

# Telomere Length Among the Elderly and Oldest-Old

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Human chromosomes terminate in a number of repeats of the sequence TTAGGG. At birth, each chromosome end is equipped with approximately 15 kb of telomere sequence, but this sequence is shortened during each cell division. In cell cultures telomere shortening is associated with senescence, a phenomenon that has also been observed in normal adult tissues, indicating that telomere loss is associated with organismal ageing. Previous work has established that the rate of telomere loss in humans is age dependent, and recent work shows a sex-specific difference in telomere length and shortening in individuals over the age span of 20 to 75 years. Here, terminal restriction fragment lengths on DNA purified from whole blood were measured to examine the mean telomere length in a cross-sectional cohort of 816 Danish individuals of age 73 to 101 years. In this age group, females show a linear correlation between telomere length and age, whereas the pattern tends to be nonlinear (quadratic in age) for males. This difference in telomere length dynamics between the 2 sexes may be caused by several different mechanisms, including differences in selection by mortality, differences in leukocyte population or different telomerase expression pattern.

Linear chromosomes, found in all higher organisms, have a specialized structure, termed the telomere, at each end. The telomeres participate in several functions, including meiotic chromosome segregation and chromosome silencing. Telomeres are also important in cellular ageing, where the primary function is to protect against chromosomal end-to-end fusions (Blackburn, 1994; Greider, 1996; Zakian, 1995). This is achieved by an intricate interaction between the telomere DNA sequence, consisting of tandem repeats of the motif TTAGGG, and a number of proteins, including TRF1, TRF2, Tankyrase, hRap1, TIN2, Ku and WRN (Bilaud et al., 1997; Broccoli et al., 1997; Chong et al., 1995; Hsu et al., 1999; Kim et al., 1999; Opresko et al., 2004; Smith et al., 1998). Several of

these proteins interact with the telomeric DNA and bend it into a loop, thereby forming a circular structure at both ends of the linear, chromosomal DNA (Greider, 1999; Griffith et al., 1999). Mimicking the circular chromosome structure found in prokaryotes, this is believed to be a very stable construction that explains the inability of the telomere-protected DNA strands to fuse and thereby form dicentric and ring-shaped chromosomes.

In the different human cell types that have so far been investigated, the mean telomere length at birth is between 5 kb and 20 kb. As a consequence of the DNA replication process, the tips of the telomeres are, however, eroded at each cell division. This phenomenon, described by Olovnikov (1973) and called the end replication problem, is believed to drive replicative senescence in human cells and is consequently assumed to be an important factor in the ageing process. There are, however, cell lines that do not exhibit telomere shortening, and thus do not enter replicative senescence when cultured. Germ line cells (Wright et al., 1996) and most cancer cell lines do not show telomere shortening, and it has also been demonstrated that certain stem cell lines do not show telomere shortening (Hiyama et al., 1995). These cell lines express telomerase, a ribonucleoprotein that has reverse transcriptase activity, and is capable of elongating the telomeres by adding TTAGGG repeats at the 3'-ends of the chromosomes.

In most somatic cells in humans, telomerase activity is repressed in contrary to germ line cells, where active telomerase maintains telomere length. In certain somatic cells, however, active telomerase has been demonstrated, indicating that these cells need an

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extended proliferative capacity. So far active telomerase has been observed in normal somatic cells such as lymphocytes, stem cells in the skin and in intestinal mucosa (Bachor et al., 1999; Broccoli et al., 1995; Counter et al., 1995; Harle-Bachor & Boukamp, 1996; Wright et al., 1996). Active telomerase has also been found in more than 90% of all cancers. In the fraction of cancers that do not express telomerase, it is believed the telomeres are elongated by the Alternative Lengthening of Telomeres (ALT) pathway, a mechanism that is currently under intensive investigation (Opresko et al., 2004).

The observation that telomere length correlates to replicative history and potential survival has long been of interest in both gerontological and oncological research. Thus in peripheral blood leukocytes, telomere length in newborns is typically around 16kb, but telomere loss throughout life erodes this amount to only approximately 8kb in individuals at the age of 72 years (Frenck et al., 1998).

Investigations have shown that the rate of telomere loss varies with age, that is, the rate of loss is much more pronounced in newborns than in the middle-aged — possibly correlating with the observed cellular proliferation rate (Frenck et al., 1998; Zeichner et al., 1999). A study has also demonstrated the presence of a statistically significant difference in telomere length between the two sexes (Benetos et al., 2001). The authors report that females throughout the age span 20 to 75 years constantly possess longer telomeres than males at the same age. Telomere length beyond the age of 75 years was not investigated by this group.

In the present cross-sectional study, sex-specific differences in telomere length in the oldest individuals were investigated in a cohort of 816 individuals aged 73 to 101 years, and we demonstrate that when comparing telomere length measured on DNA purified from whole blood, a statistically significant difference between the sexes persists up to about 80 years of age, but is lost in the age span from 80 years to beyond 92 years of age due to stabilization of the mean telomere length in males. Only a limited number of individuals over 100 years of age participated in this study, but the data obtained for this group seem to suggest that the sex difference reappears in individuals 100 years and more due to the increased telomere shortening rate in males.

## Materials and Methods

The DNA upon which this study is based is obtained from three different study populations:

### The Longitudinal Study of Aging Danish Twins (LSADT)

In 1995, LSADT began by assessing all cooperating Danish twins aged 75 years and older (a sample of 2401 individuals) who were registered in the population-based nationwide Danish Twin Registry (Bathum et al., 2001; Skytthe et al., 2003). The assessment which was a home-based 2-hour multidimensional

interview, including cognitive and physical performance tests, was repeated in 1997 including all twins who participated in 1995 as well as a sample of previously unassessed twin pairs who were between 73 and 76 years old in 1997. The 1997 survey comprised 2172 individuals corresponding to a participation rate of 79%. We subsequently asked individuals from pairs where both twins participated in the interview study ( $n = 974$ ) to allow a trained technician to visit them in their homes and draw a sample of blood. A total of 689 individuals provided a blood sample and, of these, telomere length could be determined for 607 individuals aged 73 to 94 years (Christensen et al., 2000).

### The Danish 1905 Cohort Study

In order to study nonagenarians, all Danes born in 1905 were invited in 1998 to participate in a survey similar to the LSADT and carried out by the same lay interviewers. Population-based registers were used to evaluate representativeness. Participants and nonparticipants were highly comparable with regard to marital status, institutionalization and hospitalization patterns, but men and rural area residents were more likely to participate than women and urban residents. Despite the known difficulties of conducting surveys among the very old, the study showed that it was possible to conduct a nationwide survey, including more than 2000 fairly representative nonagenarians, using lay interviewers (Nybo et al., 2001). A total of 181 full blood samples were obtained from participants from one county (Funen) and telomere length was determined for a total of 167 of these individuals aged 92 to 93 years.

### The Longitudinal Danish Centenarian Study

This study is a nationwide survey of all persons living in Denmark who celebrated their 100th birthday during the period April 1, 1995 to May 31, 1996. The residence of all centenarians in the study population was identified through the Civil Registration System by the personal identification number of each centenarian. Approximately 2 weeks after their 100th birthday, all centenarians received a letter explaining the study, and they were asked permission to let a geriatrician and a geriatric nurse visit them in order to interview them and carry out a physical examination including phlebotomy. A total of 207 centenarians participated (Andersen-Ranberg et al., 2001; Bathum et al., 1998; Bladbjerg et al., 1999; Bruunsgaard et al., 2003; Gerdes et al., 2000). A year and a half later, the surviving participants were revisited and a total of 62 blood samples were obtained. Telomere length was determined for a total of 42 of these individuals aged 101 years.

In all the three surveys, informed consent was obtained from the participants after the nature and possible consequences of the study had been explained to them. Hence, a total of 816 blood samples contained enough DNA of sufficiently good quality to generate reliable terminal restriction fragment (TRF)

data. The age span of the examined individuals ranged from 73 years to 101 years, and the sex distribution was 261 males (mean age 80.6 years) and 555 females (mean age 81.8 years). The data from the LSADT 1997 survey represent twins, some of these constituting intact twin pairs.

Red blood cells were removed by osmotic lysis, and DNA was extracted from peripheral blood using standard procedures. In brief, leukocytes were pelleted by standard techniques, as previously described (Shannon et al., 1989; Shannon et al., 1992). Using this method, DNA is prepared from a population of peripheral blood leukocytes consisting of lymphocytes, monocytes, neutrophils and eosinophils.

### Telomere Length Analysis

The methods for measurement of mean terminal restriction fragment (TRF) length have been described elsewhere (Allsopp et al., 1992; Vaziri et al., 1993). In brief, telomere length was determined by standard TRF analysis, which is based on southern blotting. Overnight, 6  $\mu$ g DNA was digested by the restriction endonucleases *Hinf*I (6 U) and *Rsa*I (6 U) following the manufacturer's instructions (Boehringer Mannheim), and subsequently subjected to gel electrophoresis (0.6% agarose, 18 V for 18 hours in 1 $\times$ TAE [40mM Tris-Acetate, 1mM EDTA]). On each gel, three marker lanes (one on each side and one in the middle) were loaded with biotinylated *Hind*III digested phage lambda DNA. Following electrophoresis, the gel was stained in ethidiumbromide and photographed (Eagleeye), and the DNA was transferred to nylon membranes (Boehringer Mannheim) using a Pharmacia vacublotter. The membrane was then incubated overnight at 65°C with 5 ng of a biotinylated oligo complementary to the telomere sequence (C3TA2)3 and washed in 2  $\times$  SSC/0.1% SDS for 2  $\times$  5 minutes at room temperature and in 0.2  $\times$  SSC/0.1% SDS for 2  $\times$  15 minutes at room temperature. Following a 1 to 5 minute rinse in 0.1 M maleate buffer, pH 7.5/0.3% Tween 20 at room temperature, and 30 minutes incubation in 5 $\times$  blocking buffer at room temperature, the membrane was incubated in 1:2500 streptavidin-AP (Boehringer Mannheim) for 30 minutes at room temperature. The filter was developed by adding 20 drops of CSPD and incubating for 5 minutes.

The membranes were visualized by 30 minutes of exposure and 30 minutes of dark subtraction in a BioRad Fluorimager. BioRads Quantity One program was used to generate a grid, where each lane was segmented into 40 blocks of identical size. In each segment, the average density was calculated, and the data set was exported to MS Excel, which was used to calculate the mean telomere length from each blood sample.

The statistical analysis of the telomere length data was done using Stata 8.2 (StataCorp, 2005). Regression analyses were performed, with telomere length as the dependent variable and age, age<sup>2</sup> and sex

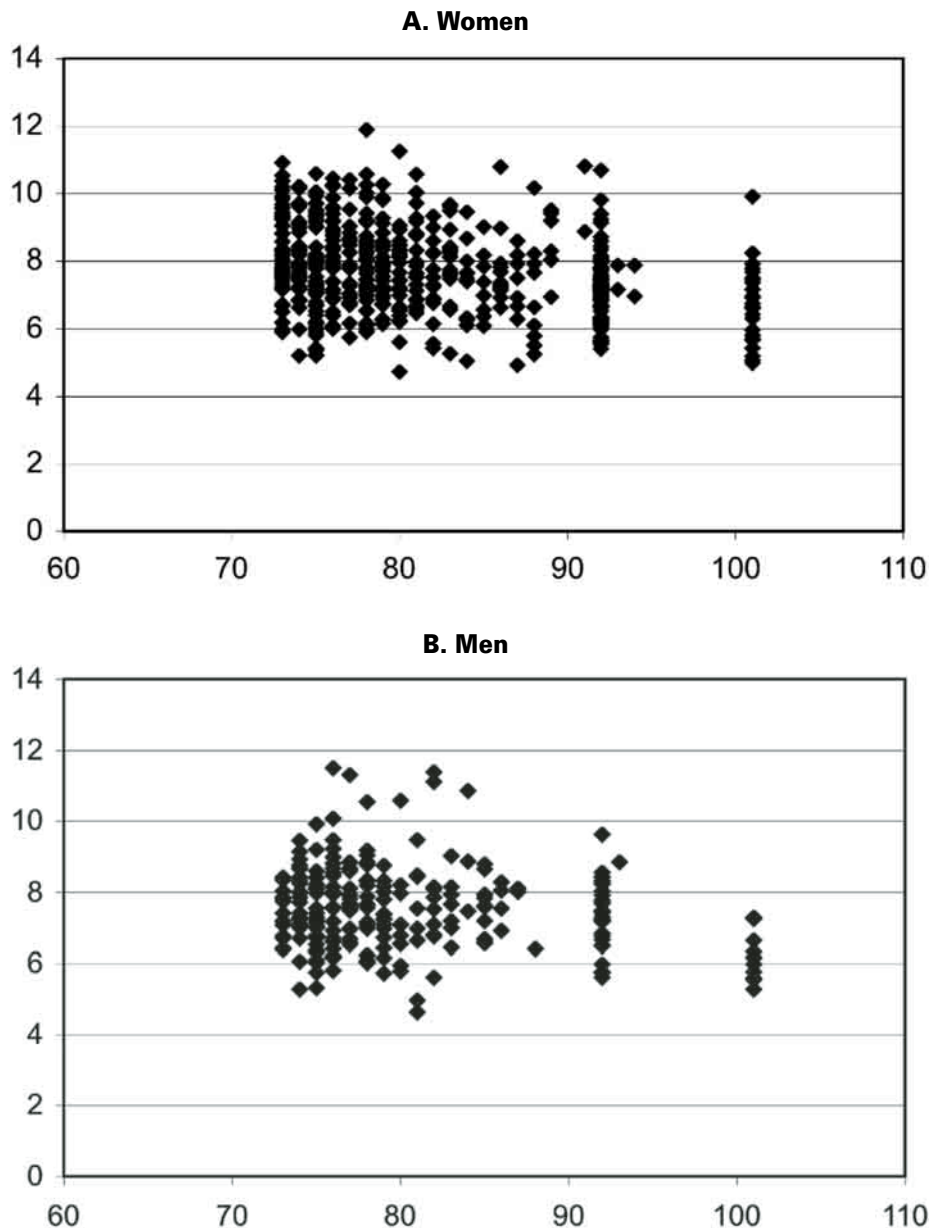
as independent variables. As the data partly pertains to twins, a correlation between twins in a pair would lead to an underestimation of standard errors, if using the traditional procedure for obtaining these. Therefore, these regression analyses were performed using the 'cluster' option in Stata, with a unique pair number as the cluster variable. For the nontwins, pseudo-pair numbers were attributed to each single individual.

### Results

Figure 1 shows graphical representations of the whole data set, split on females (Figure 1A) and males (Figure 1B). The blood samples were collected from three different populations, of which two only include individuals of very narrow age spans, namely a cohort of individuals aged 92 years (The Danish 1905 cohort) and a cohort of individuals aged 101 years (The Longitudinal Danish Centenarian Study). As a consequence, the age distribution is not even over all ages. It is particularly apparent that there is a lack of individuals in the 95 to 100 years age interval. Pooling all age groups together, the mean telomere length in the male participants (mean age 80.6 years) was found to be 7.54kb (*SEM* .07) and in the female participants (mean age 81.8 years) 7.72kb (*SEM* .05), revealing a small but statistically significant difference in telomere length between the two sexes. Considering the rather large age span in our study group, these mean values are, however, of limited interest.

As it has been firmly established that the decline in telomeric length in adult humans proceeds linearly (Frenck et al., 1998; Rufer et al., 1999), we investigated if this linear decline is present equally in both sexes in our sample of very old individuals. First, a common regression for both sexes showed that age<sup>2</sup> was not statistically significant. A regression with age and sex gave a change in telomere length of -40.9 bp/year (95% Confidence Interval [CI]: -51.4 — -30.4 bp/year, *p* < .0005). This analysis also showed a statistically significant sex difference, with age-adjusted telomere lengths being 232.5 bp longer in females (95% CI: 44.3–420.6 bp, *p* < .0005). As sex-specific plots (Figure 2) of the association between telomere length and age showed a linear pattern for females and a nonlinear pattern for males, sex-specific regressions were also performed. In females a linear association was found, as age<sup>2</sup> was nonsignificant as expected. The slope of the line amounts to -44.7 bp/year (posthoc 95%CI: -57.8 — -31.6 bp/year, *p* < .0005). Males, on the other hand, exhibit a more complex pattern, with age as well as age<sup>2</sup> being statistically significant. The coefficients were 566.4 bp/year (posthoc 95% CI: 193.5–939.3 bp/year, *p* = .003) for age and -3.5 bp/year<sup>2</sup> (posthoc 95% CI: -5.7 — -1.3 bp/year<sup>2</sup>, *p* = .002) for age<sup>2</sup>.

In summary, we find that in the age group 73 to 79 years, males have significantly shorter telomeres than females, a difference that disappears in the age interval 80 to beyond 92 years, and may reappear beyond the



**Figure 1**

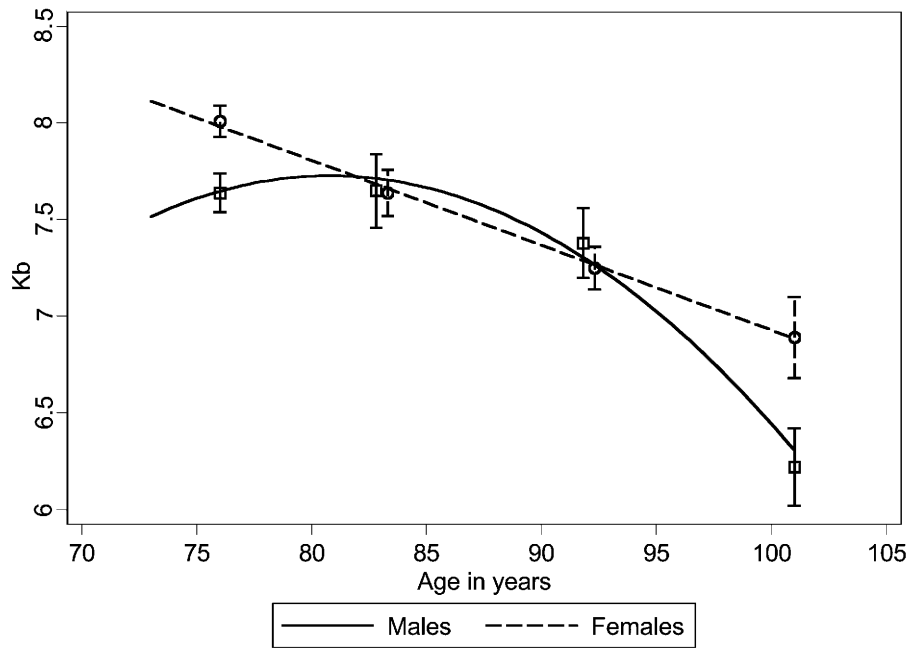
Female (A) and male (B) telomere length as a function of donor age, as determined by TRF analysis on genomic DNA obtained from whole blood, each point representing one individual.

age of 100 years. Combining the data with that of Benetos et al.'s (2001) study yielded a very monotonic linear decline for females while the male patterns were irregular at the oldest age (Figure 3).

### Discussion

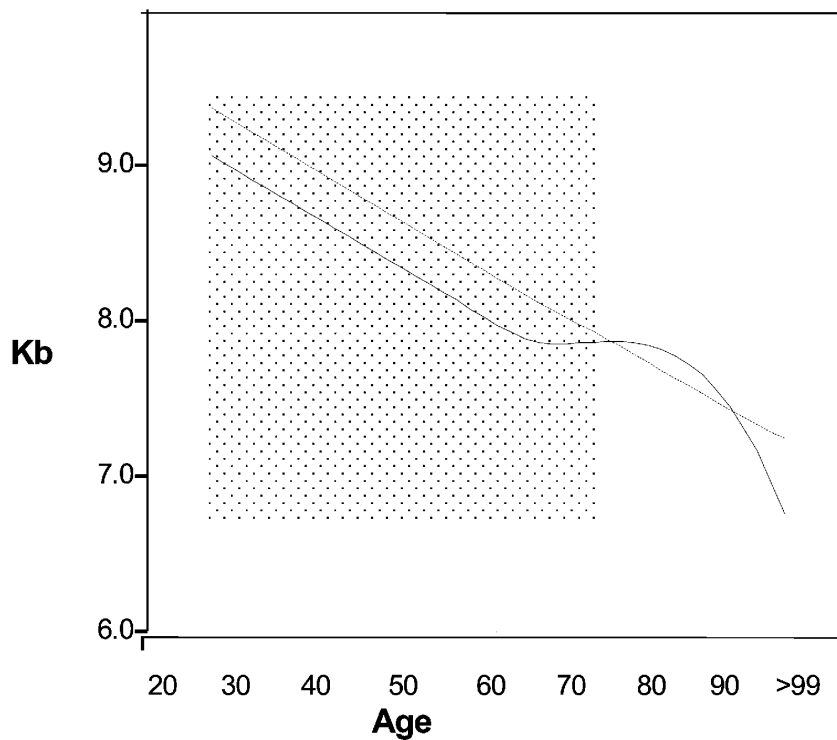
In the present communication telomere length was measured on purified DNA from blood cells obtained from 816 individuals aged 73 to 101 years. For practical reasons we had only purified DNA and not fresh cells available and chose therefore to measure telomere length by the well-established TRF assays and not by one of the recently developed fast assays based on

quantitative hybridization or polymerase chain reaction (PCR; Nakagawa et al., 2004). The TRF assay has the theoretical disadvantage that it measures a block of subtelomeric DNA in addition to the true telomeres, which can cause errors especially if this subtelomeric block varies from person to person. We do not, however, consider this source of error as significant, simply because we could verify in our material the significant sex difference found by other groups in the age group up to 75 years. With regards to the assays based on quantitative hybridization and PCR we have tried these with little success. These assays are based on measurements of telomere



**Figure 2**

Female and male mean telomere length values are depicted for age groups 73–79, 80–89, 90–98 and beyond 100 years together with best fitted curves. Equation for males: telomere length =  $-15.1718 + 0.5664 \cdot \text{age} - 0.0035 \cdot \text{age}^2$



**Figure 3**

Graph showing compiled data from this study and the study of Benetos et al. (2001; shaded gray).

It is apparent that the females (upper line) throughout the observed age span maintain a constant rate of telomere erosion. Males (lower line) maintain a constant rate of telomere erosion from young and until approximately 73 years of age. After 73 years of age, the telomere length of the examined males becomes less predictable.

amounts, and therefore sensitive to precise pipetting. Our experience is that it is extremely difficult to pipette purified DNA with sufficient precision, and because of this we returned to the TRF assay.

Previous works have shown that the rate of telomere loss in human blood cells varies with age (Frenck et al., 1998; Rufer et al., 1999; Zeichner et al., 1999), and three phases with different rates of telomere loss have been suggested (Frenck et al., 1998): from birth to approximately 4 years of age a drastic telomere loss is evident (250 to 1000 bp/year), most likely as a consequence of the high rate of proliferation. The telomere rate loss then enters a plateau with a relatively modest loss of telomere mass (less than 30 bp/year) that lasts until young adulthood, after which time telomere loss again increases (30 to 50 bp/year). In other words, the telomere length is not governed by a rigorous loss of telomere sequence each day of living, but is dependent upon other factors — most likely the cellular proliferation rate undergone in the different stages of life.

Even if based on post hoc analyses, the sex-specific results reveal some interesting details. The telomere loss observed in females (45 bp/year) is in accordance with previous studies (e.g., Benetos et al., 2001; Frenck et al., 1998; Slagboom et al., 1994). In the males, however, we observed an unexpected pattern: the mean telomere length in the age-grouped males in the interval 73 to ~90 years of age exhibits no pronounced decline in mean telomere length, as the linear and quadratic effects of age tend to cancel each other, while from ~90 years and up we observe a drastic erosion of the telomeres (~120 bp/year). This finding suggests that there may be more than the aforementioned three phases of telomere shortening, as a fourth phase at the oldest age exists in males.

Our finding, that males in the lower ages in our age interval have shorter telomeres than females, corresponds with the finding reported by Benetos et al. (Benetos et al., 2001), who investigated telomere lengths in both males and females in the age interval 20 to 75 years. In a large series he demonstrated that females at all ages had a mean telomere length about 0.4kb longer than males. When plotting our data into the graph shown in Benetos et al. (2001; Figure 3), it is striking that the linear fits plotted by Benetos et al. ends exactly at the mean telomere length values obtained for our data group 73 to 79 years. Furthermore, the best linear fit for our female data is an extension of the best linear fit for the females in Benetos et al. (2001). These sex differences are in agreement with the male–female difference in lifespan, if telomere length is of importance for lifespan. By combining our data with the data from Benetos et al., it therefore seems reasonable to postulate that females maintain the same rate of telomere decline per year in the age interval 25 years to beyond 100 years of age, while the pattern in males may be different.

The cause of the apparent plateau in the telomere erosion curve that is found in this study among males age 73 to 90, though post hoc, could be a random finding. However, by analyzing the twin data in this material (Bischoff et al., 2005) we found some indication of different heritability of telomere length in males and females, although the difference was statistically nonsignificant. From a theoretical point of view several explanations can be considered. First, the male plateau may be caused by selection by mortality. In other words, when males reach a given telomere length, mortality may increase among males with shorter telomeres. Such a selection would generate a plateau in the telomere length/age curve, because the disappearance of males with short telomeres would result in an increase in the mean value of the remaining males. It has been suggested in a recent publication that there is excess mortality in individuals with shorter telomeres beyond the age of 60 years (Cawthon et al., 2003), but the telomere-dependent mortality in the two sexes was found to be similar, at least when calculated for all age groups together. Whether this mortality is higher in males during the period where the male mean telomere length is stabilized remains to be seen. It should also be emphasized that our data are cross-sectional and therefore could be influenced by cohort effects.

Another possible reason for the male mean telomere length stabilization for almost two decades could be a gradual change in composition of the nucleated blood cells that supply the DNA for the TRF analysis. It is known that leukocyte composition changes with age (Rufer et al., 1999) but nothing is known about sex-specific differences in these changes. It can also be speculated that males reach a critically low telomere length around the age of 73 years, inducing lymphocyte telomerase, which could then maintain the male telomere length around 7kb to 8kb for an extended period of time. In females, the telomere erosion throughout life would not diminish telomere length critically before the age of 90 years under this hypothesis, and thus the telomerase elongation of critically short telomeres is not switched on until then. In other words, male white blood cells would depend on telomerase activity before ~70 years of age, whereas females become dependent on telomerase activity only after age ~90. There is no data available at present on sex-specific differences in telomerase activity in white blood cells.

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