Telomeres in Molecular Epidemiology Studies

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Abstract

Telomeres are long nucleotide repeats and protein complexes at the ends of chromosomes that are essential for maintaining chromosomal stability. They shorten with each cell division and therefore telomere length is a marker for cellular aging and senescence. Epidemiologic research of telomeres investigates the role that these genetic structures have in disease risk and mortality in human populations. This chapter provides an overview of the current telomere epidemiology research and discusses approaches taken in these investigations. We also highlight important methodological considerations that may affect data interpretation.

Keywords

Telomere; telomere length; epidemiology; association studies

Introduction

Telomeres have been an attractive biological marker for longevity and health since the discovery that they shorten with normal cell division (1). Telomere studies in humans have ranged from understanding telomeric structure, function, and regulatory mechanisms, to evaluating their role in aging, disease risk and mortality. At the end of 2013, the search term "telomeres" in PubMed returned more than 17,000 publications.

The first (2), published in 1950, reported the existence of a structure that was first hypothesized by Hermann Muller in the 1930s to cap the end of the chromosome. Since then, the number of telomere-related publications has grown rapidly (Figure 1). Telomere studies have been largely conducted in the fields of cancer and aging as noted by the number of publications in those fields. As of early 2014, the search term "telomeres and cancer" returned 5,586 publications and "telomeres and aging" returned 3,340 publications.

Epidemiology is derived from three Greek words: *epi*, meaning "among", *demos*, meaning "people" and *logos* meaning "study." In total, they mean "the study of what is among the people," with a focus on health-related events. The science of epidemiology further expanded from describing distribution and patterns, to identifying etiological factors and evaluating effects of diseases in a defined population.

The epidemiological literature of telomeres over the past decade features studies focused on 1) describing telomere length (TL) variation by demographic characteristics such as age (3), gender or race(4); 2) evaluating the association between rare and common inherited

telomeric defects and disease risk or severity(5–7); 3) evaluating the effect of environmental exposures and lifestyle factors on TL (*e.g.* stress (8, 9), exercise (10), smoking (11, 12), certain chemicals (13), and others); and 4) determining the role telomeres may play in certain outcomes such as mortality (14), or age-related diseases such as cancer (15), cardiovascular diseases (16), or others (17). Few behavioral intervention studies have been implemented to date; they include a study evaluating the role of meditation training on telomerase activity (18) and a study of the role of a lifestyle intervention on TL (19).

In this chapter, we discuss challenges related to study design in telomere epidemiology research, limitations related to telomere measurement methods and surrogate tissue use in most studies. Table 1 presents the definitions of some epidemiological terms (20, 21) introduced in this chapter.

Telomere Length in Accessible Tissue as a Surrogate for the Disease Site

Many epidemiological studies measure TL in peripheral blood leukocytes. While leukocyte TL may be the specific biomarker of interest in some studies, in others, it may be a surrogate for a specific tissue. Data comparing TL in peripheral blood leukocytes and in different somatic tissues from the same individual are limited. The use of leukocyte TL as a surrogate for the tissue of interest may be affected by the tissue specific proliferation rate as well as the susceptibility to environmental factors affecting TL regulation in that specific tissue. Leukocytes are a heterogeneous cell population, formed mainly by lymphocytes and granulocytes. Notably, lymphocytes have shorter telomeres than granulocytes (22).

Studies comparing TL in different tissues from normal individuals have found differences in absolute TL between tissues but strong correlations are present. For instance, in a study measuring TL in leukocytes, skeletal muscle, skin, and subcutaneous fat from the same 87 adult individuals, the authors found high degrees of correlation within individuals but noted that leukocytes had the shortest telomeres (23). Similar findings have been reported when comparing TL in leukocytes, skin, and joint tissues in a small study of elderly patients with hip fractures (24). A Japanese study measured TL in cerebral cortex, kidney, liver and myocardium in over 100 autopsies from individuals of different ages, including neonates through centenarians, and also found high intra-individual correlations between different tissue TL measurements (25); myocardial tissue had the longest TL while liver and renal cortex had the shortest. Finally, a study compared average leukocyte TL and buccal TL in young (18–26 years of age) and old (66–75 years of age) adults (26). The authors found that leukocyte TL was significantly shorter than buccal TL in both age groups. As expected, leukocyte TL was significantly shorter in the older group compared with the younger group, but surprisingly there were no differences in buccal TL between the two age groups (26). Of note, other factors, such as increased body mass index (BMI) or tobacco smoking, that have been associated with shortening of leukocyte TL (12), were not accounted for in that study.

Some studies have compared TL measured in different tissues from individuals with specific illnesses. TL was measured in leukocytes, buccal cells and fibroblasts in individuals with dyskeratosis congenita (DC), an inherited telomere biology disorder where telomeres are exceedingly short (below the 1st percentile of normal individuals of the same age) (5, 7). The

authors reported strong correlations between the TL in these different types of cells, with leukocytes having the shortest telomeres (27). TL has also been measured in somatic tissues from cancer patients, comparing tumor tissue to adjacent normal in most cases. Telomeres were shorter in prostate cancer cells than in adjacent non-malignant cells (28). Moreover, shorter TL in prostate cancer-associated stromal cells and larger TL variation in prostate cancer cells were associated with poor outcomes. Telomere shortening has also been reported in the early stages of breast carcinogenesis (29) and was correlated with more aggressive subtypes of breast cancer (30). Shorter telomeres have also been observed in tissues near breast tumors compared with distant normal breast tissue (31, 32) suggesting a TL field effect in the adjacent non-malignant breast tissue. Variation in TL in adjacent non-malignant breast tissues was suggested to be a prognostic factor of breast cancer recurrence (33). Finally, findings from a study that measured TL in colon tissues from patients diagnosed with ulcerative colitis, a chronic inflammatory disease of the colon, and healthy controls suggested shorter colonic TL in patients than healthy controls (34). Notably, telomere shortening has also been observed in adenomas and colorectal cancer tissues as compared with normal adjacent or colon distal tissues (35, 36). The field effect suggested in the breast cancer studies did not appear to be present in a colon cancer and adenoma study (36).

Overall, there is evidence of strong correlation between leukocyte TL and TL measured in other somatic tissues from the same individuals, which is not surprising given that TL is highly heritable (37). It is important to note that the absolute TL differs between tissues, and telomeres tend to be shorter in leukocytes compared with other evaluated tissues. This suggests that the dynamics of TL are likely to be tissue-specific, reflecting tissue-specific replicative capacity. Future research is required to evaluate the relationship between TL measured in surrogate tissues and in tissues affected by the disease of interest.

Considerations in Observational Studies of Telomere Length

Association studies of TL and disease have become an important area in molecular and genetic epidemiology. Like other genetic and biological marker studies, telomere epidemiology relies on the common observational study designs, including cross-sectional, case-control, and cohort studies. Table 2 summarizes the purpose and characteristics of each design, which should be carefully considered during study design and data interpretation.

Cross-sectional studies in telomere epidemiology have been used to assess the relationship between TL and demographic characteristics, such as behavioral lifestyles or environmental exposures. For example, cross-sectional study design has been used to evaluate the relationship between TL and chronological age (reviewed in (38)), and its distribution by sex and race (4). Overall, studies have shown that older age (39), White race (4), and male gender (4, 40) are associated with shorter telomeres, but the strength of association varied by participants' age and method of TL measurement (41). The association between TL and other factors, such as smoking, physical activity or alcohol use, has been inconsistent (11, 12, 42). The inconsistencies found in the published studies may be related to the fact that TL at the time of the study may not reflect the current state of an exposure, as measured in a cross-sectional study (43). Cross-sectional studies are often highly feasible but limited by

their inability to determine a temporal relationship between TL and factors of interest (*i.e.*, outcome may have preceded TL changes in this situation). Despite this limitation, cross-sectional studies are valuable tool for hypothesis generation.

Cross-sectional studies are useful in evaluating the relationship between genetic variation in telomere genes and disease risk since an individual germline genome does not change over time. Single nucleotide polymorphisms (SNPs) in the *TERT* gene locus has been associated with peripheral arterial disease (44) and several cancers (45, 46); variation in the *OBFC1* gene predicted cardiovascular mortality in women (47); and variation in both *TERT* and *TEP1* genes were associated with male infertility (48). In these situations, investigators suggest that genetic variation can be thought as a surrogate of TL (49). This is based on a connection between TL and SNPs in several genome-wide association studies that identified associations between genetic variation in some known telomere biology genes (*TERT*, *TERC*, and *OBFC1*) and TL (50–52). However, many more studies are required before making a direct connection between these SNPs and TL.

Case-control and prospective studies are commonly used analytic approaches in evaluating whether TL is a biomarker or risk factor for age-related diseases. The main difference between the two approaches is the timing of TL assessment in relation to the disease of interest. In case-control design, TL is measured after the disease develops and is compared to individuals who are free of the disease (controls). The selection of the control population is crucial; it should be representative to the source population that gave rise to the cases to avoid possible selection bias. Details about control selection processes can be found elsewhere (53). In prospective design (cohort, nested case-control, or case-cohort) studies, TL is measured in disease-free individuals who are followed-up overtime and disease rate is then assessed in relationship to baseline TL. Because classic cohort studies are expensive and usually require long follow-up time, prospective nested case-control and case-cohort studies are valuable because they include advantages present in both case-control and cohort studies (53). In this approach, cases and controls are selected from an existing cohort in whom biological samples have been collected in the past and prior to disease development. Controls in nested case-control design are a random sample from subjects who have not developed the disease at the time of case diagnosis, but are a sample from all cohort participants in case-cohort. The biospecimen collection precedes disease development, thus, TL precedes disease diagnosis in these studies.

Association studies of TL and cancer are an example of why study design is crucial in the conduct and interpretation of TL epidemiology studies. Many studies have suggested that short telomeres are associated with cancer risk (15, 54). However, these data were primarily derived from case-control studies, which could be subject to reverse-causation bias. The meta-analysis by Wentzensen and colleagues showed significant differences between findings of TL and cancer associations in case-control studies compared with cohort studies (odds ratio (OR) =2.90, 95% confidence interval (CI) =1.75–4.80 in case-control studies *vs.* OR=1.16, 95% CI=0.87–1.54 in prospective studies (15). This observed difference could be, at least partially, explained by a possible reverse-causation bias in case-control studies; *i.e.*, cancer precedes telomere shortening and the observed difference in TL between cases and controls is due to the disease status. This bias is less of a concern in prospective studies. In

all prospective studies, it is important to assess the lag-time between sample collection and disease development, since chronic diseases such as heart disease or even cancer can develop over a long period of time. If DNA samples are collected close to the date of diagnosis, the disease could already be present, but not diagnosed, and therefore this relationship will still suffer from possible reverse-causation.

Another example on how the choice of the study design can influence the findings comes from epidemiological studies evaluating age-related telomere attrition rate. Most of these studies have been cross-sectional in nature; only few longitudinal studies have been reported. Cross-sectional studies attempt to estimate such attrition rate based on a single measurement of TL in subjects of different ages. They assume that the telomere attrition rate is constant across different ages and is similar in all individuals. The age range of study participants (telomere erosion varies across ages, with faster erosion during childhood), their gender, race distribution, and survival effect may also affect these estimates (55, 56). Longitudinal studies have calculated the rate of TL attrition based on 2 measurements at a 3 to10-year time interval and suggest that between 38 and 48 base pairs of telomeres are lost per year (38). When cross-sectional and longitudinal assessment of TL attrition rate were simultaneously compared, longitudinal TL attrition varied between individuals with 10% of study participants experiencing TL elongation overtime (57). Whether TL elongation over time is a true phenomenon or an artifact caused by measurement error is yet to be determined (58). Long follow-up periods, large sample sizes, and multiple TL measurement points are needed to thoroughly understand the age-related decline in TL.

Epidemiological Evaluation of the Role of Telomeres in Human Health

While it is clear that telomeres play an important role in human health, there is significant variability in the extent to which aberrant telomere biology is connected to disease. For examples patients with the prototypic telomere biology disorder, DC, are at high risk of bone marrow failure, pulmonary and liver fibrosis, immunodeficiency, and certain cancers (59, 60). Patients with DC have TL below the first percentile for age.

In contrast, association studies of TL and disease in the general population evaluate TL that are significantly different between cases and controls but still within the normal range of TL (*i.e.*, not as short as DC). As discussed above, studies of the general population, suggest that short telomeres are associated with mortality (14, 61), cancer (15), cardiovascular diseases (16), infections (17) and inflammatory disorders (62). But similar to all epidemiology research, the question remains "do shorter than average telomeres directly cause health problems in the general population?"

Making a valid causal inference from observational epidemiological studies is complicated mainly by possible bias associated with study design, or the presence of uncontrolled (or unknown) confounding. A confounding factor in an exposure-outcome relationship is a variable that is associated with both the exposure and the outcome (63). Age is an example of a common confounder in observational studies of age-related diseases such as cancer, where the observed association is affected by the unbalanced age distribution between comparison groups. Figure 2 provides a schematic description of the relationship between

TL (exposure), age (confounder), and cancer (outcome). In population studies, age only explains a small percentage of the observed inter-individual variation in TL, ranging from 8% (64) to 29% (65) but it is still an important confounder in TL studies. For instance, in a recent study evaluating the role of TL in cancer risk in a large population-based study, age adjustment contributed to a 70% attenuation in the calculated hazard ratio (unadjusted Hazard Ratio; HR=1.74 *vs.* adjusted HR=0.98) of cancer incidence in individuals with short *versus* long telomeres (64). TL is also associated with sex (66), race (4), body mass index (67), and tobacco smoking (12), many of which are known risk factor for age-related diseases and therefore have to be accounted for when evaluating the relationship between TL and such diseases.

While confounding may mislead a true association and should be eliminated, valid interacting factors can be of biological importance. Interaction or effect modification describes a relationship between two or more factors, in which they alter the effect of each other with respect to the outcome (63). Some important examples of interacting factors have been noted in TL epidemiology studies. Fu and colleagues found that liver cirrhosis modified the association between hepatocellular carcinoma (HCC) and leukocyte TL (68). Liver cirrhosis is a strong risk factor for HCC in patients with chronic hepatitis C and B viruses. In that study, significant associations between leukocyte telomeres and HCC risk was restricted to non-cirrhotic patients (OR = 3.54, 95% CI 1.58–7.93 *vs.* OR = 0.95, 95% CI 0.55–1.64, in patients with liver cirrhosis). Identifying interactions with biological relevance can provide a risk stratification tool that could be valuable for screening or treatment strategies. In another example, Puterman *et al.* (69) found that physical activity can modify the observed inverse association between TL and perceived stress, suggesting that exercise can protect from the negative biological effect of stress on human cells, as reflected by TL. A subsequent intervention trial suggested that lifestyle changes, including stress management and physical activity, may affect TL over time (19). Testing for interactions is important, but, as in all studies, the biologic plausibility of the interactions and the findings needs to be considered.

Evaluating the relationship between TL, risk factors, and disease in a multidimensional way is attractive for an in-depth understanding of the actual role TL plays in human diseases. One example is through mediation analysis (70). The mediation model identifies factors that are in the causal pathway between the study variable and outcome. Through it, a researcher can quantify the total effect of an exposure on the outcome of interest, and can calculate how much of this association is through the mediator of interest. Figure 3 illustrates a theoretical example in which TL is in the causal pathway between age and cancer, and extends this to show that this model can accommodate multiple factors (*e.g.* oxidative stress). Several investigators have suggested that telomere dysfunction may causally mediate the association between psychological stress and cardiovascular diseases (71), or lifestyle factors and risk of type 2 diabetes (72). Mediation analysis have been used in few small studies to evaluate if TL could explain some of the epidemiological observations that are not yet well understood including the relationship between childhood socioeconomic status and the high risk of respiratory infections in adulthood (73) and a possible connection between consanguinity and mental illnesses (74).

The Effect of Telomere Length Method of Measurement on Study Results

Several methods have been used to measure TL, each with its strengths and limitations reviewed in detail elsewhere (75–77). In this section, we focus on issues that may impact validity of TL-disease association studies comparing the three most commonly used TL measurement methods in epidemiological and clinical research. Southern blots of terminal restriction fragments (TRF) are considered the gold standard for TL measurement and are used to calibrate other methods (78). TL has also been successfully measured using real-time quantitative polymerase chain reaction (qPCR) (79, 80). Both southern blot and qPCR techniques measure TL in extracted DNA, and therefore, can be used for stored samples commonly available for epidemiological studies. However, qPCR is more attractive for large-scale studies because it is high throughput, needs small amounts of DNA, and is relatively inexpensive. Both methods are limited by their reported lack of sensitivity to detect very short telomeres, and by the fact that their TL measurement is an average across population of cells per sample (77, 81). On the other hand, flow cytometry with fluorescent *in situ* hybridization (flow FISH) TL measurement of leukocytes is very accurate and provides cell population-specific measure (82), but its utility in population-based studies is limited by its need for viable cells.

The correlation of TL values between these three measurement methods is generally modest. In an epidemiological study of 681 elderly individuals, the correlation coefficient (r) between TL data generated by Southern blot and qPCR was weak (r=0.52) (41). This is significantly weaker than reported correlations between TL measured by the two methods in the initial validation studies of either qPCR monoplex assay (r=0.82) (79), or qPCR monochrome multiplex assay (r=0.91) (80). Our data comparing TL measured by qPCR and flow FISH in 52 normal individuals showed similar result (r=0.47) (83). No data are available for the correlation between flow FISH and Southern blot TL beyond the strong correlation reported in the initial flow FISH study (r=0.9) (82).

The weak correlations between methods may partially explain some of the inconsistencies observed in the published literature when different TL measurement methods are used. For example, a large cohort study measuring relative TL using qPCR reported significant excess in cancer mortality associated with short telomeres (n=787, HR=11.1 in the shortest *versus* longest tertile) (84). However, a second study (85) measuring TL using Southern blot analysis reported no association between TL and cancer mortality (n=1,136; HR=1.2, in the shortest *versus* longest quartile). In a meta-analysis including TL-obesity studies the authors reported that the results from studies that used the Southern blot assay to measure TL were more consistent than those that used qPCR (67). Similar inconsistencies between studies evaluating the association between qPCR relative TL and breast cancer has been observed (two showed an association with long telomeres, three with short telomeres, and three showed no association) (15). Of interest, this variation existed across different study designs (case-control and prospective studies) (15), reflecting a possible role for measurement variability between laboratories, and highlighting the need to standardize qPCR laboratory procedure in relative TL measurement. In a study comparing blinded duplicates blood samples for Southern blot TL and qPCR relative TL, results from Southern blot assay were found to be more consistent than that of qPCR (coefficient of variation (CV) for Southern

blot =1.7% *vs.* 6.5% for qPCR) (65). Of note, qPCR relative TL measurements may be sensitive to DNA quality, quantity, and extraction method (86).

These differences in TL measurement methods illustrate the importance of minimizing technique-related measurement error, and thus limit misclassification bias. Simple measures that can help with this include 1) distributing samples from both comparisons groups (case/control, exposed/unexposed) on the same plate; 2) avoiding the use of DNA samples of unknown source or unknown extraction method; 3) using a single method of DNA extraction or, at least, match the cases and controls on the extraction method.

Of note, recent research suggest a need to switch from measuring average TL to a more biologically relevant measurement of TL based on the proportion of short telomeres in cells (77). *In vitro* and animal studies suggest that this measurement may be a better indicator of cell aging than the average TL, which can be effected by the presence of very long telomeres in some cells (87, 88).

Analytical Considerations in Telomere Length Epidemiology Studies

Determining the number of individuals included in a study is one of the important early decisions made during study design. Things to be taken in account when calculating sample size include (89): study design, scale of measurement of the response variables (*i.e.*, are we comparing means or proportions?), the difference we want to detect (larger sample sizes are needed to detect smaller differences), the population inter-individual variability of the measure of interest (the more homogeneous the population, the smaller the number of individuals needed), the desired level of statistical significance (the smaller the p-value the larger the sample size needed), and study power (*i.e.*, probability to detect differences when exist; the higher the desired power, the larger the sample size needed).

For TL studies, a large enough sample size is especially important because inter-individual TL heterogeneity and age variability must be included in the calculations. Using simulations, it was shown that longitudinal designs may have more power to observe TL association with age-related diseases, because they can capture the actual TL attrition rate (43).

Adjusting for confounders is crucial in epidemiological studies. Several methods are used, some at the design level and others are used during the analysis. At the design level, comparison groups (*e.g.*, cases and controls) are often matched by potential confounders (*e.g.*, age). A disadvantage of a matched design is that strict matching (one-to-one) limits our ability to evaluate the relationship of the matched variable with the study outcome. At the analysis level, regression models are the most commonly used method to control for confounding. However it is important to note that limitations may exist (reviewed in detail in (90)) including: 1) the adjusted effect size can be invalid if the distribution of the potential confounder greatly differs between studied groups (*e.g.*, when certain age groups are not presented in the control groups, usually very young or very old); 2) multivariable model misspecification can lead to biased results, such as when the model doesn't account for some important nonlinearities. Nonlinearity has been observed in TL relationship with age in several studies (39, 91). For instance, a study of 835 individuals found that a piecewise

linear curve with breakpoints hinged at 1 and 18 years of age provided the best fit to the data as compared with linear, quadratic, or cubic curves (39).

Another widely used method to control for confounding, mostly to adjust for age, is calculating age-standardized TL using an independent population (5, 7). Again selecting a large enough reference population for each age category is important to ensure capturing the TL variability.

Conclusions

Epidemiological studies are crucial in understanding the role of telomere biology in human aging and disease. In this chapter we have summarized some important epidemiologic concepts and issues that can affect the interpretation of the results from epidemiological studies of TL. Careful selection of an appropriate study design, an adequate sample size, and an accurate TL measurements assay are important factors that can significantly affect the findings of the study. Future research to compare TL measurement from different assays in large studies is needed. This will aid the development of more reliable assays for large epidemiological studies. It is also paramount to measure TL from multiple samples collected in longitudinal epidemiological studies with long follow-up times to precisely estimate the age-related telomere attrition rate and how it relates to disease risk. Including large enough sample from different age groups is necessary to enhance our understanding of telomere dynamics during the life span. Telomere measurement in somatic tissues will provide information the organ-specific aging rate. Additionally, standardization of available measurement methods is crucial to allow comparing between studies. Recent research calls for new methods that measure the proportion of short telomeres within cells rather than the current average TL measurement. Such an assay could provide many research opportunities and help to better understand the role of telomeres in health and disease.

In summary, proper epidemiological research has the potential to clarify the role telomeres play in disease risk and may eventually guide preventive strategies, identify modifiable targets, and aid in the development of diagnostic and prognostic biomarkers.

Acknowledgment

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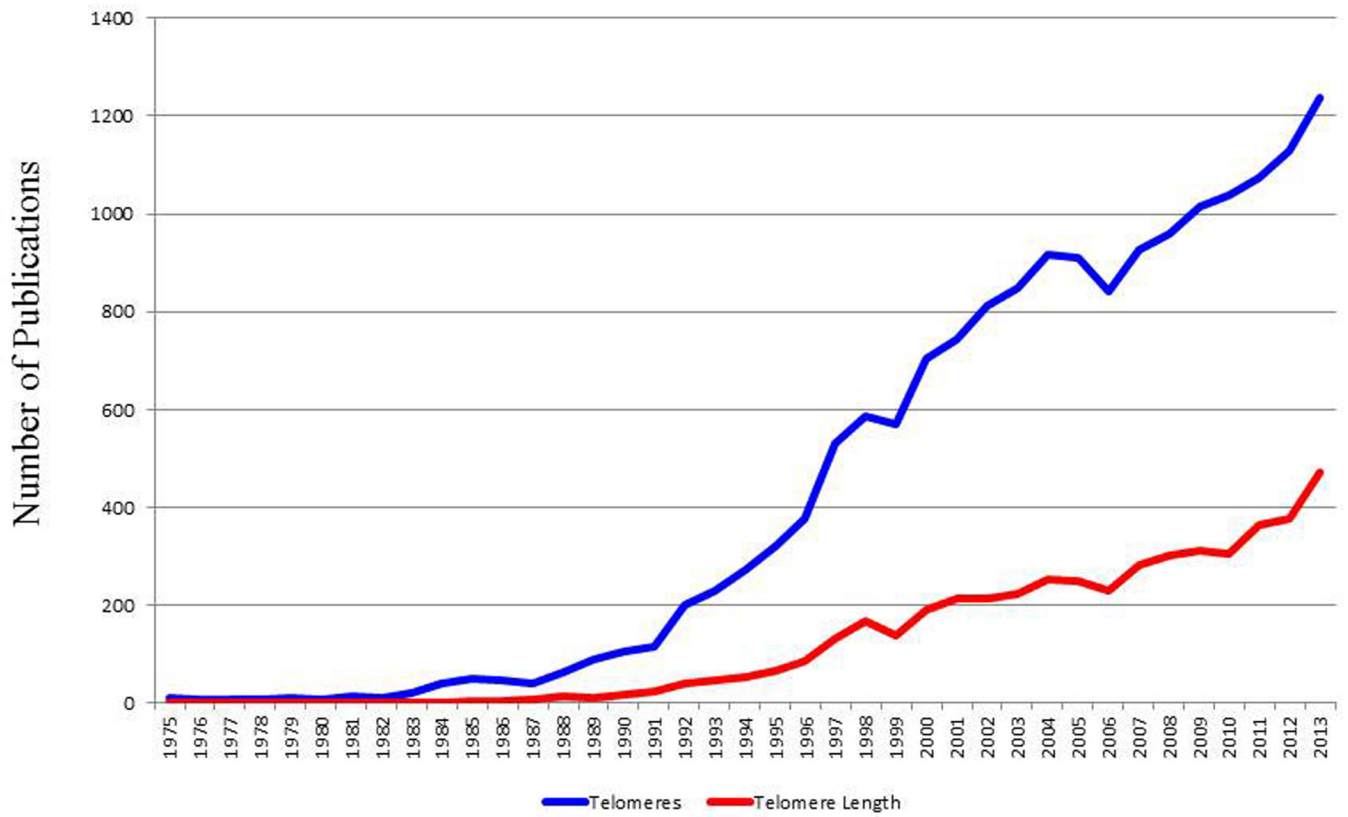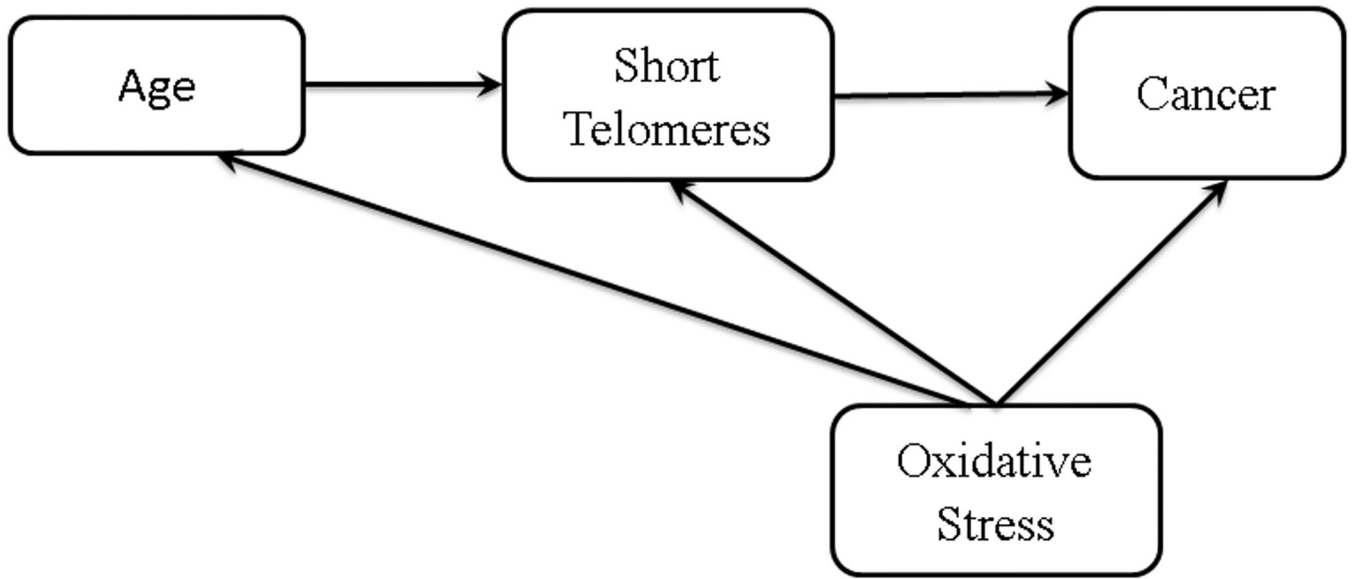
Figure 1:
Number of telomere-related PubMed indexed publications and the observed rising trend overtime
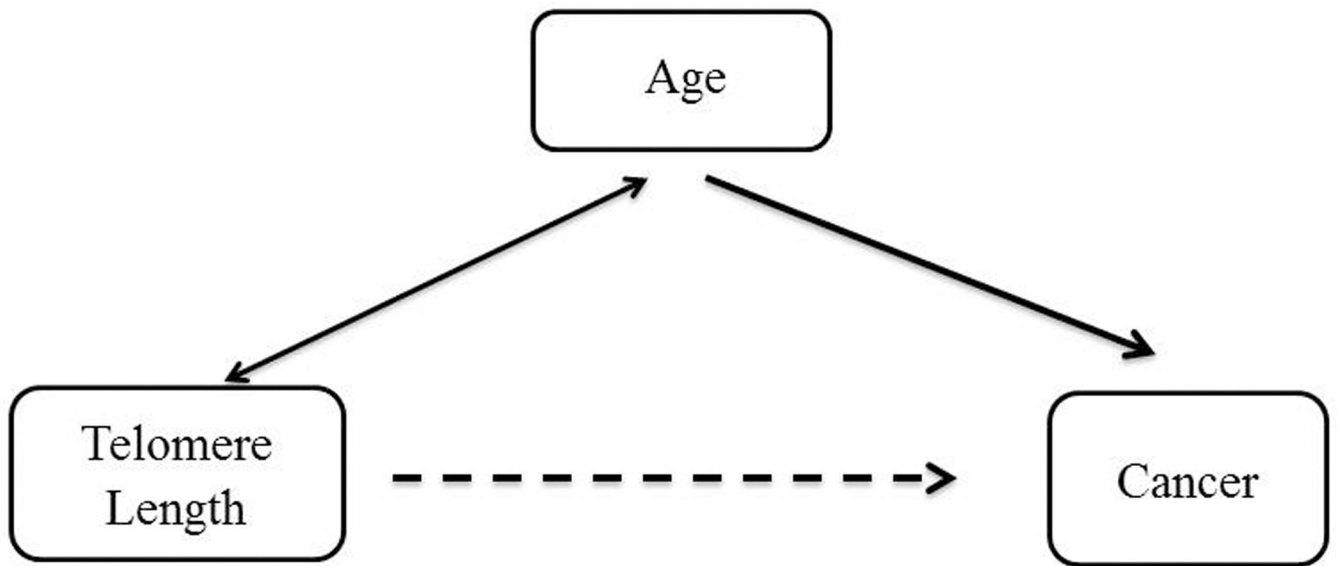
Figure 2:
A schematic diagram describing confounding. The bidirectional arrow represents a non-causal association, the unidirectional arrow represents a causal association, and the dashed line represents the observed association

Figure 3:

A schematic diagram describing mediation where short telomeres are in the causal pathway of age and cancer

Table 1:

Definitions of some epidemiological and statistical terms

Term	Definition
Confounding	The distortion of the effect of an exposure on an outcome due to the association of another factor (confounder) with the exposure and the outcome.
Bias	Systematic deviation of results or inferences from truth
Temporal relationship	Exposure always precedes the outcome
Biological plausibility	The association is coherent with firmly established knowledge on pathobiological processes
Mediator variable	A variable that occurs in the pathway from an exposure to an outcome.
Correlation Coefficient (r)	A measure of association that indicates the degree to which two variables have a linear relationship. It ranges between −1 and 1; r=0 means no correlation
Coefficient of variation (CV)	A measure of the spread of the data defined as the ratio of the standard deviation to the mean. Usually expressed as a percentage
Odds Ratio (OR)	The ratio of the odds of exposure among the cases to the odds of exposure among controls; it is commonly used in case-control studies
Hazard Ratio (HR)	The ratio of the hazard rates comparing exposed to unexposed groups. Hazard rate is the number of new cases developing per unit person-time at risk
Confidence Interval (CI)	A range of values about a point estimate that indicates the degree of statistical precision that describes the estimate. A wider interval indicates less precision

Table 2.

Observational study designs

Study Design	Purpose	Characteristics
Cross-sectional	• To assess the prevalence of an exposure or conditions • Sometimes used to evaluate the association between study variables (exposures and outcomes)	• Simple to conduct • Suitable for assessing multiple exposures and outcomes • Information on exposure and outcome are obtained at the same time; cannot assess temporality
Case-control	• To compare odds of exposure (*e.g.*, telomere length) in diseased and not diseased populations	• Retrospective study (recruitment happens after disease develop) • Suitable for rare diseases • Suitable for assessing multiple exposures • Controls need to be representative of the population that gave rise to the cases; otherwise subject to selection bias • Subject to reverse causation • Subject to recall bias
Cohort	• To compare disease incidence over time in exposed *versus* non-exposed individuals	• Prospective study (participants are free from outcome at the start of the study) • Suitable for assessing multiple outcomes • Long follow-up times and large samples are needed for rare outcomes; it can be costly • Subject to bias from non-response or losses to follow-up