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Citation
Sanchez-Guerra, M., Y. Zheng, C. Osorio-Yanez, J. Zhong, Y. Chervona, S. Wang, D. Chang, et al. 2015. “Effects of particulate matter exposure on blood 5-hydroxymethylation: results from the Beijing truck driver air pollution study.” Epigenetics 10 (7): 633-642. doi:10.1080/15592294.2015.1050174. http://dx.doi.org/10.1080/15592294.2015.1050174.

Published Version
doi:10.1080/15592294.2015.1050174

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Effects of particulate matter exposure on blood 5-hydroxymethylation: results from the Beijing truck driver air pollution study

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Keywords: DNA methylation, Epigenetics, Particulate Matter, 5-hydroxymethylcytosine, 5-methylcytosine

Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; Al, aluminum; BC, black carbon; BMI, body mass index; Ca, calcium; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; Fe, iron; FWER, family-wise error rate; HPLC, high-performance liquid chromatography; K, potassium; PM, particulate matter; PM2.5, particulate matter ≤ 2.5 μm; PM10, particulate matter ≤ 10 μm; S, sulfur; Si: silicon; TET, ten-eleven translocation enzymes; Ti, titanium and Zn: zinc.

Previous studies have reported epigenetic changes induced by environmental exposures. However, previous investigations did not distinguish 5-methylcytosine (5mC) from a similar oxidative form with opposite functions, 5-hydroxymethylcytosine (5hmC). Here, we measured blood DNA global 5mC and 5hmC by ELISA and used adjusted mixed-effects regression models to evaluate the effects of ambient PM10 and personal PM2.5 and its elemental components—black carbon (BC), aluminum (Al), calcium (Ca), potassium (K), iron (Fe), sulfur (S), silicon (Si), titanium (Ti), and zinc (Zn)—on blood global 5mC and 5hmC levels. The study was conducted in 60 truck drivers and 60 office workers in Beijing, China from The Beijing Truck Driver Air Pollution Study at 2 exams separated by one to 2 weeks. Blood 5hmC level (0.08%) was ~83-fold lower than 5mC (6.61%). An inter-quartile range (IQR) increase in same-day PM10 was associated with increases in 5hmC of 26.1% in office workers (P = 0.004), 20.2% in truck drivers (P = 0.014), and 21.9% in all participants combined (P < 0.001). PM10 effects on 5hmC were increasingly stronger when averaged over 4, 7, and 14 d preceding assessment (up to 132.6% for the 14-d average in all participants, FI < 0.001). PM10 effects were also significant after controlling for multiple testing (family-wise error rate; FWER < 0.05). 5hmC was not correlated with personal measures of PM2.5 and elemental components (FWER > 0.05). 5mC showed no correlations with PM10, PM2.5, and elemental components measures (FWER > 0.05). Our study suggests that exposure to ambient PM10 affects 5hmC over time, but not 5mC. This finding demonstrates the need to differentiate 5hmC and 5mC in environmental studies of DNA methylation.

Introduction

Urban air pollution is a major public health concern that, according to the World Health Organization, is responsible for ~3.7 million deaths annually, representing 6.7% of all deaths worldwide.¹ Governments and regulatory agencies have made concerted efforts to abate air pollution emissions, specifically by regulating particulate matter (PM) levels.²-⁴ However, further abatements are constrained by high marginal costs.⁵,⁶ Moreover, limited resources are available to identify individuals who are affected by air pollution and ascertain their risks. Biomarkers that reflect adverse exposures could be vital for identifying people

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Submitted: 02/25/2015; Revised: 04/24/2015; Accepted: 05/06/2015
http://dx.doi.org/10.1080/15592294.2015.1050174

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who are affected by PM exposure before it becomes clinically evident.

Epigenetic features like DNA methylation are increasingly being implicated as sensitive to environmental exposures, and therefore could serve as important biomarkers for exposure effects.7-12 DNA methylation modifications, both in specific genes and in non-coding repetitive elements, have been shown to be altered following PM exposure.13-18 Traditional DNA methylation involves the transfer of a methyl group to the carbon 5 position of cytosine to produce 5-methylcytosine (5mC). However, research has shown that the Tet family of cytosine oxygenase enzymes is involved in oxidizing 5-methylcytosine into 5-hydroxymethylcytosine (5hmC). In contrast to 5mC, which is often associated with suppressed gene expression, 5hmC has been found to be specifically enriched in expressed genes, and may play a role in activating and/or maintaining gene expression.19-21 Therefore, distinguishing these 2 forms of methylation is critical for understanding the potential effects of methylation changes.

Because the standard technologies for DNA methylation analysis, including those based on bisulfite treatment of DNA, fail to separate 5hmC from 5mC,22-24 (providing instead a composite measure of the sum of 5mC and 5hmC), most epigenetic studies have not differentiated 5hmC from 5mC. This differentiation, however, is particularly critical for air pollution studies, because PM exposures can introduce oxidative stress. Oxidative stress can alter the activity of the Ten-11 translocation (TET) protein family members, which catalyze enzymatic reactions that generate 5hmC from 5mC.25 Further, Chia et al. suggested that pro-oxidant environmental exposures can increase the global genomic content of 5hmC.25 However—while previous studies have reported that PM exposure is negatively associated with 5hmC or total methylation levels—no study to date has evaluated whether PM exposure changes 5hmC levels in human DNA.

In the present study, we determined the genomic content of both 5hmC and 5mC in a study of 2 highly exposed groups with different occupations, i.e., office workers and truck drivers, in Beijing, China. Beijing has one of the highest air pollution levels worldwide.28 We used measures of ambient PM less than 10 μm in diameter (PM10) from ambient monitors across Beijing City, as well as personal measures of both PM2.5 and elemental components using personal samplers. We hypothesized that PM exposure—by facilitating the oxidation of 5mC into 5hmC—would result in increased blood levels of 5hmC. The findings underscore the necessity for distinguishing these 2 types of DNA methylation to achieve a better understanding of the effects of environmental exposures and the roles of epigenetic mechanisms in non-communicable diseases development due to pro-oxidant exposures.

## Results

### Characteristics of the study participants

The participants in the Beijing Truck Driver Air Pollution Study have been described elsewhere.13,30-32 Truck drivers were moderately but significantly older than office workers. Truck drivers had higher BMI, reported more pack-years of smoking, and smoked more cigarettes during the examination time.

### Ambient PM10 mass and personal PM2.5 mass and elemental components

Office workers and truck drivers were examined on days with average ambient PM10 mass of 118.1 ± 52.8 and 124.9 ± 51.2 μg/m³ for the 2 groups, respectively (Table 1, P=0.33 for

### Table 1. Levels of ambient PM10 on and before the examination days, and personal PM2.5 and elemental components of PM during work hours

| Time window | Office workers | Truck drivers |
|-------------|----------------|---------------|
| 24-h mean  | PM10 (μg/m³) | PM10 (μg/m³) |
| 118.1 ± 52.8 | 118.0 ± 52.0 | 24.1 ± 8.3 |
| 119.0 ± 32.0 | 131.6 ± 18.4 | 70.9 ± 22.4 |
| 117.9 ± 24.1 | 185.9 ± 32.0 | 124.9 ± 32.0 |
| 119.0 ± 19.3 | 114.0 ± 29.8 | 12.4 ± 4.2 |
| 93.4 ± 22.4 | 115.1 ± 34.5 | 8.9 ± 2.3 |
| 243.3 ± 43.3 | 114.5 ± 34.5 | 1.0 ± 0.2 |
| 84.5 ± 43.3 | 136.5 ± 34.5 | 0.9 ± 0.2 |
| 131.6 ± 84.5 | 165.0 ± 34.5 | 0.8 ± 0.2 |
| 185.9 ± 131.6 | 186.0 ± 165.0 | 0.7 ± 0.2 |
| PM2.5 | PM2.5 |
| 94.3 ± 68.2 | 128.6 ± 70.9 |
| 7.1 ± 4.2 | 17.1 ± 6.9 |
| 0.53 ± 0.25 | 1.35 ± 0.94 |
| 0.32 ± 0.17 | 2.07 ± 2.23 |
| 0.38 ± 0.19 | 1.00 ± 0.63 |
| 0.72 ± 0.74 | 1.33 ± 1.05 |
| 6.28 ± 5.25 | 8.67 ± 4.97 |
| 0.79 ± 0.55 | 2.37 ± 1.78 |
| 0.00 ± 0.00 | 0.06 ± 0.04 |
| 0.15 ± 0.17 | 0.27 ± 0.22 |

1Number of observations used for analysis was 103 for office workers and 109 for truck drivers (missing values were from missing %5hmC or %5mC measures, since we did not have samples from some individuals).

2P-values were obtained from Wilcoxon-Mann-Whitney tests.
difference in PM$_{10}$ mass between the 2 groups). Table 1 also reports the multi-day averages of ambient PM$_{10}$ mass on and before the examination days (including means of 4, 7, and 14 d).

Personal levels of PM$_{2.5}$ mass, and elemental components (BC, Al, Ca, Fe, K, S, Si, Ti, and Zn) measured during 8 work h on examination days were significantly higher in truck drivers ($P<0.001$ for all exposures). However, all personal exposure metrics showed largely overlapping distributions between the 2 groups.

Ambient PM$_{10}$ mass and personal exposure measures (PM$_{2.5}$ mass, BC, and elemental components) showed variable degrees of correlation (Fig. 1). In particular, the 4 time-window averages of ambient PM$_{10}$ mass showed strong-to-moderate correlations with each other. As expected, correlations were higher for the closer averages (i.e., 24-h and 4-d average, or 4-d and 7-d average). Personal exposures, which all were measured on particles sampled during 8 work h on the days of examination, mostly showed medium to high correlations, but had moderate or little correlation with ambient PM$_{10}$ mass.

Descriptive statistics of 5hmC and 5mC levels

The characteristics of the 60 office workers and 60 truck drivers by blood levels of 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) are presented in Table 2.

The geometric mean of 5hmC (0.08%; 95% CI: 0.08 – 0.09) was ~83-fold lower than the geometric mean of 5mC (6.61%; 95% CI: 6.24 – 7.01). The 5hmC values ranged between 0.02% (min) and 0.29% (max); 5mC ranged between 3.29% (min) and 10.19% (max). The levels of 5hmC and 5mC in blood DNA were significantly correlated with each other (Pearson’s correlation=0.35, $P=0.001$ on the first examination day; Pearson’s correlation=0.22, $P=0.021$ on the 2nd examination day) (Fig. 2).

The geometric means of 5hmC levels (Table 2) did not differ between office workers (0.09%; 95% CI: 0.08 – 0.10) and truck drivers (0.08%; 95% CI: 0.07 – 0.09, $P=0.28$). Males had significantly higher 5hmC than females (0.10%; 95% CI: 0.09 – 0.11, and 0.06%; 95% CI: 0.06; 0.07 respectively, $P<0.001$). Further, 5hmC decreased moderately and non-significantly with participant age ($P=0.94$). Former smokers and current smokers had marginally higher 5hmC than never smokers ($P=0.082$). 5hmC tended to fluctuate across the days of the week ($P=0.035$) and increased with higher dew point ($P=0.048$), whereas BMI ($P=0.58$) and temperature ($P=0.40$) were not related with 5hmC.

The geometric mean of 5mC levels (Table 2) also did not differ between office workers (6.47%; 95% CI: 6.19 – 6.77) and truck drivers (6.75%; 95% CI: 6.46 – 7.06, $P=0.19$). As for 5hmC, 5mC levels were higher in males (6.93%; 95% CI: 6.67 – 7.21) than females (6.10%, 95% CI: 5.80 – 6.41, $P<0.001$). 5mC also decreased slightly with age ($P=0.069$), but was not associated with BMI, smoking, day of week, temperature, or dew point ($P > 0.1$). Based on our results, 5hmC measurements exhibited less day-to-day variability (about 28 times lower median intra-individual variability), compared to 5mC (Supplemental Figure S1). These findings suggested that global 5hmC in our study population was likely to be a relatively stable DNA modification compared to 5mC, which is in line with previous studies.33

Effects of ambient and personal exposures on blood genomic levels of 5hmC and 5mC

We used mixed-effects regression models adjusted for age, gender, BMI, smoking status, day of week, temperature, and dew point to evaluate the associations of 5hmC with ambient and personal exposure levels (Table 3). An IQR increase in ambient PM$_{10}$ levels on the examination days was associated with an increase in blood 5hmC equal to 26.1% in office workers (95% CI: 7.9 – 47.4, $P=0.004$), 20.2% in truck drivers (95% CI: 4.0 – 39.0, $P=0.014$), and 21.9% in all participants combined (95% CI: 10.3 – 34.7, $P < 0.001$). The correlations between 5hmC and ambient PM$_{10}$ mass increased in strength with increasing number of days included in multi-day averages. Among office workers, an IQR increase ambient PM$_{10}$ mass was associated with increased 5hmC of 27.7% (95% CI: 9.0 – 49.5, $P=0.003$) with the 4-d average, 43.2% (95% CI: 15.6 – 77.4, $P=0.001$) with the 7-d average, and 152.1% (95% CI: 59.8 - 297.8, $P < 0.001$) with the 14-d average. In truck drivers, an IQR increase of ambient PM$_{10}$ mass was associated with increased 5hmC of 34.1% (95% CI: 13.3 – 58.6, $P=0.001$) with the 4-d average, 39.9% (95% CI: 11.6 – 75.5, $P=0.005$) with the 7-d average, and 122.6% (95% CI: 49.9 – 230.6, $P < 0.001$) with the 14-d average. In all participants combined, an IQR increase in ambient PM$_{10}$ mass was associated with increased 5hmC of 28.1% (95% CI: 15.3 – 42.2, $P < 0.001$) with the 4-d average, 37.3% (95%...
CI: 18.8 – 58.6, \( P < 0.001 \) with the 7-d average, and 132.6% (95% CI: 75.6 – 208.0, \( P < 0.001 \)) with the 14-d average. To account for the multiple dependent statistical tests conducted in our analysis, we calculated permutation-based \( P \)-values adjusted for multiple comparisons using FWER. At FWER < 0.05, all the different time windows of ambient PM10 mass remained highly significant for office workers, truck drivers, and all participants combined. The increasingly larger cumulative effects of PM10 on 5hmC with more days before the examination days suggest that the effect of PM10 on 5hmC tend to build up in time. Although an IQR increase BC was significantly associated with increased 5hmC of 27.0% (95% CI: 6.5 – 51.4, \( P=0.009 \)) in office workers and 14.6% (95% CI: 3.5 – 26.9, \( P=0.010 \)) in truck drivers, at the same FWER threshold, none of the personal measures of exposures (PM2.5 mass, BC, and elemental components during 8 work h on examination days) was associated with 5hmC.

We conducted the same set of statistical analyses in relation to 5mC (Table 4). 5mC did not show significant associations with ambient PM10 mass, personal PM2.5 mass, BC, or elemental components at FWER < 0.05.

### Discussion

In this study of truck drivers and office workers in Beijing, China, we found that exposure to ambient PM10 mass was associated with increased levels of 5hmC, while no association was observed with 5mC. The effects of ambient PM10 mass levels on 5hmC were progressively higher in relation to longer time windows of exposure, possibly due to accumulation of effects over time. Consistent with this time-dependent dynamics, short-term (8-h) personal exposures to PM2.5, BC, Al, C, K, Fe, S, Si, Ti,
and Zn were not associated with either blood global 5hmC or 5mC.

As expected based on previous analysis of human blood,24 we found that the levels of 5hmC were much lower than those of 5mC. We found higher global 5mC in males than females, consistent with previous studies conducted in Chinese populations and in a Belgian population.26,35,36 findings were similar for global 5hmC. However, the ratio of 5mC/5hmC was higher in our data compared to previous studies. In our study, 5hmC was ~83-fold lower than 5mC. In 48 participants in the US Strong Heart Study, 5hmC levels were just ~2.5-fold lower than 5mC, with median values of 0.12% for 5hmC and 0.32% for 5mC in DNA isolated from frozen buffy coat, and 0.15 for 5hmC and 0.32 for 5mC in frozen whole blood.34 In a study of 48 healthy men from Spain, 5hmC levels were 10-fold lower than 5mC in DNA from whole blood (0.09% for 5hmC vs. 0.90% for 5mC). We note that 5hmC levels in our Beijing population (0.08%) were remarkably similar to those reported in these previous studies in Spain (0.09%) and the United States (0.12%). Although, the 5mC (6.61%) levels differed between 7-fold and 21-fold across the 3 studies; the levels found in this study are more similar to those reported by De Prins et al. (4.3%).26

### Table 3. Percent changes on 5-hydroxymethylcytosine associated with an interquartile range (IQR) increase in exposure levels*

| Study group | % | 95% CI | P-value | Adj. | % | 95% CI | P-value | Adj. | % | 95% CI | P-value | Adj. |
|-------------|---|--------|---------|------|---|--------|---------|------|---|--------|---------|------|
| **Office Workers** | | | | | | | | | | | | |
| PM10 (μg/m³) from ambient monitors on the examination days (24-h average) and multi-day averages on and before the examination days | | | | | | | | | | | | |
| 24-h average | 26.1 | (7.9; 47.4) | 0.004 | 0.025 | 20.2 | (4.0; 39.0) | 0.014 | 0.042 | 21.9 | (10.3; 34.7) | <0.001 | <0.001 |
| 4-d average | 27.7 | (9.0; 49.5) | 0.003 | 0.019 | 34.1 | (13.3; 58.6) | 0.001 | 0.002 | 28.1 | (15.3; 42.2) | <0.001 | <0.001 |
| 7-d average | 43.2 | (15.6; 77.4) | 0.001 | 0.011 | 39.9 | (11.6; 75.5) | 0.005 | 0.014 | 37.3 | (18.8; 58.6) | <0.001 | <0.001 |
| 14-d average | 152.1 | (59.8; 297.8) | <0.001 | 0.001 | 122.6 | (49.9; 230.6) | <0.001 | 0.001 | 132.6 | (75.6; 208.0) | <0.001 | <0.001 |
| **Truck Drivers** | | | | | | | | | | | | |
| PM2.5 | 9.0 | (−3.7; 25.6) | 0.16 | 0.79 | 10.0 | (−6.8; 29.8) | 0.26 | 0.92 | 7.9 | (−2.1; 18.9) | 0.13 | 0.60 |
| BC | 27.0 | (6.5; 51.4) | 0.009 | 0.18 | 3.1 | (−9.5; 17.4) | 0.65 | 0.99 | 7.9 | (−2.1; 18.9) | 0.13 | 0.60 |
| Al | 1.1 | (−29.5; 44.9) | 0.95 | 0.99 | 8.3 | (−3.8; 22.0) | 0.19 | 0.83 | 5.7 | (−4.0; 16.4) | 0.26 | 0.85 |
| Ca | −1.9 | (−57.1; 124.3) | 0.96 | 0.99 | 3.2 | (−3.7; 10.6) | 0.38 | 0.98 | 3.2 | (−3.0; 9.8) | 0.32 | 0.91 |
| Fe | 3.0 | (−24.9; 41.3) | 0.85 | 0.99 | 4.7 | (−6.3; 16.9) | 0.42 | 0.99 | 2.9 | (−5.9; 12.6) | 0.53 | 0.99 |
| K | 10.0 | (−3.9; 25.9) | 0.17 | 0.80 | 3.3 | (−8.1; 16.0) | 0.59 | 0.99 | 4.3 | (−3.6; 12.7) | 0.30 | 0.89 |
| S | 14.3 | (−1.1; 32.2) | 0.074 | 0.56 | 9.5 | (−7.8; 30.1) | 0.31 | 0.95 | 9.1 | (−1.2; 20.5) | 0.087 | 0.49 |
| Si | −18.4 | (−38.9; 9.1) | 0.17 | 0.99 | 9.5 | (−2.3; 22.7) | 0.12 | 0.69 | 4.2 | (−4.9; 14.1) | 0.38 | 0.96 |
| Ti | −12.6 | (−32.9; 13.7) | 0.32 | 0.99 | 14.6 | (3.5; 26.9) | 0.010 | 0.16 | 8.2 | (−0.7; 18.0) | 0.075 | 0.45 |
| Zn | −0.4 | (−14.4; 15.8) | 0.96 | 0.99 | 0.3 | (−12.3; 14.7) | 0.96 | 0.99 | −1.4 | (−9.5; 7.5) | 0.75 | 0.99 |

*Adjusted for age, sex, BMI, smoking status, day of week, and average of temperature and dew point values corresponding to the average of exposure metrics.

1Number of observations used for analysis was 103 for office workers, 109 for truck drivers, and 212 for all participants.

2P-values were calculated using mixed-effects regression models.

3Adjusted P-values were estimated by a resampling-based approach, which takes dependent structures among exposures into consideration. This approach controls family-wise error rate (FWER), i.e., probability of having at least one false positive among the whole set of comparisons at a given significance level.
### Table 4. Percent changes on 5-methylcytosine associated with an interquartile range (IQR) increase in air particle levels

| Study group | Office Workers | Truck Drivers | All participants |
|-------------|----------------|---------------|-----------------|
| % | 95% CI | P-value | Adj. | % | 95% CI | P-value | Adj. | % | 95% CI | P-value | Adj. |
| PM$_{10}$ (μg/m$^3$) from ambient monitors on the examination days (24-h average) and multi-day averages on and before the examination days | | | | | | | | | | | |
| 24-h average | −1.2 (−9.0; 7.2) | 0.77 | 0.99 | 1.8 (−4.5; 8.6) | 0.58 | 0.91 | −0.4 (−5.2; 4.6) | 0.87 | 0.99 |
| 4-d average | 3.3 (−4.3; 11.5) | 0.41 | 0.71 | −2.7 (−9.9; 5.1) | 0.49 | 0.99 | −0.1 (−5.0; 4.9) | 0.98 | 0.99 |
| 7-d average | 6.0 (−4.2; 17.3) | 0.26 | 0.52 | −3.0 (−12.5; 7.5) | 0.57 | 0.99 | 0.3 (−6.3; 7.3) | 0.94 | 0.99 |
| 14-d average | 2.5 (−17.9; 28.0) | 0.83 | 0.99 | 1.9 (−16.4; 24.3) | 0.85 | 0.99 | −1.8 (−14.7; 13.1) | 0.80 | 0.99 |

- 95% CI: 95% confidence interval
- P-value: Probability value
- Adj.: Adjusted for age, sex, BMI, smoking status, day of week, and average of temperature and dew point values corresponding to the average of exposure metrics.

*Adjusted for age, sex, BMI, smoking status, day of week, and average of temperature and dew point values corresponding to the average of exposure metrics.

†Number of observations used for analysis was 102 for office workers, 106 for truck drivers, and 208 for all participants.

‡P-values were calculated using mixed-effects regression models.

§Adjusted P-values were estimated by a resampling-based approach, which takes dependent structures among exposures into consideration. This approach controls family-wise error rate (FWER), i.e., probability of having at least one false positive among the whole set of comparisons at a given significance level.

5hmC content in HEK293 cells. Chia et al. have proposed that ROS affects 5hmC patterns via metabolic alterations influencing the tricarboxylic acid cycle and thereby activating TET and other chromatic modifying proteins. We note that our study was based on global measures of genomic content of 5hmC and 5mC. It is also expected. While the 2 marks often co-exist, 5hmC is significantly enriched at gene transcription start sites coinciding with TET1 on genes with high CpG-content. Additionally, 5hmC exhibits lower levels in repetitive elements and minor satellites compared with 5mC. Therefore, 5hmC and 5mC have distinct genomic distributions; the different localizations of 5hmC and 5mC reflect their distinct biological functions and might help to explain the differential effects of PM observed in our study.
have comparatively weaker effects, explaining the lack of associations in our study. Taken together, since personal PM$_{2.5}$ measurement were only collected for 8-hr average, we are not able to draw a definite conclusion on the impact of short-term PM$_{2.5}$ exposure on 5hmC. However, it is possible that PM$_{10}$ and PM$_{2.5}$ may have distinct biologic effects on 5hmC due to their differences in composition and size. Future toxicological studies are required to ascertain the mechanistic underpinning of the different effects of PM$_{10}$ and PM$_{2.5}$ on 5hmC.

Our results suggested potential effect modification by occupation (truck driver/office workers), which might imply that office workers were more sensitive/vulnerable to the effect of BC on 5hmC, compared to truck drivers due to unknown physiological/sociological factors. However, due to limited sample size we did not have enough statistical power to detect effect modification.

We recognize that our study is subject to a number of limitations. We cannot exclude false negative findings or chance findings due to the relatively small sample size; however, all our findings were significant using a stringent cutoff of 0.05 for FWER, limiting the chance of false-positive results. While our study population may not be representative of the general population, consistent internal validity and adjustment for various lifestyle factors and demographic characteristics allow for moderate generalizability. Our main findings are based on stationary measures of ambient PM$_{10}$, which are used as a proxy of personal exposure. Based on the study operations, it is reasonable to assume that the measurement error occurred independent of the outcome (global 5mC and 5hmC in blood DNA). Therefore, the misclassification was likely non-differential and can only bias the results toward to null. Previous studies have also shown that the error introduced by using data from stationary monitors usually leads to underestimating of the effects of air pollution. Moreover, we used multivariable models to control potential confounders, while residual confounding due to unmeasured variables is not unlikely, chances that the observed association reflected bias resulting from residual confounding are minimized. Finally, due to the lack of blood cell counts data, we cannot exclude actual epigenetic variation from exposure-related differences in cellular composition. It is possible that the change in methylation status was partially due to changes in cell composition following PM$_{10}$ exposures.

Conclusion

In summary, our study suggests that exposure to ambient PM$_{10}$ affects blood genomic content of 5hmC over time, but not of 5mC. If confirmed, this finding would indicate the need to differentiate 5hmC and 5mC in environmental studies of DNA methylation. Further studies are warranted to evaluate the patterns of 5hmC in relation to PM across the human epigenome and to identify relevant biological pathways for human health effects.

Methods

Study population and design

The Beijing Truck Driver Air Pollution Study, conducted between June 15 and July 27, 2008, included 60 truck drivers and 60 indoor office workers. All study participants worked and lived in the Beijing metropolitan area and had held their current jobs for at least 2 years. Truck drivers and indoor office workers were similarly distributed by age, sex, smoking, and education level. In-person interviews using a detailed questionnaire were conducted to collect information on demographics, lifestyle, and other exposures. Information on time-varying factors, including alcohol consumption and smoking status, was obtained for past usual exposure as well as for each examination day. Because PM levels vary on a day-to-day basis, we examined all participants on 2 workdays separated by a 1-2 week period. Individual written informed consent was obtained from all participants before enrollment in the study. Institutional Review Board approval was obtained at all participating institutions before study participant recruitment.

Sample collection and DNA global methylation analysis (5hmC and 5mC)

All blood draw procedures were conducted at approximately the same time of the day between 4-6 pm (after participants completed a full work-day) to eliminate confounding due to diurnal variation. Their whole blood was collected in EDTA tubes and processed within 2 h. The samples were centrifuged at 2,500 rpm to separate the buffy coat and stored at −80°C until analysis. Wizard Genomic DNA Purification Kit (Promega, Madison, WI) was used to extract total DNA from 200 µL buffy coat. One hundred nanograms of genomic DNA in each reaction were used to measure global %5hmC and %5mC by specific ELISA. 5hmC was measured using the Quest 5hmCTM DNA ELISA Kit. 5mC was measured using the 5mC DNA ELISA Kit (both kits from Zymo Research, Orange, CA, USA). Analyses were conducted according to the manufacturer’s protocols, and all experiments were run in triplicate. Blood DNA samples were randomized across plates to limit potential bias from plate effects, and laboratory personnel were blinded to exposure groups and exposure study. Both kits are highly sensitive and specific to the 5mC modification of concern, with no cross-reactivity for the other 5mC modification. The within- and between-assay coefficients of variation were 3.9% and 10.8%, respectively, for 5hmC; and 3.01% and 14.5%, respectively, for 5mC. The detection limit for per 100 ng input DNA was 0.02% for 5hmC and 0.5% for 5mC.

Ambient PM$_{10}$ mass data

Ambient PM$_{10}$ data during the study period were obtained from the Beijing Municipal Environmental Protection Bureau (http://www.bjepb.gov.cn/). To determine individual-level PM$_{10}$ exposures, we used the aggregate measures (the daily averages of PM$_{10}$ levels across all the 27 monitoring stations) as surrogate measures of individual-level exposure in Beijing on that specific day. We then constructed the exposure matrix for each individual.

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based on the time window of interest. In detail, we calculated the individual-level exposure for 1-day mean (PM$_{10}$ on examination day), 4-d mean (average of PM$_{10}$ mass on the examination day and on the 3 d before examination), 7-d mean (average of PM$_{10}$ on the examination day and on the 6 days before examination), and 14-d mean (average of PM$_{10}$ on the examination day and on the 13 d before examination). We obtained daily outdoor temperature data for Beijing city from the National Oceanic and Atmospheric Administration online database.

**Personal PM$_{2.5}$ mass and elemental component measurements**

We measured average personal PM$_{2.5}$ on both examination days using gravimetric samplers worn by the study participants during 8 h of work. The air sampler was carried in a belt pack with the inlet clipped near the breathing zone. Each air sampler setup included an Apex pump (Casella Inc., Bedford, UK), a Triplex Sharp-Cut Cyclone (BGI Inc., Waltham, Massachusetts, USA), and a 37-mm Teflon filter placed on top of a drain disc and inside a metal filter holder. The filters were kept under atmosphere-controlled conditions before and after sampling and were weighed with a microbalance (Mettler-Toledo Inc., Columbus, Ohio, USA). A time-weighted average of PM$_{2.5}$ concentrations was calculated by dividing the change in filter weight before and after sampling by the volume of air sampled. We found high reproducibility of PM$_{2.5}$ measures ($r=0.944$) in replicate samples on a subset of 24 participants who wore 2 monitors at the same time (data not shown). The blackness of the same filters was used to measure PM$_{2.5}$ and was assessed using an EEL Model M43D blackness reflectometer, applying the standard black-smoke distance. We assumed a factor of 1.0 for converting the absorption coefficient to black carbon (BC) mass, which was then divided by the sampled air volume to calculate average BC exposure concentration. BC is a combustion by-product contained in PM that has been used as a surrogate measure for PM from smoke stain reflectometer, applying the standard black-smoke distance. We assumed a factor of 1.0 for converting the absorption coefficient to black carbon (BC) mass, which was then divided by the sampled air volume to calculate average BC exposure concentration. BC is a combustion by-product contained in PM that has been used as a surrogate measure for PM from gasoline- and, especially, diesel-powered motor vehicles, and others like coal combustion and biomass burning.

Elemental components of PM were measured from the PM collected on the filters using an XRF PANalytical Epsilon 5 analyzer (Almelo, Netherlands), as described previously. We selected the 8 elements, i.e., potassium (K), sulfur (S), iron (Fe), silicon (Si), aluminum (Al), zinc (Zn), calcium (Ca), and titanium (Ti), that showed the highest reproducibility ($r=0.75$) in replicate samples from the subset of 24 participants who wore 2 monitors at the same time (data not shown).

**Statistical analysis**

In the present study, we examined each participant on 2 different days. Therefore, measures may lack independence within participants. To account for this data feature, we used mixed-effects regression models with random intercept in all analyses (PROC MIXED in SAS 9.3, SAS Institute Inc., Cary, NC).

To evaluate the association of short-term variations on air particle with 5hmC and 5mC, the following mixed-effects model was used:

$$ Y_{ij} = \beta_0 + \beta_1 \text{(Airparticle)}_{ij} + \beta_2 X_{2ij} + \ldots + \beta_n X_{nij} + \xi_j + e_{ij} $$

where $Y_{ij}$ is the measures of 5hmC or 5mC for the $j$th participant measured on the $i$th examination day; $\beta_0$ is the overall intercept; $\beta_i$ is the regression coefficient for exposure variable, i.e., air particle level; $\beta_2 \ldots \beta_n$ are the regression coefficients for the time-dependent covariates (i.e., temperature and dew point) or time-independent covariates (i.e., occupation group, gender, age, BMI, and smoking status) included in multivariate models; $\xi_j$ is the random effect for the participant, and $e_{ij}$ is the residual error term. A two-sided p level of less than 0.05 was considered significant. To account for multiple hypothesis testing where the underlying tests are dependent, we conducted a single-step permutation max $T$ procedure within the 4 ambient PM$_{10}$ measures, and within the other personal monitor measures (PM$_{2.5}$, BC, and elemental component), respectively. The conventional used Benjamini-Hochberg False Discovery Rate correction assumes that all hypotheses should be independent, which was not the case in our study. In fact, the 4 PM$_{10}$ time windows are inherently inter-related, as the shorter moving averages are embedded in the longer moving averages. The personal monitor measures (PM$_{2.5}$, BC, and constituents) are not independent as BC and constituents are all analyzed on the same PM$_{2.5}$ sample collected from the same device and same filter. Adjusted p-values were calculated from 1,000 permutations in a way that matches the null hypothesis. This method strongly controls FWER (family-wise error rate), which is the probability of having at least one false positive among the whole set of comparisons. All analyses were performed in SAS 9.3 (SAS Institute Inc., Cary, NC).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Funding**

This work has been supported by the NIEHS (R21ES020984, R21ES020010, R01ES021733). MSG has been financially supported by the Fundación México en Harvard, A.C and Consejo Nacional de Ciencia y Tecnología (CONACYT-Mexico). Additional support was received from center grants from the NIEHS (ES00002) and the US EPA (R-82735301).

**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

**Authors’ Contributions**

SW, CD, PAB, JS, WZ, LH, and AAB designed the study and supervised the study operations. CD, JPM, AD, and XZ prepared the study protocols and oversaw their implementation.
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