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Black Carbon Exposures, Blood Pressure, and Interactions with Single Nucleotide Polymorphisms in MicroRNA Processing Genes

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BACKGROUND: Black carbon (BC) is a marker of traffic pollution that has been associated with blood pressure (BP), but findings have been inconsistent. MicroRNAs (miRNAs) are emerging as key regulators of gene expression, but whether polymorphisms in genes involved in processing of miRNAs to maturity influence susceptibility to BC has not been elucidated.

OBJECTIVES: We investigated the association between BC and BP, as well as potential effect modification by single nucleotide polymorphisms (SNPs) in miRNA processing genes.

METHODS: Repeated measures analyses were performed using data from the VA Normative Aging Study. Complete covariate data were available for 789 participants with one to six study visits between 1995 and 2008. In models of systolic and diastolic BP, we examined SNP-by-BC interactions with 19 miRNA-related variants under recessive models of inheritance. Mixed-effects models were adjusted for potential confounders including clinical characteristics, lifestyle, and meteorologic factors.

RESULTS: A 1-SD increase in BC (0.415 µg/m³) was associated with 3.04 mmHg higher systolic (95% confidence interval [CI], 2.29–3.79) and 2.28 mmHg higher diastolic BP (95% CI, 1.88–2.67). Interactions modifying BC associations were observed with SNPs in the DICER, GEMIN4, and DiGeorge critical region-8 (DGCR8) genes, and in GEMIN3 and GEMIN4, predicting diastolic and systolic BP, respectively.

CONCLUSIONS: We observed evidence of effect modification of the association between BP and 7-day BC moving averages with SNPs associated with miRNA processing. Although the mechanisms underlying these associations are not well understood, they suggest a role for miRNA genesis and processing in influencing BC effects.

KEY WORDS: black carbon, blood pressure, epigenetic mechanisms, gene–environment interactions, genetic polymorphisms. Environ Health Perspect 118:943–948 (2010). doi:10.1289/ehp.0901440

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Exposure to particulate air pollution has been associated with cardiovascular morbidity and mortality in numerous epidemiologic studies (Brook 2008; Pope et al. 2004). Black carbon (BC), a combustion by-product, is a widely used marker of traffic pollution and has been linked to cardiac and ventricular arrhythmias (Rich et al. 2005), ST-segment depression (Gold et al. 2000), decreased flow-mediated vascular reactivity (O’Neill et al. 2005), lowered heart rate variability (Schwartz et al. 2005), and increased cardiovascular mortality (Maynard et al. 2007). Growing evidence suggests that traffic-related pollution, including BC, may be driving the cardiotoxic effects observed in response to air pollution exposures (Hoffmann et al. 2007). A few recent studies have examined associations between particles and blood pressure (BP), and although positive associations have been observed (Aurchinello et al. 2008; Ihald-Mulli et al. 2001; Zanobetti et al. 2004), both inverse (Harrabi et al. 2006) and null (Jansen et al. 2005; Madsen and Naftstad 2006) associations have also been reported.

MicroRNAs (miRNAs) are small, non-coding RNAs that repress or inhibit gene expression by targeting messenger RNA (mRNA) (Mattick and Makunin 2006; Zhang 2008). Evidence suggests that miRNAs affect pathogenic pathways including angiogenesis (Suarez and Sessa 2009), redox signaling (Brewer and Shah 2009; Urbich et al. 2008), and stress response (van Rooij et al. 2007). Much of the existing miRNA-related literature has focused on cancer outcomes (Esquela-Kerscher and Slack 2006; Hu et al. 2008, 2009; Luthra et al. 2008; Yang et al. 2008). However, there is growing evidence that the dysregulation of cell-signaling pathways associated with miRNA is a key factor affecting heart disease (Chen et al. 2008; Cheng et al. 2007; Divakaran and Mann 2008; Ikeda et al. 2007; Zhang 2008). Additionally, studies in controlled environments have demonstrated cardiovascular effects due to loss of function of miRNA processing genes (Asada et al. 2008; da Costa Martins et al. 2008; Suarez et al. 2007). For the current study, we genotyped participants for potentially functional single nucleotide polymorphisms (SNPs) involved in the processing and formation of miRNAs. We then investigated the association between air pollution and BP as well as potential differences in susceptibility by SNP carrier status. We hypothesized that polymorphisms in genes that regulate miRNA processing could modify effects of BC on BP, a marker of autonomic function and cardiovascular health.

Materials and Methods

Study population. Our study participants were members of the Veterans Affairs Normative Aging Study (NAS). This is an ongoing longitudinal study of aging established in 1963, details of which have been published previously (Bell et al. 1966). Briefly, the NAS began as a closed cohort of 2,280 male volunteers from the greater Boston area, 21–80 years of age at entry, who enrolled after an initial health screening determined that they were free of known chronic medical conditions. All participants provided informed consent. Collection of blood samples for genetic analysis began in the late 1990s; 942 participants were still actively participating and provided BP and blood samples for some or all miRNA-related SNPs, which were successfully genotyped. Participants were reevaluated every 3–5 years using detailed on-site physical examinations and questionnaires.

Physical parameters and medical history. Study center visits occurred after an overnight fast and abstention from smoking. Physical examination included measurement of height and weight, with body mass index (BMI) calculated as weight (kilograms)/height (meters squared). Questionnaires evaluated lifestyle factors and medication use. Type 2 diabetes was classified based on physician’s diagnosis or fasting blood glucose ≥ 126 mg/dl.

BP measurements. At each clinical visit, a physician measured BP using a standard

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mercury sphygmomanometer with a 14-cm cuff. Systolic BP (SBP) and fifth-phase diastolic BP (DBP) were measured in each arm to the nearest 2 mmHg while the participant was seated. The means of the right and left arm measurements were used as the BP measurement of each participant for analytical purposes. Although there was no specific rest period prior to measurement of BP, SBP and DBP were measured immediately after a complete patient history was taken with the subject seated.

SNP selection and genotyping. SNPs were selected based on previously published work investigating associations between genes involved in miRNA processing and disease (Horikawa et al. 2008; Yang et al. 2008). These SNPs were chosen because of overlap in pathways involved in cancer processes related to autonomic function through cell signaling, apoptosis, angiogenesis, and inflammation. Genotyping was performed using multiplex polymerase chain reaction assays designed with Sequenom SpectroDESIGNER software (Sequenom, Inc., San Diego, CA). The extension product was then spotted onto a 384-well spectroCHIP before analysis in the MALDI-TOF mass spectrometer (Sequenom, Inc.). Duplication was performed on 5% of the samples. The 24 SNPs analyzed for this study were all successfully genotyped. After genotyping, we excluded those SNPs for which fewer than 10 participants were homozygous variant carriers ([rs1106042 in H1W1, rs3742330 in DICER, rs417309 in DiGeorge critical region-8 (DGCR8), rs636832 in Argonaute 1 (Ago1)]) and also those with a Hardy–Weinberg p-value < 0.05 (rs10719 in DROSH), leaving a total of 19 SNPs in 10 genes.

**Table 1.** Descriptive statistics at baseline for participants included in analysis (*n* = 789).a

| Study characteristic | Value |
|----------------------|-------|
| **BP measurements**  |       |
| SBP (mmHg)           | 132.0 ± 18.4 |
| DBP (mmHg)           | 76.8 ± 10.9  |
| **Clinical measures**|       |
| Age at visit (years) | 72.3 ± 7.5  |
| BMI (kg/m²)          | 28.0 ± 4.1  |
| Pulse (beats/min)    | 71.1 ± 8.1  |
| Hypertension diagnosis| 542 (81.8) |
| Hypertension medication use | 478 (72.1) |
| Two or more drinks per day | 199 (25.2) |
| Diabetesb            | 182 (23.1) |
| **Education (years)**|       |
| ≤ 12                 | 280 (35.5)  |
| 13–16                | 372 (47.2)  |
| > 16                 | 137 (17.3)  |
| **Smoking status**   |       |
| Never               | 237 (30.0)  |
| Current             | 47 (6.0)    |
| Former              | 505 (64.0)  |
| **Air pollution and weather data**|         |
| Seven-day moving average BC (µg/m³) | 0.98 ± 0.415 |

Values are mean ± SD or no. (%).

*Corresponding to a 0.415-µg/m³ increase in 7-day average BC concentrations. 
*Model 1 was adjusted for age, BMI, smoking status, season, 7-day moving average of BC, and 7-day moving average of apparent temperature. 
*Model 2 was adjusted for education, blood urea nitrogen, age, BMI, pulse, smoking status, pack-years, median income, apparent temperature, alpha blocker use, beta blocker use, calcium channel blocker use, angiotensin receptor blocker use, ACE inhibitor use, diuretics, season, two or more drinks per day, and diabetes diagnosis.

We performed sensitivity analyses to determine whether our results were robust to changes in the covariates adjusted for in the models and also whether there were significant effects due to missing data. To address attrition in the study, we used inverse probability weighting to determine whether reweighting these observations with only one or two repeated measures significantly changed our results (Hernan et al. 2006). This was done by modeling the probability of having only one measurement or only two measurements using the covariates described in model 2 and treating those participants with three or more visits as the reference group. We then used the inverse of the predicted values as the weights. We also examined associations adjusting only for covariates selected in model 1 to determine whether the significance and or magnitude of our adjusted results changed. Linkage disequilibrium (LD) between SNPs in the same gene that met the significance criterion were assessed using the LDPrplotter tool (Innate Immunity in Heart Lung and Blood Disease; http://www.pharmgkb.org/IIPGA2/Bioinformatics/).

**Results.** Of the 2,280 men who originally entered the cohort in 1963, complete covariate data were available on participants who took part in one to six examinations during the study period. Because the NAS comprises over 95% white participants, we restricted our analysis to white individuals based on self-report. There were 942 participants with some or all microRNA-processing genotyping data and BP measurements. Of these, BC data were available for 799 participants who reside in Massachusetts. All visits occurred between 1995, when pollutant monitoring began, and 2008. Our full models (model 2) include data from the 798 participants with complete covariate data and some or all genotyping data. In this group, 645 (82%) participants had at least two study visits from 1995 to 2008; 475 (60%) had three or more visits. Our study population was composed entirely of males, most of whom were former cigarette
smokers (Table 1). The mean age (± SD) of study participants was 72.3 ± 7.5 years and mean BMI was 28.0 ± 4.1 kg/m². Average SBP and DBP were 132 ± 18.4 mmHg and 76.8 ± 10.9 mmHg, respectively. We evaluated the association of SBP and DBP with ambient BC and expressed the results as the mmHg change associated with a 1-SD increase in BC (equivalent to 0.415 µg/m³) (Table 2). In our fully adjusted models (model 2), we found that a 1-SD increase in BC concentration was associated with 3.04-mmHg higher SBP (95% CI, 2.29–3.79; p = 0.003) and a 2.28-mmHg higher DBP (95% CI, 1.88–2.67; p < 0.001). These associations were attenuated compared with our model 1 analyses, which were adjusted for a subset of potential confounders. In model 1 analyses, we observed that a 1-SD change in BC was associated with 3.52-mmHg (95% CI, 2.77–4.26) and 2.72-mmHg (95% CI, 2.31–3.12) changes in SBP and DBP, respectively.

The complete list of the 19 SNPs analyzed is described in Table 3. We genotyped 24 SNPs and excluded those SNPs that, in fewer than 10 study participants, were homozygous carriers of the variant allele (rs1106042 in H1WI, rs3742330 in DICER, rs1713099 in DGCRR, rs6368382 in AGO1) and those in which Hardy–Weinberg equilibrium was not met at the 0.05 level (rs10719 in DROSHA), leaving a total of 19 SNPs in 10 genes. First, we examined the main effects of the SNPs of interest within this population. In our fully adjusted models, none of these SNPs were associated with BC at the 0.05 level. We examined BC-by-SNP interactions, and results are reported in Table 4.

In models of SBP, interactions with BC were observed for 10 genes. First, we examined the main effects of the SNPs of interest within this population. In our fully adjusted models, none of these SNPs were associated with BC at the 0.05 level. We examined BC-by-SNP interactions, and results are reported in Table 4.

In models of SBP, interactions with BC were observed for two SNPs. For homozygous variant carriers of rs197414, a 1-SD change in BC was associated with 9.82-mmHg lower DBP (95% CI, –18.68 to –0.95), whereas in wild-type and heterozygous carriers, we observed a 3.07-mmHg increase in DBP (95% CI, 2.32–3.82); however, there were only 10 homozygous carriers of this variant and the CI of these carriers is very wide. We also observed 5.58-mmHg higher BP (95% CI, 3.01–8.14) among DGCR8 rs1062923 homozygous recessive carriers in response to a 1-SD change in BC, but only 2.87 (95% CI, 2.10–3.65) in heterozygotes and homozygous wild-type carriers. Because this SNP had a Hardy–Weinberg p-value of 0.05, we approach interpretation of these results with caution.

We observed statistically significant interactions for four SNP-by-BC interactions predicting DBP. In all of these analyses, smaller changes in BP were observed for carriers of the homozygous variant genotype. A 1-SD change in BC was associated with 1.48-mmHg higher DBP (95% CI, 0.78–2.18) for homozygous variant carriers and 2.57 mmHg (95% CI, 2.12–3.02) in others. The rs13078 DICER SNP was associated with 0.13-mmHg higher DBP (95% CI, 0.08–0.18).

Table 4. Effect modification of the association between a 1-SD increase in BC concentrations and BP by gene variants related to miRNA processing under recessive models.6

| Gene | SNP | MAF | p-interaction | p-interaction | p-interaction |
|------|-----|-----|--------------|--------------|--------------|
| DBP  | rs13078 | 0.85 | 0.006 | 0.002 | 0.004 |
| rs197414 | 0.005 | 0.017 | 0.03 | 0.008 |
| AGO1 | rs1062923 | 0.024 | 0.04 | 0.006 |
| rs7813 | 0.045 | 0.04 | 0.027 |

Table 3. SNPs in miRNA processing genes included in analysis.

| Gene | RS number | SNP position | Alleles | Role | Amino acid change | MAF (%) |
|------|-----------|--------------|---------|------|------------------|---------|
| Gem-associated protein 3 | rs197414 | chr1:112110646 | C/A | Coding exon | Arginine/serine | 0.12 |
| (GEMIN3) (ODX20) | rs197388 | chr1:112099005 | T/A | Promoter | 0.18 |
| rs197412 | chr1:112110476 | T/C | Coding exon | Isoleucine/threonine | 0.38 |
| AGO1 (EIF2C1) | rs956961 | chr1:36140367 | A/G | Intron | 0.15 |
| DROSHA (RNASEN) | rs68784 | chr5:3168895 | G/C | Promoter | 0.18 |
| Exportin 5 (XPO5) | rs11077 | chr6:43598925 | C/A | 3' UTR | 0.40 |
| AGO2 (EIF2C2) | rs4691280 | chr8:141715696 | C/A | Promoter | 0.18 |
| rs784567 | chr12:52180732 | C/T | Promoter | 0.49 |
| Tar-RNA binding protein 2 (TARBP) | rs13078 | chr14:94625500 | T/A | 3' UTR | 0.18 |
| DICER (DGCR8) | rs17813 | chr17:5945806 | T/C | Coding exon | Cysteine/arginine | 0.43 |
| rs1062923 | chr17:5959817 | T/C | Coding exon | Isoleucine/threonine | 0.19 |
| rs3744741 | chr17:595892 | T/C | Coding exon | Arginine/glutamine | 0.11 |
| rs4968104 | chr17:596255 | T/A | Coding exon | Valine/glutamic acid | 0.27 |
| rs910925 | chr17:596297 | G/C | Coding exon | Glycine/alanine | 0.42 |
| DiGeorge syndrome critical region gene 8 (DGCR8) | rs357 | chr17:18743931 | G/A | 3' UTR | 0.25 |
| rs1640299 | chr17:18743939 | G/T | 3' UTR | 0.48 |
| Ras-related nuclear protein (RAN) | rs14035 | chr12:129972194 | C/T | 3' UTR | 0.28 |

Abbreviations: HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency; RS, reference SNP.

*Gene name (abbreviation), official gene symbol. **HWE p = 0.08. ***HWE p = 0.05.
BP in carriers of the homozygous variant (95% CI, –1.65 to 1.91) and 2.32 (95% CI, 1.91–2.72) in others. Under a recessive model, homozygous carriers of the variant alleles of rs7813 and rs910925, both in GEMIN4, were both associated with lower BP in response to a 1-SD increase in BC compared with others. Both these SNPs produced very similar magnitudes of effect and were found to be in high LD ($r^2 = 0.9$). As a sensitivity analysis, we also investigated whether there were significant associations in models adjusted only for model 1 variables, and the same SNPs achieved significant results. We also tested our associations using inverse probability weighting (IPW) to address attrition and found that our most significant results for both SBP and DBP became more significant, although changes associated with the IPW analysis were modest. We report the model 1 $p$-values and IPW $p$-values for sensitivity additionally in Table 4.

**Discussion**

We observed that BC concentration averaged over the 7 days preceding each study center visit was positively associated with SBP and DBP in a cohort of elderly men. Furthermore, SNPs in miRNA processing genes modified the BC associations with BP that we observed. Our analysis focused on potentially functional SNPs located in genes involved in the processing of miRNAs. To our knowledge, this is the first study to investigate the role of polymorphisms in miRNA processing genes to be identified as effect modifiers of an association between air pollution and cardiovascular response.

We selected these SNPs because of their involvement in the biogenesis and processing of miRNA. The transcription and processing of miRNAs has been described in detail elsewhere (Bartel 2004). Briefly, miRNAs are processed in a multistep pathway (Figure 1). First, long primary transcripts coded within the introns of protein-coding genes are transcribed into primary miRNAs (pri-miRNAs) and the introns of protein-coding genes are transcribed into primary miRNAs (pri-miRNAs). Then, the pre-miRNA stem-loop (approximately 70–100 nt) is formed. Through a series of steps mediated by DROSHA and DGCR8, the pre-miRNA stem-loop (approximately 70–100 nt) is formed. After export from the nucleus via the RAN-GTP complex and XPO5, these pre-miRNAs undergo modification by DICER, which guides the miRNA into the RNA-induced silencing complex (RISC), where the miRNA strand anneals to the 3′ untranslated regions (UTRs) of target miRNAs, promoting translational repression or mRNA degradation.

**Figure 1.** Major steps in miRNA processing. Long primary transcripts coded within the introns of protein-coding genes are transcribed into primary miRNAs (pri-miRNAs). Then DROSHA and the DGCR8 complex mediate the formation of the pre-miRNA stem-loop. After export from the nucleus via the RAN-GTP complex, these pre-miRNAs undergo modification by DICER within the cytoplasm to generate an ~22-nt duplex from the loop complex, which comprises miRNA and its complement. AGO2, GEMIN3, and GEMIN4 then interact with miRNA to form a ribonucleoprotein, which guides the miRNA into the RISC. In the RISC, where the miRNA strand anneals to the 3′ UTRs of target mRNAs, promoting translational repression or mRNA degradation.

It has been shown that miRNAs, which are generally associated with silencing or suppression of target gene transcripts (negative regulation), may become activators of gene expression during stress (Rocha 2007; Yang and Paschen 2008). Animal studies have also shown that miRNA expression is down-regulated in lungs of rats exposed to cigarette smoke, which is consistent with the hypothesis that alterations of the miRNA control mechanism can occur even after relatively short periods of exposure (Izzotti et al. 2009). Other forms of epigenetic regulation, such as DNA methylation, have been associated with exposure to traffic particles (Baccarelli et al. 2009). Recently, miRNA processing genes, such as Dicer, have been associated with endothelial function and angiogenesis (Suarez et al. 2007). None of the interactions identified were significantly protective of both SBP and DBP. Although BC interactions with SNPs in DGCR8, Dicer, and GEMIN4 were observed in models of SBP, only interactions with single SNPs in GEMIN3 and GEMIN4 were predictive of SBP. The Dicer and DGCR8 SNPs for which we observed interactions predicting DBP are both located within 3′ UTRs. These regions are not translated directly into the protein; however, they are thought to play a role in mRNA stability. Additionally, these polymorphisms may affect miRNA binding to the 3′ UTR of target miRNAs. Previously, Dicer knockdown increased activation of the endothelial nitric oxide synthase pathway (Suarez et al. 2007). Dicer is thought to be more generally involved in miRNA processing, whereas DGCR8 is thought to be more specific to particular miRNAs (Han et al. 2006). DGCR8 is required for the maturation of miRNA primary transcripts at the initial stages. A recent study found that deletion of DGCR8 in cardiomyocytes leads to left ventricular malfunction progressing to a dilated cardiomyopathy and premature lethality (Rao et al. 2009), which suggests a central role of this gene in cardiovascular function. Lack of Dicer activity in knockout studies has been suggested to be associated with defective blood vessel formation (Bernstein et al. 2003; Yang et al. 2005). However, no associations predicting SBP have been observed for the particular SNPs that we observed interacting with BC.

In our analyses, we found that BC interactions with SNPs in GEMIN4 were associated with SBP and DBP. Interactions with one SNP in GEMIN3, rs197414, was also associated with SBP. These genes code for proteins associated with the survival motor neuron
and we have examined associations with SNPs in metal-processing genes and heart rate variability (Park et al. 2006; Ren et al. 2010). Recently, we reported that polymorphisms in oxidative defense genes did not significantly modify the association of BC with BP, although there were trends in that direction (Mordukhovich et al. 2009). Taken together, these studies suggest that these pathways may be important for air pollution effects, although confirmation for other related outcomes and in other cohorts will be required.

In another recent paper, we demonstrated that BC exposure is associated with epigenetic changes—specifically, a reduction in LINE-1 methylation (Baccarelli et al. 2009). miRNAs are other important epigenetic mechanisms of gene expression control. Here we explored a novel pathway, polymorphisms in genes responsible for processing miRNAs, and found evidence for effect modification of BC on BP. Again, it will be important to determine whether these same polymorphisms modify effects on other end points and in other cohorts.

Although we believe our findings suggest altered susceptibility to BC exposures resulting from alterations in miRNA-processing related genotypes, we acknowledge that there are some limitations to our analysis. Despite these observations, we are not able to ascertain whether the polymorphisms described here modify the associations with BC because they are more susceptible to downregulation or because their basal activity makes individuals more susceptible to downstream changes in biological function. It is possible that both mechanisms may be at work. Additionally, it is also possible that observed associations could be attributable to LD between the genotyped SNPs and some other causal variant. For each of the SNP-by-BC interactions examined, we limited our investigation to associations under a recessive model of inheritance to maximize power to detect interactions, and therefore we may have overlooked some true associations that would only be observed under other additive or dominant models of inheritance. On the other hand, we also face the problem of multiple comparisons, as we examined multiple SNPs in the miRNA pathway. We tested interactions with 19 SNPs in this study, and multiple testing is subject to false positives. However, whereas studies of large numbers of genes incorporate corrections for multiple testing, we did not test separate independent hypotheses. Rather, we tested consistency of the pattern of association examined and not just the significance of the most significant association for the 10 genes examined.

We also acknowledge that there may be some misclassification of exposure in our analysis. Our study uses stationary measures of air pollution to represent personal exposures.

Prior research indicates that when looking at longitudinal air pollution, most error is of the Berkson type. To the extent that this error is classical, simulation studies have shown that it is highly unlikely to bias away from the null even in the presence of covariates. This indicates that this exposure misclassification may lead to an underestimation of the health effects of air pollution (Zeger et al. 2000). In addition, several studies, including one conducted in the greater Boston area, have found that longitudinal measures of ambient particulate concentrations are representative of longitudinal variation in personal exposures (Rojas-Bracho et al. 2000). BC concentrations are spatially heterogeneous because of the numerous local (mobile) sources. Therefore, measurement error in our BC exposure metric would likely attenuate the true association. Given that we found significant positive associations for BC, it is unlikely that this error would affect our conclusions. Our study population is homogeneous, consisting entirely of elderly men, and we have restricted our analysis to white individuals. The median distance of the participant homes from the central site monitoring station was 17.6 km. However, our findings are consistent with studies of particulate air pollution conducted in more heterogeneous populations.

Conclusions

We have used a pathway approach to investigate how the processing of miRNAs may be a susceptibility factor for traffic particle–induced cardiovascular effects. Individual miRNAs may have multiple targets; more research is needed to address the relation between BC exposure and BP among diverse study populations and to clarify the mechanisms underlying the association between BC and BP. These results provide support for the hypothesis of the toxic effects of traffic pollution as measured by BC and contribute to growing understanding about the role of epigenetic modification and response to environmental stresses such as exposure to BC. In cardiovascular research, these results may be used to explore interactions with BC. Further investigations are needed to replicate our findings in other large cohort studies.

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