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Characterization in humans of *in vitro* leucocyte maximal telomerase activity capacity and association with stress

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The goal of this study was to develop and validate a measure of *maximal telomerase activity capacity* (mTAC) for use in human studies of telomere biology, and to determine its association with measures of stress and stress responsivity. The study was conducted in a population of 28 healthy young women and men who were assessed serially across two separate days, at multiple time points, and in response to a standardized laboratory stressor. Venous blood was collected at each of these multiple assessments, and an *in vitro* mitogen challenge (phytohaemagglutinin supplemented with interleukin-2) was used to stimulate telomerase activity in leucocytes. After first establishing the optimal post-stimulation time course to characterize mTAC, we determined the within-subject stability and the between-subject variability of mTAC. The major findings of our study are as follows: (i) the optimal time point to quantify human leucocyte mTAC appears to be at 72 h after mitogen stimulation; (ii) mTAC exhibits substantial within-subject stability (correlations were in the range of r 0.68–0.82) and between-subject variability, with a high intra-class coefficient (0.70), indicating greater between-subject relative to within-subject variability; (iii) mTAC is not influenced by situational factors including time of day, cortisol, acute stress exposure and immune cell distribution in the pre-stimulation blood sample; and (iv) a significant proportion of the between-subject variability in mTAC is associated with measures of stress and stress responsivity (mTAC is lower in subjects reporting higher levels of perceived (chronic) stress and exhibiting higher psychophysiological stress reactivity). Based collectively on these findings, it appears that mTAC, as proposed and operationalized, empirically meets the key criteria to represent a potentially useful individual difference measure of telomerase activity capacity of human leucocytes.

This article is part of the theme issue 'Understanding diversity in telomere dynamics'.

1. Introduction

(a) Overview

A substantial and converging body of epidemiological, clinical and experimental evidence supports a fundamental role for the telomere biology system in the maintenance of DNA and cellular integrity, with important implications for health and disease risk across a wide range of age-related disorders [1–4].

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The telomere biology system comprises two closely interlinked components—the length of telomeres (non-coding double-stranded repeats of guanine-rich tandem DNA sequences and shelterin protein structures that cap the ends of linear chromosomes), and the activity of telomerase (the reverse transcriptase enzyme that adds telomeric DNA to telomeres) [5,6]. The majority of human epidemiological and clinical studies of the role of telomere biology in health and disease risk have focused largely on the telomere length component of this system. Relatively few studies have considered the role of telomerase. Because the expression and activity of telomerase constitutes a *critical* and *complementary* (i.e. non-redundant) component of the functional integrity of the telomere biology system, we suggest that it may be important to incorporate telomerase-related measures in studies. This, then, leads to the question of how to optimally quantify this component of the system in human epidemiological or clinical studies. The relatively few studies that have included measures of telomerase have typically measured telomerase expression or activity under basal conditions or in terms of its acute (short-term) response to systemic challenges (e.g. [7]). It is, however, challenging to reliably quantify or interpret these data for several reasons. Firstly, telomerase is typically not expressed, or expressed only at very low levels, in most resting cells (including immune cells) [8]. Secondly, telomerase levels may vary as a function of cell-cycle stage and other factors [9]. And thirdly, differences or changes in telomerase may reflect either the direct effects of states or conditions that stimulate telomerase expression (e.g. infection), or the secondary (compensatory/counter-regulatory) adaptations to states or conditions that reduce telomere length [10]. Thus, in contrast to assessment of basal telomerase, we suggest that it may be more informative to assess telomerase expression or activity in cells in response to a standardized stimulus, such as a mitogen challenge, in well-controlled *ex vivo* conditions. The potential advantage of this approach is that it may bypass the above-mentioned limitations to provide an indicator of individual differences in the *capacity* of the telomere biology system to respond to an ecologically relevant challenge. This, in principle, is similar to the information provided about the integrity of the glucose homeostatic system by serial measures of blood glucose and insulin in response to ingestion of a standardized glucose load. Accordingly, we propose that maximal telomerase activity capacity (hereinafter referred to as mTAC) may represent an individual difference measure that, by itself, or in combination with measures of telomere length, could prove to be potentially and particularly informative in studies of telomere biology, health and disease risk. For a construct such as mTAC to serve as a potentially useful individual difference measure, it should meet at least two criteria: high within-subject stability and substantial between-subject variability. Other considerations in this specific context include selection of the optimal cell population, challenge, its dose and the time course that best captures telomerase expression/activity.

Given the importance of stress and stress physiology as a likely regulator of the telomere biology system, the observation that many of the previous studies of human telomerase have been conducted in the context of stress, and the specific interest of our own research programme in the effects of stress and stress biology on health and disease risk, we additionally elected to determine within-subject stability and between-subject variability of mTAC in the context of stress. Accordingly, we used an extensively validated acute psychosocial stress challenge (the Trier Social Stress Test, TSST) to examine the within-subject stability of mTAC, and we used reliable and previously validated measures of perceived (chronic) psychological stress and individual differences in physiological stress reactivity to examine the between-subject variability of mTAC. As several physiological systems (including stress biology) exhibit chronobiological regulation, we also considered the influences of situational factors such as time of day of blood sample collection and cortisol concentration on the within-subject stability of mTAC, and of factors such as sleep quality, chronotype and the slope of the diurnal change in cortisol on the between-subject variability of mTAC. Finally, we determined the extent to which mTAC may be driven by the composition of immune cell subpopulations before mitogen stimulation.

(b) The telomere biology system

The telomere biology system is a highly evolutionary conserved system that plays a central role in maintaining the integrity of the genome and cell. As mentioned above, telomere biology refers to the structure and function of two closely interlinked entities—*telomeres*, non-coding double-stranded repeats of guanine-rich tandem DNA sequences and shelterin protein structures that cap the ends of linear chromosomes [11,12], and *telomerase*, the reverse transcriptase enzyme that adds telomeric DNA to telomeres [1,5,6].

Because DNA polymerase is unable to fully replicate the 3′-end of the DNA strand, telomeres lose approximately 30–150 base pairs (bp) with each cell division and eventually reach a critical short length, resulting in decreased recruitment of shelterin proteins to form the protective internal nucleotide loops, which, in turn, leads to cellular senescence. Once cells become senescent, they exhibit a variety of (epi)genetic and morphological changes that result in loss of cell and tissue function [13]. Shortened telomeres have been linked to several age-related disease risk factors, disease prevalence and progression [1–4,14–19], and early mortality [20,21]. Moreover, some recent reports have suggested a *causal* role for telomeres in the aetiology of many of these adverse health outcomes [22–24].

The enzyme telomerase is a ribonucleoprotein consisting of an RNA component (TR or TERC) and a catalytic protein domain (TERT). Conventional DNA polymerase machinery is unable to fully replicate the ends of linear chromosomes. The enzyme telomerase uses its own template to add short TG-rich repeats to chromosome ends, thus reversing or attenuating their gradual erosion at each round of replication [6,25]. Telomerase is regulated by epigenetic, translational and post-translational mechanisms [26]. Its expression varies during development, according to cell-cycle stage and across cell types [9,27]. Typically, telomerase activity is diminished or absent in most adult somatic cells, with the exception of cells with a strong potential for division, such as germ cells, stem cells of proliferating tissues and activated immune cells [27]. Of particular relevance here, it is well established that activated lymphocytes express high telomerase levels [8,9,28,29]. This upregulation of telomerase is believed to prevent immune cell senescence and facilitate a fast and profound clonal cell expansion. Very occasionally, cells bypass the cellular senescence and DNA damage signalling pathways described above to constitutively express high levels of telomerase, which is a characteristic feature in about 90% of all malignancies [30]. This feature of

telomerase biology is beyond the scope of the current study and is therefore not addressed here.

Telomerase not only maintains telomere length but also preserves healthy cell function. Telomerase promotes proliferation of resting stem cells, modulates signalling pathways during embryogenesis and normal adult tissue genesis, protects cellular proliferation capacity and survival under conditions of cellular stress [31], and gets excluded from the nucleus to co-localize with mitochondria to protect mitochondrial DNA and function [32,33]. Thus, because the effects of telomerase on cellular function extend beyond, and are uncoupled from, those of telomere lengthening [34], measures of telomerase expression and activity could, in addition to telomere length, provide valuable insights regarding the role of the telomere biology system in health and disease risk. Additionally, the relevance of studying variation in telomerase activity or expression in relation to organismal fitness is underscored in a recent paper by Criscuolo *et al*. [35].

(c) Basal leucocyte telomerase

Studies of the association of basal leucocyte telomerase with health, disease risk (other than cancer) and conditions such as stress have largely yielded inconsistent results. Several, but not all, studies suggest that that basal telomerase expression/activity appears to be upregulated in autoimmune disorders, with differences between active and inactive disease [36], and between early- and advanced-stage disease [37]. Findings in the context of psychiatric disorders such as depression, schizophrenia and post-traumatic stress disorder appear to be more heterogeneous [38–42]. The same pattern of mixed findings is evident in the context of chronic stress exposure, with some suggesting suppression [7,43–45] and others suggesting stimulatory effects of stress on telomerase activity [10,46]. This heterogeneity may be a consequence of the fact that telomerase is regulated in response to various factors such as cell-cycle stage [9], stress hormones [7] and inflammation [47,48], which, in turn, may introduce bias based on time of day of blood sample collection [49], current infections [47,48], physical exercise [50] or acute stress exposure status [7]. In addition, elevations in basal telomerase may reflect the counter-regulatory (compensatory, secondary) adaptations to states/conditions that reduce telomere length. For example, high telomerase in conjunction with shorter telomere length may be indicative of a physiologically stressed system [10]. Finally, because telomerase is normally expressed at very low levels in resting cells, the lower limit of detection of many telomerase assays may place constraints in terms of reliable quantification [51].

(d) Stimulated leucocyte telomerase

Hiyama *et al.* [8] were the first to describe the upregulation of telomerase in leucocytes after *in vitro* mitogen stimulation. Telomerase was detectable in very low levels in isolated peripheral blood mononuclear cells (PBMCs), but increased up to 300–1000-fold over a one-week period in cultured T cells stimulated with phytohaemagglutinin (PHA) and interleukin (IL)-2, and increased up to 30-fold over a one-week period in cultured B cells stimulated with pokeweed mitogen. Similarly, Yamada *et al.* [29] used PHA and IL-2 to stimulate PBMCs and observed over a 96 h period that telomerase activity started to increase after 24 h and peaked at 72 h.

The observation by Son *et al.* [52] that the capacity for induction of telomerase activity in T or B cells after *in vitro* stimulation varied significantly across subjects but did not change as a function of subject's age (the age of study participants ranged between birth and 94 years of age) provides the first indication that this measure may reflect a stable individual difference characteristic. This observation has since been replicated by other studies [53,54]. However, to date, only a few studies have characterized the association of measures of stimulation-induced telomerase with health and disease states [54–58]. Broadly, these studies have reported that the mitogen-stimulated leucocyte telomerase response appears to be attenuated in subjects with autoimmune conditions such as systemic lupus erythematosus [56] and rheumatoid arthritis [54,57], and unchanged in conditions such as atopic dermatitis [58] and chronic hepatitis B infection [55].

Two studies have examined the association of stimulated human leucocyte telomerase with chronic stress. An *in vitro* experiment by Choi *et al.* [59] modelled the effect of chronic stress exposure and found that co-exposure of human T cells stimulated with PHA and IL-2 to exogenous cortisol reduced hTERT transcription and inhibited telomerase production *across a 3-day period*. In a study of carers of patients with Alzheimer's disease (stress exposure group) and age-matched controls, basal PBMC and T-cell telomerase levels were increased in carers compared with controls, while no differences were observed across a 3-day period in antigen-stimulated telomerase levels (with anti-CD3/CD28 monoclonal antibody) [46].

(e) Maximal telomerase activity capacity measure: key considerations

Based on the findings and considerations discussed above, we propose that mTAC may represent an individual difference measure that, by itself, or in combination with measures of telomere length, could prove to be potentially and particularly informative in studies of telomere biology, health and disease risk. Several considerations guided our development of this measure for possible use in human epidemiological and clinical studies, including the following questions: What is the optimal cell population, optimal challenge, its optimal dose and the optimal time course that capture cell capacity for telomerase expression/activity?

We selected PBMCs as the cell population of choice because: it is relatively easy and convenient to obtain blood samples; telomere length in human studies is most commonly measured in peripheral leucocytes; and telomere length and telomerase activity are closely associated with immune function due to their important role in lymphocyte development, differentiation and replicative capacity. We, therefore, reasoned that quantifying mTAC in PBMCs could be particularly informative, and this also would increase the feasibility of the use of this measure in clinical studies (as opposed to a single more specific immune cell type such as T cells).

PBMCs were stimulated with PHA (10 $\mu g\ ml^{-1}$) supplemented with IL-2 (50 units ml^{-1}), because it induces lymphocyte (especially T cell) proliferation. In humans, telomerase is typically expressed in lymphocytes by induction of activating and proliferating pathways (e.g. T-cell clonal expansion during a viral infection). We selected the 10 $\mu g\ ml^{-1}$ PHA dose because maximal lymphocyte proliferation is known to occur at this dose [60], and we added IL-2 because of its importance in the maintenance of immune cells, including T cells, B cells and NK cells, and because it has a synergistic effect on

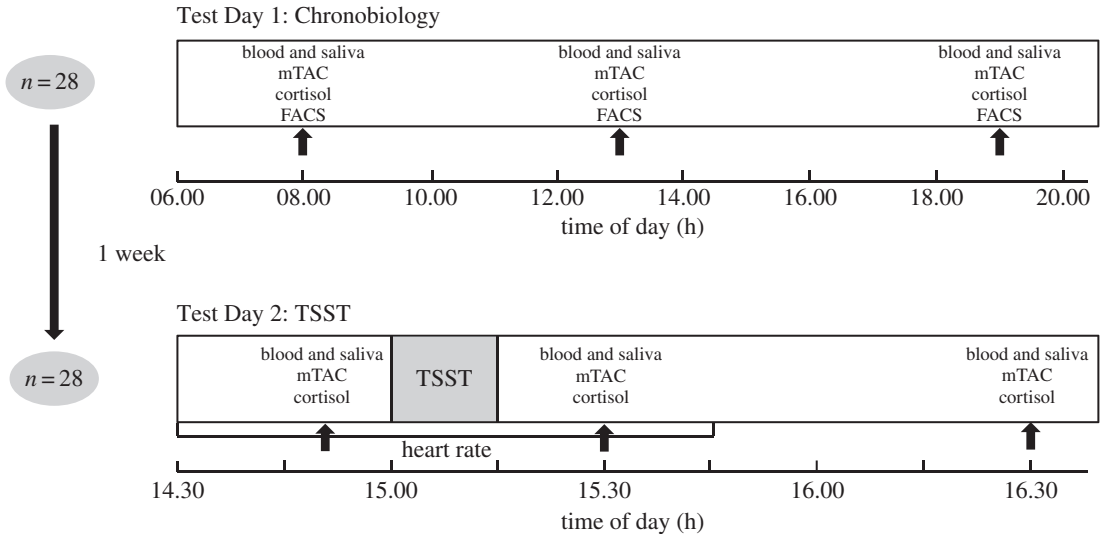

Figure 1. Study protocol: all subjects reported to the laboratory on two occasions (Test Day 1, 'Chronobiology day'; Test Day 2, 'TSST day'); mTAC, maximal telomerase activity capacity; FACS, fluorescence-activated cell scanning (flow cytometry); TSST, Trier Social Stress Test.

the proliferation of T cells and on leucocyte telomerase expression [29,61].

To establish the optimal time course for characterization of mTAC, we considered three investigational parameters: (i) telomerase activity, as quantified by the TeloTAGGG Telomerase PCR ELISA plus assay; (ii) changes in immune cell populations; and (iii) changes in cell viability. Previous studies have reported that across a time period spanning eight consecutive days after mitogen stimulation, peak leucocyte hTERT mRNA expression occurs between 3 and 5 days after stimulation [54]. Most studies, to date, have used a 3-day incubation protocol with and without the addition of IL-2, and have used PHA [53,62], anti-CD3 monoclonal antibodies [46,54,57,58] or PMA/ionomycin [52,56] as a stimulant to induce telomerase activity or hTERT mRNA expression in leucocytes or leucocyte subsets. Our criterion for characterizing the 'optimal' time was to determine the balance between the time required to enable quantification of the mTAC without producing major alterations in immune cell subpopulations and cell viability (as these changes could, in and of themselves, influence telomerase production and activity [52,63]).

(f) Goals

Thus, to summarize, the primary goals of our study were to establish the optimal time course to characterize human leucocyte mTAC, determine the within-subject stability and between-subject variability of mTAC, and determine its association with measures of stress and stress responsivity. To determine the within-subject stability of mTAC, we considered the possible influences of situational factors such as time of day of blood sample collection, cortisol concentration and acute social stress exposure. To determine the between-subject variability of mTAC, we considered the possible influence of factors including sleep quality, chronotype, the slope of the diurnal change in cortisol, perceived (chronic) stress and psychophysiological stress responsivity. Finally, we also determined the extent to which changes in mTAC may be driven by (or a reflection of) changes in immune cell subpopulations following mitogen stimulation.

2. Material and methods

(a) Participants

The study population comprised 28 young adults (14 women and 14 men, mean age 23.8 $\pm$ 3.3 (s.d.) years) recruited through announcements at universities in Berlin, Germany, and five additional adults who donated a single blood sample for the mTAC optimal time course assessment. All subjects were healthy (no acute or chronic health problems), as ascertained by self-report and confirmed by a brief clinical examination. All subjects also were non-smokers and medication-free, except for oral contraceptives (all female subjects were oral contraceptive users). The mean body mass index (BMI) was 20.9 $\pm$ 1.7 (s.d.).

(b) Study protocol

Subjects reported to our research laboratory on two occasions: Test Day 1—the 'Chronobiology day', and Test Day 2—the 'TSST day' (figure 1), with an approximately one-week interval between the first and second study visits. On both study days, participants were asked to refrain from strenuous physical exercise and consumption of alcoholic beverages for 24 h prior to the study assessments.

On Test Day 1, participants were asked to come to the laboratory after an overnight fast. Venous blood and saliva samples were collected at three time points across the day (at 08.00 h, 13.00 h and 19.00 h). Participants were asked to refrain from eating for at least 2.5 h before the 13.00 h and 19.00 h blood draws. On Test Day 2, participants were asked to come to the laboratory at 14.00 h and to refrain from eating for at least 2.5 h before that time. Participants were then exposed to the TSST. This laboratory-based protocol consists of a free speech task and a mental arithmetic task of 15 min duration performed in front of an audience and a camera [64]. This protocol is among the most extensively validated tasks for the induction and assessment of acute psychophysiological stress responses, and has been found to induce significant endocrine (cortisol and adrenocorticotropic hormone (ACTH)) and autonomic nervous system (as indexed by heart rate and blood pressure) responses in the vast majority of subjects [65]. Blood and saliva samples were obtained before (−10 min) and after (+30 and +90 min) the TSST, whereas heart rate was measured continuously (Actiheart, CamNtech) during this period. Saliva was collected by placing cotton swabs (Salivettes, Sarstedt, Nümbrecht, Germany) in the participant's mouth for 2 min, and Salivettes were immediately frozen at −80°C. Blood was

collected in citrate-containing vacutainers (BD Vacutainer) and processed as described below for various assays.

(c) Questionnaires

Participants completed several standardized and previously validated questionnaires. Perceived (chronic) psychological stress over the past month was quantified using the 10-item version of the *Perceived Stress Scale* (PSS) [66]. Chronotype was assessed using the *Morningness Eveningness Questionnaire* (MEQ) [67]. Sleep quality (global sleep quality score) was assessed using the *Pittsburgh Sleep Quality Index* (PSQI) [68].

(d) Cortisol assay

Salivary cortisol was determined using a commercial ELISA kit (Salimetrics, 1–3002), with a sensitivity of 0.007 $\mu g\ dl^{-1}$. Intra- and inter-assay coefficients of variability were 5.9% and 7.3%, respectively.

(e) Maximal telomerase activity capacity

(i) Peripheral blood mononuclear cell isolation

PBMCs were isolated from peripheral blood collected in citrate tubes through a standard Ficoll protocol with SepMate tubes (Stemcell Technologies), and live cells were counted with a haemocytometer. Cells were then frozen using a Mister Frosty freezing chamber at a concentration of 1×10^7 cells ml^{-1} in freezing medium containing fetal bovine serum (HyClone, Thermo Scientific) and 10% DMSO (Sigma-Aldrich) until subsequent stimulation experiments (for further details see the supplementary material).

(ii) Mitogen stimulation protocol

Cells were thawed and washed in RPMI 1640 medium (Gibco®) containing 10% fetal bovine serum (HyClone, Thermo Scientific). Exactly 1×10^6 PBMCs were stimulated with PHA (10 $\mu g\ ml^{-1}$) (Sigma-Aldrich) (to induce lymphocyte proliferation) [60], supplemented with IL-2 (50 units ml^{-1}) (Sigma-Aldrich) (for the maintenance of immune cells, including T cells, B cells and NK cells, and its synergistic effect on lymphocyte proliferation and leucocyte telomerase expression [29,61]). Cells were cultured at 37°C and 5% CO_2 for a period of 8 days for the time course study, and for a period of 72 h for the other studies. For each individual and each study day, cells obtained at each of the three different time points were thawed and stimulated at the same time (in the afternoon) and were cultured in the same 12-well plate (for further details see the supplementary material).

(iii) Telomerase activity

Telomerase can be quantified by determining the presence of hTERT and/or other proteins of the telomerase enzyme complex, or by measuring the activity of the telomerase enzyme. Telomerase activity can be assessed by using the telomeric repeat amplification protocol (TRAP) assay, which relies on the ability of the telomerase enzyme to add telomere repeats to specifically designed oligonucleotide primers [69]. As the induction of telomerase activity in lymphocytes has been previously determined to occur independently of changes in mRNA or protein expression [26], we elected to quantify activity (instead of expression), and did so using the TeloTAGGG Telomerase PCR ELISA plus kit (Roche), which combines a TRAP assay with detection by ELISA. The linear range of this assay was first assessed using extracts of various cell numbers (stimulated PBMCs). Cell pellets were lysed in ice-cold CHAPS lysis buffer at a concentration of 1000 cells μl^{-1} and incubated for 30 min on ice. After incubation, the lysates were centrifuged at 16 000*g* for 20 min at 4°C. An extract corresponding to 1000 cells (1 μl) was added to each PCR. Amplification products were hybridized to a DIG-labelled telomeric repeat-specific probe bound to a streptavidin-coated 96-well plate. The binding reaction was detected with an anti-DIG-peroxidase antibody, visualized by a colour reaction product and quantified photometrically. The absorbance of each sample was measured at 450 nm reading against the blank (reference wavelength 620 nm). Each negative sample was obtained by heat treatment (30 min at 94°C). The relative telomerase activities of the samples were determined by comparing the signal from the sample with the signal obtained using a known amount of control template.

To determine the optimal stimulation time to characterize human leucocyte mTAC, we conducted an *in vitro* time course study using isolated PBMCs from five volunteers. PBMCs were stimulated with PHA (10 $\mu g\ ml^{-1}$) and IL-2 (50 units ml^{-1}), and cultured in eight separate wells for 8 days. On each day, PBMC telomerase activity was assessed, and (stimulated) PBMCs were stained with fluorescent antibodies against different immune cell subpopulations and analysed using flow cytometry (see §2f).

(f) FACS flow cytometry

In a subset of 13 participants, PBMCs from each of the three assessment time points across Test Day 1 were stained with fluorescent antibodies against different immune cell subpopulations before *in vitro* stimulation. PBMCs were incubated for 15 min at 4°C, with antibodies against CD45 (Vioblue) CD3 (APC), CD4 (PerCp), CD8 (APC-io770), CD14 (FITC), CD19 (PE-Vio770), CD16 (PE) and CD56 (PE) (Miltenyi Biotec). Cells were washed in PBS containing 0.5% bovine serum albumin, fixed in 2% formaldehyde and analysed using a BD FACSCanto II with FACSDiva 6.1.3. The data analysis was performed with FlowJo 10.1r5.

(g) Statistics

Repeated-measures ANOVAs were computed to assess the effects of time (day) on change in mTAC. Greenhouse–Geisser corrections were applied and adjusted results are reported. Pearson's correlations were used to determine the within-subject variability of mTAC. In addition, the intra-class coefficient (ICC) of mTAC was computed to determine the proportion of total variation in mTAC that is attributable to variation between subjects relative to variation within subjects. The area under the curve with respect to ground (AUCg) was used to quantify total mTAC and cortisol output (see formula 2 in [70]). The slope of the diurnal change in cortisol occurring on Test Day 1 was calculated by fitting a linear regression line, which predicted the cortisol values from morning to evening. Repeated-measures ANOVAs were computed to assess the effect of time on change in cortisol and heart rate. Heart rate was measured prior to the TSST (−5 min, with an average value of 5 min duration), during the TSST (average value of 15 min duration) and +40 min after the beginning of the TSST (average value of 5 min duration). Measures of psychophysiological stress reactivity were determined by calculating the per cent change in cortisol from before to 30 min after the TSST, and the per cent heart rate increase from the resting state to the average heart rate measured during the TSST phase. mTAC may be influenced by the distribution of immune cell subpopulations present in the sample before stimulation. Thus, we examined whether mTAC varies as a function of variation in immune cell subpopulations. Immune cell subpopulations in pre-mitogen-stimulated blood and mTAC (i.e. following mitogen stimulation) were characterized in samples from each of the three time points across Test Day 1. Linear mixed models were used to predict telomerase activity as a function of each immune cell type percentage by including the repeatedly measured mTAC as the outcome and each immune cell type percentage as varying covariates. This model assessed whether total PBMC telomerase activity was, in part, a function of certain cell subpopulation frequencies while accounting for within-subject autocorrelations. For all analyses, $p < 0.05$ was considered significant. Data analysis was performed using the SPSS statistical software (SPSS 23.0, Inc., Chicago, IL, USA).

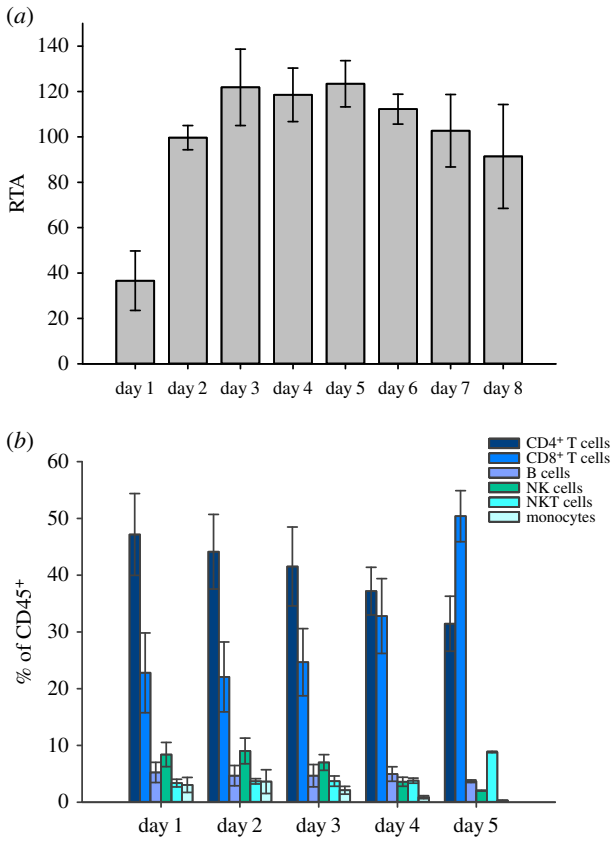

Figure 2. Time course experiment: (*a*) mean relative PBMC telomerase activity (±s.d.) of five individuals measured at days 1–8 after *in vitro* PHA/IL-2 stimulation, and (*b*) mean percentages (±s.d.) of immune cell subpopulations (calculated as percentages of $CD45^+$ cells) of five individuals measured at days 1–5 after PHA/IL-2 stimulation *in vitro*; RTA, relative telomerase activity.

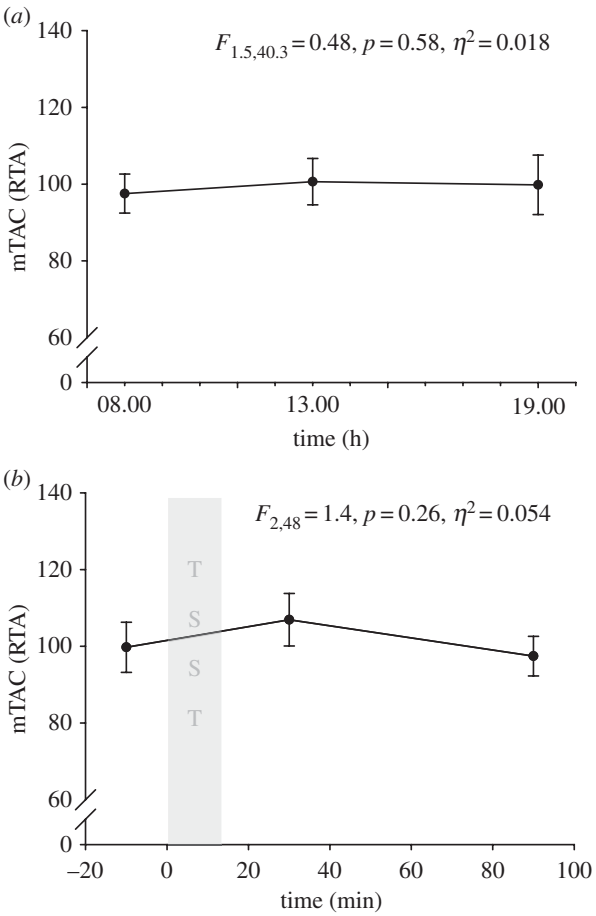

Figure 3. mTAC levels measured on both test days: (*a*) mean mTAC levels (±s.e.) in stimulated PBMCs isolated at 20.00 h, 13.00 h and 19.00 h during Test Day 1, and (*b*) before (−10 min) and after (+30 min, +90 min) the TSST (Test Day 2); RTA, relative telomerase activity.

3. Results

(a) Aim 1: optimal time course to characterize the maximal telomerase activity capacity response

As shown in figure 2*a*, maximal telomerase activity was observed on days 3, 4 and 5 of the time course study. Concurrently, the flow cytometry data indicate that changes in immune cell subtypes (specifically percentage of $CD8^+$ T cells) started occurring on and after day 4 (figure 2*b*), and that the amount of cell debris/lysis (as determined by the FCS/SSC signal) also started to increase from day 4 onwards (data not shown). Based collectively on these three sets of results (maximal telomerase activity, minimal change in immune cell subtypes and minimal cell degradation), we ascertained that a 3-day (72 h) period represents the optimal time point at which to determine PBMC mTAC in response to PHA/IL-2 mitogen stimulation challenge.

(b) Aim 2: within-subject stability of maximal telomerase activity capacity

(i) Chronobiological influences on maximal telomerase activity capacity

mTAC (i.e. PBMC telomerase activity 72 h after mitogen stimulation) was not significantly different across participants' blood samples collected serially in the morning, afternoon and evening (08.00 h, 13.00 h and 19.00 h; main effect *time*: $F_{1.5,40.3} = 0.48$, $p = 0.58$, $\eta^2 = 0.018$; figure 3*a*), suggesting no influence of chronobiology on mTAC. As expected, cortisol concentrations in these serially collected blood samples exhibited a diurnal rhythm and declined significantly from morning to evening ($F_{1.1,29.8} = 44.1$, $p < 0.001$, $\eta^2 = 0.62$). Cortisol concentration at these three time points was not associated with any of the corresponding mTAC measures. Finally, the within-subject correlations of mTAC levels from these serially obtained blood samples were strong and were in the range of r 0.68–0.82 (table 1). The intra-class coefficient of mTAC across the day (proportion of total variation in mTAC that is attributable to variation between subjects relative to variation within subjects) was 0.7, indicating substantially greater between-subject variability relative to within-subject across time variability.

Table 1. Within-subject correlations (Pearson's correlations) between mTAC levels from samples obtained at the three different time points during Test Day 1.

	mTAC 08.00 h	mTAC 13.00 h	mTAC 19.00 h
mTAC 08.00 h	1	0.82**	0.73**
mTAC 13.00 h	0.82**	1	0.68**
mTAC 19.00 h	0.73**	0.68**	1

**$p < 0.001$.

(ii) Acute stress exposure and maximal telomerase activity capacity

As expected, exposure to the TSST produced significant changes in cortisol (−10, +30, +90 min, main effect time: $F_{1.5,40.3} = 35.9$, $p < 0.001$, $\eta^2 = 0.57$) and heart rate (average heart rate before, during and after TSST, main effect time: $F_{1.6,36.6} = 80.7$, $p < 0.001$, $\eta^2 = 0.78$). There was, however, no difference in the mTAC levels between the serially collected blood samples obtained *before* (one sample) and *after* TSST exposure (two samples) (main effect time: $F_{2,48} = 1.4$, $p = 0.26$, $\eta^2 = 0.054$, figure 3*b*), suggesting that, on average, mTAC was not influenced by acute stress exposure.

(c) Aim 3: between-subject variability of maximal telomerase activity capacity: age, sex, body mass index, sleep quality and chronotype

The average of the mTAC (AUCg) measures from the serially collected blood samples across the course of the day was not significantly associated with participant age ($r = -0.27$, $p = 0.17$, $R^2 = 0.073$), BMI ($r = -0.11$, $p = 0.60$, $R^2 = 0.012$) or sex ($r = -0.047$, $p = 0.81$, $R^2 = 0.002$), and also was not related with either sleep quality (PSQI global sleep quality score, $r = -0.21$, $p = 0.29$, $R^2 = 0.044$), chronotype (MEQ score, $r = -0.10$, $p = 0.63$, $R^2 = 0.01$) or the slope of the diurnal change in cortisol ($r = -0.19$, $p = 0.35$, $R^2 = 0.036$).

(d) Aim 3: between-subject variability of maximal telomerase activity capacity: perceived stress and individual differences in physiological stress reactivity

(i) Perceived psychological stress and maximal telomerase activity capacity

Participants' score on the PSS was inversely associated with average mTAC (AUCg) (figure 4*a*; $r = -0.34$, $p = 0.08$, $R^2 = 0.12$). As the PSS is not a diagnostic instrument and there are no clinical cut-off values, we used our study population-based tertiles to categorize our participants into low-, moderate- and high-stress groups. When we compared the high perceived (chronic) stress group (upper tertile PSS score, mean score = 23 ± 2.8) with the low/medium perceived (chronic) stress group (lower two tertiles PSS score, mean score = 12 ± 3.2), there was a significant group effect of PSS score on mTAC ($F_{1,25} = 5.6$, $p = 0.026$, $\eta^2 = 0.18$), with individuals exposed to high perceived stress exhibiting significantly lower mTAC (figure 4*b*).

(ii) Biological stress reactivity and maximal telomerase activity capacity

Next, we examined the association of individual differences in physiological stress reactivity (endocrine (cortisol) and autonomic (heart rate) responses to the TSST) with average mTAC (AUCg). One subject had a cortisol response (percentage change from before to 30 min after the TSST) more than 3 s.d. above the mean and was therefore excluded from the analysis. The cortisol and heart rate responses to the TSST were moderately intercorrelated ($r = 0.60$, $p = 0.002$, $R^2 = 0.36$).

The cortisol response to the TSST was negatively related to mTAC (AUCg) (figure 5*a*, $r = -0.57$, $p = 0.004$, $R^2 = 0.32$). The heart rate response also was negatively—although not statistically significantly—associated with mTAC (AUCg) (figure 5*b*, $r = -0.34$, $p = 0.12$, $R^2 = 0.12$).

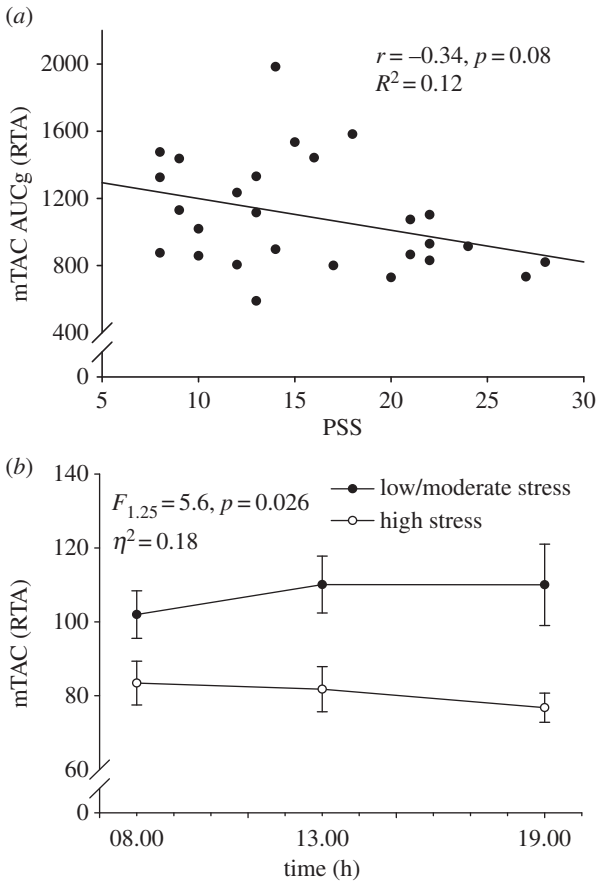

Figure 4. Perceived (chronic) stress score and mTAC: (*a*) correlation of mTAC (AUCg) with the PSS, and (*b*) mTAC levels in individuals reporting high and low/moderate stress. RTA, relative telomerase activity.

(e) Immune cell composition and maximal telomerase activity capacity

Some studies have suggested that basal [51] and stimulated [63] telomerase activities can vary by immune cell subtype. We, therefore, determined whether our measure of PBMC mTAC was influenced by the distribution of immune cell subpopulations present in the blood sample before mitogen stimulation. On Test Day 1, before *in vitro* stimulation, immune cell type frequencies were obtained from the three serially collected blood samples across the day in a subgroup of study participants ($n = 13$) [7]. The *B*-coefficients of the linear mixed models presented in table 2 reflect the change in mTAC (change in relative telomerase activity) for each percentage change in cell type of the different immune cell subtypes modelled simultaneously for all three time points. None of the relationships was significant, suggesting that this measure of mTAC is not altered as a function of immune cell type distribution present in the sample before stimulation.

4. Discussion

Based on the results of the time course study (i.e. determination of maximal telomerase activity with a minimal change in immune cell subtypes and minimal cellular degradation),

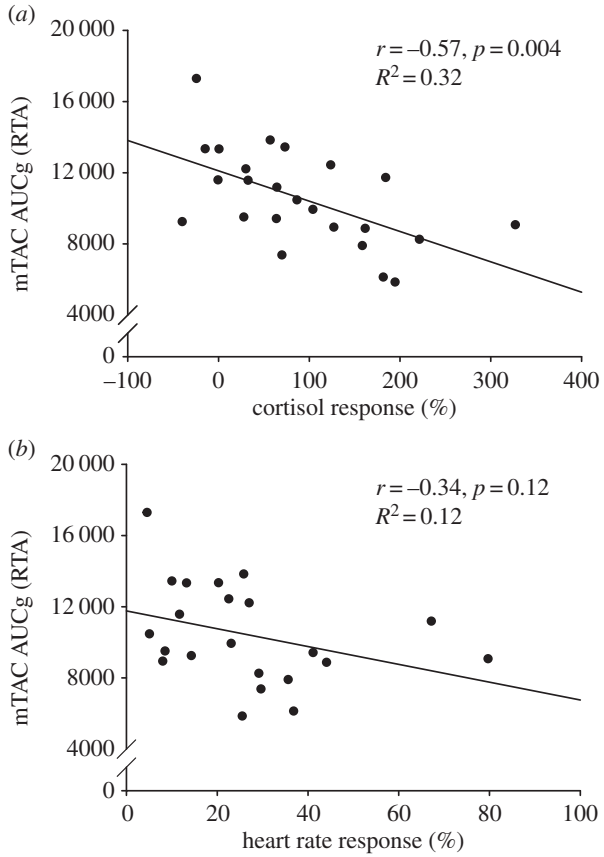

Figure 5. Correlation of mTAC (AUCg) with (*a*) the cortisol response and (*b*) the heart rate response (%) to the TSST. RTA, relative telomerase activity.

Table 2. Results of mixed models predicting mTAC by immune cell subtype percentages (%) on Test Day 1.

mTAC on Test Day 1		
cell type (%)	*B* (s.e.)	*p*-value
$CD4^+$ T cells	−0.64 (0.42)	0.14
$CD8^+$ T cells	0.92 (0.80)	0.26
B cells	0.28 (1.65)	0.87
NK cells	0.15 (0.82)	0.86
NKT cells	−0.10 (0.31)	0.98
monocytes	0.52 (0.53)	0.34

we determined that a 72 h *post-mitogen stimulation* period appears to represent the *optimal time point* at which to quantify human leucocyte mTAC. Using this optimal time point, our next set of results determined that the mTAC measure exhibits *substantial within-subject stability* across time and does not appear to be influenced by age, sex, BMI and situational factors including time of day, cortisol concentration, acute stress exposure and immune cell distribution prior to mitogen stimulation. The mTAC measure also exhibits *substantial between-subject variability*. Particularly, participants' perceived (chronic) stress level over the past one-month period accounted for 12% of the variation in the mTAC measure, with a 25% difference in mTAC between subjects reporting high compared with medium or low levels of perceived (chronic) stress. Moreover, individual differences in a key stress-related trait—psychophysiological stress responsivity (cortisol response to TSST exposure)—accounted for as much as 32% of the variation in mTAC. Finally, the mTAC ICC indicated substantially greater between-subject relative to within-subject variability. Collectively, these findings support our premise that the maximal leucocyte telomerase activity capacity construct (mTAC) empirically meets the criteria to represent a potentially useful individual difference measure.

Our finding that telomerase activity peaks between 3 and 5 days after mitogen stimulation replicates previous studies [29,63]. However, given the changes we observed after day 4 in immune cell subtypes and cell debris/lysis, we suggest that a 3-day period represents the optimal time point at which to characterize human leucoctye mTAC in response to PHA/IL-2 mitogen stimulation challenge.

In our study, mTAC was not associated with participants' age. While our study was limited in exploring this relationship given the relatively restricted age range in our cohort, we note that most previous studies of stimulated immune cell telomerase activity responses also did *not* find a significant effect of age on this measure [52–54], suggesting that mTAC may reflect a stable individual difference characteristic. Discrepancies between these findings and those of the two studies that did find an effect of age on stimulated telomerase activity [55,62] may perhaps be accounted for by differences in the mitogen stimulation protocols and the immune cell subpopulations that were assessed. In our study, there was also no significant effect on mTAC of time of day, basal cortisol concentrations assessed across the day, sex, BMI, sleep quality, chronotype and the slope of the diurnal change in cortisol. To the best of our knowledge, these factors have not been investigated/reported in previous studies using stimulated measures of telomerase expression/activity.

Our findings suggest that individual differences in mTAC are independent of percentages of different immune cell subsets present before *in vitro* stimulation. However, we cannot completely rule out the contribution of differences in immune cell composition. For example, here, we analysed the total fraction of $CD8^+$ T cells and did not discriminate between naive, effector and memory $CD8^+$ cytotoxic T cells. It has been shown in mice that effector and memory $CD8^+$ T cells express higher levels of telomerase activity after acute viral infection [71], indicating that the percentage of naive and virus-specific $CD8^+$ T cells can influence mTAC after stimulation *in vitro*. In addition, mTAC could be influenced by the percentage of senescent cells. Lin *et al.* [51] showed that, in total PBMC counts, senescent $CD28^-$ T cells had lowest telomerase activity and shortest telomere length. CD28 signalling is required for optimal telomerase upregulation, indicated by the paralleled loss of telomerase activity and CD28 expression in T cells after chronic antigen stimulation *in vitro* [63].

Previous studies have reported that chronically stressed individuals exhibit lower levels of *unstimulated* telomerase activity (e.g. [7,43–45]). Our results indicate that individuals reporting high levels of perceived (chronic) psychological stress display lower stimulated telomerase activity capacity (mTAC). A previous study by Damjanovic *et al.* [46] showed no differences in induced PBMC or T cell telomerase activity levels (anti-CD3/CD28 monoclonal antibody stimulation for 72 h) between carers of Alzheimer's patients (stress exposure group) and controls. This discrepancy with our findings may perhaps be explained by differences in the mitogen stimulation protocols that were employed, or by the fact that stress

exposure in the carers was operationalized using a depression scale, whereas we assessed stress over the past month using the Perceived Stress Scale (PSS).

Our protocol particularly stimulates cell types involved in cellular immunity, the component of the immune system that is initially affected by chronic stress exposure [72]. Chronic stress induces a shift in the production of type 1 cytokines towards type 2 cytokines, and it has been associated with blunted mitogen-induced lymphocyte proliferation and mitogen-induced IL-2 production [73], both activators of signalling pathways stimulating telomerase activity [9,61]. Therefore, the observation that higher levels of perceived (chronic) psychological stress are associated with lower mTAC may be a reflection of impairment in cell-mediated immunity. In addition, chronic stress exposure is associated with a higher level of oxidative stress [44,74]. Oxidative stress decreases telomerase activity and induces senescence (or apoptosis) via DNA damage-induced activation of the p53 pathway, probably by causing erosion of telomeres or perhaps by inducing nuclear exclusion of hTERT into mitochondria [32,33]. Senescent $CD28^-$ T cells show impaired upregulation of telomerase activity after antigen stimulation [63] and could therefore inhibit mTAC.

In our study population, a larger cortisol increase in response to an acute psychosocial stressor was related to lower mTAC. An individual's biological stress reactivity measure can be considered a stress-related trait and has been studied in the context of several stress-related pathologies and conditions [75]. Previous findings showed that greater cortisol response to an acute stressor was associated with shorter telomere length, as were higher overnight urinary-free cortisol levels and flatter daytime cortisol slope [76]. In line with our finding, exposure to exogenous cortisol has been shown to inhibit telomerase production in human T cells stimulated with PHA and IL-2 [59]. Glucocorticoids place a limit on the maximal activity of the immune system, modulate inflammatory gene transcription [77] and can either through direct action or through the modulation of cytokine release (type 1 cytokines towards type 2 cytokines) inhibit lymphocyte proliferation and NK cell activity [73], thereby influencing telomerase regulatory pathways [9,61]. Cortisol levels measured during the chronobiology test day (Test Day 1) were not associated with mTAC. However, mTAC was negatively related to the cortisol response to acute stress. It is likely that individuals experiencing persistently exaggerated stress responses are exposed to greater levels of cortisol over longer periods of time, which may have important consequences on immune system function. In a study by Epel *et al.* [7], in which basal/unstimulated telomerase activity was measured soon after a standardized stress test, the cortisol response was positively associated with telomerase activity. As discussed by these authors, telomerase activity levels may change dynamically in response to stress, probably as a protective functional response to protect telomeric regions from stress-induced acute increases in biological stress mediators such as cortisol or oxidative stress. Two key differences between this study and ours is that the Epel *et al.* study [7] did not use a mitogen challenge to stimulate telomerase expression and did not characterize *maximal* capacity of cells to express telomerase (i.e. mTAC) but measured only the short-term response (up to 90 min) to acute social stress, whereas we assessed telomerase in response to a mitogen challenge and across a 3-day period of time.

In this study, individuals displaying higher cortisol responses also showed higher heart rate responses, indicating a coupling between these stress-related endocrine and autonomic systems. Catecholamines, which are normally produced in response to acute stress by the adrenal medulla and postganglionic sympathetic nerve fibres, activate β2-receptors present on immune cells. *In vitro* studies suggest that β2-receptor stimulation by catecholamines activates the cAMP signalling pathway, inhibiting mitogen-stimulated T-cell proliferation and NK cell activation [78]. Hence, catecholamines could also inhibit leucocyte mTAC after stimulation *in vitro*.

Strengths of this study include the following: we first established the optimal mTAC time course and stimulation conditions before the development and use of the *in vitro* study stimulation protocol to address the study aims. Furthermore, to the best of our knowledge, our study is the first to systematically investigate the within- and between-subject stability of stimulated telomerase activity responses (mTAC) in women and men with respect to chronobiological and stress-related influences.

Limitations of our study include the unavailability of measures of telomere length in our study subjects, the relatively modest sample size, and the restricted range of age and BMI in our study subjects. Any interpretation of sex effects is limited by the fact that all the women in our study were using oral contraceptives. While the absence of chronobiological or acute stress effects on mTAC in our study may provide a justification for future studies against requiring multiple mTAC measures over the course of a day or in response to an acute challenge, we note, based on the within-subject correlations of serial measures of mTAC indicating a shared variance of approximately 50–65%, that measurement issues such as single versus average of multiple samples remain an important consideration. Finally, because mTAC was measured in isolated circulating immune cells (PBMCs) in the context of immune cell activation and clonal T-cell expansion, it is unclear whether this measure also reflects the capacity of haematopoietic stem and progenitor cells to induce a telomerase activation response.

As discussed earlier, only a few studies, to date, have examined the characteristics of stimulation-induced telomerase responses in the context of health and disease states [54–58]. Based on our findings in support of the premise that the maximal leucocyte telomerase activity capacity construct (mTAC) may represent a potentially useful individual difference measure, one future research direction will be to determine the prognostic value of mTAC, independently, and in combination with measures of telomere length, with respect to health, disease risk and mortality. Also, further mechanistic studies are warranted to better understand the contribution of specific immune cell populations such as memory and senescent T cells, circulating levels of other stress-related biological mediators such as catecholamines, oxidative stress and pro-inflammatory cytokines to mTAC. Future studies should also explore the characteristics of mTAC in haematopoietic stem cells [79], such as those obtained from umbilical cord blood.

In conclusion, because mTAC exhibits high within-subject stability, is not influenced by situational circumstances and shows substantial between-subject variability in relation to stress-related traits and states, this *in vitro* measure of telomerase activity may represent a potentially useful individual difference measure in studies of telomere biology and health and disease.

Ethics. The study was approved by the medical ethics committee of Charité — Universitätsmedizin Berlin and was conducted in accordance with the Declaration of Helsinki. All subjects provided their written informed consent before participation.

Data accessibility. Additional methodological details have been uploaded as part of the electronic supplementary material. The dataset supporting this article is available at: https://doi.org/10.6084/m9.figshare.5624959.

Author contributions. K.d.P. coordinated the study, participated in the design of the study, carried out the laboratory assays, participated in the data analyses and drafted the manuscript. C.H. participated in the data analysis and provided editorial assistance. I.P. performed the flow cytometry measurements and data analysis. P.D.W. participated in the analysis and interpretation of study findings, drafted portions of the manuscript and provided editorial assistance. S.E. conceived of and designed the study, participated in the analysis and interpretation of study findings, drafted portions of the manuscript and provided final editorial oversight.

Competing interests. We declare we have no competing interests.

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