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INCREASED EXPRESSION OF TELOMERE-REGULATING GENES IN ENDURANCE ATHLETES WITH LONG LEUKOCYTE TELOMERES

Running head: Telomere-regulating genes in endurance athletes

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Abstract

Leukocyte telomeres shorten with age and excessive shortening is associated with age-related cardio-metabolic diseases. Exercise training may prevent disease through telomere length maintenance though the optimal amount of exercise that attenuates telomere attrition is unknown. Furthermore, the underlying molecular mechanisms responsible for the enhanced telomere maintenance observed in endurance athletes is poorly understood.

We quantified the leukocyte telomere length and analysed the expression of telomere-regulating genes in endurance athletes and healthy controls (both n = 61), using quantitative PCR.

We found endurance athletes have significantly longer (7.1%, 208–416 nt) leukocyte telomeres and up-regulated *TERT* (2.0-fold) and *TPP1* (1.3-fold) mRNA expression compared to controls in age-adjusted analysis. The telomere length and telomere-regulating gene expression differences were no longer statistically significant after adjustment for resting heart rate and relative $\dot{V}O_{2max}$ (all $p > 0.05$). Resting heart rate emerged as an independent predictor of leukocyte telomere length, *TERT* and *TPP1* mRNA expression in stepwise regression models. To gauge whether volume of exercise was associated with leukocyte telomere length, we divided subjects into running and cycling tertiles (distance covered per week) and found individuals in the middle and highest tertiles had longer telomeres than individuals in the lowest tertile. These data emphasise the importance of cardiorespiratory fitness and exercise training in the prevention of biological aging. They also support the concept that moderate amounts of exercise training protects against biological ageing, while higher amounts may not elicit additional benefits.

Introduction

Telomeres are repetitive DNA (in mammals, 5'-TTAGGG-3') positioned at the ends of chromosomes that protect against genomic DNA degradation and chromosomal fusion events (24, 25). Due to the end replication problem, telomeres shorten in the absence of telomerase with each round of cell-division and as such telomere length is an established marker of ageing (1, 36, 71). Telomeres and six telomere-regulating proteins (telomere repeat-binding factor 1 [TRF1], telomere repeat-binding factor 2 [TRF2], TRF1-interacting nuclear factor 2 [TINF2], adrenocortical dysplasia homolog [TPP1], protection of telomeres 1 [POT1] and TRF2-interacting protein [TERF2IP]), collectively called shelterin, form nucleoprotein complexes that maintain genomic stability and regulate telomere length. Shelterin is crucial for telomerase-mediated telomere length maintenance and genomic stability, as removal of shelterin causes severe telomere and chromosomal aberrations (46, 51, 61). Telomerase is comprised of telomerase reverse transcriptase (TERT) and the telomerase RNA component (*TERC*), and can combat premature ageing by extending telomeric DNA (23, 57).

Telomere length of proliferative tissues, such as leukocytes, is longest at birth and shortening is dependent on genetic and lifestyle factors. Psychological stress (21), poor diet (63) and age-related diseases including coronary artery disease (56), obesity (68) and diabetes (55) are all associated with excessive leukocyte telomere shortening. Conversely, mounting evidence has unveiled a positive influence of physical activity levels on leukocyte telomere length (11, 20, 33, 39, 41, 52, 73). Lifestyle interventions including increases in moderate-intensity physical activity extends telomere length after a five-year period (49). Although exercise seems to

benefit telomere length, the ideal amount of exercise training for telomere length maintenance and the underlying molecular mechanisms remain elusive.

We previously reported that relative to healthy controls, ultra-marathon runners had, on average, 11% longer leukocyte telomeres, indicating that they had prevented ~16 years' worth of age-related telomere attrition (18). German National Track and Field athletes have increased TRF2 protein content and up-regulated telomerase activity in peripheral blood mononuclear cells (PBMC) compared to sedentary controls (73). Furthermore, PBMC shelterin gene (TRF1, TRF2 and POT1) expression was up-regulated after a seven day ultra-marathon event (34). Thus, shelterin and other telomere-regulating genes may underpin the longer leukocyte telomeres associated with long-term endurance exercise training. A comprehensive analysis of all shelterin and *TERT* gene expression between endurance athletes and healthy controls has not yet been performed.

Subsequently, the purpose of our study was to extend previous findings by determining whether any association between telomere length and exercise was mediated through telomere-regulating gene expression in endurance athletes. A further aim was to establish whether linear associations exist between physical activity, cardiorespiratory fitness and leukocyte telomere length.

Materials and Methods

Participants

A total of 122 Caucasian subjects were recruited from the general public and participated in this study. Subjects were deemed apparently healthy – non-smoking, not taking any medications and free from any age-related chronic diseases – according to self-reported health questionnaires. Endurance athletes (n = 61) and

recreationally active controls (n = 61), aged 18 to 55 y were analysed. Endurance-athletes trained were cyclists, triathletes, middle- or long-distance runners and ultra-marathon runners at state through to international level. Endurance athletes trained >3 times per week and had trained consistently for a minimum of one year. The apparently healthy controls were recreationally active but were not engaged in any structured aerobic or resistance exercise training.

All participants gave written informed consent and this study was approved by Federation University Australia's Human Research Ethics Committee.

Procedures

Subjects physical activity levels and psychological stress was assessed by the self-administered International Physical Activity Questionnaire (IPAQ) Long form (5) and Perceived Stress Scale (PSS) (14), respectively. Data cleaning and analysis was performed according to the IPAQ guidelines and average weekly Metabolic Equivalent of task (MET) – minutes and sitting were calculated and included as continuous variables in statistical analyses. Height, weight and body mass index (BMI) were recorded and subjects were seated for approximately 10 minutes before BP assessment. The SphygmoCor device (AtCor Medical, Australia) was used to assess brachial blood pressure, averaged from three separate measurements, taken one minute apart with subjects seated. Subjects' cardiorespiratory fitness, determined as maximal oxygen consumption ($\dot{V}O_{2max}$), was assessed through a maximal graded treadmill or cycle ergometer test via pulmonary analysis. While control subjects completed a maximal treadmill test, the endurance cyclist completed a cycle ergometer test. Triathletes obtain a comparable $\dot{V}O_{2max}$ value regardless of exercise mode (45) and as such, triathletes from the present study completed either

a cycle or treadmill test. Before maximal exercise testing subjects were fitted with a two-way breathing valve (Hans Rudolph) and expired air was collected into an online metabolic system (Moxus, Modular, USA) for O_2 and CO_2 analysis. The metabolic system was calibrated prior to each test using ambient air and gas of known composition. The treadmill commenced at 10 $km \cdot h^{-1}$ and was progressively increased by 1 $km \cdot h^{-1}$ every two minutes until volitional exhaustion. Cycle ergometer $\dot{V}O_{2max}$ tests commenced at 100 W and the load was increased by 30 $W \cdot min^{-1}$ every two minutes until pedalling cadence dropped below 50 RPM for 10 seconds or until volitional exhaustion. Subjects were asked to maintain 90–100 RPM throughout cycle ergometer-assessed exercise tests. Individual $\dot{V}O_{2max}$ was determined as the highest O_2 value averaged over 60 seconds.

Telomere length quantification

A preprandial blood sample (~20 ml) was drawn from the antecubital vein into EDTA tubes using standard phlebotomy procedures. All subjects gave a seated resting blood sample 24 to 48 hours after their last exercise session. DNA was extracted from whole-blood leukocytes using the Purelink Genomic DNA Mini Kit (Life Technologies, Australia). Telomere length was quantified using an established qPCR method (7, 17, 18, 42), previously validated by terminal restriction fragment analysis (7). Within each sample, the telomere repeat copy number (T) is compared to a single copy gene copy number (S) and expressed in arbitrary units as a (T/S) ratio. Briefly, 10µl reactions comprised of 2 × SensiFast SYBER Lo-ROX master mix (Bioline, Australia), primer sets and 10ng of DNA, were run in triplicate on the ViiA7 Real Time PCR System (Life Technologies, Australia). Either 300nM of telomere-specific forward (5'GGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT3')

and reverse (5'GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT3') primers, or 300nM of forward (5'CAGCAAGTGGGAAGGTGTAATCC3') and 500nM of reverse (5'CCCATTCTATCATCAACGGGTACAA3') primers for the 36B4 gene was used in reactions. All samples were run with a positive and no template controls on a single 384-well plate to prevent any inter-plate variability. The cycling conditions telomere assays was as follows: a hold at 95° for 10 min, followed by 40 cycles at 95° for 15 s and 58° for 1 min. As a quality control, samples were excluded from the analysis if the difference between triplicates was greater than one cycle threshold (Ct), or the average of duplicates was taken for further analysis. The intra-assay coefficient of variation between triplicate samples was 2.5% and 1.4% for the telomere and 36B4 gene, respectively.

Gene expression analysis

Leukocytes were isolated as previously described (19) and RNA was extracted using the miRVana miRNA Isolation Kit (Life Technologies, Australia), following the manufacturer's guidelines. RNA was reverse transcribed to cDNA using the High Capacity Reverse Transcription Kit (Life Technologies). Telomere-regulating gene expression was quantified using SYBR or TaqMan chemistries. Primer-sets and TaqMan Assays (Life Technologies) are outlined in Table 1. An efficiency curve was generated for each primer-set using cDNA diluted 1:2 from 50ng to 3.125ng. The qPCR product was run on an agarose gel to ensure appropriate amplicon length and a single product. Triplicate samples were run on a single 384-well plate with negative controls. The cycling conditions for primer-based assays was: a hold at 95° for 2 min, followed by 40 cycles at 95° for 5 s, 60° for 10 s and 72° for 20 s. Cycling for TaqMan assays was: a hold at 50° for 2 min and another at 95° for 20 sec, followed

by 40 cycles at 95° for 1 s and 60° for 20 s. Relative gene expression was assessed using the $2^{-\Delta\Delta Ct}$ method (38). Whilst differential gene expression between athletes and controls was represented by fold-difference, gene expression analysis involving all subjects was represented using relative gene expression compared to the control mRNA, *GAPDH*. The coefficient of variation between triplicates for each of the mRNAs ranged from 0.66 to 1.49% (Table 1).

Statistical analysis

Using data from our previous cross-sectional study (18), our *a priori* power analysis revealed we required a sample size of 88 (44 in each group) in order to achieve >90% power to detect a difference ($d > 0.7$) in leukocyte telomere length between athletes and controls (G*Power, version 3.1.5). All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 21, IBM Corp, NY). Data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Two-way independent samples *t*-tests or Mann-Whitney U-tests were used to examine differences in physical characteristics and fitness parameters, and telomere length between athletes and controls. To control for covariates, an ANCOVA was used to establish differences between athlete and control telomere length and telomere-regulating gene expression. An ANOVA was also used to determine telomere length differences between subjects divided into cycling and running distance tertiles. Spearman's correlations were used on to examine associations between physical characteristics and fitness parameters, with telomere length and telomere-regulating gene expression. Stepwise linear regression was performed to identify predictors of telomere length and telomere-regulating gene expression. Statistical significance was set at $p < 0.05$. The difference in biological age and

telomere length – expressed as nucleotides (nt) – between athletes and controls was estimated using the same calculations as described previously (18).

Results

Physical characteristics

The controls were five years younger than the athletes ($p = 0.06$). Relative to the controls, the athletes had a lower body weight, resting heart rate and had a higher cardiorespiratory fitness as indicated by their $\dot{V}O_{2max}$ and maximal treadmill speed (all $p < 0.001$, Table 2). Athletes engaged in less sitting and were more physically active compared to their non-athletic peers (all $p < 0.01$, Table 2).

Linear correlations between telomere length, age, health and exercise phenotypes

Age was not statistically correlated to telomere length in all subjects or when athletes and controls were analysed separately (all $p > 0.05$, Table 3). When athletes and controls were pooled we found weak to moderate correlations between telomere length and weight, BMI, systolic blood pressure and resting heart rate (n = 122, all $p < 0.05$, Figure 1). Furthermore, we found correlations between cardiorespiratory fitness and physical activity parameters – Metabolic equivalent of task-min per week, time spent sitting and maximal treadmill speed – and leukocyte telomere length (all $p < 0.05$, Figure 2). In athletes, years spent training was not associated with telomere length (n = 60, r = -0.12, $p = 0.37$).

Telomere length analysis

Relative to the controls, the endurance athletes had 7.1% longer leukocyte telomeres after age-adjustment (T/S ratio ± SE: 3.64 ± 0.06 v 3.38 ± 0.06, $p = 0.002$, Figure 3A). The biological age difference between endurance athletes and controls

translated to 10.4 years, meaning the athletes had prevented 10.4 years of biological ageing. We estimated the biological age difference was equivalent to the athletes possessing 208–416 nt longer telomeres compared to the controls. Compared to controls, athletes had lower body weight and resting heart rate, and a higher cardiorespiratory fitness (Table 2, all $p < 0.001$). To determine whether these phenotypes mediated the leukocyte telomere length difference found between athletes and controls, we performed an additional analysis including these phenotypes as covariates. After adjusting for age, weight, resting heart rate and relative $\dot{V}O_{2max}$, however, the difference between athletes and controls was no longer statistically significant (T/S ratio ± SE: 3.58 ± 0.08 vs 3.45 ± 0.08, $p = 0.36$).

We then performed a stepwise linear regression to determine predictors of leukocyte telomere length. After including health and fitness parameters – age, height, weight, body mass index, systolic, diastolic, mean arterial and pulse pressure, and relative $\dot{V}O_{2max}$ – in the stepwise regression model, resting heart rate emerged as the only independent predictor of leukocyte telomere length amongst athletes and controls, such that it explained 10.1% of the overall variation (B = -0.012, CI: 3.85–4.625, $p < 0.001$).

Telomere-regulating gene expression analysis

Relative to controls, endurance athletes had 2.0-fold and 1.3-fold up-regulated *TERT* (Figure 3B) and *TPP1* (Figure 3C) mRNA expression, respectively. No other telomere-regulating genes – *TERC*, *TERF2IP*, *TINF2*, *TERF1*, *TERF2* and *POT1* – were differentially regulated between athletes and controls ($p > 0.05$, Table 4). The up-regulated *TERT* and *TPP1* mRNA expression remained statistically significant after adjusting for health phenotypes ($p = 0.005$ and $p = 0.05$, respectively). After

250 further adjustment for heart rate and relative $\dot{V}O_{2max}$, however, the difference was no
251 longer statistically significant (p = 0.16 and p = 0.41). Besides *TERC* (r = -0.28, p =
252 0.003), there were no other statistically significant correlations between telomere
253 length and expression of any of the telomere-regulating genes analysed (p > 0.05,
254 Table 5). *TERT* and *TPP1* were both correlated with resting heart rate and relative
255 $\dot{V}O_{2max}$ (Figure 3D–G).
256 Again, we performed stepwise regression including health and fitness parameters
257 and found that resting heart rate was an independent predictor of *TERT* mRNA
258 expression, explaining 9.4% of the variation (Table 6). Age, height and resting heart
259 rate were independent predictors of *TPP1* mRNA expression, together explaining
260 9.5% of the variation (Table 6).
261
262 *Moderate amounts of exercise training associated with long telomeres and increased*
263 *TERT and TPP1 mRNA expression*
264 To establish associations between volume of exercise training and telomere length,
265 we divided subjects into tertiles for weekly running and cycling distance and
266 analysed telomere length. We found that age-adjusted telomere length was
267 significantly longer in subjects in the middle and highest tertiles for weekly running
268 and cycling distance (Figure 4A and B, respectively) compared to those in the lowest
269 tertile. A similar relationship was observed between weekly training distances and
270 *TERT* and *TPP1* mRNA expression (Figure 4D, E, G and H). Moreover, individuals
271 with the highest cardiorespiratory fitness had longer leukocyte telomeres, up-
272 regulated *TERT* and *TPP1* mRNA expression compared to those in the lowest tertile
273 with poor cardiorespiratory fitness (Figure 4C, F and I, respectively). No statistically
274 significant differences were found between those in the middle and highest

cardiorespiratory fitness tertiles for telomere length, *TERT* and *TPP1* mRNA expression.

Lower resting heart rate is associated with longer telomeres

To investigate the association between resting heart rate and telomere length we divided our subjects into resting heart rate tertiles and found a linear decrease in leukocyte telomere length with a higher resting heart rate (Figure 4J). Subjects with a resting heart rate below 50 beats·min^{-1}, on average, exhibited 14.4% and 8.5% longer telomeres compared to those with a resting heart rate 51–74 and >75 beats·min^{-1}, respectively (Figure 4J). A similar relationship was also observed between resting heart rate and *TERT* and *TPP1* mRNA expression (Figure 4K and L, respectively).

Discussion

Endurance athletes who regularly engage in high volumes of exercise training have preserved leukocyte telomeres (18, 33, 73) though the underlying molecular and physiological determinants remain incompletely understood. Here, we not only verified that endurance athletes have significantly longer leukocyte telomeres, but we also wanted to determine if the longer telomeres observed in athletes was caused by the modulation of gene expression in telomere length regulating genes. We found that the adrenocortical dysplasia homolog (TPP1) and TERT genes were both up-regulated in leukocytes from athletes compared to controls. The longer leukocyte telomeres and increased *TERT* and *TPP1* mRNA expression observed in endurance athletes appears to be associated with their lower resting heart rate and superior $\dot{V}O_{2max}$.

300 The majority of previous research has shown physical activity is positively correlated
301 to leukocyte telomere length (11, 20, 33, 41, 52, 73), though the optimal amount for
302 telomere length maintenance remains unclear. For instance, some researchers
303 suggest moderate amounts of physical activity is ideal for telomere maintenance (41,
304 59), whilst studies on endurance athletes – who regularly engage in strenuous
305 endurance exercise training – supports the premise that higher volumes of
306 endurance exercise is conducive to telomere protection (18, 33, 73). Here, we verify
307 previous studies (18, 33) indicating endurance athletes possess significantly longer
308 leukocyte telomeres (by 7.1%, 208–416 nt) compared to controls of average
309 cardiorespiratory fitness. Our previous investigation on ultra-marathon runners
310 revealed they had 324–648 nt longer telomeres, which translated to 16.2 years less
311 telomere attrition compared to healthy controls (18). The endurance athletes in the
312 present study were, on average, five years older than the controls yet possessed
313 longer leukocyte telomeres to a relatively similar magnitude as found in our previous
314 study (18). The average telomere length difference between endurance athletes and
315 controls from the present study indicated the endurance athletes possessed
316 telomeres as long as controls 10.4 years their junior, providing additional evidence
317 that endurance exercise training attenuates biological ageing.
318 Although previous studies (18, 33, 73) and our findings indicate endurance exercise
319 training is associated with longer telomeres, the molecular mechanisms leading to
320 longer leukocyte telomeres in endurance athletes is unclear. Up-regulation of
321 telomerase is a likely mechanism of longer telomeres in athletes. German track and
322 field and endurance athletes accumulating an average of >70 km of running per
323 week, exhibited up-regulated peripheral blood mononuclear *TRF2* mRNA and protein
324 expression, with increased telomerase activity (73). Here, we found increased whole-

blood leukocyte *TERT* and *TPP1* mRNA expression in endurance athletes. It is possible that repeat bouts of exercise training may reprogram *TERT* and *TPP1* mRNA expression, which would improve telomerase activity and processivity, and ultimately preserve telomere length. Previous analyses involving mononuclear cells (73), *TRF2* mRNA was not differentially expressed in our endurance athletes, potentially due to the different cell type studied – whole blood leukocytes. Increased mononuclear cell *TRF1*, *TRF2* and *POT1* mRNA expression was observed in endurance athletes the day after a 183-mile ultra-marathon race (34), but these shelterin genes were not differentially expressed in our athletes in a rested state. TERT is the major protein component of the reverse transcriptase, *telomerase* (9), with a known role in preventing replication-induced telomere shortening (13, 69). Interestingly, leukocyte *TERT* mRNA expression was increased (19.4-fold) after a 30-min run at 80% of $\dot{V}O_{2max}$ in healthy men (12). Therefore, considering POT1 together with TPP1 help recruit and increase the repeat processivity of telomerase (72), the increased *TERT* and *TPP1* mRNA expression found in athletes from our study and up-regulated leukocyte telomerase activity in athletes' from others (73) may contribute to the underlying molecular mechanisms by which endurance exercise training preserves leukocyte telomeres. Pathways activated by aerobic exercise training, such as the nitric oxide synthase, Akt protein kinase, insulin growth factor-1 signalling (73, 74) and p38 mitogen-activated protein kinase (40) are candidate signalling cascades that may regulate telomerase activity-dependent telomere maintenance via *TERT* activation.

Interestingly, age was not negatively correlated to leukocyte telomeres in athletes, control or pooled subjects. This may be due to the narrow age range (18–55 y) or alternatively because the controls were recreationally active. Body weight, body

mass index, systolic BP, mean arterial pressure and resting heart rate were all inversely correlated to leukocyte telomere length. Consistent with previous studies (31, 33, 44, 50), we found a positive correlation between cardiorespiratory fitness, assessed by $\dot{V}O_{2max}$ testing, and telomere length. Interestingly, *TERC* mRNA expression was inversely correlated to telomere length. Potential explanations for this finding is that elevated *TERC* mRNA expression may not be required in the absence of excessive telomere shortening, experimental noise or because *TERC* is not the rate limiting factor for telomerase activity. Providing evidence that longer leukocyte telomeres are reflective of physical performance capabilities and physical activity, we found maximal treadmill speed and physical activity were positively correlated to leukocyte telomere length. A recent randomised, controlled trial revealed reduced time spent sitting was associated with telomere lengthening in a group of sedentary older adult (68 y) men and women (65). We found time spent sitting per week was inversely correlated to leukocyte telomere length in younger (~30 y) subjects. Notably, the athletes in the present study reported sitting much less relative to controls (4.8 v 10.8 $hr \cdot day^{-1}$). It may be that the longer leukocyte telomeres possessed by endurance athletes is result of both extensive exercise training and less sedentary time (i.e. more physical activity). Therefore, these data suggest increased physical activity, cardiorespiratory fitness and limited time spent sitting contribute to telomere maintenance, in turn, protecting against cardiovascular disease and biological ageing.

We also found *TERT* and *TPP1* mRNA expression were positively and inversely correlated to $\dot{V}O_{2max}$ and resting heart rate, respectively. To our knowledge we are the first to show such a relationship between parameters of cardiorespiratory fitness – $\dot{V}O_{2max}$ and resting heart rate – and telomere-regulating gene expression. An

increase in $\dot{V}O_{2max}$ and lowering of resting heart rate are adaptations to endurance exercise training (8, 62). Interestingly, the differences in leukocyte telomere length, *TERT* and *TPP1* mRNA expression between athletes and controls was no longer statistically significant after adjustment for $\dot{V}O_{2max}$ and resting heart rate, indicating these parameters may be important for telomere length maintenance.

Exceptional arterial health and cardiac capacity (primarily stroke volume) are required for a high $\dot{V}O_{2max}$ and maybe the underlying biological mechanisms explaining the observed association with telomere length maintenance. The shorter leukocyte telomeres observed in patients with atherosclerosis is well known (7, 42, 47, 56) and shortening of leukocyte telomeres is more pronounced in individuals with atherosclerotic progression over a six (4) and ten (43) year time period. Leukocyte telomere length reflects the telomere length of haematopoietic stem cells (29), which are precursors for endothelial progenitor cells (3). Subsequently, endurance exercise training may attenuate telomere shortening in haematopoietic stem cells and, in turn, conserve the replicative potential of endothelial progenitor cells to ultimately conserve arterial health and function.

The stepwise inverse association between lower resting heart rate and leukocyte telomere length has multiple explanations. For example, exercise-training induced bradycardia involves decreased sympathetic nervous system activation and increased peripheral arterial compliance (8). Increased oxidative stress production in medulla of rats leads to sympathetic activation and hypertension (48). Telomeres are particularly vulnerable to shortening caused by inflammation (54) and oxidative stress (32, 70), and both are implicated in cardiovascular disease (10, 28, 37). Endurance athletes, however, have low circulating markers of inflammation (67) and exercise training leads to up-regulated antioxidant enzyme activity (22, 30).

Therefore, whilst speculative, ameliorated inflammation and oxidative stress, with up-regulate telomere-associated genes caused by endurance exercise training may protect against telomere shortening, but this requires additional investigation. Most studies have found a positive relationship between the amount of physical activity and leukocyte telomere length, but the optimal amount of exercise for telomere preservation is not known. Another novel aspect of our study was that after dividing subjects into tertiles for running and cycling distance covered per week, we found individuals in the middle and highest tertiles for exercise training possessed similar leukocyte telomere lengths that were longer compared to those in the lowest exercise tertile. A similar relationship was observed with *TERT* and *TPP1* mRNA expression, suggesting that exercise-induced benefits to telomere length maintenance maybe conferred by moderate and high amounts of exercise training. The practical application of these findings are that individuals who wish to maintain their leukocyte telomere length could benefit from running more than 10 km a week, but running more than 25 km a week may not provide additional telomere preservation. Similarly, cycling greater than 200 km a week may be unnecessary for telomere length maintenance, rather a minimum of 30 km cycling a week could elicit attenuate age-related telomere attrition. These data are somewhat supported by findings from epidemiological studies on physical activity measured in context with cardiovascular disease and mortality risk. A meta-analysis indicated the risk of coronary heart disease is reduced by 14% and 20% in individuals engaging in the recommended 150 and 300 minutes, respectively, of moderate-intensity physical activity per week (58). The relative risk of coronary heart disease, however, was only modestly lower in those engaging in the highest amount – 750 minutes – of physical activity per week (58). In a cohort of 55,137 adults, the relative risk reduction in all-

cause and cardiovascular mortality was reduced in runners compared to non-runners but the decreased risk was achieved with as little as running ~10 km per week (35). We found a linear relationship between leukocyte telomere length and resting heart rate. Resting heart rate has long been recognised an independent risk factor for cardiovascular disease and all-cause mortality, with higher resting heart rates eliciting a greater risk (27, 60, 66). Leukocyte telomere length is also a predictor of cardiovascular disease (7, 75) and all-cause mortality (16, 53). Therefore, it is possible that aerobic exercise training-induced telomere maintenance could occur in conjunction with lowering of resting heart rate and this, in turn, may ameliorate the risk of cardiovascular disease and mortality. This study was not designed to investigate the possible causal role exercise-induced lowering of resting heart rate has on leukocyte telomere length and disease and mortality risk. Future research should establish how improvement to cardiorespiratory fitness and a reduction of resting heart rate maintains telomere length.

We had over 90% power to detect a difference in leukocyte telomeres, which is a strength of the study. Whilst we acknowledge our data does not directly show that endurance exercise training maintains leukocyte telomere length, the alternative explanation would be that being born with long telomeres might be associated with a markedly higher cardiorespiratory performance and instinctive willingness to engage in extensive exercise training; an alternative and plausible explanation. A limitation of the study is that dietary analysis was not performed therefore we cannot account for the potential impact of diet on leukocyte telomere biology. Leukocyte protein was not collected therefore future studies should confirm the *TERT* and *TPP1* mRNA expression differences amongst athletes and controls at the translational level. Given that critically short telomeres promote cellular senescence (26), it would be

advantageous to study the percentage of short telomeres in context with physical activity and cardiorespiratory fitness, rather than mean telomere length outlined in the present study. Although our statistical analysis indicated key cardiorespiratory fitness adaptations – lower resting heart rate and superior $\dot{V}O_{2max}$ – partly explained the telomere length difference found between athletes and controls, additional studies are required to delineate the physiological mechanism. Our data was correlative and does not infer causation. Future work should focus on the molecular mechanisms regulating telomere length dynamics in context with exercise training. It will be important to determine the genetic contribution of long telomeres from the influence of exercise training. Considering $\dot{V}O_{2max}$ and resting heart rate are heritable traits, accounting for ~50% (6) and 13 to 60% (2, 15, 64) of the variation, respectively, it could be that endurance athletes from our study inherited long telomeres and their involvement in exercise training is coincidental. Longitudinal analyses are required to appreciate whether and what type of exercise training, and underlying physiological adaptations, attenuates the rate of telomere shortening in humans, to prevent biological ageing and disease.

In summary, endurance athletes possess longer leukocyte telomeres and up-regulated *TERT* and *TPP1* mRNA expression. Our findings indicate a role for $\dot{V}O_{2max}$ and lower resting heart rate in the benefits that endurance exercise training has on leukocyte telomere maintenance. We also found a plateauing effect between the amount of running and cycling distance covered per week and increasing leukocyte telomere length. Therefore, this suggests that moderate amounts of exercise (running: 10 to 25 km·week^{-1}; cycling: 30 to 200 km·week^{-1}) may be as sufficient as large amounts of exercise to prevent age-associated telomere erosion.

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Disclosures

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Tables

Table 1. Primer-sets and assay identification numbers.

Gene symbol	Primer-sets/Assay ID	CV (%)
TERT	F: GAA GAA GCC ACC TCT TTG GA	1.36
	R: AGA GAG CTG AGT AGG AAG GAG	
POT1	F: GCT CTG GCT TTG CAT CTT TG	0.82
	R: GGT GCC ATC CCA TAC CTT TAG	
TINF2	F: CAA GTC CTG AAA GCC CTG AA	1.32
	R: CTT TCT CCA GCT GAC ACA AGT A	
TPP1	F: CCA CGC TGC TTG TGT CT	1.05
	R: GCG GTC CAC CTG GAG ATA	
TERF1	F: ACC CTT GAT GCA CAG TTT GA	1.49
	R: CTG CCT TCA TTA GAA AGG TTG ATG	
TERF2	F: CAC ACC ACT GGA ATC AGC TAT C	0.66
	R: CAG GAT GGG CCA AGT TCT TT	
GAPDH (control)	F: GGG TGT GAA CCA TGA GAA GT	0.98
	R: AGT AGA GGC AGG GAT GAT GT	
TERF2IP	Hs00430292_m1	0.71
TERC	Hs03454202_s1	1.30
GAPDH (control)	Hs02786624_g1	1.03

Legend: ID, identification number (Life Technologies); CV, coefficient of variation (intra-plate).

749 Table 2. Characteristics of endurance athletes and controls.

Variable	Endurance athletes (n = 61)	Controls (n = 61)	*p*-value
Men/women (n)	46/15	47/14	
Age (y)	33.7 ± 11.03	28.7 ± 10.64	0.06
Ht (cm)	176.36 ± 10.10	173.82 ± 8.97	0.14
Wt (kg)	70.56 ± 10.69	78.65 ± 10.96	**< 0.001**
BMI (Wt/Ht^2)	22.6 ± 2.23	26.02 ± 2.95	**< 0.001**
SBP (mm Hg)	124.96 ± 10.91	125.75 ± 10.65	0.68
DBP (mm Hg)	73.44 ± 8.08	75.95 ± 9.11	0.11
PP (mm Hg)	51.52 ± 7.97	49.46 ± 9.45	0.20
MAP* (mm Hg)	90.46 ± 8.3	92.44 ± 8.73	0.20
Resting HR ($beats \cdot min^{-1}$)	51.62 ± 7.58	68.67 ± 10.62	**< 0.001**
$\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	58.77 ± 8.75	43.73 ± 7.03	**< 0.001**
Maximum treadmill speed ($km \cdot h^{-1}$)	17.02 ± 1.97	13.23 ± 1.92	**< 0.001**
Maximum wattage (w)	370.23 ± 69.38	-	**-**
PSS	12.21 ± 4.81	11.36 ± 5.74	0.39
Sitting ($min \cdot wk^{-1}$)	2010 (1290–2700)	4560 (2220–8460)	**< 0.001**
EEE ($Mj \cdot wk^{-1}$)	32.43 (23.23–55.7)	23.64 (8.92–40.65)	**0.002**
METs ($min \cdot wk^{-1}$)	6976 (4878–13116)	3528 (1556.5–7520.5)	**< 0.001**
Years trained (y)	5.5 (2.62–12)	2.25 (0–8.5)	**< 0.001**
Run distance ($km \cdot wk^{-1}$)	40 (30–60)	2.5 (0–10)	**< 0.001**
Cycle distance ($km \cdot wk^{-1}$)	150 (0–237.5)	-	**-**
Swim distance ($km \cdot wk^{-1}$)	4.5 (0–8)	-	**-**

Data are expressed as mean ± standard deviation or median (interquartile range) from two-tailed independent samples *t*-tests or Mann-Whitney U-tests.

Legend: Ht, Height; Wt, Weight; BMI, body mass index; SBP, systolic BP; DBP, diastolic BP; PP, pulse pressure (SBP-DBP); MAP, mean arterial pressure *calculated by ((2×diastolic)+systolic)÷3; HR, heart rate; $\dot{V}O_{2max}$, maximal aerobic (cardiorespiratory) fitness; PSS, perceived stress scale; EEE, estimated energy expenditure; METs, metabolic equivalent of task.

Table 3. Linear correlations between age and telomere length in athletes and controls.

	All subjects (n = 122)		Athletes (n = 61)		Controls (n = 61)	
Variable	**r**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**
Age	0.03	0.74	0.04	0.78	-0.12	0.35

Data are from Spearman's correlations.

796 Table 4. Telomere-regulating gene expression in athletes and controls ($p > 0.05$).

Gene	FD
TERC	1.27
TRF1	0.91
TRF2	0.93
TINF2	0.90
POT1	0.97
TERF2IP	1.03

797 Data are expressed as fold-difference relative to controls (FD = 1).

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Table 5. Linear correlations between telomere length and telomere-associated gene expression.

	All subjects (n = 121)	
Gene	**r**	***p*-value**
TERT	0.09	0.315
TERC	**-0.28**	**0.003**
TRF1	-0.08	0.35
TRF2	0.05	0.55
TPP1	0.12	0.25
TINF2	0.07	0.48
POT1	-0.007	0.93
TERF2IP	0.05	0.61

Data are from two-tailed Spearman's Correlation.

Table 6. Stepwise regression models for *TERT* and *TPP1* mRNA expression.

Dependent variable	Predictors	Unstandardised B-value	SE	*t*-value	*p*-value	r^2 (adj)
TERT	HR	-1.24	0.34	-3.64	< 0.001	0.094
TPP1	Age	0.33	0.16	2.01	0.047	0.095
	Height	0.45	0.18	2.15	0.01	
	HR	-0.26	0.14	-1.89	0.06	

Data are from stepwise linear regression. Variables excluded from the models for *TERT* include: age, height, weight, body mass index, systolic, diastolic, pulse and mean arterial pressure, and $\dot{V}O_{2max}$. Variables excluded from the models for *TPP1* include: weight, body mass index, systolic, diastolic, pulse and mean arterial pressure, and $\dot{V}O_{2max}$.

Legend: SE, standard error; HR, resting heart rate.

Figure legends

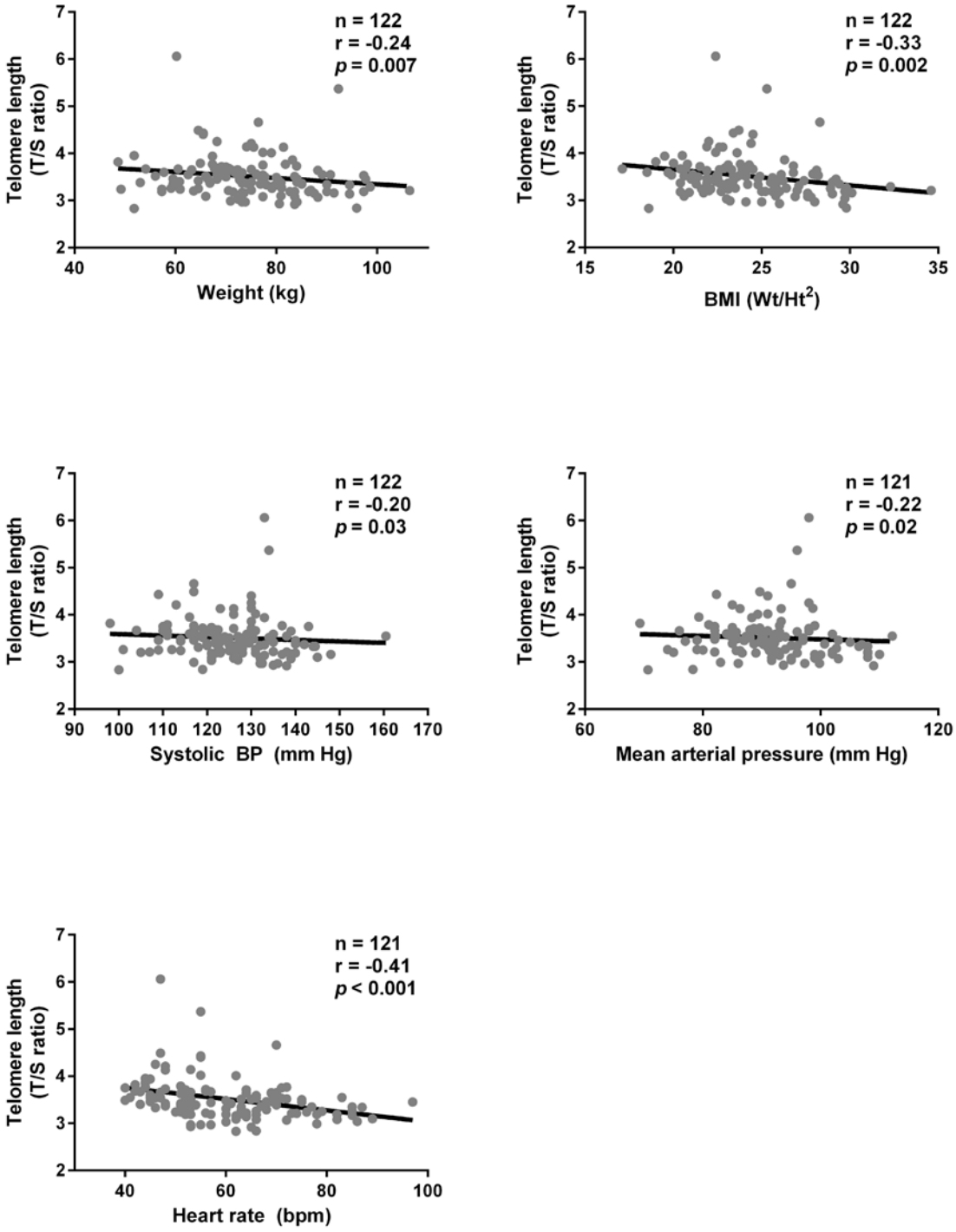
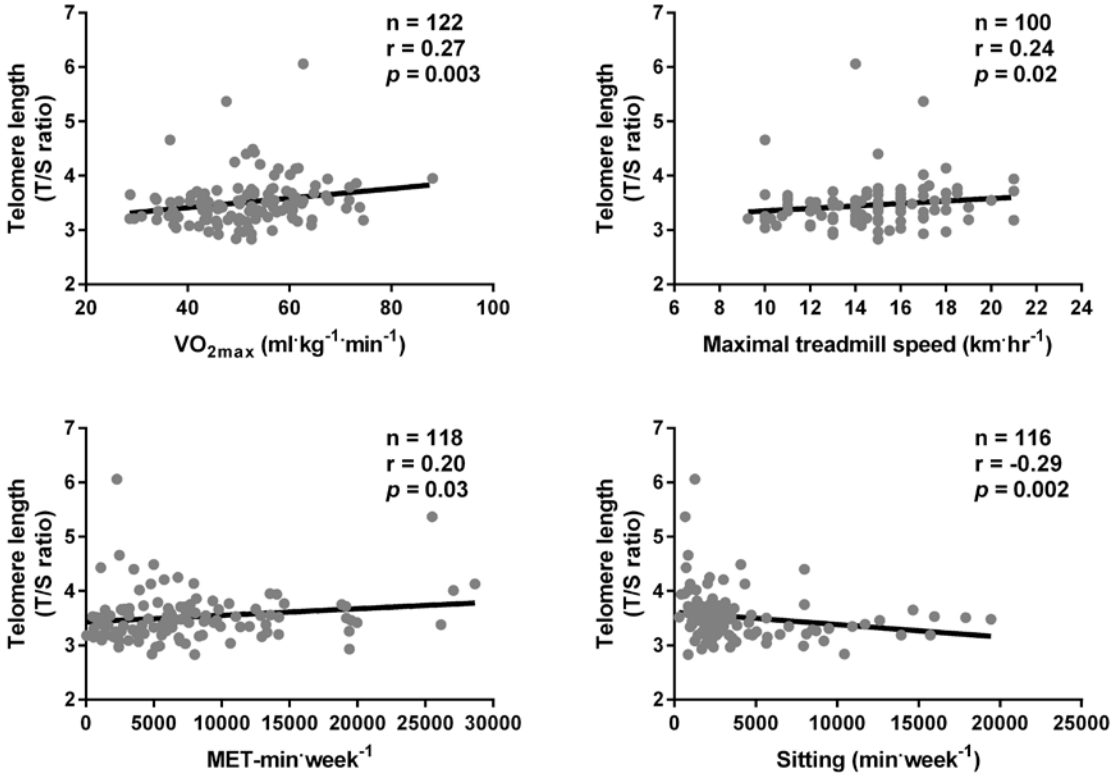


Figure 1. Linear correlations between leukocyte telomere length and health parameters. Data are from Spearman's correlations.



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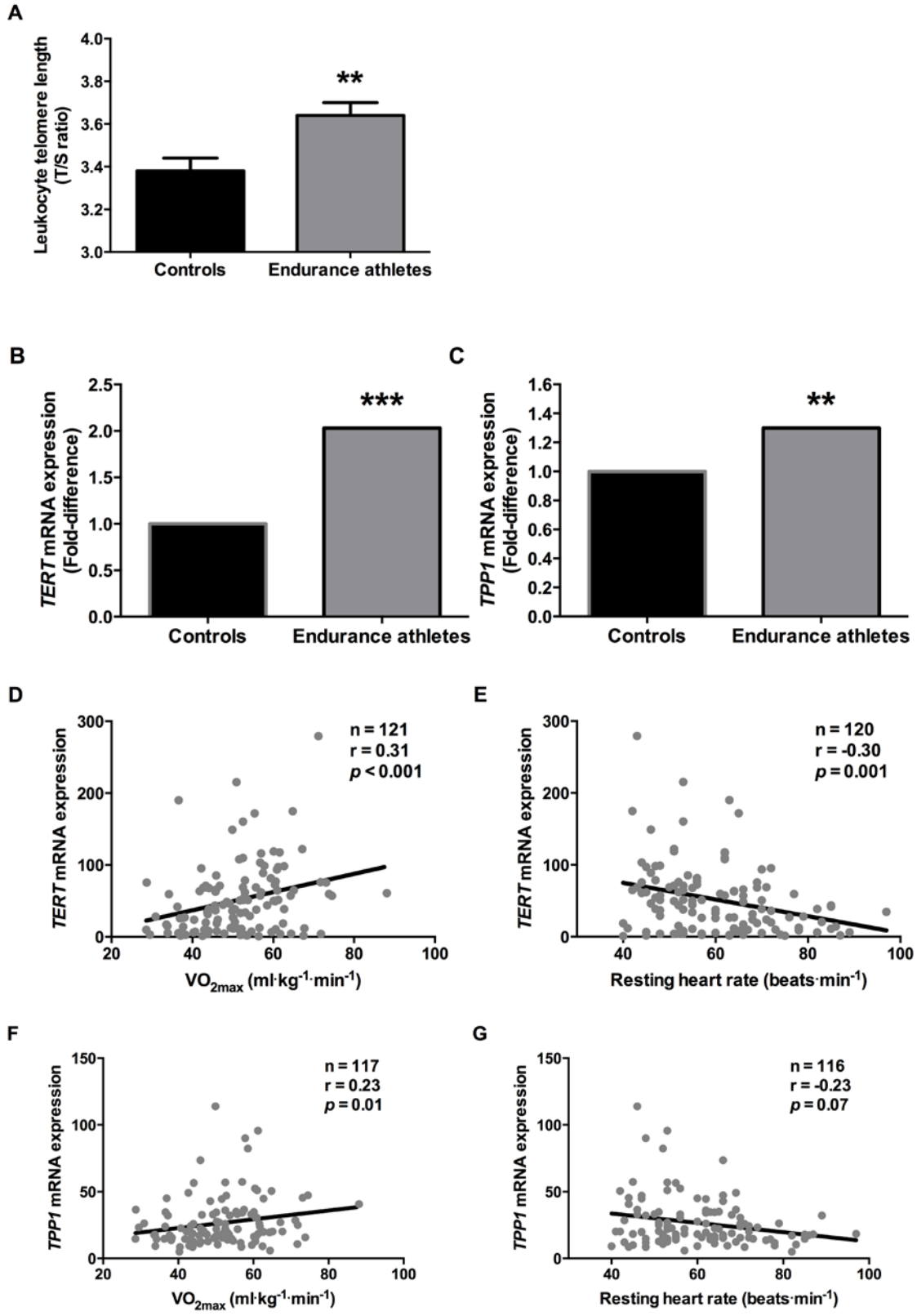
851 **Figure 2. Linear correlations between leukocyte telomere length and exercise**

852 **parameters.** Data are from Spearman's correlations.



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854 **Figure 3. Endurance exercise, telomere length, and *TERT* and *TPP1* mRNA**

855 **expression.** A) Leukocyte telomere lengths adjusted for age are from an ANCOVA

including 61 athletes and controls. Bars and whiskers indicate mean and standard error, respectively. Relative to controls, endurance athlete had increased *TERT* (B) and *TPP1* (C) mRNA expression (athletes vs controls [relative expression ± SE]: 68.31 ± 7.03 vs 34.07 ± 4.3, $p < 0.001$ and 31.39 ± 2.93 vs 21.53 ± 1.56, $p = 0.004$, respectively). Data are from Mann-Whitney U test. Correlations between *TERT* mRNA expression, $\dot{V}O_{2max}$ (D) and resting heart rate (E). Correlations between *TPP1* mRNA expression, $\dot{V}O_{2max}$ (F) and resting heart rate (G). Data are from Spearman's correlations. $^{**}p < 0.01$; $^{***}p < 0.001$.

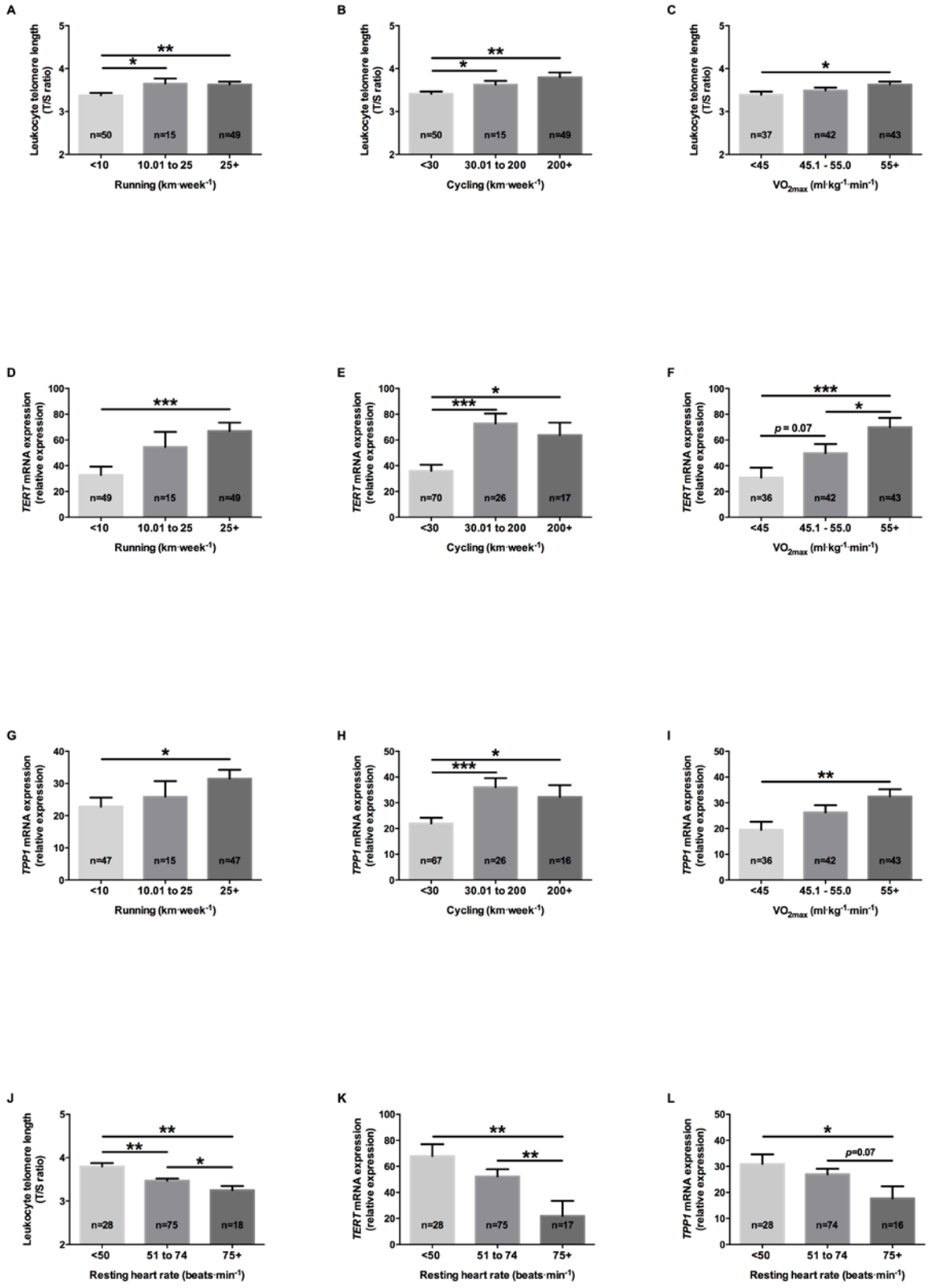

Figure 4. Moderate amounts of exercise training and lower resting hear rates are associated with longer leukocyte telomeres. Telomere length was analysed in context with running (A) and cycling (B) distance, and $\dot{V}O_{2max}$ tertiles (C). Similarly,

TERT (D, E and F) and *TPP1* (G, H and I) mRNA expression was analysed in context with running, cycling and $\dot{V}O_{2max}$, respectively. Heart rate tertiles were formed and analysed in context with telomere length (J), *TERT* (K) and *TPP1* (L) mRNA expression. Bars and whiskers indicate mean±SE from an ANCOVA, adjusted for age. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.