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Bronchial cell epigenetic aging in a human experimental study of short-term diesel and ozone exposures

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Abstract

Blood-based, observational, and cross-sectional epidemiological studies suggest that air pollutant exposures alter biological aging. In a single-blinded randomized crossover human experiment of 17 volunteers, we examined the effect of randomized 2-h controlled air pollution exposures on respiratory tissue epigenetic aging. Bronchial epithelial cell DNA methylation 24 h post-exposure was measured using the HumanMethylation450K BeadChip, and there was a minimum 2-week washout period between exposures. All 17 volunteers were exposed to ozone, but only 13 were exposed to diesel exhaust. Horvath DNAmAge [Pearson coefficient (r) = 0.64; median absolute error (MAE) = 2.7 years], GrimAge (r = 0.81; MAE = 13 years), and DNAm Telomere Length (DNAmTL) (r = –0.65) were strongly correlated with chronological age in this tissue. Compared to clean air, ozone exposure was associated with longer DNAmTL (median difference 0.11 kb, Fisher's exact *P*-value = .036). This randomized trial suggests a weak relationship of ozone exposure with DNAmTL in target respiratory cells. Still, causal relationships with long-term exposures need to be evaluated.

Keywords: DNA methylation age; particulate matter; RCT; lung tissue

Introduction

Exposure to air pollution is ubiquitous and remains a leading contributor to global morbidity and mortality. It is estimated that 99% of the world lives in areas with air quality that does not meet World Health Organization air quality guidelines and each year, air pollution exposure is associated with approximately 6.7 million premature deaths globally [1]. Diesel exhaust and ozone are two particularly toxic air pollutants [2, 3]. On a molecular level, the pathogenesis of air pollution is believed to occur through two main pathways: (i) local respiratory tissue/inflammation, and (ii) the translocation of particles and their component species across alveolar membranes into circulation contributing to systemic inflammation and other tissue toxicity [4]. More recently, researchers have utilized DNA methylation-based biomarkers of aging and health status as tools to better understand these molecular relationships while predicting morbidity and mortality risks. Existing observational studies support the hypothesis that air pollution exposure in adults, including to diesel and ozone, is associated with epigenetic age acceleration—suggesting increased morbidity and mortality risks in individuals with high exposures [5–8]. Still, many of the studies that have established this relationship involved long-term exposure windows (e.g. 1-year exposure), focused on leukocyte epigenetic aging, and are observational in nature. This is likely due to trends in aging being considered long-term phenomena unlikely to experience substantial alterations because of transient, short-term exposures. However, short-term exposure to air pollution has been associated with mortality [9] and there is evidence of short-term exposures being associated with changes in the methylome [2, 3]. Hence, if shorter-term exposures can be associated with epigenomic changes, it is plausible that they could also be associated with changes in epigenetic age. If these relationships do exist, characterizing them would be important for the broader air pollution and DNA methylation literature. Furthermore, considering that direct respiratory toxicity is

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one pathway for air pollution pathogenesis, there is also a need for studies that include respiratory target tissues, known to be sensitive to air pollutant exposure.

To better understand the impact of air pollution on human respiratory target tissues, the present study employs a randomized, single-blind, crossover study design to assess the effects of short-term diesel exhaust and ozone exposures on human bronchial epithelial cell epigenetic aging. Our study focuses on seven of the most biologically significant and well-studied epigenetic aging measures and evaluates their utility in target bronchial epithelial tissue. The Hannum [10], Horvath [11], and the SkinBloodClock [12] epigenetic biomarkers are primarily viewed as DNA methylation-based predictors of biological age, where the deviation of these predictors from chronological age is associated with morbidity and mortality. The PhenoAge [13] clock is primarily considered a biomarker of overall morbidity, while the GrimAge [14] clock is the best estimator of mortality risk. The DNA methylation Telomere Length (DNAmTL) [15] biomarker is correlated with directly measured telomere length (TL), but may reflect cell replication rather than TL itself. The epigenetic time to cancer 2 (EpiTOC2) [16] measure is a DNA methylation-based surrogate of mitotic cell divisions that predicts tissue-specific cancer risk. Given that aging is a multifactorial process, examining this evidenced-backed panel of biomarkers that each provide different information on DNA methylation-based biological aging allows for a more comprehensive understanding of epigenetic aging relationships. Therefore, in lung epithelial cells, we (i) evaluated epigenetic age prediction performance and (II) tested for associations between aging biomarkers and diesel and ozone exposures. We utilized the randomized study design to aid us in addressing the causality of any observed air pollution and epigenetic age relationships.

Results

Participant characteristics

Only 13 (76%) of participants were exposed to diesel exhaust, while all 17 participants were exposed to both ozone and clean air. Table 1 presents the characteristics of the 17 study participants. Participants had a mean (SD) chronological age of 25.3 (3.7) years, ranging from 21 to 33 years. Most participants were male (88%) and white (88%). The four participants who were not exposed to diesel were all male. These participants were younger than the remaining 13 participants who were exposed to both diesel and ozone, but this difference was not statistically significant (Wilcoxon rank sum test $P = .49$).

Table 1. Study participant characteristics

Variables	Full cohort ($n = 17$) Mean (SD) [range] or n (%)	Participants with diesel and ozone exposures ($n = 13$) Mean (SD) [range] or n (%)	Participants missing diesel exposure ($n = 4$) Mean (SD) [range] or n (%)
Chronological age (years)	25.3 (3.7) [21–33]	25.7 (3.9) [21–33]	24.0 (2.9) [21–27]
Sex			
Female	2 (12%)	2 (15%)	0 (0%)
Male	15 (88%)	11 (85%)	4 (100%)
Race			
Black	1 (6%)	0 (0%)	1 (25%)
Hispanic	1 (6%)	1 (8%)	0 (0%)
White	15 (88%)	12 (92%)	3 (75%)
First pollution exposure			
Diesel	8 (47%)	8 (62%)	–
Ozone	9 (53%)	5 (38%)	–

Performance of epigenetic clocks and effects of controlled exposures

Figure 1 displays the Pearson correlations of the epigenetic clocks, DNAmTL, and epiTOC2 with chronological age. Of the epigenetic biomarkers that we examined, three were associated with chronological age. GrimAge demonstrated the strongest correlation with chronological age with a relatively large error ($r = 0.81$; MAE = 13 years), while Horvath DNAmAge ($r = 0.64$) and DNAm TL ($r = -0.65$) had similar absolute magnitude correlations with chronological age. The correlation with DNAmTL was negative as expected given the shortening of telomeres with advancing chronological age. The MAE for Horvath DNAmAge was relatively small (MAE = 2.7 years). With respect to Pearson correlation coefficients of well-performing epigenetic aging biomarkers, DNAmTL was moderately and negatively correlated with EAA Horvath ($r = -0.53$) and EAA GrimAge ($r = -0.57$). EAA GrimAge was moderately correlated with EAA Horvath ($r = 0.50$).

Table 2 presents the median difference in bronchial epithelial cell epigenetic age for 2-h chamber exposures to diesel exhaust and ozone relative to clean air. Compared to clean air, ozone exposure was associated with longer DNAmTL (median difference 0.11 kb, Fisher's exact P-value = .036, Fig. 2c). All other epigenetic aging markers tested against the exposure paradigms were null.

Discussion

In this randomized, single-blind, crossover study, we tested the performance of epigenetic aging biomarkers in bronchial epithelial cells and examined relationships of short-term controlled diesel exhaust and ozone exposures. We observed that Horvath's DNAmAge, GrimAge, and the DNAmTL epigenetic aging biomarkers performed well, even with the small sample of participants. In this crossover trial, ozone exposure was associated with longer DNAmTL relative to clean air in target bronchial respiratory cells. We could not reject the null hypotheses for EAA GrimAge, EAA Horvath, and other epigenetic aging biomarkers that were not correlated with chronological age in bronchial tissue (Hannum, Skin and Blood, PhenoAge, and EpiTOC2).

Research examining relationships between epigenetic age and ozone is limited. We identified one such study in the Scottish-based Lothian Birth Cohort [17]. The authors examined relationships of residential-linked life course air pollution exposures with blood epigenetic aging measures (Horvath, Hannum, PhenoAge, GrimAge, and DNAmTL). Annual fine particle, ozone, sulfur dioxide, and nitrogen dioxide concentrations around 1935, 1950, 1980, 1990, and 2001 were estimated using the EMEP4UK atmospheric chemistry transport model. In their study sample, increased annual overall air pollution levels were associated with a 0.322 year EAA Horvath and 0.015 to 0.017 kb shorter DNAmTL. Annual ozone levels were not associated with EAA Horvath; however, they were associated with longer DNAmTL ranging from 0.012 kb to 0.015 kb in men and women, respectively. This latter finding agrees with our observed association of ozone exposure and longer DNAmTL although the magnitude of our association is

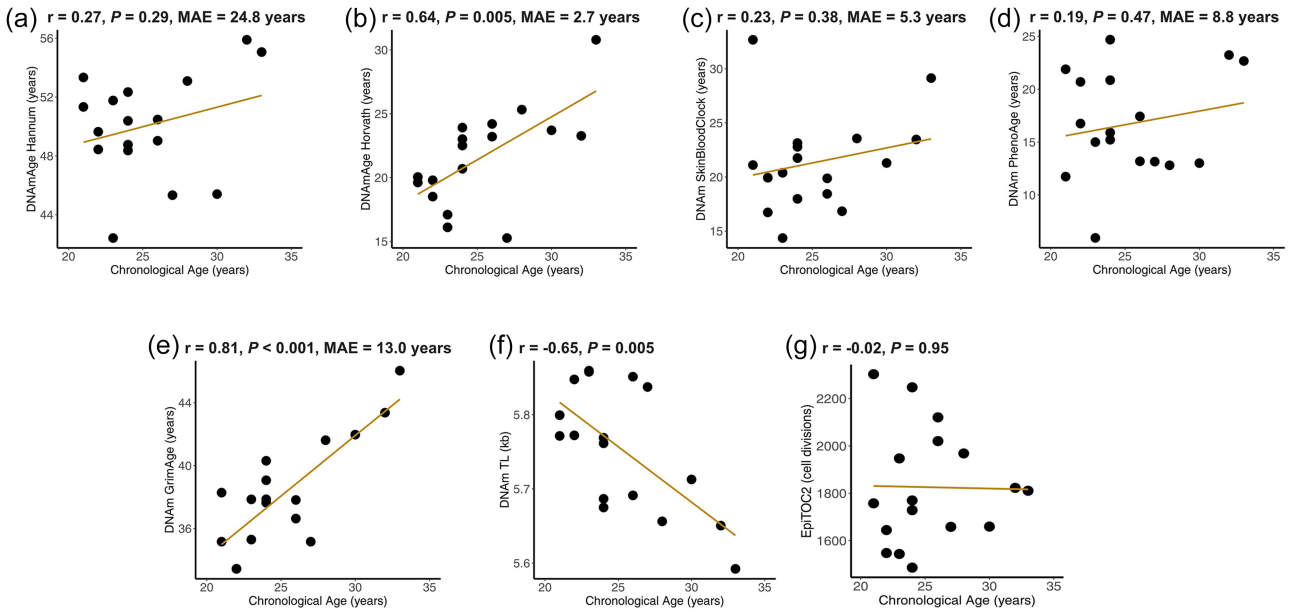

Figure 1. This figure presents the clean air chronological age and epigenetic age Pearson correlation coefficients (r) and MAEs in the study sample ($n = 17$) for DNAmAge Hannum [a], DNAmAge Horvath [b], DNAmAge SkinBloodClock [c], DNAm PhenoAge [d], DNAm GrimAge [e], DNAm TL [f], and EpiTOC2 [g].

Table 2. Estimated difference in well-performing epigenetic age biomarkers after controlled exposures compared to clean air (Obs = 47)

	Diesel ($n = 13$)		Ozone ($n = 17$)	
Aging biomarker	**Difference in medians for epigenetic age biomarker**	**Fisher's exact *P*-value**	**Difference in medians for epigenetic age biomarker**	**Fisher's exact *P*-value**
EAA Horvath (years)	0.72	0.202	−1.23	0.249
EAA GrimAge (years)	−0.72	0.920	−1.10	0.593
DNAm TL Age Adjusted (kb)	−0.03	0.322	0.11	0.036

47 Observations = 17 clean air, 17 ozone, and 13 diesel observations.
EAA = epigenetic age acceleration (residuals of regressing epigenetic age on chronological age).

at least seven times greater and observed in lung epithelial cells. Although we do not know the true reason for this difference in magnitude, possible reasons include that air pollutants have more direct toxic effects on bronchial cells than they do on peripheral leukocytes, that timing of exposure and aging biomarker measurement matters, and that the Lothian Birth Cohort study performed mean regression, while we assessed median differences. Randomized controlled studies examining blood epigenetic aging relationships will be helpful for better characterizing this relationship. Furthermore, the direction of this association may seem contradictory to the framework that adverse air pollution exposures are associated with biological age acceleration, but there are notable nuances for the interpretation of DNAmTL compared to directly measured TL. In particular, previous evidence has suggested that DNAmTL captures broader aspects of cellular aging related to telomeres, but potentially independent of telomerase activity and telomere attrition [18]. There is also some evidence that transient inflammatory events, like short-term ambient pollution exposures, are associated with lengthening telomeres [19].

Compared to the ozone literature, evidence linking diesel exposure with epigenetic aging is more robust. As noted, diesel exhaust makes up a large fraction of fine particle air pollution. Although the solid fraction of diesel exhaust is mainly composed of elemental carbon and metals, inorganic and organic gases are also important components. The previously described life course study in the Lothian Birth Cohort reports associations of fine particle mass, sulfur dioxide, and nitrogen dioxide with EAA Horvath [17]. These findings support the paradigm of air pollutants being adverse exposures that would accelerate measures of biological aging and increase morbidity and mortality risk. Observational long-term exposure and blood epigenetic age studies in cohorts in the USA [5, 7] and Germany [6] further support the conclusions made in the Scottish Lothian Cohort. With respect to short-term exposure studies, in an observational study of 3-day exposures, nitroaromatics and polycyclic aromatic hydrocarbons found in diesel exhaust were associated with blood EAA PhenoAge in an elderly Chinese Cohort [8]. The same authors went on to show that 72-h moving averages of fine particle air pollution modified short-term air pollution and blood pressure relationships, where positive associations were stronger in epigenetically older individuals [20]. In a study of 26 healthy college students in Beijing, China, naturally occurring 24-h fine particle pollution waves were associated with EAA in Horvath, Hannum, GrimAge, and epiTOC [21]. Compared to these studies, we find no significant relationships between diesel exhaust exposure and epigenetic age. This is possibly due to the even smaller sample size for this exposure due to nonspecific participant dropout. Hence, larger randomized trials where both blood and respiratory tissues are examined will help future analyses.

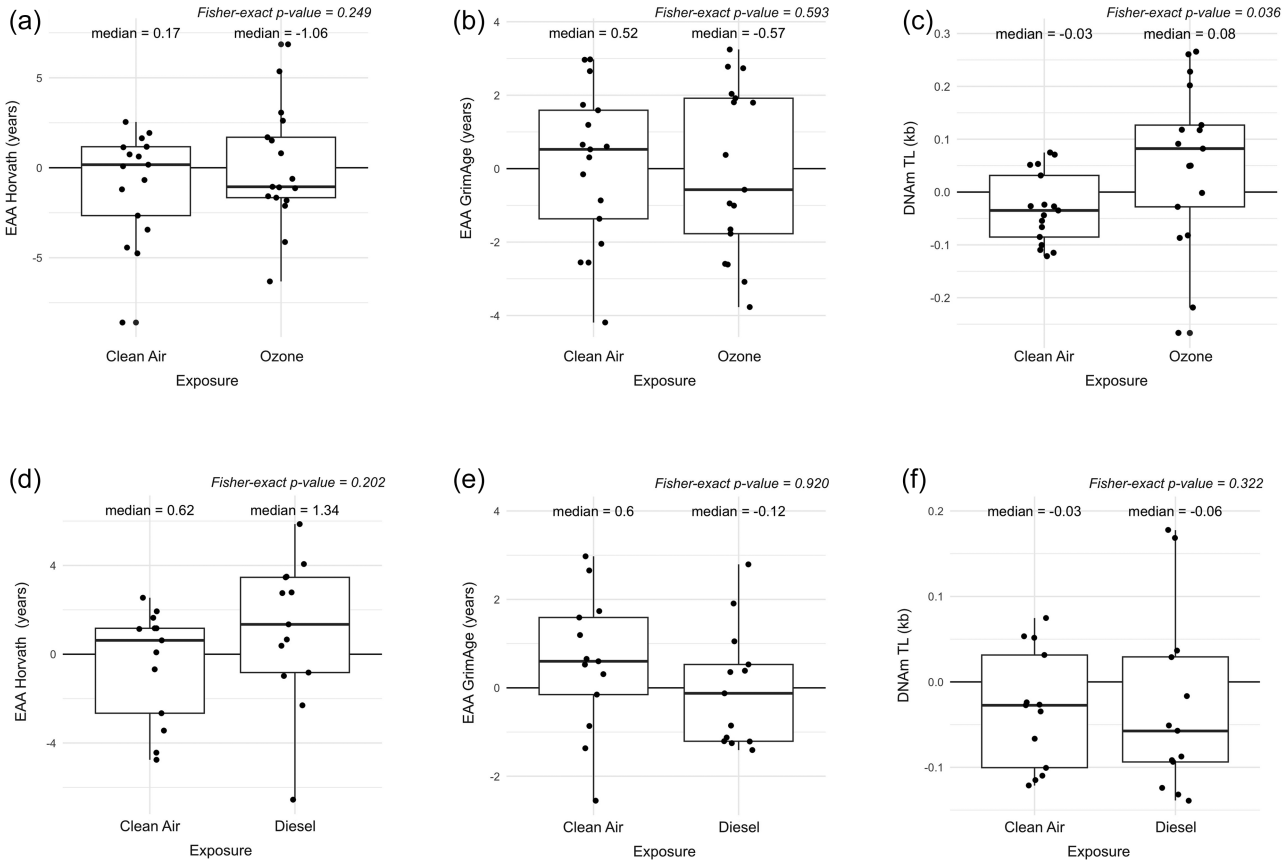

Figure 2. This figure presents the median differences for EAA Horvath [a], EAA GrimAge [b], and DNAmTL [c] among participants exposed to clean air and ozone as well as median differences for EAA Horvath [d], EAA GrimAge [e], and DNAmTL [f] among participants exposed to clean air and diesel.

Strengths of our study include its randomized crossover design and novel examination of target bronchial epithelial cell epigenetic aging; still, it has some important limitations. For instance, our study population is composed of healthy individuals, majority of which are white men from the USA. Thus, we have important limits to racial, gender, and geographical generalizability. Additionally, our observed relationships may be different in individuals with existing comorbidities. While our study sample of 17 individuals is small, our study design provides stronger insights into causal relationships when compared to observational studies. Lastly, we only measured epigenetic aging 24 h post exposure and it is possible that our observed associations represent transient changes in line with biomarker changes observed in previous controlled exposure studies.

In conclusion, the present study provides weak evidence of a causal—but possibly transient—effect of short-term ozone exposures with DNAmTL measured in human bronchial epithelial cells, a target tissue for ambient air pollutants. Nevertheless, causal relationships with long-term exposures across tissues, including peripheral blood cells, and in diverse populations are needed.

Materials and methods

Study participants and experimental design

The study sample and exposure design have been previously described in detail [2, 3]. Overall, 17 healthy participants were recruited under a contract with Westat Corporation (Rockville, MD, USA). Study participants were excluded if they were pregnant, smokers, had allergies, or cardiopulmonary disease (as determined via medical history, questionnaire, or physical exam), or had a forced vital capacity or forced expiratory volume in the first second of expiration (FEV_1) <80% predicted for their height and age. All participants were informed of the study procedures and potential risks, and all provided a written informed consent prior to enrollment. The controlled exposures were conducted at the US Environmental Protection Agency (EPA) Human Studies Facility on the campus of the University of North Carolina (UNC), Chapel Hill. The consent forms and protocol were approved by the UNC School of Medicine and the US EPA's Institutional Review Board. The study was powered to detect physiological differences, specifically changes in (FEV_1). The trial was registered on ClinicalTrials.gov (NCT01492517).

At the start of this randomized, single-blind, crossover study, 17 participants were randomly assigned an exposure order: 2 h to clean air (i.e. PM, CO, NO_2, SO_2 concentrations were below detection limit) or 0.3-ppm ozone or 0.3-ppm of diesel exhaust in separate study visits. During the 2-h exposure period, participants alternated between 15 min of rest and 15 min of exercise on a cycle ergometer. Minute ventilation was measured during exercise, and the cycle ergometer workload was adjusted to obtain a minute ventilation of approximately 25 l min^{-1} m^2 body surface area. Bronchial epithelial cells were collected 24 h after each exposure. Each exposure took place at a different study visit, and a minimum 2-week washout period between exposure experiments was implemented in the study design to avoid carry-over effects associated with earlier exposures (Fig. 3). Although participants were asked to complete all exposures, some participants did not

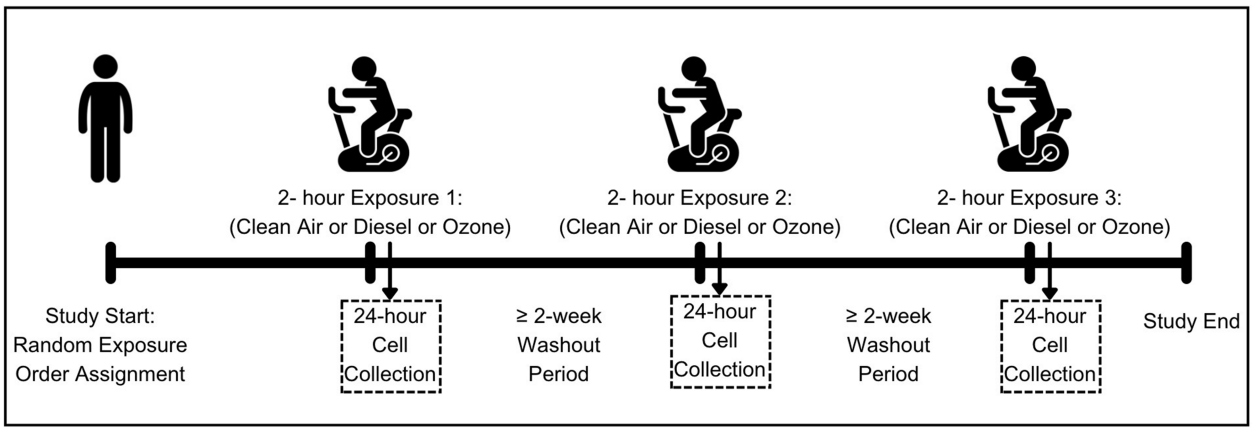

Figure 3. This figure presents the randomized crossover study design for the study sample where all 17 participants completed the clean air and ozone exposure study arms, but only 13 were exposed to diesel with no reasons provided for participant dropout.

return for certain exposures resulting in only 13 of the 17 participants being exposed to diesel exhaust. No reasons were provided for this dropout. All participants completed clean air and ozone exposures.

DNA methylation measurements and calculation of epigenetic clocks

Following a standard protocol, 24 h after each exposure, study participants underwent a research bronchoscopy with brush biopsy to collect bronchial epithelial cells [22, 23]. The cytology brushes containing epithelial cells were placed in 1.5 ml tubes with 200 µl of Trizol Buffer (ThermoFisher Scientific). DNA was extracted using the Gentra Puregene Buccal Cell Kit (Qiagen, Inc.), and DNA samples were stored at −80°C until analysis at a commercial laboratory (Expression Analysis, Durham, NC, USA). DNA methylation analysis was performed using the Illumina HumanMethylation 450 K BeadChip array, where the extracted DNA samples were placed on four chips. We performed background correction using noob. We performed dye bias correction and corrected for probe design bias arising from Type I and Type II probes with the Beta-mixture quantile normalization method [3, 24, 25]. DNA methylation beta values (i.e. the proportion of cytosine residues methylated) from 484 531 CpGs were used for analyses. EpiTOC2 was calculated from the methylation beta values using instructions and R code available at https://doi.org/10.5281/zenodo.2632938. The remainder of the epigenetic clocks were calculated by uploading the required methylation beta values to a publicly available online calculator as recommended (http://dnamage.genetics.ucla.edu).

Statistical analysis

We estimated Pearson correlations between chronological age and each of the epigenetic biomarkers from the clean air exposures. For all measures except for DNAmTL and epiTOC2, we calculated the median absolute error (MAE) in years, computed as the median absolute deviation between age predicted by each epigenetic clock and chronological age to further evaluate accuracy. Epigenetic age measures that were associated with chronological age were designated as well-performing to further evaluate effects in our study.

We tested sharp null hypotheses stating that the epigenetic age measures are the same (i) under exposures to diesel exhaust and clean air, and (II) under exposures to ozone and clean air. Instead of using distributional approximations, we constructed the null randomization distributions of the paired *t*-test statistic and calculated Fisher's exact *P*-values separately for each epigenetic age measure [26]. All statistical analyses were performed using R Version 4.2.2 (R Core Team, Vienna, Austria).

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Author Contributions

Jamaji C. Nwanaji-Enwerem (data analysis, visualization, original writing), Anne K. Bozack (data analysis, visualization, original writing), Cavin Ward-Caviness (data analysis, Supervision, Writing—review & editing), Marie-Abèle C. Bind (data analysis, Supervision, Writing—review & editing), David Diaz-Sanchez (data gathering, Conceptualization), Robert B. Devlin (data gathering, Conceptualization), Andres Cardenas (Conceptualization, Supervision, Validation, Writing—review & editing).

Conflict of interest: C.W.C. is a scientific advisor for the Clock Foundation. The Clock Foundation had no role in any aspect of this work. The remaining authors declare no conflicts of interest.

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Data Availability

The datasets used and/or analyzed in the current study are available from the study team upon reasonable request and appropriate IRB approval.

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