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Original Contribution

Air Pollution and DNA Methylation: Interaction by Psychological Factors in the VA Normative Aging Study

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DNA methylation is a potential pathway linking air pollution to disease. Studies indicate that psychological functioning modifies the association between pollution and morbidity. The authors estimated the association of DNA methylation with ambient particulate matter less than 2.5 μm in diameter ($PM_{2.5}$) and black carbon, using mixed models. DNA methylation of the inducible nitric oxide synthase gene, *iNOS*, and the glucocorticoid receptor gene, *GCR*, was measured by quantitative polymerase chain reaction pyrosequencing of 1,377 blood samples from 699 elderly male participants in the VA Normative Aging Study (1999–2009). The authors also investigated whether this association was modified by psychological factors including optimism or pessimism, anxiety, and depression. *iNOS* methylation was decreased after acute exposure to both black carbon and $PM_{2.5}$. A 1-$\mu g/m^3$ increase in exposure to black carbon in the 4 hours preceding the clinical examination was associated with a 0.9% decrease in 5-methylcytosine (95% CI: 0.4, 1.4) in *iNOS*, and a 10-$\mu g/m^3$ increase in exposure to $PM_{2.5}$ was associated with a 0.6% decrease in 5-methylcytosine (95% CI: 0.03, 1.1) in *iNOS*. Participants with low optimism and high anxiety had associations that were 3–4 times larger than those with high optimism or low anxiety. *GCR* methylation was not associated with particulate air pollution exposure.

air pollution; DNA methylation; psychology

Abbreviation: $PM_{2.5}$, particulate matter less than 2.5 μm in diameter.

Air pollution has been associated with morbidity and mortality (1, 2), and epigenetic mechanisms, such as DNA methylation, have been hypothesized as a potential pathway linking environmental exposures and disease outcomes. DNA methylation has been associated with diseases, such as cancer (3), ischemic heart disease (4), and atherosclerosis (5), and studies suggest that changes in DNA methylation may be a biomarker of disease risk (6). A limited body of evidence indicates that environmental exposures may be associated with changes in DNA methylation, but only a few studies have demonstrated associations with ambient air pollution (7–9) or second-hand smoke (10).

A growing body of literature suggests that psychological factors alter susceptibility to physical exposures (11–13). Clougherty and Kubzansky (14) have hypothesized that allostatic load may be a mechanism by which this occurs, whereby chronic psychological stress impairs the body's ability to respond to transient environmental stressors. If physical environmental stressors and psychological stressors impact health through similar physiologic mechanisms, they may act synergistically beyond their individual effect. Few studies have examined the association between air pollution and DNA methylation, and none, to our knowledge, have examined the interrelation among air pollution, psychological factors, and DNA methylation. This study is an extension of the work examining synergism between the physical and social environments.

First, we sought to examine the association between ambient particulate air pollution and DNA methylation in the promoter regions of 2 specific genes: the inducible

nitric oxide synthase gene, *iNOS*, and the glucocorticoid receptor gene, *GCR*. We selected these genes because of their relation to inflammation and the stress response. Inflammation is considered a primary mechanism linking inhalation of air pollutants to their acute health effects (15). A key step during inflammation is the chemokine-controlled leukocyte infiltration, which is regulated by inducible nitric oxide synthase-derived nitric oxide (16). The glucocorticoid receptor is also linked to the inflammatory process, as glucocorticoids exert antiinflammatory effects on many cell types (17).

Second, we examined whether certain psychological traits and symptoms altered the association between air pollution and DNA methylation. Studies indicate that stress is associated with modifications to oxidative/nitrosative pathways in the brain in response to activation of inflammatory mediators and that stress results in increased nitric oxide production in the brain and periphery (18). The glucocorticoid receptor has also been implicated in the stress response (19), and early exposure to stress in humans has been associated with increased methylation of *GCR* and an increased cortisol stress response (20). We focused on psychological factors that have been linked to respiratory and cardiovascular disease, as these outcomes have been associated with air pollution exposure. We hypothesized that poor psychological functioning (characterized by greater depression, anxiety, and pessimism, as well as lower optimism) would enhance the association between air pollution and DNA methylation.

MATERIALS AND METHODS

Study population

This study included 735 elderly men who, as of March 1999, were active participants in the VA Normative Aging Study. Established by the Veterans Administration in 1961, the Normative Aging Study cohort enrolled men 21–80 years of age from the greater Boston, Massachusetts, area who were free of known chronic medical conditions (21). Since enrollment, participants have had comprehensive clinical examinations at intervals from 3 to 5 years. Further details can be found elsewhere (22). In examinations that took place between March 1999 and December 2009, active participants agreed to donate at least 1 blood sample that was used to analyze DNA methylation. Because of *iNOS* methylation assay failures, there were 661 men with 1,176 measurements of *iNOS* methylation and 735 men with 1,466 measurements of *GCR* methylation. After exclusion of men who were seasonal residents in the Boston metropolitan area, there were 627 men with 1,105 measurements of *iNOS* methylation and 699 men with 1,377 measurements of *GCR* methylation.

Exposure variable and covariates

The air pollutant of interest was ambient particulate matter less than 2.5 μm in diameter ($PM_{2.5}$) and one of its components, black carbon, which is a measure of traffic particles. Measurements were obtained from a stationary monitoring source located at the Harvard School of Public Health, less than 1 km from the examination site where the patients' visits took place. The median distance of the participants' homes from the central site monitoring station was 20.5 km, with an interquartile range from 10.5 to 37.9 km. The maximum distance was 126 km, but 90% of participants live within 78 km of the central monitoring site.

$PM_{2.5}$ was measured continuously by using a model 1400A tapered element oscillating microbalance (Rupprecht & Patashnick Co., East Greenbush, New York), operated at 50°C with 2 model 4LPM-PM2.5 impactors before the inlet. A season-specific correction, based on collocated gravimetric samplers, was used to correct for loss of semivolatile particles in the monitor. Black carbon was measured continuously by using a model AE-14 aethalometer (Magee Scientific, Inc., Berkeley, California). Hourly data for black carbon and $PM_{2.5}$ were occasionally missing. For these times, data were imputed by use of a regression model (23). Covariate information was assessed at each medical examination. The temperature and relative humidity for each day were obtained from the National Climatic Data Center (Asheville, North Carolina).

The Life Orientation Test was used to measure dispositional optimism and pessimism. A validated instrument (24), the Life Orientation Test is made up of 12 items comprising questions such as, "If something can go wrong for me, it will." Depressive and anxious symptoms were evaluated by using the Brief Symptom Inventory, a 53-item validated questionnaire that assesses 9 primary symptom dimensions (anxiety, depression, hostility, interpersonal sensitivity, obsessive-compulsive, paranoid ideation, phobic anxiety, psychoticism, somatization) (25). Further information about these measurements can be found in the Web Appendix that, along with 10 Web-only tables, is posted on the *Journal*'s website (http://www.aje.oxfordjournals.org/). To examine the variation over time in psychological measures, we calculated the intraclass correlation coefficient for using a mean rating as the unit of analysis or the reliability for the mean score (26) by the SAS %INTRACC macro (available at http://support.sas.com/kb/25/031.html#ref).

Outcome variable

DNA methylation analyses were performed on bisulfite-treated DNA by using highly quantitative analyses based on polymerase chain reaction pyrosequencing; a 500-ng DNA sample (concentration, 25 ng/μL) was treated by using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, California) according to the manufacturer's protocol. Further details can be found in the Web Appendix (Web Tables 1–3). DNA methylation analysis measured 2 individual CpG dinucleotide positions for *iNOS* and 1 CpG dinucleotide position for *GCR*. At each CpG position, pyrosequencing was repeated twice, and the results were averaged to minimize the assay variability. All samples were analyzed consecutively by 1 laboratory technician.

Statistical analysis

Multiple moving-averaged exposures of $PM_{2.5}$ and black carbon were examined. The moving average is the mean exposure for the time period before each examination.

Because fasting blood samples are collected, blood is always drawn in the morning, at approximately 8 AM. The moving average is calculated from 8:00 AM (i.e., the 4-hour moving average includes the 4 hours immediately preceding 8:00 AM of the visit day) and, therefore, is close to the blood draw time. Separate models were created for each of the following moving average exposure windows: 4 hours and 1, 2, 3, 4, 5, 6, 7, 14, and 28 days preceding the clinical examination. These windows were chosen because they have been shown to be important in the relation between air pollution and DNA methylation (7, 9), as well as other intermediate health outcomes (27–30).

Although DNA methylation at adjacent CpG sites is usually correlated, the correlation is not perfect, and differences exist that are not due to random variability. Therefore, mixed models were used to take full advantage of the information from all the measurements in the data and to maximize statistical power by distinguishing between the different sources of variance in the data. Accordingly, mixed-effects models with 2 random intercepts were used to capture the correlation among measurements within the same subject or the same location within a promoter region. The model was as follows:

$$Y_{ijk} = \beta_0 + u_k + u_i + \beta_1 \,\text{pollutant} + \beta_2 X_2 + \ldots + \beta_p X_p, \quad (1)$$

where Y_{ijk} is the measured value of *iNOS* methylation at CpG dinucleotide position i at visit j of subject k; β_0 is the overall intercept; u_k is the separate random intercept for the subject, which captures the correlation among measurements within the same subject; u_i is the separate random intercept for each CpG dinucleotide postion, which captures the average difference between methylation at that dinucleotide position and the overall mean methylation for the gene; and $X_2 \ldots X_p$ are the covariates. Because *GCR* methylation was measured at only 1 CpG position, a simplified version of model 1 was used, which included only 1 random intercept for each subject.

The following potential confounders were chosen a priori and included baseline age; season (through sine and cosine functions of time); a linear term for time to capture long-term trends; a linear term for apparent temperature, a composite index of human discomfort due to combined heat and high humidity, calculated from air and dew point temperature (31); and the percent lymphocytes and percent neutrophils. The latter were included to control potential variation among leukocyte populations in *iNOS* and *GCR* methylation (32). A second set of covariates, which were not thought to be confounders, was also evaluated. The following were potential predictors of DNA methylation that were evaluated to improve the goodness of fit of our models: smoking (current/former/never and pack-years), body mass index, hypertension medication, alcohol intake (>2 drinks per day), diabetes mellitus, ischemic heart disease, number of years of education, and race (white/black). We used the stepwise selection procedure "stepAIC" in R, version 2.10.1 (R Foundation for Statistical Computing, Vienna, Austria), to determine which of these additional covariates improved the fit of the mixed models. After running separate models for each moving-average time window of exposure, we created models with multiple averaging time windows of exposure to determine which was the most appropriate time window of exposure. We performed a number of sensitivity analyses to test the robustness of our findings. These included restricting our population to those living within 40 km of the central air pollution monitor, including seasonal residents, using a quadratic term to model temperature, and using the mean methylation measurement from the 2 CpG sites for *iNOS*.

Finally, when we identified a main association with an air pollutant, interaction between our pollutant exposures and psychological measures was examined. The models examining interaction were restricted to the a priori potential confounders. To facilitate interpretability of the interaction term, we dichotomized the measures of optimism and pessimism by the median value. As a sensitivity analysis, optimism and pessimism were also dichotomized at the upper quartile of our population distribution, because studies examining these traits in relation to morbidity and mortality have used such cutpoints (33, 34). For depression and anxiety, a binary variable to indicate when scores were 1 standard deviation above the normal population mean (i.e., >0.50) was used, as was done previously in this cohort (35). Interaction was examined for the psychological measures by a cross-product term of high versus low and by a linear term for $PM_{2.5}$ or black carbon. All final mixed-effect models were conducted by using the PROC MIXED procedure in SAS, version 9.2, software (SAS Institute, Inc., Cary, North Carolina).

RESULTS

The mean age of participants at baseline was 72 years (Table 1). The mean level of *iNOS* methylation was 68.4% 5-methylcytosine, with a range of 24.5%–87.2% 5-methylcytosine. The 2 CpG loci in *iNOS* were moderately correlated (rho = 0.455; $P < 0.0001$). The mean level of *GCR* methylation was 46.6% 5-methylcytosine, with a range of 14.7%–87.2% 5-methylcytosine. During the study period, the mean levels of $PM_{2.5}$ and black carbon over a 24-hour period were 10.5 $\mu g/m^3$ and 0.8 $\mu g/m^3$ (Table 2). A summary of exposure levels by psychological category is provided in Web Table 4. The calculated intraclass correlation coefficients of 0.79 for optimism, 0.77 for pessimism, 0.76 for anxiety, and 0.79 for depression indicated that our psychological measures had a high degree of stability over time (10 years). Correlations among the psychological measures are provided in Web Table 5. With optimism recoded for congruence, the standardized Cronbach's alpha was 0.72 for the measures.

After forcing the aforementioned a priori chosen covariates into our models, we found that a stepwise selection procedure additionally identified diabetes mellitus status, body mass index, and hypertension medication as important predictors of *iNOS* methylation and diabetes mellitus status and race as important predictors of *GCR* methylation. Increased exposure to both black carbon and $PM_{2.5}$ was associated with decreased *iNOS* methylation. For each pollutant, both a short response and an intermediate response

Table 1. Descriptive Characteristics of Men in the VA Normative Aging Study at Baseline, Boston, Massachusetts, 1999–2009

	GCR[a] Analysis ($n = 699$)			*iNOS*[a] Analysis ($n = 627$)		
	No.	%	Mean (SD)	No.	%	Mean (SD)
Age, years			71.9 (6.8)			71.8 (6.8)
Race						
White	672	96.1		604	96.3	
Black	13	2.0		11	1.8	
Hispanic White	5	0.7		5	0.8	
Hispanic Black	1	0.1		1	0.2	
Unknown	8	1.1		6	0.9	
Body mass index[b]			28.3 (4.1)			28.1 (4.2)
Smoking status						
Never smoker	198	28.3		171	27.3	
Former smoker	471	67.4		433	69.1	
Current smoker	30	4.3		23	3.7	
Cumulative smoking, pack-years[c]			21.2 (25.3)			21.7 (25.5)
Alcohol consumption, drinks/day						
< 2	565	80.8		505	80.5	
≥ 2	128	18.3		114	18.2	
Unknown	6	0.9		8	1.3	
Taking antihypertensive medication	412	58.9		387	61.7	
Ischemic heart disease	213	30.5		195	31.1	
Diabetes mellitus	125	17.9		114	18.2	
Highest level of education, years			15.0 (3.0)			15.0 (3.0)

Abbreviation: SD, standard deviation.
[a] *GCR*, glucocorticoid receptor gene; *iNOS*, inducible nitric oxide synthase gene.
[b] Body mass index: weight (kg)/height $(m)^2$.
[c] Among current or former smokers.

were observed, with statistically significant associations for exposure during the 4-hour period preceding the examination and during the 1–4 weeks preceding it (Table 3). There was no association between increased exposure to either black carbon or $PM_{2.5}$ and *GCR* methylation (Table 3). In models for *iNOS* methylation that simultaneously included a short exposure period and an intermediate period, only the short exposure for $PM_{2.5}$ remained statistically significant, although both the short and intermediate exposures for black carbon persisted (Web Table 6). Our sensitivity analyses did not provide substantially different results from our main analyses (Web Tables 7–10).

Table 2. Air Pollution Characteristics, Boston, Massachusetts, 1999–2009

Moving Average	Black Carbon, $\mu g/m^3$		$PM_{2.5}$, $\mu g/m^3$	
	Mean	IQR	Mean	IQR
4-hour	1.26	0.92	11.56	7.57
1-day	0.84	0.55	10.51	6.86
2-day	0.80	0.49	10.39	6.25
3-day	0.75	0.46	10.17	5.46
4-day	0.72	0.38	10.11	5.26
5-day	0.73	0.35	10.15	4.57
6-day	0.75	0.34	10.13	4.47
7-day	0.76	0.34	10.12	4.21
2-week	0.76	0.29	10.15	3.63
4-week	0.76	0.27	10.18	3.32

Abbreviations: IQR, interquartile range; $PM_{2.5}$, particulate matter less than 2.5 μm in diameter.

Optimism, pessimism, anxiety, and depression were examined for interaction with both pollutants for *iNOS* methylation but not for *GCR* methylation, because there was no main association with either pollutant. There was interaction ($P = 0.05$) between black carbon and both anxiety and optimism. The final models included a cross-product term between each psychological measure and black carbon exposure in the preceding 4 hours, as well as adjustment by black carbon exposure in the preceding 2 weeks. Participants with low optimism scores had a decrease in *iNOS* methylation 3 times greater than those with high optimism scores (Table 4). Participants with high anxiety scores had a decrease in *iNOS* methylation 3 times greater than participants with low anxiety. There was also interaction ($P = 0.05$) between optimism and $PM_{2.5}$. Participants with low optimism scores had a decrease in *iNOS* methylation 4 times greater than participants with high optimism.

Table 3. Associations Between Air Pollution and Gene Promoter Methylation in the VA Normative Aging Study, 1999–2009

	% Change in 5-Methylcytosine			
	Per 1 μg/m^3 Black Carbon	95% Confidence Interval	Per 10 μg/m^3 $PM_{2.5}$	95% Confidence Interval
iNOS[a,b]				
4-hour	−0.884	−1.385, −0.382	−0.577	−1.126, −0.029
1-day	−0.440	−1.352, 0.471	−0.390	−1.094, 0.314
2-day	−0.010	−1.147, 1.128	−0.250	−1.010, 0.510
3-day	0.021	−1.330, 1.372	−0.258	−1.098, 0.582
4-day	−0.622	−2.181, 0.937	−0.554	−1.496, 0.388
5-day	−1.328	−3.064, 0.409	−0.877	−1.946, 0.192
6-day	−1.376	−3.276, 0.524	−1.068	−2.240, 0.104
7-day	−1.689	−3.738, 0.359	−1.327	−2.573, −0.081
2-week	−3.731	−6.402, −1.059	−1.550	−3.034, −0.065
4-week	−5.395	−8.941, −1.848	−1.477	−3.259, 0.306
GCR[a,c]				
4-hour	−0.363	−0.789, 0.064	−0.135	−0.615, 0.345
1-day	−0.672	−1.519, 0.176	−0.351	−0.971, 0.269
2-day	−0.865	−1.898, 0.168	−0.516	−1.190, 0.157
3-day	−1.051	−2.285, 0.184	−0.521	−1.270, 0.229
4-day	−1.407	−2.830, 0.015	−0.627	−1.482, 0.228
5-day	−0.899	−2.470, 0.672	−0.500	−1.470, 0.469
6-day	−1.452	−3.160, 0.255	−0.688	−1.752, 0.377
7-day	−1.541	−3.357, 0.276	−0.850	−1.971, 0.271
2-week	−1.064	−3.422, 1.295	−0.790	−2.132, 0.552
4-week	−0.272	−3.403, 2.858	−0.787	−2.363, 0.788

Abbreviation: $PM_{2.5}$, particulate matter less than 2.5 μm in diameter.
[a] *GCR*, glucocorticoid receptor gene; *iNOS*, inducible nitric oxide synthase gene.
[b] Models were adjusted for baseline age, season, and time trend, apparent temperature, percent lymphocytes, percent neutrophils, body mass index, diabetes mellitus, and hypertension medication.
[c] Models were adjusted for baseline age, season, and time trend, apparent temperature, percent lymphocytes, percent neutrophils, diabetes mellitus, and race.

Table 4. Associations Between Acute Air Pollution Exposure and *iNOS*[a] Promoter Methylation by Psychological Factor in the Normative Aging Study, 1999–2009

4-Hour Exposure Period	% Change in 5-Methylcytosine		$P_{interaction}$	% Change in 5-Methylcytosine		$P_{interaction}$
	Per 1 μg/m^3 Black Carbon[b]	95% Confidence Interval		Per 10 μg/m^3 $PM_{2.5}$[c]	95% Confidence Interval	
High pessimism	−1.005	−1.742, −0.268	0.867	−0.788	−1.532, −0.043	0.925
Low pessimism	−0.924	−1.580, −0.269		−0.835	−1.593, −0.077	
High optimism	−0.480	−1.134, 0.173	0.048	−0.835	−1.593, −0.077	0.050
Low optimism	−1.432	−2.168, −0.696		−1.267	−2.044, −0.490	
High depression	−0.833	−2.252, 0.586	0.878	−1.057	−2.335, 0.222	0.558
Low depression	−0.950	−1.492, −0.407		−0.650	−1.254, −0.046	
High anxiety	−2.0976	−3.419, −0.777	0.053	−1.657	−2.893, −0.422	0.101
Low anxiety	−0.6564	−1.203, −0.110		−0.548	−1.153, 0.057	

Abbreviation: $PM_{2.5}$, particulate matter less than 2.5 μm in diameter.
[a] *iNOS*, inducible nitric oxide synthase gene.
[b] Models were adjusted for baseline age, season, and time trend, apparent temperature, percent lymphocytes, percent neutrophils, and black carbon exposure in the 2 weeks prior to examination.
[c] Models were adjusted for baseline age, season, and time trend, apparent temperature, percent lymphocytes, and percent neutrophils.

Interaction between $PM_{2.5}$ and pessimism, depression, or anxiety was not statistically significant; however, for anxiety, there was a trend similar to that seen with optimism. The results were not materially different when the upper quartile of optimism and pessimism was used as a cutpoint instead of the median value.

DISCUSSION

We found that exposure to particulate air pollution was associated with decreased promoter methylation of *iNOS* but not of *GCR*. For *iNOS*, we saw associations with both short and intermediate exposure periods. This association was enhanced in individuals that were less optimistic and more anxious. Although gene expression was not measured in our study, methylation usually results in transcriptional suppression, while hypomethylation of regulatory sequences tends to correlate with gene expression (36).

Animal models have demonstrated that particles can induce inflammation and increase nitric oxide production (37). In addition, some animal and tissue studies have demonstrated an association between air pollution and DNA methylation (38, 39). Consistent with our findings, a study in rats demonstrated increased *iNOS* messenger RNA after exposure to particulate matter (40). Animal studies have also linked nitric oxide to psychological factors, such as stress and depression. In a study of white rats, chronic stress induced increases in *iNOS* expression in many parts of the brain (41). In an animal model that used chronic mild stress to mimic human depression, fluoexitine, an antidepressant, inhibited nitric oxide synthase overexpression (42).

Only a few epidemiologic studies have investigated the association between air pollution and DNA methylation. In the same cohort that we have investigated here, ambient particulate matter exposure was associated with repetitive element methylation (7, 9). Indoor stove coal usage has been associated with a higher rate of *p16* promoter region methylation (43), and a study of foundry workers showed that exposure to particulate air pollution was associated with hypomethylation of the *iNOS* gene (8). Finally, prenatal tobacco smoke exposure was associated with changes in repetitive element and gene-specific methylation in a cohort of children (10). We know of no other epidemiologic studies that have looked at psychological factors and *iNOS* methylation specifically; however, a limited number of epidemiologic studies have reported associations between psychological factors and DNA methylation of other genes (20, 44, 45).

Similar to the study in foundry workers, our study found that the association between $PM_{2.5}$ and *iNOS* was primarily a short-term association, although we also observed a persistent association between black carbon exposure in the previous 2 weeks and *iNOS* hypomethylation, beyond the acute association. In urban areas, black carbon derives primarily from exhaust emissions from vehicles (46), possibly reflecting different pathways or associations with particles of different characteristics.

We found that poor psychological functioning (i.e., more anxiety, less optimism) amplified the association between particulate matter exposure and decreased DNA methylation. Very few studies have examined the interaction between the physical and social environments, but there is limited evidence that such exposures act synergistically. In an animal model, higher air pollution exposure was associated with a rapid, shallow breathing pattern only in rats under chronic stress (47). Human epidemiologic studies have demonstrated that psychological stress may alter susceptibility to air pollution on respiratory outcomes (11–13), as well as mental health outcomes (48). Building on prior research suggesting that optimism may buffer the effects of chronic stress (49) and that pessimism is associated with depressive and anxious symptoms (50), the current study expands the literature to consider potential synergism between environmental toxicants and poor psychological functioning beyond simple measures of stress.

A potential limitation of this study is the use of a single ambient monitor to characterize exposure, which can lead to exposure error when the pollutant is spatially heterogeneous. However, concentrations of $PM_{2.5}$ have been shown to be homogeneous over a wide geographic region, and ambient $PM_{2.5}$ measurements have been shown to be a good surrogate for personal exposures. In panel studies in Boston (51, 52), where participants were longitudinally followed, longitudinal variation in ambient $PM_{2.5}$ concentrations was strongly correlated with corresponding personal $PM_{2.5}$ exposures. With respect to a particular component of $PM_{2.5}$, the spatial homogeneity may vary. Black carbon is moderately heterogeneous because of the numerous local sources (53). Classical measurement error tends to bias the effect downward, while Berkson measurement error tends to increase the standard error of the estimate. When looking at longitudinal variations in air pollution, we find that most error is of the Berkson type. To the extent that it is classical, simulation studies have shown that it is highly unlikely to bias away from the null even in the presence of covariates (54). Thus, any measurement error in our black carbon exposure metric would likely attenuate the true association. Given that we found significant associations for black carbon, it is unlikely that this error would qualitatively change our conclusions.

Our measurement of DNA methylation has some limitations. The methylation analyses were performed on white blood cell DNA. To what extent the change we observed in white blood cell DNA reflects similar modification of DNA methylation in target tissue is unclear. However, white blood cells regulate the systemic response to inflammation and, given that inflammation is a relevant mechanism in the relation we examined, measurements in white blood cells may be appropriate. We were unable to measure the expression of *iNOS*- and *GCR*-encoded RNAs and proteins in this cohort; however, other studies provide evidence that methylation of *iNOS* is functional (55). Thus, although the potential role of hypomethylation in response to the exposure remains speculative in our cohort, it is important to note that small changes in DNA methylation may be clinically meaningful. In the same cohort as we investigated here, decreased *iNOS* methylation was associated with decreased lung function (56). We saw stronger associations for longer periods of exposure versus acute exposure periods in this study, particularly for black carbon, suggesting the possibility of

cumulative effects (at least over short periods of time). Given that air pollution exposure is ubiquitous and is often high in the same populations that are under high psychological burden (14), we believe that even small changes in DNA methylation may be important for understanding the interrelation between such exposures and morbidity.

As with any observational study, the possibility of residual confounding exists. Because we investigated a time-varying exposure, we adjusted for several potential time-varying confounders (i.e., age, time trend, temperature, and season) and included a random intercept for each subject in our mixed-effects models. This should minimize unmeasured, time-invariant confounding across subjects. Our measures of optimism, pessimism, anxiety, and depression symptoms were all self-reported; however, we used validated instruments with known characteristics, and these measures had excellent reliability in this cohort (57). Although we analyzed multiple moving averages of pollutant exposure, we note that these comparisons are not completely independent hypotheses but, rather, are testing a pattern of association. Nonetheless, multiple comparisons were performed, and we urge cautious interpretation of these findings within the context of the broader body of scientific literature. Finally, this study was limited to a cohort of elderly, almost entirely white, men. The elderly, black people, and women have been found to be more susceptible to air pollution exposure (58). Therefore, our findings may not apply to other populations.

The present study indicates that ambient air pollution exposure is associated with promoter hypomethylation of *iNOS*. Individuals reporting poorer psychological functioning may be particularly susceptible to these effects. This study provides preliminary evidence that such physical and psychological stressors may work through shared mechanistic pathways and, hence, the burden of one of these stressors may alter the ability to adapt to another. Further work is needed to clarify the complex interplay between physical and psychological stressors on epigenetic programming and human health.

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