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Leukocyte telomere length and coronary artery calcification

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ABSTRACT

Objective: Leukocyte telomere length is representative of biological aging and is associated with clinical coronary artery disease but its association with coronary atherosclerosis is unclear. The objective of this study was to examine the association of telomere length with coronary artery calcification in middle aged adults.
Methods: Leukocyte telomere length was measured with a quantitative PCR-based technique and coronary artery calcification (CAC) scoring was performed on a dual-source CT scanner in a sample of 325 adults aged 40–64 years old free of previously diagnosed diabetes, CHD, stroke and cancer. We used logistic regression to determine the association of presence of CAC (Agatston score >0 versus 0) with telomere length adjusted for age, gender, race and metabolic syndrome. Finally, we examined the relation of telomere length to extensiveness of CAC.
Results: The unadjusted odds ratio of having CAC for the shortest tertile of telomere length versus the longest was 3.39 (95% CI 1.85–6.20). After adjustment for age, race, gender and metabolic syndrome the odds decreased but remained significant (OR 2.36; 95% CI 1.23–4.52). Mean telomere length was significantly shorter with more extensive coronary calcification. The correlation between telomere length and chronological age was $r = -0.19$ ($p < .001$) while the correlation between telomere length and arterial age was $r = -0.22$ ($p < .001$).
Conclusions: In conclusion, telomere length is negatively associated with the presence of coronary atherosclerosis in a low risk cohort free of previously diagnosed CVD.

1. Introduction

Telomere length has emerged as a marker that seems to represent biological aging which is the key to age-related morbidity [1]. Telomeres consist of TTAGGG tandem repeats and telomere binding proteins cap the ends of chromosomes and protect them from degradation. Telomeres become progressively shorter with each replication of somatic cells. Telomere attrition ultimately leads to a loss of replicative capacity.

Telomeres hold a unique advantage as a marker for biological aging and risk of disease development because they represent both an inherited predisposition to cell senescence as well as a cumulative lifelong burden of oxidative stress [2]. Several studies have shown that telomere length is familial [3–7]. Further, oxidative stress is associated with telomere shortening [8–10].

The relationship between telomeres and aspects of heart disease suggest that telomere length in white blood cells may be a useful marker for the biological aging of the cardiovascular system [11–13]. Recent human data show that development of atherosclerotic plaques is associated with progressive telomere shortening in vascular smooth muscle cells [14]. Shorter telomeres are also associated with hypertension, higher pulse pressure, coronary artery disease and risk of premature myocardial infarction [6,15–17]. In the Cardiovascular Health Study, there was a 3-fold increased risk of myocardial infarction and stroke associated with each kilobase shortening of telomeres [18], while in another study the risk of MI in individuals under age 50 years was higher for those with shorter telomere lengths compared to those in the highest quartile [16].

Coronary artery calcification (CAC) indicates the presence of coronary atherosclerosis and is a predictor of coronary events [19–23]. Moreover, because the amount of CAC is thought to reflect the degree of coronary atherosclerosis, CAC has been proposed as an indicator of biological or specifically, arterial age [24].

Although telomere length is suggested to be an indicator of biological age and CAC represents atherosclerotic plaque burden, the relationship between the two has not been investigated. Thus, we undertook a study to examine the relationship between telomere

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length and CAC in a sample of middle aged adults free of a history of cardiovascular disease.

2. Methods

2.1. Subjects

Subjects were recruited through health fairs, flyers and advertisements posted in the local health science university. In total 325 subjects, 40–64 years of age and free of diagnosed diabetes, CHD, stroke and cancer were studied. The subjects consisted of 184 non-Hispanic Whites (56.6%), 133 non-Hispanic Blacks (40.9%) and 8 other race/ethnicity (2.4%). This is consistent with the racial/ethnic demographics of the area. This study was approved by the Institutional Review Board of the Medical University of South Carolina.

2.2. Variables

2.2.1. Telomere length

Leucocyte telomere length was measured with a quantitative PCR-based technique that compares telomere repeat sequence copy number to single-copy gene (36B4) copy number in a given sample [25]. Duplicate DNA samples are amplified in parallel 25 μl PCR reactions comprising of 15 ng genomic DNA, 1x SensiMix NoRef SYBR Green master mix, 1x SYBR Green (Quantace, UK) and either 300 nmol/l of telomere-specific primers (forward: 5′GGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT3′; reverse: 5′GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT3′); or 300 nmol/l of the 36B4 forward primer (5′CAGCAAGTGGGAAGGTGTAATCC3′) primer and 500 nM of the 36B4 reverse primer (5′CCCATTCTATCATCAACGGGTACAA3′). All PCRs were run on a Corbett Research Rotor-Gene 6000 Real-time Thermal Cycler (Corbett Research, Cambridge, UK). The thermal cycling profile for begins with a 95 °C incubation for 10 min to activate the Taq DNA polymerase followed by cycling of 15 s at 95 °C and 1 min at 58 °C for either 20 cycles (telomere) or 30 cycles (36B4). Prior to running samples the linear range of the assay was determined by generating a standard curve using serially diluted DNA (200 ng – 1.56 ng in twofold dilutions) in quadruplicate. Both PCR reactions exhibited good linearity across this input range ($r^2 > 0.99$). Test samples were checked to confirm that they fall within this range, and any that did not were diluted as necessary and re-run. The specificity of all amplifications was determined by melting curve analysis. 48 study samples, a calibrator sample, and one no-template control sample (all in duplicate) were processed per run.

The PCR data was analyzed with the comparative quantitation approach as previously described and implemented with the Corbett Research Rotor-Gene 6000 version 1.7 analysis software [26]. During this analysis the amplification efficiency is calculated for each sample along with the mean efficiency of the run (MAE), which is used in calculating the relative concentration of each sample relative to the calibrator sample. This calculation coupled with the use of the same calibrator samples on all runs allows for any inter-run variation. This process is done for both telomere (T) and single-copy (S) gene reactions, and telomere length expressed as a ratio of the two, the T/S ratio, of the mean data from duplicate runs. All analyses were done blinded to patient characteristics.

The telomere length assay (T/S ratio) was checked for reproducibility by re-running 76 samples on a different day. The correlation (r) between the two runs was 0.946. The first tertile of telomere length was T/S ratio <1.525, the second tertile T/S ratio 1.525–1.772, and the third tertile T/S ratio >1.772. The minimum T/S ratio was 1.025 and the maximum T/S ratio was 2.623. Telomere length was normally distributed.

2.2.2. Coronary artery calcification

Coronary artery calcification scoring was performed on a dual-source CT scanner (Somatom Definition, Siemens Medical Solutions, Malvern, PA) using prospective ECG-triggering and a reconstructed section thickness of 1.5 mm without use of intravenous contrast material. Per convention, lesions with a mean attenuation of >130 Hounsfield units with an area of 1 mm squared were included in the calcium score. The Agatston score (Traditional Calcium Score) was calculated according to the method described by Agatston et al. using automated software (Circulation, Siemens) [27]. Briefly, the score is derived by measuring the plaque area and the maximal attenuation within each region of interest for each calcified coronary artery focus. The score is then calculated by multiplying the measured area of calcification per coronary segment by an attenuation coefficient based on the peak CT number (coefficient 1 for peak attenuation = 131–200 HU; coefficient 2 for peak attenuation = 201–300 HU; coefficient 3 for peak attenuation = 301–400 HU; coefficient 4 for peak attenuation ≥401 HU). The sum of the individual scores measured within the borders of each coronary artery is used to compute the final Agatston score. The radiologists reading the CT scans for computing CAC were blinded to the telomere length of the patient.

Patients were classified as having an Agatston score of 0 or an Agatston score >0 to indicate the presence of coronary calcium. Because the amount of calcium represents the extent of coronary atherosclerosis we also examined CAC as a continuous variable. Further, we also split CAC into three groups of 0, >0 up to 10, and >10. We also used the actual calcium score to estimate the arterial age of the subject. This construct represents the arterial age of the patient based on the level of atherosclerosis. Following the method proposed by McClelland we estimated arterial age with the equation *Arterial Age* = 39.1 + 7.25 ln(CAC + 1) [24].

2.2.3. Control variables

Age, gender race/ethnicity, smoking status and metabolic syndrome were assessed as potential confounding variables between telomere length and CAC. Smoking status was defined as current smoker or non-smoker. The metabolic syndrome is a multivariable measure that is associated with atherosclerosis and coronary heart disease. The metabolic syndrome was diagnosed when three or more of the following risk determinants were present: Waist circumference >102 cm for men and >88 cm for women, triglycerides ≥150 mg/dl, high-density lipoprotein cholesterol <40 mg/dl in men and <50 mg/dl in women, blood pressure ≥130/ ≥ 85 mmHg, or fasting glucose ≥110 mg/dl [28].

The Framingham risk score (FRS) is one of the most widely used CHD risk assessment tools but it has also been used as a measure to indicate the risk of atherosclerosis. A higher FRS is associated with a greater likelihood of having CAC [29–31]. Although the FRS for CHD has been computed in a variety of different ways since it was first presented, this project will use the version that was included in the ATP III [28]. The FRS from the ATP III is computed on individuals without diabetes or previous CHD. The FRS uses age, gender, measured total cholesterol, HDL cholesterol, systolic blood pressure and medications for hypertension, and current smoking status.

2.2.4. Analysis

Descriptive statistics were computed for the entire sample. We used logistic regression to determine the association of telomere length with presence of CAC (Agatston score >0 versus 0) in an unadjusted logistic regression. In an effort to determine whether telomere length has an independent relationship with CAC we conducted a second logistic regression adjusted for age, gender and race, and then a third logistic regression adjusted for age, gender, race, metabolic syndrome and smoking status. Because higher CAC

represents greater atherosclerosis we wanted to use the full range of values of CAC. We computed arterial age for each of the subjects. Neither CAC nor arterial age was used as a continuous variable since they were not normally distributed and so we computed Spearman's correlations between chronological age, CAC, arterial age and telomere length as a continuous variable. We also computed partial correlations between telomere length and CAC adjusting for age, race and gender. In the partial correlations between telomere length and arterial age we adjusted for gender and race. We did not adjust for chronological age because a chronological age measure is considered in the computation of arterial age. We also evaluated the two principal racial groups, non-Hispanic White and non-Hispanic Black for the relationship of telomere length and CAC in adjusted logistic regressions.

Mean telomere length (T/S ratio) was also calculated for three CAC categories of Agatston score 0, >0 up to 10, and >10. Unadjusted mean telomere length, mean telomere length adjusted for age, gender and race, plus mean telomere length adjusted for age, gender, race, metabolic syndrome and smoking status were calculated for each CAC group.

We computed a Pearson correlation between ln(CAC + 1) and telomere length. We also conducted unadjusted and adjusted linear regressions with ln(CAC + 1) as the dependent variable and telomere length as the independent variable. Adjustment was made for [1] age, gender and race/ethnicity, and [2] age, gender, race/ethnicity, metabolic syndrome and smoking status.

The FRS has been used as a predictor of presence of CAC and it is unclear whether the predictive value of the FRS can be improved by the addition of an individuals' telomere length. Consequently, we computed a logistic regression with the FRS on the presence of CAC and used the *C* statistic as an indicator of the explanatory power of the model. We examined the FRS as continuous 10-year risk and then again splitting the sample into individuals at low (<10%) versus intermediate/high risk (≥10%).

3. Results

The characteristics of the sample are presented in Table 1. A majority of the sample (75.8%) was overweight or obese, and 30.8% had presence of CAC (Agatston score >0).

Table 2 shows that shorter telomere length is associated with the presence of CAC in unadjusted and adjusted analyses. This indicates that telomere length is independently associated with sub-clinical atherosclerosis even after controlling for the metabolic syndrome and smoking status. Since both telomere length and CAC were collapsed into categories for the preceding analyses, further analyses examining telomere length and CAC as continuous variables were performed and yielded similar findings with shorter telomere length being associated with greater atherosclerosis. The Pearson correlation between ln(CAC + 1) and telomere length was $r = -0.21$, ($p < .001$). In an unadjusted linear regression, telomere length was correlated with ln(CAC + 1) with a parameter estimate of −1.39 ($p < .001$). Adjusting for age, gender and race/ethnicity, telomere length was correlated with ln(CAC + 1) with a parameter estimate of −0.84 ($p = .017$). Further adjustment with age, gender, race/ethnicity, metabolic syndrome and smoking status produced a parameter estimate of −0.76 ($p = .033$) for telomere length.

The Spearman correlation between telomere length and chronological age was $r = -0.19$ ($p < .001$) while the Spearman correlation between telomere length and CAC was $r = -0.22$ ($p < .001$). Similarly, the Spearman correlation between telom-

Table 1
Characteristics of the subjects by tertiles of telomere length (T/S ratio).

	Telomere length (T/S ratio)					
	First tertile		Second tertile		Third tertile	
	N	%	*N*	%	*N*	%
Age (years)						
40–52	50	27.8	61	33.9	69	38.3
53–64	57	40.4	46	32.6	38	27.0
Gender						
Male	57	39.6	46	31.9	41	28.5
Female	50	28.2	61	34.5	66	37.3
Race/ethnicity						
Non-Hispanic White	69	37.9	63	34.6	50	27.5
Non-Hispanic Black	35	26.7	40	30.5	56	42.8
Hispanic	1	25.0	2	50.0	1	25.0
Other race	2	50.0	2	50.0	0	0.0
Body mass index (kg/m^2)						
<25	34	43.6	21	26.9	23	29.5
25–29.9	37	30.3	35	28.7	50	41.0
≥30	36	30.2	49	41.2	34	28.6
Metabolic syndrome						
Yes	27	37.5	24	33.3	21	29.2
No	80	32.8	81	33.2	83	34.0
Smoking status						
Non-smoker	99	33.2	99	33.2	100	33.6
Current smoker	8	34.8	8	34.8	7	30.4
Coronary artery calcium (Agatston score)						
0	57	25.7	80	36.0	85	38.3
>0	50	50.5	27	27.3	22	22.2
Framingham risk score (10-year risk)						
<10%	91	32.8	91	32.8	95	34.3
10–20%	16	39.0	14	34.2	11	26.8
>20%	0	0.0	1	50.0	1	50.0

Table 2
Unadjusted and adjusted logistic regressions testing the association of coronary artery calcium with telomere length.

	Odds ratio (95% CI)	Odds ratio (95% CI)[a]	Odds ratio (95% CI)[b]
N	321	321	316
Telomere length (T/S ratio)			
First tertile	3.39 (1.85–6.20)	2.50 (1.32–4.73)	2.40 (1.25–4.62)
Second tertile	1.30 (0.69–2.47)	1.05 (0.54–2.07)	0.99 (0.49–2.00)
Third tertile	1.00	1.00	1.00
C statistic	0.636	0.729	0.750

[a] Adjusted for age, gender, and race.
[b] Adjusted for age, gender, race, metabolic syndrome and smoking status.

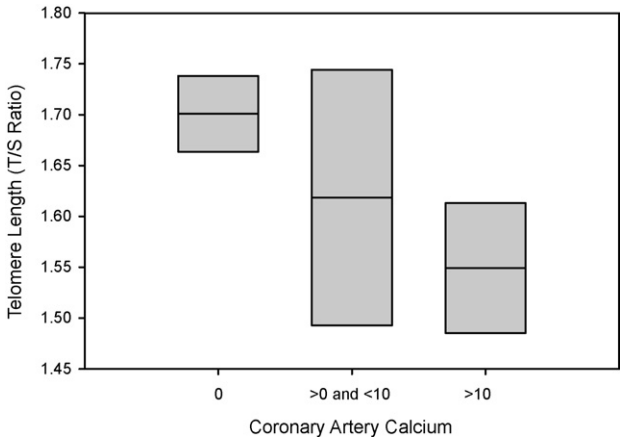

Fig. 1. Unadjusted mean telomere length (T/S ratio) and 95% confidence interval for three classes of coronary artery calcium (Agatston score).

ere length and arterial age was $r = -0.22$ ($p < .001$). The partial correlation between telomere length and CAC controlling for age, race and gender decreased slightly but remained significant ($r = -0.15$, $p = .009$). The partial correlation between telomere length and arterial age controlling for race and gender was $r = -0.19$ ($p < .001$).

Analyses of non-Hispanic Whites and non-Hispanic Blacks showed there were racial differences in the relationship of telomere length and CAC. For non-Hispanic Whites the odds ratio for the first tertile of telomere length versus the third tertile of telomere length was 2.96 (95% CI 1.17–7.47) in a logistic regression adjusted for age, gender, metabolic syndrome and smoking status. In non-Hispanic Blacks, the odds ratio for the first tertile of telomere length versus the third tertile of telomere length was 1.86 (95% CI 0.69–4.99) in an adjusted logistic regression.

Unadjusted mean telomere lengths and 95% confidence intervals are shown for three CAC categories in Fig. 1. Adjusted mean telomere lengths for the three CAC categories are shown in Table 3. In each comparison the mean telomere length for CAC of 0 was significantly different ($p < 0.05$) from that of CAC >10 indicating shorter telomere length with more extensive atherosclerosis.

When the FRS is classified as low versus intermediate/high risk the model predicting presence of CAC improves from a *C* of 0.55 to a *C* of 0.65 when telomere length is added. The odds ratio for the first tertile of telomere length versus the third tertile of telomere length in this second analysis is 3.35 (95% CI 1.82–6.16).

4. Discussion

In this study we show that there is a significant inverse relationship between telomere length and coronary artery calcification, a marker of coronary atherosclerosis in individuals with no clinical history of coronary heart disease. The findings provide additional support to findings regarding the association of telomere length with coronary artery disease and support the concept that telomere length represents a measure of biological aging.

The observation of the inverse relationship between telomere length and coronary artery calcification extends previous studies showing an association between shorter telomere lengths and clinical coronary phenotypes [17,18]. The demonstration that such an association exists in asymptomatic healthy subjects confirms that the relationship is not simply a consequence of the development of clinical disease [26]. Indeed, the strength of association of shorter telomeres with coronary calcification as indicated by the odds ratios for the tertile with the shortest telomere (>3.0) is stronger than that seen with clinical phenotypes, perhaps reflecting the more precise quantitative coronary phenotype studied [16,26,32]. Our finding, by itself, does not imply a causal association between shorter telomere length and coronary atherosclerosis. However, vascular cellular senescence is a hallmark of atherosclerosis atherosclerosis [14]. Interestingly, the association of shorter telomere length with coronary calcification was not linear; the odds ratio in the middle tertile was not significantly different from that in the highest tertile suggesting that if the relationship is causal then there may be some threshold before telomere length impacts on relevant vascular biology.

This study should also be considered in the context of other recent findings that have examined an association with shorter telomere length with carotid intima-media thickness (IMT) [33,34]. In one study, telomere length was not a significant independent determinant of IMT while in another study, there was a significant inverse association between telomere length and common carotid artery IMT in obese men. Our findings of a significant inverse relationship between telomere length and CAC may be because although CAC and IMT are both representative of atherosclerosis,

Table 3
Unadjusted and adjusted means of telomere length with extensiveness of coronary artery calcium.

	Adjusted mean telomere length (T/S ratio)[a]	Adjusted mean telomere length (T/S ratio)[b]
N	321	316
Coronary artery calcium (Agatston score)		
(a) 0	1.69[c]	1.68[c]
(b) >0 and ≤10	1.64	1.65
(c) >10	1.58	1.58

[a] Adjusted for age, gender, and race.
[b] Adjusted for age, gender, race, metabolic syndrome and smoking status.
[c] Mean T/S ratios of 'a' and 'c' are significantly different at $p < 0.05$; 'b' is not significantly different from either 'a' or 'c'.

CAC is a better predictor of both CVD and CHD than is IMT [22,35]. CAC and IMT are global atherosclerosis measures and can be used for CVD risk assessments but among asymptomatic adults, CAC is more predictive of CHD. Thus, telomere length may be more related to CHD than stroke risk.

Our analysis is based on a cross-sectional analysis and a surrogate marker of coronary events. Therefore, further prospective studies in large population-based cohorts are required before the integration of telomere length assessment into cardiovascular risk assessment in clinical practice can be recommended. In this context it is relevant to note that in an analysis of the West of Scotland Prevention Study (WOSCOPS), Brouilette et al. found an attenuation of risk in individuals with shorter telomeres who were treated with pravastatin, regardless of lipid level [26]. In contrast, individuals with the longest telomeres received no apparent benefit from statin treatment [26].

The results of the race stratified results indicated a significant relationship between telomere length and CAC among non-Hispanic Whites but not among non-Hispanic Blacks. Although, the results among non-Hispanic Blacks suggested a similar relationship to that found in non-Hispanic Whites it did not reach statistical significance. This finding may be a result of the racial differences previously discovered indicating that although non-Hispanic Blacks males have greater stroke risk and a worse cardiovascular risk profile than non-Hispanic Whites, they have a lower prevalence of CHD and presence of coronary artery calcification [36,37]. The relationship between telomere length and CAC may be more complex once racial differences are considered.

There are several limitations to this study, in addition to its modest size. Notably, we used a commonly employed volunteer-based strategy for recruitment of an asymptomatic sample. However, we did not recruit systematically on a community wide basis. Although, it is unlikely that the imaging-based phenotype we studied would have been influenced by the recruitment strategy, we cannot entirely rule out the possibility of selection bias. Generalizability of our findings therefore requires further studies in probability-based cohorts representative of the population. Furthermore as discussed earlier while our design (cross-sectional) allows us to examine the association between telomere length and atherosclerosis, it limits our ability to infer whether short telomere length leads to accelerated development of atherosclerosis over time.

In conclusion, telomere length is negatively associated with the presence of atherosclerosis. Furthermore, telomere length is more highly correlated to a measure of arterial age than chronological age, supporting its use as a measure of biological aging. Further studies are needed to evaluate the relevance of telomere length assessment in improving risk assessment strategies.

Conflict of interest

The authors of this manuscript have no conflicts of interest to disclose.

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