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Long-term exposure to traffic-related air pollution is associated with epigenetic age acceleration

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ABSTRACT

Epigenetic aging biomarkers, predicted by selected Cytosine-phosphate-Guanine (CpG) sites, might be influenced by air pollution exposure. However, evidence from longitudinal studies is still limited. This study aims to determine the associations between long-term exposure to air pollution and epigenetic aging biomarkers, and to identify vulnerable subgroups. Data was collected from the German population-based Cooperative Health Research in the Region of Augsburg (KORA) S4 survey (1999–2001) and two follow-up examinations (F4: 2006–08 and FF4: 2013–14). We measured DNA methylation (DNAm) in blood samples and calculated DNAm Age and DNAm-based telomere length (DNAmTL). We only included participants with at least two repeated measurements. Annual average concentrations of ultrafine particles (PNC), particulate matter (PM) less than 10 μm (PM_{10}), fine particles ($PM_{2.5}$), coarse particles (PM_{coarse}), soot ($PM_{2.5abs}$), nitrogen oxides (NO_2 and NO_x) and ozone (O_3) were estimated by land-use regression models. We applied linear mixed-effect regression models to assess the associations between air pollutants and epigenetic aging biomarkers, and further performed a limited epigenome-wide association study (EWAS) to examine whether air pollution influences individual CpGs. We included 4105 observations from 1651 KORA participants. In clinical models, interquartile range (IQR) increases in all air pollutants except O_3 were positively associated with accelerated DNAmGrimAge and DNAmPhenoAge. Moreover, all air pollutants showed negative associations with DNAmTL. Specifically, in ever smokers, the air pollutants were positively associated with the age acceleration of DNAmHorvathAge and DNAmPhenoAge, and inversely associated with DNAmTL with the largest effect estimates observed for $PM_{2.5abs}$. We identified two exposure-related CpGs with PM_{coarse} at a Benjamini-Hochberg false discovery rate corrected p-value <0.05 in ever smokers. Our findings suggest a robust association between long-term exposure to traffic-related air pollution with epigenetic age acceleration, especially in ever smokers. These results imply that air pollution is augmenting the negative impact of smoking on biological ageing.

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1. Introduction

Epidemiological studies indicated that air pollution exposure is associated with a higher risk of aging-related adverse health outcomes, e.g., pulmonary, cardiovascular, and neurological diseases, cancer, and mortality (Stieb et al., 2003; Raaschou-Nielsen et al., 2013; Bae et al., 2021; Hales et al., 2021; Kasdagli et al., 2022). Research investigated the potential biological mechanisms underlying those air pollution-associated adverse outcomes and reported underlying inflammatory response, oxidative stress, genetics, and epigenetics alterations as potential pathways (Peters et al., 2021). To date, emerging evidence has shown that DNA methylation (DNAm), a representative epigenetic marker, could be modulated by air pollution exposure, affecting inflammation, disease development, and exacerbation risk (Rider and Carlsten, 2019). Epigenetic aging biomarkers are estimated by DNAm but restricted to a small part of CpG sites (Hannum et al., 2013; Horvath, 2013; Horvath et al., 2018; Horvath and Raj, 2018; Levine et al., 2018; Lu et al., 2019a). DNAm-based aging biomarkers serve as valuable tools to evaluate the aging process from the molecular perspective across the lifespan, to measure the responses to the environment, and to predict health outcomes (Dhingra et al., 2018). Depending on the training method for each epigenetic aging biomarker, they could accurately predict chronological age, e.g., Hannum and Horvath epigenetic clocks, or quantify age- or disease-related outcomes, e.g., PhenoAge clocks (Horvath and Raj, 2018). Therefore, the epigenetic aging biomarkers could be potential hallmarks to link air pollution and aging-related health outcomes (Peters et al., 2021). However, evidence for the associations between ambient air pollution and epigenetic aging biomarkers from longitudinal studies in cohort settings is very limited so far.

The Hannum clock (DNAmHannumAge) is a blood-based epigenetic clock that mainly depends on age-related leukocyte composition and is more accurate for adults (Hannum et al., 2013). In contrast, Horvath's clock (DNAmHorvathAge) is a multi-tissue epigenetic clock determined across multiple tissues and cells showing better prediction throughout the human lifespan (Horvath, 2013). More recently, some new epigenetic clocks, considered the second generation of epigenetic clocks, were derived to better capture the age-related epigenetic variation from intrinsic, intra-cellular, or extrinsic aspects of the aging process, and subsequently better predict aging, morbidity, and mortality. For example, the Levine clock (DNAmPhenoAge) was built from chronological age and mortality-related blood parameters (Levine et al., 2018), while DNAmGrimAge was constructed by a two-stage method with an inclusion of risk factors (e.g., smoking and smoking pack-years) and a selection of plasma proteins (e.g., C-reactive protein), and subsequently used to regress time-to-death (Lu et al., 2019a). A new epigenetic clock, the DNAmSkinBloodAge clock, provides a better prediction of chronological age. It is more accurate when applied to blood-derived samples (Horvath et al., 2018; Sturm et al., 2019).

Telomeres are repetitive nucleotide sequences located at the end of chromosomes, and telomere length (TL) shortens with cell replications (Cong et al., 2002). Therefore, theoretically, TL has an inverse association with chronological age (Nordfjäll et al., 2010). However, the results were not consistent across different studies, and one potential reason could be the challenges on TL measurements due to specific technical limitations e.g., DNA extraction and experiment limitations (Kimura et al., 2010; Denham et al., 2014; Verhulst et al., 2015). As a complementary approach, a DNAm based TL derived from 140 CpG sites (DNAmTL) has been proposed (Lu et al., 2019b).

Some epidemiological studies have reported the associations between long-term exposure to air pollution and epigenetic aging biomarkers. A cross-sectional study based on a survey of the population-based Cooperative Health Research in the Region of Augsburg (KORA F4) observed weak associations between DNAmHorvathAge and long-term air pollution exposure. Moreover, those associations could be sex-specific because male and female participants responded differently for some of the examined measures of epigenetic aging accelerations (Ward-Caviness et al., 2016). A study on non-Hispanic white women living in the contiguous U.S. indicated associations between the acceleration of several epigenetic clocks and nitrogen dioxide (NO_2)and fine particle ($PM_{2.5}$) component profiles; however, the directions of those associations varied (White et al., 2019). In the Normative Aging Study (NAS), a longitudinal cohort consisting of only older male participants in the U.S., $PM_{2.5}$ and black carbon were associated with an increase of DNAmHorvathAge (Nwanaji-Enwerem et al., 2016). Several recent studies included some of the new epigenetic aging biomarkers, e.g., DNAmGrimAge and DNAmTL (de Prado-Bert et al., 2021; Baranyi et al., 2022; Koenigsberg et al., 2023). However, none of them investigated all of the above-mentioned epigenetic clocks and were limited with regard to the representativeness of the study population (de Prado-Bert et al., 2021; Baranyi et al., 2022). For example, Baranyi et al. included only elder participants (70–80 years old) (Baranyi et al., 2022), while Prado-Bert et al. considered children aged 6–11 years old) (de Prado-Bert et al., 2021), and Koenigsberg et al. only inculded women in their study (Koenigsberg et al., 2023).

Given the limitations of previous studies, such as cross-sectional study design, inconsistent results, and restriction on study participants (e.g., single sex, late adulthood, or children), as well as the development of novel epigenetic clocks, more studies, especially in a cohort setting with a longitudinal study design should be performed to provide more evidence for long-term air pollution-associated biological aging. Moreover, since epigenetic aging biomarkers are estimated by different methods, studies with a comprehensive assessment of various epigenetic aging biomarkers are essential for reliable results. Therefore, we performed this longitudinal study within the German population-based Cooperative Health Research in the Region of Augsburg (KORA) cohort to determine the associations between long-term air pollution exposure and three generations of DNAm-based biological aging biomarkers to better understand the underlying biological changes in a general population.

Additionally, as an individual's characteristics could influence biological aging in the long run, we explored the role of potential individual characteristics in modifying the effects of air pollution effects, including age, sex, body mass index (BMI), lifestyle factors (e.g., smoking status and physical activity) and pre-existing diseases (e.g., hypertension and diabetes). We hypothesized that long-term exposure to air pollution might accelerate the biological aging represented by the accelerations on epigenetic clocks and shorter DNAmTL, and individual characteristics might modify these effects.

2. Methods

2.1. Study design and participants

This longitudinal study was based on data of the population-based KORA cohort, conducted in the area of Augsburg, Germany. The fourth cross-sectional health survey of the KORA cohort (KORA S4, baseline) was conducted from October 1999 to April 2001, recruiting 4,261 participants aged 25–74 years with German citizenship in the city of Augsburg, Germany, and two adjacent counties. Participants were randomly selected from municipal population registries and stratified by sex and 10-year age groups. Two follow-up examinations were carried out: KORA F4 (October 2006–May 2008; n = 3,080; response rate 72.3 %) and KORA FF4 (June 2013–September 2014; n = 2,279; response rate 74.0 %). For the present analysis, we included participants with available DNA methylation data, exposures, and covariates at two or more of these study waves (**Appendix,** Fig. S1).

A computer-assisted personal interview, a self-administered questionnaire, and physical examinations were performed at each visit by trained investigators at the study center. Educational attainment was categorized into primary school, high school, and college. The continuous body-mass index (BMI) was categorized into normal weight (≤30

kg/m^2) and obesity (>30 kg/m^2). Physical activity was originally recorded in four categories: (1) regular >2 h/week; (2) ~1 h most weeks (regular); (3) ~1 h on average, not every week (irregular); (4) almost none/none. For the primary analyses, physical activity was recoded into three levels—low (categories 3–4), medium (category 2), and high (category 1). For power and interpretability in the effect-modification, physical activity was recoded to binary: yes (categories 1–3) versus no (category 4). Smoking status was recorded as current (regular or irregular smokers), former smokers (ex-smokers), and never smokers; for effect-modification analyses it was additionally recoded to binary: ever smoker (current or former smokers) versus never smoker. A written informed consent was obtained from all participants. The KORA study was approved by the ethics committee of the Bavarian Chamber of Physicians (Munich, Germany).

2.2. DNA methylation data and epigenetic clocks estimates

For KORA S4 and F4 participants, DNA was extracted from whole blood, and methylation was measured by the Infinium HumanMethylation450K BeadChip. In contrast, the Infinium HumanMethylationEPIC BeadChip was used to examine the DNA methylation for KORA FF4 participants. The bisulfite conversion and genome-wide methylation assessment were performed as previously described (Zeilinger et al., 2013).

Further quality control and pre-processing of the data were performed on the raw methylation data of KORA S4, F4, and FF4, following the CPACOR pipeline (Lehne et al., 2015), starting with the exclusion of single-nucleotide polymorphism markers, background correction using the R package minfi (Aryee et al., 2014), and subsequently setting probes to NA if the signals had a detection p-value of >0.01 or were summarized from ≤3 functional beads. Quantile normalization was performed on the signal intensity values, divided into categories by probe type and colour channel. A β-value, representing the methylation level of a given cytosine, was calculated by the ratio of the methylated signal intensity to the sum of the methylated and unmethylated signal intensities and used for further estimates of epigenetic aging biomarkers.

All epigenetic aging biomarkers were calculated by Horvath's online calculator (http://dnamage.genetics.ucla.edu/). In total, six DNAm-based aging biomarkers including DANmHorvathAge, DNAmHannumAge, DNAmPhenoAge), DNAmGrimAge, DNAmSkinBloodAge and DNAmTL were predicted by this online calculator and included in this analysis (Hannum et al., 2013; Horvath, 2013; Horvath et al., 2018; Levine et al., 2018; Lu et al. 2019a, 2019b). Except for DNAmTL, differences between each epigenetic clock and chronological age were then used as age accelerations. In addition, the white blood cell proportions (monocytes, granulocytes, natural killer cells, CD4+ T cells, CD8+ T cells, and B cells) were estimated by the Houseman method (Houseman et al., 2012).

The clocks differed in their training sets and CpG coverage. The Horvath clock was trained in 51 different tissue types, including 353 CpGs (Horvath, 2013). The Hannum and Levine clocks were developed and trained from whole blood samples consisting of 71 and 513 CpG sites, respectively (Hannum et al., 2013; Levine et al., 2018). The skin & blood clock was calculated by 391 CpGs and trained on different tissue and cell types, e.g., endothelial cells, skin, as well as blood (Horvath et al., 2018). The DNAmTL estimator was predicted by 140 CpG sites based on Lu's method (Lu et al., 2019b). Table 1 summarizes the overlap in CpGs across those epigenetic aging biomarkers. Because the EPIC array does not assay all clock CpGs, especially for the Horvath (19 CpGs missing) and Hannum (6 CpGs missing) clocks, missing values were left as NA and imputed internally by the online calculator prior to clock estimation.

2.3. Exposure assessment

Eight air pollutants were included in this analysis, including five particulate air pollutants (ultrafine particles with ≤100 nm in aerodynamic diameter, represented by particulate number concentration (PNC)), particulate matter (PM) with an aerodynamic diameter less than 10 μm (PM_{10}), coarse particles (PM_{coarse}), $PM_{2.5}$, and soot ($PM_{2.5abs}$)), and three gaseous air pollutants (nitrogen oxides (NO_2 and NO_x), and ozone (O_3)). Land-use regression (LUR) models were used to estimate the annual air pollution. Model performance was evaluated using leave-one-out cross-validation (LOOCV), a robust method for assessing predictive accuracy (Wolf et al., 2017). The adjusted model-explained variance (R^2) of the LUR models ranged from 68 % (PM_{coarse}) to 94 % (NO_2), and the adjusted leave-one-out cross-validation R^2 was between 55 % (PM_{coarse}) and 89 % (NO_2), which indicated a good model fit. More details about the process had been described in a previous publication (Wolf et al., 2017). In brief, to capture seasonal variation, air pollution measurements were conducted at 20 monitoring sites within the KORA study area. These measurements took place in three bi-weekly monitoring periods between March 2014 and April 2015, each representing a different seasonal condition (warm, cold, and intermediate). Annual average concentrations of air pollutants were subsequently calculated at each monitoring site. These concentrations were used as dependent variables in LUR models, developed using geographic information system (GIS)-based spatial predictors. Predictors included land use characteristics (e.g., residential, industrial, green space), population and building density, topography, geographic coordinates, as well as traffic-related variables (e.g., total traffic load, major road intensity). They were summarized within pollutant-specific buffers around each residence. Typical spatial scales were small buffers (~25–100 m) for traffic-related terms (e.g., road length, traffic load, nearest-road intensity) and larger buffers (~300–1000 m, up to 5 km for some land-use terms) for particulate matters and regional-scale features (e.g., industrial and green/seminatural areas). Full variable definitions and buffer sizes are described in previous publication (Wolf et al., 2017). Individual residential exposure levels were estimated by applying participants' home addresses to the fitted LUR models. Fig. S2 presents the air pollution exposure maps in the KORA cohort area (**Appendix**).

Long-term traffic noise exposure was estimated using the noise- and air-pollution information system (http://www.laermkarten.de/augsburg/), developed by ACCON GmbH. This system incorporates detailed three-dimensional ground model on roads and buildings, with rural road networks derived from Google Earth and OpenStreetMap. Traffic data were obtained from local government sources including the Bavarian Ministry of the Interior, Building and Transport, the digital street map of Augsburg, several traffic censuses and surveys. Noise levels were modeled at 4 m above ground for 2009 (urban) or 2000–2011

Table 1
Number of overlapping CpGs between the epigenetic aging biomarkers. [a]

	DNAmHorvathAge	DNAmHannumAge	DNAmPhenoAge	DNAmSkinBloodAge
DNAmHannumAge	6			
DNAmPhenoAge	41	6		
DNAmSkinBloodAge	60	5	58	
DNAmTL	0	45	1	5

[a] Although the CpGs contributing to DNAmGrimAge are known from its component DNAm surrogates, the final clock is derived through a two-stage modeling process, so individual CpG effects cannot be directly determined.

(rural). Maximum annual A-weighted equivalent continuous sound pressure levels [dB(A) Leq] for the full day (24 h) and nighttime (22:00–06:00) were estimated for each participant's residential address. If the address unavailable, the noise level from the nearest available building was assigned (Pitchika et al., 2017; Herder et al., 2023).

2.4. Statistical analyses

Basic descriptive analyses were conducted for participant characteristics and air pollutants. We performed Kruskal-Wallis tests (one-way ANOVA) for continuous variables and Pearson's Chi-squared tests for categorical variables to test for differences between the three examination waves. Pearson's correlation coefficients were calculated to determine the correlations between chronological age and epigenetic aging biomarkers, as well as between air pollutants.

Linear mixed-effects models were used with random participant-specific intercepts to examine the associations between repeated epigenetic aging biomarkers and air pollutants. In addition, we included the batch and chip numbers as random effects in the models to control the potential technical variations. Chronological age (years) was included in all models, including those with age-acceleration outcomes; DNAmTL was modeled on its native scale. In a pre-specified sensitivity excluding age from age-acceleration models, estimates were consistent with the primary analysis. Further covariates were selected based on previous studies (Ward-Caviness et al., 2016). ***Basic models*** adjusted for age, sex, estimated Houseman cell types (monocytes, natural killer cells, CD4 T+ cells, CD8 T+ cells, and B cells), and an indicator of each visit (KORA S4, KORA F4, or KORA FF4). In the ***behavioural models***, we added to the basic model by further including smoking status (never/-former/current smoker), alcohol consumption (g/day), physical activity (low/medium/high), and educational attainment (primary school/high school/college). The ***clinical models*** considered covariates of the basic models plus clinical variables, including body mass index (BMI), disease status (hypertension and diabetes), high-density lipoprotein (HDL), and low-density lipoprotein (LDL). The ***full models*** combined all covariates from both the behavioral and clinical models, and corresponding results were our main results. We assessed multicollinearity in the *full model* using variance inflation factors (VIFs) from the fixed-effects design matrix (excluding random effects). Continuous covariates were centered, categorical variables coded as indicators, and VIF <5 was considered acceptable (Appendix S1). Directed Acyclic Graph (DAG) for air pollution, confounding, and epigenetic aging was also provided in Fig. S3 (**Appendix**).

Since health effects of air pollution exposure might be closely related to those of smoking, as they sharing some of the pathways, we were interested in exploring potential differences in the effect estimates of ever and never smokers. We therefore performed all analyses on ever smokers and never smokers separately. Moreover, effect modification was investigated by including an interaction term between each air pollutant and the potential effect modifier assessed at each visit. The examined modifiers included age ($<$65 years *vs.* $\geq$ 65 years, as the age 65 years is the current official retirement age in Germany), sex (male *vs.* female), obesity (BMI $\leq$30 kg/m^2 *vs.* $>$ 30 kg/m^2), smoking status (ever (current/former) *vs.* never smoker), alcohol consumption (low vs medium vs high), physical activity (yes *vs.* no), educational attainment (low *vs.* high (high school/college)), hypertension (yes *vs.* no), and type 2 diabetes (yes *vs.* no).

We performed several sensitivity analyses in this study: 1) Since a few CpGs used for the calculation of some epigenetic aging biomarkers, e.g., Hannum clock, are not detected by the Infinium HumanMethylationEPIC BeadChip, we imputed the missing CpG sites by the website calculator, which might lead to minor bias in the corresponding epigenetic aging biomarkers between participants measured by Infinium HumanMethylation450k BeadChip (KORA S4 and KORA F4) and Infinium HumanMethylationEPIC BeadChip (KORA FF4). To reduce this bias, we selected the overlapping CpG sites existing in both chips, and imputed the missing CpGs for calculating corresponding epigenetic aging biomarkers with NA, and will be imputed with online calculator. Subsequently, we recalculated the epigenetic aging biomarkers for each visit by these updated methylation datasets (Dhingra et al., 2019); 2) We excluded estimated Houseman cell types from the models since some epigenetic aging biomarkers were not affected by the white blood cells; 3) We increased the number of included participants by including all participants with either single or repeated visits with data on air pollution, phenotypes, and methylation into this analysis; 4) We restricted our analysis to participants without changing residences throughout the entire study period; 5) We separately included the daytime and nighttime noise averages in the full models; 6) As a sensitivity analysis of effect modification, we modeled physical activity at three levels (low/medium/high) to evaluate dose–response; 7) We back-extrapolated air pollutant concentrations by calibrating LUR estimates with monitoring data to capture both spatial and temporal variation. More details about this approach were in the Appendix (Text S1); 8) We performed two-pollutant models by including two air pollutants simultaneously if their Spearman correlation was smaller than 0.7; 9) We conducted a limited epigenome-wide association study (EWAS), which restricts CpG sites to those specifically used in the predictions of epigenetic aging biomarkers to examine whether air pollution influences individual CpG. Furthermore, for the potential exposure-related CpG sites, we used Ingenuity Pathway Analysis (IPA, QIAGEN Inc.) to identify canonical pathway enriched with those CpG-annotated genes, and the pathway was determined if p-values <0.05.

Effect estimates were presented as absolute change (together with 95 % confidence intervals [95 % CI]) of the repeatedly assessed outcomes per interquartile range (IQR) increase in air pollutant concentrations, except for DNAmTL, which was presented as percent change based on the mean DNAmTL across all visits. All statistical analyses were done with R (version 4.1.2), and the p-value cut-off was set as 0.05. Results from the stratified analysis and limited EWAS were corrected by the Benjamini-Hochberg false discovery rate (FDR) with corrected p-values <0.05.

3. Results

3.1. Characteristics of study participants

Participants' characteristics are summarized in Table 2. Out of 9,620 observations from 4,261 study participants in the KORA cohort, we included 4,105 (42.7 %) observations from 1,646 (38.6 %) participants in this analysis. Specifically, 833 (50.6 %) out of the 1646 participants attended two examinations, and 813 (49.4 %) attended all three examinations.

The average age has increased from KORA S4 to KORA F4 and KORA FF4. No differences were observed between the sex distribution (p-value $= 0.78$) or for average BMI (p-value $= 0.07$) across the three studies. In contrast, the mean of alcohol consumption, HDL and LDL, the percentages of participants with hypertension or diabetes, and the counts of six Houseman-estimated white blood cell types significantly differed among KORA S4, F4, and FF4 (p-value <0.05). The mean predictions of DNAmHorvathAge, DNAmHannumAge, DNAmPhenoAge, DNAmGrimAge, and DNAmSkinBloodAge, as well as mean DNAmTL, varied throughout the three visits (p-value <0.01). In general, the chronological age and the epigenetic aging biomarkers all showed moderate to high positive correlations with each other (Pearson correlation coefficients ranged from 0.48 to 0.83), except for DNAmTL, which always showed moderate to strong negative correlations with all others (**Appendix**, Fig. S4). In addition, weak correlations were observed between the five cell types estimated by the Houseman method (**Appendix**, Fig. S5).

Table 2
Descriptive statistics of participant characteristics by study wave (total number of observations: N = 4,105).

Variable	S4 (N = 1,481) Mean ± SD/N (%)	F4 (N = 1,599) Mean ± SD/N (%)	FF4 (N = 1,025) Mean ± SD/N (%)	*p*–value
Age (years)	53.9 ± 8.9	60.7 ± 8.9	64.7 ± 8.1	<0.001
Sex (male)	736 (49.7)	783 (49.0)	495 (48.3)	0.78
Education				0.36
Primary school	873 (59.0)	919 (57.5)	567 (55.3)	
High school	338 (22.8)	368 (23.0)	240 (23.4)	
College	270 (18.2)	312 (19.5)	218 (21.3)	
BMI (kg/m^2)	27.7 ± 4.5	28.1 ± 4.8	28.2 ± 5.0	0.066
Alcohol consumption (g/day)	17.1 ± 22.1	15.7 ± 20.7	15.6 ± 20.5	0.036
Smoking status				<0.001
Never smoker	633 (42.7)	673 (42.1)	421 (41.1)	
Current smoker	554 (37.4)	697 (43.6)	473 (46.1)	
Former smoker	294 (19.9)	229 (14.3)	131 (12.8)	
Physical activity				<0.001
Low	483 (32.6)	492 (30.8)	279 (27.2)	
Medium	708 (47.8)	697 (43.6)	453 (44.2)	
High	290 (19.6)	410 (25.6)	293 (28.6)	
Hypertension (yes)	620 (41.9)	719 (45.0)	483 (47.1)	0.028
Diabetes (yes)	51 (3.4)	142 (8.9)	130 (12.7)	<0.001
HDL cholesterol (mmol/l)	1.5 ± 0.4	1.5 ± 0.4	1.7 ± 0.5	<0.001
LDL cholesterol (mmol/l)	3.7 ± 1.1	3.6 ± 0.9	3.6 ± 0.9	<0.001
DNAmHorvathAge (years)[a]	55.2 ± 8.3 (60.8)	59.1 ± 7.5 (38.1)	65.0 ± 6.1 (54.0)	<0.001
DNAmHannumAge (years)[a]	58.4 ± 9.3 (83.6)	70.4 ± 10.0 (96.5)	53.8 ± 7.7 (1.2)	<0.001
DNAmPhenoAge (years)[a]	49.5 ± 11.0 (25.0)	54.0 ± 12.5 (21.1)	52.6 ± 9.7 (3.2)	<0.001
DNAmGrimAge (years)[a]	55.3 ± 8.6 (56.9)	61.9 ± 8.8 (52.8)	64.0 ± 7.8 (36.8)	<0.001
DNAmSkinBloodAge (years)[a]	54.2 ± 9.3 (53.4)	61.8 ± 9.2 (60.2)	59.5 ± 7.7 (7.4)	<0.001
DNAmTL	7.1 ± 0.3	6.7 ± 0.3	6.8 ± 0.2	<0.001
CD8 T cells	0.11 ± 0.05	0.1 ± 0.05	0.05 ± 0.04	<0.001
CD4 T cells	0.22 ± 0.05	0.24 ± 0.05	0.19 ± 0.06	<0.001
Natural killer cells	0.03 ± 0.02	0.06 ± 0.03	0.07 ± 0.04	<0.001
B cells	0.06 ± 0.02	0.07 ± 0.02	0.05 ± 0.03	<0.001
Monocytes	0.11 ± 0.02	0.1 ± 0.02	0.07 ± 0.02	<0.001
Daytime noise (dB(A))[b]	54.84 ± 6.61 (0.7 %)	54.68 ± 6.61 (0.6 %)	54.36 ± 6.52 (6.9 %)	0.38
Nighttime noise (dB(A))[b]	45.81 ± 6.41 (0.7 %)	45.64 ± 6.41 (0.6 %)	45.31 ± 6.32 (6.9 %)	0.27

KORA = Cooperative Health Research in the Region of Augsburg; S4 = fourth cross-sectional health survey of the KORA cohort; F4 = first follow-up examination of KORA S4; FF4 = second follow-up examination of KORA S4; BMI = body mass index; HDL = high density lipoprotein; LDL = low density lipoprotein; Physical activity was defined according to the exercise time per week: Low = almost or no sporting activity, Medium = regular/irregular approx. 1 h per week, High = regularly more than 2 h per week.
Of 1646 participants in total, 833 attended two examinations, and 813 attended three examinations.
p-value was based on the Kruskal-Wallis test for continuous variables, and Pearson's Chi-squared test for categorical variables.
[a] Numbers in brackets indicate the percent of persons with positive age acceleration.
[b] Numbers in brackets indicate the missing percent of noise data in each examination.

3.2. Characteristics of air pollutants

Annual average concentrations of $PM_{2.5}$, PM_{10}, and NO_2 at participant's residences were all below the current EU air quality standards, with values of 25 μg/m^3 set for $PM_{2.5}$ and 40 μg/m^3 for both PM_{10} and NO_2 (Table 3). However, they were higher than the WHO air quality guideline values of 5 μg/m^3, 10 μg/m^3, and 10 μg/m^3 for $PM_{2.5}$, PM_{10}, and NO_2, respectively. We observed moderate to strong positive correlation between all air pollutants, except for O_3, showing negative or weak correlations with the other air pollutants. The interquartile ranges (IQR) were 1.4 μg/m^3 for $PM_{2.5}$, 2.1 μg/m^3 for PM_{10}, 1.4 μg/m^3 for PM_{coarse}, 2.0 × 10^3/cm^3 for PNC, 0.3 × 10^{-5}/m for $PM_{2.5abs}$, 7.2 μg/m^3 for NO_2, 8.8 μg/m^3 for NO_x, and 3.4 μg/m^3 for O_3. There was no correlation between the air pollutants and chronological age (**Appendix**, Fig. S6).

3.3. Associations between epigenetic aging biomarkers and long-term air pollution

DNAmTL showed robust negative associations with $PM_{2.5}$, PM_{10}, PNC, PM_{coarse}, $PM_{2.5abs}$, and NO_2 in our four regression models with different strengths of confounder adjustment. Additionally, it was significantly negatively associated with NO_x in the basic and clinical models. The acceleration of DNAmGrimAge was positively associated with $PM_{2.5}$, PM_{10}, PNC, PM_{coarse}, $PM_{2.5abs}$, NO_2, and NO_x in our basic and clinical models (*p*-value <0.05), but not in the behavioural and full models (Fig. 1). More details were summarized in Table S1 (**Appendix**). We therefore added the specific covariates of the behavioural model (smoking status, alcohol consumption, physical activity, and educational attainment) into the clinical model step by step which showed that the inclusion of smoking status shifted the results in the behavioural and full models (**Appendix,** Fig. S7). Effect estimates of all covariates used in the full models were also provided for each epigenetic aging biomarker (**Appendix,** Table S2).

3.4. Stratified analysis by smoking status (smoking-specific associations)

In ever smokers, the results showed consistent accelerating effects with DNAmHorvarthAge and DNAmPhenoAge by all air pollutants except for O_3 (Fig. 2). In addition, DNAmTLwas significantly accelerated by $PM_{2.5}$, $PM_{2.5abs}$, and NO_2 (FDR-adjust *p*-value <0.05). For the DNAmGrimAge and DNAmSkinBloodAge, we could observe stronger accelerating effects in ever smokers, however they were not statistically significant. In general, the accelerating effect on DNAmHannumAge clock were similar between ever and never smokers. Taken together, our results suggested that ever smokers might be more susceptible to air pollution exposure with regard to epigenetic age acceleration. More details of participants' characteristics of never and ever smokers and effect estimates of pollutant-related epigenetic aging biomarkers were summarized in Tables S3–S6 (**Appendix**).

3.5. Effect modification

Fig. 3 shows that the presented associations between DNAmTL and PM_{10}, PNC, PM_{coarse}, $PM_{2.5abs}$, NO_2 and NO_x were modified by hypertension (uncorrected $p < 0.001$). For other potential effect modifiers such as age, sex, obesity, alcohol consumption, educational attainment, physical activity, or diabetes, we did not observe significant differences. Effect modification analysis for the other five epigenetic aging biomarkers indicated no consistent pattern (**Appendix**, Fig. S8–S12).

3.6. Sensitivity analysis

The associations between air pollution and all six epigenetics clocks were generally robust in different sensitivity analyses (**Appendix,** Fig. S13**)**. In detail, results remained stable when excluding the CpG sites that were missing in the methylation data of KORA FF4 compared to the KORA S4 and F4 methylation data, with only small differences observed for DNAmHorvathAge and DNAmHannumAge with particulate matter exposures. Concordance between default (imputed) and overlap-only clocks was high (Pearson r = 0.88–1.0; Table S7), and 87.5 % of pollutant–clock associations were directionally consistent (**Appendix**, Table S8). Furthermore, the results remained robust without the adjustment of Houseman-estimated white cell types, or when

Table 3
Descriptive statistics and Spearman correlation coefficients of air pollution concentrations (N = 1,646).

				Spearman correlation coefficients of air pol							
Pollutant	Mean ± SD	Range	IQR	$PM_{2.5}$	PM_{10}	PNC	PM_{Coarse}	$PM_{2.5abs}$	O_3	NO_2	NOx
$PM_{2.5}$ (μg/m^3)	11.8 ± 1.0	8.4–14.3	1.4	1							
PM_{10} (μg/m^3)	16.6 ± 1.6	12.7–22.3	2.1	0.53	1						
PNC (10^3/cm^3)	7.3 ± 1.9	3.3–15.0	2.0	0.65	0.82	1					
PM_{coarse} (μg/m^3)	5.0 ± 1.0	2.6–8.5	1.4	0.57	0.78	0.76	1				
$PM_{2.5abs}$ (10^{-5}/m)	1.2 ± 0.2	0.8–1.9	0.3	0.62	0.78	0.78	0.81	1			
O_3 (μg/m^3)	39.1 ± 2.4	31.3–46.2	3.4	−0.19	0.01	−0.07	0.09	−0.15	1		
NO_2 (μg/m^3)	14.3 ± 4.6	6.9–28.2	7.2	0.72	0.72	0.77	0.83	0.87	−0.21	1	
NO_x (μg/m^3)	22.0 ± 7.6	4.0–50.5	8.8	0.76	0.75	0.90	0.76	0.74	−0.10	0.83	1

Of the total of 1646 participants, only 25 participants moved between the examinations. For participants who changed residence, the updated residential addresses were used for exposure assignment of the follow-up visits. Otherwise, the same exposure levels were assigned to the follow-up visits. $PM_{2.5}$ = particulate matter with an aerodynamic diameter less than or equal to 2.5 μm; PM_{10} = particulate matter with an aerodynamic diameter less than or equal to 10 μm; PNC = particle number concentration; PM_{coarse} = particulate matter with an aerodynamic diameter of 2.5–10 μm; $PM_{2.5abs}$ = $PM_{2.5}$ absorbance; PNC = particle number concentration; NO_2 = nitrogen dioxide; NO_x = nitrogen oxide; O_3 = ozone. Pearson's correlation coefficients were calculated to determine the correlations between air pollutants.

conducting the analysis in participants with single or repeated visits or by restricting the analysis to participants who did not move their residences during the whole study period. Also, additionally controlling for daytime and night-time traffic noise showed robust results or slightly strengthened the effect estimates of air pollution exposure. Three-level physical activity results were directionally consistent with the binary models, while the three-level stratification provided more differentiated patterns of associations between air pollution exposures and several epigenetic aging (**Appendix,** Fig. S14**)**.

Back-extrapolated exposures shifted estimates and widened CIs for $PM_{2.5}$, PM_{10}, and PNC, while associations for $PM_{2.5abs}$, NO_2, NO_x and O_3 remained stable (**Appendix,** Fig. S15**)**. In addition, the results derived from two-pollutant models were consistent with the single-pollutant models (**Appendix,** Figs. S16–S17). The results of sensitivity analysis and two-pollutant models from the stratified analysis restricted to ever smokers or never smokers were similar to our main analysis (data not shown).

In total, 1,253CpGs were used to estimate the five epigenetic aging biomarkers (DNAmHorvathAge, DNAmHannumAge, DNAmPhenoAge, DNAmSkinBloodAge, DNAmTL). In the limited EWAS, we did not observe any significant results between air pollutant exposures and the 1,253 CpGs, both in the main analysis and in the subgroup of never smokers at the FDR-corrected p-value <0.05. However in ever smokers, cg12745325 (annotated to *SLC39A5* on chromosome 12) included in the estimation of DNAmTL and cg24081819 (within *EPHX2* on chromosome 8) included in the prediction of DNAmHorvathAge were significantly associated with PM_{coarse} exposure at the FDR-corrected p-value <0.05 (Table 4). The top canonical pathway, top biofunction, and top disease identified via IPA were triacylglycerol biosynthesis, cardiovascular disease, and lipid metabolism, respectively (**Appendix,** Table S9).

4. Discussion

In this study, long-term exposure to air pollution showed accelerating effects on the epigenetic aging biomarkers. In the clinical models, we observed negative associations between DNAmTL and particulate air pollutants ($PM_{2.5}$, PM_{10}, PNC, PM_{coarse}, and $PM_{2.5abs}$) and NO_2, as well as positive associations between the acceleration of DNAmGrimAge and DNAmPhenoAge and particulate air pollutants ($PM_{2.5}$, PM_{10}, PM_{coarse}, PNC, and $PM_{2.5abs}$) and nitrogen oxides (NO_2 and NO_x). In ever-smokers, DNAmHorvathAge and DNAmPhenoAge were positively associated with all pollutants except for O_3; no consistent associations were observed for DNAmHannumAge or DNAmSkinBloodAge.

4.1. Epigenetic aging biomarkers and air pollution

The acceleration of epigenetic clocks has been developed to predict chronological or biological aging, which might indicate homeostasis dysfunction and increase the susceptibility to air pollution exposure. Evidence from other cohort studies indicates biomarker-specific heterogeneity in associations between air pollution and DNAm aging biomarkers.

Ward-Caviness et al. observed that higher accelerated epigenetic aging elevated associations between traffic-related air pollution and peripheral arterial disease, with significant interactions between DNAmHorvathAge acceleration and gasoline- and diesel-related $PM_{2.5}$ sources (Ward-Caviness et al., 2020). In a cross-sectional analysis using the first follow-up survey of KORA (KORA F4), higher annual $PM_{2.5}$ was associated with greater DNAmHorvathAge acceleration (Ward-Caviness et al., 2016). Consistently, analyses from the NAS cohort linked annual $PM_{2.5}$ to DNAmHorvathAge acceleration, and $PM_{2.5}$-associated CpGs were involved in lung-related pathways (Nwanaji-Enwerem et al., 2016). However, they did not observe significant associations between $PM_{2.5}$ and DNAmHannumAge or DNAmPhenoAge using the same NAS methylation data (Nwanaji-Enwerem et al., 2017; Wang et al., 2020). In the U.S. Sister Study, an IQR increase in NO_2 was inversely associated with DNAmHannumAge acceleration, with no associations for DNAmHorvathAge or DNAmPhenoAge and no significant results for $PM_{2.5}$ or PM_{10} (White et al., 2019). A life-course analysis in older adults observed DNAmHorvathAge acceleration at higher pollution epochs, but this finding disappeared after the adjustment for multiple testing (Baranyi et al., 2022). A study in children reported that exposure to indoor particulate matter and parental smoking were associated with DNAmSkinBloodAge acceleration, outperforming the other epigenetic aging clocks in their results (de Prado-Bert et al., 2021). So far, only a few studies have included DNAmGrimAge to explore the effects of air pollution. For example, Koenigsberg et al. reported positive associations with PM_{10} and NO_2 (Koenigsberg et al., 2023), and Shi et al. found DNAmGrimAge acceleration modified effect on the association between $PM_{2.5}$ and blood pressure (Shi et al., 2022), whereas a life-course analysis did not observe notable results between DNAmGrimAge and air pollution associations (Baranyi et al., 2022). For DNAmTL, Ward-Caviness et al. reported inverse associations between black carbon (equal to $PM_{2.5abs}$) and TL–based age acceleration in KORA F4, with replication in NAS (Ward-Caviness et al., 2016), and Baranyi et al. found shorter DNAmTL at higher $PM_{2.5}$ and NO_2 exposure (Baranyi et al., 2022).

In clinical models, higher $PM_{2.5}$, PM_{10}, PM_{coarse}, PNC, $PM_{2.5abs}$, and NO_2/NO_x were associated with greater acceleration of DNAmGrimAge and DNAmPhenoAge and shorter DNAmTL. Among ever smokers, DNAmHorvathAge and DNAmPhenoAge were positively associated with all pollutants except for O_3. We observed no consistent associations for DNAmHannumAge or DNAmSkinBloodAge. All models accounted for batch/chip effects and estimated blood-cell composition.

After behavioral covariates were included, the DNAmGrimAge associations attenuated toward the null. The stepwise inclusion implicated

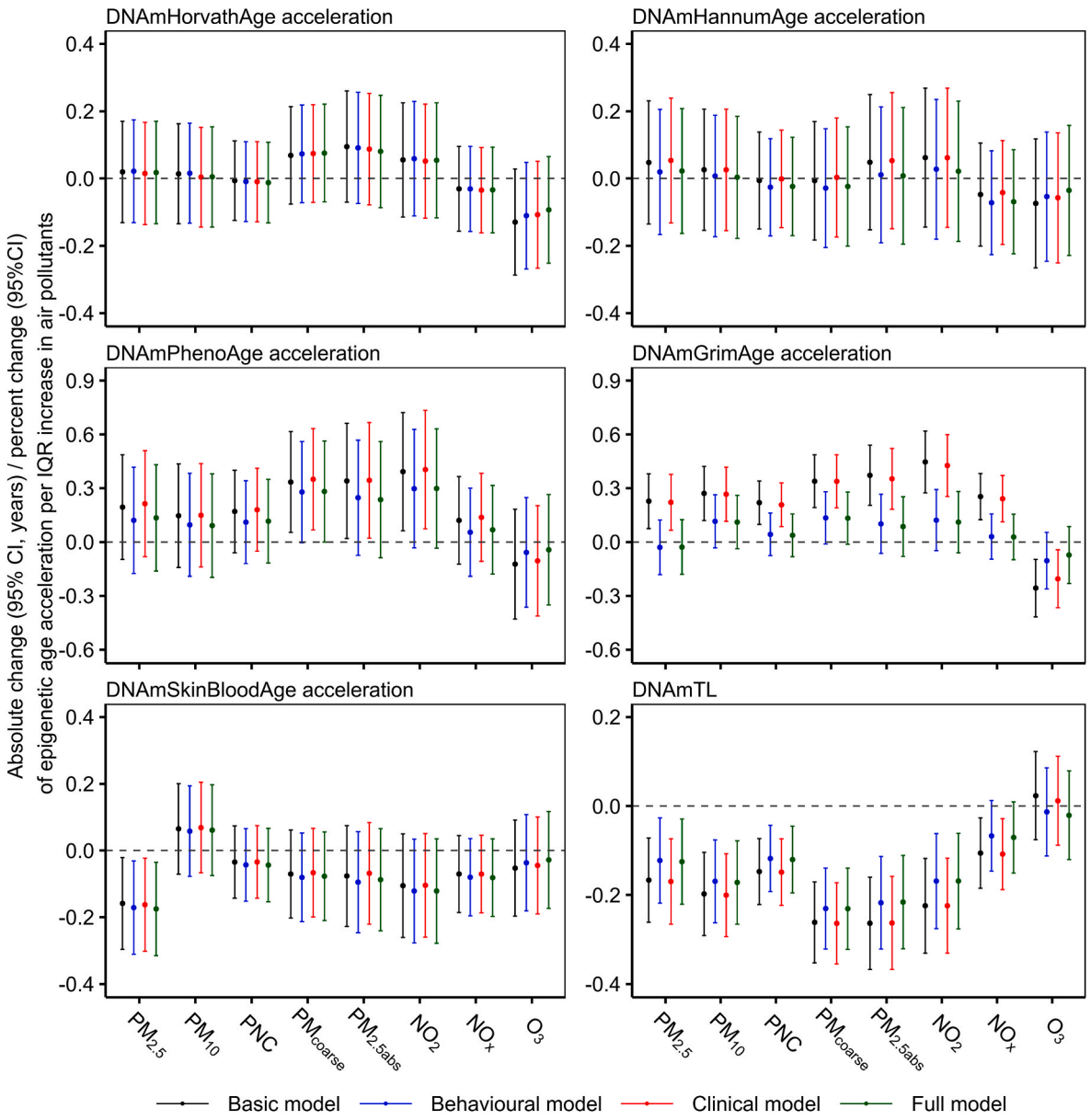

Fig. 1. Absolute change (95 % CI, years) / percent change (95 %CI) of epigenetic age acceleration per IQR increase in air pollutant concentrations with basic, behavioral, clinical, and full covariate adjustment (percent change only for DNAmTL). Covariate-adjusted linear mixed-effect regression models were used. Basic model: basic models were adjusted for age, sex, an indicator of each study wave (KORA S4, KORA F4, or KORA FF4), houseman estimated cells, batch and chip; Behavioral model: basic model further adjusted for educational attainment, alcohol consumption, smoking status (current/former/never), physical activity (low/medium/high),; Clinical model: basic model further adjusted for BMI, hypertension, diabetes, HDL, and LDL. Full model: all covariates from the behavioral and clinical model combined. $PM_{2.5}$ = particulate matter with an aerodynamic diameter less than or equal to 2.5 μm; PM_{10} = particulate matter with an aerodynamic diameter less than or equal to 10 μm; PNC = particle number concentration; PM_{coarse} = particulate matter with an aerodynamic diameter of 2.5–10 μm; $PM_{2.5abs}$ = $PM_{2.5}$ absorbance; NO_2 = nitrogen dioxide; NO_x = nitrogen oxide; O_3 = ozone. An IQR increase was 1.4 μg/m^3 for $PM_{2.5}$, 2.1 μg/m^3 for PM_{10}, 2.0 × 10^3/cm^3 for PNC, 1.4 μg/m^3 for PM_{coarse}, 0.3 × 10^{-5}/m for $PM_{2.5abs}$, 7.2 μg/m^3 for NO_2, 8.8 μg/m^3 for NO_x, and 3.4 μg/m^3 for O_3.

smoking status as the primary driver of this attenuation (Appendix, Fig. S7), consistent with GrimAge's incorporation of DNAm surrogates for smoking pack-years and mortality-related proteins. Stratified analyses indicated inverse associations with particulate air pollutants and NO_2/NO_x overall and among ever smokers for DNAmHorvathAge and DNAmPhenoAge, and pathway analysis of exposure-related CpGs in this subgroup highlighted enrichment in lipid-metabolism and cardiovascular pathways.

We analyzed DNAmTL—a methylation-derived estimate of leukocyte telomere length based on 140 CpGs—as a complement to multi-CpG clocks. In this study, shorter DNAmTL related to higher particulate matters and NO_2/NO_x overall and among ever smokers. These patterns align with inverse associations reported between black carbon (equal to $PM_{2.5}$ absorbance ($PM_{2.5abs}$)) and TL-based age acceleration in males (Ward-Caviness et al., 2016), and with the findings of shorter DNAmTL at higher exposure to $PM_{2.5}$ and NO_2 (Baranyi et al., 2022).

These patterns motivate Section 4.2, where we consider why different DNAm aging biomarkers respond variably to air pollution exposures.

4.2. *Variability of epigenetic aging biomarkers to air pollution exposures*

Responses differed by biomarker: DNAmGrimAge showed exposure associations that were behaviorally sensitive—particularly to smoking;

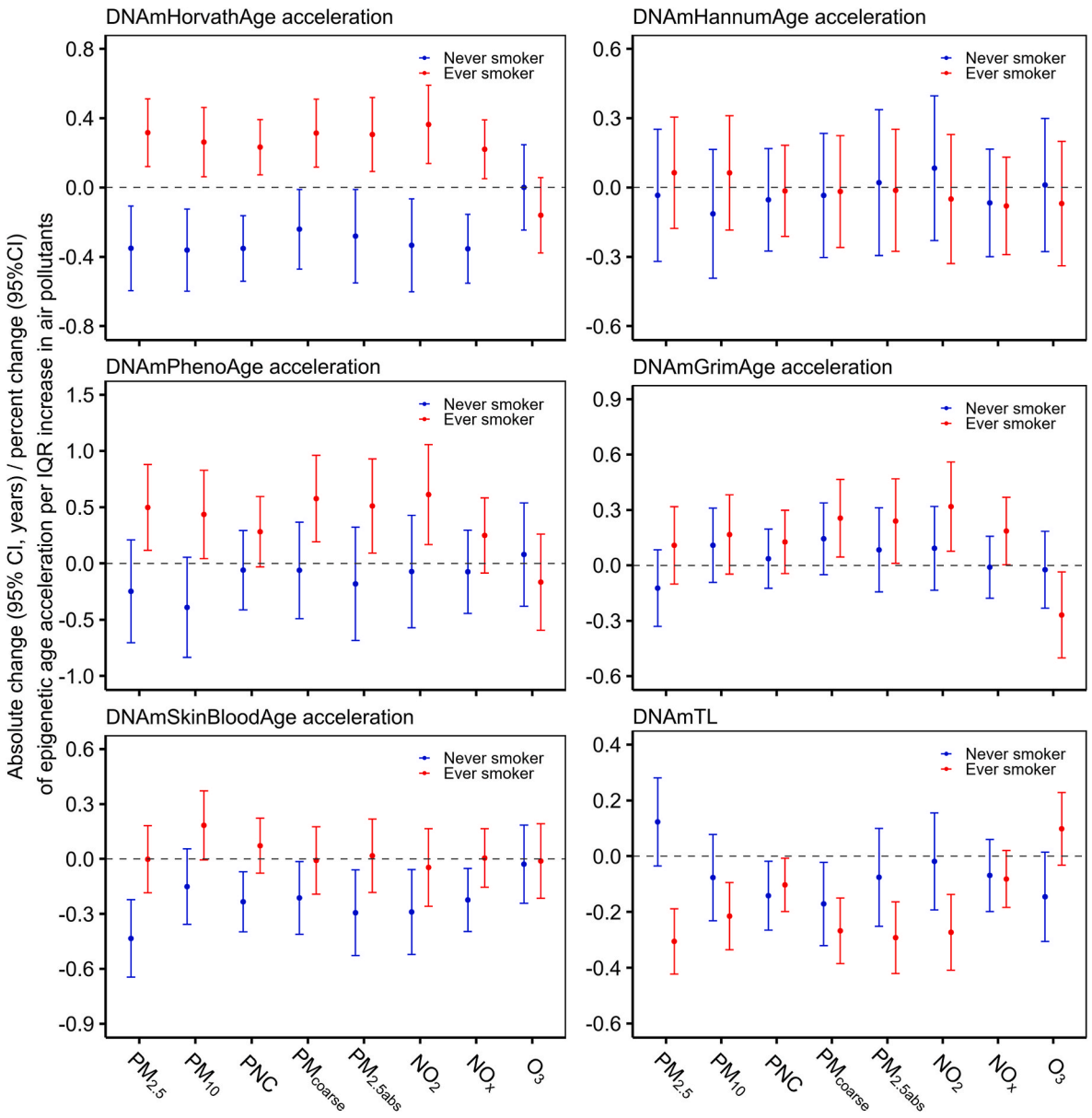

Fig. 2. Absolute change (95 % CI, years) / percent change (95 %CI) of epigenetic aging biomarkers per IQR increase in air pollutant concentrations stratified by smoking status (percent change only for DNAmTL). Covariate-adjusted linear mixed-effect regression models were used. Results from the full model adjusting for age, sex, an indicator of each study waves visit (KORA S4, KORA F4, or KORA FF4), Houseman imputed cells, batch, educational attainment, alcohol consumption, physical activity (low/medium/high), BMI, hypertension, diabetes, HDL, and LDL. Never smoking = participants who never smoked; Ever smoking = current or former smokers. $PM_{2.5}$ = particulate matter with an aerodynamic diameter less than or equal to 2.5 μm; PM_{10} = particulate matter with an aerodynamic diameter less than or equal to 10 μm; PNC = particle number concentration; PM_{coarse} = particulate matter with an aerodynamic diameter of 2.5–10 μm; $PM_{2.5abs}$ = $PM_{2.5}$ absorbance; NO_2 = nitrogen dioxide; NO_x = nitrogen oxide; O_3 = ozone. An IQR increase was 1.4 μg/m^3 for $PM_{2.5}$, 2.1 μg/m^3 for PM_{10}, 2.0 × 10^3/cm^3 for PNC, 1.4 μg/m^3 for PM_{coarse}, 0.3 × 10^{-5}/m for $PM_{2.5abs}$, 7.2 μg/m^3 for NO_2, 8.8 μg/m^3 for NO_x, and 3.4 μg/m^3 for O_3.

DNAmHorvathAge and DNAmPhenoAge displayed clearer positive associations among ever smokers; DNAmHannum and DNAmSkinBlood were largely null in this study; and DNAmTL moved inversely with air pollutants, consistent with a telomere-focused construct. Such heterogeneity is expected given differences in construct and training targets (chronological-age clocks *vs* phenotypic/mortality-linked clocks *vs* telomere-focused DNAmTL), CpG composition, training cohorts/tissues, and behavioral sensitivity, especially to smoking (Hannum et al., 2013; Horvath, 2013; Horvath et al., 2018; Levine et al., 2018; Lu et al. 2019a, 2019b).

Comparable variability appears across cohorts: DNAmHorvathAge and extrinsic epigenetic age and intrinsic epigenetic age acceleration (EEAA and IEAA) linked to NO_x and black carbon in females and inversely to PM_{10} in males, with no findings of $PM_{2.5}$ exposure (Ward-Caviness et al., 2016). In the Sister Study, only DNAmHannumAge acceleration inversely related to NO_2, and responses differed by $PM_{2.5}$ component clusters (White et al., 2019). In NAS, DNAmHorvathAge related to $PM_{2.5}$ exposure, whereas DNAmPhenoAge associated with specific components (e.g., calcium, lead) and DNAmHannumAge was null (Nwanaji-Enwerem et al. 2016, 2017; Wang et al., 2020). Taken together, these examples underscore that construct-specific sensitivity interacts with exposure composition and population.

By evaluating six epigenetic aging biomarkers side-by-side under harmonized exposure assessment and covariate adjustment, decomposing behavioral attenuation (identifying smoking as the principal driver), and stratifying by smoking status, this study helps differentiate

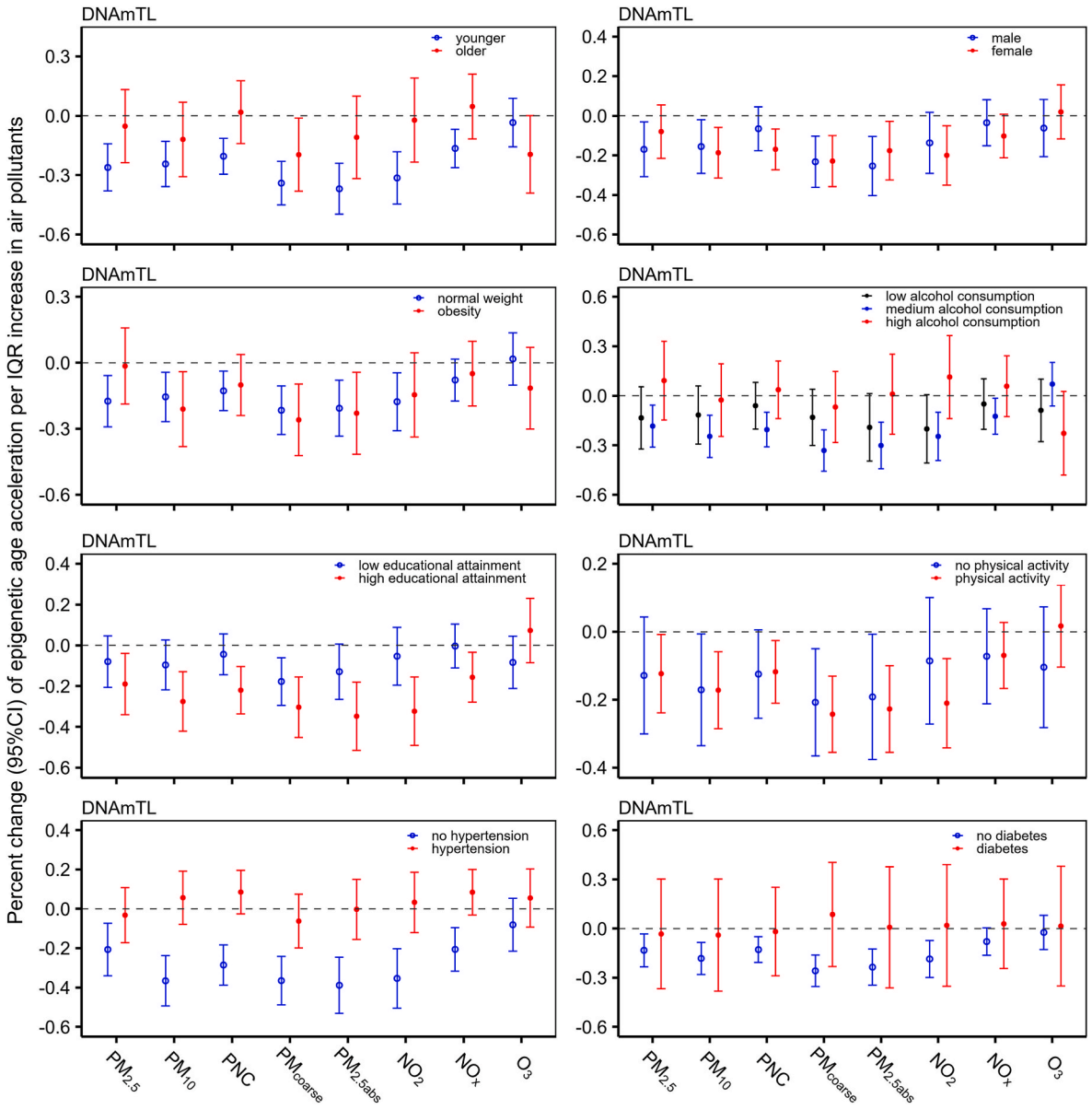

Fig. 3. Percent change (95 % CI) in DNAmTL per IQR increase in air pollutant concentrations stratified by categorized age group (≤65 vs > 65), sex, categorized BMI group, categorized group of alcohol consumption, educational attainment, physical activity, and history of diseases (hypertension, diabetes). Covariate-adjusted linear mixed-effect regression models were used. Results were from our full model adjusted by age, sex, an indicator of each study waves visit (KORA S4, KORA F4, or KORA FF4), houseman estimated cells, batch, chip, educational attainment, alcohol consumption, smoking status (ever/never), physical activity (yes/no), BMI, hypertension, diabetes, HDL, and LDL. $PM_{2.5}$ = particulate matter with an aerodynamic diameter less than or equal to 2.5 μm; PM_{10} = particulate matter with an aerodynamic diameter less than or equal to 10 μm; PNC = particle number concentration; PM_{coarse} = particulate matter with an aerodynamic diameter of 2.5–10 μm; $PM_{2.5abs}$ = $PM_{2.5}$ absorbance; NO_2 = nitrogen dioxide; NO_x = nitrogen oxide; O_3 = ozone. An IQR increase was 1.4 μg/m^3 for $PM_{2.5}$, 2.1 μg/m^3 for PM_{10}, 2.0 × 10^3/cm^3 for PNC, 1.4 μg/m^3 for PM_{coarse}, 0.3 × 10^{-5}/m for $PM_{2.5abs}$, 7.2 μg/m^3 for NO_2, 8.8 μg/m^3 for NO_x, and 3.4 μg/m^3 for O_3.

Table 4
Results from epigenome-wide association study (EWAS) restricted to CpG sites involved in predicting the respective epigenetic clocks with PM_{coarse} (ever smokers).

CpG	CHR	Gene	Beta (mean ± SD)	Regression coefficient (β)	*p*-value	FDR	Epigenetic clocks
cg12745325	12	SLC39A5	0.64 ± 0.06	−0.005	6.6×10^{-5}	0.04	DNAmTL
cg24081819	8	EPHX2	0.10 ± 0.03	0.003	7.1×10^{-5}	0.04	DNAmHorvathAge

Linear mixed-effects models, adjusted for age, sex, an indicator of each study waves visit (KORA S4, KORA F4, or KORA FF4), Houseman imputed cells, educational attainment, alcohol consumption, smoking status (current/former/never), physical activity (low/medium/high), BMI, hypertension, diabetes, HDL, and LDL. Batch and chip numbers were included as random effects in the models to control the potential technical variations.
CHR: chromosome; FDR: Benjamini-Hochberg false discovery rate.
DNA methylation in the 0–1 range can be divided in low-, moderate- and high-methylation with ranges [0–0.35], [0.35–0.65] and [0.65–1] respectively.

construct-specific from exposure-specific sensitivity. For study design, the results support using multi-clock panels when feasible, treating DNAmTL as a telomere-focused complement, and prioritizing DNAmGrimAge and DNAmPhenoAge when hypotheses target morbidity-linked or smoking-sensitive pathways.

4.3. Susceptibility factors of air pollution effects

The most recent publications showed that behavioral, lifestyle, and health factors are associated with DNA methylation age (Quach et al., 2017; Ryan et al., 2020). In Lu et al.'s study, smokers had significantly shorter DNAmTL shortened by around 0.02 kilobases per smoking pack-year (Lu et al., 2019b). Cardenas et al. reported that former smokers had increased aging accelerating effect of DNAmHorvathAge and shorter DNAmTL relative to non-smokers (Cardenas et al., 2022). We observed that our results were sensitive to adjustment for smoking and that ever smokers had a stronger negative association between air pollutants and DNAmTL as well. In addition, DNAmHorvathAge and DNAmPhenoAge showed a stronger association with all air pollutants in ever smokers compared to participants who never smoked. Epigenetic modifications are associated with aging and age-related diseases (Fraga and Esteller, 2007; Calvanese et al., 2009; Gentilini et al., 2013). For some diseases such as cancer, cardiovascular disease (CVD), and diabetes, the DNA methylation levels might be altered; therefore, chronic diseases are related to most epigenetic clocks. Individuals with chronic diseases might have a faster aging speed than healthy people (Oblak et al., 2021). In the NAS investigating the associations between long-term exposure to air pollution ($PM_{2.5}$ and BC) and epigenetic clocks, the authors found that participants with chronic diseases, e.g., coronary heart disease, hypertension, and a lifetime cancer diagnosis, had a higher mean epigenetic age than their respective counterparts (Nwanaji-Enwerem et al., 2016). However, in our main analysis and stratified analysis in never smokers, we observed inverse associations with stronger acceleration of DNAmHannumAge and shorter DNAmTL in non-hypertensive participants compared to individuals with hypertension, but those effects were attenuated in ever-smoking participants. A potential explanation might be unknown confounding factors such as anti-hypertensive medication. We found one study that reported that any anti-hypertensive medicines were associated with higher aging acceleration on DNAmHorvathAge (Gao et al., 2018). However, another study observed rather opposite results in their cross-sectional design (Kho et al., 2021). Regarding differences between women and men, some epigenetic aging biomarkers might be sex-specific, and females might experience increased aging speed after menopause (Levine et al., 2016; Ward-Caviness et al., 2016).

4.4. Enriched pathway with exposure-related CpG

An increasing number of studies investigated the effects of air pollution exposure on DNA methylation at single CpG level, and many of them reported significant exposure-related CpG sites (Panni et al., 2016; Plusquin et al., 2017; Lichtenfels et al., 2018; Gondalia et al., 2019; Lee et al., 2019; White et al., 2019; Eze et al., 2020; Wang et al., 2022). Plusquin et al. found that long-term exposures to NO_2 and NO_x were associated with several CpG sites. Moreover, they connected those CpGs with transcriptomic data, and the enriched pathways identified by the significant CpG-transcript pairs were mainly involved in the immune system (Plusquin et al., 2017). White et al.'s study also conducted a limited EWAS by restricting the CpG sites to those included in DNAmHorvathAge, DNAmHannumAge, and DNAmPhenoAge. They observed that cg22920873, annotated by the *C7orf55* gene on chromosome 7, was associated with annual exposure to PM_{10}, and the identified pathway was related to anatomical structure development, regulation of developmental process, and cell communication (White et al., 2019). By using a candidate pathway method to conduct pathway enrichment analysis, Eze et al. observed that C-reactive protein, BMI, and eGFR (estimated glomerular filtration rate) were the enriched pathways for long-term traffic-related $PM_{2.5}$ exposure (Eze et al., 2020). Our studies suggested that the CpG-annotated genes were mainly involved in lipid metabolism and cardiovascular disease. However, neither identified CpG sites nor enriched pathways out of those studies and our study were consistent. As several systemic reviews indicated that air pollution was consistently associated with alterations in DNA methylation (Alfano et al., 2018; Wu et al., 2021), more studies are needed to investigate the heterogenicity between different population and explore the potential consistent and inconsistent findings.

4.5. Strengths and limitations

To the best of our knowledge, this is the first longitudinal study to explore the associations between ambient air pollutants and DNAm-based estimators, including the first, second, and third generation of epigenetic clocks, DNAm-based telomere length, and multiple air pollutants. Robustness of findings was supported by extensive sensitivity analyses. We had the largest sample size of participants and more air pollutant exposures compared to previous studies (Nwanaji-Enwerem et al. 2016, 2017; Ward-Caviness et al. 2016, 2020; White et al., 2019; de Prado-Bert et al., 2021; Wang et al., 2021; Baranyi et al., 2022). Moreover, the KORA cohort is a well-characterized study with standardized and comprehensive methods to collect individual information, enhancing our results' reliability. The longitudinal study design with repeated measurements of biomarkers strengthened statistical power and reduced potential residual confounding from unmeasured factors. It might also provide analytical improvement to previous cross-sectional analyses despite the lack of replication by other cohorts. This study also has the strength to assess the susceptibility from both external and intrinsic factors, especially behavioral and lifestyle factors, which are known to affect the human epigenome.

A limitation of our study is that annual average concentrations of air pollutants were estimated using spatial models for 2014–15. We believe these exposure estimates are valid for the historical spatial contrasts because previous studies have shown that the spatial variation in exposure remained stable over time (Eeftens et al., 2011; Gulliver et al., 2013; Wang et al., 2013; de Hoogh et al., 2018). To address temporal mismatch, we applied a monitor-based back-extrapolation to recover year-specific absolute levels while preserving spatial contrasts. Moreover, we restricted our study to non-movers (participants who did not move within the study period) to reduce the exposure misclassification in sensitivity analyses. The robust results from non-movers validated our exposure assessment approach. Nevertheless, unavoidable measurement error from residual spatial or temporal misalignment may cause non-differential misclassification, typically attenuating associations and widening CIs.

Other limitations include cross-platform methylation measurement (450K vs EPIC) with partial CpG coverage; however, overlap-only recalculations indicated minimal impact. The observational design limits causal inference, and several pollutant–clock associations had wider confidence intervals. Generalizability may be restricted to populations with similar demographic and environmental contexts; KORA reflects a single southern German region, so external validity is strongest for middle-aged and older adults in comparable urban–rural European settings with similar sociodemographic profiles and pollution sources.

5. Conclusions

In conclusion, our study suggested that long-term exposure to traffic-related air pollution is associated with epigenetic aging biomarkers. This was particularly the case in current and former smokers, who might be a susceptible population group for air pollution exposure. The study indicates that traffic-related air pollution is potentially contributing to immunosenescence and thereby strengthens the evidence that chronic exposure to low-level air pollution impacts multiple non-communicable

diseases such as cardiometabolic and pulmonary diseases and cancer.

CRediT authorship contribution statement

Yueli Yao: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Kathrin Wolf:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Susanne Breitner:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Siqi Zhang:** Writing – review & editing, Methodology, Conceptualization. **Melanie Waldenberger:** Writing – review & editing, Methodology, Data curation. **Juliane Winkelmann:** Writing – review & editing, Funding acquisition. **Alexandra Schneider:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Annette Peters:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2025.123284.

Data availability

KORA data and biosamples are available upon request (https://helmholtz-muenchen.managed-otrs.com/external/)

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