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Telomere Length and Vascular Phenotypes in a Population-Based Cohort of Children and Midlife Adults

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Background—Telomere length has been inversely associated with cardiovascular disease in adulthood, but its relationship to preclinical cardiovascular phenotypes across the life course remains unclear. We investigated associations of telomere length with vascular structure and function in children and midlife adults.

Methods and Results—Population-based cross-sectional CheckPoint (Child Health CheckPoint) study of 11- to 12-year-old children and their parents, nested within the LSAC (Longitudinal Study of Australian Children). Telomere length (telomeric genomic DNA [T]/ β -globin single-copy gene [S] [T/S ratio]) was measured by quantitative polymerase chain reaction from blood-derived genomic DNA. Vascular structure was assessed by carotid intima-media thickness, and vascular function was assessed by carotid-femoral pulse-wave velocity and carotid elasticity. Mean (SD) T/S ratio was 1.09 (0.55) in children (n=1206; 51% girls) and 0.81 (0.38) in adults (n=1343; 87% women). Linear regression models, adjusted for potential confounders, revealed no evidence of an association between T/S ratio and carotid intima-media thickness, carotid-femoral pulse-wave velocity, or carotid elasticity in children. In adults, longer telomeres were associated with greater carotid elasticity (0.14% per 10-mm Hg higher per unit of T/S ratio; 95% CI, 0.04%–0.2%; $P=0.007$), but not carotid intima-media thickness ($-0.9\ \mu\text{m}$; 95% CI, -14 to $13\ \mu\text{m}$; $P=0.9$) or carotid-femoral pulse-wave velocity ($-0.10\ \text{m/s}$; 95% CI, -0.3 to $0.07\ \text{m/s}$; $P=0.2$). In logistic regression analysis, telomere length did not predict poorer vascular measures at either age.

Conclusions—In midlife adults, but not children, there was some evidence that telomere length was associated with vascular elasticity but not thickness. Associations between telomere length and cardiovascular phenotypes may become more evident in later life, with advancing pathological changes. (*J Am Heart Assoc.* 2019;8:e012707. DOI: 10.1161/JAHA.119.012707.)

Key Words: aging • arterial stiffness • atherosclerosis • carotid intima-media thickness • CheckPoint (Child Health CheckPoint) study • LSAC (Longitudinal Study of Australian Children) • pulse-wave velocity

Given the global burden of cardiovascular disease (CVD), considerable efforts are under way to understand the underlying cause and to identify potential biomarkers predictive of risk.¹ Cell senescence may play an important role in the progression of CVD, although the upstream drivers of CVD-associated cell death remain to be fully elucidated. One well-studied pathway to cellular senescence is mediated via telomere shortening. Telomeres are nucleoprotein structures that cap the ends of linear chromosomes. Their

shortening represents a molecular mechanism of biological aging² and occurs because of the general inability of cells to replicate the ends of linear DNA in the absence of telomerase. At a critical length, the DNA damage response drives cell senescence, resulting in inhibited tissue repair capacity and function.

Considerable evidence has linked shorter telomeres with greater risk of all-cause mortality,³ cancer,⁴ infectious disease,⁵ and CVD.^{6,7} Notably, a meta-analysis of 43 725 adults (mean

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Accompanying Data S1, Tables S1 through S4, and Figure S1 are available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.012707>

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Clinical Perspective

What Is New?

- This is the first study to examine the relationship between telomere length and vascular structure and function in a population-based cohort of children and adults.
- In midlife adults, but not children, there was some evidence that telomere length was associated with vascular elasticity.
- The novel small association between longer telomere length and higher carotid elasticity in adults is interesting; however, the strength of the association could equally be caused by chance and, hence, requires replication.

What Are the Clinical Implications?

- The clinical utility of cross-sectional telomere length assessment for cardiovascular disease risk in healthy populations of children or midlife adults is limited.
- Future studies should consider longitudinal associations of telomere length with vascular phenotypes and cardiovascular events.

age, 60 years) showed that those with the shortest telomeres in blood had a 30% to 83% higher risk of coronary artery disease relative to those with the longest telomeres.⁶ Recent studies in adults have shown accelerated telomere shortening in blood cells from patients with atherosclerosis.^{8,9} Few studies have explored this relationship in relatively healthy populations of younger individuals, despite the fact that the pathways to cardiometabolic ill health in adulthood begin much earlier in life.¹ Telomere-linked cell senescence may be on the mechanistic pathway to poor cardiovascular health, or it may represent a critical “tipping point” in the life course when overt CVD emerges.

Noninvasive measures of vascular phenotypes enable assessment of cardiovascular structure and function in the large arteries. Ultrasound and tonometry of the carotid artery can capture carotid intima-media thickness (IMT),^{10,11} carotid-femoral pulse-wave velocity (PWV), and carotid elasticity.^{12–14} Carotid IMT is a validated structural marker of subclinical atherosclerosis and correlates with total atherosclerotic burden in adults.¹⁵ Faster carotid-femoral PWV and reduced carotid elasticity are functional measures of arterial stiffness¹⁴ linked to higher risk of cardiovascular events.¹⁶ Thicker carotid IMT has previously been associated with shorter telomeres in clinical samples of older adults.^{17,18} However, recent studies of healthy adults in late life showed that telomere length was not associated with carotid IMT¹⁹ or with carotid atherosclerosis.²⁰ Functional measures have also been linked with shortened telomeres in later-life adults.^{21,22}

Although telomere length may be a potential mechanism underlying CVD across the life course, studies are conflicting⁶

and little is known of the relationship in younger adults or children. It may be that telomere length is associated with vascular function earlier than structural phenotypes.²³ Thus, exploring the relationship of telomere length with functional and structural phenotypes across the life course could enhance our understanding of the role of telomere length-driven cell senescence in the pathogenesis of CVD. We aimed to assess the associations of telomere length with measures of vascular structure and function in a population-based cross-sectional study of children and their parents.

Materials and Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Study Design and Participants

The LSAC (Longitudinal Study of Australian Children) recruited a nationally representative birth cohort in 2004 (n=5107). Participants have been followed up at 7 biennial waves spanning birth to 13 years. The CheckPoint (Child Health CheckPoint) study was an additional physical health and biomarker wave for the birth cohort, nested between LSAC's sixth and seventh waves. During the LSAC wave 6 assessment in 2014, interviewers obtained written consent from 3513 families (93% of the 3764 seen) to be contacted to participate in the upcoming CheckPoint study. Ultimately, 1874 adult-child pairs (50%) took part. Most nonparticipation (60%) was caused by inability to attend or to reschedule a visit during the short period CheckPoint study was in each location. Details of the LSAC and CheckPoint study design and recruitment have been previously described,^{24–27} and the Figure shows the flow through the study.

Data collection ran from February 2015 to March 2016. The CheckPoint study Assessment Center operated sequentially across Australia in major cities (3.5 hours, main center) and regional centers (2.5 hours, minicenter), with home visits offered to those unable to attend a center. Children and their attending parent rotated through a series of 15-minute stations, in which different aspects of health were assessed and biological samples were collected, including venous blood. A trained researcher undertook cardiovascular measures while medically trained researchers or phlebotomists collected venous blood. Samples were processed and cryopreserved within 2 hours on site. For the first 2 months, this included blood clots from plasma tubes; for logistic reasons, clots were then discontinued and replaced with a whole blood sample for the remaining centers. As each assessment center closed, samples were transported to the Murdoch Children's Research Institute in Melbourne, Australia, where they remain

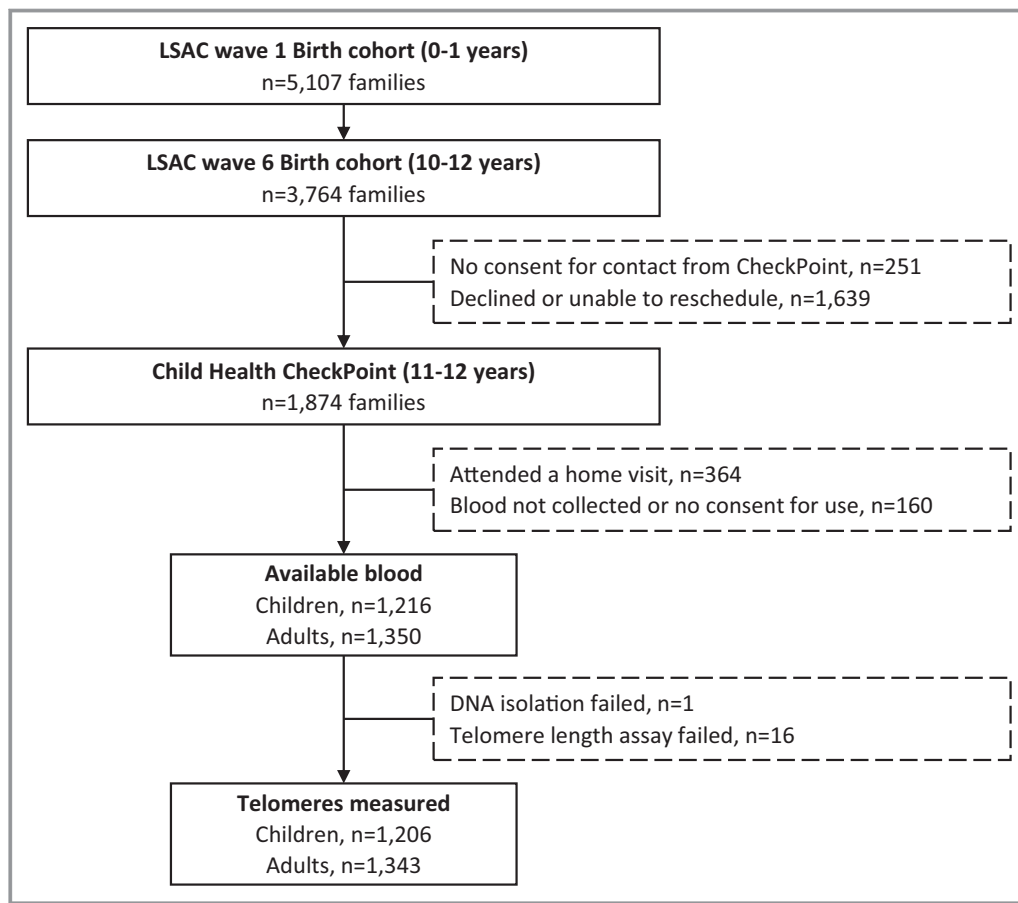


Figure. LSAC (Longitudinal Study of Australian Children) and CheckPoint (Child Health CheckPoint) study participant flow.

cryopreserved until depletion. The logistics and specified requirements of this large, national, government-owned population-based cohort precluded any participants attending repeated sessions. However, all values for all participants in both age groups are based on repeated measurements within the same session, as are the interobserver and intraobserver ratings for the observer-dependent measurements (carotid IMT and waveform quality ratings). Previous studies have confirmed similar repeatability between children and adults,^{28,29} and between sessions separated by hours to weeks.³⁰

The study was approved by the Royal Children's Hospital Melbourne Human Research Ethics Committee (33225D) and the Australian Institute of Family Studies Ethics Committee (14-26). The attending parents provided written informed consent for themselves and their child before participation, and the child provided assent.

DNA Isolation and Telomere Length Measurement

Genomic DNA was isolated from available blood using the QIAamp 96 DNA Blood Kit (Qiagen, Venlo, the Netherlands).

Purity and integrity was confirmed using the NanoDrop 2000 Spectrophotometer (NanoDrop Technologies, Middleton, WI), the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA), and gel electrophoresis. Telomere length was measured with the quantitative real-time polymerase chain reaction method, originally described by Cawthon.³¹ This method measures the amount of telomeric genomic DNA (T)/ β -globin single-copy gene (S) (T/S ratio) for each participant. The mean intra-assay variability between "T" and "S" quadruplicates used in the calculation of the T/S ratio was 1.7% (SD, 0.3%; range, 0.9%–2.6%). The interassay variability between plates was 1.7% (SD, 1.4%; range, 0.3%–6.2%). Further details on the telomere length calculation (Table S1), plate conditions, and constituents are described in Data S1 and in the Standard Operating Procedure on the CheckPoint study's website.^{32,33}

Carotid IMT and Carotid Elasticity

Carotid IMT and elasticity were measured using standardized protocols via ultrasound (Vivid i BT06 with 10-MHz linear array probe; GE Healthcare, Little Chalfont, UK).^{10,11} Ultrasounds were performed in supine position with head turned 45° to the

left. The ultrasound probe was applied to the right side of the neck at $\approx 45^\circ$ to the midline with concurrent 3-lead ECG trace. The duration of the captured real-time B-mode ultrasound cine loops was 10 cardiac cycles.

All images were reviewed for quality to select optimal loops comprising a clear near and far wall intima-media, clear lumen, straight vessel, presence of the carotid bulb, and an ECG trace. The best-quality 5 to 7 cardiac cycle section of the loops was trimmed and extracted. These loops were further processed using Carotid Analyzer (Medical Imaging Applications, Coralville, IA). Raters calibrated the images using ultrasound image markers. Maximum carotid IMT values were used in analyses, which refer to the 3 to 5 frame average of the thickest point of carotid IMT measurement over the highest quality 5- to 10-mm section from the carotid bulb. After algorithmic detection of the intima-media interface over the entire cine-loop, frames were manually adjusted as needed or rejected if the intima-media interface was unclear or blurred. The intraobserver variability for the maximum carotid IMT values was 4.9% (95% CI, 4.6%–5.2%), and the interobserver variability was 6.2% (95% CI, 5.2%–7.2%). Further details are described elsewhere.^{25,34}

Carotid elasticity was calculated from carotid artery images and expressed as a percentage change in intima-intima lumen diameter (measured from ultrasound) per change in pulse pressure (measured from SphygmoCor), as previously described (Table S1).¹³ Intima-intima lumen diameter measurement was automated using Carotid Analyzer and, thus, was rater independent; it was calculated by measuring the average intima-intima distance (subtracting near and far wall IMT measurements) on each of the 3 to 5 still frames used to calculate maximum carotid IMT. Intraobserver and interobserver variability values for maximum lumen diameter were 1.3% (95% CI, 1.2%–1.4%) and 1.6% (95% CI, 1.4%–1.9%), respectively; and values for minimum lumen diameter were 1.2% (95% CI, 1.1%–1.3%) and 1.5% (95% CI, 1.2%–1.7%), respectively. Further details are in Data S1 and prior publications.^{25,34}

Carotid-Femoral PWV and Blood Pressure

Carotid-femoral PWV and blood pressure were measured using tonometry via SphygmoCor XCEL (AtCor Medical, West Ryde, Australia), as previously described.¹⁴ Carotid-femoral PWV was determined by detecting waveforms simultaneously by a hand-held tonometer at the carotid pulse and a cuff placed on the proximal thigh, over a 10-second period. This was completed 3 times in the supine position. The distance traveled by waveforms was measured with a tape measure from the carotid pulse to the suprasternal notch, from the suprasternal notch to the right femoral pulse, and from the

femoral pulse to the top of the thigh cuff. The mean of at least 2 valid carotid-femoral PWV measurements (in m/s) was used in analyses. Systolic and diastolic blood pressure values were recorded at the brachial artery, 3 times, 1 minute apart, with either a 23- to 33-cm or a 31- to 40-cm cuff, depending on arm size. Systolic and diastolic blood pressure values were included if participants had at least 2 valid measurements. Further details of these measures are described elsewhere.^{25,35}

At the time of assessment, waveforms were assessed using the in-built quality control software in the SphygmoCor XCEL. After study completion, waveforms were further reviewed by 2 trained analysts and were excluded if they did not meet quality control standards. To assess interrater reliability, 112 individually recorded waves from a random sample of 40 participants (20 children and 20 adults) from the CheckPoint study database were presented blindly to 2 analysts for review. Pulse wave quality ratings given by each analyst (poor, adequate, or good) were compared by calculating the proportion of positive agreement between analysts. Most sample waveforms were of good quality, and none were of poor quality. The overall correlation between analysts was high ($r=0.99$). Automated measurement of carotid-femoral PWV by SphygmoCor is approved by the US Food and Drug Administration, is extensively standardized across large general populations,^{22,36,37} and is validated against other well-validated tonometry-based methods,^{38,39} as per the ARTERY (Association for Research into Arterial Structure and Physiology) PWV validation guidelines.⁴⁰ Published values for interobserver and intraobserver carotid-femoral PWV variability are 0.30 m/s (SD, 1.25 m/s) and 0.07 m/s (SD, 1.17 m/s), respectively.⁴¹ Further details are in Data S1 and are described elsewhere.^{25,35}

Potential Confounders

Several variables were considered a priori as potential confounders, including body mass index (BMI; kg/m^2),⁴² socioeconomic status,⁴³ and smoking.⁴⁴ Each of these variables has consistently been associated with telomere length, as well as being commonly known risk factors for cardiovascular health. BMI was calculated from height (standard portable stadiometer; IP0955; Invicta, Leicester, UK) and weight (InBody230 bioelectrical impedance analysis scale; Biospace Co Ltd Seoul, South Korea). For children, an age- and sex-adjusted BMI z score was calculated using the US Centers for Disease Control and Prevention growth reference charts.⁴⁵ As a measure of neighborhood socioeconomic status, we used the Socio-Economic Indexes for Areas Index of Relative Disadvantage score, which is based on the postcode of domicile of the participating family. This is a standardized score by geographic area, compiled from 2011 Australian Census data to numerically summarize the social

and economic conditions of Australian neighborhoods (national mean of 1000 and SD of 100, where higher values represent less disadvantage). Parental current smoking behavior and cigarettes smoked per day were self-reported at LSAC wave 6. Preexisting conditions were self-reported (yes/no) by questionnaire at the CheckPoint study assessment, including diabetes mellitus status, heart conditions, hypertension medication, and pacemakers. Further details of these sample measures are described elsewhere.²⁵

Other Covariates

Other covariates traditionally associated with cardiovascular outcomes were measured using the Nightingale nuclear magnetic resonance metabolomics platform (Helsinki, Finland) from blood serum. These included lipids (total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides), glucose, and the inflammation marker of glycoprotein acetyls. Further details of this platform and method are extensively described elsewhere.⁴⁶

Statistical Analysis

Stata 14.2 (StataCorp, College Station, TX) was used for all analyses. Continuous variables are presented as means and SD, whereas dichotomous variables are presented as percentages.

Associations of telomere length with vascular measures were examined by linear and logistic regression models, in children and adults separately. Assumptions for linear regression were examined using histograms and scatterplots, and they showed no discernible outliers and minimal right skewing for child's and adult's telomere length. For aim 1, linear regression models fitted continuous telomere length as the independent variable and continuous data on vascular measures as dependent variables in separate models. For aim 2, logistic regression models fitted continuous telomere length as the independent variable and elevated carotid IMT, carotid-femoral PWV, and reduced carotid elasticity as the dependent variables in separate models. Elevated carotid IMT and elevated carotid-femoral PWV were defined internally as >75th percentile for the sample. Reduced carotid elasticity was defined internally as <25th percentile.

For both aims, model 1 included adjustments for the potential lifelong confounders of sex and age, as well as for sample type (ie, whole blood or blood clot) to account for any effects of the early change in the sample collection protocol from blood clots to whole blood. Adjusted analyses included the covariates as independent variables. Model 2 additionally included adjustments for the additional potential confounders of BMI and Socio-Economic Indexes for Areas Index of

Relative Disadvantage score, plus smoking status, for adults. In model 3, we further adjusted for systolic blood pressure, lipids, glucose, and glycoprotein acetyls. Finally, we conducted sensitivity analysis that did the following: (1) further adjusted for preexisting conditions, (2) adjusted for mean arterial pressure or diastolic blood pressure in place of systolic blood pressure, and (3) stratified by, rather than adjusted for, sample type. Sensitivity analyses showed similar results to those presented below (data not shown; available on request).

Results

A total of 1874 parent-child dyads participated in the CheckPoint study (Figure). As venous blood was not collected at home visits ($n=364$ dyads), these participants were not included in the current analysis. Venous blood (whole blood or blood clot) was available for 1216 children and 1350 adults. Telomere length data were generated for 1206 children (99.2%) and 1343 adults (99.5%).

Sample Characteristics

Participant characteristics are displayed in Table 1. Children and adults were, on average, aged 11 years (SD, 0.5 years) and 44 years (SD, 5.1 years), respectively. Adults were predominantly mothers ($n=1168$; 87%), whereas there were approximately equal numbers of boys and girls. Participants came from slightly less disadvantaged and more homogeneous areas compared with the national average (mean Socio-Economic Indexes for Areas Index of Relative Disadvantage score, 1026 [SD, 61] versus 1000 [SD, 100]). Child and adult BMI scores were similar to those of the current Australian population, as recorded by the Australian Bureau of Statistics, with ≈ 1 in 4 children and 2 in 3 adults overweight or obese.⁴⁷ Compared with the general Australian population rates for similar age ranges, adults' self-report of diabetes mellitus (2.4% versus 4%) and being a current smoker (8% versus 16%) were lower⁴⁷ but CVD (2.2% versus 2.5%) was comparable.⁴⁷

Children had longer telomeres (higher T/S ratio, 1.09; SD, 0.55) than adults (0.81; SD, 0.38).³³ Adults, on average, had thicker carotid IMT than children (663 [SD, 97] μm versus 580 [SD, 46] μm) and stiffer vessels, as shown in faster carotid-femoral PWV (7.0 [SD, 1.1] m/s versus 4.4 [SD, 0.5] m/s) and lower carotid elasticity (2.4% [SD, 0.6%] versus 4.8% [SD, 0.9%] per 10-mm Hg). Further details of the distributions of vascular phenotypes are in Figure S1 and were previously described.^{34,35} Adults with self-reported diabetes mellitus and hypertension medication use had thicker carotid IMT, faster carotid-femoral PWV, and lower carotid elasticity (Table S2). Vascular phenotypes were similar in current

Table 1. Summary Characteristics of Children and Adults

Participant Characteristic	Children	Adults
Total No.	1206	1343
Age, y	11 (0.5)	44 (5.1)
Female sex, %	51	87
Height, cm	154 (8)	167 (8)
Body mass index, kg/m ²	19 (3)	28 (6)
Body mass index <i>z</i> score	0.3 (1)	...
Disadvantage score	1026 (62)	1026 (61)
Current smoking, %	...	8.2
Cigarettes smoked per day	...	2.6 (0.14)
Preexisting conditions, %		
Diabetes mellitus	0.1	2.4
Heart condition	...	2.2
Hypertension medication	...	5.1
Pacemakers	...	0.1
Systolic blood pressure, mm Hg	108 (8)	120 (13)
Diastolic blood pressure, mm Hg	63 (6)	74 (9)
Lumen diameter, mm	5.9 (0.5)	5.8 (0.6)
HDL cholesterol, mg/dL	54 (10)	56 (14)
LDL cholesterol, mg/dL	25 (6)	30 (8)
Total cholesterol, mg/dL	73 (12)	86 (16)
Triglycerides, mg/dL	105 (49)	132 (75)
Glucose, mg/dL	89 (14)	88 (19)
Glycoprotein acetylation, mmol/L	0.99 (0.13)	1.04 (0.17)
Telomere length (T/S ratio)	1.09 (0.55)	0.81 (0.38)
Vascular outcomes		
Carotid intima-media thickness, μ m	580 (46)	663 (97)
Carotid-femoral pulse wave velocity, m/s	4.4 (0.5)	7.0 (1.1)
Carotid elasticity, % per 10-mm Hg	4.8 (0.9)	2.4 (0.6)

Data are presented as mean (SD) or percentage. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; T/S ratio, telomeric genomic DNA (T)/ β -globin single-copy gene (S).

smokers and nonsmokers, noting that this may be limited by lack of smoking history. Telomere length was not related with diabetes mellitus, current smoking, and hypertension medication in adults (Table S3). Higher blood pressure was associated with longer telomeres in adults, but not in children (Table S4).

Association of Telomere Length With Carotid IMT, Carotid-Femoral PWV, and Carotid Elasticity

In linear regression models adjusted for age, sex, and sample type, children's T/S ratio showed little association with vascular measures (Table 2; model 1). Similarly, adult T/S ratio showed little association with carotid IMT or carotid-

femoral PWV (Table 2; model 1). However, in adults, longer telomeres were associated with greater carotid elasticity (0.14% per 10-mm Hg higher for each additional unit of T/S ratio; 95% CI, 0.04%–0.2%). This is equivalent to an effect size of 0.09 SDs higher carotid elasticity for each SD increase in T/S ratio. This association persisted after adjustments for sex, age, sample type, BMI, Socio-Economic Indexes for Areas Index of Relative Disadvantage score, and smoking status (0.13% per 10-mm Hg; 95% CI, 0.05%–0.20%; Table 2; model 2), as well as for systolic blood pressure, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glucose, and glycoprotein acetyls (0.09% per 10-mm Hg; 95% CI, 0.003%–0.18%; Table 2; model 3).

In both adults and children, adjusted logistic regression models showed little evidence of a difference in the odds of elevated carotid IMT, elevated carotid-femoral PWV, or reduced carotid elasticity for each unit higher T/S ratio (Table 3).

Discussion

Main Findings

In the present study, we hypothesized that telomere attrition is an upstream event of cardiometabolic disease and, as such, evidence for shortened telomeres will be present in mid-aged adults and children in association with preclinical phenotypes of poorer cardiovascular health. Furthermore, given the progressive nature of the proposed association, we predicted that the strength of association between telomere length and phenotypes will be stronger in adults relative to their children. We tested this in a population-based cohort of children and mid-life adults and found no convincing evidence of associations between shorter telomeres and adverse vascular phenotypes at either age, other than a small association between longer telomere length and increased carotid elasticity in adults.

Strengths and Limitations

Strengths of our study include the objective examination of carotid IMT, carotid-femoral PWV, and carotid elasticity with high reliability and the T/S measurements showing low replicate variation. We are confident in interpreting the child and adult findings within the same study because they were assessed at the same time point with the same protocols, equipment, and staff. For telomere length, we used a quantitative real-time polymerase chain reaction method that correlates strongly with the gold standard terminal restriction fragment analysis.³¹ However, although well suited for large epidemiological studies, this method does not quantify absolute, chromosome-specific, or vascular tissue-specific telomere length. Also, the capacity of telomere length derived

Table 2. Association of Telomere Length With Carotid IMT, Carotid-Femoral PWV, and Carotid Elasticity, in Children and Adults

Outcome	Children				Adults			
	No.	Coefficient (95% CI)	R ²	P Value	No.	Coefficient (95% CI)	R ²	P Value
Model 1*								
Carotid IMT, μm	1193	4.11 (−1.21 to 9.4)	0.04	0.13	1203	−0.85 (−14.31 to 12.61)	0.16	0.90
Carotid-femoral PWV, m/s	1167	−0.01 (−0.07 to 0.06)	0.06	0.86	1110	−0.10 (−0.27 to 0.07)	0.10	0.23
Carotid elasticity, % per 10-mm Hg	1083	−0.04 (−0.15 to 0.06)	0.01	0.42	1040	0.14 (0.04 to 0.24)	0.12	0.007
Model 2†								
Carotid IMT, μm	1192	4.67 (−0.64 to 9.99)	0.05	0.09	1198	0.46 (−12.71 to 13.64)	0.20	0.95
Carotid-femoral PWV, m/s	1166	0.01 (−0.05 to 0.07)	0.13	0.68	1106	−0.12 (−0.28 to 0.04)	0.24	0.13
Carotid elasticity, % per 10-mm Hg	1082	−0.06 (−0.17 to 0.05)	0.04	0.26	1035	0.13 (0.04 to 0.22)	0.26	0.006
Model 3‡								
Carotid IMT, μm	1082	4.89 (−0.60 to 10.37)	0.07	0.08	1082	1.17 (−12.60 to 14.94)	0.25	0.87
Carotid-femoral PWV, m/s	1070	0.02 (−0.04 to 0.08)	0.16	0.57	1031	−0.09 (−0.23 to 0.06)	0.40	0.24
Carotid elasticity, % per 10-mm Hg	1037	−0.09 (−0.19 to 0.02)	0.15	0.11	1003	0.09 (0.003 to 0.18)	0.36	0.04

IMT indicates intima-media thickness; PWV, pulse-wave velocity; R², value for the linear regression model.

*Model 1 was adjusted for sex, age, and sample type.

†Model 2 was additionally adjusted for body mass index, Socio-Economic Indexes for Areas Index of Relative Socioeconomic Disadvantage score, plus smoking status for adults.

‡Model 3 was additionally adjusted for brachial systolic blood pressure, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glucose, and glycoprotein acetylation.

from blood as a surrogate for telomere length within the vasculature itself is uncertain; however, there is evidence that telomere length is highly conserved between tissues.^{48,49} Differential uptake in LSAC and attrition in CheckPoint study led to our sample including few fathers and few less disadvantaged families than in the general Australian population. Thus, if such children and adults had both shorter telomeres and poorer vascular outcomes, this could have altered estimates and/or their precision. Finally, the cross-sectional design precluded conclusions about temporal associations.

Interpretation in Light of Other Studies

Telomere length, vascular structure, and atherosclerosis

We found little evidence of an association between telomere length and carotid IMT. This is consistent with several prior reports showing no or only marginal associations.^{17,19,20,50–52} For example, in healthy adults, telomere length was not independently associated with carotid IMT¹⁹ (n=2509; age range, 35–55 years) and atherosclerosis in the carotid arteries (n=1459; age range, 40–54 years).²⁰ Together, these studies suggest that associations between telomere length and vascular structure appear primarily later in adulthood. Moreover, there is evidence that shortened telomere length is more reliably associated with patients with CVD. For example,

shorter telomere length has been observed in aortic tissues with atherosclerotic lesions, compared with tissues without atherosclerotic lesions;⁸ and in another study, telomere length was associated with advanced-state, but not with early-stage, atherosclerosis.⁵¹ Telomere shortening might play a role late in the pathogenesis of CVD, in late adulthood when the effects of cumulative burden over the life course begin to manifest. This hypothesis would be consistent with current knowledge, in which the cellular senescence induced by the aging process is known to trigger the progressive deterioration of vascular functionality with age.⁵³

Our findings also align with evidence that shortened telomere length is associated with vascular phenotypes in those with greater CVD risk⁶ (eg, in elderly populations; mean age, 74.2 years; SD, 5.2 years),¹⁷ diabetic subjects,^{9,54} and obese men.⁵⁰ Interestingly, a recent meta-analysis of 5566 patients with coronary artery disease found an overall higher risk for coronary artery disease in older subjects (≥ 70 years), compared with younger subjects (< 70 years; relative risk, 1.9 versus 1.5), with respect to individuals in the shortest telomere tertile compared with those in the longest telomere tertile.

Telomere length, vascular function, and arterial stiffness

There was little evidence of an association between telomere length and carotid-femoral PWV in children or adults. Several

Table 3. Odds Ratio for Elevated Carotid IMT, Elevated Carotid-Femoral PWV, and Reduced Carotid Elasticity for 1 Unit Higher T/S Ratio, in Children and Adults

Outcome	Children			Adults		
	No.	Odds Ratio (95% CI)	P Value	No.	Odds Ratio (95% CI)	P Value
Model 1*						
Elevated carotid IMT	1193	1.23 (0.94–1.61)	0.13	1203	0.94 (0.65–1.37)	0.77
Elevated carotid-femoral PWV	1167	0.82 (0.60–1.13)	0.23	1110	0.88 (0.59–1.30)	0.56
Reduced carotid elasticity	1083	0.99 (0.73–1.32)	0.92	1040	0.84 (0.55–1.30)	0.44
Model 2†						
Elevated carotid IMT	1192	1.27 (0.97–1.66)	0.08	1198	0.98 (0.67–1.44)	0.92
Elevated carotid-femoral PWV	1166	0.88 (0.63–1.23)	0.47	1106	0.83 (0.54–1.28)	0.40
Reduced carotid elasticity	1082	1.02 (0.76–1.38)	0.91	1035	0.83 (0.52–1.32)	0.42
Model 3‡						
Elevated carotid IMT	1082	1.26 (0.95–1.68)	0.11	1082	1.03 (0.66–1.61)	0.89
Elevated carotid-femoral PWV	1070	0.97 (0.68–1.38)	0.86	1031	0.73 (0.43–1.24)	0.25
Reduced carotid elasticity	1037	1.16 (0.84–1.58)	0.37	1033	1.03 (0.61–1.74)	0.92

Elevated carotid IMT defined as >75th percentile, elevated carotid-femoral PWV defined as >75th percentile, and reduced carotid elasticity defined as <25th percentile. IMT indicates intima-media thickness; PWV, pulse-wave velocity; T/S ratio, telomeric genomic DNA (T)/ β -globin single-copy gene (S).

*Model 1 was adjusted for sex, age, and sample type.

†Model 2 was additionally adjusted for body mass index, Socio-Economic Indexes for Areas Index of Relative Socioeconomic Disadvantage score, plus smoking status for adults.

‡Model 3 was additionally adjusted for brachial systolic blood pressure, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glucose, and glycoprotein acetylation.

recent studies have reported an association between shorter telomeres and faster carotid-femoral PWV, but most have been in older adults (>50 years), were limited by modest sample sizes (range, 49–303 individuals),^{21,37,54} and were predominately in men.^{21,55} For example, Benetos et al showed that shorter telomere length was significantly associated with faster carotid-femoral PWV in men ($n=120$; mean age, 55 years; $r=-0.14$), but not in women ($n=73$; mean age, 56 years; $r=-0.05$).⁵⁵ Similar results were also found in a study of healthy Chinese men ($n=112$; mean age, 56 years).²¹ We cannot tell whether our lack of association between telomere length and carotid-femoral PWV reflects our preponderance of women or the younger age in our sample. Interestingly, previous findings suggest that age significantly modifies the relationship between telomere length and carotid-femoral PWV in healthy individuals,²² an effect not observed in our study.

In summary, we report a novel, albeit small, association between telomere length and carotid elasticity in adults, which remained after adjustment for a priori specified confounders. Although speculative at this point, this suggests a possibility that cellular senescence might contribute to vascular function, through the elasticity of the vasculature. One hypothesis might be that the secretory phenotype of senescent vascular cells might promote vascular degeneration through the destabilization of extracellular matrix, elastin, collagen, and smooth muscle,¹² thus modifying the elasticity

of the carotid artery. However, we suspect that it would also have effects on overall arterial stiffness and, thus, affect carotid-femoral PWV, which we did not find. This discordance warrants further investigation. We note that although vascular structure and function are considered to be related, they ultimately measure different properties of the arterial tree, both likely affected by risk factors in a nonuniform manner. It has been documented that functional changes precede the development of structural alterations in the vasculature.²³

Clinical Implications, Unanswered Questions, and Future Direction

Our findings, if replicated in other settings, question the clinical utility of measuring telomere length in healthy relatively young populations. The magnitude of the cross-sectional association between telomere length and carotid elasticity observed among adults was small. In addition, the European Society of Hypertension and the European Society of Cardiology noted that a carotid IMT of >900 μm and a carotid-femoral PWV of >12 m/s are associated with increased CVD risk,⁵⁶ thresholds not reached by our relatively healthy cohort of children and adults. This suggests that the magnitude of the associated changes is unlikely to represent clinically significant differences on CVD risk, especially in largely healthy populations of children or mid-life adults. However, telomere length might be of greater clinical

relevance at an advanced age or in association with more pronounced vascular pathological features. Moreover, it is possible that such associations could be important longitudinally, such that the rate of telomere attrition, rather than cross-sectional telomere length per se, may be a more clinically important cardiovascular risk marker. Indeed, in a recent British longitudinal study (baseline $n=2611$; mean age, 53 years; follow-up age range, 60–64 years), an association was observed between the rate of telomere attrition and thicker carotid IMT at 60 to 64 years, despite limited cross-sectional associations.¹⁸ Future studies should consider longitudinal evaluation with repeated telomere sampling to assess whether telomere attrition over time is associated with vascular phenotypes and cardiovascular events.

We cannot exclude the possibility that our findings may have arisen by chance, and might be explained by factors beyond the scope of this study, including genetic variation and other unmeasured environmental exposures. The ability of carotid ultrasounds and tonometry to effectively measure meaningful changes in vascular structure and function in healthy individuals is also uncertain. In particular, the predictive value of carotid IMT measurement in children aged 11 to 12 years without CVD risk remains unclear.⁵⁷

Conclusion

In a healthy cohort of children and mid-life adults, we report limited evidence that cellular senescence, as measured by telomere length, is associated with vascular structure or function in children or adults. The novel small association between longer telomere length and higher carotid elasticity in adults is interesting, supporting our hypothesis that telomere length may be on the mechanistic pathway toward impaired vascular function; however, the strength of the association could equally be caused by chance and, hence, requires replication. Although telomere length may be important for individuals at intermediate to high CVD risk or those at advanced disease stages, as previously reported, cross-sectional telomere length assessment appears to have limited clinical value for CVD risk in healthy populations, particularly at younger ages. The interaction between telomere dynamics and vascular phenotypes across the life course is multifaceted, with contributions from genetic variation and environmental exposures. Further longitudinal investigations, with repeated telomere and vascular phenotypic sampling, are warranted.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Blood collection and genomic DNA isolation

Whole venous blood was collected into vacutainer tubes containing ethylenediaminetetraacetic acid (known as EDTA) and immediately transported to an on-site laboratory. The blood sample was processed into aliquots within 2 hours into 1.0 mL FluidX tubes (FluidX, Cheshire, UK) and frozen in a -80 °C ultra-low temperature freezer (Thermo Fisher Scientific, Waltham, USA). Samples were transported to the Murdoch Children's Research Institute (MCRI) biobank. Genomic DNA was isolated from available blood (e.g. whole blood or blood clot) using the Qiaamp 96 DNA Blood Kit (Qiagen, Venlo, Netherlands). Samples were randomised with child and parent dyads on the same plate to minimise batch effects using a random number generator (Stata 14.2, StataCorp LLC, USA). The sample retrieval, protocol optimisation, consumable acquisition, and isolation of genomic DNA spanned April 2016 to January 2017. Purity and integrity of genomic DNA was confirmed using NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Middleton, USA), Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA) and gel electrophoresis, prior to storage in a -80 °C ultra-low temperature freezer (Thermo Fisher Scientific, Waltham, USA). Genomic DNA was also isolated from 3 sets of control samples: (1) the K562 leukemic cell line, (2) newborn cord blood and (3) human placental tissue. These control samples have previously been described as having 'shorter', 'average' and 'longer' telomeres relative to peripheral blood samples.¹⁻⁴ Genomic DNA from each of these control samples was used on all telomere assays to assess day-to-day and plate effects.

Telomere length measurement

Each sample was measured in quadruplicates comprising 4 µl of diluted genomic DNA at 5 ng/µl, 5 µl of SensiFAST SYBR No-ROX Kit master mix (Bioline, Sydney, Australia) and 0.5 µl of each forward and reverse primer at 2 µM. The primer sequences were tel1 (5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT), tel2 (5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT), bg1 (5'-GCA GGA GCC AGG GCT GGG CAT AAA AGT CA) and bg2 (5'-GGG CCT CAC CAC CAA CTT CAT CCA CGT TC). All 'T' and 'S' reactions were performed in 384-well plates on a Lightcycler 480 Instrument II (Roche, Melbourne, Australia). The cycling condition began with incubation at 95°C for 10 minutes, followed by 35 cycles of (i) 95°C for 15 seconds and (ii) 62°C for 60 seconds. The final 384-well layout included participant genomic DNA, three sets of control genomic DNA and a no-template control containing RNase-free water instead of a genomic DNA template, each present in quadruplicates. Further details are described in the standard operating procedure on the Growing Up in Australia's CheckPoint website.⁵

Calculation of telomere length

A ratio, known as the T/S ratio, is calculated by comparing the relative amount of 'T' and 'S' for each of these samples to a reference genomic DNA sample (in this case the average T/S ratio of all control genomic DNA). The final relative telomere length from each sample, based on the T/S ratio, was calculated as the change in cycle threshold (Ct) of the test sample (Table S1), normalised to the average T/S ratio of the control genomic DNA on the corresponding plate (Table S2). Hence, the final equation is shown in Table S3. If less than two successful replicates out of the quadruplicates for either 'T' and 'S' were measured then the sample data was omitted (n=16), otherwise a median was calculated, resulting in a median 'T' and a median 'S' for each sample

(n=2,549). A Ct replicate from 5 to 28 was considered successful as a Ct outside of this range was uncertain.

Telomere length replicate variability

To assess the replicate reliability, the degree of variation between replicates in the assay plate, an intra-assay coefficient of variation was calculated. The intra-assay coefficient of variation was calculated as the ratio of the pooled Ct's standard deviation (SD) from all samples (each was analysed in quadruplicate) and the overall Ct mean, and then multiplied by 100. To assess the degree of assay-to-assay and day-to-day consistency an inter-assay coefficient of variation was calculated using the pooled Ct's SD divided by the overall Ct mean of all duplicated samples, and then multiplied by 100. The mean intra-assay variability between 'T' and 'S' quadruplicates used in the calculation of the T/S ratio was 1.7 % (SD 0.3; range 0.9-2.6). The inter-assay variability between plates was 1.7 % (SD 1.4; range 0.3-6.2).

Reliability of carotid intima-media thickness (IMT) readings

Six trained raters analysed all cine-loops. Training consisted of thirty example cine-loops that were subsequently assessed for consistency by an expert rater. Inter- and intra-observer variability was assessed by reanalysing a subset of 105 randomly-selected child images four times at the end of the scoring process. Images were reassessed twice each by two raters in a balanced incomplete block design as not all raters assessed the complete subset. This allowed estimation of the repeatability of measurements made by the same rater and the reproducibility of measurements made by different raters. Image acquisition was only performed once.

In our reliability analysis, we used the modelling of repeated measurements on carotid IMT films with rater and participant as random effects to estimate between-participant variance, between-rater variance, and residual error variance. These were used to calculate intra- and inter-observer variability (the degree of measurement error proportional to the mean.) For maximum carotid IMT value, the intra-observer variability was 4.9% (95% CI 4.6-5.2) and the inter-observer variability was 6.2% (95% CI 5.2-7.2).

Reliability of carotid elasticity

Carotid elasticity measures the ability of the arteries to expand as a response to pulse pressure caused by cardiac contraction and relaxation.⁶ Carotid elasticity was calculated from carotid artery images and expressed as a percentage change in intima-intima lumen diameter per change in pulse pressure (Table I), according to previously published work from the Cardiovascular Risk in Young Finns Study⁶ and other related studies.⁷ Intima-intima lumen diameter measurement was automated using Carotid Analyser (Medical Imaging Applications, Coralville, IA, USA), rater-independent, and was calculated by measuring the average intima-intima distance (subtracting near and far wall IMT measurements) on each of the three to five still frames used to calculate maximum carotid IMT. The final calculation is shown in Table S1 (IV).

After algorithmic detection of the intima-media interface over the entire cine-loop by Carotid Analyser (Medical Imaging Applications, Coralville, IA, USA), frames were manually adjusted as needed or rejected if the intima-media interface was unclear or blurred. Intra- and inter-observer variability for maximum lumen diameter was 1.3% (95% CI 1.2-1.4) and 1.6% (95% CI 1.4-1.9). Intra- and inter-observer variability for minimum lumen diameter was 1.2% (95% CI 1.1-1.3) and 1.5% (95% CI 1.2-1.7).

Reliability of arterial pulse waveforms

Twenty children and twenty parents were randomly sampled from the SphygmoCor database and sampling was stratified by two analysts. Hence, twenty subjects were chosen from each analyst. The pulse wave analysis (PWA) waveforms had already undergone

quality checks by one of these two trained data analysts. Each child provided three individually recorded PWA and whilst temporally close they were not necessarily exchangeable and were treated as linked for rating purposes. Waves were presented to the analyst without evidence of previous quality assessment. Three waves were present for 32 children and two waves for 8 children, resulting in each analyst making 112 assessments. Waves from the same subject were identifiable as they were presented sequentially for assessment. To assess the quality of the PWA waveforms we compared the quality ratings (1 good, 2 adequate and 3 poor) assigned by each analyst.

We compared quality ratings of the PWA waveforms by calculating the proportion of positive and negative agreement between analysts. No waves were classified as being of poor quality. The positive agreement between analysts was high (0.99). There were discrepancies in quality ratings for only two waveforms and therefore the negative agreement (0.5) was likely a poor estimate. Overall both analysts agreed that all scans were of acceptable quality or above.

Table S1. Equations for the calculation of (I, II and III) telomere length and (IV) carotid elasticity.

	Equation
(I) Change in cycle threshold for test sample	$\Delta Ct_{test} = Ct_{(test, telomere)} - Ct_{(test, beta-globin)}$
(II) Change in cycle threshold for reference sample	$\Delta Ct_{ref} = Ct_{(ref, telomere)} - Ct_{(ref, beta-globin)}$
(III) Relative telomere length (T/S ratio)	$2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct_{test} - \Delta Ct_{ref})}$
(IV) Carotid elasticity (%/mmHg)	$\frac{\left(\frac{LD_{max} - LD_{min}}{LD_{min}}\right)}{\Delta PP} \times 100\%$

Table S2. Summary of adults' vascular outcomes for diabetics, current smokers and hypertension

medications users.

	Carotid IMT (μm)			Carotid-femoral PWV (m/s)			Carotid elasticity (% per 10 mmHg)		
	N	Mean (SD)	p-value*	N	Mean (SD)	p-value*	N	Mean (SD)	p-value*
Diabetes									
Yes	30	758 (170)	-	24	7.81 (1.39)	-	22	2.05 (0.63)	-
No	1286	661 (93)	0.004	1176	6.94 (1.11)	0.006	1105	2.44 (0.60)	0.008
Current smoker									
Yes	110	658 (91)	-	94	6.78 (1.08)	-	94	2.43 (0.62)	-
No	1206	664 (97)	0.51	1106	6.97 (1.12)	0.11	1033	2.44 (0.60)	0.95
Hypertension medication									
Yes	66	720 (124)	-	55	8.00 (1.40)	-	52	2.13 (0.50)	-
No	1250	660 (94)	0.0002	1145	6.91 (1.08)	<0.0001	1075	2.45 (0.60)	<0.0001

SD: standard deviation.

* Student's t-test.

Table S3. Summary of adult T/S ratios for diabetics, hypertension current smokers and medication users.

	Telomere length (T/S ratio)		
	N	Mean (SD)	p-value*
Diabetes			
Yes	32	0.76 (0.30)	-
No	1312	0.81 (0.38)	0.40
Current smoker			
Yes	110	0.77 (0.34)	-
No	1234	0.81 (0.38)	0.20
Hypertension medication			
Yes	68	0.78 (0.36)	-
No	1276	0.81 (0.38)	0.48

SD: standard deviation.

* Student's t-test.

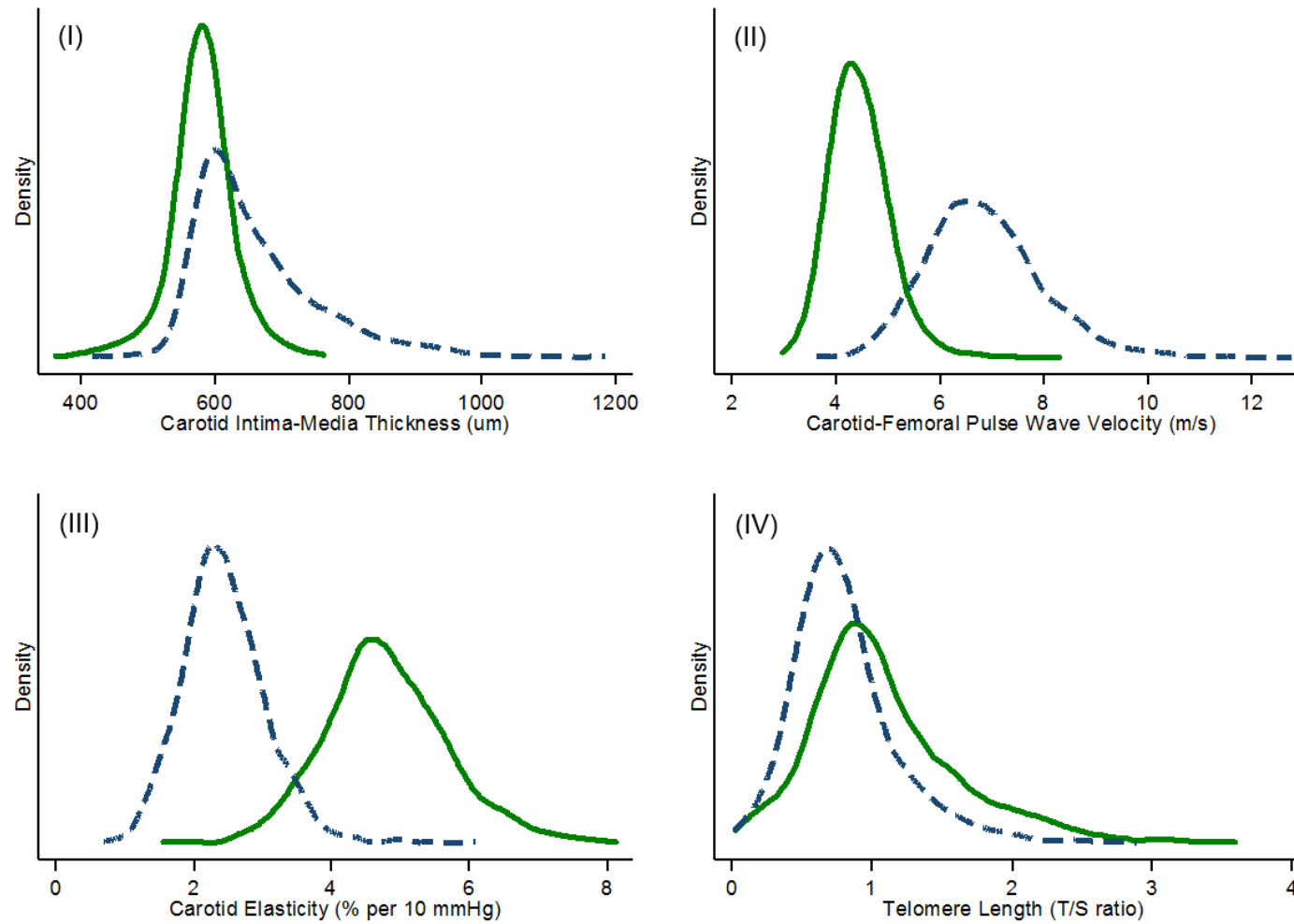
Table S4. Association of brachial systolic blood pressure (exposure) with telomere length (separately. outcome), in children and adults

per 10 mmHg	Children			Adults		
Outcome	N	Coefficient (95% CI)	p-value	N	Coefficient (95% CI)	p-value
Model 1*						
T/S ratio	1140	-0.02 (-0.06 to 0.02)	0.25	1141	-0.02 (-0.04 to -0.002)	0.03
Model 2†						
T/S ratio	1139	-0.002 (-0.04 to 0.04)	0.92	1136	-0.02 (-0.04 to -0.002)	0.03

* Model 1 adjusted for sex, age and sample type.

† Model 2 additionally adjusted for body mass index, Socio-Economic Indexes for Areas Index of Relative Socioeconomic Disadvantage score, plus smoking status for adults.

Figure S1. Children's (green, solid) and adults' (blue, dash) distribution of (I) carotid intima-media thickness, (II) carotid-femoral pulse wave velocity, (III) carotid elasticity and (IV) telomere length.



Supplemental References:

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