Genetics of onset of asthma

Chapter 2

Abstract

Purpose of review
Most asthma starts early in life. Defining phenotypes of asthma at this age is difficult as many preschool children have asthma-like respiratory symptoms. This review discusses progress in defining early wheezing phenotypes and describes genetic factors associated with the age of onset of asthma.

Recent findings
Latent class analyses confirmed transient and persistent wheezing phenotypes, and identified a novel intermediate-onset wheezing phenotype that was strongly associated with atopy and asthma at age 8 years. However, no single cross-sectional or longitudinal definition of respiratory symptoms in childhood strongly predicts asthma later in life. Genome-wide association (GWA) studies have identified a locus on chromosome 17q12–21 (encoding ORMDL3 and GSDMB) as a risk factor for predominantly childhood-onset asthma, but not for atopy, and overall not for adult-onset asthma. Other loci found by GWA studies appear to increase asthma risk both in children and adults. Atopy genes do not explain early-onset asthma.

Summary
Although most asthma starts early in life, no valid test is able to identify asthma at that age period. GWA studies have provided more insight into the unique and common genetic origins of adult-onset and childhood-onset asthma. The 17q12–21 locus is predominantly associated with childhood-onset asthma.

Abbreviations

ALSPAC - Avon Longitudinal Study of Parents and Children
AHR - Airway hyperresponsiveness
AUC - Area under the curve
ETS - Environmental tobacco smoke
ERS - European Respiratory Society
GWA - Genome-wide association
LD - Linkage disequilibrium
LLCA - Longitudinal latent class analysis
OR - Odds ratio
Introduction

Asthma is a common chronic lower respiratory disease that results from the interactions between genes and environmental factors. It affects more than 300 million people worldwide and its prevalence is still increasing globally. Asthma is characterized by chronic airway inflammation associated with variable airflow obstruction and airway hyperresponsiveness (AHR) leading to recurrent episodes of wheeze, cough, and shortness of breath. Different asthma phenotypes can be distinguished based on the presence, timing, and severity of symptoms (e.g., age of onset, nocturnal symptoms), atopy, responsiveness to triggers, and type of airway inflammation eosinophilic or neutrophilic). Recent findings indicate that genetic factors of childhood-onset asthma differ from those of adult-onset asthma. Therefore, this review will focus on recent progress made in defining phenotypes of early-onset asthma, and describe genetic factors associated with the age of onset of asthma.

Defining early-onset asthma

It is problematic to define when asthma starts, because there is no conclusive diagnostic test available at preschool age. Respiratory symptoms suggestive of asthma, such as wheeze, cough, and shortness of breath are highly prevalent in early childhood, yet not specific for asthma, and commonly occur during viral respiratory infections. In clinical practice, a history of recurrent wheezing episodes is frequently considered as the onset of asthma in children, often defined retrospectively and, hence, inaccurately.

Longitudinal studies revealed that wheezing in the first 6 years of life comprise distinct phenotypes, with dissimilar causes and outcomes. A 1995 seminal paper from the Tucson Children's Respiratory Study introduced a longitudinal wheezing classification. The 'early transient wheezing' phenotype included children with onset of wheezing during the first 3 years of life with no symptoms at age 6, the 'persistent wheezing' phenotype included children with wheezing during the first 3 years of life that continued until age 6, and the 'late-onset wheezing' phenotype included children with development of wheezing between 3 and 6 years of age.

In 2004, these wheezing phenotypes were further classified incorporating markers of atopy. 'Transient wheezing' was not associated with allergic sensitization and was associated with a slightly lower lung function, which was already present before any lower respiratory tract illness had occurred, probably caused by relatively narrow airways. 'Nonatopic wheezing' was associated with viral respiratory infections, without allergic sensitization. These children had normal lung function in infancy but slightly lower lung function in later childhood, combined with increased AHR. 'Atopic wheezing' was recognized as the classical asthma phenotype, with normal lung function early in life but impairment later in life. Earlier onset of symptoms was associated with more severe asthma.

Recently, two longitudinal birth cohort studies in the UK and the Netherlands analyzed annual reports of wheezing until age 8 and identified similar wheezing phenotype patterns using the unbiased, longitudinal latent class analyses (LLCAs). A novel wheezing phenotype ‘intermediate-onset wheeze’ was identified, characterized by a low prevalence of wheeze until the age of 1.5 years with a rapidly increasing prevalence of symptoms thereafter. Persistent, late-onset, and intermediate-onset wheezing phenotypes were strongly associated with doctor-diagnosed asthma at age 8 years compared with never, transient early, and prolonged early wheezing phenotypes. Risk factors for persistence of wheeze were atopy, lower lung function, AHR, and maternal asthma and/or allergy.
As longitudinal phenotypes can only be categorized retrospectively, an European Respiratory Society task force proposed a cross-sectional classification of wheezing illness based on triggers: episodic viral wheeze (EVW) and multitrigger wheeze (MTW). It is often thought that MTW may precede asthma, yet literature does not fully support this. Inconsistent clinical and pathological differences between these phenotypes have been reported and significant phenotypic switching between these wheezing phenotypes was observed. Therefore, this classification is of limited value when studying onset of asthma.

When does asthma start?

In most patients, asthma has its origins in early childhood. A recent Danish study of high-risk children showed that children with asthma at the age of 7 years already had increased AHR and lower lung function as neonates. These lower lung function levels progressively declined into childhood. Moreover, characteristic pathological features of asthma in airway wall biopsies, such as eosinophilic inflammation and basement membrane thickening were observed in children with MTW at age 3, preceding symptoms of wheeze in toddlers. Finally, asthma at age 22 years was predicted by late-onset or persistent wheezing at age 6, a decreased lung function, and AHR. There is still much uncertainty about the relationship, similarities, and differences between childhood and adult asthma. Comparing the characteristics of childhood and adult asthma reveals similarities between risk factors, such as environmental tobacco smoke (ETS) exposure, AHR, a family history of asthma, the role of viral infections, allergic rhinitis, atopic status, and eczema, but also differences (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Childhood-onset</th>
<th>Adulthood-onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male predominance</td>
<td>Female predominance</td>
</tr>
<tr>
<td>Symptom pattern</td>
<td>Episodic</td>
<td>Chronic and episodic</td>
</tr>
<tr>
<td>Severity</td>
<td>Early onset in childhood more severe</td>
<td>Accelerated loss of lung function</td>
</tr>
<tr>
<td>Atopy</td>
<td>Prominent role</td>
<td>Both atopic and non-atopic asthma</td>
</tr>
<tr>
<td>Type of inflammation severe asthma patients</td>
<td>Eosinophilic</td>
<td>Neutrophilic and eosinophilic</td>
</tr>
<tr>
<td>Airflow obstruction</td>
<td>During childhood more peripheral airway resistance</td>
<td>Peripheral and central airway obstruction</td>
</tr>
</tbody>
</table>

Table 1. Clinical differences between childhood-onset and adulthood-onset asthma.

Susceptibility to childhood-asthma: answers from genetic studies

Genetic factors were estimated to explain 34% of the variation in age of onset of asthma. Several candidate gene studies have provided evidence for an age-specific genetic effect. Recently, novel genome-wide association (GWA) studies have allowed to disentangle childhood-onset and adult-onset asthma susceptibility in a hypothesis-free manner (Table 2 lists genetic terminology). Thus far, 14 GWA studies have been published revealing 27 loci with genome-wide significant risk variants for the development of asthma. Half of these studies were performed in childhood-onset asthma (Table 3).
The role of the 17q12–21 locus in childhood onset

Asthma In the first GWA study on asthma, the 17q12–21 IKZF3-ZPBP2-GSDMB-ORMDL3 region was identified as an asthma susceptibility locus in childhood-onset asthma in a white population. This observation was extensively replicated\(^{34,40-42,44,47-52}\) and thus far only not confirmed in African–Americans.\(^{53}\) A remarkable finding is that the 17q12–21 SNPs have a particularly strong association with (early) childhood-onset asthma. This was first shown in a French population wherein 17q12–21 risk alleles were strongly associated with asthma when retrospectively defined as starting before the age of 12, but not with asthma that started at a later age. Subsequently, a prospective Danish study confirmed that this ORMDL3/GSDMB locus conferred an increased risk of recurrent wheeze and asthma, but only in case of early-onset disease, that is, before the age of 3 years, but not in later-onset asthma. Furthermore, an association was observed with severe exacerbations until age 6 years and AHR until age 4, but not with atopy.\(^{48}\) Other studies also indicated that the 17q12–21 locus is not an atopy-susceptibility locus.\(^{40,47,49,54,55}\) In addition, a recent study performed in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort revealed a significant association between 17q12–21 SNPs and the persistent-onset and intermediate-onset wheezing phenotypes.\(^{55}\)

A large-scale GWA meta-analysis of the GABRIEL (A Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community) consortium of European ancestry individuals classified asthmatic patients in two age-of-onset groups: before or after 16 years of age. Significant associations of ORMDL3/GSDMB SNPs were restricted to childhood-onset asthma. The SNP with the strongest association had an odds ratio (OR) of 0.64 in the childhood-onset group, compared with an OR of 1.03 in individuals with a later onset (mean difference \(P\) value: \(1.34 \times 10^{-9}\)).\(^{40}\) Two other studies reported a significant association of the 17q12–21 locus with childhood-onset asthma, but not adult-onset asthma\(^{54}\) and with severe childhood asthma.\(^{35}\) Furthermore, the effect of 17q12–21 SNPs on early-onset asthma seems to be enhanced by fetal and infant smoke exposure.\(^{47,57}\) Of interest, carriers of this risk genotype also had a
stronger association between respiratory infections and asthma onset less than 4 years of age if they had early ETS exposure. Moreover, the latter effect was also present in childhood asthma that remits in adulthood.\textsuperscript{58} In contrast, a complete restriction of the 17q12–21 variants to early-onset asthma was refuted in a study of predominantly adult-onset severe asthma (mean age of onset 21 years). Yet a genome-wide significant association with this locus was reported.\textsuperscript{44} Furthermore, a study\textsuperscript{59} performed in US asthma patients reported a similar OR for 17q12–21 risk variants in their early-onset (<4 years) and late onset (≥4 years) asthma group.

The functionality of the genes at the 17q12–21 locus is not well understood. Asthma risk alleles at this locus are strongly correlated over multiple genes and, therefore, it is difficult to discover the causal asthma variant(s). The asthma-associated 17q12–21 alleles were correlated with the transcript levels of predominantly two genes, ORM1-like 3 (Saccharomyces cerevisiae) (ORMDL3) and GSDMB, which indicates that both genes are coregulated.\textsuperscript{33,54,55,60,61} A functional SNP in this region affects the binding of the nuclear CCCTC-binding factor and, thus, alters nucleosome occupancy leading to altered regulation of transcription.\textsuperscript{60} ORMDL3 encodes for a transmembrane protein anchored in the endoplasmic reticulum. Its function might be related to endoplasmatic reticulum-mediated calcium signaling with modulation of an unfolded protein response.\textsuperscript{62,63} ORMDL3 may modify important pathways in the process of T-lymphocyte activation.\textsuperscript{64} A recent study\textsuperscript{65} in mice demonstrated that ORMDL3 is an airway epithelial gene that is activated by Th2 cytokines and allergens. Furthermore, its function has been linked to sphingolipid homeostasis.\textsuperscript{66} GSDMB may play a role in apoptosis and tumorgenesis and is expressed in epithelial cells.\textsuperscript{67}

In conclusion, although different definitions of early-onset and late-onset asthma have been used, it seems that the asthma-associated 17q12–21 polymorphisms are related to a nonatopic, more severe early-onset asthma phenotype, and associations with adult-onset asthma are less prominent.

Table 3. Published GWA studies and meta-analysis of GWA studies for childhood and adult asthma which obtained genetic associations at a genome-wide significant level, either in the original cohort, the replication cohort or in a combined analysis.
Eleven asthma genes were identified in seven GWA studies performed in populations with childhood-onset asthma. The GABRIEL GWA study found, next to the childhood-onset asthma ORMDL3/GSDMB locus, also more prominent effects in childhood-onset asthma for the IL1RL1/IL18R1 and IL33 genes.\(^7\) In contrast, HLA-DQ variants were slightly more strongly associated among the later-onset individuals, but none of these differences was significant. In childhood-onset asthma, an association with cAMP-specific 3',5'-cyclic phosphodiesterase 4D (PDE4D)\(^{34}\) and DENN/MADD Domain containing 1B (DENND1B)\(^{35}\) gene polymorphisms was identified in US white populations. PDE4D was reported to be important in signaling pathways and airway smooth muscle contraction\(^{69}\), whereas DENND1B encodes for a protein that may interact with tumor necrosis factor-a. It is expressed by natural killer and dendritic cells, which have a critical role in the inflammatory pathogenesis of asthma.\(^{35,63}\) A subsequent analysis of DENND1B variants with age of onset of asthma was performed in children of European and African ancestry, all with asthma diagnosed before age 6. This yielded contradictory results, with opposite alleles being associated in children with asthma symptoms at older age, compared with younger age, and also different alleles associated in populations of African ancestry compared with European ancestry. The latter might be due to differences in underlying genetic architecture between populations.\(^{35}\)
In Japanese childhood-onset (<15 years) asthma patients, the genes *Major histocompatibility complex, class II, DP (HLA-DP)* and *Solute Carrier Family 30 member 8 (SLC30A8)* emerged as genome-wide significant variants. 36 The HLA-DP gene product is a cell surface receptor for foreign or self-antigens, with a function in immune-related diseases. The gene seems to have a protective role in Th1-derived diseases such as diabetes type 1 and rheumatoid arthritis, which suggests that it regulates Th1/Th2 balance. 69,70 *SLC30A8* is associated with type 2 diabetes, and its link to asthma may lie in its localization in epithelial cells and epithelial integrity.71

In the Isle of Wight study, SNPs and haplotypes at chromosome 1p33-p32.31 were associated with asthma and asthma severity in children with asthma at age 10 using a pooled GWA study approach. 37 This region contained the *ATP Synthase Mitochondrial F1 Complex Assembly Factor 1 (ATPAF1)*, *C1ORF223*, and *KIAA0494* genes. In replication cohorts, wherein asthma was diagnosed during childhood, significant trends for association were identified in this linkage disequilibrium block in several ethnic groups, except in Hispanic populations. In an Australian study of adults and children, SNPs at the IL-6 receptor gene *IL6R1* and on chromosome 11q13.5 were identified as asthma susceptibility variants in individuals of European ancestry of whom 53.9% had childhood-onset asthma, defined as onset 16 years of age or less (26.1% >16 years, 20% unknown). No significant difference in strength of association of these risk alleles with age of onset was found.

Other asthma GWA studies have identified important asthma susceptibility genes, yet included both adults and children with often an unknown age of asthma onset (Table 3), thus not allowing conclusions as to differential effects according to age of onset of asthma.

In conclusion, it is difficult to put forward a solid opinion about distinct genetic origins of childhood-onset and adult-onset asthma. Most studies made no formal comparison between the two age groups. Furthermore, age of onset is subject to recall bias because it was often defined in a retrospective manner. 72 Until now, only the childhood-onset associated *ORMDL3/GSDMB* region has been replicated when age of onset was taken into account and this has not been observed so strongly for other GWA loci.

### Genome-wide association studies of age of onset asthma and wheezing phenotypes

Until now, only one GWA study reported on the association between asthma and age of onset in childhood as an outcome measurement. This study included 573 US non-Hispanic white children with a mean±SD age of asthma onset of 3.1±2.5 years 38 and replicated in three independent cohorts with European, American, and Latin American children (age of onset 2.5±2.3, 3.1±2.4, and 3.3±2.7 years, respectively). Two SNPs on chromosome 3 and 11 were associated with earlier onset of asthma using a survival analysis. The combined effect of the associated SNPs led to a mean age of onset of 3.4 in children having 0 risk alleles, compared with a mean age of onset of 2.5 years (P<0.0001) in children having at least one risk allele. Both SNPs were not located in any known gene, but one of them was close to the IL-5 receptor a gene (*IL5RA*). This receptor is selectively expressed in the bronchial muscle and shows an eosinophil-independent role in AHR. 38 Recently, a GWA study 39 performed in the ALSPAC cohort investigated whether the combined signal of asthma risk SNPs was predictive of childhood-onset asthma (<8 years). Asthma-associated SNPs from the GABRIEL GWA study (excluding ALSPAC) were used to construct a genetic prediction score. Children were classified into six LLCA-derived wheezing phenotypes 6: persistent, late-onset, intermediate-onset, prolonged early, transient early, and never/infrequent wheezing. Despite weak discrimination (area under the receiver operating characteristic curve scores≤0.60), an association was examined
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between the top 46 ranked SNPs (including mostly 17q12–21 SNPs) and two symptom-based phenotypes, early-onset persistent wheeze and intermediate-onset wheeze. Interestingly, doctor-diagnosed asthma was associated with the lower ranked SNPs, which could indicate that early-onset persistent wheeze and intermediate-onset wheeze are better predicted by the 17q12–21 locus than doctor-diagnosed asthma. However, the clinical relevance of prediction scores in the determination of disease risk is poor.\textsuperscript{39,40,43}

Genome-wide association studies of asthma and atopy: a comparison

Atopy is a strong risk factor for childhood asthma. Therefore, it is of interest to compare atopy genes that regulate total or specific IgE production, and blood eosinophils to asthma susceptibility SNPs (Table 4).\textsuperscript{73-80,81,82} Remarkably, published data suggest that early childhood asthma is not explained by atopic genetic susceptibility, as atopy genes thus far discovered through GWA studies are not detected as asthma susceptibility genes. This observation is paralleled by studies that showed no association between allergen exposure and asthma\textsuperscript{83,84}, and reports that avoidance of allergens did not lead to a reduction in asthma development.\textsuperscript{85} The causal model in which allergic sensitization directly leads to an increased risk of asthma should, therefore, be re-considered.\textsuperscript{86}

Conclusion

Although most asthma starts early in life, there is no valid test to diagnose asthma in the first years of life. GWA studies have provided more insight into the specific and common genetic origins of adult-onset and childhood-onset asthma. The 17q12–21 asthma locus is important for childhood-onset, nonatopic, more severe asthma. Other asthma risk loci do not have a strong relation with age of onset. Finally, atopy risk alleles do not explain genetic susceptibility to asthma. Future genetic studies are needed to systematically compare age of onset of asthma in large groups of patients including interactions with environmental factors, especially in critical periods, and additionally implementing the role of epigenetic mechanism. Studies that assess the effects of asthma risk SNPs on gene expression (expression quantitative trait loci) can guide further functional studies that will provide more insight into asthma pathogenesis.\textsuperscript{87} Finally, in this way, early detection of asthma using genetic markers interacting with personal and environmental factors might become feasible.

Key points

- Novel longitudinal symptom based wheezing phenotypes have been discovered by the use of latent class analyses.
- GWA studies have revealed that 17q12-21 is predominantly a childhood-onset asthma locus, with modified effects through ETS exposure.
- Two loci have been associated with age of onset in childhood asthma, one locus being close to the IL5RA gene.
- Genetic susceptibility to asthma cannot be explained by atopic risk alleles.
- Genetic profiles of childhood and adult asthma, with the combination of environmental risk factors and associated biomarkers may improve the early detection of asthma.
Table 4. Published GWA studies and meta-analysis of GWA studies for atopy and asthma related phenotypes which obtained genetic associations at a genome-wide significant level, either in the original cohort, the replication cohort or in a combined analysis.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Original Population</th>
<th>Replication Population</th>
<th>Age original&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Age start asthma</th>
<th>Age replication</th>
<th>Age start asthma</th>
<th>Gene&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Association Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Germany</td>
<td>Germany</td>
<td>52.3y (25-69)</td>
<td>Not reported</td>
<td>6-74y</td>
<td>Not reported</td>
<td><strong>FCER1A, STAT6, RAD50</strong></td>
<td>Weak association RAD50 and asthma</td>
</tr>
<tr>
<td>YKL 40 level&lt;sup&gt;d&lt;/sup&gt;</td>
<td>European American</td>
<td>European American</td>
<td>33.3y (6-92)</td>
<td>Not reported</td>
<td>4-69y</td>
<td>s6y (COAST), s6y (Chicago), Not reported (Freiberg)</td>
<td><strong>CHI3L1</strong></td>
<td>Serum YKL-40 levels and CHI3L1 associated with asthma</td>
</tr>
<tr>
<td>Eosinophil count&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Iceland, European, Asian</td>
<td>Iceland, European, Asian</td>
<td>&gt;18y</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Not reported</td>
<td><strong>IL1R1, IKZF2, GATA2, IL5, SH2B3, MYB, WDR36, IL33, GRAF2</strong></td>
<td>IL1R1 genome-wide significant associated asthma. MYB, WDR36, IL33 nominally associated with asthma, especially atopic asthma</td>
</tr>
<tr>
<td>Eosinophilic esophagitis&lt;sup&gt;f&lt;/sup&gt;</td>
<td>European American</td>
<td>European American</td>
<td>11.3±10.4y</td>
<td>Not reported</td>
<td>7.85±9.4y</td>
<td>Not reported</td>
<td><strong>TSLPR, WDFR2, 13q31.1</strong></td>
<td>Signal independent of asthma</td>
</tr>
<tr>
<td>Atopy&lt;sup&gt;g&lt;/sup&gt;</td>
<td>UK</td>
<td>European</td>
<td>45.2±20.4y</td>
<td>Not reported</td>
<td>Mean range 7.3-56.2y</td>
<td>Not reported</td>
<td><strong>FDNCD1</strong></td>
<td>Asthma not assessed</td>
</tr>
<tr>
<td>Atopic Dermatitis&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Germany</td>
<td>European</td>
<td>Mean range 8-18y</td>
<td>Not reported</td>
<td>Mean range 6-16y</td>
<td>Not reported</td>
<td><strong>IL13, HNRP</strong></td>
<td>Asthma not assessed</td>
</tr>
<tr>
<td>Allergic rhinitis/Glass sensitization&lt;sup&gt;i&lt;/sup&gt;</td>
<td>European</td>
<td>No replication</td>
<td>18-60y</td>
<td>Not reported</td>
<td>Mean range 9-54y</td>
<td>Not reported</td>
<td><strong>FCER1A, STAT6, IL13, HLA-DQA1, HLA-DQB1, DQA1</strong></td>
<td>No heterogeneity by asthma status</td>
</tr>
<tr>
<td>Total IgE&lt;sup&gt;j&lt;/sup&gt;</td>
<td>European</td>
<td>Non Hispanic white</td>
<td>Mean range 40-75y</td>
<td>Not reported</td>
<td>Mean range 9-54y</td>
<td>Not reported</td>
<td><strong>OVOL1, ACT19, KIF5A, FLG, N1e133</strong></td>
<td>Asthma not assessed, percentage asthma patients in cohorts ranges 8-18% original, 4-100% replication</td>
</tr>
<tr>
<td>Atopic Dermatitis&lt;sup&gt;k&lt;/sup&gt;</td>
<td>European</td>
<td>European</td>
<td>6mn-92y</td>
<td>Not reported</td>
<td>6mn-89y</td>
<td>Not reported</td>
<td><strong>IL1RL1, IL1RL1B, IL15RAP, GP1SM3, OR10A3-NRBP, GLB1, CCOQ0, CARD11, ZNF635, CAP2441-F004</strong></td>
<td>IL1RL1, IL1RL1B, IL15RAP genome-wide significant associated with asthma. Other loci p-values between 5x10^-7-1x10^-4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reported as mean (±SD) or range, mn=month, y=year, † Bold genes were genome wide significant (P<5x10^-8) in the original cohort, underlined genes were genome wide significant in the replication/combined analyses, normal printed genes showed nominal significance in original, replication or combined analyses, ‡Only associated with grass sensitization, not allergic rhinitis. §Study: COAST. The Childhood Origins of Asthma.
References


52. Smitt LA, Bouzigon E, Pin I, et al. 17q21 variants modify the association between early respiratory infections and asthma. Eur Respir J. 2010;36:57–64.


