DNA methylation in the ARG-NOS pathway is associated with exhaled nitric oxide in asthmatic children

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Online Data Supplement

Sample Collection for FeNO, Transport, and Analysis

Details of the protocol have been published elsewhere (1-2). Exhaled breath collection was performed at schools, usually from midmorning to noon to avoid traffic-related peaks of ambient NO and possible effects of recent eating on FeNO. Each community was visited at least twice in different seasons, to minimize confounding of location and season effects. Health status at testing was evaluated by a brief questionnaire; subjects with symptoms of acute respiratory infection within the past 3 days were excluded or rescheduled. FeNO was collected in Mylar bags after discarding dead space air (1), using a commercial apparatus (Sievers Division, GE Analytical Instruments, Boulder, CO), with target expiratory flow of 100 ml/sec. Ambient air samples, collected similarly, were used to estimate each subject's ambient NO exposure at the time of testing. Samples were stored in coolers and transported to a central laboratory for NO analysis. Between collection and analysis, the samples were transported on “blue ice” at 2 to 8 degree C. Lag time between collection and analysis ranged from 2 to 26 hours (1-2). Potential sources of measurement artifact were documented and tested statistically for influence on the results.

Buccal Sample Collection and Processing

Children were provided with two toothbrushes and instructed to brush their teeth with the first one. They were instructed to gently brush the buccal mucosa with the second toothbrush. The brush was then placed in a leak proof container that was filled with an alcohol-based fixative. Children then swished liquid throughout their mouths and expelled the fluid into a container. The majority of buccal cell specimens were collected at school under the supervision of study staff. The remaining specimens were collected at home and sent to us by mail.

Buccal cell suspensions were centrifuged at 2,000g on the day they were received in the laboratory. The pellets were stored frozen at –80°C until used for DNA extraction, at which time they were resuspended and incubated in 600 μl of lysis solution from a PUREGENE DNA isolation kit (cat #D-5000; GENTRA, Minneapolis, MN) containing 100 μg/ml proteinase K overnight at 55°C. DNA extraction was performed according to manufacturer's recommendations. The DNA samples were resuspended in the hydration solution (GENTRA) and stored at –80°C.

Selection of CpG loci in ARG/NOS genes

We examined CpG loci located in the promoter regions of *NOS1, NOS2A, NOS3, ARG1* and *ARG2.* The CpG positions were located at the following sites: chr12:117,769,133-117,769,145 for *NOS1*, chr17: 26,127,518-26,127,523 for 'Non-CpG islands' in the promoter of *NOS2A*, chr17: 26,126,265-26,126,267 for 'non-CpG island' between exons 1 and 2 of *NOS2A*, chr17: 26,120,696-26,120,703 for 'CpG islands' of *NOS2A*, chr7:150,690,770-150,690,776 for *NOS3*, chr6:131,894,358-131,894,360 for *ARG1* and chr14:68,086,547-68,086,554 for *ARG2* according to assembly of GRCh37/hg19. Primers were designed using MethPrimer software, with parameters for selection including a product size of 100~300bp, 50~60^{o}C for primer Tm, and a primer size of 20~30bp.

Pyrosequencing

We used the Pyrosequencing assay using the HotMaster Mix (Eppendorf, Hamburg, Germany) and the PSQ HS 96 Pyrosequencing System (Biotage AB, Uppsala, Sweden) (3) as described in previous work (Byun HM, 2009, hMG) (4). 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. In addition, the Pyrogram peak pattern from every sample was checked to confirm the quality of reaction.

References

E1. Linn WS, Berhane KT, Rappaport EB, Bastain TM, Avol EL, Gilliland FD. Relationships of online exhaled, offline exhaled, and ambient nitric oxide in an epidemiologic survey of schoolchildren. *J Expo Sci Environ Epidemiol* 2009;19:674-681.

E2. Linn WS, Rappaport EB, Berhane KT, Bastain TM, Avol EL, Gilliland FD. Exhaled nitric oxide in a population-based study of southern california schoolchildren. *Respir Res* 2009;10:28.

E3. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Genet* 2007;8:253-262.

E4. Byun HM, Siegmund KD, Pan F, Weisenberger DJ, Kanel G, Laird PW, Yang AS. Epigenetic profiling of somatic tissues from human autopsy specimens identifies tissue- and individual-specific DNA methylation patterns. *Hum Mol Genet* 2009;18:4808-4817.

Tables

Table E1. Primer sequences and reaction conditions for NOS and ARG genes

Gene	Primer	Sequence	Annealing Temp	PCR size(bp)
NOS1	PCR Forward	NOS1-F:AGGTTGGTAATGAAGATATTTAGAGAATAG	56.7°C	223
	PCR Reverse	NOS1-R(biotin) : TCACCCACTCATAACTAATAACCC		
	PSQ sequencing	NOS1-SP:TTTTAGGGATA		
NOS2A	PCR Forward	iNOS(23151743)-F: AAAAATAATTTTTTGGATGGTATGG	TDOWN53	177
	PCR Reverse	iNOS(23151567)-R(biotin): AAACTATCTAAAACTACCCAATCCC		
	PSQ sequencing	iNOS(23151671)-SP:TTTATAATTTTGTAG		
NOS2A	PCR Forward	iNOS(23150425)-F: TTAGGGTTAGGTAAAGGTATTTTTGTTT	TDOWN53	212
	PCR Reverse	iNOS(23150214)-R(biotin): CAATTCTATAAAACCACCTAATAATCTTAA		
	PSQ sequencing	iNOS(23150395)-SP: TAAAGGTATTTTTGTTTTAA		
NOS2A	PCR Forward	iNOS(23145018)-F: GGAAGGTAGGGAAGGAGGGGTAGTT	TNCTD	243
	PCR Reverse	iNOS(23144776)-R(biotin): AAAAATCCTACAAAACAACCTACACAACC		
	PSQ sequencing	iNOS(23144840)-SP: GAGGGGTTGGG		
NOS3	PCR Forward	NOS3-F: GGATATTTGGGTTTTTATTTA	TDOWN53	187
	PCR Reverse	NOS3-R(biotin): CAATAAAAAAAAACTCTCCA		
	PSQ sequencing	NOS3-SP: TGGGATAGGGG		
ARG1	PCR Forward	ARG1-F: GGAGTTAGTTGTTTTTATTAGA	56.7°C	200
	PCR Reverse	ARG1-R(biotin):TTTTTCCTTACCTATCCCTTTA		
	PSQ sequencing	ARG1-SP: TGTTAGAGTATGAG		
ARG2	PCR Forward	ARG2-F: GGAGAGTATAGGTTAGAGTG	56.7°C	274
	PCR Reverse	ARG2-R(biotin): AAAAACTACCCCTTAAAAAC		
	PSQ sequencing	ARG2-SP: GGGGTTGGTTGGAGG		

Table E2. Distribution of FeNO by participant characteristics

		N	Mean	SD	Median	Range	Wilcoxon p-value
Overall		940	14.6	14.4	9.8	114.3	
Ever diagnosed with asthma	No	807	13.3	11.9	9.6	102.6	0.001
	Yes	133	22.4	23.4	10.7	114.0	
Ever reported wheeze	No	659	13.2	12.4	9.5	102.3	0.01
	Yes	193	18.7	18.3	10.4	98.3	
Use of asthma medication	No	793	13.5	12.4	9.6	102.6	0.0003
	Yes	88	24.1	23.7	13.6	114.0	
Sex	Female	489	14.5	14.1	10.2	114.0	0.29
	Male	451	14.7	14.8	9.4	102.6	
Ethnicity	Non-Hispanic white	333	13.9	14.1	9.6	102.0	0.16
	Hispanic white	607	15.0	14.6	10.0	114.3	
Exposed to maternal smoking *in utero*	No	859	14.6	14.4	9.7	114.3	0.96
	Yes	51	16.0	18.3	9.8	88.5	
Exposure to paternal smoking *in utero*	No	789	14.6	14.6	9.7	114.3	0.96
	Yes	113	14.9	14.7	9.8	73.5	
Exposure to secondhand smoke	No	860	14.3	13.6	9.8	101.4	0.99
	Yes	31	14.9	18.3	10.8	101.0	

Table E3. Descriptive characteristics of the 488 selected compared to the 908 non-selected CHS participants from Year 3

		N=488	N=908	
Characteristics		**Count (%)**	**Count (%)**	**p-value****
FeNO (median)		9.5	10.3	0.002
Age (mean(SD))		8.4 (0.7)	8.3 (0.7)	0.15
Male		234 (48.0%)	446 (49.1%)	0.68
Race/Ethnicity	Hispanic White	315 (64.6%)	434 (58.4%)	0.03
	Non-Hispanic White	173 (35.5%)	309 (41.6%)	
Exposed to maternal smoking *in utero*		36 (7.6%)	63 (7.5%)	0.93
Exposure to paternal smoking *in utero*		68 (14.6%)	142 (17.1%)	0.25
Exposure to secondhand smoke		21 (4.4%)	48 (5.5%)	0.41
Ever diagnosed with asthma		54 (11.1%)	59 (6.5%)	0.003
Ever reported wheeze		92 (20.0%)	164 (19.3%)	0.74
History of respiratory allergy		242 (49.6%)	473 (52.2%)	0.36
Use of asthma medication		39 (8.3%)	62 (7.2%)	0.48
Annual family income	≤$14,999	62 (14.9%)	118 (15.7%)	0.27
	$15,000-$49,999	131 (31.4%)	266 (35.4%)	
	≥$50,000	224 (53.7%)	368 (48.9%)	
Parent/guardian education	Less than 12th grade	94 (20.1%)	174 (20.8%)	0.27
	Completed grade 12	88 (18.8%)	161 (19.3%)	
	Some college or technical school	164 (35.0%)	310 (37.1%)	
	Completed 4 years of college	60 (12.8%)	113 (13.5%)	
	Some graduate training	62 (13.3%)	77 (9.2%)	

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

**p-values are calculated using Chisq test or Wilcoxon test.

Table E4. Descriptive characteristics of the 436 selected compared to the 1398 non-selected CHS participants from Year 5

		N=436	N=1398	
Characteristics		**Count (%)**	**Count (%)**	**p-value****
FeNO (median)		10.0	9.3	0.02
Age (mean(SD))		10.3 (0.6)	10.3 (0.7)	0.98
Male		211 (48.4%)	690 (49.4%)	0.73
Race/Ethnicity	Hispanic White	280 (64.2%)	693 (59.1%)	0.06
	Non-Hispanic White	156 (35.8%)	480 (40.9%)	
Exposed to maternal smoking *in utero*		15 (3.6%)	97 (7.4%)	0.005
Exposure to paternal smoking *in utero*		42 (10.0%)	214 (16.6%)	0.001
Exposure to secondhand smoke		10 (2.5%)	53 (4.3%)	0.11
Ever diagnosed with asthma		77 (17.7%)	183 (13.1%)	0.02
Ever reported wheeze		99 (26.1%)	277 (23.2%)	0.25
History of respiratory allergy		267 (61.4%)	836 (59.8%)	0.56
Use of asthma medication		49 (12.3%)	122 (10.0%)	0.18
Annual family income	≤$14,999	61 (17.0%)	150 (13.0%)	0.12
	$15,000-$49,999	104 (29.0%)	377 (32.7%)	
	≥$50,000	194 (54.0%)	626 (54.3%)	
Parent/guardian education	Less than 12th grade	95 (23.1%)	252 (19.4%)	0.13
	Completed grade 12	70 (17.0%)	234 (18.0%)	
	Some college or technical school	134 (32.6%)	503 (38.8%)	
	Completed 4 years of college	62 (15.1%)	164 (12.6%)	
	Some graduate training	50 (12.2%)	144 (11.1%)	

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

**p-values are calculated using Chisq test or Wilcoxon test.

Need to rename tables here and in text

Table E5a. Spearman pairwise correlations for *NOS2A* CpG loci

	Position 1	**Position 2**	**Position 3**	**Position 4**	**Position 5**	**Position 6**	**Position 7**
Position 1	1.00	0.46*	0.14*	0.06	-0.06	-0.09*	-0.02
Position 2		1.00	0.06*	0.05	0.03	-0.04	-0.02
Position 3			1.00	0.12*	0.07*	-0.03	0.02
Position 4				1.00	0.34*	0.20*	0.25*
Position 5					1.00	0.30*	0.42*
Position 6						1.00	0.35*
Position 7							1.00

*p<0.05

Table E5b. Spearman pairwise correlations for *NOS1* and *NOS3* CpG loci

		NOS1			***NOS3***	
		Position 1	**Position 2**	**Position 3**	**Position 1**	**Position 2**
NOS1	Position 1	1.00	0.50*	0.63*	-0.05	-0.08*
	Position 2		1.00	0.46*	-0.02	-0.05
	Position 3			1.00	-0.08*	-0.10*
NOS3	Position 1				1.00	0.41*
	Position 2					1.00

*p<0.05

Table E5c. Spearman pairwise correlations for *ARG* CpG loci

		ARG1	***ARG2***		
		Position 1	**Position 1**	**Position 2**	**Position 3**
ARG1	Position 1	1.00	0.01	0.05	0.08*
ARG2	Position 1		1.00	0.24*	0.27*
	Position 2			1.00	0.40*
	Position 3				1.00

*p<0.05

Table E6. The association between percent DNA methylation of NOS genes and percent change in FeNO from linear regression models

Gene loci		Combined[†]		Asthmatics[*]		Non-Asthmatics[*]		P for interaction
		%difference	95%CI	%difference	95%CI	%difference	95%CI	
NOS1	Average[‡]	0.2	(-0.8, 1.2)	-1.1	(-4.2, 2.1)	0.1	(-0.9, 1.1)	0.92
	Position 1	-0.2	(-0.9, 0.6)	-1.0	(-3.1, 1.0)	-0.2	(-0.9, 0.5)	0.70
	Position 2	0.5	(-0.6, 1.5)	2.2	(-1.3, 5.8)	0.2	(-0.9, 1.3)	0.46
	Position 3	0.2	(-0.5, 0.9)	-1.3	(-3.6, 0.9)	0.2	(-0.5, 0.9)	0.91
NOS2A	Promoter average[§]	1.3	(-0.9, 3.6)	5.5	(-2.1, 13.7)	0.7	(-1.5, 3.0)	0.72
	Position 1	0.9	(-1.1, 2.9)	0.8	(-5.7, 7.8)	1.2	(-0.9, 3.2)	0.43
	Position 2	0.9	(-0.8, 2.7)	5.4	(-0.6, 11.9)	0.2	(-1.5, 1.9)	0.23
	Position 3	-0.3	(-1.4, 0.8)	-1.2	(-5.0, 2.8)	-0.6	(-1.7, 0.5)	0.71
	CpG island average[‡]	0.2	(-1.5, 1.9)	0.4	(-7.8, 9.4)	0.3	(-1.3, 2.0)	0.57
	Position 4	0.04	(-1.3, 1.4)	0.3	(-4.3, 5.0)	0.2	(-1.2, 1.6)	0.62
	Position 5	0.1	(-1.2, 1.4)	4.0	(-3.2, 11.5)	0.03	(-1.2, 1.3)	0.89
	Position 6	0.7	(-1.0, 2.4)	-4.1	(-12.0, 4.5)	1.1	(-0.6, 2.7)	0.26
	Position 7	-0.2	(-2.0, 1.7)	-0.7	(-11.9, 11.0)	0.1	(-1.7, 1.9)	0.59
NOS3	Average[‡]	-0.2	(-1.0, 0.5)	2.8	(-0.2, 5.8)	-0.3	(-1.1, 0.5)	0.56
	Position 1	-0.1	(-0.7, 0.5)	1.7	(-0.6, 4.1)	-0.2	(-0.8, 0.4)	0.59
	Position 2	-0.3	(-1.0, 0.4)	2.6	(-0.2, 5.5)	-0.2	(-0.9, 0.5)	0.69

[*]Analyses are adjusted for age, sex, race, plate, town, month of DNA collection, asthma medicine used, and education.
[†]Analyses are adjusted for age, sex, race, plate, town, month of DNA collection, asthma medicine used, education, and asthma.
[‡]Average methylation of multiple positions within the gene was used.
[§] Only NOS2A positions 1 and 2 were used for the average, since Position 3 is not in the promoter but is located between exons 1 and 2

Table E7. The association between % methylation of ARG genes and % change in FeNO, by wheeze status

Gene loci		Ever Wheeze*			Non-Wheeze			P-int†
		%difference	95%CI	N	%difference	95%CI	N	
ARG1	Position 1	-2.5	(-5.2, 0.3)	189	0.1	(-1.0, 1.2)	646	0.09
ARG2	Average‡	-6.5	(-11.3,-1.5)	176	-1.7	(-3.3, 0)	616	0.09
	Position 1	-1.9	(-5.2, 1.6)	176	-1.4	(-3.0, 0.2)	615	0.52
	Position 2	-5.2	(-8.9, -1.4)	176	-2.0	(-3.5, -0.4)	615	0.08
	Position 3	-5.9	(-10.5, -1.1)	176	-2.1	(-3.9, -0.3)	616	0.09

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

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

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

Table E8. The association between % methylation of ARG genes and % change in FeNO by asthma medication use among asthmatics only

Gene loci		Any medication use *			No medication use *			P-int**
		%difference	95%CI	N	%difference	95%CI	N	
ARG1	Position 1	-5.8	(-11.1, -0.2)	66	-1.7	(-6.4, 3.3)	53	0.78
ARG2	Average***	-18.3	(-28.3, -7.0)	59	-4.5	(-12.0, 3.6)	50	0.15
	Position 1	-20.1	(-30.7, -8.0)	59	2.9	(-4.6, 11.1)	50	0.12
	Position 2	-6.2	(-16.5, 5.4)	59	-4.0	(-11.7, 4.3)	50	0.94
	Position 3	-13.1	(-20.1, -5.4)	59	-6.9	(-12.0, -1.4)	50	0.20

*Analyses are adjusted for age, sex, race, plate, town, month of DNA collection, and education.
**P-value testing the interaction between ARG DNA methylation and asthma medicine used status in a model adjusted for age, sex, race, plate, town, month of DNA collection, asthma medicine used, and education among asthmatics.
***Average methylation of three positions within *ARG2* gene was used.