Telomerase, telomere length, and coronary artery calcium in black and white men in the CARDIA study

Candice H. Kroenke a,*, Mark J. Pletcher b, Sue Lin c, Elizabeth Blackburn c, Nancy Adler d, Karen Matthews e, Elissa Epel d,***

a Kaiser Permanent Division of Research, 2101 Webster Street, 4th floor, Oakland, CA 94612, USA
b University of California, San Francisco, Department of Epidemiology and Biostatistics, San Francisco, CA, USA
c University of California, San Francisco, Department of Biochemistry, San Francisco, CA, USA
d University of California, San Francisco, 3333 California St., Suite 465, San Francisco, CA 94118, USA
e University of Pittsburgh, Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

** Corresponding author. Tel.: +1 510 891 2710.
*** Corresponding author. Tel.: +1 415 476 7648.
E-mail addresses: candice.h.kroenke@kp.org (C.H. Kroenke), eepeel@pitt.edu (E. Epel).

A B S T R A C T

Objective: To evaluate whether telomerase activity, measured in circulating blood leukocytes, might be associated with prevalent atherosclerosis, or predict future coronary artery disease risk.

Methods and results: We examined associations of telomerase activity levels measured at year 15 in the Coronary Artery Risk Development in Young Adults (CARDIA) Study with prevalent coronary artery calcium (CAC), progressive CAC at year 20, and incident CAC between years 15 and 20, in 440 black and white men aged 33-45 years. Telomere length was also measured in a subset of participants (N=129).

In multivariate-adjusted analysis, higher quartiles of telomerase were cross-sectionally associated with greater odds of prevalent CAC at year 15 (quartile 2: OR=1.32, 95% CI: 0.54–3.23; quartile 3: OR=1.40, 95% CI: 0.60–3.30; quartile 4: OR=3.27, 95% CI: 1.39–7.71 compared with quartile 1, p-continuous=0.012) and progressive CAC at year 20, but telomerase was not significantly associated with incidence of newly detectable CAC. Higher telomerase activity levels predicted greater CAC progression at year 20 among persons with short telomere length; low telomerase and short TL predicted less CAC progression.

Conclusion: Telomerase activity in leukocytes was associated with calcified atherosclerotic plaque, and was also a predictor of advancing plaque among persons with short telomeres.

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1. Introduction

Telomeres are DNA–protein complexes consisting of short tandem DNA repeats that, along with telomere-associated proteins, protect the ends of eukaryotic chromosomes from degradation and recombination.

The enzyme telomerase both lengthens telomeres, independently promotes cell growth and longevity, and may increase in response to short telomere length (TL) in an attempt to maintain TL and thus cell integrity. Though telomerase has been shown to increase TL, and this should theoretically be cardioprotective, a growing number of studies suggest that development of atherosclerosis at the earliest stages is associated with, and may be facilitated by, telomerase activation [1]. Elevated telomerase activity in cardiomyocytes has been detected in age-related cardiomyopathy [2], in heart tissue from patients with cardiac hypertrophy [3], and in human atherosclerotic coronary artery specimens [4,5]. Gupta et al. found a suggestive association between telomerase activity in coronary artery tissue and restenosis in 23 patients undergoing coronary atherectomy [4]. Telomerase activation is associated with greater vascular smooth muscle cell proliferation, which may underlie early development of atherosclerosis [1,6–8] and telomerase inhibition is related to decreased vascular smooth muscle cell proliferation [6]. It is unclear, however, whether elevated telomerase activity measured in peripheral blood mononuclear cells (PBMCs) might be a marker of prevalent atherosclerosis, a protective factor via telomere lengthening, or a factor which increases risk of developing atherosclerosis.

The Coronary Artery Risk Development in Young Adults (CARDIA) Study provided a unique opportunity to study the association between white blood cell telomerase activity and coronary artery calcium (CAC), a measure of calcified atherosclerosis, in black and
white men of low and high socioeconomic status. We also examined the association of telomerase activity and telomere length (TL) with CAC in a subset of the participants with TL data.

2. Methods

2.1. Study population

CARDIA is a longitudinal investigation of coronary artery disease risk factors in a population aged 18–30 years at study inception. Details of study design, recruitment, and procedures have been published elsewhere [9,10]. To date, CARDIA has completed 6 examination cycles: a baseline examination during the 1985–1986 study year (n = 5115) and follow-up examinations at years 2, 5, 7, 10, 15, and 20 that achieved high retention rates.

We selected a stratified random sample of CARDIA men with a fasting (>8 h) blood sample drawn at the year 15 exam [10]. The sample was stratified by race (black and white) and socioeconomic status (SES), defined as high SES (education < 15 years and income $50,000) and low SES (education = 15 years and income <$50,000). Within these four strata (high SES black, high SES white, low SES black, low SES white), we randomly selected 600 participants and assayed telomerase; of these, 523 had viable telomerase values (i.e., cells were still living). Of these men, we measured telomere length in a subgroup (n = 154) for whom extracted DNA was available at year 15.

Coronary artery calcium (CAC) was measured in 3043 CARDIA participants at year 15, and in 3139 participants at year 20. Of those 523 men with telomerase data in our study subsample, 440 had CAC data at year 15 and 386 had CAC data at year 20. Of the 154 men with data on both telomerase and TL, 129 had CAC data at year 15 and at year 20. To maximize power, we allowed the sample size to vary across analyses. For those missing covariate data at year 15 (~1%), we assigned values from the year 10 exam.

2.2. Data collection

2.2.1. Coronary artery calcium (EBT scanning and reading protocol)

Subclinical atherosclerosis was measured via computed tomography (CT) during years 15 and 20 [11,12]. Two sequential scans were obtained using either an electron beam or multi-detector electrocardiographically gated cardiac computed tomography scanner, with a standard phantom for calibration. Image analysts blinded to participant characteristics and paired scan results calculated a total coronary artery calcium score using a modified Agatston method [13], with select overreading by a physician expert in cardiovascular imaging. Accuracy, comparability, and reproducibility of these methods have been published [14,15].

We labeled participants with any measurable CAC at year 15 as having “prevalent CAC”. For those without any measurable CAC at year 15 but with new measurable CAC at year 20, we designated those participants as having “incident CAC”. In the larger CARDIA study, nearly all of the CARDIA participants with CAC at year 15 showed progression in Agatston score (i.e., higher amounts of coronary calcium) by year 20 [12]. Biologically relevant, increasing coronary calcium is correlated with increased risk of atherosclerosis [16]. Thus, we designated the group of participants having CAC at year 20 as having “progressive CAC” or advancement of calcified atherosclerosis. This outcome included incident cases; those who had CAC at year 15, all of whom increased in levels of coronary calcium between years 15 and 20; and several cases for whom we did not have information at year 15 but would likely be similar in character to the first two groups given the pattern of CAC advancement in the overall cohort.

2.2.2. Blood draw and sample preparation

Blood samples were drawn by venipuncture, according to a standard protocol [10], at the year-15 exam, in the morning after an overnight fast (>8 h) using EDTA-containing tubes. PBMCs were purified from blood using Ficol-Paque PLUS (Amersham) according to the manufacturer’s instructions and cryopreserved and stored in liquid nitrogen until assays were performed. DNA for TL was prepared using Gentra Puregene Cell Kit (QIAGEN, Valencia, CA, USA).

2.2.3. Telomerase activity

PBMCs were thawed at 37 °C and live cells counted; 0.5 million cells were resuspended in RPMI 1640 medium and shipped overnight to Dr. Elizabeth Blackburn’s lab at the University of California, San Francisco, on ice. Cryopreserved PBMCs were thawed at 37 °C, washed with 10 ml of cold DPBS twice (PBS without Mg++ and Ca++, Biosource) and live cells were counted using a hemocytometer with the trypan exclusion method. 1 × 10⁶ viable cells were pelleted and lysed with 1 × CHAPS buffer as directed by the manual for the Trapeze kit. An extract corresponding to 5000 cells/μl was made and two concentrations, corresponding to 5000 and 10,000 cells were used for TRAP reactions for each sample to ensure linearity of the assay. The reaction was carried out according to the Trapeze kit manual and run on a 10% polyacrylamide–8 M urea sequencing gel. The gel was exposed to a phosphorimager plate overnight and scanned on a STORM 860 (GE Healthcare, Piscataway, NJ). The 293T cancer cell line was used as a positive telomerase activity control and standard; telomerase activity is expressed as equivalent of number of 293T cells (1 unit = the amount of product from one 293T cell/10,000 PBMCs). Telomerase activity was quantified using the software ImageQuant 5.2 (GE Healthcare, Piscataway, NJ).

2.2.4. Leukocyte telomere length

The telomere length measurement assay was adapted from the published original method [17,18]. Telomere length values were measured from DNA by a quantitative PCR assay that determines the ratio of telomere repeat copy number to single-copy gene copy number (T/S) ratio in experimental samples as compared with a reference DNA sample. Higher T/S ratio signifies longer mean telomere length.

2.2.5. Measurement of covariates

Educational attainment, smoking, and alcohol consumption were ascertained by self-report. An interviewer-administered follow-up questionnaire was given to those who reported drinking or smoking. Alcohol consumption was assessed as the average number of drinks per week, multiplied by ethanol concentration [19]. Participants were asked whether or not they currently smoke. Based on an interviewer-administered physical activity history, total activity was computed as the sum of frequency and intensity scores for 13 categories of vigorous and moderate activity over the previous 12 months [20,21]. Chronic burden was assessed as the number of domains (health of close others, work, finances, and relationships) in which participants reported experiencing moderately or very stressful ongoing problems lasting 6 months or longer [22].

Blood pressure was measured on the right arm with a Hawksley random zero sphygmomanometer [23]. Total cholesterol, triglycerides, and HDL were measured, and LDL was computed using the Friedewald equation [24–28]. Body mass index was calculated as weight in kilograms divided by the square of height in meters. A high-sensitivity ELISA measured year 15 serum C-reactive protein (CRP) at the Department of Pathology, University of Vermont, using a BN-II analyzer [29]. Covariates were assessed at year 15.
2.3. Statistical analysis

Using analysis of covariance (PROC GLM), we evaluated whether telomerase levels, TL, and presence of any CAC differed by race and SES. We also used analysis of covariance, adjusted for age, to compare participants on a variety of demographic, psychosocial, and lifestyle characteristics, by quartiles of telomerase (Table 1).

2.3.1. Telomerase and CAC

Because of stratified sampling and because biological relationships may vary by group, analyses were initially stratified by race and SES. However, associations did not differ substantively by group. Thus, we combined groups and obtained overall estimates for most analyses (with adjustment for race and SES). We used logistic regression (SAS PROC LOGISTIC) to evaluate associations between continuous log telomerase or quartiles of telomerase and any CAC at year 15. We further evaluated associations with progressive CAC at year 20. We also examined development of new CAC between exams (i.e., incident CAC at year 20 excluding persons with any CAC at year 15). In continuous analyses, we used the log of telomerase to improve normality of the independent variable, model stability, and the strength of the linear association.

In developing multivariate-adjusted models, we considered several covariates (see Table 1). Except for the categorical race-SES variable, covariates were modeled continuously (age, body mass index (BMI), total cholesterol, HDL cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, C-reactive protein, physical activity, alcohol intake, chronic burden, average CES-D score) or dichotomously (diabetes, current smoking). In a final model, we selected those covariates which were at least marginally associated ($p < 0.15$) either with CAC or with log telomerase, adjusted for age and race-SES strata.

2.3.2. The combination of telomerase and telomere length and CAC

Though shorter TL has been related to a higher risk of coronary heart disease [30–33], a mechanistic study by Poch et al. demonstrated the potentially proliferative influence of long (vs. short) telomeres on the development of atherosclerosis in mice [34]. It is thought that the development of atherosclerosis may not be possible with telomere exhaustion [1]. Thus, if telomeres and telomerase both have proliferative effects, we wanted to explore whether those with short TL and low telomerase might have a low CAC risk. Since the combined state of short TL and elevated telomerase has also been related to lower endogenous estrogen levels [35], and to higher levels of stress and depression [36,37], we wanted to examine whether this particular state might be related to a higher risk of CAC.

We evaluated the Spearman correlation between TL and telomerase at year 15 and used $t$-tests to compare those with and without TL data. In the subgroup of participants with measures of both telomerase and TL, we examined associations of log telomerase with CAC, stratifying by long vs. short TL (above or below median TL = 1.23). We further evaluated the combination of the two variables by evaluating the association between the cross-classification of TL and telomerase and CAC. We classified participants as having long or short TL and high or low telomerase, based on median values of each (median telomerase = 2.18 activity/10,000 cells), and then examined CAC in association with the cross-classified categories of TL and low telomerase, short TL and low telomerase, long TL and high telomerase, or short TL and high telomerase. We evaluated possible differences between groups with likelihood ratio $\chi^2$ tests, comparing models with and without the cross-classified variable. We evaluated the interactions of both the continuous and dichotomous TL and telomerase variables using Wald tests. Finally, using contrast statements, we compared the group characterized as...
having short TL and high telomerase to the other three TL/telomerase groups and also compared the group defined as having short TL and low telomerase, to the other three groups to determine whether the first group was more likely, and whether the second group was less likely, to have progressive or incident CAC.

The SAS version 9.2 statistical software (SAS Institute, Cary, NC) was used in all statistical analyses. The institutional review boards of the centers involved approved the study, and all participants gave informed written consent.

3. Results

Black men of low SES had higher levels of telomerase in their PBMC samples compared with other race-SES groups, who had similar mean telomerase levels (3.3 vs. 2.3 units of activity per 10,000 cells, p < 0.0001). Though black men comprised 40% of the sample, they were overrepresented in the highest quartile of telomerase, as were men of low SES. Prevalence of current smoking and levels of C-reactive protein were positively associated with telomerase activity. Most behavioral and cardiovascular disease risk factors, however, did not vary by quartile of telomerase activity (Table 1).

In age- and race-SES-adjusted logistic analyses, year-15 telomerase was positively associated with any CAC at year 15, OR = 1.99 per log unit greater activity, 95% CI: 1.23–3.24 (Table 2). We found no evidence of effect modification by race-SES strata (Wald $\chi^2 = 2.2$, p-interaction = 0.53, df = 3). Exclusion of men with diabetes did not diminish the association (OR = 2.18, 95% CI: 1.30–3.65). Upon adjustment for covariates, associations between telomerase and CAC were only modestly attenuated. Continuous (log) telomerase was not related to any CAC at year 20 or with development of new CAC between exams (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Outcome</th>
<th>N</th>
<th>CAC (%)</th>
<th>OR (95% CI), adjusted for age, race-SES strata</th>
<th>OR (95% CI), MV-adjusted$^b$</th>
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<tr>
<td>Year 15 coronary artery calcium</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Quartile 1 (reference)</td>
<td>115</td>
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<td>1.00</td>
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<td>Quartile 2</td>
<td>111</td>
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<td>1.40 (0.60–3.30)</td>
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<td>3.27 (1.39–7.71)</td>
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<td>Continuous log telomerase</td>
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<td>1.99 (1.23–3.22)</td>
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<td>p-Value$^c$</td>
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<td>Year 20 coronary artery calcium</td>
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<td></td>
<td></td>
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<tr>
<td>Quartile 2</td>
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<td>1.88 (1.00–3.54)</td>
<td>1.86 (0.96–3.61)</td>
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<td>Continuous log telomerase</td>
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<td>1.34 (0.90–1.99)</td>
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<td>p-Value$^c$</td>
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<td>Incident coronary artery calcium (year 15 – 20)</td>
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<td></td>
<td></td>
<td></td>
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<td>1.00</td>
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<td>19</td>
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<td>Continuous log telomerase</td>
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<td>p-Value$^c$</td>
<td></td>
<td></td>
<td>0.99</td>
<td>0.95</td>
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</table>

$^a$ The odds ratio (OR) is defined as the increased odds of CAC per unit increase in log telomerase (activity per 10,000 cells). In this sample, telomerase activity ranged from 0.4 to 17.2 and log telomerase ranged from −0.99 to 2.84.

$^b$ Multivariable-adjusted logistic models adjusted for age, race-SES strata, diabetes, total cholesterol, HDL cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, C-reactive protein, current smoking, physical activity, alcohol intake, stress burden, and chronic depression (average CES-D score for years 7, 10, and 15).

$^c$ p-Value, continuous log telomerase.

Fig. 1. Quartiles of telomerase and prevalence of coronary artery calcium at year 15, in age and race-SES adjusted analysis, p-value = 0.005 for log of continuous variable. Multiple adjustment did not markedly alter the association. Compared to the reference, adjusted odds ratios were OR = 1.32 (95% CI: 0.54–3.23) for quartile 2, OR = 1.40 (95% CI: 0.60–3.30) for quartile 3, and OR = 3.27 (95% CI: 1.39–7.71) for quartile 4, p-value for log of continuous variable = 0.005. Quartiles of telomerase were not associated with progressive coronary artery calcium at year 20. p-continuous = 0.005 (Table 2 and Fig. 1). Multiple adjustment slightly attenuated associations (p-continuous = 0.012). Though telomerase quartiles were not monotonically related to progression of CAC at year 20, those in the highest quartile of telomerase had significantly higher odds of CAC at that timepoint compared with the reference (OR = 2.14, 95% CI: 1.05–4.35 (Table 2).

The Spearman correlation between year 15 telomerase and TL was $r = −0.01$, p = 0.87. Those with and without TL data were similar except for telomerase quartile (2.4 vs. 2.7 per 10,000 cells, p = 0.05) and prevalence of smoking (30.9% vs. 20.9%, p = 0.03). In the subsample of those with information on both telomerase and TL, adjusted for both age and race-SES strata, log telomerase was positively associated with CAC at year 15 (OR = 3.00 per log unit, 95% CI: 0.86–10.5) and year 20 (OR = 2.34 per log unit, 95% CI: 0.74–7.4).
CI: 1.08–5.05), but there was no association with incident CAC (OR = 1.27, 95% CI: 0.42–3.92), similar to the pattern seen in the full sample. Similarly, among those with data on TL, higher quartiles of telomerase were associated with year 15 CAC (quartile 2, OR = 1.58 (95% CI: 0.19–13.14), quartile 3, OR = 2.35 (0.36–15.31), and quartile 4, OR = 7.18 (1.01–50.88) vs. quartile 1) and year 20 CAC (quartile 2, OR = 2.18 (95% CI: 0.59–8.04), quartile 3, OR = 2.92 (0.86–9.89), and quartile 4, OR = 4.65 (1.25–17.29) vs. quartile 1), but not incident CAC (data not shown).

However, in both cross-sectional and prospective analyses, we noted evidence of interactions with TL in stratified analyses. log telomerase was significantly, positively associated with progressive CAC at year 20 among those with short TL, OR = 7.03 (95% CI: 1.63–30.3), but was not associated among those with long TL, OR = 1.04 (95% CI: 0.44–2.46), p-interaction = 0.02 (employing log continuous TL and telomerase). We noted a similar pattern at year 15 and for analyses of incident CAC, although these analyses were underpowered and nonsignificant (data not shown). Analysis of cross-classifications of long vs. short TL and high vs. low telomerase (median splits) showed the lowest proportion with year 20 CAC (progressive and incident cases) in persons with low telomerase and short TL (14% progressive and 6% incident CAC) and the highest in persons with high telomerase and short TL (46% progressive and 25% incident CAC), whereas persons with long TL had intermediate values (31% progressive and 12% incident CAC among those with long TL and high telomerase; 30% progressive and 21% incident CAC among those with long TL and low telomerase) irrespective of telomerase levels (Fig. 2, likelihood ratio (LR) $\chi^2 = 8.4$, $p = 0.08$, p-value, test for the interaction between TL and telomerase = 0.02 for progressive CAC at year 20). Findings with incident CAC appeared similar but were underpowered and nonsignificant (data not shown).

Those with high levels of telomerase and short TL had greater progressive (Wald $\chi^2$, test for contrast = 6.0, $p = 0.01$) and incident (Wald $\chi^2$, test for contrast = 3.4, $p = 0.06$) CAC than the other three telomerase/TL groups. In contrast, those with low telomerase and short TL had lower progressive CAC (Wald $\chi^2$ test for contrast = 5.8, $p = 0.02$) and marginally lower incident CAC (Wald $\chi^2$ test for contrast = 2.3, $p = 0.13$) than other groups.

4. Discussion

We found a positive association between elevated telomerase activity and prevalent CAC, but no association with new development of CAC over five years. However, in a subset of participants for whom we had measurements of TL, we found evidence of positive associations between telomerase activity and CAC (progressive and incident) in persons with short TL, no association among those with high TL, and the lowest CAC incidence in persons with the combination of short TL and low levels of telomerase activity. Adjusting for C-reactive protein and other cardiovascular risk factors did not substantively influence associations. Patterns of association were consistent for black and white men and for persons with lower and higher SES. Our findings suggest that elevated telomerase may coincide with early CAC development and that combined information on telomerase and TL may be useful in predicting CAC development prospectively. To our knowledge, this is the first epidemiologic study examining the relationship between telomerase and atherosclerotic disease in humans, in the largest sample of men with telomerase data currently available.

Consistent with the notion of elevated telomerase coinciding with a diseased state, telomerase was positively associated with CAC cross-sectionally. Previous studies have examined small numbers of patients with well-established coronary artery disease. The positive cross-sectional association and the prospective finding for the combination of telomerase and TL with CAC lends credence to previous work suggesting that elevated levels of telomerase may promote early plaque formation [1]. The lack of independent association of telomerase with CAC prospectively is consistent with the previous notion that telomerase activity could rise markedly in the
early stages of atherosclerosis development and decline later with established disease [1].

Our results suggest, however, that the combination of telomerase and TL may be more predictive of atherosclerosis prevalence and risk than either variable alone, and further suggest the need to consider alternative mechanisms in understanding atherosclerosis development. Previous studies have found consistent inverse associations of TL with coronary heart disease (CHD) in cross-sectional [30–32] and prospective studies [33]. However, despite previous findings, it has been unclear what potential role telomeres may have in the development of coronary disease since cardiovascular death and CHD events occur years after initial development of atherosclerosis, and telomere shortening may be a consequence rather than a cause of coronary disease, augmented by the underlying proliferative effect of the chronic inflammatory response associated with greater cardiovascular risk.

Our finding of an apparently higher CAC prevalence among those with elevated telomerase and short TL is consistent with the notion that this pattern may be a marker of a diseased state. It is somewhat unclear whether this is due to the influence of disease on TL and telomerase or to the effects of cellular aging on disease. Cellular aging caused by telomere shortening could lead to CAD through a reduced ability of senescent cells to repair vascular injury, while inflammation associated with established cardiovascular disease may shorten telomeres. However, adjustment for C-reactive protein, a marker of inflammation, had little effect on the associations we detected, suggesting that inflammation as a common cause of both coronary artery disease and cellular aging may not explain our findings.

Our suggestive finding showing a lower risk of having any CAC among those with both short TL and low telomerase is consistent with the potential proliferative influence of both telomeres [34] and telomerase on one contributor to early atherosclerosis development. Nevertheless, more research is needed to clarify these associations. For one, it is necessary to ascertain what states are consistent with both disease outcomes and with optimal health. These findings need to be replicated in a larger sample and should also include women.

Study strengths include the ability to evaluate both cross-sectional and longitudinal associations in a diverse group of black and white men of low and high socioeconomic status, and to evaluate associations adjusted for cardiovascular risk factors. We had the unique opportunity to examine associations of telomere length and telomerase with the earliest development of detectable coronary artery disease.

The most notable limitation of this study was limited power to examine associations with variables characterized in part by telomere length. Further research in a larger sample could help confirm and clarify potential relationships. The relationships between PBMC telomerase and leukocyte TL with telomerase and TL in cardiomyocytes are also not firmly established. Thus, it is unclear whether these are mechanistically related in disease development or whether the former are markers for the latter, with both perhaps influenced by the same underlying disease processes. Another potential limitation is measurement error. It has been suggested that measurement of TL by quantitative PCR as compared with Southern blot may be less accurate [38]. However, previous findings suggest consistent results across these two methods [39].

Finally, as in all observational studies, it is impossible to rule out the possibility of unmeasured residual confounding. However, one of the advantages of the CARDIA study has been the measurement of numerous potential confounding variables in an enduring cohort with careful attention to methodology and high retention. Finally, our sample for this cellular aging ancillary study of CARDIA included only men, and thus our findings should not be generalized to women.

In summary, high levels of telomerase predicted higher prevalence of CAC in an epidemiologic cohort of young to middle-aged black and white men, and this association was stronger in persons with shorter telomeres. When considered in the context of prior research, our findings suggest that telomerase may be a marker of a proliferative disease state, and, in combination with short telomeres, may be a marker of greater future risk of disease. Future studies should examine cellular aging associations in women, replicate our results in men, and analyze the joint predictive power of telomerase and telomere length on future development of clinical CHD.

Disclosures

E. Epel, E. Blackburn, and J. Lin are co-founders of Telome Health, Inc., a telomere health biotechnology company.

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