

Roles of arginase variants, atopy, and ozone in childhood asthma

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Background: Arginases (encoded by *ARG1* and *ARG2* genes) might play an important role in asthma pathogenesis through effects on nitrosative stress. Arginase expression is upregulated in asthma and varies with T_H2 cytokine levels and oxidative stress.

Objective: We aimed to examine whether variants in these genes are associated with asthma and whether atopy and exposures to smoking and air pollution influence the associations.

Methods: Among non-Hispanic and Hispanic white participants of the Children's Health Study (n = 2946), we characterized variation in each locus (including promoter region) with 6 tag single nucleotide polymorphisms for *ARG1* and 10 for *ARG2*. Asthma was defined by parental report of physician-diagnosed asthma at study entry.

Results: Both *ARG1* and *ARG2* genetic loci were significantly associated with asthma (global locus level $P = .02$ and $.04$, respectively). Compared with the most common haplotype within each locus, 1 *ARG1* haplotype was associated with reduced risk (odds ratio [OR] per haplotype copy, 0.55; 95% CI, 0.36-0.84), and 1 *ARG2* haplotype was associated with increased risk (OR per haplotype copy, 1.35; 95% CI, 1.04-1.76) of asthma. The effect of the *ARG1* haplotype that was significantly associated with asthma varied by the child's history of atopy and ambient ozone ($P_{\text{interaction}} = .04$ and $.02$, respectively). Among atopic children living in high-ozone communities, those carrying the *ARG1* haplotype had reduced asthma risk (OR per haplotype copy, 0.12; 95% CI, 0.04-0.43; $P_{\text{heterogeneity across atopy/ozone categories}} = .008$).

Conclusions: *ARG1* and *ARG2* loci are associated with childhood asthma. The association between *ARG1* variation and asthma might depend on atopy and ambient ozone levels. (J Allergy Clin Immunol 2009;123:596-602.)

Key words: Air pollution, asthma genetics, atopy, gene-environment interaction, nitrosative stress

Abbreviations used

AHR:	Airway hyperresponsiveness
<i>ARG1</i> :	Arginase I gene
<i>ARG2</i> :	Arginase II gene
CHS:	Children's Health Study
MAF:	Minor allele frequency
NF:	Nuclear factor
NO:	Nitric oxide
NO ₂ :	Nitrogen dioxide
NOS:	Nitric oxide synthase
OR:	Odds ratio
PC:	Principal component
PM _{2.5} :	Particulate matter with an aerodynamic diameter 2.5 μm or less
PM ₁₀ :	Particulate matter with an aerodynamic diameter 10 μm or less
SNP:	Single nucleotide polymorphism

Both oxidative/nitrosative stress- and allergy-mediated airway inflammation play important roles in asthma pathogenesis.¹ Nitric oxide (NO) is a key mediator of nitrosative stress² because it can react with reactive oxygen species to form reactive nitrogen species (eg, peroxynitrite). Exhaled NO is also a marker of allergic airway inflammation.³ In addition to its role in airway inflammation, NO is also involved in bronchodilatation and apoptosis. NO is synthesized from L-arginine by nitric oxide synthases (NOSs); however, arginase competes with NOS and hydrolyzes L-arginine to urea and ornithine in the urea cycle. Increased arginase activity leads to the increased production of polyamines and proline, which are involved in cell hyperplasia and collagen deposition, respectively. Furthermore, increased arginase activity has been shown to inhibit NO synthesis.⁴

There are 2 isoforms of arginase, which are encoded on different chromosomes. Arginase I is encoded by *ARG1* and is located in the cytosol, whereas arginase II is encoded by *ARG2* and is located in the mitochondrial matrix.⁵ In human subjects both isoforms are expressed in airway epithelium, smooth muscle, and peribronchial and perivascular connective tissues,^{6,7} as well as in granulocytes, although not in macrophages.^{8,9}

A growing body of evidence suggests a role of arginase in asthma occurrence. Asthmatic subjects have significantly higher arginase activity than nonasthmatic subjects.¹⁰ Another study found 3-fold higher arginine levels within airway epithelial cells in asthmatic subjects compared with nonasthmatic control subjects.¹¹ Both *ARG1* and *ARG2* gene expressions have been reported to be upregulated in a mouse model of asthma.¹² Arginase I protein was highly expressed in bronchoalveolar lavage fluid cells of atopic asthmatic subjects compared with that seen in subjects with no atopy/asthma.¹² The role of few single nucleotide polymorphisms (SNPs) in *ARG1* and *ARG2* on asthma

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occurrence has been reported in only one study to date.¹³ Two highly correlated *ARG2* SNPs were associated with asthma, but no associations were found for *ARG1* SNPs. It is possible that failure to capture the haplotype diversity in these loci with few SNPs, smaller sample size available ($n = 433$), or both might have limited the opportunity to detect global associations between these genetic loci and asthma.

Based on experimental evidence, associations of arginase variants with asthma might depend on the airway inflammatory state and exposures that mediate oxidant stress, both of which are strong determinants of asthma. Arginase expression has been shown to be upregulated in murine models of asthma by IL-4 and IL-13,^{12,14,15} 2 of the T_H2 cytokines that have been strongly associated with atopic phenotypes. In addition to T_H2 cytokine-mediated allergic airway inflammation, exposures to tobacco smoke and ambient air pollutants result in oxidant stress-mediated airway inflammation and have been associated with asthma occurrence and exacerbations. Tobacco smoke has been shown to increase arginase I expression in airway epithelium among asthmatic subjects compared with that seen in nonsmoking asthmatic subjects.⁶ In this study arginase I expression was found in airway smooth muscle in smokers but not in nonsmokers. Among ambient air pollutants, exposure to high levels of ozone has been also been shown to produce oxidative stress and inflammation, which can modulate NOS isoform expression.¹⁶ Therefore it is plausible that the mechanism for the variation of the association of *ARG1* and *ARG2* variants with asthma in children with different ambient air pollutant exposures (eg, ozone and particulate matter with an aerodynamic diameter less than $2.5 \mu\text{m}$ [$\text{PM}_{2.5}$]) could be in part due to differences in arginase isoform expression.

Based on this evidence, we aimed to test 2 hypotheses: (1) variations in the *ARG1* and *ARG2* genetic loci are globally associated with childhood asthma, and (2) these associations vary by atopy, tobacco smoke exposures (*in utero* exposure to maternal smoking and exposure to secondhand smoke in early childhood), and ambient air pollutants. We tested these hypotheses in a population-based study conducted among non-Hispanic and Hispanic white children who had participated in the Children's Health Study (CHS).

METHODS

Study design and participants

The CHS recruited children attending public school in the 4th, 7th, and 10th grades in 12 southern California communities in 1993 and 1996. The communities were selected primarily on the basis of wide regional variations in ambient pollution levels. At study entry, parents or guardians of each participating student completed a self-administered questionnaire, which included detailed information about demographic and household characteristics, as well as information regarding the child's respiratory health. Details about the CHS have been described.^{17,18} The University of Southern California Institutional Review Board reviewed and approved the study. The present analysis included 2,946 children who were classified as either Hispanic ($n = 936$) or non-Hispanic ($n = 2,010$) white with known asthma status and complete genotypic data for *ARG1* and *ARG2* single nucleotide polymorphisms (SNPs).

Assessment of outcome and exposures

Children were classified as having asthma if the adult completing the questionnaire reported that a doctor had "ever diagnosed the child as having asthma." The child's atopic status was based on parental report of any allergy, hay fever, or both. A child's exposure to maternal smoking *in utero* was based on smoking by the biologic mother during pregnancy. Secondhand exposure to

smoking was based on exposure to smokers inside the house during childhood. Ambient levels of ozone, nitrogen dioxide (NO_2), particulate matter with an aerodynamic diameter of $10 \mu\text{m}$ or less (PM_{10}) and $2.5 \mu\text{m}$ or less ($\text{PM}_{2.5}$), acid vapor, and elemental and organic carbon were measured at air-monitoring sites in each of the 12 communities from 1992 onward, as previously described.^{17,18} Based on the median of average pollutant concentrations across the study communities, we categorized each exposure into high and low pollutant levels. Because PM_{10} , $\text{PM}_{2.5}$, NO_2 , acid vapor, and elemental and organic carbon were highly correlated and ozone was not significantly correlated with the former pollutants (see Table E1 in this article's Online Repository at www.jacionline.org) and because communities defined as being in the "high" or "low" pollution categories based on any of the nonozone pollutants were the same for all of the correlated pollutants, we only examined whether the associations between *ARG1* and *ARG2* haplotypes and asthma varied by high/low ozone and $\text{PM}_{2.5}$ categories.

SNP selection and genotyping

In representative non-Hispanic and Hispanic white samples from the multiethnic cohort ($n \sim 71$ each),¹⁹ 1 to 3 SNPs per kilobase were genotyped by using the Illumina Golden Gate Assay (Illumina, Inc, San Diego, Calif) to determine ethnic-specific minor allele frequencies (MAFs) and patterns of linkage disequilibrium around the 20-kb upstream and 10-kb downstream region unless another gene was located within this region. The minimum set of haplotype-tagged SNPs with MAFs of 0.05 or greater was chosen to explain more than 90% of haplotype diversity (coefficient of determination [R^2] ≥ 0.90) for each haplotype block by using the TagSNPs program (available at <http://www-hsc.usc.edu/~stram/tagSNPs.html>).²⁰ Redundant tagSNPs were genotyped to substitute for critical SNPs in the event of assay failure in the latter. SNPs were genotyped by using the Illumina BeadArray platform. Based on these criteria, we selected 6 SNPs in *ARG1* and 10 SNPs in *ARG2* for our analyses (Table I). These SNPs had call rates of greater than 90%. Details of buccal sample collection and the quality control protocol are presented in the Methods section in this article's Online Repository at www.jacionline.org.

Haplotype estimation

For each gene, high multiallelic D' (which represents the level of recombination between adjacent haplotype blocks) values between haplotype blocks allowed estimation of common haplotypes ($>5\%$ frequency in either ethnic group) across the gene using all SNPs within each locus. Haplotype frequencies of unphased *ARG1* and *ARG2* SNPs were estimated separately for Hispanic and non-Hispanic white subjects by using an SAS macro code available with the TagSNPs program. This haplotype estimation technique provides the maximum likelihood estimates of the haplotype frequencies assuming Hardy-Weinberg equilibrium.²⁰

Statistical analysis

Initially, the global association between each genetic locus and asthma was determined by using haplotypes and principal components (PCs) analyses²¹ in the overall sample, adjusting for ethnicity and other potential confounders. If the locus was significantly associated with asthma, ethnic-specific analyses were conducted to examine whether the results varied by ethnicity. Subsequently, to further investigate the global associations, we conducted SNP-based analyses to determine whether specific SNPs could account for the locus-level significance. To address multiple comparison issues across correlated SNPs, we adjusted the P values for significant SNP-based results at the locus level by using the p_{ACT} procedure (available at http://csg.sph.umich.edu/boehnke/p_act.php).²² This procedure has been shown to provide a valid adjustment of P values for multiple testing similar to the permutation test but uses significantly less computational time. Finally, modifying effects of atopy, tobacco smoke, and air pollution exposures on the associations between risk haplotypes and asthma were evaluated.

We used an additive genetic model for the SNPs for the PC- and SNP-based analyses and for haplotypes for haplotype-based analyses. In the PC-based approach, PCs (ordered by magnitude of explained variance) that explained at

TABLE I. Minor allele and haplotype frequencies of *ARG1* and *ARG2* by ethnicity

		MAF		Haplotype frequency		
Gene, SNPs	Alleles*	Non-Hispanic white	Hispanic white	Gene, haplotypes†	Non-Hispanic white	Hispanic white
ARG1				ARG1		
RS2608981	A/C	0.35	0.22	000000 (<i>ARG1h1</i>)	0.57	0.43
RS3895535	G/A	0.25	0.18	111111 (<i>ARG1h2</i>)	0.13	0.21
RS2608937	G/A	0.25	0.17	000110 (<i>ARG1h3</i>)	0.11	0.08
RS2749935	A/T	0.49	0.38	000100 (<i>ARG1h4</i>)	0.05	0.05
RS2781659	A/G	0.41	0.32	100110 (<i>ARG1h5</i>)	0.04	0.10
RS2246012	T/C	0.27	0.16	000001 (<i>ARG1h6</i>)	0.02	0.05
				Other haplotypes‡	0.08	0.08
ARG2				ARG2		
RS12885261	C/T	0.41	0.59	1000100000 (<i>ARG2h1</i>)	0.28	0.16
RS7144243	A/G	0.17	0.21	0000000010 (<i>ARG2h2</i>)	0.16	0.16
RS3759757	G/C	0.36	0.26	1000000010 (<i>ARG2h3</i>)	0.13	0.08
RS4902501	T/C	0.35	0.26	0000000100 (<i>ARG2h4</i>)	0.12	0.11
RS7156352	C/T	0.19	0.31	1111011101 (<i>ARG2h5</i>)	0.09	0.05
RS4902503	A/G	0.42	0.26	0111010100 (<i>ARG2h6</i>)	0.04	0.05
RS7140310	T/G	0.16	0.14	0011010101 (<i>ARG2h7</i>)	0.04	0.15
RS742869	G/A	0.56	0.40	0000011101 (<i>ARG2h8</i>)	0.003	0.06
RS3742879	A/G	0.25	0.30	Other haplotypes‡	0.14	0.18
RS10483801	C/A	0.31	0.19			

*Alleles are presented as major/minor alleles. For SNP location and distance between SNPs, see Table E2.

†SNP order in *ARG1* haplotypes is RS2608981-RS3895535-RS2608937-RS2749935-RS2781659-RS2246012 and in *ARG2* haplotypes is RS12885261-RS7144243-RS3759757-RS4902501-RS7156352-RS4902503-RS7140310-RS742869-RS3742879-RS10483801. Within each haplotype, 0 and 1 represent wild-type and variant alleles at the ordered SNP position, respectively. Each haplotype was abbreviated, with the gene name appearing first followed by a number.

‡Haplotypes with less than 5% frequencies are combined into the “other haplotypes” category.

least 80% of the SNP variance within the locus were used to test locus-level significance. This approach, which has been assessed previously,²¹ captures the underlying linkage disequilibrium and correlations among the SNPs within the locus. In the haplotype-based approach, uncommon haplotypes (frequencies <0.05 in both ethnic groups) were combined into an “other haplotypes” category.

Logistic regression models were fitted to compute odds ratios (ORs) and 95% CIs. Age, sex, ethnicity, child's history of atopy, parental asthma, parent/guardian's education, *in utero* exposure to maternal smoking, health insurance coverage, and community of residence were considered as potential confounders. The modifying effects of tobacco smoke exposures, ambient air pollutants, and atopy on the associations between haplotypes and asthma were evaluated by using likelihood ratio tests with appropriate interaction terms. All tests were 2-sided at a 5% significance level. We used the R program to implement the p_{ACT} procedure. The rest of the analyses were done with SAS version 9.1 (SAS Institute Inc, Cary, NC).

RESULTS

There were limited differences in minor allele and haplotype frequencies by ethnicity (Table I; for SNP location and distance between SNPs, see Table E2 in this article's Online Repository at www.jacionline.org). Haplotype block structure was similar for *ARG1* by ethnicity (see Fig E1 in this article's Online Repository at www.jacionline.org) but less so for *ARG2* (see Fig E2 in this article's Online Repository at www.jacionline.org). For *ARG1*, common haplotypes accounted for 92% of the haplotype frequencies in both ethnic groups. For *ARG2*, 86% and 82% cumulative haplotype frequencies were obtained with common haplotypes for non-Hispanic and Hispanic white subjects, respectively.

Boys were at higher asthma risk than girls (Table II). Atopy and parental asthma were positively associated with asthma. Children with asthma were more likely to have health insurance compared with children without asthma. The prevalences of asthma in Non-

Hispanic and Hispanic white children were comparable (15.7% vs 14.3%, $P = .32$).

ARG1 and asthma

Variations in the *ARG1* locus were globally associated with asthma (global $P = .04$, Table III) in the haplotype-based approach but not in the PC-based approach (see Table E3 in this article's Online Repository at www.jacionline.org). In addition, no *ARG1* SNP was associated with asthma (see Table E3). These findings suggest that a haplotype, rather than SNPs, might underlie the association with the locus. Compared with the most common *ARG1* haplotype that carried the wild-type allele for all studied SNPs, the haplotype that carried the variant allele for RS2749935 (ie, the *ARG1h4* haplotype) was associated with a 45% reduced risk of asthma (OR, 0.55; 95% CI, 0.36-0.84). These associations did not vary substantially by ethnicity.

ARG2 and asthma

Variations in the *ARG2* locus were globally associated with asthma in both haplotype (global $P = .02$, Table III) and PC-based (global $P = .01$, see Table E3) analyses. Each copy of the *ARG2h3* haplotype was associated with a 35% increased asthma risk (95% CI, 1.04-1.76) compared with the most common haplotype. Furthermore, the *ARG2h6* haplotype was associated with a 50% reduced asthma risk (OR, 0.50; 95% CI, 0.28-0.92) in non-Hispanic white subjects only. However, the *ARG1* and *ARG2* haplotype-specific ORs did not vary by ethnicity (both $P_{\text{interaction}} > .66$). In SNP-based analysis 4 of the 10 *ARG2* SNPs were associated with asthma in the combined population (see Table E3). After adjusting for multiple comparisons, each variant allele of RS3742879 was associated with a 31% increased asthma risk (adjusted $P = .007$).

TABLE II. Descriptive statistics of the study population*

	No asthma		Asthma		P value‡
	No.†	Percentage	No.†	Percentage	
Sex					
Girls	1376	55.1	201	44.7	<.0001
Boys	1120	44.9	249	55.3	
Age (y)					
≤10	1398	56.1	245	54.4	.45
11-12	468	18.7	79	17.6	
>12	630	25.2	126	28.0	
Ethnicity					
Non-Hispanic white	1694	67.9	316	70.2	.32
Hispanic white	802	32.1	134	29.8	
Parental history of asthma					
No	1950	82.8	247	57.8	<.0001
Yes	405	17.2	180	42.2	
Exposure to maternal smoking <i>in utero</i>					
No	2024	82.9	357	80.2	.17
Yes	418	17.1	88	19.8	
Exposure to secondhand smoke					
No	1710	70.9	292	66.5	.06
Yes	700	29.1	147	33.5	
Child's atopic status§					
No	1582	68.0	127	31.7	<.0001
Yes	743	32.0	274	68.3	
Annual family income (in US dollars)					
<15,500	303	14.1	48	12.1	.09
15,000-49,999	919	42.8	154	38.9	
≥50,000	924	43.1	194	49.0	
Parent/guardian education					
<12th grade	313	12.9	30	6.7	.002
12th grade	459	18.9	87	19.6	
Some college	1092	45.0	232	52.1	
College	246	10.1	44	9.9	
Some graduate	315	13.0	52	11.7	
Health insurance coverage					
No	378	15.4	35	7.9	<.0001
Yes	2078	84.6	406	92.1	

*Non-Hispanic and Hispanic white children in the CHS with complete data on *ARG1* and *ARG2* SNPs were included. For details, see the [Methods](#) section.

†Because of missing data, numbers do not always add up.

‡P values from Pearson χ^2 tests comparing children with asthma with those without asthma.

§Child's atopic status was determined based on parental report of allergy and/or hay fever.

Modifying the role of atopy, smoking, and ambient air pollutants

Atopy and ambient ozone modified the association between the *ARG1h4* haplotype and asthma ($P_{\text{interaction}} = .04$ and $.02$, respectively; [Table IV](#)) but did not influence the relationship of the *ARG2h3* haplotype and asthma. None of the SNPs or haplotypes in *ARG1* and *ARG2*, however, was associated with atopy. *In utero* and secondhand smoke exposures and ambient $\text{PM}_{2.5}$ categories (high/low) did not modify the associations between *ARG1* and *ARG2* haplotypes and asthma. Because 2 SNPs in *ARG2* (RS4902503 and RS3742879) showed significant associations (see [Table E3](#)), we further evaluated whether the associations of these SNPs with asthma varied by atopy, tobacco smoke, or air

TABLE III. Associations between *ARG1* and *ARG2* haplotypes and asthma

Gene, haplotypes	Non-Hispanic white	Hispanic white	Combined
	OR (95% CI)*	OR (95% CI)*	OR (95% CI)*
<i>ARG1</i> haplotypes†			
<i>ARG1h1</i>	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)
<i>ARG1h2</i>	0.96 (0.72-1.28)	0.85 (0.56-1.28)	0.93 (0.74-1.17)
<i>ARG1h3</i>	1.00 (0.74-1.36)	1.23 (0.76-1.98)	1.06 (0.82-1.37)
<i>ARG1h4</i>	0.61 (0.37-1.00)	0.41 (0.17-0.97)	0.55 (0.36-0.84)
<i>ARG1h5</i>	1.33 (0.85-2.09)	1.01 (0.57-1.77)	1.22 (0.87-1.72)
<i>ARG1h6</i>	0.83 (0.38-1.81)	0.57 (0.23-1.38)	0.69 (0.39-1.21)
Other haplotypes	0.96 (0.66-1.39)	0.99 (0.55-1.77)	0.95 (0.70-1.29)
Global P value‡	.39	.18	.04
<i>ARG2</i> haplotypes†			
<i>ARG2h1</i>	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)
<i>ARG2h2</i>	1.30 (0.97-1.73)	1.10 (0.67-1.82)	1.22 (0.96-1.56)
<i>ARG2h3</i>	1.38 (1.02-1.87)	1.28 (0.73-2.26)	1.35 (1.04-1.76)
<i>ARG2h4</i>	1.00 (0.72-1.39)	0.89 (0.50-1.61)	0.98 (0.74-1.30)
<i>ARG2h5</i>	0.86 (0.58-1.27)	0.68 (0.31-1.51)	0.83 (0.59-1.18)
<i>ARG2h6</i>	0.50 (0.28-0.92)	1.28 (0.60-2.71)	0.73 (0.47-1.15)
<i>ARG2h7</i>	0.74 (0.41-1.32)	0.96 (0.57-1.63)	0.88 (0.61-1.26)
<i>ARG2h8</i>	0.80 (0.09-7.18)	0.87 (0.41-1.86)	0.79 (0.40-1.54)
Other haplotypes	1.37 (0.99-1.91)	1.19 (0.71-2.00)	1.30 (0.99-1.71)
Global P value‡	.007	.89	.02

*Odds ratios and 95% CIs are calculated by using the most common haplotype as the reference and represent the risk associated per copy of the haplotype. ORs are adjusted for age, sex, ethnicity (for the combined population), child's atopic status, parental history of asthma, parent/guardian's education, *in utero* exposure to maternal smoking, exposure to secondhand smoke, health insurance coverage, and community of residence.

†For the order of SNPs in haplotypes, see the footnote of [Table I](#).

‡Global P values are from likelihood ratio tests compared with a model with no haplotype data and were based on 6df for *ARG1* and 8df for *ARG2*.

pollution exposures. None of these factors influenced the associations of these SNPs with asthma (data not shown).

Among atopic children, each copy of the *ARG1h4* haplotype was associated with reduced asthma risk (OR, 0.35; 95% CI, 0.19-0.66); however, this haplotype was not associated with asthma among nonatopic children. Among children living in high-ozone communities (annual average, >50 ppb), each copy of the *ARG1h4* haplotype was associated with reduced asthma risk (OR, 0.31; 95% CI, 0.16-0.63). In contrast, no association was found between the *ARG1h4* haplotype and asthma in children living in communities with low ambient ozone levels.

We found significant heterogeneity in the relationship of the *ARG1h4* haplotype with asthma across joint categories of atopy and ozone exposure ($P_{\text{heterogeneity}} = .008$, [Table V](#)). Among children with a history of atopy who lived in communities with high ambient ozone concentrations, each copy of the *ARG1h4* haplotype was associated with greatly reduced asthma risk (OR, 0.13; 95% CI, 0.04-0.44). Nonatopic children living in high-ozone communities or atopic children living in low-ozone communities had reduced asthma risk, but the ORs were not statistically significant.

Gene-gene interaction

We did not find any significant gene-gene interaction between *ARG1* and *ARG2* haplotypes or SNPs and asthma (not shown). Both *ARG1* and *ARG2* haplotypes showed independent effects

TABLE IV. Associations between *ARG1* and *ARG2* risk haplotypes and asthma and modifying effects of atopy, smoking exposures, and air pollution

Modifying factors	<i>ARG1h4</i> haplotype	<i>ARG2h3</i> haplotype
	OR (95% CI)*	OR (95% CI)*
Marginal effects	0.55 (0.36-0.83)	1.34 (1.06-1.68)
Children's atopy status		
No atopy (n = 1709)	0.87 (0.45-1.66)	1.38 (0.95-2.00)
Atopy (n = 1017)	0.35 (0.19-0.66)	1.17 (0.84-1.62)
P value†	.04	.61
In utero exposure to maternal smoking		
No (n = 2381)	0.53 (0.32-0.88)	1.29 (0.99-1.67)
Yes (n = 506)	0.76 (0.33-1.76)	1.37 (0.78-2.42)
P value†	.73	.74
Exposure to secondhand smoke in childhood		
No (n = 2002)	0.53 (0.31-0.90)	1.23 (0.92-1.64)
Yes (n = 847)	0.67 (0.33-1.36)	1.75 (1.16-2.64)
P value†	.81	.35
Ambient ozone categories		
Low (n = 1420)	0.90 (0.52-1.55)	1.35 (0.96-1.90)
High (n = 1526)	0.31 (0.16-0.63)	1.36 (0.99-1.86)
P value†	.02	.96
Ambient PM ₁₀ categories		
Low (n = 1545)	0.66 (0.38-1.15)	1.36 (0.99-1.87)
High (n = 1401)	0.44 (0.22-0.88)	1.32 (0.94-1.86)
P value†	.36	.91

*The odds ratios and 95% CIs for marginal effect represent asthma risk associated per copy of the haplotype. The rest of the ORs represent asthma risk associated per copy of the haplotype within each stratum of modifying factors. These ORs are adjusted for age, sex, ethnicity, child's atopic status, parental history of asthma, parent/guardian's education, *in utero* exposure to maternal smoking, exposure to secondhand smoke, health insurance coverage, and community of residence.

†P values for interactions were obtained from likelihood ratio tests and were based on 1df.

on asthma. In a model that contained both these haplotypes along with other covariates, compared with the other haplotypes within each locus, the ORs for asthma with each copy of the *ARG1h4* and *ARG2h3* haplotypes were 0.54 (95% CI, 0.36-0.83) and 1.34 (95% CI, 1.06-1.69), respectively.

Sensitivity analyses

Previously, we have reported associations with variants in genes in the oxidant stress pathway (ie, glutathione S-transferase P1 Ile105Val,²³ glutathione S-transferase M1 null,²⁴ *TNF* -308G/A,²⁵ short allele of the heme oxygenase 1 gene,²⁶ and catalase -262C>T26) and asthma/whoeeze in this cohort. In sensitivity analyses we adjusted our models with these variants and found that the *ARG1* and *ARG2* haplotypes and SNPs had independent effects (see Table E4 in this article's Online Repository at www.jacionline.org). Restricting the analysis to children with health insurance did not affect any observed associations (not shown).

DISCUSSION

Using data from a large, well-characterized population-based study, we observed significant associations between *ARG1* and *ARG2* genetic loci and asthma in children. We showed that effect of *ARG1* variants on asthma could represent a phase (haplotypic) effect. Furthermore, we found that both genetic (history of atopy)

TABLE V. Association of *ARG1h4* haplotype with asthma within strata of atopy and ambient ozone

Children's atopy status	Ambient ozone categories	<i>ARG1h4</i> haplotype		
		No.*	OR (95% CI)†	P value‡
Nonatopic	Low	59/723	1.30 (0.57-2.97)	.008
Nonatopic	High	68/859	0.55 (0.19-1.57)	
Atopic	Low	145/399	0.66 (0.31-1.40)	
Atopic	High	129/344	0.12 (0.04-0.43)	

*Numbers of children with or without asthma within each atopy and ambient ozone categories.

†The odds ratios and 95% CIs represent asthma risk associated per copy of *ARG1h4* haplotype within each atopy and ambient ozone categories. The ORs are adjusted for age, sex, ethnicity, parental history of asthma, parent/guardian's education, *in utero* exposure to maternal smoking, exposure to secondhand smoke, health insurance coverage, and community of residence.

‡P value for heterogeneity of haplotype effects across categories of atopy/ozone was obtained from a likelihood ratio test and was based on 3df.

and environmental (ambient ozone) factors influenced the asthma risk associated with *ARG1*, but not *ARG2*, variants. This is a novel finding that suggests that atopy and ambient ozone could influence the expression of these 2 genes differently.

Epidemiologic evidence of associations of these genetic loci with asthma is limited. Only 1 study to date has reported associations between 2 highly correlated *ARG2* SNPs ($R^2 = 0.937$) and asthma among Mexican children without adjusting the P values for multiple comparisons.¹³ One advantage of our study was the selection of more SNPs with greater coverage of the promoter region (20 kb upstream). This SNP selection approach allowed better identification of haplotype diversity and enabled us to evaluate the global association of each locus with asthma.

Very limited data exist on the functional significance of *ARG1* and *ARG2* SNPs. We were unable to find any published work on the functional effects of *ARG1* and *ARG2* variants. According to the Single Nucleotide Polymorphism database, there are 3 nonsynonymous and 1 synonymous SNPs in *ARG1* and 4 nonsynonymous and 2 synonymous SNPs in *ARG2*. However, the MAFs of these SNPs are 1% or less in white subjects. Given the paucity of functional data, we hypothesize that the studied genetic variants or other "causal" SNPs linked with these SNPs might affect asthma risk through nitrosative stress-mediated airway hyperresponsiveness (AHR), remodeling, or both. We have identified several putative transcription factor binding sites on some of the *ARG1* and *ARG2* SNPs in the 5' genomic regions and in introns (see Table E5 in this article's Online Repository at www.jacionline.org). These transcription factors have diverse functions on lung development, morphogenesis, and immune functions.²⁷⁻³⁰ Whether these SNPs with transcription factor binding sites affect arginase expression in the airways or leukocytes remains to be explored.

There are several potential mechanisms by which arginase could affect asthma risk. Arginase is a key enzyme in the urea cycle. In the final step of the cycle, arginase hydrolyzes L-arginine to produce ornithine and urea. Arginase and NOS compete for the common substrate L-arginine. Increased arginase activity increases the production of ornithine, which can be converted into proline and polyamines in the mitochondrial matrix. Proline is a component of collagen and mucus, and polyamines increase cell proliferation. Therefore it is plausible that increased collagen deposition and cell proliferation caused by high arginase activity

could lead to airway remodeling, a pathophysiologic hallmark of asthma.

In addition to the effects mediated by proline and polyamines, arginase overexpression affects nuclear factor (NF) κ B activity,⁴ reactive nitrogen species production,^{31,32} and respiratory physiology.³³⁻³⁵ Activation of the transcription factor NF- κ B has been implicated in asthma,³⁶ and NF- κ B inhibition attenuated allergic airway inflammation, AHR, and reduced T_H2 cytokine synthesis in a mouse model.³⁷ Reduced L-arginine levels caused by increased arginase activity could uncouple NOS and result in increased formation of peroxynitrite,^{31,32} a highly reactive nitrogen species that might promote airway inflammation. In addition, increased arginase activity has been associated with increased AHR, reduced airway smooth muscle relaxation, and increased airway remodeling in animal models.³³⁻³⁵ Furthermore, arginase inhibition promptly reversed allergen-induced AHR and airway inflammation *in vivo* in a guinea pig model.³⁸ In light of these observations and our findings, we speculate that the *ARG1* protective and *ARG2* risk haplotypes that were significantly associated with asthma could reduce and increase arginase activity, respectively, relative to the other haplotypes. This hypothesis needs to be examined further.

We found that atopy and ozone modified the relationship of *ARG1* haplotypes but did not affect the association of *ARG2* haplotypes with asthma. Existing data show that these 2 isoforms have differential expression profiles. In experimental studies T_H2 cytokines (IL-4 and IL-13) promoted expression of *ARG1* but not of *ARG2*.^{39,40} This differential expression pattern might explain our findings of a modifying role of atopy on the effect of the *ARG1* haplotype on asthma but no such modulation for *ARG2* haplotypes. Although ozone imparts oxidant stress-mediated injury to the airways, we are unaware of any published report that ozone could modulate arginase expression. However, ozone exposure induces inducible NOS in alveolar macrophages and type II alveolar epithelium in animals.⁴¹ If ozone-mediated upregulation of inducible NOS reduces L-arginine availability for *ARG1*, then it is plausible that the arginase activity could vary across *ARG1* haplotypes with ozone exposure. This could be one explanation for finding a stronger reduced risk in children carrying one *ARG1* haplotype who lived in high-ozone communities. Further research is warranted to examine the effect of ozone exposure on arginase expression.

We considered several potential sources of errors and biases in this study. Children who participated in the genetic study of the CHS by providing buccal samples differed modestly on demographic factors (age, sex, and ethnicity), socioeconomic status factors (family income, parental education, and health insurance coverage), and smoking exposures (*in utero* and childhood) from those who did not participate. We compared models with and without each covariate of interest to determine the effect of these factors on the risk estimates. None of these covariates changed the ORs by more than 5%. We used parental report of physician-diagnosed asthma in our study, and concern has been raised in that parental report might not reflect physician diagnosis and access to health care might lead to variation in the likelihood of diagnosis. To investigate this potential bias, we reviewed medical records of children with asthma and found strong evidence that parental report reflected physician diagnosis.⁴² Furthermore, restricting our analyses to children with health insurance yielded similar results (data not shown). We acknowledge that our atopy definition based on parental report of allergy, hay fever, or both could be

misclassified, but it is unlikely to be differential with respect to the genotypes and ambient ozone exposure.

In association studies involving multiethnic populations, population stratification could bias the results. To address this potential bias, we have excluded and not genotyped children who were African American (n = 163), Asian (n = 166), or of a mixed ethnic background (n = 192) because of insufficient sample sizes to conduct ethnic-specific analysis. Because our results for the genetic associations yielded similar results by ethnicity, our analytic approach of conducting pooled analyses while adjusting for ethnicity appears reasonable. In addition, restricting the analyses to non-Hispanic white subjects, which provided larger sample size for ethnic-specific associations, provided similar results. Because we could not generalize our finding to African American or Asian populations, further investigation is warranted in these populations.

To conclude, we found that both *ARG1* and *ARG2* genetic loci are associated with childhood-onset asthma. The effect of *ARG1* haplotypes on asthma might be modulated by atopy and exposure to ambient ozone. Arginase has been considered a candidate gene for asthma; however, limited studies in human subjects have been conducted. Further research is warranted to resequence the loci to identify novel variants and then determine the effect of those variants on arginase activity, airway inflammation, and AHR in human airways. This could explain the possible mechanism or mechanisms by which variants in these genes could affect asthma risk in children.

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Clinical implications: Variants in arginase genes are associated with asthma in children. Both genetic (history of atopy) and environmental (ambient ozone) factors could influence the asthma risk associated with arginase variants.

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METHODS

Buccal sample collection and processing

Children were provided with 2 toothbrushes and instructed to brush their teeth with the first one. They were instructed to gently brush the buccal mucosa with the second toothbrush. The brush was then placed in a leak-proof container that was filled with an alcohol-based fixative. Children then swished liquid throughout their mouths and expelled the fluid into a container. The majority of buccal cell specimens were collected at school under the supervision of study staff. The remaining specimens were collected at home and sent to us by mail.

Buccal cell suspensions were centrifuged at 2000g on the day they were received in the laboratory. The pellets were stored frozen at -80°C until used for DNA extraction, at which time they were resuspended and incubated in 600 μL of lysis solution from a PUREGENE DNA isolation kit (catalog no. D-5000; GENTRA, Minneapolis, Minn) containing 100 $\mu\text{g}/\text{mL}$ proteinase K overnight at 55°C . DNA extraction was performed according to the manufacturer's recommendations. The DNA samples were resuspended in the hydration solution (GENTRA) and stored at -80°C .

Laboratory protocol to prevent sample contamination

To prevent contamination in the laboratory, we used appropriate safeguards to ensure integrity of the samples. On arrival of the buccal collection kits in the

laboratory, each tube was scanned, and a unique barcode and a box location were assigned. During DNA extraction, a new set of labels with identification numbers and a box location were printed out for the long-term storage of DNA in a microtube. After extraction, the purified DNA was put back into the same box location as before, and our records were updated to reflect the change from buccal cells to purified DNA.

For genotyping purposes, we prepared dilution trays to prevent contamination of repeatedly opening up stock DNA microtubes and to maximize the use of our samples without losing volumes because the concentration of genomic DNA needed for TaqMan PCR was quite low. When preparing dilution trays, at least 2 persons were present for the bench work, one loading the samples and the other monitoring to ensure the correct sample was loaded in the correct location in a 96-well plate.

In addition, the following guidelines were always practiced: gloves were always worn when handling samples, and dilution plates or stock tubes were spun down before opening; we used deionized DNase, RNase free water, certified RNase, DNase, DNA, pyrogen, adenosine triphosphate-free tubes, and aerosol-resistant filter tips. We also had a designated post-PCR area where a different set of pipettes was used solely for post-PCR applications.

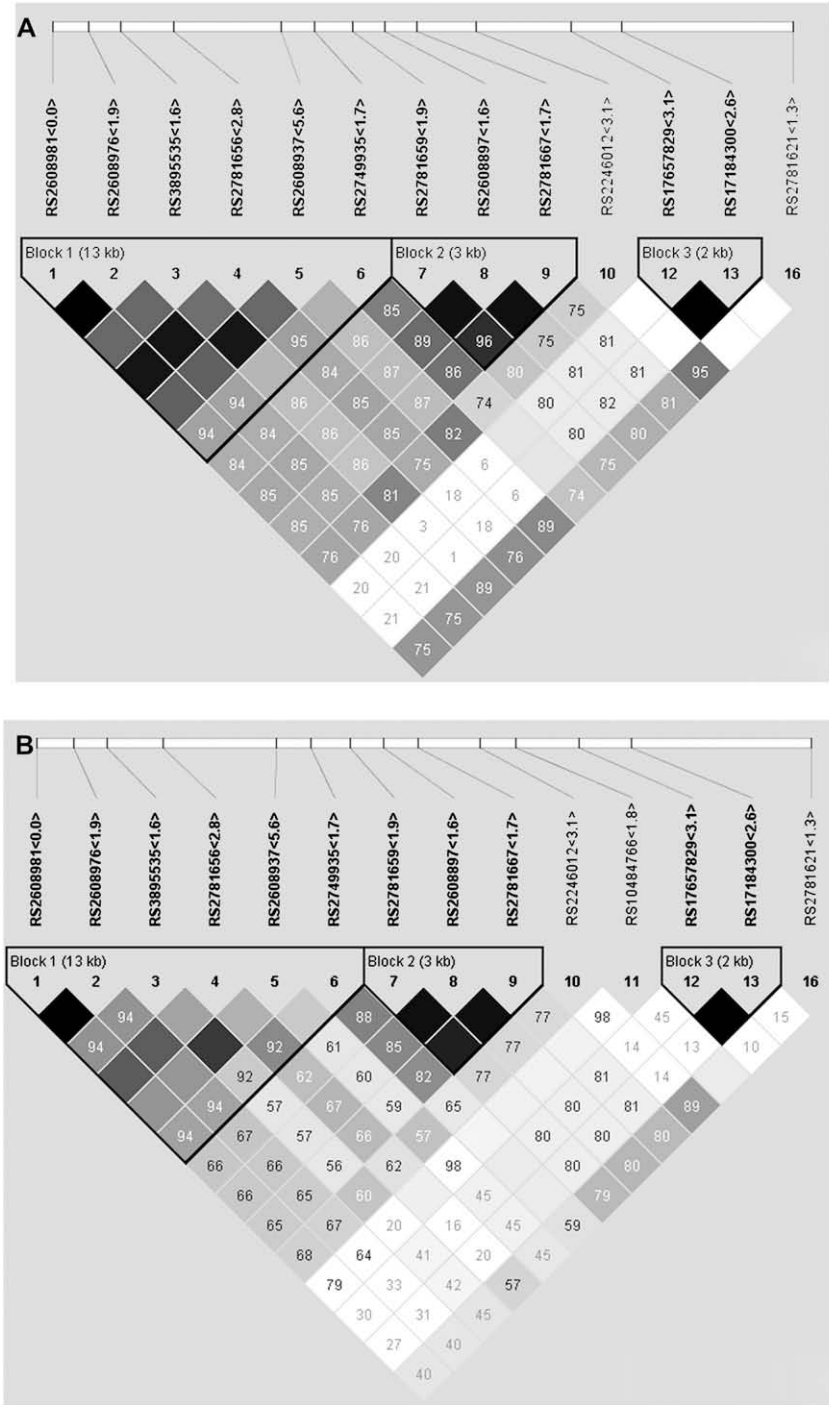


FIG E1. The linkage disequilibrium plot of *ARG1* for white subjects (**A**; non-Hispanic white subjects) and Latinos (**B**; Hispanic white subjects). The numbers in *boxes* represent the *D'* value as a percentage if it is less than 1.

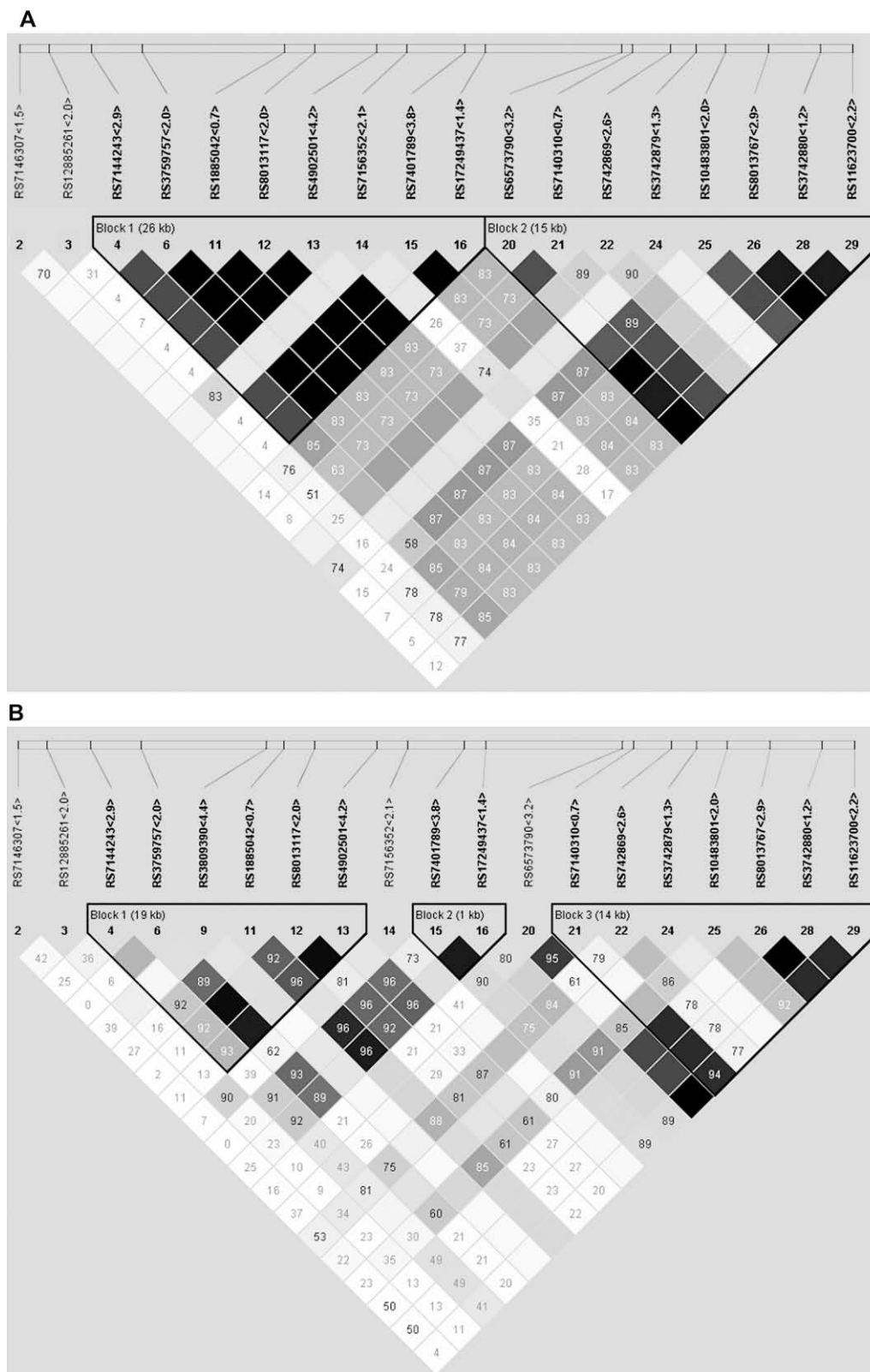


FIG E2. The linkage disequilibrium plot of *ARG2* for white subjects (**A**; non-Hispanic white subjects) and Latinos (**B**; Hispanic white subjects). The numbers in boxes represent the D' value as a percentage if it is less than 1.

TABLE E1. Correlations* of mean air pollution levels from 1992 through 2005 across the 12 communities

Pollutant	NO ₂	PM ₁₀	PM _{2.5}	Elemental carbon	Organic carbon	Acid vapor†
Ozone (10 AM-6 PM)	−0.06	0.22	0.16	−0.01	0.14	0.41
NO ₂		0.67	0.81	0.94	0.64	0.86
PM ₁₀			0.95	0.85	0.96	0.81
PM _{2.5}				0.92	0.90	0.86
Elemental carbon					0.82	0.87
Organic carbon						0.76

*Pearson correlation coefficients are presented. Each pollutant measurement represents the 24-hour average concentration, unless otherwise noted. All pairwise correlations were significant, except between ozone and other pollutants.

†Acid vapor is the sum of nitric, formic, and acetic acid.

TABLE E2. Location of the *ARG1* and *ARG2* SNPs and distance from the next SNP and the transcription starting site

Gene, SNPs*	SNP location	Distance from next downstream SNP (bp)	Distance from transcription starting site (bp)†
<i>ARG1</i>			
RS2608981	5' Genomic region	3,508	−18,126
RS3895535	5' Genomic region	8,342	−14,618
RS2608937	5' Genomic region	1,734	−6,276
RS2749935	5' Genomic region	1,939	−4,542
RS2781659	5' Genomic region	6,388	−2,603
RS2246012	Intron 2		3,785
<i>ARG2</i>			
RS12885261	5' Genomic region	2,862	−15,125
RS7144243	5' Genomic region	3,395	−12,263
RS3759757	5' Genomic region	15,758	−8,868
RS4902501	Intron 2	2,051	6,890
RS7156352	Intron 2	3,837	8,941
RS4902503	Intron 2	11,264	12,778
RS7140310	Intron 3	2,556	24,042
RS742869	Intron 4	1,708	26,598
RS3742879	Intron 7	2,005	28,306
RS10483801	Intron 7		30,311

**ARG1* is located at the chromosome 6 band q23.2 and has a genomic size of 11,104 bp with 8 exons. *ARG2* is located at the chromosome 14 band q24.1 and has a genomic size of 31,858 bp with 8 exons.

†Negative and positive numbers represent location of the SNP upstream and downstream from the transcription start site, respectively.

TABLE E3. Associations between *ARG1* and *ARG2* SNPs and asthma

SNP RS no.	Non-Hispanic white	Hispanic white	Combined
	OR (95% CI)*	OR (95% CI)*	OR (95% CI)*
<i>ARG1</i> SNPs			
RS2608981	1.06 (0.85-1.32)	0.93 (0.69-1.26)	1.01 (0.85-1.21)
RS3895535	0.99 (0.78-1.25)	0.92 (0.66-1.30)	0.96 (0.79-1.17)
RS2608937	1.03 (0.81-1.31)	0.88 (0.63-1.23)	0.98 (0.80-1.19)
RS2749935	0.99 (0.81-1.20)	0.91 (0.68-1.22)	0.96 (0.82-1.13)
RS2781659	1.09 (0.90-1.33)	1.12 (0.83-1.50)	1.10 (0.94-1.29)
RS2246012	0.95 (0.74-1.23)	0.84 (0.59-1.19)	0.91 (0.74-1.11)
Global <i>P</i> value†	.59	.87	.59
<i>ARG2</i> SNPs			
RS12885261	1.05 (0.88-1.27)	1.00 (0.75-1.34)	1.04 (0.89-1.21)
RS7144243	0.76 (0.60-0.97)	1.12 (0.77-1.62)	0.87 (0.71-1.06)
RS3759757	0.76 (0.61-0.94)	1.01 (0.74-1.37)	0.85 (0.71-1.01)
RS4902501	0.76 (0.61-0.95)	1.00 (0.74-1.36)	0.85 (0.72-1.02)
RS7156352	0.97 (0.79-1.18)	0.97 (0.67-1.39)	0.96 (0.81-1.14)
RS4902503	0.74 (0.60-0.93)‡	0.98 (0.73-1.32)	0.83 (0.70-0.98)
RS7140310	0.90 (0.68-1.18)	0.78 (0.51-1.17)	0.87 (0.70-1.09)
RS742869	0.81 (0.67-0.98)	0.88 (0.65-1.18)	0.84 (0.71-0.98)
RS3742879	1.39 (1.15-1.69)‡	1.14 (0.81-1.60)	1.31 (1.11-1.55)‡
RS10483801	0.82 (0.64-1.06)	0.76 (0.55-1.06)	0.81 (0.66-0.98)
Global <i>P</i> value†	.006	.37	.01

*ORs are adjusted for age, sex, ethnicity, child's atopic status, parental history of asthma, parent/guardian's education, *in utero* exposure to maternal smoking, exposure to secondhand smoke, health insurance coverage, and community of residence.

†Global *P* values are from likelihood ratio tests (LRTs) compared to a model with no principal components. Log-additive genetic model was used for all SNPs. The LRTs were based on 2*df* for *ARG1* loci and 3*df* for *ARG2* loci analyses except the LRT testing for the global association of Hispanic white, which was based on 4*df*.

‡ORs are statistically significant after correction for multiple comparisons. Adjusted *P* values for RS3742879 were .006 and .007 in non-Hispanic white subjects and the combined population, respectively. The adjusted *P* value for RS4902503 in non-Hispanic white subjects was .05.

TABLE E4. Associations between *ARG1* and *ARG2* haplotypes and asthma after adjusting for variants in other genes in the oxidant stress pathway*

Gene, haplotypes	OR (95% CI)†
<i>ARG1</i> haplotypes	
<i>ARG1h1</i>	1.0 (Reference)
<i>ARG1h2</i>	0.94 (0.74-1.20)
<i>ARG1h3</i>	1.10 (0.84-1.43)
<i>ARG1h4</i>	0.58 (0.37-0.91)
<i>ARG1h5</i>	1.29 (0.89-1.85)
<i>ARG1h6</i>	0.57 (0.31-1.06)
Other haplotypes	0.94 (0.68-1.30)
Global <i>P</i> value‡	.04
<i>ARG2</i> haplotypes	
<i>ARG2h1</i>	1.0 (Reference)
<i>ARG2h2</i>	1.22 (0.94-1.59)
<i>ARG2h3</i>	1.34 (1.01-1.76)
<i>ARG2h4</i>	0.95 (0.71-1.29)
<i>ARG2h5</i>	0.80 (0.55-1.16)
<i>ARG2h6</i>	0.88 (0.55-1.42)
<i>ARG2h7</i>	0.91 (0.63-1.34)
<i>ARG2h8</i>	0.71 (0.34-1.47)
Other haplotypes	1.33 (0.99-1.77)
Global <i>P</i> value‡	.05

*For these analyses, 2,669 (90.6%) of 2,946 children were available because of missing data on glutathione S-transferase P1 Ile105Val, glutathione S-transferase M1 null, short allele of heme oxygenase gene, *TNF* -308G/A, and catalase -262C>T genotypes.

†ORs are adjusted for age, sex, ethnicity, child's atopic status, parental history of asthma, parent/guardian's education, *in utero* exposure to maternal smoking, exposure to secondhand smoke, health insurance coverage, and community of residence.

‡Global *P* values are from likelihood ratio tests compared with a model with no haplotype data and were based on 6df for *ARG1* and 8df for *ARG2*.

TABLE E5. Transcription factor binding sites on *ARG1* and *ARG2* SNPs*

Gene, SNPs	Transcription factor	Transcription factor binding site
<i>ARG1</i>		
RS3895535	TBX5	tattACACCtgt
RS2608937	HNF1	tgaTTAATtattaaagaa
RS2608937	PAX4	attAATTAtta
RS2781659	HOXA7	aGATTGg
RS2781659	POU5F1 (OCT4)	gattggcagaAGAAA
<i>ARG2</i>		
RS7144243	NKX2-1 (TTF1)	cactCTTGGgag
RS4902501	CCDC6 (TST1)	ggagtatTATTCagt
RS4902503	GATA4	AAATAgaaggaa
RS4902503	ZIC2	aggGAGGTc
RS7140310	PAX2	tctgGTCAAgtctcactgt

CCDC6, Coiled-coil domain-containing protein 6; *GATA4*, GATA-binding protein 4; *HNF1*, HNF1 homeobox; *HOXA*, homeobox A7; *NKX2-1*, NK2 homeobox 1; *OCT4*, octamer-binding transcription factor 4; *PAX2*, paired box gene 2; *PAX4*, paired box gene 4; *POU5F1*, pou domain, class 5, transcription factor 1; *TBX5*, t-box 5; *TST1*, transforming sequence thyroid 1; *TTF1*, thyroid transcription factor 1; *ZIC2*, zinc finger protein of cerebellum 2.

*Transcription factor binding sites were searched by using the TRANSFAC database with the Match program for all *ARG1* and *ARG2* SNPs. The table only shows those SNPs that have a transcription factor binding site. All known transcription factors and their predicted binding sites are shown.