Telomere Length and Mortality in Elderly Men: The Zutphen Elderly Study

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Telomere shortening is a marker of aging and therefore telomere length might be related to disease progression and survival. To address these questions, we measured leukocyte telomere length (LTL) in male participants from the Zutphen Elderly Study. LTL was measured by quantitative polymerase chain reaction in 203 men: mean aged 78 years in 1993 and 75 surviving participants mean aged 83 years in 2000. During 7 years of follow-up, 105 men died. Cox proportional hazards models were used to estimate hazard ratios for all-cause and cause-specific mortality. We found that LTL declined with a mean of 40.2 bp/year, and LTL values measured in 1993 and 2000 correlated significantly ($r = .51$, $p < .001$). Longer telomeres at baseline were not predictive for all-cause mortality, cardiovascular mortality, or cancer mortality. These results suggest that LTL decreases with increasing age and that LTL is not related to mortality in men aged more than 70 years.

Key Words: Telomere length—Mortality—Zutphen Elderly Study.

Received May 7, 2010; Accepted August 25, 2010

Decision Editor: Rafael de Cabo, PhD

In human somatic cells, telomeres, the nucleoprotein structures at the end of chromosomes, shorten with each cell division leading to an irreversible growth arrest of the cell. This process of senescence is associated with the aging process, and telomeres have therefore been proposed as markers for biologic aging (1,2).

Most studies investigating telomere shortening are cross-sectional, focusing on differences between groups of participants. Unfortunately, these studies give no information on the individual rate of telomere shortening over a period of time, rendering them inconclusive on individual susceptibility. Furthermore, it can also not be concluded from these studies whether there is an association between telomere length and (disease-specific) mortality. It is therefore relevant to perform longitudinal studies in which these relationships are investigated (3–14).

Several chronic diseases have been linked to chronic oxidative stress and accelerated telomere shortening, such as cardiovascular disease (CVD) and cancer (8,15,16). Epel et al. showed that short baseline telomere length was related to greater mortality from CVD in women. For men, aged 70–79 years, the rate of telomere shortening was related to greater cardiovascular mortality (8). The association of shorter telomeres with cancer morbidity and mortality is inconsistent. Although some studies showed a relationship between short telomeres and cancer risk (17,18), there were also studies showing an inverse relationship (16,19,20).

We hypothesized that leukocyte telomere length (LTL) decreases with age and that LTL is inversely related to all-cause and cause-specific mortality. To investigate this, we prospectively evaluated the rate of telomere shortening over a period of 7 years in 203 men who were 73–91 years old at baseline as part of the Zutphen Elderly Study cohort. Furthermore, we investigated the relationship between LTL and all-cause and cause-specific mortality during 7 years of follow-up.

Materials and Methods

Study Population

The Zutphen Elderly Study consisted of 887 participants in 1985 (response rate 70.1%). Because no whole blood samples were available in 1985, we defined 1993 as our baseline measurement for the present analyses. The cohort of The Zutphen Elderly Study consists of men born between 1900 and 1920. The study started in 1960 as the Dutch contribution to the Seven Countries Study (21). Between 1985 and 2000, the men were examined every 5 years. The purpose of these surveys was to gain insight into the associations of these risk factors with physical, social, psychosocial, cognitive functions, and perceived health in men aged 65 years and older. The Zutphen Elderly Study consisted of 553 men in 1993, of whom 390 participated (response rate 71%), and
345 had a blood sample. However, only 203 (59%) buffy coats could be used for DNA isolation because 142 samples were unavailable. For the follow-up examination in 2000, 176 men enrolled in the study in Zutphen. Blood samples were available from 146 individuals. Because of the insufficient quality of the DNA of two of the participants, 144 men (82%) were finally included in the present analysis. Only 75 men had also a blood sample available both in 1993 and in 2000. Written informed consent was obtained from all participants. The Zutphen Elderly Study (in 1993 and in 2000) was approved by the Medical Ethics Committee of The Netherlands Organization for Applied Scientific Research (TNO).

LTL Measurement

Nonfasting blood samples were collected in the morning. The samples were kept cool in a box with cooling elements, and plasma and serum were obtained in the afternoon. Samples were stored in Zutphen at −30°C before transport to the National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands, which took place within a few days after collection. After arrival at the RIVM, all samples were stored at −80°C until analyses. An aliquot of 200 μL of buffy coat was used to extract genomic DNA with the QIAamp DNA Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocol. The DNA was quantified using a Nanodrop instrument (Isogen Life Science, St-Pieters-Leeuw, Belgium).

LTL was determined by quantitative polymerase chain reaction as described previously (22,23). Two master mixes were prepared: one with telomere primers and one with human β-globin primers (1× IQ SYBRgreen supermix from BioRad-Laboratories BV, Veenendaal, The Netherlands). To confirm reproducibility of the applied method, we repeated for 20 samples the leukocyte LTL measurement by using an additional reference gene, acidic ribosomal phosphoprotein PO (36B4), 300 nM forward primer (5′-CACCAAGT-GGGAAATGTAAATCC-3′), and 500 nM reverse primer (5′-CCCAATCTATCATCAACGGTCAAAC-3′). Sample DNA was pipetted in a 96-well plate at a final concentration of 10 ng/μL. Subsequently, 20 μL of the mastermix was added, and the plate was shortly centrifuged. Each sample was run in triplicates. The coefficient of variation for the triplicates of the telomere reaction was 4.11% and for the reference gene 3.03%. For the standard curve, a reference DNA sample was diluted serially to produce three concentrations of 1.25, 5, and 10 ng/μL. In every run, negative controls (MQ + mastermix) and reference samples were included. The references were derived from two different HeLa cell lines: one with relatively short telomeres (HeLa S3: 5.5 kb) and one with long telomeres (HeLa 229: 14–15 kb). HeLa cell lines were kindly provided by Prof. Alexander Bürkle, University of Konstanz, Germany. Thermal cycling profiles of the polymerase chain reaction (PCR) protocol were as follows—telomere PCR: Cycle 1 (1×): 95°C for 3 minutes (min), Cycle 2 (30×): 95°C for 15 seconds (s) and 54°C for 2 min, Cycle 3 (1×): 95°C for 1 min, Cycle 4 (1×): 65°C for 1 min, and Cycle 5 (60×): 65°C for 10 s (melt curve) and human β-globin PCR: Cycle 1 (1×): 95°C for 3 min, Cycle 2 (40×): 95°C for 15 s and 58°C for 1 min, Cycle 3 (1×): 95°C for 1 min, Cycle 4 (1×): 65°C for 1 min, and Cycle 5 (60×): 65°C for 10 s (melt curve). The PCR was performed using a BioRad MyiQ iCycler Single Color RT-PCR detection system using iQ SYBR Green Supermix, containing iTaq Polymerase, dNTPs, SYBRGreen I, and buffers (BioRad).

Collection of Data on Lifestyle and History of Chronic Diseases

Information on cigarette smoking and marital status was collected by standardized questionnaires. Information about habitual consumption of foods, including alcohol, coffee, and tea beverages, was collected in 1993 by using the cross-check dietary history method, adapted to the Dutch food consumption pattern (24). During a physical examination, height and body weight were measured according to standardized procedures. Body mass index was calculated by dividing weight (kilograms) by the square of the height (square meters). As a sedentary lifestyle was associated with shorter LTL (25), a validated questionnaire on physical activity designed for retired men (that participants were asked to complete at home) was used to calculate the total minutes spent in physical activity per week (of an intensity of more than 2 kcal/[kg·h]) (26). Information on marital status (dichotomized) and education (dichotomized into lower and higher education, the later defined as higher vocational education, college, or university) was obtained by a self-administered questionnaire. The prevalence of chronic diseases such as CVDs, cancer, and the presence of diabetes mellitus was assessed by questionnaire and verified by information from general practitioners. Information on the vital status of the participants until July 1, 2000 was obtained from municipal population registries and the participants’ general practitioners. Information was verified with either hospital discharge data or information from The Netherlands Cancer Registry. The initial coding of the causes of death was done by three physicians and the final coding by an experienced clinical epidemiologist, who were all four unaware of the outcome of the LTL measurement. CVD was defined as Codes 390–459 as the primary cause of death according to the International Classification of Diseases, Ninth Revision and cancer as Codes 140–172 and 174–208 (referring to malignant neoplasm, except nonmelanocytic skin cancers).

Statistical Analysis

All data are presented as number (percentage), mean ± SD, or median, when appropriate. The LTL values were normally distributed. The baseline characteristics of the participants
were compared according to tertiles of LTL by using the one-way analysis of variance or the chi-square test, when appropriate. The mean LTL shortening over 7 years was estimated using a t test for paired samples. The Pearson’s correlation coefficient was used to analyze LTL data from 1993 to 2000. The Kaplan–Meier curves were used to present crude all-cause and cause-specific mortality. Hazard ratios with 95% confidence intervals of all three end points were estimated by Cox proportional hazards models. Three multivariate models were used. First, we tested the association in a crude model. Subsequently, two additional multivariable models were tested. Model 1 adjusted for age, and in Model 2, we additionally adjusted for potential confounders, such as smoking (27,28), alcohol use (29), body mass index (28), education (and the related socioeconomic status) (30), marital status, physical activity (31), and the presence of chronic diseases (6,23,32–36) because they were associated with telomere length (TL) in previous studies (9,14,23).

Furthermore, we performed two sensitivity analyses in which we first repeated the Cox proportional hazards models using data of 193 men excluding 10 participants who had died within the first year of follow-up and subsequently using data from 144 men without a history of chronic diseases. Tests of linear trend across increasing tertiles of LTL were performed by using the median values of LTL for each tertile. Two-tailed \( p < .05 \) was considered statistically significant. Statistical analyses were performed with SPSS for Windows (SPSS 17.0; SPSS Inc., Chicago, IL).

**RESULTS**

Sociodemographic and clinical characteristics at baseline are presented in Table 1. The mean age at baseline was 78 years (range: 73–91 years). During 7 years of follow-up, 105 men died (51.7%), of whom 45 (42.9%) from cardiovascular causes, 36 (34.3%) from cancer (of whom 12 from lung cancer). The 75 surviving and participating men had an average age of 83.1 years (range: 79–92) in 2000. No association was found of sociodemographic and lifestyle factors with tertiles of LTL.

We analyzed paired DNA samples from 75 individuals (elderly men), which were collected in 1993 and in 2000. The mean LTL in 1993 was 5.03 kbp, whereas the mean LTL in 2000 was 4.76 kbp. The mean decline in leukocyte LTL during 7 years of follow-up was 40.2 bp/year (95% confidence interval: 26.9–53.5, \( p < .001 \)). Although most individuals showed a decrease in TL, for 12 participants (16%), we found an increase. Pearson’s correlation coefficient showed a significant relationship between LTL in 1993 and 2000 in 75 men (\( r = .51, p < .001 \)) (Figure 1). Results from the Kaplan–Meier analysis of survival according to LTL are depicted in Figure 2. No statistically significant differences in all-cause, cardiovascular, or cancer
mortality were found in relation to LTL tertiles. Table 2 summarizes the results of the survival analysis according to tertiles of LTL. Again, LTL was not related to cardiovascular, cancer, and all-cause mortality. Results from the sensitivity analyses with a lag time of 1 year showed no indication of a potential reverse causation owing to preexisting chronic diseases (data not shown).

**DISCUSSION**

In a prospective analysis among elderly men, we observed a substantial decrease in mean LTL per year between 1993 and 2000. LTL measured in 1993 was strongly correlated with LTL measured in 2000. LTL was not related to all-cause or disease-specific mortality. In previous cross-sectional studies, the decrease in average TL ranged from 31 to 63 bp/year (37–41), and in prospective studies, a mean decrease of 38–71 bp/year was observed (8,13,14). These studies included different study populations (i.e. premenopausal women, patients with colorectal carcinomas, and healthy participants) with a mean age ranged from 31.4 to 75 years. These results were in accord with the 40 bp average decrease in LTL per year in the present study. For 16% of the participants, we found an increase in TL, which is consistent with five other prospective studies. In about 10%–34% of the participants in these studies, a stabilization or increase in TL was observed with a time interval ranging from 2.5 to 12.9 years (8,13,14,42,43). However, these results should be interpreted with caution because of measurement error and random variation.

With increasing age, telomeres shorten, but they are also influenced by oxidative stress (44,45). Several inflammatory diseases, such as CVDs and cancer, have been linked to chronic oxidative stress and accelerated telomere shortening (6,16). Therefore, we hypothesized that LTL is a risk factor for all-cause or cause-specific mortality, but this was not found in the present analysis. Likewise, three studies with similar study populations, e.g. elderly and the oldest old and similar follow-up periods, found also no association between TL and mortality (42,43,46). A possible explanation for these negative findings might be that there is a higher degree of TL instability in the oldest old compared with younger populations. In two recent studies carried out in younger populations, an inverse association was observed between TL and survival (13,14). Also in studies carried out in patients with coronary artery disease, diabetes, or stroke survivors, an association was found between TL and survival (3,9,12). Other differences between the present study and the ones showing an association were follow-up period (varying from 2.5 to 15 years), a higher number of participants (ranging from 510 to 1542 participants) (6,9,13,14,47), and the method used to determine TL (3,5,7,8,12,47).

![Figure 1. Relationship between leukocyte telomere length in 1993 and 2000 for 75 elderly men.](image1)

![Figure 2. Kaplan–Meier analysis of survival according to the tertiles of leukocyte telomere length status in 203 Dutch men. In log-rank overall tests, men with a shorter telomere length had similar survival rates as compared with men with a longer telomere length.](image2)
In a study by Halaschek-Wiener and colleagues, TL was measured in exceptionally healthy elderly persons. They concluded that there was less TL variation in the healthy oldest old when compared with younger participants (48). In the study by Cawthon and colleagues, it was concluded that TL was a significant predictor of mortality for participants aged 60–74 years, but in the older age group (of 75 years or older), this association was not significant. In the present study, LTL was determined with the same method as in the study by Cawthon and colleagues (7). These data support our finding that in the oldest old, LTL is not related to mortality.

Shorter LTL has been associated with CVD (8), such as myocardial infarction (49), although not in all studies (50) and predicted death or hospitalization in patients with chronic heart failure (50). LTL has predicted also myocardial infarction and stroke in men under 73 years old (15). However, other studies, notably those with elderly cohorts, have failed to find an association between LTL and mortality (42,46). In the present study, we did also not find an association between LTL and CVD mortality. In the previous studies, a positive instead of an inverse relationship was noted between long telomeres and risk of death through cancer for breast cancer (20), skin cancer (19), and non-Hodgkin lymphoma (16). A possible explanation for this association may be that cancer is associated with increased telomerase activity that lengthens telomeres (51), which is normally very low or absent in somatic cells (52). In the present study, LTL was not related to total cancer.

Potential limitations of our study should be considered. The Zutphen Elderly Study cohort is a random sample of the general population of white Dutch elderly men, which may limit the generalizability of our findings to other ethnic groups, to women and to men of younger age. TL was determined in the total white blood cell population, which may not accurately reflect TL dynamics. Lymphocytes showed a greater age-related decline in TL (53), and recently, it was shown that subpopulations of lymphocytes display differences in TL and in telomerase activity (54). Determining TL in specific subpopulations of lymphocytes may therefore better reflect the age-related shortening of telomeres. For the individual diseases, the number of cases was too small, and therefore, we combined all CVDs and all different types of cancer, but these end points were not related to LTL. This study has also several strengths. The high reliability of our TL analysis was confirmed in 20 samples with a different reference gene (reliability coefficient of .87, p < .001), rendering potential fragmentation or loss of DNA during isolation unlikely. Finally, we had an almost complete mortality follow-up and could estimate the LTL shortening over 7 years of follow-up, whereas previous studies used mostly cross-sectional designs.

In conclusion, our data showed that the average telomere shortening is 40 bp/year in elderly men, and LTL measured 7 years apart was strongly correlated. On the other hand, we did not confirm the hypothesis that LTL is a risk factor for mortality in elderly men. Future studies should focus on TL measurement in populations with a wider age range.

**Funding**
The Zutphen Elderly Study was supported by grants from The Netherlands Prevention Foundation (Preventiefonds). Part of this study was funded by a grant from The Netherlands Brain Foundation (Hersenstichting, Nederland, 15F07(2).24).

**Conflict of Interest**
No disclosures to report.
References


