Short Telomere Length, Myocardial Infarction, Ischemic Heart Disease, and Early Death

Maren Weischer, Stig E. Bojesen, Richard M. Cawthon, Jacob J. Freiberg, Anne Tybjærg-Hansen, Børge G. Nordestgaard

Objective—We tested the hypothesis that short telomere length is associated with increased risk of myocardial infarction, ischemic heart disease, and early death.

Methods and Results—We measured leukocyte telomere length in 2 prospective studies of 19,838 Danish general population participants from the Copenhagen City Heart Study and the Copenhagen General Population Study. Participants were followed for up to 19 years for incident myocardial infarction (n=929), ischemic heart disease (n=2038), and death (n=4342). Follow-up was 100% complete. Telomere length decreased linearly with increasing age in women and men in both studies (P=7×10⁻⁷⁴ to P=3×10⁻¹²⁵). Multifactorially adjusted hazard ratios were 1.10 (95% CI 1.01–1.19) for myocardial infarction, 1.06 (1.00–1.11) for ischemic heart disease, and 1.09 (1.05–1.13) for early death per 1000–base pair decrease in telomere length. The multifactorially adjusted hazard ratios for the shortest versus the longest decile of telomere length were 1.49 (1.07–2.07) for myocardial infarction, 1.24 (1.01–1.53) for ischemic heart disease, and 1.25 (1.07–1.46) for early death.

Conclusion—Short telomere length is associated with only modestly increased risk of myocardial infarction, ischemic heart disease, and early death. (Arterioscler Thromb Vasc Biol. 2012;32:822-829.)

Key Words: acute coronary syndromes • ischemic heart disease • death • myocardial infarction • telomere length

Telomeres are protective chromosomal caps at the linear ends of chromosomes consisting of a variable number of TTAGGG repeats.¹ Telomeres shorten with each cell cycle in most cells and therefore reflect organism aging at a cellular level.¹,² Accordingly, telomere length decreases with increasing age,³,⁴ but also with male sex, smoking, adiposity, oxidative stress, UV irradiation, and low socioeconomic status.²,³,⁵,⁶ It has therefore been a matter of speculation whether short telomere length is associated with increased risk of cardiovascular disease and early death.⁵,⁸–¹⁰

Previous studies of telomere length and human disease have been limited by cumbersome techniques mainly allowing low-throughput measurements.¹¹ Furthermore, the majority of studies so far have mainly been smaller case-control studies rather than large prospective studies of unselected individuals from the general population.¹² Accordingly, there is a need for large studies of the general population with extended follow-up, with telomere length measured reliably with a high-throughput method to examine the influence of short telomere length on cardiovascular disease and early death.

We tested the hypothesis that short telomere length is associated with increased risk of myocardial infarction, ischemic heart disease, and early death. For this reason, we developed a high-throughput real-time polymerase chain reaction assay calibrated to measure absolute telomere length. Subsequently, we measured 19,838 individuals from 2 prospective studies of the Danish general population from the Copenhagen City Heart Study and the Copenhagen General Population Study, followed up to 19 years.

Methods

Study Design

The Copenhagen City Heart Study is a population-based prospective study initiated in 1976 to 1978,¹³,¹⁴ Participants aged 20 years or above were randomly invited from the Central Population Register and reexamined in 1981 to 1983, 1991 to 1994, and 2001 to 2003, with younger participants added at each reexamination. Whole blood samples from the last 2 examinations were used for DNA isolation. We included 9765 participants with available DNA samples.

The Copenhagen General Population Study is a parallel population-based prospective study initiated in 2003 and still recruiting.¹⁵ Participant selection and data collection were similar to that in

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We included the first 10,073 participants with available DNA samples. All participants in both studies were white and of Danish descent. There was no overlap of individuals between the 2 studies, thus permitting independent confirmation of findings in each study.

Covariates

Before examinations, participants filled in self-administered questionnaires concerning present and past lifestyle and health status. This was completed on the day of examination, before physical examination, blood sampling, and measurement of cardiovascular risk factors. The following covariates were obtained or measured:

- Age at examination (years)
- Gender (male/female)
- Total cholesterol levels (mmol/L)
- Triglyceride levels (mmol/L)
- High-density lipoprotein cholesterol levels (mmol/L)
- High sensitivity C-reactive protein levels (mg/L)
- Use of lipid lowering therapy (no/yes)
- Body mass index (measured weight in kilograms divided by squared measured height in meters)
- Hypertension (no/yes) (yes if participants had systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg, or reported use of antihypertensive medication)
- Diabetes mellitus (no/yes) (yes if nonfasting blood glucose was >11 mmol/L or if the participant reported having diabetes mellitus or using antidiabetic medication)
- Current smoking (no/yes), heavy alcohol intake (no/yes) (yes if female and male participants reported a weekly alcohol intake above 87.5 and 175 g, respectively), physical inactivity (no/yes) (yes if participants reported <4 hours of leisure time physical activity per week), postmenopausal (no/yes) (yes if women reported being postmenopausal or using hormone replacement therapy or were aged 60 years or above), and use of hormone replacement therapy (yes if women reported use of hormone replacement therapy).

End Points

Using the unique Danish personal identification numbers, date of death was obtained from the Danish Civil Registration System, whereas diagnoses and dates of myocardial infarction and ischemic heart disease were obtained from the national Danish Patient Registry and the national Danish Cause of Death Registry. Diagnoses were classified according to the World Health Organization International Classification of Disease (ICD), 8th revision (ICD-8 codes 410 for myocardial infarction and 410–414 for ischemic heart disease) until 1993, and ICD 10th revision thereafter (ICD-10 codes I21–I22 for myocardial infarction and I20–I25 for ischemic heart disease). Follow-up was 100% complete; that is, we did not lose track of even a single individual.

Telomere Length

We measured telomere length in DNA from leukocytes in peripheral blood, a measurement highly correlated with telomere length in cells from other tissues. Telomere length was measured on a CFX384 real-time polymerase chain reaction detection system (Bio-Rad Laboratories), using a modified monochrome multiplex quantitative polymerase chain reaction method. Failed samples were measured a second time, and a third if they failed again. Therefore, valid measurements of telomere lengths were available for more than 99.9% of participants. The interassay coefficient of variation of the internal NTERA-2 control was 2% for Ct values at mean values of 17.9 cycles for the telomere assay and 9% for absolute telomere length at the mean level of 2534 base pairs. For a complete
### Table 1. Baseline Characteristics of Participants From the General Population by Quartiles of Decreasing Telomere Length

<table>
<thead>
<tr>
<th>1st Quartile</th>
<th>2nd Quartile</th>
<th>3rd Quartile</th>
<th>4th Quartile</th>
<th>P for Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copenhagen City Heart Study</td>
<td>Copenhagen General Population Study</td>
<td>Copenhagen City Heart Study</td>
<td>Copenhagen General Population Study</td>
<td></td>
</tr>
<tr>
<td>Telomere length, kbp</td>
<td>11.7±0.9</td>
<td>12.0±0.9</td>
<td>12.2±0.9</td>
<td>12.4±0.9</td>
</tr>
<tr>
<td>No. of participants</td>
<td>248</td>
<td>254</td>
<td>259</td>
<td>264</td>
</tr>
<tr>
<td>Age, y</td>
<td>51</td>
<td>53</td>
<td>56</td>
<td>59</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.8 (5.6–6.0)</td>
<td>5.9 (5.7–6.1)</td>
<td>6.1 (5.9–6.3)</td>
<td>6.3 (6.1–6.5)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.6 (1.4–1.8)</td>
<td>1.7 (1.6–1.9)</td>
<td>1.8 (1.7–2.0)</td>
<td>1.9 (1.8–2.1)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.5 (1.3–1.7)</td>
<td>1.5 (1.3–1.7)</td>
<td>1.6 (1.4–1.8)</td>
<td>1.7 (1.5–1.9)</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>1.6 (1.4–1.8)</td>
<td>1.8 (1.6–2.0)</td>
<td>2.0 (1.8–2.2)</td>
<td>2.2 (2.0–2.4)</td>
</tr>
<tr>
<td>Lipid-lowering therapy</td>
<td>2.0 (2.0–2.2)</td>
<td>2.0 (2.0–2.2)</td>
<td>2.0 (2.0–2.2)</td>
<td>2.0 (2.0–2.2)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24 (22–26)</td>
<td>25 (23–27)</td>
<td>26 (25–28)</td>
<td>27 (26–29)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1080 (44)</td>
<td>1221 (50)</td>
<td>1372 (56)</td>
<td>1562 (64)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>72 (3)</td>
<td>81 (3)</td>
<td>105 (4)</td>
<td>156 (8)</td>
</tr>
<tr>
<td>Current smokers</td>
<td>1053 (43)</td>
<td>1137 (47)</td>
<td>1199 (49)</td>
<td>1234 (50)</td>
</tr>
<tr>
<td>Heavy alcohol intake</td>
<td>960 (41)</td>
<td>1019 (46)</td>
<td>1009 (45)</td>
<td>1076 (50)</td>
</tr>
<tr>
<td>Physical inactivity</td>
<td>938 (35)</td>
<td>1000 (40)</td>
<td>965 (40)</td>
<td>1020 (44)</td>
</tr>
<tr>
<td>Postmenopausal*</td>
<td>760 (33)</td>
<td>857 (36)</td>
<td>998 (40)</td>
<td>1061 (44)</td>
</tr>
<tr>
<td>Use of HRT*</td>
<td>204 (14)</td>
<td>208 (15)</td>
<td>216 (16)</td>
<td>221 (16)</td>
</tr>
</tbody>
</table>

Continuous variables are shown as medians (interquartile ranges), and categorical variables are shown as numbers (%). Only measured values are shown; covariates were more than 99% complete. HDL indicates high-density lipoprotein; HRT, hormone replacement therapy.

*Women only.

### Ethical Considerations

The Danish Data Protection Agency, Herlev Hospital, Copenhagen University Hospital, and a local Danish ethical committee approved the studies (KF100.2039/91 and H-KF01-144/01). All participants gave written informed consent.

### Results

#### Telomere Length and Age

Telomere length decreased linearly with increasing age in women and men in both studies of the general population ($P=7×10^{-17}$ to $P=3×10^{-125}$) (Figure 1); $R^2$ between age and telomere length ranged from 6% to 11%. Telomere length decreased by 20 base pairs per year of increase in age in the Copenhagen City Heart Study and by 14.5 base pairs in the Copenhagen General Population Study. In addition, as shown in Table 1, decreasing telomere length was also associated with male gender, increasing levels of total cholesterol, triglycerides, and C-reactive protein, decreasing levels of high-density lipoprotein cholesterol, use of lipid lowering therapy, increasing body mass index, hypertension, diabetes mellitus, current smoking, decreasing heavy alcohol intake, physical inactivity, and for women only postmenopausal status and use of hormone replacement therapy.

#### Telomere Length and Myocardial Infarction

During follow-up, 929 participants developed myocardial infarction in the 2 studies combined. Per 1000–base pair decrease in telomere length, the multifactorially adjusted hazard ratio of myocardial infarction was 1.10 (95% CI 1.01–1.19) (Table 2). Similar hazard ratios were found in an age-, gender-, and study-adjusted model; in each study alone; and in each gender separately. When stratifying in subgroups of age, gender, and biochemical and lifestyle cardiovascular risk factors, we found no evidence of interaction between
Table 2. Risk of Myocardial Infarction, Ischemic Heart Disease, and Early Death per 1000-bp Decrease in Telomere Length

<table>
<thead>
<tr>
<th></th>
<th>Combined Studies, Adjustment</th>
<th>Copenhagen City Heart Study, Adjustment</th>
<th>Copenhagen General Population Study, Adjustment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age, Gender, Study</td>
<td>Multifactorially*</td>
<td>Age and Gender</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>1.13 (1.04–1.23)</td>
<td>1.10 (1.01–1.19)</td>
<td>1.11 (1.02–1.21)</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>1.08 (1.03–1.14)</td>
<td>1.06 (1.00–1.11)</td>
<td>1.07 (1.01–1.13)</td>
</tr>
<tr>
<td>Early death</td>
<td>1.13 (1.09–1.18)</td>
<td>1.09 (1.05–1.13)</td>
<td>1.14 (1.09–1.18)</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>1.17 (1.03–1.32)</td>
<td>1.13 (1.00–1.29)</td>
<td>1.14 (1.00–1.30)</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>1.06 (0.98–1.14)</td>
<td>1.02 (0.95–1.10)</td>
<td>1.04 (0.96–1.13)</td>
</tr>
<tr>
<td>Early death</td>
<td>1.12 (1.07–1.19)</td>
<td>1.08 (1.02–1.14)</td>
<td>1.13 (1.07–1.19)</td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>1.11 (1.00–1.23)</td>
<td>1.08 (0.97–1.20)</td>
<td>1.08 (0.97–1.21)</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>1.11 (1.03–1.20)</td>
<td>1.09 (1.01–1.18)</td>
<td>1.10 (1.01–1.19)</td>
</tr>
<tr>
<td>Early death</td>
<td>1.14 (1.08–1.20)</td>
<td>1.10 (1.04–1.16)</td>
<td>1.15 (1.08–1.21)</td>
</tr>
</tbody>
</table>

*Adjusted for age, gender, study, cholesterol, triglycerides, high-density lipoprotein cholesterol, C-reactive protein, use of lipid lowering therapy, body mass index, hypertension, diabetes mellitus, smoking, heavy alcohol intake, and physical inactivity; in women, also adjusted for postmenopausal status and hormone replacement therapy.

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Discussion

After measuring telomere length using a high-throughput real-time polymerase chain reaction method in 19 838 individuals from the general population followed for up to 19 years, we observed that short telomere length was associated only modestly with increased risk of myocardial infarction, ischemic heart disease, and early death. We cannot exclude that these modest risk estimates could be explained by residual undetected confounding.

The mechanism behind these findings could be that short telomere length is a marker of a systemic degenerative phenotype arising after years of exposure to cardiovascular risk factors, causing cellular damage and resulting in an increasing frequency of tissue mitosis and thereby shorter telomere length.18 A systemic degenerative phenotype could then lead to the observed increased risk of myocardial infarction, ischemic heart disease, and early death.
Figure 2. Risk of myocardial infarction, ischemic heart disease, and early death per 1000-bp decrease in telomere length for both studies combined. Black dots indicate hazard ratios and horizontal lines indicate 95% confidence intervals. HDL indicates high-density lipoprotein; HRT, hormone replacement therapy.

We confirmed previous findings of a strong linear correlation between decreasing telomere length and increasing age as demonstrated by the validity of our high-throughput assay. In our studies of the general population, telomere length decreased by 14.5 to 20 base pairs per year. This is similar to the finding in previous reports of 14 to 29 base pairs per year, but less than that in other reports of up to 7 base pairs per year. These study differences may reflect interpopulation variation, or they may be a result of interassay variation because some former assays included subtelomeric regions.

For myocardial infarction, the present observation of association between decreasing telomere length and increased risk is in accordance with previous studies, all smaller than the present one. Following 19,284 individuals prospectively for up to 19 years, we detected 929 incident myocardial infarctions and a hazard ratio for myocardial infarction of 1.18 (95% CI 0.97–1.43) for individuals in the shortest versus longest telomere quartile, whereas the Bruneck Study, a prospective study of 800 individuals followed for 10 years, observed 43 incident myocardial infarctions and a hazard ratio for myocardial infarction of 1.18 (95% CI 0.97–1.43) for individuals in the shortest versus the longest telomere quartile. Also, the Cardiovascular Health Study, a prospective study of 388 individuals followed for 7 years, reported 36 incident myocardial infarctions and a hazard ratio for myocardial infarction of 1.55 (0.85–2.83) per 1000–base pair decrease in telomere length; whereas our comparable hazard ratio was 1.10 (1.01–1.19). Finally, a case-control study from Leicester of 203 cases and 180 controls reported an odds ratio for myocardial infarction of 2.79 (1.53–5.11) for individuals in the shortest versus the longest telomere quartile. Taken together, previous
### Table 1: Decreasing Telomere Length and Risk of Myocardial Infarction, Ischemic Heart Disease, and Early Death

<table>
<thead>
<tr>
<th>Telomere Length</th>
<th>Participants</th>
<th>Events</th>
<th>Incidence Rate per 10,000 Person-years</th>
<th>Hazard Ratio (95% CI)</th>
<th>Hazard Ratio (95% CI)</th>
<th>P-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quartiles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>4864</td>
<td>163</td>
<td>33 (28-39)</td>
<td>1.00</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>4853</td>
<td>187</td>
<td>39 (34-46)</td>
<td></td>
<td>0.95 (0.77-1.17)</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>4801</td>
<td>253</td>
<td>56 (50-63)</td>
<td></td>
<td>1.10 (0.90-1.34)</td>
<td></td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>4766</td>
<td>326</td>
<td>80 (72-89)</td>
<td></td>
<td>1.18 (0.97-1.43)</td>
<td></td>
</tr>
<tr>
<td>Ischemic Heart Disease</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>4779</td>
<td>393</td>
<td>83 (75-91)</td>
<td>1.00</td>
<td>p = 0.22</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>4739</td>
<td>444</td>
<td>98 (89-107)</td>
<td></td>
<td>0.95 (0.83-1.09)</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>4673</td>
<td>549</td>
<td>128 (117-139)</td>
<td></td>
<td>1.02 (0.89-1.16)</td>
<td></td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>4570</td>
<td>652</td>
<td>170 (158-184)</td>
<td></td>
<td>1.06 (0.93-1.20)</td>
<td></td>
</tr>
<tr>
<td>Early Death</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>4950</td>
<td>705</td>
<td>139 (129-150)</td>
<td>1.00</td>
<td>p = 4x10^-4</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>4955</td>
<td>871</td>
<td>178 (167-190)</td>
<td></td>
<td>1.00 (0.90-1.10)</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>4964</td>
<td>1173</td>
<td>248 (234-262)</td>
<td></td>
<td>1.08 (0.98-1.19)</td>
<td></td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>4969</td>
<td>1593</td>
<td>369 (351-387)</td>
<td></td>
<td>1.18 (1.08-1.29)</td>
<td></td>
</tr>
</tbody>
</table>

### Figure 3: Risk of myocardial infarction, ischemic heart disease, and early death by decreasing telomere length in quartiles and deciles for both studies combined. Black dots indicate hazard ratios, and horizontal lines indicate 95% confidence intervals. Probability values for trend are for decreasing telomere length in quartiles or deciles.
and present studies all support that decreasing telomere length is associated with increased risk of myocardial infarction, whereas the present study, with the most statistical power, suggests that the magnitude of the risk estimate is more modest than originally thought.

For risk of ischemic heart disease, the present study detected 2038 events and a hazard ratio of 1.06 (0.93–1.20) for individuals in the shortest versus longest telomere quartiles, a modest effect size compared with that in a previous study.32 Here, in the West of Scotland Primary Prevention Study, a nested case-control study of 484 cases and 1058 controls, an odds ratio for ischemic heart disease of 1.44 (1.10–1.90) was observed for individuals in the shortest versus the longest telomere tertile. However, as participants in that study were randomly assigned to treatment with statins or placebo, these results not be directly comparable to those of our study.

For risk of early death, our observed 4342 deaths and a hazard ratio of 1.09 (1.05–1.13) per 1000–base pair decrease in telomere length is similar to that of 1.22 (0.91–1.63) observed in the Cardiovascular Health Study, a prospective study of 419 individuals followed for 7 years with 156 deaths. However, a prospective study of 143 individuals aged 60 years or older followed for a maximum of 20 years, reported 101 deaths and a 1.86 (1.22–2.83)-fold mortality rate for individuals in the shortest versus the longest half of telomere length. In contrast to our findings and that of the other reports mentioned above,27,33 a prospective study of 812 individuals aged 73 years or older observed 412 deaths but no association between telomere length and overall survival.34 Taken together, the combined evidence supports that short telomere length is associated with a modest increased risk of early death.

Strengths of the present study include the large study size of 19 838 participants from the general population followed prospectively for up to 19 years. Second, telomere length was measured with a new, high-throughput assay, validated prospectively for up to 19 years. Second, telomere length was measured on more than 99.9% of available participants because of several rounds or reruns. Fourth, because of the large-scale telomere measurements. We are indebted to staff and participants of the Copenhagen City Heart Study and the Copenhagen General Population Study for their important contributions.

In conclusion, short telomere length is associated only modestly with increased risk of myocardial infarction, ischemic heart disease, and early death.

Acknowledgments

We thank laboratory technician Anja Johchumsen for assisting with the large-scale telomere measurements. We are indebted to staff and participants of the Copenhagen City Heart Study and the Copenhagen General Population Study for their important contributions.

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Disclosures

Dr Cawthon stands to profit if Telome Health, Inc, sells telomere length measurements by his assay. For this study, Dr Cawthon helped his coauthors develop a new assay for high throughput. Dr. Cawthon did not do any of the telomere length measurements on participants in the present study and declares that his involvement in the study cannot have biased any of the associations found.

References


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Supplemental Material

Supplementary Appendix

Quantitative polymerase chain reaction (PCR) assay

We measured telomere length in DNA isolated from leukocytes in peripheral blood, a measurement highly correlated with telomere length in other tissues\(^1\)\(^-\)\(^3\). Telomere length was measured on a CFX384 real-time PCR detection system (Bio-Rad Laboratories, Denmark), using a modified Monochrome Multiplex Quantitative PCR method\(^4\). In the duplex PCR, the single-copy gene ALB which encodes albumin was amplified simultaneously with the telomere template in the same well and was used as a reference to adjust for different amounts of DNA in different samples. Final concentrations of PCR reagents were 1X QuantiFast SYBR Green Master Mix (Qiagen), 20 ng DNA, 300 nmol/L forward telomere primer (5' - ACATAAGGTGGGGTTGCTTCTTGGGTTTAGTGT-3'), 300 nmol/L reverse telomere primer (5' - TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'), 350 nmol/L forward albumin primer (5' - CGGCACCGGCCGCGCGCGCTGGGGGAAATGCTGCACAGAATCCCTTG-3'), and 350 nmol/L reverse albumin primer (5' - GCCGCCGCGCGCCGCGCGCGCGCGCGCGCGAAAGCATGGTGCCTGCTTG-3') in a volume of 10 μL. The present thermal cycling profile was elaborated from the original publication\(^1\)\(^7\): Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; and Stage 3: 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 73°C with signal acquisition, 10 s at 84°C, 15 s at 87°C with signal acquisition; Stage 4: 1 cycle of 0.05 s at 65°C with signal acquisition. SYBR Green I, in QuantiFast, bind to double stranded DNA to produce a fluorescence signal. The 73°C reads provided the Ct values for the amplification of the telomere template (in early cycles when the albumin signal is still at baseline). The 87°C reads provided the Ct values for the amplification of the albumin template (at
this temperature the telomere template is fully melted). The 65°C signal acquisition in Stage 4 was used to identify wells with primer-dimer formation for rerun. On each 384-well plate, DNA from participants was examined in quadruplicates together with triplicates of an internal control and a calibrator consisting of genomic DNA from the cell lines NTERA-2 and K562, respectively (purchased from DMSZ, Braunschweig, Germany). To determine the absolute telomere length (absTL) in basepairs of our calibrator, we measured its t/s ratio relative to the reference DNA sample used in Dr. Cawthon's 2009 methods paper, and then calculated the calibrator's absTL as \([(t/s) \times (\text{absTL of the reference DNA})]\). The absTL of this reference DNA is provided by the slope of the linear regression line in Figure 5 of ref. 4, since that slope equals the change in mean Terminal Restriction Fragment length (in basepairs) by Southern blot, per unit change in t/s, and the reference DNA, by definition, has t/s = 1.0 unit. The absTL of the calibrator derived in this way was 5290 basepairs, which was comparable to previous reports for this cell line of 5360 and 6500 basepairs. Ct values are the raw results from a quantitative PCR analysis. Ct is short for cycle threshold, which is the number of PCR cycles needed to produce enough amplicons to raise the SYBR Green I fluorescence signal above a computer set threshold of the background fluorescence.

**Assay efficiency**

In setting up the method, we optimized assay efficiencies, since efficiencies of the telomere and albumin assay need to be high and similar, to derive absolute telomere length values by the \(\Delta\Delta C_t\) method. Efficiencies on a triplicate dilution series ranging from 71.8 to 4.49 ng DNA were automatically calculated by the Bio-Rad CFX384 computer software. We ran 10 DNA dilution series, one of which is shown in Supplementary Figure 1. Under ideal conditions the amount of template is doubled in each PCR cycle equaling an efficiency of 100%. Lower efficiencies may suggest problems with the assay, e.g. master mix performance or sample quality. Efficiencies above 100% indicate the formation of primer-dimers. Generally, efficiencies between 90 and 110% are
acceptable\(^7\). For both assays, that is, for telomere length and the reference gene ALB, we observed high efficiencies between 97% and 101%. The \(\Delta\Delta C_t\) method to calculate absolute telomere length, premises that efficiencies of the telomere length and albumin PCR-reactions are equal. This means that the number of PCR cycles between the Ct value of the telomere amplicon and the albumin amplicons (\(\Delta C_t\)) is independent of the amount of DNA. This was tested graphically by plotting \(\Delta C_t\) from a dilution series on the Y axis against amount of DNA (log DNA, ng) on the X axis (Supplementary Figure 2). According to guidelines, the \(\Delta\Delta C_t\) method may be used if the slope of a linear regression for this plot is less than 0.1\(^7\). We found a slope of 0.03, which indicated that \(\Delta C_t\) is constant across DNA amounts. Therefore, we were able to leave out standard curves in each individual plate during running of patient samples. Not including standard curves in each plate increased throughput and also eliminated the adverse effect of any dilution errors in creating standard curve samples\(^7\).

The \(\Delta\Delta C_t\) method for determining absolute telomere length

DNA from each participant was measured in four different wells, resulting in four Ct values for the telomere signal and four Ct values for the albumin signal. A mean of the four telomere Ct values and the four albumin Ct values were calculated, and wells producing measurements outside mean Ct values ±2 SD were excluded, and new mean Ct values for telomere and albumin were calculated after exclusion of these outlier wells. Participants with less than two valid telomere and albumin Ct values were run again. For each participant we calculated a mean Ct value for the telomere assay (C_{tel\_X}) and for the albumin assay (C_{alb\_X}). Similarly for the calibrator, we calculated means for C_{tel\_K562\_All} and C_{alb\_K562\_All} across all plates and for each individual plate (C_{tel\_K562\_plate\_X} and C_{alb\_K562\_plate\_X}). We calculated normalization factors for both assays on every plate. For the telomere assay, the normalization factor was: factor_{tel\_plate\_X} = (C_{tel\_K562\_All} / C_{tel\_K562\_plate\_X}). Next,
we calibrated \( C_{tel} \) and \( C_{alb} \) of internal controls and participant samples. For the telomere assay, the calibration was: 
\[
C_{tel\ X\ calibrated} = C_{tel\ X} * \text{factor}_{tel\ \text{plate\ X}}.
\]
We then used the \( \Delta \Delta Ct \) calculation to derive the unknown telomere length of a participant sample (X): 
\[
\Delta Ct_{X} = C_{alb\ X\ calibrated} - C_{tel\ X\ calibrated}.
\]
\[
\Delta Ct_{K562} = C_{alb\ K562} - C_{tel\ K562} \quad \text{and} \quad \Delta \Delta Ct = \Delta Ct_{X} - \Delta Ct_{K562}.
\]
The absolute telomere length of sample X was then calculated as 
\[
2^{\Delta \Delta Ct} \times 5290 \text{ basepairs}.
\]
Failed samples were measured a second round and a third if they failed again. Therefore, valid measurements of telomere lengths were available for more than 99.9% of participants.

**Assay precision**

The coefficient of variation was measured with the use of triplicates in each plate of DNA from the cell line NTERA-2, where \( \Delta Ct \) and the absolute telomere length were calculated exactly as for participant samples. Means and standard deviations were calculated for \( C_{tel\ \text{NTERA-2}} \) and absolute telomere length values. The inter-assay coefficient of variation (CV) was determined using the calculation: 
\[
\text{CV} = (\text{standard deviation}/\text{mean}) \times 100\%.
\]
Coefficients of variation for the internal control were 2% for \( C_{tel\ \text{NTERA-2}} \) at mean value of 17.9 and 9% for absolute telomere length in basepairs at the mean level of 2534 basepairs.
References


Supplement Material

Supplementary Figures Legends

Supplementary Figure I

Efficiency for the telomere assay. A screen dump from the BioRad CFX384 software shows triplicate samples of five different concentrations representing a dilution series of 71.9 to 4.49 ng DNA. The left panel shows relative fluorescence on the Y axis and number of PCR cycles on the X axis. Initially, fluorescence is low, but amplification of telomere sequence creates more double stranded DNA, thereby increasing fluorescence. The Ct value is the number of PCR cycles needed before sample fluorescence rises above the threshold line (horizontal green line). The three deviant curves marked with a black circle are from H2O templates demonstrating no significant contamination. The right panel shows the same Ct values as a function of log(DNA content per sample, ng). The linear regression is the standard curve. Based on the slope, the software calculates efficiency (E), which here is 99.8% indicating near doubling of telomere amplicons in each PCR cycle. R² was 0.997 indicating near perfect reproducibility between triplicates.

Supplementary Figure II

Telomere and albumin assay efficiencies across DNA amount per sample. ΔCt = (Ct albumin – Ct telomere) on the Y axis plotted against log DNA amount for triplicates for a dilution series ranging from 71.9 to 4.49 ng DNA. Dots indicate mean ΔCts for the triplicates. The vertical line through the dot indicates range. The dashed line shows the linear regression for ΔCt as a function of log (DNA amount). Result of the regression is shown.
Supplementary Figure I

Amplification

Standard Curve

Log(DNA content per sample)

Threshold Cycle

0.65 0.95 1.25 1.56 1.87

0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000
12000
13000
14000

0 5 10 15 20 25 30

Cycles

O Standard
X Unknown

9Y9R: E=69.0% R^2=0.997 Slope=-3.326
Supplementary Figure II

Δ Ct = 0.03*\log(DNA) + 5.28
R^2 = 0.06

Log(DNA amount per sample) (DNA amount per sample, ng)