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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.

Keywords

airway inflammation; asthma; biomarker; exhaled nitric oxide; nitrosative stress

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

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:

Introduction

Fractional concentration of nitric oxide (FeNO) in exhaled breath has been recognized as a biomarker of airway inflammation (1). Measurement of FeNO provides a non-invasive 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 are 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 among 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 (50ml/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 time of collection and FeNOmeasurement (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 estimation of determine 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 ≥ 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 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 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 Tables 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 promoter region (block 3) were associated 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 variant (*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 *NOS2A* haplotypes (block 1 h0110010, block 2 h0001000001 and h0000000100, and block 3 h0111101 and h0000000) 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 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).

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 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 hyperresponsiveness, and bronchodilator response (31). Our findings extend the earlier work and show that this SNP is an 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 non-differential 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 of 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 due to 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Associations of selected characteristics of the study population with FeNO*

	Year 1		Year 2		Mean FeNO (ppb) (95% CI) †	*P*-value‡
	N	%	N	%		
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 (≥95th 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)	

	Year 1		Year 2		Mean FeNO (ppb) (95% CI) †	*P*-value‡
	N	%	N	%		
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)	
≥$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.

Table 2

Global associations of *NOS1*, *NOS2A*, *NOS3*, *ARG1*, and *ARG2* loci with log FeNO

Genetic Locus*	df†	*N*	*P*-value§
NOS1	7	2701	0.79
NOS2A	4	2611	0.0002
NOS3	6	2720	0.79
ARG1	2	2742	0.67
ARG2	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 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.

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.

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 non-stratified models with appropriate interaction terms and were based on 1 degree of freedom.

Table 6

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

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

*Percent difference in FeNO and 95 % confidence intervals (95% CIs) associated with per variant allele and haplotype copy was 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 non-stratified models with appropriate interaction terms and were based on 1 degree of freedom.