

**Prospective associations between leukocyte telomere length and adiposity in childhood**

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**Abbreviations:** BMIZ, body mass index-for-age z-score; LMS, lambda mu sigma; LTL, leukocyte telomere length; WC, waist circumference; WHO, World Health Organization

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**ABSTRACT**

Leukocyte telomere length (LTL) is associated with obesity and may be involved in its etiology, but few studies have focused on children and most have been cross-sectional. We assessed the relation of LTL with adiposity development in a prospective study of Colombian children. We quantified LTL at enrollment in 722 children ages 5-12 years and measured anthropometry annually for a median 6 years. Using mixed effects models, we estimated changes in adiposity measures including BMI and waist circumference (WC)-for-age z-scores in relation to baseline LTL z-score. In girls, longer LTL was linearly related to a lower increase in WC z-score from ages 6 to 16 y. Every 1 SD LTL was associated with an adjusted 0.13 units lower increase in WC (95% CI: -0.23, -0.03;  $P = 0.01$ ). In conclusion, longer LTL among girls in middle childhood is associated with smaller increases in WC, an indicator of abdominal adiposity.

## INTRODUCTION

Childhood overweight and obesity represent urgent public health challenges globally; their prevalence is particularly high in Latin America.<sup>1</sup> Childhood obesity contributes to a growing burden of cardiometabolic disturbances, which may track into adulthood and lead to an increased cardiovascular risk later in life.<sup>2</sup> Hence, it is essential to identify factors that predict the development of adiposity.

Telomeres are repeated DNA sequences found at the ends of chromosomes that protect against loss of genomic DNA with each replication and cell division. It is well-established that leukocyte telomere length (LTL) in humans decreases with chronological age, inflammation, and damage to DNA from oxidative stress, and these factors appear to be related to the development of adiposity.<sup>3</sup> LTL has generally been inversely associated with obesity in children.<sup>4</sup> However, the role of LTL in the development of obesity and metabolic dysregulation in early life remains unclear. Much of the available evidence in this age group is cross-sectional, and its interpretation is limited by the possibility of reverse causation because the link between obesity and LTL is complex and likely bidirectional.<sup>3</sup> Relatedly, most previous studies of this question have not considered LTL as the exposure.

We aimed to evaluate the associations between LTL in middle childhood and subsequent changes in measures of adiposity through adolescence using prospectively collected data from the Bogotá School Children Cohort (BoSCCo) study in Colombia.

## METHODS

### *Study design and population*

The BoSCCo study design has been described previously.<sup>5</sup> Briefly, in 2006 we recruited a random group of 3,202 children aged 5-12 years to represent all those enrolled in primary schools in Bogotá, Colombia at the time. At baseline, we administered a parental questionnaire inquiring on sociodemographic characteristics, performed height and weight measurements on the children, and collected blood samples. Additional anthropometric assessments were performed annually for a median 6 years. We began measuring waist circumference (WC) one year post-baseline. Primary caregivers to all participants provided written informed consent prior to enrollment. Children provided verbal assent. The study was approved by the Ethics Committee at the National University of Colombia Medical School. The University of Michigan Health Sciences and Behavioral Sciences Institutional Review Board approved the use of data and samples from the study.

### *Laboratory methods*

Whole blood collected in EDTA tubes at baseline was separated into its components by centrifugation, and DNA was extracted from the buffy coat. Extracted DNA was cryopreserved prior to transportation to the University of Michigan for analysis. LTL was quantified in the DNA samples of a random subgroup of participants ( $n = 723$ ) as the telomere-to-single-copy-gene (T/S) ratio, using a multiplex qPCR method that has been previously published and is widely used in telomere research.<sup>6</sup> LTL measurements had high internal and external validity, as detailed elsewhere<sup>7</sup> (**Appendix 1**).

### *Data analysis*

We transformed the exposure of interest, LTL, into z-scores using the study population as the standard, as recommended,<sup>8</sup> and categorized them into quartiles. We also considered LTL z-score as a continuous variable by examining associations per 1 SD LTL z-score.

Outcomes comprised change in body mass index (BMI) and waist circumference (WC), indicators of overall and abdominal adiposity, respectively. We calculated sex-specific BMI-for-age z-scores (BMIZ) at each anthropometric assessment, according to the WHO Growth Reference for children ages 5-19 years. WC was normalized per the LMS method<sup>9</sup> using National Health and Nutrition Examination Survey data as the standard. For each outcome, we estimated change from ages 6 to 16 years, which corresponds to the age range of follow-up assessments in the cohort.

The analytic sample consisted of 722 children, after excluding one who lacked follow-up anthropometry. All analyses were stratified by sex because LTL dynamics and physical growth are sex-specific.<sup>10</sup> We modeled average growth curves for each indicator with use of restricted cubic spline models and tested their linearity with the likelihood ratio test. Spline terms were retained in the models for BMIZ, which was non-linear; but dropped from the models for WC, which followed a linear shape. For each indicator, we fitted mixed effects models with a random intercept and robust variances to account for within-child correlations of repeated measures. Predictors included LTL quartiles, age at assessment, and their interaction terms, which allows for modeling separate growth trajectories among children in each LTL quartile. Adjustment covariates included age at enrollment, maternal education level, and socioeconomic status, a household-level variable based on the city government's classification of neighborhoods. Mean adiposity indicator values at 6 and 16 years and change between these ages were estimated from these models and compared across LTL quartiles using adjusted mean differences with 95%

confidence intervals (CI). All analyses were conducted using SAS version 9.4 (Cary, NC, USA).

An a-priori two-sided alpha of  $< 0.05$  was considered statistically significant.

## RESULTS

Mean  $\pm$  SD age at baseline was  $8.6 \pm 1.7$  years; 54.4% were girls. Mean  $\pm$  SD LTL z-score was  $0.11 \pm 1.04$  in girls and  $-0.12 \pm 0.94$  in boys ( $P = 0.002$ ). Children contributed 3,701 anthropometric assessments over a median 5.4 years, with a median 5 assessments per child.

In girls, LTL was inversely associated with estimated change in WC z-score from ages 6 to 16 years in a dose-response manner (**Table 1**). WC increased with age in all LTL groups, but compared with girls in the shortest LTL quartile, those in the longest quartile had an adjusted 0.40 units lower increase in WC z-score (95% CI: -0.69, -0.10;  $P$ , trend = 0.004). Every 1 SD LTL was also associated with an adjusted 0.13 units lower increase in WC z-score (95% CI: -0.23, -0.03;  $P = 0.01$ ). LTL was not significantly associated with BMIZ in girls. In boys, LTL was inversely, linearly related to change in BMIZ (**Table 2**), although this association was not significant (adjusted  $P$ , trend = 0.18). LTL was not associated with WC in boys.

## DISCUSSION

In this prospective study of Colombian children, longer LTL in middle childhood was related to lower increases in WC through adolescence among girls. LTL was not significantly associated with any other measure of adiposity in girls or boys.

Previous studies of LTL and adiposity in children have yielded mixed results. In a cohort of Latino children, shorter LTL was prospectively associated with the development of obesity,<sup>11</sup> whereas BMIZ and WC were inversely associated with LTL in a cross-sectional study of 8 year-old European children.<sup>12</sup> In another cross-sectional study of school-age children, WC strongly predicted LTL in girls but not boys,<sup>13</sup> consistent with our main finding. Similarly, LTL was inversely related to WC in a cross-sectional study of Mesoamerican children,<sup>14</sup> in agreement with our results for girls. Additionally, in an intervention program for adolescents with overweight or obesity, longer LTL at baseline predicted larger weight loss.<sup>15</sup> By contrast, a survey of adolescents did not find associations between any measure of adiposity and LTL,<sup>16</sup> and a different investigation found that obesity was inversely related to LTL in adults but not in children.<sup>17</sup> Overall, our findings appear to support the notion that LTL in childhood may predict a slower development of abdominal adiposity in certain groups.

The mechanisms through which LTL may influence adiposity are not fully understood. Animal models suggest that LTL might impact metabolic function, including in adipose tissue. For example, in mice, disruption of a protein that is part of a protective complex at telomeres results in early onset of obesity and accumulation of abdominal fat, particularly in females.<sup>18</sup> However, it is also possible that factors such as oxidative stress and inflammation could be common causes of LTL and adiposity;<sup>3</sup> this mechanism would not represent a causal effect of LTL. Nevertheless, the biological plausibility of the connection between LTL and adiposity is



strengthened by the observation that LTL predicts multiple chronic diseases of which obesity is also a risk factor. Finally, an effect of obesity on LTL is also plausible, possibly mediated by inflammatory processes.<sup>19,20</sup>

Our study has important strengths. The prospective design allowed us to assess the temporal association between LTL and change in adiposity, minimizing reverse causation bias. Few previous studies have had a comparably long follow-up of participants, and most have not examined LTL as the exposure. In addition, we examined data from an understudied region where childhood obesity and its associated cardiometabolic risks are increasing public health challenges. Our study provides additional evidence that LTL can predict future adiposity, which suggests that it may be a useful biomarker for identifying children at risk of developing obesity and an indicator of lifestyle characteristics that might impact obesity through effects on LTL.

Several limitations should also be considered. First, because we only measured LTL at baseline, we could not assess the role of changes in this exposure throughout childhood. Furthermore, residual confounding by unmeasured common causes of LTL and adiposity in childhood, such as maternal pre-pregnancy BMI,<sup>12</sup> cannot be discarded. Because we measured LTL in a subset of the original cohort, we also cannot rule out the possibility of selection bias. Finally, generalizability may be restricted to settings with similar distributions of LTL and adiposity.

In conclusion, higher LTL in middle childhood was associated with a lower increase in WC, an indicator of abdominal adiposity, through adolescence among girls.

**COMPETING INTERESTS**

None of the authors has conflicts of interest in relation to this manuscript.

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Author contributions: JG performed the statistical analyses; JG and EV wrote the paper and have primary responsibility for the final content; MM-P, HO, and CM conducted the research; KF provided essential materials; and EV designed the research. All authors have read and approved the final version of the manuscript.

Table 1. Estimated changes in BMI-for-age z-score (BMIZ) and waist circumference z-score according to leukocyte telomere length (LTL) z-score among girls from Bogotá, Colombia.

Footnotes to Table 1

- <sup>1</sup> Median LTL z-score within each quartile.
- <sup>2</sup> Test of interaction terms between age variables and a variable representing medians of each LTL category introduced as a continuous covariate.
- <sup>3</sup> According to the WHO Growth Reference for children ages 5-19 years.
- <sup>4</sup> From mixed effects models with each anthropometric variable as the continuous outcome and linear and cubic spline terms for age, indicators for LTL quartiles, and interaction terms for age and LTL quartiles as predictors. Robust estimates of variance were specified in each model. Models for waist circumference did not include spline terms.
- <sup>5</sup> Adjusted for baseline age, maternal education, and socioeconomic status. Complete case analyses ( $n = 376$ ).

Table 2. Estimated changes in BMI-for-age z-score (BMIZ) and waist circumference z-score according to leukocyte telomere length (LTL) z-score among boys from Bogotá, Colombia.

Footnotes to Table 2

- <sup>1</sup> Median LTL z-score within each quartile.
- <sup>2</sup> Test of interaction terms between age variables and a variable representing medians of each LTL category introduced as a continuous covariate.
- <sup>3</sup> According to the WHO Growth Reference for children ages 5-19 years.
- <sup>4</sup> From mixed effects models with each anthropometric variable as the continuous outcome and linear and cubic spline terms for age, indicators for LTL quartiles, and interaction terms for age and LTL quartiles as predictors. Robust estimates of variance were specified in each model. Models for waist circumference did not include spline terms.
- <sup>5</sup> Adjusted for baseline age, maternal education, and socioeconomic status. Complete case analyses ( $n = 312$ ).

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Table 1. Estimated changes in BMI-for-age z-score (BMIZ) and waist circumference z-score according to leukocyte telomere length (LTL) z-score among girls from Bogotá, Colombia.

Variable	LTL z-score quartile, Q (median) <sup>1</sup>				<i>P</i> , trend <sup>2</sup>	Per 1 SD LTL z-score (95% CI)
	Q1 (-0.81) <i>n</i> = 98	Q2 (-0.25) <i>n</i> = 98	Q3 (0.16) <i>n</i> = 98	Q4 (1.11) <i>n</i> = 99		
BMIZ <sup>3</sup>						
Estimated mean ± SE at age 6 years <sup>4</sup>	0.18 ± 0.13	0.04 ± 0.15	-0.17 ± 0.13	0.09 ± 0.14		
Estimated mean ± SE at age 16 years	0.64 ± 0.12	0.40 ± 0.10	0.13 ± 0.11	0.39 ± 0.12		
Change from 6 to 16 years						
Mean ± SE	0.46 ± 0.14	0.36 ± 0.17	0.30 ± 0.15	0.30 ± 0.16		
Adjusted difference (95% CI) <sup>5</sup>	Reference	-0.01 (-0.47, 0.44)	-0.11 (-0.52, 0.29)	-0.07 (-0.50, 0.36)	0.77	0.00 (-0.17, 0.17)
Waist circumference Z						
Estimated mean ± SE at age 6 years	-0.18 ± 0.07	-0.23 ± 0.06	-0.13 ± 0.06	0.00 ± 0.06		
Estimated mean ± SE at age 16 years	0.29 ± 0.06	0.18 ± 0.06	0.11 ± 0.07	0.04 ± 0.07		
Change from 6 to 16 years						
Mean ± SE	0.47 ± 0.10	0.41 ± 0.09	0.24 ± 0.10	0.03 ± 0.11		
Adjusted difference (95% CI)	Reference	-0.04 (-0.32, 0.24)	-0.18 (-0.46, 0.11)	-0.40 (-0.69, -0.10)	0.004	-0.13 (-0.23, -0.03)

<sup>1</sup> Median LTL z-score within each quartile.

<sup>2</sup> Test of interaction terms between age variables and a variable representing medians of each LTL category introduced as a continuous covariate.

<sup>3</sup> According to the WHO Growth Reference for children ages 5-19 years.

<sup>4</sup> From mixed effects models with each anthropometric variable as the continuous outcome and linear and cubic spline terms for age, indicators for LTL quartiles, and interaction terms for age and LTL quartiles as predictors. Robust estimates of variance were specified in each model. Models for waist circumference did not include spline terms.

<sup>5</sup> Adjusted for baseline age, maternal education, and socioeconomic status. Complete case analyses (*n* = 376).



Table 2. Estimated changes in BMI-for-age z-score (BMIZ) and waist circumference z-score according to leukocyte telomere length (LTL) z-score among boys from Bogotá, Colombia.

Variable	LTL z-score quartile, Q (median) <sup>1</sup>				<i>P</i> , trend <sup>2</sup>	Per 1 SD LTL z-score (95% CI)
	Q1 (-0.95) <i>n</i> = 82	Q2 (-0.46) <i>n</i> = 82	Q3 (-0.02) <i>n</i> = 82	Q4 (0.72) <i>n</i> = 83		
<b>BMIZ<sup>3</sup></b>						
Estimated mean ± SE at age 6 years <sup>4</sup>	0.01 ± 0.21	0.04 ± 0.20	0.25 ± 0.18	0.16 ± 0.15		
Estimated mean <sup>4</sup> ± SE at age 16 years	-0.27 ± 0.13	-0.26 ± 0.17	-0.23 ± 0.15	-0.38 ± 0.13		
Change from 6 to 16 years						
Mean <sup>4</sup> ± SE	-0.28 ± 0.23	-0.31 ± 0.21	-0.48 ± 0.19	-0.54 ± 0.17		
Adjusted difference (95% CI) <sup>5</sup>	Reference	-0.01 (-0.59, 0.57)	-0.15 (-0.72, 0.41)	-0.32 (-0.87, 0.23)	0.18	-0.11 (-0.29, 0.06)
<b>Waist circumference z-score</b>						
Estimated mean ± SE at age 6 years	-0.11 ± 0.09	-0.04 ± 0.08	0.01 ± 0.08	-0.05 ± 0.06		
Estimated mean ± SE at age 16 years	-0.26 ± 0.08	-0.09 ± 0.07	-0.26 ± 0.07	-0.19 ± 0.06		
Change from 6 to 16 years						
Mean ± SE	-0.16 ± 0.14	-0.06 ± 0.10	-0.27 ± 0.10	-0.14 ± 0.09		
Adjusted difference (95% CI)	Reference	0.16 (-0.20, 0.51)	-0.08 (-0.43, 0.27)	0.06 (-0.28, 0.40)	0.99	-0.01 (-0.11, 0.08)

<sup>1</sup> Median LTL z-score within each quartile.

<sup>2</sup> Test of interaction terms between age variables and a variable representing medians of each LTL category introduced as a continuous covariate.

<sup>3</sup> According to the WHO Growth Reference for children ages 5-19 years.

<sup>4</sup> From mixed effects models with each anthropometric variable as the continuous outcome and linear and cubic spline terms for age, indicators for LTL quartiles, and interaction terms for age and LTL quartiles as predictors. Robust estimates of variance were specified in each model. Models for waist circumference did not include spline terms.

<sup>5</sup> Adjusted for baseline age, maternal education, and socioeconomic status. Complete case analyses (*n* = 312).

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**Appendix 1.** Telomere Research Network reporting recommendations for PCR-based telomere length measurement.

Sample type, storage, extraction, and integrity:

- Sample type
  - **Buffy coat**
- Sample storage conditions, including temperature, duration, and buffer
  - **Samples were collected in EDTA tubes and one aliquot was collected in a metal-free polypropylene tube with no anticoagulant for separation of serum. The samples were placed on ice, protected from light, and transported to the Colombian National Institute of Health in Bogotá, where buffy coat was separated and DNA extracted prior to cryopreservation at -80 °C. The DNA was then transported frozen to the University of Michigan, where LTL quantification took place.**
- DNA extraction method
  - **We extracted DNA from buffy coat using QIAmp DNA Blood Mini Kits (Qiagen, Valencia, CA, USA).**
- DNA storage conditions, including freeze-thaw cycles
  - **DNA was cryostored at -80° C in buffer AE (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0) until the time of analysis. All LTL measurements were made between 6/22/15 and 12/19/15. DNA samples were frozen after extraction and thawed before LTL measurement; thus each sample went through approximately 1-2 freeze-thaw cycles between extraction and measurement.**
- Method of documenting DNA quality and integrity
  - **None**
- Percentage of samples specifically tested for DNA quality and integrity
  - **N/A**
- For studies with repeated measures design, report the above for all time points
  - **N/A**

qPCR assay:

- State whether qPCR, MMqPCR, aTL (absolute TL/PCR based) or other PCR based method
  - **MMqPCR**
- PCR machine type
  - **Bio-Rad MyiQ Single Color Real-Time PCR Detection System**
- Source (manufacturer/home-made) of master mix and reagents, and final reaction volume
  - **10 ng of input DNA, iQTM SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and two sets of primers (Invitrogen, Carlsbad, CA, USA). The final reaction volume was 25 µl per well plate.**
- Telomere primer sequences and concentration
  - **telg, AACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT and telc, TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA, both with concentration 900 nM**
- Single copy gene name, primer sequences, and concentration



- albu, CGGCGGCGGGCGGGCGGGCTGGGCGGaaatgctgcacagaatccttg and  
albd, GCCCGGCCCGCCGCGCCCGTCCCGCCGgaaaagcatggctgcctgtt, both with concentration 900 nM
- Full PCR program description including temperature, times, and cycle numbers
  - Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; and Stage 3: 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C with signal acquisition, 10 s at 84°C, 15 s at 88°C with signal acquisition
- PCR efficiency of single copy gene and telomere primers
  - > 90% for both
- Source and concentration of control samples and standard curve
  - A single batch of human genomic DNA (Aldevron, Fargo, ND, USA) was serially diluted to make an eight-point standard curve on each plate, with concentration ranging from 1.2 to 156 ng/uL and input DNA quantities spanning a 128-fold range. Each point on the curve was run in duplicate. The curves were used to quantify the telomere (*T*) and albumin (*S*) signals from each sample and control. A single batch of pooled DNA from several samples was measured in triplicate on each plate as a control to measure inter-assay variation, and each plate also contained a no template control (water) to detect spurious amplification.
- For aTL PCR measurement only: sequence and concentration of oligo standards
  - N/A

#### Data analysis:

- Mean and standard deviation or median and range of telomere lengths
  - **0.92 ± 0.33 in girls and 0.84 ± 0.29 in boys**
- Number of sample replicates
  - **All samples were run in triplicate.**
- Level of independence of the replicates (plate vs day vs extraction)
  - **The three replicates were run on the same plate/day, but different wells.**
- Analytic method, considering replicate measurements, to determine final telomere length
  - **Standard curves were used to quantify the telomere (*T*) and albumin (*S*) signals for each reaction, and LTL was calculated as the *T/S* ratio. The median of 3 replicate measures was used as the final LTL value.**
- Method of accounting for variation between sample replicates
  - **We used the median of 3 replicates, which is the optimal method to reduce random variation (Villamor & Bosch, Ann Hum Biol, 2015;42(5):507-10), and calculated average intra-assay CV for all plates (11.3%).**
- Method for accounting for well position effects within plates
  - **We calculated the mean *T/S* ratio among samples measured at a given well position across all plates and subtracted the well-specific mean from each individual *T/S* value, as recommended (Eisenberg et al., Am J Hum Biol, 2015;27:570-5).**
- Method of accounting for between-plate effects
  - **We calculated the inter-assay CV (16.2% ) as the standard deviation of all plate-specific *T/S* means for the control sample divided by the mean of those**

**means. We also checked the control sample T/S ratios on each plate and verified that they were within a reasonable range.**

- % of samples repeated and % samples failing final QC and excluded from further analyses
  - **121/750 samples (16.1%) had at least 1 replicate rerun. 27/750 samples (3.6%) failed QC and were ultimately excluded.**
- Acceptable range of PCR efficiency for the single copy gene and telomere primers
  - **90-110%**
- ICCs of sample/study groups to address variability (not CV)
  - **We calculated ICC statistics for the triplicate measurements obtained in our laboratory (0.59).**
  - **As a test of validity, we also sent a random selection of 48 samples, covering the entire range of T/S ratios as measured at our laboratory, for testing at Dr. Elizabeth Blackburn's laboratory at the University of California, San Francisco. The agreement between the two measurements was excellent (r= 0.87).**
- T/S ratio transformed to a z score prior before comparison across methods/studies
  - **We transformed all of the T/S ratios into z-scores prior to analysis.**
- For studies with family samples or repeated measure design: analytic method to account for this
  - **Only baseline LTL measurements were used in this analysis. Outcomes with repeated measures were analyzed using linear mixed models. All models specified robust estimates of variance.**