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Environmental Exposures, Nitric Oxide Synthase Genes, and Exhaled Nitric Oxide in Asthmatic Children

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Summary

Exhaled nitric oxide (FeNO), a measure of airway inflammation, is being explored as a tool to guide asthma management in children. Investigators have identified associations of genetic polymorphisms in nitric oxide synthase genes (NOS1 and NOS3) with FeNO levels; however, none have explored whether these polymorphisms modify the relationship of environmental exposures with FeNO. The objective of this project was to evaluate the association of NOS polymorphisms and environmental exposures with FeNO levels among children with asthma. We conducted a 12 month, prospective cohort study of 225 tobacco-smoke exposed children (6 to 12 years) with doctor-diagnosed asthma. We assessed environmental exposures (tobacco, indoor allergens, & airborne particulates), polymorphisms in NOS1 (an intronic AAT tandem repeat) and NOS3 (G894T), and FeNO levels. There was no association of NOS1 or NOS3 polymorphisms with FeNO levels. There were no significant interactions of environmental exposures and the NOS1 polymorphism with FeNO levels. In contrast, there was an interaction of the NOS3 polymorphism and airborne nicotine concentration with FeNO levels ($p=0.01$). Among GG genotype individuals, nicotine exposure did not affect FeNO levels; however, among individuals with at least one T allele, higher nicotine exposure was associated with lower FeNO levels (approximately 5ppb decrease from the lowest to the highest quartile). We conclude that genetic differences may explain some of the conflicting results in studies of the effects of tobacco smoke exposure on FeNO levels and may make FeNO interpretation difficult for a subset of children with asthma.

Keywords

allergen; asthma; sensitization; tobacco smoke; air nicotine; nitric oxide synthase; inhaled corticosteroid; exhaled nitric oxide

Introduction

Exhaled nitric oxide (FeNO), a measure of airway inflammation, is being explored as a noninvasive tool to guide asthma management in children. Although there is evidence that environmental exposures and genetic variation can independently affect FeNO levels, little is known about how the interaction of environmental exposures and genetic differences may affect FeNO levels.

Nitric oxide is an intracellular messenger that is synthesized from L-arginine by nitric oxide synthases (NOS). There are three forms of these NOS enzymes (NOS1, NOS2, and NOS3), and all three are expressed in bronchial tissue.^{1,2}

Genetic polymorphisms in nitric oxide synthase genes (NOS1 and NOS3) have been associated with FeNO levels in studies of adults with asthma. Wechsler et al. found that the presence of at least one allele with <12 AAT tandem repeats in NOS1 was associated with increased FeNO levels among adults with asthma.³ However, among healthy children, Ali et al. demonstrated that the same NOS1 gene polymorphism was associated with atopy but not FeNO.⁴ In another study, a missense mutation in the NOS3 gene (G895T) was found to be associated with FeNO levels in adults with asthma.² The presence of the G allele was associated with an increase in FeNO level.² These candidate gene associations were not confirmed by other investigators, and investigators have not identified any polymorphisms in NOS2 that are associated with FeNO.⁵⁻⁷

Environmental exposures have been associated with FeNO levels. Sensitized individuals with asthma who are exposed to indoor allergens have higher FeNO levels.⁸⁻¹¹ Paradoxically, smoking has been associated with decreased FeNO levels in adults.^{12,13} The association of secondhand tobacco smoke exposure and FeNO in children, however, is mixed. Some studies found no relationship, while others demonstrated an inverse association.^{14,15} Previously, we reported that higher baseline FeNO levels, atopy, and fall season were associated with increased FeNO levels, while inhaled steroid use, summer season, and increased nicotine exposure were associated with lower FeNO levels.¹⁶

Genetic polymorphisms in NOS1 and NOS3 genes (but not NOS2) have been associated with FeNO levels in some studies while environmental exposures have been associated with FeNO levels in others; however, investigators have not explored these factors together.⁹ We sought to examine gene and environment interactions using these two candidate genetic polymorphisms.

Materials & Methods

We used data from the Cincinnati Asthma Prevention (CAP) study for this analysis. The CAP study is described in more detail elsewhere.^{17,18} Briefly, the CAP study was a randomized controlled trial to evaluate the health effects of use of air cleaners in the homes of 225 children (age 6-12 years old) who had physician diagnosed asthma (based on medical billing data) and were exposed to tobacco smoke. Children and their families provided written informed consent prior to enrollment in this year long study. This project and the CAP study were approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board.

FeNO Measurement

Trained research assistants collected exhaled air for FeNO analysis using the Mylar balloon, offline technique at the baseline, 6 month, and 12 month home visits. Briefly, children breathed through a bacterial filter attached to the mouthpiece a collecting tube which had a

charcoal filter on the inspiratory end of the tube to remove nitric oxide from the inhaled ambient air. The child exhaled slowly into the a collecting tube designed to maintain flow in the range of 50 to 80 mL/sec (Deadspace Discard Kit, Sievers Instruments, Boulder, CO, USA). The first part of each breath was not collected. We used the Model 280i nitric Oxide Analyzer (Sievers Instruments, Boulder, CO, USA) in accordance with the manufacturer's instructions for all nitric oxide analyses.¹⁹

Environmental Exposure Measures

Technicians collected environmental samples during three home visits over the study period (baseline, six months, and twelve months). Settled dust allergens were collected from the largest area of the floor in the child's bedroom using a standardized HVS-3 dust collection method at baseline.^{17,20} We analyzed dust samples for levels of dust mite (Der f 1), dog (Can f 1), cat (Fel d 1), and cockroach (Bla g 1) allergen using monoclonal ELISA technique (Indoor Biotechnologies, Charlottesville, VA, USA).²¹⁻²³ Lower detection limits was 0.01 µg/g dust for settled allergens. We measured airborne particulate concentration using a GT-321 particulate monitor (Met One Instruments, Grants Pass, OR, USA). We evaluated serum samples that were obtained by a pediatric phlebotomist at the baseline visit to determine allergen specific IgE levels using the ImmunoCap test (Pharmacia Diagnostics, Portage, MI, USA).²⁴ We considered children sensitized to an allergen if their allergen specific IgE level was at least Class I (≥0.35 kU/L, the lower detection limit). We measured tobacco exposure using a survey, biomarkers, and a nicotine dosimeter. We surveyed guardians about the cigarettes smoked per day at each home visit. We also measured hair and serum cotinine using standard techniques at each home visit.²⁵⁻²⁷ Lower detection limits for serum and hair cotinine were 0.05 ng/mL and 0.005 ng/mg respectively.²⁵⁻²⁷ Lastly, we measured airborne nicotine levels using nicotine dosimeters that were located on the back of the air cleaner machines in the main activity room at the baseline and 6-month visits, and retrieved at the 6 and 12-month visits respectively so that the first dosimeter reflected nicotine exposure from baseline to 6 months and the second 6 to 12 months. The dosimeters were analyzed for nicotine level using a standard technique.²⁸⁻³¹ The lower detection limit for nicotine was 0.2 µg/m³.³¹

Genotyping

Genomic DNA was extracted for both polymorphism assays from blood spots on Whatman FTA cards (Whatman Inc, Florham Park, New Jersey, USA) according to the manufacturer's protocols.³² Polymerase Chain Reaction (PCR) amplification of alleles containing an AAT_n repeat in intron 20 of the NOS1 gene was conducted using forward (5' – CTG GGG GCA ATG GTG TGT – 3') and reverse (5' – GAG TAA AAT TAA GGG TCA GC – 3') primers similar to other investigators.^{3,33} Primers were 6-Fam labeled. The PCR occurred in a 10 µl final reaction volume containing genomic DNA, 10 pmol 6-Fam labeled primers, and 0.1 µl of AmpliTaq Gold™ DNA polymerase (5 U/µl) (Applied Biosystems, Foster City, CA, USA). Initial incubation occurred for 12 min at 95 °C. The amplification was carried out for 30 cycles in ABI GeneAmp™ PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following parameters: denaturation at 94 °C for 1 min, annealing of primers at 58°C for 1 min, and extension at 72 °C for 1 min. We then employed a final extension at 72 °C for 10 min, followed by an overnight incubation at 4 °C. ABI PRISM® 3130XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) was used for the detection of the amplification products according to the manufacturer's protocols. The GeneMapper software v 4.0 (Applied Biosystems, Foster City, CA, USA) was used for sizing and genotyping. To determine the NOS3 G894T polymorphism, the TaqMan® (fluorogenic 50 nuclease) assay (Applied Biosystems, Foster City, CA, USA) was used for genotyping according to the manufacturer's instructions.³⁴ In analyses all 96-well plate

contained two positive controls (duplications) and one negative control (just water) for quality checking.

Additional Covariates

We surveyed the participant's guardian about the child's asthma medication use, demographic information, and housing characteristic information. We also conducted surveys about asthma history, asthma therapy, and other characteristics of the children and their families to use as covariates. Race was based on parent report.

Data Analysis

Similar to previous investigators, we coded the NOS1 allele based on whether the allele had <12 AAT repeats or ≥12 repeats so that there were three distinct genotypes for NOS1 (<12/<12, <12/≥12, and ≥12/≥12).^{3,35} The NOS3 genotype was coded into three distinct genotypes GG, GT, and TT. In analyses we evaluated these non-dominant models (3 levels) first. Then based on genotype frequencies and FeNO geometric means by genotype we explored dominant models (2 levels) for the genotypes.

The distributions of the FeNO data, cotinine data, and nicotine data were approximately log-normal, so geometric means and 95% confidence intervals (95% CI) were used to describe central tendency and dispersion. We compared groups using t-tests on the log scale and chi-square tests. We employed the standard two sided 5% level to determine statistical significance for all analyses.

FeNO levels were obtained at three time points for each child over the 12 month study; therefore, we modeled FeNO levels as a repeated measures design using a mixed-effects linear model (Proc Mixed in SAS) with subjects (children) as a random effect and subject characteristics as fixed effects. First we conducted separate analyses to evaluate the relationship between each polymorphism and FeNO. Next, we developed a full multivariable model incorporating genetic factors with environmental and covariate predictors determined to be significant in earlier studies.¹⁶ We considered gene and environment interactions for environmental risk factors identified in previous analyses.¹⁶ Although the intervention in the CAP study (air filters) did not affect FeNO levels, we included a group and a time variable in all models to account for the design effects of intervention group assignment. SAS Version 9.1 (SAS Institute, Inc., Cary, NC, USA) was used for all data analyses.

Results

Sample characteristics have been previously reported (Table 1).¹⁷ The proportion of the NOS1 <12 allele was 0.35. The proportion of the NOS3 G allele was 0.72. The NOS1 and NOS3 genotype frequencies were in Hardy-Weinberg equilibrium. Both the NOS1 and NOS3 polymorphism frequencies varied by race; however, there was no significant association of race with FeNO levels.

Based on genotype frequencies and geometric mean FeNO by genotype, the dominant model we explored for NOS1 was determined by presence of at least one low repeat allele. Presence of the T allele determined dominance in our NOS3 models. There was no statistically significant association of the main effect of NOS1 genotype or NOS3 genotype with FeNO level in either dominant or non-dominant models (Table 2). In addition there was no significant difference in FeNO trends over time by NOS1 genotypes (Table 2); however, there was a statistically significant difference in FeNO trend over time by NOS3 genotype (gene by time interaction). There was also no significant association of the main effect of

NOS1 genotype or NOS3 genotype with FeNO level in either dominant or non-dominant models when stratified by race.

In prior analyses using these data, we reported that atopy and fall season were associated with increased FeNO levels, but inhaled steroid use, summer season, and increasing nicotine exposure were associated with lower FeNO levels, adjusting for baseline age, intervention group assignment, and time.¹⁶ In those analyses, settled dust allergen levels, airborne particulate concentrations, reported tobacco smoke exposure, serum cotinine, hair cotinine, and recent upper respiratory infection were not associated with FeNO levels. When we placed NOS1 or NOS3 genotype into the multivariable model from the previous analyses, there was no statistically significant main effect of genotype.

There were no significant interactions of NOS1 polymorphisms (both non-dominant and dominant models) and environmental exposures, inhaled steroid use, season, gender, or race with FeNO levels. Potential environmental exposure interactions that were explored included airborne nicotine, reported tobacco smoke exposure, serum cotinine, hair cotinine, settled indoor allergens, and sensitization to any allergen. There was also no significant interaction of recent upper respiratory infection and NOS1 polymorphism with FeNO levels.

There was a statistically significant interaction of NOS3 polymorphism and airborne nicotine concentration with FeNO levels ($p=0.01$). Among individuals with the GG genotype, nicotine exposure did not affect FeNO levels; however, among individuals in the combined GT and TT genotype group, higher nicotine exposure was associated with decreased FeNO levels. There was also a statistically significant interaction for the non-dominant model of the NOS3 polymorphism ($p=0.04$). To further quantify this relationship, we categorized nicotine exposure into quartiles. Among those with at least one T allele, there was an approximately 5 ppb decrease in geometric mean FeNO comparing individuals in the lowest quartile of nicotine exposure to those in the highest quartile (Figure 1, $p=0.11$). This decrease is large compared to the standard deviation of FeNO for the cohort at baseline (2 ppb). Other measures of tobacco smoke exposure were not significantly associated with FeNO, nor were there significant interactions of those exposure measures with genotype. There was no significant difference in nicotine exposure by race. There was no significant difference in overall sensitization or dust mite allergen sensitization by race. African-American children were more frequently sensitized to cockroach allergen ($p<0.01$), and less frequently sensitized to cat allergen ($p<0.01$) and dog allergen ($p<0.01$) than Caucasian children.

There were no significant interactions of NOS3 polymorphisms and other environmental exposures (airborne nicotine, reported tobacco smoke exposure, serum cotinine, hair cotinine, settled indoor allergens, and sensitization to any allergen), inhaled steroid use, season, race, gender, or recent upper respiratory infection with FeNO levels. There was also no significant gene by gene interaction regardless of model.

When we stratified the NOS3 and nicotine interaction model by race we found that among African-Americans ($n=93$), this interaction was statistically significant ($p=0.001$). Among Caucasians ($n=74$) this interaction was not statistically significant ($p=0.37$). Among African Americans with at least one T allele, there was an approximately 5.7 ppb decrease in geometric mean FeNO comparing individuals in the lowest quartile of nicotine exposure to those in the highest quartile, and among those with the GG genotype, there was an approximately 3.3 ppb increase in geometric mean FeNO. Although the association was not statistically significant, among Caucasians all genotypes were associated with a decrease in FeNO when exposed to nicotine; the decrease was greater in those with at least one T allele.

The association of the three way interaction of race by NOS3 by nicotine exposure with FeNO was of borderline significance (0.053).

In a secondary analysis, when the variable sensitization to any allergen was replaced with individual allergen sensitization models (dust mite, cat, dog, and cockroach) there were no statistically significant interactions of individual allergen sensitization and NOS1 genotype with FeNO levels. There was a statistically significant interaction of NOS3 genotype and dust mite sensitization with FeNO levels in the dominant genotype model (Figure 2, $p=0.04$), but this association was not as strong in the non-dominant genotype model ($p=0.06$). There were no statistically significant interactions of NOS3 genotype and any of the other allergen sensitizations with respect to FeNO levels.

Discussion

We found no direct association between the NOS1 (AAT repeat allele) or NOS3 (G895T) polymorphism and FeNO levels in children with asthma, but we did find that a NOS3 polymorphism modified the effect of nicotine exposure on FeNO levels. This interaction may be race specific. Overall, children with the GT or TT genotype of NOS3 who had higher nicotine exposure had decreased FeNO levels, while FeNO levels among children with the GG genotype were not affected by nicotine exposure. These results suggest that genetic differences may explain some of the conflicting results in prior studies that evaluated the effects of tobacco exposure on FeNO levels. Moreover, these differences may need to be considered when using FeNO in managing children with asthma.

The genotype frequencies of NOS1 and NOS3 alleles in our cohort were similar to that reported in other cohorts.^{2,33,35} Consistent with some studies, there was no association of genetic polymorphisms in NOS1 or NOS3 with FeNO levels. While Wechsler et al. demonstrated an association between the AAT repeat polymorphism in NOS1 and FeNO levels in adults with asthma, Leung et al. did not find an association of the NOS1 polymorphism and FeNO levels among Chinese children with asthma.^{3,5} Similarly, van Gravesande et al., demonstrated an association between the G894T polymorphism in NOS3 and FeNO levels in adults with asthma.² However, among Chinese children with asthma Leung et al. did not find an association, and Hollá et al. did not find an association in adults with asthma.^{5,6} Our study is the first to explore the relationship of environmental exposure to tobacco and NOS polymorphisms with FeNO.

Several studies have demonstrated an effect of environmental exposures on FeNO levels. Studies evaluating the effect of tobacco smoke exposure have been mixed, with some studies noting an inverse association and others documenting no association.^{14,15,36} We found that individuals with the GT and TT genotypes of NOS3 have decreased FeNO when exposed to nicotine. There was an approximately 5 ppb decrease in geometric mean FeNO comparing individuals in the lowest quartile of nicotine exposure to those in the highest quartile, and this decrease is clinically relevant compared to the standard deviation of FeNO for the cohort at baseline (2 ppb). The difference in response to nicotine that we found among those with different NOS3 polymorphisms may explain some of the conflicting results in prior studies of tobacco exposure and FeNO levels because those studies did not also consider genetic differences. We found that nicotine exposure was associated with FeNO, but only among children with GT and TT genotypes. In contrast, the other tobacco smoke measures in this study were not related to FeNO levels. It is possible that airborne nicotine is a better measure of the fraction of tobacco smoke that directly affects the lung or nitric oxide synthesis than the other measures of tobacco exposure. Alternatively, our finding that air nicotine affects FeNO may be spurious. However, there is mounting evidence for an interaction of tobacco smoke exposure and NOS3 gene polymorphisms in

relation to other health outcomes. Investigators exploring cardiovascular disease risk as well as breast cancer risk found interactions in polymorphisms in NOS3 and cigarette smoke.^{37,38} Collectively, these findings suggest that there may be a role of NOS3 in susceptibility to tobacco smoke.

The NOS3 polymorphism we evaluated was a mutation in exon 7 of the NOS3 gene. There is evidence to suggest that the mutation has functional significance. Wang et al. found a slight but linear increase in NOS3 mRNA and protein levels with the presence of one or two of the T alleles suggesting an allele dose effect; however, the enzyme activity decreased with the presence of the T allele.³⁹ While the sample size was small and the association was not statistically significant, it suggests that there may be a functional significance to this polymorphism. Our finding of a decreased FeNO among individuals with the GT and TT genotypes exposed to increasing nicotine exposure may reflect a decrease in enzyme activity due a combination of genetic factors and environmental exposure. Investigators have also demonstrated that the environment, specifically, cigarette smoking is associated with reduced expression of NOS3 in pulmonary arteries.⁴⁰ Thus, there are several mechanisms that could lead to decreases in FeNO levels among these individuals.

In our secondary analysis we found that there may be an interaction of dust mite sensitization and NOS3 polymorphism with FeNO levels. Among individuals with the GG genotype, dust mite sensitization was associated with higher FeNO levels compared to the individuals that were not sensitized. There was no difference in FeNO based on dust mite sensitization among individuals with a T allele. This genotype and sensitization relationship with FeNO is different from the genotype and nicotine exposure relationship. In this case, GG individuals were the ones for whom dust mite sensitization affected FeNO levels. While the significance of this association was weaker it suggests that the effects of allergen sensitization and nicotine exposure on FeNO may manifest themselves through different mechanisms.

There are some limitations to this study. First, this cohort only examined children with asthma who were exposed to tobacco smoke. Thus, it may not be generalizable, and it could have underestimated the effect of NOS3 because there were no unexposed children in the study. Still, there was a wide range of exposure to tobacco smoke in this cohort so it encompasses a large group of potential exposure levels. Moreover, a majority of children with asthma have measurable levels of tobacco smoke exposure, thus this cohort may be generalizable to a majority of children with asthma.⁴¹ A second limitation is that we did not have a single expiratory flow restriction during our FeNO collection, but we attempted to maintain flows within a narrow range. This means that findings relating to our FeNO levels may not be comparable to groups using a specific flow rate. The American Thoracic Society regulations were being established at the time of FeNO measurement in this study, thus the methods used reflected standards for the time and closely mirror current recommendations.⁴² A third limitation is that our sample size may not have been adequate to provide us with the power to explore gene by gene interactions, and the sample was not large enough to explore gene by gene by environment interactions. It is possible that having more than one susceptibility gene may result in differing FeNO levels in response to environmental exposures. A fourth limitation is that similar to other association studies, our results could be confounded by population stratification. While there were differences in allele frequency by race, there was no association of race with FeNO so the potential for population stratification is minimal.^{43,44} A fifth limitation is that of multiple comparisons. To overcome this limitation, our analyses will need to be replicated in other populations.

We have demonstrated that there is no direct association of FeNO levels with the candidate genetic polymorphisms in NOS1 or NOS3 that we tested among tobacco smoke exposed

children with asthma. However, individuals with the GT and TT genotypes of NOS3 have decreased FeNO if they are exposed to nicotine. These genetic differences may explain some of the conflicting results in earlier studies that evaluated the effects of tobacco exposure on FeNO levels. Future studies evaluating FeNO should consider the effect of gene and environment interactions on FeNO levels. FeNO may be more representative of exposures in subpopulations of people with asthma. Moreover, these results suggest that differences in genetics may affect the ease of interpretation of FeNO levels in the management of children with asthma.

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Abbreviations

FeNO	exhaled nitric oxide
CAP	Cincinnati Asthma Prevention

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Figure 1. Geometric Mean FeNO by Airborne Nicotine Exposure and NOS3 Genotype (adjusted for baseline age, intervention group, time, allergen sensitization, season, and inhaled steroid use)

Figure 2. Geometric Mean FeNO by Dust Mite Allergen Sensitization and NOS3 Genotype (adjusted for baseline age, intervention group, time, allergen sensitization, season, and inhaled steroid use)

Table 1

Characteristics and Nicotine Exposure of Study Participants (limited to those with FeNO data at baseline, N=203)

Characteristics	N (%)
Male	130 (64)
Female	73 (36)
Race	
African American	112 (55.2)
Caucasian	86 (42.4)
Other	5 (2.4)
Income	
<\$20,000	89 (45.6)
\$20-40,000	63 (32.3)
>\$40,000	43 (22.1)
Age (Mean $\pm$ Std dev)	9.4 $\pm$ 1.8
Allergen Sensitized	
Dust Mite	91 (45.7)
Cat	77 (38.7)
Dog	34 (17.1)
Cockroach	61 (30.7)
1 or more allergen sensitization	108 (65.3)
Inhaled Steroid Use (by report)	
Baseline	50 (24.8)
6 Months	46 (24.9)
12 Months	43 (21.5)
Oral Steroid Burst in the Previous Three Months	
Baseline	65 (32.2)
6 Months	40 (19.9)
12 Months	51 (25.5)
Airborne nicotine in first 6 months ($\mu\text{g}/\text{m}^3$) (Geometric Mean, range)	0.5 (0.001, 15.8)
Airborne nicotine in second 6 months ($\mu\text{g}/\text{m}^3$) (Geometric Mean, range)	0.5 (0.002, 13.5)
NOS1 Genotype	
<12/<12	28 (14.8)
$\square$ <12/ $\geq$ 12	80 (42.3)
$\geq$ 12/ $\geq$ 12	81 (42.9)
NOS 3 Genotype	
GG	98 (53.3)
GT	71 (38.6)
TT	15 (8.2)

Table 2

Geometric Mean of FeNO by Genotype at Each Visit (adjusted for group and time)

Genotype	0	6	12	p-value
NOS 1				
<12/<12	13.7 (10.7, 17.5)	12.5 (9.8, 15.9)	14.6 (11.4, 18.6)	0.40 ^a
□<12/≥12	11.5 (9.9, 13.3)	11.6 (10.0, 13.5)	12.8 (11.0, 14.8)	0.88 ^b
≥12/≥12	12.9 (11.2, 14.9)	12.9 (11.2, 14.8)	13.6 (11.8, 15.7)	
NOS 1				
□<12/<12 or <12/≥12	12.0 (10.6, 13.6)	11.8 (10.4, 13.4)	13.2 (11.7, 15.0)	0.43 ^a
≥12/≥12	12.9 (11.2, 14.9)	12.9 (11.2, 14.8)	13.6 (11.8, 15.7)	0.67 ^b
NOS 3				
GG	13.2 (11.6, 15.0)	12.4 (10.8, 14.1)	12.9 (11.3, 14.7)	0.78 ^a
GT	11.5 (9.8, 13.4)	12.6 (10.8, 14.7)	14.7 (12.7, 17.2)	0.05 ^b
TT	11.6 (8.4, 16.0)	10.8 (7.8, 15.1)	12.7 (9.0, 17.9)	
NOS 3				
GG	13.2 (11.6, 15.0)	12.4 (10.8, 14.1)	12.9 (11.3, 14.7)	0.87 ^a
GT/TT	11.5 (10.0, 13.2)	12.3 (10.7, 14.1)	14.4 (12.5, 16.5)	0.02 ^b

^a p-value for differences across genotypes (gene main effect)^b p-value for differences in trends over time across genotypes (gene*time)