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METHODS

Do leukocyte telomere length dynamics depend on baseline telomere length? An analysis that corrects for 'regression to the mean'

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Abstract Leukocyte telomere length (LTL) shortens with age. Longitudinal studies have reported accelerated LTL attrition when baseline LTL is longer. However, the dependency of LTL attrition on baseline LTL might stem from a statistical artifact known as regression to the mean (RTM). To our knowledge no published study of LTL dynamics (LTL and its attrition rate) has corrected for this phenomenon. We illustrate the RTM effect using replicate LTL measurements, and show, using simulated data, how the RTM effect increases with a rise in stochastic measurement variation (representing LTL measurement error), resulting in spurious increasingly elevated dependencies of attrition on baseline values. In addition, we re-analyzed longitudinal LTL data collected from four study populations to test the hypothesis that LTL attrition depends on baseline LTL. We observed that the rate of LTL attrition was proportional to baseline LTL, but correction for the RTM effect reduced the slope of the relationship by 57 % when measurement error was low (coefficient of variation ~2 %). A modest but statistically significant effect remained however, indicating that high baseline LTL is associated with higher LTL attrition even when correcting for the RTM effect. Baseline LTL explained 1.3 % of the variation in LTL attrition, but this effect, which differed significantly between the study samples, appeared to be primarily attributable to the association in men (3.7 %).

Keywords Leukocyte telomere length · Sex · Age · 'Regression to the mean' · Longitudinal studies

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Introduction

Telomere length is increasingly attracting attention as a potential biomarker of aging and health. Longitudinal studies of telomere length are of particular interest because they provide information on potential determinants of telomere shortening. One pattern that is typically reported in such studies is that attrition of leucocyte telomere length (LTL) is positively correlated with baseline LTL [e.g. 1–4]. Such a finding is of interest, for example, because longer telomeres are associated with better survival prospects [see 5 for a meta-analysis], and this pattern would suggest that any advantage from having longer telomeres might diminish with age since it would cause initial differences in LTL to diminish with age.

Notably, the correlation between LTL attrition and baseline LTL is likely to be at least partly due to a statistical artifact known as "regression to the mean"[RTM; see e.g. 6–8]. This effect arises when there are two successive measurements of LTL (X1 and X2), and one assesses the

correlation between LTL attrition (X2–X1) and baseline LTL (X1). As X1 and X2–X1 are statistically not independent (because X1 contributes to both axes), any measurement error in X1 is reflected in both axes, generating the RTM effect. This problem arises regardless of the trait that is being studied, and RTM is consequently a well-recognized phenomenon. For example, the search string "regression to the mean" yielded >500 papers in the Web of Science (as 'Topic'), and >31.000 hits in Google Scholar. Disregarding RTM may lead to unjustified inferences on the relation between baseline value and rate of change. Thus, correction for RTM is required when examining the effect of baseline LTL on its attrition rate.

Giltay et al. [9] noted that the correlation between LTL attrition and baseline LTL could be a statistical artifact (without actually mentioning the term 'regression to the mean'), and they supported their argument with a simulation study. However, they did not provide a test of the effect of baseline LTL on LTL attrition that corrected for the RTM effect. Here we (1) illustrate the RTM effect using duplicate measurements of LTL in the same DNA sample, meaning that there is no real difference in LTL, (2) use simulated data to show how the strength of the RTM effect depends on the level of stochastic variation in measurements (i.e. predominantly measurement error in our context), and (3) then re-analyze three data sets to address the question of whether the rate of LTL shortening depends on initial LTL when controlled for the effects of RTM. Previous studies have reported variation in telomere attrition with respect to age [10, 11] and sex [11, 12] and we therefore take these factors into account in the latter analysis.

Methods

LTL data

We used LTL data from three population-based studies ("populations") that were set up with the aim to gain insight into LTL dynamics from longitudinal data: the Jerusalem Lipid Research Clinic (LRC) study, the Bogalusa Heart Study and the Evolution de la Rigidite Arterielle (see Table 1 for references). Telomere length in all study samples was measured in a single laboratory (AA) by Southern blots of the terminal restriction fragments [13]. In all studies, duplicate measurements of the same DNA sample were resolved on different gels and occasions. Baseline and follow-up samples from the same individual were resolved on adjacent lanes on the same gel, and without considering sex (i.e. these were distributed over gels). The inter-assay coefficients of variation of the LTL measurement were 2.0–2.1 %. The correlation between successive LTL measurements was high (all $r > 0.93$) in all four studies (Table 1). The data set published by Chen et al. [22] contained both Caucasian Americans and African Americans and LTL dynamics were previously found to differ between these groups [14]. We therefore treated them as separate samples in our analyses.

Simulation data

To illustrate the RTM effect, and in particular how it depends on the extent to which subsequent measurements are correlated, we generated three bivariate normal distributions that differed in the strength of the correlation between the subsequent measurements. Data were generated using the function mvrnorm in R [15]. The chosen levels of correlation ($r = 0.4$, 0.65 and 0.95) approximately cover the range encountered in longitudinal LTL studies. For illustrative purposes we used the data of one of the study samples (the Jerusalem LRC, Table 1) for the average value and standard deviation as well as the average LTL shortening between the successive measurements. However, the results can be generalized and are independent of the chosen average and standard deviation.

Table 1 Descriptives of the three data sets

	LRC	ERA	BHS—White Americans	BHS—African Americans
Initial age (years)	30.08 ± 0.80	58.09 ± 9.76	31.18 ± 4.65	30.81 ± 4.83
Follow up (years)	13.05 ± 0.77	9.47 ± 0.52	12.36 ± 1.75	12.43 ± 2.00
Baseline LTL (kb)	7.333 ± 0.673	6.452 ± 0.558	7.074 ± 0.671	7.668 ± 0.707
CRS	0.958	0.963	0.959	0.934
Proportion male	0.666	0.670	0.358	0.284
N	620	185	204	67
Reference	[20]	[21]	[22]	

Numbers are mean ± standard deviation

LRC the Jerusalem Lipid Research Clinic, *BHS* the Bogalusa Heart Study, *ERA* the Evolution de la Rigidite Arterielle, *CRS* correlation of successive samples

Statistical analysis

Data were analyzed with General Linear Models using JMP® 9.0 (SAS Institute Inc., Cary, NC, USA, 1989–2007). LTL attrition between successive samples was corrected for the RTM effect following "Methods" section in Berry et al. [6]. This entails adjusting the difference between successive measurements by subtracting the change that is expected as a result of the regression effect, which is estimated using the correlation between successive measurements (see "Appendix" for the equation). The equation involves mean centering of the values for both measurements, and hence the average corrected change is equal to zero (when this is confusing, e.g. in presentations, this can be remedied by adding the difference between the averages of the first and second measurement to all corrected changes).

Results

Duplicate measurements

Using all baseline LTL measurements that were done in duplicate on different gels (n = 1,006), we plotted the difference between the two measurements against the value of the first measurement for each of our study populations (Fig. 1). All four correlations were negative (Fig. 1), with an effect size r that is significant with a sample size from ≈200. We find this result despite there being on average no difference between the two duplicates, and notwithstanding the high quality of the measurements as evidenced by the low inter-assay coefficient of variation. Thus, even when comparing high quality duplicate measurements, changes from one sample to the next tend to be positive when the initial value was low and negative when the initial measurement was high. This resulting negative correlation (Fig. 1) solely represents the RTM effect.

Impact of measurement repeatability

To evaluate how the RTM effect depends on the similarity between subsequent measurements we generated three data sets that differed in how strongly subsequent measurements were correlated (Fig. 2, left panels). For each of the three data sets we calculated the change in LTL from the baseline to the follow up, and plotted these values against the baseline LTL (Fig. 2, right panels). From these figures it is clear that the change in LTL is correlated with the baseline LTL ($-0.55 < r < -0.14$), mirroring the findings for the duplicate LTL measurements (Fig. 1). More importantly, as the correlation between the baseline and follow-up

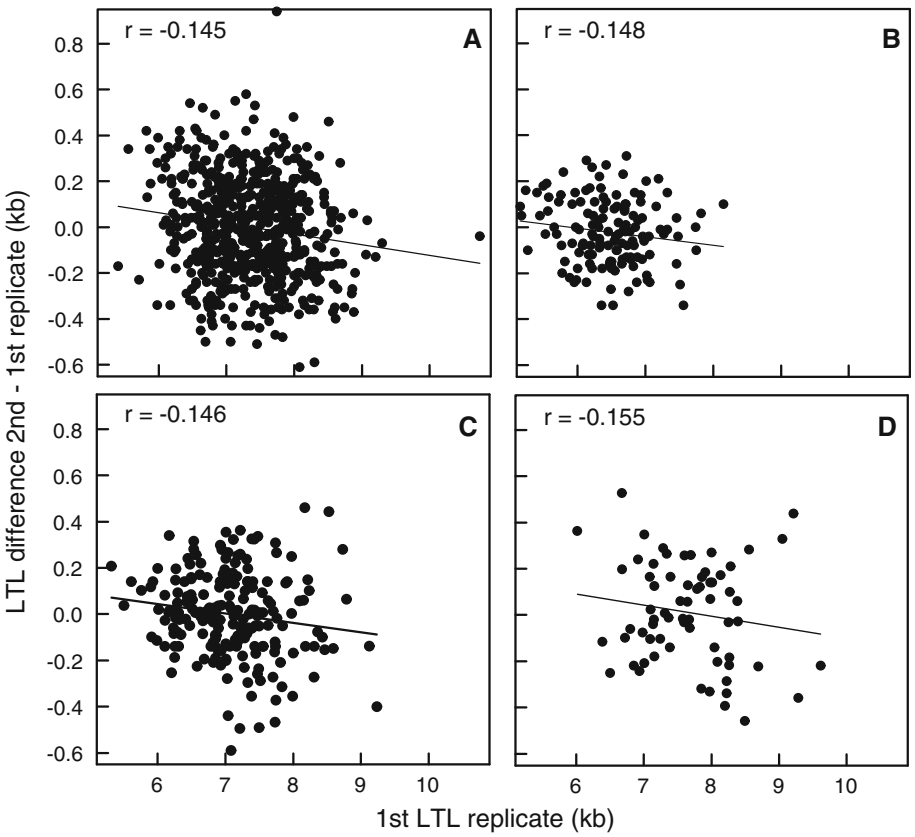

Fig. 1 The regression to the mean effect illustrated using duplicate telomere measurements of each baseline sample that were run on different gels. **a** LRC (N = 598), **b** ERA (N = 137), **c** BHS, Caucasians (N = 204), **d** BHS, African Americans (N = 67). See Table 1 for details of the studies but note that sample sizes are slightly lower than in Table 1 because not in all cases were both replicate measurements successful. Results displayed some stochastic variation, depending on which replicate is taken as baseline. We therefore randomized this 30 times and here show examples that are representative of the average result

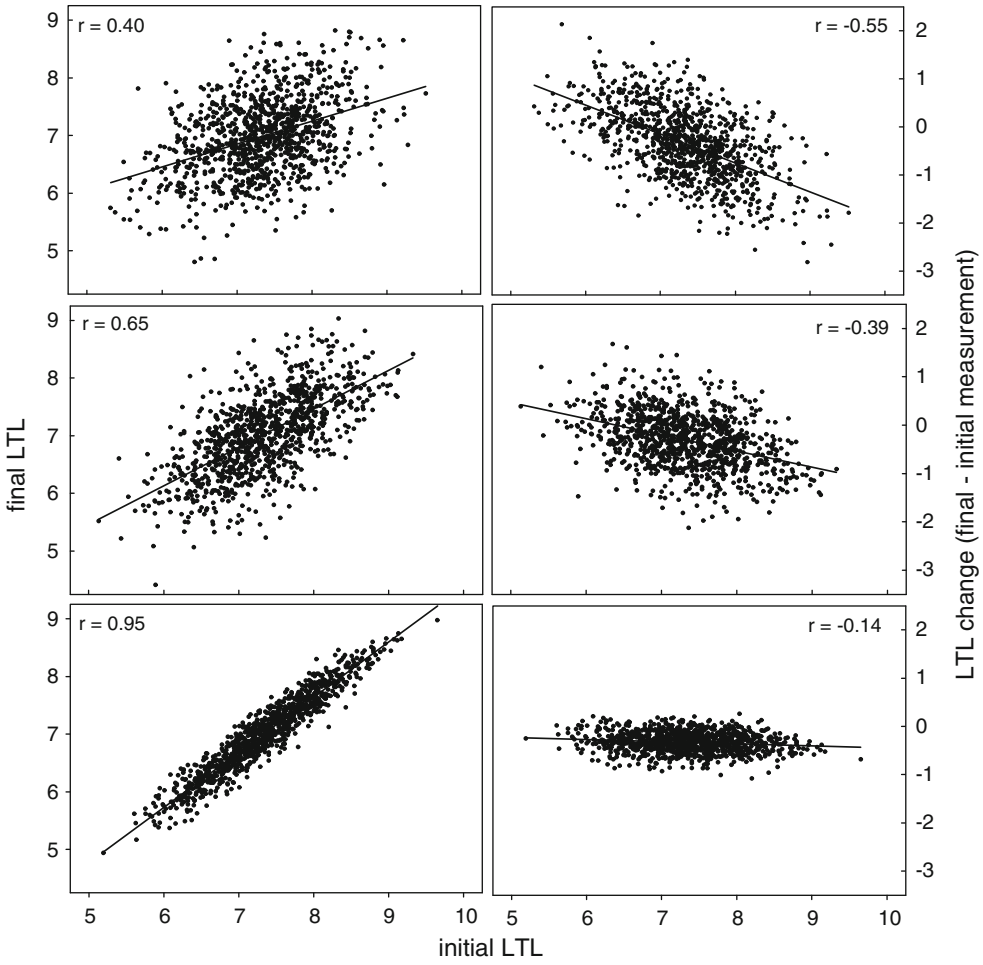

Fig. 2 Simulated data, with average baseline LTL and LTL attrition as in the LRC study population (Table 1). The *panels* on the *left* show the correlation between the baseline and the follow-up LTL measurements in the simulated data. The *panels* on the *right* show the correlation between the baseline LTL and the change in LTL from the baseline to the follow-up measurements (negative values indicate that LTL became shorter)

measurements becomes stronger from top to bottom, the RTM effect becomes progressively weaker, as indicated by the diminishing correlation coefficient from a strong spurious correlation of −0.55 to one of −0.14. Note, however, that even when the correlation between successive measurements is high (r = 0.95, as in the studies analyzed below), there is still a non-negligible RTM effect of r = −0.14 that would be considered statistically significant with a sample size of ≈200. These results illustrate that the RTM effect increases strongly with increasing stochastic variation due to measurement error and/or other sources.

Correcting for RTM

To illustrate and verify the effectiveness of the method we used to correct for the RTM effect we first applied this method to the simulation data described in the previous section (Fig. 2). For each of the three data sets in Fig. 2, with r-values of −0.55, −0.39, and −0.14 respectively, correction for the RTM-effect yielded correlations close to zero (r = −0.004, 0.024, 0.017 respectively, note the positive sign of the latter two values). We can therefore assume that our chosen method effectively corrects for the RTM effect.

Pooling all four populations, and controlling statistically for 'population' and the time elapsed between baseline and follow-up samples, we tested whether LTL attrition was dependent on baseline LTL. To illustrate the effect of correcting for the RTM effect, we carried out these analyses for both the uncorrected LTL attrition, and LTL attrition that was corrected for RTM. Correcting for RTM more than halved the slope of this effect (Table 2), illustrating how the RTM effect inflates this correlation in a real data set. However, the effect of baseline LTL on LTL attrition remained highly significant even when correcting LTL attrition for the RTM effect (Table 2). Thus, we conclude that on average the rate of LTL attrition per unit time increases with increasing baseline LTL over and above the RTM effect. Initial age did not explain a significant part of the variance when added to the model in Table 2b ($F_{1,\ 1,069} = 0.5$, $P = 0.5$) and neither did participant's sex ($F_{1,\ 1,069} = 0.11$, $P = 0.7$). These results did not change when analyzed separately for the four study populations.

Table 2 Statistical analyses (GLM) of telomere attrition in relation to the baseline LTL (kb) and the time interval (years) between successive samples

Variable	A. Uncorrected values		B. Corrected values	
	Slope (s.e.)	F (d.f.)	Slope (s.e.)	F (d.f.)
Baseline LTL	−0.077 (0.009)	79.9 (1)	−0.033 (0.009)	14.7 (1)
Interval (years)	−0.027 (0.005)	29.2 (1)	−0.027 (0.005)	29.1 (1)
Study population		14.6 (3)		9.7 (3)

Analyses were carried out separately for telomere attrition values A. before and B. after correction for regression to the mean (RTM). Study population is included as a factor to allow intercepts to differ between the populations (see Table 3 for analyses that also allow slopes to differ between the populations). For both analyses: N = 1,076, d.f. error = 1,070. All effects are significant at $P < 0.0001$

Table 3 Sex specific statistical analyses (GLM) of telomere attrition corrected for regression to the mean in relation to baseline LTL and the time interval between successive samples in A. women and B. men

Variable	Slope (s.e.)	F (d.f.)	*P*
A. Women			
Baseline LTL (kb)	−0.003 (0.01)	0.05 (1,441)	0.82
Interval (years)	−0.025 (0.007)	10.67 (1,441)	0.001
Study population		2.89 (3,441)	0.035
Added to model above:			
Pop × Baseline LTL		3.09 (3,438)	0.027
B. Men			
Baseline LTL (kb)	−0.055 (0.011)	24.90 (1,623)	<0.0001
Interval (years)	−0.033 (0.007)	22.54 (1,623)	<0.0001
Study population		10.45 (3,623)	<0.0001
Added to model above:			
Pop × Baseline LTL		0.42 (3,620)	0.74

For both sexes first a model is shown without interactions, and subsequently the results obtained for the interaction between study population and baseline LTL is shown when added to this model

The four populations differed significantly in average LTL attrition rate, as evidenced by the significant variation between populations in intercept (Table 2, column B). As a follow up to this finding, we tested whether the dependence of LTL attrition on baseline LTL also differed between the four populations. The interaction between population and baseline LTL proved to be statistically significant when added to the model in Table 2b ($F_{3,\ 1,067} = 4.19$, $P = 0.006$), indicating noticeable inter-population variation in the relationship between baseline LTL and LTL attrition. The source of this variation could be manifold, but age and LTL at baseline measurement do not seem likely candidates since these variables do not consistently differ between the BHS study and the other populations. There is, however, a large difference in the proportion of women in the different studies (Table 1), and we therefore verified to what extent the overall emerging pattern was sex dependent. When adding sex and the interaction between sex and baseline LTL to the model in Table 2b, we found this interaction to be statistically significant ($F_{1,\ 1,068} = 7.30$, $P = 0.007$). To investigate this finding in more detail we analyzed data on the sexes separately (Table 3) and found that baseline LTL was on average correlated with LTL attrition in men, but not in women (Table 3). Furthermore, this relationship varied significantly between populations in women (predominantly attributable to BHS vs. the other 3 study populations), but not in men (Fig. 4).

Discussion

Longitudinal studies in humans reported that LTL shortens at a higher rate with age in individuals with longer LTL [e.g. 1–3], but to our knowledge none of these analyses corrected for the RTM effect. We illustrated this effect using duplicate measurements, where there can be no question that any pattern must be a statistical artifact because the replicates do not differ systematically in LTL. This analysis showed the RTM effect to be strong (Fig. 1), despite the relatively high consistency of the measurements ($r > 0.9$). Such a finding underlines the importance of controlling for the RTM when examining the effect of baseline LTL on LTL attrition. We further illustrated how the magnitude of the RTM effect increases with increasing stochastic noise due to measurement error and other sources (Fig. 2). Laboratory error in telomere measurement varies considerably between methods [16, 17], and the RTM effect will be stronger when measurements are less accurate, resulting in spuriously high correlations of LTL attrition with baseline values. However, even when measurement accuracy is high (and hence stochastic effects are relatively small) the RTM effect is substantial, and thus correcting for RTM is always necessary when evaluating changes in telomere length or other traits over time in relation to the baseline value.

Although the RTM effect was strong, when correcting for this effect we still find that LTL attrition increases with increasing baseline LTL (Fig. 3). The magnitude of this effect is such that with a 1 kb longer LTL at baseline, over the measurement period its effect on LTL attrition is approximately equivalent to the effect of a 1 year longer interval between measurements (Table 2b). Thus, controlling for baseline LTL in longitudinal LTL studies may be useful to increase statistical power by reducing residual

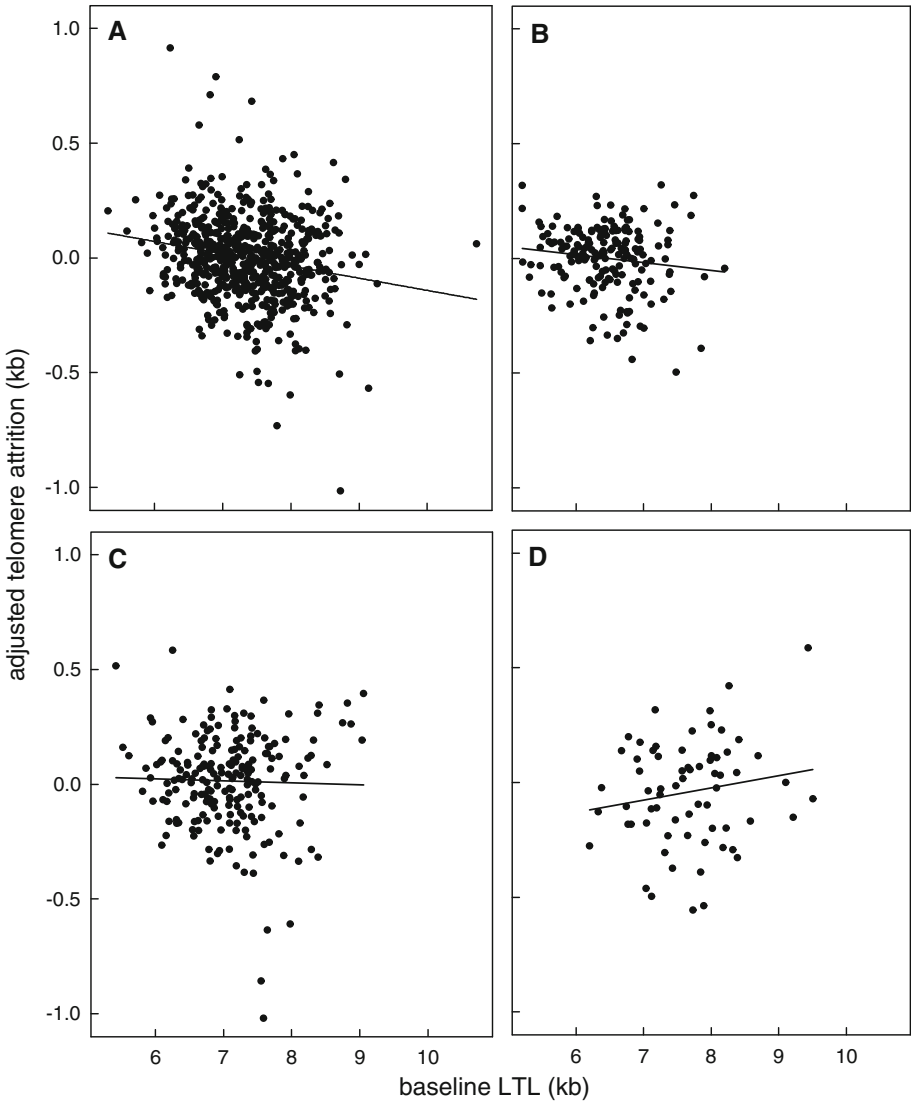

Fig. 3 LTL attrition adjusted for regression to the mean in relation to baseline LTL in the four study populations. **a** LRC, **b** ERA, **c** BHS, Caucasians, **d** BHS, African Americans (see Table 1 for details). Note that in the correction procedure the changes in LTL are scaled to average zero. In the analysis also the interval between the successive measurements was taken into account (Table 2), but the data in this figure were not corrected for the latter effect

variation, but when doing so one should correct for the RTM effect to obtain unbiased estimates.

Evidence supporting the conclusion that longer telomeres lose base pairs at a higher rate also comes from observations of telomere dynamics in other species. For instance, in jackdaws *Corvus monedula*, a small crow species, TRF measurements in erythrocytes showed that individuals lost more base pairs per unit time from their longer telomeres than from their shorter telomeres [18]. In this context, Grasman et al. [19] developed a telomere-shortening model assuming that longer telomeres are more vulnerable due to being a larger target for damaging agents such as reactive oxygen species, and this model could well replicate the observed pattern in jackdaws. The same model could potentially also explain the effects of age and baseline LTL on LTL attrition in adult humans, which would support the hypothesis that there is a shared mechanism, but this remains to be investigated.

In conclusion, we observed a pattern of a modest increase in LTL attrition with increasing baseline LTL when populations and sexes were pooled and the analysis was corrected for RTM. While this pattern was more or less uniform in men of the four populations, in women there was significant variation between the study populations with Afro-American women differing strongly from the rest (Fig. 4), yielding an average not deviating significantly from zero. Since the difference between the sexes was detected in a post hoc analysis rather than the test of an a priori stated hypothesis, and there are no obvious candidate hypotheses to explain this detail in our results, our finding requires confirmation in other populations for us to be convinced that the sex difference we documented can be generalized. Clearly, the inclusion of more study populations would resolve this issue,

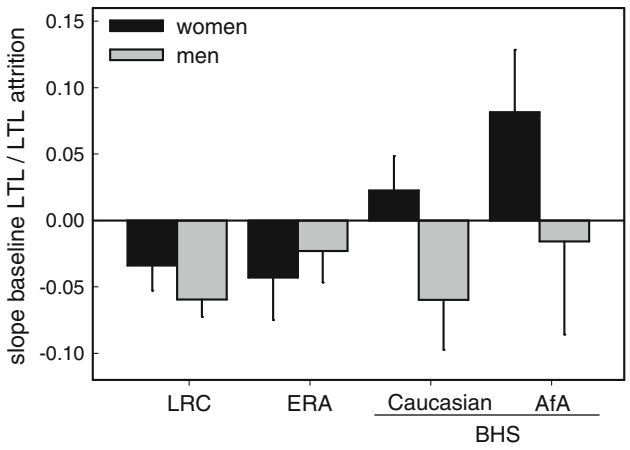

Fig. 4 Slope (±s.e.) of the relationship between baseline LTL and LTL attrition as in the model in Table 3, but calculated separately for each study population. In women the slope did overall not significantly deviate from zero, but varied significantly between populations, while in men the slope was overall significantly negative and did not significantly vary between populations (see Table 3 for details of the analysis and Table 1 for details on the different studies)

as well contribute more generally to the exploration of possible causes of the variation between populations in the rate of telomere attrition in general, and its dependence on baseline LTL.

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Appendix

The change from the baseline measure X_1 to follow up measure X_2 is adjusted for the regression to the mean effect to yield a corrected value D as follows:

$$D = \rho(X_1 - \bar{X}_1) - (X_2 - \bar{X}_2)$$

where

$$\rho = \frac{2rS_1S_2}{S_1^2 + S_2^2}$$

in which r is the correlation between X_1 and X_2.

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