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Leukocytes of Exceptionally Old Persons Display Ultra-short Telomeres

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Running Lead: Telomeres in the Exceptionally Old

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Abstract

With a view to understanding the association between leukocyte telomere length and the human lifespan, we performed genome-wide telomere length analyses by the terminal restriction fragment length (TRFL) and single molecule telomere length analysis (STELA) of the X and Y chromosomes in leukocytes of exceptionally old (aged 90-104 years) and younger (aged 23-74 years) individuals. We found that the mean TRFL of 82 exceptionally old individuals was within a range projected by age-dependent TRFL attrition of 99 younger individuals. However, compared with the younger individuals, exceptionally old persons exhibited peaking of the TRFL distribution with overrepresentation of ultra-short telomeres. These findings were confirmed by the STELA. Women had longer mean TRFL than men (6.10 kb vs. 5.86 kb) and exceptionally old women exhibited fewer ultra-short telomeres than exceptionally old men. Our results have implications for gerontological studies of the limitation of lifespan in humans.

Key words: aging, centenarians, mortality, replication, lifespan, senescence, telomere.

Introduction

While numerous interactions between genes and the environment appear to bring on aging in general, only a few biological variables may determine mortality and hence the lifespan (age at death from natural causes) of 'exceptionally old' people. Though exceptionally old people live in diverse environments and cultures and have different habits (4,25,39), they share attributes that suggest the role of familial (4,25,38) if not genetic (30,32,40,41) factors in their unusual longevity. Two of these are low prevalence of aging-related diseases at younger ages (4) and compressed morbidity prior to death (22, 28). Compressed morbidity does not exclude the presence of a host of aging-related diseases (2, 12) but exceptionally old individuals have either survived these diseases or lived a life that is relatively free of them.

The compressed morbidity of the exceptionally old suggests that perhaps there are only a few factors that may ultimately bring about the demise of humans who draw closer to the individual's maximal lifespan (IMLS). The IMLS is defined as the oldest age that the individual can attain, based on his/her genetic endowment under favorable environmental circumstances that reduce extrinsic mortality. With imminent mortality, biological factors that forecast the IMLS may therefore rapidly accrue in exceptionally old individuals. These factors may include ultra-short telomeres in leukocytes.

Telomere attrition ultimately precipitates replicative senescence in cultured somatic cells — an eventuality that is brought about not by the mean telomere length on the p and q arms of all chromosomes, but by a subset of telomeres with the shortest length (27,46). Leukocyte telomere shortening with age largely reflects the replicative history of hematopoietic stem cells (HSCs), progenitor cells and circulating leukocytes, a process that is influenced by many factors (10,11,14,18,21,24,31,35,42,45). In theory, a subset of telomeres in leukocytes or HSCs could shorten to a critical length that might impose replicative impasse and consequently define the IMLS.

To gain a better insight into leukocyte telomere dynamics at the fringe of the human lifespan, we compared the distributions of telomere lengths in leukocytes of exceptionally old individuals and younger adults of a wide age range, and assessed the contribution of ultra-short telomeres to the mean leukocyte telomere length. We hypothesized that those individuals who approach their IMLS display a surge in ultra-short telomeres in their leukocytes and that this phenomenon might forecast mortality. In this project we report on the distribution of telomere length in leukocytes of exceptionally old individuals.

Materials and Methods

Individuals

The study population consisted of 179 unrelated Caucasians (114 women and 65 men) aged 23-104 years, living in the five districts of the Campania region, a geographically and administratively well-defined Mediterranean area located on West Coast of Southern Italy. Only individuals originally coming from Campania were enrolled. Individuals whose grandparents had not been original habitants of Campania were excluded. For the enrollment of the exceptionally old individuals, a written permission with project explanation has been submitted to Campania communities in order to obtain a demographic list of individuals born between 1900 and 1913. Individuals were contacted at home or in their institution to arrange for the physical examination and blood collection.

All individuals were in apparently good health with normal fasting blood glucose, according to American Diabetes Association criteria (1), normal liver, kidney and thyroid functions, as determined by a medical history, physical examination, and routine screening laboratory analyses. They signed informed consent approved by the Ethics Committee of the Second University of Naples.

Measurements of the Terminal Restriction Fragment (TRF) Length (TRFL):

DNA was extracted from leukocytes using NucleoSpin Blood Kits (Macherey-Nagel Inc, Duren, Germany). Integrity of the DNA was assessed through electrophoresis of 0.5 μg DNA on 1.0% agarose gels (200 V for 2 hr) and staining

with ethidium bromide. The absence of contaminating endonucleases was confirmed in a subset of DNA samples from individuals of different ages by treating 0.5 µg DNA suspended at 37 ^{o}C overnight in buffer, comprising (in mmol/L): Tris-acetate 33, Mg-acetate 10, K-acetate 66, DTT 0.5, pH 7.9 (Roche, Indianapolis, IN) and comparing its migration with untreated DNA.

Samples were digested overnight with restriction enzymes Hinf I (10 U) and Rsa I (10 U) (Roche). DNA samples (3 µg each) and 4 DNA ladders (1 kb DNA ladder plus λ DNA/Hind III fragments; Invitrogen, Carlsbad, CA) were resolved on a 0.6% agarose gel (20 cm x 20 cm) at 50 V (GNA-200 Pharmacia Biotech). After 16 hr, the DNA was depurinated for 15 min in 0.25 N HCl, denatured 30 min in 0.5 mol/L NaOH/1.5 mol/L NaCl and neutralized for 30 min in 0.5 mol/L Tris, pH 8/1.5 mol/L NaCl. The DNA was transferred for 1 hr to a positively charged nylon membrane (Roche) using a vacuum blotter (Boeckel Scientific, Feasterville, PA). The membranes were hybridized at 65 ^{o}C with the telomeric probe [digoxigenin 3'-end labeled 5'-$(CCTAAA)_3$] overnight in 5 x SSC, 0.1% Sarkosyl, 0.02% SDS and 1% blocking reagent (Roche). The membranes were washed 3 times at room temperature in 2 x SSC, 0.1% SDS each for 15 min and once in 2 x SSC for 15 min. The digoxigenin-labeled probe was detected by the digoxigenin luminescent detection procedure (Roche) and exposed on X-ray film.

We scanned all autoradiographs and digitized the TRF signal between MW of 1-20 kb, largely because the TRF signal of exceptionally old individuals frequently

extended below 2 kb (Figure 1A). We fixed as the background the signal at the nadir of the low MW region (Figure 1A, B). The OD values vs. DNA migration distances were converted to OD (adjusted for background)/MW vs. MW. The mean (m) TRFL was then calculated accordingly (between the nadir and 20 kb). The coefficient of variation (CV) of samples resolved on different gels on different occasions was 1.76%.

Next, we obtained empirical distribution curves for each individual (Figure 1C) by fitting the data for each individual by least squares to 4-parameter logistic dose response distribution curves, $y= a_0+a_1/[1+(x/a_2)^{a3}]$, using TableCurve 2D software (SYSTAT Software Inc., Richmond, CA). The parameters a_0 and a_1 were not constrained to 0 and 1; however, in all cases, the fitted parameters closely matched these values (mean ± SD); a_0= -0.02 ± 0.027, and a_1 = 1.05 ± 0.036. The median of the distribution is a_2, and a_3 is a shape parameter. The logistic curves fit the data slightly better than 4-parameter lognormal; Weibull distribution curves (37), with intercept and height parameters corresponding to a_0 and a_1, fit marginally better than the logistic curves but are less parsimonious models. We note that the mean r^2 values exceeded 0.999 for all alternatives. The maximum first derivative of the fitted curve (density at the mode; maximal slope) was used to capture each individual's distribution. This relative frequency value is inversely related to the narrowness of the distribution.

Single Telomere Length Analysis of the p arms of the X (Xp) and Y (Yp) chromosomes (STELA$_{XY}$): We have performed STELA$_{XY,}$ because among the few available primers for telomeres on different chromosomes the ones for Xp,Yp have been shown to generate the most reproducible results (8).

The STELA was performed by modifications of a previously described method (7,8). DNA was digested with EcoRI for 1 hr at 37 ^{o}C, quantified with Hoechst 33258 and diluted to 10 ng/μL in 10 mmol/L Tris (pH 7.5). 10 ng of genomic DNA was ligated to 10 nmol/L mixture of the 6 "telorettes" (8) for 12 hours at 35 ^{o}C using 0.5 U T4 DNA ligase in 10 μL of the supplier's ligase buffer. For PCR, the DNA was diluted to the point where only a small number of molecules were amplified per reaction, which empirically corresponded to an input of approximately 250 pg. PCR was performed using expand Long Template PCR system (system 2) (Roche) in 10 μL reaction, which included 0.1 μmol/L "teltail" and telomere-adjacent primers. We cycled the reaction with GeneAmp PCR System (Applied Biosystems) under the following condition: 28 cycles of 95 ^{o}C for 15 sec, 56 for 30 sec and 66 ^{o}C for 10 min. Nine PCR reactions were performed for each DNA sample. Amplified samples were analyzed on 0.5% Tris-acetate-EDTA agarose gels and probed with random-prime, digoxigenin-labeled PCR-amplified fragment specific to the XpYp telomere (using XpYpE2 and XpYpB2 primers). The primers used were teltail = 5'-TGCTCCGTGCATCTGGCATC, telorette = 5'-teltail-(CCCTAAC)', where the sequence in parentheses is CCCTAAC, CCTAACC, CTAACCC, TAACCCT, AACCCTA or ACCCTAA,

XpYpE2 = 5'-TTGTCTCAGGGTCCTAGTG, XpYpB2 = 5'-TCTGAAGTGGACC(A/T)ATCAG. They were synthesized and HPLC purified by Operon Biotechnologies, Inc., Huntsville AL.

Statistical Analysis: Individuals were categorized into three age groups: 25-40 (mean age for men=32.6 years, women=31.0 years), 41-67 (mean age for men=52.4 years, women=51.8 years) and 90-104 years (mean age for men=95.9 years, women=96.4 years). Analysis of variance was used to compare the mean mTRFLs of age groups and of males and females within age groups. To compare mTRFLs in men and women with adjustment for the sex difference in age distribution, we performed two-way ANOVA with age group as a categorical factor and included age-sex interaction in the model. The SAS procedure PROC GLM was used to compute least squares means with standard errors and to test for the significance of the factors. The values are two-tailed; p values for pair wise comparisons were adjusted using Sidak's refinement of Bonferroni's inequality.

Simulations: Two models for telomere attrition (34) were used to simulate change in telomere length with age ΔT (bp yr^{-1}): 1) constant loss $\Delta T=-\Delta T_0$, and 2) loss proportional to the telomere length T (bp) in addition to a constant loss $\Delta T=-((1-f_s)T+\Delta T_0)$. Maximum Likelihood Estimation (MLE) was used to quantify the model parameters ΔT_0 (constant telomere loss, (bp yr^{-1})) and f_s (shortening factor (yr^{-1})) based on the mTRFL data. A logistic dose response peak curve (i.e. the

first derivative of the cumulative logistic dose-response curve) was fitted to the average TRFL frequency distribution data for the youngest age group.

The objective function to be minimized by MLE was a weighted nonlinear least squares error criterion, based on the difference between the model predictions of the mTRFL at different ages between 32.6 and 104 and the observed mTRFL. The variances of the most optimal model parameters were obtained from the diagonal of the inverse of the Fisher Information Matrix (FIM), calculated with respect to the parameters in the optimum. The models were fitted to the observed mTRFL data.

We initiated simulations at the mean age of the youngest group (32.6 years), ending at age 104 years. To this end, we fitted the distribution of the mTRFL frequencies for the youngest age group. Afterwards we calculated a mean mTRFL for each age from the predicted distributions. This model outcome was fitted to the mTRFL data. To validate both models, we compared the model-predicted TRFL distributions at the average ages of the three groups (32.6, 52.2 and 96.3 years) to the observed data distributions.

The simulations and parameter estimation were carried out in MATLAB 6.5 (The Mathworks, Inc.). The Nelder-Mead method of MATLAB's Optimization Toolbox version 2.2 was used for fitting the models.

Unless indicated, data are presented as mean ± SD.

Results

TRFL: The mTRFL in leukocytes exhibited considerable variation among individuals of the same age. Cross-sectionally the mTRFL clearly displayed an age-dependent shortening (Figure 2A). The mean mTRFL of the exceptionally old group was significantly shorter than that of the youngest (25-40 years) and the middle (41-67 years) groups (Figure 2B)**.** The slopes of the regression lines describing the relation between the mTRFL and age without and with the exceptionally old individuals (ages 25-67 and 25-104 years, respectively) were essentially the same (33.0 bp/year vs. 30.6 bp/year). The observed mean mTRFL of the exceptionally old individuals was within the range projected by a linear rate of erosion in the younger individuals.

Two-way ANOVA showed that after adjustment for age group, the mean mTRFL of women was longer than that of men (mean ± SE: 6.10 ± 0.08 kb vs 5.86 ± 0.09 kb, $p = 0.038$) (Figure 2C).

Given that the TRFL distribution somewhat deviated from normality, we also examined the regressions generated by the median of the TRFL (Figure 1, appendix). The slopes of the regression lines generated without and with the exceptionally old individuals were 31.2 bp/year and 39.2 bp/year. The difference between the slopes was not statistically significant.

Figure 3A displays the empirical frequency distributions of mTRFLs from 1-20 kb across all individuals within the 3 age groups. The lines are 4-parameter logistic dose response peak curves fitted to average frequencies for each age group. The middle group demonstrated a shift to the left (towards lower MW) in the distribution compared to the youngest group. However, the exceptionally old group manifested not only a shift to the left in the MW distribution, compared to the two other groups, but also a higher peak (maximal slope), of the frequency distributions. This is numerically depicted by the bar graph in Figure 3B; the higher peak in concert with a shift to the lower MW in the exceptionally old group indicates the overrepresentation of ultra-short telomeres in their leukocytes. We note that exceptionally old women showed fewer ultra-short telomeres than exceptionally old men (Figure 3C).

We used simulation to further explore the TRFL distribution. Model 1 (Figure 3D), which assumed only an autonomous telomere loss, predicted a shift in the mTRFL distribution to lower MW with increasing age, while the shape of the distribution remained the same. Model 2 (Figure 3E), which comprised both an autonomous loss and a loss proportional to the telomere length (i.e., longer telomeres shorten more rapidly than shorter telomeres), predicted a shift in the distribution and also a higher peak. The higher peak was already observed in the middle age group compared with the youngest group. Thus, model 2 was more compatible with the empirical findings, but not entirely. The empirical findings (Figure 3A) demonstrated that a higher peak in the frequency distribution was

hardly noticeable in the middle group but was quite profound in the exceptionally old group.

Single Telomere Length Analysis of Xp,Yp (STELA$_{XY}$): Notwithstanding the variation in telomere length on the p and q arms of different chromosomes, as age-dependent telomere erosion occurs in parallel in all telomeres, the results of the STELA$_{XY}$ should correlate with the mean telomere length, a finding documented by others (7,8). Given that the STELA provides a distribution of single telomeres, we examined whether exceptionally old individuals display to a greater extent ultra-short telomeres than do younger individuals.

Figure 4 illustrates STELA$_X$ from 41 (A) and 102 (B) year old women. Of note are the numerous ultra-short telomeres (< 3 kb) and the shift in the frequency distribution towards telomeres with markedly short lengths in the centenarian (C, D).

Figure 5 summarizes frequency distribution data of STELA$_{XY}$ derived from 15 exceptionally old individuals and 15 younger individuals. The exceptionally old individuals demonstrate a considerable shift in the frequency distribution, which was non-symmetric, of the STELA$_{XY}$ towards shorter telomeres. (median values 2.96±0.78 kb vs. 4.43±0.73 kb, $p<0.0001$). Moreover, there was a highly significant correlation between median Xp, Yp telomere length and mTRFL (Figure 6).

Discussion

Three conclusions can be made based on our findings. First, as expressed by the mTRFL, leukocyte telomere length of exceptionally old individuals was that predicted by the age-dependent telomere attrition in younger individuals. Second, mTRFL distribution in leukocytes from exceptionally old individuals exhibited overrepresentation of ultra-short telomeres compared with the two younger groups. Third, women had longer mTRFLs than men, in agreement with previous findings (10,11,21,31,35,44), and the TRFL distribution of exceptionally old women showed fewer ultra-short telomeres compared with exceptionally old men.

The deviation of the observed data from the two simulation models suggests an increase in the exceptionally old of ultra-short telomeres over and above that predicted by telomere attrition with age in the younger cohorts. The results of the $STELA_{xy}$ (Figures 4 and 5) confirm this conclusion. We postulate that in exceptionally old individuals the build up of ultra-short telomeres suggests that HSCs, progenitor cells or subsets of peripheral leukocytes approach replicating senescence. We would like to emphasize, nonetheless, that at present there is no *in vivo* evidence for replicative senescence as a determinant in the lifespan of HSCs in elderly humans, but such a phenomenon was observed in cultured cells (8,33) and was predicted through simulation (37). Additional studies have suggested that the shortest telomeres were associated with the triggering of senescence in cultured cells (27, 46).

There is little evidence that in epidemiological settings leukocytes provides better information than lymphocytes of the link between telomere dynamics and aging (reviewed in 6). We have elected to perform this study in leukocytes not to miss ultra-short telomeres from leukocyte subsets that may not be present in lymphocytes. That said, our finding paves the way to the painstaking search to identify the leukocyte subsets that may account for this phenomenon. However, this might be a tall task for subsets with small number of circulating cells. We note that identifying by Q-FISH and karyotypes analysis the specific chromosome(s) with the shortest telomeres in leukocyte subsets of the exceptionally old may be difficult, as well. Karyotype analysis entails culturing the cells to obtain those in metaphase, but the cells with the shortest telomeres are the ones most likely to reach replicative impasse and thus not enter metaphase. But regardless of which leukocyte subsets and chromosomes account for the build-up of ultra-short telomeres in exceptionally old individuals, the timing of this phenomenon may ultimately forecast the IMLS.

Chronic inflammation enhances leukocyte turnover, whereas oxidative stress increases telomeric loss per replication (reviewed in 5). Together, these processes would accelerate leukocyte telomere attrition. If inflammation (20) and oxidative stress (9) figure in the human lifespan, the ultimate life histories of both exceptionally old and ordinary people would be recorded in their leukocyte telomeres. This proposition bears a far-reaching ramification to the arithmetic of

life expectancy of the populations of industrialized nations in the 21st century. In spite of the well-established linear increase in record life expectancy during the last two centuries (36), there is likely to be a cap to human longevity (15). In part, this cap might be delineated by the ultra-short telomeres of leukocytes. When set against compressed morbidity, the substantial increase in ultra-short telomeres in leukocytes of exceptionally old individuals suggests the convergence of telomere length to a threshold beyond which no cell replication takes place.

Although leukocyte telomere length is in large measure heritable (3,31,35,42), telomere attrition rate is markedly influenced by environmental factors (10,17,19,23,36,43). Therefore, assuming that telomere length and the presence of ultra-short telomeres predicts the IMLS, these variables are the outcome of both genetic determinism and the environment.

We have included exceptionally old individuals in our study because of their compressed morbidity and imminent mortality, but these individuals belong to a highly selected group. Many younger individuals died earlier (from natural causes), perhaps due in part to the accumulation of ultra-short telomeres in their leukocytes. This might have happened because they had inherited relatively short telomeres at birth, their leukocyte telomere length shortened at a rapid pace, or both.

We also note a shortcoming of this work, namely, a two-decade gap between the exceptionally old individuals and the middle group. However, age-dependent leukocyte telomere attrition in the present study (across the gap) is within the range reported for adults, including elderly individuals, based on cross-sectional and longitudinal analyses (11,18,21,42,43). It is hence unlikely that the inclusion of mTRFL from 70-90 years-old individuals would substantially modify the regression lines describe in Figure 2. No matter what telomere parameters of the 70-90 years old individuals might have shown, if included, the conclusion of the study will stand, namely, that exceptionally old persons display ultra-short telomeres. Observing ultra-short telomeres during the 8^{th} and 9^{th} decades would not weaken the conclusion of this study; instead, it would only extend the conclusion to a wider age-range of the elderly. We note in this regard the controversy of whether or not the mean telomere length is associated with mortality in the elderly (13.16,26,29,34).

Our findings invite investigations of how ultra-short telomeres and the mean telomere length in leukocytes compare to other biomarkers of aging in their ability to forecast mortality in the elderly.

Perspectives and Significance

Interest in the field of human telomere biology has had a remarkable ascendancy during the last decade. There are two closely related, yet unresolved questions at the core of this field: (i) As telomeres are short in humans compared to most other mammals, do short telomeres serve to restrict cellular proliferation, contributing to the relative resistance of humans to cancer? (ii) Is the short telomere trait an evolutionary trade-off expressed in resistance to cancer vs. curtailment of the human lifespan? If the answers to these two questions are in the affirmative, it is reasonable to propose that future research focuses not on extending human lifespan past the evolutionary barrier but rather on improving the human healthspan within the lifespan bounds. Study of telomere dynamics and their relationship with survival in exceptionally old humans might provide critical information regarding this fundamental issue.

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Figure Legends

Figure 1: Illustration of the distribution of leukocytes telomere length: Panel A depicts a Southern blot from a 95-year-old female. The red lines mark the scan limits used for determining the TRFL. Panel B presents the OD vs. migration distances derived from panel A. Panel C presents the corresponding OD (adjusted for background) normalized by MW (blue plot). The red line is the empirical distribution curve obtained from the scatter plot (blue plot). The black dashed line is the fitted 4-parameter logistic dose response curve.

Figure 2: mTRFL shortening with age. For A, closed symbols=males, open symbols=females; red triangles=individuals 25-40 years old, green triangles=individuals 41-67 years old, blue circles=individuals 90-104 years old. The continuous line is a linear regression for the entire cohort and the dashed line is a linear regression for individuals 25-67 years old. Within age group results are described in B and C. The overall p value for B is by a one-way ANOVA. Sidak's test shows significant difference between the mean mTRFL of the exceptionally old individuals and means of mTRFL of both the middle and youngest groups (both p values < 0.0001). The mTRFL of the middle group was significantly shorter than that of the youngest group ($p < 0.0003$). In C, the overall p value for sex effect is by a two-way ANOVA (sex, age group); p values for differences between males (M) and females (F) within age groups are displayed. Though p values for male-female differences within age groups were not

significant, females had consistently longer mTRFL, so that the overall male-female difference for the entire cohort was significant.

Figure 3: Frequency Distributions and Simulations of TRF lengths. A. displays empirical data using 4-parameter logistic dose response peak curves fitted to average frequencies for each age group. The maximum first derivatives from each individual fitted cumulative curve ('slopes') were averaged within age groups and are shown in B and C. D Simulation displaying the predicted TRFL distributions at the average ages of the 3 groups (32.6, 52.2 and 96.3 years) from model 1 with only a constant telomere loss with age. The estimated parameter was: ΔT_0 = 30.8 ± 1.4 [b yr^{-1}]. E. Simulation displaying the predicted TRFL distributions from model 2 with a rate of telomere attrition proportional to the telomere length, in addition to a constant loss with age. The estimated parameters were: f_s = 0.9958 ± 0.0002 [yr^{-1}] and ΔT_0 = 6.1 ± 1.6 [b yr^{-1}]. For B, the overall p is by a one-way ANOVA. * denotes significant difference from each of the other two age groups. For C, the overall p for gender effect is by a two-way ANOVA (gender, age group). Within age group, p values for differences between males (M) and females (F) are also shown.

Figure 4: Illustration of single telomere length analysis (STELA) of Xp telomeres from 41 year-old (A, C) and 102 year-old (B, D) females. STELA products are presented in A and B (for A and B, L denotes MW ladder). Frequency

distributions of single telomeres are presented in C and D; the mean lengths of single Xp chromosomes for each distribution are noted.

Figure 5: Summary of frequency distributions of Xp,Yp telomeres derived from 15 exceptionally old individuals (ages 90-102; blue) and 15 of their younger individuals (ages 33-66; orange).

Figure 6: Correlations of the median telomere length of Xp,Yp with the mTRFL Individuals presented in Figure 5. Blue symbols= exceptionally old individuals (ages 90-102) and orange symbols= younger individuals (ages 33-66).

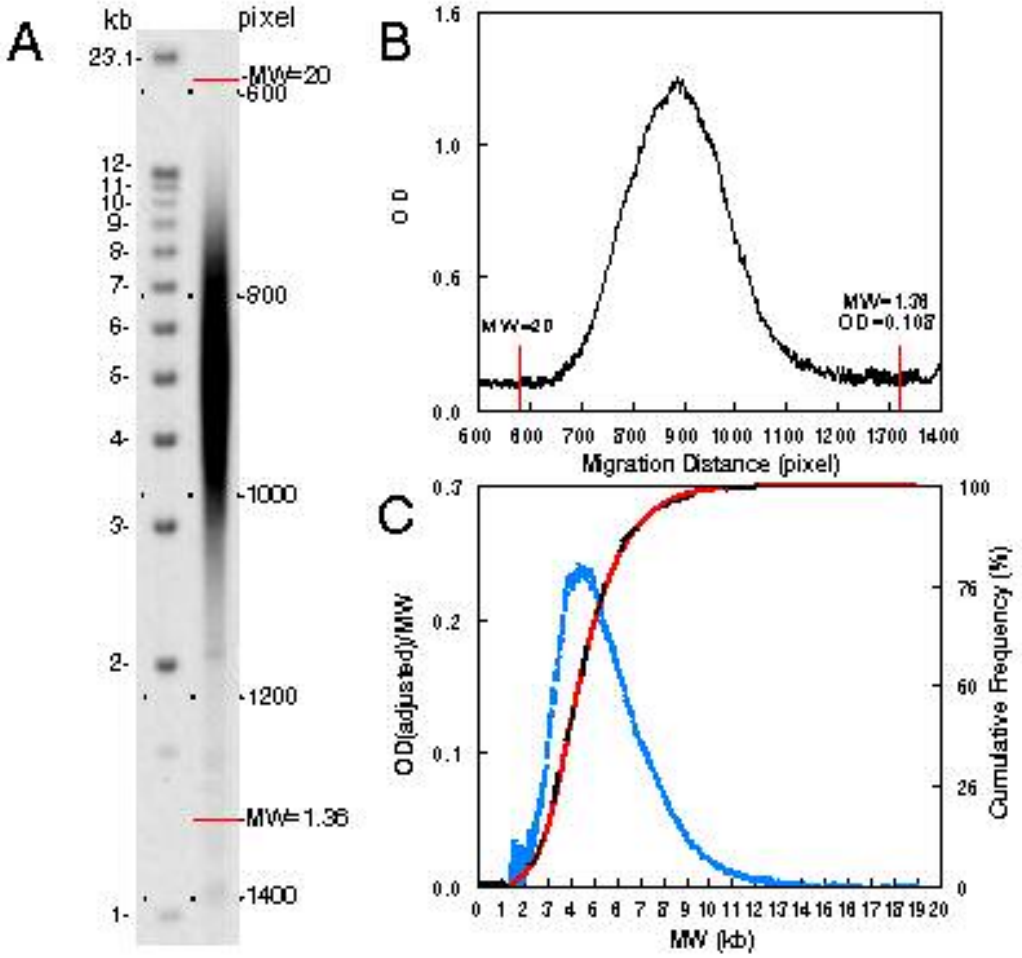
A
kb
pixel
23.1-
MW=20
600
12-
11-
10-
9-
8-
7-
800
6-
5-
4-
1000
3-
2-
1200
MW=1.36
1-
1400
B
1.6
1.0
0.6
0.0
O D
MW=20
MW=1.36
OD=0.105
600 700 800 900 1000 1100 1200 1300 1400
Migration Distance (pixel)
C
0.3
0.2
0.1
0.0
OD(adjusted)/MW
100
75
50
25
0
Cumulative Frequency (%)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
MW (kb)

Figure 1

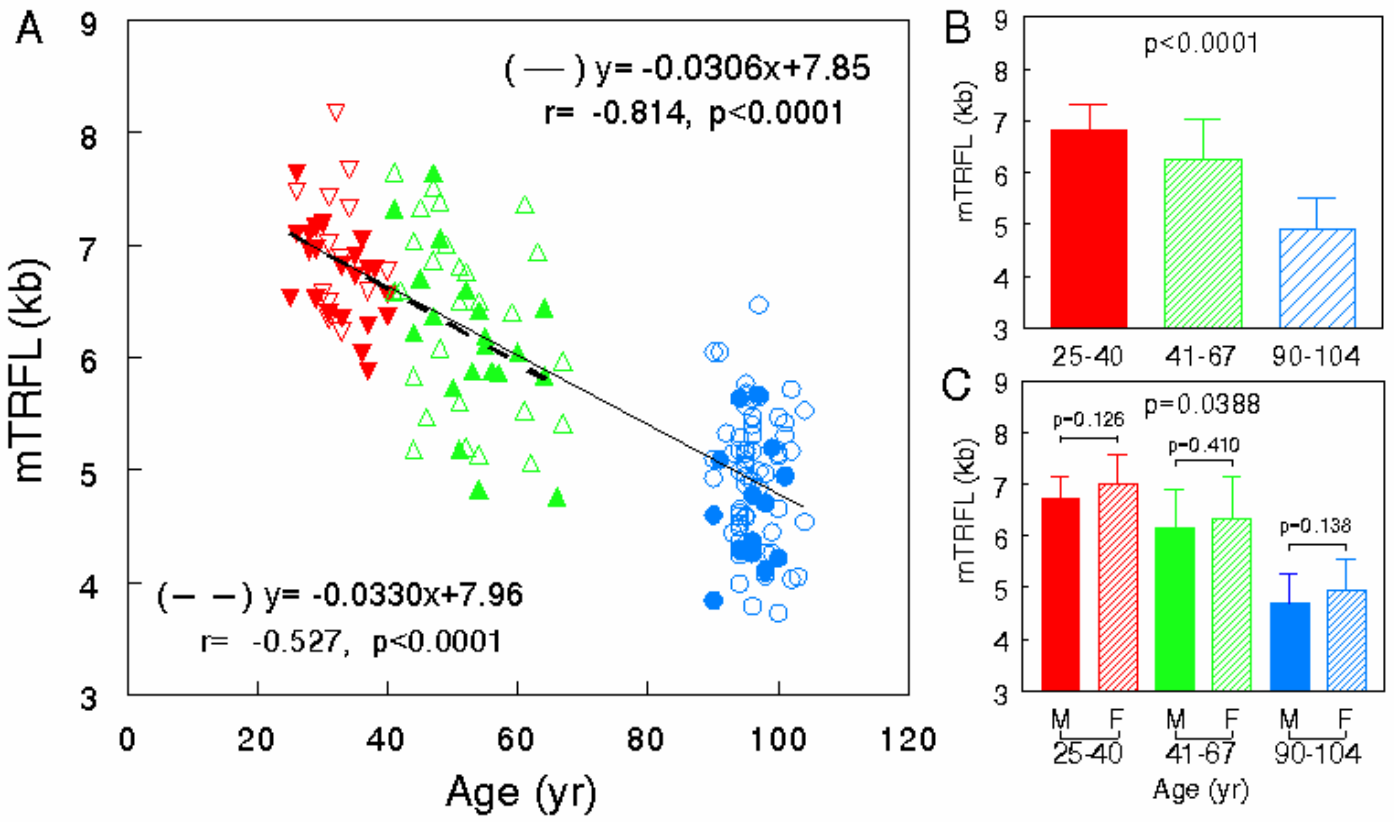
A
B
C
(——) y= -0.0306x+7.85
r= -0.814, p<0.0001
(— —) y= -0.0330x+7.96
r= -0.527, p<0.0001
mTRFL (kb)
Age (yr)
p<0.0001
25-40
41-67
90-104
p=0.0388
p=0.126
p=0.410
p=0.138
M F M F M F
25-40
41-67
90-104
Age (yr)

Figure 2

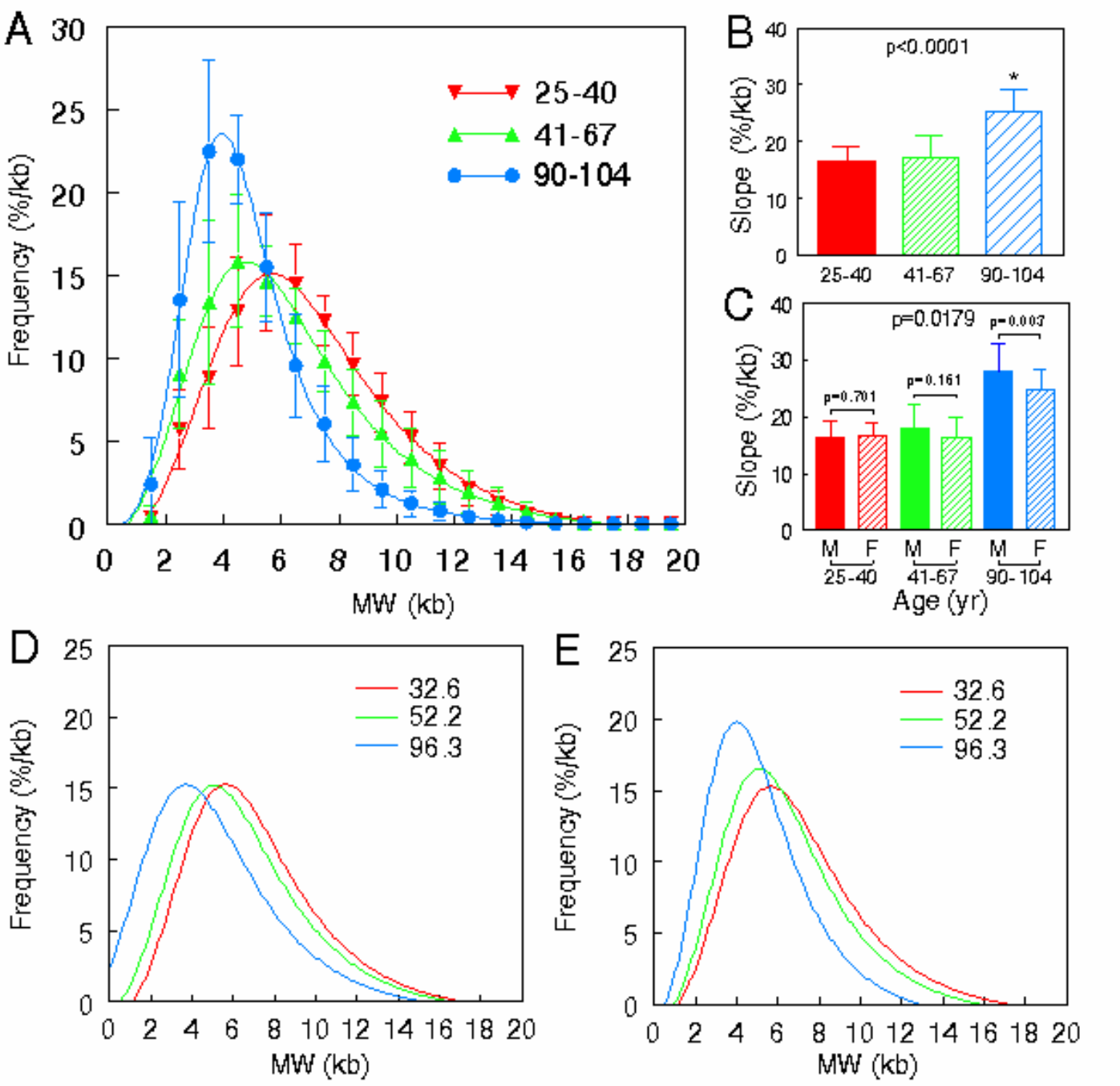
A
Frequency (%/kb)
MW (kb)
25-40
41-67
90-104
B
Slope (%/kb)
p<0.0001
*
25-40
41-67
90-104
C
Slope (%/kb)
p=0.0179
p=0.003
p=0.701
p=0.161
M F M F M F
25-40 41-67 90-104
Age (yr)
D
Frequency (%/kb)
MW (kb)
32.6
52.2
96.3
E
Frequency (%/kb)
MW (kb)
32.6
52.2
96.3

Figure 3

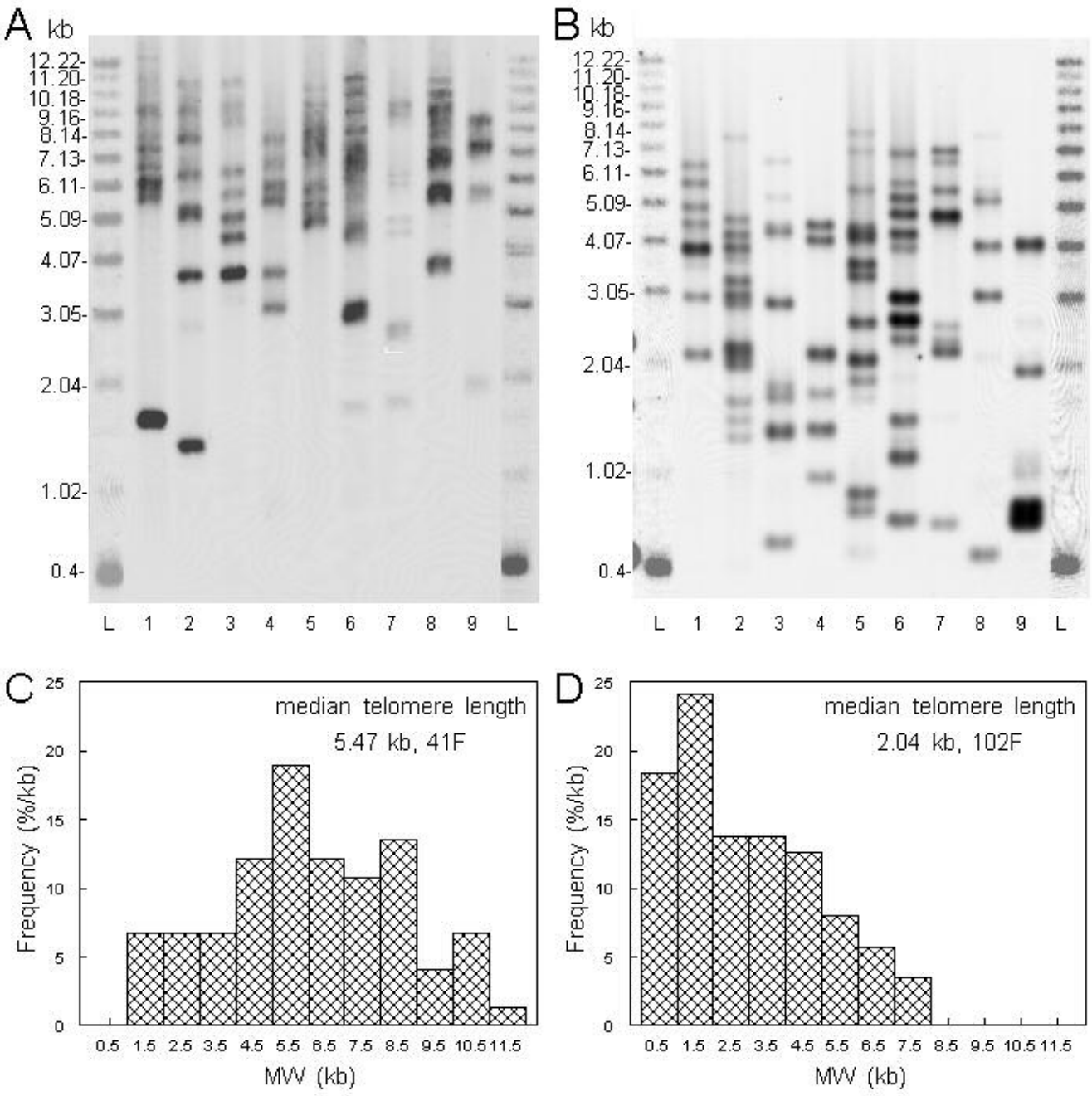
A kb
12.22-
11.20-
10.18-
9.16-
8.14-
7.13-
6.11-
5.09-
4.07-
3.05-
2.04-
1.02-
0.4-
L 1 2 3 4 5 6 7 8 9 L
B kb
12.22-
11.20-
10.18-
9.16-
8.14-
7.13-
6.11-
5.09-
4.07-
3.05-
2.04-
1.02-
0.4-
L 1 2 3 4 5 6 7 8 9 L
C
median telomere length
5.47 kb, 41F
Frequency (‰/kb)
0 5 10 15 20 25
0.5 1.5 2.5 3.5 4.5 5.5 6.5 7.5 8.5 9.5 10.5 11.5
MW (kb)
D
median telomere length
2.04 kb, 102F
Frequency (‰/kb)
0 5 10 15 20 25
0.5 1.5 2.5 3.5 4.5 5.5 6.5 7.5 8.5 9.5 10.5 11.5
MW (kb)

Figure 4

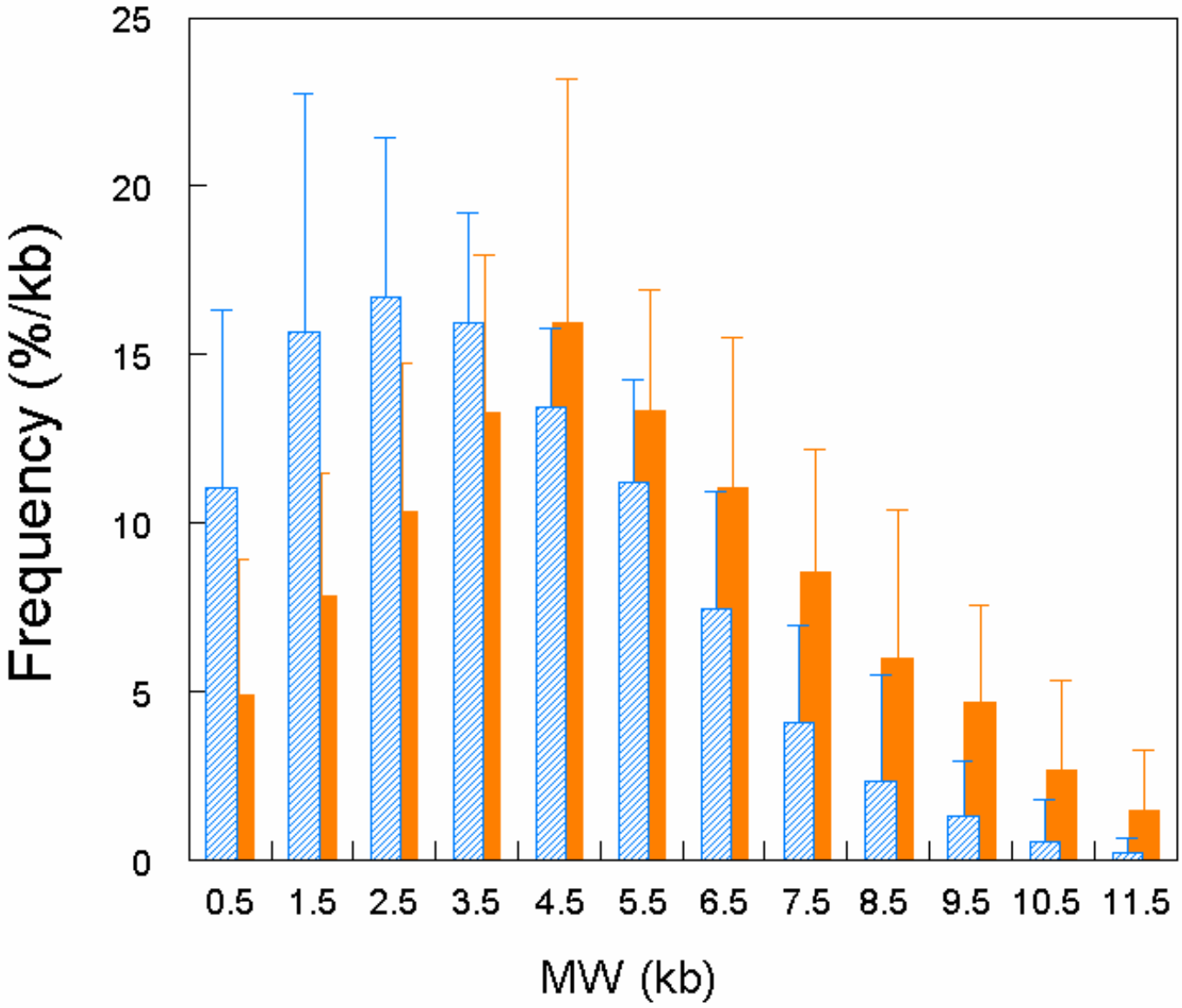
25
20
15
10
5
0
Frequency (%/kb)
0.5
1.5
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5
MW (kb)

Figure 5

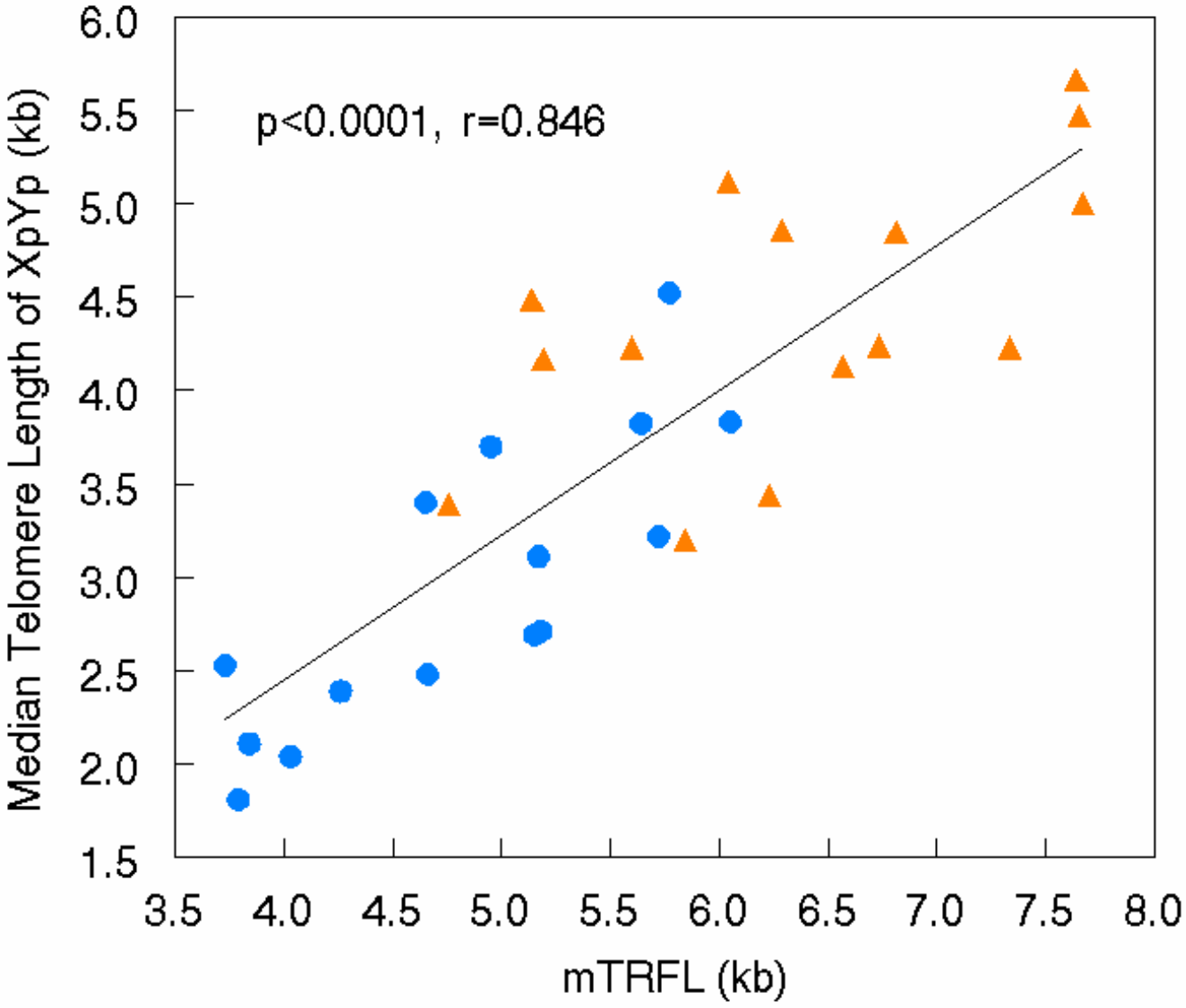
p<0.0001, r=0.846
Median Telomere Length of XpYp (kb)
mTRFL (kb)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0

Figure 6

Appendix

Figure 1, Appendix: Median TRFL shortening with age. For A, closed symbols=males, open symbols=females; red triangles=individuals 25-40 years old, green triangles=individuals 41-67 years old, blue circles=individuals 90-104 years old. The continuous line is a linear regression for the entire cohort and the dashed line is a linear regression for individuals 25-67 years old. Within age group results are described in B and C. The overall p value for B is by a one-way ANOVA. Sidak's test shows significant difference between the mean median TRFL of the exceptionally old individuals and means of median TRFL of both the middle and youngest groups (both p values < 0.0001). In C, the overall p value for sex effect is by a two-way ANOVA (sex, age group); p values for differences between males (M) and females (F) within age groups are displayed. Though p values for male-female differences within age groups were not significant, females had consistently longer median TRFL, so that the overall male-female difference for the entire cohort was significant.

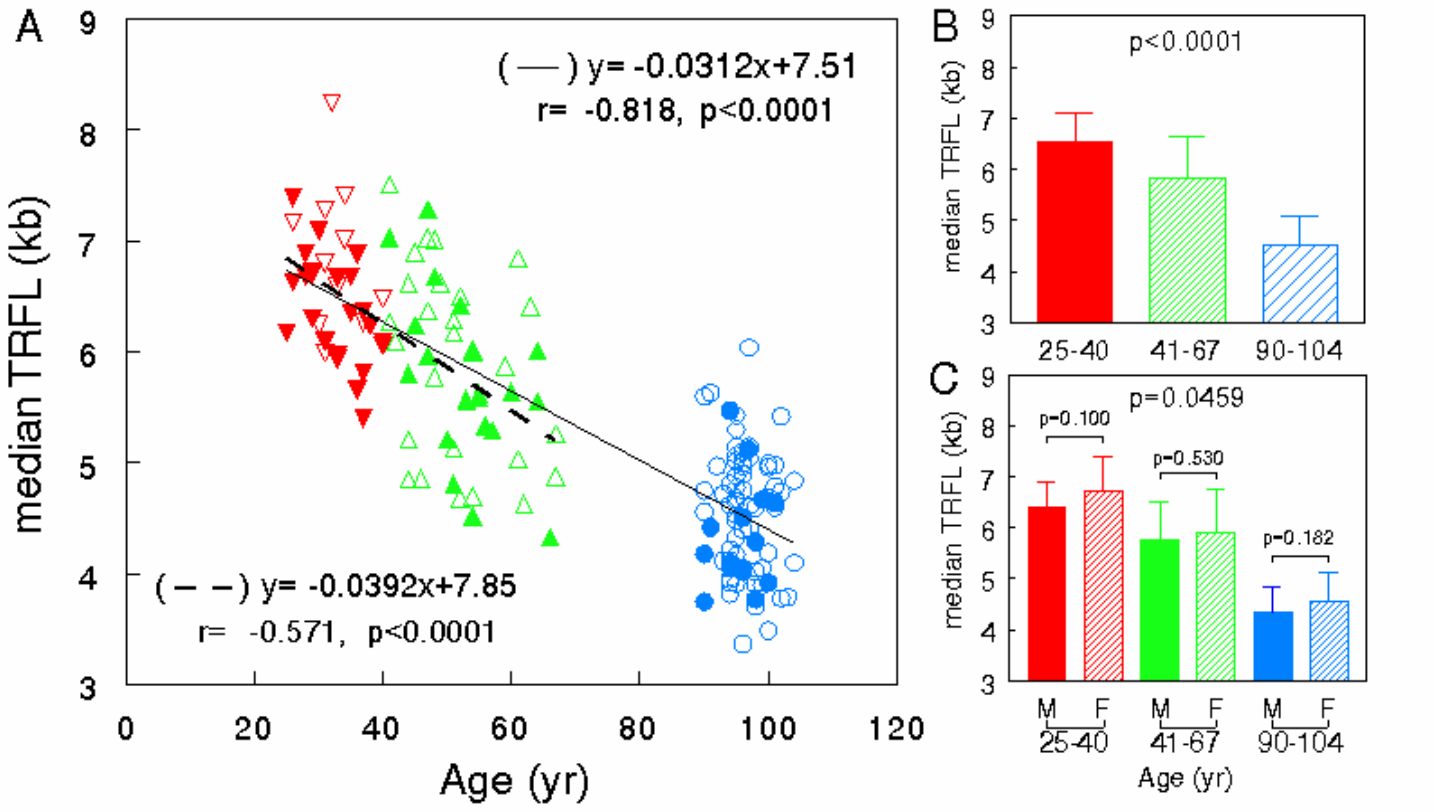

Figure 1, Appendix