Predicting asthma phenotypes: characterization of IL1RL1 in asthma

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General introduction

Chapter 1
Asthma

Asthma is among the most frequent chronic diseases in children worldwide, with 100,000 children being affected in the Netherlands. There is an increase in asthma prevalence in the Western world, which is accompanied by the observation that more people are concomitantly affected by one or more other allergic disorders such as eczema, rhinitis and food allergy. Asthma is a multifactorial disease caused by a complex interaction between multiple genetic and environmental influences. The disease is characterized by ongoing airway inflammation associated with variable airflow obstruction, bronchial hyperresponsiveness (BHR) and airway remodeling. Asthma symptoms include episodes of wheeze, cough, and shortness of breath and occur by exposure to various triggers, such as allergens, (viral) infections, specific weather conditions or exercise.

Compared to healthy peers, children with asthma experience a lower quality of life, and it is the leading cause of emergency room visits, hospitalizations, school absenteeism, and school underperformance. It also affects their psychological functioning, physical activity and their quality of life of that of their parents. Approximately 250,000 people die prematurely from asthma each year, with asthma mortality rates in children ranging from 0.0-0.7 per 100,000. Moreover, the disease has a considerable economic burden with high medical costs due to emergency room visits/hospitalizations and use of pharmaceutical agents.

Obtaining better insight in factors causing asthma will lead to better prevention strategies and improvement in therapeutic, personalized management, which is greatly warranted.

Diagnosis of asthma

Thirty to 50% of preschool children experience symptoms suggestive of asthma, for instance they report recurrent wheeze or cough. However, only approximately 30% of these children with respiratory symptoms at preschool age will develop asthma at a later age. Due to the non-specific symptoms of asthma, it is hard to diagnose asthma or to determine which child will develop the disease and which child will not. Furthermore, there is no specific diagnostic test in childhood for asthma and lung function tests are often unreliable in preschool children.

Specific phenotypes of asthma

Asthma has a large phenotypic heterogeneity. Different asthma phenotypes can be distinguished based on the presence, timing, and severity of symptoms, such as age of onset and nocturnal symptoms, in combination with the presence of atopy, responsiveness to triggers, and type of airway inflammation.

In the past, multiple approaches have been used to group different asthma phenotypes, in which cluster analyses of mostly adults has led to the identification of 5 specific groups based on age, gender, lung function, the presence of atopy, health care utilization and body mass index. Pediatric asthma differs from adult asthma in multiple ways, therefore it is important to determine specific phenotypes in children and adults separately.
Data driven approaches and longitudinal latent class analyses in children from two different birth cohorts have led to the identification of longitudinal wheezing phenotypes.\textsuperscript{12,13} A study performed in two large birth cohorts identified 5 specific wheezing phenotypes, i.e. 1) never/infrequent wheeze, 2) transient early wheeze, 3) intermediate onset wheeze, 4) late onset wheeze and 5) persistent wheeze.\textsuperscript{13} In one birth cohort, transient wheeze was split into transient early wheeze and prolonged early wheeze. All those phenotypes were differentially associated with asthma, atopy, BHR and lung function at the age of 8 years, with intermediate onset, late onset and persistent wheeze being associated with an increased risk for doctor’s diagnosis of asthma and sensitization against common allergens, both at age 8 (Figure 1, Savenije et al.).\textsuperscript{13}

In asthma, there has traditionally been thought to be a shift, or predilection, toward a Th2-cytokine profile resulting in eosinophilic inflammation characteristic of asthma. Aeroallergens or other environmental insults activate airway epithelial cells that release activators such as IL-25, IL-33 or TSLP, which in turn activate immune cells such as dendritic cells, Th2 cells and Innate type 2 lymphoid cells. The latter two are responsible for the increased production of type 2 cytokines such as IL-4, IL-5 and IL-13, leading to downstream cascade of inflammatory events (Figure 2, Fahy JV).\textsuperscript{14–16} Type 2 cytokines are thought to be responsible for IgE overproduction by B-cells, chemo-attraction of effector cells (mast cells, eosinophils and basophils), development of BHR and remodeling of the airway epithelium, all characteristics of asthma.\textsuperscript{15,16} Type 2 associated asthma starts most frequently during childhood (childhood-onset asthma) and is usually well responsive to corticosteroids.\textsuperscript{16,17} However, nowadays there is increasing evidence that a large proportion of asthma patients has airway inflammation driven by other mechanisms. Cellular research on gene expression in bronchial brushes has shown evidence for distinct Th2 high and Th2 low asthma phenotypes.\textsuperscript{17–19} In line with this, a classification based on eosinophilic and non-eosinophilic asthma phenotypes has also been described.\textsuperscript{9,20,21} Those phenotypes have been found to have different airway histopathology, airway structure, mechanisms of airway dysfunction, time of onset of symptoms and different response to asthma treatment.

Novel phenotypic strategies have attempted to cluster asthma patients based on 1) association with environmental factors (air pollution, cigarette smoke), 2) specific symptoms or clinical characteristics (age of onset, obesity) and 3) the presence of certain biomarkers (eosinophilic, neutrophilic). One example is the definitions proposed by Hekking et al. (Figure 3)\textsuperscript{18}, who suggested 13 distinct clinical phenotypes of asthma.
Identification of specific (biological) phenotypes is helpful in better understanding of disease pathogenesis and susceptibility as well as starting personalized treatment. Moreover, the identification of asthma phenotypes aids the interpretation of asthma associated genes and can lead to more targeted genetic and perhaps subsequent intervention studies.

**Figure 2.** Type 2 immune responses characteristic for asthma. Airway epithelial derived cytokines, such as interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP), induces the expression of OX40 ligand (OX40L) on dendritic cells (DCs). This leads to their mobilization to nearby lymph nodes where they activate naive CD4+ T cells to an IL-4-competent state. These IL-4-competent T cells migrate to B cell zones and differentiate into T follicular helper (TFH) cells and move into the circulation to mature to Th2 cells. IL-4-secreting TFH cells in parafollicular B cell zones mediate IgE class-switching in B cells, whereas TH2 cells that migrate to the airway epithelium and to the subepithelial mucosa secrete IL-5 and IL-13 to mediate inflammatory and remodelling changes in the airway mucosa that predispose an individual to asthma and to asthma exacerbations. Reprinted from Fahy JV, with permission.16
General introduction

Figure 3. Grouping of asthma phenotypes in adulthood, divided by environmental factors, symptoms, clinical characteristics and eosinophilic versus non-eosinophilic airway inflammation. Reprinted from Hekking et al., with permission.18

The (epi)genome

The genome is the complete set of a person’s nucleic acid sequences, encoded as DNA within 22 chromosome pairs and one pair of sex chromosomes. DNA is located in the nucleus of each cell and in small DNA molecules within mitochondria. Human DNA is composed of base pairs that form a double helix structure when joined together. There are four bases; the base adenosine (A) always binds with thymine (T), and the base cytosine (C) always binds with guanine (G). Somatic cells of humans consist of six billion base pairs. Amino acids are formed from translation of three base pairs and they are the cornerstones to build proteins. DNA consists of coding and non-coding DNA, in which only coding DNA, around 1.5% of the entire DNA, is responsible for the production of proteins. Proteins are formed by transcription from DNA to mRNA followed by translation from mRNA to protein. Transcription and translation of a single gene results in one protein or different protein isoforms. The human genome contains approximately 25,000 genes, but several biological processes (such as alternative mRNA splicing) can lead to the formation of many more unique proteins than the number of genes.22,23

The DNA of individuals differs only on 0.1% of this genetic variation, leading to variation in about 12 million base pairs. A variation in one base pair between persons that is prevalent in the population is called a single nucleotide polymorphism (SNP) (Figure 4), whereas rare variants (allele frequency <1%) are called mutations. The frequency at which the second most common allele is present in a population is called the minor allele frequency (MAF).22,24

SNPs in close physical proximity are frequently inherited together which results in a correlation between specific variants. This correlation is called linkage disequilibrium (LD, expressed as r² or D’) when the frequency of association of different SNPs is higher than what would be expected if the SNPs were independently inherited from each other.25
Besides genetics, the study of genes, genetic variation, and heredity, there has become more and more attention to epigenetics, which is the study of heritable changes in gene function that do not involve changes in the DNA sequence.\textsuperscript{23,24} This can involve for example changes that affect gene expression and function. Mechanisms responsible for such changes are DNA methylation, histone modification and the presence of small or long non-coding mRNAs. DNA methylation is the process in which a methyl group (CH\textsubscript{3}) is added to carbon 5 in a cytosine base to create 5-methylcytosine, which affects the regulation of gene expression. Methylation usually occurs at the sequence CG (5'-C-phosphate-G-3') (CpG) and regions with a high frequency of CpG sites (CpG islands) are highly regulatory units.

Genetic association studies are performed to identify SNPs that are associated with diseases or specific traits (Figure 5). Different variants of a SNP called alleles, are being compared between patients (cases) and non-affected individuals (controls) (Figure 6). Allelic variation can regulate levels of mRNA expression (called expression Quantitative Trait Locus, eQTL), DNA methylation at CpG sites (methylation Quantitative Trait Locus, meQTLs), protein levels (protein Quantitative Trait Locus, pQTLs) or protein function.\textsuperscript{24}
**General introduction**

Figure 5. Flow chart of genetic association studies that can be performed to identify single nucleotide polymorphisms (SNPs) that are associated with methylation (such as with CpG sites, meQTL), expression (such as with mRNA, eQTL), or protein (such as with receptors, pQTL). eQTM, expression quantitative trait methylations; pQTM, protein quantitative trait methylations.

SNPs in genes and CpG sites on genes associated with the disease can be discovered by performing genome wide association studies (GWAS) or epigenome wide association studies (EWAS).

Thus, by combining the results of these association studies, more knowledge can be gained on how all processes influence each other (e.g. expression and protein) and the biological pathways underlining specific disease associations. In addition, these studies allow us to assess effects on asthma treatment and asthma phenotypes.

Figure 6. The concept of genetic association testing in which the differences in allele frequency between cases and controls is being tested. Significant findings are identified as risk/protective alleles for the trait being tested.

**(Epi)genetics of asthma**

Genetic family based and twin studies have shown that the heritability of asthma is around 60%.\(^\text{26}\) Identification of the genetic factors that are responsible for asthma is difficult, since the disease is highly polygenic; disparate genes are leading to the same disease in different subjects. Publication of the HapMAP project\(^\text{27}\) and the 1000 genomes project\(^\text{28}\) led to a detailed catalogue of human genetic variation, with the presence of sequence variants of more than 2000 participants from a number of different ethnicities. With this data it has become more accessible with increase in power to perform large hypothesis free genome wide association studies (GWAS). Over more than 90 GWAS have been performed in asthma, with the identification of more than 850 associated variants in multiple susceptibility genes.\(^\text{29}\) Recently, two consortia performed large meta-analyses of published GWAS on asthma\(^\text{30}\) and allergic diseases (either asthma, hay fever or eczema)\(^\text{31}\), which led to doubling of the number of genetic risk factors that are associated with asthma. The TAGC consortium meta-analysis performed in 142,000 subjects from different ethnicities has identified 18 asthma associated loci. Moreover, 136 independent genetic asthma and allergy variants were discovered in the meta-analyses of the SHARE consortium performed in 360,000 subjects.
Besides the identification of genes that are associated with asthma, epigenome wide studies (EWAS) have additionally been performed, showing that epigenetic regulation, such as methylation, has a strong association with asthma development as well. Epigenetics changes with exposures and with age, moreover it can specifically occur in one or a few cell types, therefore it is important to consider methylation stages of different cells at different stages in life.

The discovery of (epi)genetic associations with asthma is promising, but the step from association to the mechanisms leading to the development of the disease is complex. To make it more complex, some genes will be more specifically related to the time of onset of the disease, while others are more associated with severity or response to treatment. In addition, gene-gene, gene-environment and more recently gene-epigenetic interactions have been described.

**IL1RL1, asthma associated gene**

In 2008, Reijmerink et al. from our group were the first to describe the association of SNPs in the interleukin-1 receptor–like 1 (IL1RL1), and IL18R1 gene with asthma and associated phenotypes.40 In 2009, a strong conformation of IL1RL1 as a new asthma gene, was provided by a GWAS on peripheral blood eosinophilia in the Icelandic population, and subsequently association with asthma was found in ten populations.41 A year later this gene was reported to be associated with (childhood onset) asthma in one of the largest consortium based GWAS on asthma.34 This finding has been reproduced by multiple studies, with (SNPs) in IL1RL1 being associated with asthma and other atopic traits.40,43–50 Because of a complex LD structure in the IL1RL1 region it has been difficult to identify the true causal variants related to asthma.42

**IL1RL1** (also called ST2) is part of the Toll-like/IL-1 receptor superfamily with expression on inflammatory cells present in the lung. The gene is located on 2q12, consisting of a distal and proximal promoter and 13 exons. Alternative splicing leads to four different isoforms: IL1RL1-a (soluble ST2, sST2), which can be measured in serum; a transmembrane receptor, IL1RL1-b (S2TL); and two less well-characterized isoforms, isoform 3 and IL1RL1-c (ST2V) (Figure 7, Dijk et al. adjusted).54

**Figure 7.** The interleukin-1 receptor-like 1 (IL1RL1) gene (GRCh37/hg19, chr2:102,927,962–102,968,497) with transcript annotation of IL1RL1-a, the soluble variant (sST2) (ENST00000311734.6), IL1RL1-b, the transmembrane variant (ST2L) (ENST00000233954.5), and two less well known variants, IL1RL1-c (ST2V) (ENST00000427077.1) and isoform 3 (ENST00000404917.6). Adjusted from Dijk et al.54
**IL1RL1** is the receptor for Interleukin-33 (IL-33), a cytokine that is important in the regulation of several allergic disorders. Binding of IL-33 to the receptor complex of IL1RL1-b and IL-1 receptor accessory protein (IL-1RAcP) present on Th2 cells, basophils and mast cells and type 2 innate lymphoid cells leads to an inflammatory signaling cascade with the release of pro-inflammatory Th2 cytokines that facilitate inflammation in the lung. In contrast IL1RL1-a is thought to serve as a decoy receptor, and sequestering IL-33 leading to blockade of its function (Figure 8, Grotenboer et al.).

In this thesis, we hypothesize that SNPs and methylation of **IL1RL1** are associated with a disbalance in **IL1RL1** gene expression, resulting in increased **IL1RL1-b** transcription and upregulation of pro-inflammatory Th2 responses in asthma. In addition, downregulation of **IL1RL1-a** levels may possibly attenuate Th2 inflammation. Finally, in patients with asthma induced by **IL1RL1** dysfunction particularly, the disease might be characterized by specific Th2 inflammatory (bio)markers such as eosinophilic inflammation and an increase in fractional exhaled nitric oxide (FeNO).
Pharmacogenetics
Asthma treatment is based on a stepwise protocol, yet the different phenotypes as mentioned above are currently treated very similar. Most asthma medications (ICS, short- and long-acting b2-agonists, and leukotriene receptor antagonists) are effective in most patients. Notwithstanding this, there is still a considerable proportion of asthma patients that responds less to these treatments or exhibits side-effects.\textsuperscript{58} It is thought that this heterogeneous response to medication might be genetically determined. Pharmacogenetics is the study of how genetic variations influence drug response, with recent candidate and GWAS studies discovering multiple genes that exert an effect on asthma treatment response.\textsuperscript{59}

Recently, molecular insight into asthma development has led to the identification of new therapeutic regimens focused on for example cytokine inhibition, such as anti-IgE, anti-IL-5 or anti-IL-13, all with the aim to downregulate the inflammatory response.\textsuperscript{60} Eosinophilic, Th2 associated airway inflammation has been associated with responsiveness to ICS in asthma.\textsuperscript{17,61} Since \textit{IL1RL1} is thought to be important in type 2 inflammatory responses, it might be that patients with specific \textit{IL1RL1} risk variants represent responders to ICS therapy. Identification of such genetic variants could lead to better, personalized treatment.

Prediction of asthma
Accurately predicting which child will develop asthma is important for primary prevention and for improvements in asthma diagnosis. Yet it is extremely difficult at childhood to diagnose asthma, this may lead to over-treatment with steroids in children that do effectively not have asthma, but also to a late diagnosis and possibly under-treatment, which is associated with reduced lung growth, a high personal burden due to recurrent symptoms, school absence and consequently a low quality of life.\textsuperscript{62,63} Several prediction models based on personal and environmental factors have been developed to improve the early diagnosis of asthma\textsuperscript{64–66} All these predictions are based on preschool children with respiratory symptoms, a subjective measure. Next to symptoms, asthma is characterized by variable airflow obstruction, an objective measure. We know that the impairment of lung function at one month after birth is a risk factor for asthma at age 8.\textsuperscript{67} Further, approximately 75\% of all children with mild-to-moderate severe asthma have abnormal patterns of lung growth and early decline of lung function.\textsuperscript{68} Up to now there is no risk model that can predict the development of asthma directly after birth when no symptoms have yet occurred. Attempts have been made to develop genetic risks scores for asthma.\textsuperscript{69,70} However, prediction models made thus far have no sufficiently sensitive or specific clinical value. Since the multifactorial origin of asthma, with personal- and environmental factors as well as genetic factors being involved, it might be that better prediction can be achieved by combining all these factors into one predictive score.

Study groups
To identify (epi)genetic associations and link those to phenotypic traits or search for interactions with environmental factors large cohorts and studies are needed. For the purpose of this study we were able to use data from different studies, like birth cohorts, family studies and case-control studies. These studies originated from all over the world and multiple ethnicities were included. Information present in the cohorts included complete phenotypic, perinatal, familial, (epi) genetic and protein data.

For the investigation in heterogeneous diseases such as asthma there is the need to use large study groups. By combining data from multiple cohorts, when for example performing meta-analyses or replication studies, we were able to increase power in our research. Harmonization of definitions, serum measurements or genetic data is important in achieving a well powered and reliable outcome. The included cohorts in this thesis are summarized in Table 1.
In almost all the studies performed for this thesis we used data from the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study. This is a multicenter Dutch prospective birth cohort, which was initiated in 1996. Children were recruited from the north (Groningen and surrounding), middle (Utrecht and surrounding) and west (Rotterdam and surrounding) in the Netherlands. 7862 pregnant women (2779 with allergy and 5083 without allergy) were invited to participate in the study. At the end 3963 live-born children participated in the study (1327 with a mother with allergy were defined as high-risk, and 2726 children with a mother without allergy were defined as low-risk). Questionnaires for parental completion, partly based on the International Study of Asthma and Allergies in Childhood core questionnaires, were sent to the parents during pregnancy, when the infant was 3 months old, yearly from 1 until 8 years, and at ages 11, 14 and 17 years. Subgroups of high-risk children and low-risk children were selected for an extensive medical examination at age 4, 8, 12 and 16 years. Blood or a buccal brush was used to obtain DNA data.

The PIAMA cohort is a great example of how birth cohorts can help in the investigation of risk factors related to disease such as house dust mites for allergy or sugar-containing beverages for asthma. The fact that the cohort has participants from rural and more urban areas provides the ability for example to investigate the effect of air pollution differences on asthma development throughout childhood and adolescence. The identification of the aforementioned longitudinal phenotypes, which was possible because of the intensive follow-up present, is furthermore an example of the power of this study.

The effectiveness of collaboration between cohorts is proven by the studies we performed in cohorts as part of the Mechanisms of the Development of Allergy (MeDALL) project and the Pharmacogenomics in Childhood Asthma (PiCA) consortium. The MeDALL project consists of 14 European birth cohorts (with PIAMA being one of them), including 44,010 participants, which were followed up between pregnancy and age 20 years. With usage of a standardized MeDALL Core Questionnaire and a strictly defined definition for asthma, there is a high improvement in linking epidemiologic, clinical, and functional research among studies, needed to understand the complex mechanisms behind multifactorial diseases.

For the pharmacogenetic study described in this thesis we used data from 5 cohorts of the PiCA consortium, an international collaboration cohort, integrating a total of 21 studies worldwide, with the inclusion of 14,227 children/adolescents from 12 different countries. The consortium contains 619 users of ICS who have data on asthma symptoms, exacerbations and treatment response. This research project provides a unique chance to discover (pharmaco)genetic differences between multiple ethnicities which could lead to novel precision based asthma medicine.
Table 1. Description of the cohorts and data used for this thesis.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Cohort name</th>
<th>Study design</th>
<th>Ethnicity (predominancy)</th>
<th>Data used</th>
<th>N</th>
<th>Chapter in thesis</th>
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<td>Prospective birth cohort</td>
<td>Caucasian (Dutch)</td>
<td>Phenotypic, environmental, perennial, eosinophils, genetic, methylation, protein</td>
<td>2007, 1913, 1677, 1966</td>
<td>Chapters 3, 5, 7 and 9</td>
</tr>
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<td>Children, Allergy, Milieu, Stockholm, an Epidemiological survey</td>
<td>Prospective birth cohort</td>
<td>Caucasian (Swedish)</td>
<td>Phenotypic, environment, perennial, genetic, methylation, protein</td>
<td>385</td>
<td>Chapters 5 and 9</td>
</tr>
<tr>
<td>INMA</td>
<td>Infancy Medio Ambient cohort</td>
<td>Prospective birth cohort</td>
<td>Caucasian (Spanish)</td>
<td>Phenotypic, genetic, methylation, protein</td>
<td>322</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>ALSPAC</td>
<td>AvanLongitudinal Study of Parents and Children</td>
<td>Prospective birth cohort</td>
<td>Caucasian (English)</td>
<td>Phenotypic, genetic</td>
<td>2247, 521</td>
<td>Chapters 3 and 7</td>
</tr>
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<td>Dutch Asthma Genome-wide Association study</td>
<td>Combined trio, case-control family study</td>
<td>Caucasian (Dutch)</td>
<td>Phenotypic, eosinophil, IgE, lung function, genetic, protein</td>
<td>909, 1885</td>
<td>Chapters 4 and 5</td>
</tr>
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<td>CASP</td>
<td>Genetics of Severe Asthma Phenotypes study</td>
<td>Asthma cohort</td>
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<td>1536</td>
<td>Chapter 4</td>
</tr>
<tr>
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<td>Caucasian (English)</td>
<td>Phenotypic, genetic</td>
<td>1025</td>
<td>Chapter 4</td>
</tr>
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<td>Normal Values of Inflammatory Variables from Healthy Subjects study</td>
<td>Multicenter patient lung tissue cohort</td>
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<td>Lung tissue, genetic</td>
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<td>Chapter 4</td>
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<td>NORM</td>
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<td>Prospective observational study</td>
<td>Caucasian (Dutch)</td>
<td>Bronchial biopsy, bronchial brushing, genetic</td>
<td>77</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>PACMAN</td>
<td>The Pharmacogenetics of Asthma Medication in Children, Medication with Anti-inflammatory effects cohort</td>
<td>Asthma children cohort</td>
<td>Caucasian (Dutch)</td>
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<td>820</td>
<td>Chapter 6</td>
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<td>Asthma case-control study</td>
<td>Hispanic (Latino)</td>
<td>Phenotypic, asthma medication use, genetic</td>
<td>876</td>
<td>Chapter 6</td>
</tr>
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<td>Asthma case-control study</td>
<td>African American</td>
<td>Phenotypic, FEV, FeNO, asthma medication use, genetic</td>
<td>325</td>
<td>Chapter 6</td>
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<td>Effectiveness and Safety of Treatment with Asthma Therapy in children study</td>
<td>Asthma children cohort</td>
<td>Caucasian (Slovene)</td>
<td>Phenotypic, asthma medication use, genetic</td>
<td>107</td>
<td>Chapter 6</td>
</tr>
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<td>Effectiveness and Safety of Treatment with Asthma Therapy in children study</td>
<td>Asthma case-control study</td>
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<td>Phenotypic, asthma medication use, genetic</td>
<td>104</td>
<td>Chapter 6</td>
</tr>
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<td>Chapter 6</td>
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*The PIAMA, BAMSE and INMA cohort are part of the Mechanisms of the Development of Allergy (MeDALL) project. #The PACMAN, GALAII, SACE, SLOVENIA and ESTATe cohort are all part of the Pharmacogenomics in Childhood Asthma (PiCA) consortium.

Aims of this thesis

The first aim of this thesis is to improve our understanding of the role of (epi)genetics in specific asthma phenotypes, with a special focus on the IL1RL1 gene. The second aim of this study is to gain more insight on the role of IL1RL1 in the effect of ICS treatment in asthma. And finally, the third aim is to investigate the added value of genetics in asthma prediction.

Scope of the thesis

Chapter 2 gives an overview of the progress in defining early wheezing phenotypes and describes genetic factors associated with the age of onset of asthma. Furthermore a comparison between asthma and atopy associated genes is made.
In Chapter 3 we show the results of a candidate gene study performed in two birth cohorts in whom we investigated the association of the \textit{IL33-IL1RL1} pathway with asthma and longitudinal wheezing phenotypes. The phenotypes used have been identified and studied previously by our group as described above, with the important finding that specific phenotypes show different associations with lung function, sensitization and asthma. In this chapter we also searched for the association of gene-gene interactions within the \textit{IL33-IL1RL1} pathway with asthma and the asthma associated wheezing phenotypes.

With the use of three independent asthma cohorts and re-sequencing data we investigated in Chapter 4 the association of independent \textit{IL1RL1} asthma related genetic variants to specific features of asthma as defined by clinical and immunological measures. Moreover, with the use resequencing data and in vitro studies we tried to identify SNP driven mechanisms that may contribute to the identified genetic association signals in lung and airway structural cells.

Since the (epi)genetic regulation of \textit{IL1RL1} protein expression has not been established we focus in Chapter 5 on the association between \textit{IL1RL1} genetic variants, \textit{IL1RL1} blood DNA methylation and serum IL-1RL1-a protein levels. With this study, performed in one adult cohort and three birth cohorts, we aimed to identify causal pathways in asthma relating to genetic variation in \textit{IL1RL1}, white blood cell \textit{IL1RL1} methylation and \textit{IL1RL1}-a protein expression.

The translation of genetic and functional data related to a disease back to the patients is of great importance. In Chapter 6 we therefore investigated the role of \textit{IL1RL1} genetic variants on exacerbations, asthma control, FeNO levels and FEV1% predicted in multi-ancestry asthma patients during ICS treatment. Furthermore we aimed to identify whether there is a pharmacogenetic effect of \textit{IL1RL1} SNPs on ICS treatment response in asthma patients.

In Chapter 7 we tried to replicate experimental data in animals in human studies. In this chapter we focus on the \textit{transient receptor potential ankyrin-1} (\textit{TRPA1}) gene, which has been thought to play a key role in promoting airway inflammation in asthma and may mediate effects of paracetamol on asthma. We investigated in two large birth cohorts the association between \textit{TRPA1} gene variants with childhood asthma and total IgE concentration. Especially, we searched for interactions between \textit{TRPA1} and prenatal paracetamol exposure on these outcomes.

Asthma prediction might be improved with the identification of novel biomarkers, more specific definitions of asthma subphenotypes and better understanding of the genetic and biological pathways involved in asthma. In Chapter 8 we investigated in preschool wheezers whether serum \textit{IL1RL1}-a levels can be used to predict asthma, with a special focus on asthma with elevated FeNO, as a marker of eosinophilic asthma. We compared this to the commonly used Asthma Prediction Index (API).

In Chapter 9 we aimed to generate a prediction model for asthma in the first 8 years of life based on the combination of family, perinatal, environmental and genetic risk factors. Several prediction models based on personal and environmental factors have been developed to improve early diagnosis of asthma. With this study set out to investigate the added value of genetics at predicting asthma at birth.
References


General introduction
Chapter 2

F. Nicole Dijk, Johan C. de Jongste, Dirkje S. Postma, and Gerard H. Koppelman