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RESEARCH ARTICLE

Differential DNA methylation in the brain as potential mediator of the association between traffic-related $PM_{2.5}$ and neuropathology markers of Alzheimer's disease

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Abstract

INTRODUCTION: Growing evidence indicates that fine particulate matter ($PM_{2.5}$) is a risk factor for Alzheimer's disease (AD), but the underlying mechanisms have been insufficiently investigated. We hypothesized differential DNA methylation (DNAm) in brain tissue as a potential mediator of this association.

METHODS: We assessed genome-wide DNAm (Illumina EPIC BeadChips) in prefrontal cortex tissue and three AD-related neuropathological markers (Braak stage, CERAD, ABC score) for 159 donors, and estimated donors' residential traffic-related $PM_{2.5}$ exposure 1, 3, and 5 years prior to death. We used a combination of the Meet-in-the-Middle approach, high-dimensional mediation analysis, and causal mediation analysis to identify potential mediating CpGs.

RESULTS: $PM_{2.5}$ was significantly associated with differential DNAm at cg25433380 and cg10495669. Twenty-four CpG sites were identified as mediators of the association between $PM_{2.5}$ exposure and neuropathology markers, several located in genes related to neuroinflammation.

DISCUSSION: Our findings suggest differential DNAm related to neuroinflammation mediates the association between traffic-related $PM_{2.5}$ and AD.

KEYWORDS
Alzheimer's disease, DNA methylation, neuropathology, traffic-related fine particulate matter

Highlights

- First study to evaluate the potential mediation effect of DNA methylation for the association between PM2.5 exposure and neuropathological changes of Alzheimer's disease.
- Study was based on brain tissues rarely investigated in previous air pollution research.
- Cg10495669, assigned to RBCK1 gene playing a role in inflammation, was associated consistently with 1-year, 3-year, and 5-year traffic-related PM2.5 exposures prior to death.
- Meet-in-the-middle approach and high-dimensional mediation analysis were used simultaneously to increase the potential of identifying the differentially methylated CpGs.
- Differential DNAm related to neuroinflammation was found to mediate the association between traffic-related PM2.5 and Alzheimer's disease.

1 | BACKGROUND

Exposure to traffic-related air pollution (TRAP) is a significant contributor to public health burden with various well-characterized and emerging detrimental health effects.[1] Fine particulate matter ($PM_{2.5}$), which has been regulated by the National Ambient Air Quality Standards (NAAQS) as a criteria air pollutant since 1997 in the United States,[2] is an important component of TRAP mainly resulting from tailpipe exhaust, brake wear, tire wear, and resuspended dust.[3] A previous study has demonstrated $PM_{2.5}$ from traffic emissions has higher toxicity compared to other natural sources in terms of oxidative potential, cell viability, genotoxicity, oxidative stress, and inflammatory response.[4] The literature to date demonstrates that exposure to $PM_{2.5}$ is associated with a series of neurological disorders, including dementia and Alzheimer's disease (AD).[5,6]

AD is the most common cause of dementia and its hallmark pathologies include the accumulation of beta-amyloid (Aβ plaques) outside neurons and aggregation of hyperphosphorylated tau protein (neurofibrillary tangle, NFT) inside neurons in the brain.[7] In the United States, 9.30 and 75.68 million people are estimated to develop clinical AD or preclinical AD by 2060,[8] and the total direct medical costs of AD at the national level is estimated to reach $259 billion by 2040.[9] Due to the growing public concern with these substantial increases in the prevalence of AD, investigations on interventions to prevent progression and onset of AD have targeted the potentially modifiable risk factors of AD, including air pollution.[10]

Different biological pathways have been discussed underlying the association between air pollution and AD development. $PM_{2.5}$ exposure might directly infiltrate the brain[11] and accelerate AD pathogenesis and development via neuroinflammation, oxidative stress, and Aβ accumulation.[12] Increasing evidence from human and animal studies proposes that perturbations in DNA methylation (DNAm), which regulate the expression of genes, are associated with indicators of AD as well as $PM_{2.5}$ exposure. However, the tissue specificity of DNAm has limited the ability of previous studies to formally investigate mediation.

While there is no conclusive evidence of an association between AD and DNAm in blood,[13] a growing body of evidence suggests robust association in brain tissues.[13] DNAm alterations in a number of genes were observed to be associated with AD pathology and neuroinflammation in brain tissues, such as amyloid precursor protein (*APP*),[14] microtubule-associated protein tau (*MAPT*),[14] apolipoprotein (*APOE*) promoter region,[15] homeobox A3 (*HOXA3*),[16] interleukin-1 beta (*IL-1β*),[17] interleukin-6 (*IL-6*),[17] and claudin-5 (*CLDN5*) genes.[18]

The association of $PM_{2.5}$ with DNAm in blood has been extensively studied,[19] and one study found that DNAm in interleukin-10 (*IL-10*), *IL-6*, tumor necrosis factor (*TNF*), toll like receptor 2 (*TLR2*) genes, which play key roles in neuroinflammation,[20] was significantly altered in response to short-term exposure to $PM_{2.5}$ and its species.[21] However, to the best of our knowledge, no human studies have been published on the association between $PM_{2.5}$ exposure and DNAm in the brain,

which is the most relevant tissue when studying AD. The only evidence to date comes from in-vivo and in-vitro studies. Tachibana et al. demonstrated with a mouse model that prenatal exposure to diesel exhaust altered DNAm in brain tissues collected from 1- and 21-day-old offspring and the differentially methylated CpG sites were enriched in the gene ontology (GO) terms related to neuronal development.[22] Wei et al. exposed human neuroblastoma cells to $PM_{2.5}$ collected at a near-road site and found that DNAm was hypermethylated in the promoter regions of neurexin 1 (*NRXN1*) and neuroligin 3 (*NLGN3*) genes encoding synaptic neuronal adhesion molecules that mediate essential signaling at the synapse.[23]

Given the limited evidence of an association between $PM_{2.5}$ exposure and DNAm in the brain, the mediating role of DNAm for the association between $PM_{2.5}$ and AD pathology has not been well studied. Only one study investigated DNAm in mouse brain; these investigators failed to find evidence for DNAm as a potential mediator of the association between particulate matter exposure and increased cytokines and Aβ levels associated with early AD-like pathology.[24]

The current study investigated the relationship among $PM_{2.5}$, DNAm, and AD neuropathology in the *post mortem* human brain among brain donors of the Emory Goizueta AD Research Center (ADRC) brain bank. We recently showed a significant association between traffic-related $PM_{2.5}$ exposure and increased AD neuropathology in this dataset.[25] To elucidate the biological mechanisms for this association, we here investigated whether differential DNAm in the prefrontal cortex tissues mediates the association between long-term exposure to traffic-related $PM_{2.5}$ and the levels of AD-related neuropathological markers. This hypothesis was tested using a combination of the meet-in-the-middle (MITM) approach and high-dimensional mediation analysis.

RESEARCH IN CONTEXT

1. **Systematic review:** Growing evidence indicates fine particulate matter ($PM_{2.5}$) exposure as a risk factor for Alzheimer's' disease (AD), but the underlying mechanisms have been insufficiently investigated. Several studies have investigated associations between $PM_{2.5}$ exposure and DNA methylation (DNAm) levels in blood or between DNAm levels in the brain and AD neuropathology. However, no human study has explored differential DNAm in the brain as a potential mediator of the association between air pollution and AD.
2. **Interpretation**: In an autopsy cohort, we detected multiple CpG sites in prefrontal cortex tissues that mediated associations between $PM_{2.5}$ exposure and AD-related neuropathology markers. Some of these probes are located in genes related to neuroinflammation and neuroinflammation-mediated necroptosis in brain tissues, implicating neuroinflammation a potential underlying mechanism of $PM_{2.5}$ neurotoxicity.
3. **Future directions**: Future research should investigate whether these changes in DNAm could also be detected in other more accessible tissues to consequently serve as early biomarkers of disease.

2 | METHODS

2.1 | Study design

The current cross-sectional analysis included study participants recruited by the Emory Goizueta ADRC. The ADRC was founded in 2005 and has maintained a brain bank to facilitate AD research. The study participants were research participants evaluated annually, and others were patients treated by Emory Department of Neurology physicians and diagnosed clinically with AD (biomarker defined) or probable AD. The prefrontal cortex tissues were obtained from the participants who had consented to donate biospecimens to the ADRC brain bank. There were 1011 donors enrolled by the third quarter of 2020. After applying the following inclusion criteria, 264 donors remained eligible for the current study (Figure S1): (1) the availability of residential addresses within Georgia (GA) state; (2) age at death equal to or over 55 years; (3) deceased after 1999 (due to the availability of air quality data); (4) no missing values in neuropathology outcomes and key covariates including age at death, race, sex, educational attainment, and APOE genotype. Among these donors, genome-wide DNAm was measured in 161 available samples from the donors deceased after 2007, and after quality control, 159 were included in the current analysis. Written informed consent was provided for all donors, and samples were obtained following research protocols approved by the Emory University Institutional Review Board.

2.2 | Neuropathology assessment

The ADRC performed thorough neuropathologic evaluations on the brains of all donors using established comprehensive research evaluations and diagnostic criteria.[26] These neuropathological assessments include a variety of stains and immunohistochemical preparations, as well as semi-quantitative scoring of multiple neuropathologic changes by experienced neuropathologists using published criteria.[27] In this project, AD-related neuropathological changes were evaluated using Braak stage, Consortium to Establish a Registry for AD (CERAD) score, and a combination of Amyloid, Braak stage, and CERAD (ABC) score which were developed based on the Aβ plaques and NFTs.[28] Braak stage is a staging scheme describing NFTs with six stages (Stage I-VI) with a higher stage indicating a wider distribution of NFTs in brain. CERAD score describes the prevalence of Aβ plaques with four levels from no neuritic plaques to frequent. ABC score combines the former two (along with the Thal score for Aβ plaque distribution

across various brain regions)[29] and is transformed into one of four levels: not, low, intermediate, or high level of AD neuropathologic changes.

2.3 | Air pollution assessment

Annual concentrations of traffic-related $PM_{2.5}$ were estimated for the 20-county area of Metropolitan Atlanta, Georgia, for 2002–2019 using two air quality models with one covering the years 2002–2011 and the other for 2012–2019 (see details below). The spatial resolution of the $PM_{2.5}$ data were 250 × 250 m (for 2002–2011) and 200 × 200 m (for 2012–2019). The grid cells of the corresponding side length were evenly distributed throughout the study area. The process for estimating 2002–2011 $PM_{2.5}$ concentrations was previously published.[30,31] Briefly, a calibrated Research LINE-source dispersion (R-LINE) model for near-surface releases was applied for calculating annual averages of traffic-related $PM_{2.5}$. The model yielded a normalized root mean square error of 24% and a normalized mean bias of 0.3% by comparing with the estimates of the receptor-based source apportionment Chemical Mass Balance Method with Gas Constraints.[30] For estimating 2012 to 2019 $PM_{2.5}$ concentrations, we trained a land-use random forest model based on the 2015 annual concentrations of traffic-related $PM_{2.5}$ obtained from Atlanta Regional Commission,[32] road inventory and traffic monitoring data shared by the Georgia Department of Transportation,[33] land cover data accessed via the National Land Cover Database,[34] and ambient $PM_{2.5}$ data obtained from Atmospheric Composition Analysis.[35] The random forest model was trained with the R package *randomForest*,[36] and two user-defined parameters (i.e., the number of trees and the number of variables randomly tried at each split) were determined by a balance of the efficiency and the out-of-bag R2 value. The final model reached an out-of-bag R2 of 0.8 and a root-mean-square deviation of 0.2 μg/m3. This model was used to predict annual traffic-related $PM_{2.5}$ for 2012–2019 with a spatial resolution of 200 m. More details can be found elsewhere.[25] Finally, we spatially matched geocoded residential addresses to the centroid of the closest grids and calculated the individual long-term exposures as the average of specific exposure windows (1-, 3-, and 5-years prior to death). The estimated $PM_{2.5}$ concentrations of different exposure windows served as an approximate proxy of individuals' long-term exposure, with a subsidiary goal of assessing the robustness of the hypothesis.

2.4 | Genome-wide DNA methylation

DNA was isolated from the fresh frozen prefrontal cortex in 161 samples using the QIAGEN GenePure kit. DNAm was assessed with the Illumina Infinium MethylationEPIC BeadChips in batches of 167 prefrontal cortex samples including six replicates. The raw intensity files were transformed into a dataset that included beta values for each of the CpG sites, and these beta values were computed as the ratio of the methylated signal to the sum of the methylated and unmethylated signals, which ranged from 0 to 1 on a continuous scale. Pre-processing and statistics were done using R (v4.2.0). We followed a validated quality control and normalization pipeline as previously published.[37] The detailed data processing and sample quality control can be found in the Supplementary Methods. One hundred and fifty-nine samples passed the quality check, and after excluding SNP probes, XY probes, and other low-quality probes, 789,286 CpG sites remained. The final DNAm beta values were further normalized to reduce the probe type differences and corrected by *ComBat* to remove the batch effect before the downstream analysis.[38] We estimated the cell-type proportions (neuronal vs. non-neuronal cells) for each sample using the most recent prefrontal cortex database and the R package *minfi*.[39,40]

2.5 | Covariate assessment

Individual-level demographic characteristics (sex, race [Black vs. White], educational attainment [high school or less, college degree, and graduate degree], age at death, APOE ε4 genotype) were obtained from the medical records. APOE ε4 genotype was continuous with a 3-point scale (0 = no ε allele, 1 = one ε4 allele, and 2 = two ε4 alleles). Area Deprivation Index (ADI) for each donor was estimated at the residential address as a proxy for neighborhood socioeconomic status, based on a publicly available database at the level of the Census Block Group for 2015.[41] *Post mortem* interval (hours) of sample collection was provided by our lab collaborators. The confounding structure was determined according to literature review and our previous studies, which was illustrated by directed acyclic graphs (DAGs) in the Supplement (Figure S2). Briefly, the minimum adjustment set for the association between $PM_{2.5}$ exposure and DNA methylation contained sex, race, age at death, educational attainment, and ADI. Furthermore, PMI and cell-type proportions were also included due to their substantial impact on DNA methylation. The minimum adjustment set for the association between DNA methylation and neuropathology markers contained sex, race, age at death, educational attainment, APOE ε4 genotype, PMI, and cell-type proportions.

2.6 | Statistical analysis

Previously, we found higher residential $PM_{2.5}$ exposure was associated with increased AD neuropathology in the Emory Goizueta ADRC brain bank.[25] To identify DNAm patterns in brain tissue that potentially mediate the association between $PM_{2.5}$ exposure and increased neuropathology markers, we (1) conducted an epigenome-wide association study (EWAS) for the long-term $PM_{2.5}$ exposures 1-, 3-, and 5-years prior to death and then investigated whether any differentially methylated CpG sites that were significantly associated with $PM_{2.5}$ exposure in the EWAS were also associated with increased neuropathology markers; and (2) conducted a combination of the MITM approach and high-dimensional mediation analysis (HDMA) to identify any mediating CpGs that did not reach genome-wide significance in the EWAS of $PM_{2.5}$ (Figure 1). The MITM approach and

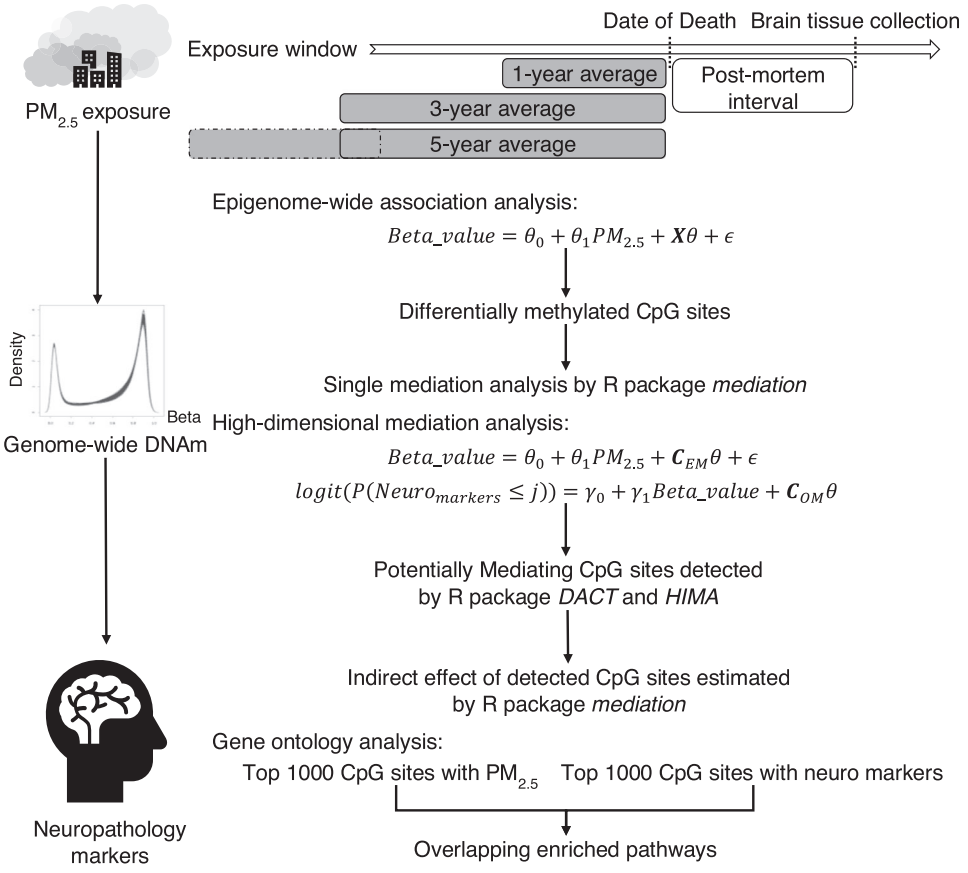


FIGURE 1 Graphical overview of the analytical strategy to evaluate the potential mediating CpG sites in the current analysis. *X*, C_{EM}, and C_{OM} denote the matrices of covariates for each association.

HDMA work complementarily to maximize the detention of potential mediators.

First, we conducted an EWAS to assess associations of long-term $PM_{2.5}$ exposures 1-, 3-, and 5-years prior to death, and methylation levels of CpG sites. Specifically, we used robust multiple linear regression models as implemented in the R package *MASS* to identify differentially CpG sites associated with $PM_{2.5}$ exposures.[42] To account for measured confounding factors, we included sex, race, educational attainment, age at death, PMI, ADI, and proportion of neuronal cells in the model. Potential batch effect and other unwanted variations were further corrected using the R packages *sva*[43] (estimating surrogate variables included in the EWAS model as covariates) and *Bacon*.[44] The *sva* was used to obtain surrogate variables to be included in the models. To account for multiple testing, the Bonferroni threshold was used for statistical significance ($0.05/789{,}286 = 6.33 \times 10^{-8}$), while no cutoff was applied on the magnitude of DNA methylation difference.[45]

Any CpG sites that were significantly associated with $PM_{2.5}$ exposure were then investigated for their associations with neuropathology markers. These associations were extracted from an EWAS of each neuropathology marker (CERAD, Braak stage, ABC score) with methylation levels of all CpG sites, using robust multiple linear regression models with the neuropathology markers converted to continuous outcomes and DNAm beta values of CpG sites as exposures, adjusting for sex, race, educational attainment, age at death, PMI, APOE genotype, and proportion of neuronal cells. We used *Bacon*[44] to control for unmeasured confounding and bias due to the minor inflation/deflation indicated by raw *p*-values.

For the MITM, we compared the 1000 most significant CpGs from the two sets of EWAS on all CpG sites for $PM_{2.5}$ exposures and neuropathology markers to identify the differentially methylated CpG sties that were associated with both exposures and outcomes. In other words, the raw *p*-values of all 789,286 CpG sites were sorted increasingly, which were derived from the two set of EWAS models conducted on $PM_{2.5}$ exposure and neuropathology markers, respectively. We selected the CpG sites among the lowest 1000 for both $PM_{2.5}$ exposure and neuropathology markers. The MITM approach is widely used in high-dimensional setting to identify intermediate biomarkers.[46]

Then, we conducted an HDMA using the R packages *HIMA* and *DACT* to identify any potential mediating CpG sites between $PM_{2.5}$ exposure and neuropathology from all 789,286 CpG sites. *HIMA* is an R package for estimating and testing high-dimensional mediation effects for omics data, which adopts the multiple mediator model's framework with reducing the dimensionality of omics data via sure independence screening and minimax concave penalty.[47] The divide-aggregate composite null test (*DACT*) is a more recent method for

HDMA, which utilizes the Efron empirical null framework to calculate a weighted sum of *p*-values obtained from exposure-mediator (EWAS of $PM_{2.5}$ exposure as described above) and mediator-outcome (EWAS of neuropathology markers as described above) models for testing the significance of all mediators.[48] We corrected for multiple testing in *HIMA* and *DACT* using the *Bonferroni* method. Last, for the mediating CpG sites identified by either *HIMA* or *DACT*, we used the R package *mediation* to conduct a causal mediation analysis obtain their indirect effects.[49–51] The *mediation* is a frequently used tool which implements the mediation methods and suggestions proposed by Imai et al.[52,53] The average causal mediation effect (i.e., indirect effect) and total effect estimated by *mediation* were summarized for the CpG sites with positive indirect effects that were in line with the hypothesized adverse effect of traffic-related $PM_{2.5}$ on neuropathology markers. In contrast to the MITM approach described earlier, HDMA examine multiple mediators together in a framework of mediation analysis, which allowed us to ascertain the extent to which the particular indirect effects were associated with the mediators.

To aid the interpretation of model results, we conducted a gene ontology analysis using the R package *missMethyl* based on the top 1000 CpG sites with lowest raw *p*-values.[54] The gene ontology analysis was conducted for the EWAS results of $PM_{2.5}$ exposure as well as for the EWAS results of the three neuropathology markers. All CpG sites were annotated using an online annotation data for the "IlluminaHumanMethylationEPIC."[55] Additional functional insight on single CpG sites was obtained by searching the corresponding CpG site in publicly available databases, including EWAS catalog[56] and GoDMC.[57]

All analyses were completed in R (v4.2.0).

3 | RESULTS

3.1 | Study population characteristics

A total of 159 donors were included in the current analysis, and their demographic characteristics and neuropathologic markers are described in Table 1. The average age of death was 76.6 years (SD = 9.98) and 56% of the study population were male. The study population was predominantly white (89.3%) and well-educated with 123 (78.7%) completing college or more and living in less deprived neighborhoods (ADI: mean = 36.3, SD = 24.2). The prevalence of the APOE ε4 allele (56% with at least one APOE ε4 allele) in this population was much higher than that in the general population in the United States.[58]

As illustrated by the 1-year traffic-related $PM_{2.5}$ exposure (Figure 2A), donors living in urban areas had a higher level of $PM_{2.5}$ exposure compared to those living in suburban areas. The median of 1-year exposure was 1.21 μg/m3 [interquartile range (IQR) = 0.78]. As $PM_{2.5}$ concentrations have decreased over the last decades, 3- and 5-year exposures were slightly higher (3-year exposure: median = 1.32 μg/m3 [IQR = 0.74], 5-year exposure: median = 1.39 μg/m3 [IQR: 0.81]) (Figure 2B).

TABLE 1 Selected population characteristics among the donors included in the current analysis.

Parameter	*N* = 159
Age at death, mean (SD)	76.6 (9.98)
Sex, No. (%)	
Female	70 (44.0)
Male	89 (56.0)
Race, No. (%)	
Black	17 (10.7)
White	142 (89.3)
Educational attainment, No. (%)	
High school or less	36 (22.6)
College degree	76 (47.8)
Graduate degree or more	47 (29.6)
Area Deprivation Index, mean (SD)	36.3 (24.2)
APOE genotype	
No ε4 allele	70 (44.0)
Single ε4 allele	68 (42.8)
Two ε4 allele	21 (13.2)
Post mortem interval (hours), mean (SD)	11.7 (9.68)
Proportion of neuronal cells (%), mean (SD)	31.9 (8.21)
Braak stage, No. (%)	
Stage 1	16 (10.1)
Stage 2	11 (6.9)
Stage 3	20 (12.6)
Stage 4	17 (10.7)
Stage 5	22 (13.8)
Stage 6	73 (45.9)
CERAD score, No. (%)	
No	35 (22.0)
Sparse	4 (2.5)
Moderate	10 (6.3)
Frequent	110 (69.2)
ABC score, No. (%)	
Not	15 (9.4)
Low	29 (18.2)
Intermediate	22 (13.8)
High	93 (58.5)

Abbreviations: ABC, a combination of Amyloid, Braak stage, and CERAD (ABC) score; AD, Alzheimer's disease; APOE, apolipoprotein E; CERAD, Consortium to Establish a Registry for AD; SD, standard deviation.

3.2 | Association between $PM_{2.5}$ exposure and DNAm in the brain

After correcting for multiple tests and adjusting for bias and measured and unmeasured confounding, two CpG sites (cg25433380 and

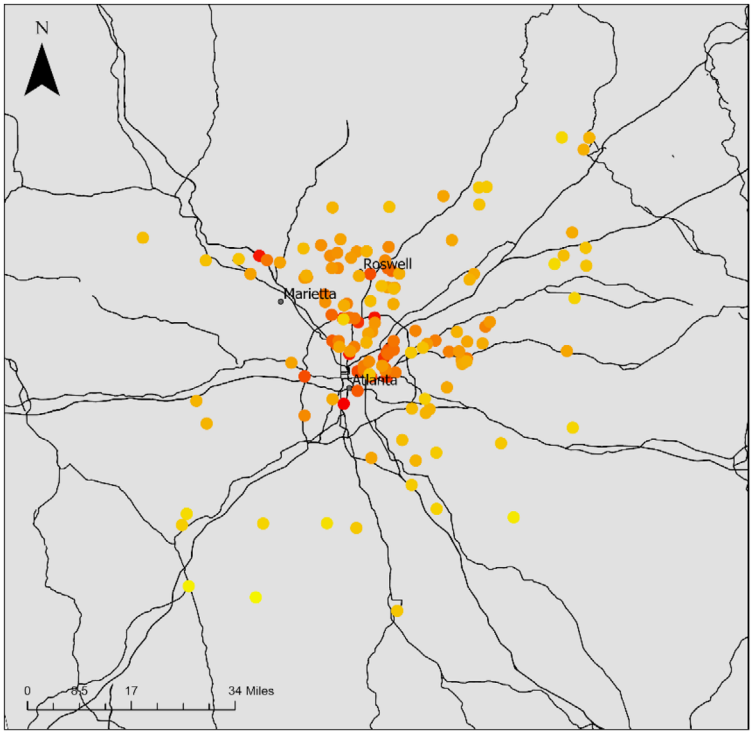
(A) Map of Metropolitan Atlanta

1-year $PM_{2.5}$ exposure:

Min — Max

United States Highways:

Cities over 50,000 residents:



(B) Statistics of individual averaged annual $PM_{2.5}$ exposures

	$PM_{2.5}$ (μg/m³)		
	1-year	3-year	5-year
Min	0.18	0.34	0.33
Median	1.21	1.32	1.39
Max	3.27	3.44	3.55
IQR	0.78	0.74	0.81

FIGURE 2 Statistics and distribution of $PM_{2.5}$ exposures in Metropolitan Atlanta (study area), Georgia, United States. (A) Map of Metropolitan Atlanta with individual 1-year averaged annual $PM_{2.5}$ exposure. The dots denote the donors' residential address and are colored according to their $PM_{2.5}$ exposures as showed in the legend. Red means a higher exposure level. (B) Statistics of individual averaged annual $PM_{2.5}$ exposures for 1-, 3-, and 5-years.

cg10495669) were consistently associated with $PM_{2.5}$ across different exposure windows (Figure 3, Table 2; summary statistics for all 789,286 CpG sites are provided as Tables S1-S3 in spreadsheets). For example, a 1 μg/m3 increase in 1-year $PM_{2.5}$ exposure was associated with 0.0065 increase in the DNAm beta value of cg25433380 ($p = 1.58 \times 10^{-8}$). The cg25433380 and cg10495669 are on chromosome 9 and 20, respectively, and cg10495669 is assigned to the gene encoding RanBP-type and C3HC4-type zinc finger-containing protein 1 (*RBCK1*). The two CpG sites were not significantly associated with any neuropathology markers (Table 2).

3.3 | MITM approach and high-dimensional mediation analysis

For the MITM approach, we explored the overlapping CpG sites among the top 1000 CpG sites for the EWAS of $PM_{2.5}$ and the EWAS of neuropathology markers (results presented in Tables S4-S6 in spreadsheets) and identified four overlapping CpG sites (Table S7). Specifically, DNAm in cg01835635 (*APOA4* gene) was associated with CERAD score as well as $PM_{2.5}$ exposure for the 1- and 3-year exposure windows. DNAm in cg16342341 (*SORBS2* gene) was associated with CERAD score as well as 1-year $PM_{2.5}$ exposure. Two other CpG sites (cg09830308 and cg27459981) were also among the top 1000 CpGs for both EWAS. However, due to their opposing directions of effect estimates in the exposure- and outcome-EWAS, which is biologically not plausible, they are considered false positive findings.

The HDMA via *HIMA* did not identify any CpG sites as significant mediators. In the HDMA using a combination of *DACT* and causal mediation analysis, we identified 22 CpG sites to mediate the positive association between $PM_{2.5}$ exposure and ABC score (Table 3), while none were observed for Braak stage and CERAD score. Seven CpG sites were associated with two exposure windows, and fifteen with a single exposure window. Of note, one of the seven CpG sites, cg16342341 (*SORBS2*), was also identified in the MITM approach described above. The total effect estimated for all mediation analyses was positive but insignificant in this subsample of the cohort (see Christensen et al. (2024) for the significant total effect in the full cohort).[25] The summary statistics for all CpG sites detected by *DACT* are summarized in the Supplement (Table S8).

3.4 | Secondary analyses

A gene ontology analysis was conducted for the top 1000 CpG sites associated $PM_{2.5}$ and for the top 1000 CpG sites associated with the neuropathology markers. None of the KEGG pathways reached significance after correcting for multiple tests. Therefore, we summarized the top 10 KEGG pathways for each of the $PM_{2.5}$ exposures or neuropathology markers in the Supplement (Table S9). One pathway, which

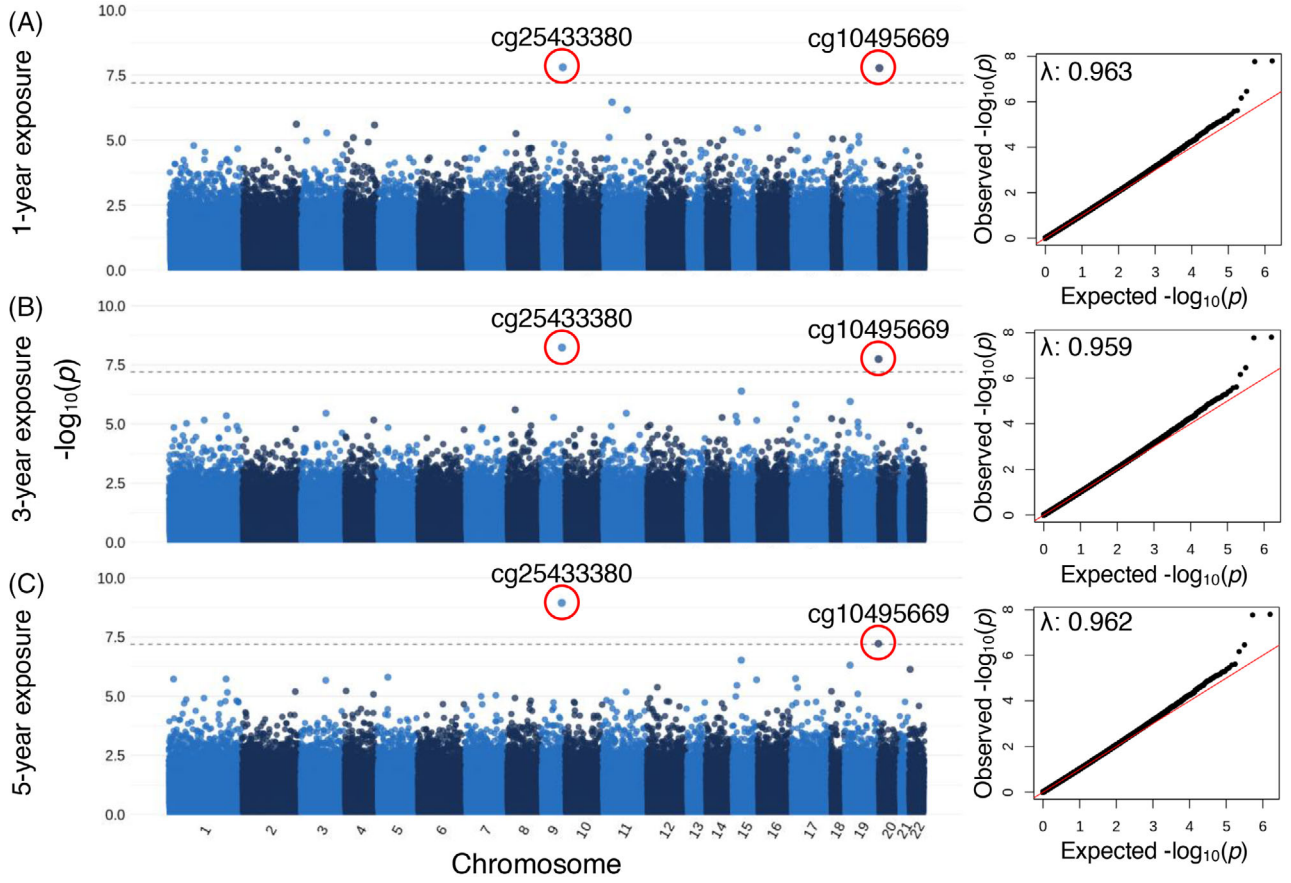


FIGURE 3 Manhattan and QQ plots for the epigenome-wide association of $PM_{2.5}$ exposures (A) 1-year/(B) 3-year/(C) 5-year average exposure prior to death, and DNA methylation in *post mortem* frontal cortex tissue. λ denotes the inflation factor. Adjusted for covariates: age at death, sex, race, educational attainment, *post mortem* interval, area deprivation index, and cell type composition. Unmeasured confounding and bias were adjusted with surrogate variable analysis and R package *Bacon*. Bonferroni threshold: 0.05/789,286.

is the longevity regulating pathway, was associated with both 3-year exposure to $PM_{2.5}$ and CERAD score. Eight genes (*HSPA1A*, *HSPA1L*, *IRS1*, *KRAS*, *NRAS*, *RPTOR*, *IRS2*, *ATG5*) in this pathway were enriched by differentially methylated CpG sites that were associated with 3-year $PM_{2.5}$ exposure, and 10 genes (*ADCY3*, *ADCY5*, *NFKB1*, *PRKAG2*, *RPTOR*, *TSC2*, *EHMT1*, *ULK1*, *AKT1S1*, *ATG5*) with CERAD score. Of note, *AKT1S1* was also among the genes that were identified in the HDMA (DACT and causal mediation analysis).

4 | DISCUSSION

In the current study of 159 donors from the Emory Goizueta ADRC brain bank, we identified differential DNAm in prefrontal cortex tissues at two CpG sites to be significantly associated with long-term $PM_{2.5}$ exposure. Two CpG sites (cg25433380 [intergenic] and cg10495669 [RBCK1]) were consistently associated with all exposure windows of traffic-related $PM_{2.5}$, after controlling for measured and unmeasured confounding. While cg25433380 and cg10495669 were not associated with increases in neuropathology markers, we identified 4 CpG sites that overlapped between the top 1000 CpG sites associated with $PM_{2.5}$ and neuropathology markers (MITM approach) and 22 CpG sites that mediated the adverse effect of $PM_{2.5}$ exposures on AD-related neuropathology markers using HDMA. In addition, the longevity regulating pathway, was found to be enriched by differentially methylated CpG sites associated with $PM_{2.5}$ (3-year exposure window) and CERAD score.

Although there is a growing body of research on $PM_{2.5}$-associated DNAm patterns in the human blood,[19] this is the first study showing an association between $PM_{2.5}$ exposure and differential DNAm in the brain (cg25433380 and cg10495669). Scarce evidence related to air pollution has been reported so far on cg25433380. On the other hand, higher DNA methylation levels of cg10495669 in nasal cells have been associated with 1-year ambient $PM_{2.5}$ exposure among 503 children from Project Viva in Massachusetts state.[59] *RBCK1*, the gene which cg10495669 is assigned to, is involved in carcinogenesis and inflammation pathways. The overexpression of *RBCK1* was observed in multiple cancer cells, including renal, colorectal, and breast cells, in in-vitro experiments.[60–62] The knockdown of *RBCK1* in renal cancer cells may induce p53 expressions, and thus, Yu et al. proposed a model in which *RBCK1* promoted the ubiquitination and degradation of p53, a protein playing a major role in DNA damage response.[62] The impairment of p53 expression and activity might participate in neurodegeneration, as p53 can bind to genes that regulate the expression

TABLE 2 CpGs associated with traffic-related $PM_{2.5}$ exposure prior to death and their association with neuropathology markers.

CpG	chr	Position	Gene		Coefficients[a]	*p*-Values[b]
A. CpGs with $PM_{2.5}$ exposures						
cg25433380	9	388,531	Intergenic	1-year exposure	0.0065	1.58×10^{-8}
				3-year exposure	0.0066	5.82×10^{-9}
				5-year exposure	0.0063	1.12×10^{-9}
cg10495669	20	137,531,767	*RBCK1*	1-year exposure	0.0127	1.69×10^{-8}
				3-year exposure	0.0128	1.78×10^{-8}
				5-year exposure	0.0114	5.96×10^{-8}
B. CpGs with neuropathology markers						
cg25433380	9	388,531	Intergenic	Braak stage	0.08	0.729
				CERAD	0.05	0.629
				ABC	0.04	0.825
cg10495669	20	137,531,767	*RBCK1*	Braak stage	0.02	0.929
				CERAD	0.12	0.397
				ABC	0.09	0.593

Abbreviations: chr, chromosome; $PM_{2.5}$, fine particulate matter; RBCK1, RanBP-type and C3HC4-type zinc finger-containing protein 1.
[a]The coefficients for $PM_{2.5}$ exposures represent the change in the beta values of CpG sites associated with a one-unit increase in the exposures; the coefficients for neuropathology markers represent the change in the neuropathology markers associated with a one-interquartile-range increase in the beta values of CpG sites.
[b]The Bonferroni threshold: $0.05/789{,}286 \approx 6.33 \times 10^{-8}$.

of synaptic proteins, neurite outgrowth, and axonal regeneration, which indicated a neuroprotective role against AD development.[63] In addition, *RBCK1*, as part of linear ubiquitin chain assembly complex, can regulate the proinflammatory-cytokines-induced nuclear factor kappa B (NF-kB) activation which serves as a pivotal mediator of inflammatory responses.[64,65] NF-kB activation is a common feature of many neurodegenerative diseases,[66] and the increased expression and/or activation of NF-kB has been largely observed in *post mortem* studies of AD patients.[67] However, the two CpG sites were not found to be associated with any neuropathology markers in the current analysis. More research is warranted on the physiological function of cg25433380 to clarify its potential role in $PM_{2.5}$-related pathological changes, and the impact of $PM_{2.5}$ on the two CpG sites needs to be verified with a larger sample size and donors of more diverse disease stages from preclinical to severe dementia.

We identified two CpG sites (cg01835635 and cg16342341) that overlapped between the top 1000 CpG sites associated with both $PM_{2.5}$ and neuropathology markers via MITM approach. It is worth noting that the MITM approach used in the present study was hypothesis-generating, as the overlapping features were derived from the top 1000 CpG sites associated with exposures or outcomes without multiple comparison corrections. The findings need to be verified in an independent target analysis.

Cg16342341, assigned to the Sorbin and SH3 domain-containing protein 2 gene (*SORBS2*), was also identified as a potential mediator in the HDMA, where it mediated the association of all $PM_{2.5}$ exposure windows with ABC score. As *SORBS2* is well known for its role in AD and neuroinflammation[68,69] and has also been associated with $PM_{2.5}$ exposure in rats,[70] our findings contribute to the growing body of evidence of *SORBS2* expression playing a role in $PM_{2.5}$ associated changes in neuropathology markers of AD. *SORBS2* was found to repress IL-6 and TNF-α expression in the mouse embryonic fibroblasts,[71] and Chen et al. demonstrated that the level of *SORBS2* was lower in the brains of AD model mice compared to wild type mice,[68] implying a role of *SORBS2* in regulating neuroinflammation. In a human study of families that multiply affected by AD, Lee et al. reported that genetic variation in *SORBS2* was associated with age at onset of AD.[69] While evidence on the association between $PM_{2.5}$ exposure and *SORBS2* is more scarce, Chao et al. reported that prenatal exposure to $PM_{2.5}$ caused upregulation of microRNAs targeting the *SORBS2* gene in fetal rat cortex tissues.[70]

In addition to cg16342341, we identified 21 other CpGs as potential mediators of the association between long-term exposure to traffic-related $PM_{2.5}$ and ABC score using HDMA, and two of these CpGs have been previously reported in association with AD. Differential methylation in cg07963191, assigned to the dual 3′,5′-cyclic-AMP and -GMP phosphodiesterase 11A gene (*PDE11A*), mediated the adverse effect of the average $PM_{2.5}$ exposure 3 years prior to death on the ABC score. *PDE11A* pertains to the phosphodiesterase family that plays an essential role in neuroplasticity and neuroprotection.[72] Differential methylation in cg27297993, assigned to the gamma-aminobutyric acid B receptor 1 gene (*GABBR1*), mediated the adverse effect of the average $PM_{2.5}$ exposure 3 and 5 years prior to death on the ABC score. *GABBR1* is the main inhibitory neurotransmitter in the central nervous system, which was reported to be downregulated in the brains of AD patients.[73] Iwakiri et al. observed a negative correlation between

TABLE 3 Indirect effect estimated by causal mediation analysis via the R package *mediation* of CpG sites selected by high-dimensional mediation analysis for the associations between $PM_{2.5}$ exposure and ABC score[a].

CpG	chr	Gene	Exposure[b]	*DACT* *p*-values[c]	ACME[d]	Total effect[e]
cg23932332	1	*DUSP10*	3-year	4.3×10^{-8}	0.056 (0.005, 0.150)	0.086 (−0.110,0.280)
	a		5-year	2.9×10^{-8}	0.060 (0.002, 0.170)	0.104 (−0.081,0.310)
cg08512806	1	*TARBP1*	3-year	5.3×10^{-8}	0.058 (0.008, 0.130)	0.084 (−0.107,0.300)
			5-year	3.8×10^{-8}	0.063 (0.009, 0.130)	0.102 (−0.080,0.310)
cg10705045	2	*RNF144A*	5-year	2.6×10^{-8}	0.063 (0.001, 0.140)	0.109 (−0.079,0.310)
cg17275287	2	*Intergenic*	3-year[f]	3.4×10^{-9}	0.085 (0.019, 0.170)	0.079 (−0.118,0.300)
			5-year	2.0×10^{-9}	0.089 (0.020, 0.180)	0.097 (−0.093,0.300)
cg07258300	2	*CYP27C1*	3-year	5.4×10^{-8}	0.080 (0.020, 0.150)	0.083 (−0.107,0.300)
cg05532414	2	*Intergenic*	3-year	6.2×10^{-8}	0.071 (0.004, 0.170)	0.090 (−0.084,0.320)
cg07963191	2	*PDE11A*	3-year	3.1×10^{-8}	0.061 (0.005, 0.140)	0.080 (−0.103,0.300)
cg26109897	4	*TBC1D14*	3-year	2.1×10^{-8}	0.085 (0.010, 0.190)	0.090 (−0.098,0.310)
cg26877022	4	*POLR2B*	3-year	4.5×10^{-9}	0.080 (0.015, 0.180)	0.089 (−0.092,0.310)
			5-year	1.2×10^{-8}	0.077 (0.011, 0.180)	0.107 (−0.079,0.310)
cg16342341	4	*SORBS2*	1-year[f]	1.3×10^{-9}	0.097 (0.021, 0.180)	0.034 (−0.168,0.230)
			3-year	5.4×10^{-9}	0.076 (0.017, 0.160)	0.080 (−0.106,0.280)
			5-year	1.6×10^{-9}	0.078 (0.017, 0.150)	0.098 (−0.093,0.320)
cg17444747	5	*COL23A1*	5-year	3.2×10^{-8}	0.074 (0.015, 0.150)	0.098 (−0.085,0.290)
cg27297993	6	*GABBR1*	3-year	8.3×10^{-9}	0.064 (0.009, 0.140)	0.084 (−0.091,0.300)
			5-year	9.2×10^{-9}	0.066 (0.003, 0.140)	0.103 (−0.076,0.300)
cg00829961	8	*Intergenic*	3-year	1.3×10^{-8}	0.075 (0.009, 0.170)	0.092 (−0.092,0.310)
			5-year	3.2×10^{-8}	0.075 (0.012, 0.170)	0.110 (−0.078,0.330)
cg02987635	10	*C10orf11*	3-year	4.1×10^{-8}	0.063 (0.004, 0.150)	0.079 (−0.099,0.300)
cg06805557	11	*APBB1*	5-year	4.1×10^{-8}	0.062 (0.007, 0.130)	0.101 (−0.104,0.300)
cg19969778	11	*SIAE; SPA17*	3-year	8.9×10^{-9}	0.065 (0.008, 0.130)	0.080 (−0.108,0.310)
			5-year	1.8×10^{-8}	0.063 (0.010, 0.130)	0.098 (−0.092,0.310)
cg20713102	15	*ZSCAN2*	5-year	5.0×10^{-8}	0.074 (0.014, 0.160)	0.106 (−0.083,0.310)
cg09088153	15	*Intergenic*	3-year	4.6×10^{-8}	0.072 (0.013, 0.150)	0.089 (−0.094,0.320)
cg27181554	16	*SEPX1*	1-year[f]	1.7×10^{-8}	0.084 (0.021, 0.180)	0.039 (−0.162,0.270)
			3-year	2.7×10^{-8}	0.069 (0.015, 0.150)	0.085 (−0.108,0.280)
cg20389589	16	*FAM57B*	3-year	2.9×10^{-8}	0.069 (0.003, 0.160)	0.084 (−0.120,0.290)
cg06832209	16	*ADGRG3*	3-year	4.2×10^{-8}	0.078 (0.015, 0.160)	0.089 (−0.101,0.280)
cg00633834	19	*AKT1S1; TBC1D17*	5-year	3.7×10^{-8}	0.081 (0.017, 0.160)	0.095 (−0.090,0.290)

Abbreviations: ACME, average causal mediated effect (i.e., indirect effect); chr, chromosome; $PM_{2.5}$, fine particulate matter.

[a]All CpG sites that were selected by DACT and had a positive ACME were associated with ABC score. No positive associations were found for Braak stage and CERAD score.

[b]Only the exposure windows were shown for which significant indirect effects were found.

[c]The *p*-values of mediation effect testing conducted by DACT.

[d]The ACME was associated with a one-interquartile-range increase in beta values of CpG sites.

[e]Effect estimates, associated with a 1-unit increase, of PM2.5 exposures on neuropathology markers. The total effect was obtained by adding the indirect effects and direct effects together.

[f]The CpG sites had opposing direct and indirect effect estimates, resulting in a lower total effect compared to ACME. The direct effect is in contrast with the positive total effect and biologically not plausible. Therefore, these associations are most likely false positive hits.

GABBR1 and NFT formation in the hippocampus of 16 aged subjects, suggesting that the increased or stable expression of *GABBR1* may contribute to neuronal resistance to AD development.[74] Note that in Table 3, the total effect was obtained by adding the indirect effects and direct effects together. That higher indirect effects than total effects equaled a negative direct effect of $PM_{2.5}$ on neuropathology markers, which is not biologically plausible. The findings need to be interpreted with caution.

To derive more functional insights from the mediating CpG sites, we conducted gene ontology analysis based on the KEGG pathway database for the top 1000 CpGs associated with $PM_{2.5}$ exposure or neuropathology markers.[54] Proline-rich AKT1 substrate 1 (*AKT1S1*) was one of the genes enriched in the longevity regulating pathway which was found to overlap between $PM_{2.5}$ exposure and CERAD score. Of note, differential DNA methylation in cg00633834, which is assigned to *AKT1S1*, was also identified as a potential mediator in the HDMA. *AKT1S1* can activate mammalian target of rapamycin (mTOR)—mediated signaling pathways when phosphorylated,[75] and mTOR signaling was observed to have higher activity in AD brains.[76] As mTOR plays a role in maintaining the balance between protein synthesis and degradation, Salvatore Oddo suggested a critical role of mTOR in the accumulation of Aβ and tau proteins over the course of AD development from early to late stage.[76]

The current analysis employed the MITM approach and HDMA simultaneously to maximize the potential of identifying the differentially methylated CpG sites lying on a pathway from $PM_{2.5}$ exposure to AD-related neuropathology. The application of the MITM approach was based on the investigation of epigenomics versus $PM_{2.5}$ exposures and AD-related neuropathology versus epigenomics, which lent credibility to the association between $PM_{2.5}$ exposure and AD-related neuropathology by breaking it down and linking it up with DNAm.[77] In other words, the MITM approach was a conceptually straightforward extension of the causal step strategy in which investigators estimate the individual paths first, including exposure-to-mediator and mediator-to-outcome, and then manually identify the overlapping metabolic features.[78] Furthermore, while conventional methods of multiple testing correction (e.g., Bonferroni method) may overlook potentially relevant CpG sites, especially given a small sample size, the MITM approach serves as a supplement by taking into account the biological relevance regardless of their statistical significance.[77] However, the MITM approach assumes that all intermediate variables are independent, which is not always the case in many real-world scenarios. The HDMA focused more on quantifying the indirect effect of the mediator. Compared to the MITM approach, *HIMA* examines multiple mediators in one model and utilizes variable selection techniques to identify important mediators.[47] *DACT* created a divide-aggregate composite-null test which yields less conservative results and accounted for possible correlations among the multiple tests by adopting Efron's empirical null inference framework.[48] Admittedly, we did not observe many consistencies, except for cg16342341 (*SORBS2*), between the two approaches. Further research is warranted with a large sample size.

Our study has several strengths. We established for the first time a potential mediation effect of DNAm for the association between $PM_{2.5}$ and neuropathological changes of AD. Although false discovery is a problem in high-dimensional settings, we minimized the possibility of false discovery by verifying the indirect effect of CpG sites identified by HDMA using causal mediation analysis. The neuropathological changes of AD were quantified via multiple markers, including Braak stage, CERAD score, and ABC score, which covers the essential components (i.e., NFTs and Aβ plaques) for the neuropathological diagnosis of AD. Further, the neuropathology markers were assessed by experienced neuropathologists at Emory Goizueta ADRC following a standardized protocol, which minimized the misclassification bias of outcomes. Finally, the high-resolution $PM_{2.5}$ exposure assessment model enabled the characterization of spatial variation in individual exposure and reduced the potential measurement error.[79]

Our study is not without limitations. First, the temporal sequence between mediators (DNAm changes) and outcomes (AD neuropathology) could not be clearly defined because both were assessed *post mortem*. Second, traffic-related $PM_{2.5}$ exposure was estimated based on the residential address of donors at death. Moving shortly prior to death could have introduced measurement errors in exposure assessment, and the selection of exposure windows was arbitrary, as the disease process of AD may start many years before death and vary by patients. Third, the results are from a single brain bank and donors with a high *APOE* ε4 carrier rate and a high prevalence of dementia, so the generalizability should be tested in other brain bank or autopsy cohorts, and the implication on the onset of Alzheimer's disease needs further investigation. Fourth, even though most of the study population was White, and we controlled for race, the ancestry effect on DNA methylation might persist as residual confounding. Fifth, the current analysis only focused on the health effect of traffic-related $PM_{2.5}$, while $PM_{2.5}$ from other sources or other air pollutants such as nitrogen oxides or ozone might also play a role in AD and influence or confound the present findings.[80,81] Furthermore, $PM_{2.5}$ is a complex mixture and its composition varies by geographic region. In our analysis, we cannot determine which $PM_{2.5}$ components, such as heavy metals, were driving the association with AD.[82] Thus, this study may not capture a comprehensive picture of the overall health impact of air pollution on neuropathology, and the findings may have limited generalizability to regions or populations where non-mobile sources of $PM_{2.5}$ are dominant. Sixth, while the sample size of 159 brain samples was relatively large considering the challenges in collecting such samples, the high dimensionality of the genome-wide DNAm data raises concerns about the reliability of our findings. We had previously investigated the total effect of $PM_{2.5}$ on neuropathology markers with a slightly larger sample size and observed that traffic-related $PM_{2.5}$ exposure was significantly associated with CERAD score at autopsy, but we did not observe any significant total effect estimates in the present analysis, which indicated an insufficient sample size as well. Additional research using a larger sample size is necessary to confirm and validate our results. Finally, while the present findings suggested a mediation role of neuroinflammation pathways for the impact of $PM_{2.5}$ exposure on neuropathology, it is important to note that we did not directly assess

proinflammatory factors or the expression of related genes in brain tissues in this study. Further research (e.g., measurement of inflammatory cytokines, transcriptomics, and metabolomics research) is warranted to validate their involvement.

5 | CONCLUSIONS

Using a combination of the MITM approach, high-dimensional mediation analysis, and causal mediation analysis, we identified several CpG sites mediating the adverse effects of long-term exposure to traffic-related $PM_{2.5}$ exposure on the levels of AD-related neuropathology markers among prefrontal cortex tissues from 159 donors. Of note, several of these CpGs were identified by more than one approach and located in genes related to neuroinflammation and neuroinflammation-mediated necroptosis. Our findings provide important information on the biological mechanisms underlying $PM_{2.5}$ toxicity in AD pathogenesis. Future studies evaluating the mediating role of DNAm on AD-related outcomes should consider: (1) performing the analysis among early-stage AD patients or patients with mild cognition impairment to further illustrate the role of $PM_{2.5}$ in AD etiology; (2) performing genome-wide DNAm together with transcriptomics, proteomics, and/or metabolomics to capture a holistic picture of the underlying mechanism. Furthermore, it would be of interest to investigate whether these changes in DNAm could also be detected in other more accessible tissues to consequently serve as early biomarkers of AD.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. Author disclosures are available in the supporting information.

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