OBSTETRICS

Impact of intrauterine tobacco exposure on fetal telomere length

Hamisu M. Salihu, MD, PhD; Anupam Pradhan, PhD; Lindsey King, MPH, CHES, CCRP; Arnut Paothong, PhD; Chiaka Nwoga, MPH, CPH, CCRP; Phillip J. Marty, PhD; Valerie Whiteman, MD

OBJECTIVE: We sought to investigate whether maternal smoking during pregnancy affects telomere length of the fetus.

STUDY DESIGN: Pregnant women were recruited on hospital admission at delivery. A self-report questionnaire and salivary cotinine test were used to confirm tobacco exposure. Neonatal umbilical cord blood samples were collected, and genomic DNA was isolated from cord blood leukocytes and was analyzed for fetal telomere length based on quantitative polymerase chain reaction. A ratio of relative telomere length was determined by telomere repeat copy number and single copy gene copy number (T/S ratio) and used to compare the telomere length of active, passive, and nonsmokers. Bootstrap and analysis of variance statistical methods were used to evaluate the relationship between prenatal smoking status and fetal telomere length.

RESULTS: Of the 86 women who were included in this study, approximately 69.8% of the participants were covered by Medicaid,

and 55.8% of the participants were black or Hispanic. The overall mean T/S ratio was 0.8608 ± 1.0442. We noted an inverse relationship between smoking and fetal telomere length in a dose-response pattern (T/S ratio of nonsmokers that was more than passive smokers that was more than active smokers). Telomere length was significantly different for each pairwise comparison, and the greatest difference was between active and nonsmokers.

CONCLUSION: Our results provide the first evidence to demonstrate a positive association between shortened fetal telomere length and smoking during pregnancy. Our findings suggest the possibility of early intrauterine programming for accelerated aging that is the result of tobacco exposure.

Key words: bootstrapping, cotinine, fetal telomere length, tobacco exposure, umbilical cord blood

Cite this article as: Salihu HM, Pradhan A, King L, et al. Impact of intrauterine tobacco exposure on fetal telomere length. Am J Obstet Gynecol 2014;211:●●●●.

F etal exposure to tobacco smoke has been linked consistently to adverse pregnancy outcomes,[1] which include low birthweight, preterm delivery, premature rupture of membranes, placenta previa, placenta abruption, perinatal death, and sudden infant death syndrome.[1-4]

Telomeres are complex nucleotide sequences that protect the end of chromosomes from deterioration and play a critical role in cellular division.[5-7] Over time, telomeres shorten,[8] eventually

reaching a critical short length that leads to apoptosis.[9] This shortening serves as a biomarker for cellular and biologic aging, longevity, and disease development.[5-8] Shortened telomere lengths are associated with adverse health outcomes, such as cardiovascular disease,[10] Alzheimer's disease,[11] cancer,[5-7] and early death.[7]

Accelerated telomere shortening can be caused by environmental factors such as cigarette smoking.[12,13] Several studies have shown a relationship between

tobacco exposure and shortened telomere length in adults.[14] In contrast to nonsmokers, the telomere length of smokers decreases significantly overtime, which indicates increased biologic age.[15,16] This dose-effect relationship between lifespan cumulative smoke exposure and telomere length in adults is well-established.[15]

The impact of adverse environmental exposures on telomere length appears to start at a very early stage of human development.[17,18] One study conducted by Theall et al[19] indicated a relationship

between prenatal tobacco exposure and telomere shortening in children with the use of salivary DNA samples. The study found that mean salivary telomere length was significantly shorter among children who were exposed to prenatal tobacco than in those who were not exposed.

To our knowledge, there have been no studies that have examined a relationship between prenatal tobacco exposure and human fetal telomere length, which limits our understanding of how early the telomere biologic system is affected by smoke exposure. In this study, we sought to investigate whether maternal smoking during pregnancy affects telomere length of the fetus. We hypothesize that prenatal exposure to tobacco will lead to shorter fetal telomere length, which is a marker for potential adverse health outcomes as the child grows and develops.

METHODS
Study population
This project was a nested study from a prospective cohort from July 2011 to September 2012. Study participants were recruited among women who were admitted for labor and delivery at university-affiliated hospital in Tampa, FL. Obstetrics care services at this hospital are provided by faculty members of the Department of Obstetrics and Gynecology at the University of South Florida. An unpublished hospital statistical report from 2008 showed that women who deliver at this hospital are typically of low socioeconomic status. Few patients have private health insurance; most patients are either uninsured or covered by Medicaid. It was approximated that 15% of women who deliver at the study site smoked actively during pregnancy.

Eligible participants included women ≥18 years old who delivered singleton full-term live births with no evidence of congenital and/or chromosomal anomalies. Trained research staff acquired consent from the patients before delivery. Before approaching a potential participant, we made sure that the patient was competent to provide informed consent. Both English- and Spanish-speaking women who were not under duress were approached for potential enrollment. Information pertaining to sociodemographic characteristics and newborn measures was abstracted from hospital electronic medical records. This study was approved by the University of South Florida Institutional Review Board and ethics committee on Aug. 26, 2010, and Oct. 28, 2010.

Data from this study are part of a larger clinical trial study. This study was a tertiary aim of the parent study that sought to explore genetic and epigenetic programming of fetuses who were exposed to tobacco smoke. We wanted to better understand the biologic mechanism underlying maternal tobacco consumption and suboptimal pregnancy outcomes. Cord blood was collected at delivery for subsequent DNA extraction, methylation, and telomere analyses. Thus, this analysis was planned and the controls selected were synonymous with the control subjects chosen for the parent study.

Smoking exposure measures
A smoking questionnaire and a salivary cotinine test were used to determine maternal smoking status. The smoking questionnaire was administered verbally by the research staff before or after delivery but before hospital discharge. It contained 17-multiple choice questions that were related to tobacco use, environmental smoke exposure, and cessation. Only question 1 ("Did you smoke before you found out you were pregnant?") and question 2 ("Do you currently smoke?") were used for this study to establish self-report smoking status.

For biologic confirmation, exposure to nicotine was measured by a test of maternal saliva for the presence of cotinine with the use of a rapid semiquantitative screening test called NicAlert (Jant Pharmacal Corporation, Encino, CA). Cotinine, a major metabolite of nicotine, has been shown to be more precise than carbon monoxide monitoring for the measurement of smoking status.[20,21] The test strip displays 7 levels; each level represents a range of cotinine concentrations. Per manufacturer guidelines, individuals with a reading of negative or level 0 are considered nonusers of tobacco products (cotinine concentrations <10 ng/mL); individuals with a level 1 result are considered light active smokers or heavy passive smokers (10-30 ng/mL), and individuals with levels 2-6 results are considered active smokers (>30 ng/mL).

To define smoking status for this study, a combination of self-report and semiquantitative saliva test results was used, which created 3 groups. Individuals who tested levels 1-6 on the saliva cotinine screen and who answered "Yes" to either or both questions 1 and 2 were considered active smokers. The active smokers category also included those who tested within levels 2-6, regardless of their response on the self-report questionnaire. Conversely, nonsmokers tested at level 0 on the saliva screen and answered "No" to both questions 1 and 2 on the self-report. Passively exposed smokers were those whose cotinine scores were equal to 1 and who answered "No" on the self-report questionnaire to both questions 1 and 2. Nine participants were excluded because the hospital medical record system was used to determine smoking status because the questionnaire could not be obtained. However, the medical record smoking status was not accurate for these 9 patients because their smoking status was entered as "smoker," but their cotinine level was 0.

DNA extraction and telomere length measures
At birth, umbilical cord blood was collected from full-term infants with a device called the Umbilicup (DeRoyal Industries Inc, Powell, TN). Genomic DNA was isolated from 200 μL of Buffycoat of cord blood collected in BD EDTA tubes (DNeasy Blood and Tissue Kit; Qiagen, Valencia, CA) as per manufacturer's directions. The isolated DNA was quantified with the use of Nanodrop 2000c (Thermo Scientific, Waltham, MA) and stored at 4°C until the time of assay. The relative telomere lengths were then quantified by a comparison of the cycle threshold ($\varDelta$Ct) obtained by quantitative polymerase chain reaction

(qPCR) performed in triplicate wells with the use of the MX3000P qPCR thermal cycler (Stratagene, La Jolla, CA).[21,22]

The relative telomere lengths in the DNA samples (telomere repeat copy number [T/S] ratio) were determined with GoTaq qPCR Master Mix reagent (Promega, Madison, WI) with 20 ng DNA as a template from cord blood. We adopted a similar strategy described by Cawthon[23] to determine the relative telomere lengths by qPCR for each DNA samples that were obtained. The factor by which each sample differed from a reference DNA sample is its ratio of telomere repeat copy number to a single copy gene copy number. The relative quantities of telomere repeats (T) and the single copy gene (S) were measured by plotting the respective qPCR-generated (ΔCt) values in the standard curve that was obtained from an arbitrary human DNA Ct by qPCR. Therefore, the relative telomere length for each experimental sample is the ratio of the telomere to single copy gene (T/S). Consequently T/S equals 1 when anonymous DNA is identical to the reference gene in ratio of its relative copy number to a single gene copy number. To determine the absolute copies of repeats, telomere (T) was divided by absolute copy of the single copy gene 36B4 (S). The length of the hexametric repeats for each sample analyzed was expressed as T/S ratio; the Cawthon ratio was expressed in kilo bases.[22,23]

The telomere primer concentrations were tel1 270 nM; tel2, 900 nM. The final 36B4 (single copy gene) primer concentrations were 36B4u, 300 nM; 36B4d, 500 nM. The primer sequences (from 5′ →3′) were tel1, GGTTTTTGAGGGT-GAGGGTGAGGGTGAGGGTGAGGGT; tel2, TCCCGACTATCCCTATCCCTATC CCTATCCCTATCCCTA; 36B4u, CAG-CAAGTGGGAAGGTGTAATCC; 36B4d, CCCATTCTATCATCAACGGGTACAA. The thermal cycling profile for both single copy gene and the telomere started with 2 minutes at 95°C for hot start activation followed by 40 cycles of 15-second denaturation at 95°C and annealing/ extension at 54°C for 2 minutes.

The standard curves of the telomere length and the single copy gene 36B4

were generated by the plotting of the ΔCt values from qPCR reactions of a reference human DNA that was diluted serially 6 points from 1.9-60 ng. Thereafter, in all DNA samples, the ΔCt values for telomere repeat gene and the single copy gene were determined. Telomere length variations can be described with 3 different variant analyses: T/S ratio (direct use of the obtained ΔCt values), relative T/S ratio (RTS; a log derivation of the ΔCt values with the use of the standard curve intercept), and the Cawthon ratio ([1910.5 × log T/S ratio] + 4157).

RTS and T/S ratio have a perfect positive correlation (r = +1.0) based on the formula used to convert T/S to RTS ratio:

$$RTS = 4157 + 1910.5 \times TS$$

Based on a simple regression between Cawthon and T/S ratios:

$$Cawthon = 4482.26 + 1323.31 \times TS$$
with $R^2 = 0.8834$

Cawthon and T/S ratios are also correlated highly with correlation equal to 0.8834. The 3 variables that were obtained to express telomere length (T/S, RTS, and Cawthon ratios) showed very high correlations (Figure 1). Consequently, we elected to use T/S ratio to compare the telomere length of the 3 groups because this is the most commonly reported measure of telomere length.[19,24,25]

Statistical analysis
We used analysis of variance (ANOVA) for differences in continuous outcomes to compare maternal sociodemographic and delivery characteristics among the 3 groups (nonsmokers, passive smokers, and active smokers). For the categoric variables, we applied a χ^2 test to assess differences in proportions. Where expected cell size was <5, we used Fisher exact test.

In our analysis, we restricted T/S ratio to values between 0 and 3. Negative values and values >3 were meaningless outliers and were excluded. Because of the scarcity of amount of genomic DNA from participants and the sample size,
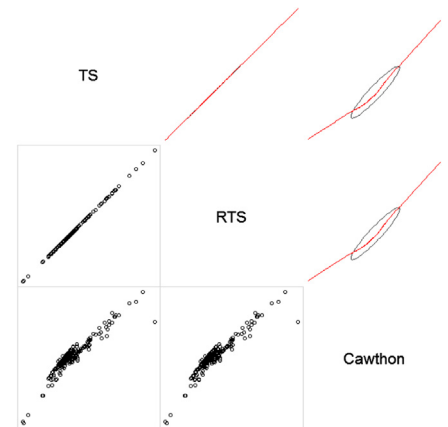
The 3 variables that were obtained to express telomere length (T/S, RTS, and Cawthon ratios) showed very high correlations.

RTS, relative T/S ratio; *T/S*, telomere repeat copy number ratio.

Salihu. Tobacco exposure and fetal telomere length. Am J Obstet Gynecol 2014.

each individual sample was run in triplicate to determine the T/S ratio. A mean of all telomere Ct and the single copy gene Ct (36B4) was calculated. Wells that produced measurements outside the mean Ct values (±3 SD) were excluded, and new mean Ct values for telomere and 36B4 were calculated after exclusion of these outlier wells. The outliers were the range of sample deviation or the data which did not fit in the tendency of the Time Series that was observed. Similarly, the calibrator, which was the measurement of relative quantities of telomere repeats (T) and single copy gene (S) from an arbitrary human DNA, was set up across all plates for the standard curve.

Because of the small sample size, the small difference detected, and the high variance of T/S ratio across the 3 groups, we decided to apply a resampling method called *bootstrapping* in combination with repeated ANOVA for each sampled unit. Bootstrapping is a robust alternative to traditional methods because it requires fewer assumptions and provides more accurate inferences, particularly when the sample size is small.[26] The method involves repeatedly sampling from an observed data set with replacement a finite number of times.[27]

Then, the sampling distribution of the desired statistic is derived from each sampled unit. The distribution of the collection of these individual statistics provides a framework to approximate the overall mean value.[28]

For our case, we conducted simulation analysis to derive precise estimates using the data that we had collected that involved the following processes: (1) We resampled with replacement from the original dataset. (2) We calculated the mean of T/S ratio for each of 3 smoking categories, using simulation technique. (3) We performed repeated ANOVA and derived the probability value. (4) We performed Tukey's (HSD [honest significant difference] post hoc) test across the 3 groups and derived the probability value for each pairwise comparison. (5) Finally, we repeated steps 1-4 ten thousand (10,000) times. The simulation procedure is illustrated as a flow chart in Figure 2.

The ANOVA model is represented by the following formula:

$$TS_{hij} = \mu + \alpha_h + \tau_j + (\alpha\tau)_{hj} + b_{i(h)} + e_{hij}$$

μ = grand mean is a fixed parameter
α_h = effect of group and is a fixed parameter. $\sum_{h=1}^{3} \alpha_h = 0$
τ_j = effect of sample and is a fixed parameter. $\sum \tau_j = 0$
$(\alpha\tau)_{hj}$ = interaction effect of sample and group is a fixed parameter and $\sum_{h=1}^{3} \sum (\alpha\tau)_{hj} = 0$

$b_{i(h)}$ = individual difference component, $b_{i(h)} \sim NID(0, \sigma_b^2)$
e_{hij} = random error, $e_{hij} \sim NID(0, \sigma_e^2)$

This model tests the following hypothesis:

$$H_0 : \alpha_{Non} = \alpha_{Pass} = \alpha_{Smoke} = 0$$

Statistical analyses were performed with the use R software (version 3.0.2; www.R-project.org).

RESULTS

Of the 86 women included in this study, 29% women were nonsmokers; 36% women were passive smokers, and 35% women were active smokers. The Table shows a detailed sociodemographic description and delivery variables of the study sample, with all the selected characteristics being similar across the 3 groups of women. The population in this study was largely underprivileged, with 69.8% of the participants covered by Medicaid and 15.1% reporting no insurance coverage. Approximately 67.4% of the sample was unmarried, and 55.8% was black or Hispanic. There was no statistical difference in mean gestational age among the 3 groups (40 ± 1.32 weeks for nonsmokers; 40 ± 1.15 weeks for passive smokers; 40 ± 1.25 weeks for active smokers) at the time of delivery (P = .581). Passive smokers had the highest mean body mass index of 37 ± 12.88 kg/m² followed by active smokers

(35 ± 8.12 kg/m²); nonsmokers had the lowest body mass index (31 ± 4.50 kg/m²). The differences between the 3 groups were not statistically significant (P =.100). The overall mean birthweight of infants was 3311 ± 486.02 g (P = .657). The overall mean T/S ratio was 0.8608 ± 1.0442 (SD).

Figure 3, A shows the mean distribution of T/S ratio within the study sample before we performed the bootstrapping approach. Figure 3, B shows the same results after the bootstrapping was applied. By visual inspection, the data distribution appeared smoother after bootstrapping, and the mean T/S ratio of nonsmokers had the greatest value as indicated by its location farthest to the right. This is followed by the curve for passive smokers, then the lowest was that of active smokers.

The histogram in Figure 4 represents the histogram of all probability values that were generated with the use of repeated ANOVA from the 10,000 bootstrapping simulations that were performed. The Figure shows that, in 9999 of the 10,000 iterations, the probability value was < .05 and that only in a single iteration was the probability value > .05. Hence, the result represents conclusive evidence that there was significant group effect with respect to T/S ratio in the study. It means that the differences in T/S ratio across the 3 groups was significant at least 99.9% of the time.

Tukey's HSD procedure was used for pairwise comparisons among the 3 groups. The results are presented in Figure 5 for the 10,000 probability values that were generated from the bootstrapping procedure. The results showed that, of the 10,000 simulations for the comparisons between passive vs nonsmokers (Figure 5, A), the probability value was < .05 in 5895 iterations (58.95%). This indicates a significant difference between the 2 groups (ie, passive vs nonsmokers) after adjustment for multiple comparisons. For the comparison between active vs nonsmokers (Figure 5, B), we observed that 9998 of 10,000 iterations yielded a probability value on each occasion of < .05 (99.98%). This result was the most impressive significant difference to be
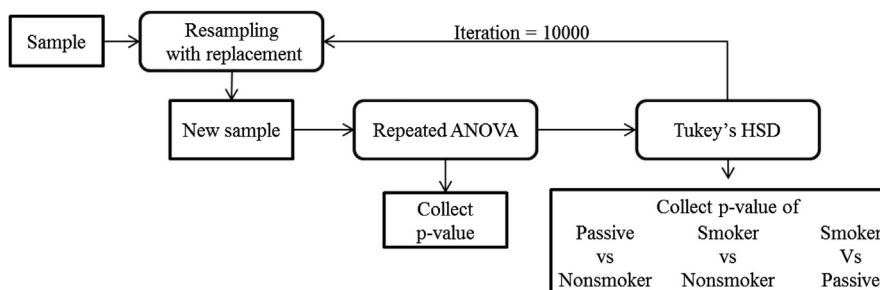
We conducted simulation analysis to derive precise estimates using the data that we had collected. The simulation procedure steps are illustrated in this Figure.

ANOVA, analysis of variance; *HSD*, honest significant difference.

Salihu. Tobacco exposure and fetal telomere length. Am J Obstet Gynecol 2014.

TABLE
Maternal sociodemographic and delivery measures among the 3 groups

Maternal and newborn characteristics	Nonsmoker (n = 25)	Passive smoker (n = 31)	Active smoker (n = 30)	P value
Maternal age, y[a]	25 ± 4.38	25 ± 5.42	25 ± 4.64	.807
Gestational age, wk[a]	40 ± 1.32	40 ± 1.15	40 ± 1.25	.581
Previous pregnancies, n[a]	2 ± 1.74	2 ± 1.74	2 ± 1.45	.543
Maternal body mass index, kg/m2[a]	31 ± 4.50	37 ± 12.88	35 ± 8.12	.100
Birthweight, g[a]	3248 ± 480.71	3312 ± 363.22	3373 ± 614.15	.657
Head circumference at birth, cm[a]	35 ± 1.38	34 ± 1.57	35 ± 2.03	.581
Cotinine, n (%)				
Score 0	25 (29.1)	0	0	.000
Score 1	0	31 (36.1)	18 (20.9)	
Score >1	0	0	12 (14)	
Marital status, n (%)				
Married	10 (11.6)	10 (11.6)	8 (9.3)	.599
Single	15 (17.4)	21 (24.4)	22 (25.6)	
Race, n (%)				
Black	3 (3.5)	11 (12.8)	9 (10.5)	.051
White	10 (11.6)	4 (4.7)	13 (15.1)	
Hispanic	9 (10.5)	10 (11.6)	6 (7.0)	
Other	3 (3.5)	6 (6.9)	2 (2.3)	
Insurance, n (%)				
Medicaid	16 (18.6)	20 (23.3)	24 (27.9)	.401
No insurance	3 (3.5)	7 (8.1)	3 (3.5)	
Private	6 (7.0)	4 (4.7)	3 (3.5)	

[a] Data are given as mean ± standard deviation.

Salihu. Tobacco exposure and fetal telomere length. Am J Obstet Gynecol 2014.

observed after the influence of multiple comparisons was taken into account.

Finally, for the pairwise comparison between active vs passive smokers (Figure 5, C), we obtained a probability value of < .05 in 9095 of 10,000 iterations (90.95%). These results further confirm that fetal telomere length differed significantly across the 3 groups in a dose-response pattern (shortest among active smokers followed by passive smokers and longest among nonsmokers).

COMMENT
In this study, we found a positive relationship between smoking during the prenatal period and shortened telomere length of the fetus at birth. These findings were validated with the use of a robust statistical tool (bootstrapping). This finding is further confirmation that exposure to smoking during pregnancy could impact the health of the fetus because telomere shortening has been associated with adverse health conditions during childhood, adolescence, and adult life.

Although other studies demonstrate the association between tobacco smoke and shortened telomeres in adults,[13,15] there is a paucity of data regarding the telomeric effects of smoking on the pediatric population. Entringer et al[29] evaluated the impact of maternal stress on umbilical cord telomeres and documented a 540–base pair shortening in infants to mothers with stress in comparison with those who experienced less stress. Theall et al[19] demonstrated that children who were exposed to tobacco in utero had a 1-unit shorter telomere length than those who were not exposed.

The long-term implications of shortened telomeres at birth are tremendous. Our study evaluated umbilical cord telomeres as a reflection of the in-utero environment. Abnormal telomere lengths have been implicated in various pediatric conditions. Sawyer et al[30] documented abnormal telomeres and increased telomerase activity with pediatric solid tumors. Shortened telomeres were also documented in neuroblastoma by Binz et al[31] and Ohali et al.[32] Subsequently, shortened telomeres and telomerase activity were implicated as playing a potential role in childhood malignancies by Polychronoulou and Koutroumba.[33] However, none of these publications highlighted cigarette smoking as a possible etiologic factor. Whether in-utero exposure to the multiple toxins in cigarettes would further increase the onset and/or severity of these conditions remains unknown. However, because shortened telomeres are associated with cellular senescence, one can speculate that antenatal shortening would place an inordinate risk for long-term adverse health outcomes.

This study was not designed to determine the effects of smoking on maternal telomeres. Epel et al[34] evaluated the telomeric changes with life stresses in women. They documented that excessive perceived life stress shortened telomere levels to the equivalent of women 1 decade older. Two separate reviews by Butt et al[35] and Fyrquist et al[36] linked shortened telomeres with various cardiovascular conditions that included atherosclerosis and carotid and peripheral vascular disease. Fyhrquist et al[37] also documented that a faster progression of diabetic nephropathy was associated with shortened telomeres of >8 kb and a higher percentage of shortened telomeres. Whether these processes in the adult could be accelerated by the

FIGURE 3
Histogram of mean of T/S for all 3 groups
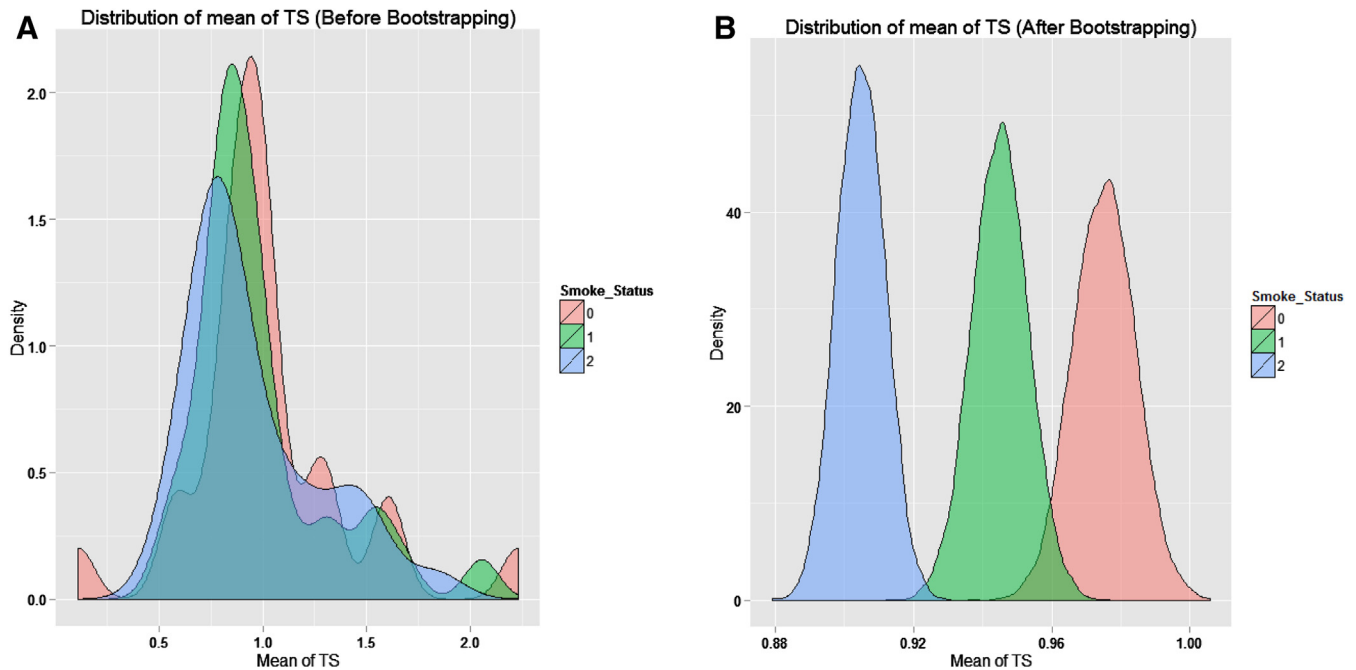
A, The mean distribution of T/S ratio within the study sample before the bootstrapping approach was applied. **B,** The same results after the bootstrapping approach.

T/S, telomere repeat copy number ratio.

Salihu. Tobacco exposure and fetal telomere length. Am J Obstet Gynecol 2014.

FIGURE 4
Histogram of probability value of repeated analysis of variance

The histogram represents all probability values that were generated with the use of repeated analysis of variance from the 10,000 bootstrapping simulations that were performed.

*Salihu. Tobacco exposure and fetal telomere length. Am J Obstet Gynecol 2014.*

physiologic changes of pregnancy is unknown. Although research in the male population does not necessarily have the same impact on women, Strandberg et al[38] documented that shortened telomeres (<5 kb) were associated with being overweight and smoking among men.

Clearly, the findings in our study reaffirm the necessity to seek more innovative avenues of preventing smoking during pregnancy because current methods of smoking cessation interventions have not been successful.[39] Although identification of the most effective ways of preventing smoking and passive exposure during pregnancy are challenging, it remains central and critical in making any meaningful impact on our ability to improve birth outcomes early in life to avert adult-onset disease. Some of the most popular strategies that are used in smoking cessation programs and are targeted toward pregnant women include advice and counseling, behavioral cognitive therapies, feedback

on the health of the developing fetus, measurement of biomarkers of tobacco smoke exposure, pharmacotherapy such as nicotine replacement therapy (NRT), social support, and hypnosis; however, data found only a modest reduction in prenatal smoking in these programs.[39,40] Furthermore, the safety and efficacy of NRT in pregnant women is unclear.[39] However, some investigators have suggested that the benefits outweigh the risks. Although NRT exposes the fetus to nicotine, it does not expose the fetus to the other harmful chemicals in cigarette smoke[40]; more studies are needed to ascertain whether NRT alone or in combination with behavioral therapy is a safe and effective cessation strategy for pregnant smokers.

A strength of our study is that this is the first evidence to show a correlation between umbilical cord blood telomere shortening and smoking during pregnancy; however, there are some limitations that deserve attention. One of the
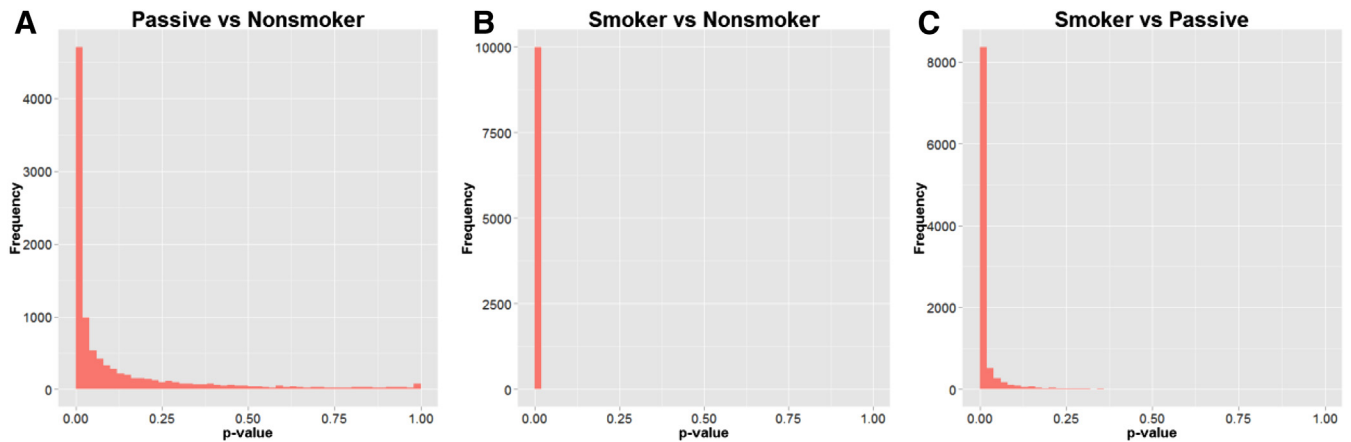
The results of Tukey's HSD procedure for pairwise comparisons among the 3 groups are presented for the 10,000 probability values that were generated from the bootstrapping procedure.

HSD, honest significant difference.

Salihu. Tobacco exposure and fetal telomere length. Am J Obstet Gynecol 2014.

---

limitations in this study is the small sample size; more studies that will use a larger sample size are needed to confirm our findings and to provide a more generalizable result. As a result of paucity of numbers, we were not able to do subgroup analysis, but future studies should look at that possibility to strengthen the level of evidence. Additionally, we thought it was important to note that, because of the inherent stigma of smoking while a woman is pregnant, we found it likely for the women to falsify tobacco use on a self-report questionnaire. For this reason, our definition of *active smokers* also included women in whom only a biologic saliva cotinine test resulted in levels 2-6. To account for participants who might have smoked in early gestation before learning of the pregnancy, we found it necessary to include the following question from the tobacco exposure questionnaire: "Did you smoke cigarettes before you found out you were pregnant?" Because women commonly have their first prenatal visit and confirm their pregnancies at 8-12 weeks, it is plausible that exposure to tobacco, even in early gestation, might have already caused irreversible effects on telomere length. Finally, this analysis could not account for the complicated mode of inheritance of

telomere length. Smoking behavior tends to run in families, which suggests a genetic contribution to the risk of becoming a smoking mother. Without having measured the telomere length of the women in the study, we cannot comment on the maternal telomere length contribution that may or may not have compromised the telomere length potential of the offspring. Although beyond the scope of this current study, further work should address the interaction between intrauterine exposure to smoke and maternal telomere variation on newborn telomere length.

In summary, our results provide the first evidence to demonstrate shortened telomere length among fetuses who were exposed to smoking during pregnancy and suggest prenatal smoking as a preventable cause of accelerated chromosomal aging. The potential long-term implications of neonatal "genetic aging" and maternal "genetic aging" are potentially enormous, which underscores the importance of more innovative and effective smoking cessation interventions during pregnancy. ■

REFERENCES

1. Salihu HM, Aliyu MH, Pierre-Louis BJ, Alexander GR. Levels of excess infant deaths attributable to maternal smoking during pregnancy in the United States. Matern Child Health J 2003;7:219-27.
2. Salihu HM, Wilson RE. Epidemiology of prenatal smoking and perinatal outcomes. Early Hum Dev 2007;83:713-20.
3. Russell T, Crawford M, Woodby L. Measurements for active cigarette smoke exposure in prevalence and cessation studies: why simply asking pregnant women isn't enough. Nicotine Tob Res 2004;6(suppl2): S141-51.
4. Magee SR, Bublitz MH, Orazine C, et al. The relationship between maternal-fetal attachment and cigarette smoking over pregnancy. Matern Child Health J 2014;18:1017-22.
5. Bojesen SE. Telomeres and human health. J Intern Med 2013;274:399-413.
6. Astrup AS, Tarnow L, Jorsal A, et al. Telomere length predicts all-cause mortality in patients with type 1 diabetes. Diabetologia 2010;53: 45-8.
7. Wang H, Chen H, Gao X, et al. Telomere length and risk of Parkinson's disease. Mov Disord 2008;23:302-5.
8. Shalev I, Entringer S, Wadhwa PD, et al. Stress and telomere biology: a lifespan perspective. Psychoneuroendocrinology 2013;38:1835-42.
9. Frenck RW, Blackburn EH, Shannon KM. The rate of telomere sequence loss in human leukocytes varies with age. Proc Natl Acad Sci U S A 1998;95:5607-10.
10. Fitzpatrick AL, Kronmal RA, Gardner JP, et al. Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. Am J Epidemiol 2007;165:14-21.
11. Zhang J, Kong Q, Zhang Z, Ge P, Ba D, He W. Telomere dysfunction of lymphocytes in patients with Alzheimer disease. Cogn Behav Neurol 2003;16:170-6.

12. Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 2010;464:520-8.

13. Morlá M, Busquets X, Pons J, Sauleda J, MacNee W, Agustí AG. Telomere shortening in smokers with and without COPD. Eur Respir J 2006;27:525-8.

14. Babizhayev MA, Yegorov YE. Smoking and health: association between telomere length and factors impacting on human disease, quality of life and life span in a large population-based cohort under the effect of smoking duration. Fundam Clin Pharmacol 2011;25:425-42.

15. Valdes AM, Andrew T, Gardner JP, et al. Obesity, cigarette smoking, and telomere length in women. Lancet 2005;366:662-4.

16. Huzen J, Wong LS, van Veldhuisen DJ, et al. Telomere length loss due to smoking and metabolic traits. J Intern Med 2014;275:155-63.

17. Huang J, Okuka M, McLean M, Keefe DL, Liu L. Effects of cigarette smoke on fertilization and embryo development in vivo. Fertil Steril 2009;92:1456-65.

18. Huang J, Okuka M, Lu W, et al. Telomere shortening and DNA damage of embryonic stem cells induced by cigarette smoke. Reprod Toxicol 2013;35:89-95.

19. Theall KP, McKasson S, Mabile E, Dunaway LF, Drury SS. Early hits and long-term consequences: tracking the lasting impact of prenatal smoke exposure on telomere length in children. Am J Public Health 2013;103(suppl1): S133-5.

20. Jarvis MJ, Tunstall-Pedoe H, Feyerabend C, Vesey C, Saloojee Y. Comparison of tests used to distinguish smokers from nonsmokers. Am J Public Health 1987;77:1435-8.

21. Benowitz NL. Biomarkers of environmental tobacco smoke exposure. Environ Health Perspect 1999;107(suppl2):349-55.

22. Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. Association between telomere length in blood and mortality in people aged 60 years or older. Lancet 2003;361:393-5.

23. Cawthon RM. Telomere measurement by quantitative PCR. Nucleic Acids Res 2002;30: e47.

24. Aviv A, Hunt SC, Lin J, Cao X, Kimura M, Blackburn E. Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR. Nucleic Acids Res 2011;39:e134.

25. Drury SS, Theall K, Gleason MM, et al. Telomere length and early severe social deprivation: linking early adversity and cellular aging. Mol Psychiatry 2012;17:719-27.

26. Porter S, Rao ST, Ku J, Poirot RL, Dakins M. Small sample properties of nonparametric bootstrap t confidence intervals. J Air Waste Manag Assoc 1997;47:1197-203.

27. Preacher KJ, Hayes AF. Asymptotic and resampling strategies for assessing and comparing indirect effects in multiple mediator models. Behav Res Methods 2008;40: 879-91.

28. Efron B. Bootstrap methods: another look at the jackknife. Ann Statist 1979;7:1-26.

29. Entringer S, Epel ES, Lin J, et al. Maternal psychosocial stress during pregnancy is associated with newborn leukocyte telomere length. Am J Obstet Gynecol 2013;208:134.e1-7.

30. Sawyer JR, Roloson GJ, Bell JM, Thomas JR, Teo C, Chadduck WM. Telomeric associations in the progression of chromosome aberrations in pediatric solid tumors. Cancer Genet Cytogenet 1996;90:1-13.

31. Binz N, Shalaby T, Rivera P, Shin-ya K, Grotzer MA. Telomerase inhibition, telomere shortening, cell growth suppression and induction of apoptosis by telomestatin in childhood neuroblastoma cells. Eur J Cancer 2005;41: 2873-81.

32. Ohali A, Avigad S, Ash S, et al. Telomere length is a prognostic factor in neuroblastoma. Cancer 2006;107:1391-9.

33. Polychronopoulou S, Koutroumba P. Telomere length variation and telomerase activity expression in patients with congenital and acquired aplastic anemia. Acta Haematol 2004;111:125-31.

34. Epel ES, Blackburn EH, Lin J, et al. Accelerated telomere shortening in response to life stress. Proc Natl Acad Sci U S A 2004;101: 17312-5.

35. Butt HZ, Atturu G, London NJ, Sayers RD, Bown MJ. Telomere length dynamics in vascular disease: a review. Eur J Vasc Endovasc Surg 2010;40:17-26.

36. Fyhrquist F, Saijonmaa O, Strandberg T. The roles of senescence and telomere shortening in cardiovascular disease. Nat Rev Cardiol 2013;10:274-83.

37. Fyhrquist F, Tiitu A, Saijonmaa O, Forsblom C, Groop PH, Group FS. Telomere length and progression of diabetic nephropathy in patients with type 1 diabetes. J Intern Med 2010;267:278-86.

38. Strandberg TE, Saijonmaa O, Tilvis RS, et al. Association of telomere length in older men with mortality and midlife body mass index and smoking. J Gerontol A Biol Sci Med Sci 2011;66: 815-20.

39. Lumley J, Chamberlain C, Dowswell T, Oliver S, Oakley L, Watson L. Interventions for promoting smoking cessation during pregnancy. Cochrane Database Syst Rev 2009: CD001055.

40. Coleman T. Reducing harm from tobacco smoke exposure during pregnancy. Birth Defects Res C Embryo Today 2008;84:73-9.