Prenatal stress and newborn telomere length

Nicole M. MARCHETTO, MD¹, Ms. Rebecca A. GLYNN, BS², Ms. Mackenzie L. FERRY, BS², Ms. Maja OSTOJIC, BS², Sandra M. WOLFF, MD¹, Ruofan YAO, MD¹, Mark F. HAUSSMANN, PhD²

¹College of Medicine, Drexel University, Philadelphia, Pennsylvania, USA

²Department of Biology, Bucknell University, Lewisburg, Pennsylvania, USA

*These authors contributed equally to this work

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Corresponding Author: Mark F. Haussmann, PhD. mfh008@bucknell.edu

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Abstract

Background: The Developmental Origin of Health and Disease hypothesis is based on the premise that many chronic diseases have their roots in fetal development. Specifically, maternal stress during pregnancy is associated with altered fetal development and many adverse long-term health outcomes. While the mechanisms underlying this effect are currently unclear, at the cellular level, one possible mediator is the regulation of telomere length. Telomere dynamics appear to play a role in disease progression, and an adverse intrauterine environment may contribute in the establishment of short telomeres in newborns. In accordance with this, recent work reported that prenatal stress is significantly associated with shorter mean newborn telomere length. However, this finding has yet to be replicated and currently we know nothing about whether different size classes of telomeres within the telomere length distribution are differentially affected by prenatal stress. Examining telomere length frequency distributions is important, because the shortest telomeres in the distribution appear to be the most indicative of telomere dysfunction and thus, the best predictors of mortality and morbidity in humans.

Objectives: We investigated the effects of intrauterine exposure to maternal stress over the whole course of gestation on newborn mean telomere length and telomere length frequency distributions.

Study Design: We conducted a prospective cohort study of 24 mother-newborn dyads at an urban teaching hospital. Pregnant women with nonanomalous, uncomplicated pregnancies were recruited and assessed in the third trimester of gestation. Maternal psychosocial stress was quantified using the Holmes & Rahe Stress Scale and categorized as high stress (>300 points) or low stress (<300 points) exposure. Newborn telomere length was measured from cord blood at delivery using the Telomere Restriction Fragment assay.

Results: We found a significant negative association between maternal stress and newborn telomere length ($\beta = -0.463$, $P = 0.04$). Newborns whose mothers experienced a high level of stress during pregnancy had significantly shorter telomere length (6.98 ± 0.41 kb) compared to newborns of mothers with low stress (8.74 ± 0.24 kb; $t = -3.99$, $P = 0.003$). Moreover, the difference in newborn telomere length between high stress and low stress mothers was due to a shift in the telomere length distribution, with the high stress group showing an under-representation of longer telomeres and an over-representation of shorter telomeres.

Conclusion: Our findings replicate those of other recent studies and additionally show, for the first time, that the prenatal stress-associated difference in newborn mean telomere length is due to a shift in the overall telomere distribution.

Key Words: Developmental Origins of Health and Disease, Fetal Programming, Maternal stress, Prenatal stress, Telomere length

Introduction

Converging evidence from epidemiological, clinical and experimental studies suggests that exposure to suboptimal conditions in early life can produce long-term effects on health and disease susceptibility (1,2). The developing fetus is especially sensitive to intrauterine perturbations (3), and this has led to the developmental origins of health and disease hypothesis (DOHaD), which posits that an individual's long-term risk of disease is dependent, in part, on the quality of the intrauterine environment (4). While a number of intrauterine factors may contribute to the long-term risk of disease, fetal exposure to maternal stress appears to represent a particularly salient insult (5,6). The mechanisms that link prenatal stress exposure to disease decades later are still unclear, but recent work suggests that at the cellular level telomere dynamics may represent one such underlying mechanism (2,7).

Telomeres are protective complexes of non-coding, repetitive DNA and shelterin proteins that cap chromosomes and promote chromosomal stability. As cells divide, telomeres shorten due to both incomplete replication and through oxidative damage. Telomere length (TL) varies between chromosomes within each cell and among cells within a tissue due to different replicative histories and past levels of cellular damage. This results in a distribution of TLs within the tissue of an individual (8). While there are mechanisms in place to rebuild the shortest telomeres, such as the enzyme telomerase (9-11), if a telomere has undergone a critical degree of shortening, it can lead to cellular senescence. Senescent cells lose the ability to replicate and cease to divide or undergo apoptosis, contributing to the aging phenotype and susceptibility to disease (12,13). Based upon this, measuring the average TL of the TL distribution has gained traction as a biomarker for cellular function and aging and can serve as an early predictor of disease onset for cardiovascular disease (14), stroke (15), Alzheimer's disease (16), diabetes mellitus (17), childhood autism (18), as well as overall mortality risk (19,20).

Given the role that telomere dynamics may play in disease progression, current efforts have turned to identifying the mechanisms that cause variation in TL (21). TL is a function of both the initial setting of TL (22) and TL attrition over time (14,23). Little is currently known about the causes of variation in the initial setting of TL, but one possibility is that the intrauterine environment plays an important role in the establishment of initial (newborn) TL (24,25). In accordance with this hypothesis, recent work by Entringer et al. (2013) showed that prenatal stress was significantly associated with shorter mean newborn leukocyte TL. However, this finding has yet to be replicated. Moreover, while average TL is the most commonly-used biomarker to describe TL variation among individuals in a population, it is not the mean TL, but rather the shortest telomeres that lose function and initiate cellular senescence (26). Therefore, characterizing the entire distribution of TL within a sample from an individual may provide particularly valuable information about the relative increases in the number of short telomeres, which is more indicative of telomere dysfunction than measures of mean TL (25).

In this study, we investigated the effects of intrauterine exposure to maternal stress over the whole course of gestation on newborn mean TL and TL frequency distributions. Thus, our study builds on previous work exploring prenatal stress and TL in order to replicate and expand

on these previous studies (24,25). The notable additional contributions of our study involve the characterization of maternal stress over the entirety of gestation and also its effect on the entire frequency distribution of telomeres. The use of TL frequency distributions provides a better and more meaningful resolution on putative prenatal stress effects, since the shortest telomeres are the most indicative of telomere dysfunction and the best predictors of mortality and morbidity in humans (27).

Methods

Subject Details:

The study cohort of pregnant women was enrolled at an urban teaching hospital in Philadelphia, PA, between April and August 2013. Eligible women were between the ages of 18-35 and delivered a single viable non-anomalous infant. Women were excluded if the pregnancy was complicated by medical conditions that could affect fetal growth, such as hypertension, diabetes and smoking; if they developed any condition that required steroid therapy; or if they developed chorioamnionitis. Medical records were reviewed to obtain sociodemographic characteristics, medical history, and to determine eligibility pertaining to maternal age at birth, maternal pre-pregnancy weight, obstetric complications during pregnancy, length of gestation, weight, height, and birth weight adjusted for gestational age. Financial incentive or compensation was not offered. The result was a sample of 24 mother-newborn dyads. The sociodemographic and newborn characteristics of this population are displayed in Table 1. The study was approved by Drexel University Institutional Review Board and informed, written consent was obtained from all participants.

Measures:

Maternal Stress

When the mother presented at the hospital for delivery, maternal psychosocial stress was assessed using the Holmes and Rahe Stress Scale questionnaire (also referred to as the Social Readjustment Rating Scale). This measurement scale has been called the ‘gold standard’ for stress assessment (28) and has been shown to correlate with health outcomes in a variety of studies (29,30). The 43-item dichotomous survey objectively ascertained whether the mothers had experienced life events within the past year that are likely to initiate a physiological stress response (Supplemental material). Each survey event is associated with a numerical value, and this scale was used to assess the cumulative stress the mother experienced over the course of her pregnancy. A score of 300+ indicates the mother is at high risk of stress-induced illness, while scores lower than 299 correlates with lower risk levels of stress-induced illness (30,31).

Cord Blood Telomere Length

Telomeres were measured with the Telomere Restriction Fragment (TRF) assay and the procedure was carried out according to previous studies (32,33). Briefly, DNA was extracted from newborn umbilical cord blood using the Puregene Blood Core Kit B following the manufacturer's specifications (Qiagen). DNA integrity was assessed through the use of integrity

gels (34), and then cord blood TL was measured using the telomere restriction fragment assay (34). Ten µg of DNA was digested using 1.0 µl of RsaI (New England Biolabs, R0167L) and 0.2 µl of HinfI (New England Biolabs, R0155M) in CutSmart Buffer (New England Biolabs, B7204S) overnight at 37°C. The digested DNA was separated using pulsed field gel electrophoresis (3 V/cm, 0.5–7.0 s switch times, 14 °C) for 19h on a 0.8% non-denaturing agarose gel. The gel was then dried without heating and hybridized overnight with a ³²P-labelled oligo (5'-CCCTAA-3') that binds to the 3' overhang of telomeres. Hybridized gels were placed on a phosphorscreen (Amersham Biosciences, Buckinghamshire, UK), which was scanned on a Storm 540 Variable Mode Imager (Amersham Biosciences). We used densitometry (ImageQuant 5.03v and ImageJ 1.42q) to determine the position and strength of the radioactive signal in each of the lanes compared to the molecular marker (1 kb DNA Extension Ladder; Invitrogen, CA, USA; 20).

A major advantage of the TRF assay is that it provides frequency distributions of telomere length for each sample (27). The resulting plots allow visualization of the relative abundances of TRFs at each molecular weight (MW), providing useful information on where differences in TL occur among groups. For each individual sample, area under the optical density curve was calculated in 1kb intervals from 1-20 kb, and each interval was divided by the area under the curve for the entire distribution. The background was fixed as the nadir of the low MW region on the gel (<1 kb). Relative abundances of TRFs in each of the MW classes were log transformed and then fit by least squares fourth order polynomial regression with the MW classes (1-20 kb).

Statistical Analysis:

As determined by the Shapiro-Wilk test, neonatal cord blood TL was normally distributed ($W = 0.99$ $P = 0.97$). To examine the association between maternal psychosocial stress during pregnancy and newborn cord blood TL adjusted for the effects of other possible determinants, a linear regression model was employed that included the effects of maternal stress, maternal age, gestational age at birth, and birth weight (adjusted for gestational age). To further explore how the magnitude of maternal stress affects newborn cord blood TL, the study population was divided into two groups based on their stress score (high: >300 points, or low: <299 points). Demographics for the stress groups were compared using chi-square for dichotomous variables. We used a t-test accounting for unequal variances to compare cord blood TL between the stress groups. Mean $\pm$ standard deviation is shown.

Frequency distributions of TL for the high and low stress groups were produced following Haussmann et al. (32). We compared skewness and kurtosis values of the distribution following Jemielity et al. (35), which were not normally distributed, and so were analyzed using a Wilcoxon Kruskal-Wallis test. All statistical analyses were performed in JMP (v11.1.1).

Results

After accounting for the effects of other potential factors that might influence newborn cord blood TL (maternal age, gestational age at birth, and birth weight), our linear regression model showed a significant negative association of maternal stress exposure on newborn cord

blood TL ($\beta = -0.463$, $P = 0.04$; Figure 1; Table 2). Further, newborns whose mothers had experienced high levels of stress within the past year had significantly shorter average cord blood TL (6.98 ± 0.89 , $n = 6$) compared to mothers experiencing relatively lower levels of stress during pregnancy (8.74 ± 1.05 , $n = 18$; $t = -3.99$, $P = 0.003$; Figure 1).

Interestingly, the difference in mean cord blood TL between the high stress and low stress groups was due to a shift in the telomere distribution, with the high stress group showing an under-representation of longer telomeres, and an over-representation of relatively shorter telomeres (Figure 2). These differences in distribution shape were significant, as indicated by a significantly greater kurtosis value (Chisquare = 5.67, $P = 0.01$) and a skew trending toward shorter telomeres (Chisquare = 2.82, $P = 0.09$) in the high stress group.

Comment

Here we show that after accounting for the potential effects of maternal age, gestational age, and birth weight, that maternal stress was significantly and independently associated with newborn mean cord blood TL. The offspring of mothers who experienced high levels of maternal stress had mean cord blood telomeres that were on average 1.76 ± 0.48 kb shorter than the offspring of mothers who experienced lower levels of stress during gestation. To place the differences between the stress groups in context, routine smoking (23 pack-yr) resulted in a 1.5 kb difference in TL between smokers and non-smokers, suggesting that high levels of maternal stress may have a similar cumulative affect on TL as does daily smoking (36). While this type of comparison can help place our results into context, we emphasize that due to the modest sample size of our study, comparison of absolute differences between the groups should be made cautiously. While high variability in TL was present in this newborn population, this variability is consistent with previous findings (25,37,38).

The number of human studies reporting a relationship between TL and survival are steadily growing (19,20,39-43). Interestingly, in conjunction with the human epidemiologic literature, there are an increasing number of studies in the field of evolutionary ecology that report significant correlative relationships between TL or telomere shortening rates and survival prospects (44-47). Regardless of the taxa studied, the sensitivity of TL as a biomarker to predict age-related diseases and mortality appears to differ based on the subject's age when TL is measured. Longitudinal studies have revealed that telomere loss occurs at a faster rate early in life (22,47-49), and it is believed that this early attrition is due to increased proliferation and growth during early development (50). A recent longitudinal study indicated an independent association between shorter telomeres in early childhood and arterial wall thickness in later life, suggesting that relatively short telomeres in early childhood are a biomarker for vascular disease risk later in life (51). Additionally, a study in birds showed that TL measured in the first month of life is a stronger predictor of survival and overall health than TL measured in adulthood five years later (52). Another recent longitudinal study in birds reported that offspring TL was dependent in part on the infection status of their mother, suggesting that maternal condition plays a significant role in the initial setting of offspring TL (53). Taken together, these studies suggest that the human newborns in our study that experienced a more stressful intrauterine environment

may have experienced a degree of cellular aging by the time of birth that could have significant effects on aging and subsequent health and disease susceptibility later in life. This association between the prenatal environment and health consequences later in life is consistent with the DOHaD hypothesis and provides further motivation to investigate telomeres as a potential mechanism by which stress influences the prenatal environment and the development of chronic disease later in life.

Our study provides a detailed analysis showing that the difference in mean cord blood TL in our high and low stress mothers appears to be due to a shift in the overall TL frequency distribution, so that the high stress group has an over representation of shorter telomeres and an under representation of longer telomeres compared to the low stress group. It is reasonable to expect that the shift toward shorter TLs in the high stress group places these offspring at a higher risk for telomere dysfunction and subsequent disease. The proportion of short telomeres in previous studies has been shown to reflect the level of genomic instability within a tissue (8,13,54). Generally, a distribution shift toward shorter telomeres may suggest that telomeres of all lengths in the distribution were equally affected; however, the significant effect on the kurtosis of the distribution suggests that the longer telomeres were preferentially shortened. Longer telomeres may be more likely shortened because their size makes them a larger target for shortening caused by oxidative damage (55,56). Future work could expand upon the relationships found here by using single-telomere length analysis (STELA) in order to detect whether prenatal stress increases the number of extremely short telomeres in the length ranges that trigger both cellular senescence and telomere dysfunction (8,13).

The effect of maternal stress on offspring TL may be mediated, in part, through maternal stress hormones. While we were unable to measure circulating glucocorticoids in pregnant mothers in this study, it is possible that mothers with higher stress scores had higher glucocorticoid levels (57). The pioneering work of Epel and colleagues has established that chronic stress in humans results in an increased rate of telomere shortening (58). Further work reported that elevated glucocorticoids produce negative effects on telomeres, suggesting that these hormones mediate the destructive effect of stress on telomere maintenance (59). Experimental evidence is in accordance with these findings, as a recent study in chickens showed that experimentally elevating glucocorticoid levels during prenatal development results in shorter TL in young individuals (32). These higher levels of glucocorticoids also appeared to increase levels of oxidative stress (32). Because, telomeres are particularly vulnerable to oxidative damage (21,60), it is possible that the effect of prenatal stress and elevated glucocorticoids on TL may be mediated through oxidative stress. Additionally, glucocorticoids may impact telomere loss through their effects on the telomere-restorative enzyme, telomerase (58,61,62). We currently need more experimental work to better understand how prenatal glucocorticoids impact telomere dynamics and its long-term consequences on overall health and survival. We also need to better understand how the developing fetus can regulate their exposure to maternal glucocorticoids and thereby alter fetal programming. For example, recent work in an avian

model suggests that a metabolic buffer can metabolize maternally-derived glucocorticoids, to alter the developmental endocrine environment (63), and thereby temper prenatal stress effects.

In summary, we found that higher levels of maternal stress are associated with shorter average cord blood TL in newborns and a shift in the overall frequency distribution of telomeres, indicating that the high stress group had an accumulation of short telomeres. More experimental work is needed to determine the molecular underpinnings linking maternal stress and telomere dynamics in offspring. In addition, future studies that incorporate information regarding the frequency of short telomere will help to determine the risks associated with prenatal stress on telomere dynamics and subsequent health and disease susceptibility.

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Table 1. Maternal and newborn characteristics

Maternal characteristics	Mother-newborn dyads, n = 24
Sociodemographic	
Age (y), mean $\pm$ SD	25.1 $\pm$ 4.0
BMI	29.5 $\pm$ 8.5
Race/ethnicity	
Non-Hispanic white	13% (n = 3)
African American	75% (n = 18)
Hispanic	13% (n = 3)
Newborn characteristics	
Gestational age at birth (wk), mean $\pm$ SD	40.0 $\pm$ 1.1
Birthweight (g), mean $\pm$ SD	3298 $\pm$ 510

Table 2. Regression model predicting newborn cord telomere length (TRF length, kb)

Variable	Unstandardized β (95% CI)	SE of β	P value	Standardized β	VIF
Maternal stress	-0.005 (-0.009 to -0.001)	0.002	0.04	-0.463	1.219
Maternal age	0.068 (-0.089 to 0.198)	0.069	0.44	0.174	1.298
Gestational age at birth	0.121 (-.397 to 0.639)	0.247	0.63	0.104	1.217
Birthweight (adjusted for gestational age)	-0.0001 (-0.002 to 0.001)	0.001	0.15	-0.328	1.217

Figure 1. Maternal stress at delivery and newborn cord blood telomere restriction fragment length ($\beta = -0.463$, $P = 0.04$). The dotted line divides the study population into offspring with mothers who had high stress levels during the year prior to giving birth (>300), and those whose mothers had low stress levels during the year prior to giving birth (<299).

Figure 2. Distributions of telomere restriction fragment lengths from newborn cord blood samples in the low stress and high stress groups. Plotted are mean ($\pm$ standard deviation) relative frequencies of telomere restriction fragments (%) at a given telomere length (kb). Data are jittered to allow for visualization of error bars.

Figure 1

Figure 2