

Longitudinal changes of telomere length and epigenetic age related to traumatic stress and post-traumatic stress disorder.

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Running title: Age and PTSD

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Abstract

Several studies have reported an association between traumatic stress and telomere length suggesting that traumatic stress has an impact on aging at the cellular level. A newly derived tool provides an additional means to investigate cellular aging by estimating epigenetic age based on DNA methylation profiles. We therefore hypothesise that in a longitudinal study of traumatic stress both indicators of cellular aging will show increased aging. We expect that particularly in individuals that developed symptoms of post-traumatic stress disorder (PTSD) increases in these aging parameters would stand out.

From an existing longitudinal cohort study, ninety-six male soldiers were selected based on trauma exposure and the presence of symptoms of PTSD. All military personnel were deployed in a combat zone in Afghanistan and assessed before and 6 months after deployment. The Self-Rating Inventory for PTSD was used to measure the presence of PTSD symptoms, while exposure to combat trauma during deployment was measured with a 19-item deployment experiences checklist. These groups did not differ for age, gender, alcohol consumption, cigarette smoking, military rank, length, weight, or medication use. In DNA from whole blood telomere length was measured and DNA methylation levels were assessed using the Illumina 450K DNA methylation arrays. Epigenetic aging was estimated using the DNAm age estimator procedure.

The association of trauma with telomere length was in the expected direction but not significant ($B=-10.2$, $p=0.52$). However, contrary to our expectations, development of PTSD symptoms was associated with the reverse process, telomere lengthening ($B=1.91$, $p=0.018$). In concordance, trauma significantly accelerated epigenetic aging ($B=1.97$, $p=0.032$) and similar to the findings in telomeres, development of PTSD symptoms was inversely associated with epigenetic aging ($B=-0.10$, $p=0.044$). Blood cell count, medication and premorbid early life trauma exposure did not confound the results.

Overall, in this longitudinal study of military personnel deployed to Afghanistan we show an acceleration of ageing by trauma. However, development of PTSD symptoms was associated with telomere lengthening and reversed epigenetic aging. These findings warrant further study of a perhaps dysfunctional compensatory cellular aging reversal in PTSD.

1.

Introduction:

In addition to a wealth of literature about the molecular sequelae of exposure to traumatic stress in humans, recent studies have identified telomere shortening as one of the alterations associated to traumatic stress, (for review see (Shalev *et al.* 2013)).

Telomeres are repeats of the nucleotides TTAGGG about 3 to 20 kB long at the end of chromosomes and have a function in protecting functional genetic code of the chromosomes against shortening due to duplication of DNA by DNA-polymerase that is part of normal cell division. Telomere length decreases with age, and the renewed interest in telomeres is partly due its association with longevity. Another age indicator that has recently been developed is the age estimator based of methylation of CpG sequences in the genome (Horvath *et al.* 2012; Horvath, 2013). DNA methylation is an epigenetic mechanism that plays a role in tissue type specification and is strongly related to aging (Boks *et al.* 2009; Horvath *et al.* 2012). Recently Horvath (Horvath, 2013) identified 353 CpG loci that are routinely investigated in commercial available DNA methylation arrays that predict age with high accuracy. Interestingly, recent studies have firmly established that DNA methylation also plays a role in several diseases and is associated to environmental exposures and particularly traumatic stress (for reviews see [Vinkers, submitted]).

Considering that both telomere length and epigenetic age may be associated to trauma exposure, it is of interest to investigate this in a longitudinal design, which, in contrast to previous cross sectional studies, provide a higher level of evidence for a causal relationship. However, from the perspective of clinical utility, it is even more interesting to incorporate development of post-traumatic stress disorder (PTSD) symptoms as outcome in such an analysis in order to interpret the role of any trauma related changes in age-related parameters in disease aetiology of this psychiatric disorder. We hypothesized that both telomere shortening and accelerated epigenetic age would be positively associated with trauma exposure, and that development of PTSD symptoms would be associated with an even stronger acceleration of these aging parameters.

2.

Method

2.1

Subjects

We analysed longitudinal changes in a selected subgroup from a large, prospective cohort of 1032 Dutch military personnel deployed to Afghanistan, (see van Zuiden et al. 2011). Blood samples and standardized measures of Posttraumatic Stress Disorder (PTSD) symptoms were collected before and 6 months after deployment. The Self-Rating Inventory for PTSD (SRIP) was used to measure the presence of PTSD symptoms. The SRIP has a good reliability (Cronbach's alpha between 0.90 and 0.94) and validity (0.82 correlation to the Mississippi scale for combat-related PTSD) (Keane et al. 1988, Hovens et al. 2000). Exposure to combat trauma during deployment was assessed with a 19-item deployment experiences checklist as reported previously (van Zuiden *et al.* 2009). This assessment provides a range of self reported traumatic experiences that occur as part of deployment and include direct combat. From the entire cohort, 96 male participants were selected based on Dutch ethnicity, high or low levels of traumatic stress exposure and high and low levels of PTSD symptoms. This selection resulted in 64 participants with high combat trauma exposure (mean: 8.0 events \pm 2.7) of which half had high levels PTSD symptoms at follow up (SRIP: 44.7 \pm 8.7), and half low scores (24.9 \pm 2.6). , and 32 participants with low combat trauma exposure (mean: 0.4 events \pm 0.5) and low levels of post-deployment PTSD symptoms at follow up (SRIP: 25.4 \pm 3.7). These groups did not differ for age, gender, alcohol consumption, cigarette smoking, military rank, length, weight, or medication use. Traumatic experiences during childhood were assessed using the 27-item Dutch version of the self-report version of the Early Trauma Inventory (ETI) (Bremner *et al.* 2007). This questionnaire assesses exposure to potentially traumatic experiences before the age of 18 years (general trauma, physical abuse, emotional abuse and sexual abuse) (Hovens *et al.* 2002; Witteveen *et al.* 2006). This study was conducted in accordance with the Declaration of Helsinki and all participants gave written informed consent.

Table 1 shows the demographic and clinical characteristics of the participants. For the current analysis, trauma was defined as score of more than 1 on the trauma experience checklist. This pre-set dichotomisation is based on the selection of participants with low or high levels of trauma to increase power (Boks et. al. 2007) leading to a bivariate distribution of trauma (see supplementary figure 1 for histogram). PTSD symptoms were analysed as increase in PTSD symptoms as a continuous indicator. Missing values at baseline for three participants were imputed using linear regression based single imputation with ZIL score at follow up, pre-deployment trauma and age as constraints. Imputed values showed over 0.9 correlation with median ZIL baseline values. Imputation is a superior way to handle missing data as it avoids issues of non-randomness of missings and conserves power (Donders et. al. 2006). For transparency we also report results using case-wise deletion. *Laboratory work*

Whole blood EDTA samples were obtained from all participants. Blood cell-count was obtained using standardized flowcytometry procedures in our hospital. DNA extraction was conducted using standard salting procedures. DNA concentration was assessed using riboGreen, and integrity using BioAnalyser. Bisulphite conversion was conducted using Zymo kits under standard procedure. DNA methylation levels were assessed using Illumina 450K DNA methylation arrays, interrogating over 450,000 CpG loci per sample at single-nucleotide resolution. It covers 99% of RefSeq genes and 96% of CpG islands, with an average of 17 CpG sites per gene region distributed across the promoter, 5'UTR, first exon, gene body, and 3'UTR. Baseline and follow up were positioned on the same array and exposure to trauma and PTSD outcomes were equally distributed over the 16 arrays to reduce any batch effects to the minimum.

Baseline and follow up samples were positioned on the same plate, and PTSD and trauma conditions were equally distributed across plates. Telomere length was determined in duplicate using the q-PCR method described by Cawthon *et al.* (Cawthon, 2002), modified with synthetic standards as described by O'Callaghan and Fenech (O'Callaghan and Fenech, 2011) to measure absolute telomere length. Assays were performed using a Bio-rad cfx-96 Real-Time PCR Detection System. 36B4 was used as single copy gene (S) to normalize for DNA input. The following primers were used: 36B4 FW (CAGCAAGTGGGAAGGTGTAATCC) and 36B4 RV (CCCATTCTATCATCAACGGGTACAA) at a final concentration of 1.25 μ M and 0.75 μ M, respectively. Telomere length (T) was measured using the following primers: Telo 1

(CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT) and Telo 2 (GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT), both at a final concentration of 0.5 μ M. For each PCR reaction 10 μ l SYBRgreen PCR mastermix (Biorad) and 35 nanogram of DNA was used, in a total volume of 20 μ l per well. A standard curve was established on each 96-well plate by serial dilution of a known amount of oligonucleotide for either 36b4 or telomere. The R^2 of each standard curve was >0.95 . The thermal cycling PCR reaction was initiated with a 95°C incubation for 10 minutes followed by 35 cycles of 95°C for 5 seconds, 59°C for 10 seconds and 72°C for 2 minutes. In total 5 plates for telomere and 5 plates for 36B4 were run.

2.2

Quality control and calculation of age parameters

Absolute quantification of 36B4 and telomere was based on the threshold cycle (Ct) of each well and the standard curve on the respective plate. Twenty-two samples with a difference in Ct value of > 1 were excluded. The mean of the quantified duplicates was used to calculate the ratio between telomere and 36B4 (T/S). Since 36B4 is a single copy gene per genome, this calculation represents the absolute telomere length of each sample per genome copy. Although of minor importance due to sample distribution we investigate plate effects over the 5 used plates making boxplots.

Epigenetic age (DNAm Age) was calculated from the DNA methylation data using the publicly available scripts (Horvath, 2013). In short normalisation of the relevant CpG methylation levels is conducted using a mixed effect model implemented in the BMIQ package (Teschendorff *et al.* 2013), and based on the DNA methylation levels age of a tissue is predicted based on regression coefficients obtained from numerous training sets (Horvath, 2013).

2.3

Analysis

Stratified longitudinal analysis were conducted using linear regression with baseline methylation level as covariate (outcomeT2~outcomeT1+ trauma+ PTSD+ time interval), time interval was included as covariate to adjust for small but potential relevant differences in time interval. By including trauma and PTSD symptoms in the same model we estimate the independent contribution of trauma and PTSD. Assumptions for linear regression were evaluated using inspection of residuals. Differences in cell type composition between inclusion and the follow up were analysed using repeated measures analysis of variance (ANOVA). The association to childhood trauma was investigated separately by adding the childhood trauma measures to the above model. To investigate whether differences were already present before deployment and the results merely reflect change fluctuations we analysed the association to trauma and PTSD symptoms at baseline using linear regression, but with telomere length and DNAm age at baseline as outcomes. The potential influence of cigarette smoking was investigated by excluding participants with documented change in smoking behaviour from the analysis. The influence of alcohol use was investigated by modelling increase or decrease in alcohol consumption relative to unchanged alcohol use in models for telomere length and mDNA age. Because of potential batch effects in the telomere length we modelled a random effect for the 5 plates using the nlme package in R (Pinheiro *et al.* 2014).

3

Results

Table 1: Demographic and clinical characteristics of the participants

	All (n=96)	Low trauma (N=32)	High trauma (N=67)
Age (sd)	27.04 (9.15)	25.1 (8.1)	27.4 (9.3)
Mean Trauma score at follow up (sd)	5.5 (4.2)	0.4 (0.5)	8.0 (2.7)
PTSD symptoms at follow up Mean SRIP score (sd)	32.3 (11.0)	25.1 (3.7)	35.8 (11.7)
Change in PTSD symptom score	5.9 (11.1)	-0.3 (4.9)	8.9 (11.0)
Time to follow-up (months)	13.5 (3.7)	13.4 (3.7)	13.5 (3.2)

3.1

Association of trauma and PTSD with change in telomere length

Trauma exposure and increase in telomere length were not significantly associated (B=-10.17, p= 0.528). However, increase in PTSD symptoms was associated with lengthening of telomeres (B=1.91, p= 0.018) in the mixed model analysis. Table 2 shows the full model for the analysis of change in telomere length.

Table 2: Association of PTSD symptoms and Trauma with telomere length post deployment.

	Beta	SE	t-value	p-value
Pre-deployment Telomere length	0.35	0.11	3.13	0.003
Time interval	1.95	1.94	1.01	0.318
Trauma	-10.17	16.03	-0.63	0.528
PTSD symptoms	1.91	0.78	2.44	0.018

Bold signifies significance at 0.05 level

3.2

Association of trauma and PTSD with change in epigenetic aging (DNAm age)

As a second measure of age acceleration we analysed change in DNAm age. We found a pattern comparable to the telomere length analysis. Trauma was significantly associated with an increase of DNAm age ($B=1.97$, $p=0.032$). However, similar to the relationship of telomere length, increase in PTSD symptoms were associated with a relative *decrease* in DNAm age ($B=-0.10$, $p=0.044$) in a model with marked explained variance (Adjusted R squared= 0.87, $F(4,86)=146.4$, $p<0.001$). Table 3 shows the full model for the analysis of DNAm age.

Table 3: Association of PTSD and Trauma with DNAm age post deployment.

	Beta	SE	t-value	p-value
Pre-deployment DNAm age	1.01	0.04	23.42	<0.001
Time interval	0.13	0.11	1.27	0.206
Trauma	1.97	0.90	2.18	0.032
PTSD symptoms	-0.10	0.05	-2.04	0.044

Bold signifies significance at 0.05 level

3.3

Analysis of quality control and association with biological age

Several analyses were conducted to investigate the quality of our measures. For telomere length we investigated the correlation between T1 and T2 which was high ($r=0.7$, $p<0.001$). However there were significant differences between the 5 plates (see supplementary material for figure S2). The most likely explanation are differences in the performance of the standards. We adjusted for these differences by using a mixed effect model with plate number as random factor (Liao and Lewis, 2000). Telomere length was not associated with

age ($B=-0.48$, $p=0.48$). However DNAm age was almost perfectly associated with age at inclusion ($r=0.99$, $p<0.001$). See supplementary figure 3 for a scatterplot.

3.4

Cell type composition

Cell type composition is of importance because it is potentially associated with DNA methylation as well as with telomere length. Given that that the cell composition may also be related to our main determinants (trauma and PTSD), it can be a potential confounder. We investigated cell type composition using flowcytometry analysis as implemented in the clinical laboratory of our University Medical Center that we used previously (van Zuilen *et al.* 2009). Figure 3 shows that cell type composition at inclusion and follow up. Repeated measure Analysis of Variance (ANOVA) showed no significant differences. Although the DNA methylation age predicting tool is validated across tissue types (Horvath, 2013) , we investigated the correlation with DNAm age at inclusion and follow up with DNAm age estimates. Correlations were smaller than 0.15. Similarly the correlation with telomere length was smaller than 0.15 for all blood cell composition measures.

3.5

Medication, smoking and alcohol and missing values analysis

Sixty-nine of the 96 participants did not use any medication at inclusion nor at follow up. Eight participants used medication before and after deployment, 4 used medication at inclusion but not at follow up, and 8 patients started medication after inclusion. Started medication included: antibiotics in 4 cases, antihistamines in 3 cases and one case of a benzodiazepine prescription. Stopped medication included: antibiotics in one case, antihistaminic in two cases and one case of oral isotretinoin. To investigate potential confounding by medication we reran the analysis with only participants that were without medication at any time points. Results reflected loss of power but were basically unchanged; the association of PTSD symptoms with change in telomere length was ($B=2.18$, $p=0.011$), the association with DNAm age with trauma ($B=2.3$, $p=0.036$) and association of DNAm age with PTSD symptoms also remained significant ($B=-0.14$, $p=0.023$).

Smoking was analysed in a subgroup of 19 participants of which smoking at both timepoints was recorded. Excluding six participants that stopped smoking from the analysis retained the main findings; the association of PTSD symptoms with change in telomere length was ($B=1.62$, $p=0.040$), the association with DNAm age with trauma ($B=2.03$, $p=0.035$) and association of DNAm age with PTSD symptoms also remained significant ($B=-0.10$, $p=0.042$). Change in alcohol intake was available in 56 participant. Alcohol use was recorded in 4 levels of use. We identified increase alcohol use in 15 participants, decrease in 10 and unchanged alcohol use in 31. To investigate potential influence we analysed the association of increase or decrease alcohol use with unchanged use as reference using two dummy variables. Change or decrease in alcohol use did not contributed significantly to change in telomere length or DNAm Age. Excluding 7 participants that stopped or commenced alcohol use retained the association of PTSD symptoms with change in telomere length was ($B=1.64$, $p=0.041$), the association of DNAm age with trauma ($B=2.13$, $p=0.021$) and association of DNAm age with PTSD symptoms also remained significant ($B=-0.12$, $p=0.011$).

Finally, analysis of the data using listwise exclusion of missings retained the reported associations (Telomere length and PTSD ($B=2.39$, $p=0.006$); trauma with epigenetic aging ($B=2.32$, $p=0.010$) and PTSD symptoms with epigenetic aging ($B=-0.14$, $p=0.003$).

3.6

Association with childhood trauma

Addition of childhood trauma measure to the analysis of telomere length and DNAm age showed no association of pre-deployment trauma with change in telomere length or DNAm age, nor was it associated with these outcome measures at baseline. Pre-existing trauma score ranged from 0 to 11 (mean= 3.1, $sd=2.7$) but was associated to neither of the outcomes (telomere length; $B=0.16$, $p=0.95$, DNAm Age; $B=0.13$, $p=0.43$), while the association with trauma and PTSD symptoms remained unchanged.

3.7

Association at baseline

Analysis of the association of both outcomes at baseline showed no significant relationships. For telomere length at baseline the association with trauma was $B=12.4$, $p=0.37$, for PTSD; $\beta=-0.92$, $p=0.37$. For the association with DNAm age the association with trauma was; $\beta=-0.25$, $p=0.9$, for PTSD; -2.19 , $p=0.41$, model statistics: $F(2,92)=0.42$, $p=0.66$, $R^2=0.001$.

Discussion

In this unique longitudinal study in Dutch military personnel before and after deployment to a combat zone in Afghanistan, we studied an age indicator derived from DNA methylation levels (DNAm age) and telomere length. We found that trauma was not associated with decrease in telomere length, but was associated with accelerated DNAm aging. Therefore the association of these measures with trauma are in the expected direction as previous studies that have reported similar association of decreased telomere length with trauma (Epel *et al.* 2004; Tyrka *et al.* 2010) .

In contrast to our expectation we also found that development of post traumatic stress disorder (PTSD) symptoms was significantly associated with *increased* telomere length and *decreased* DNAm aging. Previous cross sectional studies in PTSD have suggested an association with decreased telomere length (Ladwig *et al.* 2013; Malan *et al.* 2011; O'Donovan *et al.* 2011) although sometimes only in interaction with pre-existent youth trauma (Zhang *et al.* 2013). Although the relationship of the two aging parameters with PTSD is in the opposite direction as previously reported, these findings are not without precedent. There are also previous reports of an increase activity of telomerase (the enzyme that lengthens telomeres) due to chronic exposure to stress in rats (Beery *et al.* 2012). In a human longitudinal experimental study, Epel et al (Epel *et al.* 2010) effectively show that leukocyte telomerase activity increases in response to a laboratory stressor. Studies that have reported an association of telomere length and PTSD were all cross sectional. The question therefore rises whether there may be a difference between the short-term and long-term response in aging parameters, particularly since we here report on newly developed PTSD symptoms. Our results are consistent with a model whereby aging parameters increase in response to trauma but for those who develop symptoms of PTSD the reverse is true. At least during the first six months during which symptoms of PTSD develop. It remains possible that this age decrease (as a maladaptive state), subsides later on. Only further longitudinal studies with dense time series can further illuminate whether such age decrease subsides and how this relates to symptom perseverance .

The equivocal relationship between telomere length, trauma and disorders is underscored by several other studies. There are for instance also negative reports on the association between trauma and telomere length. Investigators of the Dunedin cohort study did not find an association with trauma but shortening in externalising disorder in men only (Shalev *et al.* 2014). This also points to the importance of gender in these analyses. There may be gender differences, and as we only included males in our study, we cannot be sure that our findings can be extrapolated to women. Interestingly, there are also several environmental factors that may impact on telomere length such as the use of Lithium that increases telomere length (Martinsson *et al.* 2013) and vigorous physical activity that protects against telomere shortening by traumatic stress (Puterman *et al.* 2010).

The use of the DNAm age method is new and presents an intriguing new tool for the study of disease aetiology and biomarkers (Horvath, 2013). The tool has been extensively validated and is an accurate predictor of age and morbidity (Horvath, 2013) and in our data almost perfectly correlated with age. The utility for studies of disease aetiology can only be assumed, but there are several promising studies underway.

Strengths of the current study are the longitudinal design and the use of two independent measures of accelerated aging (DNAm age and telomere length). Also, many potential confounds for such as cell type composition, gender, and medication, alcohol use and smoking, and baseline characteristic are unlikely to explain our results. However several limitations warrant caution when interpreting the results. Although the longitudinal design is only vulnerable for confounding by variables that changed in between measurements we cannot rule out residual confounding for instance by variation in the delay between trauma and blood draw or other unmeasured variables. Foremost, the selection of predominantly male military serviceman with a Dutch genetic background, enriched for development of PTSD symptoms may not reflect the general population. It is therefore possible that the age reversal presented here may not generalise to other populations. A serious limitation is the large differences in telomere length between plates and dropout due to large differences between two replication samples. Although design and analysis rule out influences on the significance of the reported associations, it does preclude interpreting the absolute telomere lengths. In other words: the presented absolute quantification of telomere length may not be accurate and only serves as comparison between participants.

Overall we here present two measures of cellular aging of which change is associated with development of PTSD symptoms in the opposite direction of their association to trauma exposure. The exact meaning of these remarkable findings remains unclear until replication in more dense time series have been obtained. However it does open a new etiological perspective on PTSD whereby cellular aging may be (temporarily) disrupted.

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