

Genetic variations in nitric oxide synthase and arginase influence exhaled nitric oxide levels in children

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Abstract

Background: Exhaled nitric oxide (FeNO) is a biomarker of airway inflammation. In the nitric oxide (NO) synthesis pathway, nitric oxide synthases (encoded by *NOS1*, *NOS2A*, and *NOS3*) and arginases (encoded by *ARG1* and *ARG2*) compete for L-arginine. Although FeNO levels are higher in children with asthma/allergy, influence of these conditions on the relationships between variations in these genes and FeNO remains unknown. The aims of the study were to evaluate the role of genetic variations in nitric oxide synthases and arginases on FeNO in children and to assess the influence of asthma and respiratory allergy on these genetic associations.

Methods: Among children (6–11 years) who participated in the southern California Children's Health Study, variations in these five genetic loci were characterized by tagSNPs. FeNO was measured in two consecutive years ($N = 2298$ and 2515 in Years 1 and 2, respectively). Repeated measures analysis of variance was used to evaluate the associations between these genetic variants and FeNO.

Results: Sequence variations in the *NOS2A* and *ARG2* loci were globally associated with FeNO ($P = 0.0002$ and 0.01 , respectively). The *ARG2* association was tagged by intronic variant rs3742879 with stronger association with FeNO in asthmatic children (P -interaction = 0.01). The association of a *NOS2A* promoter haplotype with FeNO varied significantly by rs3742879 genotypes and by asthma.

Conclusion: Variants in the NO synthesis pathway genes jointly contribute to differences in FeNO concentrations. Some of these genetic influences were stronger in children with asthma. Further studies are required to confirm our findings.

Fractional concentration of nitric oxide (FeNO) in exhaled breath has been recognized as a biomarker of airway inflammation (1). Measurement of FeNO provides a noninvasive tool to assess oxidative and nitrosative stress in the airways. Nitric oxide (NO) is synthesized by nitric oxide synthase (NOS) from L-arginine. In competition with NOS for this critical NO substrate, arginase also utilizes L-arginine in the urea cycle. In humans, three isoforms of NOS (encoded by *NOS1*, *NOS2A*, and *NOS3*) and two isoforms of arginase (encoded by *ARG1* and *ARG2*) have been identified. All three NOS isoforms (i.e., neuronal NOS or nNOS encoded by *NOS1*, endothelial NOS or eNOS encoded by *NOS3*, and inducible NOS or iNOS encoded by *NOS2A*) are expressed in airway epithelium (2, 3). Among arginase isoforms, arginase I is located in the cytosol, whereas arginase II is located

in the mitochondrial matrix (4). Both isoforms are expressed in the airway epithelium, smooth muscle, and peribronchial and perivascular connective tissues (5, 6).

To date, a limited number of studies have reported on the associations of selected putatively functional single nucleotide polymorphisms (SNPs) or microsatellite variants in *NOS* genes and FeNO with inconsistent results (7–12). To the best of our knowledge, the influences of *ARG* variants on FeNO have not been reported. Among the susceptibility factors, atopic conditions (asthma, allergic rhinitis) are associated with higher FeNO (13, 14). In this study, we aimed to investigate common variants within the *ARG* and *NOS* genetic loci as determinants of FeNO levels in children. We aimed to test three hypotheses: (i) DNA sequence variation in the *ARG* and *NOS* genetic loci is globally associated with FeNO levels,

(ii) variants in these loci have joint effects on FeNO levels, and (iii) associations between variants in these loci and FeNO vary by child's history of asthma and allergy. We tested these hypotheses in a population-based study conducted between Hispanic and non-Hispanic white children who had participated in the southern California Children's Health Study (CHS).

Methods

Design and study population

Subjects were participants in a cohort of the Children's Health Study established in 2003. Details about the study design have been described elsewhere (15). The University of Southern California Institutional Review Board approved the protocol. Briefly, children were recruited from 13 Southern California communities when they were in kindergarten or first grade (5–7 years old). Although FeNO data were available irrespective of race/ethnicity, genetic data were only available on Hispanic and non-Hispanic white children. Therefore, the present analysis is limited to these two ethnic groups. Children had FeNO measurement in two consecutive school years: 2004–2005 (Year 1; $N = 2298$) and 2005–2006 (Year 2; $N = 2515$). Because 2040 children had FeNO data on both years, there are 2773 children available for the combined analysis.

FeNO measurement

Details of the FeNO collection and quality control approaches have been reported earlier (16, 17). FeNO was measured using the offline technique by collecting breath samples in bags at 100 ml/s expiratory flow rate following the ATS guidelines (18). In a subsample ($N = 361$) for whom both offline and online (50 ml/sec flow) techniques were used, online FeNO levels was predicted reliably (model adjusted $R^2 = 0.94$) using a statistical model that incorporated offline FeNO, ambient NO, and lag time between the time of collection and FeNO measurement (16). In the present analysis, predicted online FeNO data obtained from that model were used.

Selection of genetic variants

Details of haplotype-tagged single nucleotide polymorphism (htSNPs) selection and genotyping methods have been presented in the Supporting information. Each genetic locus was defined as 20 kb upstream and 10 kb downstream of the gene. A minimum set of htSNPs with minor allele frequency ≥ 0.05 were chosen that explained $>90\%$ of the haplotype diversity for each haplotype block using the TagSNPs program (available at <http://www-hsc.usc.edu/~stram/tagSNPs.html>) (19).

Based on these criteria, we genotyped 30 SNPs in *NOS1*, 24 in *NOS2A*, 10 in *NOS3*, 6 in *ARG1*, and 10 in *ARG2* loci using the Illumina BeadArray platform (Tables S1–S4). In addition, we genotyped 233 ancestry informative markers (AIMs) to differentiate ancestry to address population stratification issues. We used the STRUCTURE program (a free

software package available at <http://pritch.bsd.uchicago.edu/structure.html>) to differentiate four major ancestral populations (African, European, American Indian, and East Asian, shown in Fig. S1). Details of the basic algorithm of the program (20–23) and the methods utilized in similar multiethnic populations have been published elsewhere (24, 25).

We evaluated the genetic loci using single nucleotide polymorphism (SNPs), whereas majority of the previous work focused on the role of variations in microsatellites on FeNO levels (7–12). To follow up and validate earlier work, we additionally determined the repeat lengths of *NOS1* intron 20 (AAT)_n and exon 29 (CA)_n repeats, *NOS2A* (CCTTT)_n repeat and -/AAAT insertion (rs12720460) in the promoter region, and one *NOS3* 27 base pair repeats in intron 4 (see Supporting information and Table S5 for more details). To minimize the number of statistical tests, we evaluated the associations of these variants with FeNO by using the cut-points that were used by the aforementioned investigators.

Haplotype estimation

For each locus represented by multiple haplotype blocks, presence of high multiallelic D' (>0.8) between adjacent blocks allowed the estimation of determining common haplotypes ($>5\%$ frequency) using all SNPs within those blocks. Haplotype frequencies were estimated for each ethnic group separately using SAS macro code available with the TagSNPs program (19). For details, see the Supporting information.

Assessment of covariates

Race/ethnicity, physician diagnosis of asthma, history of respiratory allergy (allergic rhinitis and/or hay fever), asthma medication use during the previous 12 months [rescuer (i.e., as-needed bronchodilators) or controller (i.e., regular long-acting bronchodilators, corticosteroids, or leukotriene inhibitors) or both], and exposure to secondhand tobacco smoke (SHS) were based on parental reports. Height and weight were measured on the day of test. Age- and sex-specific percentiles based on the Centers for Disease Control and Prevention body mass index (BMI) growth charts (<http://www.cdc.gov/NCCDPHP/dnpa/growthcharts/resources/sas.htm>) were used to categorize BMI.

Statistical analysis

Predicted online FeNO was not normally distributed and was natural-log-transformed (mentioned as FeNO henceforth). Repeated measures analysis of variance was used with autoregressive correlation structure to account for within subject correlation in FeNO (PROC MIXED in SAS) to compute estimates of the association between the genetic variants and FeNO level. To examine the contribution of each gene to variation in FeNO, we first tested the global association between variation in each genetic locus and FeNO using principal component (PC) analysis based on the tagSNPs using appropriate likelihood ratio tests (LRTs) and following methods described earlier (26). In the PC-based approach, a

set of PCs (ordered by magnitude of explained variance) that explained at least 80% of the tagSNP variance within the locus was used to test global associations with FeNO levels. All models were adjusted for age, race/ethnicity, an index of genetic ancestry, asthma, history of respiratory allergy, SHS exposure, respondent education, community of residence, and hour and month of FeNO collection, as these factors were *a priori* potential confounders and were associated with FeNO in our data (all *P*-values < 0.10).

We next examined the contribution of specific variation in the loci that were globally associated with FeNO. Haplotype- and SNP-based analyses were conducted using additive genetic models. Two analytic approaches were utilized for haplotype-based analysis. In the first modeling approach, we examined the effects of all common haplotypes (haplotype frequency $\geq 5\%$) on FeNO using the most common haplotype

as the reference group. In the second modeling approach, we conducted single haplotype models to evaluate the association of FeNO with a particular haplotype relative to all other haplotypes. This was performed to reduce the number of haplotypes that would be tested for gene–gene interaction. For the SNP-based analysis, *P*-values for SNPs within a genetic locus were further adjusted by Bonferroni correction for multiple testing.

The influences of asthma and respiratory allergy were evaluated using LRTs with appropriate interaction terms restricting such assessment of interaction to genetic variants that showed significant associations within loci that were globally associated with FeNO. Because *NOS2A* haplotypes and *ARG2* rs3742879 SNP were associated with FeNO, we further examined the joint effects of *NOS2A* haplotypes and the *ARG2* rs3742879 SNP on FeNO. All tests were two-sided

Table 1 Associations of selected characteristics of the study population with FeNO*

	Year 1		Year 2		Mean FeNO (ppb) (95% CI) [†]	<i>P</i> -value [‡]
	<i>N</i>	%	<i>N</i>	%		
Age (years) [mean (range)] [§]	2298	7.9 (6–10)	2515	8.8 (7–11)	34.9% (34.5–35.2%)	<0.0001
Sex						
Girls	1173	51.0	1283	51.0	12.66 (12.29, 13.05)	0.61
Boys	1125	49.0	1232	49.0	12.53 (12.15, 12.91)	
Ethnicity						
Non-Hispanic white	905	39.4	975	38.8	11.99 (11.59, 12.41)	0.0003
Hispanic white	1393	60.6	1540	61.2	12.99 (12.64, 13.35)	
Asthma						
No	2091	91.0	2200	87.5	12.16 (11.89, 12.44)	<0.0001
Yes	207	9.0	315	12.5	16.61 (15.63, 17.66)	
Respiratory allergy						
No	1101	47.9	1108	44.1	11.51 (11.16, 11.87)	<0.0001
Yes	1196	52.1	1405	55.9	13.60 (13.22, 14.00)	
Exposure to secondhand Smoke						
No	2047	90.6	2244	89.6	12.54 (12.24, 12.84)	0.08
Yes	212	9.4	259	10.4	13.37 (12.48, 14.34)	
BMI percentile						
Underweight (<5th percentile)	52	2.3	56	2.2	12.55 (11.13, 14.14)	0.38
Normal (5th to <85th percentile)	1407	61.2	1543	61.4	12.70 (12.37, 13.04)	
Overweight (85th to <95th percentile)	407	17.7	385	15.3	12.17 (11.63, 12.73)	
Obese (≥ 95 th percentile)	432	18.8	531	21.1	12.67 (12.12, 13.26)	
Respondent education						
<12th grade	475	21.6	516	21.5	13.59 (12.96, 14.24)	0.0005
12th grade	404	18.4	456	19.0	12.42 (11.80, 13.06)	
Some college	838	38.1	908	37.7	12.68 (12.24, 13.14)	
College	263	11.9	281	11.7	11.84 (11.11, 12.63)	
Some graduate	221	10.0	244	10.1	11.54 (10.76, 12.38)	
Annual family income						
<\$15 500	297	15.0	320	14.9	13.49 (12.71, 14.31)	0.003
\$15 000–\$49 999	663	33.4	708	33.0	12.86 (12.37, 13.38)	
$\geq$ \$50 000	1025	51.6	1119	52.1	12.11 (11.72, 12.50)	

*Sample included Hispanic and Non-Hispanic white children in the Children's Health Study with available genetic and FeNO data, the latter collected between October 2004 and June 2005 (Year 1; *N* = 2298) and October 2005 and June 2006 (Year 2; *N* = 2515). Numbers do not always add up because of missing data.

[†]Geometric mean and 95% CIs are obtained from a univariate repeated measure analysis of variance (PROC MIXED in SAS).

[‡]*P*-values from repeated measure analysis of variance testing overall association of the variable with FeNO level.

[§]For age (continuous), mean and range and percent difference in FeNO per year is presented with 95% CIs.

at a 5% significance level. We used SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA) for all analyses.

Results

Sociodemographic characteristics

Children were 6–10 years of age in Year 1 and 7–11 years of age in Year 2 of FeNO measurements with nearly equal proportion of boys and girls (Table 1). Age, asthma, respiratory allergy, and Hispanic white ethnicity were statistically significantly associated with higher FeNO level; however, sex and BMI were not significantly associated with FeNO. Children with a low socioeconomic background (represented by parental education and annual family income) had lower FeNO. Children exposed to SHS had higher FeNO than those who were unexposed; however, this association was marginally significant ($P = 0.08$). American Indian ancestry was associated with 18.5% higher FeNO (95% CI: 7.3%–30.8%; $P = 0.0007$); however, European, African, and East Asian ancestries were not significantly associated with FeNO. Sociodemographic characteristics of the subjects in the current analysis were similar to all non-Hispanic and Hispanic white children at cohort entry and to those with genetic data (Table S6).

Global associations

Variations in the *NOS2A* ($P = 0.0002$) and *ARG2* ($P = 0.01$) loci were globally associated with FeNO (Table 2). Variations in *NOS1*, *NOS3*, and *ARG1* loci were not associated with FeNO. After adjusting for multiple testing, four *NOS2A* SNPs and one *ARG2* SNP were significantly associated with FeNO (described below and presented in Table S7 for *NOS1*, Table S8 for *NOS2A*, Table S9 for *NOS3*, Table S10 for *ARG1* and Table S11 for *ARG2* SNP based results). These results were similar by ethnicity (not shown).

NOS2A variants and FeNO

Haplotypes in the *NOS2A* coding (block 2 h0010000100 and h0001000001), promoter (block 3 h1000010, h0111101, and

h0000000), and downstream (block 1 h0110010) regions explained the haplotype block-specific associations with FeNO (Table 3). Further details of the haplotype block-specific findings are presented in the Supporting information. For *NOS2A*, variants in three highly correlated SNPs (i.e., rs8081248, rs2297512, and rs2274894; all Spearman correlation coefficient > 0.9) in the downstream region (block 1) and rs2531872 in the promoter region (block 3) were associ-

Table 3 Associations between *NOS2A* haplotypes and FeNO

Haplotypes*	Percent difference in FeNO (95% CI) [†]	P-value [‡]
Haplotype block 1 (coding and downstream region)		
h0110010	Reference	
h1000000	7.2 (3.1, 11.5)	0.0004
h1001101	5.8 (1.0, 10.9)	0.02
h0000000	8.0 (3.1, 13.0)	0.001
Other haplotypes	4.4 (−0.6, 9.8)	0.09
h0110010 vs. others	−6.2 (−9.1, −3.2)	0.00007
Haplotype block 2 (predominantly coding region)		
h0101100010	Reference	
h1010111001	−0.8 (−6.0, 4.7)	0.77
h0010000100	2.8 (−2.2, 8.0)	0.27
h0010000101	−1.6 (−7.5, 4.8)	0.62
h0101000010	−4.8 (−10.3, 0.9)	0.10
h0000000100	−5.9 (−11.1, −0.5)	0.03
h1001011001	2.4 (−4.3, 9.6)	0.49
h0001000001	9.1 (0.6, 18.4)	0.03
Other haplotypes	−1.2 (−6.2, 3.9)	0.63
h0000000100 vs. others	−6.0 (−10.3, −1.5)	0.01
h0001000001 vs. others	10.0 (2.0, 18.5)	0.01
Haplotype block 3 (upstream/promoter region)		
h0111101	Reference	
h1000010	5.3 (1.6, 9.2)	0.005
h0000010	2.8 (−1.9, 7.7)	0.25
h0000000	6.1 (1.6, 10.7)	0.007
Other haplotypes	6.8 (−4.2, 19.1)	0.23
h0111101 vs. others	−4.7 (−7.7, −1.7)	0.003
h1000010 vs. others	2.7 (−0.4, 5.8)	0.09
h0000000 vs. others	3.0 (−0.8, −6.8)	0.12

*SNP order in *NOS2A* haplotypes is rs4796017–rs8081248–rs2297512–rs2297518–rs9797244–rs2274894–rs1137933 for haplotype block 1, rs4462652–rs2297520–rs944725–rs9895453–rs8072199–rs3794766–rs16949–rs3730013–rs10459953–rs2779248 for haplotype block 2, and rs4795080–rs2779253–rs1889022–rs10853181–rs2531866–rs1014025–rs2531872 for haplotype block 3. Within each haplotype (h), '0' and '1' represent the common and the variant alleles at the ordered SNP position, respectively. For modeling approach for haplotype-based analysis, see the Statistical analysis section of the Methods.

[†]Percent difference in FeNO and 95% confidence intervals (95% CIs) associated with per copy of haplotype were obtained. All models include adjustment for covariates listed in the 'Methods' section.

[‡]P-values for the association between the haplotypes and FeNO.

Genetic Locus*	df [†]	N	P-value [‡]
<i>NOS1</i>	7	2701	0.79
<i>NOS2A</i>	4	2611	0.0002
<i>NOS3</i>	6	2720	0.79
<i>ARG1</i>	2	2742	0.67
<i>ARG2</i>	3	2735	0.01

*For details of SNPs included within each locus/haplotype block, see Tables S1 through S4 in the Supporting information.

[†]Degrees of freedom (df) reflect the number of principal components that accounted for ≥80% of the locus variance.

[‡]Global P-values are from likelihood ratio tests based on the degrees of freedom presented. All models include adjustment for covariates listed in the 'Methods' section.

Table 4 Associations between ARG2 haplotypes and SNP and FeNO

Haplotypes*	Percent difference in FeNO (95% CI) [†]	P-value [‡]
h1000100000	Reference	
h0000000010	-6.6 (-11.0, -1.9)	0.006
h1000000010	-4.0 (-9.4, 1.8)	0.17
h0000000100	0.7 (-4.4, 6.1)	0.79
h1111011101	0.0 (-6.5, 6.9)	0.99
h0111010100	-3.9 (-10.9, 3.7)	0.30
h0011010101	1.4 (-4.1, 7.1)	0.63
h0000011101	-3.6 (-11.1, 4.6)	0.38
Other haplotypes	-2.0 (-6.9, 3.1)	0.44
rs3742879	-4.8 (-7.7, -1.7)	0.02 [§]

*SNP order in ARG2 haplotypes is rs12885261–rs7144243–rs3759757–rs4902501–rs7156352–rs4902503–rs7140310–rs742869–rs3742879–rs10483801. Within each haplotype, '0' and '1' represents the common and the variant alleles at the ordered SNP position, respectively. Within each haplotype, '0' and '1' represent the common and the variant alleles at the ordered SNP position, respectively.

[†]Percent difference in FeNO and 95% confidence intervals (95% CIs) associated with each haplotype compared to the most common haplotype (reference) and per variant G allele for rs3742879 were obtained. All models include adjustment for covariates listed in the 'Methods' section.

[‡]P-values for the association between the haplotypes or SNP and FeNO.

[§]P-value for rs3742879 is adjusted for multiple testing using Bonferroni correction.

ated with lower FeNO after adjusting for multiple testing (Bonferroni corrected $P < 0.05$; Table S8). Therefore, tests for gene–gene interactions for NOS2A were restricted to these six haplotypes and to one of the three highly correlated SNPs (i.e., rs8081248) and rs2531872.

ARG2 haplotypes and FeNO

Compared to the most common haplotype, one ARG2 haplotype (h0000000010) that contained the variant allele in rs3742879 (A > G; intronic SNP) was associated with lower FeNO (Table 4). After adjusting for multiple testing, variant G allele in rs3742879 was associated with lower FeNO (Bonferroni corrected $P = 0.02$).

Joint effects of ARG2 and NOS2A variants on FeNO

We found that FeNO levels depended on variants in both ARG2 and NOS2A. The association of NOS2A block 3 h1000010 haplotype with FeNO varied significantly by ARG2 rs3742879 (P -interaction = 0.009; Table 5). Among children with rs3742879 AA genotype, the NOS2A haplotype was associated with higher FeNO. In contrast, among children carrying the rs3742879 GG genotype, the haplotype was associated with lower FeNO. The effects of the other

Table 5 Associations between NOS2A promoter haplotype and FeNO within each ARG2 rs3742879 genotype

ARG2 rs3742879 A > G genotypes	NOS2A Block 3 h1000010
AA	
Percent difference in FeNO*	5.6 (1.3, 10.1)
N	1487
P-value [†]	0.01
AG	
Percent difference in FeNO*	0.9 (-3.8, 5.8)
N	1044
P-value [†]	0.71
GG	
Percent difference in FeNO*	-8.9 (-17.6, 0.7)
N	215
P-value [†]	0.07
P-interaction [‡]	0.009

*Percent difference in FeNO and 95% confidence intervals (95% CIs) associated with per copy of haplotype were obtained from models stratified by ARG2 rs3742879 genotypes. All models include adjustment for covariates listed in the 'Methods' section.

[†]P-values for the association between the haplotypes and FeNO within each ARG2 rs3742879 genotype.

[‡]P-values for interaction for ARG2 rs3742879 by NOS2A haplotypes using additive genetic model were obtained from likelihood ratio tests from nonstratified models with appropriate interaction terms and were based on 1 degree of freedom.

NOS2A haplotypes (block 1 h0110010, block 2 h0001000001 and h0000000100, and block 3 h0111101 and h00000000) and the NOS2A SNPs that showed significant associations with FeNO did not vary by ARG2 rs3742879 (not shown).

Influence of asthma and respiratory allergy

Asthma status was an important factor for determining the contribution of these genetic variants to FeNO levels. The association between ARG2 rs3742879 and NOS2A block 3 haplotype h1000010 and FeNO varied by asthma (both P -interaction ≤ 0.05 ; Table 6). The ARG2 rs3742879 variant G allele was associated with lower FeNO, and the NOS2A block 3 haplotype h1000010 was associated with higher FeNO, and these associations were stronger in children with asthma. Respiratory allergy did not appear to play a role in the genetic associations, as the associations between NOS2A and ARG2 variants and FeNO did not vary by the history of respiratory allergy (not shown).

Other NOS variants and FeNO

We did not observe any significant associations between NOS1 intron 20 (AAT)_n and exon 29 (CA)_n, NOS2A promoter (CCTTT)_n and -/AAAT insertion, and NOS3 27 base pairs repeat and FeNO (Table S12).

Table 6 Association between *NOS2A* and *ARG2* variants and FeNO in children with and without asthma

	Asthma status		
Genetic variant	No	Yes	<i>P</i> -interaction [†]
<i>NOS2A</i> block 3 h1000010			
Percent difference in FeNO*	1.6 (−1.4, 4.7)	11.7 (−0.8, 25.8)	0.05
<i>N</i>	2455	343	
<i>P</i> -value	0.31	0.07	
<i>ARG2</i> RS3742879			
Percent difference in FeNO*	−3.5 (−6.5, −0.4)	−13.7 (−23.5, −2.7)	0.01
<i>N</i>	2472	348	
<i>P</i> -value	0.03	0.02	

*Percent difference in FeNO and 95% confidence intervals (95% CIs) associated with per variant allele and haplotype copy were obtained from models stratified by asthma. All models include adjustment for covariates listed in the 'Methods' section.

[†]*P*-values for interaction for asthma by haplotypes were obtained from likelihood ratio tests from nonstratified models with appropriate interaction terms and were based on 1 degree of freedom.

Discussion

We found that common variants in the NO synthesis pathway genes jointly contribute to variation in FeNO levels in children. Some of these genetic influences were stronger in children with asthma. These novel findings provide evidence that joint evaluation of the genetic variants in the NO synthesis pathway genes is needed to understand interindividual differences in FeNO levels in children.

Genetic variations in the *NOS2A* but not in the *NOS1* and *NOS3* were the determinants of FeNO. These findings are consistent with earlier observations that iNOS is the major determinant of NO synthesis in the airways (2). In an earlier study, we reported that promoter haplotypes in *NOS2A* (h1000010 and h0111101) are associated with asthma incidence and lung function growth (27). Experimental studies have found regulatory/enhancer elements within the upstream (28) as well as in the downstream 3'-untranslated region (29) of *NOS2A* that control iNOS expression or mRNA stability. Our findings are consistent with these experimental data suggesting that these *NOS2A* haplotypes may have affected FeNO level through differential gene expression. Further experimental studies are warranted to define the functional basis for differential expression or other mechanisms that could account for our observations.

ARG2 rs3742879 variant was associated with lower FeNO with stronger effect in children with asthma. We also found that this SNP modified the effect of *NOS2A* promoter haplotype h1000010 on FeNO. Although functional significance of this SNP remains to be investigated, this tagSNP has been associated with asthma (30, 31), airway hyper-responsiveness, and bronchodilator response (31). Our findings extend the earlier work and show that this SNP is an important determinant of FeNO, particularly in children with asthma. The mechanism for the observed gene–gene interaction may be because of competition for a common substrate, L-arginine, as arginase has been associated with reduced NO synthesis by inhibiting iNOS expression at the level of translation (32). Further work is warranted to understand the functional

significance of the independent and joint effects of *ARG2* and *NOS2A* variants on FeNO.

Interpretation of our results requires the consideration of some study limitations. In this large, population-based study where elementary school children were recruited from classrooms, we did not collect blood samples in the field setting to determine atopy status. Based on our experience in the CHS communities, nearly 50% parents would have declined participation had we collected blood samples. In addition, parental report of asthma and respiratory allergy may have resulted in misclassifications; however, it is unlikely that such reporting would be differential with respect to genotypes. Therefore, such nondifferential misclassification may have attenuated the effect estimates. Although our haplotype-tagged SNP selection approach was able to characterize the genetic loci and we observed associations with haplotypes and tagSNPs, the functional significance of these genetic variants remains unknown. It is possible that these variants are either functional or linked with the causal variants.

Conducting multiple tests is a concern in any genetic association study. To minimize false-positive findings from exhaustive mining of the data, in the present study, we implemented the following strategies at study design and data analysis stages. We only included the genes that are unequivocally the most proximally located in the NO synthesis pathway. Rather than assessing each variant separately, we conducted tests for global association with FeNO. Prior findings also support the biological relevance for these variants in asthma and related atopic phenotypes (27, 30, 31). Furthermore, we adjusted the *p*-values for SNP-based analysis for multiple testing. For testing interactions, we only evaluated variants that were associated with FeNO and examined the influence asthma and allergy – factors that have been consistently shown to be associated with FeNO in earlier research.

Replication of genetic associations in independent populations is required to limit false-positive findings and to provide robustness of the genetic associations although such replication is often problematic because of issues relating to sample size, effect size, allele frequencies, population admixture, and

gene–environment and gene–gene interaction (33). Lack of an independent replication sample did not allow us to replicate our findings. Although confirmation of our findings are needed in future studies, the strong biological knowledge base for the genes involved in airway NO production provides strong prior support of our findings.

The strengths of this study include large population-based sample of children with both genetic and FeNO data available in two successive years. In contrast to all previously published papers that evaluated selected *NOS* variants, we have evaluated the role of the genetic locus for the genes in the NO synthesis pathway on FeNO. We have also minimized population stratification by adjusting for ancestry in all our models.

We conclude that *NOS2A* and *ARG2* variants are the important genetic determinants of FeNO levels in children. Some of the variants in these loci have joint effects on FeNO. Child's asthma status influences some of these associations. These findings suggest that pathway-based approach may be necessary in evaluating the impact of genetic and other determinants of FeNO.

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Conflict of interest

The authors do not have any conflict of interest on this paper.

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Authorship contribution

MTS conducted the statistical analysis and wrote the manuscript; TMB, EBR, TI, KB, WJG, and FDG contributed in analysis and interpretation of the data and revised the manuscript for intellectual content; FDG conceived and designed the study and EBR maintained the database.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution of ancestral contribution between (A) non-Hispanic ($N = 1066$) and (B) Hispanic white ($N = 1707$) children.

Table S1. Minor allele and block-specific haplotype frequencies of *NOS1* by ethnicity.

Table S2. Minor allele and block-specific haplotype frequencies of *NOS2A* by ethnicity.

Table S3. Minor allele and block-specific haplotype frequencies of *NOS3* by ethnicity.

Table S4. Minor allele and haplotype frequencies of *ARG1* and *ARG2* by ethnicity.

Table S5. Forward and reverse primers and probes for *NOS1*, *NOS2A* and *NOS3* variants genotyped in the laboratory.

Table S6. Comparison of selective baseline characteristics between non-Hispanic and Hispanic white children at cohort entry, those with genotype data and those with any FeNO measured in Years 1 and 2.

Table S7. Associations between *NOS1* SNPs and FeNO.

Table S8. Associations between *NOS2A* SNPs and FeNO.

Table S9. Effects of *NOS3* SNPs and FeNO.

Table S10. Effects of *ARG1* SNPs and FeNO.

Table S11. Effects of *ARG2* SNPs and FeNO.

Table S12. Associations with *NOS1*, *NOS2A*, and *NOS3* putative functional variants and FeNO.

Data S1. Methods.

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**Genetic Variations in Nitric Oxide Synthase and Arginase Influence
Exhaled Nitric Oxide Levels in Children**

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Online Supplement (Supporting information)

METHODS

Study design

Of the 5774 children available at cohort entry, 4,819 (83.5%) were Hispanic and non-Hispanic whites (1). Among them, 3440 (71.4% of 4,819) were included in the Illumina BeadArray assay for genotyping. Questionnaires distributed to children at school and completed by parents at study entry and then annually, were used to determine children's demographic and allergy/asthma characteristics.

In Year 1 of FeNO measurement (Year 3 of the study), consent was obtained and testing was performed on 2792 children. After exclusion of breath samples that developed problems during storage, 2710 subjects had valid measurements. African American (N=59), Asian (N=93) or children with mixed ethnic background (N=167) with available FeNO data were not genotyped in this study because of insufficient sample sizes to conduct ethnic-specific analysis. Of the 2,391 non-Hispanic and Hispanic white, genotyping data were unavailable for 93 children. Therefore, 2,298 children with Hispanic and non-Hispanic white ethnic background had both genotype and FeNO data in Year 1.

In Year 2 of FeNO measurement (Year 4 of the study), testing was performed on 2,948 children. Of them, 2,605 were non-Hispanic and Hispanic white. Genotyping data were unavailable on 90 children. Therefore, 2,515 children with Hispanic and non-Hispanic white ethnic background had both genotype and FeNO data in Year 2. So, in the final analysis, we have 2,773 subjects with 2,040 with FeNO data on both Years 1 and 2.

Sample Collection for FeNO, Transport, and Analysis

Details of the protocol have been published elsewhere (2, 3). Exhaled breath collection was performed at schools, usually from midmorning to noon to avoid traffic-related peaks of ambient NO and possible effects of recent eating on FeNO. Each community was visited at least twice in different seasons, to minimize confounding of location and season effects. Health status at testing was evaluated by a brief questionnaire; subjects with symptoms of acute respiratory infection within the past 3 days were excluded or rescheduled. FeNO was collected in Mylar bags after discarding dead space air (2), using a commercial apparatus (Sievers Division, GE Analytical Instruments, Boulder, CO), with target expiratory flow of 100 ml/sec. Ambient air samples, collected similarly, were used to estimate each subject's ambient NO exposure at the time of testing. Samples were stored in coolers and transported to a central laboratory for NO analysis. Between collection and analysis, the samples were transported on “blue ice” at 2 to 8 degree C. Lag time between collection and analysis ranged from 2 to 26 hours (2, 3). Potential sources of measurement artifact were documented and tested statistically for influence on the results.

Buccal Sample Collection and Processing

Children were provided with two 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 2,000g 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 (cat #D-5000; GENTRA, Minneapolis, MN) containing 100 $\mu\text{g/ml}$ proteinase K overnight at 55°C . DNA extraction was performed according to manufacturer's recommendations. The DNA samples were resuspended in the hydration solution (GENTRA) and stored at -80°C .

Lab Protocol to Prevent Sample Contamination

To prevent contamination in the lab, we employed appropriate safeguards to ensure integrity of the samples. Upon arrival of the buccal collection kits in the lab, each tube was scanned and a unique barcode and a box location were assigned. During DNA extraction, a new set of labels with ID# and 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 in order 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 people were present for the bench work, one person 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, dilution plates or stock tubes were spun down before opening; we used deionized DNase, RNase free water, certified RNase, DNase, DNA, pyrogen, ATP free tubes and aerosol-resistant filter tips. We also had a designated post-PCR area where a different set of pipettes were used solely for post-PCR applications.

SNP Selection and Genotyping

In representative non-Hispanic and Hispanic white samples from the Multiethnic Cohort (n~71 each) (4), 1-3 SNPs/kb were genotyped using the Illumina Golden Gate Assay to determine ethnic-specific minor allele frequencies (MAFs) and patterns of linkage disequilibrium (LD) 20kb upstream and 10kb downstream region unless another gene was co-located within this region. A minimum set of haplotype-tagging SNPs (htSNPs) with MAFs ≥ 0.05 were chosen to explain >90% of haplotype diversity ($R^2 \geq 0.90$) for each haplotype block using the TagSNPs program (available at <http://www-rcf.usc.edu/~stram/tagSNPs.html>). Redundant tagging SNPs were genotyped to substitute for critical SNPs in the event of assay failure. SNPs were genotyped using the Illumina BeadArray® platform. Based on these criteria, we selected 30 SNPs in the *NOS1*, 24 in the *NOS2A*, 10 in the *NOS3*, 6 in *ARG1* and 10 in *ARG2* loci (Table S1 to S4). These SNPs had call rates >90%. To address population stratification issues, we genotyped 233 ancestry informative markers (AIMs) SNPs that have been used in similar population to differentiate four major ethnic study populations (African, European, American Indian, and East Asian) (5). In addition, we determined the repeat lengths of *NOS1* intron 20 (AAT)_n and exon 29 (CA)_n repeats, *NOS2A* (CCTTT)_n repeat and -/AAAT insertion (rs12720460) in the promoter region and one *NOS3* 27 base-pair repeats in intron 4 because these variants were evaluated previously (6-11).

Genotyping

Genomic DNA was extracted from buccal mucosal cells using PUREGENE™ DNA purification kit (Gentra Systems, Minneapolis, MN). The genotyping for *NOS1* (CA)_n, and (AAT)_n, *NOS2A* AAAT insertion and (CCTTT)_n and *NOS3* 27bp repeats were performed using fluorescence PCR and GeneScan® analysis (Applied Biosystems, Foster City, CA) and by following previous methods (12-14). The primers and labels used for these assays are presented in Table S5. To reduce non-specific PCR product, the Touchdown PCR method was applied and the fluorescence PCR was amplified on a GeneAmp PCR system 9600. In the Touchdown PCR method, the annealing temperature is initially set at 69 °C then gradually decreased over a period of 16 cycles to 57 °C. Finally, PCR was performed over 30 cycles of 45 s at 95 °C, 1 min at 56 °C, and 1 min at 72 °C. The size of each NOS repeats in each fluorescence-labeled PCR product was determined with GeneScan Analysis Software (Applied Biosystems). Five known samples and four duplicates were run in each experiment as positive and within-plate controls, respectively. Genotypes of twelve homozygous samples were also confirmed by automatic sequencing for all repeats (BigDye version 3.1, 377XL DNA sequencer, Applied Biosystems) and also gel assay for *NOS3* 27bp repeat (14).

Selection of the cut-points for the repeats and insertion/deletion variants

The cut-points were based previously published papers on asthma/atopy and other inflammatory diseases. We dichotomized *NOS1* intron 20 (AAT)_n at ≥12 repeats (<12/<12, <12/≥12 and ≥12/≥12) (6, 10), *NOS1* exon 29 (CA)_n at either 17(<17/<17, <17/≥12 and ≥17/≥17) or 18 repeats (<18/<18, <18/≥18 and ≥18/≥18) (15), *NOS2A* promoter (CCTTT)_n at 14 repeats (<14/<14, <14/≥14 and ≥14/≥14) (16). *NOS2A* -/AAAT insertion/deletion variant was

categorized into “no insertion/no insertion” (*ni/ni*), “no insertion/insertion” (*ni/ins*) and “insertion/insertion” (*ins/ins*) (17), and *NOS3* 27 base-pair repeat was categorized in *bb* (5/5 repeats), *ba* (5/4 repeats) and *aa* (4/4 repeats) (18). In order to minimize the number of tests in our analyses, we conducted *a priori* hypothesis testing with previously reported repeat length cut-offs. We did not attempt to conduct additional analyses to identify other cut-offs that could have provided significant associations.

Haplotype estimation

For each locus represented by multiple haplotype blocks, presence of high multiallelic D' (>0.8) between adjacent blocks allowed estimation of determine common haplotypes ($>5\%$ frequency) using all SNPs within those blocks. Haplotype frequencies were estimated separately for Hispanic and non-Hispanic white subjects using SAS macro code available with the TagSNPs program (19).

There were limited differences in haplotype frequencies by ethnicity for the *NOS* and *ARG* loci (Tables S1 to S4). Because *NOS1* and *NOS2A* loci were larger in size (148.6kbp and 73.8kbp, respectively) and had low multiallelic D' s (<0.8) between some adjacent haplotype blocks, haplotypes were estimated for 4 blocks for *NOS1* and 3 blocks for *NOS2A* loci. The promoter regions of *NOS1* and *NOS2A* were contained in corresponding haplotype blocks 4 and 3, respectively. Only one haplotype block was evident in *NOS3* (containing the promoter region) and haplotypes were estimable for this block. For *ARG1* and *ARG2*, high multiallelic D' between adjacent haplotype blocks allowed estimation of common haplotypes using all SNPs within each locus.

RESULTS

NOS2A haplotypes and FeNO: In *NOS2A* haplotype block 1 (containing SNPs from exon 10 to 8.8kb downstream of the gene), compared to the most common haplotype (h0110010), all other haplotypes were positively associated with FeNO level (Table 3). To reduce the degrees of freedom in subsequent analyses, the impact of the most common haplotype on FeNO was then evaluated, and as expected, h0110010 was associated with lower FeNO ($P=0.00007$).

Two haplotypes (h0000000100 and h0001000001) in *NOS2A* haplotype block 2 (containing SNPs from 277bp upstream of the gene to intron 9) were significantly associated with FeNO level (Table 3). For the promoter haplotypes for *NOS2A* (haplotype block 3, extending up to 19.7kb upstream of the genetic locus), the two most common haplotypes (h1000010 and h0111101 comprising more than 60% haplotype frequencies) were mirror images of each other. Compared to the most common haplotype (h0111101), haplotype h1000010 and h0000000 were significantly associated with higher FeNO (Table 3). Because the common haplotypes were positively associated with FeNO, when haplotype h0111101 was evaluated alone compared to all other haplotypes, it was significantly associated with lower FeNO.

FIGURE S1. Distribution of ancestral contribution among (A) non-Hispanic (N=1,066) and (B) Hispanic white (N=1,707) children. The X-axes represent proportion of the sample within each ethnic group, whereas the Y-axes represent proportion on ancestral contribution from each of the four ancestral populations.

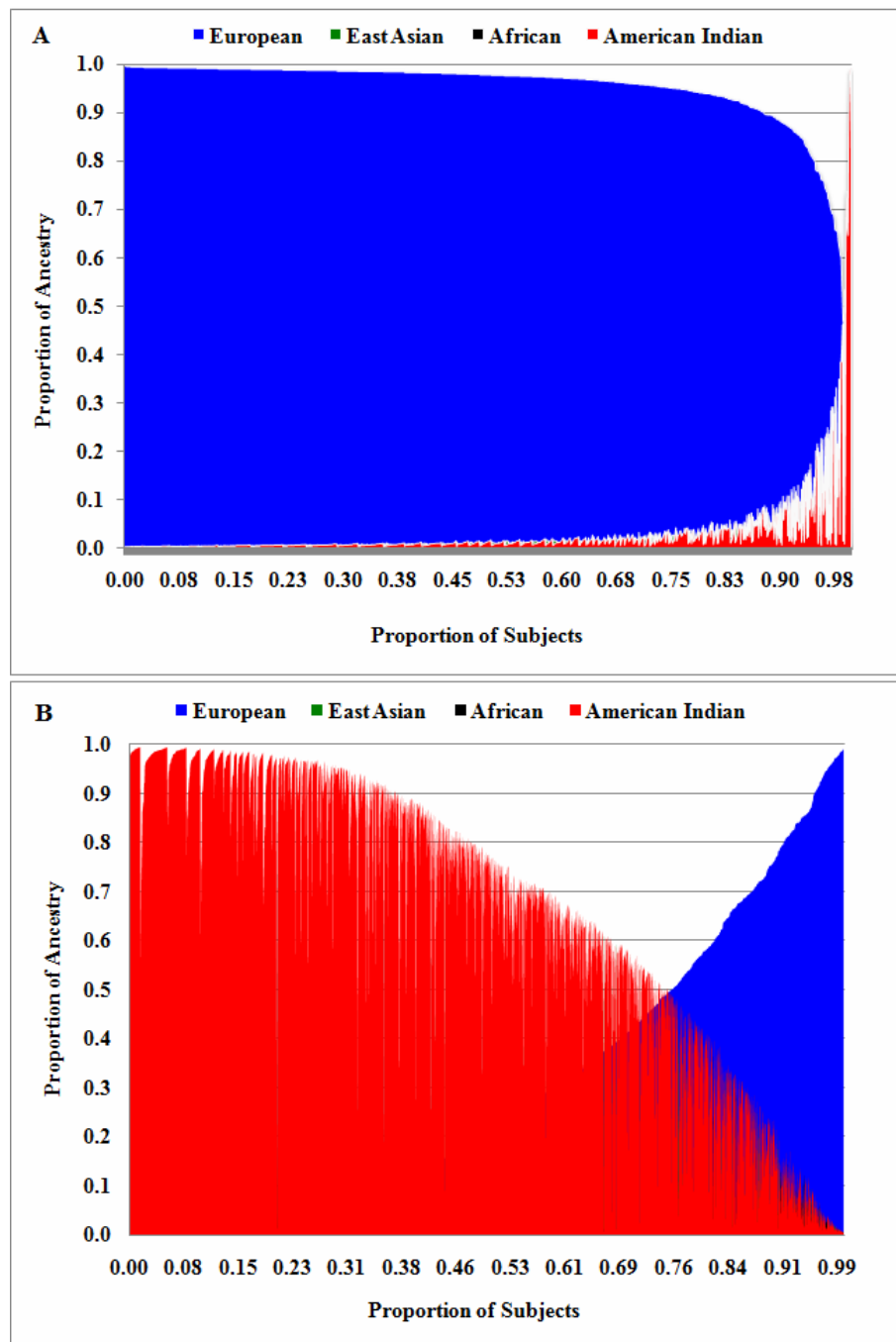


TABLE S1. Minor allele and block-specific haplotype frequencies of *NOS1* by ethnicity

SNPs	Minor allele Frequency		Haplotypes [*]	Haplotype frequency	
	Non-Hispanic white	Hispanic white		Non-Hispanic white	Hispanic white
Haplotype block 1					
rs9658570	0.10	0.32	h0010001000	0.50	0.31
rs816361	0.27	0.24	h0101100001	0.15	0.11
rs816363	0.59	0.39	h1001010000	0.09	0.30
rs904658	0.34	0.56	h0010001001	0.08	0.05
rs1353939	0.21	0.20	h0101100000	0.04	0.07
rs9658474	0.14	0.38	Other haplotypes [†]	0.14	0.15
rs816353	0.59	0.38			
rs2650163	0.10	0.11			
rs816358	0.09	0.06			
rs4767524	0.32	0.26			
Haplotype block 2					
rs2293054	0.30	0.48	h010000010	0.34	0.22
rs7964845	0.56	0.37	h010101000	0.17	0.10
rs7139256	0.20	0.15	h101011001	0.15	0.11
rs9658351	0.42	0.56	h000100100	0.09	0.07
rs532967	0.18	0.15	h100101000	0.09	0.21
rs552268	0.49	0.50	h100100100	0.01	0.11
rs11068445	0.12	0.19	Other haplotypes [†]	0.15	0.18
rs561712	0.39	0.29			
rs1879419	0.18	0.15			
Haplotype block 3					
rs12578547	0.25	0.46	h01010	0.34	0.24
rs570234	0.38	0.27	h10001	0.20	0.30
rs1004356	0.18	0.11	h00001	0.15	0.13
rs693534	0.39	0.39	h00100	0.12	0.08
rs1123425	0.43	0.47	h00101	0.05	0.02
			h10010	0.01	0.11
			Other haplotypes [†]	0.13	0.12
Haplotype block 4 (promoter region)					
rs9658247	0.43	0.47	h010000	0.19	0.26
rs1879417	0.40	0.31	h001000	0.19	0.28
rs4767535	0.46	0.45	h010100	0.18	0.10
rs816292	0.44	0.49	h101000	0.13	0.08
rs7301107	0.28	0.19	h100000	0.11	0.07
rs12811576	0.05	0.05	h101100	0.05	0.05
	0.05	0.04	h100100	0.05	0.03
			Other haplotypes [†]	0.11	0.12

* SNP order in *NOS1* haplotypes is rs9658570-rs816361-rs816363-rs904658-rs1353939-rs9658474-rs816353-rs2650163-rs816358-rs4767524 for haplotype block 1, rs2293054-rs7964845-rs7139256-rs9658351-rs532967-rs552268-rs11068445-rs561712-rs1879419 for haplotype block 2, rs12578547-rs570234-rs1004356-rs693534-rs1123425 for haplotype block 3, and rs9658247-rs1879417-rs4767535-rs816292-rs7301107-rs12811576 for haplotype block 4 (promoter region). Within each haplotype, ‘0’ and ‘1’ represents the common and the variant alleles at the ordered SNP position, respectively.

† Haplotypes with <5% frequencies are combined into the “other haplotypes” category.

TABLE S2. Minor allele and block-specific haplotype frequencies of *NOS2A* by ethnicity

SNPs	Minor allele Frequency		Haplotypes*	Haplotype frequency	
	Non-Hispanic white	Hispanic white		Non-Hispanic white	Hispanic white
Haplotype block 1 (coding and downstream region)					
rs4796017	0.43	0.47	h0110010	0.32	0.34
rs8081248	0.42	0.40	h1000000	0.20	0.29
rs2297512	0.38	0.38	h1001101	0.17	0.12
rs2297518	0.20	0.15	h0000000	0.16	0.14
rs9797244	0.19	0.15	Other haplotypes [†]	0.15	0.12
rs2274894	0.39	0.38			
rs1137933	0.24	0.16			
Haplotype block 2 (predominantly coding region)					
rs4462652	0.24	0.16	h0101100010	0.24	0.15
rs2297520	0.40	0.33	h1010111001	0.15	0.09
rs944725	0.41	0.42	h0010000100	0.12	0.20
rs9895453	0.49	0.44	h0010000101	0.10	0.07
rs8072199	0.43	0.28	h0101000010	0.09	0.11
rs3794766	0.24	0.16	h0000000100	0.09	0.12
rs16949	0.24	0.16	h1001011001	0.06	0.05
rs3730013	0.34	0.43	h0001000001	0.02	0.05
rs10459953	0.35	0.30	Other haplotypes [†]	0.15	0.16
rs2779248	0.39	0.32			
Haplotype block 3 (upstream/promoter region)					
rs4795080	0.28	0.38	h0111101	0.35	0.31
rs2779253	0.37	0.32	h1000010	0.27	0.37
rs1889022	0.37	0.32	h0000010	0.18	0.11
rs10853181	0.37	0.32	h0000000	0.17	0.19
rs2531866	0.35	0.31	Other haplotypes [†]	0.02	0.02
rs1014025	0.46	0.49			
rs2531872	0.37	0.32			

* SNP order in *NOS2A* haplotypes is rs4796017-rs8081248-rs2297512-rs2297518-rs9797244-rs2274894-rs1137933 for haplotype block 1, rs4462652-rs2297520-rs944725-rs9895453-rs8072199-rs3794766-rs16949-rs3730013-rs10459953-rs2779248 for haplotype block 2, and rs4795080-rs2779253-rs1889022-rs10853181-rs2531866-rs1014025-rs2531872 for haplotype

block 3. Within each haplotype, ‘0’ and ‘1’ represents the common and the variant alleles at the ordered SNP position, respectively.

[†] Haplotypes with <5% frequencies are combined into the “other haplotypes” category.

TABLE S3. Minor allele and block-specific haplotype frequencies of *NOS3* by ethnicity

SNPs	Minor allele Frequency		Haplotypes*	Haplotype frequency	
	Non-Hispanic white	Hispanic white		Non-Hispanic white	Hispanic white
rs3778872	0.17	0.10	h010000	0.33	0.29
rs3807370	0.36	0.24	h001000	0.16	0.34
rs1036145	0.34	0.30	h100001	0.14	0.09
rs10260501	0.25	0.41	h100010	0.13	0.08
rs3918226	0.09	0.05	h001001	0.09	0.07
rs3918169	0.15	0.14	h100101	0.08	0.05
rs1799983	0.15	0.14	Other haplotypes [†]	0.09	0.08
rs3918188	0.36	0.23			
rs2566514	0.26	0.50			
rs743507	0.25	0.17			
rs3800787	0.38	0.54			
rs1077872	0.35	0.28			
rs11760487	0.15	0.10			

* SNP order in *NOS3* haplotypes is rs3807370-rs1036145-rs10260501-rs3918226-rs3918169-rs1799983. Within each haplotype, ‘0’ and ‘1’ represents the common and the variant alleles at the ordered SNP position, respectively. Haplotype estimation for the remainder of the SNPs (i.e., rs3918188, rs2566514, rs743507, rs3800787, rs1077872, and rs11760487) was not possible because they were not in a haplotype block (i.e., not in linkage disequilibrium).

[†] Haplotypes with <5% frequencies are combined into the “other haplotypes” category.

TABLE S4. Minor allele and haplotype frequencies of *ARG1* and *ARG2* by ethnicity

Gene, SNPs	Minor allele Frequency		Gene, Haplotypes*	Haplotype frequency	
	Non-Hispanic white	Hispanic white		Non-Hispanic white	Hispanic white
<i>ARG1</i>			<i>ARG1</i>		
rs2608981	0.35	0.22	000000	0.57	0.43
rs3895535	0.25	0.18	111111	0.13	0.21
rs2608937	0.25	0.17	000110	0.11	0.08
rs2749935	0.49	0.38	000100	0.05	0.05
rs2781659	0.41	0.32	100110	0.04	0.10
rs2246012	0.27	0.16	000001	0.02	0.05
			Other haplotypes [†]	0.08	0.08
<i>ARG2</i>			<i>ARG2</i>		
rs12885261	0.41	0.59	1000100000	0.28	0.16
rs7144243	0.17	0.21	0000000010	0.16	0.16
rs3759757	0.36	0.26	1000000010	0.13	0.08
rs4902501	0.35	0.26	0000000100	0.12	0.11
rs7156352	0.19	0.31	1111011101	0.09	0.05
rs4902503	0.42	0.26	0111010100	0.04	0.05
rs7140310	0.16	0.14	0011010101	0.04	0.15
rs742869	0.56	0.40	0000011101	0.003	0.06
rs3742879	0.25	0.30	Other haplotypes [†]	0.14	0.18
rs10483801	0.31	0.19			

* 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’ represents wild-type and variant alleles at the ordered SNP position, respectively.

[†] Haplotypes with <5% frequencies are combined into the “other haplotypes” category.

TABLE S5. Forward and reverse primers and probes for *NOS1*, *NOS2A* and *NOS3* variants genotyped in the laboratory

Gene	Variant	Primer direction	Primer sequences (fluorescent labels)
<i>NOS1</i>	(CA) _n	Forward Primer	5'-CAAGCTCTCTGAGCCAGTCC-3' (5'-fluorescent-labeled with 6FAM)
		Reverse Primer	5'- GACACGTTTCTTGGCATTGA -3'
<i>NOS1</i>	(AAT) _n	Forward Primer	5'-GCAATGGTGTGTTGGTGAAC-3' (5'-fluorescent-labeled with VIC)
		Reverse Primer	5'-GCCAGGTGTTTCGAGACTAGC-3'
<i>NOS2A</i>	AAAT insertion	Forward Primer	5'-TGCCACTCCGCTCCAG-3'
		Reverse Primer	5'-GGCCTCTGAGATGTTGGTCTT-3' (5'-fluorescent-labeled with 6FAM)
<i>NOS2A</i>	(CCTTT) _n	Forward Primer	5'-TGGAAGCCTACACTGCATTC-3' (5'-fluorescent-labeled with NED)
		Reverse Primer	5'-CACTGCACCCTAGCCTGTCT-3'
<i>NOS3</i>	27bp tandem repeat	Forward Primer	5'-CCTGGTTATCAGGCCCTATG-3' (5'-fluorescent-labeled with HEX)
		Reverse Primer	5'-AGGCTGCTCCTGCTACTGAC-3'

TABLE S6. Comparison of selective baseline characteristics among non-Hispanic and Hispanic white children at cohort entry, those with genotype data and those with any FeNO measured in Years 1 and 2

	Cohort entry (N=4819)		With genetic data (N=3440)		With FeNO data (N=2773)	
	N	(%)	N	(%)	N	(%)
Age (yrs)						
4-5	1190	(24.7)	850	(24.7)	671	(24.2)
6	2338	(48.5)	1654	(48.1)	1324	(47.7)
7-8	1291	(26.8)	936	(27.2)	778	(28.1)
Sex						
Girls	2334	(48.5)	1691	(49.2)	1405	(50.7)
Boys	2481	(51.5)	1749	(50.8)	1368	(49.3)
Ethnicity						
Non-Hispanic White	1742	(36.2)	1292	(37.6)	1066	(38.4)
Hispanic White	3077	(63.8)	2148	(62.4)	1707	(61.6)
Exposure to secondhand smoke						
No	4276	(94.6)	3110	(94.8)	2535	(95.4)
Yes	245	(5.4)	171	(5.2)	121	(4.6)
BMI percentile						
Underweight (<5 th percentile)	627	(13.0)	386	(11.2)	300	(10.8)
Normal (5 th to <85 th percentile)	2917	(60.5)	2121	(61.7)	1732	(62.5)
Overweight (85 th to <95 th percentile)	635	(13.2)	472	(13.7)	370	(13.3)
Obese (>95 th percentile)	640	(13.3)	461	(13.4)	371	(13.4)
Respondent education						
<12 th grade	1102	(24.4)	726	(22.1)	693	(25.1)
12 th grade	903	(19.9)	623	(19.0)	501	(18.2)
Some college	1686	(37.3)	1266	(38.6)	1008	(36.5)
College	457	(10.1)	360	(11.0)	303	(11.0)
Some graduate	375	(8.3)	303	(9.2)	253	(9.2)
Annual family income (in US dollars)						
<15,500	732	(18.2)	465	(15.8)	729	(26.6)
15,000 - 49,999	1454	(36.2)	1027	(35.0)	810	(29.5)
≥50,000	1825	(45.6)	1442	(49.2)	1203	(43.9)

* Numbers do not always add up because of missing data.

TABLE S7. Associations between *NOS1* SNPs and FeNO

rs number	Percent difference* (95% CI)	P-value*	Percent difference† (95% CI)	P-value†
rs9658570	3.0 (-0.4, 6.5)	0.08	-1.2 (-4.7, 2.3)	0.49
rs816361	-1.4 (-4.8, 2.1)	0.43	-0.6 (-3.9, 2.7)	0.72
rs816363	-1.1 (-4.0, 1.8)	0.46	1.5 (-1.5, 4.5)	0.33
rs904658	0.7 (-2.2, 3.7)	0.64	-2.4 (-5.2, 0.6)	0.11
rs1353939	-2.5 (-6.1, 1.3)	0.20	-2.2 (-5.6, 1.4)	0.23
rs9658474	1.7 (-1.4, 5.0)	0.29	-2.4 (-5.6, 0.8)	0.14
rs816353	-0.5 (-3.4, 2.4)	0.73	2.4 (-0.6, 5.4)	0.12
rs2650163	0.4 (-4.4, 5.5)	0.88	1.3 (-3.3, 6.1)	0.58
rs816358	0.2 (-5.2, 5.9)	0.95	1.8 (-3.4, 7.3)	0.51
rs4767524	-2.2 (-5.4, 1.1)	0.18	-0.4 (-3.5, 2.8)	0.79
rs2293054	1.0 (-2.0, 4.1)	0.51	-1.8 (-4.7, 1.2)	0.23
rs7964845	-0.2 (-3.1, 2.8)	0.90	2.0 (-0.9, 5.0)	0.18
rs7139256	-4.0 (-7.8, -0.2)	0.04	-1.2 (-4.9, 2.6)	0.52
rs9658351	3.8 (0.9, 6.9)	0.01	1.1 (-1.7, 4.1)	0.43
rs532967	-2.5 (-6.4, 1.5)	0.21	-0.2 (-4.0, 3.7)	0.91
rs552268	-0.4 (-3.4, 2.7)	0.81	-0.7 (-3.5, 2.1)	0.62
rs11068445	3.0 (-1.0, 7.2)	0.14	2.3 (-1.5, 6.3)	0.24
rs561712	-1.5 (-4.5, 1.7)	0.35	-0.1 (-3.1, 2.9)	0.94
rs1879419	-2.4 (-6.3, 1.7)	0.24	-0.4 (-4.2, 3.6)	0.84
rs12578547	1.0 (-1.9, 4.1)	0.50	-2.1 (-5.0, 0.9)	0.18
rs570234	-1.1 (-4.2, 2.1)	0.50	0.6 (-2.4, 3.7)	0.68
rs1004356	1.5 (-2.8, 6.1)	0.50	1.8 (-2.4, 6.2)	0.41
rs693534	0.5 (-2.5, 3.6)	0.77	0.7 (-2.2, 3.6)	0.65
rs1123425	0.8 (-2.1, 3.8)	0.59	0.3 (-2.4, 3.1)	0.82
rs9658247	-0.7 (-3.7, 2.5)	0.68	1.3 (-1.7, 4.4)	0.40
rs1879417	-0.4 (-3.3, 2.6)	0.78	0.5 (-2.3, 3.3)	0.73
rs4767535	-0.7 (-3.6, 2.3)	0.66	-0.9 (-3.7, 1.9)	0.51
rs816292	-1.9 (-5.3, 1.7)	0.30	0.5 (-2.9, 4.0)	0.76
rs7301107	-2.7 (-9.0, 4.2)	0.44	0.0 (-6.2, 6.6)	1.00
rs12811576	-3.9 (-10.6, 3.2)	0.27	-0.5 (-7.0, 6.5)	0.88

*Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained without any covariate adjustment.

[†]Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained. All models include adjustment for covariates listed in the ‘Methods’ section.

TABLE S8. Associations between NOS2A SNPs and FeNO

rs number	Percent difference* (95% CI)	P-value*	Percent difference† (95% CI)	P-value†
rs4796017	5.7 (2.6, 9.0)	<.0000001*	4.9 (1.9, 7.9)	0.001‡
rs8081248	-6.2 (-9.0, -3.2)	<.0000001*	-6.1 (-8.7, -3.3)	0.00003‡
rs2297512	-6.0 (-8.9, -3.1)	<.0000001*	-5.8 (-8.5, -3.0)	0.00006‡
rs2297518	0.8 (-3.2, 4.9)	0.71	2.8 (-1.1, 6.8)	0.16
rs9797244	1.0 (-3.0, 5.2)	0.62	2.7 (-1.1, 6.7)	0.17
rs2274894	-5.8 (-8.7, -2.9)	<.0000001*	-5.8 (-8.5, -3.0)	0.00006‡
rs1137933	-1.7 (-5.5, 2.2)	0.38	0.3 (-3.3, 4.1)	0.87
rs4462652	-1.6 (-5.2, 2.2)	0.40	0.6 (-3.0, 4.3)	0.75
rs2297520	-3.3 (-6.3, -0.2)	0.04	-2.2 (-5.1, 0.8)	0.14
rs944725	2.3 (-0.7, 5.5)	0.14	2.0 (-0.9, 5.0)	0.17
rs9895453	-0.5 (-3.5, 2.6)	0.76	0.2 (-2.6, 3.2)	0.87
rs8072199	-1.8 (-4.9, 1.4)	0.26	0.4 (-2.6, 3.6)	0.79
rs3794766	0.2 (-3.5, 4.1)	0.90	2.4 (-1.2, 6.1)	0.20
rs16949	0.7 (-3.0, 4.6)	0.71	2.9 (-0.7, 6.6)	0.12
rs3730013	1.4 (-1.7, 4.6)	0.38	-0.5 (-3.3, 2.5)	0.75
rs10459953	-3.2 (-6.3, 0.0)	0.05	-2.8 (-5.8, 0.2)	0.07
rs2779248	2.0 (-1.2, 5.3)	0.23	3.9 (0.8, 7.1)	0.01
rs4795080	4.6 (1.4, 8.0)	0.004	2.4 (-0.6, 5.5)	0.12
rs2779253	-5.4 (-8.4, -2.3)	0.001‡	-4.2 (-7.0, -1.2)	0.006
rs1889022	-5.4 (-8.4, -2.4)	0.001‡	-4.2 (-7.1, -1.3)	0.005
rs10853181	-5.7 (-8.7, -2.6)	0.0003‡	-4.3 (-7.2, -1.4)	0.004
rs2531866	-6.0 (-9.1, -2.8)	0.0003‡	-4.5 (-7.5, -1.5)	0.004
rs1014025	2.5 (-0.5, 5.6)	0.11	1.9 (-1.0, 4.8)	0.20
rs2531872	-6.1 (-9.1, -3.1)	0.0001‡	-4.8 (-7.6, -1.9)	0.002‡

*Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained without any covariate adjustment.

†Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained. All models include adjustment for covariates listed in the ‘Methods’ section.

‡ P-values are statistically significant after Bonferroni correction.

TABLE S9. Effects of NOS3 SNPs and FeNO

rs number	Percent difference* (95% CI)	P-value*	Percent difference† (95% CI)	P-value†
rs3778872	-1.4 (-5.6, 3.1)	0.54	-0.5 (-4.6, 3.8)	0.81
rs3807370	-0.9 (-4.1, 2.5)	0.60	1.8 (-1.4, 5.1)	0.27
rs1036145	-0.3 (-3.5, 3.1)	0.87	0.4 (-2.7, 3.6)	0.80
rs10260501	2.4 (-0.7, 5.6)	0.14	-0.1 (-3.1, 3.0)	0.93
rs3918226	0.3 (-5.5, 6.4)	0.92	3.6 (-2.0, 9.6)	0.21
rs3918169	0.7 (-3.5, 5.2)	0.74	-0.3 (-4.3, 3.9)	0.89
rs1799983	-1.3 (-4.7, 2.1)	0.44	1.3 (-1.9, 4.7)	0.43
rs3918188	-2.7 (-5.9, 0.5)	0.10	-0.6 (-3.8, 2.6)	0.70
rs2566514	3.7 (0.8, 6.7)	0.01	-0.4 (-3.3, 2.6)	0.80
rs743507	-2.2 (-5.9, 1.6)	0.25	-0.1 (-3.7, 3.6)	0.94
rs3800787	3.6 (0.7, 6.6)	0.02	1.1 (-1.7, 4.0)	0.45
rs1077872	-1.7 (-4.8, 1.5)	0.29	-1.2 (-4.2, 1.8)	0.42
rs11760487	0.0 (-4.5, 4.8)	0.99	1.0 (-3.3, 5.6)	0.65

*Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained without any covariate adjustment.

†Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained. All models include adjustment for covariates listed in the ‘Methods’ section.

TABLE S10. Effects of *ARG1* SNPs and FeNO

rs number	Percent difference* (95% CI)	P-value*	Percent difference† (95% CI)	P-value†
rs2608981	2.1 (-1.1, 5.4)	0.20	-0.2 (-3.3, 2.9)	0.88
rs3895535	1.2 (-2.4, 4.9)	0.53	-0.9 (-4.2, 2.6)	0.62
rs2608937	1.7 (-1.9, 5.4)	0.36	-0.6 (-4.0, 2.8)	0.71
rs2749935	1.5 (-1.5, 4.6)	0.34	0.2 (-2.7, 3.1)	0.91
rs2781659	2.3 (-0.8, 5.4)	0.16	1.0 (-1.9, 4.0)	0.49
rs2246012	1.0 (-2.4, 4.6)	0.57	-0.5 (-3.8, 2.9)	0.77

*Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained without any covariate adjustment.

†Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained. All models include adjustment for covariates listed in the ‘Methods’ section.

TABLE S11. Effects of *ARG2* SNPs and FeNO

rs number	Percent difference* (95% CI)	P-value*	Percent difference† (95% CI)	P-value†
rs12885261	-0.6 (-3.5, 2.4)	0.67	1.8 (-1.1, 4.8)	0.24
rs7144243	-2.3 (-6.1, 1.6)	0.24	0.0 (-3.6, 3.9)	0.98
rs3759757	3.4 (0.1, 6.8)	0.04	2.7 (-0.4, 5.9)	0.09
rs4902501	2.7 (-0.6, 6.1)	0.11	1.9 (-1.1, 5.1)	0.22
rs7156352	-0.5 (-4.0, 3.2)	0.79	2.0 (-1.5, 5.5)	0.27
rs4902503	4.1 (0.9, 7.4)	0.01	2.0 (-1.0, 5.2)	0.19
rs7140310	-0.4 (-4.5, 3.9)	0.85	0.6 (-3.3, 4.7)	0.76
rs742869	4.3 (1.3, 7.3)	0.005‡	2.5 (-0.3, 5.4)	0.09
rs3742879	-5.3 (-8.4, -2.1)	0.001‡	-4.8 (-7.7, -1.7)	0.002‡
rs10483801	3.9 (0.5, 7.5)	0.02	2.3 (-1.0, 5.7)	0.17

*Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained without any covariate adjustment.

†Percent difference in FeNO and 95 % confidence intervals (95% CIs) and P-values for the association between the SNPs and FeNO were obtained. All models include adjustment for covariates listed in the ‘Methods’ section.

‡ P-values are statistically significant after Bonferroni correction.

TABLE S12. Associations of *NOS1*, *NOS2A* and *NOS3* putative functional variants and FeNO

Gene	Variant	Cut-points	N	Percent difference* (95% CI)	P-value*
<i>NOS1</i>					
	(AAT)n	12	2711	-1.7 (-4.5, 1.3)	0.27
	(CA)n	17	2709	-0.2 (-3.3, 3.0)	0.91
	(CA)n	18	2709	1.1 (-3.8, 6.3)	0.66
<i>NOS2A</i>					
	AAT insertion		2723	-1.5 (-6.2, 3.4)	0.54
	(CCTTT)n	14	2718	0.5 (-3.6, 4.8)	0.81
	(CCTTT)n	15	2718	-2.3 (-6.1, 1.6)	0.24
<i>NOS3</i>					
	27 base-pair repeat	4	2706	0.0 (-4.3, 4.5)	0.99
	rs1799983	additive	2770	1.3 (-1.9, 4.7)	0.43

* Percent difference in FeNO and 95 % confidence interval (95% CIs) associated with each

functional variant was obtained. All models include adjustment for covariates listed in the

‘Methods’ section.

‡P-values for the association between the functional variants and FeNO.

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