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

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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 (TAO).

Methods: We conducted a large-scale meta-analysis of 9 genome-wide association studies of TAO (total of 5462 asthmatic patients with a broad range of age of asthma onset and 8424 control subjects of European ancestry) performed by using survival analysis techniques.

Results: We detected 5 regions associated with TAO at the genome-wide significant level ($P < 5 \times 10^{-8}$). We evidenced a new locus in the 16q12 region (near cylindromatosis turban tumor syndrome gene *[CYLD]*) and confirmed 4 asthma risk regions: 2q12 (IL-1 receptor–like 1 *[IL1RL1]*), 6p21 *(HLA-DQA1)*, 9p24 *(IL33)*, and 17q12-q21 (zona pellucida binding protein 2 *[ZPBP2]*–gasdermin A *[GSDMA]*). Conditional analyses identified 2 distinct signals at 9p24 (both upstream of *IL33*) and 17q12-q21 (near *ZPBP2* and within *GSDMA*). Together, these 7 distinct loci explained 6.0% of the variance in TAO. In addition, we showed that genetic variants at 9p24 and 17q12-q21 were strongly associated with an earlier onset of childhood asthma ($P \leq .002$), whereas the 16q12 single nucleotide polymorphism was associated with later asthma onset ($P = .04$). A high burden of disease risk alleles at these loci was associated with earlier age of asthma onset (4 vs 9-12 years, $P = 10^{-4}$).

Conclusion: The new susceptibility region for TAO at 16q12 harbors variants that correlate with the expression of *CYLD* and nucleotide-binding oligomerization domain 2 *(NOD2)*, 2 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. (J Allergy Clin Immunol 2016;138:1071-80.)

Key words: *Asthma, age of onset, genetics, genome-wide association study, survival analysis, conditional analysis,* CYLD, NOD2

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Abbreviations used
CYLD: Cylindromatosis (turban tumor syndrome)
eQTL: Expression quantitative trait locus
GSDMA: Gasdermin A
GWAS: Genome-wide association study
IL1RL1: IL-1 receptor–like 1
LCL: Lymphoblastoid cell line
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
ZPBP2: Zona pellucida binding protein 2

The prevalence of asthma has dramatically increased over the past decades in high-income countries, affecting 5% to 16% of persons worldwide.[1] It is the most common chronic disease among children, and a decrease in age of asthma onset has been documented recently.[2]

Asthma is a complex and heterogeneous disease with variable clinical expression over the lifespan.[1] It is now well recognized that asthma is not a single disease but rather a collection of different phenotypes that might represent different manifestations of a common underlying pathologic process or might be separate disease entities.[3] One of the simplest characteristics that can be used to differentiate disease phenotypes is age at onset.[4,5] Indeed, asthma displays different characteristics according to the lifetime period during 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, more common in women, and generally less allergic.[3]

The risk of 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 a more specific definition of asthma incorporating the age of disease onset. A genome-wide linkage screen conducted for time to asthma onset (TAO) in French families revealed 2 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 2 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[12] was found to be restricted to early-onset asthma.[13,14]

Instead of stratifying the data according to age of disease onset with an arbitrary threshold, one can integrate the age of onset in modeling asthma risk by using survival analytic methodologies applied to both asthmatic and nonasthmatic subjects. The goal of the present study was to identify the genetic determinants underlying TAO in a large meta-analysis of 5462 asthmatic patients and 8424 control subjects from 9 independent European-ancestry populations.

METHODS

Populations

We studied 13,886 subjects of European ancestry from 9 independent studies (1 birth cohort, 5 population-based studies, and 3 family studies) that were part of the GABRIEL European consortium on the genetics of asthma.[14] A brief description of these studies with appropriate references is provided in the Methods section and Table E1 in this article's Online Repository at www.jacionline.org. All of these studies had age of asthma onset and imputed genetic data available.

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

TAO definition

The definition of asthma was based on report of doctor's diagnosis, on standardized questionnaires, or both (see the Methods section in this article's Online Repository). To model TAO, we used age of onset or age at first wheeze for patients with asthma, whereas in subjects who were free of disease on examination, we used age at last examination.

Genotyping

Genotyping, the single nucleotide polymorphism (SNP) imputation process, and quality control (QC) criteria (for subjects and SNPs) for each study are described in Table E1. All data sets were genotyped at Centre National de Génotypage (Evry, France) as part of the European GABRIEL asthma consortium.[14] QC and imputations were performed independently for each study. Genome-wide imputations were conducted with MACH 1.0 software,[15] with reference haplotype panels from HapMap2. SNPs with imputation quality scores (R^2) of 0.5 or greater and minor allele frequencies of 1% or greater were kept for analysis. Then, to further investigate the regions associated with TAO 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 subjects from the 9 cohorts were included in the present study. In each data set association between TAO and individual SNPs was investigated under an additive genetic model by using a Cox proportional hazards regression model adjusted for sex and the first 4 principal components to account for population structure. A robust sandwich estimation of variance[16] was used in family data to take into account familial dependencies. Moreover, because of the complex sampling design of the GABRIELA study, survey regression techniques were used for this study to estimate robust SEs (*svy* command in Stata software). Proportional hazard assumptions for the main SNP effect were tested and never rejected. GWASs of TAO were first conducted in each of the 9 data sets separately and then combined through a meta-analysis to increase power and obtain more robust findings. Meta-analyzed hazard ratios and 95% CIs were calculated by using a fixed-effect (inverse variance) model. The Cochran Q statistic was calculated to assess the heterogeneity of the SNP effect across studies. If heterogeneity was evidenced, a random-effect model was fitted. All analyses were performed with Stata software (version 13.1; StataCorp, College Station, Tex). After the meta-analysis, we only kept meta-analysis summary statistics of SNPs included in at least 66% of the studies (>6 of the 9 studies) 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 a P value of 5×10^{-8} or less 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 reanalyzed separately these regions in each of the 9 studies. For that purpose, we added the region's 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 by using the same strategy as

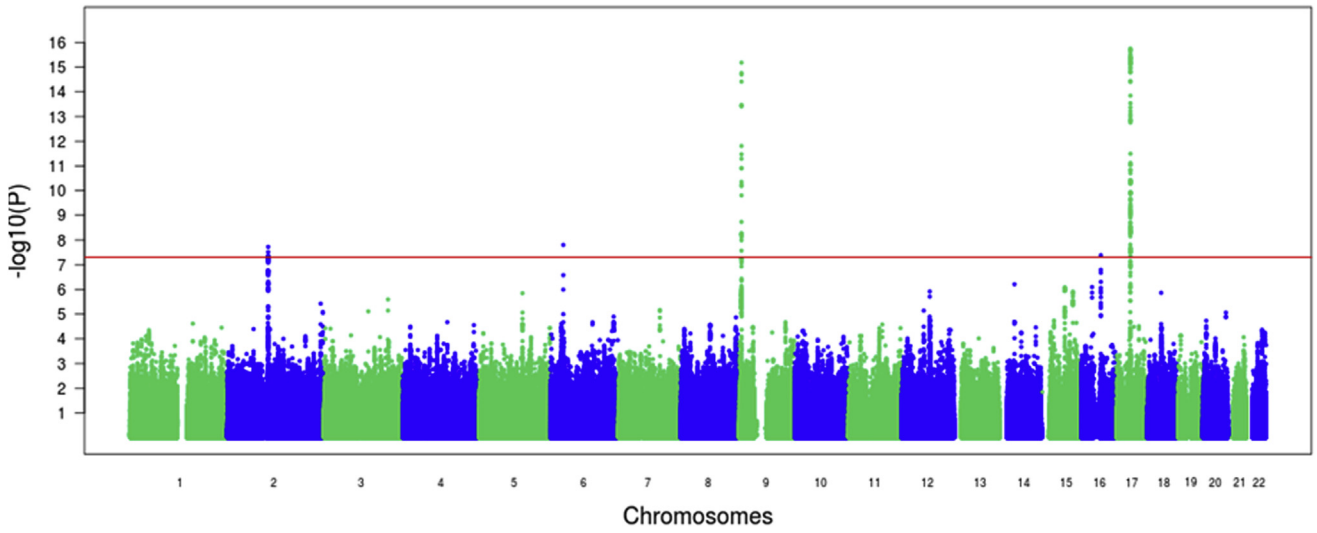

FIG 1. Manhattan plot showing association *P* values of the genome-wide association results for TAO from the meta-analysis. The $-\log_{10}$ of the *P* value for each of 2,387,926 SNPs *(y-axis)* is plotted against the genomic position *(x-axis)*. The *solid red line* indicates the genome-wide significance threshold of a *P* value of 5×10^{-8}.

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. The length of the explored regions was based on regional association plots and ranged from 200 to 500 kb depending on recombination hotspots.

Expression quantitative trait locus analysis and functional annotations

We queried whether significant SNPs (or their proxies) associated with TAOs at a *P* value of 5×10^{-8} or less 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 the Methods section in this article's Online Repository).[17-23]

Functional annotations of significant SNPs (or their proxies) were obtained by using Encyclopedia of DNA Elements 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 asthmatic patients whether each of the SNPs associated with TAO were also associated with age of asthma onset by using a nonparametric rank test, followed by a nonparametric equality of medians test. In a second step we assessed the cumulative effect of risk alleles of SNPs found to be 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 the adjusted hazard ratio estimated in asthmatic patients only. The associations were tested in 8 studies for which we had access to raw data (all data sets except the Avon Longitudinal Study of Parents and Children) by 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 asthmatic patients and 8,424 nonasthmatic subjects). Asthmatic patients had a mean age of asthma onset of 12.5 years (range, 0.5-75 years; see Fig E1 in this article's Online Repository at www.jacionline.org) and a mean age of 26.8 years at examination (mean per study ranging from 9.1-51.3 years), and 52.6% were male. Nonasthmatic subjects had a mean age of 32.4 years at examination (mean per study ranging from 8.9-55.8 years), and 49% were male (see Table E1).

Genetic variants associated with TAO

The Manhattan and quantile-quantile plots of the meta-analysis of TAO GWAS results are shown in Fig 1 and Fig E2 in this article's Online Repository at www.jacionline.org, respectively. A total of 155 SNPs were associated with TAO at a genome-wide significance level of a *P* value of less than 5×10^{-8}. These SNPs clustered into 5 distinct chromosomal regions (Table I) that included a new risk locus on 16q12 (near *CYLD*, 1 SNP) and 4 established risk loci for asthma: 2q12 (IL-1 receptor–like 1 *[IL1RL1]–IL18R1*, 7 SNPs), 6p21 (near *HLA-DQA1*, 1 SNP), 9p24 (flanking *IL33*, 25 SNPs), and 17q12-q21 (121 SNPs spanning 389 kb, with the main signal located near zona pellucida binding protein 2 *[ZPBP2]*). The regional association plots for these genome-wide associated loci are shown in Fig 2[26] and Fig E3 in this article's Online Repository at www.jacionline.org, and the forest plots for the top signal in each region are shown in Fig E4 in this article's Online Repository at www.jacionline.org. Three additional loci were associated with TAO at a suggestive significance threshold ($5 \times 10^{-8} < P < 10^{-6}$, Table I): mitogen-activated protein kinase kinase kinase kinase 4 (*MAP4K4*; 2q11-q12), RAR-related orphan receptor A (*RORA*; 15q22), and IL-4 receptor (*IL4R*; 16p12-p11).

To determine whether any of the 5 TAO loci harbored additional association signals, we performed conditional association analysis in each region. For this analysis, a threshold *P* value of 2.1×10^{-5} or less was used to declare significance, corresponding to a Bonferroni threshold for 2382 independent tests. These analyses evidenced 2 secondary signals (Table II and see Fig E5 in this article's Online Repository at www.jacionline.org): (1) rs413382 in the 9p24 region at 73 kb of *IL33* ($P = 9.7 \times 10^{-6}$ after conditioning on the top SNP and

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

Chromosome	Marker	Position*	Nearest gene or genes (kb distance)	Effect/reference alleles†	Effect frequency	Time to asthma onset: n = 13,886		
						Hazard ratio (95% CI)	*P* value‡	P_{Het} value§
Loci with genome-wide significance ($P \leq 5 \times 10^{-8}$)								
2q12	rs10208293	102,966,310	*IL1RL1*‖	G/A	0.73	1.14 (1.08-1.19)	3.1×10^{-8}	.26
6p21	rs9272346	32,604,372	*HLA-DQA1 (0.8)*	A/G	0.59	1.13 (1.08-1.17)	1.6×10^{-8}	.12
9p24	rs928413	6,213,387	*IL33 (2)*	G/A	0.25	1.19 (1.13-1.25)	6.5×10^{-16}	.15
16q12	rs1861760	50,857,693	*CYLD (22)*	A/C	0.04	1.28 (1.17-1.40)	4.2×10^{-8}	.11
17q12-q21	rs9901146	38,043,343	*ZPBP2 (9)* *GSDMB (17)*	G/A	0.51	1.18 (1.13-1.22)	1.9×10^{-16}	.17
Suggestive loci ($5 \times 10^{-8} < P < 10^{-6}$)								
2q11-q12	rs12468899	102,426,140	*MAP4K4*‖	G/A	0.69	1.12 (1.09-1.16)	1.7×10^{-7}	.89
15q22	rs11071559	61,069,988	*RORA*‖	C/T	0.85	1.16 (1.10-1.24)	8.3×10^{-7}	.96
16p12-p11	rs1805013	27,373,980	*IL4R*‖	T/C	0.05	1.22 (1.13-1.32)	8.0×10^{-7}	.37

*Position in base pairs: build 37.3, National Center for Biotechnology Information.
†For the calculation of hazard ratios, effect alleles were designated as risk alleles. *Effect frequency* denotes the frequency of the effect allele.
‡*P* values obtained from the single-SNP Cox model for TAO 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 the *P* value of the Cochran Q statistic across studies.
‖The SNP is located within the reported gene.

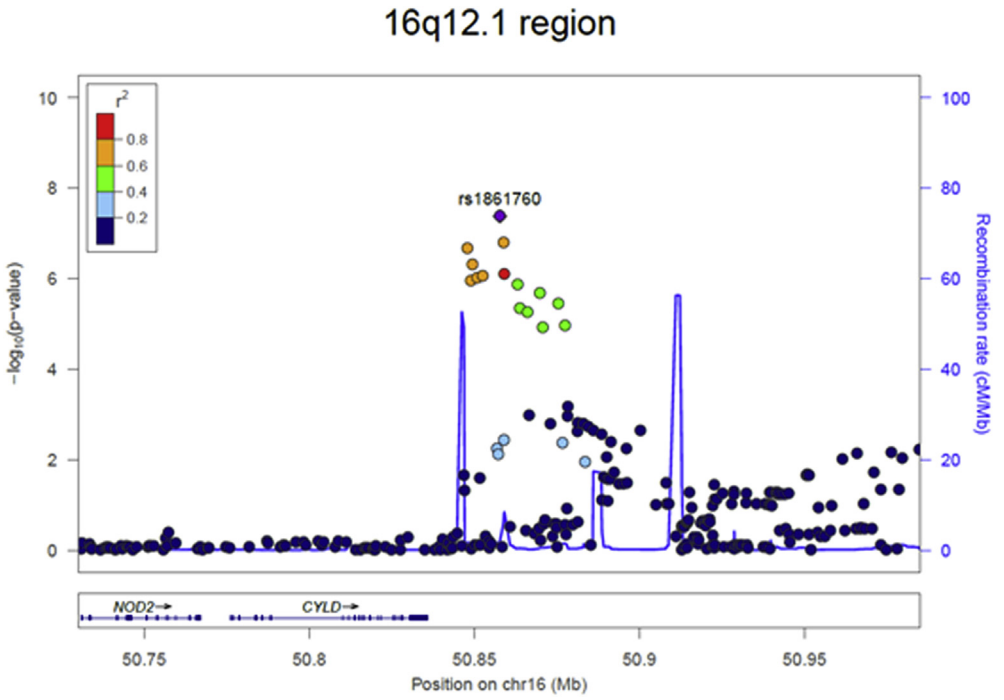

FIG 2. Regional association plot of the 16q12 region using Locuzoom software.[26] SNPs are plotted with their *P* values ($-\log_{10}$ values, *left y-axis*) as a function of genomic position *(x-axis)*. Estimated recombination rates *(right y-axis)* taken from the 1000 Genomes Project (EUR) are plotted to reflect the local linkage disequilibrium structure around the top associated SNP *(purple circle)* and correlated proxies (according to a blue to red scale from and r^2 value of 0-1).

$P = 5.9 \times 10^{-8}$ in the primary meta-analysis) and (2) rs3859192 in the 17q12-q21 region within gasdermin A (*GSDMA*; $P = 4.0 \times 10^{-6}$ after conditioning on the top SNP and $P = 1.5 \times 10^{-13}$ in the primary meta-analysis). In contrast, at the 2q12, 6p21, and 16q12 regions, inclusion of the most significant TAO 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 ($3.8 \times 10^{-8} \leq P \leq 2.6 \times 10^{-7}$) located in an intergenic region encompassing the lead SNP rs1861760 (see Table E2 and Fig E6 in this article's Online Repository at www.jacionline.org). These SNPs were in moderate to high linkage disequilibrium with rs1861760 ($0.71 \leq r^2 \leq 0.81$) and thus did not represent independent signals from that top hit. Similar

TABLE II. Secondary signals associated with TAO after stepwise conditional analysis in 9p24 and 17q12-q21 regions

Chromosome	Marker	Nearest gene (kb distance)	Position*	Effect/ reference alleles†	Effect frequency	Single-SNP analysis			Fitted SNP(s)		
						Hazard ratio (95% CI)	*P* value‡	P_{Het}§	Hazard ratio (95% CI)	*P* value‡	P_{Het}§
9p24 region									**rs928413**		
9	rs413382	*IL33 (73)*	6,142,948	A/C	0.80	1.15 (1.08-1.22)	5.9×10^{-8}	.84	1.13 (1.06-1.20)	9.7×10^{-6}	.80
9	**rs928413**	*IL33 (2)*	6,213,387	G/A	0.25	1.19 (1.13-1.25)	6.5×10^{-16}	.15	—	—	—
17q12-q21 region									**rs9901146**		
17	**rs9901146**	*ZPBP2 (9)*	38,043,343	G/A	0.51	1.18 (1.13-1.22)	1.9×10^{-16}	.17	—	—	—
17	rs3859192	*GSDMA*‖	38,128,648	T/C	0.48	1.16 (1.12-1.21)	1.5×10^{-13}	.90	1.11 (1.06-1.15)	4.0×10^{-6}	.74

For these 2 regions, this table contains the top TAO SNP in boldface (rs928413 and rs9901146 respectively) and the most significant SNP in the conditional analysis after fitting the lead SNP in the region.

*Position: Position in base pairs: build 37.3, National Center for Biotechnology Information.

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

‡*P* values are obtained from the Cox model of TAO adjusted for sex and principal components.

§P_{Het} reflects the *P* value of the Cochran Q statistic across studies.

‖The SNP is located within the reported gene.

TABLE III. Main cis-eQTL results for the top SNPs in genome-wide associated regions from the meta-analysis of TAO

Locus	SNP* (LD with top SNP)	Alleles (reference/ effect)	Gene(s)	Range of *P* values	Tissue	Source‡
2q12	**rs10208293**	G/A	*IL18RAP, IL18R1*	2.5×10^{-13} to 9.8×10^{-198}	Blood, LCLs	Blood eQTLs, eQTL Browser
6p21	**rs9272346**	G/A	*HLA-DQA1/DQA2/DQAS1/ DQB1/DQB2, HLA-DRA/ DRB1/DRB5/DRB6, TAP2*	1.3×10^{-6} to 2.1×10^{-121}	LCLs, lung, blood	eQTL_Chicago,GTEx, blood eQTLs
16q12	**rs1861760**	C/A	*NOD2*	3.6×10^{-11}	Blood	Blood eQTLs
	rs5743266† ($D' = 1$, $r^2 = 0.02$)		*CYLD, NOD2*	5.0×10^{-9} to 3.2×10^{-120}	Blood	Blood eQTLs
	rs7205760† ($D' = 1$, $r^2 = 0.005$)		*CYLD, NOD2*	2.8×10^{-6} to 4.0×10^{-15}	Lung, blood	Lung eQTLs, blood eQTLs
17q12-q21	**rs9901146**	A/G	*GSDMB, ORMDL3*	3.8×10^{-6} to 9.8×10^{-198}	Blood, LCLs	Blood eQTLs, GTEx, eQTL Browser, eQTL_Chicago
	rs3859192	C/T	*GSDMA, GSDMB, ORMDL3*	1.1×10^{-7} to 2.5×10^{-12}	Lung, LCLs	GTEx, eQTL Browser

We focused on eQTLs measured in blood, lymphoblastoid cell lines, and lung tissue.

LCL, Lymphoblastoid cell line; *LD*, linkage disequilibrium.

*Top genome-wide significant SNPs in TAO meta-analysis and secondary associations identified by conditional analyses are indicated in boldface.

†Haplotype reconstruction was done with 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),[18] Blood eQTL Browser (nontransformed peripheral blood samples),[20] Lung eQTLs (lung tissue),[17] GTEx eQTL Browser v4 (several tissues, among which were blood and lung tissue),[23] and eQTL Chicago Browser (LCLs).[19,21,22]

analyses conducted in the 4 other TAO-associated regions also supported our original findings and did not find evidence for any additional independent signal in these regions.

Overall, the 7 distinct SNPs (5 top SNPs and 2 secondary SNPs) associated with TAO showed low heterogeneity between studies ($P > .11$) and together explained 6.0% of the variance in TAO.

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 5 top SNPs and 2 secondary signals (and their proxies) were (1) tagging potentially deleterious SNPs, (2) located in regulatory elements, and (3) reported to influence the expression of 1 or more of the nearby genes (eQTLs at $P < 5 \times 10^{-5}$). We focused on the new TAO risk locus at the 16q12 region. Functional annotations for the remaining 6 loci are presented in the Results section in this article's Online Repository at www.jacionline.org, and eQTL data are presented in Table III[17-23] and Table E3 in this article's Online Repository at www.jacionline.org.

The 16q12 TAO-associated variants are located in an intergenic region delimited by 2 recombination hotspots on each side near *CYLD* (22 kb downstream). rs1861760 maps to the 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 the expression of nucleotide-binding oligomerization domain 2 *(NOD2)* in blood (Table III and see Table E3).[17,20]

Relationship between TAO-associated variants and age of asthma onset

To investigate whether TAO-associated SNPs influence age of asthma onset, in asthmatic patients we compared the distribution of age of asthma onset according to the number of risk alleles at

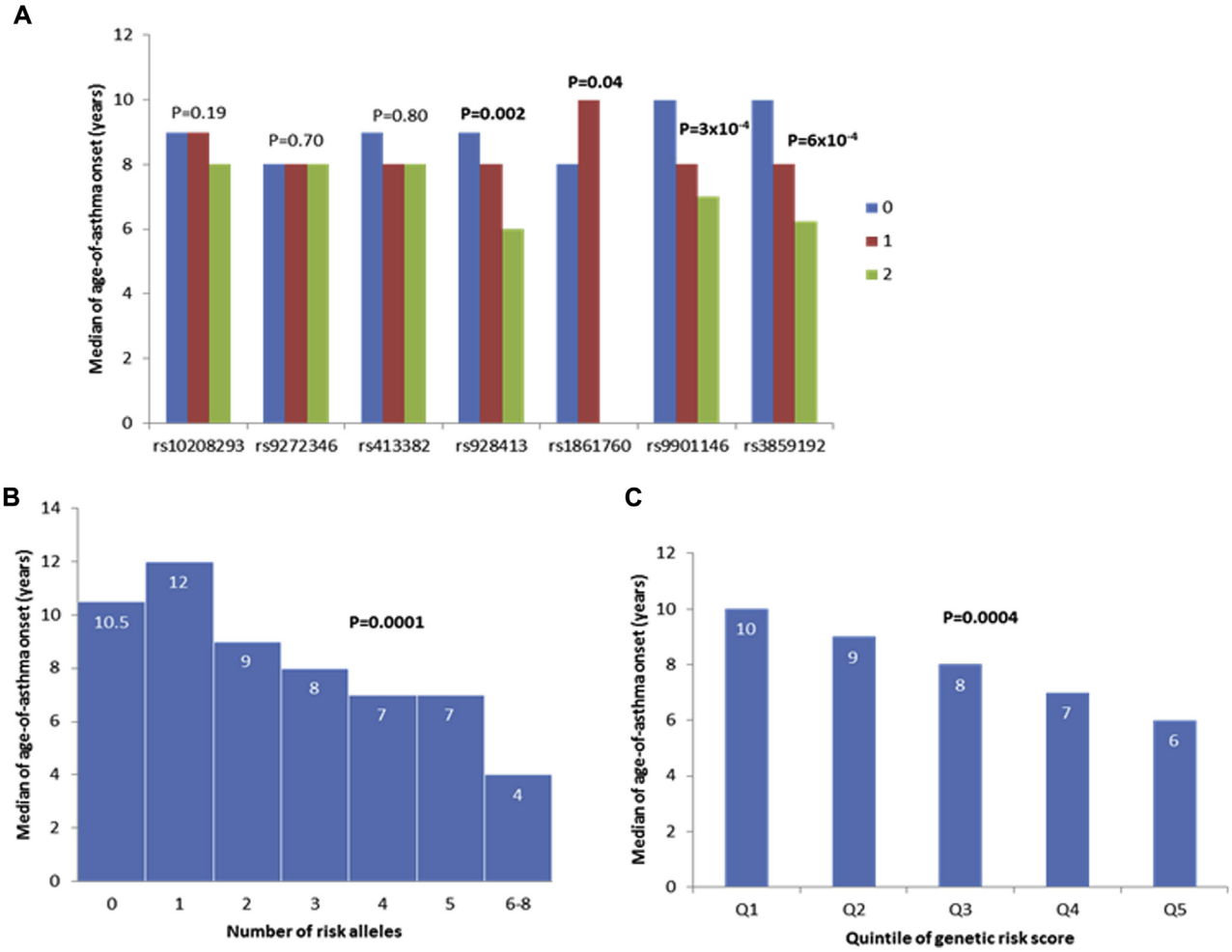

FIG 3. Relationship between TAO-associated SNPs and age of asthma onset. **A,** Association between age of asthma onset and genotypes at individual loci. **B,** Median of age of asthma onset as a function of the total number of risk alleles of SNPs found associated with the age-of-asthma onset and carried by asthmatic subjects. **C,** Median of age of asthma onset by quintile of genetic risk score.

each of the 7 main and secondary TAO-associated SNPs (Fig 3). Asthmatic patients carrying 1 or 2 copies of the risk allele at 17q12-q21 SNPs (rs9901146 and rs3859192) or at 9p24 rs928413 had a younger age of asthma onset than noncarriers (median of 6-8 vs 10 years [$P \leq 6 \times 10^{-4}$] and 6-8 vs 9 years [$P = .002$], respectively), whereas those having at least 1 copy of the rs1861760 risk allele at 16q12 had a later age of asthma onset than noncarriers (median of 10 vs 8 years, $P = .04$). No significant difference was found for the other 3 SNPs. We evidenced that an increased number of risk alleles at these 4 SNPs was associated with a younger age of asthma onset (median of 12 years for carrying 1 risk allele to 4 years for carrying 6-8 risk alleles, $P = 10^{-4}$). Finally, we detected a strong association between age of asthma onset and the polygenic risk score (from a median of 10 years in the first quintile to 6 years in the last quintile, $P = 4 \times 10^{-4}$).

Comparison of TAO GWAS results with previous asthma GWASs

To investigate the effect 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 9 cohorts included in the present study (see Table E4). We also investigated the GABRIEL top SNPs in our TAO meta-analysis (see Table E4).[14] We observed a strong decrease in heterogeneity of the SNP effect across studies in our TAO analysis ($P_{Het} \geq .11$) compared with the asthma binary trait analyzed in the same data sets ($P_{Het} \geq .004$), as well as in all GABRIEL data sets ($P_{Het} \geq .0009$), especially in the 9p24 and 17q12-q21 regions. The association signals were always more significant in TAO analysis compared with the binary trait analysis in the same data sets. This increase in 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 2 loci (*HLA* and *GSDMA*) at the genome-wide significance level 7 TAO-associated loci. Conversely, at the genome-wide significance level, the present TAO analysis identified 4 of the 6 main published GABRIEL regions[14] and events at higher significance for the 9p24 and 17q12-q21 regions (100- to 10^4-fold) compared with GABRIEL significance levels. The 2 remaining GABRIEL loci not detected by our TAO analysis were those with weaker effects (odds ratio, 1.12 for rs744910 in 15q22 and rs2284033 in 22q13) in the GABRIEL meta-analysis.[14]

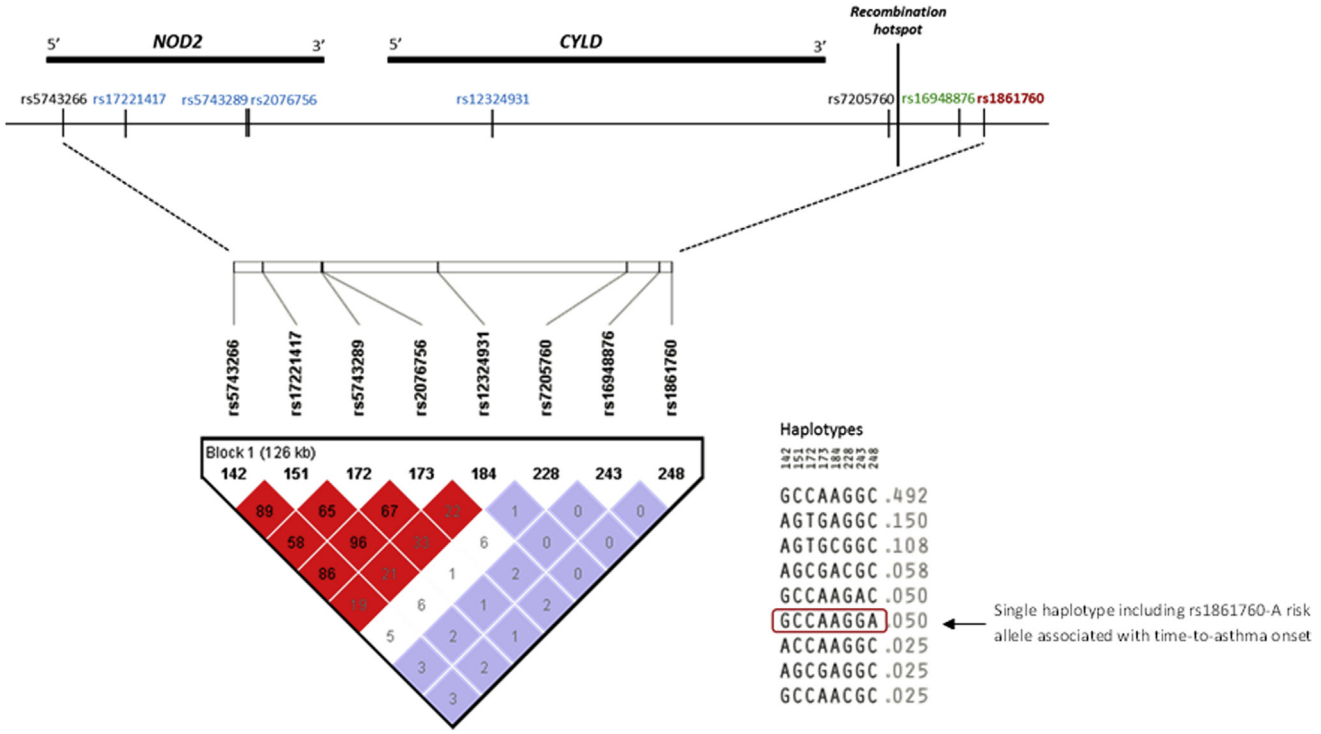

FIG 4. Map of the 16q12 region (build 37.3 position: 50,723,355 to 50,860,722) and haplotype reconstruction for SNPs found to be associated with inflammatory bowel disease (among which was Crohn disease, *blue*), leprosy *(green)*, or asthma *(red)* or with expression of *CYLD* or *NOD2 (black)*. The linkage disequilibrium plot was obtained by using the Hapmap2 CEU reference sample from Haploview[37] (values and colors reflect r^2 and D′ values, respectively). The 16q12 top SNP (rs1861760) associated with TAO is indicated in boldface.

Finally, we evaluated whether previously reported susceptibility loci for asthma[27] were associated with TAO in our meta-analysis (see Table E5 in this article's Online Repository at www.jacionline.org). 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 9 nonreplicated signals, 3 SNPs (or some proxies) were not available in our data, and the remaining 6 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).[11,28,29]

DISCUSSION

By taking into account age of asthma onset in an asthma association analysis, in this large meta-analysis including both asthmatic and nonasthmatic subjects (adults and children), we identified a new susceptibility locus at 16q12 associated with TAO and confirmed the involvement of 6 other distinct loci belonging to 4 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 near *CYLD* and *NOD2* and also maps to a binding site of FOXJ1, a transcription factor associated with allergic rhinitis.[30] Genetic variants located in a 130-kb region around rs1861760 were reported to be associated with immune-related diseases: inflammatory bowel diseases (Crohn disease) and leprosy.[31-36] Interestingly, haplotype reconstruction (Fig 4[37]) showed that the TAO rs1861760-A risk allele was always associated with SNP alleles that conferred a decreased risk of Crohn disease (rs17221417-C, rs5743289-C, and rs2076756-A located in *NOD2* and rs12324931-A located in *CYLD)*[31-33,36,38] and of leprosy (rs16948876-G located in intergenic region at 2 kb from rs1861760).[34] Indeed, GWASs revealed common genetic susceptibility loci for asthma and other immune-related disorders, suggesting shared molecular pathways involved in their cause; however, opposite alleles appear to be at risk.[39] Interestingly, an opposite effect of the rs1861760-A allele is also observed at the gene expression level. Thus the TAO risk allele at rs1861760 correlated with both expression of *CYLD* and *NOD2* in blood, although with an opposite effect.[20] However, this TAO risk allele was only associated with increased *CYLD* expression in lung tissue.[17] *CYLD* encodes a deubiquitinating enzyme that regulates diverse physiologic processes, including immune response and inflammation.[40] *CYLD* mainly acts as a negative regulator of nuclear factor-κB *(NFkB1)* to protect the host from an overreactive inflammatory response.[40] Conversely, *NOD2*, which plays an important role in the innate immune response to intracellular bacterial LPSs, activates the *NFkB1* pathway.[41] 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.[42,43] Moreover, the FOXJ1 transcription factor that binds to the genomic region encompassing the 16q12 TAO-associated SNP (rs1861760) was described to inhibit *NFkB1* activity.[44] Recently, *CYLD* has been shown to regulate lung fibrosis in mice by inhibiting TGF-β signaling through a decrease of SMAD3 protein stability.[45] Of interest, *SMAD3* has

been reported to be associated with asthma in previous GWASs.[14]

Defining the phenotype is an important consideration because phenotypic heterogeneity can reduce the power of GWASs.[46] In the present analyses we studied the variability of TAO in both asthmatic and nonasthmatic subjects based on survival analysis methods. The information used for such analysis was the age of onset in asthmatic patients and the age at last examination or death in nonasthmatic subjects. In such a model unaffected subjects represent censored observations because 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 and disease status (affected/unaffected), led to a decrease in genetic heterogeneity across studies and an increase in the power to detect association signals (on a 10^6-fold increase compared with the disease status–only analysis). More specifically, increased evidence of association was observed in regions in which 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.[13] 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 might help disentangle the genetic heterogeneity of asthma.

Our study included both children and adults with asthma. Age of disease onset might be subject to recall bias, especially among subjects who are furthest from the time of first symptoms (eg, adults with 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 2 independent studies, including the European Community Respiratory Health Survey, which was part of the present study.[47,48] Erroneous recall of age of asthma onset is unlikely to have significantly affected the results because we observed little genetic heterogeneity across studies (eg, childhood-onset asthma reported by either adults or children).

It was suggested that some genetic variants can 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[13,14] 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 the GABRIEL meta-analysis 9p24 SNPs were more strongly associated with early-onset (before age 16 years) than late-onset (after age 16 years) asthma, but this difference was not significant.[14] 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 vs 9-12 years). This difference in asthma onset might reflect the difference in patterns of onset of disease.[49] Indeed, we evidenced in the GABRIELA study that subjects with persistent early wheezing carried more risk alleles than subjects with transient early wheezing, and we confirmed the previous association between persistent early wheezing and 9p24 and 17q12-q21 loci (data not shown). The 17q12-q21 genetic variants were reported to be associated with the persistent childhood wheeze phenotype, whereas 9p24 variants were mostly associated with intermediate-onset wheeze but also with persistent early wheeze.[50,51] Moreover, 17q12-q21 SNPs were associated with fraction of exhaled nitric oxide levels in children but not 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.[51-57]

In summary, we identified 5 regions harboring 7 distinct signals associated with TAO, including the 16q12 region, which is reported for the first time. Several lines of evidence suggest that *CYLD* and *NOD2*, which are 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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Key messages

- **16q12 genetic variants are associated with TAO and correlate with *CYLD* and *NOD2* expression.**
- **Genetic variants at 9p24 (upstream of *IL33*) and 17q12-q21 (nearby *ZPBP2* and within *GSDMA*) are associated with an earlier asthma onset, whereas variants at 16q12 are associated with 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.**

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