JNCI J Natl Cancer Inst (2015) 107(6): djv074

doi:10.1093/jnci/djv074
First published online April 10, 2015
Article

ARTICLE

Peripheral Blood Leukocyte Telomere Length and Mortality Among 64 637 Individuals From the General Population

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Abstract

Background: Short telomeres in peripheral blood leukocytes are associated with older age and age-related diseases. We tested the hypotheses that short telomeres are associated with both increased cancer mortality and all-cause mortality.

Methods: Individuals (n = 64 637) were recruited from 1991 onwards from two Danish prospective cohort studies: the Copenhagen City Heart Study and the Copenhagen General Population Study. All had telomere length measured by quantitative polymerase chain reaction and the genotypes rs1317082 (*TERC*), rs7726159 (*TERT*), and rs2487999 (*OBFC1*) determined. The sum of telomere-shortening alleles from these three genotypes was calculated. We conducted Cox regression analyses and instrumental variable analyses using the allele sum as an instrument. All statistical tests were two-sided.

Results: Among 7607 individuals who died during follow-up (0–22 years, median = 7 years), 2420 had cancer and 2633 had cardiovascular disease as causes of death. Decreasing telomere length deciles were associated with increasing all-cause mortality ($P_{trend} = 2^{*}10^{-15}$). The multivariable-adjusted hazard ratio of all-cause mortality was 1.40 (95% confidence interval [CI] = 1.25 to 1.57) for individuals in the shortest vs the longest decile. Results were similar for cancer mortality and cardiovascular mortality. Telomere length decreased 69 base pairs (95% CI = 61 to 76) per allele for the allele sum, and the per-allele hazard ratio for cancer mortality was 0.95 (95% CI = 0.91 to 0.99). Allele sum was not associated with cardiovascular, other, or all-cause mortality.

Conclusion: Short telomeres in peripheral blood leukocytes were associated with high mortality in association analyses. In contrast, genetically determined short telomeres were associated with low cancer mortality but not with all-cause mortality.

Telomeres consist of protein and tandem repeats of TTAGGG nucleotides positioned at the chromosome tips. During DNA replication in normal mitosis, the telomeric DNA shortens because of the end-replication problem (1). When telomeres reach a critically short length, the cells become senescent and are unable to divide further. This process limits uncontrolled cellular division and, therefore, is believed to protect against cancer (2).

Short telomeres are associated with older age, male sex, and adverse lifestyle factors such as smoking and obesity (3). Telomere length in leukocytes has been investigated as a potential biomarker for age-related diseases such as cardiovascular disease (4,5), chronic obstructive pulmonary disease (6–8), and diabetes (9,10). It is also a marker of all-cause mortality in some (4,11,12) but not all studies (13).

Received: July 30, 2014; **Revised:** December 10, 2014; **Accepted:** February 24, 2015

Telomerase is activated in some cancer cells and enables their maintenance of telomere length contributing to the immortalization of cancer cells (2,14). There is, however, conflicting evidence regarding short telomere length and the risk of cancer and the potential association with increased mortality after a cancer diagnosis. Whereas some found an association between short telomeres and increased cancer risk (15,16), others did not (17). Similarly, some found reduced survival after a cancer diagnosis in patients with short telomeres (17–20), whereas others did not (21).

Observational analyses of the association between telomere length and mortality are confounded by the strong association of both telomere length and mortality with age, sex, adverse lifestyle, and socioeconomic characteristics (3,4). However, recent studies have identified single nucleotide polymorphisms (SNPs) in genes associated with telomere length (22–25). This enables genetic and, thus, unconfounded analyses of the association between telomere length and mortality.

In a study of 64 637 individuals, we tested the hypothesis that short telomeres measured in peripheral blood leukocytes are associated with increased cancer mortality and all-cause mortality observationally as well as genetically.

Methods

Study Design

We studied 56 216 participants from the Copenhagen General Population Study who were included between 2003 and 2009 and 8421 participants from the Copenhagen City Heart Study who were included between 1991 and 1994. In these Danish general population studies (4,6), Copenhagen residents were invited to complete a questionnaire and to undergo a physical examination. Blood samples were drawn for biochemical measurements and DNA isolation. There was no overlap of individuals between the two studies. Only white Danish individuals with an available telomere length measurement and a determined genotype for the studied SNPs were included (Supplementary Figure 1, available online). All participants gave written informed consent, and Danish ethics committees approved the studies (H-KF01-144/01 and KF100.2039/91).

Telomere Length Measurement

Telomere length was measured in DNA isolated from peripheral blood leukocytes using the Qiagen blood kit (26). We used a modified monochrome multiplex quantitative polymerase chain reaction (PCR) method (27), described previously (4,6), as this is the only method available for large-scale measurement (3). We used the reference single-copy gene for albumin to adjust for different amounts of DNA in samples. DNA from participants was analyzed in quadruplicates. The absolute telomere length was derived after calibration with measurement on K562 cell line DNA, for which telomere length was set to 5290 base pairs as determined by southern blotting (27) and which was included in each plate. For a complete description, see the Supplementary Methods (available online).

Genotyping

The study population was genotyped for rs1317082, rs7726159, and rs2487999. We selected these three SNPs as they were the three most significant hits with the largest effect sizes for SNPs associated with telomere length in a study that included samples from the Copenhagen City Heart Study (24). These SNPs are located in genes related to telomere biology: rs1317082 is located at 3q26.2 near the *TERC* gene, rs7726159 is located at 5p15.3 in the *TERT* gene, and rs2487999 is located at 10q24.3 in the *OBFC1* gene. *TERT* and *TERC* encode for telomerase reverse transcriptase and telomerase RNA template, which is necessary for elongation of telomeres (28). *OBFC1* encodes for part of the CST complex, which regulates telomerase activity (29). We used the Taqman method (Applied Biosystems, Life Technologies, Carlsbad, CA) to genotype individuals from the Copenhagen General Population Study. For a complete description, including details regarding primers and probes, see Supplementary Methods and Supplementary Table 1 (available online). Genotyping of individuals from the Copenhagen City Heart Study was performed using an Illumina custom genotyping chip (30) (Illumina). All genotypes were in Hardy-Weinberg equilibrium (chi^2 test: P = .98 for rs1317082, P = .90 for rs7726159, and P = .58 for rs2487999).

The allele sum was defined as the sum of the telomere length–shortening alleles for each of the three genotypes, thus theoretically ranging from 0 (3x0) to 6 (3x2). We combined individuals with a sum of 0 and 1 alleles into one group, as only 38 individuals had 0 telomere length–shortening alleles.

Mortality Endpoints

We used the Danish Central Person Registry to obtain date of death for all participants who died before April 23, 2013. All-cause mortality was defined as any death regardless of cause of death. None of the participants were lost to follow-up, and those emigrating (n = 286) were censored at the date of emigration.

We further classified all deaths according to the cause of death using the national Danish Register of Causes of Death, which is based on death certificate information reported by the attending physician at the hospital or in general practice when a person dies in Denmark. We used ICD-8 and ICD-10 diagnoses to classify all deaths as cancer mortality, cardiovascular mortality, and death from other causes. In Denmark, it is possible to list up to three causes of death on the death certificate, and some participants had causes of death in more than one category. Therefore, the sum of deaths from cancer, cardiovascular disease, and other causes exceeds the total number of deaths. Cause of death was not available after December 31, 2011, and the 1184 deaths after this date were classified as unspecified cause of death.

Covariates

All baseline characteristics were recorded or derived from data collected at the day of examination based on the self-administered questionnaire, physical examination, and blood sample. We selected covariates a priori based on a previous study indicating that these variables are most likely to contribute to mortality (31). Body mass index was calculated from measured weight (kg) divided by the height squared (m^2). Systolic blood pressure was measured at the physical examination. Smoking status, alcohol consumption in grams per week (12 g alcohol ~ 1 unit), and physical activity level were self-reported. Pack-years, the consumption of 20 cigarettes or equivalent per day for one year, were calculated on the basis of the information from the questions about current and former smoking habits. Standard hospital colorimetric assays measured plasma total cholesterol (mmol/L). For multivariable adjustments, missing continuous values were imputed

according to age and sex (<1%), and missing categorical values were assigned to a missing category (<0.5%).

Statistical Analyses

We used Stata version 12.0 (StataCorp, College Station, TX). All statistical tests were two-sided. Level of significance was a P value of less than .05, unless multiple comparisons were made, in which case we used Bonferroni correction. For trend tests, individuals were categorized in study-specific deciles according to decreasing telomere length coded 1 to 10 (the first decile consisted of individuals with the longest telomeres) or according to allele sum coded 1 to 6 (the first group represented individuals with the genetic predisposition to the longest telomeres).

Association Analyses

We used Cox regression analyses. For these analyses, follow-up began at the examination and blood sampling and ended at death, emigration, or April 23, 2013, whichever came first. Multivariable models were adjusted for age, sex, body mass index, systolic blood pressure, smoking status, tobacco consumption, alcohol consumption, physical activity, and cholesterol level. We assessed risk according to telomere length decile and used the decile group with the longest telomere length as the reference group. We assessed the proportional hazards assumption using Schoenfeld residuals; no major violations were observed. All statistical tests were two-sided.

Genetic Analyses

For the Cox regression models, we also examined risk per 200-base pair decrease of telomere length on a continuous scale. This arbitrary choice represents roughly a 10-year increase in age (4,6) and, thus, would be expected to infer a considerable increased mortality. We examined mortality according to allele sum, as well as per allele for each of the three SNPs, in unadjusted Cox regression analysis. The odds ratio of mortality for a genetically determined 200-base pair decrease in telomere length was derived by instrumental variable analysis using the control function estimator from a two-stage regression analysis (32). The first stage was a linear regression of the allele sum as a continuous instrument on telomere length. The second stage was a logistic regression of the predicted values of telomere length (including the estimated residuals from the first-stage linear regression in the second stage logistic regression) on mortality. The first stage regression analysis yielded a partial R-square of 0.005 and an F-value of 342; an F-value above 10 indicates sufficient statistical strength of the genetic information to be used as an instrument (33). This Mendelian randomization design is further described in Figure 1.

Sensitivity Analyses

We accounted for competing risk of death by calculating subhazard ratios by the method of Fine and Gray (34) using noncancer mortality as competing event for cancer mortality, noncardiovascular mortality as competing event for cardiovascular mortality, and cancer and cardiovascular mortality as competing event for death from other causes; participants were only followed up until December 31, 2011. For stratified analyses we tested for interaction with a likelihood ratio test that compared the main effect model with a model also including a two-factor interaction term.

Results

In this sample of 64 637 individuals from the general population, telomere length decreased by 17.4 base pairs (95% confidence interval [CI] = 17.0 to 17.8) per year increase in age ($P < 1*10^{-300}$) (Supplementary Figure 2, available online). Decreasing telomere length decile was statistically significantly associated with all potential confounders (Table 1). Notably, the allele sum was not associated with any confounders (Table 1; Supplementary Table 2, available online).

A total of 7607 individuals (12%) died during follow-up; 2420 had cancer and 2633 had cardiovascular disease as causes of death. Median follow-up time was seven years (range = 0 to 22 years). Decreasing telomere length decile was associated with increased all-cause mortality in age-adjusted analysis ($P_{trend} = 3*10^{-30}$), which attenuated after multivariable adjustment ($P_{trend} = 2*10^{-15}$) (Figure 2). For individuals in the tenth (with the shortest telomere length) vs the first decile, the multivariable adjusted hazard ratio of mortality was 1.40 (95% CI = 1.25 to 1.57). Results were similar for cancer mortality, cardiovascular mortality, death from other causes, and unspecified cause of death (Supplementary Table 3, available online).

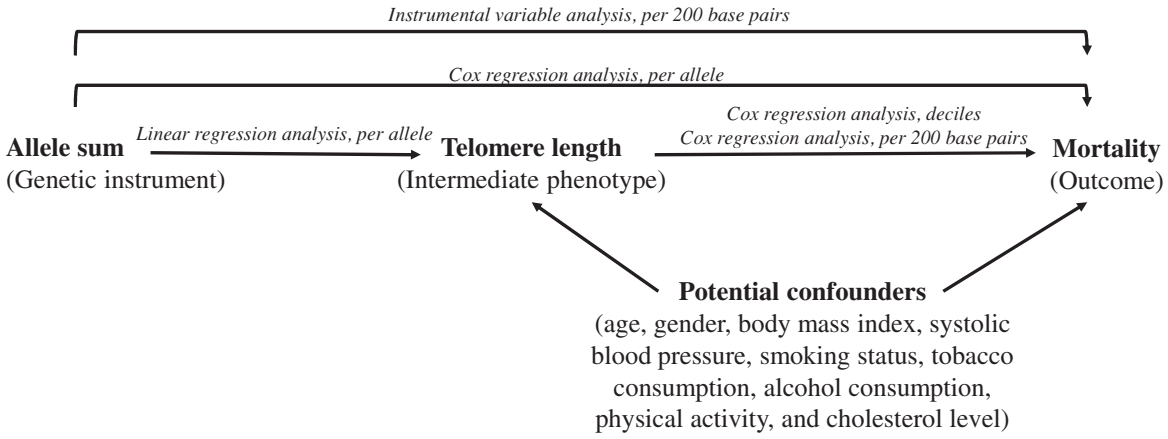

Figure 1. Mendelian randomization design (modified from Solovieff et al. [40]). Allele sum is used as a genetic instrument to test whether short telomeres are likely to cause increased mortality or whether the association between telomere length and mortality is more likely to be because of confounding factors or reverse causation. We assume that: 1) the genetic instrument (allele sum) is robustly associated with the intermediate phenotype (telomere length); 2) the genetic instrument (allele sum) is unrelated to confounding factors that bias the relationship between the intermediate phenotype (telomere length) and the outcome (mortality); and 3) the genetic instrument (allele sum) is related to the outcome (mortality) only through its association with the intermediate phenotype (telomere length).

Table 1. Baseline characteristics of the 64 637 participants according to telomere length

	Telomere length decile, no.											
Characteristic	1st (longest) 6465	2nd 6464	3rd 6464	4th 6464	5th 6464	6th 6463	7th 6463	8th 6464	9th 6464	10th (shortest) 6462	P_{trend}	P_{trend} allele sum*
Median telomere length, bp	5116	4073	3745	3512	3329	3169	3003	2828	2647	2331		
Age, y, median (IQR)	50 (42–60)	52 (43–62)	54 (45–63)	55 (45–64)	57 (46–66)	58 (48–67)	59 (49–69)	61 (51–70)	63 (53–72)	66 (56–74)	$<1*10^{-300}$	.38
Male sex, %	41	42	43	44	45	46	46	46	47	50	$1*10^{-36}$	.31
Body mass index, kg/m^2, median (IQR)	25.0 (22.7–27.9)	25.1 (22.8–27.9)	25.3 (22.9–28.2)	25.4 (23.0–28.2)	25.3 (23.0–28.2)	25.6 (23.2–28.5)	25.8 (23.3–28.8)	25.9 (23.4–28.7)	26.0 (23.5–28.7)	26.1 (23.6-29.0)	$4*10^{-99}$	.16
Systolic blood pressure, mmHg, median (IQR)	134 (120–149)	135 (123–150)	136 (124–150)	137 (125–152)	139 (125–153)	140 (125–155)	140 (127–155)	140 (128–156)	141 (129–157)	143 (130–159)	$2*10^{-226}$	.05
Ever smoker, %	55	59	60	61	62	63	64	65	65	68	$2*10^{-64}$	.95
Pack-years (ever smokers), median (IQR)	14 (5–28)	15 (6–30)	17 (7–30)	18 (8–31)	18 (8–32)	20 (8–34)	20 (8–35)	21 (9–36)	23 (10–36)	24 (10–40)	$3*10^{-156}$	.77
Alcohol consumption, g/week, median (IQR)	84 (24–156)	84 (36–168)	84 (36–168)	84 (36–168)	96 (36–180)	84 (36–180)	96 (36–180)	96 (36–192)	96 (36–192)	96 (36–192)	$4*10^{-36}$	.86
Leisure time physically inactive, %	45	45	47	47	47	47	47	49	50	49	$6*10^{-15}$	.45
Cholesterol, mmol/L, median (IQR)	5.5 (4.8–6.2)	5.6 (4.9–6.3)	5.6 (4.9–6.4)	5.6 (4.9–6.4)	5.6 (4.9–6.4)	5.6 (4.9–6.4)	5.7 (5.0–6.5)	5.7 (5.0–6.5)	5.7 (5.0–6.5)	5.7 (5.0–6.5)	$4*10^{-40}$	.53

* See Supplementary Table 2. *P* values are considered significant if less than .006 after Bonferroni adjustment (.05/9 = .006). bp = base pairs; IQR = interquartile range.

Telomeres shortened with increasing number of alleles when analyzing the sum ($P_{trend} = 1*10^{-104}$) (Figure 3) and each of the three individual SNPs (Supplementary Table 4, available online); the average per-allele decrease was 69 base pairs (95% CI = 61 to 76).

In Cox regression models the per-allele hazard ratio for cancer mortality was 0.95 (95% CI = 0.91 to 0.99) for the combined allele sum, 0.90 (95% CI = 0.82 to 0.98) for rs2487999 (*OBFC1*), 0.96 (95% CI = 0.90 to 1.02) for rs7726159 (*TERT*), and 0.96 (95% CI = 0.90 to 1.03) for rs1317082 (*TERC*) (Figure 4). Neither the allele sum nor any of the three SNPs individually were associated with cardiovascular mortality, death from other causes, unspecified cause of death, or all-cause mortality. Different combinations of the three SNPs yielded similar results (Supplementary Table 5, available online).

The hazard ratio for cancer mortality for a 200-base pair shorter telomere length was 1.02 (95% CI = 1.01 to 1.03) in

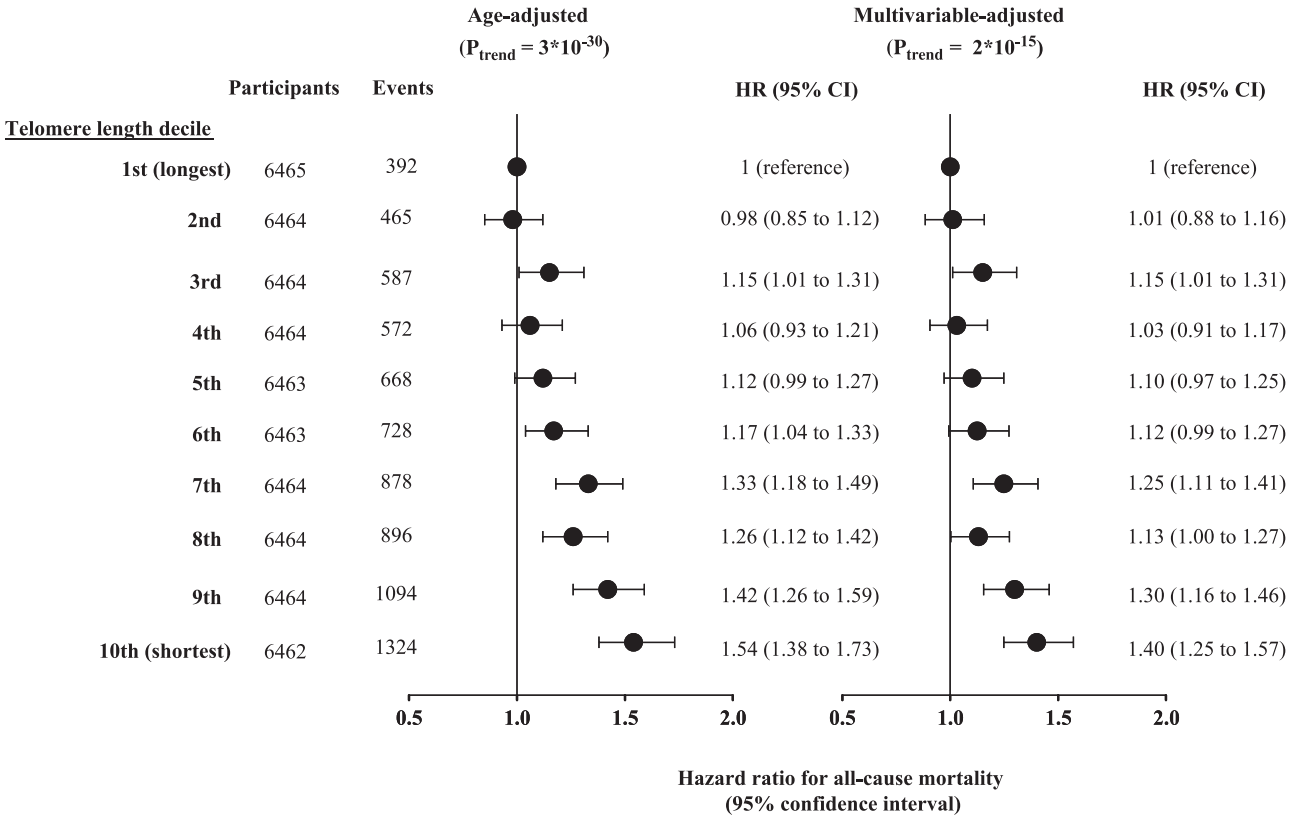

Figure 2. Risk of all-cause mortality in the 64637 participants from the general population according to telomere length deciles in age-adjusted and multivariable-adjusted Cox regression analysis. Multivariable models were adjusted for age, sex, body mass index, systolic blood pressure, smoking status, tobacco consumption, alcohol consumption, physical activity, and cholesterol level. All statistical tests were two-sided.

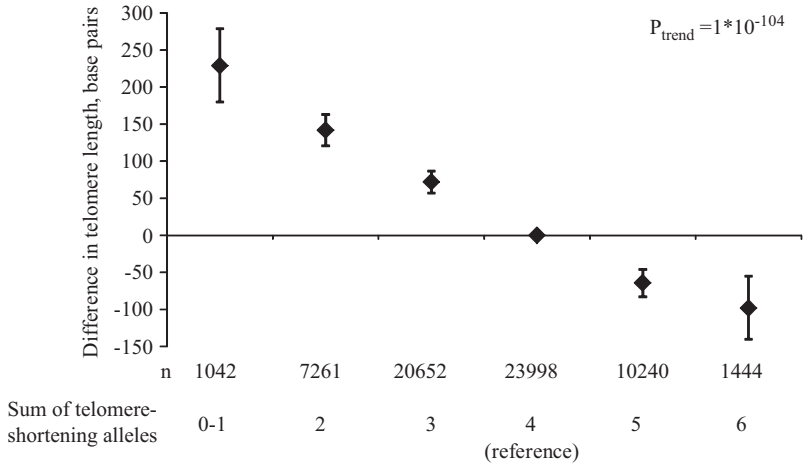

Figure 3. Difference in telomere length (mean, standard error) according to allele sum as the sum of the three genotype scores with an allele sum of 4 as the reference. rs2487999 (*OBFC1*) genotype: 0 = TT, 1 = CT, 2 = CC; rs7726159 (*TERT*) genotype: 0 = AA, 1 = AC, 2 = CC; rs1317082 (*TERC*) genotype: 0 = AA, 1 = AG, 2 = GG. Allele sum 0 and 1 are combined to one category because only 38 individuals had an allele sum of 0. See also Supplementary Tables 2 and 4 (available online) for estimates of the individual genotypes.

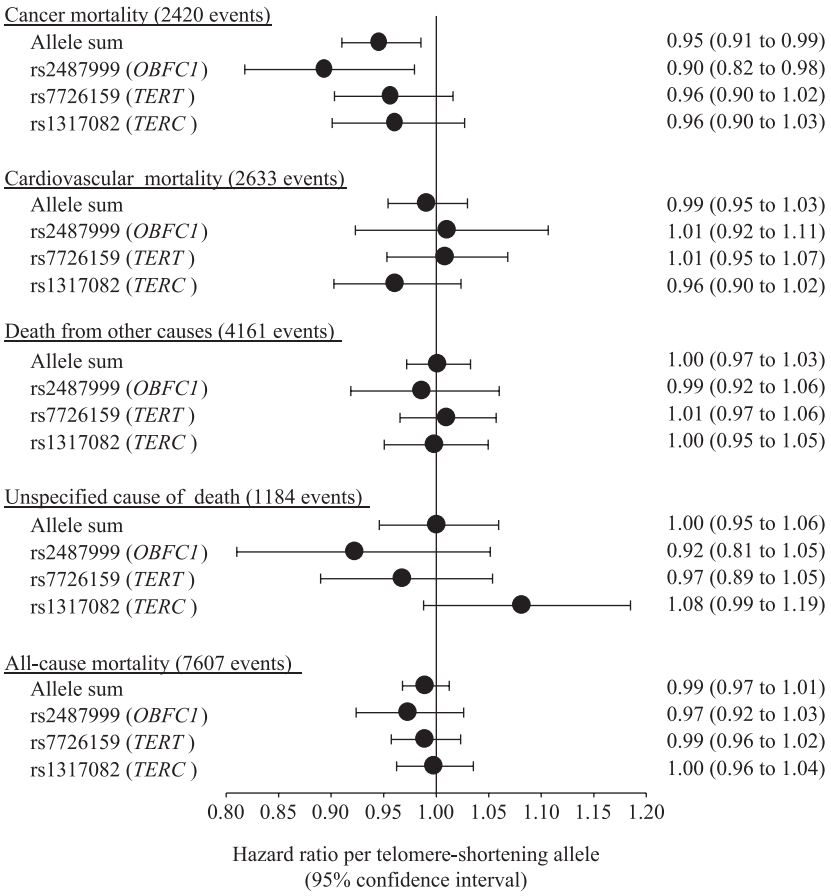

Figure 4. Risk of cause-specific and all-cause mortality per telomere length–shortening allele in the 64 637 participants from the general population. Allele sum constructed from rs2487999 (*OBFC1*), rs7726159 (*TERT*), and rs1317082 (*TERC*) genotypes: rs2487999 (*OBFC1*) genotype: 0 = TT, 1 = CT, 2 = CC; rs7726159 (*TERT*) genotype: 0 = AA, 1 = AC, 2 = CC; rs1317082 (*TERC*) genotype: 0 = AA, 1 = AG, 2 = GG. The total sum of cause-specific deaths exceeds the total of 7607 deaths because some participants had more than one cause of death listed on the death certificate.

multivariable-adjusted observational analysis (Figure 5). Results of observational analyses were similar for cardiovascular mortality, death from other causes, unspecified cause of death, and all-cause mortality. In contrast, a 200-base pair decrease in genetically determined telomere length was associated with decreased cancer mortality (odds ratio = 0.86, 95% CI = 0.76 to 0.96) (Figure 5) but not with cardiovascular mortality, death from other causes, unspecified cause of death, or all-cause mortality.

The results for cause-specific mortalities remained similar when we accounted for competing risk of death from other causes (Supplementary Figure 3, available online). Also, when we only allowed for one cause of death per individual, results were similar (results not shown).

In stratified multivariable-adjusted observational analyses for cancer mortality for a 200-base pair decrease in telomere length according to population, age, sex, body mass index, systolic blood pressure, smoking status, alcohol consumption, level of physical activity, and cholesterol level, we did not find evidence of interaction (Supplementary Figure 4, available online). In similarly stratified genetic analyses, all odds ratios for cancer mortality were robustly below 1, and we did not detect evidence of interactions.

Discussion

In this study of 64 637 individuals from the general population, we have confirmed (11,20,35) and repeated (4,17) previous findings of an observational association between short telomeres and high mortality, including cancer mortality. Also, and in contrast, we found that genetically short telomeres were associated with low cancer mortality but not low cardiovascular mortality, death from other causes, or all-cause mortality. This implies that genetically long telomeres are associated with increased cancer mortality. This is a novel finding. Supplementary Figure 5 (available online) summarizes our results for cancer and all-cause mortality.

Although unexpected, our seemingly paradoxical finding might be less puzzling when we consider the biology of telomere shortening and maintenance. Short telomeres as well as cancer mortality have been shown to be associated with increasing age, male sex, smoking, obesity, and several age-related diseases (3,16). Thus, the observational finding could be explained by common factors leading independently to both telomere shortening and increased cancer mortality without short telomere length per se mediating the increased mortality. Therefore, observational analyses are likely to be confounded. Conversely, a genetic disposition to long telomeres could affect cancer mortality through increased telomere length maintenance in certain cell types, which would otherwise have undergone senescence and apoptosis. All three genotypes examined in this study are related to telomere biology: two of them presumably through the TERT and TERC components of telomerase, and the third presumably through the CST complex, which regulates telomerase activity. Maintenance of telomeres is crucial for the long-term development of tumors, because telomerase

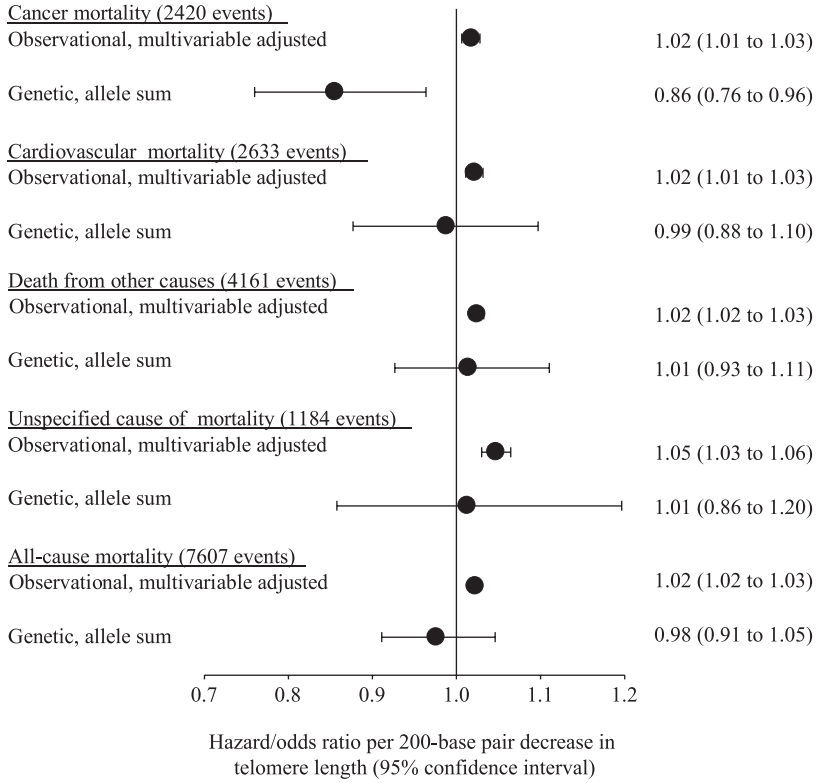

Figure 5. Observational hazard ratio and genetic odds ratio of cause-specific and all-cause mortality per 200-base pair decrease in telomere length in 64 637 individuals from the general population. Multivariable-adjusted models were adjusted for age, sex, body mass index, systolic blood pressure, smoking status, tobacco consumption, alcohol consumption, physical activity, and cholesterol level. The sum of cause-specific deaths exceeds the total of 7607 deaths because some participants had more than one cause of death listed on the death certificate.

activity, which is usually low or absent in normal somatic cells, has been found in most types of human cancers (2,14). Thus, our study might suggest that cancer cells arising in an individual with very few telomere-shortening alleles may have inherited this individual's good telomere maintaining capability and, consequently, this individual with genetically long telomeres will have increased cancer mortality simply because the cancer cells keep dividing and the cancer grows more, finally leading to death of the patient. The opposite directions of the association in our observational and genetic analyses can be compared with what was found for antioxidant supplements for prevention of early death. Based on animal models as well as observational studies, antioxidant supplements were long suggested to have a life-prolonging effect, but randomized trials including a large Cochrane review found no evidence to support such an effect (36). In fact, beta-carotene and vitamin E supplements seemed to increase mortality.

The assumptions for Mendelian randomization studies listed in the legends of Figure 1 seem to be met in this study. Assumption 1: The allele sum used as genetic instrument is constructed from three SNPs, which are highly associated with telomere length even in other studies using another telomere length measurement technique (23,25). They are located on three different chromosomes and thus unlikely to be genetically linked. This means that the effect of each of the three genotypes is likely only counted once, when adding the number of alleles together. Also, the three genes all encode for known and important components of complexes involved in telomerase activity and telomere maintenance. Although the instrument only explained 0.5% of the variation in telomere length, it had a very high F-value of 342. Assumption 2: We did not find any indication of an association between the allele sum and confounding factors. Assumption 3: The allele sum should only affect mortality through the telomere length. Although some SNPs in *TERT* were recently reported to be associated with risk of different cancers (30,37), the rs7726159 SNP used here was not independently among those.

The strength of our study includes the large sample size of 64 637 individuals with accurate information on date of death for all individuals who died before April 23, 2013, which enabled up to 22 years of follow-up for all-cause mortality. Our study also has some inherent limitations that are related to the applied methods including use of a Register of Causes of Death that does not rely solely on findings from autopsies but is based on the judgment of the attending physician. However, an inaccurately determined cause of death would tend to pull results towards the null hypothesis and thus is unlikely to explain our findings on cancer mortality. The registration of all-cause mortality is highly reliable, because it is mandatory by law in Denmark and therefore is considered to be 100% complete.

There are currently no standard calibrators and, thus, no definite traceability for telomere length measurements (3,38). We used quantitative PCR to measure telomere length, because this is the only practical method for large-scale studies like ours. Compared with southern blotting, which was the first method for telomere length measurement, quantitative PCR measures the average cumulative amount of TTAGGG repeats relative to the diploid genome and not telomere length per se. Because southern blotting usually measures the mean and the distribution of fragments containing TTAGGG repeats, including subtelomeric segments, the two methods are not directly comparable,

and the r^2 correlation between the two methods varies according to publications from 36% to greater than 80% (38). However, our single-center telomere length measurement had low imprecision and showed the expected very strong association with age, with a *P* value of less than $1^{*}10^{-300}$. Another limitation with regard to our telomere length measurement is that we measured mean peripheral leukocyte telomere length, and this may not necessarily reflect telomere length in all cells of the body. Earlier studies indicate, however, that leukocyte telomere length is correlated with telomere length in other cell types (39).

Admittedly, the choice of a 200-base pair telomere shortening is arbitrary and could also have been 20 base pairs or 1000 base pairs. However, as telomere length is a continuous measure, any other choice of shortening would only change the scale of the estimate, but not the *P* value.

In conclusion, short telomeres in peripheral blood leukocytes are associated with high mortality in association analyses. In contrast, genetically determined short telomeres are associated with low cancer mortality but not with all-cause mortality. This implies that genetically determined long telomeres are associated with high cancer mortality. We speculate that long telomeres may represent a survival advantage for cancer cells, allowing multiple cell divisions leading to high cancer mortality.

Funding

This work was supported financially by the Chief Physician Johan Boserup and Lise Boserup's Foundation, the Copenhagen County Foundation, and Herlev Hospital, Copenhagen University Hospital.

Notes

Author contributions: LR, BGN, and SEB initiated the study and collected data. LR did statistical analyses supervised by BGN and SEB. All authors analyzed and interpreted results. LR drafted the manuscript, which was scrutinized by the other authors, all of which accepted the final submitted manuscript.

We thank laboratory technician Anja Jochumsen for assisting with the telomere measurements. The authors are indebted to the staff and the participants in the Copenhagen General Population Study and the Copenhagen City Heart Study.

The authors declare that they do not have any conflict of interest.

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