Associations of smoking indicators and cotinine levels with telomere length: \textit{National Health and Nutrition Examination Survey}

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\textbf{ABSTRACT}

The influence of smoking exposure on telomere length with a focus on the impact of race has rarely been discussed. We performed a cross sectional analysis into the associations of smoking indicators with leukocyte telomere length (LTL) by race among 5864 nationally representative sample of US adults (≥20 years). Data from 1999 to 2002 National Health and Nutrition Examination Survey was used for the analysis. Smoking indicators were assessed by interviews and serum cotinine levels. LTL was quantified by polymerase chain reaction. Multiple linear regressions were used to assess the association with adjustment for covariates, sample weights and design effects separately for Whites, Blacks and Mexican Americans. The intensity of smoking, measured by the average number of cigarettes consumed per day, was negatively associated with LTL among Whites (β: −3.87, 95% CI: −5.98 to −1.21) and among Blacks (β: −15.46, 95% CI: −29.79 to −2.12) participants. Compared with cotinine level < 0.05 ng/ml, cotinine level ≥3 ng/ml was associated with shorter LTL (β: −77.92, 95% CI = −143.05 to −11.70) among Whites, but not among Blacks. We found increased number of cigarette consumption to be associated with shorter LTL in both Blacks and Whites, indicating that the impact of smoking on life-shortening diseases could partly be explained by telomere biology. Increased cotinine concentration, however, was associated with shorter LTL only among Whites, but not among Blacks. This differential relationship that we observed may have implications in interpreting cotinine as an objective biomarker of smoking exposure across races and warrant additional prospective investigation.

\textbf{1. Introduction}

Smoking is a critical environmental hazard and a major public health problem. Since smoking is associated with a wide variety of age-related diseases, aging is considered an important comparative measure of health outcomes with regard to smoking exposure. Human telomeres, which are nucleoprotein structures that cap the ends of chromosomes and protect them from degradation and maintain genome integrity, play a critical role in aging process (Blackburn, 2000; Blackburn, 2001). Telomeres generally shorten progressively with every cell division over the lifespan and thus, telomere length typically declines with advancement of age (Aubert and Lansdorp, 2008; Blasco, 2005). Telomere length also reflects the balance between cytotoxic stressors and antioxidant defense mechanisms (Houben et al., 2008; von Zglinicki, 2002). Therefore, telomere attrition in circulating white blood cells serves as a marker for cumulative oxidative stress, inflammation and, consequently, shows the pace of biological aging. Smoking has been hypothesized to promote telomere shortening and accelerated aging because of the increased oxidative stress burden caused by the large amount of free radicals and reactive oxygen species produced by smoking toxicities (Thorne et al., 2009; van der Vaart et al., 2004).

Cigarette smoke also contains a large amount of other chemical compounds, such as nicotine, acrolein, formaldehyde, and carcinogens (van der Vaart et al., 2004). Among them, nicotine's metabolite, cotinine, is often used as a biomarker of smoke exposure. Cotinine is formed from nicotine in a reaction catalyzed by the enzyme CYP2A6

\textbf{Abbreviations:} LTL, Leukocyte telomere length; NHANES, National Health and Nutrition Examination Survey; NCHS, National Center for Health Statistics; CDC, Centers for Disease Control and Prevention; bp, Base pairs; CRP, C-reactive protein; CI, Confidence interval

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(Benowitz et al., 2004; Pérez-Stable et al., 1998). Cotinine has a half-life of 15–20 h, compared to that of nicotine’s 0.5–3 h; therefore, cotinine is a more stable marker of exposure to tobacco smoke than nicotine itself and has been used as an objective index of tobacco exposure (Benowitz et al., 2009; Benowitz, 1996).

A number of previous studies have shown that telomere length is shorter in the peripheral blood leukocytes of smokers than in those of nonsmokers (Babizhayev and Yegorov, 2011; Valdes et al., 2005; Huzen et al., 2014; Zhang et al., 2016; Bendix et al., 2014). Although cotinine is regarded as an objective biomarker of smoking, little is known about the associations between cotinine and telomere length. We found one study conducted exclusively among White current smokers that examined this relationship and found no association (Robertson et al., 2013). It is however, well established that cigarette-smoking behavior and nicotine metabolism differs substantially by race in the United States (Darby et al., 2008; Ross et al., 2016). Blacks tend to have higher cotinine levels with the same level of smoking exposure because of lower CYP2A6 activity compared to Whites (Mwenifumbo et al., 2005; Kim, 2016). The three categories of cotinine levels were 1) cotinine < 0.05 ng/ml, 2) cotinine 0.05–2.99 ng/ml, and 3) cotinine ≥ 3 ng/ml. Cotinine was also used as a dichotomized variable in which the cut-off was 10 ng/mg (Jain, 2017).

2.3. LTL measurements

LTL relative to standard reference DNA (T/S ratio) was measured using the quantitative polymerase chain reaction method (Cawthon, 2002; Lin et al., 2010). The formula to convert the T/S ratio to base pairs (bp) was 3274 + 2413 × (T/S) (Cawthon, 2002; Lin et al., 2010). Analytical methods and quality control procedure for LTL quantification have been described in detail on the NHANES website and in supplementary section (National Health and Nutrition Examination Survey, 2001).

2.4. Other covariates

According to self-reported race information participants were categorized as non-Hispanic Whites, non-Hispanic Blacks, and Mexican Americans. Age was calculated in years. Educational attainment was dichotomized as less than high school and more than high school. Marital status was categorized as married/partnered (yes/no). Participants were considered physically active if they were involved in vigorous activity over the past 30 days (yes/no). Body mass index or BMI (weight in kg/height in meter (Blackburn, 2001)) was calculated using height and weight measurements, and individuals with BMI ≥ 30 were considered obese (WHO, 1995). Participants’ dietary intake of total calories (energy) was measured in kilocalories per 24 h. Information on existing chronic disease conditions (yes/no), including diabetes, high blood pressure, congestive heart failure, asthma, malignancy and blood C-reactive protein (CRP) concentration, was also included.

2.5. Data analysis

All analyses were adjusted for the sampling design and used sample weights from the subsample of NHANES participants who provided DNA. Characteristics of were summarized by race using descriptive statistics. We then built different linear regression models to assess the relationships between smoking indicators, which served as the independent variables and the dependent variable, LTL. Smoking indicators included smoking status (never/past/current, never-smokers as reference group), intensity of smoking (number of cigarettes smoked daily among current smokers), filter type of current smokers (mentholated or not, nicotine content and tar content), secondhand smoking (if someone smoked at home, for never-smokers) and serum cotinine levels (both as categorical and continuous variables). We used sequential models to control for potential confounders. Model 1 was adjusted for age (continuous). Model 2 was further adjusted for sex; educational level; marital status; obesity; physical activity; alcohol consumption;
total calorie intake; and chronic disease conditions, including diabetes, high blood pressure, congestive heart failure, asthma, malignancy and CRP levels. Models with cotinine levels were additionally adjusted for smoking status. Given prior evidence of race/ethnic differences in tobacco smoking status, cotinine level and LTL, we estimated models stratified by race. We also tested for the statistical interaction between categories of tobacco exposure (smoking status and cotinine levels) and race on LTL. To examine the variation by sex, the interaction between tobacco exposure and sex was also checked. To evaluate the interactions, we added main effects and cross-product terms to the regression after adjusting for all the variables previously mentioned. p-Values for each interaction term and F-tests comparing full and reduced models (with and without the interaction term) were used to test the statistical significance of the interaction terms.

3. Results

Among the 5864 participants (51.0% women) with ages ranging from 20 to 84 (mean age 46.22) years, 3328 (56.75%) were Whites, 1061 (18.10%) were Blacks and 1474 (25.15%) were Mexican Americans. We found no evidence of effect modification by sex in associations between smoking and LTL. Consistent with our hypothesis, the interaction between smoking exposure and race remained significant; hence, we present our results stratified by race. Table 1 shows the weighted distributions of study population characteristics stratified by race. Whites were older (mean age 47.34 years) than Blacks (mean age 42.68 years) or Mexican Americans (38.27 years). Blacks had longer LTLs (mean 5949.31 bp) than Whites (mean 5778.25 bp) or Mexican Americans (mean 5785.87 bp).

The smoking behaviors of the participants are presented by race in Table 2. Approximately 28.70% of Blacks, 24.86% of Whites and 21.28% Mexican Americans were current smokers. The average number of cigarettes consumed by current smokers was considerably higher among Whites (19.45/day) than among Blacks (12.67/day) and Mexican Americans (9.42/day). Approximately 79% of all Black smokers use menthol cigarettes, a rate that was nearly 6 times higher than that of White smokers and 8 times higher than that of Mexican American smokers. Smoke exposures, as calculated by serum cotinine concentration, were also relatively higher among Blacks compared to Mexican Americans and Whites. Approximately 38% of Blacks had cotinine levels ≥3 ng/ml, compared to 29% of Whites and 22% of Mexican Americans.

Multivariate regression analysis showed no statistically significant association between self-reported smoking status, secondhand smoking and type of filter used with LTL (Table 3). However, among self-reported current smokers, the intensity of smoking, as measured by the average number of cigarettes consumed per day, was negatively and significantly associated with LTL in the White and Black populations. Consumption of each cigarette was equivalent to a loss of approximately 4 bp (regression coefficient, β: −3.87, 95% CI: −5.98 to −1.21, p-value < 0.05) and 15 bp (β: −15.46, 95% CI: −29.79 to −2.12, p-value < 0.05) for Whites and Blacks, respectively. No association was observed among Mexican Americans.

The associations between different cotinine groups and LTL by race are presented in Table 4. In the age-adjusted model, we found that compared with cotinine < 0.05 ng/ml, cotinine ≥3 ng/ml was associated with shorter LTL among Whites. On average, participants with cotinine levels ≥3 ng/ml had telomeres that were shorter by approximately 92 bp (β: −92.40, 95% CI: −150.99 to −33.81, p-value < 0.01) compared to those with cotinine levels < 0.05 ng/ml category, with a significant trend across the exposure categories, suggesting a dose-response phenomenon (for the trend across cotinine categories, p-value = 0.007). Further adjustment for other covariates slightly attenuated the regression coefficient, but the association remained significant (β: −77.92, 95% C.I = −143.05 to −11.70, p-value < 0.01). The association between cotinine levels and LTL, however, was not significant among Blacks and Mexican Americans. Analysis performed with cotinine category 0–9.99 ng/ml versus ≥10 ng/ml also showed that increased cotinine levels were associated with shorter LTL among Whites only (Table 4). When we repeated the analysis with cotinine as a continuous variable (ng/ml), a similar pattern of association was observed. A 1-ng/ml increase in cotinine levels was significantly associated with an average −0.30 bp decrease in telomere length among Whites only (β: −0.30, 95% CI = −0.50 to −0.11, p-value = 0.001).

4. Discussion

In this study, we used representative data from the U.S. population and showed that smoking intensity, as measured by the number of cigarettes consumed, was associated with a shorter LTL for the White and Black populations. Our finding is in line with previous research that showed that smoking more cigarettes per day was associated with shorter LTL (Latifovic et al., 2016; Muezzinler et al., 2015; Astuti et al., 2017). Several potential mechanisms can help explaining the link. Cigarette smoke can suppress the number of peripheral blood eosinophils and can induce a wide range of proinflammatory responses, leading to...
increased systemic inflammation causing lymphocyte turnover, which has been linked with LTL shortening (O’Donovan et al., 2011). Chemical components of cigarette can produce free radicals, trace heavy metals, quinones, and oxidizing radicals (Stedman, 1968; Hoffmann et al., 2001). They are capable of initiating or promoting oxidative stress, which may also lead to subsequent and progressive oxidative damage and cause aging, age-related pathologies and LTL shortening (Valdes et al., 2005; Huzen et al., 2014).

Despite this biological plausibility, we found no difference in LTL among self-reported current, former and never smokers for any race. Several previous studies that used only self-reported smoking status as exposure, have also failed to show association between smoking and LTL (Weischer et al., 2014; Nordfjall et al., 2008; Batty et al., 2009). Since tobacco use history is a multi-dimensional phenomenon and is subjected to recall bias, addressing smoking status conventionally as current/former/never may not capture smoking exposure accurately and could be a reason behind this lack of association. To understand the complexities of smoking history, using other smoking related characteristics such as intensity, duration, type of smoking together with biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance. Therefore, to capture a comprehensive impact of smoking on LTL, in addition to smoking status, in-biomarkers is of central importance.

It was somewhat surprising that we were able to confirm significant links between smoking intensity and short LTL for both Whites and Blacks, but we found no association between cotinine levels and LTL among Blacks. While plasma cotinine is believed to reflect nicotine intake, it is possible that this absence of association among Blacks was due to biological differences in nicotine metabolism across race. Prior studies have described racial variations in the rate of metabolism of nicotine and cotinine (Benowitz et al., 2004; Pérez-Stable et al., 1998). Studies have also reported that for the same daily level of cigarette smoking, higher serum cotinine concentrations have been detected in Blacks than in Whites (Derby et al., 2008; Ross et al., 2016). We also found that, despite smoking fewer cigarettes than Whites, Blacks had higher serum cotinine levels. As mentioned before, we speculate that the CYP2A6 enzyme activity plays a critical role in the variation of cotinine levels by race. To validate our speculation, we calculated CYP2A6 activity from the NHANES 2009–2010 dataset by race, since information on caffeine metabolites was not available for the NHANES 1999–2002 dataset that we used for the current analysis.
found CYP2A6 activity to be higher in Whites than in Blacks (method of calculation and detailed results are given in supplementary section). These data are consistent with previous research that also showed lower CYP2A6 enzyme activity in Blacks compared to Whites, suggesting a slower metabolism and clearance of nicotine and cotinine and subsequent accumulation of cotinine (Mwenifumbo et al., 2005; Nakajima et al., 2006). Cotinine levels can also be affected by different tobacco products used by different ethnic groups. The majority (79%) of Black smokers smoked menthol cigarettes, compared to only 14% of White smokers in our study. Menthol can compete for the same metabolic pathway with nicotine, thus affecting the metabolism of cotinine (Benowitz et al., 2004; MacDougall et al., 2003). These observations provide a mechanistic explanation for the increased variability in cotinine to cotinine. CYP2A6 enzyme activity data was not available. We therefore measured the CYP2A6 enzyme activity from NHANES 2002–2004 and found relatively slower activity among Blacks. The large multiethnic population sample allowed us to report our results stratified by race. This is important because smoking behaviors, smoking biology and telomere length vary by race. We used cotinine, a biological biomarker, as the surrogate for smoking exposure to assess the smoking-LTL relationship rather than relying only on self-reported smoking status. This allowed us to capture a broader spectrum of smoking exposure. Additionally, our study was conducted in a representative sample of U.S. adults, suggesting that our findings may be generalized to the entire U.S. population. Finally, rich covariate data allowed for comprehensive adjustments of sociodemographic, lifestyle, biological and disease status variables, which are potential confounders of the relationship between smoking and LTL. Limitations of the present study include its cross-sectional design, and therefore, no inferences can be made on causality in relation to smoking and LTL. We used a single measure of cotinine, which might not necessarily reflect chronic nicotine exposure over time and might not be reflective of comprehensive smoking exposure. Additionally, CYP2A6 enzyme activity is needed to assess accurate rate of metabolism of nicotine to cotinine. CYP2A6 enzyme activity data was not available. We have however, measured the CYP2A6 enzyme activity from NHANES 2009–2010 cohort, and found relatively slower activity among Blacks. Therefore, difference in CYP2A6 enzyme activity across races and our

| Race-specific association between cotinine (by category and concentration) and telomere length among participants in NHANES 1999–2002 (N = 5864). |
|---------------------------------------------|----------------|----------------|----------------|
| By cotinine categories                     |                |                |                |
| Non-Hispanic Whites                        |                |                |                |
| 0–0.049 ng/ml                              | 3328           | 0.007          | 0.07           |
| ≥0.05–2.99 ng/ml                           | 1529           | Ref            | 0.749          | 0.59 |
| ≥3 ng/ml                                   | 989            | −10.55         | −105.86, 84.77 | 6.77 |
| ≥3 ng/ml                                   | 901            | −92.40        | −150.99, −33.81 | 7.79 |
| Mexican Americans                          |                |                |                |
| 0–0.049 ng/ml                              | 1475           | 0.08           | 0.15           |
| ≥0.05–2.99 ng/ml                           | 782            | Ref            | 0.08           | 0.15 |
| ≥3 ng/ml                                   | 414            | 57.71          | −57.9, 173.32  | 63.53 |
| ≥3 ng/ml                                   | 279            | 90.78          | −2.50, 182.06  | 75.28 |
| By cotinine concentration                   |                |                |                |
| Non-Hispanic Whites                        |                |                |                |
| 0–0.049 ng/ml                              | 3328           | 0.007          | 0.07           |
| ≥0.05–2.99 ng/ml                           | 2474           | Ref            | 0.749          | 0.59 |
| ≥3 ng/ml                                   | 854            | −87.29         | −133.27, −41.31 | 74.25 |
| Non-Hispanic Blacks                        |                |                |                |
| 0–0.049 ng/ml                              | 1061           | Ref            | 0.08           | 0.15 |
| ≥0.05–2.99 ng/ml                           | 701            | Ref            | 0.08           | 0.15 |
| ≥3 ng/ml                                   | 359            | 21.57          | −92.46, 135.21 | 20.10 |
| Mexican Americans                          |                |                |                |
| 0–0.049 ng/ml                              | 1475           | 0.08           | 0.15           |
| ≥0.05–2.99 ng/ml                           | 1225           | Ref            | 0.08           | 0.15 |
| ≥3 ng/ml                                   | 250            | 75.45          | −13.58, 172.49 | 85.08 |
| By cotinine levels                          |                |                |                |
| Non-Hispanic Whites                        |                |                |                |
| 0–0.049 ng/ml                              | 3328           | 0.007          | 0.07           |
| ≥0.05–2.99 ng/ml                           | 2474           | Ref            | 0.749          | 0.59 |
| ≥3 ng/ml                                   | 854            | −87.29         | −133.27, −41.31 | 74.25 |
| Non-Hispanic Blacks                        |                |                |                |
| 0–0.049 ng/ml                              | 1061           | Ref            | 0.08           | 0.15 |
| ≥0.05–2.99 ng/ml                           | 700            | Ref            | 0.08           | 0.15 |
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| ≥3 ng/ml                                   | 250            | 75.45          | −13.58, 172.49 | 85.08 |
| No. of participants Age-adjusted Multivariable-adjusted |
| β coef95% CI P for trendβ coef95% CI P for trend |
| 3328 | 0.007 | 0.07 |
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| 901 | −92.40 | −150.99, −33.81 |
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| 700 | Ref | 0.08 |
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| 1475 | 0.08 | 0.15 |
| 1225 | Ref | 0.08 |
| 250 | 75.45 | −13.58, 172.49 |

**Table 4**

Abbreviation: CI, confidence interval.

Values are adjusted β coefficients. Analyses were performed with adjustment for sample weights and design effects.

* Adjusted for age.

** Adjusted for sex, educational level, marital status, body mass index, smoking status, physical activity, alcohol consumption, total calorie intake, chronic disease conditions including diabetes, high blood pressure, congestive heart failure, asthma, cancer or malignancy and CRP levels.

a Adjusted for age.

b Adjusted for age.

c Adjusted for age.

p < 0.05.

Our study has several strengths. Many of the studies reporting the impact of tobacco smoking on LTL were performed with smaller sample sizes, and race-specific reports of the association between smoking and LTL are almost absent. The large multiethnic population sample allowed us to report our results stratified by race. This is important because smoking behaviors, smoking biology and telomere length vary by race. We used cotinine, a biological biomarker, as the surrogate for smoking exposure to assess the smoking-LTL relationship rather than relying only on self-reported smoking status. This allowed us to capture a broader spectrum of smoking exposure. Additionally, our study was conducted in a representative sample of U.S. adults, suggesting that our findings may be generalized to the entire U.S. population. Finally, rich covariate data allowed for comprehensive adjustments of sociodemographic, lifestyle, biological and disease status variables, which are potential confounders of the relationship between smoking and LTL. Limitations of the present study include its cross-sectional design, and therefore, no inferences can be made on causality in relation to smoking and LTL. We used a single measure of cotinine, which might not necessarily reflect chronic nicotine exposure over time and might not be reflective of comprehensive smoking exposure.
inability to control for this variable could influence our result.

5. Conclusion
In the current study, data from a large representative sample of US were examined for race differences in the variation in telomere length associated with multiple indicators of cigarette smoking. No association was observed when self-reported smoking categories such as categories of current, former, never smoking was used as exposure.

Nevertheless, we found a significant association between increased number of cigarette consumed and shorter LTL in both Blacks and Whites, indicating that the impact of smoking on life-shortening diseases could partly be explained by telomere biology. Our results also indicate the possibility of differential impact of smoking biomarker cotinine on LTL by race. In our study, cotinine concentration was associated with reduced LTL only among Whites, not among Blacks. This observation may have implications in interpreting cotinine as an objective biomarker of tobacco smoke exposure and possibly explaining differences in aging process or other smoking-related disease risks in different races. The association of smoking exposure with telomere length has been previously studied. However, despite the differences in LTL, smoking behavior and nicotine metabolism among different races, little information is available on race-specific variation in telomere length due to smoking. Our study addresses gap and provides information on race specific associations between several smoking indicators and LTL. Nevertheless, prospective longitudinal studies with repeated measurements of smoking status, cotinine and telomere length measuring possible role of differential effects of smoking exposure on telomere attrition, and comparison among different ethnic and racial groups is needed to confirm the causality of our findings.

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Declaration of Competing Interest
No conflicts of interest declared.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmedr.2019.100895.

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