Supplementary Online Content

Willeit P, Willeit J, Mayr A, et al. Telomere length and risk of incident cancer and cancer mortality. *JAMA*. 2010;304(1):69-75.

This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods

Study participants

The population of Bruneck is exclusively white and of heterogeneous geographic origin, with sizeable segments of the population being of either northern Italian or Austro-German descent. Population mobility within the Bruneck area is low, 0.2% per year at the time of the study. The official population register contains information obtained from the national census and is continuously updated regarding births, deaths, and changes of residence. The study population was recruited as a sex- and age-stratified random sample of all inhabitants aged 40 to 79 years: age groups were formed by decades and 125 women and 125 men from each age group were selected using a computer-based random number generator. At baseline, 936 men and women participated and 919 participants with complete data assessment formed the primary study population. Of these 63 died between 1990 and 1995. In 1995, 30 did not attend the re-evaluation, whereas the remaining 826 individuals participated (919-63-30=826). The latter two groups (30 versus 826) did not differ with respect to age, sex or baseline clinical characteristics, thus making relevant attrition bias and compromise in external validity unlikely. Follow-up until 2005 was 100% complete for clinical endpoints including cancer. The follow-up period for all participants except those who died was 120 (±1) months because re-evaluations were performed exactly every five years always over a period of about six weeks.

Clinical examination and history

All study participants underwent an ambitious examination program and completed a standardized questionnaire on medical history and various lifestyle factors, including current smoking, alcohol consumption, and physical activity. Diabetes was coded present according to the WHO definition (fasting blood glucose ≥126mg/dl, two-hour glucose level ≥200mg/dl after 75g oral glucose load, or definite pre-established diagnosis of diabetes). Pack years of smoking in current smokers and ex-smokers were defined as the average number of packs smoked daily multiplied by years of smoking. Body mass index was calculated as weight divided by height squared (kg/m^2). Physical activity was assessed with the Baecke score (sports index).[1] Average alcohol consumption was quantified in grams per day.[2] Finally, socioeconomic status was assessed on a three-category scale (low, medium, high) based on information about occupational status and educational level of the person with the highest income in the household. High socioeconomic status was assumed if the participant had ≥12 years of education and/or an occupation with an average monthly income ≥ $2000 (1995 salary). Low socioeconomic status was defined as ≤8 years of education and/or an average monthly income ≤$1000. All participants not meeting these criteria were classified into the medium socioeconomic status category. Chronic infection was assessed by a two-step procedure as described in detail elsewhere[3,4] and comprised chronic respiratory infections, COPD with recurrent infect exacerbation, recurrent lower urinary tract infections, pyelonephritis, chronic pancreatitis, diverticulitis, periodontitis, recurrent bacterial skin infections and diabetic foot ulcers. Overall, one-third of the entire population suffered from chronic infections.

Efficiency correction of the quantitative PCR method

Differences in quantitative PCR efficiency between samples were corrected with a mathematical algorithm developed by Pfaffl et al.[5,6] In general, the quantitative PCR method amplifies a defined DNA section and amplification kinetics are registered via a fluorescent signal. If the reaction is 100% efficient, the existing amplicons are copied completely and fluorescence doubles with every cycle. However, it has been shown that several factors, like phenol, ethanol, hemoglobin, and heparin impede the qPCR[7] and, thus, 100% efficiency is a rare condition. For instance, if efficiency is only 50%, the measured signal will rise by 1.5 with every cycle. Considering the exponential increase, even slight differences in efficiency have a striking impact on quantitative PCR.

The quantitative PCR kinetics in the exponential phase of the PCR can be described as $F_n = F_0 \times (1 + Eff)^n$ with F_0 and F_n being the amount of fluorescence measured at the very beginning and after n PCR cycles, respectively, and Eff being the efficiency of the PCR reaction. By log-transforming the equation, an equation similar to the general linear equation $y = d + k \times x$ is formed: $\log F_n = \log F_0 + n \times \log(1 + Eff)$. On a log-transformed ordinate, this formula describes the linear regression line of data points in the exponential phase, with $\log F_0$ being the intercept and $\log (1 + Eff)$ the slope of the line (red line in eFigure 6).

In our study, we used the software LinRegPCR 7.2 published in March 2003 for efficiency computation.[8] As depicted in eFigure 7, the program analyzes the log-linear part of the PCR by using linear regression and calculates intercept and slope. In detail, an iterative algorithm searches for lines consisting of at least four and no more than six data points with the highest R^2 value and a slope close to the maximum slope. Efficiency is determined by inserting the calculated slope in the following equation:

$$\text{slope} = \log(1 + Eff) \Leftrightarrow 10^{slope} = 1 + Eff \Leftrightarrow 10^{slope} - 1 = Eff$$

Calculation of T/S ratios

A control sample was added to each plate to account for inter-assay variability.[9] The relative T/S ratio is described as the ratio of the telomere to a single copy gene (SCG) in one sample, set in relation to the same ratio in the control DNA. In keeping therewith, the formula for calculating the relative T/S ratios can be stated as:

$$F_n = F_0 \times (1 + Eff)^n$$
$$\Rightarrow F_{Ct(tel)} = F_{0_{tel}} \times (1 + Eff_{tel})^{Ct(tel)}$$
$$\Rightarrow F_{Ct(SCG)} = F_{0_{SCG}} \times (1 + Eff_{SCG})^{Ct(SCG)}$$

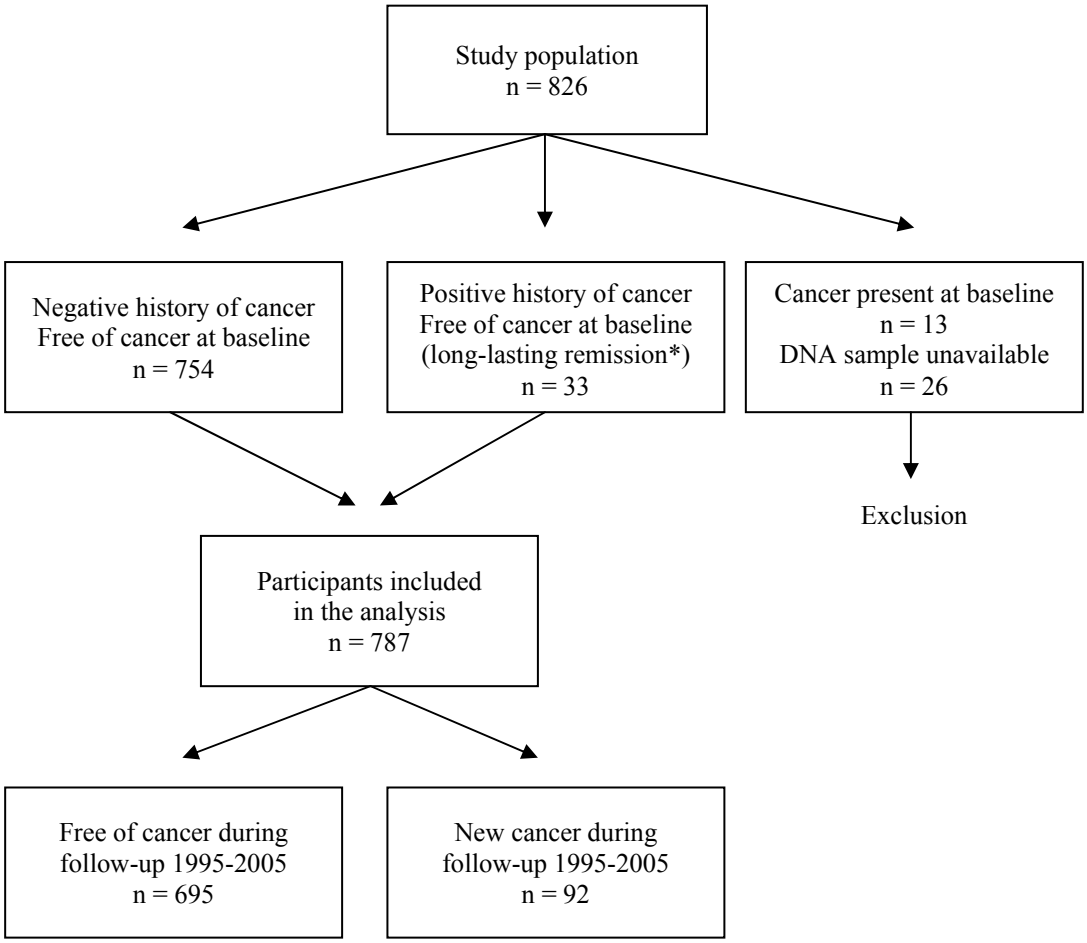
$$T/S\ ratio\ (sample) = \frac{F_{Ct(tel)} \times \left(1 + Eff_{tel,sample}\right)^{-Ct(tel,sample)}}{F_{Ct(SCG)} \times \left(1 + Eff_{SCG,sample}\right)^{-Ct(SCG,sample)}}$$
$$T/S\ ratio\ (control) = \frac{F_{Ct(tel)} \times \left(1 + Eff_{tel,control}\right)^{-Ct(tel,control)}}{F_{Ct(SCG)} \times \left(1 + Eff_{SCG,control}\right)^{-Ct(SCG,control)}}$$

$$\Rightarrow Relative\ T/S\ ratio = \frac{\left(1 + Eff_{tel,sample}\right)^{-Ct(tel,sample)}}{\left(1 + Eff_{SCG,sample}\right)^{-Ct(SCG,sample)}} \ / \ \frac{\left(1 + Eff_{tel,control}\right)^{-Ct(tel,control)}}{\left(1 + Eff_{SCG,control}\right)^{-Ct(SCG,control)}}$$

Southern Blot

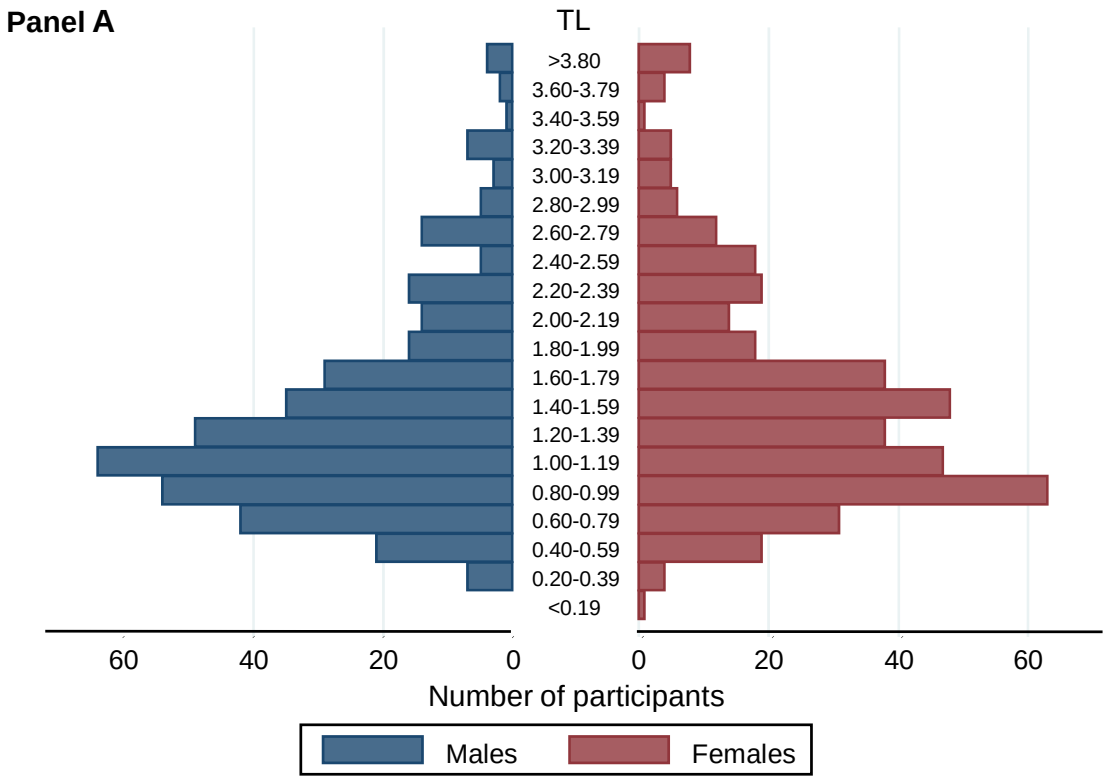
5µg of genomic DNA were digested overnight with the restriction enzymes RsaI/HinfI (Roche) and loaded onto a 0.5% agarose gel with 0.5µl marker (DIG-labeled Marker VII, Roche). After electrophoresis, gels were depurinated, denaturated, and neutralized. Capillary transfer was performed overnight with 10X SSC using positively charged nylon membranes (Roche). Membranes were briefly rinsed with 2X SSC, cross-linked for 3min under UV light, washed for 30 min with 2X SSC at 65°C and prehybridized with hybridization buffer (5X SSC, 1% blocking reagent by Roche, 0.02% SDS, 0.1% sarcosyl) for 4.5 hours at 65°C. Hybridization took place overnight at 65°C using 10pmol/ml DIG-labeled probe $(CCCTAA)_3$ (Microsynth) in hybridization buffer. Membranes were washed three times for 15 minutes at room temperature and 15 minutes at 65°C with 2X SSC/0.1% SDS. Detection of DIG-labeled probe and marker was performed using the DIG Luminescent Detection Kit for Nucleic Acids (Roche) according to the manufacturer's instructions. After 2 h of exposure, films (GE Healthcare) were developed and fixed (Kodak). Density measurements were performed with the Quantity One software by BioRad.

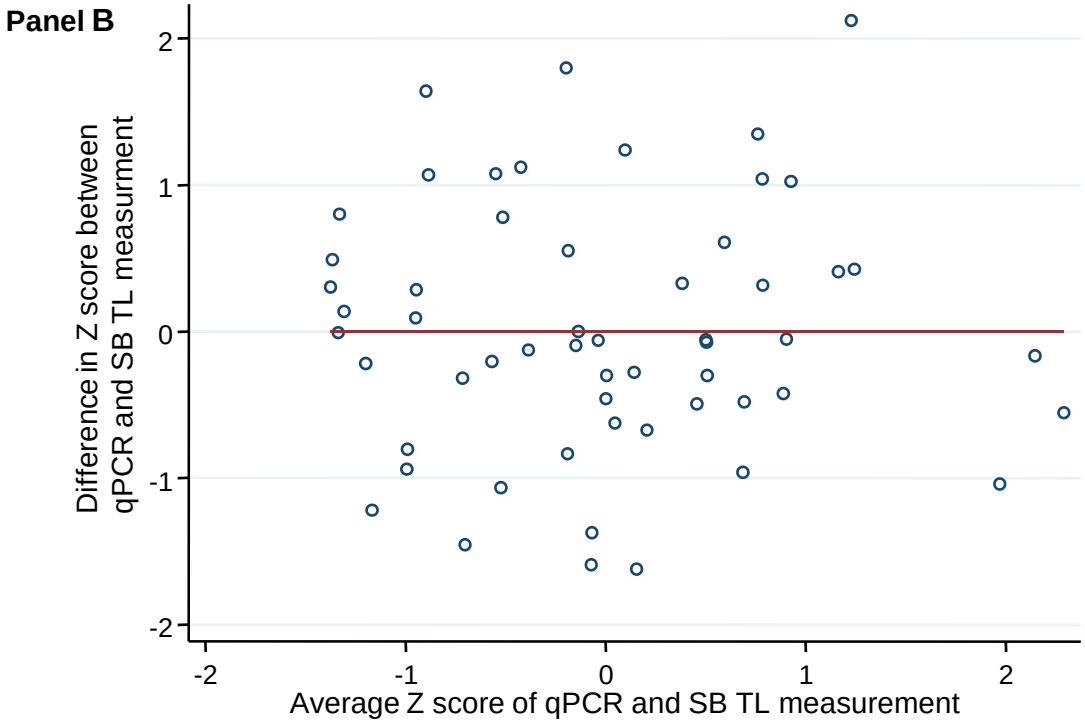
eFigure 1: Flow-chart of study population



*These patients were considered to be cured of cancer at baseline. However, four of the 33 patients with prior cancer (renal cell carcinoma n = 1, uterine cancer n = 3) subsequently developed a second cancer (colorectal cancer n = 2, gallbladder cancer n = 1, pancreatic cancer n = 1).

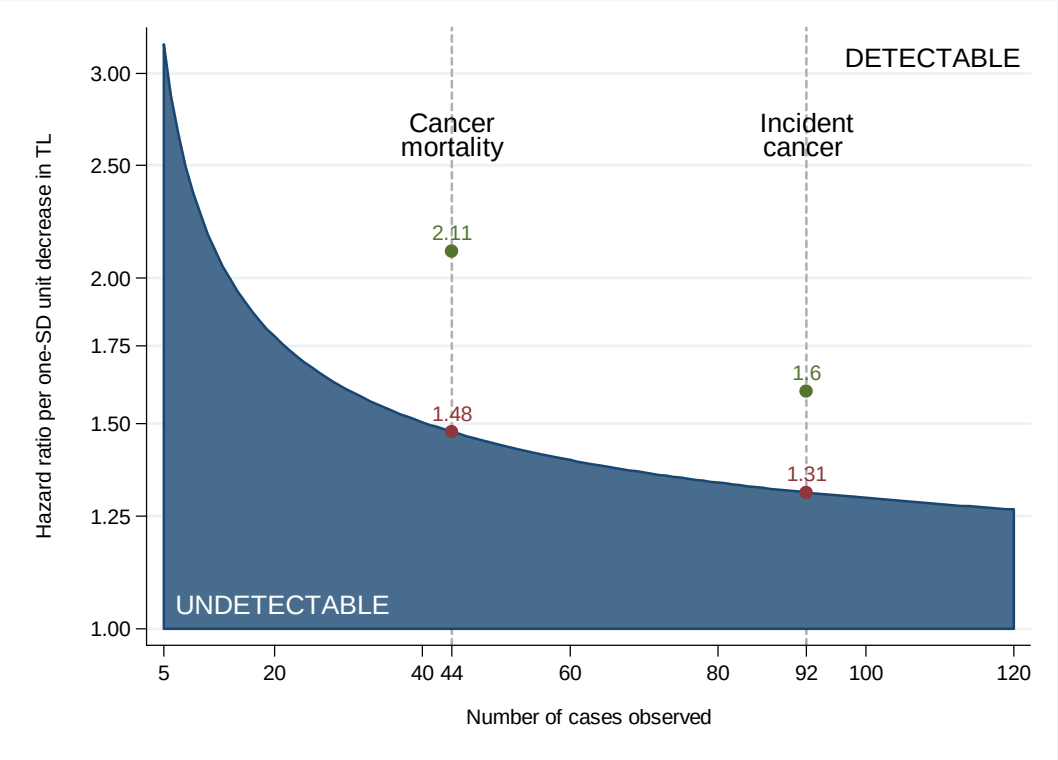
eFigure 2: Distribution of telomere length (T/S ratio) in the study population (N = 787) (Panel A) and comparison of telomere length assessed by quantitative PCR (T/S ratios) and Southern Blot (TRF) in a random sub-sample of 56 participants (Panel B). The latter graph shows a Bland-Altman Plot.





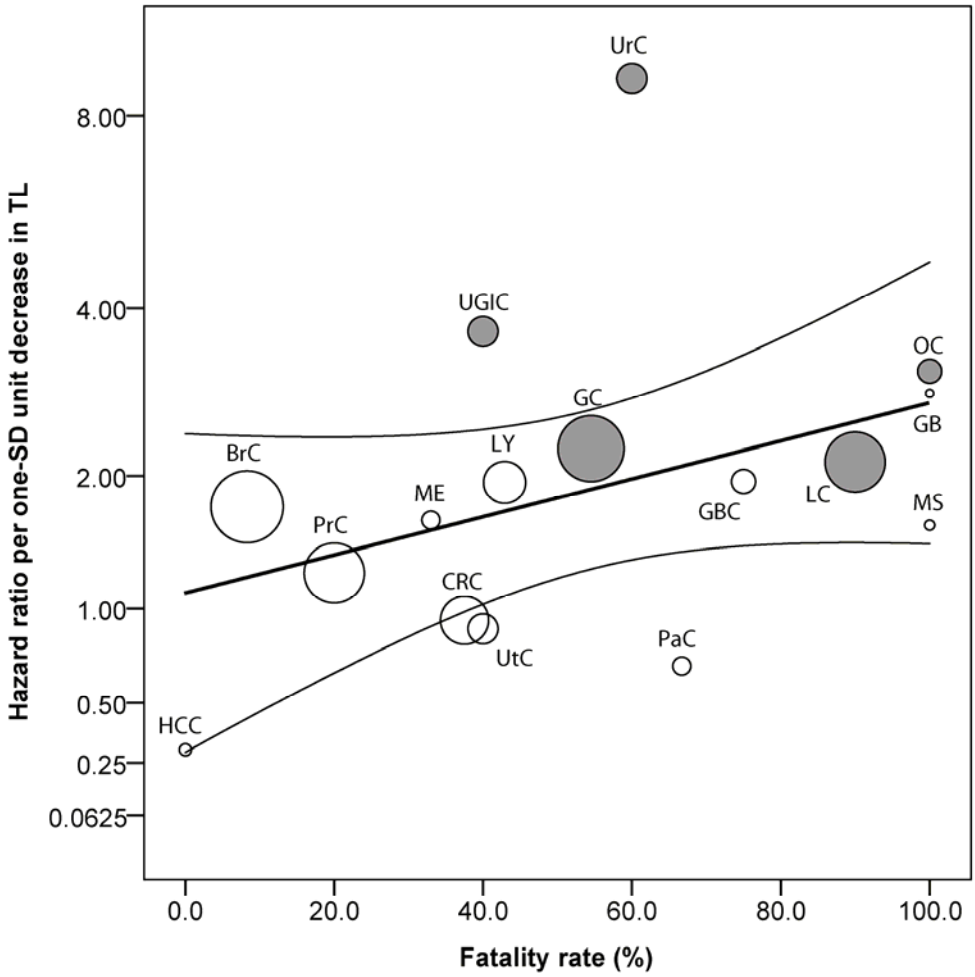
The Bland-Altman Plot indicates that there is no proportional measurement error for either method and no association between magnitude and variation of measurements. Since T/S ratios and TRF use different units, both were Z-transformed.

eFigure 3: Minimum hazard ratio detectable with a power of 80% (α=0.05 for one-sided hypothesis testing) (y axis) as a function of the number of endpoints observed (x axis) .



The sample size calculation was adjusted for participants' characteristic based on the expectation that multiple regression of telomere length on all covariates (see methods) results in an R^2 of 0.0841. For example, the HR detectable for a specific cancer type with ten incident cases amounts to 2.26. The number of endpoint events in our study (n=44 for cancer mortality and n=92 for cancer incidence) are marked on the x axis, as are the minimum HRs detectable (red dots) and the observed HRs (green dots).

eFigure 4: Hazard ratios for the manifestation of specific cancer subtypes (y axis) plotted against the respective fatality rate (x axis).



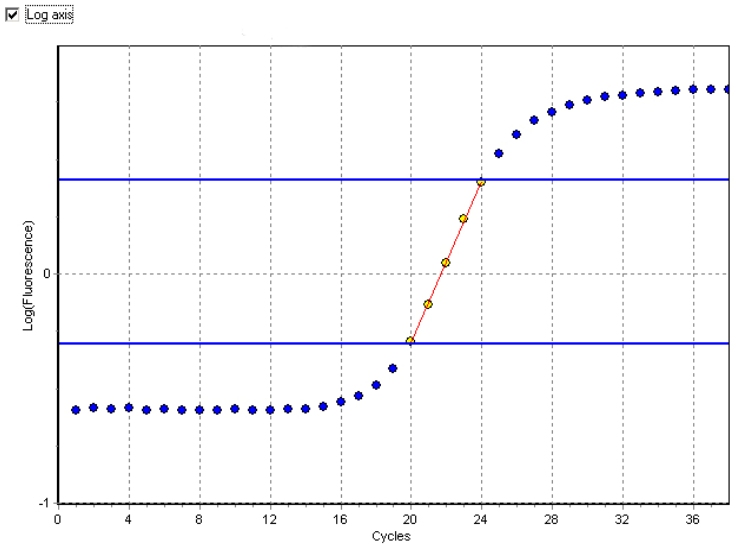
HRs of each specific cancer were calculated for a one SD unit decrease in $\log_e$-transformed telomere length. The curves display the regression line (95%CI) fit between $\log_e$-transformed HR of specific cancers (weighted by the number of events observed) and the respective fatality rate (slope ß=0.086 for a 10% increase in fatality rate; 1.09-fold HR for a 10% increase in fatality rate; P<.001). Overall this graph indicates that the predictive significance of telomere length for cancer manifestation increases with given fatality rates. The area of the circles reflects the number of incident cases for each cancer type, circles in grey indicate statistical significance at a P<0.05 level.

Abbrevations: BC, breast cancer; CRC, colorectal cancer; GB, glioblastoma; GBC, gallbladder cancer; GC, gastric cancer; HCC, hepatocellular carcinoma; LC, lung cancer; LY, lymphoma; ME, melanoma; MS, meningosarcoma; OC, ovarian cancer; PaC, pancreatic cancer; PrC, prostate cancer; UGIC, upper gastrointestinal cancer; UrC, urothelial cancer; UtC, uterine cancer.

eFigure 5: Changes in telomere length (TL) between 1995 and 2005 for female and male study participants according to baseline telomere length

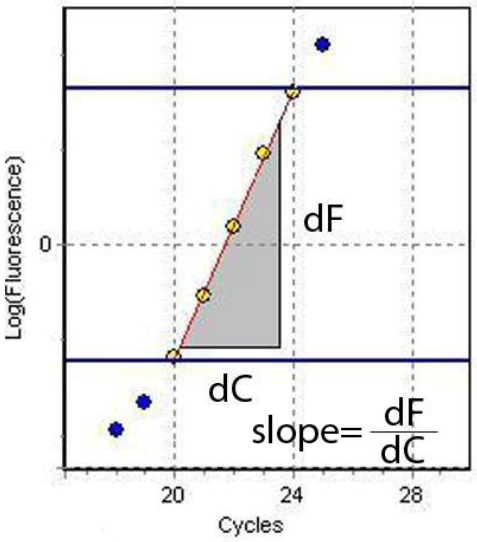
Telomere length at the 1995 baseline was divided into five groups (<7000 base pairs; 7000-7999; 8000-8999; 9000-9999; ≥10000) and plotted against the respective telomere length at the 2005 follow-up investigation. Dots are mean values in each group. Numbers of individuals in the five groups are as follows: female (26, 122, 78, 48, and 22) and male (13, 106, 81, 41, and 21) in 2005.

eFigure 6: Rise of the fluorescent signal in a quantitative PCR



The ordinate has a logarithmized scale. For details, see section on efficiency correction of the quantitative PCR method.

eFigure 7: Triangle for computation of the slope



For details, see section on efficiency correction of the quantitative PCR method.

eTable. Baseline characteristics in the entire study population (left column) and separately in participants with and without incident cancer during follow-up (1995-2005)

Characteristic[a]	Total Participants (N = 787)	Incident Cancer		*P* Value[a]
		No (n = 695)	Yes (n = 92)	
Age, mean (95 %CI), y	62.6 (61.8-63.3)	62.0 (61.1-62.8)	67.0 (64.9-69.1)	<.001
Male sex, No. (%)	388 (49.3)	336 (48.4)	52 (56.5)	.14
Social status, No. (%)				.90
Low	473 (60.1)	416 (59.9)	57 (62.0)	
Middle	177 (22.5)	158 (22.7)	19 (20.6)	
High	137 (17.4)	121 (17.4)	16 (17.4)	
		Mean (95% CI)[b]		
Telomere length[c]				
Relative T/S ratio assessed by quantitative PCR	1.48 (1.43-1.54)	1.53 (1.47-1.59)	1.12 (1.01-1.23)	<.001
Southern Blot, kB	8.05 (7.97-8.12)	8.11 (8.02-8.19)	7.57 (7.42-7.71)	<.001
Lifestyle and cancer risk factors				
Alcohol consumption, g/d[d]	10.7 (0-33.5)	9.0 (0-31.0)	21.3 (3.9-49.8)	<.001
Current or ex-smokers, No. (%)	353 (44.9)	304 (43.7)	49 (53.3)	.08
Pack-years of smoking[d]	0 (0-26)	0 (0-24.5)	9.6 (0-35)	.04
Physical activity via sport index	2.37 (2.31-2.43)	2.39 (2.33-2.46)	2.18 (1.99-2.37)	.03
Body mass index[e]	25.7 (25.4-26.0)	25.8 (25.6-26.1)	24.7 (24.0-25.5)	.01
Diabetes, No. (%)	93 (11.8)	77 (11.1)	16 (17.4)	.08
LDL cholesterol, mmol/L	3.76 (3.69-3.83)	3.80 (3.72-3.87)	3.50 (3.33-3.68)	.008
High-sensitivity CRP, nmol/L[d]	16.2 (8.6-30.5)	15.2 (7.6-28.6)	21.9 (9.5-48.6)	.01
Chronic infection, No. (%)	242 (30.8)	202 (29.1)	40 (43.5)	.005
Interleukin 6, pg/mL	8.2 (6.9-9.4)	8.0 (6.7-9.3)	9.7 (6.1-13.3)	.37
25-hydroxyvitamin D, nmol/L	79.7 (77.5-81.9)	80.5 (78.2-82.8)	73.6 (66.9-80.3)	.05

Abbreviations: CI, confidence interval; CRP, C-reactive protein; LDL, low-density protein; PCR, polymerase chain reaction.
SI conversion factors: To convert CRP to mg/L, divide by 9.524; LDL cholesterol to mg/dl, divide by 0.0259; 25-hydroxyvitamin D to ng/mL, divide by 2.496.
[a]Calculated with the χ^2 or Fisher exact test or with independent-samples T test.
[b]Unless otherwise indicated.
[c]Transformation of relative T/S ratio (quantitative PCR) to telomere length in kilobases (kB) was performed with the formula: telomere length in kB = 6.016 + 1.365 x (relative T/S ratio). For further explanations, see eMethods.
[d]Values are expressed as median (interquartile range).
[e]Calculated as weight in kilograms divided by height in meters squared.

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