Identification of a new locus at 16q12 associated with time-to-asthma onset

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ABSTRACT

Background: Asthma is a heterogeneous disease in which age-of-onset plays an important role.

Objective: We sought to identify the genetic variants associated with time-to-asthma onset.

Methods: We conducted a large-scale meta-analysis of nine genome-wide association studies of time-to-asthma onset (total of 5,462 asthmatics with a broad range of age-of-asthma onset and 8,424 controls of European ancestry) performed using survival analysis techniques.

Results: We detected five regions associated with time-to-asthma onset at genome-wide significant level (P<5x10^{-8}). We evidenced a new locus in 16q12 region (near cylindromatosis turban tumor syndrome gene (CYLD)) and confirmed four asthma risk regions: 2q12 (IL1RL1), 6p21 (HLA-DQA1), 9p24 (IL33) and 17q12-q21 (ZPBP2-GSDMA). Conditional analyses identified two distinct signals at 9p24 (both upstream of IL33) and at 17q12-q21 (near ZPBP2 and within GSDMA). These seven distinct loci explained together 6.0% of the variance in time-to-asthma onset. In addition, we showed that genetic variants at 9p24 and 17q12-q21 were strongly associated with an earlier onset of childhood asthma (P≤0.002) whereas 16q12 SNP was associated with a later asthma onset (P=0.04). A high burden of disease risk alleles at these loci was associated with earlier age-of-asthma onset (4 years versus 9-12 years, P=10^{-4}).

Conclusion: The new susceptibility region for time-to-asthma onset at 16q12 harbors variants that correlate with the expression of CYLD and NOD2 (nucleotide-binding oligomerization domain 2), two strong candidates for asthma. This study demonstrates that incorporating the variability of age-of-asthma onset in asthma modeling is a helpful approach in the search for disease susceptibility genes.
Key Messages:

- 16q12 genetic variants are associated with time-to-asthma onset and correlate with
  \textit{CYLD} and \textit{NOD2} expressions.

- Genetic variants at 9p24 (upstream of \textit{IL33}) and 17q12-q21 (nearby \textit{ZPBP2} and within \textit{GSDMA}) are associated with an earlier asthma-onset whereas variants at 16q12 are
  associated with a later asthma onset.

- Taking into account the variability of age-of-asthma onset in disease modeling can
  increase the power of identifying new genes involved in asthma physiopathology.

Capsule summary:

This large-scale meta-analysis of nine genome-wide association studies identified 16q12
 genetic variants associated with time-to-asthma onset that correlate with \textit{CYLD} and \textit{NOD2}
 expressions, two strong candidate genes implicated in inflammation.

Keywords: Asthma, age-of-onset, genetics, genome-wide association study, survival analysis,
 conditional analysis, \textit{CYLD}, \textit{NOD2}

Abbreviations:

\textit{CYLD}: Cylindromatosis (turban tumor syndrome)

\textit{eQTL}: Expression quantitative trait locus

\textit{GSDMA}: Gasdermin A

\textit{GWAS}: Genome-wide association study

\textit{HLA-DQA1}: Major histocompatibility complex, class II, DQ alpha 1

\textit{HR}: Hazard ratio

\textit{IL1RL1}: Interleukin 1 receptor-like 1
IL33: Interleukin 33

LCL: Lymphoblastoid cell line

LD: Linkage Disequilibrium

NFkB1: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

NOD2: Nucleotide-binding oligomerization domain containing 2

QC: Quality control

SNP: Single nucleotide polymorphism

TAO: time-to-asthma onset

ZPB2P2: Zona pellucida binding protein 2
INTRODUCTION

The prevalence of asthma has dramatically increased over the past decades in high-income countries, affecting 5–16% of people worldwide.\(^1\) It is the most common chronic disease among children and a decrease in the age-of-asthma onset has been recently documented.\(^2\) Asthma is a complex and heterogeneous disease with variable clinical expression over the life span.\(^1\) It is now well recognized that asthma is not a single disease but rather a collection of different phenotypes which may represent different manifestations of a common underlying pathological process or may be separate disease entities.\(^3\) One of the simplest characteristics that can be used to differentiate disease phenotypes is the age at onset.\(^4,5\) Indeed, asthma displays different characteristics according to the lifetime period at which it occurs.\(^6\) Early age of onset is more frequently associated with a family history of asthma, allergy sensitization and clinical response to triggers, whereas late-onset disease is associated with eosinophilic inflammation and obesity, is more common in women, and is generally less allergic.\(^3\)

The risk of developing asthma has a strong genetic component, with estimated heritability ranging from 35 to 95%.\(^7\) Genome-wide association studies (GWASs) have been successful in identifying more than 20 loci associated with asthma.\(^8\) However, the genetic factors identified to date account only for a small part of the genetic component of the disease.\(^1\) This hidden heritability might be linked to the phenotypic heterogeneity of asthma.\(^9\) The vast majority of GWASs conducted until now have analyzed asthma as a binary phenotype. A few genetic studies have considered more specific definition of asthma incorporating the age of disease onset. A genome-wide linkage screen conducted for time-to-asthma onset in French families revealed two regions, 1p31 and 5q13, potentially linked to this phenotype.\(^10\) A single GWAS has been performed on age-of-asthma onset in asthmatic children and led to the identification of two loci, not found by the previous asthma GWASs; these loci on chromosomes 3p26 and 11q24 were associated with an earlier onset of childhood asthma.\(^11\) Moreover, the effect of
17q12-q21 genetic variants, identified by the first GWAS of asthma,\textsuperscript{12} was found to be restricted to early-onset asthma.\textsuperscript{13,14} Instead of stratifying the data according to age-of-onset of disease using an arbitrary threshold, one can integrate the age-of-onset in modeling asthma risk, by using survival analysis methodology applied to both asthmatic and non-asthmatic subjects. The goal of the present study was to identify the genetic determinants underlying time-to-asthma onset in a large meta-analysis of 5,462 asthmatics and 8,424 controls from nine independent European-ancestry populations.
METHODS

Populations

We studied 13,886 individuals of European ancestry from nine independent studies (one birth cohort, five population-based and three family studies) which were part of the GABRIEL European consortium on the genetics of asthma. A brief description of these studies with appropriate references is provided in the online repository and in Table E1. All of these studies had the age of asthma onset and imputed genetic data available.

For all studies, ethical approval was obtained from the appropriate institutional ethic committees and all individuals or child's legal guardians provided written informed consent.

Time-to-asthma onset definition

Definition of asthma was based on report of doctor’s diagnosis and/or on standardized questionnaires (see online repository). To model time-to-asthma onset (TAO), we used age of onset or age at first wheeze for individuals who developed asthma, while in individuals who were free of disease upon examinations, we used age at last examination.

Genotyping

Genotyping, single nucleotide polymorphism (SNP) imputation process and quality control (QC) criteria (for individuals and SNPs) for each study are described in Table E1 in the online repository. All datasets were genotyped at Centre National de Génotypage (CNG, Evry, France), as part of the European GABRIEL asthma consortium. Quality control and imputations were performed independently for each study. Genome-wide imputations were conducted using MACH 1.0 software, with reference haplotype panels from HapMap 2. SNPs with imputation quality score (Rsq) ≥ 0.5 and minor allele frequency ≥ 1% were kept for analysis. Then, to further investigate the regions associated with time-to-asthma onset at...
the genome-wide significant level, we used imputed data from the 1000 Genomes Project and applied the same SNP QC criteria.

**Statistical analysis and strategy of analysis**

After the study-specific QC, a total of 13,886 individuals from the nine cohorts were included in the present study. In each dataset, association between time-to-asthma onset and individual SNPs was investigated under an additive genetic model using a Cox proportional-hazards regression model adjusted for sex and the first four principal components to account for population structure. A robust sandwich estimation of the variance\textsuperscript{16} was used in family data to take into account familial dependencies. Moreover, due to the complex sampling design of the GABRIELA study, survey regression techniques were used for this study to estimate robust standard errors (‘svy’ command in Stata). Proportional hazard assumptions for the main SNP effect were tested and never rejected. Genome-wide association studies of time-to-asthma onset were first conducted in each of the nine datasets separately, and then combined through meta-analysis in order to increase power and to obtain more robust findings. Meta-analyzed hazard-ratios (HRs) and 95% confidence intervals (CIs) were calculated using a fixed-effect (inverse variance) model. To assess heterogeneity of SNP effect across studies, the Cochran’s Q statistic was calculated. If heterogeneity was evidenced, a random-effect model was fitted. All analyses were performed using Stata\textsuperscript{®} version 13.1 (STATA Corp., College Station, Texas, USA). After the meta-analysis, we only kept SNPs for which at least 66% of the studies (≥6 studies among the 9 studies) participated to the summary statistics in the meta-analysis, to reduce the rate of false-positive findings. The meta-analysis results were obtained for a total of 2,387,926 SNPs. We used the classical threshold of $P$-value $\leq 5 \times 10^{-8}$ to declare a meta-analyzed SNP effect as genome-wide significant.
Conditional analysis to uncover distinct signals at TAO-associated loci

To identify distinct TAO-associated SNPs in each region harboring genome-wide significant signals, we re-analyzed separately these regions in each of the nine studies. For that purpose, we added the region top SNP into the primary Cox model as a covariate and tested the effect of each other SNP of that region. Then, the results were meta-analyzed using the same strategy as the primary GWASs. If a secondary signal was detected in a region, a second run of conditional analyses was performed to check for a third distinct signal in that region. Length of explored regions was based on regional association plots and ranged from 200 kb to 500 kb depending on recombination hotspots.

eQTL analysis and functional annotations

We queried whether significant SNPs (or their proxies) associated with time-to-asthma onset at P≤5x10^{-8} and potentially secondary signals from conditional analysis were expression quantitative trait loci (eQTLs). We used existing eQTL databases in multiple tissues (especially blood and lung) for populations of European ancestry (see online repository).^{17-23} Functional annotations of significant SNPs (or their proxies) were obtained using ENcyclopedia Of DNA Elements (ENCODE) data^{24} provided by the HaploReg tool.^{25}

Relationship of TAO-associated loci with age-of-asthma onset

In a first step, we investigated in asthmatics whether each of the SNPs associated with TAO were also associated with age-of-asthma onset using a non-parametric rank test followed by a non-parametric equality-of-medians test. In a second step, we assessed the cumulative effect of risk alleles of SNPs found associated with the age-of-asthma onset at step 1. For that purpose, we used either the number of risk alleles or the quintiles of a polygenic score distribution. The polygenic risk score is the weighted sum of the number of age of asthma
onset associated alleles with weight being the log of adjusted hazard ratio estimated in asthmatics only. The associations were tested in eight studies for which we had access to raw data (all datasets except ALSPAC) using a cox proportional hazard model adjusted on sex and principal components.
RESULTS

Description of populations

A total of 13,886 subjects were included in the present study (5,462 asthmatics and 8,424 non-asthmatics). Asthmatics had a mean age-of-asthma onset of 12.5 years (ranging from 0.5 to 75 years, Figure E1), a mean age of 26.8 years at examination (mean per study ranging from 9.1 to 51.3 years) and 52.6% were males. Non-asthmatics had a mean age of 32.4 years at examination (mean per study ranging from 8.9 to 55.8 years) and 49% were males (Table E1).

Genetic variants associated with time-to-asthma onset

The Manhattan and the Q-Q plots of the meta-analysis of time-to-asthma onset GWAS results are shown in Figure 1 and Figure E2 in the online repository respectively. A total of 155 SNPs were associated with time-to-asthma onset at the genome-wide significance level of \( P<5\times10^{-8} \). These SNPs clustered into five distinct chromosomal regions (Table I), that included a new risk locus on 16q12 (nearby CYLD (cylindromatosis turban tumor syndrome), 1 SNP) and four established risk loci for asthma: 2q12 (IL1RL1-IL18R1, 7 SNPs), 6p21 (nearby HLA-DQA1, 1 SNP), 9p24 (flanking IL33, 25 SNPs) and 17q12-q21 (121 SNPs spanning 389 kb, and with the main signal located near ZPBP2). The regional association plots for these genome-wide associated loci are shown in Figures 2 and E3, and the forest plots for the top signal in each region are shown in Figure E4. Three additional loci were associated with time-to-asthma onset at a suggestive significance threshold \( 5\times10^{-8}<P<10^{-6} \); Table I): MAP4K4 (2q11-q12), RORA (15q22) and IL4R (16p12-p11).

To determine whether any of the five TAO loci harbored additional association signals, we performed conditional association analysis in each region. For this analysis, a threshold of \( P\leq2.1\times10^{-5} \) was used to declare significance, corresponding to a Bonferroni threshold for
2,382 independent tests. These analyses evidenced two secondary signals (Table II and Figure E5 in the online repository): 1) rs413382 in 9p24 region at 73 kb of IL33 (P=9.7x10^{-6} after conditioning on top SNP and P=5.9x10^{-8} in the primary meta-analysis) and 2) rs3859192 in 17q12-q21 region within GSDMA (P=4.0x10^{-6} after conditioning on top SNP and P=1.5x10^{-13} in the primary meta-analysis). In contrast, at 2q12, 6p21 and 16q12 regions, the inclusion of the most significant time-to-asthma onset GWAS SNP as a covariate in association analysis resulted in nearly complete reduction of the association signal in these regions, suggesting that there was no evidence for a second distinct genetic factor in these regions.

To obtain a denser map of the new TAO 16q12 locus, we repeated association analyses using 1000 Genomes Project imputed SNPs. These analyses strengthened our original finding with additional signals (3.8x10^{-8} \leq P \leq 2.6x10^{-7}) located in an intergenic region encompassing the lead SNP rs1861760 (Table E2 and Figure E6). These SNPs were in moderate to high LD with rs1861760 (0.71 \leq r^2 \leq 0.81) and thus did not represent independent signals from that top hit. Similar analyses conducted in the four other TAO-associated regions also supported our original findings and did not evidence any additional independent signal in these regions.

Overall, the seven distinct SNPs (five top SNPs and two secondary SNPs) associated with TAO showed low heterogeneity between studies (P>0.11) and explained together 6.0% of the variance in time-to-asthma onset.

**Functional annotations and effect on gene expression**

To provide some insights into the potential molecular mechanisms underlying the TAO-associated variants, we queried whether the five top SNPs and two secondary signals (and their proxies) were 1) tagging potentially deleterious SNPs, 2) located in regulatory elements, and 3) reported to influence the expression of one or more of nearby genes (eQTLs at P<5x10^{-5}). We focused on the new TAO risk locus at 16q12 region. Functional annotations
for the remaining six loci are presented in the online repository and eQTL data are presented in Table III (main results) and in Table E3 in the online repository.

The 16q12 TAO-associated variants are located in an intergenic region delimited by two recombination hotspots on each side, near CYLD (22 kb downstream). The rs1861760 maps to FOXJ1 and SOX binding sites. This SNP and/or its proxies correlate with the expression of CYLD in both blood and human lung tissues and of NOD2 (nucleotide-binding oligomerization domain 2) in blood\(^{20}\) (Table III for main results and Table E3 in the online repository).\(^{17,20}\)

**Relationship between TAO-associated variants and age-of-asthma onset**

To investigate whether TAO-associated SNPs influence the age-of-asthma onset, we compared, in asthmatics, the distribution of age-of-asthma onset according to the number of risk alleles at each of the seven main and secondary TAO-associated SNPs (Figure 3). Asthmatic subjects carrying one or two copies of risk allele at 17q12-q21 SNPs (rs9901146 and rs3859192) or at 9p24 rs928413 had a younger age-of-asthma onset than non-carriers (median 6-8 years *versus* 10 years (P≤6x10\(^{-4}\)) and median 6-8 years *versus* 9 years (P=0.002), respectively), whereas those having at least one copy of rs1861760 risk-allele at 16q12 had a later age-of-asthma onset than non-carriers (median 10 years *versus* 8 years; P=0.04). No significant difference was found for the other three SNPs. We evidenced that an increased number of risk alleles at these four SNPs was associated with a younger age-of-asthma onset (median 12 years for carrying one risk allele to 4 years for carrying 6-8 risk alleles; P=10\(^{-4}\)). Finally, we detected a strong association between the age-of-asthma onset and the polygenic risk score (from median 10 years in first quintile to 6 years in last quintile; P=4x10\(^{-4}\)).

**Comparison of time-to-asthma onset GWAS results with previous asthma GWASs**
To investigate the impact of taking into account the age-of-asthma onset in disease modeling through survival analysis, we explored whether the top TAO SNPs were associated with asthma modeled as a binary trait in the nine cohorts included in the present study (Table E4, see online repository). We also investigated GABRIEL top SNPs in TAO meta-analysis (Table E4). We observed a strong decrease of heterogeneity of SNP effect across studies in TAO analysis ($P_{\text{Het}} \geq 0.11$) compared with asthma binary trait analyzed in the same datasets ($P_{\text{Het}} \geq 0.004$) as well as in all GABRIEL datasets ($P_{\text{Het}} \geq 0.0009$), especially in 9p24 and 17q12-q21 regions. The association signals were always more significant in TAO analysis as compared with the binary trait analysis in the same datasets. This increase of significance level was very high: 100-fold for 2q12 and 16q12, and $10^4$ to $10^6$-fold for 9p24 and 17q12-q21. In fact, the asthma binary trait analysis only detected two loci ($HLA$ and $GSDMA$) at the genome-wide significance level out of the seven TAO-associated loci. Conversely, the present TAO analysis identified at the genome-wide significance level four of the six main published GABRIEL regions, and event at higher significance for 9p24 and 17q12-q21 regions (100 to $10^4$-fold) as compared to GABRIEL significance levels. The two remaining GABRIEL loci not detected by our TAO analysis were those with the weaker effects (OR=1.12 for rs744910 in 15q22 and rs2284033 in 22q13) in GABRIEL meta-analysis.

Finally, we evaluated whether previously reported susceptibility loci for asthma were associated with time-to-asthma onset in our meta-analysis (see online repository, Table E5). Among the 21 loci detected in European populations, 12 were replicated at 5% in our TAO meta-analysis with the same direction of effects. Among the nine non-replicated signals, three SNPs (or some proxies) were not available in our data, and the remaining six loci had been reported for specific phenotypes: asthma exacerbation, age-of-asthma onset per se in asthmatic children only (quantitative trait) or childhood asthma (binary trait).
By taking into account the age-of-asthma onset in asthma association analysis, we identified in this large meta-analysis including both asthmatic and non-asthmatic individuals, adults and children, a new susceptibility locus at 16q12 associated with time-to-asthma onset and confirmed the involvement of six other distinct loci belonging to four regions in asthma pathogenesis (2q12, 6p21, 9p24 and 17q12-q21). Genetic variants at 9p24 and 17q12-q21 were strongly associated with an earlier onset of childhood asthma whereas the 16q12 lead SNP was associated with a risk of later onset asthma.

The most significant 16q12 genetic variant (rs1861760) is located nearby CYLD and NOD2 and also maps to a binding site of FOXJ1, a transcription factor associated with allergic rhinitis. Genetic variants located in a 130-kb region around rs1861760 were reported associated with immune-related diseases: inflammatory bowel diseases (Crohn’s disease) and leprosy. Interestingly, haplotype reconstruction (Figure 4) showed that the TAO rs1861760-A risk allele was always associated with SNP alleles that conferred a decreased risk of Crohn’s disease (rs17221417-C, rs5743289-C and rs2076756-A located in NOD2 and rs12324931-A located in CYLD) and of leprosy (rs16948876-G located in intergenic region at 2 kb from rs1861760). Indeed, GWASs revealed common genetic susceptibility loci for asthma and other immune-related disorders, suggesting shared molecular pathways involved in their etiology; however opposite alleles appear to be at risk. Interestingly, an opposite effect of rs1861760-A allele is also observed at the gene expression level. Thus, TAO risk-allele at rs1861760 correlated with both the expression of CYLD and NOD2 in blood but with an opposite effect. However, this TAO risk allele was only associated with increased CYLD expression in lung tissue. CYLD encodes a deubiquitinating enzyme that regulates diverse physiological processes including immune response and inflammation. CYLD mainly acts as a negative regulator of nuclear factor-κB (NFκB1) to protect the host
from an over-reactive inflammatory response. Conversely *NOD2*, which plays an important role in the innate immune response to intracellular bacterial lipopolysaccharides (LPS), activates the *NFkB1* pathway. *NFkB1* is a pleiotropic transcription factor that acts as a key regulator of immune and inflammatory genes, and activation of the *NFkB1* pathway has been implicated in airway inflammation and asthma. Moreover, *FOXJ1* transcription factor that binds to the genomic region encompassing 16q12 TAO-associated SNP (rs1861760) was described to inhibit *NFkB1* activity. Recently, *CYLD* has been shown to regulate lung fibrosis in mice by inhibiting transforming growth factor-β-signaling (TGF-β) through a decrease of SMAD3 protein stability. Of interest, *SMAD3* has been reported to be associated with asthma by previous GWAS.

Defining the phenotype is an important consideration because phenotypic heterogeneity can reduce power of GWAS. In the present analyses, we studied the variability of time-to-asthma onset in both asthmatics and non-asthmatics based on survival analysis methods. The information used for such analysis was the age-of-onset in asthmatics and the age at last examination or death in non-asthmatics. In such a model, unaffected subjects represent censored observations as they are still at risk for disease, being perhaps too young to exhibit the trait. This approach, which allowed combining the age-of-asthma onset together with the disease status (affected/unaffected) led to decrease the genetic heterogeneity across studies and increase the power to detect association signals (upon $10^6$-fold increase as compared to the disease status only analysis. More specifically, increased evidence of association was observed in regions where age-of-asthma onset explained at least in part the genetic heterogeneity such as the 17q12-q21 locus for which a restricted SNP effect to a particular group of age-of-onset (early childhood onset asthma) was demonstrated. Moreover, this analysis led to the identification of a new locus at 16q12 near *CYLD* and of an additional signal in the 9p24 region. These results support the hypothesis that a better consideration of
the phenotypic heterogeneity of asthma may help to disentangle the genetic heterogeneity of asthma.

Our study included both children and adult asthmatic subjects. Age-of-disease onset may be subject to recall bias, especially among individuals who are the furthest from the time of the first symptoms (e.g. adults who suffered of asthma in childhood) because it is often defined in a retrospective manner. However, high accuracy of the self-reported year of asthma onset by adult subjects has been shown by two independent studies among which the European Community Respiratory Health Survey, which was part of the present study. Erroneous recall of the age-of-asthma onset is unlikely to have significantly affected the results since we observed little genetic heterogeneity across studies (e.g. childhood onset asthma reported by either adults or children).

It was suggested that some genetic variants might influence asthma in an age-specific manner. Among TAO-associated SNPs, we confirmed the association of 17q12-q21 SNPs with an early age-of-asthma onset and evidenced for the first time that the top 9p24 genetic variant near IL33 was also associated with early childhood-onset asthma (median age-of-onset of 6-8 years in risk-allele carriers). Indeed, in GABRIEL meta-analysis, 9p24 SNPs were more strongly associated with early-onset asthma (before 16 years) than late-onset asthma (after 16 years), but this difference was not significant. Conversely, genetic variants at the new susceptibility locus, 16q12, conferred a risk of later onset asthma (median age-of-onset of 10 years in risk-allele carriers). Moreover, we evidenced that a high burden of disease risk alleles at these loci is associated with earlier age-of-asthma onset (4 years versus 9-12 years). This difference in asthma onset may reflect difference in patterns of onset of disease. Indeed, we evidenced in the GABRIELA study that persistent early wheezing subjects carried more risk alleles than transient early wheezing subjects, and we confirmed previous association between persistent early wheezing and 9p24 and 17q12-q21 loci (data not shown). The 17q12-q21
genetic variants were reported associated with persistent childhood wheeze phenotype whereas 9p24 were mostly associated with intermediate onset wheeze but also with persistent early wheeze.\textsuperscript{48,49} Moreover, 17q12-q21 SNPs were associated with fractional exhaled Nitric Oxide (FeNO) levels in children but not in adults, childhood severe asthma and allergic rhinitis, and 9p24 SNPs were associated with childhood severe asthma, asthma-plus-rhinitis, atopic asthma, allergy and eosinophil counts.\textsuperscript{50-55}

In summary, we identified five regions harboring seven distinct signals that were associated with time-to-asthma onset, among which the 16q12 region that is reported for the first time. Several lines of evidence suggest that \textit{CYLD} and \textit{NOD2} located in that region are strong candidate genes for asthma. This study demonstrates that incorporating the variability of age-of-asthma onset in disease modeling is a useful strategy to uncover new disease genes.
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exposure, (g) genetic and molecular biology, (m) meteorology, (n) nutrition, (o) occupational
health, (p) pneumology, (pa) physical activity, (pd) pediatrics, (s) statistics.
REFERENCES


31. Wellcome Trust Case Control C. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 2007; 447:661-78.


46. Toren K, Palmqvist M, Lowhagen O, Balder B, Tunsater A. Self-reported asthma was biased in relation to disease severity while reported year of asthma onset was accurate. J Clin Epidemiol 2006; 59:90-3.


Table I. Top SNPs in main loci associated with time-to-asthma onset at genome-wide ($P \leq 5 \times 10^{-8}$) and suggestive significance levels ($5 \times 10^{-6} < P < 10^{-5}$)

<table>
<thead>
<tr>
<th>Loci with genome-wide significance - $P \leq 5 \times 10^{-8}$</th>
<th>Time to asthma onset - $N=13,886$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome</td>
<td>Marker</td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
</tr>
<tr>
<td>2q12</td>
<td>rs10208293</td>
</tr>
<tr>
<td>6p21</td>
<td>rs9272346</td>
</tr>
<tr>
<td>9p24</td>
<td>rs928413</td>
</tr>
<tr>
<td>16q12</td>
<td>rs1861760</td>
</tr>
<tr>
<td>17q12-q21</td>
<td>rs9901146</td>
</tr>
</tbody>
</table>

*Position in base pairs (bp) – build 37.3 NCBI
For the calculation of the hazard ratios, effect alleles were designated as risk alleles. Effect Freq denotes frequency of the effect allele, CI confidence interval, and Ref reference allele.

‡P-value obtained from single-SNP Cox model for time-to-asthma onset adjusted for sex and principal components (fixed-effect model when there was no significant evidence of heterogeneity or random-effect model otherwise)

**P-Het reflects P-value of Cochran’s Q statistic across studies

***SNP is located within reported gene
Table II. Secondary signals associated with time-to-asthma onset after stepwise conditional analysis in 9p24 and 17q12-q21 regions. This table contains, for these two regions, the top time-to-asthma onset SNP in bold (rs928413 and rs9901146 respectively) and the most significant SNP in the conditional analysis, after fitting the lead SNP in the region.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Marker</th>
<th>Nearest gene (kb distance)</th>
<th>Position*</th>
<th>Effect/Ref Alleles†</th>
<th>Effect Freq</th>
<th>Single SNP analysis</th>
<th>Fitted SNP(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hazard Ratio</td>
<td>P†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[95% CI]</td>
<td></td>
</tr>
<tr>
<td>9p24 region</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>rs413382</td>
<td>*IL33 (73)</td>
<td>6142948</td>
<td>A/C</td>
<td>0.80</td>
<td>1.15 [1.08-1.22]</td>
<td>5.9x10^-8</td>
</tr>
<tr>
<td>9</td>
<td>rs928413</td>
<td>*IL33 (2)</td>
<td>6213387</td>
<td>G/A</td>
<td>0.25</td>
<td>1.19 [1.13-1.25]</td>
<td>6.5x10^-16</td>
</tr>
<tr>
<td>17q12-q21 region</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>rs9901146</td>
<td>*ZPB2 (9)</td>
<td>38043343</td>
<td>G/A</td>
<td>0.51</td>
<td>1.18 [1.13-1.22]</td>
<td>1.9x10^-16</td>
</tr>
<tr>
<td>17</td>
<td>rs3859192</td>
<td><em>GSDMA</em>**</td>
<td>38128648</td>
<td>T/C</td>
<td>0.48</td>
<td>1.16 [1.12-1.21]</td>
<td>1.5x10^-15</td>
</tr>
</tbody>
</table>

*Position: Position in base pairs (bp) - build 37.3 NCBI

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34
†For the calculation of the hazard ratio, effect alleles were designated as risk alleles. Effect Freq denotes frequency of the effect allele, CI

‡P-values are obtained from Cox model of time-to-asthma onset adjusted for sex and principal components

**P-Het reflects P-value of Cochran’s Q statistic across studies

***SNP is located within reported gene
Table III. Main cis-eQTLs results for the top SNPs in genome-wide associated regions from the meta-analysis of time-to-asthma onset. We focused on eQTLs measured in blood, lymphoblastoid cell lines (LCLs) and lung tissue.

<table>
<thead>
<tr>
<th>Locus</th>
<th>SNP*</th>
<th>Alleles</th>
<th>Gene(s)</th>
<th>Range of P-values</th>
<th>Tissue</th>
<th>Source††</th>
</tr>
</thead>
<tbody>
<tr>
<td>2q12</td>
<td>rs10208293</td>
<td>G/A</td>
<td>IL18RAP, IL18R1</td>
<td>2.5x10^-13-9.8x10^-198</td>
<td>Blood, LCLs</td>
<td>Blood eQTLs, eQTL Browser</td>
</tr>
<tr>
<td>6p21</td>
<td>rs9272346</td>
<td>G/A</td>
<td>HLA-DQA1/DQA2/DQAS1/DQB1/DQB2, HLA-DRA/DRB1/DRB5/DRB6, TAP2</td>
<td>1.3x10^-6-2.1x10^-121</td>
<td>LCLs, Lung, Blood</td>
<td>eQTL_Chicago,GTEx, Blood eQTLs</td>
</tr>
<tr>
<td>16q12</td>
<td>rs1861760</td>
<td>C/A</td>
<td>NOD2</td>
<td>3.6x10^-11</td>
<td>Blood</td>
<td>Blood eQTLs</td>
</tr>
<tr>
<td></td>
<td>rs5743266&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
<td>CYLD, NOD2</td>
<td>5.0x10^-9-3.2x10^-120</td>
<td>Blood</td>
<td>Blood eQTLs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(D'=1, r²=0.02)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs7205760&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
<td>CYLD, NOD2</td>
<td>2.8x10^-6-4.0x10^-15</td>
<td>Lung, Blood</td>
<td>Lung eQTLs, Blood eQTLs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(D'=1, r²=0.005)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17q12-q21</td>
<td>rs9901146</td>
<td>A/G</td>
<td>GSDMB, ORMDL3</td>
<td>3.8x10^-6-9.8x10^-198</td>
<td>Blood, LCLs</td>
<td>Blood eQTLs, GTEx, eQTL Browser, eQTL_Chicago</td>
</tr>
<tr>
<td></td>
<td>rs3859192</td>
<td>C/T</td>
<td>GSDMA, GSDMB, ORMDL3</td>
<td>1.1x10^-7-2.5x10^-12</td>
<td>Lung, LCLs</td>
<td>GTEx, eQTL Browser</td>
</tr>
</tbody>
</table>

* Top genome-wide significant SNPs in time-to-asthma onset meta-analysis and secondary associations identified by conditional analyses are indicated in bold.
Haplotype reconstruction was done using Haploview; the effect allele of the top SNP (A-rs1861760) is always transmitted with the effect allele of its proxy (G-rs5743266 and G-rs7205760).

Interrogated databases: eQTL Browser (LCLs of British subjects with asthma or eczema), Blood eQTL Browser (non-transformed peripheral blood sample), Lung eQTLs (lung), GTEx eQTL Browser v4 (several tissues among which blood and lung), and eQTL Chicago Browser (LCLs).
**FIGURE LEGENDS**

**Figure 1.** Manhattan plot showing the association P-values of the genome-wide association results for time-to-asthma onset from the meta-analysis. The $-\log_{10}$ of the P-value for each of 2,400,368 SNPs (y-axis) is plotted against the genomic position (x-axis). The solid red line indicates the genome-wide significance threshold of P=$5\times 10^{-8}$.

**Figure 2.** Regional association plot of the 16q12 region using Locuzoom software. SNPs are plotted with their P-values ($-\log_{10}$ values, left y-axis) as a function of genomic position (x-axis). Estimated recombination rates (right y-axis) taken from 1000G (EUR) are plotted to reflect the local LD structure around the top associated SNP (purple circle) and correlated proxies (according to a blue to red scale from $r^2=0$ to 1).

**Figure 3.** Relationship between time-to-asthma onset associated SNPs and age-of-asthma onset. Part A) Association between age-of-asthma onset and genotypes at individual locus; Part B) Median of age-of-asthma onset as a function of the number of individual’s risk allele burden; Part C) Median of age-of-asthma onset by quintile of genetic risk score.

**Figure 4.** Map of 16q12 region (build 37.3 position: 50,723,355-50,860,722) and haplotype reconstruction for SNPs found associated with inflammatory bowel disease (among which Crohn’s disease, blue), leprosy (green) or asthma (red) or with the expression of CYLD or NOD2 (black). Linkage disequilibrium plot was obtained using Hapmap2 CEU reference sample from Haplovie\textsuperscript{57} (values and colors reflect r$^2$ and D’ respectively). The 16q12 top SNP (rs1861760) associated with time-to-asthma onset is indicated in bold.
Figure 1.
Figure 2.
Figure 3.

Part A

Median of age of asthma onset (years)

rs10208293  rs9272346  rs413382  rs928413  rs1863760  rs9901146  rs3859192

P = 0.19  P = 0.70  P = 0.80  P = 0.002  P = 0.04  P = 3 x 10^-7  P = 6 x 10^-4

0  1  2

Part B

Median of age of asthma onset (years)

Number of risk alleles

0  1  2  3  4  5  6+8

P = 0.0001

Part C

Median of age of asthma onset (years)

Quintile of genetic risk score

Q1  Q2  Q3  Q4  Q5

10  9  8  7  6  P = 0.0004
Figure 4.

Single haplotype including rs1861760: A risk allele associated with time to asthma onset.