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Genetic and Epigenetic Variations in Inducible Nitric Oxide Synthase Promoter, Particulate Pollution and Exhaled Nitric Oxide in Children

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Abstract

Background—Inducible nitric oxide synthase (iNOS, encoded by *NOS2*) is the major enzyme for nitric oxide synthesis in airways. As such, measurement of exhaled nitric oxide (FeNO) provides an *in vivo* assessment of iNOS activity. Short-term exposure to air pollution, haplotypes and DNA methylation in *NOS2* promoter have been associated independently with iNOS expression and/or FeNO.

Objective—We aimed to examine the effects of ambient air pollutants, *NOS2* promoter haplotypes and *NOS2* promoter methylation on FeNO level in children.

Methods—We selected 940 participants in the Children's Health Study who provided buccal samples and had undergone FeNO measurement on the same day. DNA methylation was measured using a bisulfite-polymerase chain reaction Pyrosequencing assay. Seven single nucleotide polymorphisms captured the haplotype diversity in the *NOS2* promoter. Average particulate matter with aerodynamic diameter 2.5μm and 10μm (PM_{2.5} and PM₁₀), ozone and nitrogen dioxide levels 7 days before FeNO measurement were estimated based on air pollution data obtained at central monitoring sites.

Results—We found interrelated effects of PM_{2.5}, *NOS2* promoter haplotypes and iNOS methylation on FeNO. Elevated 7-day average PM_{2.5} exposure was associated with lower iNOS methylation ($P=.01$). *NOS2* promoter haplotypes were globally associated with *NOS2* promoter methylation ($P=6.2 \times 10^{-8}$). There was interaction among one common promoter haplotype, iNOS methylation level and PM_{2.5} exposure on FeNO ($P_{\text{interaction}}=.00007$).

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The authors have no conflict of interest to disclose.

Conclusion—Promoter variants in *NOS2* and short term PM_{2.5} exposure affect iNOS methylation. This is one of the first studies showing contributions of genetic and epigenetic variations in air pollution mediated phenotype expression.

Keywords

air pollution; biomarker; DNA methylation; epigenetics; genetics; gene-environment interaction; nitrosative stress

INTRODUCTION

A large body of evidence indicates that oxidative and nitrosative stress-mediated airway inflammation is critically involved in development of asthma. Although ambient air pollution induces airway inflammation, measurement of the degree of acute or chronic inflammation in airways of children is difficult. Some of the methods for assessing airway inflammation involve invasive procedures (i.e., bronchial biopsies, bronchoalveolar lavage) or can be done successfully in 60-80% children (e.g., induced sputum).¹⁻² Measurement of fractional concentration of nitric oxide in exhaled breath (FeNO) allows non-invasive assessment of airway inflammation in children.³⁻⁷ The findings that FeNO predicts future risk of asthma and wheeze in children and adults⁸⁻¹⁰ suggest that FeNO is also an intermediary phenotype in the relationship between airway inflammation and development of asthma.

Atopic disease conditions (asthma, allergy), genetic factors and environmental exposures are important determinants of FeNO. Using data from the southern California Children's Health Study (CHS), we found that short term-exposure to particulate matter (PM_{2.5} and PM₁₀) and ambient ozone (O₃) are associated with higher FeNO.¹¹ A number of other studies have also documented that short term-exposures to PM_{2.5}, PM₁₀, O₃ and nitrogen dioxide (NO₂) are associated with higher FeNO.¹²⁻¹⁸ In terms of genetic determinants of FeNO, using data from the CHS, we found that among the three isoforms of nitric oxide synthases (*NOS1*, *NOS2*, and *NOS3*) that produce NO from L-arginine, only *NOS2* genetic variants were significantly associated with FeNO.¹⁹ Data from the CHS provide evidence that the two of the most common promoter haplotypes in *NOS2* are important determinants of respiratory health effects, as these haplotypes were associated with FeNO, asthma incidence and lung function growth in children.¹⁹⁻²⁰

In addition to clinical conditions, environmental exposures and genetic susceptibility, a CpG methylation locus in the *NOS2* promoter region has been shown to influence iNOS expression, with lower methylation associated with higher expression.²¹ One study found that workers had a significant decrease in *iNOS* promoter methylation in whole blood following 3-days of work in a steel plant with high PM exposures.²² Although not reported for *NOS2*, sequence variants in several genes have shown to influence DNA methylation in their respective promoter regions (*cis* effects).²³⁻²⁶ Tarantini and colleagues did not report the influence of genetic variants in *NOS2* promoter region on iNOS promoter methylation.²² Whether air pollution effects on FeNO are mediated by *NOS2* promoter methylation and whether promoter haplotypes influence such associations remains unknown.

Based on our previous findings of associations between FeNO and short-term air pollution exposures and *NOS2* promoter haplotypes and a role of environmental exposures on *NOS2* promoter methylation, we set out to understand the role of air pollution exposures on iNOS promoter methylation and the role of air pollution exposure, genetic and epigenetic variations in *NOS2* promoter on FeNO. Specifically, we hypothesized that (1) short-term air pollution exposures and promoter haplotypes in *NOS2* influence iNOS promoter

methylation, (2) iNOS promoter methylation affect FeNO level and (3) air pollution exposures, *NOS2* promoter haplotypes and methylation levels jointly influence FeNO levels. We tested these hypotheses in a study that was conducted in 940 non-Hispanic and Hispanic white children who had buccal cells collected the day of their FeNO measurements.

METHODS

Subjects

This study was nested in the ongoing Children's Health Study (CHS).²⁷ Children had FeNO measurement in three consecutive school years: 2004-2005 (Year 1), 2005-2006 (Year 2), and 2006-2007 (Year 3). For the purpose of this study, a subset of 940 non-Hispanic white and Hispanic white children who had buccal cells collected the day of FeNO collection were selected for DNA methylation analysis. Additional detail of the subject selection is provided in the online supplement. The institutional review board for human studies at the University of Southern California approved the study protocol and parents or legal guardians consented for all study subjects.

Measurement of Exhaled Nitric Oxide

Details of the FeNO collection and quality control approaches have been reported earlier.²⁸⁻²⁹ 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 recommended guidelines.⁵ In Year 3, FeNO was measured using the online technique with EcoMedics CLD-88-SP analyzers at 50 ml/s expiratory flow-rate following recommended guidelines.³⁰ Furthermore, in Year 3, we measured FeNO using both the offline and online techniques in 361 children to develop a model to derive predicted online FeNO level using the offline level. Using the offline FeNO together with ambient NO and the hours between time of collection and FeNO measurement, we could reliably predict the online FeNO levels (model adjusted $R^2 = 0.94$).²⁸ In the current study, we used the predicted online FeNO level for children with FeNO measurements in Years 1 and 2, whereas the online Year 3 FeNO was used for children who had FeNO measured in Year 3.

Assessment of Covariates

Race/ethnicity, annual family income, parental education and exposure to secondhand tobacco smoke (SHS) were based on parental reports. A child's exposure to maternal smoking *in utero* was based on smoking by biological mother during pregnancy. Height and weight were measured at the day of FeNO testing. 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 into underweight, normal, overweight and obese. Children were classified as having asthma if the adult completing the questionnaire reported that a doctor had "ever diagnosed the child as having asthma." Child's history of respiratory allergy was based on parental report of any rhinitis and/or hay fever. Height and weight were measured on the day of test.

SNP Selection, Genotyping and Haplotype Estimation

In representative non-Hispanic and Hispanic white samples from the Multiethnic Cohort (n~71 each),³¹ 1-3 single nucleotide polymorphism (SNPs) per 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 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-hsc.usc.edu/~stram/tagSNPs.html>). Redundant htSNPs were genotyped to substitute for critical SNPs in the event of assay failure. SNPs were genotyped using the Illumina BeadArray platform. For the present analysis, we selected 7 SNPs in *NOS2* promoter region. These SNPs had call rates >99%.

Haplotype frequencies were estimated separately for Hispanic and non-Hispanic white subjects using a SAS macro code available with the TagSNPs program. This haplotype estimation technique provides the maximum likelihood estimates of the haplotype frequencies assuming Hardy-Weinberg equilibrium.³²

Determination of iNOS Promoter Methylation

We used the Pyrosequencing assay using the HotMaster Mix (Eppendorf, Hamburg, Germany) and the PSQ HS 96 Pyrosequencing System (Biotage AB, Uppsala, Sweden)³³ as described previously.³⁴ The PCR and Pyrosequencing primer sequence are shown in Table E1 in the online supplement. As a quality control check to estimate the bisulfite conversion efficiency, we placed duplicate genomic DNA samples on each bisulfite conversion plate to estimate the internal plate variation of bisulfite conversion and the Pyrosequencing reaction. Conversion efficiency was greater than 95%. We also added universal PCR products amplified from cell line DNA on each Pyrosequencing plate to check the run-to-run and plate-to-plate variation in performing Pyrosequencing reactions. The coefficient of variation for the inter-plate control DNA was small (i.e., 2.11%) across the plates. In addition, the Pyrogram peak pattern from every sample was checked to confirm the quality of reaction. The methylation site was 8,091bp down-stream to the nearest *NOS2* promoter SNP (rs4795080; Figure E1 in the online supplement).

Air Pollution Exposure Assessment

Air pollution data were obtained from central monitoring sites in each study community operated by local air pollution control agencies in conformance with US Environmental Protection Agency (US EPA) monitoring requirements. At each monitoring site, 24-hr average measurements for particulate PM_{2.5} were obtained daily or every third day at or nearby by the community air monitoring sites. In addition, hourly PM_{2.5} measurements were collected at selected community air monitoring sites. Continuous hourly average measurements were made for PM₁₀, O₃, and NO₂. When pollution data were not available for certain days, attempts were made to fill the gaps by using data from nearby monitors provided that the monitors were not more than 7km apart and the measurements from the monitors were reasonably well correlated ($0.5 < r^2 < 0.95$, depending on site and season) with each other. Daily 24-hour averages of PM_{2.5}, PM₁₀, and NO₂ and daily 10AM-6PM averages of O₃ were extracted to calculate cumulative average exposure levels 7 days prior to the FeNO test date.

Statistical Analysis

FeNO (range 2.5-116.7 ppb) was not normally distributed and was natural-log-transformed. No transformation for the iNOS promoter methylation was required, as the data were normally distributed. Linear regression models were utilized to examine the effect of short-term particulate pollution, *NOS2* promoter haplotypes and iNOS methylation on FeNO levels. All models were adjusted for age, sex, ethnicity, asthma, respiratory allergy, parental education, secondhand tobacco smoke exposure, community of residence, month of FeNO collection, and experimental plate (for Pyrosequencing reactions). We presented the results for a difference in 5 μ g/m³ particulate pollution level and 5% difference in iNOS methylation across subjects. The influence of the common *NOS2* promoter haplotypes (frequencies > 5%) on iNOS methylation was evaluated using an additive genetic model. The joint effects of short-term PM_{2.5} exposure, *NOS2* promoter haplotypes and iNOS

methylation on FeNO levels were evaluated using likelihood ratio tests (LRTs) with appropriate interaction terms where the exposure, methylation and haplotypes were centered at their respective mean levels for better interpretations of the results. All tests were two-sided at a 5% significance level. We used SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA) and R (a statistical software which is freely available at <http://cran.r-project.org/>) for all analyses.

RESULTS

Subjects of the study were Hispanic and non-Hispanic white children between 6 and 11 years of age (Table I). The sample had nearly equal proportions of boys and girls while two-thirds of the subjects were Hispanic white. About 14% of children had asthma and 55% had a history of respiratory allergy (rhinitis and/or hay fever). Consistent with previous literature, we found that increasing age and history of asthma and respiratory allergy were associated with higher FeNO. Few children were exposed to SHS and nearly 40% of the subjects were overweight or obese; however, these factors were not significantly associated with FeNO.

The mean concentrations of pollutants were $13.8\mu\text{g}/\text{m}^3$, $30.2\mu\text{g}/\text{m}^3$, 19.0ppb, and 35.1ppb for $\text{PM}_{2.5}$, PM_{10} , NO_2 and O_3 , respectively with a wide range in exposure levels (Figure I, and Table E2 in online supplement). There was moderate correlation between $\text{PM}_{2.5}$ and PM_{10} concentrations (Spearman partial correlation coefficient adjusted for community and month = 0.78), whereas correlations between any other pollutants were weaker (Table E2 in online supplement). Average iNOS promoter methylation was 51.3% (standard deviation of 4.3%) and ranged from 30.5% to 63.3% (Figure II).

Short-term particulate matter exposure was associated with lower iNOS methylation. We found that per $5\mu\text{g}/\text{m}^3$ increase in 7-day average $\text{PM}_{2.5}$ exposure, there was 0.30% lower iNOS methylation ($P = .01$, Table II). Although exposure to PM_{10} was associated with lower iNOS promoter methylation in the bivariate analysis (percent methylation in iNOS promoter per $5\mu\text{g}/\text{m}^3$ increase in 7-day average exposure = -0.10 ; $P = .046$), the association did not remain statistically significant after adjusting for potential confounders ($P = .38$). Exposures to O_3 and NO_2 were not significantly associated with iNOS promoter methylation. Although iNOS methylation was associated with lower FeNO, the association was not statistically significant (Table E3 in the online supplement).

We found that *NOS2* DNA sequence variation was associated with difference in CpG methylation levels (Global $P = 6.2 \times 10^{-8}$, Table III). Compared to children who carried the haplotype that contained no variant allele at any of the SNPs (h0000000 or H1), those who carried any other haplotypes had lower iNOS promoter percent methylation. Haplotypes h0111101 [H2] and h1000010 [H3] are the two most common haplotypes that differed at each SNP position. Children carrying H2 and H3 haplotypes had significantly lower iNOS promoter percent methylation compared to those with the H1 haplotype.

We found that *NOS2* DNA sequence variants and CpG methylation levels influenced the relationship between $\text{PM}_{2.5}$ exposure and FeNO ($P_{\text{interaction}} = .03$ and $.006$, respectively; Table IV). However there was no significant interaction between promoter haplotypes and methylation for FeNO ($P_{\text{interaction}} = .75$). These results indicate that, at a given $\text{PM}_{2.5}$ exposure level, children carrying the H3 haplotype had higher FeNO level than those who carried no copy of the H3 haplotype. Further analysis revealed that, in children with the highest 10th percentile of iNOS methylation (over 56.63%), $\text{PM}_{2.5}$ exposure was significantly associated with higher FeNO (Percent difference in FeNO per $\mu\text{g}/\text{m}^3$ higher

exposure = 35%; P -value = .0002; whereas at lower methylation levels, $PM_{2.5}$ exposure was not significantly associated with FeNO.

We found that $PM_{2.5}$ exposure, *NOS2* DNA sequence variation and CpG methylation levels jointly influenced FeNO levels ($P_{\text{interaction}} = .00007$, Table V). Children carrying at least one copy of the haplotype had significantly higher FeNO levels if they had higher $PM_{2.5}$ exposure with and without lower iNOS methylation compared to children who carried no copy of the H3 haplotype and had an average $PM_{2.5}$ exposure and average iNOS methylation (Figure III).

Among the other common haplotypes, the H2 haplotype also showed significant joint effects with $PM_{2.5}$ exposure and iNOS methylation for FeNO (P -for interaction = 0.005), with the effects opposite to that was found for the H3 haplotype. However, this finding resulted from the inverse correlation between H2 and H3 haplotype (Spearman correlation coefficient = -0.43 ; $P < .0001$). We conducted two sensitivity analyses to determine which haplotype had joint effects with methylation and exposure on FeNO. In the analysis that was restricted to children with no copy of the H3 haplotype ($N = 414$), we did not find any joint effects of the H2 haplotype with $PM_{2.5}$ and iNOS methylation on FeNO ($P_{\text{interaction}} = .41$). In contrast, the interactive effect of the H3 haplotype, iNOS methylation and $PM_{2.5}$ exposure on FeNO remained statistically significant ($P_{\text{interaction}} = .0009$) even in a smaller sample of children who did not carry any H2 haplotype ($N = 377$). The results from these sensitivity analyses showed that the H3 haplotype had independent joint effects with $PM_{2.5}$ and iNOS methylation on FeNO. There were no interactive effects of iNOS methylation, $PM_{2.5}$ exposure and H1 or H4 haplotypes on FeNO (both $P_{\text{interaction}} > .37$).

In further sensitivity analyses, we did not find statistically significant difference in the distribution of age, gender, asthma, respiratory allergy, ethnicity, community of residence by Pyrosequencing plate. In addition, none of the findings was influenced by gender, ethnicity, asthma, respiratory allergy, study community, month or year of FeNO measurement, and Pyrosequencing plate. All analysis without any adjustment for covariate yielded essentially similar results to those obtained from the multivariate models that are presented in Tables II through V. Finally, restricting the analysis to FeNO data collected using the offline technique in Years 1 and 2 yielded very similar results.

DISCUSSION

Our findings show that *NOS2* promoter haplotypes and 7-day average $PM_{2.5}$ exposure before collection of DNA influence iNOS promoter methylation. Furthermore, *NOS2* genetic and epigenetic variations and short-term $PM_{2.5}$ exposure jointly affected FeNO levels. This is a novel finding that suggests that genetic, epigenetic and environmental factors jointly influence an intermediate phenotype on the pathway to adverse effects on respiratory health.

Our results extend the findings of Tarantini et al²² and show that $PM_{2.5}$ exposure influences iNOS promoter methylation. Data from experimental studies have shown that the *NOS2* gene is highly induced by cigarette smoke and particulate pollution resulting in higher NO expression in lung³⁵⁻³⁶ and systemic circulation.³⁷ Our study findings points to the possibility that pollutant mediated effects on NO expression could be mediated reduced expression from lower methylation in iNOS promoter. Our results of no significant effect of PM_{10} on iNOS methylation are in agreement with the findings by Tarantini and colleagues.²² Because $PM_{2.5}$ could be deposited and retained in the distal airways,³⁸⁻⁴⁰ this smaller size fraction may have mediated the observed reduction in methylation that was observed among the steel plant workers in the study by Tarantini and colleagues.²²

NOS2 promoter haplotypes were determinants of iNOS promoter methylation level across study participants. A number of studies have documented such allele-specific methylation (majority were *cis* effects) in the human genome;^{23-24, 41-42} however, to the best of our knowledge, influence of *NOS2* genetic variants on iNOS methylation has not been reported earlier. The effect of htSNPs derived haplotypes on methylation provides one explanation for our previously observed associations with asthma, lung function and FeNO.¹⁹⁻²⁰

*NOS2*H3 haplotypes, short-term PM_{2.5} exposure and iNOS methylation level jointly influenced FeNO level. Three lines of evidence provide biological plausibility of the observed effects. First, lower methylation at this locus has been associated with higher iNOS expression.²¹ Secondly, as the gene name indicates, iNOS expression is highly inducible by PM_{2.5} exposure,⁴³⁻⁴⁵ and a number of studies have documented that short term-PM_{2.5} exposure is associated with higher FeNO.¹²⁻¹⁸ Finally, we have previously found in a larger sample that this haplotype is associated higher FeNO level.¹⁹ Therefore, it is plausible that higher PM_{2.5} exposure and lower iNOS methylation would lead to higher iNOS expression resulting in higher FeNO level in children carrying this haplotype.

One of the strengths of the present study is that FeNO may have been a good proxy measure for *in vivo* iNOS expression from the airway, while accounting for the *NOS2* genetic and epigenetic variations, environmental exposure and other complex, unmeasured biological networks that regulate NO production in the airway. As such, availability of an objectively measured phenotype (FeNO) on a large sample of children allowed us to examine the effect of genetic and epigenetic variations in a gene (*NOS2*) that is the major catalyst of NO synthesis in the airways.

Interpretation of our results requires the consideration of some study limitations. Our use of buccal mucosal cells, although an accessible surrogate for respiratory tract epithelial cells, is a potential study limitation. Multiple cell types (bronchial epithelium, macrophages, etc) in the airways express iNOS, and collection of such cells on a large sample of children, particularly when FeNO measurements were made, was infeasible. In this context, buccal mucosal cells, an aero-digestive tract epithelium, provided an easily measurable surrogate for airway epithelium when measuring DNA methylation.⁴⁶ In addition, studies comparing expression profiles in buccal, bronchial and nasal epithelial cells in the context of tobacco smoke exposure mediated effects have demonstrated striking similarities, providing further support for the use of buccal cells as a useful surrogate for airway epithelium.⁴⁷⁻⁴⁸ Further studies are warranted to evaluate exposure, genetic and epigenetic effects on gene expression in nasal and bronchial epithelia.

We selected 940 children from a total of 1,309 eligible subjects on whom we had buccal DNA and FeNO measurement on the same day in Years 1 through 3. To evaluate the potential for any selection bias for such selection strategies, we compared the sociodemographic and clinical data of the present study population with those who were eligible but not selected for methylation assay and all Hispanic and non-Hispanic white subjects with FeNO data. As only 16 subjects from 19 eligible subjects were selected in Year 2, we limited the comparisons for Years 1 and 3 (Tables E4 and E5 in the online supplement). These comparisons showed that the current study population differed somewhat on the distribution of asthma, age and parental education from the non-selected and overall study population. We have adjusted for these factors in all of our models. Furthermore, we evaluated the influence of age, asthma, and parental education by excluding subjects within a category of these variables at a time. These sensitivity analyses did not show any particular influence of any of these factors. In light of these observations, it is not likely that subject selection could explain the findings.

The ethnic-specific haplotype frequencies in the present study population were similar to the frequencies observed in the overall population.¹⁹⁻²⁰ However, genotyping data were only available on Hispanic and non-Hispanic white subjects, so the study findings may not be generalizable to other ethnic groups.

Finally, 504 of the 940 (53.6%) of the study participants had their FeNO measured using the offline technique, and the rest had their measurement through the online technique. However, difference in FeNO measurement technique is unlikely to account for the observed findings, as we were able to reliably predict online FeNO value using the offline data that could for 94% of the variability of the online FeNO level.²⁸ Furthermore, the interactive effects of PM_{2.5}, *NOS2*H3 haplotype and iNOS methylation remained statistically significant ($P_{\text{interaction}}=0.01$) when the analysis was restricted to the FeNO data that were obtained from the online method (i.e., Year 3 data).

In summary, our finding show that short term particulate matter exposure was associated with lower iNOS promoter methylation, and that common promoter sequence variants in *NOS2* significantly affects iNOS methylation. Finally, our findings provide evidence that joint evaluation of genetic and epigenetic variations and air pollution exposure has the potential to identify novel pathways for their role in phenotype expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMI	Body mass index
CHS	Children's Health Study
CI	confidence interval
FeNO	Fractional concentration of nitric oxide in exhaled breath
htSNPs	haplotype-tagged SNPs
iNOS	inducible nitric oxide synthase
LD	Linkage disequilibrium
MAF	minor allele frequencies
NO	nitric oxide
NOS1	nitric oxide synthase isoform 1
NOS2	nitric oxide synthase isoform 2

NOS3	nitric oxide synthase isoform 3
OR	odds ratio
PM_{2.5}	particulate matter with an aerodynamic diameter <2.5µm
PM₁₀	particulate matter with an aerodynamic diameter <10µm
SHS	Secondhand tobacco smoke
SNP	single nucleotide polymorphism
US EPA	United States Environmental Protection Agency

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Clinical Implications

Exhaled nitric oxide is a biomarker of airway inflammation. Air pollution exposure and genetic and epigenetic variation in the inducible nitric oxide synthase gene jointly influence exhaled nitric oxide level in children.

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FIGURE I. Distribution of the 7-day average PM_{2.5} (triangles, $\mu\text{g}/\text{m}^3$), PM₁₀ (circles, $\mu\text{g}/\text{m}^3$), NO₂ (squares, ppb) and 10AM-6PM O₃ (diamonds, ppb) exposure levels before FeNO testing among study participants during 2004-2007.

FIGURE II.

Distribution of percent methylation at the iNOS promoter. The X-axis of represents the percent methylation in buccal DNA. The Y-axis represents the percent of the sample.

FIGURE III.

Joint effects of *NOS2* H3 haplotype, iNOS methylation and 7-day average PM_{2.5} exposure on FeNO. Data are presented by number of H3 haplotype copy with available sample sizes for analysis. The X-axis shows (methylation, PM_{2.5} exposure) with population average, 5% and 10% lower methylation level than average, and population average, 5μg/m³ and 10μg/m³ higher PM_{2.5} exposure levels than average.

TABLE I

Associations of selected characteristics of the study population with FeNO *

	N	(%)	Geometric Mean FeNO (ppb) (95% CI) [†]	P-value [‡]
Age (years) [mean (range)] ^{**}	9.3	(6.4-11.7)	15.2 (10.4 - 22.2)	.004
Sex				
Girls	489	(52.0)	12.9 (11.0 - 15.1)	.49
Boys	451	(48.0)	12.6 (10.7 - 14.7)	
Ethnicity				
Hispanic White	607	(64.6)	12.4 (10.6 - 14.5)	.31
Non-Hispanic White	333	(35.4)	13.1 (11.1 - 15.4)	
Asthma				
No	807	(85.8)	11.0 (9.6 - 12.7)	<.0001
Yes	133	(14.2)	14.7 (12.3 - 17.6)	
History of respiratory allergy				
No	418	(44.5)	12.0 (10.2 - 14.1)	.007
Yes	522	(55.5)	13.5 (11.6 - 15.7)	
Exposure to secondhand smoke				
No	860	(96.5)	12.8 (11.6 - 14.2)	.91
Yes	31	(3.5)	12.7 (9.9 - 16.2)	
Body mass index categories				
Underweight (<5th percentile)	16	(1.7)	13.8 (9.8 - 19.3)	.90
Normal (5th to <85th percentile)	550	(58.5)	12.4 (10.9 - 14.3)	
Overweight (85th to <95th percentile)	183	(19.5)	12.6 (10.8 - 14.7)	
Obese (≥ 95th percentile)	191	(20.3)	12.2 (10.5 - 14.2)	
Parental education				
<12 th grade	193	(21.6)	13.5 (11.3 - 16.2)	.85
12 th grade	163	(18.2)	12.7 (10.7 - 15.1)	
Some college	302	(33.7)	12.7 (10.8 - 14.9)	
College	125	(14.0)	12.7 (10.5 - 15.3)	
Some graduate	112	(12.5)	12.2 (10.0 - 14.9)	
Annual family income				
<\$15,000	126	(15.9)	13.7 (11.5 - 16.4)	.23
\$15,000 - \$49,999	240	(30.3)	12.6 (10.6 - 15.0)	
\$50,000	425	(53.7)	12.0 (10.1 - 14.1)	

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

[†] Geometric mean and 95% confidence intervals (CIs) are adjusted for all variables in the table and community of residence and month of FeNO collection.

[‡] P-values testing overall association of the variable with FeNO level.

^{**} Mean (range) and percent difference with 95% CIs in FeNO are presented.

TABLE II

Short-term air pollution exposures and iNOS promoter methylation

Air pollution exposure [*]	Percent methylation (95% CI) [*]	P-value
PM _{2.5}	−0.30 (−0.54 to −0.06)	.01
PM ₁₀	−0.07 (−0.22 to 0.08)	.38
NO ₂	0.10 (−0.25 to 0.45)	.57
O ₃	−0.02 (−0.35 to 0.32)	.92

^{*} All 7-day cumulative average air pollution exposures before FeNO testing were based on 24 hr averages, except for ozone, for which an average of 10am-6pm concentrations was used.

[†] Difference in percent methylation with 95% confidence intervals in iNOS promoter is provided per 5μg/m³ increase in PM_{2.5} and PM₁₀ and per 5ppb of NO₂ and O₃ exposure levels using multivariate linear regression with the exposures centered at their mean levels. Each model was adjusted for age, sex, ethnicity, asthma, respiratory allergy, parental education, community of residence, month of FeNO collection, *NOS2* promoter haplotypes, and experimental plate (for Pyrosequencing reactions). Separate models were used to examine the effects of each pollutant on iNOS methylation. The statistically significant associations are in bold.

[‡] P-value for the association of pollution exposure with iNOS promoter methylation.

CI, confidence interval.

TABLE III

NOS2 promoter haplotypes and iNOS promoter methylation

<i>NOS2</i> haplotypes *	Haplotype frequency		Percent methylation (95% CI) †	Global <i>P</i> ‡
	Hispanic white	Non-Hispanic white		
h0000000 (H1)	0.19	0.16	Ref.	6.2×10^{-8}
h0111101 (H2)	0.32	0.37	-1.55 (-2.07 to -1.02)	
h1000010 (H3)	0.36	0.25	-1.21 (-1.72 to -0.70)	
h0000010 (H4)	0.11	0.19	-0.49 (-1.12 to 0.14)	
Other haplotypes **	0.02	0.03	-1.42 (-2.69 to -0.16)	

* SNP order in *NOS2* promoter haplotypes is rs4795080-rs2779253-rs1889022-rs10853181-rs2531866-rs1014025-rs2531872 3. Within each haplotype, '0' and '1' represents the common and the variant alleles at the ordered SNP position, respectively.

† Percent methylation differences with 95% confidence intervals per haplotype copy compared to the h0000000 haplotype from a multivariate model and the *P*-value for the global association of *NOS2* promoter haplotypes (4df) with iNOS promoter methylation from this model are shown where the haplotypes were centered at their respective mean levels. The model was adjusted for age, sex, ethnicity, asthma, respiratory allergy, parental education, secondhand tobacco smoke exposure, community of residence, month of FeNO collection, 7-day average PM_{2.5} exposure, and experimental plate (for Pyrosequencing reactions). The statistically significant associations are in bold.

‡ *P*-value for the global association of *NOS2* promoter haplotypes (4df) with iNOS promoter methylation.

** Haplotypes with <5% frequencies are combined into the "other haplotypes" category.

CI, confidence interval; *NOS2*, inducible nitric oxide synthase gene;

TABLE IV

Influence of *NOS2* H3 promoter haplotype and iNOS promoter methylation on the relationship of 7-day average PM_{2.5} exposure with FeNO

Factors*	Estimate (95% CI) [†]	P-value [‡]
Joint effects of PM _{2.5} exposure and H3 haplotype		
H3 haplotype	0.030 (−0.032 to 0.091)	.03
PM _{2.5} exposure	0.001 (−0.040 to 0.042)	
PM _{2.5} exposure × H3 haplotype	0.044 (0.005 to 0.082)	
Joint effects of PM _{2.5} exposure and iNOS methylation		
PM _{2.5} exposure	0.021 (−0.019 to 0.062)	.006
iNOS methylation	−0.025 (−0.080 to 0.030)	
PM _{2.5} exposure × iNOS methylation	0.044 (0.013 to 0.075)	

* The 'x' between factors represents interaction terms.

[†] Estimates (95% confidence intervals) represent natural log transformed FeNO obtain from multivariate liner regression models where the exposure, methylation and haplotypes were centered at their respective mean levels. Each model was adjusted for age, sex, ethnicity, asthma, respiratory allergy, parental education, secondhand tobacco smoke exposure, community of residence, and month of FeNO collection. Additional adjustment for experimental plate (for Pyrosequencing reactions) was done for the analysis that included iNOS methylation. The estimates per 5μg/m³ increase in PM_{2.5} exposure, per haplotype copy and 5% increase in methylation are provided. The statistically significant associations are in bold.

[‡] P-values for interaction were based on 1 degree of freedom.

CI, confidence interval; H3, *NOS2*h1000010 haplotype; iNOS, inducible nitric oxide synthase; *NOS2*, inducible nitric oxide synthase gene.

TABLE V

Joint effects of *NOS2* H3 promoter haplotype, iNOS promoter methylation and 7-day average PM_{2.5} exposure on FeNO

Factors*	Estimates (95% CI) [†]	P-value [‡]
H3 haplotype	0.024 (−0.038 to 0.085)	.46
iNOS methylation	−0.012 (−0.068 to 0.044)	.67
PM _{2.5} exposure	0.017 (−0.024 to 0.058)	.41
H3 haplotype × iNOS methylation	−0.019 (−0.091 to 0.053)	.61
H3 haplotype × PM _{2.5} exposure	0.038 (−0.001 to 0.076)	.05
iNOS methylation × PM _{2.5} exposure	0.060 (0.027 to 0.092)	.0003
H3 haplotype × iNOS methylation × PM _{2.5} exposure	−0.093 (−0.138 to −0.047)	.00007

* The 'x' between factors represents interaction terms.

[†] Estimates (95% confidence intervals) represent natural log transformed FeNO obtain from a multivariate liner regression model where the exposure, methylation and haplotypes were centered at their respective mean levels. The model was adjusted for age, sex, ethnicity, asthma, respiratory allergy, parental education, secondhand tobacco smoke exposure, community of residence, month of FeNO collection, and experimental plate (for Pyrosequencing reactions). The estimates per 5μg/m³ increase in PM_{2.5} exposure, per haplotype copy and 5% increase in methylation are provided. The statistically significant associations are in bold. For a graphical representation of the joint effects, see Figure III.

[‡] P-values for the association of each of the main effects and interaction terms with FeNO.

CI, confidence interval; H3, *NOS2* h1000010 haplotype; iNOS, inducible nitric oxide synthase; *NOS2*, inducible nitric oxide synthase gene.