DNA Methylation in the Arginase–Nitric Oxide Synthase Pathway Is Associated with Exhaled Nitric Oxide in Children with Asthma

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***Rationale*: Genetic variation in arginase (ARG) and nitric oxide synthase (NOS) has been associated with exhaled nitric oxide (FeNO) levels in children. Little is known about whether epigenetic variation in these genes modulates FeNO.**

***Objectives*: To evaluate whether DNA methylation in ARG and NOS genes is associated with FeNO.**

***Methods*: A subset of 940 participants in the Children's Health Study were selected for this study. Children were eligible if they had FeNO measurements and buccal cells collected on the same day. CpG loci located in the promoter regions of *NOS1*, *NOS2A*, *NOS3*, *ARG1*, and *ARG2* genes were analyzed. Multiple loci in each gene were evaluated individually and averaged together. DNA methylation was measured using a bisulfite–polymerase chain reaction pyrosequencing assay. Linear regression models were used to investigate the association between DNA methylation and FeNO and whether associations differed by asthma status.**

***Measurements and Main Results*: DNA methylation in *ARG2* was significantly associated with FeNO. A 1% increase in average DNA methylation of *ARG2* was associated with a 2.3% decrease in FeNO (95% confidence interval, −4 to −0.6). This association was significantly larger in children with asthma (%diff = −8.7%) than in children with no asthma (%diff = −1.6%; $p_{int} = 0.01$). Differences in FeNO by asthma status were also observed for *ARG1* ($\%diff_{asthma} = -4.4\%$; $\%diff_{non\text{-}asthma} = 0.3\%$; $p_{int} = 0.02$). DNA methylation in NOS genes was not associated with FeNO.**

***Conclusions*: DNA methylation in *ARG1* and *ARG2* is associated with FeNO in children with asthma and suggests a possible role for epigenetic regulation of nitric oxide production.**

Keywords: arginase; nitric oxide synthase; FeNO; asthma

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Nitric oxide is involved in the pathophysiology of allergic airway diseases. Genetic variation in NOS and ARG genes is associated with fractional concentration of nitric oxide (FeNO) level, asthma pathogenesis, and lung function growth in children. Little is known about whether epigenetic variation in these genes modulates FeNO.

What This Study Adds to the Field

DNA methylation in the ARG1 and ARG2 promoters is associated with FeNO in children with asthma and indicates a role for epigenetic regulation of nitric oxide production.

Nitric oxide (NO) is involved in the pathophysiology of allergic airway diseases. Fractional concentration of NO (FeNO) is measurably higher in children with eosinophilic airway inflammation and active asthma or allergic airway diseases, conditions in which airway inflammation plays a prominent role (1–3). Based on the findings in children, FeNO has been suggested as a biomarker that reflects important aspects of airway inflammation that may be useful in the management of some children with asthma (4).

NO is synthesized from L-arginine by three NO synthase (NOS) isoforms: (*1*) neuronal NOS (nNOS encoded by *NOS1*); (*2*) endothelial NOS (eNOS encoded by *NOS3*); and (*3*) inducible NOS (iNOS encoded by *NOS2A*). The availability of intracellular L-arginine is a rate-limiting factor in NO production (5). Arginase (ARG) competes with NOS for the common substrate L-arginine. In humans, two isoforms of ARG (encoded by *ARG1* and *ARG2*) are recognized. All three NOS isoforms are expressed in airway epithelium (6–8). Among ARG isoforms, ARGI is located in the cytosol, whereas ARGII is located in the mitochondrial matrix (8). Both isoforms are expressed in the airway epithelium, smooth muscle, and peribronchial and perivascular connective tissues (9, 10). Therefore, differences in ARG and NOS expression levels or function caused by genetic or epigenetic variants have the potential to influence FeNO levels and respiratory outcomes. Emerging evidence from DNA sequence variants supports this hypothesis (11–14).

Genetic variation in NOS and ARG genes is associated with FeNO, asthma pathogenesis, and lung function growth in children (11–14). In particular, variation in SNP rs3742879 in *ARG2* was associated with decreased FeNO levels in our larger cohort (14). This inverse association was stronger in children with asthma compared with children without asthma. We also observed that a haplotype in *ARG1* was associated with reduced risk of asthma, and that this risk varied by the child's history of atopy (11). Thus, genetic variation in the promoters of ARG genes may affect gene expression and NO production.

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In addition to DNA sequence variation that affects expression patterns, epigenetic variation in NOS and ARG genes may play a role in modulating FeNO levels in children. *In vitro* studies in endothelial cells have demonstrated that increased DNA methylation in the promoter of *NOS3* is associated with decreased promoter activity (15). Paradoxically, inhibition of ARG activity was associated with increased lung inflammation and airway hyperresponsiveness in a rodent model, possibly by altering NO homeostasis (16). Taken together, the existing evidence indicates that epigenetic variation in the ARG–NOS system can perturb NO homeostasis and adversely affect children's respiratory health especially among children with asthma. Although DNA methylation of these genes in the proximal NO production pathway may have a role in respiratory tract NO homeostasis, this hypothesis has yet to be investigated.

In the present study, we investigated this hypothesis by studying the role of DNA methylation at CpG loci in NOS and ARG gene promoter regions on FeNO levels. CpG loci were selected in the promoter regions of each gene based on previous evidence of an increased likelihood for affecting gene expression or transcription. We also evaluated the association between DNA methylation and asthma, wheeze, and asthma symptoms. Because inflammatory pathways are up-regulated in asthma, the effects of DNA sequence variation are large and FeNO is higher in these groups, we hypothesized that the association between DNA methylation and FeNO is stronger in children with asthma compared with children without asthma. We also examined whether genetic variation in *ARG1* and *ARG2*, which we previously found to be associated with FeNO or asthma risk, and epigenetic variation in the same gene, were jointly associated with FeNO. We tested these hypotheses in a population-based study of children who had participated in the Southern California Children's Health Study.

METHODS

Study Population

This study was nested in the ongoing Children's Health Study (17). We sampled from 6,578 available buccal samples from 2,498 children originally recruited from kindergarten and first grades in 2002, who had at least one FeNO measurement and buccal sample collected at the same time in three consecutive school years: 2004–2005 (Year 1); 2005–2006 (Year 2); and 2006–2007 (Year 3). For the purposes of this study, selection was restricted to samples of children of non-Hispanic white and Hispanic white ethnicity who had genetic data available (n = 5,765). Further exclusions included missing FeNO measurements or measurements taken outside (n = 218); missing air pollution data or children living outside the eight towns under active follow-up (n = 1,524); missing asthma status (n = 122); and samples with little or no DNA remaining (n = 1,893) or low DNA concentration (n = 345). Of the remaining 1,663 samples from 1,137 children, a subset of 940 samples were randomly selected for this study of DNA methylation, with preference given to the earliest sample collected for those children for whom multiple samples existed. Because we based our initial selection of children with both FeNO and genetic data available, there is substantial overlap between this population and the population reported in our prior publication evaluating genetic variation and FeNO (14). The 940 children in this study are a subset of that population with the exception of 31 subjects (3.3%).

The institutional review board for human studies at the University of Southern California approved this study.

Selection of CpG Methylation Loci

We examined CpG loci located in the promoter regions of *NOS1*, *NOS2A*, *NOS3*, *ARG1*, and *ARG2* (*see* Figure E1 in the online supplement) (15, 18–22). For *ARG1*, a CpG locus in the promoter region (not in a CpG island), which was previously associated with change in expression, was selected (21). For *ARG2*, three CpG loci (Positions 1–3) in a CpG island in the promoter region were selected for analysis. This region of the promoter includes several transcription factor binding sites and numerous potential binding sites for enhancer and promoter elements (22). This region may also play a role in regulation of *ARG2* gene expression (22).

Two CpG loci in a nuclear hormone receptor regulatory sequence in exon 2 of *NOS1* were chosen for analysis, based on evidence that this nuclear hormone receptor site contributes to the regulation of *NOS1* transcription (18). Three regions within the *NOS2A* gene were selected for DNA methylation analysis. Two loci were chosen in a non-CpG island region (Positions 1 and 2) of the promoter because they were previously shown to be inversely related to NOS2A mRNA expression (19). A second region located in a non-CpG island between exons 1 and 2 (Position 3) was chosen to correspond to a previously investigated site by Tarantini and coworkers (20). The third region (Positions 4–7) was a novel locus located in a CpG island and was chosen to span transcription factor binding sites conserved in the mammal alignment (http://genome.ucsc.edu/). For *NOS3*, we examined two CpG loci located in the positive regulatory domain in the proximal promoter, which has a binding affinity with Sp1/Sp3 and has important contributions to *NOS3* transcription (23).

Polymerase chain reaction (PCR) primers targeting these loci were developed using MethPrimer software (Li LC, San Francisco, CA) (24). Primers were designed not to overlap with any repeated elements or single-nucleotide polymorphism (SNP) sites and the specificity of the primer sequence was confirmed using in-Silico PCR (Jim Kent, Santa Cruz, CA) (Table E1). More details are provided in the online supplement.

DNA Methylation

Buccal mucosal cells, an aerodigestive tract epithelium, were collected as a practical alternative for airway epithelium that express the NOS and ARG isoforms. Laboratory personnel performing DNA methylation analysis were masked to study subject information. One microgram of genomic DNA was converted using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, Orange, CA), according to the manufacturer's recommended protocol. Final elution was performed with 40 μl M-Elution Buffer (Zymo Research, Orange, CA). Bisulfite-converted DNA was stored at −70°C until further use. Methylation analyses were performed by bisulfite-PCR pyrosequencing assay using the HotMaster Mix (Eppendorf, Hamburg, Germany) and the PSQ HS 96 Pyrosequencing System (Biotage AB, Uppsala, Sweden) (25) as described in previous work (26). The output from pyrosequencing is reported as a percent of DNA methylation at each CpG locus. Additional information, including primer sequences, can be found in the online supplement.

Measurement of FeNO

Details of the FeNO collection and quality control approaches have been reported previously and are briefly summarized in the online supplement (27–29). In Years 1 and 2, FeNO was measured using the offline technique by collecting breath samples in bags at 100 ml/s expiratory flow-rate following the American Thoracic Society guidelines (30). In Year 3, online measurements were performed at 50 ml/s expiratory flow using EcoMedics CLD-88-SP analyzers, with DeNOx accessories to provide NO-free inhaled air (EcoPhysics Inc., Ann Arbor, MI, and Duernten, Switzerland), according to the manufacturer's instructions based on professional societies' recommendations (30–32). In a subsample (n = 361) for whom both offline and online (50 ml/s flow) techniques were used, online FeNO levels were 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 FeNO measurement (28). In the present analysis, predicted online FeNO data obtained from that model were used for Years 1 and 2, and online measurements were used in Year 3.

Selection of Genetic Variants

For this analysis, we evaluated one haplotype in *ARG1* (h000100) and one SNP in *ARG2* (rs3742879), either of which we previously found to be associated with either a reduced risk of asthma or decreased FeNO

levels, respectively (11, 14). Details of haplotype-tagged SNP selection and genotyping methods for this SNP and haplotype have previously been described in detail (11, 14).

Assessment of Covariates

Race and ethnicity, physician diagnosis of asthma, asthma symptoms, history of wheeze and respiratory allergy (allergic rhinitis or hay fever), asthma medication use during the previous 12 months, annual family income, parental education, and exposure to *in utero* and secondhand tobacco smoke were based on parental reports. Children were considered to have asthma if their parents reported that their child had ever been diagnosed by a physician as having asthma on the questionnaire collected at the time of the child's FeNO collection. 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 growth charts (http://www.cdc.gov/NCCDPHP/dnpa/growthcharts/resources/sas.htm) were used to categorize body mass index.

Statistical Analyses

Descriptive analyses were first conducted to examine the distribution of DNA methylation in ARG and NOS genes and FeNO by subject characteristics. Population characteristics for our selected population of 940 were compared with the unselected population (n = 1,558) to evaluate potential for selection bias. Characteristics were compared using either a Wilcoxon or chi-square test, as appropriate. Spearman correlations were used to study the pairwise correlations of percent methylation between different CpGs in the same gene. For genes with multiple CpG loci measured, percent DNA methylation at individual loci and average percent DNA methylation were analyzed. For some loci that had 20% or more values of zero methylation, analyses were also conducted using a dichotomous variable in which methylation was categorized as "unmethylated" if their percent DNA methylation value was zero; otherwise they were categorized as having "some" methylation. For categorizing average *ARG2* DNA methylation, we evaluated a score of the number of positions in *ARG2* that had some methylation.

To investigate the association between percent DNA methylation and FeNO, we fitted linear regression models adjusted for age; sex; race and ethnicity; experimental plate (for pyrosequencing reactions); town of residence; month of DNA collection; asthma status; asthma medication use; and parental education. Because FeNO values were not normally distributed, a natural log transformation was applied. Results are reported as percent changes in FeNO for a 1% difference in DNA methylation. A logistic regression model was used to evaluate the associations between DNA methylation and wheeze and asthma symptoms, bronchitis, cough, and phlegm.

To evaluate whether the association between DNA methylation of ARG and NOS genes varied by asthma or allergy status or by genetic variation in *ARG1* and *ARG2*, the appropriate statistical interaction terms were added to the linear regression model and likelihood ratio tests were conducted. *P* values were corrected for multiple testing using a Bonferroni adjustment. All tests assumed a two-sided alternative hypothesis; a 0.05 significance level; and were conducted using SAS/STAT software, version 9.2 (Cary, NC).

RESULTS

On average, children were 9 years old, two-thirds were of Hispanic white ethnicity, 14% had asthma by the time of FeNO collection, 23% reported ever wheezing, and 56% reported having history of respiratory allergies (Table 1). Median FeNO was 9.8 ppb (range = 114.3 ppb). FeNO levels were higher in children with asthma, in children who used asthma medication, and in children who ever reported wheeze (*see* Table E2). No differences in FeNO level were observed by sex, ethnicity, or exposure to *in utero* or secondhand smoke.

Only 940 out of 2,498 children were selected for this study. Children were selected primarily if they had a buccal cell sample and FeNO measurement in Years 3 or 5 with only a handful from Year 4. In Years 3 and 5, the selected population tended to have a greater number of Hispanic whites compared with non-Hispanic whites, and a greater percentage of children diagnosed with asthma (*see* Tables E3 and E4). The larger proportion of children with asthma resulted from the restriction of the population to participants with genome-wide association data available, because children with asthma were overselected in our previous genome-wide association study. Median FeNO was slighter lower in Year 3 and higher in Year 5, but the range of data was consistent across groups.

TABLE 1. DESCRIPTIVE CHARACTERISTICS OF THE 940 SELECTED CHS PARTICIPANTS*

Characteristic	Count (%)
Age, mean (SD)	9.3 (1.1)
Male	451 (48)
Race and ethnicity	
Hispanic white	607 (64.6)
Non-Hispanic white	333 (35.4)
Exposed to maternal smoking *in utero*	51 (5.6)
Exposure to paternal smoking *in utero*	113 (12.5)
Exposure to secondhand smoke	31 (3.5)
Ever diagnosed with asthma	133 (14.2)
Ever reported wheeze	193 (22.7)
History of respiratory allergy	522 (55.6)
Use of asthma medication	88 (10)
Annual family income	
≤$14,999	126 (15.9)
$15,000–$49,999	240 (30.3)
≥$50,000	425 (53.7)
Parent or guardian education	
<12th grade	193 (21.6)
Completed grade 12	163 (18.2)
Some college or technical school	302 (33.7)
Completed 4 yr of college	125 (14)
Some graduate training	112 (12.5)

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

Distribution of percent methylation of ARG and NOS loci is shown in Table 2. In general, the non-CpG island loci in *NOS1*, *NOS2A*, and *NOS3* were heavily methylated. The proportion of methylation at CpG loci in the *ARG1* non-CpG island and in *NOS2A* Position 3 (located in a non-CpG island between exons 1 and 2) were 50–60%, whereas CpG island loci in *NOS2A* and *ARG2* were largely unmethylated. Within each gene, the multiple CpG loci measured were not strongly correlated (*see* Table E5A–E5C).

We found that DNA methylation in *ARG2* but not *ARG1* was significantly associated with FeNO levels after adjusting for covariates listed in the METHODS section (Table 3). A 1% increase in average DNA methylation of *ARG2* was associated with a 2.3% decrease in FeNO (95% confidence interval [CI], −4 to −0.6; $P = 0.001$). *ARG2* Position 3 had the strongest association with FeNO, exhibiting a 2.6% decrease in FeNO for a 1% increase in DNA methylation (95% CI, −4 to −1.1; $P = 0.001$). This association remained statistically significant using a conservative Bonferroni adjustment for the 16 CpG loci tested (corrected $P = 0.02$). In contrast, a 1% change in DNA methylation of *ARG1* was associated with only a 0.1% decrease in FeNO (95% CI, −1.1 to 0.9). DNA methylation of the NOS genes was not associated with FeNO (*see* Table E6).

We found that the association between DNA methylation in both *ARG1* and *ARG2* and FeNO levels was larger in children with asthma than those without asthma (Table 3). In analyses stratified by asthma status, an increase in percent DNA methylation was more strongly associated with FeNO level in children with asthma than in children without asthma for three of the four CpG loci evaluated in *ARG1* and *ARG2* (percent change in FeNO ranged from −4.4 to −8.7 in children with

TABLE 2. DISTRIBUTION OF PERCENT METHYLATION OF CPG LOCI IN NITRIC OXIDE SYNTHASE AND ARGINASE GENES

Gene Loci	Location	N*	Mean	SD	Interquartile Range
NOS1					
Average†	Nonisland	908	73.57	4.86	6.36
Position 1	Nonisland	905	68.65	6.48	8.84
Position 2	Nonisland	894	89.66	4.14	5.29
Position 3	Nonisland	884	62.33	6.90	8.22
NOS2A					
Promoter average†	Nonisland	896	94.32	2.59	2.99
Position 1	Nonisland	896	91.65	2.48	2.82
Position 2	Nonisland	892	97.03	3.26	4.55
Position 3	Nonisland	916	51.31	4.29	5.79
CpG island average†	Island	940	1.49	2.46	0.89
Position 4	Island	940	2.08	3.05	1.72
Position 5	Island	939	1.76	3.16	2.03
Position 6	Island	939	1.01	2.39	1.11
Position 7	Island	940	1.12	2.18	1.20
NOS3					
Average†	Nonisland	914	89.89	5.37	5.96
Position 1	Nonisland	914	89.16	7.00	7.32
Position 2	Nonisland	914	90.61	5.97	6.27
ARG1					
Position 1	Nonisland	921	64.37	4.60	6.05
ARG2					
Average†	Island	876	1.84	2.55	1.34
Position 1	Island	876	1.29	2.74	1.32
Position 2	Island	875	1.57	3.01	1.58
Position 3	Island	875	2.64	3.12	2.38

* Sample size varies as a result of quality control screening for pyrosequencing reactions.

† Average methylation of multiple positions within the gene was used.

asthma vs. 0.3 to −1.8 in children without asthma) (Table 3). When DNA methylation across the three *ARG2* CpG loci was averaged, similar associations were observed. Because the three CpG loci in *ARG2* had very low levels of methylation in general, we evaluated the associations between the presence of any methylation compared with no methylation across the three loci. Individuals with asthma who had some DNA methylation in *ARG2* had lower levels of FeNO compared with individuals with no *ARG2* methylation and this association was most apparent in children with asthma (percent change in FeNO ranged from −29.6 to −34.6) for all three loci (Table 4). Moreover, within children with asthma, the largest difference in FeNO was observed in children who had some methylation at all three loci compared with one or two loci (Table 5).

We also evaluated the association between DNA methylation and FeNO among children with wheeze and by medication use in children with asthma. Similar, but weaker, results were observed in children who reported ever wheezing compared with children who never wheezed, although tests of interaction were not statistically significant (*see* Table E7). Among the 130 children with asthma, the association between DNA methylation in *ARG1* and *ARG2* seemed stronger in medication-users, although this interaction was not statistically significant (*see* Table E8). Medication itself may alter DNA methylation leading to changes in FeNO. Alternatively, individuals with asthma who take medication have a more severe form of disease, and it is the severity of disease, rather than medication use, that drives the association.

Although we observed striking differences in the association of *ARG1* and *ARG2* DNA methylation with FeNO by asthma status, percent DNA methylation in these loci were not different between children with and without asthma or for wheeze or asthma symptoms, such as bronchitis, cough, and phlegm. We previously reported that genetic variation in *ARG2* was associated with FeNO; however, adjustment for *ARG2* SNPs did not alter the observed associations between *ARG2* DNA methylation and FeNO. Lastly, genetic and epigenetic variation in the *ARG1* and *ARG2* genes did not have synergistic effects on FeNO levels overall or within subgroups with and without asthma.

DISCUSSION

We found that DNA methylation of ARG genes was inversely associated with FeNO and the association was stronger in children with asthma than in children without asthma. These results suggest that DNA methylation of ARG genes may play a role in modulating FeNO production for individuals in whom inflammatory and oxidative stress pathways are already up-regulated.

To our knowledge, no direct research has demonstrated that DNA methylation of *ARG1* or *ARG2* affects FeNO level in humans. Conventionally, both cytosolic *ARG1* and mitochondrial *ARG2* are believed to down-regulate NO production (33). Therefore, if methylation in promoter regions leads to transcriptional silencing, we would expect to observe decreased gene expression leading to increased availability of L-arginine and increased FeNO. Paradoxically, we observed decreased FeNO, suggesting there are other factors at play that remain to be clarified.

For *ARG1*, the target CpG site was located in the promoter region in a non-CpG island. Previous studies have shown that deletion of this region decreased gene expression (21). DNA methylation and expression in *ARG1* are inversely correlated in monocytes, supporting the notion that increased DNA methylation may lead to transcriptional silencing (Hooman Allayee, personal communication). However, overall DNA methylation of *ARG1* was already high in these cells, and expression generally low, thus its relative impact on FeNO is unclear.

TABLE 3. THE ASSOCIATION BETWEEN PERCENT METHYLATION OF ARGINASE GENES AND PERCENT CHANGE IN FRACTIONAL CONCENTRATION OF NITRIC OXIDE FROM LINEAR REGRESSION MODELS, CHILDREN'S HEALTH STUDY 2004–2007

		Combined*			Children with Asthma†			Children with No Asthma			
Gene Loci		% Difference	95% Confidence Interval	N	% Difference	95% Confidence Interval	N	% Difference	95% Confidence Interval	N	P_{int}*
ARG1	Position 1	−0.1	(−1.1 to 0.9)	921	−4.4	(−8.2 to −0.3)	130	0.3	(−0.7 to 1.2)	791	0.02
ARG2	Average‡	−2.3	(−4 to −0.6)	876	−8.7	(−14.6 to −2.4)	120	−1.6	(−3.3 to 0.1)	756	0.01
	Position 1	−1.6	(−3.1 to −0.03)	876	−7.6	(−13.3 to −1.6)	120	−1.1	(−2.6 to 0.5)	756	0.02
	Position 2	−1.1	(−2.6 to 0.3)	875	−5.3	(−11.5 to 1.2)	120	−0.8	(−2.2 to 0.6)	755	0.09
	Position 3	−2.6	(−4 to −1.1)	875	−7.2	(−11.9 to −2.3)	120	−1.8	(−3.3 to −0.3)	755	0.02

* *P* value testing the interaction between arginase DNA methylation and asthma status in a model adjusted for age, sex, race,plate, town, month of DNA collection, asthma medicine used, education, and asthma.

† Analyses are adjusted for age, sex, race, plate, town, month of DNA collection, asthma medicine used, and education.

‡ Average methylation of three positions within *ARG2* gene was used.

TABLE 4. THE ASSOCIATION BETWEEN SOME DNA METHYLATION VERSUS NO DNA METHYLATION IN *ARG2* AND PERCENT CHANGE IN FRACTIONAL CONCENTRATION OF NITRIC OXIDE, CHILDREN'S HEALTH STUDY 2004–2007

	Combined			Children with Asthma*			Children with No Asthma*			
Gene Loci	% Difference	95% Confidence Interval	N	% Difference	95% Confidence Interval	N	% Difference	95% Confidence Interval	N	P_{int}†
Position 1	−8.2	(−16.6 to 1.1)	876	−34.6	(−52.4 to −10.3)	120	−2.9	(−12 to 7.1)	756	0.01
Position 2	−7.4	(−16.1 to 2.1)	875	−33.4	(−50.6 to −10.2)	120	−3.7	(−13 to 6.6)	755	0.06
Position 3	−7	(−21.3 to 9.9)	875	−29.6	(−61.4 to 28.4)	120	−1.7	(−16.8 to 16.3)	755	0.07

* Analyses are adjusted for age, sex, race, plate, town, month of DNA collection, asthma medicine used, and education.
† *P* value testing the interaction between *ARG2* DNA methylation and asthma status in model adjusted for age, sex,race, plate, town, month of DNA collection, asthma medicine used, education, and asthma combined.

In *ARG2*, the selected CpG loci were located in a CpG island in the promoter that contains numerous potential binding sites for enhancer and promoter elements, including CTCF binding sites and conserved transcription factor binding sites (CREB, C-Jun, CREBP3, and ATF) and a putative promoter enhancer SP1 binding site, and may play a role in regulation of *ARG2* gene expression (22). Conceptually, increased DNA methylation in the generally unmethylated *ARG2* promoter could alter gene expression of ARG and subsequent levels of L-arginine, the substrate for NOS. However, DNA methylation and expression were only weakly correlated in monocytes (Hooman Allayee, personal communication).

As an alternative hypothesis, CpG methylation could affect binding of ARG inhibitors or suppressors. For example, methylation of CpG sites flanking transcription factor binding sites may alter binding affinity or efficiency. In *ARG2*, a CREBP3 binding site is located six base pairs downstream of the third CpG locus we evaluated. Recent *in vitro* experiments have demonstrated that methylation of the CpG island flanking a CREB binding site in several genes can limit the binding of phosphorylated CREB and promoter activity (34–37). Because CREB is a component of both activating and repressing complexes (36, 38), a change in binding affinity from methylated CpGs could either increase or decrease gene expression. The ARG inhibitor hydroxyarginine typically increases availability of L-arginine by limiting arginine consumption by ARG, under which scenario FeNO is expected to increase. However, if promoter methylation blocks binding of this or an analogous inhibitor, the result would be to increase ARG activity, decrease arginine bioavailability, and thereby reduce FeNO.

Why the observed association between DNA methylation and FeNO is strongest in individuals with asthma remains unclear, particularly because FeNO is typically elevated in children with asthma. Our results suggest that DNA methylation of *ARG1* and *ARG2* may only affect FeNO when viewed in the context of an already up-regulated inflammatory pathway typically observed in asthma and allergic airway diseases. Both *ARG1* and *ARG2* are highly up-regulated in the asthmatic lung, suggesting altered L-arginine homeostasis is important in asthma pathogenesis (39, 40).

High ARG activity and low arginine bioavailability are believed to create a relative deficiency of beneficial bronchodilating constitutively derived NO that is important in airway hyperresponsiveness (40). However, whether low arginine bioavailability predicts FeNO levels is uncertain, because at least two studies have shown no association or paradoxically increased FeNO levels in individuals with asthma (41, 42). Not all levels of ARG expression result in inhibition of NO production and stimuli can both induce ARG and inhibit iNOS expression, making prediction of downstream consequences difficult (43). L-arginine can also be catabolized by other enzymes and regulation of and interplay between these enzymes is complex (5).

Sensitivity analyses evaluating these associations among children with wheeze, a potentially milder form of disease, and among medication-users, indicating a potentially more severe form of disease, showed consistent associations. Elevated but weaker associations were observed in children with wheeze, whereas stronger associations were observed in children who reported using asthma medications compared with children with asthma who did not use medication. Although we cannot rule out the possibility that the medication itself altered DNA methylation, we did not observe a direct association between medication use and methylation levels in our population. Together, these data suggest that the association between DNA methylation and FeNO changes with severity of disease.

Lastly, we observed no associations between DNA methylation levels in NOS genes and FeNO. Although genetic variation in these genes in known to affect FeNO, epigenetic variation may not play a traditional role in silencing transcription in these genes (12, 44, 45). Alternatively, we only evaluated five small regions within these genes, and the biologically relevant loci may have been missed. Regulation of NO homeostasis is a complex pathway in which any perturbation can affect the balance and downstream NO synthesis. Epigenetic variation adds a new and largely unexplored level of complexity to the investigation of

TABLE 5. THE ASSOCIATION BETWEEN INCREASING LEVELS OF DNA METHYLATION ACROSS *ARG2* LOCI AND PERCENT CHANGE IN FRACTIONAL CONCENTRATION OF NITRIC OXIDE

	Combined* (*n* = 876)		Children with Asthma† (*n* = 120)		Children with No Asthma† (*n* = 756)		
No. of Methylated ARG2 Loci	% Difference	95% Confidence Interval	% Difference	95% Confidence Interval	% Difference	95% Confidence Interval	P_{int}*
0–1	Reference		Reference		Reference		0.03
2	−12.3	(−22.6 to −0.6)	−20.1	(−45.3 to 16.7)	−9.7	(−20.6 to 2.8)	
3	−12.5	(−22.5 to −1.3)	−42.3	(−60.3 to −16.3)	−6.6	(−17.5 to 5.7)	
Trend	−5.6	(−10.9 to 0.1)	−24.6	(−37.5 to −9.5)	−2.3	(−8 to 3.7)	0.007

* *P* value testing the interaction between *ARG2* number of methylated loci and asthma status in model adjusted for age, sex, race, plate, town, month of DNA collection, asthma medicine used, education, and asthma.
† Analyses are adjusted for age, sex, race, plate, town, month of DNA collection, asthma medicine used, and education.

biologic mechanisms underlying NO synthesis. Further experimental studies are warranted to define the functional basis for differential DNA methylation expression or other mechanisms that could account for our observations.

Interpretation of our results requires the consideration of certain study limitations. Buccal mucosal cells, an aerodigestive tract epithelium, were used for DNA methylation analysis. Given the difficulty of collecting other tissue types in children, buccal cells provided an easily measurable surrogate for airway epithelium. Although the ideal tissue to use for assessing epigenetic changes in studies of respiratory health has not been defined, previous studies have demonstrated that buccal epithelium can serve as a surrogate tissue for the lung when measuring DNA methylation (46). In addition, studies comparing expression profiles in buccal, bronchial, and nasal epithelial cells have demonstrated striking similarities, providing further support for use of buccal cells as a useful surrogate for respiratory tract cells (47, 48).

We tested 16 different CpG loci in five distinct genes. Thus, some consideration for multiple testing is warranted. We applied a Bonferroni adjustment to our main results, which is a highly conservative approach for addressing multiple testing. Even with this conservative adjustment, our finding that percent DNA methylation in *ARG2* was associated with FeNO remained significant.

Parental report of physician-diagnosed asthma was used in our study, and concern has been raised that parental report might not reflect physician diagnosis. To investigate this potential bias, we reviewed medical records of children with asthma and found strong evidence that parental report reflected physician diagnosis (49).

We selected 940 children from a total of 2,498 potential participants; thus, the potential to have introduced some selection bias exists. However, a comparison between the two groups revealed that most of the population characteristics were similar. Although ethnicity and asthma status differed, both of these known characteristics were controlled for in the analyses and main effects of ethnicity and asthma on FeNO are consistent with main effects observed in our larger study of FeNO (1). Thus, these differences are not likely to have an impact on the study of methylation in relation to FeNO. Moreover, potential selection bias does not impede the ability to evaluate effect modification. Therefore, our results stratified by asthma status remain valid.

Lastly, two different measurements of FeNO were used in this study, one from offline and one from online measurement techniques. Although not ideal, a substantial validation study has been performed using both measurements from a subset of individuals in our population, in which we have shown that offline and online FeNO are linearly associated (28). An empirical statistical model predicting individual online FeNO from offline FeNO, ambient NO, and lag time before offline analysis gave an r^2 of 0.94; thus, we used predicted online FeNO in all analyses.

In this study, we provide some of the first evidence that differences in percent DNA methylation in the promoters of *ARG1* and *ARG2* are associated with FeNO and that these differences can be detected in DNA from buccal cells, an aerodigestive tissue cell type. The observed associations were stronger in children with asthma compared with children without asthma, suggesting that DNA methylation of ARG genes may play a greater role in NO homeostasis for individuals in whom inflammatory and oxidative stress pathways are already up-regulated. These results raise several questions worthy of further investigation, including a greater understanding of the underlying biologic mechanisms driving these associations and whether children with varying degrees of asthma severity and symptom management exhibit different levels of DNA methylation.

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