Resp_MEF_{50}_2nd to 3rd control- response to treatment according to changes in MEF_{50} after 6 months (2nd to 3rd control)

Resp_FEV_1_3rd to 4th control- response to treatment according to changes in FEV_1 after 6 months (3rd to 4th control)

Resp_FENO_3rd to 4th control- response to treatment according to changes in FENO after 6 months (3rd to 4th control)

Resp_CTRL_ 3rd to 4th control- response to treatment according to changes in asthma control after 6 months (3rd to 4th control)

Resp_MEF_{50}_3rd to 4th control- response to treatment according to changes in MEF_{50} after 6 months (3rd to 4th control)

Resp_FEV_1_diagn to 2nd control- response to treatment according to changes in FEV_1 after 12 months (recruitment to 2nd control)

Resp_FENO_diagn to 2nd control- response to treatment according to changes in FENO after 12 months (recruitment to 2nd control)

Resp_CTRL_diagn to 2nd control- response to treatment according to changes in asthma control after 12 months (recruitment to 2nd control)

Resp_MEF_{50}_diagn to 2nd control- response to treatment according to changes in MEF_{50} after 12 months (recruitment to 2nd control)

Resp_FEV_1_diagn to 3rd control- response to treatment according to changes in FEV_1 after 18 months (recruitment to 3rd control)

Resp_FENO_diagn to 3rd control- response to treatment according to changes in FENO after 18 months (recruitment to 3rd control)

Resp_CTRL_diagn to 3rd control- response to treatment according to changes in asthma control after 18 months (recruitment to 3rd control)

Resp_MEF_{50}_diagn to 3rd control- response to treatment according to changes in MEF_{50} after 18 months (recruitment to 3rd control)

Resp_FEV_1_diagn to 4th control- response to treatment according to changes in FEV_1 after 24 months (recruitment to 4th control)

Resp_FENO_diagn to 4th control- response to treatment according to changes in FENO after 24 months (recruitment to 4th control)

Resp_CTRL_diagn to 4th control- response to treatment according to changes in asthma control after 24 months (recruitment to 4th control)

Resp_MEF$_{50}$_diagn to 4th control- response to treatment according to changes in MEF$_{50}$ after 24 months (recruitment to 4th control)

9.5. Supplement 5. An overview of clustering studies attempting to identify specific asthma phenotypes

Table 31. A summary of clustering studies focusing on asthma phenotyping so far.

Study method	Major findings	Limitations of the study
k-means clustering in 3 independent asthma cohorts: N=184, N=187 and N=68 (Haldar et al. 2008)	Identified 5 distinct asthma clusters: (i) early onset atopic, (ii) obese non-eosinphilic, (iii) benign, (iv) early symptom predominant and (v) inflammation predominant	Did not include physiological measures of airway obstruction (eg. FEV_1) in the analysis
Latent class analysis (LCA) on 2 large study cohorts: N=641 and N=1895 (Siroux et al. 2011)	Identified 4 distinct asthma phenotypes: (i) active treated allergic childhood-onset asthma, (ii) active treated adult-onset asthma, (iii) and (iv) inactive or mild asthma, with differences in atopy status and age of onset	Included more limited clinical information in the analysis than the previously described study
Machine learning and k-means clustering analysis on a large number of clinical variables (N=112) in a large cohort of patients (N=378), including healthy controls (Wu et al. 2014)	Identified 6 distinct asthma clusters: (i) to (ii) primarily healthy and mild asthmatics, (iii) to (vi) patients with severe asthma: (iv) early-onset allergic with low lung function and eosinophilia, (v) later-onset, mostly severe asthma with nasal polyps and eosinophilia, and (vi) persistent inflammation and exacerbations despite high systemic corticosteroid use.	Used a large number of clinical variables (N=112), which may not always be feasible in a clinical setting. Findings need to be replicated and confirmed in larger independent cohorts.
Ward's minimum-variance hierarchical clustering method- bottom-up and Ward`s linkage approach (Moore et al. 2010) in the SARP cohort (severe asthma, mostly adults, N=726)	Identified 5 distinct clusters: (i) early onset atopic asthma with normal lung function treated with two or fewer controller medications and minimal health care utilization (HCU), (ii) early-onset atopic asthma and preserved lung function but increased medication requirements and HCU, (iii) mostly older obese women with late-onset nonatopic asthma, moderate reductions in FEV1, and frequent oral corticosteroid use to manage exacerbations,	Biomarkers such as bronchial challenge test (PC20 metacholine), FeNO, total IgE, blood eosinophils and sputum eosinophils and neutrophils were collected in a smaller subset of participants (100 or less) does not give much insight into the pathophysiological mechanisms of specific phenotypes

Table 31. continued

	(iv) and (v) severe airflow obstruction with bronchodilator responsiveness but differ in to their ability to attain normal lung function, age of asthma onset, atopic status, and use of oral corticosteroids	
Hierarchical cluster analysis by Ward's method, followed by κ-means cluster analysis on 2 independent adult cohorts- COREA, N=724 and SCH, N=1843 (Kim et al. 2013)	Indicated 4 asthma subtypes: (i) smoking asthma, (ii) severe obstructive asthma, (iii) early-onset atopic asthma, (iv) late-onset mild asthma	Appropriateness of the variables used needs to be verified, did not use patterns of airway inflammatory cells as variables for cluster analysis, did not consider quality of life as a variable
Fuzzy partition-around-medoid for initial clustering of the ADEPT and U-BIOPRED data, for validation of identified phenotypes, the GLMnet-classification model of ADEPT-asthma baseline clinical clusters was applied to classify the ADEPT-asthma subjects using data from baseline and 3, 6, and 12 month follow-up visits and baseline data of U-BIOPRED participants (Loza et al. 2016)- both cohorts were adults (N=156, N=82, longitudinal data N=397)	Identified 4 independent clusters: (i) mild, good lung function, early onset, low-inflammatory, predominantly Type-2, phenotype, (ii) moderate, hyper-responsive, eosinophilic phenotype, moderate asthma control, mild airflow obstruction and predominant Type-2 inflammation, (iii) mixed severity, predominantly fixed obstructive, non-eosinophilic and neutrophilic" phenotype, moderate asthma control and low Type-2 inflammation, (iv) severe uncontrolled, severe reversible obstruction, mixed granulocytic phenotype, moderate Type-2 inflammation.	May not represent actual real-life situtation since participants were not randomly recruited from the general asthma population. Did not include smokers and obese/morbidly obese participants.
Hierarchical clustering and PCA in a pediatric cohort- N=613 (Deliu et al. 2018)	Identified 5 clusters: (i) difficult asthma, (ii) early-onset mild atopic, (iii) early-onset mild non-atopic, (iv) late-onset, and (v) exacerbation-prone asthma	Reproducibility and stability of certain models was poor
Ward`s hierarchical clustering on an adults cohort recovering from an exacerbation- N=320 (Qiu et al. 2018)	Identified 4 clusters: (i) predominnatly female with sputum neutrophilia and a small degree of airflow obstruction and early-onset asthma, (ii) predominantly female, nonsmoking, high sputum eosinophilia, poor lung	Reflects differences in recovery from exacerbations, not in pathophysiological or clinical features of the underlying disease. Still, it is very informative when it comes to predicting exacerbations

Table 31. continued

	function and pO_2 in arterial blood on admission, (iii) predominnatly female with sputum neutrophilia and a reduction in FEV1, (iv) exclusively male smoking subjects, high sputum eosinophilia and severe airflow obstruction.	
k-means clustering in a pediatric asthma cohort (Taiwanese Consortium of Childhood Asthma Study- TCCAS), N=351 (Su et al. 2018)	Identified five distinct phenotypes of childhood asthma characterized by either eosinophil-predominant or neutrophil-predominant inflammatory characteristics. The gene expression profile analysis, noted significant differences for neutrophil-predominant asthma. Additionally, in an independent inhaled corticosteroid (ICS) response cohort, neutrophil-predominant asthma may be associated with corticosteroid nonresponsiveness.	Clustering was performed only on 12 objective laboratory tests, possibly omitting relevant clinical data
Unsupervised statistical learning techniques- exploratory factor analysis (EFA) and hierarchical clustering (HC) in a cohort of 383 children with asthma (Prosperi et al. 2013)	Different methods yielded different results in cluster assignments (particularly daa encoding and transformation), slightly favouring EFA	Aggregation bias caused by the discretization of skewed variables, the use of mixed data types, didn`t perform a discrimination analysis of the original variables with respect to each clustering
LCA on a cohort of adult asthmatics- Northern Finnish Asthma Study- NoFAS, N=1995 (Makikyro et al. 2017)	Identified 4 clusters according to severity in women: controlled, mild asthma; partly controlled, moderate asthma; uncontrolled asthma, unknown severity, and uncontrolled, severe asthma, as well as 3 clusters in men: controlled, mild asthma; poorly controlled asthma, unknown severity, and partly controlled, severe asthma.	Used questionnaire data only, no objective measurements
Supervised learning algorithms including feed-forward neural networks (NN), support vector machines (SVM), andrandom forests (RF) on a cohort of pre-school	Identified 3 different classes (Wheeze, Wheeze +, Other), with recurrent chest infections as the strongest feature in the models with dependent outcome	A large proprtion of missing data (25% per patient on average)

Table 31. continued

children with wheezing- PSW cohort, N=150, mean age=33,66 months (Belgrave et al. 2017)		
Linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA) on 3 cohort in the CARE Network trial- Pediatric Asthma Controller Trial (PACT), N=285; Characterizing Response to Leukotriene Receptor Antagonist and Inhaled Corticosteroid (CLIC), N=144; Best ADd-on Therapy Giving Effective Response (BADGER), N=182 replicating SARP clusters refering to treatment response (Chang et al. 2014)	For all 3 cohorts 4 SARP clusters were replicated: (i) late- onset, normal lung, (ii) early- onset, normal lung, (iii) early onset/comorbidity and (iv) early- onset, severe lung.	Smaller number of participants assigned to the early-onset/comorbidity and early-onset/severe-lung clusters; the retrospective nature of the study limited the analyzable characteristics
Spectral clustering in the pediatric Childhood Asthma Management Program (CAMP), N=1041, longitudinal follow-up for 48 months (Howrylak et al. 2014)	Identified 5 reproducible clusters on the basis of 3 groups of features: atopic burden, degree of airway obstruction, and history of exacerbation. Cluster grouping predicted long-term asthma control (measured by the need for oral corticosteroids, OCS) or additional controller medications, as well as longitudinal differences in pulmonary function	Evaluated only children and since pediatric and adult asthma might represent 2 different disease states with different pathogenic mechanisms and natural histories, clusters may not apply to adult asthmatics; didn`t inlcude children with severe asthma; clinical outcomes in the clusters are somewhat limited because of their small sample size and modest differences; retrospective nature of the study

Table 31. continued

Unsupervised cluster analysis for 3-dimensional data (nonnegative sparse parallel factor analysis) in a birth cohort Copenhagen Prospective Studies on Asthma in Childhood 2000 (COPSAC 2000), N=398 (Schoos et al. 2017)	Identified 7 snesitization trajectories: age- and allergen-specific patterns in the COPSAC 2000 birth cohort data: (i) dog/cat/horse, (ii) timothy grass/birch, (iii) molds, (iv) house dust mites, (v) peanut/wheat flour/mugwort, (vi) peanut/soybean, and (vii) egg/milk/wheat flour. Asthma was solely associated with pattern 1. All 7 patterns were verified in an independent birth cohort BAMSE (Scandinavian birth cohort, Children, Allergy, Milieu, Stockholm, Epidemiology, N=3051)	The at-risk nature of the COPSAC 2000 participants (children born to mothers with asthma) because this population can differ from unselected populations with regard to allergen exposures; differences in study design between BAMSE and COPSAC 2000 , including age of assessment of and age of assessment and diagnosis of clinical outcomes