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Epigenome-wide association study on ambient $PM_{2.5}$ exposure in Han Chinese, the NSPT study

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ABSTRACT

Ambient $PM_{2.5}$ exposure has been recognized as a major health risk and related to aging, cardiovascular, respiratory and neurologic diseases, and cancer. However, underlying mechanism of epigenetic alteration and regulated pathways still remained unclear. The study on methylome effect of $PM_{2.5}$ exposure was quite limited in Chinese population, and cohort-based study was absent. The study included blood-derived DNA methylation for 3365 Chinese participants from the NSPT cohort. We estimated individual $PM_{2.5}$ exposure level of short-medium-, medium- and long-term, based on a validated prediction model. We preformed epigenome-wide association studies to estimate the links between $PM_{2.5}$ exposure and DNA methylation change, as well as stratification and sensitive analysis to examined the robustness of the association models. A systematic review was conducted to obtain the previously published CpGs and examined for replication. We also conducted comparison on the DNA methylation variation corresponding to different time windows. We further conducted gene function analysis and pathway enrichment analysis to reveal related biological response. We identified a total of 177 CpGs and 107 DMRs associated with short-medium-term $PM_{2.5}$ exposure, at a strict genome-wide significance ($P < 5 \times 10^{-8}$). The effect sizes on most CpGs tended to cease with the exposure of extended time scale. Associated markers and aligned genes were related to aging, immunity, inflammation and carcinogenesis. Enriched pathways were mostly involved in cell cycle and cell division, signal transduction, inflammatory pathway. Our study is the first EWAS on $PM_{2.5}$ exposure conducted in large-scale Han Chinese cohort and identified associated DNA methylation change on CpGs and regions, as well as related gene functions and pathways.

1. Introduction

Air pollution is an environmental hazard that has been globally recognized as a major health risk and resulted in millions of deaths (Landrigan et al., 2018). Ambient $PM_{2.5}$, the fine particulate matter with an aerodynamic diameter less than 2.5 μm, is one of the toxic air pollution components and a concern that has been associated with death (Lim et al., 2020) and aging (Yin et al., 2021), and multiple tissue-specific diseases including cancer (Wu et al., 2021), cardiovascular (Hayes et al., 2020), respiratory (Zhao et al., 2020) and neurologic

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diseases (Shou et al., 2019). Meanwhile, biological responses resulted from $PM_{2.5}$ exposure were reportedly involved inflammation (Chu et al., 2019), oxidative stress (Piao et al., 2018), and mitochondrial dysfunction (Gao et al., 2022). $PM_{2.5}$ can be inhaled deeply into the lung, penetrate the epithelium and reach the cardiovascular system (Brook et al., 2010), and previous epidemiological studies in the field of epigenetic effects have identified $PM_{2.5}$ exposure associated DNA methylation (DNAm) patterns as well as the underlying disruption or regulation mechanism, yielding valuable insights on the systematic effects of $PM_{2.5}$ exposure. For instance, investigation on global methylation level (De Prins et al., 2013) or differential methylation in key regulatory regions (Tantoh et al., 2020) demonstrated the epigenetic effects of $PM_{2.5}$ exposure. In addition, a few epigenome-wide association studies (EWAS) identified DNAm alteration on specific CpGs related to $PM_{2.5}$ exposure, and linked to genes involved in inflammation, oxidative stress and respiratory function (Breton et al., 2016; Chi et al., 2016; Dai et al., 2017; de FC Lichtenfels et al., 2018; Eze et al., 2020; Gao et al., 2019; Li et al., 2018; Mostafavi et al., 2018; Panni et al., 2016; Plusquin et al., 2017; Sayols-Baixeras et al., 2019; Wang et al., 2022; Zhong et al., 2017).

While these studies collectively contribute to the understanding of the epigenetic effects of $PM_{2.5}$ exposure, heterogeneity existed in both the diverse study designs and the findings (detailed in section 3.4). Specifically, previous studies observed $PM_{2.5}$ exposure with a variety of time windows, from hours, days, monthly, yearly, up to 10 years, and in multiple time windows, as well as in pre-pregnancy. However, little conclusion had been drawn on comparing the shared or different pattern of short-, medium- and long-term $PM_{2.5}$ exposure. Additionally, heterogeneity might also result from various race or population, which could act as confounders of both $PM_{2.5}$ level and DNAm profiles. As most of the studies has been conducted in Caucasian in Europe or America, understanding of the methylome effect of $PM_{2.5}$ exposure in Chinese population was limited, and especially, cohort-based study was absent. Therefore, the link between $PM_{2.5}$ exposure and DNAm in Chinese is far from clear and needs to be adequately investigated. Facing the gap of the existing literature and seeking to fill in the puzzle for more comprehensive knowledge, our study contributes to the field by performing EWASs on $PM_{2.5}$ exposure conducted in large-scale Han Chinese cohort, as well as the first study leveraging DNAm effect of $PM_{2.5}$ exposure from 1 month to 10 years.

With a sample size of 3365 Han Chinese individual, we used a validated prediction model and generated individual-level $PM_{2.5}$ exposure measurement of various time windows from short-medium- to long-term. We performed EWAS on $PM_{2.5}$ exposure and identified differential DNAm sites and regions. A systematic review was conducted to obtain the previously published CpGs, which were examined for replication in our study. Also, we investigate how DNAm varies corresponding to short-medium-, medium- and long-term exposure. We furtherly overlapped the identified genes with previous associated studies and performed enrichment analysis, to reveal the underlying mechanism and response to $PM_{2.5}$ exposure.

2. Material and methods

2.1. Study population

Our study was conducted in the Nation Survey of Physical Traits (NSPT) cohort, which was a population-based cohort study which enrolled participants of Chinese nationality in four sampling times from different suburban regions of China: Taizhou, Jiangsu in August 2015; Zhengzhou, Henan in July 2017; Nanning, Guangxi in March 2018 and Taizhou, Jiangsu in March 2019 (Figure S1.A). Individuals were recruited as volunteers by random selection, and those with any critical illness were excluded from recruitment. Residential street address of each participant at the time point of recruitment was collected by questionnaire and used for $PM_{2.5}$ prediction. The individual DNAm level was obtained using the Illumina Infinium Methylation EPIC BeadChips from blood samples, with 811,876 CpG probes retained after quality control. Outputs were the beta values that represent the percentage of methylation for every CpG probe (detailed in Supplementary). Covariates incorporated in our study were collected by the personal questionnaire which includes sex, age, smoking status, smoke pack year, passive smoking, alcohol consumption, education and household income. BMI was derived from on-site measured height and weight. Genetic principal components (PCs) were calculated by principal component analysis on genotypes, to reveal and adjust for potential population structure. Blood leukocytes fractions (B cells, $CD4^+$ and $CD8^+$ T cells, NK cells, monocytes and neutrophils) were estimated based on DNAm measurement using EpiDISH (Zheng et al., 2018). A total of 3365 individuals with complete street address, matched DNAm data and other individual information were included for follow-up analysis (Figure S1.B). The study was approved by the Ethics Committees of Fudan University (14117) and the Shanghai Institutes for Biological Sciences (ER-SIBS-261410), and all participants provided written informed consents.

2.2. $PM_{2.5}$ exposure assessment

2.2.1. $PM_{2.5}$ predictions

$PM_{2.5}$ concentrations were estimated by random forest method at a daily level and 1 km × 1 km resolution during 2005–2019 in mainland China with full spatiotemporal coverage. The details of model development and evaluation process were described and published in previous studies (Meng et al., 2021; Shi et al., 2023) and summarized here. Random forest models were trained with ground $PM_{2.5}$ measurements served as a dependent variable, and Multi-Angle Implementation of Atmospheric Correction aerosol optical depth (MAIAC AOD), MERRA-2 simulated $PM_{2.5}$ concentrations, meteorological parameters, land use data and population density served as independent variables. The overall ten-fold cross validation R^2 and root-mean-square error (RMSE) values between $PM_{2.5}$ measurements and predictions at daily level of random forest models were 0.84 and 16.08 $\mu g/m^3$, respectively. The monthly and annual mean $PM_{2.5}$ concentrations were calculated for each grid cell at 1-km spatial resolution. The $PM_{2.5}$ concentrations of grid cells were assigned to each participant according to the individual residence information.

2.2.2. Derived $PM_{2.5}$ exposure in different time windows

To optimize exposure windows, monthly mean $PM_{2.5}$ concentrations in the prior 12 months of sampling points and annual mean $PM_{2.5}$ concentrations in the prior 10 years of sampling points were predicted for each participant. Based on the monthly mean $PM_{2.5}$ predictions in 12 months, we calculated the mean value of each ascending time windows, from the current month of the sampling point, to the previous 12 months by a one-month step, and defined these measurements as short-medium-term $PM_{2.5}$ exposure. In the following analysis, the $PM_{2.5}$ exposure level in the current month of sampling points was described as "the current month", the one month prior to the sampling points as "the previous month", and the average from current to the X month prior as "X months prior".

We also inferred the one-year and one-year-prior $PM_{2.5}$ predictions as medium-term exposure, and the average of prior ten years measurement (described as "10 years prior") as long-term exposure. The derived short-to-medium-, medium- and long-term exposures were included in the following EWAS analysis.

2.3. Statistical analysis

2.3.1. Epigenome-wide association analysis

We conducted EWAS using a generalized linear regression model (GLM) to capture the correlation between DNAm level at each probe and $PM_{2.5}$ exposure with *limma* R package (Ritchie et al., 2015). Age, sex,

smoking status (not, former and current smoker), smoke pack year, BMI, sampling points (Taizhou2015; Zhengzhou2017; Nanning, 2018 and Taizhou2019), blood leukocytes fractions and the first ten genetic PCs were included as covariates in the EWAS model. Multiple testing was corrected for by using a commonly recognized epigenome-wide significance threshold $P = 5 \times 10^{-8}$. We performed a comparative association test using generalized linear mixed effect regression model (GLMEM) with duplicateCorrelation() function from *limma*, by incorporating sampling points as random effect terms instead of fixed effect term in prior GLM, to account for non-independence within the sample.

Moreover, we furtherly performed subgroup stratified EWASs and meta-analysis, to investigate heterogeneity across different sampling points (Supplementary section 2). We also conducted sensitive analysis to investigate the confounding effect of the covariate choice in EWAS model, by excluding each covariate in the primary model, as well as additionally including potential factors (Supplementary section 3).

2.3.2. Differentially methylated regions (DMRs)

In addition to the analyses of effect on individual CpG, we performed DMR analyses to investigate the effect of $PM_{2.5}$ exposure on regional methylation. We applied two algorithms, *dmrff* (Suderman et al., 2018) and *comb-p* (Pedersen et al., 2012). Both methods used the result of summary statistic data of EWAS on single probe level as input. In details, for *comb-p*, DMRs with Sidak-corrected p-value below 0.01 and including at least three probes within 500 bp were considered significant. *Dmrff* function starts by identifying all candidate regions and then shrinks the regions by calculating statistics for all sub-regions. Regions composed of at least 1 nominal significant CpG and consistent direction of effect within 500bp as the candidate region. $P < 0.01$ after the Benjamini-Houchberg adjustment was considered as significant. To reduce false positives, we leverage the results identified by both methods as DMRs.

Moreover, to estimate if $PM_{2.5}$ exposure affects DNAm level on specific genomic regions, we calculated the arithmetic mean of beta-values across all probes in each of the regions (gene bodies, promoters, CpG islands, shelves, shores, and OpenSeas) and defined as regional methylation level. Regional annotation was performed with *annotatr* R package (Cavalcante and Sartor, 2017). Multivariable linear regression models were used to test the association between $PM_{2.5}$ exposure and regional methylation levels, adjusted with the same covariates in the EWAS models.

2.4. Functional analysis on identified markers

$PM_{2.5}$ associated CpGs were assigned to genes using *annotatr* R package. The mapped genes were annotated with related traits from previous association studies, by querying against EWAS atlas (https://ngdc.cncb.ac.cn/ewas/atlas), EWAS catalog (http://ewascatalog.org/) and GWAS catalog (https://www.ebi.ac.uk/gwas/) databases. We furtherly performed enrichment analysis on $PM_{2.5}$ associated genes on Metascape (https://metascape.org/) to identified enriched pathway.

3. Results

The NSPT cohort consisted of 3365 Han Chinese individuals, of which 524 were recruited in Taizhou in 2015, 951 in Zhengzhou in 2017, 1402 in Nanning in 2018 and 488 in Taizhou (2019). Matched $PM_{2.5}$ exposure level, blood sample and other personal information were available for all participants. The population consisted of 1253 (37.24%) male participants and 2112 (62.76%) female participants, and aged from 18 to 83 years old (mean ± SD = 50.29 ± 12.62). A total of 484 (38.63%) of the male participants and 2086 (98.77%) female participants were non-smoker. Characteristics summary of all personal information were shown in Table 1.

Table 1
Characteristics summary of individual in NSPT cohort.

		Pooled	Taizhou, 2015	Zhengzhou, 2017	Nanning, 2018	Taizhou, 2019
Sample size, N		3365	524	951	1402	488
Sex, N (%)						
	Female	2112 (62.76)	316 (60.30)	567 (59.62)	889 (63.41)	340 (69.67)
	Male	1253 (37.24)	208 (39.40)	384 (40.38)	513 (36.59)	148 (30.33)
Age, Mean (sd)		50.29 (12.62)	48.19 (12.75)	43.88 (13.29)	54.60 (11.06)	52.63 (9.21)
BMI, Mean (sd)		24.51 (3.54)	24.66 (3.68)	25.13 (3.67)	23.75 (3.29)	25.33 (3.38)
Smoke status, N (%)						
	Never smoker	2570 (76.37)	385 (73.47)	723 (76.02)	1083 (77.25)	379 (77.66)
	Former smoker	149 (4.43)	31 (5.92)	39 (4.10)	63 (4.49)	16 (3.28)
	Current smoker	646 (19.20)	108 (20.61)	189 (19.87)	256 (18.26)	93 (19.06)
Smoke pack year, Mean (sd)		5.38 (15.51)	5.02 (13.44)	4.06 (11.88)	6.4 (18.73)	5.42 (13.25)
Passive smoking						
	Yes	1789 (53.16)	284 (54.20)	441 (46.37)	788 (56.20)	276 (56.56)
	No	1576 (46.84)	240 (45.80)	510 (53.63)	614 (43.80)	212 (43.44)
alcohol consumption, N (%)						
	Rarely	2755 (81.87)	400 (76.34)	800 (84.12)	1164 (83.02)	391 (80.12)
	Once a week	199 (5.91)	26 (4.96)	84 (8.83	76 (5.42)	13 (2.66)
	2-3 times a week	82 (2.44)	0 (0)	33 (3.47)	37 (2.64)	12 (2.46)
	>3 times a week	313 (9.30)	98 (18.70)	25 (2.63)	118 (8.42)	72 (14.75)
	NA	16 (0.48)	0 (0)	9 (0.95)	7 (0.50)	0 (0)
Education, N (%)						
	Uneducated	291 (8.65)	86 (16.41)	23 (2.42)	97 (6.92)	85 (17.42)
	Primary	862 (25.62)	131 (25.00)	110 (11.57)	467 (33.31)	154 (31.56)
	Junior secondary	1270 (37.74)	192 (36.64)	305 (32.07)	586 (41.80)	187 (38.32)
	Senior secondary	535 (15.90)	64 (12.21)	209 (21.98)	222 (15.83)	40 (8.20)
	Tertiary and above	405 (12.03)	51 (9.73)	303 (31.86)	29 (2.07)	22 (4.51)
	NA	2 (0.06)	0 (0)	1 (0.11)	1 (0.07)	0 (0)
Annual Household income in CNY, N (%)						
	<2500	47 (1.40)	7 (1.34)	3 (0.32)	36 (2.57)	1 (0.20)
	2500–4999	64 (1.90)	7 (1.34)	2 (0.21)	53 (3.78)	2 (0.41)
	5000–9999	162 (4.81)	17 (3.24)	23 (2.42)	116 (8.27)	6 (1.23)
	10,000–19,999	284 (8.44)	21 (4.01)	53 (5.58)	189 (13.48)	21 (4.30)
	20,000–34,999	480 (14.26)	65 (12.40)	125 (13.14)	238 (16.98)	52 (10.66)
	>35,000	1953 (58.04)	403 (76.91)	733 (77.08)	480 (34.24)	337 (69.06)
	NA	375 (11.14)	4 (0.76)	12 (1.26)	290 (20.68)	69 (14.14)

3.1. $PM_{2.5}$ exposure level across all time windows

The distribution of the calculated mean $PM_{2.5}$ exposure level suggested an increasing trend in variation as the time window expand (Fig. 1A). When stratified by sample subgroups, the inter-group variants escalated in longer timescale exposure, corresponding to the raising trend in pooled data. Meanwhile, the intra-group variants revealed relatively minor alteration in each time window for all sub-groups, except the Zhengzhou 2017, with exceeding variations in most time windows (Fig. 1B, Figure S2). The difference of $PM_{2.5}$ exposure and low-to-moderate inter-group overlap suggested an inter-group heterogeneity and spatially differed seasonal patterns, which could be explained by the seasonal, years and geographical difference. While the exposure levels peaked in November to February, and decreased in July and August in all region, the distribution showed unsynchronized peaks according to the recruitment time, as well as different levels according to geographical regions and years (Fig. 1B, Figure S2). Compared to the WHO (Organization, 2021) and national guideline (2012), the $PM_{2.5}$ exposure levels in Taizhou (2015), Zhengzhou 2017; Taizhou 2019 were all higher than the recommended annual air quality guideline level (35 $\mu g/m^3$), while in Nanning (2018), the exposure levels were comparative to the guideline level. Also, we observed a relatively higher correlation for time windows within 4 months or above, but rather lower correlation between 2 sides (Fig. 1C).

3.2. Epigenome-wide analyses

EWAS identified methylation levels on 175 CpGs significantly associated ($P < 5 \times 10^{-8}$) with $PM_{2.5}$ exposure in four short-medium time windows, of which 50 CpGs associated with exposure of the current month, 48 CpGs associated with exposure of the previous month, 147 CpGs associated with exposure of 1 month prior and 23 CpGs with exposure of 2 month prior, respectively (Fig. 2 and Table S1A). Meta-analysis on the subgroup stratification EWASs additionally identified 2 CpGs (Supplementary section 2 and Table S1A). We furtherly performed a regression test on the 177 identified CpGs using generalized linear mixed effect model (GLEME). By incorporating sampling points as random effect terms in GLMEM, we observed that the betas were less differential from zero, but overall concordant between GLM and GLMEM utility. P values on several CpGs were reduced, which resulted in a total of 37 CpGs remained genome-wide significant (Figure S3, Table S2). Taken together, the identified CpGs in EWAS with GLM were overall robust in a GLMEM regression. Moreover, EWAS on medium- and long-term $PM_{2.5}$ exposure collectively identified one significant CpG cg03801758 (Figure S4, Table S1B), which however, were lack of significance in the prior EWASs on short-medium-term exposure ($P > 0.05$). To enhance clarity on the epigenetic effect of short-medium $PM_{2.5}$ exposure, we included the 177 CpGs associated with $PM_{2.5}$ exposure in four short-medium time windows as the primary outcome for follow-up analysis.

After subgroup stratification, 23 of the 177 CpGs showed inter-group consistency, with betas at same direction and nominal significance ($P < 0.05$) (Figure S5.A), and a total of 67 CpGs shared betas with same direction across all sub-groups. Meanwhile, several CpGs showed diverse methylation alteration across the 4 subgroups. Specifically, a proportion of CpGs were significantly associated in samples from Nanning (2018) subgroup, while showed negligible or reversed effect in other subgroups (Figure S5.B).

Sensitive analysis with the leave-one-out tests demonstrated that the effect sizes on the identified CpGs were robust to intrinsic and extrinsic factors including age, sex, smoking (both active and passive smoking), BMI, population genetic PCs, alcohol consumption, education level and household income. On the other hand, the effect sizes on several CpGs were sensitive to blood leukocytes fractions, which implies that different blood cells may exhibit varying patterns of methylation changes on the identified CpGs, in response to $PM_{2.5}$ exposure, suggesting a blood-cell-type specific effect of $PM_{2.5}$ exposure (Figure S6).

3.3. Comparison on methylation effect across moving time windows of $PM_{2.5}$ exposure within one year

Of the 177 CpGs, 66 were associated with at least 2 short-medium-term exposure and 8 were associated with all four short-medium-term exposure, with genome-wide significance. We conduct a further comparison on the identified CpGs across moving time windows within 1 year as well as one-year-medium- and 10-years-long-term (Fig. 3). All CpGs showed consistent beta direction in response to short-medium-term $PM_{2.5}$ exposure within 3 months. With the extension of time scale, the effect size on most CpGs tended to drift towards zero and did not reach nominal significant ($P > 0.05$). Additionally, one CpG, cg03801758, was found significant and showed hypermethylation effect with 9 months average exposure and above, including 1-year mid-term exposure and 10-years long-term exposure (Fig. 3, Figure S4, Table S1B). This CpG cg03801758 also reached nominal significance ($P < 0.05$) in previous 2–9 months average exposure but was not significant ($P > 0.05$) within one month prior.

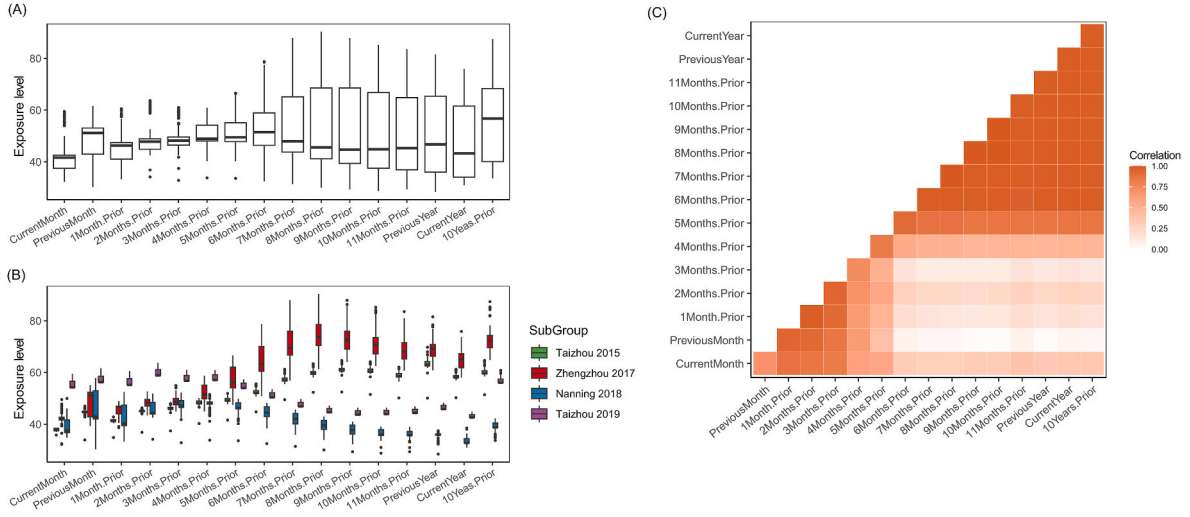

Fig. 1. Distribution of $PM_{2.5}$ exposure level across time windows. (A) Bar plot of the overall distribution of $PM_{2.5}$ exposure level. (B) Bar plot of the distribution of $PM_{2.5}$ exposure level in each subgroup. (C) Correlation of $PM_{2.5}$ exposure level across time windows.

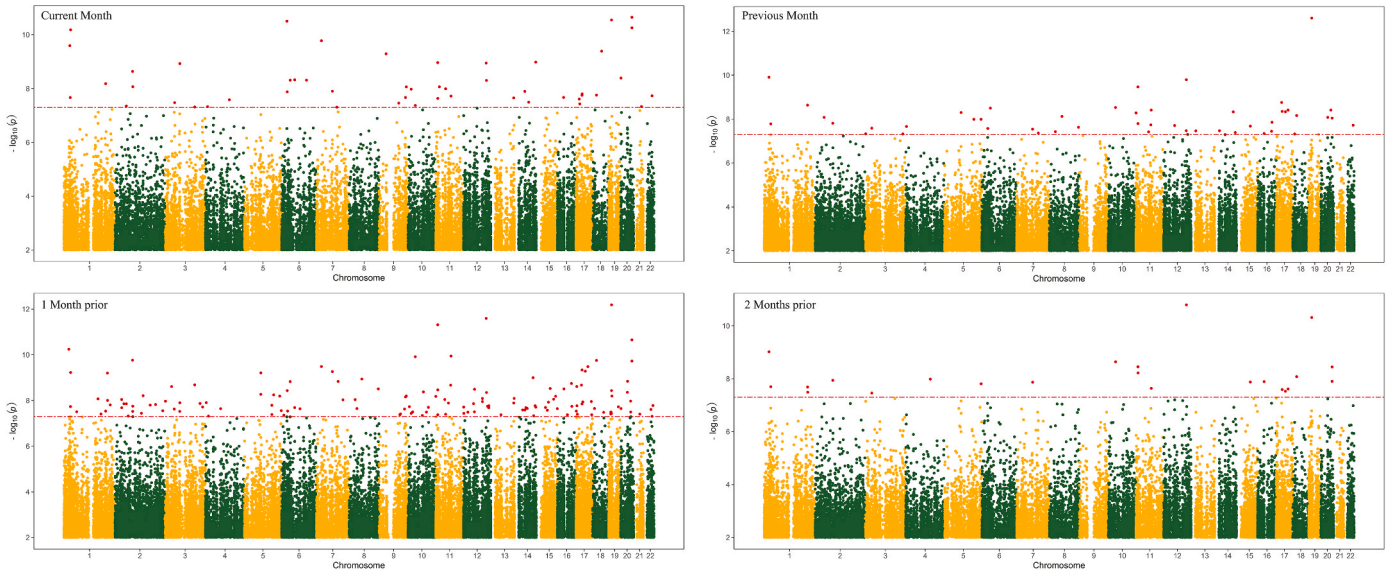

Fig. 2. Manhattan plot of EWAS on $PM_{2.5}$ exposure in four short-medium time windows. The red line corresponds to the genome-wide significant threshold ($P \leq 5 \times 10^{-8}$). Significant CpGs were plotted in red. Yellow and green represented chromosomes.

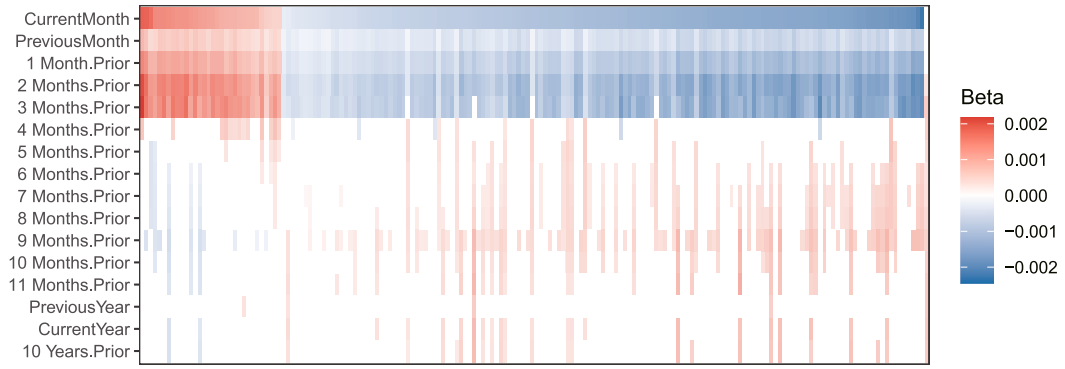

Fig. 3. Comparison of effect size estimate on identified CpG across time windows. The columns represented for a total of 178 CpGs, of which 177 CpGs associated with short-medium-term exposure, and one cg03801758 on the rightmost column. Red and blue represented hypermethylation and hypomethylation in response to $PM_{2.5}$ exposure, respectively.

3.4. replication of previously published CpGs associated with $PM_{2.5}$

For independent replication, we examined for validation on the reported CpGs from the previous EWAS on $PM_{2.5}$ exposure. Based on the search strategy (described in Supplementary section 4), we initiated from 14 manuscripts of studies on DNAm effect of $PM_{2.5}$ or $PM_{2.5}$ component (Table S3). Afterwards, following the inclusion criterion, we finally included 2 EWASs that focused on 1-month $PM_{2.5}$ exposure (Panni et al., 2016; Wang et al., 2022), and 3 EWASs on 1-year $PM_{2.5}$ exposure (Chi et al., 2016; Dai et al., 2017; Plusquin et al., 2017). We obtained the reported CpGs from the studies, of which 1657 CpGs from (Panni et al., 2016), 2498 from (Wang et al., 2022), 5 from (Chi et al., 2016), 10 from (Plusquin et al., 2017), and 17 from (Dai et al., 2017) were included in NSPT study. An overlap of 11 CpGs were both reported in the two studies on 1-month average $PM_{2.5}$ exposure (Table S4), and none overlap were reported in the 3 EWASs on 1-year $PM_{2.5}$ exposure. The 177 identified CpGs from NSPT cohort did not overlap with the previously published ones. We furtherly performed a validation test to see if the previously published CpGs could be replicated in our results with betas in consistent direction and at a nominal significance ($P < 0.05$). A total of 208 out of 1657 and 281 out of 2498 previously published CpGs were replicated in our short-medium-term EWAS, and 3 out of 37 replicated in medium-term EWAS results. Specifically, cg15297799, which were reported by both two previous studies, were replicated in all 4 short-medium-term EWASs, while another 3 CpGs were replicated by one of the short-medium-term EWASs, and the other 7 CpGs were not replicated (Figure S7, Table S4).

Permutation tests on the replication revealed that the reported 1-month exposure associated CpGs from (Panni et al., 2016) were significantly differential methylated compared to background CpGs ($P_{t.test} < 0.01$), and up to 9328 out of 10,000 iterations determined the significance (Fig. 4); while the replication on 1-month exposure associated CpGs from (Wang et al., 2022) and on 1-year exposure associated CpGs did not pass the permutation tests, with $P_{t.test} > 0.05$ for the majority of the iterations (results not shown).

3.5. Differentially methylated regions (DMRs) analysis

In addition to CpG-level EWAS study, we also focus on DMR analysis to raise the statistical power of detecting methylation changes by combining information from multiple CpGs rather than evaluating sites one by one (Breton et al., 2017). By applying *dmrff* and *comb-p*, we identified 107 overlapped DMRs in relation to short-medium-term $PM_{2.5}$ exposure, and mapped to 80 genes (Table S5C). A set of 9 genes (*KDM2B, KLF1, LAX1, NR0B2, RASSF2, TLDC2, TRIB1, VKORC1L1, ZBTB48*) annotated by significant DMRs was consistently observed across four

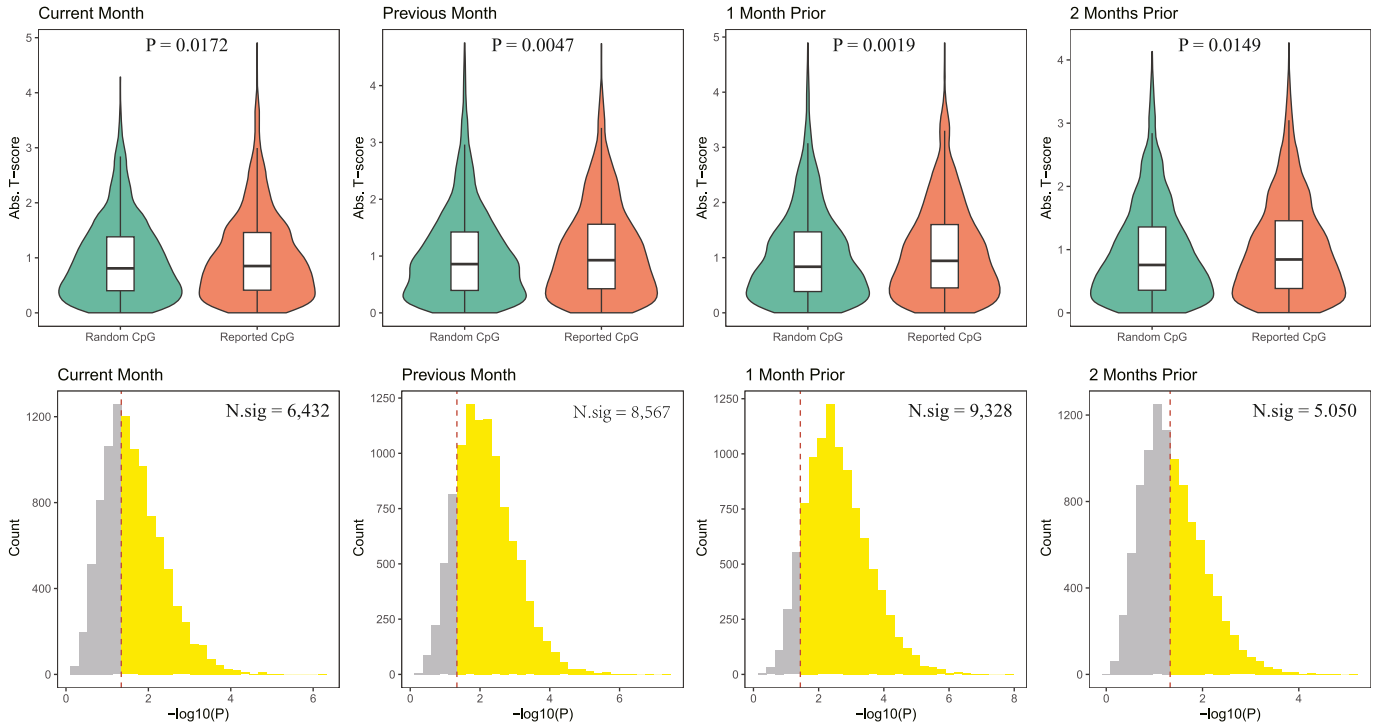

Fig. 4. Permutation tests on the replication of 1657 reported CpGs. (A) Violin plots of single permutation test. T-score of 1657 CpGs was the ratio of beta to its standard error obtained from our EWAS results, and a larger absolute T-score represented more significance. P value was derived from one-sided *t*-test between T-scores of 1657 reported CpGs and 1657 random CpGs. (B) Histograms of 10,000-times-iterated permutation tests. Red dot line represented significance of P = 0.05. N. sig represented the number of tests revealed more significance for reported CpGs than random.

exposure windows and another 14 genes (*ACOT7, C5orf63, DHFR, FAM13A, FGD4, GFOD1, HVCN1, IGF1R, KLHDC7B, LDLRAD3, PSMD13, SLC12A4, SMG6, VTI1A*) were observed in at least 3 exposure windows. Although a DMR does not necessarily contain significant CpGs, we spoted that 6 significant CpGs (cg03144560, cg20323725, cg04272309, cg10653259, cg06304167, cg26136776) identified in EWAS were also located within DMRs.

Moreover, by leveraging the genomic region annotation, we tested for association on the average methylation level of genomic components, and spotted significantly hypo-methylation on promoters, gene bodies and CpG shores, associated with short-medium-term $PM_{2.5}$ exposure (Table S6).

3.6. Gene annotation and enrichment analysis

The associated markers were mapped to 134 genes (Table S7). By leveraging databases of association studies, we overlapped the mapped genes with previous related GWAS and EWAS (Fig. 5 and Table S7). A total of 48 genes had been previously reported affected by $PM_{2.5}$ or $PM_{2.5}$ element exposure, which revealed certain consistency between our results and previous studies on $PM_{2.5}$ exposure. In addition, we observed a series of genes associated with nitrogen dioxide (NO_2) exposure or other chemical exposure, and 43 genes reported in EWAS on tobacco smoking, electronic cigarettes as well as on smoking initiation, cessation and maternal smoking. Meanwhile, many of the genes were reportedly associated with various health outcomes in prior EWASs or GWASs, including aging, immune-related traits and inflammation disease, metabolic disease, nervous system diseases and cancers.

These results suggested that $PM_{2.5}$ exposure might share DNAm alteration patterns or downstream gene regulation with other airborne pollutant and chemical pollutants such as NO_2 and smoking. The biological response related to PM2.5 exposure might also affect the development of aging, immune and inflammation disease, metabolic disease, neurodegenerative disease and cancers.

We conducted enrichment analysis for assigned genes from EWASs and DMRs. The top enriched pathways included cell cycle, actin filament reorganization, cell division and intracellular signal transduction as well as signal pathway involving rho GTPase and IL-18. Other pathways included the regulation of cell adhesion and migration, sulfur compound and phosphate as well as modification-dependent macromolecule metabolic process, lysosome, forebrain development, cellular component organization, organelle disassembly and maternal process involved in female pregnancy (Fig. 6). Moreover, we observed a set of five genes *PTPN12* (cg19643792), *UBB* (cg00960580), *EPS15L1* (cg14210191), *PTEN* (cg01740552) and *ITCH* (cg18058448) enriched on *EGF/EGFR* signaling pathway (Table S8), which has been recently reported as a key mechanism of the tumor-promoting effect of $PM_{2.5}$ exposure (Hill et al., 2023).

4. Discussion

We proposed a workflow with a) modeled prediction on individual level $PM_{2.5}$ exposure measurements based on cohort data of healthy participants, b) epigenome wide association study to identity related DNAm change, c) sensitive analysis to investigate robustness and heterogeneity, d) association analysis to test the methylation change at clusters of CpG sites within genomic regions and e) follow-up functional analysis to reveal underlying biological mechanism. To our knowledge, this study is the first cohort-based EWAS on $PM_{2.5}$ exposure in Chinese population. With a relatively large sample size and solid statistic power, we identified epigenome-wide significant signals associated with individual $PM_{2.5}$ exposure level from CpG-level EWASs and cluster-level DMR analyses.

Overall, gene annotation suggested that $PM_{2.5}$ exposure related genes involved in aging, immunity, inflammation and carcinogenesis, metabolic disease and nervous system related diseases. These showed consistent patterns that have been observed in previous studies (Wang et al., 2020; Yin et al., 2021; Zhou et al., 2016). Specifically, a large

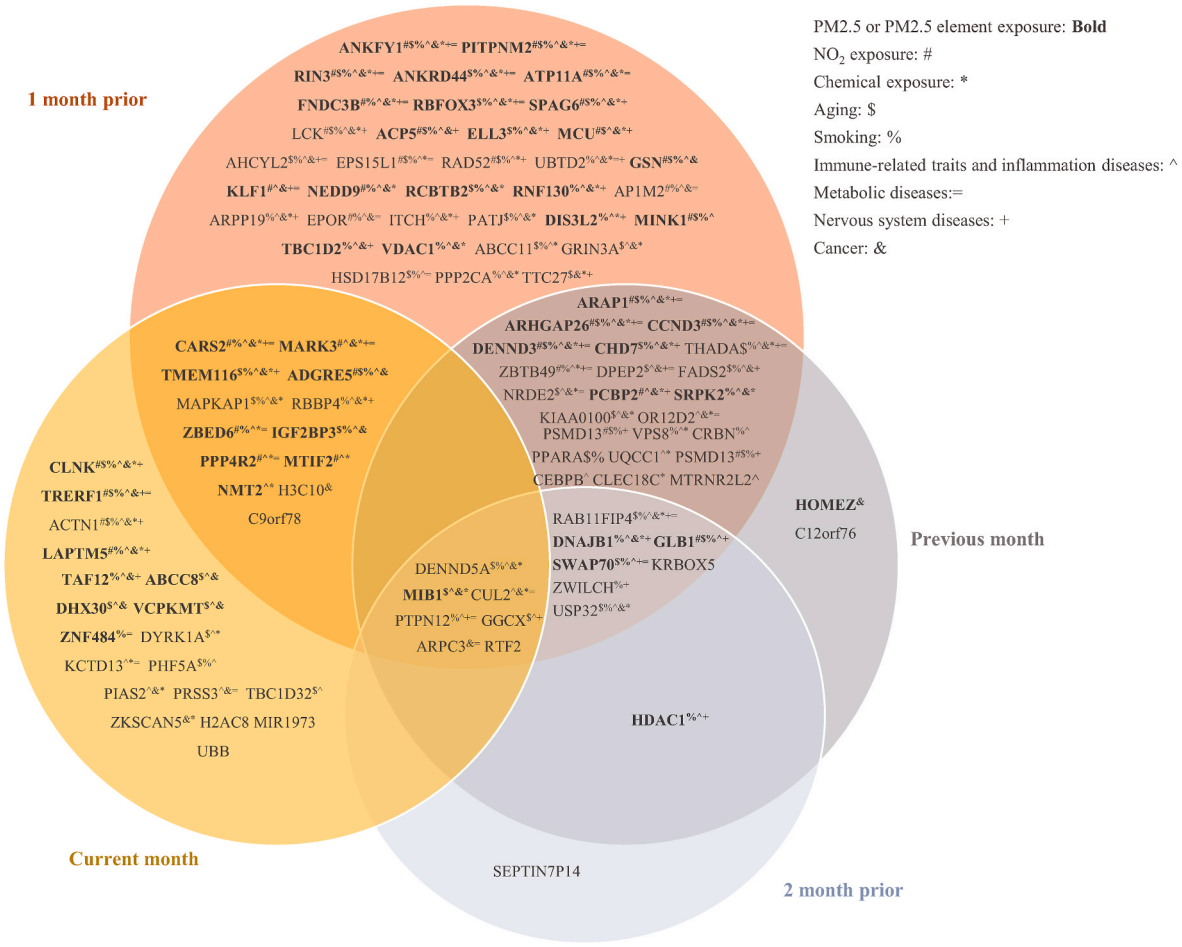

Fig. 5. Venn diagram of genes identified from EWAS on $PM_{2.5}$ exposure and overlapping with previous association studies. Bold and superscripts indicated that the gene was previously reported to be associated with certain traits.

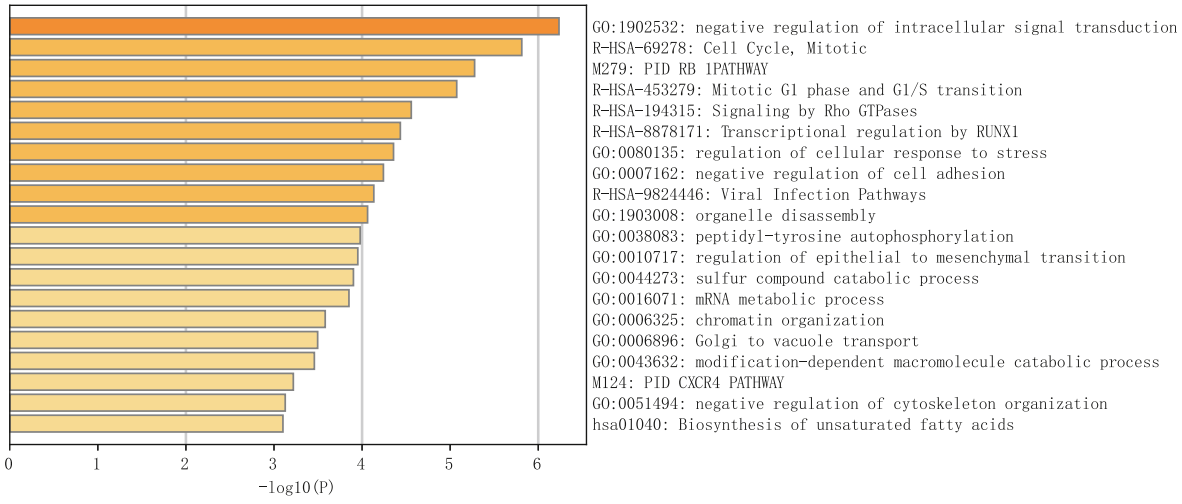

Fig. 6. Enriched pathways of $PM_{2.5}$ exposure related genes. Enrichment analysis was conducted on the mapped genes. The P value was derived by comparing the observed frequency of genes involving in a pathway term with the frequency expected by chance.

proportion of the identified genes were associated with immune-related traits and inflammation disease, which were well-recognized mechanism induced by $PM_{2.5}$ exposure, and documented in both previous mechanistic and epidemiological studies. For example, Yang et al. reported $PM_{2.5}$ exposure exacerbated asthma, of which the effect was regulated by interleukins (Yang et al., 2020). A noticeable association between $PM_{2.5}$ exposure and rheumatoid arthritis have been proposed (Adami et al., 2021; Park et al., 2021) and also highlighted by a most recent EWAS study on $PM_{2.5}$ exposure (Wang et al., 2022). And Liu, Cong et al. pointed a positive association between $PM_{2.5}$ exposure and diabetes mellitus type 2 risk, which may be explained by the fact that $PM_{2.5}$ exposure-induced inflammatory response increase may insulin

antagonism (Liu et al., 2022). Besides, many identified genes were also reported to be related with nervous system diseases. Previous epidemiological studies have shown that air pollution may cause systemic inflammation, microglia activation, oxidative stress and neuro inflammation (Haghani et al., 2020) which provides a biological rationality and potential mechanism for the observed association between exposure and subsequent risk of neurodegenerative and neurodevelopmental diseases. Several studies have revealed that exposure to $PM_{2.5}$ are linked to an increased risk of metabolic disorders in mice model (Ran et al., 2021). Additionally, we found overlaps between the identified genes and the associated genes from previous EWAS on $PM_{2.5}$ exposure, NO_2 exposure and smoking, as well as multiple chemical exposure, suggesting that various toxicological particles might be related in epigenomic effect, trigger similar biological responses likewise, or even share certain risks on health outcomes.

Enrichment analysis identified biological functions related to cell cycle, cell division and inflammatory pathway. Oxidative stress induced by $PM_{2.5}$ is considered to be an important mechanism of $PM_{2.5}$ mediated toxicity. We also observed several compounds metabolic processes were significantly enriched. Many organic chemicals on the surface of $PM_{2.5}$ can be metabolized and activated into reactive electrophilic metabolites, which may produce or increase intracellular reactive oxygen species (Suo et al., 2020; Torres-Ramos et al., 2011). Due to the exposure of $PM_{2.5}$, reactive oxygen species interact with biological macromolecules (such as plasma lipid, protein and DNA), producing various adverse effects on cells, damage cellular structure and function, and result in triggering the disturbance of cellular response, such as cell adhesion and migration. Several significant pathways and underlying mechanism have been adequately supported by previous studies. A most recent study reported that the interaction of $PM_{2.5}$ exposure and mutation on the driver gene *EGFR* significantly promote the incidence of lung cancer (Hill et al., 2023). We observed a gene set enriched in *EGF/EGFR* signaling pathway and specifically, the coding protein of *EPS15L1* participated in the phosphorylation of *EGFR* and regulated the activation of downstream signaling. These findings support the tumor-promoting role for $PM_{2.5}$ exposure in *EGFR* driven lung cancer, suggesting a mechanism mediated by DNAm modification. Jeong et al. identified a possible mechanism of $PM_{2.5}$ exposure induced lung toxicity, involving growth factor receptor (Jeong et al., 2017). Pan et al. have demonstrated the toxicity of Pb, one of the $PM_{2.5}$ elements, which induced an overexpression of Rho GDP-dissociation inhibitor 2 (RhoGDI2) in mice (McCracken et al., 2010). What's more, neurodevelopment is a most concerning health outcome affected by $PM_{2.5}$ exposure, and increasing evidence have revealed $PM_{2.5}$ exposure may damage the developing brain and contribute to neurodevelopmental disorders.

On top of the identified genes and pathways, our study provided insight in the effect of $PM_{2.5}$ exposure on DNAm across different time windows. To date, most of the studies focused on $PM_{2.5}$ exposure of single period, and only a very limited number of studies investigated the shared and differed pattern across short-, medium- and long-term $PM_{2.5}$ exposure (Gao et al., 2019; Mostafavi et al., 2018; Panni et al., 2016). Sergi et al. performed a replication test between previously published short-term exposure associated CpGs and 10 years $PM_{2.5}$ exposure (Sayols-Baixeras et al., 2019). Although daily measurement on $PM_{2.5}$ was absent in our study, and thus we failed to examine the effect of short-term $PM_{2.5}$ exposure within monthly time window, we did obtain the broadest range of time windows, including monthly, annual and 10-years exposure. We found the methylation levels on the identified CpG sites changed acutely only under short-medium-term $PM_{2.5}$ exposure. Meanwhile, the one CpG associated with medium-term exposure did not response to short-medium-term exposure. This suggested that the short-medium-term exposure and medium-to long-term exposure may trigger different biological response. We also noticed that short-medium-term $PM_{2.5}$ exposure was associated with a relatively larger amount of CpGs compared with medium- or long-term exposure. Such pattern was supported by results from previous studies as well. Panni et al. and Wang et al. identified 1819 and 2717 CpGs associated with monthly $PM_{2.5}$ exposure (Panni et al., 2016; Wang et al., 2022), respectively, while EWAS on medium- and long-term exposure reported association of no more than double digits (Chi et al., 2016; Dai et al., 2017; Plusquin et al., 2017). This might suggest that the methylome effect of $PM_{2.5}$ was mostly temporary and did not retain for a period over one year. Additionally, experimental and observational studies both indicated association between methylation changes and rapid $PM_{2.5}$ exposure even under days or hours. (Bellavia et al., 2013; Bruniquel and Schwartz, 2003; Chen et al., 2016). Previous studies reported human erythrocytes normally have a life span of 100–120 days. Changes in methylation levels brought about by the renewal of blood cells may have corresponding with our results that most significant CpGs were identified from exposure window within 2 months to some extent (Muzykantov, 2010). However, it is worth discussing that a residence-based long-term $PM_{2.5}$ prediction might be less accurate if an individual was based at multiple places during the long period, and thus the prediction was not able to reflect the true long-term $PM_{2.5}$ exposure levels.

We conducted multiple sensitive analyses to assess the robustness of the identified CpGs (detailed in Supplementary). To explore the influence of samples in each sub-group, we compared the results from pooled data, with the effect size estimates from group stratified EWAS and the leave-one-group-out models (Fig. S5). Combined, we found that the identified CpGs from pooled data were mostly affected by samples from Nanning and Zhengzhou groups. A relatively modest sample size in Taizhou (2015) group might lead a low statistic power to detect the differential methylation level. Also, there were disparities on $PM_{2.5}$ exposure levels across subgroups, as well as a confounding effect of subpopulation on methylation profiles, which might result in the heterogeneity, even though we have adjusted the group index as covariance. Such confounding effects of subgroups were mixed and hardly distinguishable, which we speculated might partially resulted from regional $PM_{2.5}$ components difference, supported by (Dai et al., 2017) that different particulate components of $PM_{2.5}$ have various methylation targets. Despite of heterogeneity, several CpGs shared consistency between the two main subgroup, Nanning and Zhengzhou, and also reached significance in meta-analysis. Besides, sensitive analysis revealed that the effect size estimated of the identified CpGs were not confounded by any of the covariates, except blood leukocytes fractions (Fig. S6). This demonstrated the robustness of the methylation change on the identified CpGs.

We performed a comprehensive review on previous EWASs on $PM_{2.5}$ exposure, followed by replication test and permutation test. Heterogeneity existed across studies, as little overlap was observed on reported CpGs from each study, nor between our identified CpGs and previous results. This might result from population disparity, different measurement on $PM_{2.5}$ exposure, or any unmeasured confound. $PM_{2.5}$ exposure, as a general measurement of fine particles, might masked the complexity of toxicology of various component, and led to study-specific results (Dai et al., 2017; Wang et al., 2022). Also, as pointed out by Mostafavi et al. methylation change induced by $PM_{2.5}$ might be subtle and thus likely to be undetected with limited statistical power (Mostafavi et al., 2018). Nevertheless, a moderate proportion of identified CpGs from (Panni et al., 2016) were replicated in our results, and permutation test determined that the significance was not arisen by random chance. And despite lacking overlap on associated CpGs with published ones, we identified genes that has been previously reported associated with $PM_{2.5}$ exposure, which suggested that methylome effect of $PM_{2.5}$ exposure might share similarity on gene level across studies and cohorts.

We would like to acknowledge the limitation of this study. First of all, we only collected the residential address at the time point of recruitment, but not the whole address history for the past 10 years. Therefore, there was a potential misclassification risk for the residence-based $PM_{2.5}$ prediction, especially for medium- and long-term, as the prediction might be less accurate if an individual was based at multiple

places during the period. Also, the disparity between ambient $PM_{2.5}$ measurement and individual $PM_{2.5}$ exposure could be affected by the frequency of outdoor activity, as those who were more outdoor active would expose to more severe ambient $PM_{2.5}$, which might bring error to exposure measurement. Secondly, the epigenetic effects of mid- and long-term PM2.5 exposure were much less significant compared to short-medium-term exposure. We reasoned that the limited dispersion of sampling points and residency could resulted in less variation of medium- and long-term PM2.5 exposure (Fig. 1), which might not be sufficient enough to identified potential associated CpGs in EWASs. Thirdly, gene expression and immune markers were not available in the current stage of NSPT cohort, and thus we were not capable to examine the role of identified CpGs in gene expression regulation or immune reaction with paired omics data. Fourthly, DNAm was profiled in blood leukocytes; the role of identified CpGs, genes or pathways may not apply in all $PM_{2.5}$ associated health outcomes, especially tissue-specific diseases. Last but not least, the inconsistency across subgroups and time windows, as well as the lack of cross-study replication or external validation implied a potential risk of false positive finding. More valid studies are expected to cross examinate the methylation effect of short-, intermediate- and long-term $PM_{2.5}$ exposure.

Our study did show certain notable strengths. For one, we applied a validated prediction model to obtain individual $PM_{2.5}$ exposure. It was more cost efficient and practical compared with trial study with laboratory conditions or personal monitoring devices, which made it possible to measure $PM_{2.5}$ exposure in cohort study, and performed EWAS with a relatively large sample size and statistical power. It was also more precise than station monitoring measurement, with higher spatial resolution. Besides, we conducted a proper systematic review, aiming at leveraging the current understanding on the methylome effect of $PM_{2.5}$ exposure. What's more, we made comparison on the exposure from monthly to 10 years' time window, and provided advanced insight in the pattern of methylation change induced by short-, medium- and long-term $PM_{2.5}$ exposure.

5. Conclusions

In conclusion, this study conducted the first EWASs of monthly exposure in Han Chinese population. With a relatively large sample size and adequate statistic power, we identified 177 CpGs and 107 DMRs associated with short-medium-term $PM_{2.5}$ exposure. $PM_{2.5}$ associated markers were related to aging, immunity, inflammation and carcinogenesis, as well as various toxicological particles. Enriched pathways were mostly involved in cell cycle and cell division, signal transduction, inflammatory pathway, biological compound metabolic process, cell adhesion and migration as well as forebrain development and maternal process.

Building upon the insights gleaned from this study, several promising avenues for future research emerge. Firstly, given the adaptability and flexibility of our PM2.5 exposure prediction model, future investigations conducted in cohorts with larger and mixed population could be beneficial to achieve more enhanced results. Secondly, comparative studies across diverse demographic groups could shed light on the observed disparity across the findings of existing EWASs on $PM_{2.5}$. Additionally, longitudinal studies are warranted to explore the dynamic of the epigenetic effects of $PM_{2.5}$ exposure. Lastly, grounded in findings of pathways acknowledged by ours and prior studies, further investigations through multi-omics integration will be beneficial, to advance the understanding on how DNAm mediate the biological response of $PM_{2.5}$ exposure.

CRediT authorship contribution statement

Xiyang Cai: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Qinglin Song:** Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Xia Meng:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Kaixuan Li:** Formal analysis. **Su Shi:** Formal analysis, Methodology. **Li Jin:** Resources, Supervision. **Haidong Kan:** Conceptualization, Supervision, Writing – review & editing. **Sijia Wang:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The summary data were provided in the attached supplementary.

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Appendix A. Supplementary data

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