Prognostic Value of Leukocyte Telomere Length in Patients With Stable Coronary Artery Disease
Data From the Heart and Soul Study

Ramin Farzaneh-Far, Richard M. Cawthon, Beeya Na, Warren S. Browner, Nelson B. Schiller, Mary A. Whooley

Background—Telomere shortening has been proposed as a marker of biological aging. Whether leukocyte telomere length is associated with mortality among patients with stable coronary artery disease (CAD) is unknown.

Methods and Results—We measured leukocyte telomere length in 780 patients with stable CAD in a prospective cohort study. Participants were categorized by quartiles of telomere length. Hazard Ratios (HRs) and 95% confidence intervals were calculated for all-cause mortality, heart failure (HF) hospitalization, and cardiovascular (CV) events. After 4.4 years of follow-up there were 166 deaths. Compared with participants in the highest telomere length quartile, those in the lowest quartile were at increased risk of death (age-adjusted HR 1.8; 95% CI 1.2 to 2.9). After multivariate adjustment for clinical (HR 2.1; CI 1.3 to 3.3), inflammatory (HR 2.0; CI 1.2 to 3.2), and echocardiographic (HR 1.9; CI 1.0 to 3.5) risk factors, patients in the lowest quartile of telomere length remained at significantly increased risk of death compared to those in the highest quartile. Patients in the lowest quartile of telomere length were also at significantly increased risk of HF hospitalization (HR 2.6; CI 1.1 to 6.0) but not CV events (HR 1.7; CI 0.9 to 3.5).

Conclusions—Reduced leukocyte telomere length is associated with all-cause mortality in patients with stable CAD. The prognostic value of short telomeres in predicting death is not completely captured by existing clinical, inflammatory, and echocardiographic markers of risk.

Key Words: telomere ■ aging ■ leukocyte ■ prognosis ■ coronary

Telomeres are specialized tandem DNA repeat sequences (TTAGGG), located at the ends of eukaryotic chromosomes which protect somatic cells from genomic instability during mitotic cell proliferation.1,2 During each cell division, DNA polymerase cannot fully replicate the 3’ end of linear DNA, resulting in progressive telomere shortening.3 After a critical degree of telomere shortening, loss of chromosomal integrity results in replicative senescence and apoptosis.4 Initial telomere length at birth is widely variable and determined by both genetic and environmental factors.5–10 In 1973 Olovnikov proposed that the process of telomere attrition may provide the basis for a “biological clock” which integrates the cumulative effect of environmental stressors independently of chronological age.11

Several lines of evidence link telomere attrition with coronary artery disease (CAD). Inflammation and oxidative stress, two key factors in the pathogenesis of atherosclerosis, are both associated with accelerated telomere shortening.12,13 Coronary endothelial cells in patients with CAD have shorter telomeres than those derived from age-matched non-CAD patients.14 The enzyme telomerase, which partially restores telomere length, is downregulated in vascular smooth muscle cells in atherosclerotic plaques.15

Prior studies have linked leukocyte telomere shortening with an increased risk of mortality and cardiovascular events in individuals with no prior history of atherosclerosis.16,17 However, there are few longitudinal data examining the prognostic value of leukocyte telomere length in the context of other clinical, inflammatory, and echocardiographic variables. We sought to investigate the relationship between leukocyte telomere length and mortality in 780 patients with stable CAD in a prospective cohort study and to determine whether leukocyte telomere length provides incremental prognostic value beyond existing clinical, inflammatory, and echocardiographic markers of risk.

Methods

Participants

The Heart and Soul Study is a prospective cohort study investigating the influence of psychosocial factors on cardiovascular events in
outpatients with stable CAD. The enrollment process for the Heart and Soul Study has been previously described. Eligible participants were recruited from outpatient clinics in the San Francisco Bay Area if they met at least 1 of the following inclusion criteria: (1) history of myocardial infarction, (2) angiographic evidence of at least 50% stenosis by area in at least 1 coronary artery, (3) evidence of exercise-induced ischemia by treadmill ECG or stress nuclear perfusion imaging, (4) history of coronary revascularization. Individuals were excluded if they had a history of myocardial infarction in the past 6 months, deemed themselves unable to walk 1 block, or if they were planning to move out of the local area within 3 years.

The study protocol was approved by the following Institutional Review Boards: the University of California San Francisco Committee on Human Research, the Research and Development Committee at the San Francisco VA Medical Center, the Medical Human Subjects Committee at Stanford University, the Medical Subjects Committee at the VA Palo Alto Health Care System, and the Data governance Board of the Community Health Network of San Francisco. All participants provided written informed consent. Between September 2000 and December 2002, a total of 1024 participants enrolled in the study. Of these, 960 provided DNA samples for analysis. A further 180 samples were excluded because significant evaporation occurred when heat-denaturing the first 3 plates of DNA, and the resulting telomere length data were poorly correlated \( (r=0.35) \) with duplicate (quality control) samples on other plates. Telomere length data for the remaining 780 samples correlated well with duplicate (quality control) samples \( (r=0.82) \). There were no differences in patient characteristics between subjects included and excluded from the telomere length analysis.

Telomere Length Assay

Genomic DNA was isolated according to standard procedures from peripheral blood leukocytes collected at the baseline study visit and stored at -70 degrees Celsius. Purified DNA samples were diluted in 96-well microtiter source plates to a fixed concentration of 1.75 ng/ul. Relative mean telomere length was measured from DNA by a quantitative polymerase chain reaction (PCR)-based assay that compares mean telomere repeat sequence copy number to a reference single copy gene copy number as previously described. For each DNA sample, we performed 6 quantitative PCR runs: 3 "T" runs (for telomere repeat sequence copy number) and 3 "S" runs (for single copy gene copy number). The reference single copy gene used in this study was human \( \beta \)-globin.

The telomere-specific primers were: forward, 5'-GGTTTGGGTTTGGGTTTGGGTTTGGGTT3' and reverse, 5'-GCGTTGTTTGTGTTGTTGTTGTTGTTG3'. The \( \beta \)-globin-specific primers were: forward, 5'-GCGTTGTTTGTGTTGTTGTTGTTG3' and reverse, 5'-GGTTTGGGTTTGGGTTTGGGTTTGGGTT3'. The thermal cycling protocol for telomere PCR was: 95 degrees C for 5 minutes for initial denaturation followed by 25 cycles of 95 degrees C x 15 seconds, 54 degrees C \( \times \) 60 seconds followed by signal acquisition. The thermal cycling protocol for \( \beta \)-globin PCR was: 95 degrees C for 5 minutes for initial denaturation followed by 35 cycles of 95 degrees C x 15 seconds, 58 degrees C \( \times \) 15 seconds followed by signal acquisition.

A standard curve derived from serially-diluted reference DNA was generated for each of the 3 T runs and each of the 3 S runs. T and S were measured as the number of nanograms of reference DNA found to match the experimental sample for copy number of the targeted template (the number of telomere repeats for T runs, and the number of single copy gene copies for S runs). The average of the 3 T measurements was divided by the average of the 3 S measurements to calculate the average T/S ratio, which has previously been found to be highly consistent with independent measurement of telomere length by Southern blot terminal restriction fragment analysis. The coefficient of variation for the average T/S ratio in our study was 9.5%. For quality control purposes, we repeated the above procedure on 26 duplicate samples that were plated separately. All measurements were performed in a blinded fashion without knowledge of the clinical data.

Echocardiographic Measurements

All patients underwent complete resting 2-dimensional echocardiography and Doppler examination using an Acuson Sequoia ultrasound system (Siemens Medical Solutions) with a 3.5-MHz transducer. Standard parasternal short-axis and apical 2- and 4-chamber views were obtained and planimetered to determine end-diastolic and end-systolic volumes. The left ventricular ejection fraction (LVEF) was calculated as \( \text{end diastolic volume - end systolic volume}/\text{end diastolic volume} \). Diastolic dysfunction was defined as the presence of at least 1 of the following: impaired relaxation defined as a ratio of peak mitral early diastolic to atrial contraction velocity (E/A) of \( \leq 0.75 \) with systolic dominant pulmonary vein flow; pseudonormal filling defined as 0.75 \( < \) E/A \( < \) 1.5 with diastolic dominant pulmonary vein flow; restrictive filling defined as an E/A of 1.5 or greater with diastolic dominant pulmonary vein flow. Left ventricular mass was calculated using a truncated ellipsoid equation as previously validated.

Other Measurements

Baseline demographics, age, sex, and self-reported ethnicity were recorded. Cardiovascular comorbidities including hypertension, diabetes, hyperlipidemia, and smoking status were determined by self-report of medical history. Medication use was determined by having patients bring bottles to the study appointment during which study personnel recorded all medications. Participants were weighed and measured without shoes. Body mass index (BMI) was calculated. All participants were instructed to bring their medication bottles to the study appointment where study personnel recorded all current medications. Fasting serum chemistry samples were used to measure HDL cholesterol, LDL cholesterol, and C-reactive protein (CRP). HDL- and LDL-cholesterol levels were measured in a clinical laboratory setting. CRP was measured using the Roche Integra high-sensitivity assay (Roche) in 229 participants and (because of a change in the laboratory) the Beckman Extended Range high-sensitivity CRP assay (Beckman) in the remaining 551 participants. The Roche Integra assay uses an immunoturbidimetric technique, has been standardized against the World Health Organization reference, and compared with the Dade nephelometric method (correlation coefficient = 0.997). The lowest detectable CRP measurement with this assay was 0.025 mg/dL. The interassay coefficient of variation was 3.2%. The Beckman Extended Range assay also uses an immunoturbidimetric technique with a detection limit of 0.20 mg/L. The interassay coefficient of variation was 6.7%. The Beckman Extended Range assay is highly correlated with the Roche Integra assay (correlation coefficient = 0.99).

Outcomes

We conducted annual telephone interviews with participants or their proxies regarding recent emergency room visits, hospitalizations, or death. Medical records, death certificates, and coroner’s reports were reviewed by 2 independent and blinded adjudicators. If the adjudicators agreed on the outcome classification, their classification was binding. If they disagreed, a third blinded adjudicator reviewed the event and determined the outcome classification.

All-cause mortality was determined by review of death certificates. Hospitalization for HF was defined as a minimum 1-night hospital stay for a clinical syndrome comprising at least 2 of the following: paroxysmal nocturnal dyspnea, orthopnea, elevated jugular venous pressure, pulmonary rales, third heart sound, and cardiomegaly or pulmonary edema on chest roentgenography. These clinical signs and symptoms must have represented a clear change from the baseline clinical status of the participant and must have been accompanied by either failing cardiac output as determined by peripheral hyperperfusion (in the absence of other causes such as sepsis or dehydration) or peripheral or pulmonary edema requiring intravenous diuretics, inotropes, or vasodilators. Nonfatal myocardial...
dial infarction (MI) was defined by the American Heart Association diagnostic criteria. Stroke was defined as a new neurological deficit not known to be secondary to brain trauma, tumor, infection, or other causes. CV death was defined as a death, occurring during the same hospitalization in which an acute MI was documented, or death occurring within 1 hour of the onset of terminal symptoms not explained by other etiologies. CV events were defined as the composite of nonfatal MI, stroke, and CV death. For all analyses, the outcome variable was time to first event. The mean length of follow-up was 4.4 years. Ascertainment of outcomes was achieved in 99% of participants.

### Statistical Analysis

Because observed telomere lengths had a skewed distribution, the statistical analyses were performed on ln-transformed data. We categorized leukocyte telomere length (average T/S ratio) into quartile groups a priori. Differences in baseline characteristics were compared with the use of ANOVA for continuous variables and the chi-squared test for dichotomous variables, as appropriate. We used Cox proportional hazards models to examine the association between telomere length and cardiovascular outcomes, and verified the proportionality assumption of all models. For multivariable models, covariates were chosen a priori based on known clinical (age, gender, ethnicity, BMI, current smoking, systolic and diastolic blood pressure, LDL-cholesterol, HDL-cholesterol, diabetes, history of congestive heart failure), inflammatory (CRP), and echocardiographic (LVEF, Diastolic Dysfunction) markers of risk. Participants were censored at date of first event or last contact, whichever came first. To explore potential modifying effects of cardiac medications, we tested for statistical interactions between telomere length and the use of statins, aspirin, beta blockers, renin-angiotensin inhibitors (ACE-Is), and angiotensin receptor blockers (ARBs). We also tested for interactions between telomere length and ethnicity, and telomere length and gender. Statistical analysis was performed using SAS software version 9.1 (SAS Institute Inc). The authors take responsibility for the integrity of the data. All authors had full access to the data, except R.M.C, who was blinded to the clinical data. All authors have read and agree to the manuscript as written.

### Results

The baseline characteristics of the study population categorized by telomere length quartiles are shown in Table 1. Participants with shorter telomere length were older than those with longer telomere length. Participants with shorter telomere length were also more likely to have lower BMI,
lower BSA, prior stroke, lower LVEF, and echocardiographic evidence of diastolic dysfunction. There were no significant differences in gender, ethnicity, or history of hypertension, congestive heart failure (CHF), diabetes, or MI across quartiles of telomere length. Moreover there was no significant difference in CRP levels.

During a mean follow-up of 4.4 years there were 166 deaths, 99 hospitalizations for heart failure (HF), and 235 CV events. The number of participants with outcome events separated by telomere length quartile and the corresponding age-adjusted HRs are shown in Table 2. After age-adjustment, patients in the shortest telomere quartile were at significantly increased risk of all-cause mortality (age-adjusted HR 1.8; 95% CI 1.2 to 2.9). Each standard deviation decrease in log telomere length was associated with a 20% increase in all-cause mortality (age-adjusted HRs are shown in Table 2. After age-adjustment, patients in the shortest telomere quartile remained at significantly increased risk of all-cause mortality (age-adjusted HR 1.2, 95% CI 1.0 to 1.4; \( P = 0.02 \)).

After multivariate adjustment for clinical risk factors, participants in the shortest telomere quartile remained at significantly increased risk of all-cause mortality (adjusted HR 2.1; 95% CI 1.3 to 3.3) than participants in the longest telomere quartile (Table 3, Model 1). After the addition of a marker of systemic inflammation (CRP) or echocardiographic indices of systolic and diastolic function into the Cox regression model, participants in the shortest telomere quartile remained at significantly increased risk of death (HR 2.0; CI 1.2 to 3.2 and HR 1.9; CI 1.0 to 3.5 respectively). In the same multivariable model, patients in the lowest quartile of telomere length were also at significantly increased risk of HF hospitalization (HR 2.6; CI 1.1 to 6.0) but not CV events (HR 1.7; CI 0.9 to 3.5).

We found no evidence that the association of telomere length with adverse outcomes differed in users and nonusers of statins, aspirin, \( \beta \)-blockers, or renin-angiotensin inhibitors (all probability values for interaction \( > 0.05 \) for adverse outcomes in age-adjusted models). There was no difference in mean telomere length between users and nonusers of statins, aspirin, \( \beta \)-blockers, or renin-angiotensin inhibitors (all probability values \( > 0.05 \)). We also found no significant interaction between telomere length and ethnicity (\( P = 0.52 \)) or male gender (\( P = 0.37 \)) for adverse outcomes in age-adjusted models.

**Discussion**

In this large prospective study of patients with stable CAD, we found that leukocyte telomere length is associated with mortality independently of chronological age, clinical factors, CRP, and echocardiographic variables. Additionally, we found no significant interactions between the use of cardio-protective medications and leukocyte telomere length for adverse outcomes.

Cawthon et al measured leukocyte telomere length in 143 healthy blood donors age 60 to 97 years and found poorer survival in those with shorter telomeres, largely attributable to increased deaths from infection and cardiovascular disease. However, 2 subsequent studies found that leukocyte telomere length was not associated with morbidity and mortality in very elderly individuals when chronological age was taken into account. Our results support an independent association between telomere shortening and all-cause mortality and extend these findings for the first time to a

<table>
<thead>
<tr>
<th>Table 2. No. (%) of Participants With Adverse Outcomes by Quartile of Telomere Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartiles of Telomere Length</td>
</tr>
<tr>
<td>All-cause mortality</td>
</tr>
<tr>
<td>HF hospitalization</td>
</tr>
<tr>
<td>CV events (MI, stroke, or CV death)</td>
</tr>
</tbody>
</table>

**Table 3. Adjusted HRs for Adverse Outcomes After Adjustment for Clinical (Model 1), Clinical+CRP (Model 2), and Clinical+Echocardiographic Variables (Model 3)**

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Model 1* ( P ) Value</th>
<th>Model 2† ( P ) Value</th>
<th>Model 3‡ ( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cause mortality Quartile I vs IV</td>
<td>2.1 (1.3–3.3)</td>
<td>0.003</td>
<td>2.0 (1.2–3.2)</td>
</tr>
<tr>
<td>Per SD ↓ in log telomere length</td>
<td>1.3 (1.1–1.5)</td>
<td>0.005</td>
<td>1.2 (1.1–1.4)</td>
</tr>
<tr>
<td>HF hospitalization Quartile I vs IV</td>
<td>2.1 (1.1–3.8)</td>
<td>0.02</td>
<td>2.0 (1.1–3.7)</td>
</tr>
<tr>
<td>Per SD ↓ in log telomere length</td>
<td>1.3 (1.0–1.5)</td>
<td>0.04</td>
<td>1.2 (1.0–1.5)</td>
</tr>
<tr>
<td>CV events (MI, stroke, or CV death) Quartile I vs IV</td>
<td>1.7 (1.0–3.0)</td>
<td>0.07</td>
<td>1.7 (0.9–2.9)</td>
</tr>
<tr>
<td>Per SD ↓ in log telomere length</td>
<td>1.1 (0.9–1.4)</td>
<td>0.20</td>
<td>1.1 (0.9–1.4)</td>
</tr>
</tbody>
</table>

*Adjusted for age, gender, ethnicity, LDL cholesterol, HDL cholesterol, systolic blood pressure, diastolic blood pressure, BMI, stroke, smoking, diabetes, and CHF.
†Adjusted for all variables in Model 1 + Log CRP.
‡Adjusted for all variables in Model 1 + LVEF and diastolic dysfunction.
prospectively-studied cohort of patients with stable CAD. These observations support the hypothesis that, in CAD, leukocyte telomere length reflects biological aging and may integrate multiple genetic and environmental factors as a final common pathway of cellular stress. Several factors important in the pathogenesis and progression of atherosclerosis are known to accelerate telomere attrition. These include inflammation, oxidative stress, and endocrine aberrations. Further basic and epidemiological studies are needed to elucidate the underlying mechanisms of telomere attrition in this patient population.

Our results differ from prior studies in individuals at risk of atherosclerosis in that we observed no relationship between telomere length and CRP. One possible explanation is that in the preclinical phase of disease, systemic inflammation promotes both atherogenesis and leukocyte telomere attrition. However, once CAD is established other genetic and environmental factors have greater influence in determining the rate of telomere shortening. Whether leukocyte telomere shortening contributes directly to the progression of CAD at the cellular and molecular level cannot be determined from our results.

We also found a significant inverse association between telomere length and hospitalization for heart failure. This finding provides prospective validation of a recent study by van der Harst et al which demonstrated shorter telomeres in patients with CHF compared with age-balanced controls. In that study, telomere length was incrementally shorter in the presence of advanced disease and an ischemic etiology. Further studies are warranted to investigate the interplay between systemic inflammation, atherogenesis, telomere shortening, and heart failure.

Brouillette et al used a nested case-control approach in the West of Scotland Primary Prevention Study to show that the risk of developing CAD was highest in individuals with short telomeres and that this risk was substantially attenuated by treatment with pravastatin. By contrast, in the present study, we found no interaction between statin use and telomere length, and no difference in mean telomere length between users and nonusers of statins. Prior studies have also shown an association between telomere attrition and obesity. In contrast, we found lower BMI in patients with shorter telomeres. The explanation for this discrepancy is unclear but suggests an inverse U-shaped relationship between BMI and telomere length. Among our study population, lower BMI (and shorter telomere length) may be a marker of greater cardiovascular disease severity.

Among the strengths of the present study is the measurement of multiple potential confounding variables including inflammatory markers and echocardiographic parameters of systolic and diastolic function. The study design allowed us to prospectively investigate the prognostic value of leukocyte telomere length in a large cohort of comprehensively-pheno-typed patients with CAD. However, several limitations should be considered in the interpretation of our results. First, telomere length in leukocytes derived from peripheral blood...
sampling does not necessarily reflect alterations in telomere length and function in the atherosclerotic plaque or myocardium.30,31 There are limited data to suggest general correlation between telomere length in leukocytes and other tissues. Although no studies have directly compared leukocyte telomere length with endothelial cell telomere length, there is evidence that telomere shortening contributes to endothelial cell senescence and may be accelerated by oxidative stress.32,33 Second, our results are derived from a single measurement of telomere length at the outset of a prospective cohort study. As such, we are unable to determine the rate of change of telomere length which would likely provide further insights into the significance of telomere attrition in this population.34 Furthermore, a single measurement of leukocyte telomere length cannot distinguish between chronic low-level stressors and a highly-stressful single prior event. Third, we did not evaluate the impact of telomere-associated proteins such as telomerase on the prognostic value of leukocyte telomere length. Fourth, we are unable to account for inherited differences in leukocyte telomere length between individuals. However, recent evidence suggests that early telomerase activity may “homogenize” telomere lengths such that longer telomeres shorten more quickly than short telomeres.35,36 Finally, the question of whether telomere shortening is merely an epiphenomenon, or whether it plays an active role in the progression of coronary atherosclerosis, cannot be answered on the basis of our results.37

In summary, we found that leukocyte telomere length in peripheral blood leukocytes is associated with mortality among ambulatory individuals with stable CAD. The prognostic value of leukocyte telomere length in this population is not fully captured by existing clinical, inflammatory, and echocardiographic markers of increased risk. Future studies should be aimed at investigating the mechanisms and significance of the association between telomere length and adverse outcomes in patients with stable CAD.

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Disclosures

None.

References


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