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Associations of Heavy Metals with Activities of Daily Living Disability: An Epigenome-Wide View of DNA Methylation and Mediation Analysis

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Background: Exposure to heavy metals has been reported to be associated with multiple diseases. However, direct associations and potential mechanisms of heavy metals with physical disability remain unclear.

Objectives: We aimed to quantify associations of heavy metals with physical disability and further explore the potential mechanisms of DNA methylation on the genome scale.

Methods: A cross-sectional study of 4,391 older adults was conducted and activities of daily living (ADL) disability were identified using a 14-item scale questionnaire including basic and instrumental activities to assess the presence of disability (yes or no) rated on a scale of dependence. Odds ratios (ORs) and 95% confidence intervals (CI) were estimated to quantify associations between heavy metals and ADL disability prevalence using multivariate logistic regression and Bayesian kernel machine regression (BKMR) models. Whole blood–derived DNA methylation was measured using the HumanMethylationEPIC BeadChip array. An ADL disability-related epigenome-wide DNA methylation association study (EWAS) was performed among 212 sex-matched ADL disability cases and controls, and mediation analysis was further applied to explore potential mediators of DNA methylation.

Results: Each 1-standard deviation (SD) higher difference in $\log_{10}$-transformed manganese, copper, arsenic, and cadmium level was significantly associated with a 14% (95% CI: 1.05, 1.24), 16% (95% CI:1.07, 1.26), 22% (95% CI:1.13, 1.33), and 15% (95% CI:1.06, 1.26) higher odds of ADL disability, which remained significant in the multiple-metal and BKMR models. A total of 85 differential DNA methylation sites were identified to be associated with ADL disability prevalence, among which methylation level at cg220000984 and cg23012519 (annotated to *IRGM* and *PKP3*) mediated 31.0% and 31.2% of manganese-associated ADL disability prevalence, cg06723863 (annotated to *ESRP2*) mediated 32.4% of copper-associated ADL disability prevalence, cg24433124 (nearest to *IER3*) mediated 15.8% of arsenic-associated ADL disability prevalence, and cg07905190 and cg17485717 (annotated to *FREM1* and *TCP11L1*) mediated 21.5% and 30.5% of cadmium-associated ADL disability prevalence (all $p < 0.05$).

Discussion: Our findings suggested that heavy metals contributed to higher prevalence of ADL disability and that locus-specific DNA methylation are partial mediators, providing potential biomarkers for further cellular mechanism studies. https://doi.org/10.1289/EHP10602

Introduction

High exposure to heavy metals has been reported to be adversely associated with neurodegenerative,[1] cardiovascular,[2] or respiratory disease.[2,3] However, these single system analyses might fail to capture the associations between heavy metals and overall health, especially for older adults.[4] As an indication of overall health for older adults, activities of daily living (ADL) disability represents the common functional consequence of multiple chronic diseases on individual difficulty in carrying out activities to maintain their independence.[5] People reporting ADL disability are likely to have impairment in motor, cognitive, or psychiatric conditions, which are common functional consequence of subclinical process and chronic diseases, such as arthritic, cerebrovascular, diabetes, or cardiovascular diseases.[6] Due to the rapidly growing number of the world's aging population, ADL disability prevalence has increased and raised worldwide concern about its increasing burden on health care costs among older adults.[7] However, to our knowledge, only one study has evaluated the associations between cadmium and lead and disability, reporting significant relationships between lead and decreased functional dependence.[8] Therefore, studies are urgently warranted to assess the associations and potential mechanisms of multiple heavy metals with ADL disability.

A growing body of studies suggests that DNA methylation, currently one of the most frequently investigated epigenetic mechanisms, is associated with various outcomes in response to environmental exposures.[9–11] Most previous studies hypothesized that methylation of a candidate gene was associated with adverse health consequences of heavy metals. For example, Nourian et al. investigated the role of APOE and ACKR3 methylation in the association of arsenic with multiple sclerosis.[12] In addition, heavy metals, including manganese,[13] copper,[14] zinc,[15] arsenic,[16] cadmium,[17] and lead,[18] have been reported to modulate neurotoxic-related pathways *in vitro* and *in vivo*. For example, manganese exposure might trigger JAK2-STAT3 signaling pathway in microglia, leading to resultant neuroinflammation and neuronal loss, as suggested by an *in vitro* study using HAPI microglial cells.[13] However, knowledge on the exact biological pathways underlying multiple adverse effects of heavy metals remains inconclusive. Growing genome-wide methylation assays allow exploration of DNA methylation changes in response to exposures and diseases on the epigenome-wide scale with no preconceived research hypotheses (e.g., Meng et al.,[19] which might provide new sights and biomarkers for formulating potential pathophysiological mechanisms in a hypothesis-free environment. To our knowledge, no prior studies have assessed the epigenome-wide associations of DNA methylation with ADL disability and further linked the DNA methylation with heavy metals. The role of DNA methylation in mediating the association between metals and ADL disability among older adults has not been investigated.

In the present study, we conducted a population study of 4,391 older adults and determined concentrations of blood metals (manganese, copper, zinc, arsenic, cadmium, and lead) to explore

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the associations between heavy metals and ADL disability. DNA methylation levels at >850,000 sites across the genome were measured in whole blood. As a noninvasive substitute, blood specimens are typically much more convenient than internal tissues to obtain from human subjects, and whole blood–derived DNA methylation has been widely used in the published studies.[20,21] Consistent changes of DNA methylation between tissues and blood samples support the inference that DNA methylation in blood can mirror corresponding methylation signatures in internal tissues,[22,23] suggesting the potential of blood DNA methylation as a surrogate measure of methylation at less accessible internal tissues.[24] Therefore, we investigated the associations of ADL disability-related whole blood DNA methylation with heavy metals. Mediation analysis has been widely used in epidemiological studies to disentangle biological mechanisms that link an exposure to an outcome under a counterfactual framework.[25] The role of blood DNA methylation in mediating associations between heavy metals and ADL disability was further assessed among older adults.

Methods

Study Population

The study participants originated from three towns (Wuzhuan, Sanshi, and Donglan) of the Hongshuihe Basin in Guangxi, China. A cross-sectional survey was carried out from August 2016 to July 2018. Local residents were invited to take part in the survey in appointed clinics, in consideration of variations in local medical conditions, scattered villages in each town, and investigation coherence in the study. Participants were eligible if: *a*) they had been living in the study area for more than 10 y and *b*) they were ≥60 y old at the time of enrollment. Individuals were excluded for the following reasons: *a*) they were aphasic, deaf, or blind; *b*) they had psychiatric disturbances with use of psychotropic drugs; or *c*) they had been diagnosed with severe organic diseases such as malignant tumors. Each participant was administered a detailed structured questionnaire, and information such as demographic and socioeconomic characteristics, and lifestyle habits were obtained through face-to-face interviews. Education attainment was classified as less than primary school, primary school, and high school and above. Participants who had always smoked ≥1 cigarette per day or drank alcohol ≥1 time per week over the past 6 months were defined current smokers and current drinkers, respectively; former smokers and former drinkers were those who had quit smoking or drinking at the time of interview; individuals without smoking or drinking in his or her lifetime were classified as nonsmokers and nondrinkers. Individuals who had suffered a prolonged (≥3 nights per week for more than 3 months) and abnormal inability to obtain adequate and uninterrupted sleep were defined as having insomnia. After an overnight fast, each participant went through anthropometric and clinical examination by trained physicians and provided their fasting venous blood samples. A 10.0-mL blood sample was drawn with one ethylenediamine tetraacetic acid (EDTA) anticoagulation tube and one vacuum coagulation tube. A total of 4,621 local residents age ≥60 y were recruited in the study between 2016 and 2018. With an exclusion of 230 participants who failed to complete required examinations, 4,391 participants were finally included in the current study. In addition, we measured genome-wide DNA methylation in a pilot study ($n = 240$) from the participants; among whom there were 106 ADL disability cases, and sex-matched controls ($n = 106$) were selected from the 240 participants that had DNA methylation measured. An ADL disability-related epigenome-wide DNA methylation analysis was conducted to find potential mediating DNA methylation sites. All participants in our study were informed and provided their written consent. The research protocol was approved by the ethics committee of Guangxi Medical University.

Assessment of ADL Disability

A variety of questionnaires were designed to measure disability irrespective of function-related conditions, among which the Katz's index of ADL (KI) and Lawton-Brody Instrumental ADL (LB-IADL) have been widely used in epidemiological studies (e.g., Ruiz et al. 2020,[26] Lv et al. 2021,[27] and many other functional scales have been built on the basis of the two scales).[28] KI is a six-item scale of basic activities, including bathing, dressing, toileting, transferring, eating, and continence, which was the standard yet less restrictive tool with well-established credibility.[29] In addition to the basic activities, instrumental activities of daily living were assessed using LB-IADL scale, with eight items listed: the ability to perform telephone calls, shopping, transportation, handing medications, managing finances, doing the laundry, preparing meals, and housekeeping.[30]

Assessment tools for the present study included basic and instrumental activities using KI and LB-IADL to measure the presence of functional limitation or disability rated on a scale of dependence. To improve the ability to detect subtle differences, modified approaches to scoring have been used from dichotomous rating to a 4-point scale (independence, some difficulty, some assistance, and dependence) in a hierarchical order. In the present study, ADL disability (yes/no) was identified if individuals self-reported needing assistance or dependence in any domains of the 14 daily activities.

Metals Determination

The concentrations of blood manganese, copper, zinc, arsenic, cadmium, and lead were measured using inductively coupled plasma mass spectrometry (ICP-MS; Thermo Scientific). Briefly, frozen aliquots of 500 μL blood sample from each subject were taken from a −80°C refrigerator, and then samples were left to set at −20°C for 8 h and 4°C for 8 h, for melting slowly without damaging blood erythrocytes. Afterward the samples were brought to room temperature for balance and mixed sufficiently using vortex oscillator, diluted with a nitric acid solution containing 0.1% (v/v) nitric acid, 1% (v/v) n-butanol, and 0.1% (v/v) Triton for full nitrification. The final fully mixed solutions were injected into a single quadrupole ICP-MS (iCAP RQ; Thermo Scientific) with kinetic energy discrimination mode. The concentrations of blood metals were identified and quantified using daily standard curves. The quality control was performed by the certified reference materials (Seronorm Trace Elements Whole Blood RUO No. 210,105, 210,205, and 210,305; ALS Scandinavia) and standard reference material (SRM1640a; Trace Elements in Natural Water from Natural Institute of Standard Technology) to assess the method accuracy and instrument stability. Three levels of certified reference materials and SRM1640a were measured every 20–25 samples and assured the measured concentrations were in agreement with the certified concentration range of each metal before subsequent sample assay. The range of the limit of detection (LOD) of blood manganese, copper, zinc, arsenic, cadmium, and lead was from 0.000 μg/L to 0.118 μg/L, and there were no samples measured below the LODs of those metals (shown in Table S1).

Genome DNA Methylation Assay

The genomic DNA from clotted blood was extracted using DNeasy Blood and Tissue Kit (Qiagen) and bisulfite converted 500 ng of DNA using the Zymo EZ DNA Methylation-Gold kit.

DNA methylation levels of prepared samples were then measured following the manufacturer's guide and protocol for the Infinium HumanMethylationEPIC BeadChip array (Illumina, Inc.). Last, EPIC BeadChips were scanned with the iScan system (Illumina, Inc.), and a measure of DNA methylation at >850,000 CpGs was detected. Samples were randomized across and within plates to minimize potential batch effects. The raw IDAT files of methylated and unmethylated probe intensity were produced from Illumina GenomeStudio software.

Quality control for filtering at sample-level and probe-level were conducted as follows. There were no samples having 10% or more sites with a detection p-value >0.01. Meanwhile, CpG sites with a detection p-value >0.01 in one or more samples were removed ($n = 17{,}231$). We removed CpG sites with bead counts of <3 in at least 5% of samples ($n = 4{,}211$). A mass of cross-reactive CpG sites ($n = 8{,}464$) or sites extending to single-nucleotide polymorphisms (SNPs) with mirror allele frequency >0.05 ($n = 7{,}447$), as well as some non-CpG sites ($n = 2{,}586$) or sites annotating to the sex chromosomes ($n = 18{,}280$) were removed. A total of 807,699 CpGs passing quality control in 212 samples were included in the subsequent analysis. In a further step, BMIQ was applied to normalize the raw data due to InfII and InfII probes bias, and batch effects of plates were also adjusted using the ComBat function in R.

Statistical Analysis

Concentrations of blood manganese, copper, zinc, arsenic, cadmium, and lead were $\log_{10}$-transformed due to right-skewed distributions (shown in Table S2). Multivariate logistic regression models were carried out to assess the odds ratios (ORs) and 95% confidence interval (CI) of ADL disability, which were estimated by 1-standard deviation (SD) higher difference in $\log_{10}$-transformed metals as continuous variables. Both single-metal and multimetal models were conducted to check whether these metals were indeed independently associated with ADL disability, and six metals were individually or simultaneously included in the two types of models, respectively. Considering correlations and collinearity between metals, we further used Bayesian kernel machine regression (BKMR) to confirm the results from single-metal and multimetal regression models. The R package BKMR conducts a Bayesian inference for the probit regression model (BKMR-P) by using the variable selection method with 10,000 iterations of Markov chain Monte Carlo algorithm. The posterior inclusion probabilities (PIP) obtained from the BKMR-P quantify the relative importance of each exposure in the model; they are a ranking measure to see how much the data favor the inclusion of a variable in the model. Visual inspections on the trace plots of model parameters was used to monitor convergence. The function h() is an exposure–response function that accommodates nonlinearity and/or interaction among the mixture components. Several additional sensitivity analyses were conducted in the present study to evaluate the robustness of our results. Due to the different magnitudes of blood metals, exposures were z-scored on the same scale in the BKMR-P model as a sensitivity analysis to assess the robustness of our results. Associations of metals with ADL disability were conducted in the subgroup that had DNA methylation measured using the multivariate logistic regression model as a sensitivity analysis. To assess the associations of metals with ADL disability type, we also performed sensitivity analysis in individuals with basic ADL (BADL) disability and instrumental ADL (IADL) disability, respectively. To control potential confounding, all models were adjusted for sex (male or female), age (continuous), body mass index (BMI, continuous), education (less than primary school, primary school, high school and above), annual income [<10,000 renminbi (RMB), ≥10,000 and <30,000 RMB, and ≥30,000 RMB], smoking status (current smokers, former smokers, nonsmokers), drinking status (current drinkers, former drinkers, nondrinkers), and insomnia (yes or no) based on the prior knowledge of factors affecting blood metals concentrations and ADL disability.[31,32] In the analysis of total population ($N = 4{,}391$) and the subset that had DNA methylation measured ($n = 212$), data were complete for variables used in the study.

To investigate the association between ADL disability and DNA methylation on individual CpG sites across the genome, we fitted linear regression models with logit transformed methylation beta values using the limma package. We performed surrogate variable analysis (SVA) to adjust for unknown biological and technical effects using the SVA package and included surrogate variables (SVs) as covariates for each CpG in genome-wide methylation analyses. Houseman cell proportions (CD8T, CD4T, NK, B cells, monocytes, and granulocytes) were also estimated and used as adjustment variables in the regression models.[33] An epigenome-wide DNA methylation association study (EWAS) was prone to suffer inflation and bias of test statistics,[34] and a Bayesian method using R package BACON was proposed and widely used to control the amount of bias and inflation.[35] Inflated test statistics (lambda) was corrected using BACON, and p-values were estimated after correction for inflation and bias in the study. Previous studies of epigenome-wide analyses of DNA methylation using BACON package reported lambda varying from 0.79 to 1.40,[36,37] which supported the confidence of our study (lambda:1.29). A false discovery rate (FDR) threshold was determined at $\alpha = 0.05$ and delta beta was determined at $|\text{delta beta}| = 0.2$ as the threshold for epigenome-wide testing. CpG sites were plotted by a volcano plot and those passing the threshold were considered genome-wide significant. The distributions of ADL disability associated CpGs were compared across the genomic features. The intergenic CpGs were annotated to the nearest genes using the matchGenes function of the DMRcate package. The epigenome-wide analysis for the differentially methylated region (DMR) associated with ADL disability were also conducted using DMRcate package. Significant CpG sites associated with ADL disability were enriched for gene ontology (GO) terms or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using missMethyl package with a FDR <0.05.

Linear mixed models were performed to evaluate the association of DNA methylation with ADL disability-related metals, and participants recruited in different towns were included as the random intercept effect in the model. Methylation levels at significantly differential CpGs associated with ADL disability were then tested for their association with blood manganese, copper, arsenic, and cadmium level, respectively, to further explore ADL disability-related and metals-related CpG sites for subsequent mediation analysis. The potential mediation of CpG sites linking ADL disability with metals was assessed by mediation analysis using the bootstrapping method in the "mediation" package. We performed two models to test the mediating effects as follows, and their estimates were used as input for the mediation function:

$$M = \alpha_0 + \alpha_{metal} X_{metal} + \alpha_C X_C + e_1$$

and

$$Y = \nu_0 + \nu_{metal} X_{metal} + \nu_{methylation} X_{methylation} + \nu_C X_C + e_2.$$

The first model is the mediator model, which examined the association between blood metals and DNA methylation, and the second model is the outcome model, which assessed the combined

association between blood metals and the mediator (DNA methylation) and the ADL disability prevalence. Significant mediation by DNA methylation was considered present when the p-value of the mediating effect was <0.05. The proportion of mediation by DNA methylation was calculated as the following formula: Prop. Mediated $= [\alpha_{metal} \times \nu_{metal} \div (\alpha_{metal} \times \nu_{methylation} + \nu_{metal}) \times 100]$.

Data analysis was performed using R (version 3.3.6; R Development Core Team).

Results

Basic Characteristics

The basic characteristics of 4,391 participants are depicted in Table 1. Most of the participants were female (~59.5%), and the mean age was 69.0 y (SD: 7.74). Individuals with ADL disability were more likely to be female and older. Individuals with ADL disability were more likely to have an education less than primary school, earn an annual income <10,000 RMB, be noncigarette smokers and nonalcohol drinkers, and report insomnia. Median values of blood manganese, copper, zinc, arsenic, cadmium, and lead across all participants were 22.1, 864, 10,850, 2.25, 3.63, and 51.3 μg/L, respectively. In comparison with controls, concentrations of manganese, copper, arsenic, and cadmium were significantly higher in those with ADL disability (all $p < 0.05$).

Association between Blood Metals and ADL Disability

In the single-metal models, each 1-SD higher difference in $\log_{10}$-transformed manganese, copper, arsenic, and cadmium was associated with a significantly higher prevalence of ADL disability, and the ORs were 1.14 (95% CI: 1.05, 1.24), 1.16 (95% CI: 1.07, 1.26), 1.22 (95% CI: 1.13, 1.33), and 1.15 (95% CI: 1.06, 1.26), respectively (shown in Table 2). Because metals might be correlated with each other, we herein conducted the multiple-metal model including six metals, and the result was consistent with single-metal models, where significant associations with ADL disability were also observed for manganese, copper, arsenic, and cadmium (shown in Table 2).

For eliminating collinearity between metals, we further used BKMR models to confirm the results from logistic regression models. The joint effect was statistically significant when all metals were at or above their 50th percentile, as compared with when all metals were at their median values, and higher probit of ADL disability was observed at higher levels of the joint exposures (Figure 1A; Excel Table S1). Additionally, significant associations with ADL disability were also observed for manganese, copper, and arsenic when other metals set at their 25th, 50th, and 75th percentile, though the association of cadmium was approaching significance (p-values: 0.50 and 0.53) when other metals set at their 50th and 75th percentile (Figure 1B and Excel Table S2). The univariate exposure–response functions showed a suggestion of significantly linear association of manganese, copper, arsenic, and cadmium with ADL disability (Figure 1C; Excel Table S3). Exposure–response slopes for relationships between metal pairs and probit of ADL disability appeared to be either parallel or approximately parallel, except for arsenic and cadmium, indicating potential interactions between arsenic and cadmium in their association with ADL disability prevalence (Figure 1D; Excel Table S4). The analyses yielded similar results when blood metals concentrations were handled on the same scale in the BKMR-P model (shown in the Figure S1 and Excel

Table 1. General characteristics and blood metals levels of the study population between 2016 and 2018 in Guangxi, China ($N = 4,391$).

	Total	Non-ADL disability	ADL disability	p-Value
No.	4,391	3,430	961	—
Sex [n (%)]				
Male	1,779 (40.5)	1,547 (45.1)	232 (24.1)	<0.001
Female	2,612 (59.5)	1,883 (54.9)	729 (75.9)	
Age [y (mean ± SD)]	69.0 ± 7.74	68.7 ± 6.51	74.4 ± 9.85	<0.001
BMI [kg/m^2 (mean ± SD)]	22.0 ± 3.22	22.1 ± 3.14	21.6 ± 3.47	0.010
Education attainment [n (%)]				
Less than primary school	2,004 (45.6)	1,372 (40.0)	632 (65.8)	<0.001
Primary school	1,461 (33.3)	1,199 (35.0)	262 (27.2)	
High school and above	926 (21.1)	859 (25.0)	67 (7.00)	
Annual income [n (%)]				
<10,000 RMB	1,697 (38.6)	1,189 (34.7)	508 (52.8)	<0.001
≥10,000 and <30,000	1,151 (26.2)	913 (26.6)	238 (24.8)	
≥30,000 RMB	1,543 (35.1)	1,328 (38.7)	215 (22.4)	
Cigarette smoking [n (%)]				
Current smokers	695 (15.8)	613 (17.9)	82 (8.50)	<0.001
Former smokers	55 (1.30)	54 (1.60)	1.00 (1.00)	
Nonsmokers	3,641 (82.9)	2,763 (80.5)	878 (91.5)	
Alcohol drinking [n (%)]				
Current drinkers	1,004 (22.8)	879 (25.6)	125 (13.0)	<0.001
Former drinkers	30 (0.70)	26 (0.80)	4 (0.40)	
Nondrinkers	3,357 (76.5)	2,525 (73.6)	832 (86.6)	
Insomnia [n (%)]				
Yes	1,504 (34.3)	1,132 (33.0)	372 (38.7)	0.001
No	2,887 (65.7)	2,298 (67.0)	589 (61.3)	
Blood metals [μg/L (median IQR)]				
Manganese (Mn)	22.1 (9.20)	21.8 (9.06)	23.4 (9.87)	<0.001
Copper (Cu)	864 (182)	855 (179)	892 (189)	<0.001
Zinc (Zn)	10,850 (2550)	10,900 (2550)	10,760 (2550)	0.330
Arsenic (As)	2.25 (1.07)	2.23 (1.08)	2.32 (1.10)	0.007
Cadmium (Cd)	3.63 (3.62)	3.57 (3.58)	3.93 (3.70)	<0.001
Lead (Pb)	51.3 (28.4)	51.3 (28.8)	51.2 (27.6)	0.650

Note: Data were complete for all variables and presented as n (%) for categorical data, mean ± SD for parametrically distributed data, or median (IQR) for nonparametrically distributed data. p-Values were estimated by Student's t-test or Mann-Whitney U test for continuous variables according to the data distribution and chi-square test for categorical variables. —, no data; ADL, activities of daily living; BMI, body mass index; IQR, interquartile range; RMB, renminbi; SD, standard deviation.

Table 2. Associations of blood metal with prevalence of ADL disability in single-metal and multimetal models for participants between 2016 and 2018 in Guangxi, China.

	ORs (95% CIs) for ADL disability per SD higher difference in $\log_{10}$-transformed metals for all subjects ($N = 4{,}391$)		ORs (95% CIs) for ADL disability per SD higher difference in $\log_{10}$-transformed metals for subgroup ($n = 212$)	
Variables	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Single-metal models				
Manganese (Mn)	1.14 (1.05, 1.24)	0.002	1.15 (1.01, 1.32)	0.003
Copper (Cu)	1.16 (1.07, 1.26)	<0.001	1.16 (1.01, 1.34)	0.001
Zinc (Zn)	1.02 (0.945, 1.11)	0.582	1.00 (0.87, 1.16)	0.964
Arsenic (As)	1.22 (1.13, 1.33)	<0.001	1.25 (1.08, 1.45)	<0.001
Cadmium (Cd)	1.15 (1.06, 1.26)	0.001	1.16 (1.02, 1.33)	0.002
Lead (Pb)	1.09 (1.00, 1.19)	0.050	1.10 (0.969, 1.26)	0.134
Multimetal model				
Manganese (Mn)	1.16 (1.06, 1.26)	0.001	1.15 (1.01, 1.31)	0.003
Copper (Cu)	1.18 (1.08, 1.29)	<0.001	1.17 (1.03, 1.35)	0.001
Arsenic (As)	1.17 (1.06, 1.28)	<0.001	1.22 (1.11, 1.33)	<0.001
Cadmium (Cd)	1.10 (1.00, 1.20)	0.049	1.11 (1.01, 1.21)	0.045

Note: Concentrations of blood manganese, copper, zinc, arsenic, cadmium, and lead were $\log_{10}$-transformed due to right-skewed distributions. The OR and 95% CI of ADL disability were estimated by 1-SD higher difference in $\log_{10}$-transformed metals as continuous variables. *p*-Values of single-metal were estimated using multivariate logistic regression models with the inclusion of each metal individually, and *p*-values of multimetal models derived from including all six metals simultaneously in the multivariate logistic regression model. All models were adjusted for sex (male or female), age (continuous), BMI (continuous), education (less than primary school, primary school, high school and above), annual income (<10,000 RMB, ≥10,000 and <30,000, ≥30,000 RMB), smoking status (current smokers, former smokers, non-smokers), drinking status (current drinkers, former drinkers, non-drinkers), and insomnia (yes or no). ADL, activities of daily living; BMI, body mass index; CI, confidence interval; OR, odds ratio; RMB, renminbi; SD, standard deviation.

Tables S5–S8). The BKMR-P analysis estimated that manganese, copper, arsenic, and cadmium showed higher PIP, with values above 0.5, consistent with the single- and multi metal models (shown in Table S3).

Sensitivity analysis in the subgroup that had DNA methylation measured showed similar results with total population, where significantly positive relationships were visually evident between blood manganese, copper, arsenic, and cadmium and higher

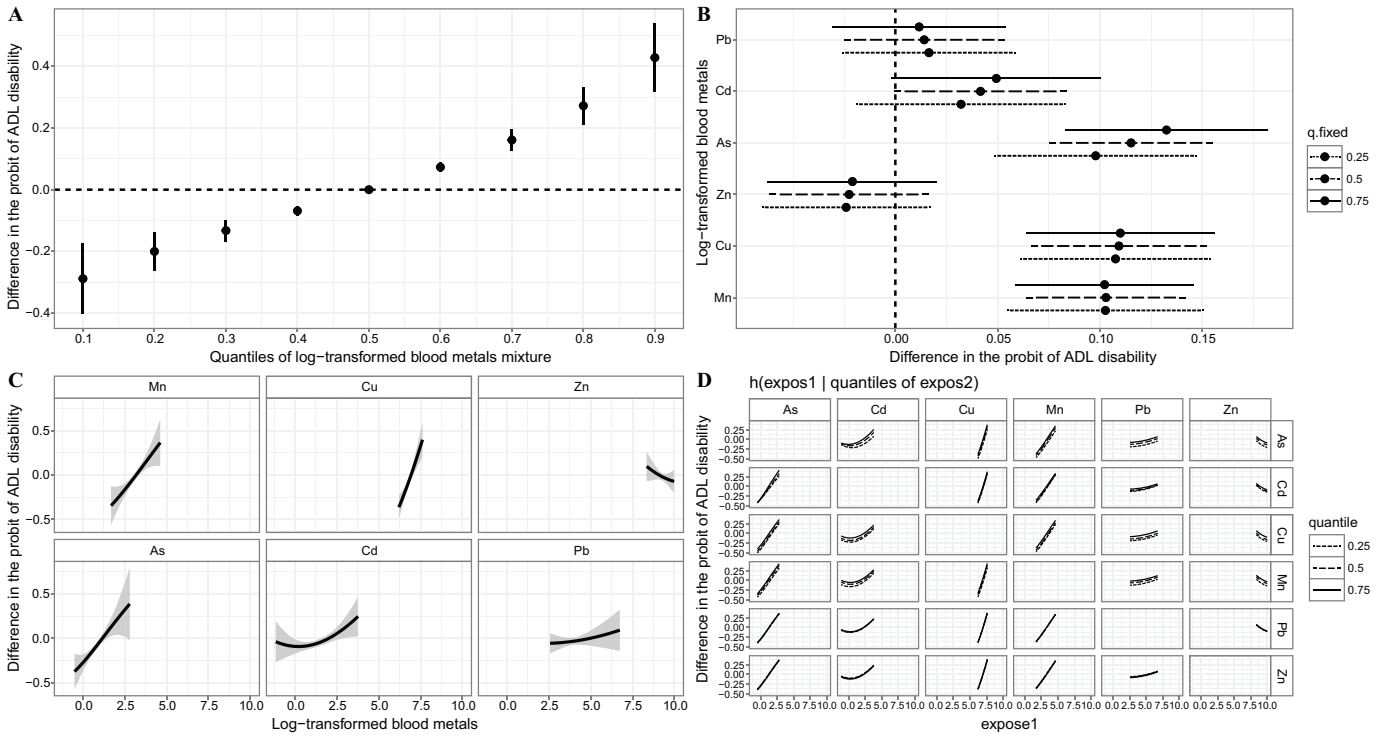

Figure 1. Associations between heavy metals mixture and prevalence of activities of daily living (ADL) disability estimated by Bayesian kernel machine regression (BKMR) for participants between 2016 and 2018 in Guangxi, China ($N = 4{,}391$) (see Excel Tables S1–S4 for corresponding numeric data). Models were adjusted for sex (male or female), age (continuous), BMI (continuous), education (less than primary school, primary school, high school and above), annual income (<10,000 RMB, ≥10,000 and <30,000 RMB, ≥30,000 RMB), smoking status (current smokers, former smokers, nonsmokers), drinking status (current drinkers, former drinkers, nondrinkers), and insomnia (yes or no). (A) Overall association between the heavy metal mixture (estimates and 95% confidence intervals) and ADL disability. This figure plots the estimated difference in the probit of ADL disability when exposures are at a particular percentile (*x*-axis) in comparison with when exposure are all at the 50th percentile. (B) Single pollutant association (estimates and 95% confidence intervals). This plot compares the probit of ADL disability when a single pollutant is at the 75th vs. 25th percentile, when all the other exposures are fixed at either the 25th, 50th, and 75th percentile. (C) Univariate exposure–response functions and 95% confidence bands for each metal with the other pollutants fixed at the median. (D) Bivariate exposure–response functions for one metal when another metal fixed at either the 25th, 50th, or 75th percentile and the remaining metals are fixed at the median. Note: ADL, activities of daily living; BMI, body mass index; RMB, renminbi.

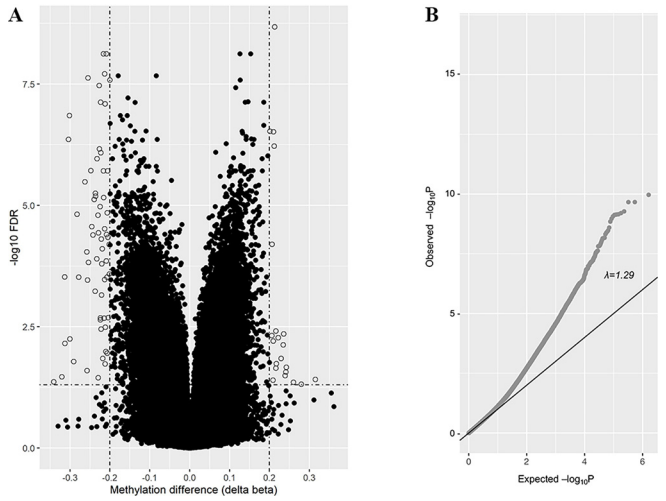

Figure 2. Volcano plot of the association between DNA methylation and ADL disability in the epigenome-wide analysis (A) and QQ plot for epigenome-wide association study (EWAS) performed for ADL disability (B) among participants between 2016 and 2018 in Guangxi, China ($n = 212$) (see Table S4 for corresponding numeric data). In the volcano plot, the x-axis indicates the association of DNA methylation at each CpG site with ADL disability, and y-axis indicates the false discovery rate value. The empty dots represent genome-wide significant CpG sites passing the threshold of a false discovery rate threshold at $\alpha = 0.05$ and |delta beta| at 0.2. In the QQ plot, the graph represents the deviation of the observed p-values plotted against the expected values from a theoretical distribution. The genomic inflation of the analyses was calculated using BACON package and the lambda (λ) of the model was 1.29. Note: ADL, activities of daily living; EWAS, epigenome-wide association study; QQ, quantile–quantile.

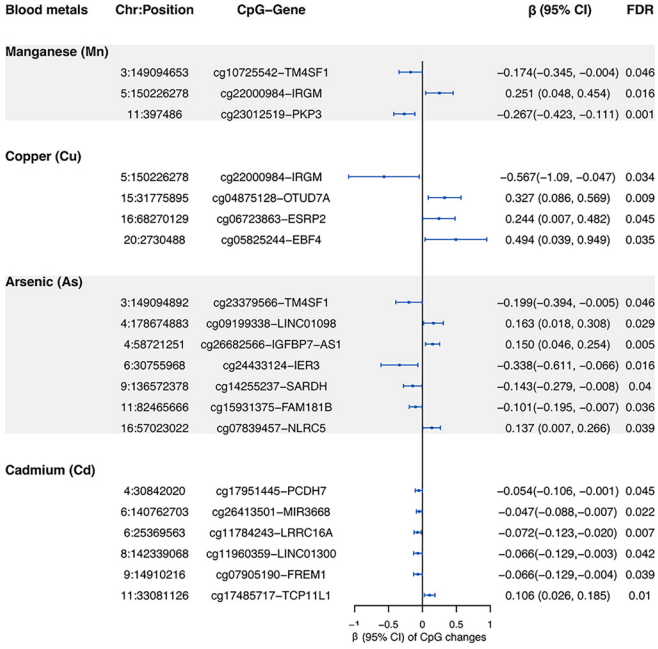

Figure 3. Associations of metals with ADL disability-associated DNA methylation for participants between 2016 and 2018 in Guangxi, China ($n = 212$). Adjusted models were adjusted for sex (male or female), age (continuous), BMI (continuous), education (less than primary school, primary school, high school and above), annual income (<10,000 RMB, ≥10,000 and <30,000 RMB, ≥30,000 RMB), smoking status (current smokers, former smokers, nonsmokers), drinking status (current drinkers, former drinkers, nondrinkers), and insomnia (yes or no). The β and 95% CI of DNA methylation changes were estimated by 1-SD higher difference in $\log_{10}$-transformed metals as continuous variables. Note: ADL, activities of daily living; BMI, body mass index; CI, confidence interval; RMB, renminbi; SD, standard deviation.

prevalence of ADL disability in the subgroup (shown in Table 2). The associations of blood metals with BADL disability and IADL disability were also quantified, respectively (Figure S2). We observed similar associations for IADL disability that were positively associated with higher manganese (OR = 1.14; 95% CI: 1.05, 1.24), copper (OR = 1.16; 95% CI: 1.07, 1.25), arsenic (OR = 1.22; 95% CI: 1.13, 1.32), and cadmium (OR = 1.15; 95% CI: 1.06, 1.26), though significant associations of BADL disability were observed with manganese (OR = 1.06 ; 95% CI: 1.01, 1.11) and arsenic (OR = 1.25 ; 95% CI: 1.17, 1.33). Given that only manganese, copper, arsenic, and cadmium contributed to higher prevalence of ADL disability, the subsequent analyses were restricted to the four metals.

Association between DNA Methylation and ADL Disability

In our genome-wide methylation analysis, 85 differentially methylated probes (DMPs) with ADL disability were identified (shown in Table S4; Figure 2). A total of 64 hypomethylated DMPs and 21 hypermethylated DMPs were associated with higher prevalence of ADL disability. Genomic distributions of 85 DMPs across different genomic regions were not randomly distributed, where the majority were located in the gene body, intergenic region, TS1500, and 5′UTR (shown in Figure S3). The distribution of hypermethylated DMPs was mainly enriched within the islands (57%) region, whereas the majority of hypo-methylated DMPs (78%) were located in the open sea region. Epigenome-wide analysis for the differentially methylated region (DMR) associated with ADL disability showed that the top 15 independent DMRs were identified to be associated with ADL disability at an FDR <0.0001 (shown in Table S5) and contained CpGs annotated to genes involved in neurotoxic-related pathways.

We performed an analysis of KEGG and GO pathway (shown in Figure S4; Tables S6 and S7) on the significant CpG sites associated with ADL disability to help test the relationships between these sites with ADL disability. GO analysis showed that the functions of these sites were enriched in the biological processes of neuronal signaling and associated with neuronal axons, synapses, and ion channel activities, which were crucial in brain homeostasis involved in the ADL disability process.[38] The KEGG pathway enrichment analysis also showed that significant sites were mainly enriched in the MAPK, calcium, cAMP, and Ras signaling pathways, which play important roles for diverse cellular functions in the pathology of ADL disability such as inflammatory reactions, apoptosis, neurodevelopment, and cognition.

Mediation Analysis of DNA Methylation Linking Heavy Metals to ADL Disability

We then assessed the associations of blood manganese, copper, arsenic, and cadmium with 85 ADL disability-associated DNA methylation CpGs and found three CpGs (cg107255, cg220000984, and cg23012519) significantly associated with manganese, four CpGs (cg04875128, cg05825244, cg06723863, and cg22000984) significantly associated with copper, seven CpGs (cg07839457, cg09199338, cg14255237, cg15931375, cg23379566, cg24433124, and cg26682566) significantly associated with arsenic, and six CpGs (cg07905190, cg11784243, cg11960359, cg17485717, cg17951445, and cg26413501) significantly associated with cadmium (FDR <0.05, shown in Figure 3).

As presented in Table 3, mediation analysis of DNA methylation showed that cg220000984 (annotated to immunity-related GTPase M, *IRGM*) and cg23012519 (annotated to plakophilin 3, *PKP3*) mediated 31.0% and 31.2% of the association between

Table 3. Mediating effects of DNA methylation related to blood metals and ADL disability for participants between 2016 and 2018 in Guangxi, China ($n = 212$).

Blood metals	Mediators	Gene description	Estimated natural direct effects (NDE, 95% CI)	Estimated natural indirect effect (NIE, 95% CI)[a]	Proportion mediated (%)[b]	p-Values of mediation[d]
Manganese	cg10725542	*TM4SF1*	0.541 (−0.156, 1.27)	0.041 (−0.037, 0.146)	7.04	0.540
	cg22000984	*IRGM*	0.385 (−0.330, 1.13)	0.173 (0.037, 0.350)	31.0	0.039
	cg23012519	*PKP3*	0.395 (−0.331, 1.14)	0.179 (0.039, 0.357)	31.2	0.026
Copper	cg04875128	*OTUD7A*	0.953 (−0.650, 2.60)	−0.032 (−0.398, 0.321)	NA[c]	—
	cg05825244	*EBF4*	0.909 (−0.690, 2.55)	0.03 (−0.203, 0.281)	3.19	0.340
	cg06723863	*ESRP2*	0.733 (−0.878, 2.38)	0.352 (0.033, 0.786)	32.4	0.042
	cg22000984	*IRGM*	1.695 (−0.023, 3.50)	−0.488 (−0.991, −0.090)	NA[c]	—
Arsenic	cg07839457	*NLRC5*	0.616 (−0.059, 1.32)	0.007 (−0.086, 0.105)	1.12	0.157
	cg09199338	*LINC01098*	0.697 (0.022, 1.41)	−0.116 (−0.258, −0.012)	NA[c]	—
	cg14255237	*SARDH*	0.862 (0.156, 1.61)	−0.184 (−0.371, −0.033)	NA[c]	—
	cg15931375	*FAM181B*	0.616 (−0.060, 1.32)	0.005 (−0.088, 0.100)	0.810	0.171
	cg23379566	*TM4SF1*	0.581 (−0.093, 1.29)	0.042 (−0.031, 0.142)	6.74	0.253
	cg24433124	*IER3*	0.577 (−0.107, 1.29)	0.108 (0.010, 0.243)	15.8	0.038
	cg26682566	*IGFBP7-AS1*	0.725 (0.043, 1.45)	−0.131 (−0.288, −0.013)	NA[c]	—
Cadmium	cg07905190	*FREM1*	0.193 (−0.169, 0.560)	0.053 (0.003, 0.122)	21.5	0.023
	cg11784243	*LRRC16A*	0.218 (−0.136, 0.579)	0.033 (−0.024, 0.102)	13.2	0.380
	cg11960359	*LINC01300*	0.231 (−0.126, 0.596)	0.047 (−0.002, 0.118)	16.9	0.219
	cg17485717	*TCP11L1*	0.205 (−0.157, 0.573)	0.090 (0.021, 0.180)	30.5	0.010
	cg17951445	*PCDH7*	0.212 (−0.144, 0.575)	0.032 (−0.010, 0.093)	13.1	0.203
	cg26413501	*MIR3668*	0.241 (−0.110, 0.599)	−0.004 (−0.038, 0.027)	NA[c]	—

Note: —, no data; ADL, activities of daily living; CI, confidence interval; NDE, natural direct effect; NIE, natural indirect effect.
[a]Mediating (indirect) effect represented effects mediated through DNA methylation and was calculated as a statistical relationship using the "product of coefficients" that multiplies changes in DNA methylation with blood metals and changes in ADL disability with DNA methylation from the "mediation" package.
[b]The proportion of mediation by DNA methylation was calculated as the following formula: Prop. Mediated $= [\alpha_{metal} \times \nu_{metal} \div (\alpha_{metal} \times \nu_{methylation} + \nu_{metal}) \times 100]$.
[c]NA represents that proportion mediated cannot be calculated when there are opposite signs between the direct effect and the mediated effect.
[d]p-Values of mediation was evaluated by the significance test of "product of coefficients" using "mediation" package in R.

blood manganese and ADL disability, cg06723863 (annotated to epithelial splicing regulatory protein 2, *ESRP2*) mediated 32.4% of the association between copper and ADL disability, cg24433124 (nearest to immediate early response 3, *IER3*) mediated 15.8% of the association between arsenic and ADL disability, cg07905190 (annotated to FRAS1 related extracellular matrix 1, *FREM1*), and cg17485717 (annotated to t-complex 11 like 1, *TCP11L1*) mediated 21.5% and 30.5% of the association between cadmium and ADL disability (all p-values of mediating effect <0.05).

Discussion

In the present study, we found for the first time, to our knowledge, that blood manganese, copper, arsenic, and cadmium were significantly associated with higher prevalence of ADL disability from both logistic regression and BKMR models. Locus-specific DNA methylation (annotated to *IRGM*, *PKP3*, *ESRP2*, *IER3*, *FREM1*, and *TCP11L1*) were partial mediators of the association.

Our findings have substantial public health implications. Metals exposure has long been a major environmental health issue that has attracted worldwide attraction. A growing body of research has reported the potential health effects of widespread metals exposure on the public, such as adults,[39] children,[40] and workers.[41] However, the older adult population, the world's fastest-growing demographic group, is more susceptible to multiple metals exposure but has long been understudied in the environmental health.[42] Our findings highlight the public concern regarding the association between metals and ADL disability among older adults and suggest potential mechanisms of DNA methylation on the genome scale, which provides substantial clues and novel insights for further cellular mechanism studies. Effective measures are urgently needed for exposure-reduction policies to reduce the burden of ADL disability, particularly for Chinese older populations, who on average experience higher metals exposure than Western populations,[43,44] underscoring the high priority in potentially pathophysiological biomarkers of locus-specific DNA methylation to lower prevalence of ADL disability related to widespread metals exposure.

Humans are actually exposed to multiple metals in the environment, and growing evidence indicates that coexposure to heavy metals exert varied health effects.[45,46] Few epidemiological studies have considered the joint associations between heavy metals mixture and ADL disability prevalence. Our findings of BKMR reported a strong and linear positive association between the whole heavy metal mixture and ADL disability, indicating synergistic effects of manganese, copper, zinc, arsenic, cadmium, and lead on ADL disability. Potential interactions between arsenic and cadmium associated with ADL disability were suggested in the study. Cadmium and arsenic are widely coexisting pollutants in the environment, which might easily cause multisystem toxicity. Exposure of mouse embryos to cadmium and arsenic during neurulation resulted in differences in the expression of genes related to neurogenesis in a dose-dependent manner.[47] Consistent with our study, Pei et al. found that cadmium and arsenic can synergistically lead to reproductive and developmental toxicity of *C. elegans*.[48] The interaction between cadmium and arsenic is related to physicochemical properties and toxic mechanisms. It was found that both cadmium and arsenic are positively charged ions, which have high affinity for electron-rich thiol and selenol residues in proteins, and can stably bind to them, affecting protein and enzyme activities.[49]

Although manganese is necessary for human health, exposures to high manganese levels are toxic.[50,51] In our study, blood manganese concentration for study participants was 22.1 μg/L, which was much higher than the normal ranges (4–15 μg/L) for adults reported by the Agency for Toxic Substances and Disease Registry.[52] Prior studies of adverse effects resulting from manganese exposure in humans mainly involved cardiovascular,[53] metabolic,[54] and neurological diseases.[55] Our study first evaluated the relationship between manganese and ADL disability prevalence among old adults. Similar findings emerged from animal studies demonstrating that chronic environmental exposure to manganese can have detrimental effects such as neuropathological features of Parkinson disease.[56] The mechanisms underlying the association

of ADL disability with manganese are not completely understood, whereas our findings indicated that methylation changes to *IRGM* and *PKP3* might serve as the intermediate pathways. It has recently been found that inflammation response and autophagy played pivotal roles in manganese-induced cell toxicity in a rat model of manganism.[57] *IRGM* encodes a member of the interferon-inducible GTPase family, which has important functions in the innate immune response by regulating autophagy formation in response to intracellular pathogens.[58] *PKP3* encodes a member of the armadillo protein family with function in both signal transduction and cell adhesion, and its dysregulation has been implicated in transcriptional activation of proinflammatory cytokines.[59,60] Our finding suggested that DNA methylation of *IRGM* and *PKP3* might contribute to ADL disability of older adults through inflammation response and autophagy induced by manganese.

Similar to manganese, we observed positive relationships between copper and ADL disability in the present study. Copper is an essential element required for normal functioning, but excess of copper also resulted in adverse physical and psychological effects.[61] It is reported that copper toxicity may appear slowly over the years, resulting in functional disorders such as difficulty walking, tremors, and memory problems. Supporting our results, Younesi et al. found that individuals with ADL disability had higher levels of serum copper than the control group (168.2 μg/L vs. 151.9 μg/L).[62] Recently, epigenetic variations have been suggested as one of the biological mechanisms underlying the health effects of copper. Our study provides additional evidence for the potential genome methylation underlying the association between copper and ADL disability, where methylation changes to *ESRP2* (cg06723863) may serve as the intermediate pathways. *ESRP2* is a member of epithelial splicing regulatory proteins that regulate alternative splicing in epithelial cells.[63] *ESRP2* and its isoform *ESRP1* play important roles in the process of epithelial-specific RNA splicing. Loss of these splicing factors leads to epithelial-to-mesenchymal transition (EMT), suggesting that DNA methylation of *ESRP2* might contribute to ADL disability through EMT induced by copper.[63]

The toxicity of arsenic has been shown to have multiple deleterious effects, which include a high degree of toxicity to various organ systems.[45] High arsenic exposure was found to be associated with disruption of the blood–brain barrier, resulting in behavior dysfunction in rats.[64] However, few epidemiological studies have explored the association of arsenic exposure with ADL disability in older adults, and our study adds to the currently limited evidence. Mechanistic studies of arsenic toxicity have demonstrated the role of reactive species generation, single-strand breaks, and DNA base damage in the detrimental effects of arsenic exposure.[65] We tested mediation by DNA methylation across the genome and found that methylation at cg24433124 showed suggestive evidence for mediation. The nearest gene to cg24433124 was *IER3*, functioning in the protein of cells from tumor necrosis factor type-α (TNFα) and inducing apoptosis.[66] It has been reported that the functions of *IER3* in apoptosis can resulted in nuclear and mitochondrial localization as well as the functional phosphorylation site, which play crucial roles in various diseases.[67] In the present study, the methylation of cg24433124 may have affected *IER3* expression. The mediating role of cg24433124 methylation indicated that *IER3* may be involved in the toxicity of arsenic on ADL disability and provided evidence that apoptosis plays a crucial role in the pathways of ADL disability induced by arsenic.

Previous studies have reported that cadmium exposure was associated with dysfunction in perception and memory.[68,69] Supporting our study, a growing body of evidence showed that an increased prevalence of age-related diseases is associated with cadmium.[70,71] It is reported that cadmium can damage mitochondria and promote the peroxidation of cellular lipids in the parietal cortex, resulting in damage to the structure and function of neuronal cell membranes.[72,73] Our findings provide additional evidence for the potential genome methylation underlying the association between cadmium and ADL disability, where methylation changes to *FREM1* and *TCP11L1* may serve as the intermediate pathways. *TCP11L1* is a member of *TCP11* and encodes T-complex protein 11-like protein 1.[74] The function of the *TCP11L1* domain remains unclear, but it might be related to signal transduction. *FREM1* encodes a large transmembrane extracellular matrix protein and plays a significant role in epithelial-stromal adhesion.[75] It is reported that *FREM1* expression was associated with the activation of the EGFR and PI3K signaling pathways,[76] indicating oxidative stress might be involved in the toxicity of cadmium on ADL disability.

Our study has several strengths. First, we assessed the mediating role of DNA methylation across the genome and identified for the first time, to our knowledge, that locus-specific differential DNA methylation acted as partial mediators linking metals with ADL disability, providing important clues for further mechanism studies. Moreover, the EPIC array measures more than 850,000 CpGs in the present study, almost twice the CpGs in the prior studies with 450K array, which improves the coverage within intergenic regions, enhancers, and distal regulatory elements and allows for a more complete exploration on the epigenome for the potential DNA methylation mechanism. Finally, this study with a relatively large-scale population enables us to establish a relationship between metals exposure and ADL disability in older adults. The robustness of findings in the study was verified by multiple analyses, such as the approach of BKMR and sensitivity analysis.

Several limitations must be considered when interpreting the results of our study. A major limitation of our study is that, like other observational studies, our study has not been able to establish a causal link between associations. Yet, the participants in the study were local residents who had been living in the study area for more than 10 y, indicating their living environment remained relatively stable. If exposure were continuous and steady, concentrations of blood metals might reach a steady-state exposure, indicating that it seems improbable for the reverse associations that ADL disability resulted in major dietary and lifestyle changes with different metals exposures.[77,78] Additionally, absence of an external validation set on verifying our results was another limitation in our study. However, an external validation set may also further change the current finding due to variations in study population characteristics. Possibly due to similar population characteristics, cg05825244 (annotated to *EBF4*) was identified to be significantly associated with copper in an EWAS study of 1,243 Chinese individuals,[79] which was consistent with our results. Other EWAS studies associated with manganese, arsenic and cadmium, which were performed in populations such as American mother–infant pairs,[79] Bangladeshi adults,[80] and American Indian tribes,[20] however, did not reported similar genes. We have additionally performed an additional analysis of KEGG and GO pathway, where significant sites enriched mainly involved diverse cellular functions such as neuronal axons, synapses, and ion channel activities that were crucial in brain homeostasis involved in the ADL disability process.

Arsenic exposure may include exposure to the inorganic forms of arsenic, organic forms of arsenic, or both. The organic forms of arsenic such as arsenobetaine originate from seafood, which are consumed quite popular in China. In fact, before the formal investigation began, we surveyed the lifestyles and diet habits of local residents we aimed to recruit. We found that their dietary patterns included rice, meat, eggs, vegetables, and fungi, but very little seafood. In the narratives of the presurveyed participants, the place they resided was a remote county in Guangxi

and located in the mountainous area far away from the sea. As a result, the questionnaire of the formal investigation did not include the intake frequency of seafood as an investigated item. It has been reported that blood arsenic concentrations range from 5 to 10 μg/L in areas where consumption of seafood is common.[82] In a population in northern Argentina with no known exposure to inorganic arsenic and very little seafood intake, the average blood arsenic concentration was 2.2 μg/L in adults.[83] Although arsenic species were not measured in our study, the concentration of measured total arsenic (2.25 μg/L) in the participants was comparable to 2.2 μg/L, indicating participants in our study rarely had been exposed to organic arsenic.

Conclusion

Higher manganese, copper, arsenic, and cadmium exposures were associated with higher prevalence of ADL disability for older adults. This epigenome-wide association study of DNA methylation highlighted potential mechanisms of locus-specific differential DNA methylation on the genome scale, providing substantial clues and novel insights for potentially pathophysiological biomarkers. Further studies are warranted to corroborate our findings and illuminate the underlying pathways.

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