Aus dem Institut für Medizinische Psychologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Characterization in humans of *in vitro* leukocyte maximal telomerase activity capacity (mTAC) and association with stress

zur Erlangung des akademischen Grades

Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

von

Karin de Punder

aus Purmerend

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Abstract (German)

Hintergrund: Telomerase, ein zelluläres Transkriptionsenzym, das der Telomerverkürzung entgegenwirkt und wichtig ist für gesunde Zellfunktion, ist ein vielversprechender Biomarker hinsichtlich pathologischer Folgen von Stress auf zelluläre Alterungsprozesse. Bisherige Studien zur Messung von Telomeraseaktivität oder -expression haben dies meistens unter basalen Bedingungen durchgeführt. Basale Telomerase-Werte sind jedoch aufgrund der niedrigen Expressionsrate und der dynamischen Regulation der Telomerase in Leukozyten sehr schwierig zu messen und zu interpretieren. Im Gegensatz zur basalen Expressionsrate kann Telomerase-Aktivität im Rahmen eines ökologisch-validen *Challenge Tests* wie der *in vitro* Mitogen-Stimulation quantifiziert werden. Ein potentieller Vorteil dieses Verfahrens ist, dass dadurch die o.a. Einschränkungen der Interpretierbarkeit der basalen Telomerase-Aktivität umgangen werden können, und man somit die Möglichkeit hat, individuelle Unterschiede zu erfassen in der Kapazität des Telomersystems auf einem immunologischen Stimulus zu reagieren.
Ziel: Das Ziel der Studie bestand in der Entwicklung und Validierung eines Maßes der maximalen Telomerase-Aktivitätskapazität (mTAC) zur Nutzung in Humanstudien im Bereich der Telomer-Biologie. Außerdem wurde der Zusammenhang dieses Maßes mit Stress bzw. Stressreaktivität untersucht.
Methoden: Zuerst wurde der optimale post-stimulative Zeitverlauf zur mTAC-Charakterisierung mit einem *in vitro* Mitogen-Challenge-Protokoll (PHA ergänzt durch Interleukin(IL)-2) bestimmt. Danach wurden sowohl mTAC in Leukozyten und Cortisolkonzentrationen im Speichel von 28 jungen gesunden Proband/innen zu verschiedenen Messzeitpunkten im Verlauf des Tages und im Rahmen eines standardisierten Paradigmas zur Induktion psychosozialen Stresses im Labor gemessen. Darüber hinaus wurde in einem Teil der Stichprobe Durchflusszytometrie verwendet, um die Immunzell-Verteilung vor Stimulation zu bestimmen. Die wahrgenommene (chronische) Stressbelastung wurde durch die *Perceived Stress Scale* erfasst.
Resultate: Der optimale Zeitpunkt zur Quantifizierung von mTAC in humanen Leukozyten liegt bei 72 Stunden nach Mitogen-Stimulation. mTAC weist eine erhebliche Stabilität innerhalb von Personen auf und wird nicht durch situationsbedingte Faktoren wie Tageszeit, Cortisol, akute Stressexposition und Immunzell-Verteilung beeinflusst. Ein signifikanter Anteil der Varianz zwischen Personen in mTAC wird durch chronische Stressbelastung und biologische

Stressreaktivität erklärt. Insbesondere waren mTAC-Werte bei Personen mit hoher wahrgenommener Stressbelastung um 25% niedriger im Vergleich zu Probanden mit mittlerem oder niedriger wahrgenommener Stressbelastung. Außerdem erklärten individuelle Unterschiede in der Cortisolreaktion auf Laborstressexposition 32% der Varianz von mTAC.
Fazit: Basierend auf diesen Ergebnissen kann man schlussfolgern, dass mTAC einen nützlichen individuellen Marker für stressbezogene Humanstudien im Bereich der Telomerbiologie darstellt.

Abstract (English)

Background: Telomerase, a cellular reverse transcriptase enzyme that can counteract telomere shortening and, in addition, preserves healthy cell function, constitutes a promising target for research into the pathological effects of stress on cellular aging. Previous studies that have included measures of telomerase have typically measured telomerase expression or activity under basal (resting) conditions. It is, however, challenging to reliably quantify or interpret these data because leukocyte telomerase is typically expressed at very low levels and is dynamic in nature. In contrast to basal measures, telomerase activity can be quantified in response to an ecologically-valid challenge such as mitogen stimulation *in vitro*. 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 immunological challenge.

Objective: The aim of this study was to validate an *in vitro* measure of leukocyte 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.

Methods: First, the optimal post-stimulation time course to characterize mTAC was established using an *in vitro* mitogen challenge (phytoheamagglutinin (PHA) supplemented with interleukin(IL)-2). Next, mTAC was measured in leukocytes and cortisol concentrations were assessed in saliva obtained from 28 healthy young women and men at different times of the day and before and after a standardized laboratory stressor. In addition, immune cell distributions prior to mitogen stimulation were determined by flow cytometry in a subset of the participants. Perceived (chronic) stress also was assessed using the *Perceived Stress Scale*.

Results: The optimal time point to quantify human leukocyte mTAC was 72 hours after mitogen stimulation. mTAC exhibited substantial within-subject stability across time and was not influenced by situational factors including time of day, cortisol concentration, acute stress exposure, and immune cell distribution. A significant proportion of the between-subject variability in mTAC was associated with measures of stress and stress responsivity. Particularly, there was a 25% difference in mTAC between subjects reporting high compared to medium or low levels of perceived (chronic) stress. Also, individual differences in the cortisol response to stress-exposure accounted for as much as 32% of the variation in mTAC.

Conclusion: Based collectively on these findings, it appears that mTAC may represent a potentially useful individual difference measure in stress-related studies of the human telomere biology system.

Affidavit

I, Karin de Punder certify under penalty of perjury by my own signature that I have submitted the thesis on the topic: „Characterization in humans of *in vitro* leukocyte maximal telomerase activity capacity (mTAC) and association with stress“. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.
All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

_____ _____

Date Signature

Detailed Declaration of Contribution

Karin de Punder had the following share in the following publication:

Publikation: Karin de Punder, Christine Heim, Ingo Przesdzing, Pathik D. Wadhwa, Sonja Entringer (2018). Characterization in humans of *in vitro* leukocyte maximal telomerase activity capacity (mTAC) and association with stress. *Philos Trans R Soc Lond* ***B*** *Biol Sci.* 5;373(1741) pii: 20160441. *http://dx.doi.org/10.1098/rstb.2016.0441*

Contribution in detail:

Karin de Punder helped writing the ethical application for this study. She participated in the design of the study and recruited all the study participants. She planned and coordinated the study and guided and prepared the participants during both test days. In addition, she carried out the laboratory assays (leukocyte isolation and stimulation, telomerase measurements, salivary cortisol measurements, antibody stainings for the flow cytometry experiments), performed the statistical data analyses and drafted the manuscript. She also made the tables and figures presented in the manuscript.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Excerpt from the Journal Summary List (ISI Web of KnowledgeSM)

Journal Data Filtered By: **Selected JCR Year: 2016** Selected Editions: SCIE,SSCI Selected Categories: **"BIOLOGY"** Selected Category Scheme: WoS **Gesamtanzahl: 72 Journale**

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	Physics of Life Reviews	1,327	13.840	0.003180
2	BIOLOGICAL REVIEWS	9,669	11.615	0.017300
3	PLOS BIOLOGY	26,893	9.797	0.067500
4	eLife	15,730	7.725	0.130380
5	BMC BIOLOGY	4,588	6.779	0.016120
6	**PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES**	**36,908**	**5.846**	**0.069600**
7	FASEB JOURNAL	42,242	5.498	0.057660
8	BIOSCIENCE	15,467	5.378	0.013660
9	PROCEEDINGS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES	44,463	4.940	0.088440
10	BIOESSAYS	9,199	4.441	0.016940
11	QUARTERLY REVIEW OF BIOLOGY	3,683	4.250	0.001500
12	Current Opinion in Insect Science	665	3.660	0.002870
13	JOURNAL OF BIOLOGICAL RHYTHMS	2,617	3.500	0.004220
14	Geobiology	1,798	3.462	0.004150
15	BIOELECTROCHEMISTRY	3,916	3.346	0.003820
16	JOURNAL OF EXPERIMENTAL BIOLOGY	30,619	3.320	0.036880
17	Biology Letters	8,242	3.089	0.021760
18	Biology Direct	1,507	2.856	0.005180
19	Science China-Life Sciences	1,621	2.781	0.004510
20	Interface Focus	1,272	2.693	0.005870
21	ASTROBIOLOGY	2,414	2.603	0.005550
22	SAUDI JOURNAL OF BIOLOGICAL SCIENCES	1,095	2.564	0.002480

23	CHRONOBIOLOGY INTERNATIONAL	4,215	2.562	0.007100
24	RADIATION RESEARCH	8,394	2.539	0.007920
25	RADIATION AND ENVIRONMENTAL BIOPHYSICS	1,468	2.398	0.002460
26	AEROBIOLOGIA	1,166	2.202	0.001060
27	JOURNAL OF THERMAL BIOLOGY	2,614	2.157	0.003440
28	JOURNAL OF THEORETICAL BIOLOGY	19,308	2.113	0.022280
29	Biology Open	1,457	2.095	0.008260
30	CRYOBIOLOGY	3,949	1.996	0.003490
31	INTERNATIONAL JOURNAL OF RADIATION BIOLOGY	4,417	1.992	0.004350
32	BIOLOGICAL BULLETIN	4,803	1.950	0.002280
33	BIOELECTROMAGNETICS	2,760	1.933	0.002570
34	Advances in Experimental Medicine and Biology	14,818	1.881	0.029570
35	COMPUTERS IN BIOLOGY AND MEDICINE	3,760	1.836	0.006550
36	JOURNAL OF RADIATION RESEARCH	2,270	1.788	0.004620
37	AMERICAN JOURNAL OF HUMAN BIOLOGY	2,959	1.780	0.005110
38	BIOLOGICAL RESEARCH	1,230	1.692	0.001630
39	BIOSYSTEMS	2,471	1.652	0.002850
40	MATHEMATICAL MEDICINE AND BIOLOGY-A JOURNAL OF THE IMA	469	1.610	0.000920
41	International Journal of Astrobiology	475	1.598	0.001340
42	BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH	4,568	1.578	0.004000

43	JOURNAL OF MATHEMATICAL BIOLOGY	4,399	1.566	0.006540
44	BioScience Trends	614	1.545	0.001230
45	EXCLI Journal	514	1.462	0.001120
46	BIOMETRIKA	18,608	1.448	0.015690
47	JOURNAL OF BIOSCIENCES	2,495	1.422	0.003410
48	COMPUTATIONAL BIOLOGY AND CHEMISTRY	1,025	1.331	0.001780
49	BIOMETRICS	19,952	1.329	0.014450
50	ELECTROMAGNETIC BIOLOGY AND MEDICINE	573	1.272	0.000960
51	BULLETIN OF MATHEMATICAL BIOLOGY	3,434	1.263	0.004440
52	MATHEMATICAL BIOSCIENCES	4,736	1.246	0.004820
53	ANNALS OF HUMAN BIOLOGY	1,865	1.240	0.002600
54	JOURNAL OF ETHNOBIOLOGY	422	1.217	0.000490
55	Journal of Biological Research-Thessaloniki	198	1.200	0.000330
56	MICROSCOPY RESEARCH AND TECHNIQUE	4,509	1.147	0.003980
57	COMPTES RENDUS BIOLOGIES	2,111	1.100	0.002640
58	TURKISH JOURNAL OF BIOLOGY	1,049	1.038	0.001480
59	Central European Journal of Biology	744	1.016	0.001670
60	ORIGINS OF LIFE AND EVOLUTION OF BIOSPHERES	1,224	1.000	0.001260
61	JOURNAL OF BIOLOGICAL EDUCATION	519	0.946	0.000420
62	FOLIA BIOLOGICA	529	0.939	0.000630
63	JOURNAL OF AGRICULTURAL BIOLOGICAL AND ENVIRONMENTAL STATISTICS	809	0.852	0.001780
64	HUMAN BIOLOGY	1,809	0.825	0.001080
65	THEORY IN BIOSCIENCES	338	0.778	0.000800
66	BIOLOGIA	1,756	0.759	0.002500

67	JOURNAL OF THE HISTORY OF BIOLOGY	482	0.737	0.000640
68	PROCEEDINGS OF THE BIOLOGICAL SOCIETY OF WASHINGTON	1,269	0.667	0.000520
69	BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY	1,826	0.644	0.001650
70	CRYOLETTERS	890	0.628	0.000780
71	BIOLOGICAL RHYTHM RESEARCH	494	0.624	0.000590
72	FOLIA BIOLOGICA-KRAKOW	333	0.581	0.000400
73	ACTA BIOLOGICA HUNGARICA	448	0.506	0.000420
74	REVISTA DE BIOLOGIA TROPICAL	2,017	0.495	0.002090
75	BRAZILIAN JOURNAL OF BIOLOGY	1,932	0.479	0.002330
76	Open Life Sciences	35	0.448	0.000080
77	Theoretical Biology Forum	23	0.421	0.000070
78	JOURNAL OF BIOLOGICAL SYSTEMS	395	0.390	0.000540
79	Archives of Biological Sciences	725	0.352	0.001370
80	AMERICAN BIOLOGY TEACHER	552	0.318	0.000290
81	ZHURNAL OBSHCHEI BIOLOGII	206	0.313	0.000150
82	BIOLOGY BULLETIN	421	0.299	0.000560
83	Bioscience Journal	544	0.267	0.001410
84	PERIODICUM BIOLOGORUM	289	0.184	0.000250

Decision Letter

The decision letter does not appear in the electronic version of my paper for reasons of data protection.

Characterization in humans of *in vitro* leukocyte maximal telomerase activity capacity (mTAC) and association with stress.

Karin de Punder[1], Christine Heim[1,2], Ingo Przesdzing[3], Pathik D. Wadhwa[4,5,6,7,8] & Sonja Entringer[1,6,8]

[1]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health (BIH), Institute of Medical Psychology, Berlin, Germany

[2]Department of Biobehavioral Health, College of Health and Human Development, Pennsylvania State University, Pennsylvania, USA

[3]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health (BIH), Department of Experimental Neurology and Center for Stroke Research Berlin (CSB), Berlin, Germany

Departments of [4]Psychiatry & Human Behavior, [5]Obstetrics & Gynecology, [6]Pediatrics, [7]Epidemiology, and the [8]Development, Health and Disease Research Program, University of California, Irvine, School of Medicine, Irvine, CA, USA

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Address correspondence to:
Sonja Entringer
Institute for Medical Psychology
Charité University Medicine
Luisenstr. 57
10117 Berlin
Germany
Tel: +49 30 450 529 222
Fax: +49 30 450 529 990
Email: sonja.entringer@charite.de

OR

Pathik D. Wadhwa, MD, PhD.
Professor of Psychiatry & Human Behavior, Obstetrics & Gynecology, Pediatrics, and Epidemiology
Director, UC Irvine Development, Health and Disease Research Program
University of California, Irvine, School of Medicine
3117 Gillespie Neuroscience Research Facility (GNRF)
837 Health Sciences Road
Irvine, CA 92697
Tel: (949) 824-8238
Fax: (949) 824-8218
E-mail: pwadhwa@uci.edu

Summary

The goal of the present 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 (PHA supplemented with interleukin(IL)-2) was used to stimulate telomerase activity in leukocytes. 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 follow: *1*) The optimal time point to quantify human leukocyte mTAC appears to be at 72 hours after mitogen stimulation; *2*) mTAC exhibits substantial within-subject stability (correlations ranged between r's 0.68 – 0.82) and between-subject variability, with a high intra-class coefficient (ICC = 0.70), indicating greater between-subject relative to within-subject variability; *3*) 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 *4*) 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 leukocytes.

1. Introduction

1.1. 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]. The telomere biology system comprises of two closely interlinked components – the length of telomeres (TL; 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 (see, 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 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. In order 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 program 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. Since 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. Last, we determined the extent to which mTAC may be driven by the composition of immune cell subpopulations before mitogen stimulation.

1.2. The telomere biology system

The telomere biology system is a highly evolutionarily 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. Shortened telomeres have been linked to several age-related disease risk factors, disease prevalence and progression [1-4, 13-18] and early mortality [19, 20]. Moreover, some recent reports have suggested a *causal* role for telomeres in the etiology of many of these adverse health outcomes [21].

The enzyme telomerase is a ribonucleoprotein consisting of a 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 utilizes 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, 22]. Telomerase is regulated by epigenetic, translational and posttranslational mechanisms [23]. Its expression varies during development, cell cycle stage, and across cell types [9, 24]. 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 [24]. Of particular relevance here, it is well established that activated lymphocytes express high telomerase levels [8, 9, 25, 26]. This up-regulation 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 signaling pathways described above to constitutively express high levels of telomerase, which is a characteristic feature in about 90% of all malignancies [27]. 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 signaling pathways during embryogenesis and normal adult tissue genesis, protects cellular proliferation capacity and survival under conditions of cellular stress [28], and gets excluded from the nucleus to co-localize with mitochondria to protect mitochondrial DNA and function [29, 30]. Thus, because the effects of telomerase on cellular function extend beyond, and are uncoupled from, those of telomere lengthening [31], measures of telomerase expression and activity could, in addition to telomere length, provide valuable insight regarding the role of the telomere biology system in health and disease risk.

1.3. Basal leukocyte telomerase

Studies of the association of basal leukocyte 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 basal telomerase expression/activity appears to be up-regulated in autoimmune disorders, with differences between active and inactive disease [32], and between early-and advanced-stage disease [33], Findings in the context of psychiatric disorders such as depression, schizophrenia, and posttraumatic stress disorder (PTSD) appear to be more heterogeneous [34-38]. The same pattern of mixed findings is evident in the context of chronic stress exposure, with some suggesting suppression [7, 39-41], and others suggesting stimulatory effects of stress on telomerase activity [10, 42]. 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 [43, 44], which, in turn, may introduce bias based on time of day of blood sample collection [45], current infections [43, 44], physical exercise [46], 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 TL may be indicative of a physiologically stressed system [10]. Lastly, 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 [47].

1.4. Stimulated leukocyte telomerase

Hiyama *et al* [8] were the first to describe the up-regulation of telomerase in leukocytes 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 1 week-period in cultured T-cells stimulated with PHA and IL-2, and increased up to 30 fold over a 1 week-period in cultured B-cells stimulated with Pokeweed mitogen. Similarly, Yamada *et al* [26] 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* [48] 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 age) provides the first indication that this measure may reflect a stable individual difference characteristic. This observation has since been replicated by other studies [49, 50]. However, to date, only few studies have characterized the association of measures of stimulation-induced telomerase with health and disease states [50-54]. Broadly, these studies have reported that the mitogen stimulated leukocyte telomerase response appears to be attenuated in subjects with autoimmune conditions such as systemic lupus erythematosus [52], rheumatoid arthritis [50, 53], and unchanged in conditions such as atopic dermatitis [54] and chronic hepatitis B infection [51].

Two studies have examined the association of stimulated human leukocyte telomerase with chronic stress. An *in vitro* experiment by Choi *et al* [55] modeled 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 caregivers of patients with Alzheimer's disease (stress exposure group) and age-matched controls, basal PBMC and T-cell telomerase levels were increased in caregivers compared to controls, while no differences were observed across a 3-day period in antigen-stimulated telomerase levels (with anti-CD3/CD28 monoclonal antibody) [42].

1.5. Maximal Telomerase Activity Capacity (mTAC) 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 captures 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 leukocytes; 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 types such as T-cells).

PBMCs were stimulated with phytohemagglutinin (PHA, 10 µg/ml) supplemented with IL-2 (50 units/ml), 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 µg/ml PHA dose because maximal lymphocyte proliferation is known to occur at this dose [56], 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 the proliferation of T cells and on leukocyte telomerase expression [26, 57].

To establish the optimal time course for characterization of mTAC we considered 3 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 8 consecutive days after mitogen stimulation, peak leukocyte hTERT mRNA expression occurs between 3 to 5 days after stimulation [50]. Most studies to date have used a 3-day incubation protocol with and without the addition of IL-2, and have used PHA, [49, 58] CD3-monoclonal antibodies [42, 50, 53, 54], or PMA/Ionomycin [48, 52] as a stimulant to induce telomerase activity or hTERT mRNA expression in leukocytes or leukocyte subsets. Our criteria for characterizing the 'optimal' time was to determine the balance between the time required to enable quantification of the maximal telomerase activity capacity without producing major alterations in immune cell subpopulations and cell viability (as these changes could, in and of themselves, influence telomerase production and activity [48, 59].

1.6. Goals

Thus, to summarize, the primary goals of our study were to establish the optimal time course to characterize human leukocyte maximal telomerase activity capacity (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. Last, 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. Materials and Methods

2.1. Participants

The study population comprised 28 young adults (14 women and 14 men, mean age 23.8 ± 3.3 (SD) years) recruited through announcements at universities in Berlin, Germany, and 5 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 contraceptives users). Mean body mass index (BMI) was 20.9 ± 1.7 (SD). 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 written informed consent before participation.

2.2. Study protocol

Subjects reported to our research laboratory on two occasions: Test Day 1 – the "chronobiology day"; and Test Day 2 – the "Trier Social Stress Test (TSST) day" (see **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 24 h prior 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 8 a.m., 1 p.m., and 7 p.m.). Participants were asked to refrain from eating at least 2.5 h before the 1 p.m. and 7 p.m. blood draws. On Test Day 2, participants were asked to come to the laboratory at 2 p.m. and to refrain from eating at least 2.5 h before that time. Participants were then exposed to the Trier Social Stress Test (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 [60]. 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, ACTH), and autonomic nervous system (as indexed by heart rate and blood pressure) responses in the vast majority of subjects [61]. Blood and saliva samples were obtained before (-10 min) and after (+30 min, +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.

2.3. 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)* [62]. Chronotype was assessed using the *Morningness Eveningness Questionnaire* (MEQ) [63]. Sleep quality (global sleep quality score) was assessed using the *Pittsburgh Sleep Quality Index* (PSQI) [64].

2.4. Cortisol assays

Salivary cortisol was determined using a commercial ELISA kit (Salimetrics, 1-3002), with a sensitivity of 0.007 µg/dL. Intra-assay and inter-assay coefficients of variability were 5.9% and 7.3%, respectively.

2.5. Maximal telomerase activity capacity (mTAC)

Peripheral blood mononuclear cell (PBMC) 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 hemocytometer. Cells were then frozen using a Mister Frosty freezing chamber at a concentration of 1 x 10^7 cells/ml in freezing media of fetal bovine serum (HyClone, Thermo Scientific) and 10% DMSO (Sigma-Aldrich) until subsequent stimulation experiments (for more details see Supplemental material)

Mitogen stimulation protocol: Cells were thawed and washed in RPMI 1640 medium (Gibco®) containing 10% fetal bovine serum (HyClone, Thermo Scientific). Exactly 1 x 10^6 PBMCs were stimulated with phytohemagglutinin (PHA) (10 µg/ml) (Sigma-Aldrich) (to induce lymphocyte proliferation) [56], supplemented with IL-2 (50 units/ml) (Sigma-Aldrich) (for the maintenance of immune cell, including T-cells, B-cells and NK cells, and its synergistic effect on lymphocyte proliferation and leukocyte telomerase expression [26, 57]). Cells were cultured at 37˚C and 5% CO2 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 more details see Supplemental material).

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 oligo-nucleotide primers [65]. Since the induction of telomerase activity in lymphocytes has been previously determined to occur independent of changes in mRNA or protein expression [23], 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 and incubated for 30 min on ice. After incubation, the lysates were centrifuged at 16,000 x g for 20 min at 4°C. An extract corresponding to 1,000 cells (1 µl) was added to each

PCR reaction. 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 color 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 to the signal obtained using a known amount of control template.

In order to determine the optimal stimulation time to characterize human leukocyte mTAC, we conducted an *in vitro* time course study using isolated PBMCs from 5 volunteers. PBMCs were stimulated with PHA (10 µg/ml) and IL-2 (50 units/ml) and cultured in 8 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 analyzed using flow cytometry (see section 2.6.).

2.6. 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 4ºC, with antibodies against CD45 (Vioblue) CD3 (APC), CD4 (PerCp), CD8 (APC-io770), CD14 (FITC), CD19 (PE-Vio770), CD16 (PE), and CD56 (PE) (Miltenyl Biotec). Cells were washed in PBS containing 0.5% bovine serum albumin, fixed in 2% formaldehyde, and analyzed using a BD FACSCanto II with FACSDiva 6.1.3. The data analysis was performed with FlowJo 10.1r5.

2.7. 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 [66]). 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 percent change in cortisol from before to 30 min after the TSST, and the percent heart rate increase from resting state to 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 percent by including the repeatedly measured mTAC as the outcome and each immune cell type percent 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-values < .05 were considered significant. Data analysis was performed using SPSS statistical software (SPSS 23.0, Inc., Chicago, IL, USA).

3. Results

3.1. Aim 1: Optimal time course to characterize the maximal telomerase activity capacity (mTAC) response

As shown in **Figure 2a**, maximal telomerase activity was observed on day 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 2b**), and that the amount of cell debris/lysis (as determined by the FCS/SSC signal) also started to increase from day 4 onwards. Based collectively on these 3 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.

3.2. Aim 2: Within-subject stability of mTAC

3.2.1. Chronobiological influences on mTAC

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 (8 a.m., 1 p.m., 7 p.m.; main effect time: $F_{1.5, 40.3} = .48$, $p = .58$, $\eta^2 = .018$, see **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 ($F_{1.1, 29.8} = 44.1$, $p < .001$, $\eta^2 =$

.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 was strong and ranged between r's 0.68 – 0.82 (see **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.

3.2.2. Acute stress exposure and mTAC

As expected, exposure to the TSST produced significant changes in cortisol (-10, +30, +90 min, main effect time: $F_{1.5, 40.3} = 35.9$, $p < .001$, $\eta^2 = .57$) and heart rate (average heart rate before, during, and after TSST, main effect time: $F_{1.6, 36.6} = 80.7$, $p < .001$, $\eta^2 = .78$). There was, however, no difference in the mTAC levels between the serially-collected blood samples obtained *before* (1 sample) and *after* TSST exposure (2 samples) (main effect time: $F_{2, 48} = 1.4$, $p = .26$, $\eta^2 = .054$, see **Figure 3b**), suggesting that on average, mTAC was not influenced by acute stress exposure.

3.3. Aim 3: Between-subject variability of mTAC: age, sex, BMI, 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 ($r = -.27$, $p = .17$, $R^2 = .073$), BMI ($r = -.11$, $p = .60$, $R^2 = .012$) or sex ($r = -.047$, $p = .81$, $R^2 = .002$), and also was not related with either sleep quality (PSQI global sleep quality score, $r = -.21$, $p = .29$, $R^2 = .044$),chronotype (MEQ score, $r = -.10$, $p = .63$, $R^2 = .01$) or the slope of the diurnal change in cortisol ($r = -.19$, $p = .35$, $R^2 = .036$).

3.4. Aim 3: Between-subject variability of mTAC: Perceived stress and individual differences in physiological stress reactivity

3.4.1. Perceived psychological stress and mTAC

Participants' perceived (chronic) stress score (PSS) was inversely associated with average mTAC (AUCg) (Figure 4a, ($r = -.34$, $p = .08$, $R^2 = .12$). Since 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, mean score = 23 ± 2.8) with the low/medium perceived (chronic) stress group (lower 2 tertiles PSS, mean score = 12 ± 3.2) there was a significant group effect of PSS on mTAC ($F_{1, 25} = 5.6$, $p = .026$, $\eta^2 = .18$), with individuals exposed to high perceived stress exhibiting significantly lower mTAC (**Figure 4b**).

3.4.2. Biological stress-reactivity and mTAC

Next, we examine 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 (percent change from before to 30 min after the TSST) more than 3 SD above the mean and was therefore excluded from the analysis. The cortisol and heart rate responses to the TSST were moderately inter-correlated ($r = .60$, $p = .002$, $R^2 = .36$).

The cortisol response to the TSST was negatively related to mTAC (AUCg) (**Figure 5a**, $r = -.57$, $p = .004$, $R^2 = .32$). The heart rate response also was negatively – although not statistically significantly - associated with mTAC (AUCg) (**Figure 5b**, $r = -.34$, $p = .12$, $R^2 = .12$).

3.5. Immune cell composition and mTAC

Some studies have suggested that basal [47] and stimulated [59] 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, RTA) for each percent change in cell type of the different immune cell subtypes modeled simultaneously for all three time points. None of the relationships were significant, suggestion 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 minimal change in immune cell subtypes and minimal cellular degradation), we determined that a *72h post mitogen stimulation* period appears to represent the *optimal time point* at which to quantify human leukoctye 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 1-month period accounted for 12% of the variation in the mTAC measure, with a 25% difference in mTAC between subjects reporting high compared to 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 intra-class coefficient (ICC) indicated substantially greater between-subject relative to within-subject variability. Collectively, these findings support our premise that the maximal leukocyte 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 to 5 days after mitogen stimulation replicates previous studies [26, 59]. 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 leukoctye 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 [48-50], suggesting that mTAC may reflect a stable individual difference characteristic. Discrepancies between these findings and those of the 2 studies that did find an effect of age on stimulated telomerase activity [51, 58] may perhaps be accounted for by differences in the mitogen stimulations 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 analyzed the total fraction of CD8+ T-cells and did not discriminate between naïve, 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 [67], indicating that the percentage of naïve 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* [47] showed that in total PBMC, senescent CD28- T-cells had lowest telomerase activity and shortest telomere length. CD28 signaling is required for optimal telomerase up-regulation, indicated by the paralleled loss of telomerase activity and CD28 expression in T-cells after chronic antigen stimulation *in vitro* [59].

Previous studies have reported that chronically stressed individuals exhibit lower levels of *unstimulated* telomerase activity (e.g., [7, 39-41]). 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* [42], showed no differences in induced PBMC or T-cell telomerase activity levels (anti-CD3/CD28 monoclonal antibody stimulation for 72 h) between caregivers 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 caregivers was operationalized using a depression scale, whereas we assessed perceived stress over the past month using the perceived stress scale.

Our protocol particularly stimulates cell types involved in cellular immunity, the part of the immune system that is initially affected by chronic stress exposure [68]. 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 [69], both activators of signaling pathways stimulating telomerase activity [9, 57]. 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 [40, 70]. 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 [29, 30]. Senescent CD28- T-cells show impaired up-regulation of telomerase activity after antigen stimulation [59], 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 [71]. Previous findings showed that greater cortisol responses to an acute stressor was associated with shorter telomere length, as was higher overnight urinary free cortisol levels and flatter daytime cortisol slope [72]. 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 [55]. Glucocorticoids place a limit on the maximal activity of the immune system, modulate inflammatory gene transcription [73], 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 [69], thereby influencing telomerase regulatory pathways [9, 57]. Cortisol levels measured during the chronobiology test day (test day 1) were not associated to 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*, in which basal/unstimulated telomerase activity was measured soon after a standardized stress test, the cortisol response was positively associated with telomerase activity [7]. As discussed by these authors, telomerase activity levels may change dynamically in response to stress, likely 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 the present 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 fibers, activate β2-receptors present on immune cells. *In vitro* studies suggest that β2-receptor stimulation by catecholamines activates the cAMP signaling pathway, inhibiting mitogen-stimulated T-cell proliferation and NK-cells activation [74]. Hence, catecholamines could also inhibit leukocyte mTAC after stimulation *in vitro*.

Strengths of the present 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 *vs* average of multiple samples remain an important consideration. Lastly, 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 hematopoietic 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 [50-54]. Based on our findings in support of the premise that the maximal leukocyte 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, on mTAC. Future studies should also explore the characteristics of mTAC in hematopoietic stem cells [75], 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.

5. Author's contributions

KdP coordinated the study, participated in the design of the study, carried out the laboratory assays, participated in the data analyses, and drafted the manuscript. CH participated in the data analysis and provided editorial assistance. IP performed the flow cytometry measurements and data analysis. PDW participated in the analysis and interpretation of study findings, drafted portions of the manuscript, and provided editorial assistance. SE 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.

6. Competing interests

We have no competing interests to declare.

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Tables

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 8 a.m.	**mTAC 1 p.m.**	**mTAC 7 p.m.**
mTAC 8 a.m.	1	.82**	.73**
mTAC 1 p.m.	.82**	1	.68**
mTAC 7 p.m.	.73**	.68**	1

**indicates p-value < .001

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 (SE)*	*p-value*
CD4+ T-cells	-.64 (.42)	.14
CD8+ T-cells	.92 (.80)	.26
B-cells	.28 (1.65)	.87
NK-cells	.15 (.82)	.86
NKT-cells	-.10 (.31)	.98
Monocytes	.52 (.53)	.34

Figure captions

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.

Figure 2. Time course experiment**:** a) mean relative PBMC telomerase activity (± SD) of 5 individual measured at day 1-8 after *in vitro* PHA/IL-2 stimulation and b) mean percentages (± SD) of immune cell subpopulations (calculated as percentages of CD45+ cells) of 5 individuals measured at day 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 (± SE) in stimulated PBMCs isolated at 8 p.m., 1 p.m. and 7 p.m. during Test Day 1, and *b*) before (-10 min) and after (+30 min, +90 min) the TSST (Test Day 2); RTA: relative telomerase activity.

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

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

Figure 1

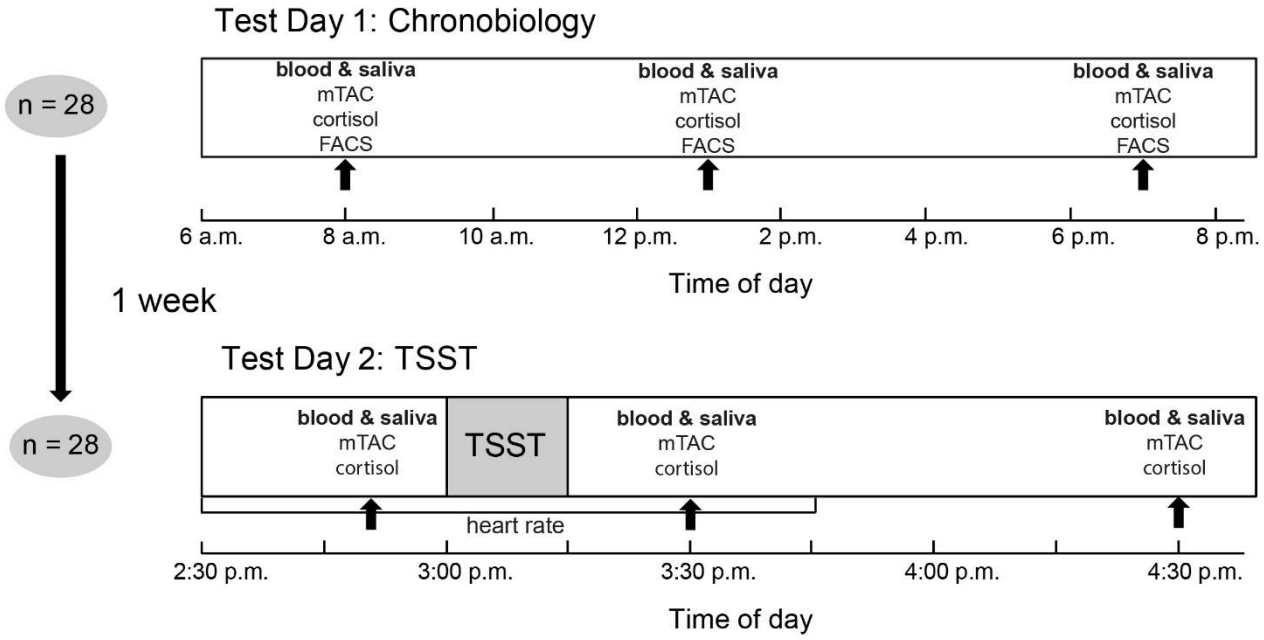

Test Day 1: Chronobiology
n = 28
blood & saliva
mTAC
cortisol
FACS
blood & saliva
mTAC
cortisol
FACS
blood & saliva
mTAC
cortisol
FACS
6 a.m.
8 a.m.
10 a.m.
12 p.m.
2 p.m.
4 p.m.
6 p.m.
8 p.m.
Time of day
1 week
Test Day 2: TSST
n = 28
blood & saliva
mTAC
cortisol
TSST
blood & saliva
mTAC
cortisol
blood & saliva
mTAC
cortisol
heart rate
2:30 p.m.
3:00 p.m.
3:30 p.m.
4:00 p.m.
4:30 p.m.
Time of day

Figure 2

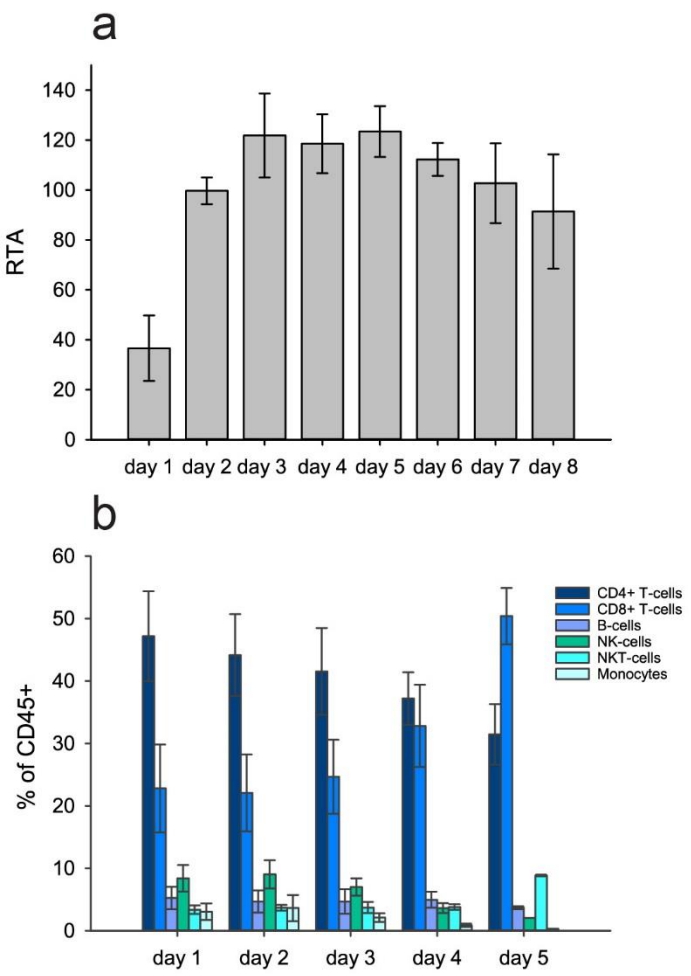

a
RTA
140
120
100
80
60
40
20
0
day 1 day 2 day 3 day 4 day 5 day 6 day 7 day 8
b
% of CD45+
60
50
40
30
20
10
0
day 1
day 2
day 3
day 4
day 5
CD4+ T-cells
CD8+ T-cells
B-cells
NK-cells
NKT-cells
Monocytes

Figure 3

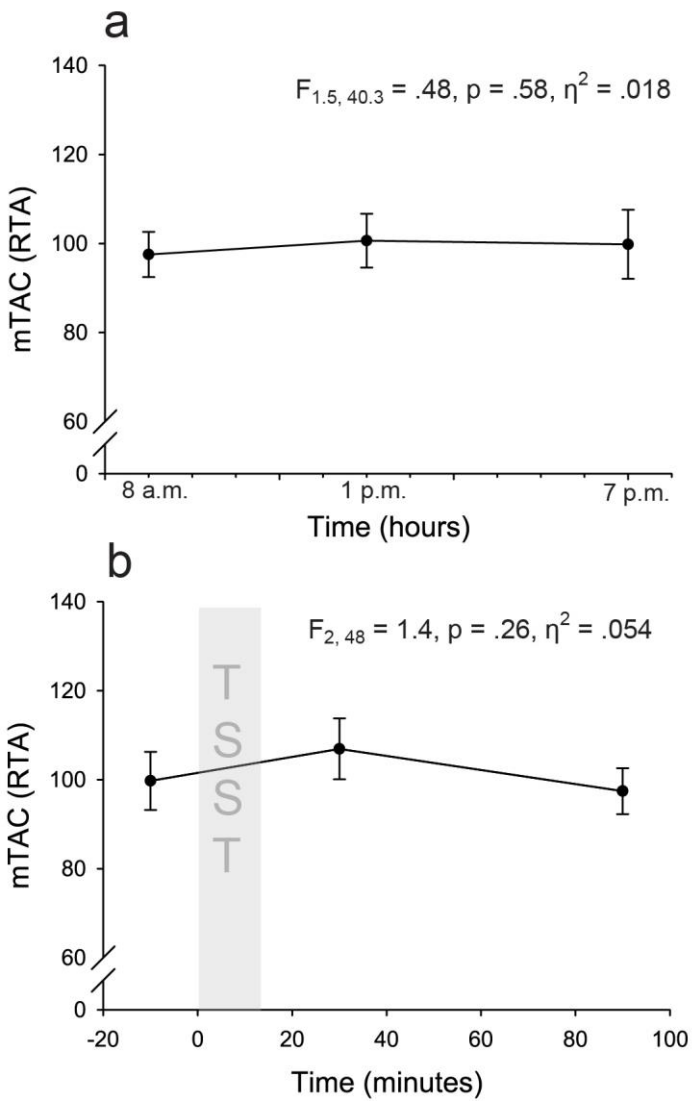

a
$F_{1.5, 40.3}$ = .48, p = .58, η^2 = .018
mTAC (RTA)
140
120
100
80
60
0
8 a.m.
1 p.m.
7 p.m.
Time (hours)
b
$F_{2, 48}$ = 1.4, p = .26, η^2 = .054
T
S
S
T
mTAC (RTA)
140
120
100
80
60
0
-20
0
20
40
60
80
100
Time (minutes)

Figure 4

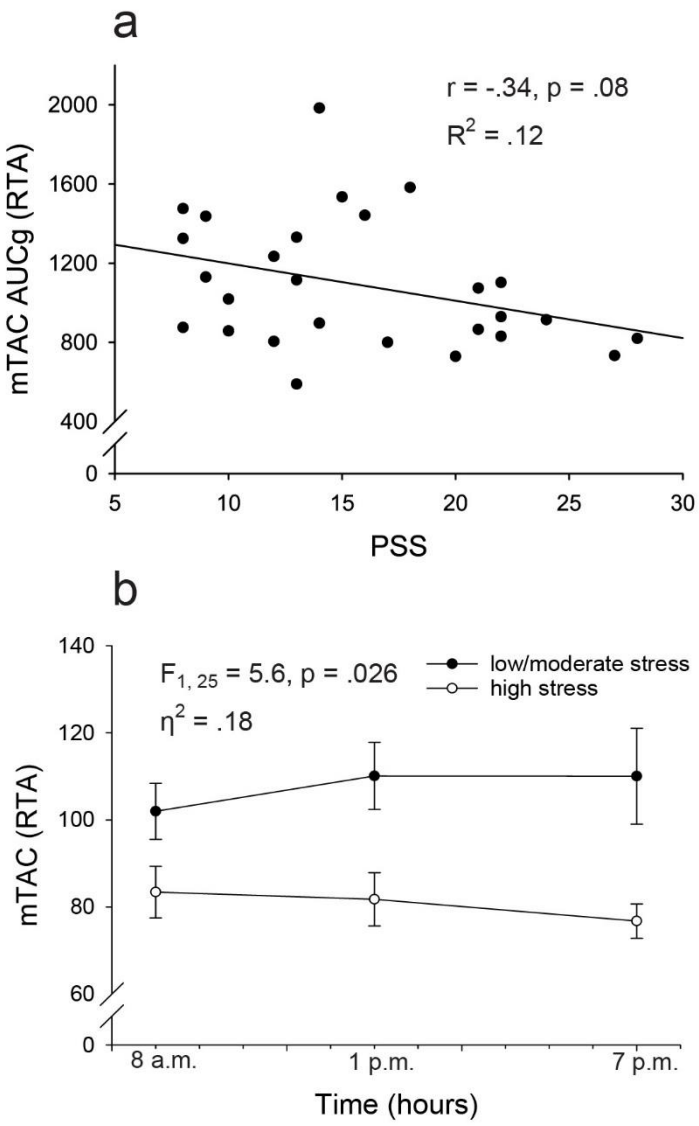
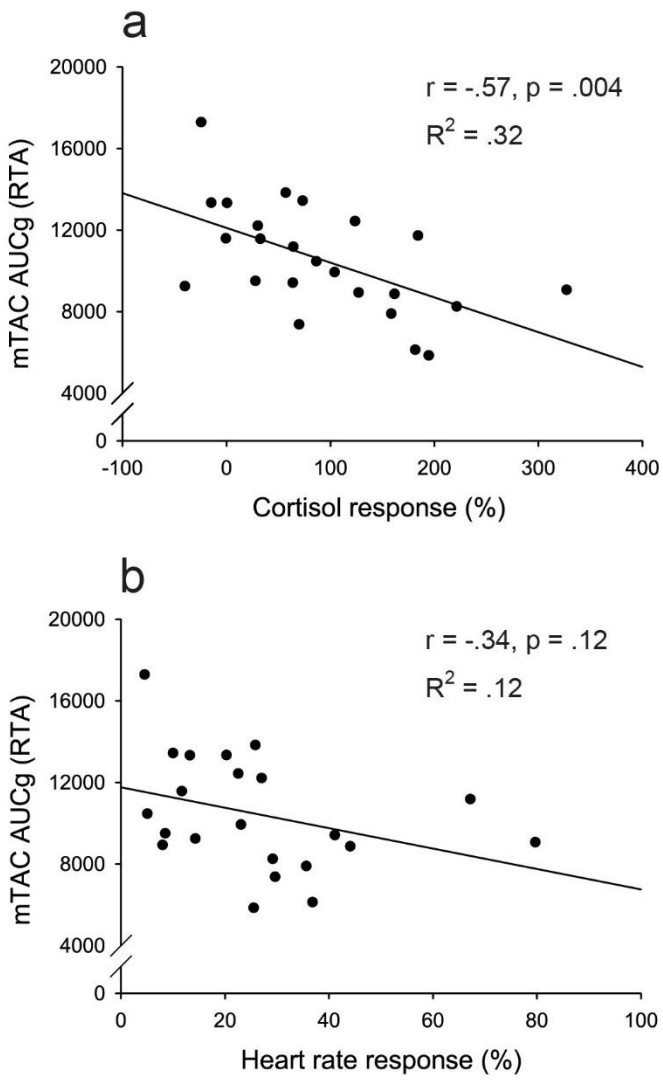

a
r = -.34, p = .08
R^2 = .12
mTAC AUCg (RTA)
2000
1600
1200
800
400
0
5
10
15
20
25
30
PSS
b
$F_{1, 25}$ = 5.6, p = .026
η^2 = .18
low/moderate stress
high stress
mTAC (RTA)
140
120
100
80
60
0
8 a.m.
1 p.m.
7 p.m.
Time (hours)

Figure 5


a
r = -.57, p = .004
R^2 = .32
mTAC AUCg (RTA)
20000
16000
12000
8000
4000
0
-100
0
100
200
300
400
Cortisol response (%)
b
r = -.34, p = .12
R^2 = .12
mTAC AUCg (RTA)
20000
16000
12000
8000
4000
0
0
20
40
60
80
100
Heart rate response (%)

Supplemental material

Supplemental material: Protocol for isolation, stimulation and lysis of PBMC

Materials (listed or equivalent)

BD Vacutainer 9NC (sodium citrate) 6ml (2x), Becton Dickinson (Franklin Lakes, USA), 366575

Falcon 50mL conical centrifuge tubes, Corning (Corning, USA), 352070

Falcon 15mL conical centrifuge tubes, Corning (Corning, USA), 352097

Sepmate-50 (or Sepmate-15 for smaller volumes of blood), Stemcell Technologies (Cologne, Germany), 15450

Ficoll, GE Healthcare Life Sciences (Little Chalfont, UK), 17-144-002

PBS ready to use (sterile), VWR International (Radnor, USA), K812-500 ml

Fetal bovine serum (FBS), HyClone (South Logan, USA), SH3007102

Dimethyl sulfoxide (DMSO, sterile), Sigma-Aldrich (Saint Louis, USA), D2438-5X10ML

Hemacytometer, Sigma-Aldrich (Saint Louis, USA), Z359629-1EA

Trypan blue, Sigma-Aldrich (Saint Louis, USA), T8154

RPMI-1640 medium, Gibco, Life Science Technologies (Carlsbad, USA), 21875-034

Phytohaemagglutinin (PHA), Sigma-Aldrich (Saint Louis, USA), L8754-1MG

Interleukin(IL)-2, Sigma-Aldrich (Saint Louis, USA), I7908-10KU

TeloTAGGG Telomerase PCR ELISA plus kit, Roche (Basel, Switzerland), 12013789001

Equipment

Biosafety cabinet

Centrifuge

Mr. Frosty freezing container with isopropanol

-80 °C freezer and/or liquid nitrogen freezer

Solutions

Freezing medium: FBS containing 10% DMSO

Culture medium: RPMI-1640 medium containing 10% FBS

PBMC isolation protocol

1. Procedures should be performed in a biosafety cabinet under sterile conditions.
2. Retrieve whole blood.

3. Dilute 1:1 with sterile PBS.
4. Fill Sepmate-50 tube with 15 ml Ficoll.
5. Carefully pipet the diluted blood on the Ficoll layer.
6. Spin down for 10 min at 1200 x g with the brake on.
7. Poor the supernatant within 2 sec in new 50 ml falcon tube and add PBS until 40 ml.
8. Spin down for 10 min at 300 x g (brake on), remove the supernatant and tap the bottom of tube to loosen the cell pellet.
9. Fill up the tube with 10 ml of PBS.
10. Spin down at 200 x g for 10 min (brake on), remove the supernatant and tap the bottom of tube to loosen the cell pellet.
11. Dissolve the pellet in exactly 1 ml of sterile PBS (mix thoroughly by resuspending).
12. Count cells and calculate the amount of cells/ml using a hemacytometer.
13. Spin down cells at 250 x g for 10 min (brake on), remove the supernatant and tap the bottom of tube to loosen the cell pellet.
14. Dissolve pellet in freezing medium (1 ml of freezing medium / 1 x 10^7 cells).
15. Divide the cells dissolved in the freezing medium over 2 (or more) sterile cryovials and freeze using a Mister Frosty at -80 ºC. Optional: Cryovials can be transferred to liquid nitrogen tank after 1 day.

PBMC stimulation protocol

1. Procedures should be performed in a biosafety cabinet under sterile conditions.
2. Thaw cells (quickly) in a 37 ˚C water bath and transfer each thawed cell suspension to an appropriately labeled 15 ml tube and add pre-warmed (37 ˚C) culture medium diluting 10 x the original volume.
3. Centrifuge at 250 x g for 10 minutes at RT, remove the supernatant and tap the bottom of tube to loosen the cell pellet.
4. Resuspend cells in exactly 1 ml of culture medium.
5. Count live cells using a hemacytometer.
6. Dilute the cells in the appropriate amount of culture medium in order to obtain a cell concentration of exactly 1 x 10^6 cells/ml.
7. Pipet 1 ml of cell suspension (1 x 10^6 cells/ml) in the well of a 12-well plate; so each well contains 1 x 10^6 cells.
8. Add 5 µl of Il-2 (10 KU/ml) per ml/well (final concentration = 50 units/ml).
9. Dissolve 1 mg of PHA (Sigma-Aldrich) in 1 ml of PBS (stock concentration = 1 mg/ml).

10. Add 10 µl of PHA (1 mg/ml) per ml/well (final concentration = 10 µg/ml) and incubate 72 hours at 37C° and 5% CO2.

Lysis protocol

1. Procedures should be performed under nuclease free conditions.
2. Take out lysis reagent (Roche TeloTAGGG Telomerase PCR ELISA plus kit) from freezer and let it thaw to 4 ºC. Keep on ice.
3. Label the appropriate amount of sterile (RNase free) 1.5 ml Eppendorf tubes.
4. Remove cells from the wells with a 1000 µl pipet (resuspend a few times to remove aggregates) and place into Eppendorf tubes.
5. Spin down tubes at 300 x g for 10 min, RT, remove the supernatant and tap the bottom of tube to loosen the cell pellet.
6. Dissolve pellet in exactly 1 ml of PBS and carefully resuspend and/or shortly vortex.
7. Count live cells with hemacytometer.
8. Transfer a volume corresponding to exactly 200,000 cells to the corresponding Eppendorf tube:

*required cell volume (µl) = total volume (1000 µl) * 200,000 / total amount of cells*

9. Cool centrifuge and spin down the 200.000 cells for 5 min, 3000 x g at 4 ºC, remove supernatant and keep pellet on ice.
10. Dissolve pellet in 200 µl of cold lysis buffer (Roche TeloTAGGG Telomerase PCR ELISA plus kit).
11. Resuspend pellet and shortly vortex, keep on ice for 30 min.
12. Centrifuge for 20 min, 16.000 x g at 4 ºC.
13. Carefully remove 175 µl of the supernatant, transfer in a sterile labeled (RNase free) 1.5 ml Eppendorf tube and store at -80 ºC.
14. Telomerase activity is measured in the lysates following the instructions of the Roche TeloTAGGG Telomerase PCR ELISA plus kit.

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

List of Publications

Original and Review Articles

Karin de Punder, Christine Heim, Ingo Przesdzing, Pathik D. Wadhwa, Sonja Entringer (2018). Characterization in humans of *in vitro* leukocyte maximal telomerase activity capacity (mTAC) and association with stress. *Philos Trans R Soc Lond **B** Biol Sci*. 5;373(1741) pii: 20160441. Impact factor: 5,846

Sonja Entringer, **Karin de Punder**, Claudia Buss, Pathik D. Wadhwa (2018). The fetal programming of telomere biology hypothesis: An update. *Philos Trans R Soc Lond **B** Biol Sci*. 5;373(1741) pii: 20170151. Impact factor: 5,846

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Karin de Punder, Leo Pruimboom (2015). Stress induces endotoxemia and low-grade inflammation by increasing barrier permeability. *Front. Immunol.* 6:223. Impact factor: 6,429

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Daleke-Schermerhorn MH, Felix T, Soprova Z, Ten Hagen-Jongman CM, Vikström D, Majlessi L, Beskers J, Follmann F, **de Punder K**, van der Wel NN, Baumgarten T, Pham TV, Piersma SR, Jiménez CR, van Ulsen P, de Gier JW, Leclerc C, Jong WS, Luirink J (2014). Decoration of outer membrane vesicles with multiple antigens by using an autotransporter approach. *Appl Environ Microbiol*. 80(18):5854-65. Impact factor: 3,807

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Diane Houben, Caroline Demangel, Jakko van Ingen, Jorge Perez, Lucy Baldeón, Abdallah M. Abdallah, Laxmee Caleechurn, Daria Bottai, Maaike van Zon, **Karin de Punder**, Tridia van der Laan, Arie Kant, Ruth Bossers-de Vries, Peter Willemsen, Wilbert Bitter, Dick van Soolingen, Roland Brosch, Nicole van der Wel and Peter J. Peters (2012). ESX-1 mediated translocation to the cytosol controls virulence of mycobacteria. *Cell Microbiol.*14(8):1287-98. Impact factor: 4,554

Eveline M. Weerdenburg, Abdallah M. Abdallah, Suman Mitra, **Karin de Punder**, Nicole N. van der Wel, Steve Bird, Ben J. Appelmelk, Wilbert Bitter, Astrid M. van der Sar (2011). ESX-5-deficient Mycobacterium marinum is hypervirulent in adult zebrafish. *Cell Microbiol*.14(5):728-39. Impact factor: 4,554

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Book Chapters

Sonja Entringer, **Karin de Punder**, Glenn Verner, Pathik D. Wadhwa (2017). Fetal programming of telomere biology: Role of maternal nutrition, obstetric risk factors and suboptimal birth outcomes in Diet, nutrition, and fetal programming, Nutrition and Health pp.569-593. *Springer International Publishing AG*.

Published Abstracts

Karin de Punder, Christine Heim, Ingo Przesdzing, Pathik D. Wadhwa, Sonja Entringer (2017). Characterization of *in vitro* leukocyte maximal telomerase activity capacity (mTAC) as a stress-related measure for human studies. *Psychoneuroendocrinology.* 83S:1-89

Karin de Punder, Judith Overfeld, Peggy Dörr, Katja Dittrich, Sibylle M. Winter, Nina Kubiak, Gergana Karaboycheva, Christine Heim (2017). Maltreatment is associated with elevated C-reactive protein levels in 3 to 5 year-old children. *Brain Behav Immun.* 66:e17-e18

Karin de Punder, Christine Heim, Pathik D.Wadhwa, Sonja Entringer (2017). Characterization of *in vitro* leukocyte maximal telomerase activity capacity (mTAC) in humans: Association with chronic stress exposure and stress-reactivity. *Brain Behav Immun.* 66:e17

Maaike van Zon, **Karin de Punder**, Astrid van der Sar, Roland Brosch, Rogelio Hernandez Pando, Anita Grootemaat, Wikky Tigchelaar-Gutter, Nicole van der Wel (2017). Subcellular localization of *M. tuberculosis in vivo* and effect of the adaptive immunity. *Ultrastruct Pathol.* 41(1):133

Karin de Punder, Judith Overfeld, Peggy Dorr, Katja Dittrich, Sibylle M. Winter, Nina Kubiak, Gergana Karaboycheva, Christine Heim (2016). Exposure to child maltreatment is associated with elevated stress and immune mediators in children aged 3–5 years. *Psychoneuroendocrinology.* 71S:1–78

Karin de Punder, Christine Heim, Pathik D.Wadhwa, Sonja Entringer (2016). *In vitro* stimulated leukocyte telomerase activity is associated with chronic stress exposure. *Psychoneuroendocrinology.* 71S:1–78

Judith Overfeld, Karin Nadig-Haynes, **Karin de Punder**, Peggy Dorr, Katja Dittrich, Sibylle M. Winter, John-Dylan Haynes, Claudia Buss, Christine Heim (2016). Hippocampal volume in children aged 3–5 years with verified maltreatment exposure. *Psychoneuroendocrinology.* 71S:1–78

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Acknowledgements

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