

REVIEW

Human telomere biology: pitfalls of moving from the laboratory to epidemiology

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Accepted 5 July 2006

Remarkable progress has been made during the last 2 decades in understanding telomere biology at the molecular and cellular levels. Clinical epidemiology research of human telomeres, in contrast, is a discipline just coming into its own. The most important observation in studying human telomere biology is that telomere length is highly variable among humans. Here we explain some of the reasons for this variability and propose several principles that should be considered in conducting epidemiological telomere research. Ignoring these principles could lead to misleading conclusions.

Keywords Telomere, leukocyte, lymphocytes, ageing

Telomeres are tandem repeats of DNA sequences that cap the ends of chromosomes.¹ Human telomere research has explored whether telomere length provides information, over and above chronological age, when assessing the ageing of the individual and the predilection to age-related diseases.² Recently, telomere research has moved rapidly from the lab to clinical- and population-based studies, which have observed that shorter mean telomere length in leukocytes is associated with cardiovascular disease,^{3–9} indices of obesity and insulin resistance,^{6,7,10–12} dementia,^{13,14} cigarette smoking,^{10,15} and a host of other maladies. Whether mortality in the elderly is also associated with shortened telomere length is still an open question.^{9,16,17} These observations are highly relevant, yet in an increasing number of studies little attention has been paid to potential biases and problems leading to discrepant results. Here we review some of the pitfalls and their causes in this exciting area of ageing research that could affect the study of many age-related traits.

Background

In post-mitotic tissues, such as skeletal muscle, telomere length hardly changes with age, but it shortens in proliferative tissues because of the inability of DNA polymerases to copy completely the lagging strand of DNA during cell replication.¹ In cultured

somatic cells lacking telomerase, different rates of telomere shortening are brought about by variation in not only the pace of cellular proliferation—a higher proliferation leads to an increased rate of telomere erosion—but also the amount of telomere repeats that are lost with each cell division. Thus, telomere dynamics (telomere length and attrition rate) may be useful in ageing research only if factors that affect it are involved in the ageing process.

Potential confounders and their causes

As peripheral leukocytes have been the main target of human telomere research, most of what is known about human telomere dynamics *in vivo* is based on these cells.² Leukocyte telomere length is a complex trait that is shaped by genetic,^{15,18–21} epigenetic,²² and environmental determinants.^{10,12,15,23,24} What's more, whereas age-adjusted telomere length is longer in leukocytes of adult women than men,^{3,6,15,19,21} at birth telomere length is equivalent in boys and girls.²⁵ Thus, sex modifies leukocyte telomere length in humans and contributes to its variability among individuals, though the sex effect on telomere length and its relation with metabolic indices may change with ageing.^{6,11}

Telomere length seems to chronicle the replicative history of leukocytes in the individual because, as seen with their conduct in cultured cells, telomeres undergo erosion with replication of leukocytes and their progenitor cells. Chronic inflammation, with its heightened leukocyte turnover rate, would thus result in a greater loss of leukocyte telomere repeats over time. Similarly, chronic or repeated oxidative stress, which is often linked to inflammation, increases telomeric erosion with each replication,^{26,27} and thereby contributes to overall telomere

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shortening. Accordingly, age-adjusted telomere length in leukocytes may be associated with diseases or environmental factors linked to chronic increases in oxidative stress and inflammation.^{6–12,15,23} However, chronic inflammatory states and infections that are not considered to be strictly linked to ageing also may display short leukocyte telomere length. Subjects with these conditions should be considered for exclusion from studies that chart leukocyte telomere dynamics with a view to explore the biology and epidemiology of human ageing. Yet these conditions underscore the tenet that inflammation and oxidative stress are determinant in leukocyte telomere dynamics *in vivo*. For instance, leukocyte telomere length is relatively short in systemic lupus erythematosus²⁸ and rheumatoid arthritis.²⁹ Patients with these diseases are highly prone to cardiovascular diseases,^{30,31} which are associated with short leukocyte telomeres.^{3–9} In HIV, subsets of leukocytes, CD8⁺ T lymphocytes, in particular, show not only shorter telomeres but also evidence of replicative impasse,^{32–34} evidently due to chronic viral antigen-driven proliferation. Further, cigarette smoke increases oxidative stress and inflammation^{35–38} and smokers exhibit shortened leukocyte telomere length.^{10,15}

Sample sizes and power considerations

The inter-individual variation in leukocyte telomere length at birth and the effects of genetic factors, sex, inflammation, and the environment on telomere attrition give rise to considerable variation in age-adjusted leukocyte telomere length throughout life. It follows that large numbers of subjects are required in cross-sectional analyses to uncover potential links between telomere parameters and ageing-related diseases.

Consider a cross-sectional study that wishes to infer differences in telomere erosion where one models telomere

length as a linear function of age. The slope of the fitted line corresponds to the yearly rate of telomere length erosion (β). (The calculations required to estimate confidence intervals around β are delineated in Appendix.) Here we provide specific illustrations, using leukocyte telomere length data from the largest cohort studied until now to estimate β from cross-sectional data or comparison of case and control groups. Findings about telomere biology in subsets of this cohort have been published elsewhere.^{10,11,18,24} We have computed the overall variance in telomere length, expressed by the mean length of the terminal restriction fragments (TRFs), in 2450 normal unselected women aged 18–79 from the TwinsUK cohort ($s_T^2 = 0.478$) and the variance in age for these subjects for various age ranges (Table 1). We have also calculated for various values of β (20–40 bp/yr) and various age ranges the minimum sample size needed for the lower confidence interval to be $\beta > 0.01$ (in bp/yr).

Table 1 illustrates, for instance, that if the relationship between telomere length and age is studied cross-sectionally in <100 individuals, an age span of 30 yr will be needed to assess whether the erosion rate at 20 bp/yr is significantly greater than zero. To assess the significance of the same erosion rate at the age range of 10 yr, a sample size of ~600 individuals is required.

Table 2 displays the minimum numbers of subjects required for a given age range to determine by cross-sectional analysis in a case–control study whether the extrapolated β s of two groups are significantly different. The numbers of subjects needed to show statistical significance between the two groups differ considerably for samples depending on their age range; both the values of and the difference in β s affect the sample size. For instance, suppose that a sustained surge in oxidative stress over 10 yr triples β from 20 to 60 bp/yr in a study group, a total number of 1176 subjects (588 in each group, Table 2) will be required to demonstrate a significant difference in cross-sectional analysis. In contrast, only 70 subjects (35 in each group) will be needed to show the same effect over a 50-yr span. As these calculations are based solely on female data, the variance, and sample size needed in a mixed sex sample may be larger.

Longitudinal data that measure the actual telomere attrition rate require much smaller sample sizes. We assume a standard deviation in yearly telomere attrition of 25.6 bp/yr based on published data from the Bogalusa Heart Study¹² and, for simplicity, that variance in telomere attrition rate is constant between groups. We find that the sample sizes required to detect the same level of difference in yearly telomere erosion (Table 3) are approximately five times smaller than those needed in a cross-sectional setting with a very wide age range (Table 2).

Table 1 Minimum sample size required for the telomere attrition rate (β), expressed in bp/yr, to be significantly greater than zero

Age range studied (in yr)	Corresponding sampling variance in age (s_a^2) ^a	Min sample size for β —95% CI > 0.01 bp/yr		
		20 bp/yr	30 bp/yr	40 bp/yr
5	2.1	2265	2060	578
10	7.9	584	262	149
20	30.3	155	73	41
30	63.4	76	35	21
40	102.3	48	23	15
50	140.9	35	18	12
60	170.9	30	15	10

^aEstimated in the TwinsUK cohort.

Table 2 Minimum sample size needed in each of the case and control groups to compare the telomere attrition rates (β s), expressed in bp/yr, assuming that individuals are studied at 10- or 50-yr age range in a cross-sectional study

10 yr age range					50-yr age range				
<i>N</i> required	β_1	95% CI	β_2	95% CI	<i>N</i> required	β_1	95% CI	β_2	95% CI
4135	15	12.50–22.50	30	22.51–37.50	235	15	7.52–22.48	30	22.52–37.48
9320	20	15.00–24.99	30	25.01–34.99	525	20	15.01–24.99	30	25.01–34.99
2330	20	10.01–29.99	40	30.01–49.99	133	20	10.03–29.97	40	30.03–49.97
588	20	0.08–39.92	60	40.08–79.92	35	20	0.13–39.87	60	40.13–79.87

Table 3 Minimum sample size needed in each of the case and control groups to compare the telomere attrition rates (β s), expressed in bp/yr, in a longitudinal study assuming a standard deviation in telomere attrition rate of 25.6 bp/yr

Longitudinal Study SD=25.6 bp/yr				
N required ^a	β_1	95% CI	β_2	95% CI
46	15	7.61–22.39	30	22.61–37.39
104	20	15.09–24.91	30	25.09–34.91
26	20	10.17–29.83	40	30.17–49.83
7	20	1.06–38.94	60	41.06–78.94

^aSample size required in each of the two groups being compared for 80% power and $P < 0.05$ using a two sided t -test.

Selection (survival) bias

Selected mortality on telomere dynamics is another important factor in observational, cross-sectional studies of the elderly. Leukocyte telomere dynamics has been linked with ageing and diseases of ageing,^{3–14,16} so that individuals with ageing-related disorders or those prone to age faster appear to have relatively short leukocyte telomere length. It follows that (if the observations are correct) these individuals are more likely to die at a younger chronological age than their peers, leaving older survivors with relatively longer telomeres than expected for the general population. Thus, cross-sectional observations may over-estimate telomere length in the elderly and there may be less variation within this group. An analogy to this is the decline in ApoE4 relationship with Alzheimer disease in the extremely elderly.³⁹ Cigarette smoking may be another example that directly relates to the telomere field. Smoking is associated with shorter leukocyte telomere length,^{8,13} but this is not readily apparent in the elderly,^{6,17} perhaps because of survival effect; those smokers that survived to an old age might be different from their peers who succumbed because of their habit to a host of cardiovascular diseases, emphysema, and cancer. For these reasons, case–control disease studies of the elderly have to be closely matched for age and results from extreme elderly cohorts interpreted with caution.^{9,16,17}

Temporal life course factors

Most of the indices of inflammation and oxidative stress, as well as markers of metabolic abnormalities, such as insulin resistance, reflect the immediate health status of the individual, while telomere length in leukocytes (and other proliferating cells) is a historical record of telomere length at birth and its attrition afterwards up to the point of the blood collection. Therefore, leukocyte telomere length may not correlate with current acute indices of inflammation and oxidative stress even though these variables may exert a profound impact on telomere erosion. One assumes, however, that when indices of inflammation and oxidative stress reflect a long-standing process, in due course they would exhibit association with leukocyte telomere length in cross-sectional analysis.

Similarly, an association between leukocyte telomere length and state-of-the-moment indices of ageing or diseases of ageing may abruptly disappear because of the contemporary alterations in the levels of these indices. For instance, menopause is marked by a sudden decline in the levels of ovarian steroid

hormones, which may influence telomere attrition in women. Different factors may influence telomere dynamics during the pre- and post-menopausal period.¹¹

Leukocyte cell types

Ageing is characterized by reconfiguration of the immune system with shifts in the relative numbers of leukocyte subsets.^{40–43} Therefore, variation in the mean leukocyte telomere length in older individuals may arise not only from telomere dynamics but also from other ageing-related changes in the distribution of leukocyte subsets. Leukocytes are derived from hematopoietic stem cells that give rise to common lymphoid progenitors (committed to T and B lymphocytes and natural killer cells) and common myeloid progenitor committed to two more lineages (granulocytes and monocytes). Peripheral blood mononuclear cells (PBMCs), which comprise T and B lymphocytes, natural killer cells, and monocytes, therefore, are more homogeneous than leukocytes. Telomere attrition is more rapid during early childhood than later in life and in adults lymphocytes have shorter telomeres than granulocytes.⁴⁴ In addition, though the proportions of lymphocytes and granulocytes change considerably upon transition from childhood to adulthood, they remain remarkably constant in healthy adults across all age groups, including the elderly, who continue to maintain normal peripheral leukocyte counts (lymphocytes, granulocytes, etc).⁴⁵

In adults, ageing-related changes include altered proportions of subsets such as memory vs naïve and CD4⁺ vs CD8⁺ T-cells.^{43,46–51} As telomere length may indeed differ within these subsets, reconfiguration of cell subsets with ageing probably confounds telomere analysis in PBMCs more so than in leukocytes. This is because in leukocytes, granulocytes, the relative number of which changes little with ageing, would ‘buffer’ ageing-related changes in the proportions of lymphocyte subsets. Having said this, there is little evidence that leukocytes are more suited than PBMCs to explore telomere dynamics in clinical/epidemiological settings. More importantly, in studying human telomere biology *in vivo* the key issue is not the relative homogeneity of a given cell preparation but the solution to the following central question: Would deciphering age-dependent telomere dynamics in a given cell type, a tissue, or an organ help to better understand human ageing and longevity? It is clear, nonetheless, that assessing telomere dynamics in leukocyte subsets may be essential to tackle specific questions of relevance. For instance, as indicated above, telomere dynamics in CD8⁺ T lymphocytes may explain features of HIV.^{32–34} The monitoring of telomere dynamics of monocytes would also be insightful in atherosclerosis, since this vascular disease is associated with monocyte activation.

Longitudinal studies

Clearly, a longitudinal study design is the optimal approach to explore the telomere-ageing relationship. However, even if the currently sparse longitudinal data did become more frequent, modelling telomere dynamics in this way may not be as simple as it seems. Recent studies suggest that the loss of telomere repeats is proportional to the length of telomeres,^{16,52} perhaps because longer telomeres provide a bigger target for

free radicals. In addition, the variances of both telomere length and yearly attrition rates could be dependent on age and on telomere length.

Measurement methods

Only scant attention has been given to the reliability and applicability for clinical research of the various techniques used to measure telomere length. It is simply impossible to reproduce in clinical settings the uniform and narrow circumstances of laboratory research. A basic researcher may be interested in the effect of factor x on telomere length. She then treats five cell strains with factor x , then measures telomere length on the same day and the same run in the five control cell strains and the five x -treated, experimental strains. She pays little attention to the absolute length of telomeres, as her interest is whether factor x elongates (relatively increases) or shortens (relatively decreases) telomere length. Epidemiological research cannot be performed in the same fashion, as it may entail the measurements of telomere length in hundreds if not thousands of DNA samples. Strict quality control, using internal and external references, is essential to ascertain not only reproducibility (on different occasions and in different runs) but also precision of the measurements.

Suppose the coefficient of variation (CV) for the measurement of telomere length by a given method is $\sim 2\%$. For a mean TRF length of say 7.5 kb, this amounts to a standard deviation of the measurement of 150 bp (0.15 kb). Given that telomere attrition is $\sim 20\text{--}30$ bp/yr, a 2% deviation from the true value of telomere length is equivalent to an error of at least 5 yr in the biological age of the individual (assuming that telomere length is a valid bio-indicator of ageing). However, the CV is given by $100 \times \text{SD}/\text{mean}$ of the measurement, so if the telomere length is characterized by a normal distribution, only 68% of the measured values will fall within the CV. In fact, the 95% confidence intervals for the measurement of 7.5 kb will be 7.21–7.79 kb. The difference between these two extremes corresponds to a deviation of >20 yr in putative biological age.

Additionally, one must carefully consider which method of telomere measurement is the most applicable for a given epidemiological study. For instance, quantitative fluorescence *in situ* hybridization (Q-FISH)⁵³ and flow cytometry and FISH(flow-Fish)⁵⁴ require intact cells (or nuclei) for the measurement of a telomere signal, a feature that will limit the feasibility and scope of many studies. The range of CV for the flow-FISH for measurements, performed on different occasions, is $\sim 5\text{--}20\%$.^{44,55,56} The length of the TRFs by Southern blot analysis⁵⁷ typically provides a measure, in absolute terms, of not only the length of telomeres but also the sub-telomeric segments that extend to the nearest restriction sites on each chromosome, though this drawback has been rectified recently.⁵⁸ The Southern blot method provides not only the mean length of the TRFs but also their distribution. The reported CV range for the mean TRF length by this method is 0.9–12%.^{6,17} The real-time PCR⁵⁹ technique is faster and cheaper and measures the amount of telomeric repeats (without the sub-telomeric segments). It can only provide the mean of the amount of telomere repeats and its reported CV is $\sim 5.8\%$. Its drawback is that results are expressed not in absolute terms but in relation to a reference gene, which is

presumed to be stable. In consequence, ageing-related changes in the DNA may also impair the precision of the PCR method and cause increasing problems in the very elderly.

We do not know, for instance, the reason why PCR-based analyses show a decline in the rate of telomere attrition in the elderly,^{9,16} while the Southern method^{20,60} and flow-FISH analysis^{44,61} do not show such a decline. Is the discrepancy related to the different methods used, the nature of the cohorts, or measurement errors? Measurement errors generally produce parameter bias and also result in reduced power for testing associations.⁶² However, both in longitudinal and cross-sectional linear models it is possible to correct for regression dilution and thus to get more accurate estimates of the relationship between telomere attrition and age.^{62,63}

Conclusion

The merit of any epidemiological research rests on a design that incorporates the appropriate sample size, recognizes the confounders, and employs reliable methods. Information about telomere biology in human leukocytes imparts a new dimension to the present understanding of human ageing. However, epidemiological telomere research has many pitfalls, primarily related to the considerable inter-individual variability in telomere length. Accordingly, a subset of published studies has used either inadequate numbers of subjects, too narrow age ranges, or both to be meaningful. Potential confounders include the effects of a host of environmental factors (the list of which is ever expanding), inflammatory diseases and chronic infection at any point in life, the effect of gender and perhaps menopause, and survivor bias. Moreover the cumulative effect of confounders has to be considered—not just their current status. Finally, the method used to measure telomere length can be critical. Despite these caveats, if carefully conducted, telomere research is likely to provide crucial insights into the ageing process and age-related disease mechanisms in humans.

Acknowledgements

A.A. ageing research is supported by NIH grants AG021593 and AG020132, and The Healthcare Foundation of New Jersey. A.M.V. and T.D.S. telomere research is funded in part by the Wellcome Trust grant ref 074951.

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Appendix

Our calculations are based on standard linear regression theory.⁶⁴

Let age (denoted by a) be the independent variable and leukocyte telomere length (denoted by τ) be the dependent variable. There are n telomere length measurements T_1, T_2, \dots, T_n to each of which corresponds an age measurement a_1, a_2, \dots, a_n . We assume that τ is a linear function of a , modelled as $T_i = \alpha + \beta a_i$, and let S_{aa} be the sum of squares of the a_i 's, such that $S_{aa} = \sum (a_i - \bar{a})^2 = (n-1)s_a^2$, where \bar{a} and s_a^2 denote the sampling mean and variance of age, respectively. Similarly, $S_{TT} = (n-1)s_T^2$ denotes the sum of squares of the telomere lengths, where s_T^2 is the sampling variance of τ .

The yearly rate of telomere attrition is then the slope of the linear regression, which can be estimated by the following equation:

$$\hat{\beta} = \left(\sum a_i T_i - n\bar{a}\bar{T} \right) / S_{aa} \quad (1)$$

and the 95% confidence intervals of β are given by the following equation:

$$2\hat{\beta} \pm t_{(n-2)} \sqrt{\frac{S_{aa}S_{TT} - \hat{\beta}^2}{(n-2)S_{aa}^2}}, \quad (2)$$

where $t_{(n-2)}$ is the two-tailed 95th percentile from Student's- t distribution with $n - 2$ degrees of freedom.

From Equation (2) we can precisely determine the yearly rate of telomere attrition in base pairs (bp)/yr, (β), if S_{aa} (a function of the variance in age and of sample size) is large. In fact, the confidence intervals are directly proportional to the variance in telomere length and inversely proportional to the variance in age.

For specific values of the sampling variances of telomere length, age, and β we can therefore calculate the minimum sample size required so that the confidence intervals of the slope differ from zero. Because age and telomere length are negatively correlated, the values of β are negative (but for simplification, they are presented in absolute terms).