Role of Inducible Nitric Oxide Synthase on Asthma Risk and Lung Function Growth During Adolescence

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ABSTRACT

Background: Inducible nitric oxide (NO) synthase (iNOS, encoded by *NOS2A*) produces NO in response to environmental stimuli, which can result in nitrosative stress. Because nitrosative stress affects respiratory health, we hypothesized that variants in *NOS2A* are associated with asthma incidence and lung function growth during adolescence.

Method: In this prospective study, spirometric testing was performed at school and a presence or abscence of asthma was ascertained annually by questionnaire among children participating in the southern California Children's Health Study. We genotyped 24 SNPs of the *NOS2A* region (with 7 promoter SNPs in one haplotype block), spanning 20kb upstream and 10kb downstream. Association between the *NOS2A* region and asthma or lung function growth was tested using genetic block-specific principal component and haplotype analyses. This study was restricted to children with Latino and Caucasian ancestry for analyses of both asthma (N=1,596) and lung function growth (N=2,108).

Result: A pair of "yin-yang" haplotypes in the promoter region showed strong association with new-onset asthma and lung function growth. The "yin" haplotype (H0111101) was associated with 44% increased asthma risk (p-value=0.003) and reduced FEV_1 growth from 10-18 years of age (-29.46 ml, p-value=0.07), whereas, the "yang" (H1000010) haplotype was associated with 23% reduced asthma risk (p-value=0.13) and better FEV_1 growth (43.84 ml, p-value=0.01). Furthermore, the increased asthma risk associated with H0111101 was restricted to children with *GSTM1* 'null' genotype (interaction p-value=0.002, HR, 1.89, 95%CI, 1.34-2.60).

Conclusion: Common haplotypes in the *NOS2A* promoter are associated with new-onset asthma and lung function growth. These effects are stronger in adolescents with the *GSTM1* 'null' genotype.

INTRODUCTION

Studies have shown that oxidative stress is an important determinant of respiratory health.[1-3] Both increased incidence of asthma and reduced lung function growth during adolescence have been associated with environmental and genetic determinants of oxidative stress.[4-5] Like reactive oxygen species (ROS), reactive nitrogen species (RNS) can also produce cellular injury through nitrosative stress.[6] Inducible nitric oxide synthase (iNOS) plays an important role in determining nitrosative stress, as it produces large amounts of nitric oxide (NO) in response to environmental stimuli.[6]

NO is a gaseous-signaling molecule that is involved in a spectrum of biological processes. In response to proinflammatory cytokines and lipopolysaccharides, epithelial cells and T-lymphocytes produce NO through inducible NOS (iNOS).[6] NO reacts with superoxides (O^{--}) to form the highly reactive peroxynitrites ($ONOO^{-}$) that have been shown to produce airway inflammation[7] and cause airway-remodeling.[8-9] Furthermore, fractional concentration of nitric oxide in exhaled breath, a marker of airway inflammation,[10] is predominantly of iNOS origin.[11] Increased expression of iNOS may play a role in inflammation of the upper and lower airways, and contribute to both allergic rhinitis and asthma.[12]

Despite the evidence supporting the potential importance of iNOS in respiratory health, limited information is available about the role of DNA sequence variations in *NOS2A* that encodes iNOS. *NOS2A* is located at chromosome 17q11.2-q12, a region identified by genome-wide association tests as a possible region determining asthma and atopy.[13] A large number of variants in NOS2A have been identified that have the potential to affect expression or function. The induction of *NOS2A* is complex and the promoter region, which contains a number of variants, extends up to 16kb upstream of the gene.[14] There is limited evidence that repeat polymorphisms in the promoter region are associated with atopic conditions.[15-18] In addition to the repeat polymorphisms, single nucleotide polymorphisms (SNPs) also exist in the promoter region that are not in strong linkage disequilibrium (LD) with the repeat polymorphisms.[19] To date, a single study involving Czech adults reported association between SNPs in the NOS2A promoter region and atopy and asthma severity.[16]

In this study, we investigated the role of variations in the *NOS2A* gene with asthma pathogenesis and lung function growth in children. We hypothesized that genetic variants of *NOS2A*, especially those in the promoter region, are associated with new-onset asthma during adolescence and lower lung function growth, due to their likely effect on nitrosative stress. Assessing the effects of the *NOS2A* variants across these two respiratory outcomes provided an opportunity to evaluate the consistency in the findings. Furthermore, because the formation of peroxynitrite is dependent on the availability of reactive oxygen species, we hypothesized that the associations between *NOS2A* polymorphisms and respiratory outcomes vary by genetic determinants of oxidative stress (*GSTM1*, *GSTP1*, *CAT* and *HMOX1*) that have been previously reported to be associated with lung function growth or asthma pathogenesis in this cohort.[4 20-21] We investigated these hypotheses in a cohort of Latino and Caucasian children who participated in the Children's Health Study (CHS).[22-23] Some of the results have been reported in abstract form.[24]

METHODS

(Further details are available in the Online Supplement)

Subjects and Materials

Details concerning the design, methods, and characteristics of the CHS cohort have been presented previously.[22-23] Briefly, in 1993, fourth-, seventh- and tenth-grade children were enrolled into the study in each of 12 communities in Southern California. A second cohort of fourth-grade children was recruited from the same schools in 1996. Study subjects were followed annually until high school graduation. Detailed health and socio-demographic data were collected annually.

As in previous CHS genetic analyses,[4] this study was restricted to children of Latino and Caucasian ancestry with available genetic data. The analysis of new-onset asthma included 1,596 children who were free of asthma and wheeze at baseline. Similarly,[25] the analysis of lung function was restricted to the two cohorts of fourth-grade children (mean age 10 years [SD=0·44]) who were recruited in 1993 (cohort 1, N=912) and 1996 (cohort 2, N=1,196) and had similar lung function growth patterns.

New-Onset Asthma

Children with no prior history of asthma at study entry, who subsequently reported physician-diagnosed asthma at annual follow-up, were classified as having new-onset asthma. Children were also interviewed annually about the use of inhaled medications. We defined a restricted group of new-onset cases with recent inhaler use for sensitivity analyses (N=116).

Lung Function measurement

Data on children's lung function (LF) were collected by trained field technicians using a standardized protocol during annual school visits. Maximal-effort spirometry and standing height and weight were measured. Details regarding the lung function testing protocol have been published previously.[22] Three measures of lung function were included in this analysis: forced vital capacity (FVC), forced expiratory volume in the first second (FEV_1) and forced expiratory flow over the mid-range of expiration (FEF_{25-75}).

NOS2A Haplotype Block Determination

Most of the upstream regulatory and promoter region was contained in a single block (Figure 1: Block 7) with a low multi-allelic D′(0.34) with the adjacent block (Figure 1: Block 6). Blocks 1 through 6 had higher multi-allelic D′(>0.70). Therefore, the remaining region (block 1-6) was analyzed as 2 segments. The first segment was comprised of blocks 1 through 3 and the second segment of blocks 4 through 6 (Figure 1).

Haplotype frequencies of unphased *NOS2A* SNPs of the promoter region and the two coding segments for Latino and Caucasian subjects were estimated using tagSNPs. The estimated number of copies of each haplotype was used as a proxy for the true haplotype, a single imputation procedure that provides unbiased estimates and appropriate confidence intervals. Four haplotypes in Block 7 explained at least 97% of the variability of this block in both ethnic groups. Furthermore, a "yin-yang" pair of haplotypes (h0111101 and h1000010), two high-frequency haplotypes that have completely mismatching SNP nucleotides at every SNP location,[28] explained at least 65% of the variability of this promoter block in Latino and Caucasian population.

Statistical methods

To assess the global association of variation across the locus with asthma occurrence or lung function growth, the groups of common SNPs and haplotypes were tested using both principal component (PC)[31] and haplotype approaches. The statistical significance was based on the likelihood ratio tests (LRT) comparing the full model (model with genetic data adjusted for all necessary adjustment variables) to the base model (model with all necessary adjustment variables without any genetic data). As indicated by Gauderman et.al., a block -specific PC analysis was preferred over a whole locus approach.[31] PCs explaining at least 80% of the variance of each of the three regions (two PCs for segment 1, four PCs for segment 2 and one PC for the promoter region block (Block 7)) were modeled to determine the overall statistical significance of each region across outcomes (Figure 1).

The haplotype model consisted of all the haplotypes with frequency >5% and 'other' haplotype, with the most common haplotype as the reference group. We also tested the effect of one and two copies of the identified haplotypes across the respiratory outcomes, using all other haplotypes as the reference group. After identifying a region that showed an overall statistically significant association with the respiratory outcomes, we tested associations between individual SNPs contained in that region and the respiratory outcomes. To address the issue of multiple testing of the correlated SNPs, the single SNP analyses were adjusted for multiple testing using the P_{ACT} method.[32]

New-Onset Asthma Analysis

We fitted Cox proportional hazard regression models, with the timescale defined as the follow-up time with sex- and age- (integer years) specific baseline hazards. All models were adjusted for community and ethnicity markers. Associations are expressed as hazard ratio (HR) and 95% confidence limits (CL).

Lung Function Analysis

A hierarchical mixed effects model was used to relate eight-year growth in each lung function measure to *NOS2A*, with a basic structure that has been previously described.[3] Random effects for the intercept and eight-year growth parameters were included at the subject level. We estimated and tested the effect of *NOS2A* on eight-year lung function growth from age 10 to 18. Because the findings were similar for all three lung function measures (Table E5), we present the results for FEV_1 only.

Sensitivity analyses

Other variables available from questionnaire data were evaluated for potential confounding in both asthma and lung function models, but ultimately removed from final analysis since they did not alter the results. Based on our previous publications, we also considered the 'null' deletion of *GSTM1*,[20] GSTP1-Ile105Val,[20] (GT)n repeats of HMOX1, and CAT-262C>T [4] as possible confounders.

Test of Heterogeneity

To test whether other genes involved in the oxidative pathway (*GSTM1*, *GSTP1:Ile105Val*, *HMOX1* and *CAT*) modified the association between *NOS2A* and respiratory outcomes, models with and without appropriate interaction terms were considered. In the presence of statistically significant heterogeneity among subgroups, stratified analysis was performed.

All hypothesis testing was conducted assuming a 0.05 significance level and a two-sided alternative hypothesis. All analyses were conducted using SAS software

Version 9.1.3 (SAS Institute, Cary, NC). We used p_ACT_seq. R program in R software (V 2.21) to address the issue of multiple testing (http://csg.sph.umich.edu/boehnke/p_act.php).

RESULTS

Table 1: Selected baseline characteristics of children who were included in the new-onset asthma and lung function analyses

	Children included in incident asthma analysis (N=1,596)	Children included in LF Analysis (N=2,108)
Age Group		
7-9 Years	875(54.8)	1,603(76.0)
10-11 Years	335(21.0)	505(24.0)
>11 Years	386(24.2)	-
Sex		
Girls	894(56.0)	1,086(51.5)
Boys	702(44.0)	1,022(48.5)
Latino	554(34.7)	710(33.7)
Overweight	395(24.7)	536(26.5)
Family History of Asthma	222(13.9)	430(21.7)
History of Atopy*	567(35.5)	916(48.4)
History of Asthma at Study Entry	0(0)	310(12.0)
Household ETS Exposure	186(19.0)	361(17.9)
In utero Exposure to Smoking	219(13.7)	354(17.2)
Pests of any kind	1,195(74.9)	1,627(83.4)
Pets of any kind	1,302(81.6)	1,728(82.0)
Children with Health Insurance	1,313(82.3)	1,781(86.3)
Annual Family Income (in US dollars) †		
≤14,999	174(10.9)	263(14.3)
15,000-49,999	584(36.6)	768(41.7)
≥50,000	597(37.4)	812(44.1)
Highest Parental Education Level†		
Less than High School	210(13.2)	222(10.9)
College	1,122(70.3)	1,567(74.3)
Graduate	208(13.0)	242(11.9)

* Children with any history of allergy, hay fever or rhinoconjunctivitis were defined as 'atopic'.

† Numbers do not add up to 100% due to missing information.

Baseline Characteristics

The majority of children included in the asthma (N=1,596) or lung function (N=2,108) analyses were 9 years of age at study entry and of Caucasian descent (Table 1). Compared to children in the asthma analysis, those in the lung function analysis were more likely to have family history of asthma (21.7% vs. 13.9%) and history of atopy

(48.4% vs. 35.5%) at baseline as the asthma analysis was restricted to children without any history of wheeze or asthma diagnosis at baseline. We did not observe any other significant differences between the two populations at baseline and 998 children were common to both analyses.

One hundred and fifty cases of new onset asthma were diagnosed during the follow-up period. The crude incidence rate of asthma was 16.3/100 person-years and showed little difference between Caucasians (IR=16.1/1000 person-years) and Latinos (IR = 16.6/1000 person-years). The prevalence of wheeze and prevalent asthma in the lung function analysis group was 34.7% and 15.0% at baseline, respectively. The mean FEV_1 growth for boys and girls over the 8-year follow-up time was 2,441 ml and 1,367 ml, respectively.

Table 2: Overall associations between *NOS2A* and new-onset asthma or FEV_1 growth (in ml) assessed using Haplotype based analysis.

NOS2A Region	Haplotype	New-Onset Asthma		8-year growth in FEV_1	
		HR† (95%CL)	P-Value§	Estimate‡ (95%CL)	P-Value§
Segment 1 (Block 1-3)	h0110010	Ref.	0.06	Ref.	0.46
	h1000000	0.72(0.51,1.01)		9.57 (-31.99, 51.14)	
	h1001101	1.30(0.93,1.8)		27.64 (-19.76, 75.04)	
	h0000000	1.00(0.71,1.42)		12.40 (-34.78, 59.57)	
	Other*	0.92(0.62,1.37)		-25.24 (-75.13, 24.65)	
Segment 2 (Block 4-6)	h0101100010	Ref.	0.25	Ref.	0.71
	h1010111001	1.21(0.81,1.81)		-6.64 (-61.13, 47.84)	
	h0010000100	0.66(0.41,1.05)		31.86 (-21.68, 85.40)	
	h0010000101	1.30(0.84,2.03)		-12.89 (-74.98, 49.20)	
	h0101000010	0.79(0.48,1.30)		-32.94 (-92.81, 26.92)	
	h0000000100	1.13(0.73,1.74)		-2.89 (-61.28, 55.51)	
	h1001011001	1.07(0.63,1.82)		31.14 (-41.84, 104.12)	
	h0001000001	0.99(0.49,1.99)		10.34 (-82.78, 103.47)	
	Other*	0.96(0.64,1.45)		1.07(-55.16, 57.30)	
Block 7	h0111101	Ref.	0.02	Ref.	0.10
	h1000010	0.66(0.49,0.88)		48.90 (11.60, 86.20)	
	h0000010	0.84(0.60,1.18)		3.86 (-41.95, 49.68)	
	h0000000	0.62(0.44,0.88)		17.04 (-26.77, 60.85)	
	Other*	0.85(0.42,1.74)		1.30 (-107.56, 110.17)	

* Haplotypes with less than 5% frequencies are grouped into the 'Other' category.
†HR and 95%CL are based on fitting the Cox proportional hazard model (for detail see 'Method').
‡ Estimate and 95%CL are based on hierarchical mixed effects model (for detail see 'Methods').
§P-values are based on a likelihood ratio test comparing models with and without genetic data.

Main Effect of *NOS2A*

Utilizing a PC -based analysis for global tests, we observed that Block 7 (containing seven SNPs of the promoter region) was associated with both increased risk of new-onset asthma (p-value=0.002) and decreased FEV_1 growth (p-value=0.02) during adolescence (Table E6). The haplotype-based analysis for global association also showed a similar pattern of association (Table 2). Compared to the common haplotype, h0111101, all other haplotypes were protective for new-onset asthma. The h0111101 haplotype was associated with increased risk of new-onset asthma (HR: 1.44, 95%, p-value=0.003) and lower FEV_1 growth (by 29.5 ml, 95% CL, p-value=0.07) compared to all other haplotypes (Table 3). This haplotype also showed a dose-dependent effect for asthma risk (p-value=0.01). Children with one or two copies of this haplotype were at 1.49 (95% CL, 1.03, 2.14) and 2.08 (95% CL, 1.25, 3.45) fold increased risk of new-onset asthma, respectively, compared to children without this haplotype. The other member of the "yin-yang" pair, h1000010, was associated with higher eight-year FEV_1 growth (p-value=0.01) and showed a dose-dependent pattern (p-value=0.02).

Table 3: Association between *NOS2A* promoter 'yin-yang' haplotypes and new-onset asthma or FEV_1 growth (in ml)).

NOS2A Region	Haplotype	New-Onset Asthma		8-year growth in FEV_1	
		HR (95%CL)*	P-Value‡	Estimate (95%CL)†	P-Value‡
h0111101	None	Ref.	0.003	Ref.	0.07
	At least one	1.44 (1.13,1.82)		-29.46 (-61.27, 2.36)	
h0111101	None	Ref.	0.01	Ref.	0.15
	1 copy	1.49 (1.03-2.14)		-40.69 (-85.04, 3.66)	
	2 copies	2.08 (1.25,3.45)		-46.82 (-118.29, 24.65)	
h1000010	None	Ref.	0.06	Ref.	0.01
	At least one	0.77 (0.59,1.01)		43.84 (11.39, 76.28)	
h1000010	None	Ref.	0.09	Ref.	0.02
	1 copy	0.86 (0.61,1.21)		28.65 (-15.62, 72.93)	
	2 copies	0.46 (0.21,1.00)		107.74 (31.54, 183.93)	

*HR and 95%CL are based on the fitting Cox proportional hazard model (for detail see 'Method').

† Estimate and 95%CL are based on the hierarchical mixed-effects model (for detail see 'Methods').

‡ P-value is based on a likelihood ratio test comparing models with and without genetic data.

Compared to children not carrying this haplotype, those with one or two copies of this haplotype had 28.6 (95% CL:-15.62, 72.93) or 107.7 (95% CL, 32.54, 183.93) ml higher FEV_1 growth over an eight-year period. A marginally significant protective trend for new-onset asthma was also observed among carriers of this haplotype. The risk of asthma decreased by 14% and 54% among carriers of 1 or 2 copies of the h1000010 haplotype.

SNP Analysis

Investigation of the single SNPs corresponding to the haplotypes for the upstream promoter region (Block 7) demonstrated significant associations (Table E7) consistent with haplotype results; therefore, we report the results from h0111101 and h1000010 analyses only.

Sensitivity Analysis

The observed association between the h0111101 and respiratory outcomes (new-onset asthma and FEV_1 growth) were not substantially affected by adjustment for potential confounders (Tables E8 and E9: Model 1). The associations between h0111101/h1000010 and respiratory outcomes were not confounded by the variants of genes involved in oxidative stress and asthma, i.e. *GSTM1*, *GSTP1*, *HMOX1* and *CAT* [4] (Tables E8 and E9: Model 2).

To assess whether the effect of the haplotypes on FEV_1 growth was due to reduced lung function level in children with asthma, we investigated the effects only among children without prevalent or new-onset asthma (N=1672). In this asthma -free population, the beneficial effect of h1000010 remained mostly unchanged (49.3 ml increase in FEV_1 growth; 95% CL: 10.9, 87.7); however, the detrimental effect of h0111101 was less pronounced (15.3 ml decrease in FEV_1; 95%CL -53.9, 23.2).

To investigate whether the associations were consistent in independent groups of children, we performed stratified analyses for the two fourth-grade cohorts in the study populations independently recruited in 1993 and 1996. These cohorts were from the same communities and schools and thus had similar environmental exposure and socio-economic characteristics. The effect estimates for both new-onset asthma and FEV_1 growth were mostly similar across cohorts (Table E3 and E4). In each of the cohorts and ethnic groups, h0111101 (Table E3) was associated with increased risk of new-onset asthma (HR range: 1.2-1.7) and lower FEV_1 growth (FEV_1 growth range: -13.8 to -51.8 ml). In contrast, h1000010 (Table E4) was associated with decreased risk of new-onset asthma (HR range, 0.68 to 0.89) and higher FEV_1 growth in all the cohorts and ethnic groups (FEV_1 growth range, 32.4 to 54.3 ml).

Joint effects with *GSTM1* null status

Lastly, we evaluated whether the association between the haplotypes of interest and respiratory disease was modified by *GSTM1* status. The association between h0111101 haplotype and new-onset asthma varied by *GSTM1* 'null' genotype (p-value= 0.002, Table 5). The increased risk of new-onset asthma for h0111101 carriers was restricted to children who lacked GSTM1 (HR: 1.89, 95% CI: 1.3-2.6). For FEV_1, those with the h0111101 haplotype and *GSTM1* 'null' genotype were also associated with the worst growth pattern (FEV_1 growth: -53.19, 95%CL:-99.63,-6.75). The protective effect of h1000010 on asthma risk or FEV_1 growth did not vary by *GSTM1* genotype. The haplotype effect was not modified by the presence of functional variants in other oxidative stress genes, i.e. *GSTP1*, *HMOX1* or *CAT*.

Table 4: Association between the *NOS2A* promoter haplotypes and new-onset asthma or FEV_1 growth, by *GSTM1* genotype.

Haplotype		*GSTM1*	New-onset asthma		8-year growth in FEV1	
			HR (95%CL)*	Interaction P-Value‡	Estimate†(95% CL)	Interaction p-value‡
h0111101	None	Present	Ref.	0.002	Ref.	0.32
	At least one	Present	0.89 (0.58-1.37)		-4.64 (-50.20,40.91)	
	None	null	Ref.		Ref.)	
	At least one	null	1.89 (1.34-2.60)		-53.19 (-99.63,-6.75)	
h1000010	None	Present	Ref.	0.42	Ref.	0.53
	At least one	Present	0.85 (0.54-1.34)		39.85 (-5.81,85.50)	
	None	null	Ref.		Ref.	
	At least one	null	0.68 (0.48-0.98)		49.75 (1.60,97.91)	

*HR and 95%CL are based on fitting the Cox proportional hazard model (for detail see 'Method').
† Estimate and 95%CL are based on the hierarchical mixed-effects model (for detail see 'Methods').
‡ Interaction p-value was based on a likelihood ratio test by comparing models with and without interaction terms.

DISCUSSION

In this prospective study of new-onset asthma and lung function growth during childhood, we found that DNA sequence variation in the promoter region of the *NOS2A* plays a potentially important role in respiratory health and development. We identified a pair of "yin-yang" haplotypes (h0111101 and h1000010) that accounted for more than 65% of the variation in the promoter region. The "yin" haplotype (h0111101) was associated with increased risk for asthma and poor lung function growth. In contrast, the "yang" haplotype (h1000010) was associated with higher lung function and lower asthma risk. Furthermore, our analyses suggest that promoter variation in *NOS2A* has independent effects on asthma occurrence and lung function. These effects were consistent across two different respiratory outcomes, different internal cohorts and two different ethnic groups.

Although *NOS2A* has been of interest in asthma research, most of the studies did not use SNPs and none of the studies investigated its role on lung function growth. Most of the studies involved a putative functional repeat polymorphism of the promoter region, $(CCTTT)_n$, that was associated with atopic status in Japanese adults;[17] but not in Chinese children,[18] Czech adults,[16] or in Indian children and adults.[15] In the present study, we found no evidence for an effect of this repeat polymorphism on asthma or lung function growth (data not shown). To the best of our knowledge, only one previous study has examined the role of SNPs in the promoter region on respiratory health, and associations with atopy and asthma severity were reported.[16] Thus it is hard to compare our current finding with previous publications on *NOS2A*. However, the consistent effects of the yin-yang haplotypes for the two different respiratory outcomes suggest that more than one variant in this region may underlie the observed associations with asthma and lung function.

Besides being consistent across outcomes, the effects of the haplotypes were also independent of the respiratory outcomes. The detrimental effect of h0111101 and beneficial effect of h1000010 on lung function were observed among children with and without a history of asthma. Although the magnitude of the effect of the haplotypes was associated with an apparently small effect (1% change in lung function growth during adolescence in boys and 2% in girls), they may nevertheless play an important role in susceptible populations with low lung function levels. Furthermore, given the multi-factorial nature of lung function growth and asthma, any genetic or environmental risk factors are not expected to have large individual effects.

Accumulating evidence indicates that nitrosative stress, like oxidative stress, plays an important role in airway pathobiology.[7] Because the effects of variants in *NOS2A* on respiratory health may depend on levels of oxidative stress and gene variants in oxidant defense pathways modulate levels of oxidative stress,[33] we hypothesized that *NOS2A* may interact with variants in oxidant defense genes like *GSTM1*, which have also been associated with asthma occurrence[21] and lung function growth.[20] Our observation that the detrimental effect of h0111101 on asthma risk was most apparent among children with a common *GSTM1* variant lacking enzyme activity supports this hypothesis. If h0111101 results in increased NO production and formation of peroxynitrites, as we have speculated, a larger effect might be expected in *GSTM1* null individuals. Lower NO production in the h1000010 might result in a protective effect irrespective of *GSTM1* status. Although levels of oxidative stress may be important, other pathways that involve

GSTM1 may also contribute. In the current study, the *NOS2A* associations were independent of variation in *GSTP1*, *HMOX1* and *CAT*, genes that are also involved in the oxidant defense pathways and have been shown to be associated with new-onset asthma and lung function. This implies that other functions of GSTM1, such as electrophil conjugation with glutathione, may be important in the interactions with *NOS2A* variants. As no functional data are currently available for the SNPs and the haplotypes studied, detailed sequence analyses followed by mechanistic studies examining different combination of SNPs in the region are needed to identify the specific variants that account for the haplotype associations.

One of the strengths of this study is the prospective follow-up of large numbers of school-age children with annual assessment of asthma diagnosis and lung function measurements in a consistent manner. The associations were robust and highly significant in both PC- and haplotype-based analyses, and the individual SNPs were also significant after adjusting for multiple testing using the p_ACT method.[32] Furthermore, population admixture is an unlikely explanation of our findings as the incidence rate of new-onset asthma and lung function growth rate during adolescence did not vary by ethnicity and the main effects of the SNPs were similar in the ethnic-specific analysis (Table E3 & E4). Furthermore, all analyses were adjusted for ancestry factors (based on 233 ancestry informative-marker SNPS) in addition to the traditional self-identified race identifier, thus controlling for any confounding effect of population stratification.

One potential limitation of our study, accuracy of self-reported new-onset asthma assignment, was addressed by excluding any child with a history of wheezing at study entry from the analyses to minimize any major misclassification. A recent study noted that children as young as seven years of age can provide information regarding their asthma with acceptable level of validity and reliability.[34] Furthermore unless the diagnostic accuracy varied by genotype, error in determining asthma status would likely attenuate the risk estimates and, therefore, would not explain our observed associations. The associations were similar in sensitivity analyses restricted to cases that recently used inhaled medication and was statistically significant for the association between h1000010 and new-onset asthma (Table E8: Model 3). Moreover, the observed associations of the promoter SNPs and haplotypes were consistent for lung function growth, which is an objective measurement of respiratory health and not susceptible to diagnostic or reporting bias. Therefore, our results are unlikely to be explained by misclassification of outcome.

We considered the potential effects of selection bias, as genetic data were available for about two-thirds of the initial cohort. As we have described previously,[4] demographic and socioeconomic factors, exposure to maternal smoking during pregnancy and secondhand smoke after birth, and household factors showed modest differences between participants and non-participants. However, adjustment for these factors did not explain our results (Table E8: Model 1), indicating that selection bias based on these or related factors is unlikely to explain our findings.

We conclude that genetic variants of the *NOS2A* promoter region play a role in the respiratory health of children during their adolescence. The role of regulation and function of *NOS2A* expression in asthma pathogenesis and lung function growth and the joint effects with *GSTM1* merit further investigation. Future experimental studies are necessary to define and test variants found by detailed resequencing of the promoter region of *NOS2A* to better understand its role in NO synthesis and respiratory health.

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COMPETING INTERESTS

None.

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Figure 1. The linkage disequilibrium (LD) plot and haplotypes of the NOS2A gene (including the promoter region) for Europeans using data from the International HapMap Project. For the LD plot, the triangles represent the haplotype blocks. Haplotype frequencies within each block are shown below the blocks and the multiallelic D' between blocks are shown between haplotypes.

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ONLINE DATA SUPPLEMENT
Role of Inducible Nitric Oxide Synthase on Asthma Risk and Lung Function Growth During Adolescence

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METHODS

Subjects and Material

Initially in 1993, 3,681 students from fourth, seventh, and tenth grade classrooms were enrolled into the study from 12 communities in southern California (selected on the basis of different ambient pollution levels) [1,2]. Another 497 students who were in the same classrooms as participants were recruited in 1994. In 1996, an additional 2,081 fourth grade children from the same communities and schools were recruited. All students were followed until high school graduation. Thus the tenth, seventh and fourth grade cohorts recruited in 1993 were followed until 1995, 1998 and 2001 respectively. The 1996 cohort was followed until 2004.

At baseline, parental questionnaire responses were used to classify subjects (N=6,259) according to race and ethnicity. Analyses were restricted to children of Latino (N=1,743) or Caucasian descent (N=3,425) due to insufficient representation of other races (African American=345, Asian=308 and Others=438). For asthma analyses, children with any history of asthma (N=832), wheezing (N=965) or missing information for wheeze or asthma history at baseline (N=259) were excluded. We further excluded another 624 children who had less than one follow-up visit, which was needed to assess new-onset asthma. *NOS2A* data were available from 65.2 % (N=1,596) of the remaining eligible 2,448 children.

For lung function analyses, besides the race/ethnicity restriction, all analyses were further restricted to 3,260 children who were in 4th grade at study entry (children recruited in 1993 [N=1,522] and 1996 [N=1,738]); because, these children could have a total of 8 years of follow-up to measure lung function growth. We further excluded another 94 children who had no lung function measurements. *NOS2A* data were available from 66.7 % (N=2,108) of the remaining eligible 3,166 children.

Parents or guardians of each participating student provided written informed consent, and completed a self-administered questionnaire, which provided information on socio-demographic and household characteristics and personal health. Pulmonary-function data were obtained yearly by trained field technicians, who traveled to study schools to undertake a maximum effort of spirometry on the children, using the same equipment and testing protocol throughout the study period. During the time of pulmonary testing, the trained field technicians conducted personal interviews with each of the participants to gather respiratory health data including diagnosis of asthma by a physician and medication use for asthma. Children who did not complete the questionnaires or interview, due to absence during visits at schools, were interviewed by telephone using the same instruments. Children who moved from a study school area during a school year were interviewed by telephone to collect information for the year that they moved and were not interviewed in subsequent years. Children who dropped out of the study were censored at the date of their last interview year. Children who reported asthma at any interview were censored at the midpoint of the year of diagnosis. The study protocol was approved by the Institutional Review Board for human studies at the University of Southern California, and written informed consent was provided by a parent or legal guardian for all participants.

Sociodemographic and Medical History Information

Selected aspects of children's medical histories and family histories of asthma and allergy were collected at study entry. Personal history of allergy included any history of

hay fever, allergic rhinitis, allergies to food or medicine, inhaled dusts, pollen, molds, animal fur or dander, or skin allergies not including poison ivy and oak. Family history of asthma was defined as either biological parent having been diagnosed with asthma. During annual school visits, students' height and weight were measured and recorded using standard protocols. Children's exposure to smoking *in utero* and second hand smoking (SHS) exposure at home was determined from questionnaire data completed by the parent/guardian at the time of study entry. Children also provided information regarding personal smoking as well as exposure to SHS at home annually during a private interview. Body mass index (BMI) was calculated as weight (kg)/height^2 (m^2). We categorized BMI into age- and sex-specific percentiles based on the Centers for Disease Control (CDC) BMI growth charts using one-month age intervals. Participants with BMI at or above the 85th percentile were classified as overweight/at risk of overweight. Based on prior knowledge, we examined pets (dogs, cats, birds and other furry animals), pests, air conditioning, use of a gas stove, household water damage, mold and mildew on household surfaces, house plants, carpeting in bedrooms, heating source, age of the home and humidifier use as potential confounding covariates for household and indoor exposures.

Buccal Cell Collection and Processing

Children were provided with two toothbrushes and instructed to brush their teeth with the first and gently brush the buccal mucosa with the second toothbrush. The second 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 returned by mail.

Buccal cell suspensions were centrifuged on the day they were received in the laboratory. The pellets were stored frozen at –20^{0}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 (cat #D-5000; GENTRA, Minneapolis, MN) containing 100 μg/ml proteinase K overnight at 55^{0}C. DNA extraction was performed according to manufacturer's recommendations. The DNA samples were resuspended in an aqueous solution and stored at –20^{0}C.

SNP selection and genotyping

For *NOS2A*, we initially identified a set of SNPs from phase I HapMap data for Caucasians (http://www.hapmap.org) and NCBI SNP database (gene view in dbSNPs http://www.ncbi.nlm.nih.gov/SNP) with a SNP density of 1–3 SNPs/kb over a region 20kb upstream and 10kb downstream of *NOS2A*. SNPs were selected based on validation status, Illumina design score, and functional potential of the SNPs. The selected SNPs were first genotyped by the Illumina Golden Gate Assay in a representative sample of 71 Latino and 71 Caucasian participants in the Multi-Ethnic Cohort population [3]. The LD pattern for both ethnic groups were similar (Figure E1). htSNPs were then chosen using TagSNPs (available at http://www-rcf.usc.edu/~stram/tagSNPs.html), a program that implements an expectation maximization (EM) algorithm approach, by finding the minimum set of SNPs (within a block) which would have $R^2_h \geq 0.85$ for all haplotypes with an estimated frequency of ≥ 5% in either ethnic group[4]. We also examined the pairwise correlation between SNPs and selected additional tagSNPs to ensure adequate

coverage of all SNPs and to provide redundant coverage in the event of assay failure for critical SNPs. This SNP list was refined in an iterative manner to provide coverage with SNPs that had adequate performance on the Illumina BeadArray platform. After genotyping was completed, we excluded SNPs with poor performance (n=1: RS1060826), or that were monomorphic (n=2: RS3729662 and RS3729717) or with MAF<0.05 (n=4: RS3730017, RS16966563, RS8067177 and RS3730014). In the final SNP list, we included 24 SNPs defining the coding and the promoter region of *NOS2A* (Table E1). These SNPs had an overall SNP call rate of 90% or more.

All the SNPs were initially tested to observe whether they were in Hardy-Weinberg equilibrium. Linkage disequilibrium between different SNPs was measured by D' and squared correlation coefficients (R^2). All measures were calculated for both ethnic groups (Latino and Caucasian) separately as well as combined. As the LD structure (Figure E1), minor allele frequencies (Table E1), haplotype frequencies (Table E2) and effect estimates (Table E3 and E4) were largely similar in both ethnic groups, they were combined for all subsequent analyses.

Oxidative Stress Genes

The deletion polymorphism of *GSTM1* was determined using TaqMan methodology as previously described [5]. Genotyping of GSTP1 Ile-105Val (rs1695) [5] and CAT-262C>T (rs1001179) [6] was performed using the TaqMan Allelic Discrimination (AD) assay (Applied Biosystems, Foster City, CA). For HMOX-1, the size of $(GT)_n$ repeats in each fluorescence-labeled PCR product was determined with capillary electrophoresis and GeneScan Analysis Software (Applied Biosystems) [6].

Adjustment for Population Stratification

To address potential confounding by population stratification, four coefficients of ancestry variables were included in the models in addition to the self-described race/ethnicity variables [7,8].These ancestry variables were constructed from four principal components derived from a set of 233 unlinked ancestry informative markers. Controlling for these ancestry variables provided adjustment for population stratification beyond adjustment for typical self-reported racial and ethnic categories.

Lung Function Analysis

A hierarchical mixed-effects model was used to relate 8-yr growth in each lung function measure to *NOS2A*, with a basic structure that has been previously described. [9] Growth patterns in lung function were modeled using linear splines, parameterized so that 8-yr growth in lung function was estimated jointly with other model parameters. Random effects for the intercept and 8-yr growth parameters were included at the subject level. We estimated and tested the effect of *NOS2A* on 8-year lung function growth at age 18. The model allowed for separate growth curves for each sex, race, ethnicity, cohort, and baseline-asthma subgroup. The model also included adjustments for ethnicity markers, height, height^2, body mass index (BMI), BMI^2, current asthma status, exercise or respiratory illness on the day of the test, any tobacco smoking by the child in the last year, and indicator variables for field technician. Because the findings were similar for all three lung function measures (Table E5), we present the results for FEV_1 only.

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Figure E1: Ethnic Specific LD Pattern of *NOS2A* for Latinos and Caucasians. The vertical line marks the boundary between the promoter and coding region.

Table E1: Minor Allele Frequency of *NOS2A* SNPs by Ethnicity

NOS2A Region	RS #	Position	Minor Allele Frequency (%)	
			Non-Hispanic White	Hispanic White
Segment 1 (Block 1-3)	RS4796017	23099118	0.4316	0.4666
	RS8081248	23106091	0.4166	0.4008
	RS2297512	23116682	0.3806	0.3775
	RS2297518	23120724	0.1980	0.1499
	RS9797244	23121258	0.1940	0.1517
	RS2274894	23123298	0.3862	0.3811
	RS1137933	23130059	0.2402	0.1639
Segment 2 (Block 4-6)	RS4462652	23131525	0.2380	0.1634
	RS2297520	23132167	0.4032	0.3266
	RS944725	23133698	0.4052	0.4229
	RS9895453	23134884	0.4862	0.4425
	RS8072199	23140975	0.4308	0.2793
	RS3794766	23146048	0.2415	0.1604
	RS16949	23148826	0.2420	0.1632
	RS3730013	23150045	0.3391	0.4313
	RS10459953	23151645	0.3520	0.2989
Promoter Region (Block 7)	RS4795080	23159761	0.3847	0.3149
	RS2779253	23161377	0.2751	0.3759
	RS1889022	23162483	0.3676	0.3236
	RS10853181	23163745	0.3687	0.3228
	RS2531866	23167291	0.3683	0.3190
	RS1014025	23168866	0.3531	0.3107
	RS2531872	23171379	0.4568	0.4883

Table E2: : Haplotype frequencies for NOS2A regions, by ethnicity

	Haplotypes	Non-Hispanic White (%)	Hispanic White (%)
Segment 1 (Block 1-3)	h0110010	0.32	0.34
	h1000000	0.20	0.29
	h1001101	0.17	0.12
	h0000000	0.16	0.13
	Cumulative frequency	0.85	0.88
Segment 2 (Block 4-6)	h0101100010	0.24	0.15
	h1010111001	0.15	0.09
	h0010000100	0.12	0.21
	h0010000101	0.10	0.07
	h0101000010	0.09	0.11
	h0000000100	0.08	0.12
	h1001011001	0.06	0.05
	h0001000001	0.02	0.05
	Cumulative frequency	0.85	0.84
Block 7	h0111101	0.35	0.30
	h1000010	0.27	0.38
	h0000010	0.18	0.11
	h0000000	0.17	0.19
	Cumulative frequency	0.97	0.98

Table E3: Haplotype analysis for the association between *NOS2A* promoter haplotype **h0111101** and incident asthma and FEV_1, by cohort and by ethnicity

	H1011110	New-Onset Asthma HR (95%CL)	8-Year FEV_1 Growth Estimate (95%CL)
Cohort 1	None	Ref.	Ref.
	At least one	1.20 (0.82-1.75)	-48.23 (-99.5,3.05)
Cohort 2	None	Ref.	Ref.
	At least one	1.71 (1.18,2.45)	-10.50 (-56.89,35.88)
Non-Hispanic White	None	Ref.	Ref.
	At least one	1.34 (0.98,1.82)	-11.84 (-56.64,32.97)
Hispanic White	None	Ref.	Ref.
	At least one	1.63 (1.09,2.42)	-45.14(-98.66,8.37)

Cohort 1: 4^{th} graders recruited in 1993.
Cohort 2: 4^{th} graders recruited in 1996.

Table E4: Haplotype analysis for the association between *NOS2A* promoter haplotype **h0100001** and incident asthma and FEV_1, by cohort and by ethnicity

	H0100001	New-Onset Asthma HR (95%CL)	8-Year FEV_1 Growth Estimate (95%CL)
Cohort 1	None	Ref.	Ref.
	At least one	0.89 (0.58,1.34)	59.8 (7.2,112.4)
Cohort 2	None	Ref.	Ref.
	At least one	0.71 (0.46,1.07)	38.4 (-8.6,85.4)
Non-Hispanic White	None	Ref.	Ref.
	At least one	0.82 (0.58,1.17)	37.6 (-9.3,84.4)
Hispanic White	None	Ref.	Ref.
	At least one	0.68 (0.44,1.05)	54.3 (2.2,106.5)

Cohort 1: 4^{th} graders recruited in 1993.
Cohort 2: 4^{th} graders recruited in 1996.

Table E5: Main effect of two common *NOS2A* promoter haplotypes on the three lung function growth measures

Haplotype		Estimate* (ml) (95%CL) for 8-year growth in FEV1	MMEF	FVC
h0111101	None	Ref.	Ref.	Ref.
	At least one	-29.5 (-61.3,2.4)	-48.0 (-110.7,14.8)	-27.6 (-65.0,9.8)
h1000010	None	Ref.	Ref.	Ref.
	At least one	43.8 (11.4,76.3)	105.6 (41.9, 169.3)	36.8 (-1.3,74.9)

*Estimate and 95%CL are based on hierarchical mixed effects model (for detail see 'Methods').

Table E6: Overall associations between *NOS2A* segments and new-onset asthma or FEV_1 growth (in ml) assessed using Principle component (PC) analysis.

NOS2A Region	Degree of Freedom for likelihood ratio test (LRT)	New-Onset Asthma P-Value*	8-year growth in FEV_1 P-Value*
Segment 1 (Block 1-3)	2	0.06	0.96
Segment 2 (Block 4-6)	4	0.80	0.23
Block 7	1	0.002	0.02

* P-values are based on a likelihood ratio test comparing models with and without genetic data.

Table E7: Association between SNPs in *NOS2A* in the promoter region (Block 7) and new-onset asthma or FEV_1 growth (in ml)

SNP RS number	New-Onset Asthma		8-year growth in FEV_1	
	HR (95%CL)*	P-Value‡	Estimate (95%CL)†	P-Value‡
RS4795080	0.74(0.57,0.96)	0.02	42.82 (10.76,74.89)	0.03
RS2779253	1.46(1.17,1.82)	0.0009	-29.42 (-59.63,0.79)	0.07
RS1889022	1.44(1.15,1.80)	0.001	-26.45 (-56.89,3.98)	0.09
RS10853181	1.47(1.18,1.84)	0.0007	-30.13 (-60.37,0.11)	0.07
RS2531866	1.48(1.17,1.86)	0.001	-31.41 (-62.79,-0.03)	0.06
RS1014025	0.79(0.63,0.99)	0.04	31.21 (2.13,60.29)	0.06
RS2531872	1.49(1.19,1.86)	0.0005	-32.24 (-64.44,-4.03)	0.07

*HR and 95%CL are based on fitting the Cox proportional hazard model (for detail see 'Method').

† Estimate and 95%CL are based on the hierarchical mixed-effects model (for detail see 'Methods').

‡ P-values were adjusted for multiple testing using p_ACT method

Table E8: Haplotype analysis for the association between *NOS2A* promoter and incident asthma, adjusted for additional covariates

	Haplotype	New-Onset Asthma HR (95%CL)
	h0111101	
Base Model*	None	Ref.
	At least one	1.44 (1.13,1.82)
Model 1†	None	Ref.
	At least one	1.42 (1.09,1.86)
Model 2‡	None	Ref.
	At least one	1.37 (1.06,1.76)
Model 3§	None	Ref.
	At least one	1.27 (0.97,1.67)
	h1000010	
Base Model*	None	Ref.
	At least one	0.77 (0.59,1.01)
Model 1†	None	Ref.
	At least one	0.81 (0.60,1.08)
Model 2‡	None	Ref.
	At least one	0.76 (0.57,1.01)
Model 3§	None	(Ref)
	At least one	0.71 (0.52,0.97)

* We fitted Cox proportional hazard model to test the association between the haplotypes and new-onset asthma with age- and sex-specific baseline hazard. All models were adjusted for community of residence, ethnicity and Q-factors.

†Model 1: was additionally adjusted for parental educational attainment, family income, birth weight, gestational age, overweight, health insurance, parental history of asthma, pets, humidifier use, other household characteristics and exposure to indoor-combustion sources including secondhand smoke.

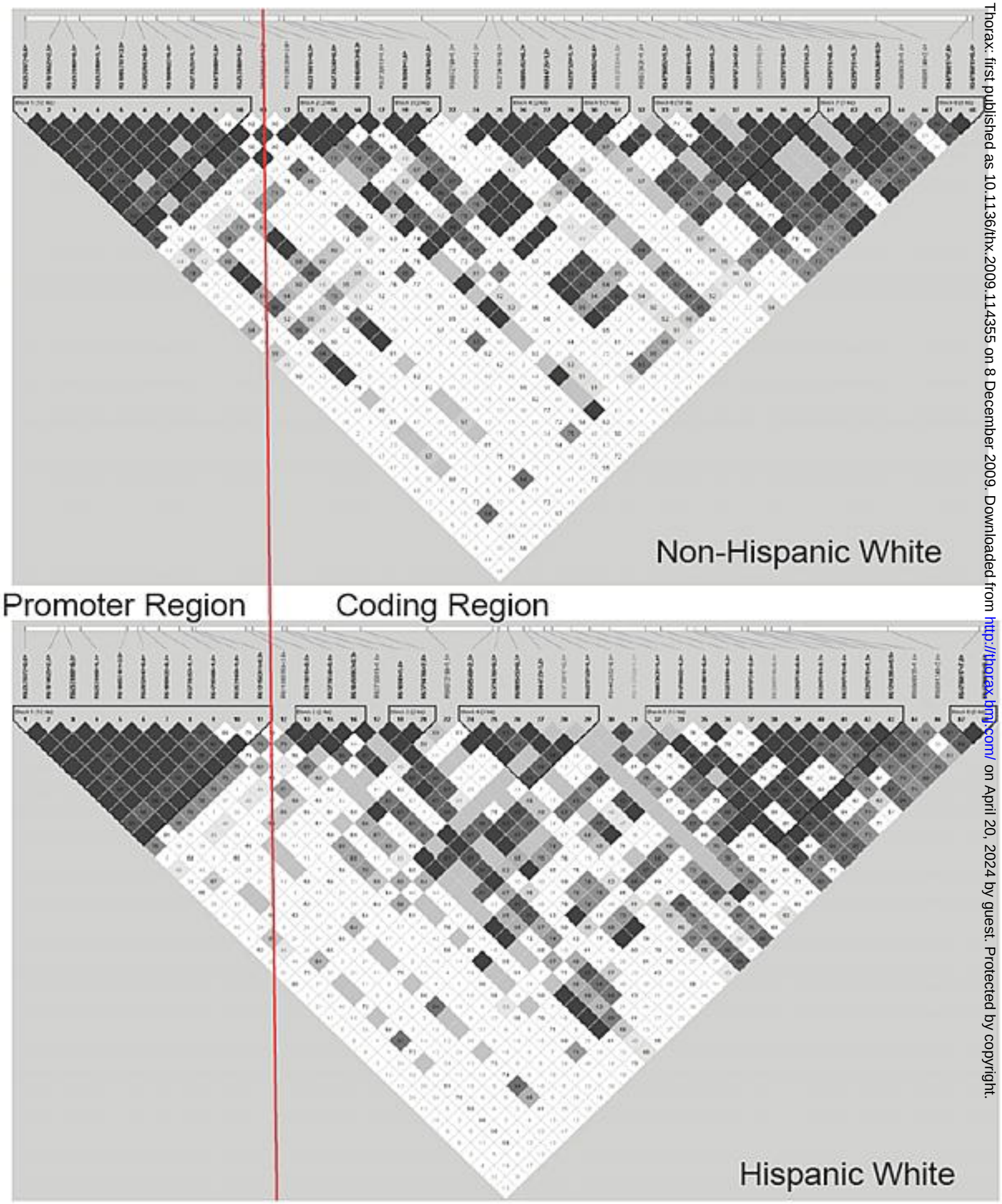
‡Model 2: was additionally adjusted for *GSTM1 'null', GSTP1(Ile105Val), HMOX1 and CAT* polymorphisms

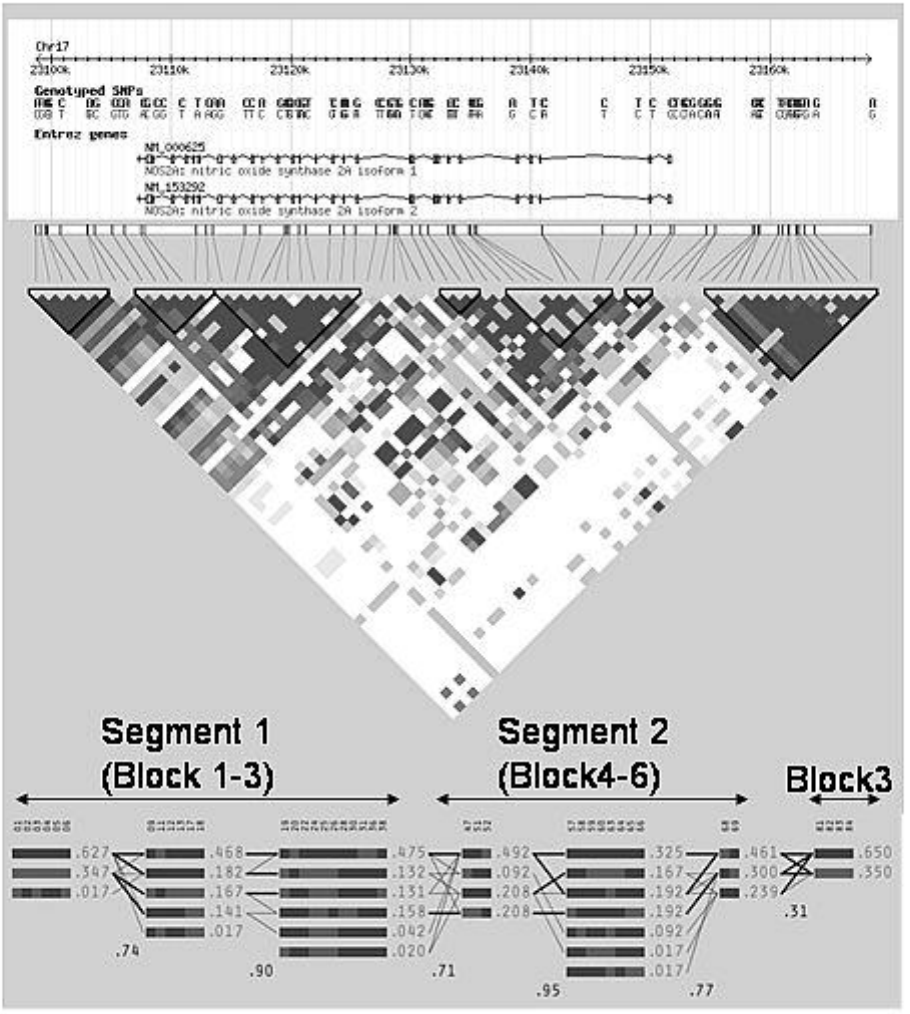
§ Model 3: had asthma definition restricted to children with asthma diagnosis and using inhaler medication (N=116 asthma cases).

Table E9: Haplotype analysis for the association between *NOS2A* promoter and FEV_1 growth (in ml), adjusted for additional covariates

	Haplotype	8-Year FEV_1 Growth Estimate (95%CL)*
		h0111101
Base Model*	None	Ref.
	At least one	-29.46 (-61.27,2.36)
Model 1†	None	Ref.
	At least one	-29.6 (-65.4,6.23)
Model 2‡	None	Ref.
	At least one	-24.0 (-59.7,11.76)
		h1000010
Base Model*	None	Ref.
	At least one	43.84 (11.39,76.28)
Model 1†	None	Ref.
	At least one	49.3 (12.8,85.7)
Model 2‡	None	Ref.
	At least one	50.7 (14.5,87.0)

* A hierarchical mixed-effects model was used to relate 8-yr growth in FEV_1. The base model allowed for separate growth curves for each sex, race, ethnicity, cohort, and baseline-asthma subgroup with adjustments for 'Q factors', height, height^2, BMI, BMI^2, current asthma status, exercise or respiratory illness on the day of the test, any tobacco smoking by the child in the last year, and indicator variables for field technician.
†Model 1 was additionally adjusted for in utero tobacco smoke exposure, indoor combustion sources such as a gas stove, pets, parental educational attainment, and environmental tobacco smoke.
‡Model 2 was additionally adjusted for *GSTM1* 'null', *GSTP1* (Ile105Val), *HMOX1* and *CAT* polymorphisms.




Chr17
23100k
23110k
23120k
23130k
23140k
23150k
23160k
Genotyped SNPs
Entrez genes
NM_000625
NOS2A: nitric oxide synthase 2A isoform 1
NM_153292
NOS2A: nitric oxide synthase 2A isoform 2
Segment 1
(Block 1-3)
Segment 2
(Block4-6)
Block3
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.347
.017
.468
.182
.167
.141
.017
.74
.475
.132
.131
.158
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.020
.90
.492
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.208
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.71
.325
.167
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.092
.017
.017
.95
.461
.300
.239
.77
.650
.350
.31