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# Psychological states and physical fates

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# Chapter 3

## Stressful life events and leukocyte telomere attrition in adulthood: a prospective population-based cohort study

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*Submitted*

## ABSTRACT

### Background

Telomere attrition might be one of the mechanisms through which psychosocial stress leads to somatic disease. To date it is unknown if exposure to adverse life events in adulthood is associated with telomere shortening prospectively. In the current study we investigated whether life events are associated with shortening of telomere length (TL).

### Methods

Participants were 1094 adults (mean age 53.1 years, range 33-79) from the PREVEND cohort. Data were collected at baseline (T1) and at two follow-up visits after 4 years (T2) and 6 years (T3). Life events were assessed with an adjusted version of the List of Threatening Events (LTE). TL was measured by monochrome multiplex quantitative PCR at T1, T2, and T3. A linear mixed model was used to assess the effect of recent life events on TL prospectively. Multivariable regression analyses were performed to assess whether the lifetime life events score or the score of life events experienced before the age of 12 predicted TL cross-sectionally. All final models were adjusted for age, sex, BMI, presence of chronic diseases, frequency of sports, smoking status, and level of education.

### Results

Recent life events significantly predicted telomere attrition prospectively (B=-0.031, p=.007). We were not able to demonstrate a significant cross-sectional relationship between the lifetime LTE score and TL. Nor did we find exposure to adverse life events before the age of 12 to be associated with TL in adulthood.

### Conclusions

Exposure to recent adverse life events in adulthood is associated with telomere attrition prospectively.

## INTRODUCTION

Psychosocial stress is a well-known risk factor for various psychiatric and somatic disorders, including major depressive disorder [1] and cardiovascular disease (CVD) [2, 3]. In attempts to unravel the relationship between psychosocial stress and adverse health, much attention has been given to the functioning of three important stress-responsive systems; the hypothalamic pituitary adrenal axis [4, 5], the autonomic nervous system [4, 6], and the immune system[7-9]. In 2004 a novel pathway through which stress can adversely influence health was identified when a study was published that demonstrated an association between psychosocial stress and short telomere length [10].

Telomeres are TTAGGG nucleotide tandem repeats at the ends of chromosomes in eukaryotic cells. As DNA polymerases cannot copy the end of the DNA strand, telomeres progressively shorten with each cell division. When telomeres become critically short this causes chromosomal instability[11] and cellular senescence [12]. Consequently telomere length is considered a biological marker of aging [13]. Although a special enzyme telomerase exists which is capable of lengthening telomeres, in most somatic cells only limited amounts of this enzyme are present [11]. Telomere shortening is predictive of increased mortality rate [14, 15] and various age-related diseases, such as cancer[16] and Alzheimer's disease [14].



According to the recent literature review by Price et al. [17] most studies investigating the relationship between psychosocial stress and telomere length found a significant negative association, although some studies also failed to demonstrate any relationship. Furthermore, the authors point out that except for one study[18] all studies investigating the relationship between psychosocial stress and telomere length have been cross-sectional [17]. This is problematic because of the high variability in telomere length between individuals which is present at birth, and gender differences in rate of change in telomere length, limiting the power of studies utilizing only one time point[17, 19]. Moreover, when studying processes such as the effects of psychosocial stress on biological systems the real interest lies in *change* over time.

The only longitudinal study on the effects of psychosocial stress on telomere length until now has been performed in children. We know from life-course epidemiology that for certain change effects 'critical periods' or 'sensitive periods' exist for some biological processes [20]. Thus, it is currently unknown if psychosocial stress in adulthood has the same damaging effect on telomere attrition as it has in childhood. Furthermore, it might be that repair mechanisms, such as telomere elongation by the enzyme telomerase[13], prevent short term stressors from leaving a permanent mark on the telomere length. In this case it might be that cumulative exposure to life events over life is needed to create an adverse outcome [20]. In the current study we want to investigate the multiple possibilities mentioned above and have formulated four hypotheses. Firstly, we postulate that recent adverse life events in adulthood are associated with telomere attrition over time prospectively. Secondly, we hypothesized that the rate of change in telomere length associated with recent adverse life events is moderated by cumulative exposure to adverse life events over life. Thirdly, we hypothesize that life events experienced in childhood have long-lasting negative effects on telomere length that can still be detected in adulthood. Finally, we hypothesize that the

cumulative exposure to stressful life events over life is associated with shorter telomeres cross-sectionally.

# METHODS

## Study population

Our study has been performed in a cohort derived from Prevention of REnal and Vascular ENd stage Disease (PREVEND), a population cohort study originally designed to investigate microalbuminuria as a risk factor for renal and cardiovascular disease. The recruitment of participants for PREVEND has been extensively described elsewhere[21]. The PREVEND baseline sample consisted of 8,592 subjects randomly selected from the population of the city of Groningen, the Netherlands, with oversampling for albuminuria (T1). Selection of subjects for the present study was aimed at recruiting a representative sample from the general population of Groningen, while simultaneously rectifying PREVEND's oversampling for albuminuria. Research assistants approached participants in the PREVEND study during their visit to the outpatient clinic during follow-up (T2) (2,554 participants). Questionnaires were completed by a total of 1,094 participants (43%), forming the population cohort of the present study. There was no significant difference in gender, age, or scores on a 12-item neuroticism scale between PREVEND participants who were invited to participate in the present study but declined and PREVEND participants who agreed to participate . The sample consisted of 588 females (53.7%) and 506 males (46.3%). Their mean age was 53.1 years (SD = 11.4 years, range 33-79) and their ethnicity was predominantly Caucasian. Follow-up measurements took place between January 2002 and November 2003 (T2) and approximately two years later, between April 2004 and November 2006 (T3). Average time between T1 and T2 was 4.1years; average time between T2 and T3 was 2.4 years. Follow-up measurements were completed by a total of 976 participants (89%) at T3. The study was approved by the local Medical Ethical Committee for human research of the University Medical Center Groningen. All participants provided written informed consent for participation in this study.

## Adverse life events

Stressful life events were assessed by means of the Dutch version of the List of Threatening Events (LTE), a 12-item self-report questionnaire [22]. The LTE comprises 12 major categories of stressful life events that were selected for their established long-term consequences. It assesses the occurrence of events such as the death of a loved one, losing one's job, or being hospitalized for a physical disease. The original LTE covers the last six months, but we used an adjusted version of the LTE with response categories for the previous year and for five age categories: "0-12 years", "13-18", "19-39", "40-60", and "> 60" [23]. Respondents were instructed not to include the last year when scoring the age categories since this period was covered by a separate response category. For each response category, participants indicated whether or not each of the 12 different life events occurred (yes/no). We also included an open item for "other events". We checked this open item for events that actually belonged to one of the 12 life event categories, and corrected the data when this was the case. The

current version of the LTE has been extensively validated as described elsewhere [23]. In brief, participants filled out the LTE at two occasions; once at T2 and once at T3. The stability of the retrospective reporting of life events was satisfactory. The test-retest correlation for the lifetime LTE score was large: 0.606. The construct validity of the list is indicated by its positive associations with psychological distress, mental health problems, and neuroticism.

The LTE total score for each age category is the sum of the item scores for that age category. We calculated the "lifetime LTE score" by adding the total scores of all age categories that were completed at the time of assessment. The "childhood LTE score" was calculated by adding the different life events that took place before the age of 12. Underreporting of adverse life events is an established bias of retrospective questionnaires [24]. To correct for underreporting of life events in the childhood LTE score and the lifetime LTE score, we scored a life event as 1 (took place) if it was reported at either T2 or T3, and as 0 (did not take place) if it wasn't reported at any of these two measurement occasions. Furthermore, we calculated two "recent LTE scores" for life events that took place in the recent year, i.e. one for events in the year before T2 and one for events in the year before T3.



## Telomere length

Fasting blood samples were collected from all participants by a nurse during a visit to the research facilities at T1, T2, and T3. In case of flu or a febrile temperature, blood collection was postponed to a later time. DNA was extracted from leukocytes. In order to prevent batch effects, the samples of the three different time points (T1,T2, and T3) were randomly assigned for DNA extraction. The same extraction method was used for all samples with a standard kit according to manufacturer's instructions (QIamp 96 DNA blood kit, catalogno 51162 of Qiagen, Venlo, the Netherlands. Telomere length (TL) was measured by a monochrome multiplex quantitative PCR method, whereby the telomere and single copy gene are amplified in the same tube[25]. Samples were run in triplicate measured in the same well position on different plates. The intra-assay coefficient of variation was 2% (T), 1.9% (S) and 4.5% (T/S ratio). Reproducibility data was obtained for 216 subjects from PREVEND and good agreement between T/S ratios was observed ($R^2$=0.99, P<0.0001, inter-run CV 3.9%)[26]. The samples of the three time points were equally divided over the PCR schedule to reduce potential time or seasonal effects. The calibrator sample used was made up of a mixture of DNAs from young adult individuals (around 25 years). There was a highly significant decline in T/S ratio with age in PREVEND (B = -.0047, SE = .0004, p<0.001) confirming the internal validity of the assay. Telomere length in the present study was available for 1019 people at T1 (93.1%) and for 982 people at T3 (89.8%). At T2 for a large part of the cohort DNA was not available and telomere length could thus be determined only for a subset of the population (N=445, 40.7%). Likelihood-based methods can still provide reliable estimates if there is missingness in the outcome variable [27, 27]. Thus, we used maximum likelihood estimation in all models involving the T2 data as is explained in more detail below.

## Statistical analyses

*Covariates*

The following covariates were selected for their known association with life events or

telomere length: gender [28], age [29], BMI [30], the presence of chronic diseases, smoking [30], frequency of sports [31], and level of education [32]. We had information about the presence of a chronic disease in the following categories: coronary heart disease (CHD), cerebrovascular accident (CVA), diabetes mellitus, chronic liver disease, chronic kidney disease, malignancy, rheumatoid arthritis, COPD or asthma, severe skin disease, severe bowel disease lasting > 3 months. All somatic diseases, except for diabetes, CHD, and CVA were self-reported diseases that were present in the previous year. Diabetes was defined as the use of antidiabetic treatment according to self-report or pharmacy data. CHD and CVA were defined as self-report of CHD/CVA upon inclusion in the study and/or confirmed occurrence of CHD/CVA as registered Dutch national registry of hospital discharge diagnoses between inclusion and date of visit to the research facilities at T2. Smoking was divided into six categories namely none, 1-5, 6-10, 11-15, 16-20, >20 cigarettes/day. Frequency of sports was defined as: I don't exercise, I exercise once per week, or I exercise twice or more per week. Educational level was categorized as: none, lower secondary education or less, higher secondary education, or tertiary education. In the statistical models smoking, frequency of sports, and level of education were entered as dummy variables with the lowest category serving as a reference.

### *Adverse life events in adulthood and telomere attrition*

Our data had a hierarchical structure, that is, repeated measurements were nested within individuals. Therefore we chose a statistical model that takes into account the non-independence of observations. We used a linear mixed model to test the hypotheses that recent adverse life events in adulthood are associated with telomere attrition over time prospectively, and secondly that this effect is moderated by cumulative exposure to adverse life events. All models contained telomere length (T2 and T3) as the dependent variable and had the "recent LTE score" (the year before T2 and the year before T3) as the predictor variable. Moreover, all models were adjusted for sex, age, BMI, presence of chronic diseases, smoking, frequency of sports, level of education, telomere length at baseline (T1), and time in years between measurement occasions. Our model can thus be viewed as an analysis of covariance, adjusting telomere length at follow-up for baseline telomere length. [33] All predictors were time-varying except sex, telomere length at baseline, level of education, and number of chronic diseases at T2. A random intercept was used to account for nesting of observations within individuals. In the first model we tested the hypothesis that life events in the previous year were associated with telomere attrition. In the second model, effect modification analysis was used to examine whether associations of recent life events with telomere length were dependent on the level of lifetime exposure to stressful life events. This model was identical to model described above, except for the addition of the "recent LTE score" by "lifetime LTE score" cross-product term. The maximum likelihood method was used for model estimation. The outcome variable was checked for normality and was natural log transformed to meet the assumptions of a normal distribution. Results were considered statistically significant for a two-sided P-value <0.05. All models were analyzed using the nlme package [34] in R, version 2.15.2 [35].

### *Childhood and lifetime adversity and telomere length in adulthood*

To test our hypotheses that exposure to adverse life events during childhood or cumulative exposure to stressful life events over life is associated with shorter telomere length in

adulthood, we performed multivariable regression analysis with as a predictor variable either the "childhood LTE score" or the "lifetime LTE score" respectively. All models were adjusted for gender and age. The outcome variable was telomere length at T3. The outcome variable was checked for normality and was natural log transformed to meet the assumptions of a normal distribution. The final models were also adjusted for BMI, presence of chronic diseases, smoking, frequency of sports, and level of education. To illustrate the influence of the covariates, we also present results of models that are only adjusted for gender and age. Results were considered statistically significant for a two-sided P-value <0.05. All models were analyzed using R, version 2.15.2 [35].

*Exploratory analyses*

Previous longitudinal studies investigating TL have reported both the possibility of telomere attrition and telomere lengthening [29, 36-38]. Most studies defined attrition as a decrease in TL >15%, and lengthening as an increase in TL >15% between baseline and follow-up measures. We investigated the dynamics of TL in our cohort using the same definitions.



# RESULTS

## Descriptive statistics

General characteristics of the study population can be found in table 1. As mentioned above we used a decrease in TL >15% and an increase in TL >15% between baseline and follow-up measures as a definition of telomere attrition and lengthening respectively. Over an average time of 6.0 years between baseline and follow-up 63.0% of participants showed decrease in TL, 6.3% remained stable, and 30.7% showed lengthening of telomeres.

## Adverse life events in adulthood and telomere attrition

The results of the analysis testing the hypothesis that recent adverse life events in adulthood are associated with telomere attrition can be found in table 2. The recent LTE score was a significant predictor of telomere attrition (coefficient= -.028, SE=.011, p=.012) in a random intercept model adjusted only for baseline telomere length and gender and age. Likewise, in a fully adjusted random intercept model, the recent LTE score predicted a significant decrease in telomere length. Age was also a significant predictor of telomere attrition. The different categories of smoking status, gender, and level of education, however, did not predict changes in TL. We also tested the hypothesis that the effect of life events in the previous year (recent LTE score) was dependent on the lifetime LTE score by adding an interaction term between these two variables to the model. There was no significant interaction between the recent LTE score and the lifetime LTE score (p=.716).

**Table 1.** General characteristics of the study population at T2

| | N=1094 |
|---|---|
| Gender (Female, %) | 53.7 |
| Age, mean (SD) | 53.1 (11.4) |
| Race (%) | |
| White | 97.6 |
| Black | 0.6 |
| Asian | 0.7 |
| Other | 1.0 |
| Lifetime LTE score, mean (SD) | 7.2 (3.8) |
| Recent LTE score* (%) | |
| 0 events | 75.9 |
| 1 event | 14.6 |
| 2 events | 5.9 |
| 3 events | 2.8 |
| 4 or more events | 0.9 |
| Childhood LTE score* (%) | |
| 0 events | 60.7 |
| 1 event | 26.7 |
| 2 events | 8.0 |
| 3 events | 3.0 |
| 4 or more events | 1.6 |
| Telomere length at T1, mean (SD) | 0.417 (0.276) |
| Level of education (%) | |
| None | 4.7 |
| Low | 26.5 |
| Middle | 27.1 |
| High | 41.7 |
| Smoking yes (%) | 23.9 |
| Smoking cigarettes/day (%) | |
| None | 76.1 |
| 1-5 | 4.3 |
| 6-10 | 3.9 |
| 11-15 | 6.4 |
| 16-20 | 5.4 |
| > 20 | 3.8 |
| Exercise (%) | |
| I don't exercise | 51.9 |
| I exercise once per week | 27.7 |
| I exercise twice or more times per week | 20.4 |
| Body mass index (kg/m$^2$) mean (SD) | 26.5 (4.1) |
| Chronic diseases (%) | |
| healthy | 80.9 |
| 1 chronic disease | 15.6 |
| 2 chronic diseases | 2.6 |
| 3 chronic diseases | 0.6 |
| 4 chronic diseases | 0.2 |
| 5 chronic diseases | 0.1 |

*SD = standard deviation, IQR = interquartile range, * = data not normally distributed thus for descriptive purposes only we created categories.*

**Table 2.** Mixed model predicting telomere length at T2 and T3 by recent life events, adjusting for baseline telomere length

| N=843 | coefficient | SE | p-value |
|---|---|---|---|
| telomere length T1 | 0.195 | 0.037 | <.001 |
| Age | -0.003 | 0.001 | .003 |
| Recent life events | -0.031 | 0.011 | .007 |
| Gender (female) | 0.024 | 0.019 | .217 |
| Sports | | | |
| once per week | -0.030 | 0.023 | .194 |
| twice or more/week | 0.027 | 0.025 | .267 |
| Smoking | | | |
| 1-5 cig | -0.002 | 0.051 | .962 |
| 6-10 cig | -0.010 | 0.050 | .838 |
| 11-15 cig | 0.005 | 0.040 | .887 |
| 16-20 cig | -0.094 | 0.045 | .039 |
| More than 20 cig | -0.057 | 0.062 | .363 |
| BMI | -0.007 | 0.003 | .009 |
| Chronic diseases | -0.006 | 0.020 | .823 |
| Education | | | |
| Low | -0.024 | 0.049 | .626 |
| Middle | -0.042 | 0.050 | .404 |
| High | -0.015 | 0.049 | .759 |
| Time | 0.006 | 0.008 | .463 |

*BMI= body mass index, SE= standard error. Note that intercept and random intercept are not shown*



## Childhood and lifetime adversity and telomere length in adulthood

To assess whether exposure to adverse life events between the ages of 0-12 years has a long-lasting effect on telomere length that can still be detected in adulthood, we investigated whether the childhood LTE score was negatively associated with TL in adulthood at T3. Multivariable regression analysis showed no relationship between the childhood LTE score and TL at T3 in the fully adjusted model (B= -.002, SE=.013, p=.936), nor in a model adjusted only for gender and age (B= -.001, SE=.012, p=.880).

Furthermore, we tested if the cumulative exposure to stressful life events over life was associated with telomere length. The results of multivariable regression analysis showed that the lifetime LTE score was not significantly associated with TL at T3 in the fully adjusted model B=.000, SE=.003, p=.852.), nor in a model adjusted only for gender and age (B=.000, SE=.003, p=.959). Only age and BMI were significant negative predictors of TL at T3.

## DISCUSSION

To our knowledge, this is the first longitudinal population-based study demonstrating that exposure to adverse life events in adulthood is associated with telomere attrition. The effect of recent life events on telomere attrition was not moderated by the cumulative exposure to stressful life events over life. We did not find a cross-sectional relationship between the lifetime LTE score or the childhood LTE score and TL.

There are several strengths and limitations of the current study that need to be taken into consideration when interpreting our results. The first major strength of this study is that it was conducted in a large population-representative cohort, which increases the generalizability of our findings. The second strength is the prospective nature of the design, allowing us to adjust for telomere length at baseline (T1) and therefore estimate change in telomere length over time.

Our finding that recent life events are prospectively associated with telomere attrition is in agreement with the results from the only other prospective study in this area of research which was performed in children [18]. It is interesting that, although the life events in our study were of a very different nature than child abuse and took place in very different age categories, the effects on telomere length are the same. As opposed to the results of our longitudinal analysis, we did not find a cross-sectional relationship between the lifetime LTE score or the childhood LTE score and TL. Several explanations for this difference in findings might be offered. Firstly, at birth there is already a high variability in telomere length between individuals [19]. This limits the power to detect environmental effects and might explain the often small effects reported in cross-sectional study psychophysiological research. A longitudinal study may focus on within-individual change and is thus less affected by large natural differences in telomere length between individuals. Some authors even claim that a cross-sectional study on telomere length would require five times the sample size a longitudinal study would need [39]. Nonetheless, also for the cross-sectional part of our study the sample size was fairly large.

Secondly, the lifetime LTE is a retrospective questionnaire that asks participants to report events that happened sometimes decades ago. Results from retrospective questionnaires suffer from recall bias. The literature shows that retrospective questionnaires mainly carry the risk of underreporting of events [24]. This would lead to a loss of power to demonstrate a true effect of lifetime LTE score or the childhood LTE score on TL. We have tried to obviate this drawback by administering the same questionnaire twice and by scoring any event that was reported either at the first or second time the questionnaire was filled out. Another limitation of the lifetime LTE score is that every event could only be scored once in every age category. Especially at old age it is not unthinkable that some events happen more than once. This would lead to an underestimation of the true number of life events. These limitations might in part explain the discrepancy in our findings between the cross-sectional and longitudinal part of our study, as the longitudinal part suffers less from recall bias.

Thirdly, telomere length is said to be dynamic and might depend upon both eroding (e.g. oxidative stress) and protective factors (e.g. anti-oxidants and telomerase). This is

illustrated by the fact that telomere attrition per month is larger on the short term (after 6 months follow-up) than on the long term (after 10 years follow-up) [38], indicating that repair mechanisms might play a role. In rats and humans there is evidence that exposure to uncontrollable stress [40] or depression [41] leads to higher blood cell telomerase activity than in healthy unstressed controls. In contrast, there is also a study in which telomerase activity was reduced in caregivers versus non-caregiving controls [10]. It might be that the body is able to counteract telomere erosion for a certain amount of time, such as during a short term depression or after a stressful life event, but that repair systems become exhausted as stress exposure becomes more chronic. Although upregulation of the telomerase enzyme under certain conditions is a possibility, there is great controversy of whether telomere lengthening as reported in many studies [29, 36-38], including ours[42] really exists. A recently published study demonstrated that the reported telomere lengthening in longitudinal studies is most likely an artifact of laboratory measurement error, which is exacerbated by a short follow-up time[43]. In our study we used monochrome multiplex quantitative PCR and had an average follow-up time of 6 years. Although our measurement error was not that large and we demonstrated good reproducibility of results[26], our method has a larger measurement error than for instance southern blot[44]. From the graphs in the paper of Steenstrup and colleagues it becomes clear that if we had run our samples in duplicate we would have misclassified about 30% of the population as telomere lengtheners. We have, however, run our samples in triplicate, therefore reducing measurement error further than is exemplified in their paper. Furthermore, they classify any increase above zero as lengthening. We classify only those people that have at least a 15% increase in TL from baseline as lengtheners, which should reduce the chance of misclassification. Nonetheless, we acknowledge that measurement error is a problem in our field that deserves much more attention as a possible explanation for the reported results than is currently the case. We propose that future prospective studies should measure both telomere length and telomerase activity simultaneously. If telomerase activity is indeed higher in telomere lengtheners, this would give support for a biological explanation for telomere lengthening in addition to the role of measurement error.

In conclusion, this is the first study to demonstrate that exposure to recent adverse life events in adulthood is associated with decreased telomere length prospectively. Telomere attrition might thus be one of the mechanisms by which psychosocial stress exerts negative effects on health.

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