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1 ORIGINAL ARTICLE

Alterations in the telomere length distribution and the subtelomeric methylation status in human vascular endothelial cells under elevated temperature in culture condition

Toyoki Maeda · Guan Jing-Zhi ·
Masamichi Koyanagi · Naoki Makino

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Abstract Temperature-associated alteration in the telomere lengths of vascular endothelial cells has not been well investigated. Telomere length of human umbilical vein endothelial cells (HUVECs) cultured at a high temperature (42 °C) was analyzed. Here described are heat-associated phenotypical alterations of human vascular endothelial cell under prolonged heat stress in terms of telomere length, telomerase activity, and the expression of telomere associated proteins and heat shock proteins. The genomic DNA extracted from HUVECs cultured for 3 days under 42 °C was digested with methylation-sensitive and -insensitive isoschizomers and was subjected to genomic Southern blot probed with a telomere DNA fragment. Their telomere lengths and telomere length distributions were analyzed. Telomerase activity and the expressions of telomere-associated RNA, telomere-associated proteins (TERC, TERT, TRF1, and TRF2), and heat shock proteins (Hsp60, Hsp70, and Hsp90) were also analyzed. At 42 °C, cell growth was suppressed and the cell senescence rate was transiently elevated. A proportional decrease in the number of long telomeres was observed transiently at 42 °C. A trend of subtelomeric hypomethylation and lowered telomerase activity were observed at 42 °C after 3-day culture. The altered phenotypes on day 1 seemed reactive responses for cell protection to heat, and those on day 3 seemed exhausted reactions after 3-day culture. Maintained expression was observed in Hsps, TRF2, and TERC. These altered phenotypes might contribute to cell-survival under prolonged heat stress.

Keywords Heat stress · Vascular endothelial cell · Telomere · Subtelomere · DNA methylation

A1 T. Maeda and G. Jing-Zhi equally contributed to this article.

T. Maeda (✉) · M. Koyanagi · N. Makino
The Department of Cardiovascular, Respiratory and Geriatric Disease, Kyushu University Beppu Hospital, Beppu, Oita 874-0838, Japan
e-mail: maedat@beppu.kyushu-u.ac.jp

G. Jing-Zhi
The 309th Hospital of Chinese People's Liberation Army, Beijing, China

Introduction

Telomeres consist of repetitive DNA sequences with accessory protein components (TRF1, TRF2, and others) capping the terminals of chromosomes [1]. It is well known that telomere DNA shortening occurs during every cell cycle due to the duplication process that produces slightly shorter DNA strands. In addition, the DNA methylation status, one of the genomic epigenetic conditions, in telomeric region has been reported to alter in response to human telomere length changes [2–6]. Telomere length in somatic cells is negatively affected by stress factors [6]. Both pathological mental and physical stress accelerate telomere attrition [6]. Telomere shortening occurs in somatic cells with aging due to the occurrence of many rounds of the cell cycle and pathological stress [7]. On the other hand, a telomere-elongating cellular mechanism functions in limited cases. Telomerase consists of a protein component composed of reverse transcriptase (TERT) and an RNA component (TERC). Telomerase contributes to telomere elongation or telomere length maintenance in unique cell populations with active mitotic potential, such as cancer cells, stem cells, and reproductive cells. Generally, however, the telomere activity is suppressed to low levels in somatic cells and is not adequate to prevent the

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telomere shortening that occurs during cell division. Telomerase activity is also affected by stress factors. However, the effects of heat stress on telomere length, subtelomeric methylation status, or telomerase activity have not been well studied. Human vascular endothelial cells have been used to analyze pathological stress-related changes in cell biology [8–10]. Yet there have been no reports that address the telomeric changes in vascular endothelial cells occurring under heat stress. Although the effects of transient heat stress on cells have been well investigated, no reports evaluating prolonged heat stress lasting more than 1 day have been published. Heat stress in vascular endothelial cells also induces the expression of various kinds of stress response genes, representatively, heat shock proteins (Hsps) [11, 12]. Hsps are ubiquitously synthesized in virtually all species and it is hypothesized that they might have beneficial health effects [13]. In response to stress stimuli, mammalian cells activate a signaling pathway leading to the transient expression of Hsp. Hsps are a family of proteins serving as molecular chaperones that prevent the formation of nonspecific protein aggregates and assist proteins in the acquisition of their native structures. Physiologically, Hsps play a protective role in the homeostasis of the vessel wall consisting of endothelial cells and smooth muscle cells [14]. We analyzed telomere DNA length, telomerase activity, and the expressions of telomere-associated components, and heat shock proteins under heat stress conditions using human vascular endothelial cells exposed for 1 day or longer to heat in culture.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA, USA). The cells were cultured in endothelial cell growth medium (Clonetics Corp.) at 37 or 42 °C and 5 % CO_2 in a gelatin-coated flask (Iwaki Glass, 2 Chiba, Japan). Culture media were refreshed every 24 h. On day 1 or 3, the cells were collected and were counted using a hemocytometer. Population doublings (PDs) were calculated using the formula: PD = [log (expansion)/log2], where expansion was the number of cells harvested divided by the initial number of cells seeded.

Senescence-associated β-galactosidase (SA-β-Gal) expression

The cells were washed in PBS, fixed for 10 min at room temperature in 2 % formaldehyde/0.2 % glutaraldehyde, and incubated at 37 °C (no CO_2) with fresh SA-β-Gal staining solution containing 1 mg/mL of X-gal, pH 6.0 for 12 h. One hundred cells were scored from each well (plate) using a light microscope.

Telomere detection

Telomere detection was performed as previously described [4]. Briefly, cell DNA (0.1 μg) was digested with methylation-insensitive or -sensitive isoschizomers, *Msp*I or *Hpa*II, at 37 °C for 2 h, and was subjected to Southern blot hybridization probed with telomere DNA $(TTAGGG)_n$. The autoradiogram was captured on an Image Master, and the telomere length was then assessed quantitatively (Fig. 1). Every sample was measured in triplicate.

Terminal restriction fragment (TRF) length analysis

Telomere length distribution was analyzed by comparing the telomere length using a telomere percentage analysis with three intervals of length (>9.4, 9.4–4.4 and <4.4 kb) as defined by a molecular weight standard as previously described [4]. The percent of the stratified intensity in each molecular weight range of a Southern blot result smear was measured for each sample. The mean TRF was estimated using the formula $S(OD_i - \text{background})/S(OD_i - \text{background}/L_i)$, where OD_i

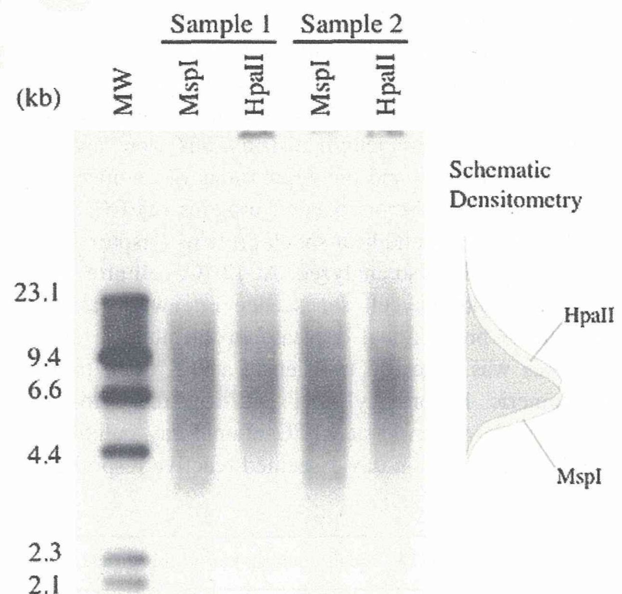

Fig. 1 Densitometric analysis of the isoschizomeric TRFs of *Msp*I- and *Hpa*II-digest. Representative Southern blot results of two samples digested with *Msp*I or *Hpa*II are shown. *Gray areas* on the *right* side depict *Hpa*II-densitometry and *Msp*I-densitometry, respectively

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is the chemiluminescent signal and L_i is the length of the TRF fragment at position i.

Semiquantitative RT-PCR for TERC RNA

Total RNA samples were extracted using RNAzol B (Tel-test). mRNA for human telomerase RNA component (TERC) was determined by RT-PCR using a DIG detection system (Roche Applied Science). Each human cDNA was produced by RT-PCR according to each human-derived sequence. For the amplification of β-actin cDNA, the forward primer β-actin (205 bp) 5′-CCTTCCTGGGCATGGA GTCCT-3′ and the reverse primer 5′-GGAGCAATGATCT TGATCTTC-3′ were used according to the published human TERC cDNA sequence [15]. And TERC forward primer 5′-TCTAACCCTAACTGAGAAGGGCGTAG-3′ and the reverse primer 5′-GTTTGCTCTAGAATGAACGG TGGAAG-3′ were used [16]. The values for TERC mRNA levels were normalized to the β-actin mRNA level in the same sample. The PCR products were directly synthesized from 2 μg of total RNA isolated from each sample using the Superscript one-step RT-PCR system with Platinum *Taq* (Invitrogen) and gene-specific primers according to the recommendations provided by the supplier. The PCR products were amplified through 15 cycles of chain reaction where the amplification is at an exponential phase. The PCR products were analyzed by agarose gel electrophoresis (1.3 %) followed by staining with ethidium bromide and scanning with Gel-Doc (Bio-Rad). For semiquantitative PCR, β-actin was used as an internal control to evaluate total RNA input, as described by our group [17].

Western blot and other analyses

Cells from a dish were homogenized with 100-μl lysis buffer (100 mM Tris pH 6.8, 4 % SDS, 20 % glycerol containing the protease inhibitor, M phenylmethanesulfonyl fluoride, 0.1 mM, leupeptin, 0.1 μl, and aprotinin, 0.1 μl). Gel electrophoresis was used to separate 10-μg protein on a 10 % SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (162-0112, Bio-Rad Laboratories, Hercules, CA, USA) blocked with 5 % dry milk or blocking solution for Western blot (Roche). Membranes were blocked and incubated with antibodies against telomerase reverse transcriptase (TERT) (Rockland), TRF1 (Imgenex), TRF2 (Cell Signaling), Hsp60 (Assay designs), Hsp70 (Assay designs), Hsp90 (Enzo), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology). Detection was performed with secondary horseradish peroxidase-conjugated antibodies (Chemicon) and the ECL detection system as previously described [18]. The relative expression levels were determined compared to that of GAPDH.

Telomerase activity

Telomerase activity was examined by means of a modified telomerase repeat amplification protocol (TRAP) method with TeloChaser (Toyobo, Osaka, Japan), as previously described [19].

Statistical analysis

Assays were repeated three times and analyzed statistically. The normality of the data was examined with the Kolmogorov–Smirnov test and the homogeneity of variance with the Levene Median test. If both the normal distribution and equal variance tests were passed, intergroup comparisons were performed using a two-way analysis of variance (ANOVA) test followed by all pairwise multiple comparison procedures using Tukey's post hoc test. The data are expressed as the mean ± standard deviation. The criterion for the significance is $p < 0.05$.

Results

Population doubling (PD) and cell senescence

The PD value of the HUVECs was assessed on day 1 and day 3 of culture. At 37 °C, the PD value increased to ~1.6 on day 1 and was found to be maintained at that level on day 3. At 42 °C, the PD value initially increased to ~1.2; however, it decreased steeply to ~0.2 on day 3 (Fig. 2a).

Senescence-associated β-galactosidase (SA-β-Gal) expression was observed in ~2 % of cells on day 1 and ~1 % of cells on day 3 at 37 °C and in ~4 % of cells on day 1 and ~2 % of cells on day 3 at 42 °C (Fig. 2b).

Such an initial increase and delayed decrease of cell senescence rate indicated that many cells entered cell senescence stage followed by cell death 3-day-cultured under heat. The proportion of senescent cells in the cell population which survived for 3-day heat of 42 °C was not less than that at 37 °C for 3 days. This indicates that the heat-sensible cells diminished on day 1 and day 3 at 42 °C, and the heat-tolerant cells remained on day 3 at 42 °C.

The mean TRF level and its distribution

The mean TL of the HUVECs was measured to assess the degree to which high-temperature conditions affect telomeric DNA (Fig. 3a). The mean *Msp*I-TRF values were 9.2 ± 1.0 kb at 37 °C for 1 day, 8.7 ± 0.3 kb at 37 °C for 3 days, 8.3 ± 1.0 kb for 1 day at 42 °C, and 8.9 ± 0.6 kb for 3 days at 42 °C. The mean *Hpa*II-TRF values were 10.1 ± 0.7 kb at 37 °C for 1 day, 9.6 ± 0.3 kb at 37 °C for 3 days, 9.3 ± 1.1 kb at 42 °C for 1 day, and 9.0 ± 1.0 kb

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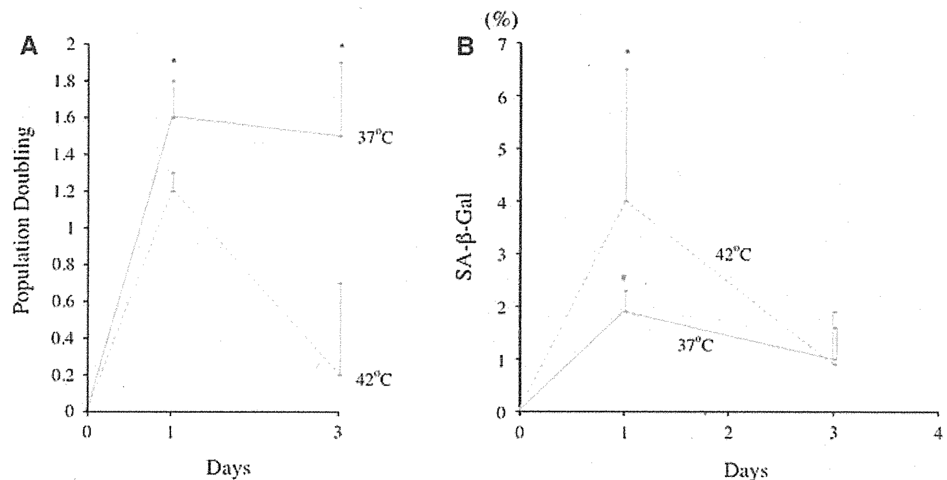

Fig. 2 The population doubling (PD) and the ratio of senescence-associated β-galactosidase (SA-β-Gal) staining of HUVECs cultured in the presence of H_2O_2. **a** The PD on day 1 and day 3 at 37 and 42 °C. The *horizontal bars* are standard deviations. $^{*}p < 0.05$, at 37 vs. 42 °C. **b** The percentages of SA-β-Gal-positive cells. $^{*}p < 0.05$, at 37 vs. 42 °C. $^{\#}p < 0.05$, on day 1 vs on day 3

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at 42 °C for 3 days. The subtracted *Hpa*II–*Msp*I TRF values were 0.9 ± 1.0 kb at 37 °C for 1 day, 0.9 ± 0.4 kb at 37 °C for 3 days, 1.0 ± 0.9 kb at 42 °C for 1 day, and 0.1 ± 0.7 kb at 42 °C for 3 days. Thus, the mean TRFs did not altered at 42 °C, whereas the subtracted TRF *Hpa*II–*Msp*I was lower at 42 °C (0.1 ± 0.7 kb) than at 37 °C (0.9 ± 0.4 kb) on day 3 ($p = 0.04$). The % intensity of telomere length distribution (>9.4, 9.4–4.4, <4.4 kb) was as follows: 51 ± 8, 47 ± 6, 2 ± 3 % of *Msp*I at 37 °C for 1 day, 46 ± 1, 51 ± 2, 3 ± 2 % of *Msp*I at 37 °C for 3 days, 39 ± 11, 59 ± 10, 2 ± 1 % of *Msp*I at 42 °C for 1 day, 49 ± 5, 48 ± 3, 4 ± 2 % of *Msp*I at 42 °C for 3 days, 63 ± 5, 36 ± 5, 1 ± 1 % of *Hpa*II at 37 °C for 1 day, 60 ± 1, 38 ± 2, 2 ± 2 % of *Hpa*II at 37 °C for 3 days, 52 ± 14, 47 ± 14, 2 ± 2 % of *Hpa*II at 42 °C for 1 day, and 58 ± 5, 37 ± 3, 6 ± 4 % of *Hpa*II at 42 °C for 3 days. (Fig. 3b, c) The difference between the telomere length distribution between *Msp*I and *Hpa*II was as follows: 12 ± 6, −11 ± 4, −1 ± 4 % at 37 °C for 1 day, 14 ± 1, −13 ± 2, −1 ± 2 % at 37 °C for 3 days, 13 ± 5, −13 ± 5, 0 ± 3 % at 42 °C for 1 day, 9 ± 4, −11 ± 2, 2 ± 3 % at 42 °C for 3 days, >9.4, 9.4–4.4, <4.4 kb, respectively (Fig. 3d). The telomere length was affected significantly in *Msp*I-distribution and in *Hpa*II–*Msp*I-subtracted distribution. At 42 °C on day 1, long telomeres (>9.4 kb) decreased ($p = 0.02$) and middle-sized telomeres (9.4–4.4 kb) increased ($p = 0.03$). The amount of short telomeres (<4.4 kb) was not significantly affected. These changes in TL distribution disappeared on day 3, suggesting that cells bearing altered telomere length distribution diminished up to day 3. The alteration of subtelomeric methylation status appeared on day 3, which is a trend of subtelomeric hypomethylation of long telomeres ($p = 0.02$) (Fig. 3d).

Telomerase activity

The telomerase activity of the HUVECs was evaluated at 37 °C and 42 °C using TRAP assays (Fig. 4). The average value of TPG at 37 °C was set at 1 (1 ± 0.66 for 1 day and 1 ± 0.2 for 3 days). The relative levels of TPG at 42 °C were 1.32 ± 0.84 on day 1 and 0.57 ± 0.28 on day 3. Therefore, the relative level of telomerase activity in the HUVECs at 42 °C was maintained on day 1 ($p = 0.71$); however, it significantly decreased on day 3 ($p = 0.03$). The level of telomerase activity decreased under prolonged heat stress at 42 °C.

Expression of telomere-associated components and others

All telomere-associated components (TERT, TRF1 and TRF2) except TERC were upregulated on day 1 and downregulated on day 3. The expression of TERC did not seem to be affected by heat (Fig. 5; Table 1).

All analyzed heat shock proteins (Hsp60, Hsp70 and Hsp90) were upregulated on day 1 and downregulated on day 3 at 42 °C. However, only Hsp70 maintained a significantly higher expression level at 42 °C than at 37 °C.

Discussion

Biological stress has been reported to induce negative effects on the maintenance of telomere length in various cells [1, 20]. However, heat stress-associated telomeric changes have not been well investigated thus far. In the present study, vascular endothelial cells were used to analyze telomere-associated alterations induced at 42 °C.

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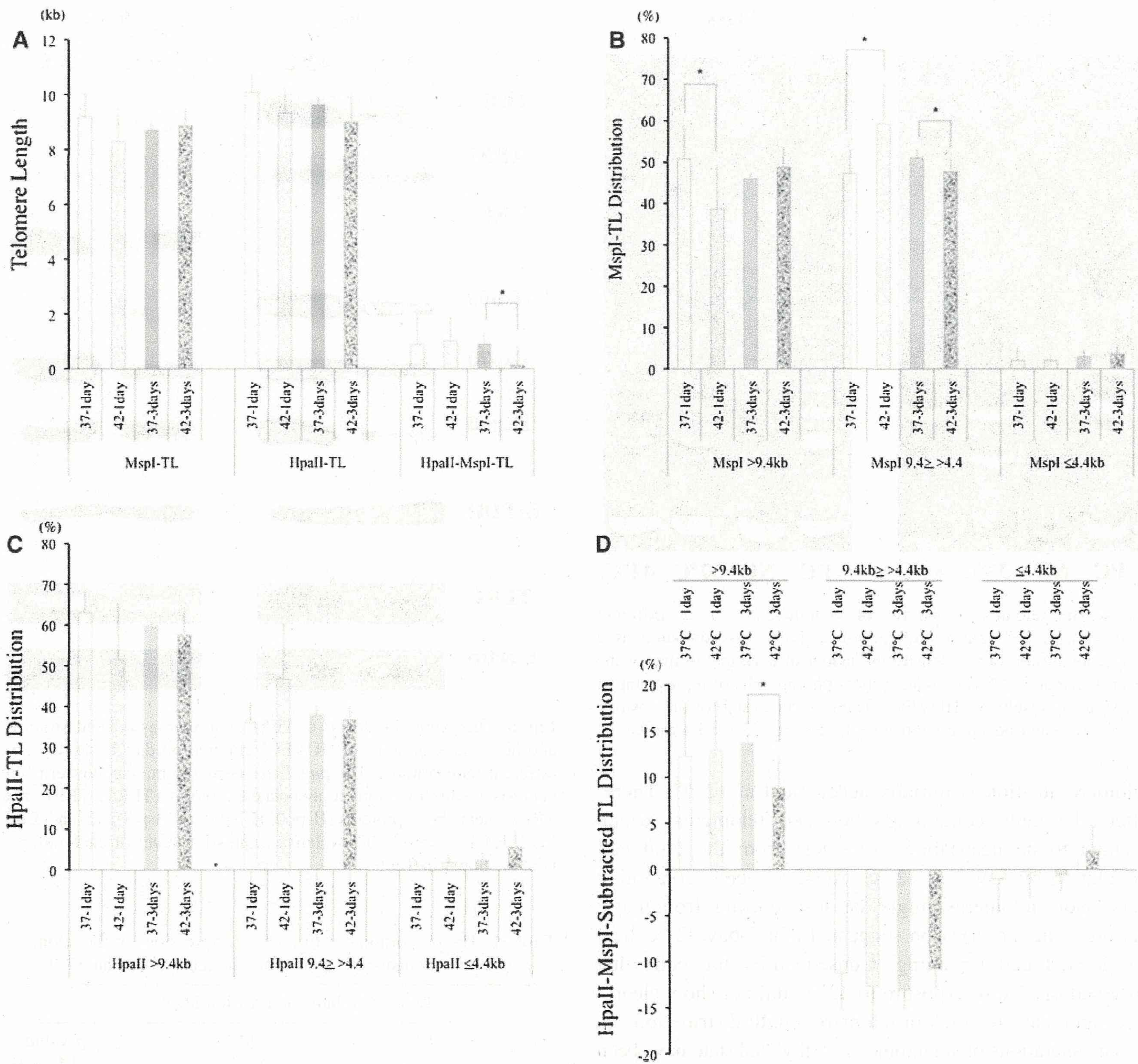

Fig. 3 The telomere length, the telomere length distribution of HUVECs exposed at different temperatures. The mean telomere lengths (**a**) and the telomere length distributions (**b–d**) are shown. The *Hpa*II–*Msp*I-subtracted percentages of telomere length range indicated (>9.4, 9.4–4.4, or <4.4 kb) are shown. Notice that only the longest telomere range (>9.4 kb) showed a significant difference between 37 and 42 °C (**d**). The *horizontal bars* represent the standard deviation. The terminal restriction fragment lengths are presented as the mean values ± standard deviation. The *horizontal bars* represent the standard deviation. A significant difference was observed between the control cells at 37 °C and those at higher temperatures. $*p < 0.05$ vs at 37 °C

289 Cell growth was found to be suppressed at 42 °C, espe-
290 cially on day 3. The proportion of senescent cells increased
291 on day 1 at 42 °C, then returned to a low level as observed
292 at 37 °C on day 3. This observation indicates that the heat-
293 sensitive cell population decreased in size during the 3-day
294 exposure to 42 °C and the heat-resistant population sur-
295 vived beyond day 3. To the best of our knowledge, this is
296 the first report to assess alterations in telomere length
297 distribution under prolonged heat stress. Furthermore, the
298 effects on cells of transient heat stress have been reported
299 to occur within several hours; however, no reports have
300 evaluated prolonged heat stress lasting more than 1 day.

301 In the present study, the telomere length and the subt-
302 elomeric methylation status were analyzed in heat-exposed
303 cultured cell, to assess whether the heat stress suppresses or
304 accelerates aging-associated phenotypes. Regarding telo-
305 mere length distribution, the number of long telomeres
306 decreased and the number of medium-sized telomeres
307 (4.4–9.4 kb) increased at 42 °C on day 1. These telomeric
308 changes disappeared by day 3. These results indicate that

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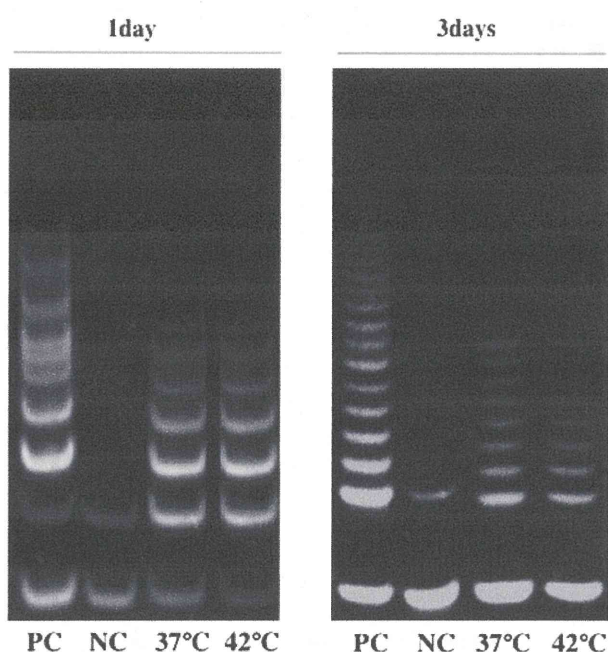

Fig. 4 The telomerase activity of endothelial cells at different temperatures. The relative telomerase activity was compared as a proportional ratio of the density the ladder of a sample to that of the mean value at 37 °C. The *panels* show photographs of representative TRAP assay results for HUVECs. The materials used for the positive control (pc) and negative control (nc) were provided with the kit

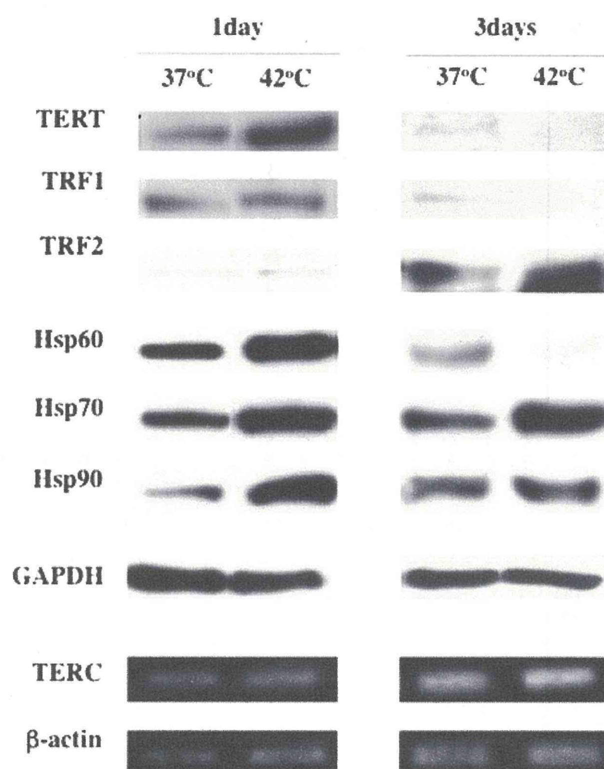

Fig. 5 The expression levels of TERC, telomere-associated proteins, and heat shock proteins of HUVECs cultured on day 1 and day 3 at different temperatures. The *panel* shows representative Western blot analysis results of telomere-associated proteins (TERT, TRF1, and TRF2), heat shock proteins (Hsp60, Hsp70, and Hsp90), and a TERC RNA RT-PCR result. The relative expression level of each component is shown in Table 1

309 telomere attrition is initially accelerated at 42 °C. There-
310 after, the cells containing shortened telomeres, which
311 seemed to be heat-labile, were lost from the total cell
312 population by day 3 at 42 °C. At 42 °C, cells containing
313 very short telomeres might be lost starting from early
314 periods. This observation suggested that 3-day 42 °C heat
315 accelerated cellular aging. Consequently, the cells that
316 survived the 3-day exposure to 42 °C did not show clearly
317 any significant features in telomere length distribution.
318 The alterations of subtelomeric methylated state have been
319 observed along with aging-associated telomeric changes in
320 human peripheral leukocytes. The decrease of long telomeres
321 with hypomethylated subtelomere and the increase of short
322 telomeres with hypomethylated subtelomere have been
323 observed as a typical aging-associated telomeric change [4–6].
324 In this study, the observed heat-induced subtelomeric hy-
325 pomethylation status on day 3 seemed to be a young pattern,
326 suggesting that old cells, which were heat-labile, were elimi-
327 nated during the 42 °C heat exposure for 3 days. Cells having
328 survived after the heat-exposure showed a young pattern of
329 subtelomeric methylation status. The heat exposure of 42 °C
330 firstly accelerated aging-associated telomeric changes and
331 finally eliminated the cells bearing the old pattern of telomeric
332 status.
333 The difference of expression pattern between TRF1 and
334 TRF2 can also be associated with the altered subtelomeric

Table 1 The proportional values of the expressions of the telomere-associated components and heat shock proteins at 42 and 37 °C

	42/37 °C-relative expression level		
	1d	3d	p value (1d vs 3d)
TERT	1.80 ± 0.32*	0.25 ± 0.14*	<0.01
TERC	0.95 ± 0.27	1.86 ± 1.66	0.44
TRF1	1.43 ± 0.19	0.44 ± 0.25	<0.01
TRF2	3.93 ± 1.45*	1.19 ± 1.07	0.06
Hsp60	2.35 ± 0.58*	0.17 ± 0.11	0.02
Hsp70	2.73 ± 0.08*	1.46 ± 0.08	<0.01
Hsp90	3.35 ± 1.43	0.89 ± 1.46	0.08

1d 1 day, *3d* 3 days

* $p < 0.05$, at 42 vs 37 °C

335 methylation status after 3-day exposure to heat. At 42 °C,
336 TRF1 was down-regulated but TRF2 was not. The heat-
337 induced subtelomeric hypomethylation might occur along
338 with the lowered expression level of TRF1. TRF1 has been
339 reported to negatively control the telomerase-associated

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340 telomere length maintenance [21, 22], while TRF2 is associated with stabilizing the telomere structure [23, 24]. From this context, the lowered expression of TRF1 would be beneficial for maintaining telomere length under high-temperature conditions. In addition, the disequilibrium of the expression level between TRF1 and TRF2 might lead to structural change of telomere. This might induce unstable telomere structure accompanying demethylation of genomic DNA neighboring to telomere. In addition, it has been reported that heat shock can elicit a transient alteration of the higher-order structure of specific heterochromatic regions and induce the transcriptional activation of silent portions of the genome [25]. The same mechanism could be applied to the subtelomeric region under heat stress and consequently lead to hypomethylated status there. The prolonged high-temperature condition of 42 °C for 3 days seemed to impair various kinds of protein expression and damage cells. We showed here the phenotypical characteristics of the survived cells through a prolonged heat stress condition, i.e., maintained telomere length, subtelomeric hypomethylation of long telomeres, maintained TERC expression, and maintained TRF2 expression. TRF2 has been believed to play key roles in telomere maintenance [26, 27]. A recent report suggests roles for TRF2 protein in DNA repair in addition to chromatin reorganization and telomere maintenance [28]. TRF2 has also been suggested to protect young neurons against death induced by DNA-damaging agents [29]. Thus, TRF2 affects cell survival and differentiation by modulating DNA damage pathways, and gene expression, and the elevated expression of TRF2 could be beneficial for cell survival. Other than telomere-associated components, Hsps were also affected by heat exposure. The difference of protein expression levels drastically changed from day 1 at 42 °C. Some of these responses seemed to contribute to cell protection against heat stress. All analyzed proteins were upregulated on day 1 at 42 °C. This indicated an acute reactive response to heat stress. Protein expression activated at 42 °C on day 1 was preserved in TRF2 and Hsp70 on day 3. TERC expression also remained activated. Hsp90 expression was moderately preserved on day 3. Hsp70s function as molecular chaperones, assisting in protein synthesis, folding, assembly, trafficking between cellular compartments, and degradation [30, 31]. They are expressed constitutively and induced in response to various types of stress, including heat shock, ischemia, oxidative stress, glucose deprivation, and exposure to toxins [32]. Hsp70 protects cellular elements from injury by reducing oxidation, inflammation and apoptosis and by refolding damaged proteins. The results of the present study suggested that the expression of Hsp70 conferred survival advantages under prolonged heat exposure. Hsp70 increases also in response to heat shock in the cardiovascular system [33]. Hsp70 rapidly accumulates after heat shock and can increase as much as eightfold in rat hearts after whole animal heat shock [34, 35]. In fact, Amrani et al. [36] have suggested that the increase in rat hearts after whole animal heat shock occurs primarily in the vascular endothelium, which is associated with improved recovery of endothelial function from cardioplegic arrest. Leger et al. [37] indicated that the primary site of Hsp70 induction after whole animal heat shock is in the blood vessels. Hsp70 improves the viability of stressed vascular smooth muscle cells, possibly via its chaperone functions [38]. The beneficial effects of Hsp70 on cell viability demonstrated in the present study may also provide survival advantages for stressed vascular endothelial cells. Maintained upregulation of Hsp90 might also support cell survival. Heat shock protein 90 (Hsp90) is induced in response to cellular stress and stabilizes client proteins involved in cell cycle control and proliferative/anti-apoptotic signaling. Tanespimycin, an Hsp90 inhibitor, reduces tumour cell survival in vitro. In multiple myeloma, Hsp90 inhibition affects multiple client proteins that contribute to tumour cell survival, including elements of the PI3/Akt, STAT3, and MAPK signalling pathways. Hsp90 inhibition also abrogates the protective effect of bone marrow stromal cells and inhibits angiogenesis and osteoclastogenesis [39]. Thus, maintained expression of some proteins under prolonged heat observed in the present study are potentially able to support heat-tolerance. In summary, prolonged heat stress conditions such as those that occur at 42 °C for 3 days give rise to cell damage with transient aging-like alterations in length distribution and subtelomeric methylation. Cell survival under prolonged heat stress may be associated with the maintenance of upregulation of TRF2, Hsp70, and Hsp90. Further study is necessary to elucidate the relationship between these factors and the cell survival mechanism through prolonged heat shock. 428

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Conflict of interest None.

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シンポジウム1

1-1. 別府市高齢者における温泉利用の実態と既往歴との関連の調査について

前田豊樹
九州大学病院別府病院　内科

The Relationship Between Hot-spring Bathing and Medical History of aged Beppu Citizens

Toyoki MAEDA
Kyushu University Hospital, The department of Internal Medicine

目的：別府は、世界に冠たる温泉地域で、約2,300の源泉が存在する。一地域の温泉源泉の数としては世界一を誇る。また別府には泉質全11種類のうち10種類が温泉入浴に利用されており、これほど多種類の温泉が密集している地域は世界的にも希有である。九州大学病院別府病では、この地の利を生かし、超高齢化が進む本邦において、歴史ある温泉治療の高齢者慢性疾患への治療応用に向けて、温泉の医学的効用を確認する研究を開始した。これは、65歳以上の別府市民を対象に、既往疾患とこれまでの温泉の利用状況をアンケートにより調査して、病気に対する温泉の予防効果を確認するものである。なお、本研究は、別府市、別府市医師会、九州大学病院別府病院が協働で進めており、厚生労働省の循環器疾患・糖尿病等生活習慣病対策総合研究事業（温泉利用が健康づくりにもたらす総合的効果についてのエビデンスに関する研究）の一環である。

方法：平成24年11月現在、別府市在住の65歳以上の高齢者が約35,000人で、うち20,000人を無作為に選びアンケート用紙を郵送して調査した。アンケートは、年齢、性別、利用してい

温泉と健康に関するアンケート

次の質問にお答え下さい。あてはまる番号に ○ をつけて下さい。カッコ内はわかる範囲で書き込んで下さい。また不明の場合は、「不明」とお書き下さい。

問1　性別

①男性　②女性

問2　年齢

①65～69歳　②70～74歳　③75～79歳　④80～84歳　⑤85歳以上

問3　日頃温泉を利用していますか。（※週1回以上温泉を利用される方は ① に ○ をつけて下さい。）

①はい　②いいえ（沸かし湯など）　⇒問4から問9もご回答下さい。

↓

「①はい」と答えた方にお尋ねします。それはどのような温泉ですか。
ア・イ・ウ・エから 1つ選んで下さい。

ア）温泉施設（温泉名　　　　）例：市営○○温泉・区営□□温泉
民間温泉施設など

イ）自宅（自家泉源）（泉質　　　　）
※泉質がわかれば記入して下さい。

ウ）自宅（引き湯）（お住まいの町名　　　　）
次の該当する給湯会社名等に ○ をつけて下さい。

①日本地熱興業（株）　②（財）別府市綜合振興センター
③泉都温泉給湯（株）　④杉乃井リゾート（株）
⑤（株）岡本製作所（ラクテンチ）　⑥鉄輪温泉管理組合
⑦その他（　　　　）　⑧わからない

エ）その他（マンション等の共同浴場など）（泉質　　　　）
※泉質がわかれば記入して下さい。

問4　温泉の利用頻度について（※ほとんど温泉に入らない方は ① に ○ をつけて下さい。）

①月に1回以下　②週1回　③週に2～3回　④週4～5回　⑤日に1回以上

問5　入浴時間（浴槽につかっている時間の1日の合計）

①10分以下　②11～20分　③21～30分　④31分以上

問6　温泉の利用期間（※日頃温泉を利用していない方は ① に ○ をつけて下さい。）

①10年未満　②10～20年未満　③20～30年未満　④30～40年未満
⑤40年以上

問7　入浴する時間帯

①午前9時より早い　②9～12時　③13～18時　④19時～

問8　今までかかったすべての病気に ○ をつけてください。
（ここ1年で新たにかかった病気には ◎ をつけて下さい。）

①がん　②心筋こうそく・狭心症　③不整脈　④高血圧　⑤脳卒中　⑥痛風
⑦ぜんそく　⑧糖尿病　⑨高脂血症　⑩じん臓病　⑪うつ病　⑫慢性肝炎
⑬膠原病（関節リウマチなど）　⑭アレルギー（病名　　　　）
⑮その他の病気（病名　　　　）

問9　がんにかかった方にお尋ねします。かかったすべてのがんに ○ をつけて下さい。
（ここ1年でかかったがんは ◎ をつけて下さい。）

①胃　②大腸　③すい臓　④肝臓　⑤乳房　⑥子宮　⑦卵巣　⑧肺
⑨喉頭　⑩甲状腺　⑪皮膚　⑫じん臓　⑬ぼうこう　⑭前立腺
⑮脳しゅよう　⑯白血病・悪性リンパ腫　⑰骨や軟骨
⑱その他のがん（病名　　　　）

※アンケートに対するご意見やコメントなどございましたらご記入下さい。

ご協力ありがとうございました。

Fig. 1　温泉と健康に関するアンケート

る温泉の種類、利用頻度、一回入浴時間、利用年数、利用時間帯と、15項目に及ぶ既往疾患を多肢選択してもらう形式で行った（Fig. 1)。H25年2月末の時点で、11,146通（55.7%）の回答を受け取った。現時点での解析結果を報告する。得られた回答について対象既往疾患としては、今回本邦の3大疾病である、がん、虚血性心疾患、脳卒中に絞って解析した。温泉利用状況については、温泉利用頻度、一回入浴時間、利用年数を解析対象とした。

結果：温泉利用頻度については、毎日入浴する人が5022人、そうでない人が5404人であり、がん、虚血性心疾患、脳卒中の既往者はそれぞれ587人（11.7%)、331人（6.6%)、109人（2.2%）と652人（12.1%)、411人（7.6%)、171人（3.2%）であった（Fig. 2)。がん、虚血性心疾患、脳卒中のいずれにおいても、毎日温泉入浴群で既往が少ない傾向にあったが、とくに虚血性心疾患と脳卒中の既往は、毎日温泉入浴する群で有意に低かった。一回入浴時間では、20分以上の7182人と20分未満の3582人の2群で比較すると、がん、虚血性心疾患、脳卒中の既往者はそれぞれ848人（11.8%)、542人（7.5%)、182人（2.5%）と408人（11.39%)、221人（6.2%)、105人（2.9%）であった（Fig. 3)。このうち虚血性心疾患の既往者は、20分以上の温泉入浴者で有為に少なかった。さらに、温泉利用年数についても解析したが、3疾患の既往者の割合と利用年数の間に一定の関係は見いだされなかった。

考察：虚血性心疾患と脳卒中における、温泉利用頻度ならびに一回入浴時間の関連から、連日、一定時間以上温泉入浴することで、全身の血管の拡張により循環動態が好ましい状態に保たれ、梗塞性疾患を減らした可能性を伺わせる一方、現時点では、脳卒中や心筋梗塞を患ったために温泉入浴頻度や入浴時間が減った可能性も除外できない。また、このことから類推される温泉利用期間と循環器疾患の既往との関係は見いだせなかった。今後は、こういったことを区別すべく、性別、年齢層を区切って再検討するなどのほか、入浴している温泉の種類、すなわち泉質ごとにどのような傾向があるかを探り、温泉独自の効果の検証を進める必要がある。

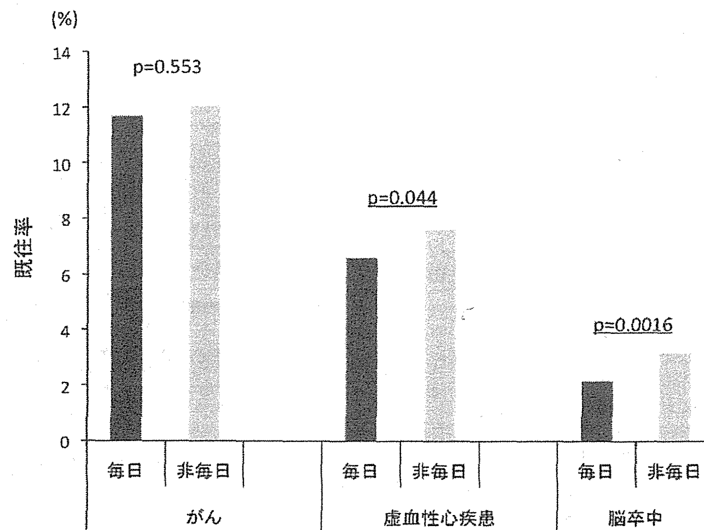

Fig. 2　毎日温泉利用者と非毎日温泉利用者における3大疾病の既往率の比較

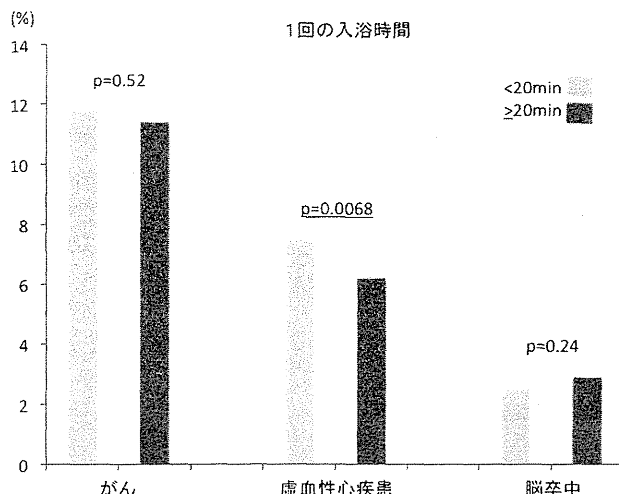

Fig. 3　一回温泉入浴時間が 20 分以上と 20 分未満の群間の 3 大疾病の既往率の比較

シンポジウム 1

1-2. 炭酸ガス入浴剤と健康

工藤道誠

花王株式会社　HHC 研究センター
パーソナルヘルスケア研究所

Health and Artificial CO_2 Bathing Agent

Michinari KUDOH

KAO Corporation R&D-Personal Health Care Research

【目的】

炭酸ガス入浴剤は炭酸ガスの血管拡張作用に起因する温浴効果により疲労回復、冷え症の緩和をはじめとした多くの効果・効能が報告されているが、発汗機能への影響は知られていない。一般的に、ヒトが繰り返し暑熱環境に暴露されると体温調節能力に適応的変化が生じ、熱放散能が向上（発汗機能の向上）する結果、より大きな暑熱負荷に耐えられるようになることが知られている（暑熱順化）が、日常のリラックスを目的とした入浴では暑熱負荷の強度が低いため、発汗機能の亢進は起こらないとされている。そこで、本研究においては炭酸ガスの皮膚からの浸透を促進する技術を用いた炭酸ガス高浸透入浴剤の温浴効果及びその連浴が発汗機能に及ぼす影響を明らかにすることを目的とした。

【方法】

1. 炭酸ガス高浸透入浴剤の温浴効果試験

健常人男性 5 名を対象に炭酸ガス高浸透入浴剤を用いて、40℃/10 分間、全身浴を行わせ、レーザードップラー血流計にて皮膚血流量、温度ロガーにて直腸温、発汗計にて発汗量の測定を行った。

2. 炭酸ガス高浸透入浴剤の連浴試験

健常人男性 10 名（23.2 ± 1.4 歳）を対象に炭酸ガスの皮膚からの浸透を促進する技術を用いた炭酸ガス入浴剤を用いて、4 週間の連浴（40℃/10 分間、全身浴）を行わせた。連浴前（0 週）、連浴開始後 4 週間の時点で、40℃/10 分間、炭酸ガス入浴剤の全身浴にて発汗量、鼓膜温を測定した。発汗の測定は、発汗波カプセルを左前胸部、上腕部に装着した後、カプセル内を窒素ガスで換気した後、カプセルから流出する窒素

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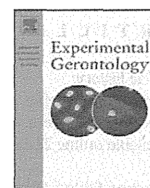

Gender and telomere length: Systematic review and meta-analysis ☆

Michael Gardner [a], David Bann [b], Laura Wiley [c], Rachel Cooper [b], Rebecca Hardy [b], Dorothea Nitsch [d], Carmen Martin-Ruiz [c], Paul Shiels [e], Avan Aihie Sayer [f], Michelangela Barbieri [g], Sofie Bekaert [h], Claus Bischoff [i], Angela Brooks-Wilson [j], Wei Chen [k], Cyrus Cooper [f], Kaare Christensen [i], Tim De Meyer [h], Ian Deary [l], Geoff Der [m], Ana Diez Roux [n], Annette Fitzpatrick [o], Anjum Hajat [n], Julius Halaschek-Wiener [j], Sarah Harris [l], Steven C. Hunt [p], Carol Jagger [c], Hyo-Sung Jeon [q], Robert Kaplan [r], Masayuki Kimura [s], Peter Lansdorp [t], Changyong Li [u], Toyoki Maeda [v], Massimo Mangino [w], Tim S. Nawrot [x], Peter Nilsson [y], Katarina Nordfjall [z], Giuseppe Paolisso [g], Fu Ren [u], Karl Riabowol [aa], Tony Robertson [m], Goran Roos [z], Jan A. Staessen [x], Tim Spector [w], Nelson Tang [ab], Brad Unryn [aa], Pim van der Harst [ac], Jean Woo [ad], Chao Xing [ae], Mohammad E. Yadegarfar [c], Jae Yong Park [q], Neal Young [af], Diana Kuh [b], Thomas von Zglinicki [c], Yoav Ben-Shlomo [a,*], the Halcyon study team

[a] *School of Social and Community Medicine, University of Bristol, Canynge Hall, Bristol, UK*
[b] *MRC University Unit for Lifelong Health and Ageing, University College London, UK*
[c] *Institute for Ageing and Health, Newcastle University, UK*
[d] *Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, UK*
[e] *Institute of Cancer Sciences, University of Glasgow, UK*
[f] *Medical Research Council Lifecourse Epidemiology Unit, University of Southampton, UK*
[g] *Department of Geriatric and Metabolic Diseases, Second University of Naples, Italy*
[h] *Clinical Research Center, Ghent University, Belgium*
[i] *Danish Aging Research Center, University of Southern Denmark, Denmark*
[j] *Genome Sciences Centre, BC Cancer Agency, British Columbia, Canada*
[k] *Tulane Center for Cardiovascular Health, Tulane University Health Sciences, New Orleans, United States*
[l] *Centre for Cognitive Ageing and Cognitive Epidemiology, University of Edinburgh, UK*
[m] *Medical Research Council/Chief Scientist Office Social and Public Health Sciences Unit, Glasgow, UK*
[n] *Department of Epidemiology, Center for Integrative Approaches to Health Disparities, University of Michigan, United States*
[o] *Department of Epidemiology, University of Washington, Seattle, United States*
[p] *Cardiovascular Genetics Division, University of Utah School of Medicine, Salt Lake City, United States*
[q] *Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University Daegu, Republic of Korea*
[r] *Department of Epidemiology and Population Health, Albert Einstein College of Medicine, New York, United States*
[s] *The Center of Human Development and Aging, University of Medicine and Dentistry of New Jersey, United States*
[t] *Division of Hematology, Department of Medicine, University of British Columbia, Vancouver, Canada*
[u] *Department of Anatomy, Liaoning Medical University, Liaoning Province, People's Republic of China*
[v] *Department of Molecular and Cellular Biology, Kyushu University, Oita, Japan*
[w] *Twin Research and Genetic Epidemiology Unit, King's College London, UK*
[x] *Division of Hypertension and Cardiovascular Rehabilitation, Department of Cardiovascular Sciences, University of Leuven, Belgium*
[y] *Department of Clinical Sciences Medicine, University Hospital, Malmö, Sweden*
[z] *Department of Medical Biosciences, Umeå University, Sweden*
[aa] *Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Calgary, Canada*
[ab] *Department of Chemical Pathology, Faculty of Medicine, The Chinese University of Hong Kong, People's Republic of China*
[ac] *Department of Cardiology, University Medical Center Groningen, The Netherlands*
[ad] *Department of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong, People's Republic of China*
[ae] *Eugene McDermott Center for Human Growth and Development, University of Texas Southwestern Medical Center, United States*
[af] *National Heart, Lung, and Blood Institute, Bethesda, United States*

☆ Précis: There is an association between gender and telomere length, with females having longer telomeres than males.

* Corresponding author at: School of Social and Community Medicine, University of Bristol, 39 Whatley Road, Canynge Hall, BS8 2PS, UK. Tel.: +44 117 928 7206; fax: +44 117 928 7325.
E-mail address: Y.Ben-Shlomo@bristol.ac.uk (Y. Ben-Shlomo).

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ABSTRACT

Background: It is widely believed that females have longer telomeres than males, although results from studies have been contradictory.
Methods: We carried out a systematic review and meta-analyses to test the hypothesis that in humans, females have longer telomeres than males and that this association becomes stronger with increasing age. Searches were conducted in EMBASE and MEDLINE (by November 2009) and additional datasets were obtained from study investigators. Eligible observational studies measured telomeres for both females and males of any age, had a minimum sample size of 100 and included participants not part of a diseased group. We calculated summary estimates using random-effects meta-analyses. Heterogeneity between studies was investigated using sub-group analysis and meta-regression.
Results: Meta-analyses from 36 cohorts (36,230 participants) showed that on average females had longer telomeres than males (standardised difference in telomere length between females and males 0.090, 95% CI 0.015, 0.166; age-adjusted). There was little evidence that these associations varied by age group ($p = 1.00$) or cell type ($p = 0.29$). However, the size of this difference did vary by measurement methods, with only Southern blot but neither real-time PCR nor Flow-FISH showing a significant difference. This difference was not associated with random measurement error.
Conclusions: Telomere length is longer in females than males, although this difference was not universally found in studies that did not use Southern blot methods. Further research on explanations for the methodological differences is required.

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1. Introduction

Telomeres are nucleoprotein complexes at chromosome ends, where the DNA component is a repetitive stretch of (TTAGGG), which caps and protects the end of the chromosome. Some studies have found that shorter telomeres are associated with obesity (Nordfjall et al., 2008a), gender (Bekaert et al., 2007), lower socioeconomic position (Cherkas et al., 2008), smoking (Valdes et al., 2005) and mortality (Cawthon et al., 2003). Hence telomere length has been proposed as a useful index of biological age (Hunt et al., 2008), although this has been called into question (von Zglinicki, 2012). The present study focuses on the association with gender.

In the literature there are inconsistencies in the association between gender and telomere length. Some studies (Nawrot et al., 2004; Bekaert et al., 2007; Fitzpatrick et al., 2007) have found white blood cell telomeres to be longer in women than men. Several hypotheses have been postulated to explain this association (Nawrot et al., 2004; Mayer et al., 2006; Barrett and Richardson, 2011). One is the action of oestrogen (Mayer et al., 2006). An oestrogen-responsive element is present in telomerase reverse transcriptase (hTERT) (Nawrot et al., 2004), hence oestrogen might stimulate telomerase to add telomere repeats to the ends of chromosomes. Furthermore, telomeres are particularly sensitive to oxidative stress (von Zglinicki, 2002) and women produce fewer reactive oxygen species than men (Nawrot et al., 2004). It has been suggested that women might also metabolise reactive oxygen species better because of oestrogen (Nawrot et al., 2004), due to its antioxidant properties (Carrero et al., 2008). However, other studies have found that it is not always the case that telomere length is longer in females than males (Hunt et al., 2008; Shiels et al., 2011) or even the reverse (Adams et al., 2007). At birth, one study found that there was little difference in telomere length between the sexes (Okuda et al., 2002), but another study found that female newborns had longer telomeres than males (Aubert et al., 2012). In another study, (Hunt et al., 2008) no difference was detected in the telomere length of women and men in the younger Bogalusa Heart Study cohort (19–37 years), but in the older Family Heart Study cohort (30–93 years) telomeres were longer in women than men. Hence the association between gender and telomere length might vary by age. Whilst telomere length is inversely related to chronological age in humans (Shiels et al., 2011), there are concerns about how robust telomere length is as a biomarker of ageing (Shiels, 2010; Shiels et al., 2011).

Existing studies of the association of gender and telomere length in humans have a number of limitations. For example, some of the studies are small e.g. (Benetos et al., 2001) and hence may not have sufficient power to detect gender differences in telomere length. Furthermore, there are methodological differences between assay methods (Aviv et al., 2006), with Southern Blot providing a mean terminal restriction length for DNA fragments containing the telomeric DNA stretch plus sub-telomeric regions of variable length and sequence composition and real-time PCR measuring actual telomere -repeat length relative to a reference gene (Aviv et al., 2006). The most frequent cell types used in studies on telomere length are either whole blood (leukocytes made up of lymphocytes, monocytes and granulocytes) or peripheral blood mononuclear cells (PBMCs made up of lymphocytes and monocytes). In adults, lymphocytes have shorter telomeres than granulocytes (Aviv et al., 2006), hence it is important to assess whether the association between gender and telomere length varies by cell type. A literature search and qualitative meta-analysis (Barrett and Richardson, 2011) found that at a qualitative level, males tended to have shorter telomeres than females. However, no systematic review of the literature has been done to examine the association between gender and telomere length.

We carried out a systematic review and meta-analyses to test the hypothesis that in human populations females have longer telomeres than males and that this association becomes stronger with increasing age. Furthermore, we also investigated whether the association between gender and telomere length varied by method of measurement of telomere length or cell type. Our study has several advantages over the earlier review: (a) our study is a systematic review and meta-analysis; (b) has standardised effect estimates; (c) has more rigorous methodology including exploration of sources of heterogeneity. We hypothesised that there would be an association between gender and telomere length, with females having longer telomeres than males and that this association would become stronger with increasing age.

2. Methods

We undertook a systematic review of the published literature following the meta-analysis of observational studies in epidemiology (MOOSE) guidelines (Stroup et al., 2000) and the PRISMA statement (Moher et al., 2009) and we include a completed PRISMA checklist (Supplementary Data 1). Full review protocol is available in Supplementary Data 2.

2.1. Selection criteria

Eligible observational studies had a minimum number of 100 participants and measures of telomere length for both males and females.

Participants were those of any age (from newborns through to oldest old) who were community dwelling at time of measurement of telomere length and who were not part of a diseased group (including healthy controls from case-control studies). Hence we excluded cancer tissue studies with measurements of telomere length and those control participants who were recruited from hospital in-patients.

2.2. Literature search and data extraction

Searches of the electronic databases MEDLINE and EMBASE (up to November 2009) were performed using text word search terms and explosion MeSH terms (Supplementary Data 2) by MG. Searches were restricted to studies of humans. Fig. 1 shows the identification of published studies. Combining the results of the MEDLINE and EMBASE electronic searches and removing duplicate records left abstracts of 6822 unique records to be screened by three authors (DB, MG and LW). Each of the three authors (DB, MG and LW) were responsible for screening one-third of the titles and abstracts of these 6822 papers and any considered to be 'uncertain' were independently screened by a second author. Sixty-one research papers were retrieved for full data extraction using a standardised data extraction form. Two authors (from DB, MG and LW) independently extracted the information and data of relevance from each of the 61 papers and any differences between the two sets of information extracted were resolved through discussion. The standardised data extraction included methods of measurement of telomere length, cell type, details of measurement error, details of sample recruitment, descriptive characteristics and unadjusted, age-adjusted and fully-adjusted measurements of differences in telomere length between females and males.

2.2.1. Data requested from eligible studies

We contacted the corresponding authors of the 61 eligible published research papers and asked them to complete standardised results tables or provide the data for us to analyse (Supplementary Data 3). We requested the regression coefficient representing the age-adjusted difference in telomere length between females and males, along with corresponding standard error. We asked that the study authors calculate regression coefficients using linear regression with raw telomere length as the outcome variable. We also requested that the study authors provide the mean age and telomere length (plus standard deviations) of males, females and total sample. After sending out one reminder, we received responses from 24 of the published research

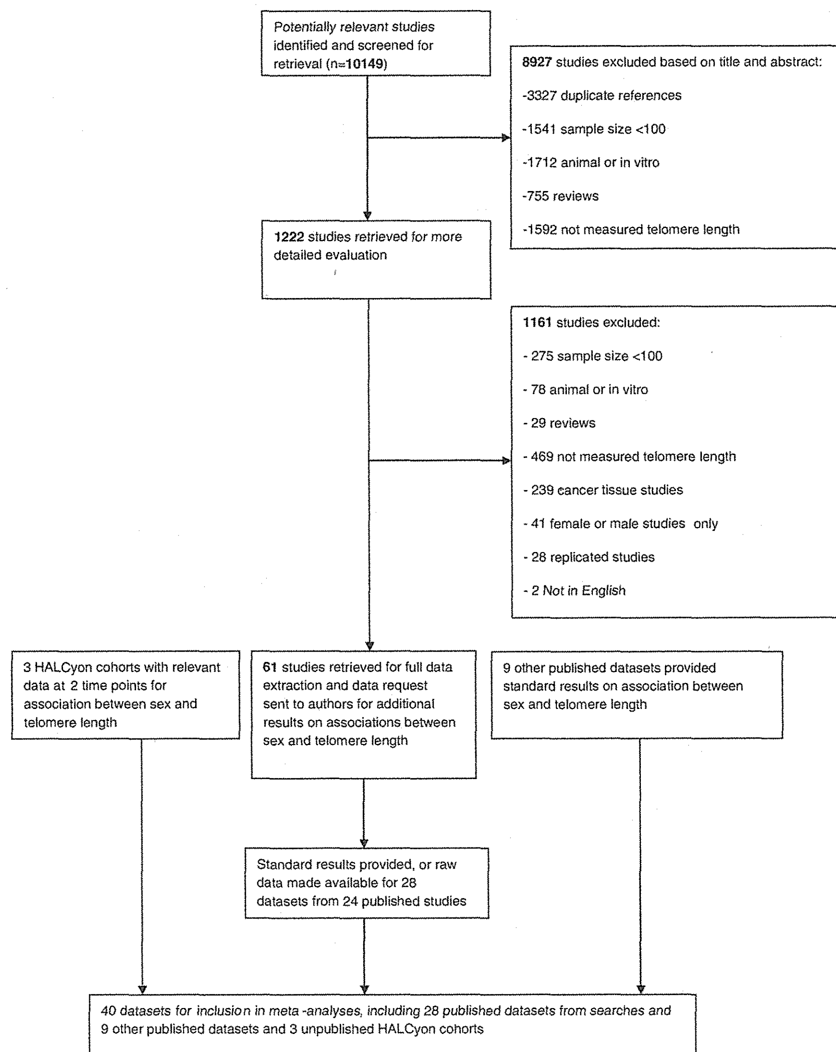

Fig. 1. Flow diagram for identification of published studies and for showing contact with authors for published and unpublished studies for inclusion in review.

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papers (28 datasets) (Fig. 1). Authors of 17 research papers provided standard sets of results and authors of another 7 research papers provided datasets for us to analyse.

2.3. Inclusion of other studies

2.3.1. HALCyon cohort studies

We included unpublished data from three of the nine UK cohort studies involved in the HALCyon collaboration. These were the Hertfordshire Ageing Study (HAS) (Syddall et al., 2010), the Lothian Birth Cohort 1921 (LBC1921) (Deary et al., 2004) and the MRC National Survey of Health and Development (NSHD) (Kuh et al., 2011). These studies had telomere length data available at two time points up to 10 years apart.

2.3.2. Other relevant studies

We contacted the corresponding author of an additional study identified through the literature search (Yamaguchi et al., 2005) which included at the time unpublished reference group telomere length data. The corresponding author subsequently provided us with the published reference group telomere length data (Aubert et al., 2012) for both granulocytes and lymphocytes. Furthermore, following e-mail contact with the corresponding authors of the 61 eligible published research papers, the corresponding authors of two unpublished studies: HyperGEN (Mangino et al., 2012) and the PREVEND Study (Huzen et al., 2011) contributed datasets (two from the HyperGEN study). An additional study (Newcastle 85 + study (Martin-Ruiz et al., 2011)) was identified through the HALCyon collaboration (www.halcyon.ac.uk) and a further study (The West of Scotland Twenty-07 Study (Der et al., 2012)) was identified through the FALCon collaboration (www.nshd.mrc.ac.uk/collaborations/falcon.aspx). The West of Scotland Twenty-07 Study contributed three datasets. Corresponding authors of the HyperGEN (Mangino et al., 2012), PREVEND (Huzen et al., 2011), Newcastle 85 + (Martin-Ruiz et al., 2011) and The West of Scotland Twenty-07 (Der et al., 2012) studies provided us with standard sets of results and each of these studies are now published. Hence these other relevant studies contributed an additional nine datasets from five separate studies.

So in total there are 40 datasets (28 datasets from published searches; 9 datasets from other relevant studies and 3 unpublished Halcyon cohort studies) from 32 separate studies.

2.4. Telomere length measures

Telomere length was measured by terminal restriction fragment (TRF) Southern Blot, real-time PCR or Flow-FISH (Aviv et al., 2006) in the eligible studies. Flow-FISH is a cytogenetic technique requiring intact cells to quantify the length of telomeres (Aviv et al., 2006). Cell types were whole blood, peripheral blood mononuclear cells (PBMC), lymphocytes or granulocytes (Aviv et al., 2006). Whole blood (leukocytes) cells were made up of lymphocytes, monocytes and granulocytes. These were prepared by red cell lysis and/or centrifugation of whole blood without Ficoll (buffy coat). PBMC (lymphocytes and sometimes variable fraction of monocytes) were prepared by whole blood centrifugation through a Ficoll cushion or gradient. Lymphocytes or granulocytes were used when specific cell fractions were isolated during the Flow-FISH method.

2.5. Statistical methods

We performed a two stage meta-analysis. Firstly equivalent models were run within each cohort. This involved using linear regression models to analyse the association between gender and telomere length adjusted for age. We made males the baseline group so that the regression coefficient represented the difference in telomere length for females compared to males. Some studies measured absolute telomere length in kb and others measured Telomere-to-Single Copy Gene (T/S) ratio (Real-time PCR). Furthermore, those laboratories using Real-time PCR methodology might use different reference samples (Horn et al., 2010) and also might change their reference sample when it runs out. To overcome such differences we standardised the regression coefficients (and corresponding standard error) by dividing both regression coefficient and standard error by the standard deviation of telomere length.

Secondly, the cohort specific standardised regression coefficients and standard errors were pooled using random effects meta-analyses (DerSimonian and Laird, 1986). We ran meta-analyses on results from models with age-adjusted as a continuous term and then from models with age-adjusted in quartiles. We included data from 36 datasets (published and unpublished studies) in our meta-analyses and included the remaining four datasets in sensitivity analyses. This was because two studies (Aubert et al., 2012; Halaschek-Wiener et al., 2008) had data on both granulocytes and lymphocytes and two studies (Nordfjall et al., 2008a,b) included the MONICA cohort study which was part of the more recent NSHDS cohort study which also included the VIP and MSP cohort studies (Nordfjall et al., 2009). Hence we wanted to make sure that the same sample was not included more than once within a meta-analysis. Furthermore, the 3 HALCyon studies and another study (Nordfjall et al., 2009) had repeat telomere length measures (time 1 and time 2). We thus ran meta-analyses first including these studies at time 1 and then including these four studies at time 2. We investigated between study heterogeneity using I^2 and Q statistics (Higgins and Thompson, 2002; Higgins et al., 2003). We examined potential sources of heterogeneity for age group (above versus below the median age), method of measurement of telomere length and cell type by stratifying random effects meta-analyses by each of these factors and by running meta-regression analyses (Thompson and Sharp, 1999). For meta-regression analyses, we used post-estimation Wald tests to obtain F ratios and p values. To address non-linearity and variation with age we undertook a meta-regression of effect size on mean age and tested a quadratic or cubic relationship. We used funnel plots to assess publication bias and tested the symmetry of the funnel plots using Egger's test (Egger et al., 1997).

We undertook a series of sensitivity analyses: (1) for the studies where we had access to the dataset, we repeated the test of association between gender and telomere length using transformed telomere length data ($\log_e$) and compared this to the results from meta-analysis using raw data for telomere length, to address the issue of positive skewness in telomere length measurements; (2) we excluded those studies where there was no detail of how the healthy participants were sampled and repeated the meta-analyses; (3) we repeated analyses using lymphocyte data rather than granulocyte data for two studies (Aubert et al., 2012; Halaschek-Wiener et al., 2008); (4) we replaced results from (Nordfjall et al., 2009) with data from either (Nordfjall et al., 2008a) or (Nordfjall et al., 2008b) to avoid using the same study population in meta-analyses more than once and repeated the analyses; (5) we repeated meta-analyses including only those studies who reported intra-assay or inter-assay coefficient of variation (CV); (6) we repeated meta-analyses having excluded those studies which had telomere length measured by any laboratory which appeared to have outlier values.

2.6. Methodological checks

As the data from the Newcastle group contributed to the apparent heterogeneity, a series of post hoc methodological checks was performed by this group.

3. Results

Forty datasets contributed results to this review. Table 1 presents the characteristics of these studies. Three studies (Hunt et al., 2008; Chen

Table 1
Characteristics of studies included in the review[a].

Reference and study name or recruitment method	Number of participants males females	Age males females	Telomere length males female	Method to measure telomere length	Cell type
Published studies from searches					
Adams et al. (2007)	108	50 (−)	5.55 kb (1.25)	Real-time PCR	PBMC
NewcastleThousand Families Study, UK	172	50 (−)	4.69 kb (1.02)		
Aubert et al. (2012)	405	43.6 (29.9)	7.97 kb (1.59)	Flow-FISH	Granulocytes
Healthy controls	403	43.6 (30.0)	8.09 kb (1.52)		
No details of how recruited, Canada					
Aubert et al. (2012)	421	43.6 (29.9)	6.95 kb (2.07)	Flow-FISH	Lymphocytes
Healthy controls	414	43.6 (30.0)	7.26 kb (2.04)		
No details of how recruited, Canada					
Bekaert et al. (2007)	1218	46.1 (5.9)	7.79 kb (0.71)	TRF Southern Blot	Whole blood
Asklepios study cohort, Belgium	1291	45.9 (6.0)	7.96 kb (0.73)		
Bischoff et al. (2006)	260	80.5 (7.4)	7.54 kb (1.15)	TRF Southern Blot	Whole blood
The Danish 1905 Cohort Study	552	81.8 (7.8)	7.73 kb (1.25)		
Longitudinal Study of Aging Danish Twins					
The Longitudinal Danish Centenarian Study					
Cawthon et al. (2003)	72	73.8 (8.0)	1.04 T/S (0.15)	Real-time PCR	Whole blood
Utah residents who gave blood, USA	71	71.8 (6.8)	1.09 T/S (0.16)		
Chen et al. (2009) (Blacks)	62	39.0 (4.1)	7.55 kb (0.70)	TRF Southern Blot	Whole Blood
Bogalusa Heart Study, USA	128	37.0 (5.3)	7.60 kb (0.81)		
Chen et al. (2009) (Whites)	208	38.5 (4.5)	7.05 kb (0.70)	TRF Southern Blot	Whole blood
Bogalusa Heart Study, USA	264	37.5 (4.8)	7.11 kb (0.74)	TRF Southern Blot	Whole blood
Cherkas et al. (2008)	247	48.1 (13.9)	6.61 kb (0.67)		
(Data used from larger	3009	48.7 (13.0)	7.01 kb (0.68)		
Mangino et al., 2009 publication)					
UK Adult Twin Registry, UK					
Cronkhite et al. (2008)	99	55.7 (16.0)	5.83 kb (0.66)	TRF Southern Blot	Whole blood
No details of how recruited, USA	102	55.8 (15.8)	5.97 kb (0.48)		
Diez Roux et al. (2009)	467	65.4 (9.6)	0.82 T/S (0.17)	Real-time PCR	Whole blood
Multi-ethnic study of Atherosclerosis, USA	514	65.1 (9.9)	0.87 T/S(0.18)		
Halaschek-Wiener et al. (2008)	69	78.1 (19.1)	6.49 kb (0.96)	Flow-FISH	Granulocytes
Population based lists, Canada	131	78.4 (18.0)	6.61 kb (0.83)		
Halaschek-Wiener et al. (2008)	69	78.1 (19.1)	4.95 kb (1.20)	Flow-FISH	Lymphocytes
Population based lists, Canada	131	78.4 (18.0)	5.26 kb (1.34)		
Hunt et al. (2008) blacks	216	52.3 (10.7)	6.95 kb (0.64)	TRF Southern Blot	Whole blood
Family Heart Study, USA	409	53.8 (11.0)	7.16 kb (0.63)		
Hunt et al. (2008) whites	1170	56.6 (13.4)	6.70 kb (0.65)	TRF Southern Blot	Whole blood
Family Heart Study, USA	1433	57.4 (13.1)	6.86 kb (0.67)		
Jang et al. (2008)	334	59.2 (6.2)	2.22 T/S (1.05)	Real-time PCR	Lymphocytes
Primary Care/GP register, Korea	152	58.7 (7.2)	2.03 T/S (1.20)		
Kaplan et al. (2009) blacks	74	73.0 (5.6)	6.31 kb (0.69)	TRF Southern Blot	Whole blood
Cardiovascular Health Study, USA	140	72.3 (5.0)	6.62 kb (0.66)		
Kaplan et al. (2009) whites	613	75.6 (5.5)	6.19 kb (0.56)	TRF Southern Blot	Whole blood
Cardiovascular Health Study, USA	842	74.9 (4.9)	6.38 kb (0.62)		
Kimura et al. (2007)	56	55.7 (25.5)	6.02 kb (0.99)	TRF Southern Blot	Whole blood
Individuals Campania region, Italy	106	76.7 (25.9)	5.57 kb (1.03)		
Maeda et al. (2009)	89	44.6 (10.2)	8.77 kb (1.78)	TRF Southern Blot	PBMC
No details of how recruited, Japan	58	43.8 (11.0)	9.54 kb (1.96)		
Martin-Ruiz et al. (2005)	184	89.9 (3.2)	4.44 kb (0.92)	Real-time PCR	PBMC
The Leiden 85-plus study, The Netherlands	495	89.8 (2.9)	4.26 kb (0.90)		
Nawrot et al. (2004)	119	42.9 (15.7)	6.78 kb (0.70)	TRF Southern Blot	Whole blood
Family Based Cohort, Belgium of the Flemish Study on Environment, Genes and Health Outcomes	152	41.7 (17.3)	6.94 kb (0.67)		
Nordfjall et al. (2005)	65	57.9 (15.3)	0.70 T/S (0.29)	Real-time PCR	PBMC
No details of how recruited, Sweden	69	53.4 (15.0)	0.75 T/S (0.26)		
Healthy individuals in 49 unrelated families					
Nordfjall et al. (2009)					
NSHDS cohort study, Sweden					
Time 1	722	47.1 (5.6)	0.70 T/S (0.21)	Real-time PCR	PBMC
	568	44.4 (7.0)	0.69 T/S (0.23)		
Time 2	361	57.1 (5.6)	0.59 T/S (0.16)	Real-time PCR	PBMC
	284	54.4 (6.9)	0.64 T/S (0.20)		
Nordfjall et al. (2008a)	197	47.1 (14.5)	0.62 T/S (0.14)	Real-time PCR	PBMC
MONICA cohort study, Sweden	335	43.8 (13.0)	0.70 T/S (0.19)		
Nordfjall et al. (2008b)	514	55.3 (11.7)	0.63 T/S (0.19)	Real-time PCR	PBMC
MDCC/Monica cohorts, Sweden	475	49.3 (13.6)	0.69 T/S (0.19)		
Ren et al. (2009)	53	44.7 (27.6)	11.7 kb (1.4)	TRF Southern Blot	Whole blood
Healthy individuals with ancestors living in region for ≥3 generations, Tibet	52	37.2 (24.7)	11.7 kb (1.4)		
Unryn et al. (2005)	51	54.2 (14.5)	7.02 kb (0.80)	TRF Southern Blot	PBMC
Random digit telephone dialling, Canada	74	58.4 (12.0)	7.09 kb (0.57)		
Van der Harst et al. (2007)	145	66.1 (8.5)	1.09 T/S 0.32	Real-time PCR	Whole blood
	38	66.7 (10.3)	1.14 T/S 0.41		

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Table 1 (*continued*)

Reference and study name or recruitment method	Number of participants males females	Age males females	Telomere length males female	Method to measure telomere length	Cell type
University Genetic Database					
Healthy non-blood related relatives of patients with Crohn's disease, Netherlands					
Woo et al. (2008)	976	72.8 (5.0)	8.81 kb (1.62)	Real-time PCR	Whole Blood
Health Survey recruited from community centres for elderly and housing estates, China	1030	72.0 (5.2)	9.35 kb (2.26)		
Additional datasets					
HALCyon cohorts					
The Hertfordshire Ageing Study (Syddall et al., 2010), UK					
Wave 1	388	67.5 (2.4)	5.32 kb (1.54)	Real-time PCR	PBMC
	269	67.5 (2.2)	5.10 kb (1.69)		
Wave 2	165	76.7 (2.4)	3.86 kb (1.20)	Real-time PCR	PBMC
	112	76.3 (2.1)	4.00 kb (1.55)		
Lothian birth cohort 1921 (Deary et al., 2004)					
Wave 1	210	79.1 (0.6)	4.23 kb (0.44)	Real-time PCR	PBMC
	287	79.1 (0.6)	3.98 kb (0.36)		
Wave 3	72	86.6 (0.4)	4.43 kb (0.43)	Real-time PCR	PBMC
	74	86.6 (0.4)	4.00 kb (0.60)		
(Harris et al., 2006 published but used larger Lothian Birth Cohort 1921 dataset)					
NSHD birth cohort 1946 (Kuh et al., 2011)					
Age 53	1324	53.5 (0.2)	5.87 kb (1.94)	Real-time PCR	PBMC
	1336	53.5 (0.2)	5.42 kb (1.89)		
Age 60–64	501	63.3 (1.2)	4.19 kb (1.28)	Real-time PCR	PBMC
	557	63.4 (1.1)	4.37 kb (1.33)		
Der et al. (2012)					
The West of Scotland, UK					
Twenty-07 study					
1970s cohort	362	36.6 (0.4)	0.85 T/S (0.22)	Real-time PCR	Whole Blood
	412	36.6 (0.4)	0.87 T/S (0.20)		
1950s cohort	379	56.9 (0.9)	0.77 T/S (0.18)	Real-time PCR	Whole Blood
	469	56.9 (0.7)	0.79 T/S (0.19)		
1930s cohort	235	75.9 (0.6)	0.68 T/S (0.19)	Real-time PCR	Whole Blood
	309	76.0 (0.6)	0.71 T/S (0.18)		
Huzen et al (2011)	4027	50.4 (12.9)	1.05 T/S (0.33)	Real-time PCR	Whole Blood
The PREVEND Study, Netherlands	4027	48.1 (12.3)	1.08 T/S (0.34)		
Mangino et al. (2012) blacks	108	52.2 (8.8)	7.05 kb (0.78)	TRF Southern Blot	Whole Blood
HyperGEN, USA	116	54.5 (9.1)	7.07 kb (0.64)		
Mangino et al. (2012) whites	612	51.9 (14.2)	6.68 kb (0.60)	TRF Southern Blot	Whole Blood
HyperGEN, USA	628	53.4 (13.1)	6.80 kb (0.61)		
Martin-Ruiz et al. (2011)	295	85.5 (0.5)	3.32 kb (1.14)	Real-time PCR	PBMC
Newcastle 85+ study, UK	456	85.5 (0.4)	3.05 kb (1.06)		

[a] Age and telomere lengths are presented as mean (standard deviation).

et al., 2009; Kaplan et al., 2009) each had telomere length data stratified for black and white participants and these data are presented separately. Mean age ranged from 36.6 ± 0.4SD years in The West of Scotland Twenty-07 Study to 89.9 ± 3.2SD years in the Leiden 85+ Study. The method of measurement of telomere length was TRF Southern Blot in 17 datasets, Real-time PCR in 19 datasets and Flow-FISH in four datasets. Telomere lengths were measured in whole blood (27 datasets), PBMC (8 datasets), granulocytes (2 datasets) and lymphocytes (3 datasets).

3.1. Meta-analyses

Overall summary estimates of effects for the associations between gender and telomere length are detailed in Table 2. Meta-analyses from the 36 cohorts (36,230 participants) showed that gender was associated with telomere length, with females having longer telomeres on average than males (standardised difference in telomere length between females and males 0.090, 95% CI 0.015, 0.166, $p = 0.02$; age-adjusted; Fig. 2). These results included the 3 HALCyon studies and Nordfjall et al., 2009 at time 1. When these analyses were repeated but including these 4 studies at time 2 rather than time 1 (where the difference in mean ages between times 1 and 2 ranged from 7.5 years to 10 years), the associations between gender and telomere length were present, again showing that females had longer telomeres than males ($p < 0.001$).

3.2. Heterogeneity

There was evidence of substantial heterogeneity between studies ($I^2 = 91.4\%$, 95% CI 89.0, 93.2, $p < 0.001$) (Table 2). There was little evidence that the association between gender and telomere length varied by age group (F ratio = 0.00, $p = 1.00$; Table 2; Fig. 3) but did vary by the method of telomere length measurement (F ratio = 5.72, $p = 0.007$; Table 2; Fig. 4). There was moderate heterogeneity between studies using the TRF Southern Blot method ($I^2 = 68.8\%$, 95% CI 48.5, 81.1, $p < 0.001$) and high heterogeneity between studies using the Real-time PCR method ($I^2 = 93.2\%$, 95% CI 90.6, 95.1, $p < 0.001$) and the summary estimates of effect showed longer telomeres in females than males only for the TRF Southern Blot method. There was little evidence that the associations between gender and telomere length varied by cell type (F ratio = 1.31, $p = 0.29$; Table 2; Fig. 5). The results in Table 2 include the 3 HALCyon studies and Nordfjall et al., 2009 at phase 1. When these analyses were repeated but including these 4 studies at phase 2, there was again little evidence that the associations varied by age group (F ratio = 0.06, $p = 0.80$) or by cell type (F ratio = 0.99, $p = 0.41$) but there was still evidence (albeit weaker) that the association between gender and telomere length varied by method to measure telomere length (F ratio = 3.10, $p = 0.06$). There was little evidence that the association between telomere length and gender had a non-linear variation with age (data not shown). For comparison purposes for the TRF Southern Blot method, the overall summary estimates of

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Table 2
Overall summary estimates of effect for the associations between gender and telomere length from stratified random effects meta-analyses.

Stratification	No[a]	ES[b] females–males	95% CI	(Age-adjusted) p-value	I^2	p-value[c]
None	36	0.090	0.015, 0.166	0.02	91.4%	<0.001
Mean age[d] (years)						
≤55.8	18	0.091	−0.018, 0.200	0.10	92.4%	<0.001
>55.8	18	0.090	−0.021, 0.210	0.11	90.2%	<0.001
					F ratio	p-value
Overall[e]					0.00	1.00
Measurement method						
Real-time PCR	17	−0.047	−0.167, 0.072	0.44	93.2%	<0.001
TRF Southern Blot	17	0.240	0.173, 0.307	<0.001	68.8%	<0.001
Flow-FISH	2	0.077	−0.016, 0.171	0.11	0.0%	0.65
					F ratio	p-value
Overall[e]					5.72	0.007
Cell Type[f]						
Whole Blood	27	0.132	0.050, 0.215	0.002	91.9%	<0.001
PBMC	6	−0.070	−0.354, 0.215	0.63	87.9%	<0.001
Lymphocytes	1	−0.194	−0.461, 0.072	0.15	–	–
Granulocytes	2	0.077	−0.016, 0.171	0.11	0.0%	0.65
					F ratio	p-value
Overall[e]					1.31	0.29

[a] Number of studies.
[b] Standardised regression coefficient (standardising for telomere length) representing the age-adjusted difference in telomere length between females and males. Here males are the baseline group so that the coefficient represents the difference in telomere length for females and males. Hence if females have longer telomeres this would be positive.
[c] p-value is obtained from the heterogeneity χ^2.
[d] Stratified by the mean age above and below the median age.
[e] Overall test for heterogeneity between subgroups by undertaking meta-regression and giving F ratio and p-values.
[f] Cell types are whole blood (leukocytes: lymphocytes, monocytes and granulocytes) and Peripheral Blood Mononucleocytes (PBMC: enriched for lymphocytes), lymphocytes or granulocytes. Random effects meta-analyses were used throughout.

the absolute difference in telomere length between females and males was 176 bp (95% CI 131, 221, $p < 0.001$; age-adjusted).

3.3. Publication bias

The funnel plots (data not shown) and Egger test (bias $= -1.52$, $p = 0.15$) did not show strong evidence for small study bias.

3.4. Sensitivity analyses

We found no evidence that the association between gender and telomere length differed whether we used raw data for telomere length, or whether it was transformed ($\log_e$) (data not shown). Excluding studies where there were no details of how the healthy participants were sampled had little effect on the findings. The Egger test using these studies, showed that there was again no strong evidence for small study bias (bias $= -1.76$, $p = 0.18$; including phase 1 data). Repeating the analyses including lymphocyte data rather than granulocyte data for the two studies with both data made little difference to the associations (data not shown). Replacing (Nordfjall et al., 2009) study with data from either (Nordfjall et al., 2008a) or (Nordfjall et al., 2008b) again had little effect on the associations. Eight out of 22 studies reported values for intra-assay CV and 4 out of 22 studies reported values for inter-assay CV. Repeating the analyses but including only those 10 studies who reported intra-assay or inter-assay CV in their studies, showed that gender was strongly associated with telomere length, with females having longer telomeres than males.

4. Discussion

4.1. Explanation of findings

The results of these meta-analyses showed that gender was associated with telomere length, with females having longer telomeres on average than males, even though significant heterogeneity between studies was detected. There was little evidence that the strength of the associations varied by age group or by cell type. However, the association between gender and telomere length did vary by measurement methods. The summary estimates of effect showed longer telomeres in females than males only for the TRF Southern Blot method, but not for the Real-time PCR nor the Flow-FISH method. However, there was significant heterogeneity between studies, particularly for the Real-time PCR method ($I^2 = 93.2\%$) but also for TRF Southern Blot ($I^2 = 68.8\%$) methodologies. Moreover, heterogeneity was also confirmed by more recent large Real-time PCR studies which did (Weischer et al., 2012) or did not (Needham et al., 2013) identify shorter telomeres in males than females.

These results suggest two alternative conclusions: (i) women have longer telomeres than men, and the failure of the PCR and Flow-FISH methods to detect this difference reproducibly is related to higher experimental variability of these techniques, or (ii) there is no consistent gender difference in telomere length but a methodological bias specific to the Southern blot technique.

The overall outcome of the present systematic review argues in favour of the first conclusion. In addition, a number of biological plausible arguments for longer telomeres in women than in men have been made. These include the action of oestrogen which can stimulate the production of telomerase and might be protective against reactive oxygen species damage (Aviv, 2002) and the heterogametic sex hypothesis (Barrett and Richardson, 2011) stating that any deleterious recessive alleles on the X chromosome of the heterogametic sex (males in humans (XY)), will have no compensatory allele, unlike in females where the second chromosome might compensate (Austad, 2006). Hence, shorter telomeres in males might arise if the unguarded X chromosome in males contains inferior telomere maintenance alleles (Barrett and Richardson, 2011).

It has been suggested that longer telomere length in women than men might arise from a slower rate of telomere attrition in women (Okuda et al., 2002). In both cross-sectional (Bekaert et al., 2007) and longitudinal studies (Chen et al., 2011) the rate of leukocyte telomere length shortening was slower in women than men. However, the associations were not strong and hence need to be confirmed in larger studies (Chen et al., 2011). In the present study, there was little evidence

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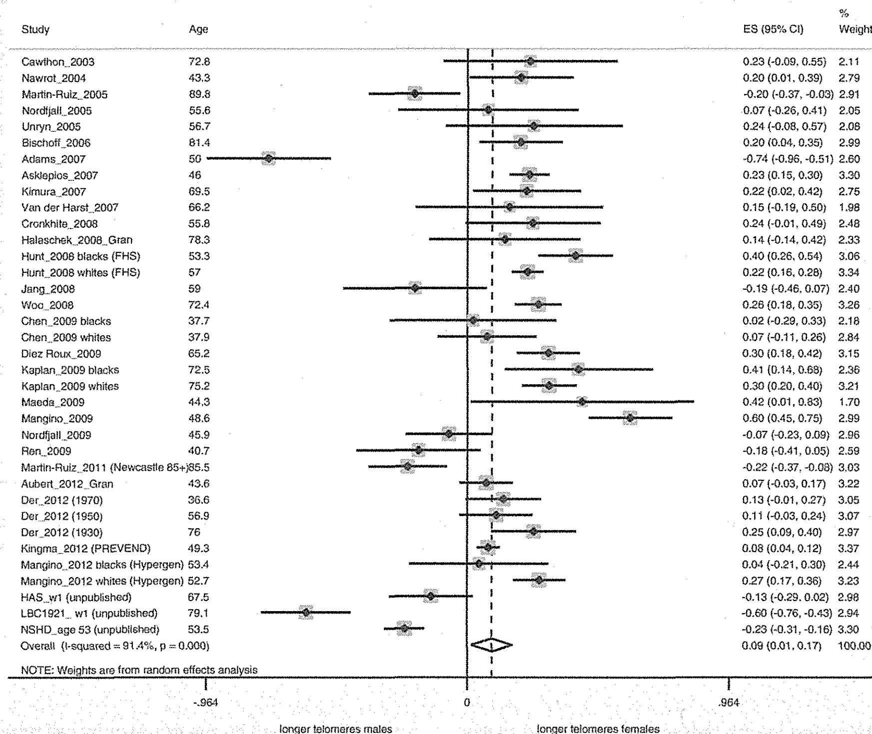

Fig. 2. Meta-analysis for the association between gender and telomere length adjusted for continuous age.

that the strength of the association between gender and telomere length varied by age group (above versus below the median age of 55.6 years). It is not clear whether gender dependent differences in telomere length are already present at birth: One study found that there was little difference in telomere length measured by TRF Southern Blot between the sexes at birth (Okuda et al., 2002), while a recent study using Flow-FISH found that female newborns had longer telomeres than males (Aubert et al., 2012).

It has been suggested that longer telomeres might be a cause of improved fitness in women, manifest in longer lifespan and lower risk for cardiovascular disease and cancers (Aviv, 2002). However, the picture might be more complex: While short leukocyte telomere length has been associated with cardiovascular risk factors (Benetos et al., 2001; Bekaert et al., 2007; Fitzpatrick et al., 2007; Nordfjall et al., 2008b; Shiels et al., 2011) and cardiovascular disease (Brouilette et al., 2007; Fitzpatrick et al., 2007), it did not predict risk for enhanced cancer incidence in carefully controlled prospective studies (Weischer et al., 2013) and for some cancers including breast cancer, long rather than short telomeres were associated with increased risk (Pellatt et al., 2013). While males die at a higher rate at virtually every age (Austad, 2006), associations of telomere length with mortality have shown contradictory results (Cawthon et al., 2003; Bischoff et al., 2006; Fitzpatrick et al., 2011). Finally, females show higher incidence and prevalence of many age-related diseases (except cardiovascular disease and cancer) and disabilities (Collerton et al., 2009). Many of these including, for example, multiple-morbidity (Sanders et al., 2012), rheumatoid arthritis (Costenbader et al., 2011) or depression (Puterman et al., 2013) have been associated with short telomeres. Together, these data do not suggest a general association between gender-specific telomere length and fitness in domains other than cardiovascular disease.

A large part of the heterogeneity of the real-time PCR studies can be attributed to a single group. The von Zglinicki group repeatedly reported longer telomeres in men than in women (Deary et al., 2004; Martin-Ruiz et al., 2005, 2011; Adams et al., 2007; Syddall et al., 2010; Kuh et al., 2011). A post hoc analysis excluding these studies (Deary et al., 2004; Martin-Ruiz et al., 2005, 2011; Adams et al., 2007; Syddall et al., 2010; Kuh et al., 2011) now showed longer telomeres in females than males for the Real-time PCR method (standardised difference in telomere length between females and males 0.134, 95% CI 0.053, 0.215, $p = 0.001$). However, there was still evidence of moderate heterogeneity (e.g. Real-time PCR $I^2 = 72.9\%$). To address the possibility of methodological errors, a number of methodological checks were performed by the von Zglinicki group. Coding errors were ruled out by independent cross-checks. Inter-assay CV were measured repeatedly and always found to be below 6%. PCR efficiencies were estimated both from standard curves and as single-well efficiency. Plate-to-plate efficiency variation was below 1% for the reference gene PCR and below 2.7% for telomere PCR. The intra-plate single-well efficiency CV was always below 3%. Finally, telomere length of the Newcastle 85+ study participants (which showed longer telomeres in men than women PBMCs) was repeated using whole blood and a different reference gene with the same result. Interestingly, Southern blot telomere length studies in patient cohorts done by the von Zglinicki group showed a tendency towards longer telomeres in women in agreement with the Southern blot studies included in the present review (von Zglinicki et al., 2000; Martin-Ruiz et al., 2006). Thus, there is no obvious methodological