THESIS

TELOMERE LENGTH AS A BIOMARKER OF EXPOSURE TO INDOOR WOODSTOVE SMOKE IN RURAL HONDURAS: A FEASIBILITY FIELD STUDY

Submitted by

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ABSTRACT

TELOMERE LENGTH AS A BIOMARKER OF EXPOSURE TO INDOOR WOODSTOVE SMOKE IN RURAL HONDURAS: A FEASIBILITY FIELD STUDY

Telomeres, the natural ends of linear chromosomes, are important for maintaining genome stability. Telomere length is an inherited trait influenced by a host of lifestyle and environmental factors, which have been shown to accelerate the rate of telomere shortening, and thus of aging. Indoor air pollution is one of the environmental factors known to influence the length of telomeres. It has been reported that people exposed to this kind of contamination, have an increased risk for pulmonary diseases, cardiovascular diseases and cancer. The accumulation of evidence correlating telomere length with different diseases and chronological age supports the use of short telomere frequency as an informative biomarker of general health status and aging. Epidemiological studies suggest that increased frequencies of nuclear aberrations (micronuclei, buds) are also correlated with exposure to air pollution.

Here, we confirm the feasibility of conducting field studies to evaluate telomere length in populations exposed to indoor air pollution in rural Honduras, and begin to address the question of whether telomere length can be used as an informative biomarker of exposure to indoor woodstove smoke. Buccal mucosa basal (stem-like) cells were collected from 100 exposed individuals in the field (prior to intervention); samples were shipped to US (CSU) for assessment of average telomere length (TL) and frequency of short telomeres. Results were correlated with age for all participants, and with total number of nuclear aberrations in a subset (20 individuals). Initial analyses suggest that frequencies of short telomeres, rather than average telomere length,

correlate with total number of nuclear aberrations in those assumed to be the most exposed individuals. These preliminary findings require correlation with actual particulate matter exposures, as well as confirmation in a larger cohort (studies on-going)

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Multiple environmental contaminants, e.g. air pollutants, have been associated with telomere shortening (Terry et al., 2008; Hou et al., 2012; Shay, 2016; Naing et al, 2017). One example of air pollution is smoke emission from indoor wood-burning cookstoves, used by some communities in developing countries. There are also studies suggesting a positive correlation between woodstove smoke exposure and development of age-related diseases; e.g., pulmonary diseases, cardiovascular diseases, and cancer (Bruce et al., 2000; Kampa et al., 2008; Clark et al., 2010; Martin et al.,2014). These increased risks are also correlated to telomere length variation (Serrano et al.,2004; Bailey et al., 2006; Terry et al., 2008; Shay, 2016).

Telomeres are the specialized terminal structures of linear chromosomes that have essential roles in maintaining genome stability (Figure 1). Telomeres protect the physical ends of chromosomes from degradation, preventing chromosomal end-to-end fusion, and from being recognized as DNA damage (De Lange, 2005; Bandaria et al., 2016, Blackburn, 2016). Another essential function of telomeres is to facilitate the complete replication of the genetic information contained within chromosomes. Telomeric repeats progressively erode—approximately 50 to 200 base pairs with each cell division—in part, due to the end-replication problem; i.e., conventional replication machinery is not able to copy the DNA completely up to the very end of linear chromosomes (Lindsey et al.,1991; De Lange, 2005; Blackburn et al., 2015). Therefore, normal human cells with telomeres of about 10 to 12 Kb in length, can divide 50 to 60 times on average before undergoing cell cycle arrest. Natural telomere shortening is a slow process, underling how normal eukaryotic cells experience a limited replicative life. When telomeres become critically short, the cells enter a permanent cell cycle arrest known as senescence (Harley

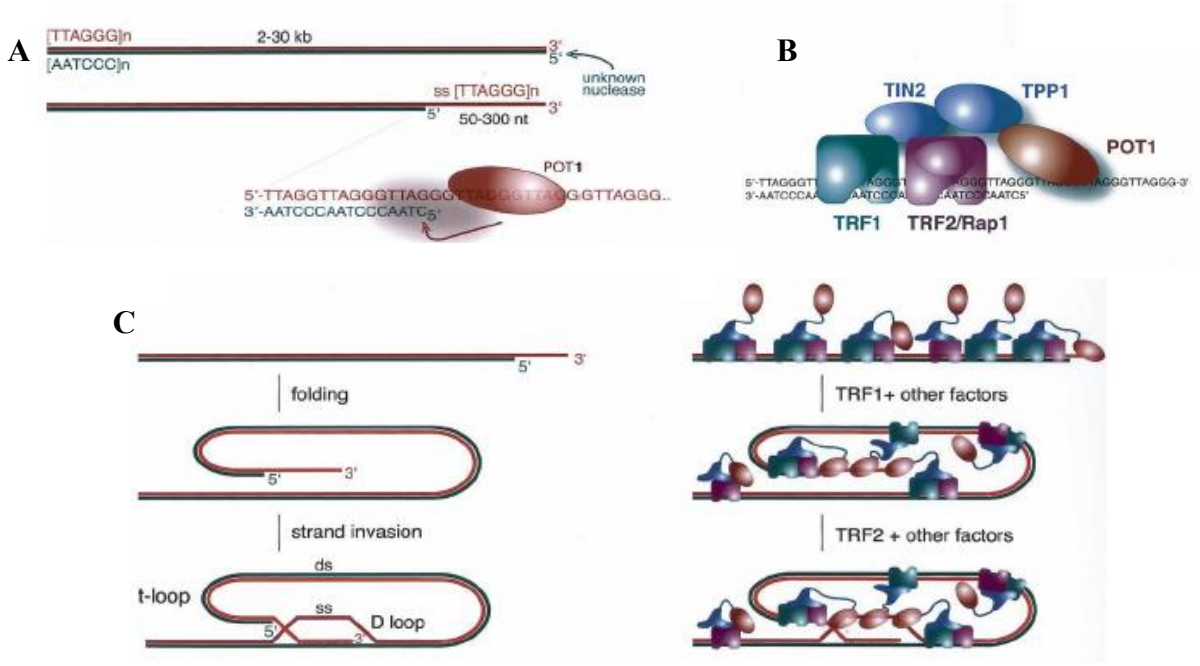


Figure 1. Schematic representation of telomere structure. A) DNA component, which consist in repetitive G-rich sequences. B) Shelterin. Protein complex composed by six proteins interacting among them and with double and single stranded DNA. C) The interaction DNA-proteins allows the DNA fold over itself hiding the 3'overhang (single stranded DNA) in telomere characteristic protective structure. Image modified from De Lange, 2005.

et al., 1990; Campisi, 2013). Accumulation of senescent cells eventually compromises the appropriate functioning of different tissues by disrupting cell tissue exchange, triggering aging and/or age related diseases (Campisi, 2013; Blasco et al.; Zhao et al., 2014). Organisms are born with a genetically determined telomere length (Takubo et al., 2017), as well as a defined number of stem cells (Watt et al., 2000; Fuchs et al., 2004; Moore et al., 2006). An increase in cellular turnover at the tissue level, e.g., to replace dead or damaged cells, eventually leads to exhaustion of stem cell niches (Watt et al.,2000; Fuchs et al., 2004; Moore et al., 2006; Zhao et al.,2014).

Cookstove smoke has been studied as a contributing factor that can accelerate cellular turnover in tissues like buccal mucosa (Martin et al. 2014), with the consequent increment of stem cell divisions, resulting in exacerbation of telomere shortening and premature aging of the exposed tissues. Together, such findings suggest a promising possibility, that of using telomeres length—specifically the abundance of short telomeres (Hemann et al., 2001; Vera et al., 2012)—as an informative biomarker of deleterious health effects resulting from a wide range of lifestyle stresses and environmental exposures.

Here, our intent was to evaluate the feasibility of using telomere length as a biomarker of indoor woodstove smoke exposure as part of an on-going study in rural Honduras. Buccal mucosa tissue (cheek swabs) from individuals exposed to indoor woodstove smoke were collected in the field for microscopic examination of basal cells (at CSU). Buccal mucosa basal (stem-like) cells divide to generate cellular progeny (progenitor cells) that give rise to more differentiated cells, which reside at the base of this stratified tissue (Figure 2). Because the basal cells have not experienced a high number of cell divisions, they represent a reliable biomarker for the rate of telomere shortening and accelerated telomere shortening resulting from contaminant exposure (Thomas et al., 2009). This feature of basal cell allowed us to avoid

potential bias, misinterpretations and/or artifacts, which could be introduced by examining more rapidly dividing cell populations, or shorter contaminant exposure times.

Buccal mucosa is a protective oral tissue that reflects contaminants exposures and life styles factors, which has been widely used for the study of chromosome instability and DNA damage (Thomas et al. 2009). Thus, use of buccal mucosa allowed assessment not only of telomere length, but also of nuclear aberrations (micronuclei, buds), which are known to increase with DNA damage. Importantly for field studies, buccal mucosa also represented an easily accessible tissue for sampling, requiring neither invasive procedure nor causing undue stress to the individual.

We performed cell-by-cell analysis using quantitative interphase Telo-FISH to assess any potential association of indoor woodstove smoke exposure and telomere length changes. Interphase telomere fluorescent *in situ* hybridization (Telo-FISH; Figure 3) is a high-resolution telomere length measurement approach that facilitates determination of average telomere length, as well as individual telomere length distributions and abundance of short telomeres (Meeker et al., 2002; Canela et al., 2007; Aubert et al., 2012; Vera et al., 2012; Montpetit et al., 2014). The Telo-FISH methodology uses Peptide Nucleic Acid (PNA) telomere probes, and combines fluorescence microscopy with digital image acquisition (Poon and Landsdorp, 2001). PNA probes are synthetic small oligonucleotides (15-18-mers) with high affinity for single-stranded DNA sequences. PNA synthetic probes were designed such that each PNA probe recognized three telomeric repeats (TTAGGG), and were directly labeled with fluorochromes. This strategy provides a directly proportional relationship between fluorescent intensity (brightness) of hybridized telomeric probes and length of the telomere (Vera et al., 2012).

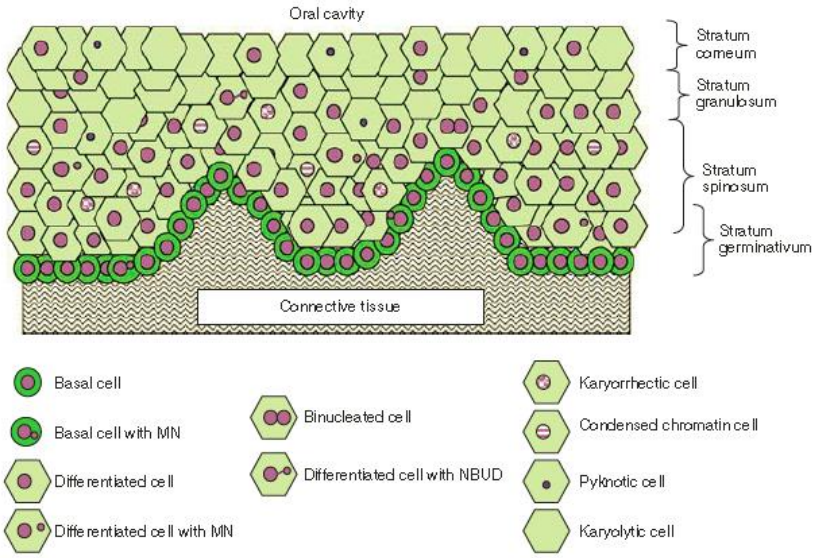


Figure 2. Normal buccal mucosa tissue structure with its four typical layers. Graph extracted from Thomas et al.,2009.

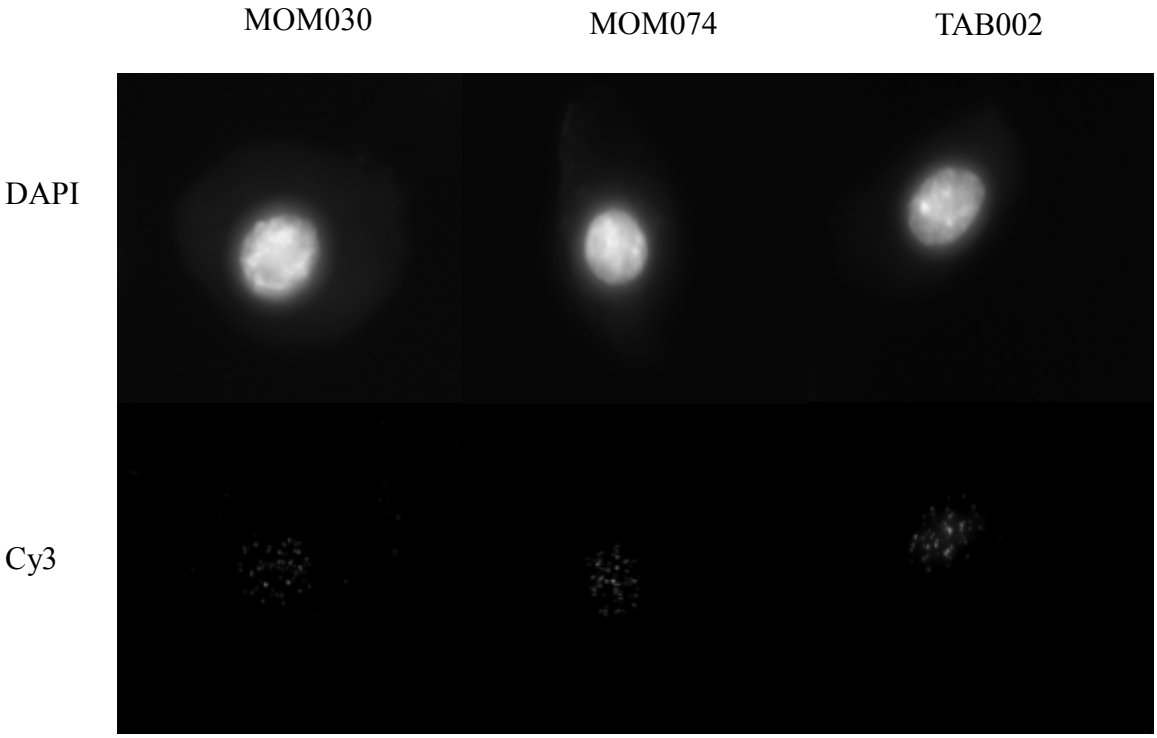


Figure 3. Image capture examples for telomere length measurements. Basal cells from three different individuals identified as: MOM030, MOM074, and TAB002. Top panel: DAPI for nuclear stain. Bottom panel: PNA probe fluorescently labeled with Cy3 fluorochrome for telomere visualization.

Importantly, interphase Telo-FISH also lends itself well to convenient "in field" sampling conditions and associated limited lab accessibility, as well as to evaluation of nuclear aberrations. Total nuclear aberrations are commonly used as biomarker of DNA damage in epidemiological studies (Thomas et al., 2009) and have been associated with exposure to environmental contaminants such as air pollution, specifically in buccal mucosa cells (Pastor et al., 2003; Thomas et al.,2009; Bolognesi et al., 2013). Therefore, in conjunction with determination of telomere length, total nuclear aberrations were also quantified in a subset of collected samples (n=20). Total nuclear aberrations (TNA) were scored in 2000 cells/individual and included: 1) micronuclei (MNi), small bodies formed when DNA is fragmented and not incorporated into the main nucleus (i.e., lagging fragments), and 2) nuclear buds (NBUDs), which appear as secondary nuclei near or attached to the main nucleus (Figure 4; Thomas et al.,2009).

Telomere length results were correlated with age for all participants and with total number of nuclear aberrations in selected subset. Initial analyses suggest that frequencies of short telomeres, rather than average telomere length, correlate with total number of nuclear aberrations in those assumed to be the most exposed individuals. These preliminary findings require correlation with actual particulate matter exposure (Clark et al., 2010), as well as confirmation in a larger cohort (studies on-going).

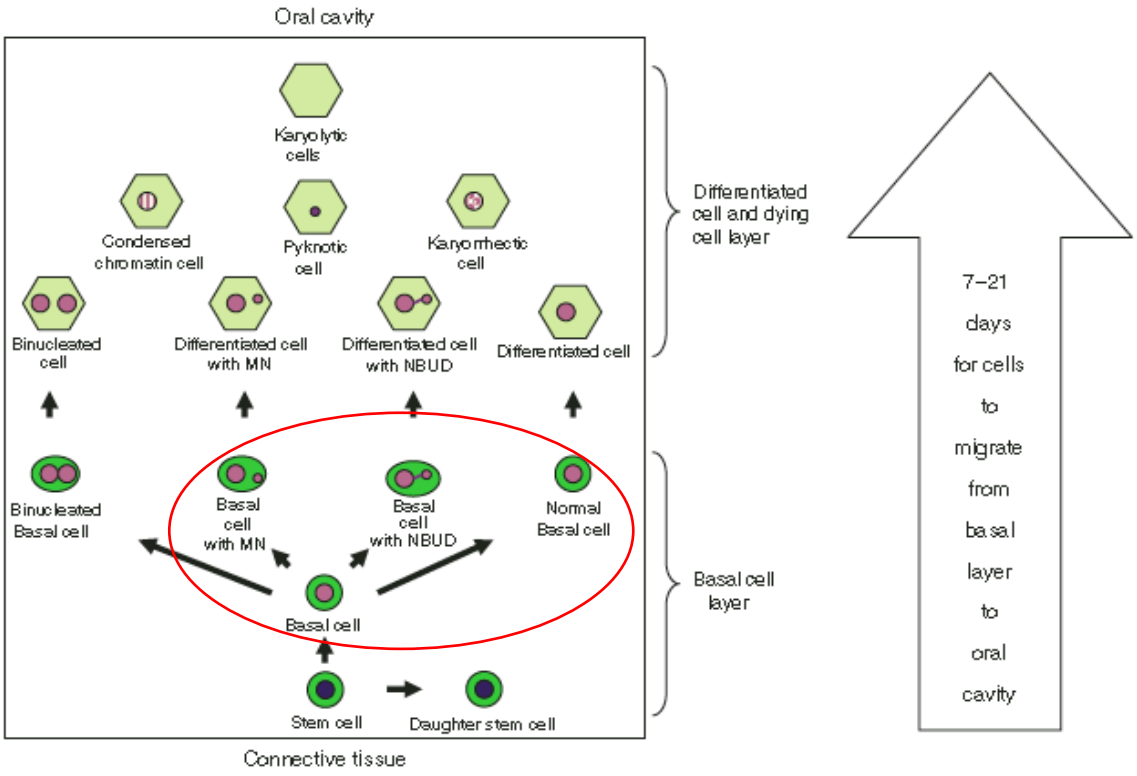


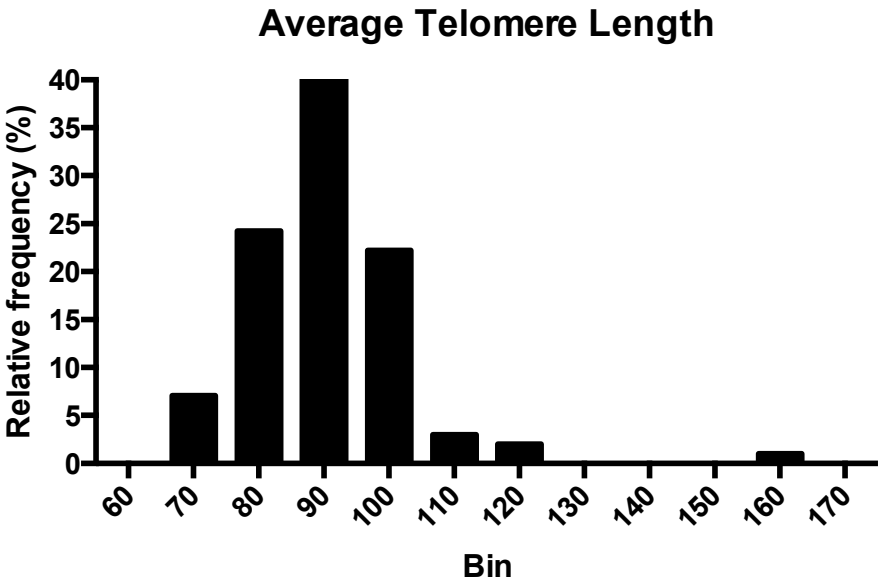
Figure 4. Origin sequence of the probable different cell types present in the buccal mucosa tissue showing the time relation with the differentiation events. Inside the red circle are the target cells for this study, Basal cell/Normal basal cell for telomere length measurement and Basal cell with MN/NBUD for nuclear aberrations. Image modified from Thomas et al.,2009.

RESULTS

Telomere length (TL) measurements and analysis

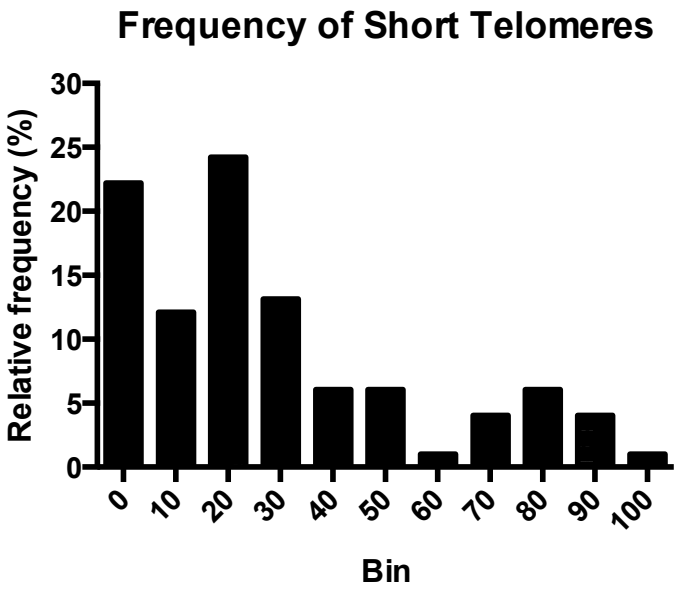
As part of an on-going study in rural Honduras (Clark), telomeres length (TL) was measured in buccal mucosa progenitor basal cells collected from 100 participants exposed to indoor woodstove smoke. Average TL for each participant was established, as was the frequency distribution of average TL for the population (Figure 5). Although participants ranged in age from 25-55 years old, approximately 86% of the individuals had similar average telomere length (average=90; range 80-100), with only 7% having shorter telomeres, and 6% having longer telomeres than average.

We then generated frequency distributions to appreciate the percentage of short telomeres per individual, which was more informative than average TL. For the entire cohort, a binomial distribution of percent short telomeres was observed (Figure 6); i.e., ~50% of the individuals had a high frequency of short telomeres, and ~50% had a low frequency of short telomeres, which did not correlate with age (e.g., young vs old).



	Average Intensity TL
Total number of values	99
Number of excluded values	0
Number of binned values	99
Minimum	68.2
25% Percentile	83.3
Median	90.5
75% Percentile	95.55
Maximum	163.2
Mean	90.3848
Std. Deviation	12.2603
Std. Error of Mean	1.23221
Lower 95% CI of mean	87.9396
Upper 95% CI of mean	92.8301

Figure 5. Histogram of average TL frequency distribution of the total analyzed cohort, including descriptive statistic table. The histogram shows that most of the individuals have an average in a range of 80 to 100 telomere fluorescent intensity units (Bin).



	% Short Telomeres
Total number of values	99
Number of excluded values	0
Number of binned values	99
Minimum	0.0
25% Percentile	7.79
Median	20.47
75% Percentile	41.73
Maximum	99.55
Mean	28.3485
Std. Deviation	27.0818
Std. Error of Mean	2.72182
Lower 95% CI of mean	22.9471
Upper 95% CI of mean	33.7498

Figure 6. Histogram of frequency of short telomeres distribution of the total analyzed cohort, including descriptive statistic table. The frequency distribution of % short telomeres show significant variation among the exposed participants.

Total nuclear aberration quantification

Total nuclear aberrations (micronuclei and buds) in buccal mucosa cells (Figure 7) are commonly used in epidemiological studies as biomarkers of air pollution exposure, as they directly correlate with other biomarkers commonly used in the field, such as particulate matter, also known as particulate pollution (Pastor et al., 2003; Thomas et al.,2009). Quantification of total nuclear aberrations (TNA) in a subset of samples (n=20) from the entire cohort is presented as a frequency histogram (Figure 8), which illustrates a distribution of individuals with and without TNA, ranging from no TNA (5%) up to a maximum value of 29 TNA (5%), with a median value of 4.5 and a mean of 7.1.

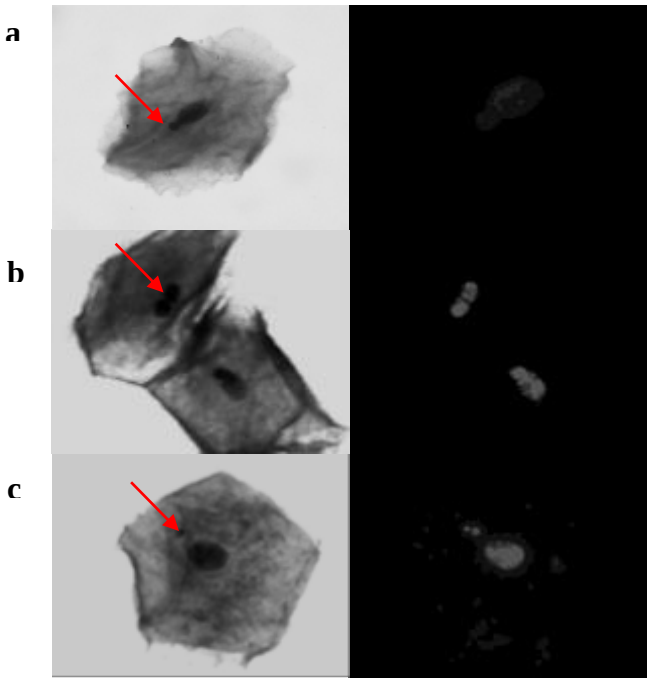
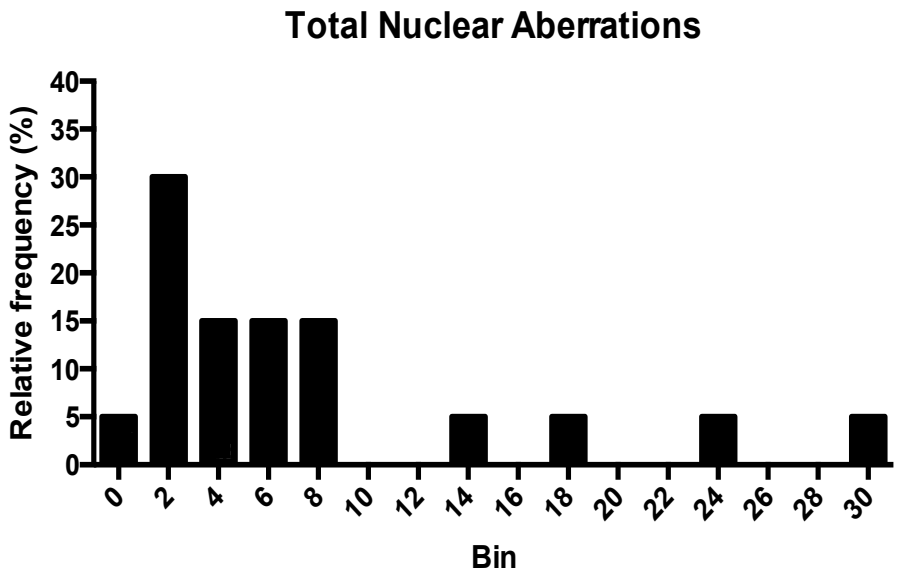


Figure 7- Differentiated buccal mucosa cells, the image show (signaled by arrows) examples of nuclear bud (a) (NBUD), broken egg cell(b) quantified as NBUD; and (c) micronuclei (MNi).



	Total Nuclear Aberration
Total number of values	20
Number of excluded values	0
Number of binned values	20
Minimum	0.0
25% Percentile	2.0
Median	4.5
75% Percentile	7.75
Maximum	29.0
Mean	7.1
Std. Deviation	7.75208
Std. Error of Mean	1.73342
Lower 95% CI of mean	3.47191
Upper 95% CI of mean	10.7281

Figure 8. Histogram of total nuclear aberrations distribution of the analyzed group (n=20), including descriptive statistic table. The graph shows the total nuclear aberrations distribution including participants with and without TNA ranging from no TNA to 29 TNA.

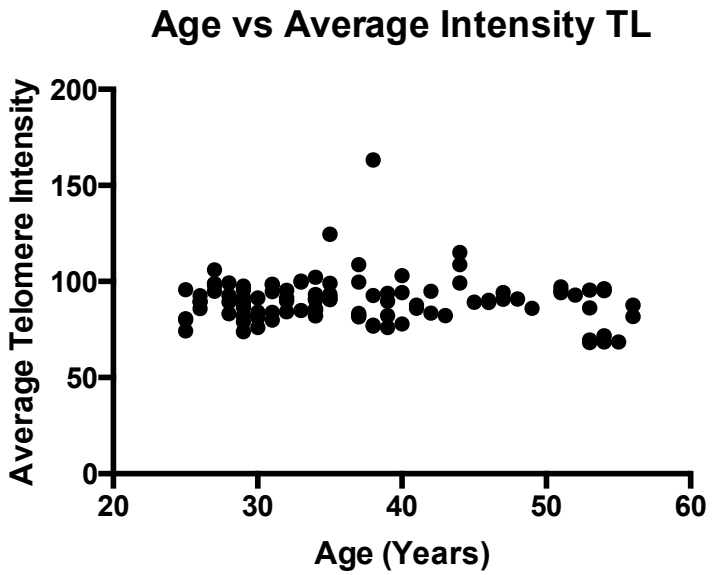
Correlation analyses

We next sought to identify other factors influencing the observed variations of telomere length in the individuals of this exposed population. To evaluate possible influence of age in our results, we correlated average telomere length with the age of each participant (ranged 25-55 years old; n=100). Calculated Pearsons and Spearman correlation coefficients did not find correlation between age and average telomere length (Figure 9). Similarly, there was no correlation between age and frequency of short telomeres (Figure 10), allowing us to eliminate age as a confounding factor in this cohort. No correlation of TL with age is consistent with expectations, as buccal mucosa stem-like basal cells have telomerase activity and undergo a low turnover rate by definition, so minimal TL shortening occurs in this cell type. Further, the age range under consideration (25-55 years old) has been reported as a plateau region for telomere shortening; i.e., no significant variation in TL has been observed (Aubert et al., 2008).

We also evaluated the correlation between average telomere length and the percentage of short telomeres to confirm the relative dependence of these two parameters (Figure11). As expected a negative correlation was observed, thus validating our measurements. It also becomes obvious that individuals with same average telomere length present significant differences in percentage of short telomeres, a finding consistent with our previous studies (Zahran et al., 2015).

Lastly, we analyzed possible correlations between average telomere length or percent short telomeres and TNA in a subset of individuals (n=20), and found no correlation between any of the parameters (Figures 12 and 13).

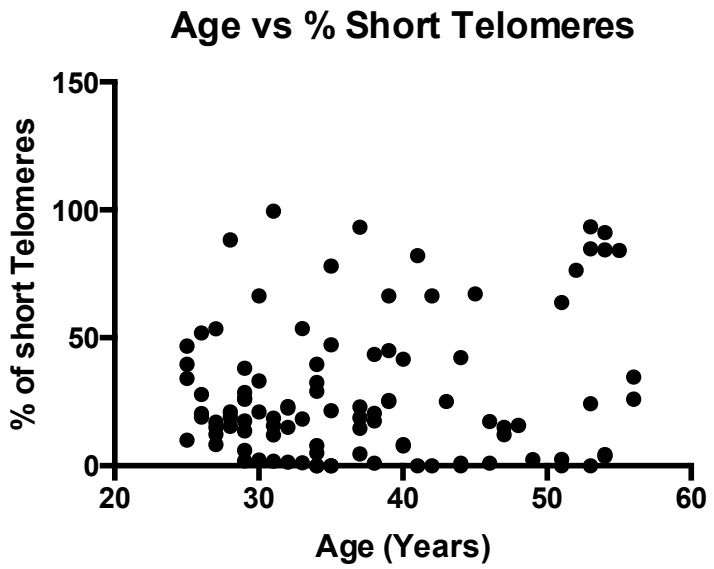
In summary; neither TL and age, nor TL and TNA were not correlated in any statistically significant way. Importantly, percent short telomeres and average TL were negatively correlated (as percent short telomere increases, average TL decreases) validating the methodology used.



Pearson's Coef.	Age vs. Average Intensity TL
P value	
P (two-tailed)	0.3402
P value summary	ns
Significant? (alpha = 0.05)	No
Number of XY Pairs	99

Spearman's Coef.	Age vs. Average Intensity TL
P value	
P (two-tailed)	0.5702
P value summary	ns
Exact or approximate P value?	Approximate
Significant? (alpha = 0.05)	No
Number of XY Pairs	99

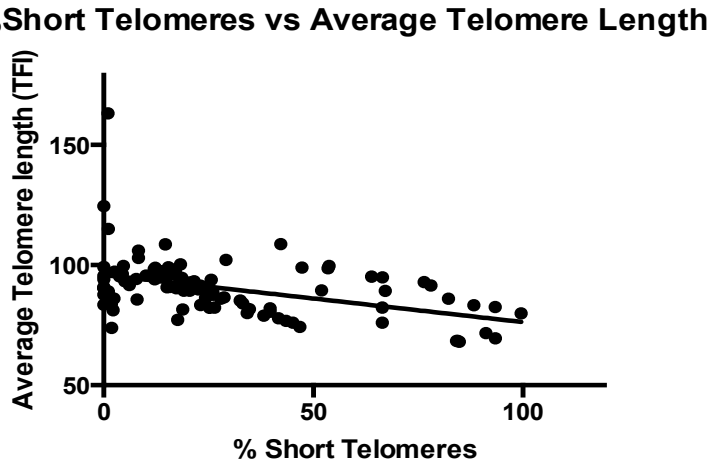
Figure 9. Scatter plot showing the result for age-average telomere length correlation. Average telomere length was not correlated to age in this cohort. Confirming age is not a confounding factor in the studied population because of the plateau phase in telomere shortening rate.



Pearson's Coef.	Age vs. % Short Telomeres
P value	
P (two-tailed)	0.0990
P value summary	ns
Significant? (alpha = 0.05)	No
Number of XY Pairs	99

Spearman's Coef.	Age vs. % Short Telomeres
P value	
P (two-tailed)	0.9334
P value summary	ns
Exact or approximate P value?	Approximate
Significant? (alpha = 0.05)	No
Number of XY Pairs	99

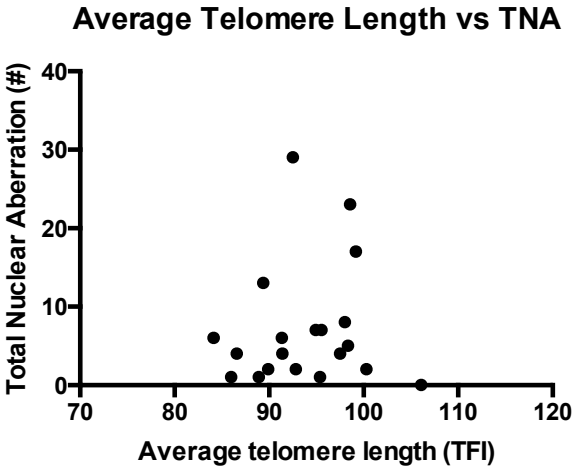
Figure 10. Scatter plot showing the correlation between for age and short telomere percentage. No correlation was found between these parameters.



	% Short Telomeres vs. Average Intensity TL
Pearson r	
r	-0.4343
95% confidence interval	-0.5818 to -0.2591
R squared	0.1886
P value	
P (two-tailed)	< 0.0001
P value summary	****
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	99

	% Short Telomeres vs. Average Intensity TL
Spearman r	
r	-0.4299
95% confidence interval	-0.5821 to -0.2485
P value	
P (two-tailed)	< 0.0001
P value summary	****
Exact or approximate P value?	Approximate
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	99

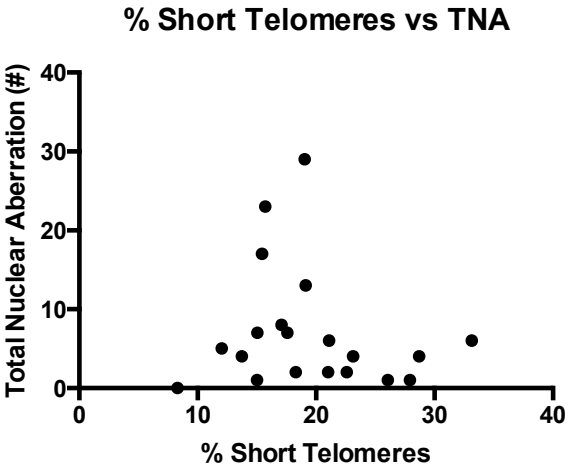
Figure 11. Scatter plot showing the negative correlation between short telomere percentage and average telomere length. For each person, average telomere length includes all telomeres length intensities, and short telomere percentage consider the fraction of telomeres with low intensity combined to the rest of telomere length from the intensity distribution per person.



	Average Intensity TL vs. Total Nuclear Aberration
Pearson r	
r	0.08879
95% confidence interval	-0.3682 to 0.5112
R squared	0.007884
P value	
P (two-tailed)	0.7097
P value summary	ns
Significant? (alpha = 0.05)	No
Number of XY Pairs	20

	Average Intensity TL vs. Total Nuclear Aberration
Spearman r	
r	0.1209
95% confidence interval	-0.3521 to 0.5448
P value	
P (two-tailed)	0.6115
P value summary	ns
Exact or approximate P value?	Approximate
Significant? (alpha = 0.05)	No
Number of XY Pairs	20

Figure 12. Average telomere length and total nuclear aberrations scatter plot. TNA were quantified in a subpopulation of participants. We found no correlation between average telomere length and TNA.



	% Short Telomeres vs. Total Nuclear Aberration
Pearson r	
r	-0.1549
95% confidence interval	-0.5591 to 0.3088
R squared	0.02398
P value	
P (two-tailed)	0.5144
P value summary	ns
Significant? (alpha = 0.05)	No
Number of XY Pairs	20

	% Short Telomeres vs. Total Nuclear Aberration
Spearman r	
r	-0.1119
95% confidence interval	-0.5383 to 0.3602
P value	
P (two-tailed)	0.6387
P value summary	ns
Exact or approximate P value?	Approximate
Significant? (alpha = 0.05)	No
Number of XY Pairs	20

Figure 13. Short telomere percentage and total nuclear aberrations scatter plot showing no correlation between them in a total of 20 individuals (same individuals from figure 12).

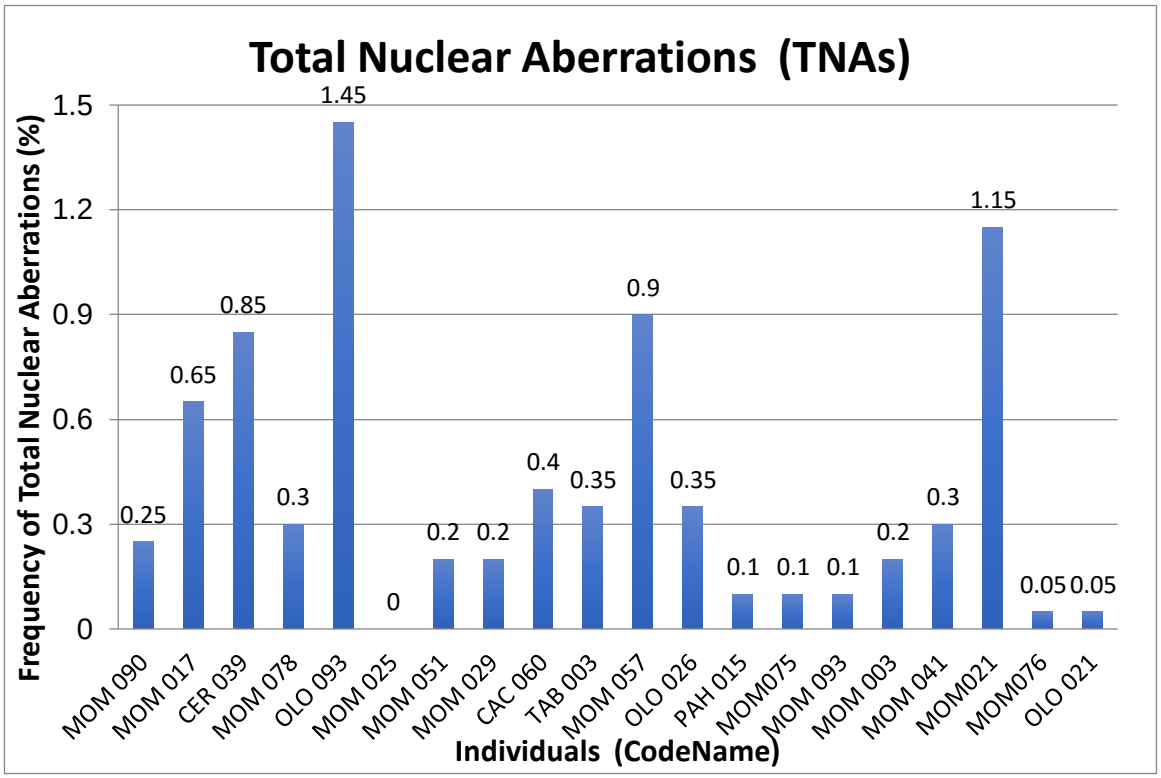
Results interpretation

We speculated that modeling the appearance of short telomeres or the increase in the frequency of cells with short telomeres due to a "catalyst" (e.g., exposure) would improve interpretation of these results. Since the link between external contaminants, in this case indoor woodstove smoke, and short telomeres is indirect, we chose the presence of total nuclear aberrations (TNA) in the entire buccal mucosa cell population as a direct measure of air pollution exposure to establish a correlation. It has been shown that high levels of exposure are necessary to increase TNAs (Thomas et al., 2009). Thus, we reasoned that the level of exposure (high vs low) to indoor woodstove smoke could be inferred based on TNAs; i.e., TNA provide a valuable link between woodstove smoke exposure and increased frequency of short telomeres.

Epidemiology studies have established 0.3% as an accepted background frequency of TNA in non-exposed control groups (Thomas et al. 2009). Extrapolation of this normal frequency to our exposed cohort allowed us to establish two groups—one considered normal or relatively low exposure, and one more highly exposed population. A background frequency of 0.3% correspond to 6 aberrations per 2,000 cells (equivalent to 3 per 1,000 or 0.3%). Therefore, one group consisted of individuals having a low number of TNA (values under 0.3%), and the other group consisted of individuals having a high number of TNA (values above 0.3%) (Figure 14). It is noteworthy that this two groups were not arbitrarily formed based on any statistical assumption, but rather were formed based on biological assumption, as higher levels of smoke exposure have been shown to produce increased numbers of TNA.

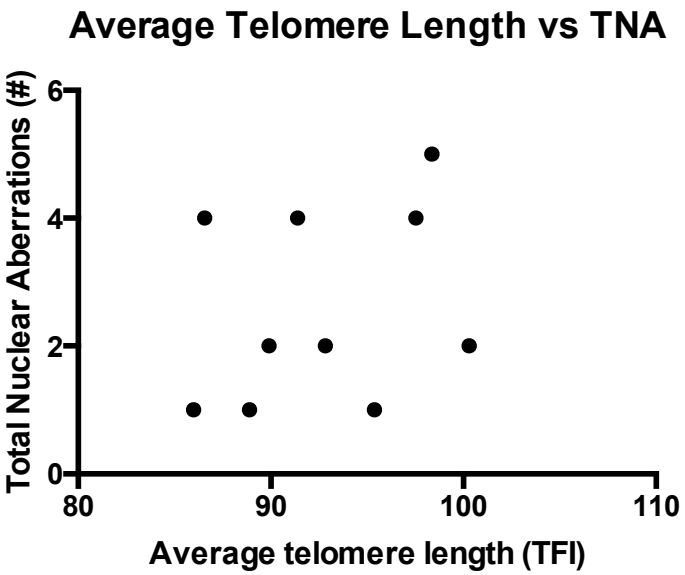
There was no correlation between average TL and either lower or higher number of TNA (Figures 15 and 17). For individuals presenting lower numbers of TNA, there was also no correlation between percent short telomeres and TNA (Figure 16). However, for individuals who

presented higher numbers of TNA, a positive correlation between percentage of short telomeres and TNA was found (Figure 18). Based on our assumption that TNAs reflect woodstove smoke exposure level, and although a small sample size, this positive correlation between percentage of short telomeres and TNA provides support for using of percentage of short telomeres—rather than average TL—as an informative biomarker of indoor woodstove smoke exposure in field studies.



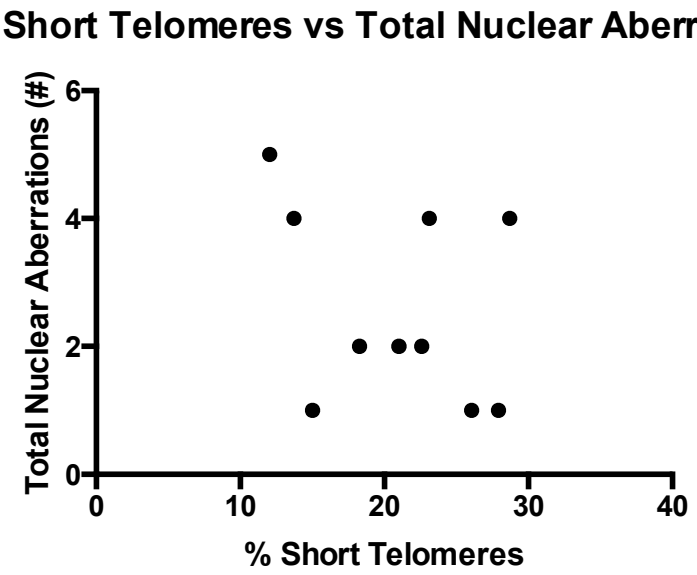
	Total Nuclear Aberration
Total number of values	20
Number of excluded values	0
Number of binned values	20
Minimum	0.0
25% Percentile	2.0
Median	4.5
75% Percentile	7.75
Maximum	29.0
Mean	7.1
Std. Deviation	7.75208
Std. Error of Mean	1.73342
Lower 95% CI of mean	3.47191
Upper 95% CI of mean	10.7281

Figure 14. Histogram showing low and high number of TNAs with respect to 0.3% normal background level (Thomas et al. 2009).



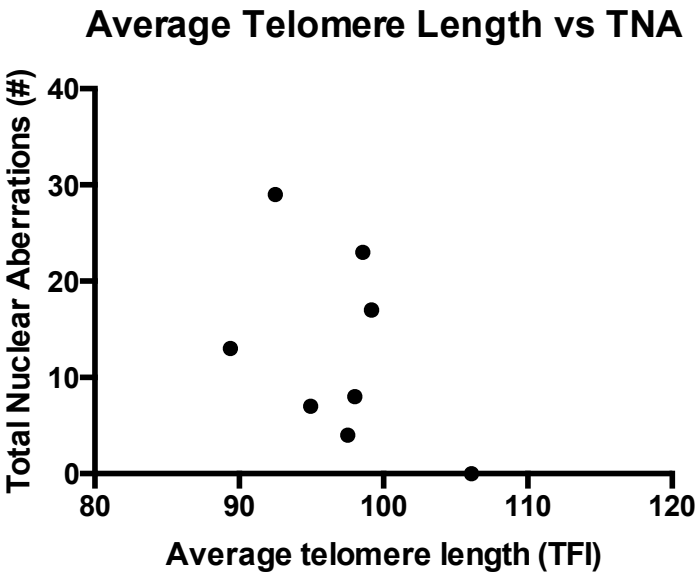
	Average TL vs. total aberrations
Spearman r	
r	0.3713
95% confidence interval	
P value	
P (two-tailed)	0.2911
P value summary	ns
Exact or approximate P value?	Exact
Significant? (alpha = 0.05)	No
Number of XY Pairs	10

Figure 15. Scatter plot showing no correlation between average telomere length and number of total nuclear aberration (TNA) in individuals who present low number of TNA.



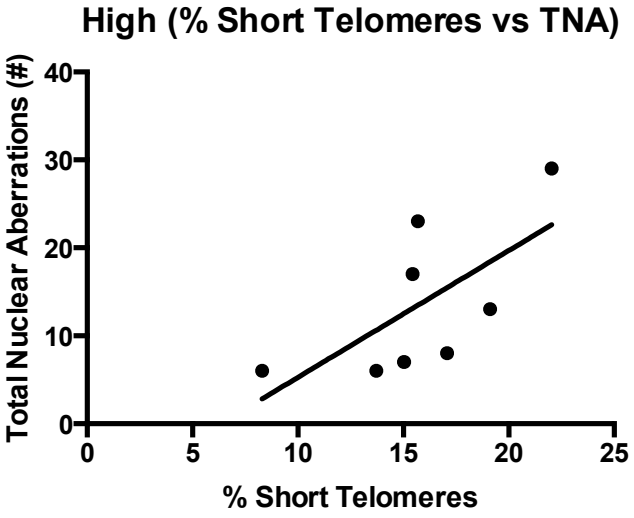
	short_telo vs. total aberrations
Spearman r	
r	-0.3210
95% confidence interval	
P value	
P (two-tailed)	0.3707
P value summary	ns
Exact or approximate P value?	Exact
Significant? (alpha = 0.05)	No
Number of XY Pairs	10

Figure 16. Scatter plot showing no correlation between short telomere percentage and the 10 individuals who present low number of TNA.



	Average TL vs. total aberrations
Spearman r	
r	-0.2857
95% confidence interval	
P value	
P (two-tailed)	0.5008
P value summary	ns
Exact or approximate P value?	Exact
Significant? (alpha = 0.05)	No
Number of XY Pairs	8

Figure 17. Scatter plot showing no correlation between average telomere length and individuals (n=8) with high number of TNA.



	short_telo vs. total aberrations
Spearman r	
r	0.8095
95% confidence interval	
P value	
P (two-tailed)	0.0218
P value summary	*
Exact or approximate P value?	Exact
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	8

Figure 18. Scatter plot showing positive correlation for percentage of short telomere and TNA for those individuals who have a high number of TNA (n=8).

DISCUSSION

It has previously been shown that air pollution can accelerate the rate of telomere shortening, (Hoxha, et al., 2009; Hou, et al., 2012; Zhang, et al., 2013). The study presented here represents the first evaluation of whether telomere length can be used as an informative biomarker of indoor woodstove smoke exposure in rural Honduras. Insights into the potential contribution of indoor air pollution from wood-burning stoves to telomere length shortening, and thus to general health status, are reported.

Overall, we demonstrate the feasibility of such field studies, finding that the percent of short telomeres—rather than average telomere length—may be a potentially informative biomarker of individual indoor smoke exposure. Ease of collection of buccal mucosa in the field, as well as selection of basal (stem-like) cells under the microscope, were shown to be preferred for TL analyses, as age was not a confounding factor.

Total nuclear aberrations (micronuclei and buds) are well-accepted biomarkers of DNA damage, commonly used to evaluate exposure to high levels of smoke; specifically, increased frequencies of total nuclear aberrations (TNA) in buccal mucosa cells are indicative of air pollution exposure (Thomas et al., 2009). Although data on woodstove smoke exposure levels for participants in the current study were not yet available, we utilized TNA frequencies to segregate a subset of 20 individuals into two groups; one with lower than background numbers of TNA, and one with higher. For individuals with lower numbers of TNA, there was no correlation between percent short telomeres and TNA (Figure 15). On the other hand, for individuals with higher numbers of TNA there was a positive correlation between the percent of short telomeres and TNA (Figure 16). Therefore, initial findings suggest that frequencies of short

telomeres, rather than average TL, correlate with number of TNA in those assumed to be the most exposed individuals. These preliminary results require correlation with actual particulate matter exposures, as well as confirmation in a larger cohort (studies on-going).

MATERIALS AND METHODS

Sample collection and processing

Buccal mucosa is an accessible tissue for no invasive cell sample collection. The samples were collected "in field" from different rural communities at Honduras. The samples were taken from the right and left inner cheeks, using a different small headed toothbrush per cheek, by applying a circular motion to the area. Then, both toothbrushes were submerged in a 30ml sterile sample and transport tube (Biomedical Marketing Associates, Wexford, PA) containing 20 ml of fixative and transport medium (Fisher HealthCare™ PROTOCOL™ Saccomano Fluid, Thermo Fisher Scientific). This allows the sample to be pre-fixed, keeping them in good conditions up to one month. Then the samples were shipped from Honduras to U.S.

Once in the lab, the fixation process is completed. First, samples were vortexed, and the toothbrushes extracted from the tube. Next, the we centrifuged the samples and discarded the supernatant, leaving about two milliliters, which allow us disaggregated the possible cell clumps and aggregates with a syringe and needle. Finally, the cells were pelleted, washed, re-suspended with 75 mM hypotonic Potassium Chloride buffer (KCl, Fisher Chemical, Fair Lawn, NJ, USA) at 37^0C for 30 minutes and then fixed with fresh prepared Carnoy solution (3:1 v/v Methanol: Glacial Acetic Acid; Fisher Chemical, Fair Lawn, NJ, USA, and Mallinckrodt Chemicals, Phillipsburg, USA, respectively). At this point, the processed samples can be stored for years or slides can be dropped to be used for telomere length measurement using Telo-FISH technique. After the fixation was completed, we dropped on clean glass microscope slides (Platinum Line™ Mercedes Medical, Sarasota, FL), air dried and overnight sated.

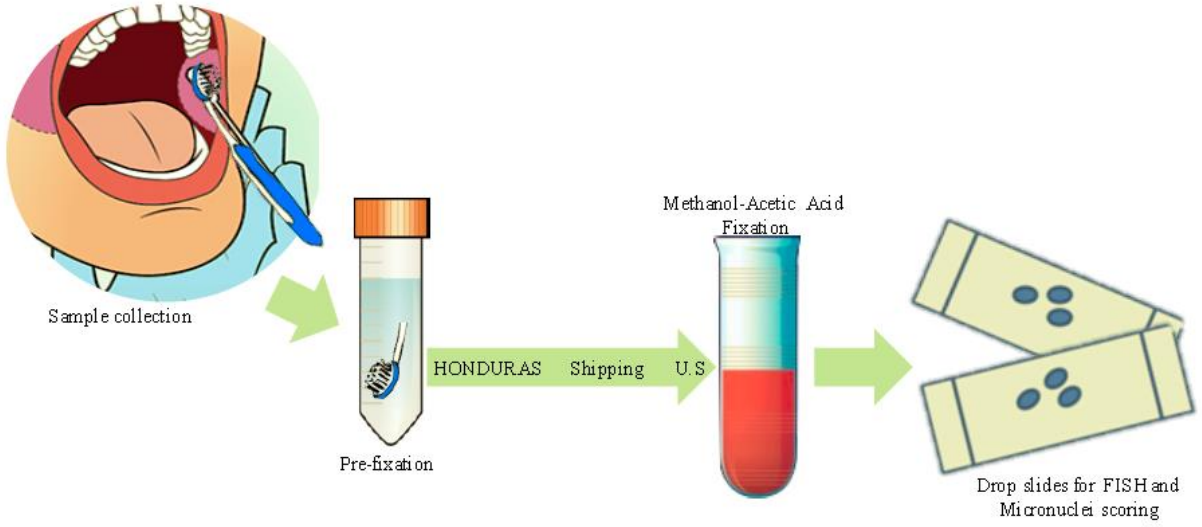


Figure 19. Schematic flow chart of sample collection and processing

Cell culture

Human foreskin keratinocytes, immortalized hTERT-BJI (ATCC, Manassas, VA, USA), were cultivated in DMEM (Corning Cellgro, Manassas, VA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma Aldrich), as recommended by manufacturing grown, in 95% air and 5% CO_2 incubator at 37^0C. This cell line was trypzinized (Trypsin-EDTA, Gibco® by Life Technologies, Canada), collected in 15 ml-Falcon tubes, pelleted, re-suspended, and treated with 75 mM hypotonic buffer Potassium Chloride (KCl, Sigma Aldrich), at 37^0C for 30 minutes.

Finally, the cell suspension was fixed with fresh prepared Carnoy solution, 3:1 v/v Methanol: Glacial Acetic Acid, (Fisher Chemical, Fair Lawn, NJ, USA, and Mallinckrodt Chemicals, Phillipsburg, USA, respectively). Clean glass microscope slides (Platinum Line™ Mercedes Medical, Sarasota, FL) were dropped, air dried and overnight sated. This sample was used as control for telomere length measurement using Telo-FISH technique.

Collection of Interphase and Quantitative-Fluorescence "in situ" Hybridization analysis (Q-FISH)

To obtain interphases cells, the collected samples were processed, and the fixed cell suspensions were dropped on glass microscope slides (Platinum Line™ Mercedes Medical, Sarasota, FL) as described in previous section. Then the slides were re-hydrated by placing them in a Coplin jar containing 1X PBS for 15 minutes at room temperature.

Q-FISH staining was performed following current lab protocol. To visualize telomeres, we use PNA Cy3-labeled telomeric probes ($TTAGGG_3$, Biosynthesis, Lewisville, TX) prepared by diluting 5 µl of probe in 36 µl of formamide (Sigma Aldrich), 12 µl of 0.05 M TRIS buffer, 2.5 µl of 0.1 M KCl (Sigma Aldrich), and 0.6 µl of 0.1 M Magnesium Chloride (MgCl, Sigma

Aldrich). Probe mix (50 µl) was applied to each slide, which was then cover-slipped and denatured at 75°C (5 min). Slides were incubated at 37°C for 2 h, then washed in a series of 43.5°C washes for 2.5 min each; washes twice in: 50% formamide in 2X sodium citrate (SSC); then twice in: 2X SSC; and finally, 2 washes of: 2X SSC + 0.1% NP40. Slides were counterstained for nuclei visualization with 50 µl of DAPI (4',6-diamidino-2-phenylindole; Prolong Gold Anti-fade, Invitrogen, Carlsbad, CA), cover-slipped (Platinum Line ® Cover glass 22x40 #1). Slides were processed in sets of five, four samples plus one control, to limit variability between sample runs.

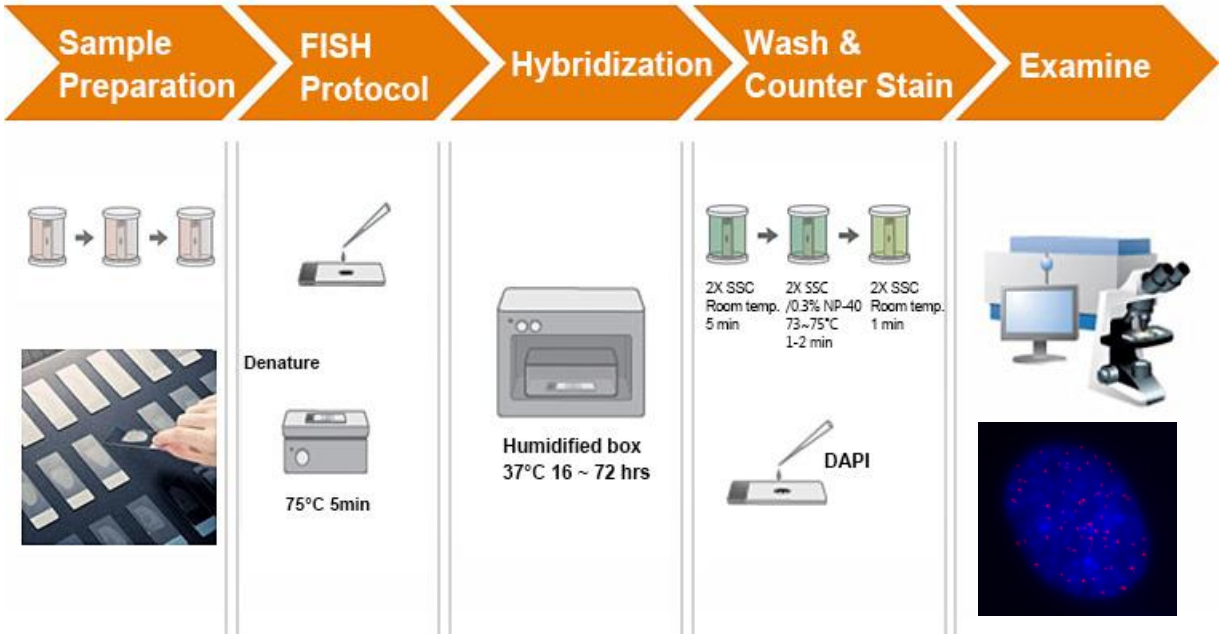


Figure 20. Graphic example of slide preparation to be used in Interphase Telo-FISH. Picture modification from original at http://www.creative-bioarray.com/protocol/fluorescence-in-situ-hybridization-fish-Protocol.htm

Imaging

The images were acquired with a Zeiss Axio Imager Z2 microscope (Carl Zeiss, Thornwood, NY, USA) equipped with a Coolsnap ES camera (Photometrics, Tucson, AZ, USA) and running Metamorph software (Molecular Devices, Sunnyvale, CA, USA). The imaging was performed by random manual selection of 50 interphases from each sample slide, using 100X magnification and immersion oil objective. We took 26 individual stack images with 0.2 um steps size down and up from the better focal plane. The images were captured in two different channels—DAPI and Cy3—and stored for later analysis.

Prior to every sample imaging, images of 0.2 μm orange fluorescent beads (540/560, Molecular Probes) slides using Cy3 wavelength were captured stored. These images were used to verify the saturation levels, exposure time and to calibrate the system intensity. In addition, we took images of our biological control slides, prepared by dropping hTERT-BJI cell line (ATCC, Manassas, VA, USA) and identically prepared as sample slides. All the measurements were based on area and fluorescent intensity.

Images processing

The analysis of telomere fluorescence intensities was made with free downloaded software called TELOMETER, an ImageJ plugin. Telomere signals from target cells were analyzed using customizing settings for this specific cell type. A telomere frequency distribution histogram was generated based on the telomere fluorescent intensities for each sample.

Total nuclear aberration quantification

To perform the quantification of total nuclear aberrations, we used an aliquot of single cell suspension of 20 samples from the total (n=100). We dropped 20 µl onto clean glass microscope slides (Platinum Line™ Mercedes Medical, Sarasota, FL) and checked their quality before the staining. We quantify total nuclear aberration of 2000 cells per sample slide. The slides preparation was made by placing them for 1 minute in a Coplin jar with 50% Ethanol (Fisher Chemical, Fair Lawn, NJ, USA) and then transferred to a Coplin jar with 20% Ethanol (1 minute). Then, washed for 2 minutes with MilliQ water and 30 minutes of incubation in 5M Chloridric Acid (Mallinckrodt Chemicals, Phillipsburg, NJ, USA). Finally, rinsed under running tap water and placed them for 60 minutes in a Coplin jar with Schiff's reagent (Sigma Aldrich), at room temperature, protected from light. The slides were washed under running tap water followed by a quick rinse in MilliQ water. We counter stain the slides with 0.2% p/v Light Green for 15-20 seconds, rinse and let them dry. These slides can be stored at 4^0C for later examination, and nuclear aberrations (MNi + NBUDs) quantification.

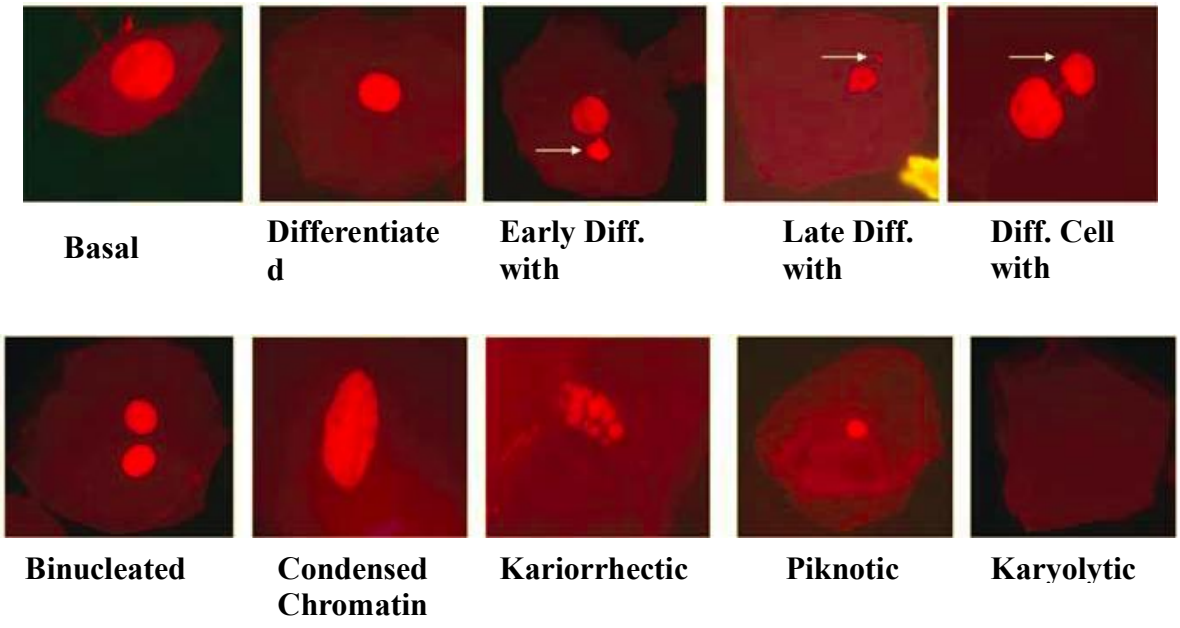


Figure 21. Buccal mucosa cell population. Example of the different cells types (Feulgen stain, fluorescent microscopy visualization) found in the analyzed samples. Image modified from Thomas et al.,2009.

REFERENCES

Aubert, Geraldine et al. “Telomere Length Measurement - Caveats and a Critical Assessment of the Available Technologies and Tools.” *Mutation research* 730.1-2 (2012): 59–67. *PMC*. Web. 14 Feb. 2017.

Aubert, Geraldine and Lansdorp, Peter M. “Telomeres and Aging.” *Physiol Rev* 88 (2008): 557-579.

Bailey, Susan M., and John P. Murnane. “Telomeres, Chromosome Instability and Cancer.” *Nucleic Acids Research* 34.8 (2006): 2408–2417. *PMC*. Web. 10 Feb. 2017.

Bandaria, Jigar N. et al. “Shelterin Protects Chromosome Ends by Compacting Telomeric Chromatin.” *Cell* 164.4 (2016): 735-746. *PMC*. Web. 11 Feb. 2017.

Blackburn, Elizabeth H. et al. “Human Telomere Biology: A Contributory and Interactive Factor in Aging, Disease Risk, and Protection.” *Science* 350.6265 (2015): 1193-1198. *PMC*. Web. 11 Feb. 2017.

Bolognesi, Claudia, and Michael Fenech. "Micronucleus assay in human cells: lymphocytes and buccal cells." *Genotoxicity Assessment: Methods and Protocols* (2013): 191-207.

Bolognesi, Claudia, et al. "The HUMN xl scoring criteria for different cell types and nuclear anomalies in the buccal micronucleus cytome assay–An update and expanded photogallery." *Mutation Research/Reviews in Mutation Research* 753.2 (2013): 100-113.

Bruce, N. et al. “Indoor Air Pollution in Developing Countries: A Major Environmental and Public Health Challenge.” *Bulletin of the World Health Organization* 78.9 (2000): 1078–1092. Print.

Campisi, Judith. “Aging, Cellular Senescence, and Cancer.” *Annual review of physiology* 75 (2013): 685–705. *PMC*. Web. 10 Feb. 2017.

Canela, Andrés et al. “High-Throughput Telomere Length Quantification by FISH and Its Application to Human Population Studies.” *Proceedings of the National Academy of Sciences of the United States of America* 104.13 (2007): 5300–5305. *PMC*. Web. 14 Feb. 2017.

Cawthon, Richard M. “Telomere Measurement by Quantitative PCR.” *Nucleic Acids Research* 30.10 (2002): e47. Print.

Clark, Maggie et al. “Indoor air pollution, cookstove quality, and housing characteristics in two Honduran communities.” *Environmental Research* 110 (2010): 12-18.

Coluzzi, Elisa et.al. “Oxidative Stress Induces Persistent Telomeric DNA Damage Responsible for Nuclear Morphology Change in Mammalian Cells.” *PLoS ONE* 9.10 (2014): e1 10963. *PMC*. Web. 11 Feb. 2017.

De Lange, Titia. “Shelterin: the protein complex that shapes and safeguards human telomeres.” *Genes & Development* 19 (2005): 2100-2110. Print.

Epel, Elissa S. et al. “Accelerated Telomere Shortening in Response to Life Stress.” *Proceedings of the National Academy of Sciences of the United States of America* 101.49 (2004): 17312–17315. *PMC*. Web. 11 Feb. 2017.

Fuchs, Elaine, Tudorita Tumbar, and Geraldine Guasch. "Socializing with the neighbors: stem cells and their niche." *Cell* 116.6 (2004): 769-778.

Harley, Calvin B., A. Bruce Futcher, and Carol W. Greider. "Telomeres shorten during ageing of human fibroblasts." *Nature* 345.6274 (1990): 458.

Hemann, Michael T., et al. “The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability.” *Cell* 107.1 (2001): 67-77. *PMC*. Web. 14 Dec. 2016.

Hou, Lifang et al. “Air Pollution Exposure and Telomere Length in Highly Exposed Subjects in Beijing, China: A Repeated-Measure Study.” *Environment international* 48 (2012): 10.1016/j.envint.2012.06.020. *PMC*. Web. 9 Mar. 2017.

Houben, Joyce MJ, et al. "Telomere length assessment: biomarker of chronic oxidative stress?" *Free Radical Biology and Medicine* 44.3 (2008): 235-246.

Hoxha, Mirjam, et al. "Association between leukocyte telomere shortening and exposure to traffic pollution: a cross-sectional study on traffic officers and indoor office workers." *Environmental Health* 8.1 (2009): 41.

Javed, A. et al. “In situ immunofluorescence analysis: immunofluorescence microscopy.” *Methods in molecular biology (Clifton, N.J.) 285* (2004): 23-28.

Kampa, Marilena and Elias Castanas. “Human health effects of air pollution.” *Environmental Pollution* 151.2 (2008): 362-367. Web. 11 Feb. 2017.

Lindsey, Janet, et al. "In vivo loss of telomeric repeats with age in humans." *Mutation Research/DNAging* 256.1 (1991): 45-48. Web. 2 Oct. 2016.

Martin, WJ et al. “Household air pollution from cookstoves: impacts on health and climate.” *Respiratory medicine: global climate change and public health* 7 (2014): 237–255. Available from: http://link.springer.com/chapter/10.1007/978-1-4614-8417-2_13

Mather, Karen Anne et al. “Is telomere length a biomarker of aging? A review.” *J Gerontol A Biol Sci Med Sci* 66A.2 (2011): 202-213. Web. 11 Feb. 2017.

Meeker, Alan K. et al. “Telomere Length Assessment in Human Archival Tissues: Combined Telomere Fluorescence *in Situ* Hybridization and Immunostaining.” *The American Journal of Pathology* 160.4 (2002): 1259–1268. Print.

Moore, Kateri A., and Ihor R. Lemischka. "Stem cells and their niches." *Science* 311.5769 (2006): 1880-1885.

Pastor, S. et al. “Biomonitoring of European populations occupationally exposed to pesticides: use of micronuclei as biomarkers.” *Mutagenesis* 18 (2003): 249-258.

Serrano, Antonio L., and Vicente Andrés. "Telomeres and cardiovascular disease." *Circulation Research* 94.5 (2004): 575-584. Web. 27 Feb. 2017.

Shay, Jerry W. “Role of Telomeres and Telomerase in Aging and Cancer.” *Cancer Discovery* 6 (2016): 584-593. Downloaded from cancerdiscovery.aacrjournals.org. 14 Feb. 2017.

Takubo, Kaiyo et al. “Telomere Lengths are Characteristic in Each Human Individual.*”* *Experimental Gerontology* 37.4 (2002): 523-531. *PMC*. Web. 11 Feb. 2017.

Terry, Dellara F. et al. “Association of Longer Telomeres with Better Health in Centenarians.” *The journals of gerontology. Series A, Biological sciences and medical sciences* 63.8 (2008): 809–812. Print.

Thomas, Philip, et al. "Buccal micronucleus cytome assay." *Nature protocols* 4.6 (2009): 825-837.

Vera, Elsa, and Maria A. Blasco. “Beyond Average: Potential for Measurement of Short Telomeres.” *Aging (Albany NY)* 4.6 (2012): 379–392. Print.

Watt, Fiona M., and Brigid LM Hogan. "Out of Eden: stem cells and their niches." *Science* 287.5457 (2000): 1427-1430.

Zahran, Sammy, et al. "Stress and telomere shortening among central Indian conservation refugees." *Proceedings of the National Academy of Sciences*112.9 (2015): E928-E936.

Zhang, Xiao, et al. "Republished: Environmental and occupational exposure to chemicals and telomere length in human studies." *Postgraduate medical journal* 89.1058 (2013): 722-728.

Zhao, Zhenrong et al. “Telomere Length Maintenance, Shortening, and Lengthening.” *Cellular Physiology* 229 (2014): 1323-1329. *PMC*. Web. 11 Feb. 2017.