

A genome-wide survey of CD4⁺ lymphocyte regulatory genetic variants identifies novel asthma genes

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Background: Genome-wide association studies have yet to identify the majority of genetic variants involved in asthma. We hypothesized that expression quantitative trait locus (eQTL) mapping can identify novel asthma genes by enabling prioritization of putative functional variants for association testing. **Objective:** We evaluated 6706 cis-acting expression-associated variants (eSNPs) identified through a genome-wide eQTL survey of CD4⁺ lymphocytes for association with asthma. **Methods:** eSNPs were tested for association with asthma in 359 asthmatic patients and 846 control subjects from the Childhood Asthma Management Program, with verification by using family-based testing. Significant associations were tested for replication in 579 parent-child trios with asthma from Costa Rica. Further functional validation was performed by using formaldehyde-assisted isolation of regulatory elements (FAIRE) quantitative PCR and chromatin immunoprecipitation PCR in lung-derived epithelial cell lines (Beas-2B and A549) and Jurkat cells, a leukemia cell line derived from T lymphocytes. **Results:** Cis-acting eSNPs demonstrated associations with asthma in both cohorts. We confirmed the previously reported association of *ORMDL3/GSDMB* variants with asthma (combined $P = 2.9 \times 10^{-8}$). Reproducible associations were also observed for eSNPs in 3 additional genes: fatty acid desaturase 2 (*FADS2*; $P = .002$), N-acetyl- α -D-galactosaminidase (*NAGA*; $P = .0002$), and Factor XIII, A1 (*F13A1*; $P = .0001$). Subsequently, we demonstrated that *FADS2* mRNA is increased in CD4⁺ lymphocytes in asthmatic patients and that the associated eSNPs reside within DNA segments with histone modifications that denote open chromatin status and confer enhancer activity. **Conclusions:** Our results demonstrate the utility of eQTL mapping in the identification of novel asthma genes and provide evidence for the importance of *FADS2*, *NAGA*, and *F13A1* in the pathogenesis of asthma. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Asthma, CD4⁺ lymphocytes, regulatory variants, expression quantitative trait locus, haplotype, integrative genomics

Genetic factors influence a subject's asthma susceptibility, with heritability estimates ranging from 36% to 79%.¹ Among the various genetic mapping techniques used to identify the genetic determinants of complex diseases, genome-wide association studies (GWASs) have been most fruitful. Individual studies and 2 meta-analyses^{2,3} have identified common sequence variants in at least 10 genes that are consistently associated with asthma in children and adults, including the Zona pellucida-binding protein 2 (*ZBPBP2*), Gasdermin-B (*GSDMB*), ORM1-like protein 3 (*ORMDL3*) locus (17q), IL-1 receptor-like 1 (*IL1RL1*) (2q), *IL33* (9p), and thymic stromal lymphopoietin (*TSLP*) (5q). Although some age- and ethnicity-specific trends were noted, these loci are widely reproducible and represent important advances in our understanding of asthma. However, as with virtually all complex diseases, the identified asthma loci explain only a small proportion of the total estimated genetic risk,⁴ suggesting that novel approaches are required to identify other genetic variants underlying this "missing heritability."

Expression quantitative trait locus (eQTL) mapping seeks to identify genetic variants that regulate gene expression by considering the distributions of gene transcript abundance

Abbreviations used

Asthma BRIDGE:	Asthma Biorepository for Integrative Genomic Exploration
CAMP:	Childhood Asthma Management Program
ChIP:	Chromatin immunoprecipitation
eQTL:	Expression quantitative trait locus
eSNP:	Expression-associated variant
<i>F13A1</i> :	Factor XIII, A1
<i>FADS1</i> :	Fatty acid desaturase 1
<i>FADS2</i> :	Fatty acid desaturase 2
FAIRE:	Formaldehyde-assisted isolation of regulatory elements
FDR:	False discovery rate
GWAS:	Genome-wide association study
LD:	Linkage disequilibrium
<i>NAGA</i> :	N-acetyl- α -D-galactosaminidase
PUFA:	Polyunsaturated fatty acid
qPCR:	Quantitative PCR
SNP:	Single nucleotide polymorphism

for thousands of genes (measured by using microarrays) as individual quantitative traits.⁵ From more than 20 such studies, expression-associated variants (eSNPs) have been mapped for thousands of expressed genes, explaining, on average, 5% to 15% of the observed variation in gene expression. Although many eSNPs demonstrate widespread effects across diverse tissue types, tissue-specific patterns are often observed. Importantly, we and others have demonstrated that compared with random single nucleotide polymorphisms (SNPs), identified eSNPs are more likely to be associated with complex disease traits.^{6,7} In a study of the HapMap lymphoblastoid cell lines, Nicolae et al⁶ reported an approximately 2-fold enrichment for eSNPs among disease-associated variants. Similarly, in primary peripheral blood CD4⁺ T cells, we found the prevalence of eSNPs among disease-associated SNPs was 63% higher than the genome-wide average.⁷ These findings suggest that eQTL identification could be leveraged to prioritize SNPs for association testing and for localization of functional genetic variants.

Given the central role of the CD4⁺ lymphocyte in allergic responses and the pathogenesis of asthma,^{8,9} we hypothesized that eSNPs identified from CD4⁺ lymphocytes would be enriched for asthma-susceptibility variants, thus enabling identification of novel variants and their associated candidate genes from existing GWAS data. We tested this hypothesis in a study of childhood asthma, the results of which we report here. Some of the results of this study have been previously reported in the form of an abstract.¹⁰

METHODS

We provide a brief description of the cohorts below, with details shown in the supplementary text in this article's Online Repository at www.jacionline.org. The Institutional Review Board of Brigham and Women's Hospital approved these studies. Subject recruitment and procedures for the cohorts have been previously described.^{7,11,12}

The Childhood Asthma Management Program: Discovery population

The Childhood Asthma Management Program (CAMP) was a multicenter, randomized, double-blind, placebo-controlled clinical trial established to investigate the effects of inhaled anti-inflammatory asthma medications.¹¹

Children enrolled in CAMP had mild-to-moderate persistent asthma based on the demonstration of airway responsiveness to methacholine ($PC_{20} \leq 12.5$ mg/mL) and at least 2 of the following: asthma symptoms at least 2 times per week, use of an inhaled bronchodilator at least twice weekly, or use of daily asthma medication for at least 6 months in the year before screening.¹¹ DNA was available for 403 parent-child trios and an additional 66 singletons of self-reported non-Hispanic white ancestry with mild-to-moderate persistent asthma, and therefore 469 self-reported white CAMP subjects were genotyped with Illumina Infinium II HumanHap 550 or Human610W-Quad BeadChips (Illumina, San Diego, Calif). Nonasthmatic control subjects were selected from 1533 subjects in the Illumina's iControlDB resource, as previously described.¹³ We generated a genetically matched case-control cohort using genetic matching,¹⁴ resulting in a final data set of 359 asthmatic patients and 846 control subjects and 547,497 SNPs.

In 200 CAMP participants we collected peripheral blood $CD4^+$ lymphocyte RNA and performed a genome-wide eQTL analysis for cis-acting eSNPs, as previously described in detail.⁷ Expression profiling was performed with the Illumina Human Ref8 v3 array. Similar to observations by others, our prior eQTL analysis demonstrated a precipitous decrease in the prevalence of cis-acting eQTLs away from the target gene locus, with a substantial increase in false discovery rates (FDRs) when considering variants more than 50 kb from the transcript.^{6,7} Thus to limit the potential for type I error inflation, we limited our search space to SNPs with frequencies of 10% or greater situated within 50 kb of the transcript. This analysis resulted in the identification of 6706 cis-acting eSNPs regulating 1585 genes.⁷ Given our prior studies demonstrating enrichment of susceptibility variants for eSNPs, in this study we prioritized these 6706 variants for asthma association testing in the CAMP cohort. An overview of our study design is shown in Fig E1 in this article's Online Repository at www.jacionline.org.

CAMP: Asthma susceptibility eSNP association testing

Case-control asthma association analysis was performed for the 6706 eSNPs detected in $CD4^+$ lymphocytes by using PLINK,¹⁵ with EIGENSTRAT adjustment,¹⁶ and retested in a subset of 403 available parent-child trios using PBAT. Haplotype association testing was conducted with Haploview (for asthma) and *haplo.score* (for expression traits).

Replication of genetic associations in the Genetic Epidemiology of Asthma in Costa Rica Cohort

Replication studies were performed in 579 parent-child trios recruited as part of the Genetic Epidemiology of Asthma in Costa Rica cohort. Details on subject recruitment and study protocols have been published elsewhere.¹² In brief, children aged 6 to 14 years were included in the study if they had asthma (a physician's diagnosis of asthma and ≥ 2 respiratory symptoms or asthma attacks in the previous year) and a high probability of having 6 or more great-grandparents born in the Central Valley of Costa Rica, which increased the likelihood that children would be descendants of the founder population of the Central Valley.¹²

Only the variants that demonstrated a nominal association with asthma in CAMP ($P < .01$) were tested for association with asthma susceptibility in the Costa Rican cohort by using family-based methods in PBAT. The transmitted/untransmitted ratio for the significant associations were identified in PLINK. Results were considered significant when identical associations (ie, same allele, phenotype, and direction of genetic effect) were identified in both populations. The Fisher combined P value method was applied to assess the cumulative significance of association across populations.¹⁷

Validation: Expression of novel asthma genes between asthmatic patients and nonasthmatic control subjects in Asthma Biorepository for Integrative Genomic Exploration

Expression of the fatty acid desaturase 2 (*FADS2*), N-acetyl- α -D-galactosaminidase (*NAGA*), and Factor XIII, A1 (*F13A1*), genes was compared

TABLE I. Baseline phenotypic characteristics of index children in the CAMP and Costa Rican cohorts

Variable	CAMP (n = 359)	Costa Rica (n = 579)
Age (y)	8.8 (2.1)	9.1 (1.8)*
Female sex	137 (38%)	222 (41%)
Height (cm)	132.7 (13.3)	131.0 (11.2)
Baseline prebronchodilator FEV ₁ (L)	1.6 (0.5)	1.7 (0.5)
Baseline prebronchodilator FEV ₁ /FVC ratio (%)	79.1 (8.3)	82.6 (7.2)

FVC, Forced vital capacity.

*Mean (SD) or count (frequency) was reported.

between asthmatic patients (n = 300) and nonasthmatic control subjects (n = 122) based on gene expression profiling in $CD4^+$ lymphocytes from the Asthma Biorepository for Integrative Genomic Exploration (Asthma BRIDGE). Asthma BRIDGE is a multicenter collaborative effort to develop well-characterized translational genomic datasets for asthma in North America.¹⁸ Samples were collected through October 2011 from among more than 14,000 subjects studied by the EVE Consortium, providing a broad representation of the North American asthmatic population. Genome-wide gene expression data (Illumina Human HT-12 v4 array) was generated from asthma-relevant primary cell types, including peripheral blood $CD4^+$ lymphocytes. Differential expression of *FADS2*, *NAGA*, and *F13A1* was determined by using a linear model adjusted for age, sex, and race with the limma package in Bioconductor.¹⁹

Functional validation: Chromatin enrichment studies

Human Beas-2B (no. CRL-9609; ATCC, Manassas, Va) and A549 (no. CCL-185, ATCC) cells were cultured in complete DMEM medium, and Jurkat (TIB-152, ATCC) cells were cultured in RPMI 1640 medium, all of which were supplemented with 10% FBS, penicillin (50 U/mL), streptomycin (50 μ g/mL), and gentamicin (10 μ g/mL). Additional details can be found in the [supplemental text](#) in this article's Online Repository.

For formaldehyde-assisted isolation of regulatory elements (FAIRE) and chromatin immunoprecipitation (ChIP), followed by real-time PCR, see details in the [supplemental text](#) in this article's Online Repository. The sequence of primers used in FAIRE quantitative PCR (qPCR) and ChIP-qPCR is listed in [Table E1](#) in this article's Online Repository at www.jacionline.org.

RESULTS

Asthma association testing of eSNPs

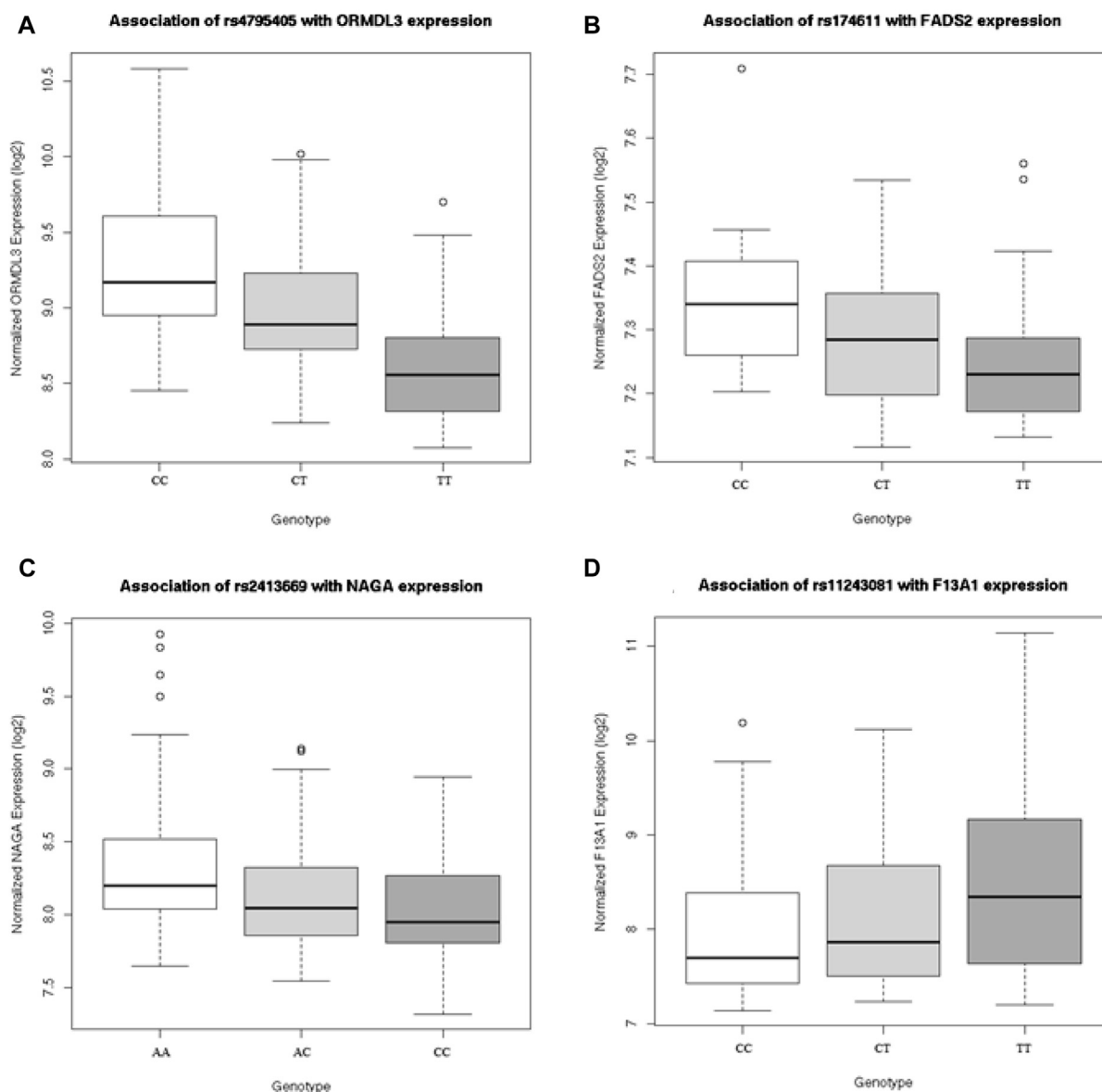
The baseline characteristics of the CAMP and Costa Rican index cases included in this study are presented in [Table I](#). Baseline characteristics were similar between the CAMP and Costa Rican cohorts, including measures of asthma severity and lung function.

Of the 6706 SNPs identified as $CD4^+$ lymphocyte cis-acting expression-associated eSNPs, 143 were associated with asthma under an additive genetic model in the CAMP cohort in either the case-control or family-based association analyses (see [Table E2](#) in this article's Online Repository at www.jacionline.org). To confirm their associations, we next genotyped these 143 variants in the Costa Rican cohort. Associations were reproduced in this independent, ethnically distinct cohort for multiple variants in 4 genes ([Table II](#)). First, we were able to replicate the association of the chromosome 17q *ORMDL3/GSDML* locus with asthma.²⁰ Specifically, 2 variants (rs4795405 and rs7216389) were strongly associated with asthma in both cohorts (Fisher

TABLE II. Replicated associations of eQTLs with asthma susceptibility in the CAMP and Costa Rican cohorts under an additive genetic model

Test of association	Gene	SNP	Chromosome	Distance (kb from transcript)	eQTL <i>P</i> value (FDR)	Minor allele	Association <i>P</i> value				Fisher combined <i>P</i> value
							CAMP	OR	Costa Rica	OR	
Case-control	<i>ORMDL3</i>	rs4795405	17	−4.6	3.0×10^{-10}	T	.0007	0.73	2×10^{-5}	0.63	2.9×10^{-8}
	<i>NAGA</i>	rs2413669	22	40.8	.0003	C	.0009	0.72	.02	0.82	.0002
Family based	<i>FADS2</i>	rs174611	11	Promoter	.0007	C	.009	1.33	.02	1.2	.002
	<i>F13A1</i>	rs11243081	6	−16.6	.0009	T	.003	1.37	.003	1.32	.0001

OR, Odds ratio.

**FIG 1.** Genome-wide significant eQTLs previously identified in CD4⁺ lymphocytes of asthmatic patients. **A**, Association of SNP rs4795405 with *ORMDL3* expression. **B**, Association of SNP rs174611 with *FADS2* expression. **C**, Association of SNP rs2413669 with *NAGA* expression. **D**, Association of rs11243081 with *F13A1* expression.

combined $P = 2.9 \times 10^{-8}$ and 3.4×10^{-7} , respectively). The T allele of rs4795405 was associated with decreased *ORMDL3* expression (Fig 1, A)⁷ and confers a decreased risk of

asthma in 2 independent cohorts. As previously reported, these variants reside on a haplotype that includes rs1293623, a functional regulatory polymorphism that alters DNA-binding

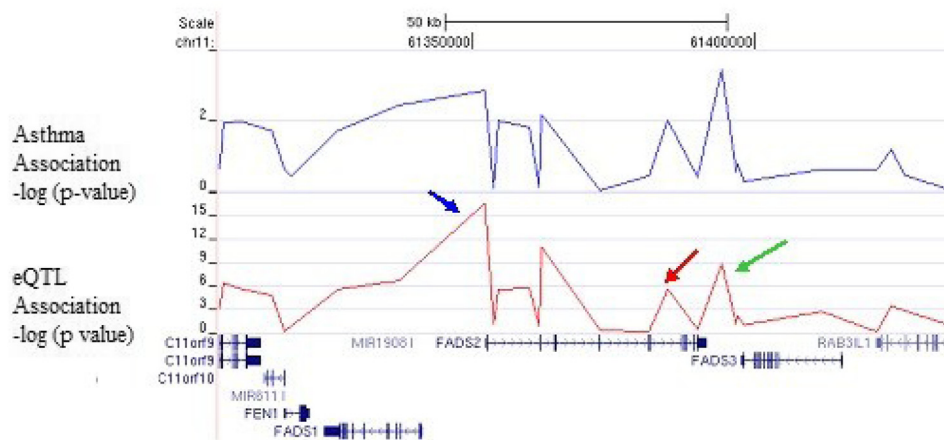


FIG 2. SNP-based eQTL analysis across the *FADS2* locus. SNP-based eQTL analysis across *FADS2*, demonstrating association of rs968567 with *FADS2* expression ($P = 10^{-15}$, 30% expression variance explained). SNP rs968567 (blue arrow), which is located in a conserved region of the promoter, demonstrates association with *FADS2* expression ($P = 10^{-15}$). SNPs rs174627 (green arrow) and rs174611 (red arrow) are in LD with rs968567 and are associated with *FADS2* expression and asthma susceptibility.

affinity with the insulator CTCF, resulting in differential expression of 3 surrounding genes: *ORMDL3*, *GSDML*, and *ZBP2*.²¹

We also found consistent association in both cohorts for 3 loci never previously associated with asthma susceptibility: the fatty acid desaturase locus (fatty acid desaturase 1 [*FADS1*]/*FADS2*) on 11q, N-acetyl galactosaminidase (*NAGA*) on chromosome 22q, and the *F13A1* locus on chromosome 6p. On chromosome 11q12.2, the minor C allele of rs174611 (C) is associated with increased expression of 2 adjacent members of the fatty acid desaturase gene cluster (*FADS1* and *FADS2*; FDR: $P = .001$ and $P = .0007$, respectively; Fig 1, B). The C allele of this variant confers an increased risk of asthma in both the CAMP and Costa Rican cohorts (Fisher combined $P = .002$). On chromosome 22q13.2, the C allele of SNP rs2413669, which is located approximately 40 kb upstream of the transcription start site of the α -N-acetylgalactosaminidase precursor gene (*NAGA*), was associated with decreased *NAGA* expression (FDR: $P = .0003$; Fig 1, C), as well as with decreased odds of asthma in both cohorts (Fisher combined $P = .0002$). On chromosome 6p25.1, the T allele of variant rs11243081 was associated with increased expression of the coagulation factor *F13A1* (FDR: $P = .0009$; Fig 1, D), as well as with increased asthma susceptibility, in both cohorts (Fisher combined $P = .0001$). Of note, the magnitudes of the effect for each of these genetic variants on asthma susceptibility were similar in both cohorts.

Haplotype testing of asthma-associated regulatory loci

Using genome-wide genotypic data available in CAMP subjects, we next tested haplotype blocks for each of the 3 novel genes identified in this study for association with gene expression and asthma susceptibility. Fig 2 shows the results of the association analyses of the *FADS2* locus. SNP rs968567, which is located in a conserved promoter region of the *FADS2* locus, was associated with *FADS2* expression ($P = 10^{-15}$), explaining 30% of its variance. In addition, the minor allele of SNP rs174611, which is associated with asthma in the CAMP and Costa Rican cohorts, explains 11% of the variance in *FADS2* gene expression

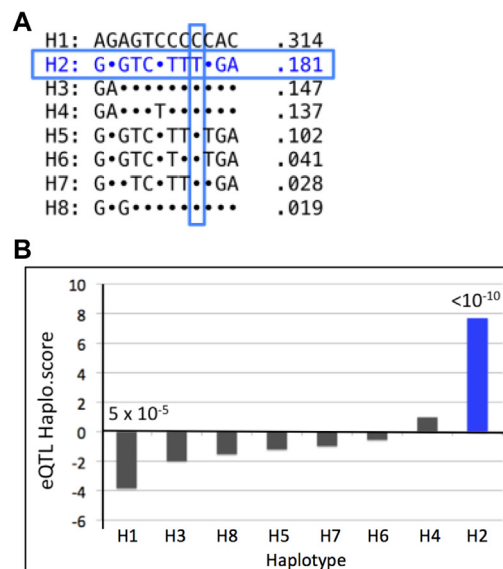


FIG 3. Haplotype analysis of the *FADS2* locus. **A**, Haplotype structure. Haplotype frequencies observed in at least 1% of CAMP subjects are listed to the right of each haplotype sequence. Dots indicate the allele present in haplotype H1, the most common haplotype. The asthma-associated rs968567 T allele is unique to and uniquely tags haplotype 2 (H2, boxed in blue), which is observed at 18.1% frequency. **B**, Haplotype-based eQTL analysis. H2 demonstrates strong association with increased *FADS2* expression ($P < 10^{-10}$), whereas haplotype 1 (H1; frequency, 31.4%) is associated with low *FADS2* expression. Other haplotypes (H3-H8) showed intermediate levels of *FADS2* expression.

($P = .0007$) and 5.3% of the expression in *FADS1* ($P = .03$). Fig 3, A, shows the haplotype structure of the *FADS2* locus. The T allele of SNP rs968567 uniquely tags a common haplotype in block 2 (H2), which is observed in 18.1% of the subjects. SNPs rs174627 and rs174611 are in linkage disequilibrium (LD) with SNP rs968567; all 3 of these variants and the *FADS* H2 haplotype are associated with asthma susceptibility in the CAMP cohort ($P = .001$ to 3×10^{-4}). Furthermore, as seen in Fig 3, B, haplotype 2 is associated with increased *FADS2* expression

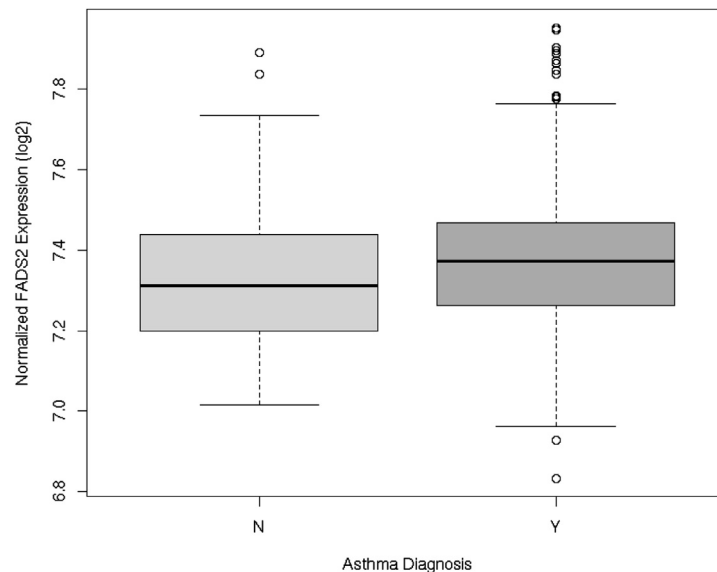


FIG 4. Differential expression of *FADS2* in asthmatic patients compared with nonasthmatic control subjects in Asthma BRIDGE. Significantly increased expression of *FADS2* in peripheral blood CD4⁺ lymphocytes of asthmatic patients compared with nonasthmatic control subjects ($P = .003$) is shown.

($P = 10^{-10}$), suggesting that this is a functional regulatory haplotype in the *FADS* region.

In contrast, the genomic region of eQTL association for *F13A1* is relatively narrow (see Fig E2 in this article's Online Repository at www.jacionline.org), despite its relatively large gene size (177 kb). Although there are 3 haplotype blocks demonstrating eQTL association (see Table E4 in this article's Online Repository at www.jacionline.org), only 1 block (block 9) is strongly associated with both *F13A* expression ($P = .0007$) and asthma ($P = .003$).

In the *NAGA* association analysis the minor allele of SNP rs2413669 explains 6% of the variation in *NAGA* expression ($P = .0003$, see Fig E3 in this article's Online Repository at www.jacionline.org). However, unlike the *FADS* and *F13A1* loci, haplotype block analysis did not help localize the functional variant: haplotype blocks in *NAGA* were not significantly associated with either asthma or *NAGA* expression ($P \geq .05$), suggesting the functional regulatory variant likely resides on more than 1 haplotype in LD with the asthma-associated eSNP rs2413669.

Validation: Differential *FADS2* expression in asthmatic patients in Asthma BRIDGE

Having demonstrated that variants that regulate gene expression in CD4⁺ lymphocytes were reproducibly associated with asthma, we next assessed whether the expression of target genes was similarly correlated with asthma status. Therefore we assessed the expression of *FADS2*, *NAGA*, and *F13A1* in 300 asthmatic patients and 122 nonasthmatic control subjects participating in Asthma BRIDGE with available expression data in peripheral blood CD4⁺ lymphocytes (see Table E3 in this article's Online Repository at www.jacionline.org for baseline characteristics of the cohort used for this analysis). Of the 3 genes evaluated, *FADS2* mRNA expression was significantly increased in asthmatic patients compared with that seen in nonasthmatic control subjects ($P = .003$, Fig 4). *FADS1* expression was also modestly increased in asthmatic patients compared with that seen in control subjects ($P = .04$), and a trend toward increased *NAGA* expression was

also observed in asthmatic patients ($P = .05$). There was no evidence of asthma-related differential expression of *F13A1* ($P = .60$).

Functional validation: Open chromatin enrichment studies

The expression-associated regulatory variants identified by using eQTL mapping are often surrogates for functional regulatory variation situated in neighboring regulatory DNA regions.^{22,23} However, given that the *FADS2* rs968567 regulatory variant uniquely tags the asthma-associated H2 haplotype and the *NAGA* rs2413669 variant (but not *NAGA* haplotypes) is associated with asthma and *NAGA* expression, it is possible that these 2 disease-associated eSNPs are not merely surrogates or tagging variants but actually represent the functional variants regulating target gene expression, thus driving the asthma association. To assess this, we first screened for active regulatory activity at the 3 loci using FAIRE-qPCR, a method used to detect open chromatin regions devoid of nucleosomes.²⁴ Given that the eQTL analysis was performed in CD4⁺ T cells, we chose the Jurkat acute T-cell leukemia cell line in addition to 2 respiratory epithelial cell lines: Beas-2B (human bronchial epithelial cells) and A549 (human alveolar type II-like pneumocyte). Three SNPs that showed association with asthma in each region were chosen for open chromatin assessments in these cell lines. As shown in Fig 5, the *FADS2*-associated SNP rs968567 showed approximately 15-fold enrichment of FAIRE signals in A549 and Jurkat cells and approximately 5-fold enrichment in Beas-2B cells compared with the negative control region ($P < .01$, unpaired *t* test), which is consistent with the previous finding that rs968567 is a functional SNP with differential binding to the transcription factor ETS domain-containing protein Elk-1 (ELK-1).²⁵ Furthermore, in the *NAGA* association region we observed strong enrichment of FAIRE signals near rs1801311 in Jurkat cells, as well as in Beas-2B and A549 cells. In contrast, FAIRE signals in SNPs around the *F13A1* region were relatively weak, suggesting these SNPs are not themselves functional but rather tag the causal variants within block 9.

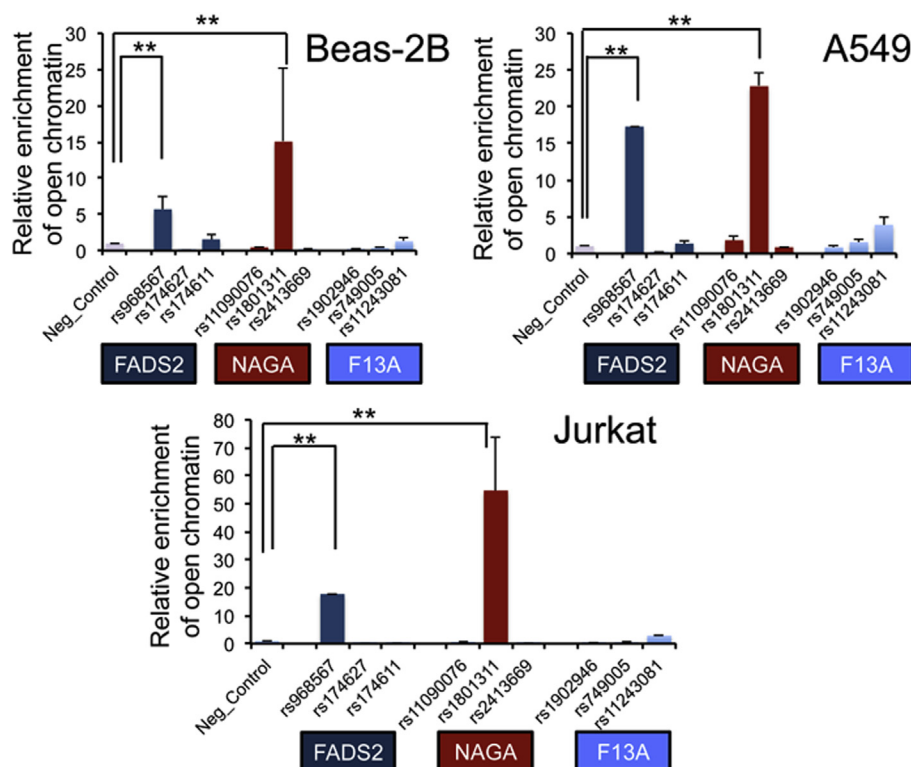


FIG 5. Functional annotation of the 3 asthma-associated regions by using FAIRE-PCR analysis. FAIRE was performed at 3 association regions near the *FADS2*, *NAGA*, and *F13A* genes in the Beas-2B, A549, and Jurkat cell lines by using real-time PCR analysis. The graph depicts the relative FAIRE signal at each locus normalized to input and negative control regions. Each locus contains 3 SNPs near eQTL signals. Means $\pm$ SDs are from 2 to 4 repeats for each SNP. ** $P < .01$, unpaired 1-way t test.

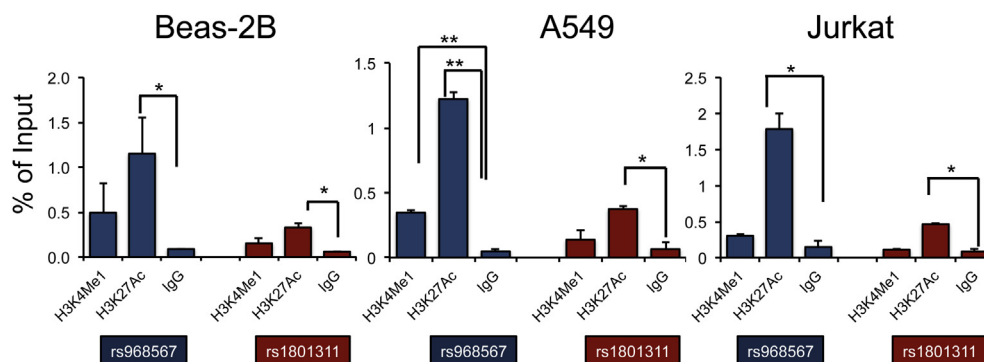


FIG 6. Enrichment of active histone marks in 2 asthma-associated regions detected by using ChIP-PCR analysis. ChIP was performed with H3K4Me1 and H3K27Ac antibodies in Beas-2B, A549, and Jurkat cell lines targeting genomic regions near 2 SNPs, rs968567 and rs1801311, in *FADS2* and *NAGA* loci, respectively. IgG was used as a negative control. Bars represent the average of 2 biological replicates with SDs. * $P < .05$ and ** $P < .01$, paired 1-way t test.

Epigenetic modifications on histone tails of nucleosomes usually indicate active or repressive transcriptional regulations.²⁶ To further confirm our finding about potential regulatory SNPs indicated by using FAIRE, we applied ChIP-PCR with antibodies targeting 2 enhancer markers, H3K4Me1 (indicating enhancer regions) and H3K27Ac (indicating active enhancer regions, promoter regions, or both), in the *FADS2* and *NAGA* regions in Beas-2B, A549, and Jurkat cells (Fig 6). Consistent with FAIRE enrichment signals, H3K27Ac showed significant enrichment at 2

SNPs we tested in *FADS2* and *NAGA* regions in all 3 cell lines, indicating active regulatory elements are contained in both the *NAGA* and *FADS2* regions near eSNPs.

DISCUSSION

Although GWASs of complex traits have offered insight into the genetic underpinnings of complex diseases, such as asthma, the discovered variants explain only a fraction of the genetic

contribution to such diseases.⁴ In part this is likely due to the stringent multiple-comparison burden imposed when testing millions of (mostly functionless) variants. Strategies that guide prioritization of functional genetic markers for testing can address this issue. We and others have demonstrated that eSNPs are more likely to be disease-associated variants,^{6,7} suggesting eQTL mapping as a promising strategy. Using asthma as a model, we sought to evaluate this possibility by testing cis-acting regulatory variants previously identified from an eQTL mapping study of peripheral blood CD4⁺ lymphocytes for association with asthma in 2 well-characterized populations. Using this integrative genomic approach in CAMP subjects, we have not only replicated the association of the 17q *ORMDL3/GSDMB* locus for association with asthma in 2 childhood asthma cohorts but have also defined 3 novel candidate loci. These later associations did not emerge from prior studies because their nominal association *P* values were less than the threshold for multiple comparisons correction. Although our application of a functional prioritization strategy using eQTL data led to consideration and successful independent replication of these loci in the Costa Rican cohort, supporting application of such strategies for identification of novel disease candidate genes, they will require follow-up replication testing to assess whether the results are generalizable. We also recognize that the relatively small size of our test and replication cohorts might have precluded identification of additional asthma variants because of limits in statistical power. Application of eQTL approaches to larger populations is warranted. In addition, consideration should be extended beyond cis-acting regulatory variants once adequately powered studies identify validated, reproducible transacting eQTLs.

Of 3 novel genes identified in this study, the greatest biological and epidemiologic support is provided for *FADS2* and the fatty acid desaturase gene cluster on chromosome 11. The *FADS1* and *FADS2* genes, respectively, encode for $\Delta 5$ - and $\Delta 6$ -desaturase, enzymes that are critical for biosynthesis of long-chain polyunsaturated fatty acids (PUFAs), which are the precursors of eicosanoid mediators that are critical to the development and resolution of allergic inflammation.^{27,28} PUFAs are synthesized from 2 essential fatty acids: linoleic acid (18:2n-6) and α -linolenic acid (18:3n-3). Desaturase enzymes introduce double bonds between defined carbons of the fatty acyl chain. $\Delta 6$ -desaturase (*FADS2*) catalyzes the first rate-limiting step in long-chain PUFA biosynthesis by causing desaturation of linoleic acid and α -linolenic acid. In our study we demonstrate association of eSNPs in *FADS2* with asthma in 2 cohorts and localize the most robust associations to a haplotype-tagging variant, rs968567, situated in the promoter of *FADS2*, which is strongly associated with *FADS2* (and, to a lesser degree, *FADS1*) expression. We also detected increased expression of *FADS2* in peripheral blood CD4⁺ lymphocytes of asthmatic patients in a large population sample, which is consistent with increased expression of *FADS2* conferred by asthma risk allele in this locus. In addition, rs968567 demonstrates strong enrichment for open chromatin status in both lymphocyte and bronchial epithelial cell lines. In hepatocytes this same variant exhibits differential binding of the ELK1 transcription factor.²⁵ This variant, and others in strong LD with it, have been strongly associated with circulating PUFAs,²⁹ total cholesterol and high-density lipoprotein levels, and omega-3 fatty acid levels.³⁰ Perhaps most pertinent in the context of

asthma pathogenesis, a recent GWAS study in a general population sample²⁹ found this *FADS2* promoter variant to explain 19% of the population variance in serum levels of arachidonic acid, the precursor for proinflammatory and anti-inflammatory prostaglandins and leukotrienes.²⁷

Because of their roles in the production of proinflammatory and anti-inflammatory eicosanoids and evidence for PUFAs in the alteration of T-cell antigen presentation,³¹ others have investigated the role of *FADS1/FADS2* polymorphisms in atopic disease, with variable results. In one candidate-gene birth cohort study of 879 children, *FADS* variants that were associated with PUFA and arachidonic acid serum levels were also associated with increased eczema risk.³² In contrast, these same variants were inversely associated with eczema and allergic rhinitis in an adult population.³³ In addition, recent work by Standl et al³⁴ demonstrated that genetic variants in the *FADS* cluster modify the effects of breast-feeding on the development of asthma, with the lowest risk noted in children who were exclusively breast-fed for the first 3 months of life. Although the biologic mechanism underlying this association is unclear, the authors speculate that carriers of the minor allele have lower fatty acid metabolism and thus less arachidonic acid levels.³⁴ Although replication of these interactions has yet to be reported, if true, such interactions could represent an important modifier of our observed associations between *FADS2* genotype and asthma susceptibility, posing greater challenges to replication in additional populations. Although none of these studies focused exclusively on asthma, making direct comparisons with our initial observations difficult, assuming a direct correlation between asthma and risk for other atopic diseases, our findings are consistent with those in the pediatric cohort, with high *FADS2* expression associated with increased asthma and atopic risk. The associations might be age dependent (explaining the different findings in adults) or modified by dietary PUFA intake. Although these possibilities must be tested in other populations, the evidence presented here, together with that from prior studies, supports *FADS2* as a biologically plausible asthma susceptibility gene.

In contrast to *FADS2*, the roles of *NAGA* and *F13A1* in asthma pathogenesis are less clear. *NAGA* is a lysosomal glycohydrolase that cleaves the α -N-acetylgalactosaminyl moieties from glycol conjugates. *NAGA* has been implicated as part of the carbohydrate-mediated mechanism of mast cell adhesion to the bronchial epithelium.³⁵ Treatment of mast cells of endo- α -N-acetylgalactosaminidase, an enzyme that cleaves disaccharides on the surface of the mast cell, results in a significant reduction in mast cell adhesion to bronchial epithelium and changes in airway responsiveness.³⁵ Moreover, a guinea pig model demonstrated increased *NAGA* released from bronchoalveolar macrophages in animals with increased airways responsiveness after high ozone exposure.³⁶ Although speculative, given the central role of macrophages and mast cell degranulation in bronchoconstriction, mucus secretion, and airway inflammation in asthmatic patients,³⁷ the asthma-associated *NAGA* eSNP might influence asthma risk through altered glycohydrolase activity.

F13A1 encodes the α subunit of Factor XIII, the last enzyme generated in the blood coagulation cascade, stabilizing blood clots by cross-linking fibrin. *F13A1* has also been implicated in the inflammatory cascade and in atopy.³⁸ When stimulated with dust mite antigen, PBMCs from patients with dust mite allergy demonstrate increased production of *F13A1*.³⁹ *F13A1* has also

been implicated as a susceptibility locus for obesity.⁴⁰ Epidemiologic data demonstrate a consistent link between asthma and obesity,⁴¹ with apparent shared genetic determinants.⁴² How *F13A1* regulatory variation confers susceptibility to asthma and whether *F13A1* represents a shared genetic determinant of both asthma and obesity remain unclear. In addition to replication in additional asthmatic populations, further characterization of the functional effect of these variants in model systems will be needed to resolve these questions.

Although we have demonstrated the utility of eQTL mapping for the identification of asthma-susceptibility variants, our studies were limited in scope, particularly in regard to the narrow focus on cis-acting regulatory variation in only one cell type: the CD4⁺ lymphocyte. Although this cell type is relevant to allergic cytokine profiles and asthma, it is only one of many cell types of potential interest. Given that a substantial proportion of cis-acting regulatory variants demonstrate tissue-specific effects, we have likely overlooked some functional variation specific to other relevant cell types, including antigen-presenting cells, granulocytes, airway smooth muscle, and bronchial epithelium. A recent eQTL study in human lung tissue samples provides complementary evidence at the 17q locus, suggesting a potential role for altered expression of *GSDMA*, in addition to the altered *ORMDL3* and *GSDMB* expression, as noted by us and others in lymphocytic lines.⁴³ Thus we view these initial efforts as the first step of a more integrative approach to disease gene mapping that considers eQTL variation across multiple tissue types, in diverse patient populations, and under disease-relevant environmental exposures and that also considers other functional annotations, including chromatin modification, CpG methylation differences, and other epigenetic modifications.⁴⁴ Such comprehensive, more holistic approaches, combined with powerful systems-based analytic tools, should provide a more complete picture of the genetic landscape in asthmatic patients.

In summary, we provide new evidence in support of *FADS2*, *NAGA*, and *F13A1* as asthma susceptibility loci. Similar to variants previously identified through GWASs and other genetic approaches, the relatively modest effect sizes conferred by these loci limit their clinical utility as diagnostic or prognostic markers. Instead, our observations motivate prioritization of these loci and their related pathways for further investigation. Of these, we find the strongest support for a common promoter polymorphism in the *FADS2* gene, which regulates *FADS2* expression, is associated with asthma and allergic phenotypes, and has been previously shown to be the major genetic determinant of plasma arachidonic acid levels, the major precursor of asthma-relevant eicosanoids. Although replication of these associations in additional populations is warranted, these findings should prompt revisiting this important pathway as a potential therapeutic asthma target.

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Key messages

- Integrative genomic approaches, including eQTL mapping, can be used to prioritize genetic variants for disease susceptibility testing.
- By using a genome-wide survey of regulatory genetic variants in peripheral blood CD4⁺ lymphocytes sampled from asthmatic patients, we identified novel asthma susceptibility loci, including *FADS1/FADS2*, *NAGA*, and *F13A1*.
- Functional variants can be further localized through a combination of eQTL mapping with assessments of chromatin status and modifications nearby eQTL SNPs.

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