## Title

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# Traffic-Related Air Pollution and Telomere Length in Children and Adolescents Living in Fresno, CA: A Pilot Study

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## Abstract

**Objective—**The main objective of this pilot study was to gather preliminary information about how telomere length (TL) varies in relation to exposure to polycyclic aromatic hydrocarbons (PAHs) in children living in a highly polluted city.

**Methods—**We conducted a cross-sectional study of children living in Fresno, California (n=14). Subjects with and without asthma were selected based on their annual average PAH level in the 12-months prior to their blood draw. We measured relative telomere length from peripheral blood mononuclear cells (PBMC).

**Results—**We found an inverse linear relationship between average PAH level and telomere length (TL) ($R^2 = 0.69$), as well as between age and TL ($R^2 = 0.21$). Asthmatics had shorter mean telomere length than non-asthmatics ($TL_{asthmatic}$=1.13, $TL_{non\text{-}asthmatic}$=1.29).

**Conclusions—**These preliminary findings suggest that exposure to ambient PAH may play a role in telomere shortening.

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**Conflict of interest (COI):** None declared

**Author Contributions**

Conceived the study, analyzed the data, and was the primary author of the paper: EL. Provided intellectual input and contributed to the writing of the paper: JB, EE, KH. Telomere length measurement and interpreting results: JL. Designed the study and provided PBMC samples: KN, MP. Provided PAH exposure data, edited and reviewed the paper: KH, BN. The authors declare that they have no conflicting interest.

## Introduction

In many urban settings, ambient air pollution is a major public health concern because of the associated burden of disease. According to the World Health Organization, outdoor air pollution is responsible for about 3.7 million deaths annually on a global basis (1). In the United States, exposure to traffic-related $PM_{2.5}$ (particulate diameter $\leq 2.5$ μm) may contribute to as much as 20% of total mortality (2). Air pollutants also appear to play an important role in the onset of many chronic diseases including asthma, lung cancer, ischemic heart disease and stroke (3–6). A number of epidemiological studies have demonstrated that exposures to particulate matter and ozone were associated with increases in cardiopulmonary mortality (7,8). Despite this mounting evidence, the exact underlying mechanisms by which air pollutants cause adverse cardiopulmonary health outcomes are not clear.

Animal studies have suggested several biological mechanisms to explain how air pollution induces disease outcomes (9). One possible mechanism is that the free radicals generated during the incomplete combustion of fossil-fuel products cause oxidative stress within the respiratory and cardiovascular systems (10). Oxidative stress occurs when free radicals exceed the relative amount of antioxidants. Reactive oxygen species (ROS), a common class of free radical, are generated with inhalation of certain air pollutants. Evidence from a number of epidemiological studies indicates that air pollution causes oxidative stress, which is capable of damaging lipids, proteins, and DNA (10–12). Since telomeres play a critical role in chromosome stability and cell viability, it is reasonable to use telomere length as a biomarker for air pollution induced cytotoxicity.

Recent studies of telomere length and exposure to high levels of traffic-related air pollutants in healthy adults have found shortening of telomeres associated with increasing air pollution levels (13,14). Telomeres are multiple short sequences of DNA located at the end of linear eukaryotic chromosomes (5′AGGGTT2′) (15). Maintenance of telomere length is important for cell viability because cells with short telomeres lose their ability to divide and become senescent or undergo apoptosis (16). In addition, telomeres protect chromosomes against inappropriate recombination and fusion with other broken chromosomes, which can potentially lead to malfunction, cancer, or cell death (15,16). Since the guanine base is more prone to be oxidized than other DNA bases, the high guanine content of the telomere sequence makes telomeric DNA vulnerable to oxidative stress (17,18).

Children may be especially vulnerable to the effects of telomeric DNA damage due to their physical development as well as developing immune system. One study has shown different telomere attrition rates among newborns, their parents, and grandparents (19). This suggests that children may have different telomere regulation than adults and thus may be differentially susceptible to effects of air pollution

As the first step towards a better understanding of the long-term health effects of traffic-related air pollution on telomere length, we conducted a pilot study to gather information about how telomere length varies in relation to air pollution, age, sex, and asthma status. In this study, we focus on polycyclic aromatic hydrocarbons (PAHs). PAHs are a class of chemical compounds characterized by fused benzene rings (20). PAHs are produced during incomplete combustion of organic matter. They exist in ambient air in both gas and particle phases (adsorbed to particulate matter). In many urban environments, motor vehicle exhaust is the main source of high-molecular-weight PAHs (four to six rings), which are more carcinogenic and mutagenic than low-molecular-weight PAHs (two- and three-rings) (21). Ambient concentrations of PAHs in the United States range from 0.02–1.2 ng/m$^3$ in rural areas, and 0.15–57.1 ng/m$^3$ in urban environments (22–25). PAHs are ubiquitous ambient air pollutants in Fresno and can be transformed into quinones in the atmosphere (25–27). Quinones can serve as catalysts in redox cycling and generate free radicals (26,27).

## Methods

All methods and procedures were approved by the institutional review boards of Stanford University and the University of California, Berkeley.

### Study subjects

Subjects were selected from a larger population of children enrolled in an ongoing study of asthma in Fresno, CA (Figure 1). They were age 11 to 18 years old, living in Fresno, California. Fresno is located in the center of the San Joaquin Valley, which is part of the Central Valley in California. Fresno is the second-most polluted city in the United States, in terms of 24-hour average $PM_{2.5}$ (28) and has a high prevalence of asthma (29). For the pilot study, 14 subjects were selected from high- and low-exposure groups, as defined by annual average 24-hour outdoor residential exposure to PAHs in the 12 months prior to their blood draw (2009–2012). The high-exposure group was defined as above the 80th percentile of PAH exposures and the low-exposure group below the 10th percentile. An equal number of subjects (n=7) were selected from the high- and low-exposure groups.

Study participants came from two related studies, the initial Fresno Asthmatic Children's Environment Study (FACES), and the subsequent Children's Health and Air Pollution Study (CHAPS). FACES was a longitudinal cohort study designed to follow children with asthma. CHAPS focused on the health risks of air pollution exposure in both asthmatic and non-asthmatic children in the San Joaquin Valley. Of the 14 subjects in the pilot, 5 were asthmatic, originally recruited for FACES, and 9 non-asthmatic subjects were recruited for CHAPS. At the baseline interview, all subjects provided detailed information on their general history and respiratory health. FACES study participants had asthma and underwent pre- and post-bronchodilator spirometry and skin prick testing for 14 aeroallergens common in the Fresno area. CHAPS subjects were defined as non-asthmatic and non-allergic if they had (1) no reported physician diagnosis of asthma, (2) normal pulmonary function test results, (3) total IgE (immunoglobulin E) <10IU/mL, and (4) negative skin test results. Further details on the study design and cohort characteristics can be found in papers published elsewhere (30–33).

### Individual PAH exposure estimates

To estimate the daily individual exposures to ambient PAHs, we developed a land use-regression model using PAH measurements from both a central monitoring site and outdoor residential samples from a subset of FACES participants' homes. .The filter-based PAH samples provided concentrations for 14 PAHs. However, we chose to use the sum of the mass concentrations of PAHs with 4-, 5- or 6-rings in this analysis as a metric representing the less volatile, particle-bound PAHs. This selected group of PAHs (PAH456) had a good correlation with the continuous measure of PAHs we were using in the spatial-temporal model. Outdoor, residential 24-hour PAH456 concentrations were used as the dependent variable in a mixed-effects regression model with a large number of independent land use and meteorological variables. Good agreement between predicted and measured concentrations of PAH456 was reported with the final model. The model parameters were used to calculate individual daily exposure to outdoor residential PAH456. More information on the model selection/parameters and field sampling of PAH456 can be found in Noth et al., 2011 (27).

## Telomere length measurement

Total genomic DNA was purified from peripheral blood mononuclear cells (PBMCs) using QIAamp® DNA Mini kit (QIAGEN, Cat#51104). The telomere length assay was adapted from the published original method by Cawthon (34,35). Telomere length was determined by relative ratio of telomere gene copy number to single copy gene copy number in each sample to reference DNA sample. The telomere thermal cycling profile consisted of:

Cycling for T(telomic) PCR: denature at 96°C for 1 second, anneal/extend at 54°C for 60 seconds, with fluorescence data collection, 30 cycles. Cycling for S (single copy gene) PCR: denature at 95°C for 15 seconds, anneal at 58°C for 1 second, extend at 72°C for 20 seconds, 8 cycles; followed by denature at 96°C for 1 second, anneal at 58°C for 1 second, extend at 72°C for 20 seconds, hold at 83°C for 5 seconds with data collection, 35 cycles.

The primers for the telomere PCR were *tel1b* [5′-CGGTTT(GTTTGG)$_5$GTT-3′], used at a final concentration of 100 nM, and *tel2b* [5′-GGCTTG(CCTTAC)$_5$CCT-3′], used at a final concentration of 900 nM. The primers for the single-copy gene (human beta-globin) PCR were *hbg1* [5′ GCTTCTGACACAACTGTGTTCACTAGC-3′], used at a final concentration of 300 nM, and *hbg2* [5′-CACCAACTTCATCCACGTTCACC-3′], used at a final concentration of 700 nM. The final reaction mix contained 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 200 μM each dNTP; 1% DMSO; 0.4× Syber Green I; 22 ng E. coli DNA per reaction; 0.4 Units of Platinum Taq DNA polymerase (Invitrogen Inc.) per 11 microliter reaction; 0.5 – 10 ng of genomic DNA. Tubes containing 26, 8.75, 2.9, 0.97, 0.324 and 0.108ng of a reference DNA (from Hela cancer cells) were included in each PCR run so that the quantity of targeted templates in each research sample can be determined relative to the reference DNA sample by the standard curve method. The same reference DNA was used for all PCR runs.

To control for inter-assay variability, eight control DNA samples were included in each run. In each batch, the the ratio of telomere to single copy gene (T/S) of each control DNA was divided by the average T/S for the same DNA from 10 runs to get a normalizing factor. This was done for all eight samples and the average normalizing factor for these samples was used to correct the participant DNA samples to get the final T/S ratio. The T/S ratio for each sample was measured twice. When the duplicate T/S value and the initial value varied by more than 7%, the sample was run a third time and the two closest values were reported. The coefficient of variation (CV) for this study was typically 2.5%.

### Statistical analysis

Linear regression was used to estimate the association between PAH456 and TL, adjusting for age, sex, race/ethnicity (Latino and White) and asthma status. In a sensitivity analysis, the oldest subject with the lowest TL was excluded.

## Results

Table 1 displays the summary characteristics of study subjects. The mean age, telomere length and PAH456 exposure are presented by sex, race/ethnicity and asthma status in Table 2. On average, TL was shorter in the higher PAH456 group; the difference in relative telomere length between the lowest and highest PAH456-exposed individual participants was 0.36.

Crude regression models for TL on age (Figure 2) and PAH456 (Figure 3) suggest inverse linear relationships for both. In a multivariable regression model, telomere length (TL) decreased by −0.14 units (95%CI: −0.25,−0.11) per one ng/m$^3$ increase in PAH456, adjusting for age, sex, race/ethnicity and asthma (Table 3). Altogether the covariates explained 83% of the variance in TL. Female participants had slightly longer mean telomeres than males ($TL_{female}$=1.25, $TL_{male}$=1.21). Asthmatic participants had shorter mean telomere length than non-asthmatic participants ($TL_{asthmatic}$=1.13, $TL_{nonasthmatic}$=1.29). The shortest telomere length (TL= 0.96) was found in the subject with the highest PAH456 exposure (4.2 ng/m$^3$). This subject was a 17 year-old Caucasian male asthmatic participant and his TL was between 1 and 2 standard deviations below the mean. After excluding this participant in sensitivity analysis, the association with PAH456 remained significant and the model R$^2$ decreased to 72%.

Asthmatic participants were exposed to higher levels of PAH456 than non-asthmatic participants (Figure 4). There were more male asthmatic participants in our sample than females and male participants were exposed to a wider range of PAH456 levels (Figure 5).

## Discussion

To the best of our knowledge, this is the first study to investigate the relationships between traffic-related air pollution, specifically ambient PAHs, and telomere length in children in the United States. We found that telomere length decreased with increasing PAH exposure among the small group of participants in this pilot study, consistent with the hypothesis that PAH exposure may cause oxidative stress that can accelerate telomere shortening. The fit of a linear model for TL and exposure to ambient PAH456 improved when adjusted for age, sex, race/ethnicity and asthma status. Therefore, our results also suggest that age, sex, and asthma status may influence the length of telomeres in children.

## Air Pollution and Telomere Length

The relationship between PAH exposure and telomere length we observed in this study of adolescents is consistent with studies in healthy adults that have shown telomere shortening with increasing air pollution levels (13,14,36–38). For example, Hoxha et al. reported mean leukocyte telomere length (LTL) among traffic officers in Milan, Italy was 1.10 (95% CI: 1.04–1.16) compared to a mean LTL in office workers of 1.27 (95% CI: 1.20–1.35) (14). In our younger participants, the mean telomere length of the subjects with the lowest PAH exposure was 1.38, whereas the telomere length of the participant with the highest PAH exposure was 0.96.

Previous studies have reported a dose-response relationship between PAH exposure and biomarkers of oxidative stress (39,40). Although preliminary pilot data, our results are consistent with the hypothesis that exposure to ambient PAHs (largely generated during combustion of diesel and gasoline fuels in Fresno) leads to oxidative stress, which in turn causes telomere shortening.

## Age and Telomere Length

Multiple studies have reported a trend of decreasing telomere length with increasing age (36–38). Most cells, with the exception of some germline and stem cells, lose their telomerase activity once they are differentiated into specific tissue or blood cells (36). In addition, there is less production of stem cells and other renewing cells with increasing age (41). In our participants, we found a weak inverse relationship between age and telomere length which could be due to the narrow age range of the subjects, or different telomere regulation in children and adolescents than that in newborns or adults. Previous studies have shown different telomere lengths and rates of telomere sequence loss with different age groups(19,36,37). Newborns had the most rapid loss of telomeres. The changes in telomere length in later life are rather gradual with advancing age. The longer telomere lengths in newborns reflect a large proportion of immature hematopoietic progenitors that have not gone through extensive proliferation relative to adults (36,41).

## Sex and Telomere Length

Female participants had slightly longer telomeres than male participants, consistent with other studies (42,43). In a meta-analysis of telomere length by sex from 36 cohorts (n=36,230), females had longer telomeres than males. Several theories have been proposed to explain telomere length difference by sex. One is related to an estrogen-responsive element that can stimulate telomerase, an enzyme that synthesizes telomere sequences and adds them to the end of chromosomes (43). Another theory is that the properties of estrogen can counteract oxidative stress by up-regulating antioxidant enzyme expression (44). Another alternative explanation for the sex difference between females and males in this pilot study may be that there were more male than female participants with asthma.

## Asthma and Telomere Length

Asthma is a chronic inflammatory disease in the airways characterized by recurring exacerbations (45). Frequent inflammatory responses and rapid cell proliferation can lead to telomere shortening (46,47). Exposure to high levels of air pollution can trigger exacerbations of asthma that could lead to telomere shortening (48–50). Although the annual average concentration of ambient PAHs was higher among the asthmatic compared to the non-asthmatic participants, it is not possible in this pilot study to make inferences about whether the shorter telomeres in asthmatic children were due to their condition or due to exposure to high levels of PAH, or both.

### Strengths and Limitations

Previous studies have reported shorter telomere length in children in relation to community stress, poverty, and social deprivation (51), but as noted above, ours is the first to address air pollution. Additional strengths of our study include a novel marker of traffic-related air pollution, PAHs, and a novel biomarker of air pollution-related cytotoxicity, telomere length. Another is our focus on children for whom relatively scant data are available on the association between air pollution and telomere length.

There are several limitations of this pilot study. The primary limitation is the small sample size. Another major limitation is that the cross-sectional design limits the ability to make temporal inferences about whether telomere length shortening occurred after exposure to air pollution.

## Conclusions

Our pilot study results suggest that telomere shortening in children may be associated with exposure to traffic-related air pollution. Greater knowledge of the impact of air pollution at the molecular level is necessary to design effective interventions and policies. Our preliminary data will inform the design of a larger study to examine the hypothesis generated from these results.

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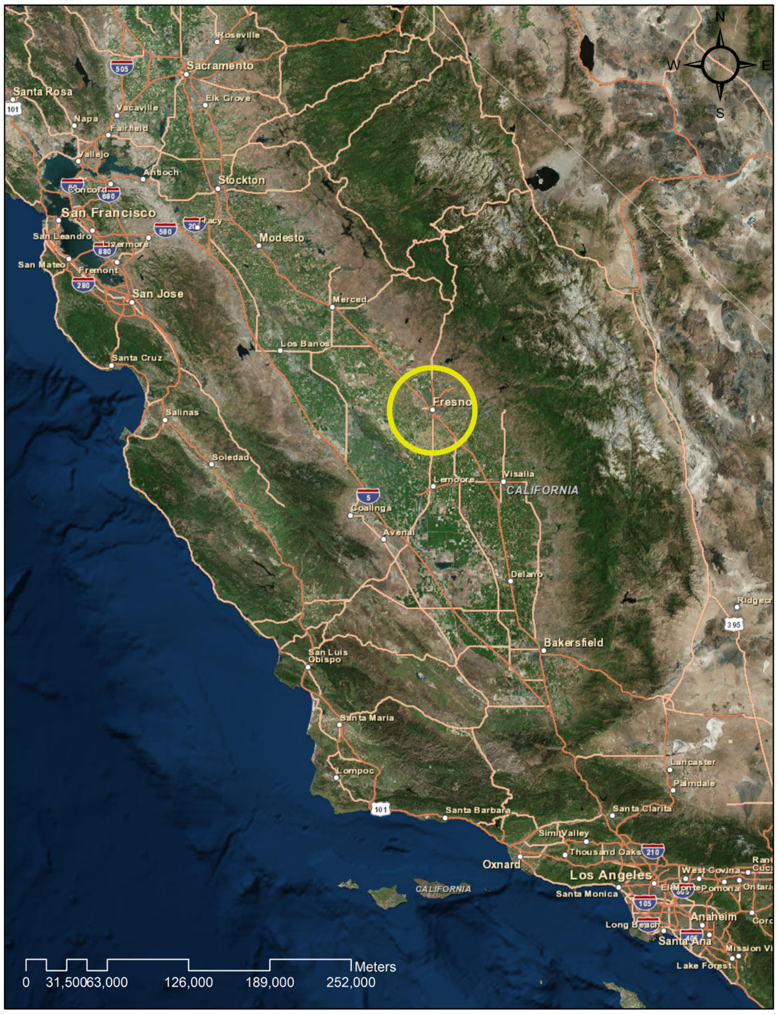

**Figure 1.**

Location of the study area.

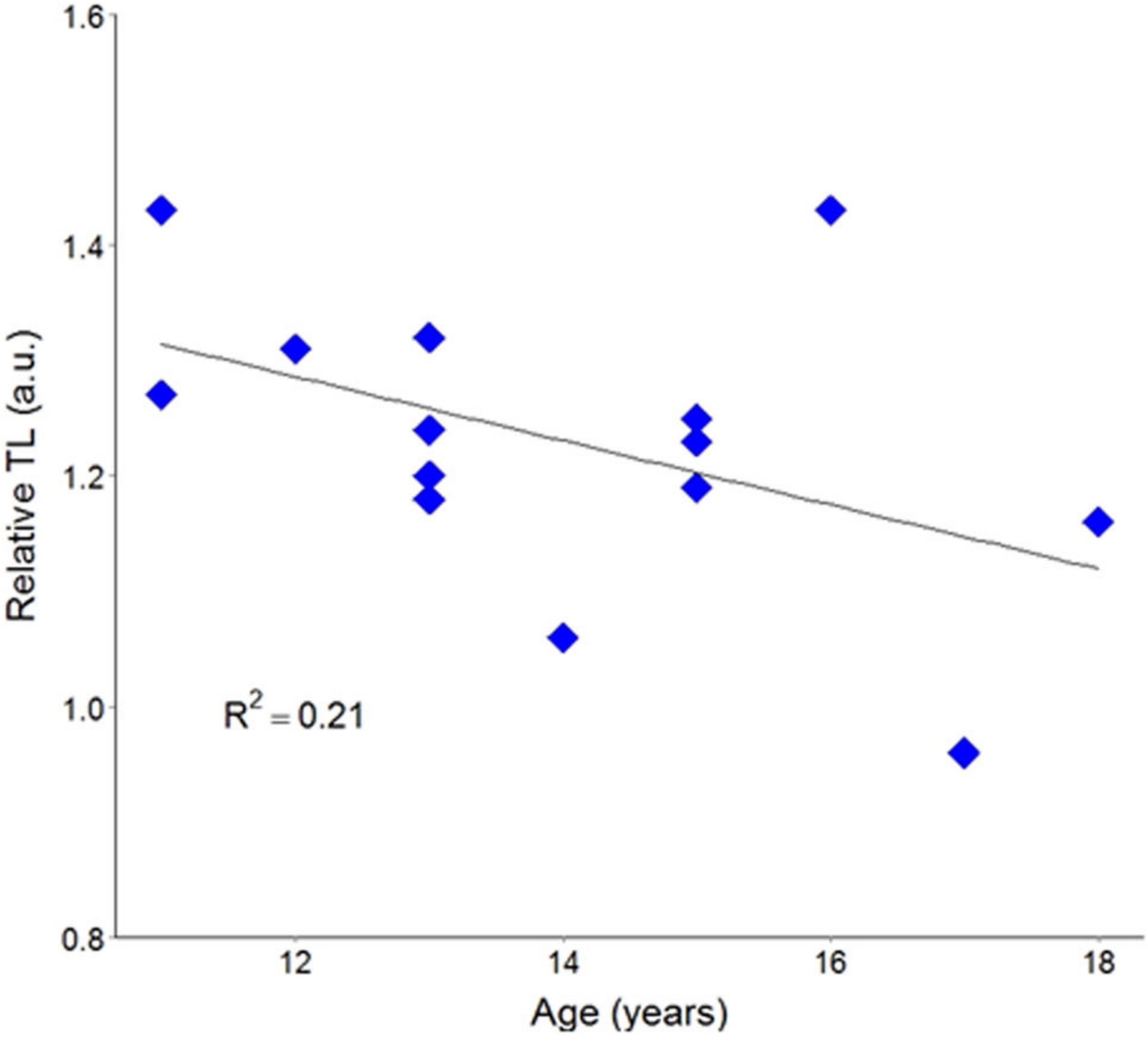

**Figure 2.**

Scatter plot for age and telomere length.

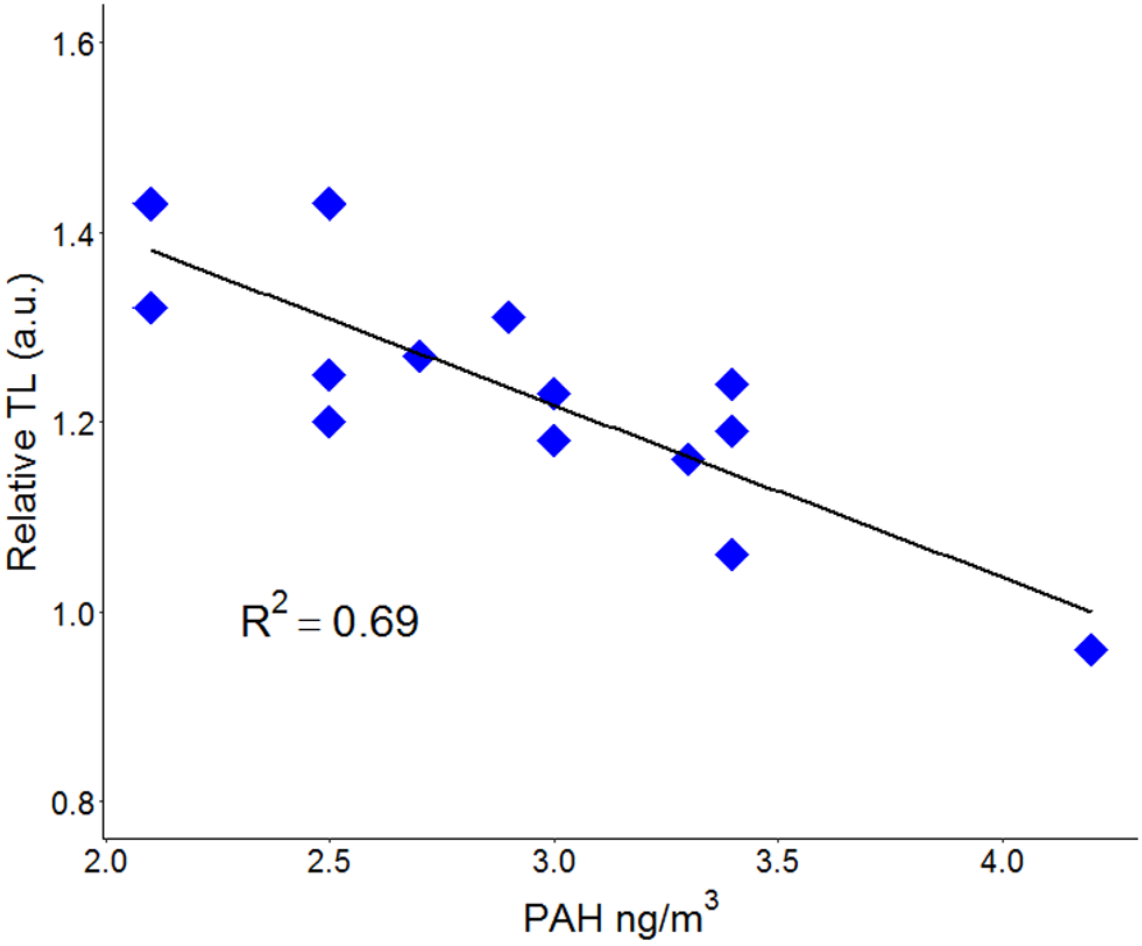

**Figure 3.**

Scatter plot for PAH exposure and telomere length.

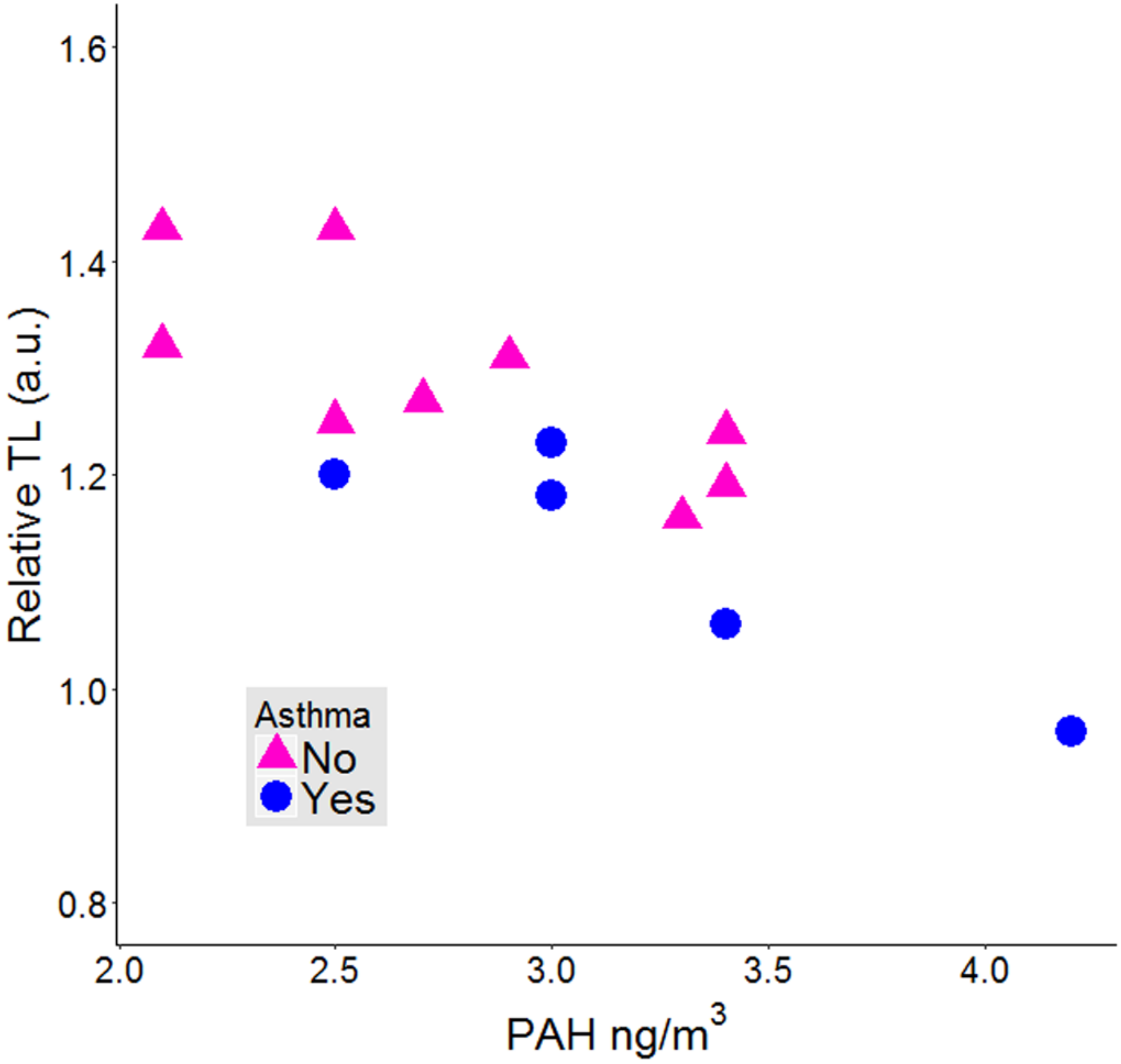

**Figure 4.**

Scatter plot for telomere length and PAH exposure by asthma status.

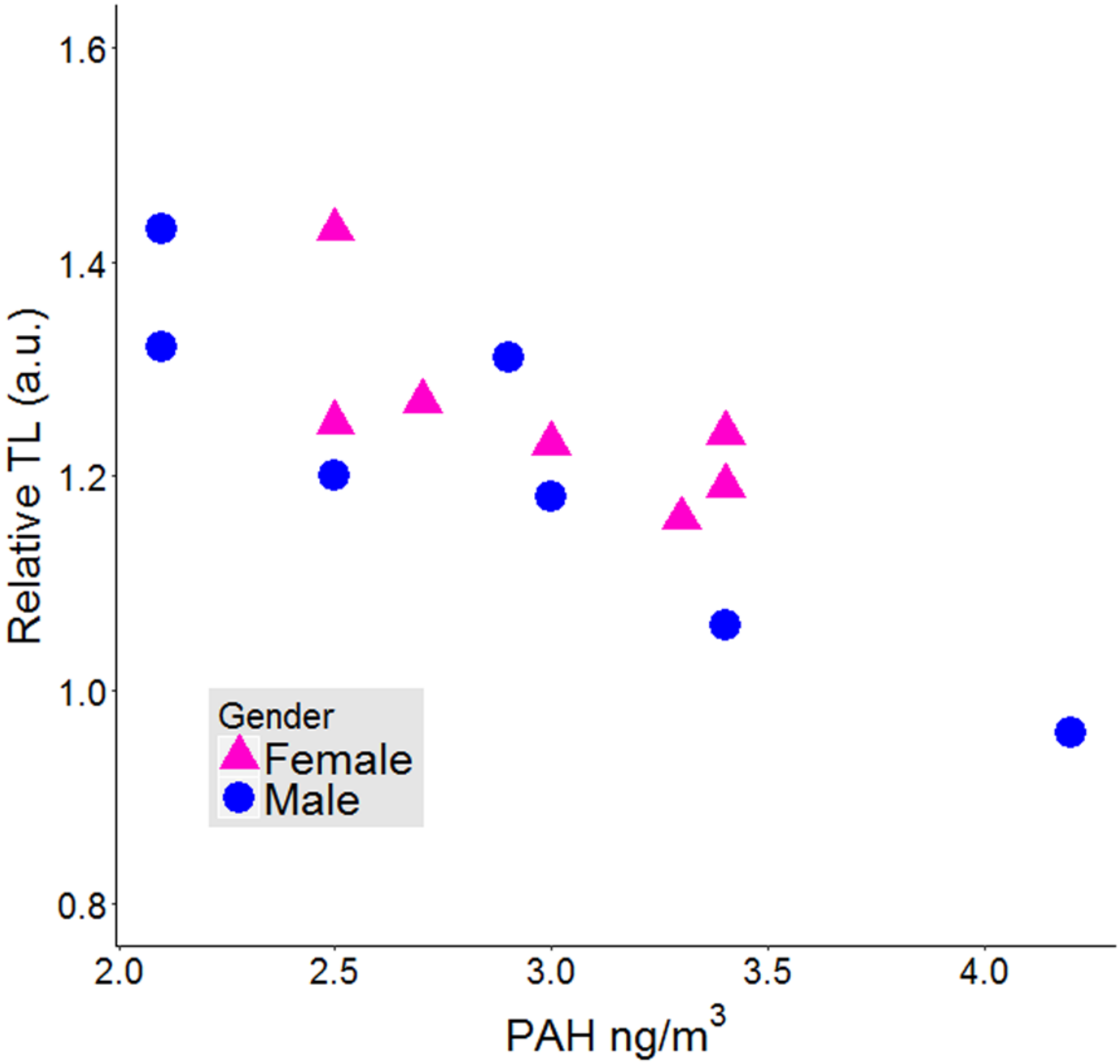

**Figure 5.**

Scatter plot for telomere length and PAH exposure by gender.

## Table 1

Summary characteristics of Fresno pilot study subjects (n=14)

| **Variable** | | | |
|---|---|---|---|
| | **Mean** | **SD** | **Range** |
| Age (years) | 14.0 | 2.11 | 11–18 |
| Telomere length (a.u.) | 1.23 | 0.13 | 0.96–1.43 |
| PAHs exposure (ng/m$^3$) | 2.98 | 0.58 | 2.1–4.2 |
| | % | | |
| Female | 50 | | |
| Asthmatic | 36 | | |
| Latino | 36 | | |

## Table 2

Mean age, telomere length and PAHs exposure by subgroups

| Subgroup | n | Age (yrs) | TL (a.u.) | PAHs (ng/m$^3$) |
|---|---|---|---|---|
| Gender | | | | |
| Male | 7 | 13.3 | 1.21 | 2.88 |
| Female | 7 | 14.7 | 1.25 | 2.97 |
| Ethnicity | | | | |
| Latino | 9 | 13.8 | 1.20 | 3.07 |
| White | 5 | 14.4 | 1.28 | 2.68 |
| Asthma | | | | |
| Asthmatics | 5 | 14.4 | 1.13 | 3.22 |
| Non-asthmatics | 9 | 13.8 | 1.29 | 2.77 |

## Table 3

Multivariable linear regression to predict telomere length (n=14)

| Predictor | Coef | St.Error | t-value | Pr(>t) |
|---|---|---|---|---|
| (Intercept) | 1.80 | 0.15 | 12.00 | <.01 |
| PAH (ng/m3) | −0.14 | 0.04 | −3.50 | 0.01 |
| Age (yr) | −0.0086 | 0.013 | −0.66 | 0.54 |
| Gender (ref group: male) | −0.04 | 0.05 | −0.80 | 0.46 |
| Race/ethnicity (ref group: white) | 0.01 | 0.05 | 0.20 | 0.79 |
| Asthma status (ref group: asthmatic) | −0.07 | 0.05 | −1.40 | 0.19 |

Residual standard error: 0.066 on 8 degrees of freedom

Multiple R-squared: 0.83, Adjusted R-squared: 0.72

F-statistic: 7.849 on 5 and 8 DF, p-value: 0.0059