The influence of exercise on telomere biology

THE INFLUENCE OF EXERCISE ON TELOMERE BIOLOGY

by

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A Doctoral Thesis

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ABSTRACT

Telomeres are repetitive nucleotide sequences located at chromosome ends; their length shortens with age and is proposed to reflect the rate of biological ageing. Negative lifestyle factors, including a lack of exercise, obesity, psychological stress, and smoking appear to increase telomere attrition rate and consequentially accelerate ageing. Conversely, positive lifestyle factors, such as exercise participation, meditation, and a healthy diet are reported to largely attenuate telomere shortening rate. The studies described in this thesis investigated the effect of various exercise modalities on telomere length (TL). Potential exercise-induced cellular mechanisms regulating telomere dynamics (e.g., telomerase reverse transcriptase (TERT) expression, inflammation, antioxidant gene expression) were also explored.

Chapter III demonstrated that a 12-week low-load, high-repetition resistance training (RT) intervention does not influence TL in sedentary middle-aged individuals. TERT gene expression also remained unchanged following the 12 -week RT intervention, although it was borderline significant ($p = 0.05$). A negative inverse association was observed between TL and serum levels of C-reactive protein (CRP) at pre and post timepoints. Furthermore, a 12-month follow-up visit revealed that TL was maintained in participants who continued to exercise using a variety of modalities. In comparison, individuals who returned to a sedentary lifestyle displayed significant telomere attrition.

Chapter IV revealed that experienced female Pilates practitioners (5.7 ± 1.5 years of training) displayed comparable TL to their sedentary aged-matched counterparts at pre (baseline) and post (12 months) timepoints. Additionally, the longitudinal analysis revealed that neither group exhibited any significant telomere shortening over the subsequent 12-month period. There was also no difference in TERT gene expression between groups at any time point. Tumour necrosis factor-α (TNF-α) serum levels were negatively correlated with TL across the whole cohort (all timepoints).

In **Chapter V**, young endurance athletes were shown to possess longer telomeres than master endurance runners, master sprinters, and middle-aged controls, which appeared to be the result of their reduced BMI and visceral fat rating. However, these differences in TL were not significant when controlled for covariates. It was also discovered that leukocyte and buccal cell TL are highly correlated in this athlete cohort. TERT gene expression was comparable between all groups and positively associated with TL.

The final study of this thesis, **Chapter VI**, discovered that young elite swimmers have shortened telomeres compared with age-matched recreationally active controls. When split by

sex, only elite female swimmers displayed significantly shorter telomeres than their recreationally active counterparts. These results suggest a negative effect of a high-level swimming competition and/or training on TL and subsequent biological ageing in females. The results of this thesis collectively suggest that a variety of exercise modalities can influence telomere dynamics, although their effects appear to be divergent and modality specific. Low-intensity exercise modalities (e.g., low-load, high-repetition RT and Pilates) do not appear to be a sufficient stimulus to regulate telomere dynamics, and exercise modalities demanding a higher intensity (e.g., swimming) may be necessary to influence TL. Nonetheless, extremely high intensities and/or doses of exercise appear detrimental to TL. Additionally, TERT gene expression does not appear to be regulated by the exercise modalities employed in this thesis.

Key words: ageing, exercise, gene expression, inflammation, telomere, TERT.

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'The stamina in the motivation is more important than the intensity of the motivation'

Arsene Wenger

PUBLICATIONS

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8-oxoG	8-oxoguanine
ACE	Angiotensin I converting enzyme
ALT	Alternative lengthening of telomeres
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATM	Ataxia telangiectasia mutated
ATP5B	ATP Synthase F1 Subunit Beta
ATR	ATM and Rad3 related
BF%	Body fat percentage
BIA	Bioelectrical impedance analysis
BMI	Body mass index
cDNA	Complementary deoxyribonucleic acid
CMJ	Countermovement jump
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
Ct	Cycle threshold
CV	Coefficient of variation
CVD	Cardiovascular disease
CYC1	Cytochrome C1
DDR	DNA damage response
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

FFM	Fat free mass
FM	Fat mass
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GPX	Glutathione peroxidase
HIIT	High-intensity interval training
hTERC	Human telomerase RNA component
hTERT	Human telomerase catalytic subunit
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IPAQ-SF	International Physical Activity Questionnaire - Short Form
kb	Kilobases
LTL	Leukocyte telomere length
LPA	Light physical activity
MC	Middle-aged controls
ME	Master endurance runners
MET	Metabolic equivalent
MMqPCR	Monochrome multiplex qPCR
MPA	Moderate physical activity
mRNA	Messenger ribonucleic acid
MS	Master sprinters
MVPA	Moderate-to-vigorous physical activity
OGG1	8-oxoguanine DNA glycosylase

PBMC	Peripheral blood mononuclear cells
POT1	Protection of telomeres 1
(Q-FISH)	Quantitative fluorescence *in situ* hybridisation
qPCR	Quantitative polymerase chain reaction
RAP1	Ras-related protein 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Resistance training
SOD	Superoxide dismutase
STELA	Single telomere length analysis
STST	Sit-to-stand test
TBW	Total body water
TERC	Telomerase RNA component
TERT	Telomerase catalytic subunit
TeSLA	Telomere Shortest Length Assay
TIN2	TERF1-interacting nuclear factor 2
TL	Telomere length
TNF-α	Tumour necrosis factor-alpha
TPP1	Tripeptidyl peptidase 1
TRF	Telomere restriction fragment
TRF1	Telomere repeat-binding factor 1
TRF2	Telomere repeat-binding factor 2
T/S	Telomere repeat copy number to single-copy gene copy number
VO_2 max	Maximal oxygen consumption

VPA	Vigorous physical activity
WEMWBS	Warwick-Edinburgh Mental Wellbeing Scale
YC	Young controls
YE	Young endurance runners
YS	Young sprinters

CHAPTER I - INTRODUCTION

1.1 Telomere biology

1.1.1 An historical overview

Eukaryotic chromosomes were long believed to be linear arrangements in which the ends were indistinguishable from the rest of their structure. This was until Hermann Muller and Barbara McClintock individually proposed the existence of a terminal region, distinct from the rest of the chromosome, that bestowed genomic stability (McClintock, 1941). Muller termed this structure the telomere, derived from the Greek *telos* (end) and *meros* (part) (Muller, 1938).

In 1961, Leonard Hayflick observed that cultured cell populations divided ~40-60 times before they experienced replicative senescence (Hayflick and Moorhead, 1961). This phenomenon, referred to as the Hayflick limit, contested the notion that normal cells were immortal in culture and was widely debated for a decade; until the concepts of telomeres and replicative senescence converged in the 1970's. Alexey Olovnikov was the first scientist to report that cells lack the ability to completely replicate chromosome ends, due to the end-replication problem (Olovnikov, 1971). Briefly, small quantities of genetic material are lost with each round of cell division, leading to the progressive shortening of telomeres. Olovnikov proposed that telomere erosion may be the cause of replicative senescence and could therefore explain the Hayflick limit (Olovnikov, 1973). Jim Watson, who also independently reported the end-replication problem, further suggested that a protective mechanism must exist in order to prevent chromosomal shortening and ensure the survival of an organism (Watson, 1972).

The initial experimental answer to the end-replication problem came courtesy of Elizabeth Blackburn and Joseph Gall in the late 1970's, when they discovered a telomeric DNA sequence (TTGGGG), repeated 20-70 times, at the terminal end of *Tetrahymena thermophila* chromosomes (Blackburn and Gall, 1978). In 1985, Blackburn went on to further uncover an enzyme that possessed 'terminal transferase' activity, capable of extending telomeric sequences, and aptly named it telomerase (Greider and Blackburn, 1987). The causal relationship between telomere length (TL) and replicative capacity was ultimately confirmed in 1998 when it was demonstrated that telomere elongation increased the replicative potential of retinal pigment epithelial cells and foreskin fibroblasts (Bodnar et al., 1998).

1.1.2 Telomere structure and function

Telomeres are repeat, non-coding DNA sequences located at chromosome ends. They function as protective caps and confer genomic stability by protecting internal regions from deterioration, end-to-end fusions, and recombination (Neidle and Parkinson, 2003). Telomeric sequences are highly conserved, organism-specific, and variable in length (Palm and de Lange, 2008). The human telomeric nucleotide sequence (5'-TTAGGG-3') is repeated thousands of times and spans 5-15 kilobases (kb) (Moyzis et al., 1988; Palm and de Lange, 2008). The majority of telomeric DNA is double-stranded, although the 3' end of eukaryotic chromosomes possesses a highly variable, guanosine-rich, 50-400 nucleotide single-strand overhang (Henderson and Blackburn, 1989). This terminal region of telomeric DNA is essential to the structure of telomere ends, folding back on itself to exist as a T-loop, in which the 3' overhang inserts into homologous double-stranded regions to form a structure termed the displacement (D)-loop (Griffith et al., 1999) (Figure 1.1.). This structure is fabricated and upheld by an array of proteins known collectively as the shelterin complex.

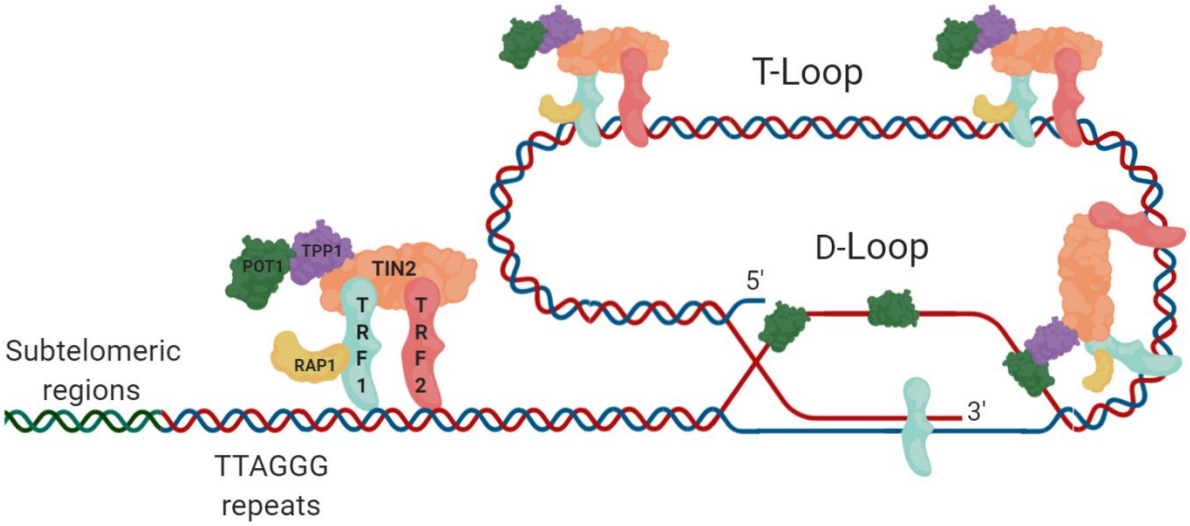

Figure 1.1. Telomere capping structure and the shelterin complex. Adapted from Maestroni (2017).

Shelterin is a multiprotein complex essential to the formation of the T-loop and is comprised of six key subunits: TRF1, TRF2, POT1, RAP1, TIN2, and TPP1 (Stansel et al., 2001). The primary function and setting of all these subunits is localised to telomeres and they typically act as negative regulators of telomerase-mediated elongation (De Lange, 2005). The shelterin complex acts as a protective cap to prevent chromosome ends from being recognised as double-stranded breaks, consequently inhibiting activation of the DNA damage response (DDR). As such, disturbances to individual subunits of the shelterin complex have been shown to affect telomere dynamics and activate the DDR (Smogorzewska and de Lange, 2002). The two main DNA damage-signalling pathways, ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR), are activated by double- and single-strand breaks, respectively. Upon activation of these pathways, kinases phosphorylate downstream targets to initiate either DNA repair, apoptosis or cellular senescence (Matsuoka et al., 2007). As telomeric DNA is single- and double-stranded, it must be able to suppress both the ATM and ATR pathways to prevent activation of the DDR throughout much of the cell cycle.

The specificity of shelterin for telomeric DNA is determined by the TRF1, TRF2 and POT1 subunits. TRF1 and TRF2 are homodimeric proteins that bind to double-stranded regions of telomeric DNA and are essential to the formation of the T- and D-loop structures (De Lange, 2010). TRF1 generally acts as a negative regulator of TL by inhibiting the action of telomerase (Van Steensel and De Lange, 1997). Thus, overexpression of this subunit results in telomere shortening (Smogorzewska et al., 2000). However, when telomere elongation is required, TRF1 recruits helicases to enable this process (Sfeir, 2012). TRF2 has a fundamental role in T-loop formation and hides the double-stranded ends of telomeres to block the ATM pathway (Stansel et al., 2001). TRF2 deletion results in the deprotection of telomeres and permanent activation of the DDR (Celli and de Lange, 2005). POT1 shelters single-stranded regions of telomeric DNA (Flynn and Zou, 2010), preventing their detection and subsequent activation of the ATR kinase pathway and cell cycle arrest (Kelleher et al., 2005).

The remaining shelterin subunits; TIN2, TPP1, and RAP1, are primarily involved in the assembly and stabilisation of the shelterin complex. TIN2 is a stabilising protein that binds to TRF1, TRF2, and the TPP1-POT1 complex, acting as a bridge between the different telomeric proteins (Takai et al., 2010). TPP1 is associated with POT1 and is fundamental in the recruitment of telomerase (Abreu et al., 2010). RAP1 is a TRF2-bining protein that acts as a negative regulator of TL (Palm and de Lange, 2008).

The interaction between telomeric DNA and the shelterin complex plays an essential role in telomere dynamics. This unique nucleoprotein arrangement forms a cap at chromosome ends and serves two key functions: 1) it impedes terminal chromosome regions from being recognised as sites of DNA damage, preventing unnecessary activation of the DDR. 2) the repeating, non-coding DNA sequences prevent the degradation of genes located at chromosome ends.

1.1.3 Telomere attrition and replicative senescence

Cultured cells are mortal and cease to replicate after ~40-60 rounds of division (Hayflick and Moorhead, 1961). This gradual and irreversible state of proliferative cessation is termed replicative senescence. With each round of cellular division telomeres shorten ~25-300 bp due to the end-replication problem (Kumar and Bhatia, 2013), which arises since the enzyme DNA polymerase operates exclusively in the 5'-3' direction. During DNA replication, the anti-parallel nature of DNA dictates double-strand formation occurs in opposing directions at the replication fork. DNA polymerase can extend the leading strand with a single primer, but the lagging strand is discontinuously elongated. These small fragments are termed Okazaki fragments, and each requires a new primer for their synthesis. When Okazaki fragments extend until they intersect the preceding RNA primer, RNA nucleotides are replaced by DNA (Blackburn and Gall, 1978). Without DNA to serve as a template for the final primer the replication machinery cannot synthesise the complementary sequence, resulting in the loss of nucleotides and a 3′ overhang gap on each new DNA molecule (Figure 1.2.).

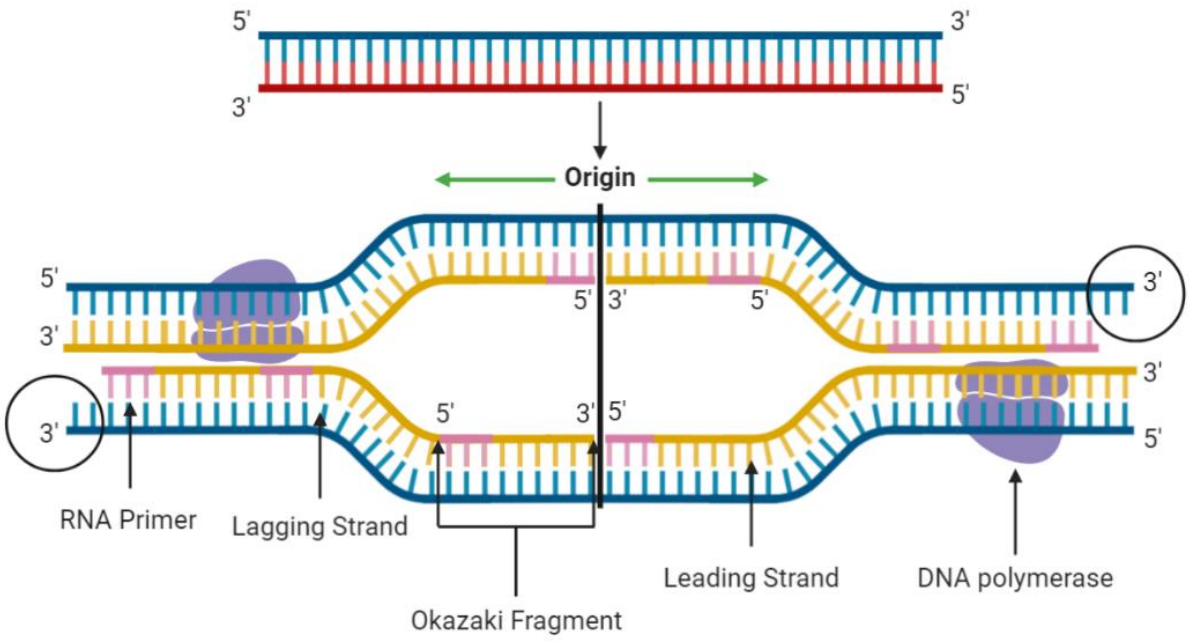

Figure 1.2. The end-replication problem. Adapted from Hug and Lingner (2006)**.**

Since telomeres shorten with cellular division, the replicative history of a cell is concurrently documented. Each successive round of DNA replication compounds the loss of chromosome ends, until telomeres reach a critical mean length (4–7 kb) (Harley et al., 1990). When telomeres become critically short, they lack the ability to assemble the shelterin complex and become 'uncapped'. This conformation resembles a double-strand DNA break and activates the ATM pathway, consequent DDR, and replicative senescence (Artandi and Attardi, 2005) (Figure 1.3.). Approximately 5–10 short telomeres are required to initiate replicative senescence in normal mammalian cells (Kaul et al., 2012; Zou et al., 2004). Telomere shortening is proposed to be one of the primary mechanisms responsible for replicative senescence (Victorelli and Passos, 2017). The accumulation of senescent cells restricts a tissues capacity for growth and repair and is proposed to contribute to tissue and organismal ageing (Van Deursen, 2014). Senescent cells are typically removed by the immune system, but this capability is diminished in humans with advancing age (Muñoz-Espín and Serrano, 2014).

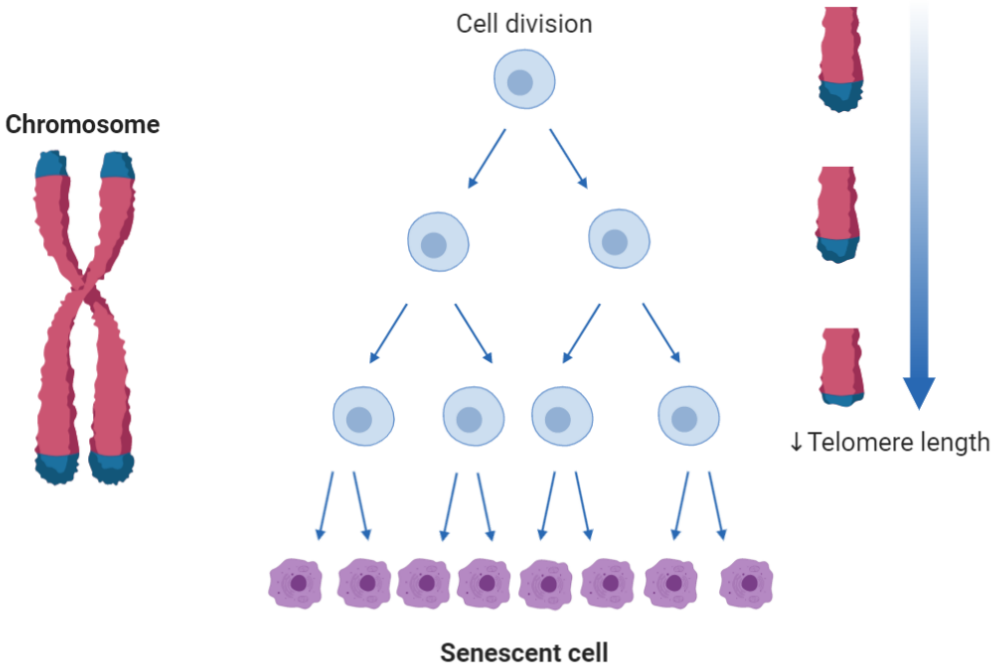

Figure 1.3. Cell division and telomere shortening. Telomeres shorten with progressive cell division. When telomeres become critically short, they cease to divide and enter senescence. Adapted from Schmidt (2020).

Although the end-replication problem is regarded to be the principal cause of telomere attrition, the loss of chromosome ends can occur via alternative DNA-damaging cellular mechanisms. For example, it has been proposed that oxidative stress is the major cause of telomere attrition in human fibroblasts (Von Zglinicki et al., 2000). Oxidative stress represents the imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defences (Betteridge, 2000). Telomeres are particularly sensitive to ROS due to their high content of guanine nucleotides, which renders them common sites of DNA damage (Kawanishi and Oikawa, 2004). To compound this problem, telomeric regions are deficiently repaired in comparison to the rest of the genome (Shen et al., 2010). The inadequate repair of telomeric breaks results in the accumulation of damaged DNA, which impedes DNA polymerases during G0 and G1 phases (Von Zglinicki et al., 2005). Consequently, telomeres may not be completely replicated and experience subsequent accelerated rates of attrition. To highlight the impact of ROS-induced DNA damage, the steady-state level of oxidatively modified guanine nucleotides is ~35% higher in senescent cells than in young cells (Chen et al., 1995). Numerous oxidative

stress genes have also been linked to telomere attrition in humans and several clinical reports show enhanced oxidative stress is associated with reduced TL (Demissie et al., 2006; Ma et al., 2013; Masi et al., 2011; Richter and Zglinicki, 2007; Starr et al., 2008; Watfa et al., 2011). Since excess ROS appear to induce telomere loss, it is unsurprising that antioxidant treatments can preserve TL and may confer protective benefits (Borrás et al., 2004; Kashino et al., 2003; Yudoh et al., 2005). Overexpression of the antioxidant superoxide dismutase (SOD) attenuates telomere attrition rate in human fibroblasts that possess a low antioxidant capacity, providing evidence that oxidative stress plays a causal role in telomere shortening (Serra et al., 2003).

Inflammation is also implicated with telomere attrition, via an increased rate in cell turnover (Bateson and Nettle, 2017), direct inhibition of telomerase activity (Beyne-Rauzy et al., 2004), and the promotion of increased oxidative stress (Khansari et al., 2009). Inflammation and oxidative stress are interdependent pathophysiological events, and certain ROS can upregulate proinflammatory gene expression (Anderson et al., 1994). Augmented expression of pro-inflammatory cytokines TNF-α, interleukin-6 (IL-6), and interferon-γ (IFN-γ) have been suggested to accelerate telomere attrition in leukocytes (O'Donovan et al., 2011; Zhang et al., 2016). Moreover, IL-6 levels are inversely correlated with TL in chronic obstructive pulmonary disease (COPD) patients (Savale et al., 2009) and TNF-α is suggested to cause telomere shortening by downregulating telomerase (Khan et al., 2012). A significant inverse association also exists between leukocyte TL (LTL) and CRP levels in individuals with type II diabetes (Al-Attas et al., 2010). Additionally, elevated levels of CRP (≥ 2 mg/L) are correlated with reduced TL in healthy older adults (Shin and Baik, 2016). Interestingly, mutations in human telomerase RNA component (hTERC) and human telomerase reverse transcriptase (hTERT) increase expression of pro-inflammatory cytokines in the lung (IL-1, IL-6, IL-10 and TNF-α) and cause premature ageing in alveolar stem cells, suggesting that the relationship between damaged telomeres and inflammation may be mutual (Chen et al., 2015). Collectively, the discussed cellular mechanisms may explain why observable telomere shortening exceeds the rate predicted by the end replication problem alone.

1.1.4 Telomerase structure and function

To circumvent telomere attrition, and consequential replicative senescence and biological ageing, certain cells express the ribonucleoprotein telomerase. Telomerase is an RNA-dependant DNA polymerase that adds specific telomeric nucleotides onto the 3' ends of linear

chromosomes to replace those that are lost with each round of cell division (Greider and Blackburn, 1987). As such, LTL is reported to only shorten at an average annual rate of 30–35 bp (Herrmann et al., 2018).

Telomerase is comprised of two essential subunits: a catalytic subunit (TERT) and an RNA subunit (TERC). The hTERT protein contains four principal domains: a telomerase N-terminal, a telomerase RNA-binding domain, a reverse transcriptase domain and a C-terminal extension (Blackburn and Collins, 2011), which are critical to enzymatic activity, processivity, and recruitment of telomerase to telomeres (Bachand and Autexier, 2001; Huard et al., 2003; Jurczyluk et al., 2011; Schmidt et al., 2014; Seimiya et al., 2000). The structure of TERT enables it to encircle the chromosome and perform its primary function of nucleotide addition. The synthesis and expression of TERT is the rate-limiting determinant of telomerase activity (Counter et al., 1998) and TERT mRNA expression is correlated with telomerase activity (Kirkpatrick et al., 2003). Suppressed hTERT expression is responsible for the minimal telomerase activity observed in somatic tissues (Collins and Mitchell, 2002), whereas overexpression of this subunit results in somatic cell immortalisation (Kogan et al., 2006).

The telomerase TERC subunit template is species-specific and determined by an organism's telomeric repeat sequence (Shippen-lentz and Blaciuuburn, 2010). Therefore, the core domain of hTERC contains the RNA template from which hTERT synthesises TTAGGG telomeric repeats (AAUCCC). Unlike hTERT, hTERC is abundant in both somatic cells and immortal cell lines (Yashima et al., 1998). However, the expression of this subunit is not indicative of telomerase activity and high levels of hTERC have been measured in cell strains that possess low telomerase activity (Shay and Wright, 2019). Nonetheless, two molecules of both TERT and TERC are required for telomerase activity *in vitro*, as they construct the catalytically active core of telomerase (Mitchell and Collins, 2000).

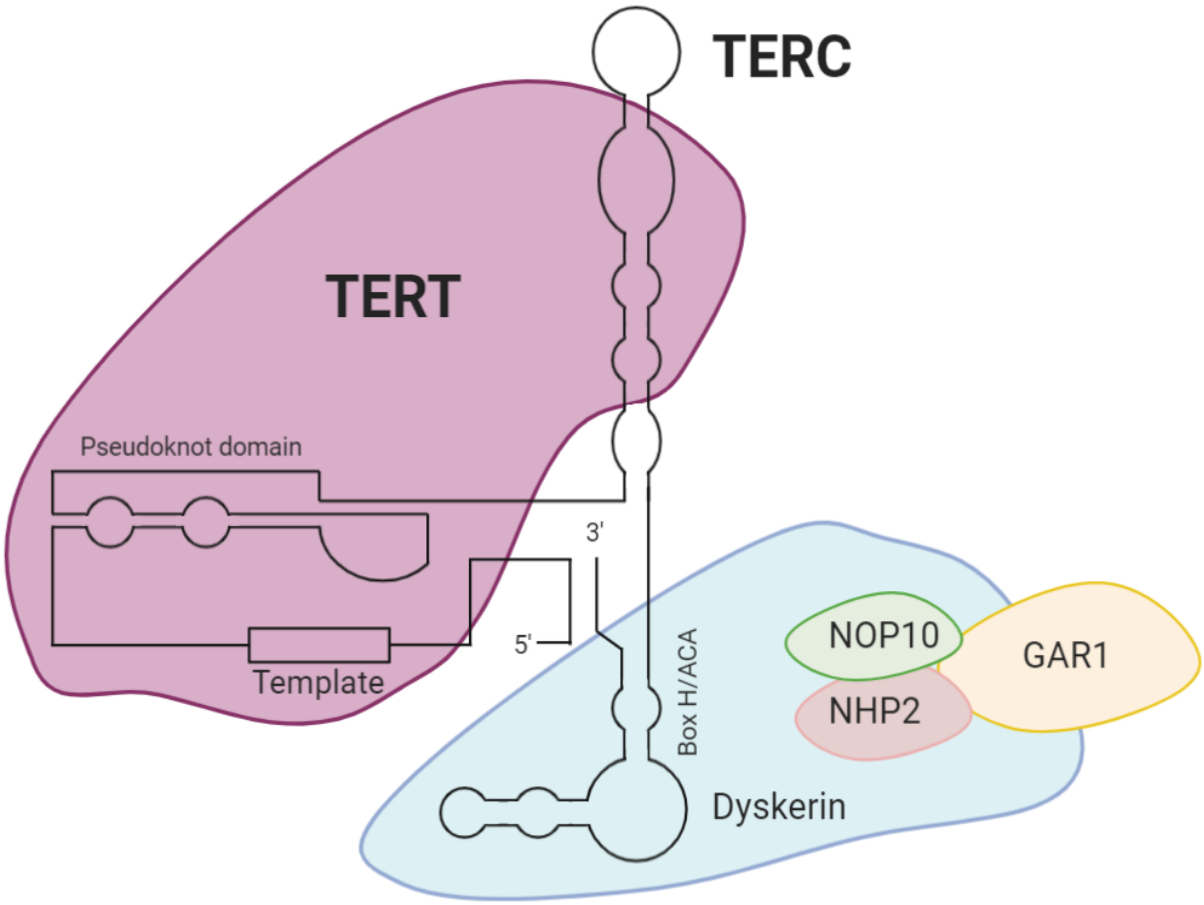

Figure 1.4. Schematic structure of the telomerase complex. Telomerase enzyme and its principal components: TERT, TERC, dyskerin, NOP10, NHP2 and GAR1. Functional regions of the TERC RNA (template, pseudoknot, Box H/ACA) and accessory proteins dyskerin, NHP2, NOP10 and GAR1 are indicated. Adapted from Garcia (2007).

1.1.5 Telomere length extension and maintenance mechanisms

The primary mechanism of TL extension occurs via a process termed repeat addition processivity (RAP); the continuous addition of nucleotide repeats to the 3' hydroxyl group by way of telomerase (Figure 1.5.).

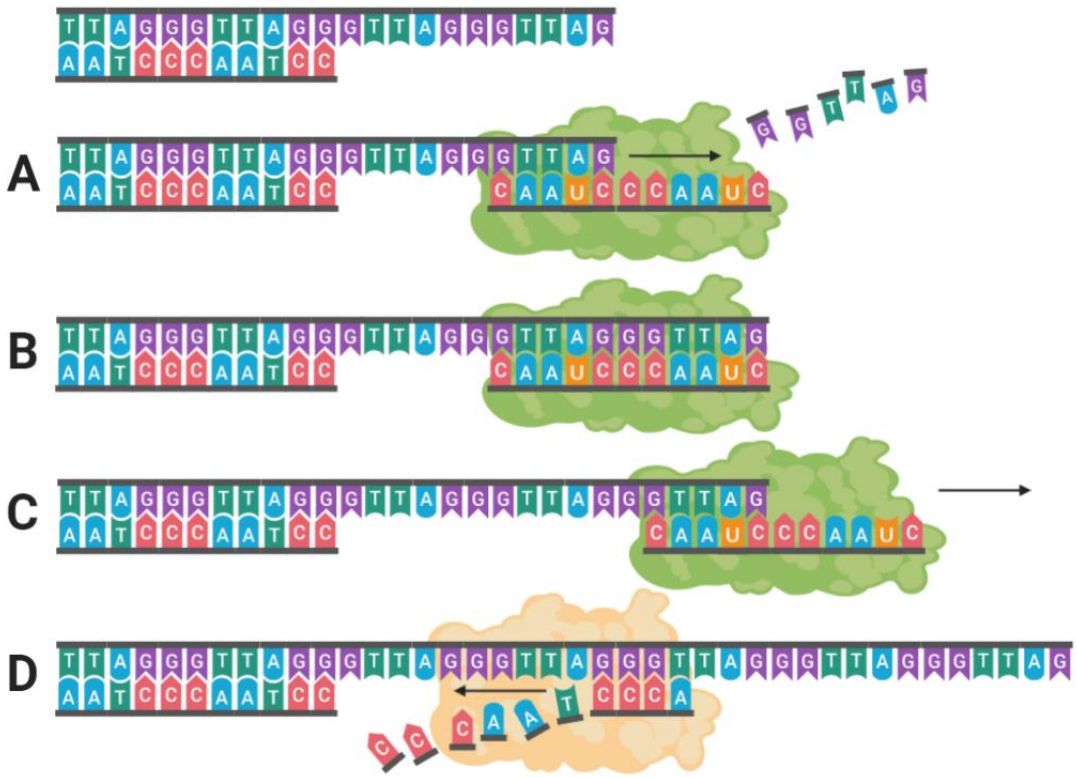

Figure 1.5. Telomerase repeat addition processivity. Precise telomeric DNA synthesis requires three key steps: recognition, elongation, and translocation. (A) telomerase binds to the 3' overhang of the chromosome that is complementary to the TERC sequence (B) telomerase elongates the 3' chromosome end by adding nucleotides until the 5' end of template RNA is reached (C) telomerase translocates on the newly synthesised DNA to initiate another round of nucleotide addition, which continues for multiple rounds (Huard et al., 2003) (D) DNA polymerase compliments the lagging strand. Adapted from Blackburn et al. (2015).

In humans, telomerase activity is limited to the germ line, adult stem, and cancer cells (Wright et al., 1996) and is typically non-existent in somatic cells (Kim et al., 1994). As a result, telomeres progressively shorten in somatic cells upon proliferation, which limits the replicative capacity of a cell and eventually culminates in replicative senescence (Allsopp et al., 1992). Low levels of telomerase have however been measured in cells of highly proliferative tissues that rely on the incessant ability to proliferate throughout our lifespan (Chiu et al., 1996; Härle-Bachor and Boukamp, 1996; Ramirez et al., 1997). Nonetheless, telomerase expression is downregulated in these cells upon further proliferation, and is only sufficient to extend their life span, not immortalise them. It has been reported that telomerase is expressed in 85% of human tumour tissues (Wright et al., 1996) and 98% of immortal cell lines (Kim et al., 1994), emphasising the importance of telomerase enzymatic activity to cell survival. Furthermore, reconstruction of telomerase activity *in vivo* increases the length of telomeric DNA and extends cellular life span (Vaziri and Benchimol, 1998).

Although telomerase is the principal mechanism of telomere lengthening, TL can also be preserved by the alternative lengthening of telomeres (ALT) pathway. This mechanism is independent of telomerase and is thought to rely on homologous DNA recombination, although the exact mechanism is not fully elucidated (Dunham et al., 2000). Overall, TL is a balance between shortening (e.g., end-replication problem and oxidative stress) and elongation events (e.g., repeat addition processivity and ALT).

1.1.6 Heritability of telomere length and association with age

The study of longitudinal variations in TL is termed telomere dynamics. Human TL is predominantly determined by genetic inheritance and is estimated to account for ~44-80% of heritability (Starkweather, 2014). The length of human telomeres is highly variable at birth and typically spans 10-12 kb (Okuda, 2002). TL is similar in both male and female newborns and synchronised across tissue types (Okuda, 2002). Additionally, the TL of newborn babies is significantly correlated with both parents and appears to be greatly influenced by paternal age at conception (Factor-Litvak et al., 2016). To highlight the importance of TL at birth, it is proposed to be the strongest predictor of expected lifespan (Heidinger et al., 2012).

Telomeres shorten ~30-35 bp per year (Herrmann et al., 2018) and longitudinal studies provide substantial evidence that TL is negatively associated with chronological age (Aviv et al., 2009; Farzaneh-Far et al., 2010; Gardner et al., 2005). TL typically follows a biphasic curve, with the

most prominent rate of erosion occurring in the initial years of life following birth (Aubert and Lansdorp, 2008). Furthermore, telomere attrition appears to be accelerated in individuals that display longer telomeres at a young age, suggesting a regulatory mechanism exists to protect the shortest telomeres and/or that longer telomeres are more vulnerable to harmful factors (Adolfsson et al., 2009). TL then remains comparatively stable during childhood and adolescence, before another phase of accelerated attrition in the senior years, which is proposed to be associated with the onset of age-related diseases (Herrmann et al., 2018).

1.1.7 Telomere length and age-associated disease

It is well established that ageing is the primary risk factor associated with the onset of numerous non-communicable disease states (Franceschi et al., 2018). Since TL is inversely correlated with chronological age, the relationship between age-associated diseases and telomeres has been extensively investigated.

Human life span is largely influenced by the development of age-associated diseases, particularly cardiovascular disease (CVD) (Herrmann et al., 2018). It has been reported that shortened telomeres are linked with atherosclerosis (Samani et al., 2001), hypertension (Aviv and Aviv, 1999), and large artery stiffness in patients with coronary heart disease (Wang et al., 2011). LTL has been proposed to predict advanced atherosclerosis and CVD risk (Willeit et al., 2010), and longer telomeres are independently associated with an improved long-term prognosis in patients with acute coronary syndrome (Perez-Rivera et al., 2012). One of the primary risk factors for CVD is diabetes, another age-associated disease independently connected to shortened telomeres (Elks and Scott, 2014; Wang et al., 2016). Telomere shortening has even been observed in individuals at the phase of impaired glucose tolerance (Adaikalakoteswari et al., 2007). Additionally, a more complex association between TL, type II diabetes, and CVD may exist, since type II diabetic patients with atherosclerotic plaques demonstrate enhanced telomere attrition compared to those without plaques (Adaikalakoteswari et al., 2007).

A variety of cancers have been associated with shortened telomeres, including bladder, oesophagal, gastric, head and neck, ovarian, renal, and colorectal cancer (Maxwell et al., 2011; Wentzensen et al., 2011). Instead of undergoing replicative senescence when telomeres become critically short, somatic cancer cells continue to divide due to the lack of normal DDR mechanisms (Wentzensen et al., 2011). This is understood to occur via an upregulation in

telomerase, which facilitates telomere stability at short lengths and enables the cell to continue proliferating (Shay and Wright, 2012).

Age-associated neurological disorders such as Alzheimer's disease (Panossian et al., 2003), dementia, and cognitive decline (Martin-Ruiz et al., 2006) are also linked with shortened telomeres. Various genetic disorders characterised by premature ageing have also been defined that involve mutations in telomerase and telomere-related genes (Herrmann et al., 2018). The most notable of these diseases is dyskeratosis congenita, which is distinguished by short telomeres and a clinical accelerated age-associated phenotype (e.g. abnormal skin pigmentation, nail dystrophy, and mucosal leukoplakia phenotype) (Heiss et al., 1998). A significant inverse correlation also exists between TL and mortality (Boonekamp et al., 2013), although this association reduces with advancing age and does not appear to be predictive of age-associated mortality in individuals >85 years old (Martin-Ruiz et al., 2005). Despite evidence that TL is a risk indicator for several different age-associated diseases across an array of heterogenous tissue types, it remains unclear as to whether telomere attrition is causally involved in the development and progression of these age-associated diseases or occurs because of their pathology.

1.1.8 Factors influencing telomere length

Substantial intra-individual differences in absolute TL have been reported across cell types (Daniali et al., 2013; Goldman et al., 2018). Nevertheless, significant correlations in TL are typically found between tissues within an individual, highlighting an intra-individual synchrony in TL across the somatic tissues of humans (Stout et al., 2017; Takubo et al., 2002). It has been suggested that differences in TL between highly proliferative (e.g., leukocytes and skin) and minimally proliferative tissues (e.g., skeletal muscle and subcutaneous fat) are established during early life, since the rate of age-dependant telomere shortening appears to be equivalent across somatic tissues in adults (Daniali et al., 2013). Significant inter-individual variation in TL has also been detected in adults (Kahl et al., 2020), and males typically display shorter telomeres than females (Sanders & Newman, 2013). This may be a result of the positive influence of estrogen on TL (Kim et al., 2012), and disparities in oxidative and inflammatory profiles.

As described in section '1.1.3 Telomere attrition and replicative senescence', oxidative stress and inflammation are proposed to influence TL. It is well documented that an individual's lifestyle choices, and environment, can substantially modify these factors (Figure 1.6.). Thus,

it is unsurprising that negative lifestyle behaviours and environments that increase oxidative stress and inflammation are linked with shortened telomeres. For example, chronic psychological stress, childhood adversity, major depressive disorders, and low educational attainment have all been associated with shortened TL (Starkweather, 2014). The elevated glucocorticoid response to stress enhances free radical formation, reduces antioxidant defences, and increases cell proliferation, all of which may contribute to telomere attrition (Haussmann and Marchetto, 2010). Moreover, cortisol reduces telomerase activity in human T lymphocytes, which could result in attenuated telomere repair (Choi et al., 2008). Smoking is also associated with enhanced telomere attrition, and the consumption of one pack of cigarettes per day for 40 years is estimated to correspond to 7.4 years of accelerated ageing (Valdes et al., 2005). Obesity, which is characterised by high oxidative stress and inflammation, is associated with shortened TL (Dankel et al., 2017; Valdes et al., 2005). Furthermore, shorter sleep duration is associated with shortened telomeres in both children (James et al., 2017) and adults (Lee et al., 2014). Children living in neighbourhoods considered to be highly disordered are reported to display shorter telomeres than those who did not live in such areas (Theall et al., 2014). Conversely, positive lifestyle choices and environments are associated with longer TL. Dietary fibre intake (Cassidy et al., 2010) and diets rich in fruits and vegetables (Mirabello et al., 2009) have both been positively associated with longer telomeres. Moreover, individuals living in closer proximity to green spaces had longer TL than their equivalents who lived in the city (Woo et al., 2009). To highlight the potential influence of environmental factors on TL during the life course, father-child correlations of TL weaken with advancing age (Nordfjäll et al., 2010). It has also been reported that only shared environmental factors, not heritable factors, are associated with TL in an elderly cohort (Huda et al., 2007). Based on this evidence, TL appears a composite characteristic that is the product of the combined effect of genetic, lifestyle, and environmental factors and is therefore considered to be a marker of biological ageing, as opposed to chronological ageing (Mather et al., 2011). However, the hierarchy and interrelation between factors that have been shown to be associated with TL is unknown. There is a clear need for a multidimensional assessment of factors related to TL, which may provide insight on the development of interventions to protect LTL.

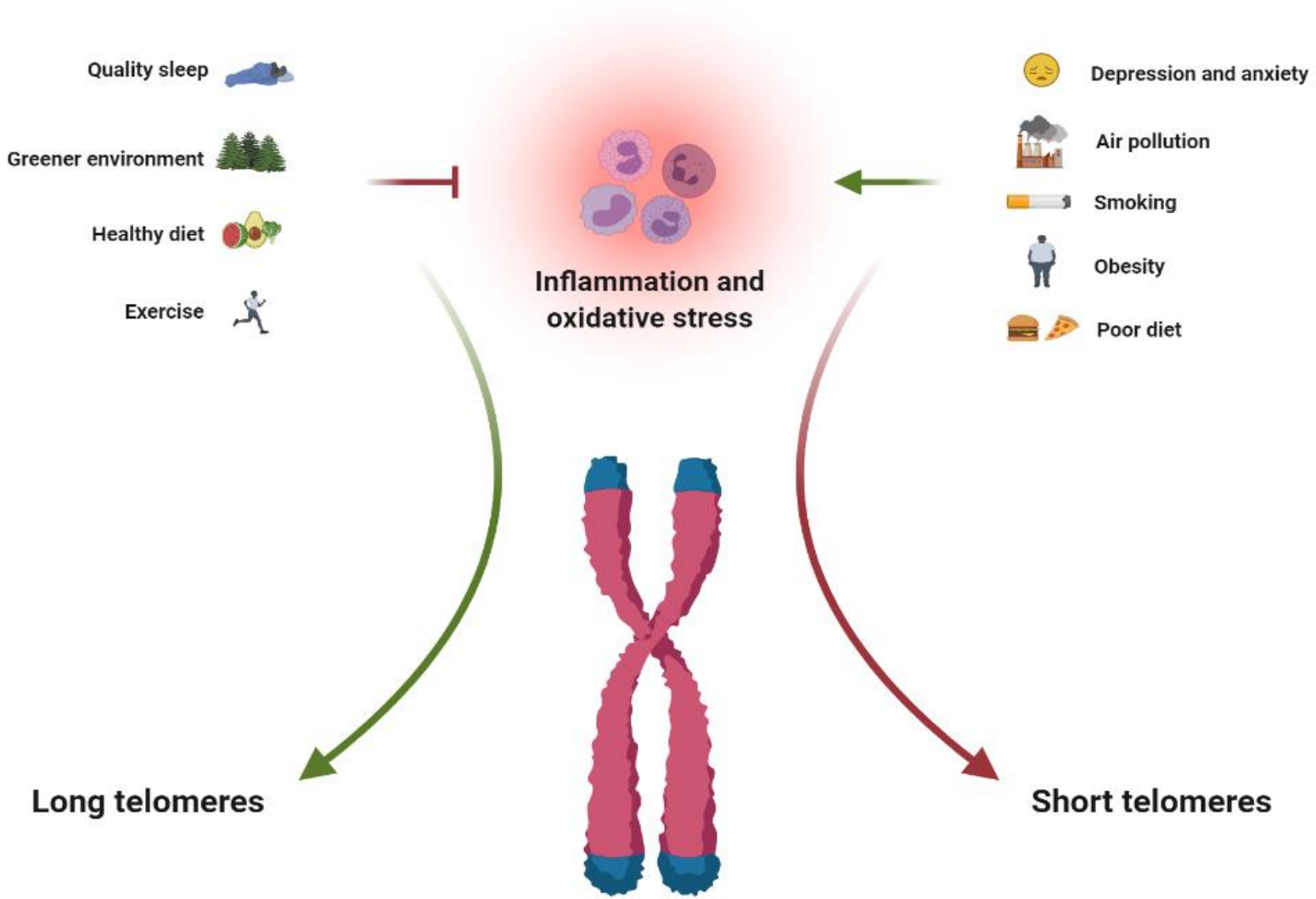

Figure 1.6. Lifestyle and environmental factors that influence telomere length. Adapted from Werner and Laufs (2018).

1.1.9 Telomere length measurement

There is no single method that can accurately, simply, and rapidly quantify TL. The two most utilised methods, Southern blot analysis and quantitative polymerase chain reaction (qPCR), are discussed in further detail below. Nonetheless, there are several alternative published methods available, each with their own unique advantages and disadvantages (see Table 1.1). The advantages and disadvantages of each method should be taken into consideration when selecting the most ideal technique for answering specific questions in telomere research, contemplating factors such as the accuracy and reliability of results, time and labour commitments, cost, quantity of material required, and information provided about average or shortest TL.

Southern blot analysis is the original method for measuring TL and is typically defined as the 'gold standard' since it is used for the validation of new TL methodologies due to its low measurement error (Aviv et al., 2011). This method quantifies the average TL in kb pairs for all the chromosomes in a sample of cells. Southern blot analysis involves digestion of genomic DNA by frequent-cutting restriction enzymes that specifically exclude telomeric and sub-telomeric regions, producing short genomic fragments and longer uncut terminal restriction fragments (TRFs). The DNA fragments are separated based on their size by agarose gel electrophoresis, with the TRFs visualised by Southern blotting, or in-gel hybridisation, using a telomeric DNA-specific probe. The varying lengths of telomeres present as a smear, of which the size and intensity is assessed by comparison to a DNA ladder of known fragment sizes to estimate average TL (Aubert et al., 2012). The primary disadvantages of this method are that it includes sub-telomeric regions, is costly and laborious, and requires large DNA quantities.

The most used TL analysis method in large human population studies is qPCR. This method is established on the ratio of two reactions and determines the factor by which an unknown DNA sample differs from a reference DNA sample in its ratio of a telomere repeat copy number (T) to a single copy gene number (S) (i.e. relative T/S ratio) (Cawthon, 2002). For example, the ratio by which the telomere signal differs from the single-copy gene signal is determined for each DNA sample. The factor by which this differs from a reference DNA sample is then determined to generate a T/S ratio. This ratio should be proportional to the average TL. Thus, if the T/S ratio equals 1, then the unknown DNA sample is equal to the reference DNA in its ratio of telomere repeat copy number to single-copy gene number. The T/S ratio of one individual compared to the T/S ratio of another signifies the relative TL differences in their DNA, making it useful for determining interindividual differences in TL within species. The most notable advantages of qPCR are that it is high-throughput and only requires nanogram

quantities of DNA. Additionally, qPCR is quicker, simpler, cheaper, and excludes sub-telomeric regions compared with the Southern blot method (Verhulst et al., 2015), but this comes at the expense of reduced reliability (inter-assay coefficient of variation (CV) 6.45% vs 1.74%, respectively) (Aviv et al., 2011). However, the singleplex qPCR method has been advanced to a monochrome multiplex (MMqPCR) assay, in which the T and S reactions are performed in the same tube. This has improved intra-assay CV (MMqPCR = 5.22% vs qPCR = 5.8%) (Aubert et al., 2012) and produces TL measurements more strongly correlated with Southern blot analysis values (R^2 = 0.844) when compared to the original singleplex method (R^2 = 0.677). (Cawthon, 2009). Still, the most notable disadvantage of qPCR is that it quantifies average TL and does not detect the shortest telomeres, which are proposed to be the primary factor for the initiation of cellular senescence (Hemann et al., 2001).

It is important to note that most human studies measure TL in a single tissue type. Whole blood is typically utilised as the desired material since sample collection is minimally invasive, easily obtained, and LTL is assumed to reflect the overall senescence state of an individual (Haupt et al., 2022). However, absolute TL appears heterogenous across different tissues, despite being positively correlated (Daniali et al., 2013). Thus, experimental findings determined using a single cell type (e.g., leukocytes) should not be generalised to other tissues and therefore only provide a limited view of whole-body telomere dynamics. Since TL has been quantified in a range of tissue types and using a variety of different methods in the current literature, this may offer some explanation for any potential discrepant findings between similar studies.

Table 1.1. Summary of alternative methods for telomere length measurement

Method	Advantages	Disadvantages
Quantitative fluorescence *in situ* hybridisation (Q-FISH)	TL can be measured in fixed tissues and cells	Many factors affect the hybridisation process; difficult to compare results across laboratories; does not provide actual TL; laborious
Flow-FISH	Useful for TL analysis of hematopoietic cells in suspension; provides TL distributions; cells can be analysed in combination with immunostaining	Laborious; cannot be performed on fixed tissues; requires specialist equipment; practically limited to peripheral blood mononuclear cells (PBMCs)
Single Telomere Length Analysis (STELA)	Measures TL of individual chromosomes with limited starting material; detects extremely short telomeres	Laborious; primers not developed for all chromosome arms; difficult to analyse very long telomeres; low throughput
Monochrome multiplex qPCR (MMqPCR)	Improved reproducibility of qPCR measurements; reduced amount of reagent required; high throughput	Only applicable to cells that are diploid and karyotypically stable; unknown whether inter-laboratory results can be compared if different reagents or single copy loci are used
Telomere Shortest Length Assay (TeSLA)	Measures shortest and average telomere length	Low throughput; laborious

1.2 Telomeres and physical activity

A lack of physical activity (PA) is associated with an increased risk of age-associated diseases and chronic conditions, including CVD, cancer, dyslipidaemia and type II diabetes (Booth et al., 2000). Conversely, participation in regular exercise is known to decrease the risk of numerous age-related diseases (Bauman and Smith, 2000), consequently reducing associated morbidity and mortality rates (Lee et al., 1997). To highlight the significance of exercise on healthy ageing, a marked, inverse relationship exists between PA and all-cause mortality (Kushi et al., 1997). Chronic mild-intensity exercise is also proven to reduce oxidative stress (Radak et al., 2008) and inflammation (Nimmo et al., 2013), two primary factors proposed to negatively influence TL (Correia-Melo et al., 2014). Although there is substantial evidence to support the various health benefits of regular exercise, the fundamental mechanisms are inadequately understood at a molecular level. Since it is well documented that both exercise and telomeres are closely aligned with the ageing process and age-associated disease, it was expected that the association between the two was explored.

1.2.1 Dose-response relationship between physical activity and telomere length

The first study to examine the relationship between PA and telomeres in humans was performed by Cherkas *et al*. and revealed a positive association between TL and leisure-time PA levels (Cherkas et al., 2008). The most active individuals in this study exhibited telomeres ~200 nucleotides longer than the least active. Furthermore, TL of the most active individuals was comparable to sedentary individuals that were 10 years younger. This finding was validated in a twin cohort discordant for PA, in which the more active twin displayed longer TL than their less active sibling (Cherkas et al., 2008). Adults with high PA levels have since been shown to possess telomeres 140 nucleotides longer than sedentary individuals, which corresponded to an estimated biological ageing advantage of 9 years (Tucker, 2017). When compared to low and moderate PA quartiles, the high PA group had a biological age advantage of 8.8 and 7.1 years, respectively. Denham *et al*. have also reported that endurance athletes in the middle and highest tertiles of distance covered per week display longer telomeres than those in the lowest tertile (Denham et al., 2015). Moreover, numerous studies have revealed a significant positive association between TL and self-reported PA (Bendix et al., 2011; Saßenroth et al., 2015; Shadyab et al., 2017b; Sillanpää et al., 2016; Silva et al., 2016; Vyas et al., 2020), movement-based behaviours, (Loprinzi et al., 2015), and ambulatory activity (Fretts et al., 2018; Shadyab et al., 2017a). Most notably, a recent systematic review and meta-analysis

comprised of eleven studies and 19,292 participants concluded that TL was positively correlated with exercise levels (Lin et al., 2019). In support of the apparent dose-response relationship between TL and PA level, several studies have reported a lack of PA is associated with shortened TL (Cherkas et al., 2006; Dankel et al., 2017; Garland et al., 2014; Venturelli, 2014).

Despite substantial evidence that indicates a positive dose-response relationship exists between PA and TL, there may be a threshold for the dose required to optimally influence telomere dynamics. Rae *et al*. revealed that the number of years running and hours spent training were inversely related with skeletal muscle TL in a cohort of experienced endurance runners, suggesting higher doses of endurance training may be related to shorter telomeres (Rae et al., 2010). In support, a 6-month PA intervention found a negative correlation between changes in time spent exercising and variations in TL in sedentary overweight men and women (Sjögren et al., 2014). Individuals engaged in moderate PA have also been reported to display longer telomeres than those who are sedentary or highly active (Ludlow et al., 2008; Savela et al., 2012). Similarly, individuals with moderate physical fitness, as defined by The American College of Sports Medicine, possessed longer telomeres than their counterparts with low and high physical fitness, suggesting moderate PA levels may confer the most positive benefits to telomere dynamics (Bastos et al., 2020). Endurance athletes experiencing exercise-associated chronic fatigue have also been shown to exhibit significantly shorter skeletal muscle TL than their healthy age-matched counterparts (Collins et al., 2003). As such, an 'inverted U hypothesis' has been described between PA and TL (Ludlow et al., 2008), in which the detected associations appear hormetic. This hypothesis opposes the previously proposed linear dose-response relationship between TL and PA level and suggests that moderate PA levels exert a more beneficial impact on telomere dynamics than extremely low and high levels (Figure 1.7.).

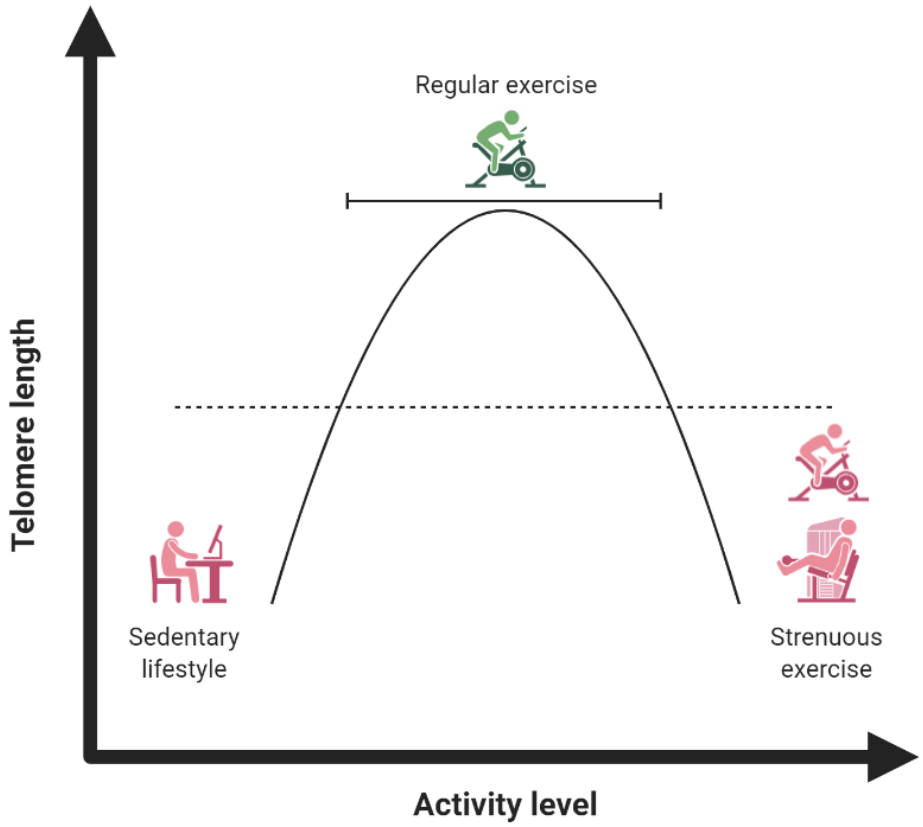

Figure 1.7. Proposed inverted U relationship between activity level and TL.

In complete contrast to findings that report a positive dose-response or inverted U relationship between PA and TL, an equal amount of studies report that no association exists (Bekaert et al., 2007; Cassidy et al., 2010; Ding et al., 2018; Farzaneh-Far et al., 2010; Laine et al., 2015; Magi et al., 2018; Mirabello et al., 2009; Ponsot et al., 2008; Rode et al., 2014; Soares-Miranda et al., 2015; Song et al., 2010; Tiainen et al., 2012; von Kanel et al., 2016; Woo et al., 2008). In a 2015 meta-analysis conducted by Mundstock *et al.*, 54% of the studies selected reported no association between PA and TL, the majority of which measured self-reported PA (Mundstock et al., 2015). Additionally, a systematic review comprised of 30 studies and 4,959 individuals revealed that there were no considerable differences in TL between physically active and inactive individuals when corrected for publication bias (Valente et al., 2021).

Although engagement in PA appears important to telomere dynamics, reducing sedentary time may also be key. Greater leisure-time screen-based sedentary behaviour is associated with shorter LTL (Xue et al., 2017). Furthermore, telomere lengthening was significantly associated with reduced sitting time in overweight individuals participating in a 6-month exercise

intervention trial (Sjögren et al., 2014). This negative correlation was only evident in the exercise training group and suggests reduced sitting time may be more beneficial than increased exercise time regarding telomere maintenance. Consistent with these findings, Denham *et al*. have reported inverse correlations between time spent sitting and LTL in endurance athletes (Denham, 2016; Denham et al., 2015). The author suggested that the longer LTL of endurance athletes may be a consequence of both endurance training and reduced sedentary time (4.8 vs. 10.8 h/day in controls). In support, it has been reported that only physically active participants who sat less and displayed higher cardiorespiratory fitness had increased odds of being in the tertile with the longest telomeres (Edwards and Loprinzi, 2017). Conversely, it has been reported that sedentary behaviour and total time spent sitting are not independently associated with LTL in a cohort of 7,813 women aged 43–70 years (Du et al., 2012).

The current literature provides conflicting results and does not provide a definitive conclusion regarding the association between PA dose and TL. Although numerous studies suggest that moderate to high levels of PA are linked with longer telomeres, a comparable amount refute such an association. If any positive association between PA and TL does exist, there is evidence to suggest that it may be dependent on continued (Laine et al., 2015; Stenbäck et al., 2019), long-term participation in exercise (>10 years) (Saßenroth et al., 2015). In summary, three different primary associations have been described to exist between PA and TL: a positive association, an inverted U relationship, and no association at all.

1.2.2 Influence of exercise modality on telomere length

Elite athletes participating in a wide variety of sport specialties have been reported to possess longer telomeres (12.4%) than inactive individuals (Muniesa et al., 2017). However, when the analysis only considered elite endurance athletes, they displayed telomeres 16% longer than control participants, suggesting exercise modalities that utilise the aerobic system may confer additional beneficial impact on TL maintenance. In support, older individuals engaged in endurance exercise displayed longer LTL than their active counterparts who participated in divergent exercise modalities (Saßenroth et al., 2015). Several studies have also provided evidence that endurance-trained adults (e.g. marathon runners, ultramarathon runners, triathletes, cross country skiers) possess longer telomeres than sedentary age-matched controls (Colon, 2019; Denham et al., 2015; Hernando et al., 2020; LaRocca et al., 2010; Østhus et al., 2012; Sousa et al., 2018; Werner et al., 2009). Ultramarathon runners have been reported to

display telomeres 11% longer than controls, which was proposed to equate to 16 years of reduced biological age (Denham et al., 2013). Nevertheless, Borghini *et al*. discovered that although chronic endurance training may protect against telomere attrition, they also observed that acute exercise (330km ultra-marathon race) can reduce salivary TL in the same cohort (Borghini et al., 2015). While most of the available literature indicates that endurance exercise training is associated with longer telomeres, there are studies within a range of tissue types (e.g., skeletal muscle, PBMC, granulocytes, lymphocytes) that report the TL of endurance athletes is comparable to sedentary age-matched controls (Denham, 2016; Mathur et al., 2013; Rae et al., 2010; Rosa et al., 2020).

Although the present literature tends to indicate a positive association between aerobic/endurance exercise and TL, most of these studies are limited by their cross-sectional design. One of the benefits of intervention studies is that they can define the effects of endurance exercise on telomere dynamics more clearly and deduce biological causality. 26 weeks of aerobic endurance training increased TL in 30-60 year olds that lacked regular PA (Werner et al., 2018). Similarly, caregivers who undertook a 24-week supervised aerobic exercise intervention experienced significant elongation in LTL (Puterman et al., 2018), and just 210 minutes of endurance training/week has been shown to increase TL over a 6 month period in both middle-aged men (Melk et al., 2014) and women (Eigendorf et al., 2019). Conversely, one year of aerobic exercise did not alter telomere attrition in healthy postmenopausal women (Friedenreich et al., 2018). Similar long-term studies utilising aerobic exercise interventions in middle-aged women have also reported no observable change in TL (Mason et al., 2013; Shin et al., 2008). Discrepant findings between what appear to be similar studies may be the result of differences in TL measurement method, DNA extraction method, sample size, cohort demographic, inclusion of confounding lifestyle factors, and tissue type utilised.

The inconsistent findings between the stated studies may also be explained by divergences in the aerobic/endurance exercise modality employed. To highlight, running-specific exercise has been previously described to be the only mode of exercise associated with longer LTL out of nine exercise modalities (e.g. bicycling, basketball etc) (Loprinzi and Sng, 2016). The authors speculated this finding may be explained by the sustained weight-bearing aspect of running, which could be optimal in activating signalling pathways associated with telomere biology. Thus, studies that utilised running aerobic exercise, compared with studies that employed cycling aerobic exercise, may have been more likely to report positive results regarding impact

on TL. Nonetheless, several of the discussed studies that display a positive association between endurance exercise and TL have included swimming, cycling and cross country skiing as exercise modalities (Colon, 2019; Denham et al., 2015; Muniesa et al., 2017; Østhus et al., 2012; Werner et al., 2009), suggesting the positive influence of endurance training on TL is unlikely to be exclusively credited to running-specific exercise.

The current literature has primarily explored the influence of endurance/aerobic exercise on TL. Thus, there is a reduced volume of investigations describing the effects of exercise modalities that use the anaerobic system (e.g., weightlifting, RT, high-intensity interval training (HIIT), sprints) on telomere dynamics. The earliest study to examine the influence of RT on telomeres reported that their length was not associated with powerlifting experience (Kadi et al., 2008). Several studies have since demonstrated no observable change in TL following RT interventions in elderly cohorts (Tosevska et al., 2016), women recovering from breast cancer (Hagstrom, 2018), and healthy inactive individuals (Werner et al., 2018). However, 12 weeks of low frequency, moderate intensity, explosive-type RT counteracted telomere shortening in elderly participants (70–75 years), indicating RT exercise may preserve TL (Dimauro et al., 2016).

Numerous short-term intervention studies that employed HIIT protocols report an increase in the LTL of sedentary individuals (Mofrad and Ebrahim, 2018; Mosallanezhad et al., 2019; Werner et al., 2018). Master sprinters have also been reported to possess longer TL than untrained age-matched controls (Rosa et al., 2020; Simoes et al., 2017). On the contrary, there was no change in sperm TL in healthy men who completed a 6-week training intervention comprised of sprint interval training performed on a cycle ergometer (Denham, 2019).

Concurrent training is the combination of aerobic exercise and RT and stimulates the molecular pathways responsible for the adaptations to each type of training (Methenitis, 2018). Interventions employing concurrent training have been shown to promote increases in LTL in pre-menopausal obese women (Brandao et al., 2020) and prevent telomere shortening in patients with MI (Saki et al., 2016). Furthermore, habitual combined aerobic and resistance exercise performed for at least 60 minutes per session, more than three times a week, for more than 12 months, has been shown to reduce telomere attrition in postmenopausal women (Kim et al., 2012).

More recently, studies have begun to investigate the relationship between TL and a greater variety of exercise modalities. Long-term yoga participation (minimum 2 years) has been

reported to positively influence telomeres (Krishna et al., 2015), although TL was unchanged following a 12-week yoga and meditation-based lifestyle intervention (Tolahunase et al., 2017). It appears that the potential beneficial influence of Yoga on TL may be mediated via a multisystemic positive effect on oxidative stress (Krishna et al., 2015). Walking activity has even been reported to be associated with longer telomeres (Tucker, 2020). However, a 14-day, 260-km, wilderness canoeing expedition that required 6–9 hours of low- to moderate-intensity daily exercise had no effect on TL (Gagnon et al., 2019), which may have been a result of the short intervention period.

Current evidence tends to highlight a positive association between endurance exercise and TL, which does not appear limited to running-specific PA. Intervention studies employing HIIT protocols have provided promising results regarding its influence on TL. On the other hand, RT does not appear to positively influence TL dynamics. This general oversight of the current literature is in line with a previous finding that aerobic-based PA, but not muscle-strengthening PA, is independently associated with TL (Loprinzi et al., 2015). Nonetheless, data concerning the mode-specific associations of PA with TL is inadequate at present, and the exercise modality that elicits the greatest influence on telomere dynamics requires further investigation.

1.2.3 Impact of exercise intensity on telomere length

Exercise intensity refers to how much energy is expended when exercising. The most accurate measure of exercise intensity is oxygen consumption (VO_2), which signifies the metabolic challenge of an exercise (Mann et al., 2013). An individual's maximum aerobic capacity is termed their maximal oxygen consumption (VO_2 max) and indicates their cardiovascular fitness. VO_2 is measured in metabolic equivalent of task (METs) (ml/kg/min). One MET is equivalent to 3.5 ml/kg/min and represents the average resting energy expenditure of a typical human being. Exercise intensity is typically categorised into three different levels: light (LPA), moderate (MPA) and vigorous (VPA). It is well established that exercise intensity can be manipulated to exert desired training adaptations on a physiological level (MacInnis and Gibala, 2017), but little is known regarding its influence on telomere dynamics.

Findings from the Berlin Aging Study II suggest that engagement in intensive sports activities appear to have the greatest impact on LTL (Saßenroth et al., 2015). In support, elite athletes competing in high-intensity sports demonstrate longer telomeres than their counterparts competing in low- and moderate-intensity sports (Sellami et al., 2021). Among 1,476 older

women, those with the highest intensity levels of leisure-time PA (≥17.00 MET-hours/week) displayed longer telomeres than those with the lowest levels (<1.25 MET-hours/week) (Shadyab et al., 2017b). Furthermore, there was no association between LPA and LTL, but there was a significant linear association of moderate-to-vigorous PA (MVPA) with LTL. Edwards *et al*. have also reported MVPA to be the only independent factor positively associated with LTL (Edwards and Loprinzi, 2017). When considered separate from total PA, increasing VPA has been described to show a significant linear trend with LTL (Latifovic et al., 2016). To highlight the potential importance of VPA, total PA was not associated with LTL in a cohort of 667 adolescents (Zhu et al., 2011). However, the average minutes of VPA per day, but not MPA, was positively associated with LTL in the same cohort of adolescents. A positive association between VPA and LTL, and lack of association between MPA and LTL, has also been reported in 6,933 U.S. adults (Ogawa et al., 2017). Still, total MET-hours/week of both MPA and VPA have been positively associated with LTL in older women (Du et al., 2012). In a cohort of 121 children with abdominal obesity, TL changes were also positively associated with METs and MVPA level and inversely associated with sedentary and LPA levels (Ojeda-Rodríguez et al., 2020). Conversely, MVPA has been reported to show no correlation with LTL in older women (Shadyab et al., 2017a). VPA during young adulthood also does not appear to have any effect on LTL in later life in former elite male athletes (Laine et al., 2015). Despite a trend for greater MVPA to be associated with longer telomeres, a recent meta-analysis concluded that active participants had longer TL than inactive individuals, irrespective of exercise intensity (Lin et al., 2019). Thus, although the current literature provides ample evidence that VPA appears to exert the most positive impact on telomere dynamics, the influence of exercise intensity on TL is diverse and requires further investigation.

1.2.4 Physical performance and telomere length

Ageing is independently associated with a decline in TL (Aviv et al., 2009) and maximal physical performance (Suominen, 2011). As such, several studies have explored the correlation between TL and components of maximal physical performance, most notably cardiorespiratory fitness. Endurance athletes exhibiting a progressive decline in performance display shorter skeletal muscle TL than healthy control athletes (Collins et al., 2003). In support, there is a growing body of literature that reports a positive association between aerobic fitness and TL (Colon, 2019; Denham et al., 2015; Kumar Dev et al., 2021; LaRocca et al., 2010; Loprinzi, 2015; Mason et al., 2013; Østhus et al., 2012; Williams et al., 2017). Improvements in aerobic

capacity even appear to be positively associated with telomere lengthening in the sperm of young males (Denham, 2019). A 2020 systematic review, which included 20 studies, concluded that better cardiorespiratory fitness was associated with an increase in TL (Marques et al., 2020). On the contrary, there are reports that aerobic capacity is not associated with leukocyte or skeletal muscle TL (Denham, 2019; Hiam et al., 2020).

Several other measures of physical performance have been positively associated with TL, including sprint performance (Simoes et al., 2017; Sousa et al., 2020), lactate threshold and running economy (Colon, 2019), lower extremity functional power (Dimauro et al., 2016)**,** muscle strength of the knee extensors (Loprinzi and Loenneke, 2016)**,** faster walking speed (Lee et al., 2013; Manoy et al., 2020; Shadyab et al., 2017b), trunk muscle endurance (Williams et al., 2017), greater walking distance (Manoy et al., 2020; Wan et al., 2019), grip strength (Woo et al., 2014), and chair-to-stand performance (Manoy et al., 2020; Soares-Miranda et al., 2015). Elite athletes, who are considered to have the highest level of physical performance, have also been reported to possess longer TL than controls (Abrahin et al., 2019; Muniesa et al., 2017). Physical ability, assessed with the Barthel index, is positively correlated with TL in older women (Maeda et al., 2011). A positive association was also found between LTL and physical performance score in women undertaking the Senior Fitness Test (Åström et al., 2019), whilst centenarians considered 'high-performing' exhibit longer telomeres than their 'low-performing' counterparts (Tedone et al., 2018).

Various studies have, however, reported that TL is not associated with numerous performance measures, such as grip strength (Baylis et al., 2015; Mather et al., 2010; Williams et al., 2017), walking performance (Sillanpää et al., 2016), balance (Gardner et al., 2013), maximal power output (Denham, 2019), chair rise speed (Gardner et al., 2013) and forced expiratory volume (Harris et al., 2006; Williams et al., 2017; Woo et al., 2014). Inverse correlations have even been detected between TL and 6 m walking speed, 6 m loaded walking, and loaded stairs climb (Dimauro et al., 2016). Furthermore, the minimum TL in the skeletal muscle of powerlifters has been inversely correlated with personal records in the squat and deadlift, suggesting a heavier load and greater performance may result in shorter TL than moderate loads (Kadi et al., 2008). A negative association has also been discovered between TL and the Short Physical Performance Battery score (Pereira et al., 2020), suggesting that telomere attrition is not necessarily associated with decreased functional capacity and that TL should not be used as an indicator of physical performance at present.

1.2.5 Effect of age and sex on exercise-mediated telomere biology

Exercise-mediated effects on telomere biology may be influenced by both age and sex. It has been demonstrated that the exercise-induced increase in hTERT expression is more pronounced in young individuals, compared with older individuals, in response to 30 minutes of cycling (Cluckey, 2017). This finding was also only evident in men, suggesting that both age and sex may be linked with decreased telomerase activation in response to exercise. Furthermore, a high volume of leisure-time PA has been associated with greater telomere shortening in women, but not in men (Jantunen et al., 2020). Exercise volume has been reported to be positively correlated with TL in men, but no such association was observed in women (Stenbäck et al., 2019).

As an indication of the potential influence of age on exercise-induced telomere biology, young footballers have been reported to display greater telomerase activity than young inactive controls, whereas elderly footballers showed equivalent telomerase activity compared with their sedentary age-matched counterparts (Hagman et al., 2020). This data could potentially explain why young elite female footballers display 22–24% longer telomeres in lymphocytes and mononuclear cells compared to young controls, whereas elderly team handball players show comparable TL to their untrained age-matched counterparts (Hagman et al., 2021). Conversely, exercise may exert less of an effect on telomere dynamics in young individuals compared with their older counterparts, since most studies report that young athletes have similar TL to their less active counterparts (LaRocca et al., 2010; Østhus et al., 2012; Sousa et al., 2018; Werner et al., 2009). For example, older individuals (65-80 years) with lifelong football participation (>40 years) possessed longer telomeres than their age-matched controls (Hagman et al., 2020). However, young footballers (18-30 years), with a history of at least 10 years of regular football training, displayed comparable telomeres to sedentary age-matched controls. Likewise, elderly ultra-trail runners (>40 years) possessed longer telomeres than age-matched sedentary controls, whilst TL was similar between ultra-trail runners and controls <40 years old (Hernando et al., 2020). The lack of association between PA and TL observed in young individuals may be explained by an absence of significant age-related telomere attrition and/or insufficient exposure to PA levels (e.g., activity years). To highlight, TL was associated with cardiorespiratory fitness in older healthy humans, but not in young participants (Marques et al., 2020). However, there is some evidence that exercise-mediated effects on telomere dynamics may begin in adolescence, particularly in females (Zhu et al., 2011). In support, recent studies employing HIIT interventions have reported increased LTL in young sedentary

students (Mofrad and Ebrahim, 2018; Mosallanezhad et al., 2019) and young elite athletes are described to possess longer telomeres than sedentary age-matched controls (Hagman et al., 2021; Muniesa et al., 2017). Nevertheless, further research is required to clearly define the influence of age and sex on the exercise-induced effects that impact telomere biology.

1.2.6 Exercise-induced telomere dynamics may be tissue-specific

As described in section '1.1.8 Factors influencing telomere length', TL, and attrition rate, is described to be tissue-specific (Aubert and Lansdorp, 2008). The variability in telomere shortening across tissue types is proposed to be the result of their divergent proliferative capacities; highly proliferative tissues (e.g., leukocytes and epithelial cells) typically display greater age-related telomere shortening than minimally proliferative tissues (e.g., skeletal muscle) (Kadi and Ponsot, 2010). Conversely, Daniali *et al*. has shown that intra-individual age-related telomere attrition is similar across somatic tissues with divergent replicative status (e.g., leukocytes, muscle, skin and fat), although there are differences in absolute length (Daniali et al., 2013). This finding suggests that there may be a shared molecular mechanism regulating TL across somatic tissue types. To date, studies investigating the relationship between exercise and TL have utilised a variety of tissue types (e.g., leukocytes, skeletal muscle, buccal cells, sperm), which limits the comparison of their results. Nonetheless, independent studies have shown that ultra-endurance athletes have longer telomeres than controls when measured in skeletal muscle (Østhus et al., 2012), leukocytes (Denham et al., 2013) and saliva (Borghini et al., 2015), suggesting possible synchrony in TL across tissue types in response to exercise.

There is evidence to suggest that the response of skeletal muscle TL to exercise may be unique, which is likely caused by their satellite cell content. For example, long-term exercise accelerates TL attrition in skeletal muscle in mice but attenuates age-related decreases in TL in cardiac and liver tissue (Ludlow et al., 2012b). Since exercise promotes muscle damage and stimulates the proliferation of satellite cells (Yin et al., 2013), and telomere attrition occurs with cell division, excessive exercise could promote an accelerated loss of telomeric DNA in skeletal muscle tissue. For example, chronic endurance running has been reported to shorten skeletal muscle TL (Rae et al., 2010), which was likely due to the increased demand for satellite cells to proliferate and repair damaged tissue. An individual's skeletal muscle satellite cell pool is reported to decrease after age 70 (Kadi and Ponsot, 2010), however, as exercise stimulates

satellite cells, it may counteract this age-related decline (Arsenis et al., 2017). Exercise appears vital to healthy ageing, as a positive correlation has been reported between the quantity of satellite cells and skeletal muscle TL in older women (Sharples et al., 2015). Satellite cell content, therefore, signifies an alternative variable that can be modified in response to exercise and may be a potential factor in the association between exercise and TL.

To date, only two studies investigating the relationship between exercise and telomeres have quantified TL in multiple tissue types. One of these studies did not directly compare TL between tissues (Laye et al., 2012). However, the second study revealed that LTL was highly correlated with skeletal muscle TL in healthy men considered to have a relatively high level of fitness (Hiam et al., 2020). This finding suggests that there may be a shared mechanism regulating TL between the two tissue types, although further research is required to ascertain whether telomeres from different tissues respond to exercise in a synchronous or tissue-specific fashion.

1.2.7 Potential cellular mechanisms of exercise-induced telomere dynamics

Preliminary insights into the cellular mechanisms of exercise-induced telomere dynamics came courtesy of pioneering studies that employed animal models. Radak *et al*. were the first to report that both mild and vigorous exercise training did not affect telomerase activity and 8-hydroxy-2′-deoxyguanosine levels in the liver and skeletal muscle of rats (Radak et al., 2001). In contrast, mice randomised to 21 days of voluntary running displayed upregulated cardiac telomerase activity, increased TERT and TRF2 mRNA expression, and reduced expression of the proapoptotic mediators cell-cycle–checkpoint kinase 2 (Chk2), p53, and p16 (Werner et al., 2008). Nonetheless, cardiomyocyte TL and LTL was comparable between the sedentary and exercising mice. During a follow-up study, Werner *et al*. replicated their previous findings regarding telomerase activity, TRF2 expression, Chk2, p16 and p53 levels, this time in the thoracic aorta of mice (Werner et al., 2009). Aortic TL was comparable between exercise and sedentary groups. Similar findings were also reported in the human arm of the study, with both young and middle-aged endurance athletes exhibiting increased telomerase activity, upregulated TRF2 expression, and downregulation of cell-cycle inhibitors Chk2, p16 and p53 in peripheral blood leukocytes compared with sedentary age-matched controls. However, long-term endurance training was only associated with longer LTL in humans, a finding which was absent in mice (Werner et al., 2009). In other rodent-based studies, long-term exercise has also

been reported to reduce age-related TL attrition in the cardiac and liver tissue of mice, but contributed to shortening in skeletal muscle TL (Ludlow et al., 2012b). Interestingly, telomerase activity was similar in the cardiac and liver tissue of exercising and sedentary mice but elevated in the skeletal muscle of active mice (Ludlow et al., 2012b). In combination, these studies provided initial promise for subsequent research in humans.

1.2.7.1 hTERT expression and telomerase activity

Telomerase can attenuate telomere attrition by adding specific nucleotides onto the 3' end of linear chromosomes (Greider and Blackburn, 1987). Since hTERT expression is correlated with telomerase activity (Kirkpatrick et al., 2003), and also considered the rate-limiting factor for enzymatic activity in somatic cells (Counter et al., 1998), exercise-induced increases in the expression of this subunit may possess the potential to stimulate telomere lengthening.

A single 30-minute bout of treadmill running at 80% VO_2 max is sufficient to upregulate hTERT mRNA expression in white blood cells (Chilton et al., 2014). Moreover, a single treadmill running session, corresponding to only ~40% of an individual's heart rate reserve, can increase PBMC telomerase activity in young healthy individuals (Zietzer et al., 2017). A significant hTERT response has also been reported in response to a single bout of cycling consistent with interval training (7 intervals at the highest absolute intensity for 1 min, followed by 2 min of recovery) (Cluckey, 2017). Cross-sectional analysis has revealed that endurance athletes with a minimum of 1 years' experience display upregulated hTERT mRNA expression (2.0-fold) and longer telomeres compared with sedentary controls (Denham et al., 2015). Conversely, seven days of ultrarunning did not change hTERT mRNA expression or telomerase activity in PBMCs (Laye et al. 2012).

Short-term intervention studies utilising HIIT interventions are reported to significantly increase telomerase activity and elongate telomeres in young non-athletes (Mofrad and Ebrahim, 2018; Mosallanezhad et al., 2019). Concurrent training has also been shown to increase telomerase activity and prevent telomere shortening in patients with myocardial infarction (Saki et al., 2016). The most comprehensive study to date investigating the relationship between PA and TL compared the effects of aerobic, HIIT, and RT interventions on LTL and telomerase activity in middle-aged individuals over a 6-month period. Werner *et al*. revealed that individuals in the aerobic and HIIT groups displayed a 2.0- to 3.0-fold

upregulation in telomerase activity, accompanied by a significant increase in LTL (Werner et al., 2018). On the other hand, telomerase activity and LTL were unchanged in individuals who performed the RT intervention. This finding suggests that distinct exercise modalities and protocols differentially may induce specific cellular pathways in circulating leukocytes.

The potential effect of exercise on telomerase activity may have a temporal aspect, since a study in middle-aged men showed a marked increase in telomerase activity, but not TL, after 3 months of endurance exercise training (Melk et al., 2014). However, a significant increase in both telomerase activity and TL was observed after 6 months. These results suggest that any potential positive effect telomerase elicits on TL may be delayed regarding observational aspects and could explain why TL remained unchanged in individuals following a 12-week yoga and meditation based lifestyle intervention despite an upregulation in telomere activity (Tolahunase et al., 2017). On the other hand, while it was initially concluded that increased telomerase activity maintained TL in men with low-risk prostate cancer following a 3-month lifestyle intervention (Ornish et al., 2008), a follow-up study revealed that telomerase activity was reduced in the lifestyle intervention group after 5 years despite an increase in TL (Ornish et al., 2013). A 24-week aerobic exercise training intervention has also been reported to significantly increase TL in caregivers, despite no change in telomerase activity (Puterman et al., 2018). Although the precise kinetics and specific influence on TL is unclear, a recent systematic review and meta-analysis concluded that exercise increases TERT expression and telomerase activity (Denham and Sellami, 2021),.

1.2.7.2 Oxidative stress

As previously described in section '1.1.3 Telomere attrition and replicative senescence', enhanced oxidative stress is associated with shortened telomeres. It is also well-established that a hormetic relationship exists between PA and oxidative stress (Radak et al., 2008); extremely low and excessive levels of exercise increase ROS production, whilst chronic mild-intensity exercise decreases the oxidative challenge to the body via upregulation of antioxidant enzymes (Gomes et al., 2012; Gomez-Cabrera et al., 2008). Thus, a direct correlation has been observed between the simultaneous increased free radical production and telomere attrition that occurs as a result of physical inactivity (Venturelli, 2014).

An acute, single bout of exercise is understood to increase oxidative stress (Radak et al., 2008). Shortened telomeres at the intermediate, and end-point, of a 330 km endurance race have been

proposed to be the consequence of this acute oxidative stress (Borghini et al., 2015). Conversely, long-term endurance running is associated with a greater redox balance status and longer TL (Sousa et al., 2018). Aerobic exercise also upregulates the DNA repair enzyme 8-oxoguanine DNA glycosylase (OGG1), which is responsible for the excision of 8-oxoguanine (8-oxoG); one of the most common DNA lesions resulting from ROS (Radák et al., 2003). Telomeric DNA is more susceptible to ROS-induced 8-oxoG formation than non-telomere sequences, likely due to the high guanosine content (Oikawa and Kawanishi, 1999). Persistent accumulation of telomeric 8-oxoG directly drives telomere attrition and impairs cell growth (Fouquerel et al., 2019). Acute 8oxoG production can even stimulate rapid senescence in the absence of telomere shortening by possibly producing oxidative base lesions which drive replication-dependent telomere fragility (Barnes et al., 2019) Thus, OGG1 may be a critical mechanistic mediator in the exercise-induced adaptation process that preserves TL. Data is currently lacking regarding the potential interconnected relationship between exercise, TL, and 8-oxoG formation/excision and warrants further investigation.

Yoga practitioners have been reported to display enhanced total antioxidant status, reduced markers of oxidative stress, and longer telomeres compared with sedentary counterparts (Krishna et al., 2015). Further analysis revealed that LTL was positively correlated with total antioxidant status and negatively correlated with markers of oxidative stress (e.g., malondialdehyde and homocysteine). In support, augmented redox homeostasis was directly correlated with reduced telomere attrition following a short-term RT intervention (Dimauro et al., 2016). LTL was also negatively correlated with the cellular redox sensor thioredoxin reductase 1 and oxidative damage marker myeloperoxidase.

On the other hand, no change in TL has been documented following a range of physical activities that reduce oxidative stress load. For example, a 14 day, 260-km, wilderness canoeing expedition evidenced no change in TL despite the increased activity of the antioxidant enzyme SOD (Gagnon et al., 2019). Furthermore, a 12-week yoga and meditation-based lifestyle intervention significantly reduced mean ROS levels and increased total antioxidant capacity, yet also had no impact on TL (Tolahunase et al., 2017). Increases in SOD and glutathione peroxidase (GPX) expression, as a result of long-term aerobic exercise (treadmill walking/running), have also been reported to have no effect on TL (Shin et al., 2008). Thus, although it is biologically plausible for exercise-mediated enhancements in redox balance to improve TL, the present literature offers inconsistent findings.

1.2.7.3 Inflammation

Inflammation is proposed to be closely aligned with telomere dynamics, primarily by increasing cell turnover rate (Bateson and Nettle, 2017). Whilst a single bout of exercise is proposed to stimulate immediate cytokine release (Suzuki, 2019), there is a wealth of evidence reporting that long-term, regular PA promotes an anti-inflammatory environment (Nimmo et al., 2013; Parsons et al., 2017; Phillips et al., 2017; Yates et al., 2012). Chronic moderate-intensity exercise has a propensity to reduce resting IL-6, TNF-α and CRP levels (Kasapis and Thompson, 2005; Moon et al., 2012; Samjoo et al., 2013). Acute exercise has been shown to stimulate the expression of insulin-like growth factor 1 (IGF-1) from skeletal muscle and liver (Berg and Bang, 2004). IGF-1 is positively associated with LTL (Barbieri et al., 2009) and suppresses vascular expression of IL-6, TNF-α and consequent oxidative stress (Sukhanov et al., 2007), providing another potential cellular target through which PA may protect TL.

To highlight the importance of an elevated pro-inflammatory environment on TL, the association between accelerated telomere attrition and lower grip strength was eradicated when adjusted for inflammaging burden (Baylis et al., 2015). In masters athletes, chronic inflammation, measured by the IL-6/IL-10 ratio, has been associated with shortened telomeres (Sousa et al., 2020). Additionally, master sprinters and endurance runners have been shown to present a better anti-inflammatory status (increased IL-10 and lowered IL-6) than their sedentary age-matched counterparts, although only the former group displayed longer telomeres than the control group (Rosa et al., 2020). In support of the lack of correlation between TL and inflammatory status in endurance runners, no correlations were found between TL and inflammatory cytokines (IL-6 and CRP) in experienced ultra-endurance athletes (Denham et al., 2013). A significant reduction in IL-6 following a yoga and meditation-based lifestyle intervention also had no effect on TL (Tolahunase et al., 2017). Interestingly, mutations in hTERC and hTERT have been shown to increase expression of pro-inflammatory cytokines (IL-1, IL-6, IL-10 and TNF- α), promoting premature ageing in alveolar stem cells (Chen et al., 2015). As such, the relationship between damaged telomeres and inflammation may be mutual, and it remains unclear as to whether decreases in inflammation influence telomere attrition or vice versa.

1.2.7.4 Shelterin expression

The shelterin complex is essential in the recruitment of telomerase to telomeric regions (De Lange, 2005). Seven days of ultrarunning has been shown to elevate mRNA expression of shelterin proteins TRF1, TRF2, and POT1 in PBMCs (Laye et al., 2012). This same study revealed that higher concentrations of TRF2 were found in skeletal muscle compared with PBMCs, indicating a differential exercise-induced response of this shelterin-associated protein between the two tissues. Endurance athletes have also been reported to also exhibit 1.3-fold upregulated TPP1 mRNA expression, and longer telomeres, than sedentary controls (Denham et al., 2015). Following an 8-week intervention study, concurrent training prevented telomere shortening and significantly increased TRF1 and TRF2 levels in patients with myocardial infarction (Saki et al., 2016). Although there is evidence to suggest an upregulation of various shelterin complex proteins in response to PA, neither a 6-month RT intervention (Dimauro et al., 2016) or 30 minutes of interval training on a cycle ergometer (Cluckey, 2017) reported any influence on TRF2 expression. Furthermore, young footballers displayed increased TRF2 and POT1 mRNA expression compared with age-matched controls, whereas elderly footballers showed comparable expression of both shelterin proteins with age-matched controls (Hagman et al., 2020). However, only elderly footballers showed longer telomeres than their age-matched counterparts despite the lack of change at the level of shelterin complex proteins.

1.2.7.5 Epigenetic regulation

A 2021 systematic review and meta-analysis that reported masters athletes possessed longer telomeres than age-matched controls concluded that this was the result of lower oxidative stress and chronic inflammation, and enhanced shelterin protein expression and telomerase activity (Aguiar et al., 2021). Nonetheless, several less notable epigenetic modifications have been proposed that may in part explain the cellular mechanistic effects of exercise on TL.

Epigenetic regulation refers to changes in gene expression that do not alter the DNA sequence. There is substantial evidence that telomere dynamics can be regulated by epigenetic modifications, such as DNA methylation and histone modification (Buxton et al., 2014; Jezek and Green, 2019; Wang, 2017). Exercise has been demonstrated to influence both DNA methylation and histone modification (Brown, 2015; Lim et al., 2020). However, research detailing genome-wide exercise-induced changes in DNA methylation and/or histone

modification is still in its infancy, and how such epigenetic alterations directly interact with telomere dynamics is not yet understood.

Telomeres can be transcribed into TERRA molecules (Azzalin et al., 2007); long non-coding RNAs that are linked to telomere maintenance (Rippe and Luke, 2015). It has been proposed that the high guanine nucleotide content of TERRA molecules may protect TTAGGG telomeric repeats from ROS (Diman et al., 2016). Experimental evidence has shown cycling endurance exercise increases TERRA levels in skeletal muscle of healthy young individuals (Diman et al., 2016), providing another potential cellular mechanism by which exercise may protect telomeres. However, a 12-week resistance exercise and nutrition intervention that significantly increased TERRA expression in sarcopenic patients ultimately had no influence on TL (Chang et al., 2020).

Small non-coding RNAs may also target shelterin mRNA and negatively regulate protein abundance (Dinami et al., 2014). For example, four micro RNAs (miRNA), that potentially focus on telomeric gene mRNA (miR-186, miR-181, miR-15a and miR-96) are significantly upregulated 60 minutes post-exercise (Chilton et al., 2014). Telomeric repeat binding factor 2 interacting protein (TERF2IP) was recognised as a prospective binding target for miR-186 and miR-96 and exhibited simultaneous downregulation to both miRNAs at the equivalent timepoint. These results offer a potential mechanistic insight into telomere dynamics and indicate miRNAs may regulate exercise-associated changes in shelterin and TL.

Despite an increasing number of studies investigating the exercise-induced molecular mechanisms that influence telomere dynamics, the precise processes are not yet fully elucidated.

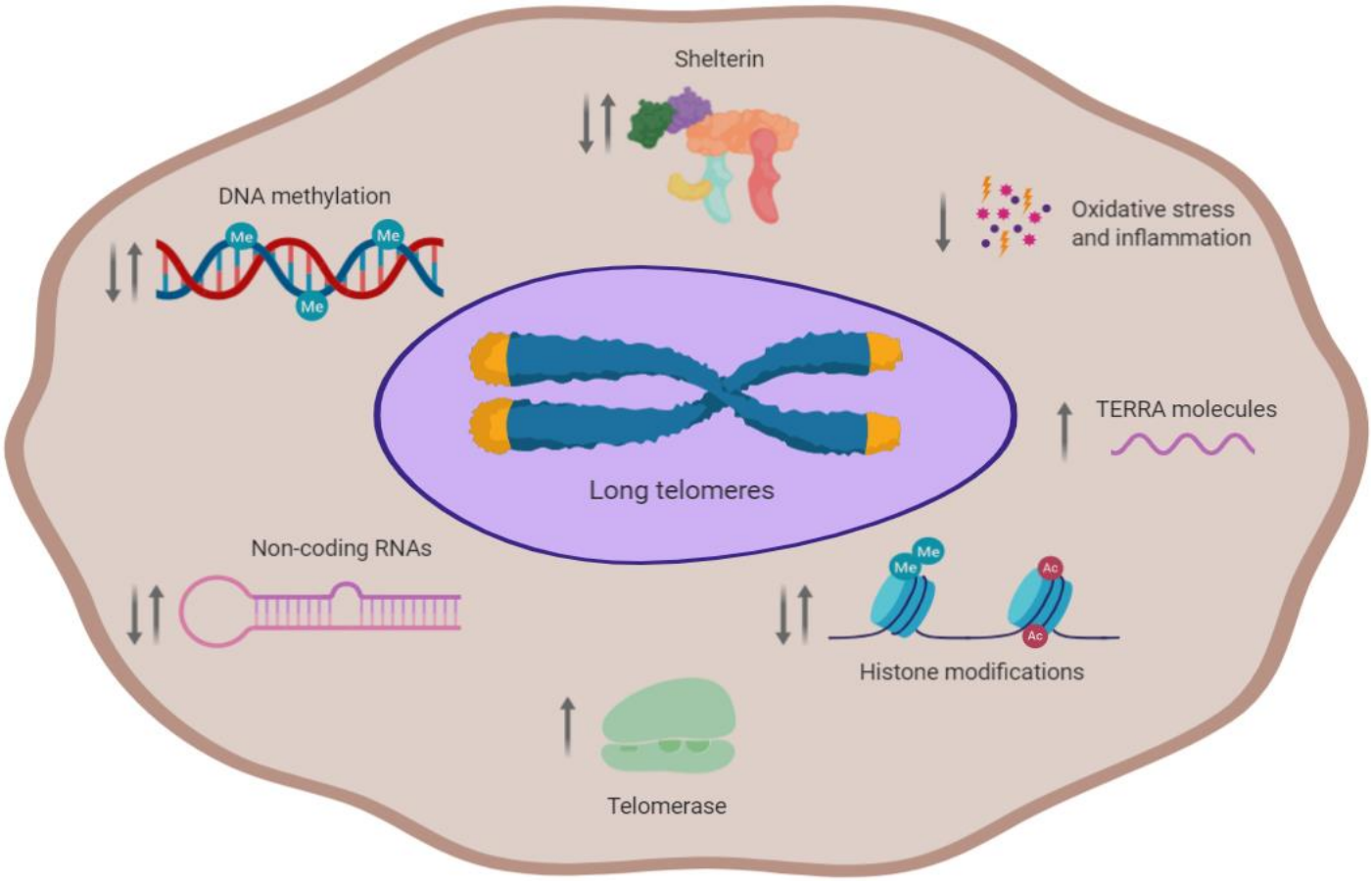

Figure 1.8. Proposed exercise-mediated cellular mechanisms that influence TL. Arrows specify whether the mechanism is increased and/or decreased by exercise training. Adapted from Denham (2016).

1.3 Summary and aims

A positive association between PA and TL is biologically credible and the studies discussed present some promising results. In summary, a higher dose of moderate-vigorous endurance exercise appears to exert the most beneficial impact on TL. However, there are several major limitations within the current literature. Most studies to date have employed a cross-sectional design, examined self-reported PA by questionnaire, primarily utilised an endurance athlete cohort, and been performed retrospectively on banked DNA samples. Subsequently, there is a paucity of intervention and longitudinal studies investigating the association between PA and TL. Additional studies are also required to elucidate the mechanistic effects of PA on TL. The discrepant findings surrounding the relationship between PA and TL in the current literature are likely to be the consequence of divergent methodological issues between studies, such as variation in the tissue type measured, method of TL measurement, study sample size, employment of different exercise protocols (e.g., modality, volume, frequency, and intensity), and the inclusion of confounding lifestyle factors (e.g., diet and psychological stress). As such, a multitude of evidential contradictions needs to be addressed.

The primary aims of this thesis are to:

1. explore how exercise modality, intensity, and dosage influence TL
2. investigate potential mechanisms of exercise-induced telomere dynamics
3. compare TL across tissue types in athletes

CHAPTER II - MATERIALS AND METHODS

2.1 Anthropometry

2.1.1 Height, body mass, BMI, and body composition measurements

Height was measured to the nearest 0.1 cm on a portable Seca 213 stadiometer (Seca GmbH, Hamburg, Germany) following a deep inhalation. Body mass was measured to the nearest 0.1 kg using a calibrated Adam CFW-150 digital scale (Adam Equipment Ltd, Milton Keynes, UK). Estimates of body fat percentage (BF%), fat mass (FM), fat-free mass (FFM), total body water (TBW), body mass index (BMI), and visceral fat rating were determined via bioelectrical impedance analysis (BIA) using a Tanita BC-418 MA segmental body composition analyser (Tanita Corporation, Illinois, USA).

2.2 Physical performance measures

2.2.1 Sit-to-stand test

The sit-to-stand test (STST) required participants to stand up from, and sit down on, an armless chair as quickly as possible 10 times. Participants folded their arms across their chest and were instructed to stand-up completely and make firm contact when sitting. A three-second countdown was given to the participants, with timing commencing on the word ‘start’ and ceasing when the participant sat after the tenth repetition. Participants were permitted two practice repetitions before their single test trial.

2.2.2 Countermovement jump

All countermovement jump (CMJ) tests were conducted on a JumpMat Pro contact mat (FSL Scoreboards LTD, Cookstown, Northern Ireland). Participants were given a visual demonstration of the CMJ prior to testing and permitted two practice jumps. Standing on the contact mat, participants were instructed to jump as high as possible and attempt to land in the same position as they took off from. Participants were required to keep their hands on their hips throughout the test and maintain extension in the hip, knee, and ankle joints during flight-time. The jump started from a standing position and participants were required to perform a short downward movement, immediately followed by an upward movement leading to take-off. The countermovement drop depth was standardised to parallel for all trials unless the

participant could not reach this depth. Each participant performed three recorded test jumps and the greatest jump height, recorded to the nearest 0.1 cm, was used for data analysis. The CV for repeated measures reliability was 8.6%.

2.2.3 Grip strength

Isometric strength of the hand and forearm muscles was measured using a Takei hand grip dynamometer (Takei Equipment Industry Company, Tokyo, Japan). The dynamometer was adjusted so that the base rested on the first metacarpal and the handle rested on the middle of the four fingers. The dynamometer handle position was recorded to ensure consistency between pre- and post-test measurements. Testing was conducted with participants seated and their feet supported, their elbow by their side and flexed to 90º, and the forearm in 0° between pronation and supination, and a neutral wrist position. Participants were required to maintain this position during the test. Testing always began with the dominant hand and participants squeezed the dynamometer with maximum isometric effort for 3-5 seconds, with verbal encouragement. No other body movement was permitted. All participants performed three tests for each hand in an alternative fashion, resting thirty seconds between attempts. Participants were allowed one practice trial per hand. The maximum value for each hand was recorded in kilograms (kg) and used for data analysis. All hand grip strength data is presented as right or left, irrespective of hand dominance. The CV for repeated measures reliability was 6.2%.

2.2.4 Isometric leg strength

Isometric leg strength was measured using a Takei back and leg dynamometer (Takei Equipment Industry Company, Tokyo, Japan). Participants stood on the footplate of the dynamometer with their knees and hips slightly flexed and their lower back maintaining an appropriate lordotic curve. Flexion at the knee continued until the leg extension angle measured 120º with a goniometer. Once in position, participants reached down until the elbows fully extended and the pull-bar was placed in their hands with the chain length adjusted accordingly. Participants were instructed to pull the bar in a vertical direction with maximal effort while maintaining the stable initial starting position. After a visual demonstration and familiarisation attempt, each participant performed three trials, with a rest period of 30 seconds between efforts. The maximal score for the three trials was used for subsequent data analysis. The CV for repeated measures reliability was 6.0%.

2.2.5 Forearm Plank

Participants were instructed to adopt the following forearm plank position; elbows in contact with the ground directly underneath the shoulders, with the humerus creating a perpendicular line to the horizontal plane. Participants forearms were neutral, and their hands were directly in front of their elbows. Participants assumed a firm anatomical body position so that only their forearms and toes supported the body. Once in this stable position, the investigator started the stopwatch and participants were instructed to maintain their stance for as long as possible. Verbal cues were provided to encourage form adherence. The test was terminated when the participant voluntarily ended the test or ceased to uphold the position. Tests were also terminated if two consecutive corrective verbal cues did not lead to a suitable improvement in form. The duration time of the test to the nearest tenth of a second was recorded. Participants only performed the test once due to the exhaustive nature of the assessment.

2.2.6 Y-Balance Test

The Y-Balance Test Kit (Functional Movement Systems, Virginia, USA) was used to assess dynamic balance. Prior to formal testing, participants were allowed three practice trials per leg in each of the three directions. The standardised testing order was as follows: right anterior, left anterior, right posteromedial, left posteromedial, right posterolateral, and left posterolateral. All testing was performed barefoot, and participants were instructed to stand on their right leg on the centre platform, with their toes behind the line and their hands on their hips. While maintaining a single leg stance, participants pushed the reach indicator box as far as possible in the direction being tested. Participants returned to the start position without putting their foot down to complete a successful trial. Following three successful trials of the right leg anterior reach, participants then repeated the procedure with the left leg (see standard test order above). Trials were repeated if any of the following occurred during the test: 1) the non-stance leg touched the floor; 2) the participant stepped off the testing platform; 3) the participant removed their hands from their hips; and 4) the participant put weight on the plastic box while sliding it forward. The distance the reach indicator box was pushed was recorded for each attempt and averaged across trials. Results were then normalised to leg length and a composite score was calculated as the sum of the maximum reach distances (cm) in the three directions, divided by three times the limb length, and then multiplied by one hundred. Participants lower-limb length was measured in the supine position, from the anterior superior

iliac spine to the most distal part of the medial malleolus using a tape measure. The CV for repeated measures reliability was 2.0%.

2.2.7 Sit and reach test

Participants were instructed to sit on the floor with both feet straight out against a sit and reach box (Cartwright Fitness, Chester, UK). All participants were required to be barefoot, had both knees pressed down to the floor and their palms facing downward. Participants stretched their arms out and reached as far forward as possible, pushing the measuring ruler with their fingertips. Participants were allowed three practice trials and one recorded trial, with 30 seconds rest in between. Participants were required to hold their final position for at least 2 seconds. The CV for repeated measures reliability was 4.5%.

2.3 Biochemical analyses

2.3.1 Blood sample collection and processing

All blood samples were drawn from the antecubital vein by a trained phlebotomist using standard procedures. Whole blood for DNA extraction was collected in a dipotassium ethylenediaminetetraacetic acid (K_2EDTA) vacutainer (Becton, Dickson & Company, New Jersey, USA) and aliquoted directly into low-bind 1.5ml microcentrifuge tubes to be immediately stored at −80°C. Anticoagulant-free vacutainers (Becton, Dickson & Company, New Jersey, USA) were used for serum isolation and kept at room temperature for 30-60 minutes (no longer than 2 hours) prior to centrifugation at 1400xg (4100 rpm) for 5 minutes (20°C). The collected material was recentrifuged using the same conditions to ensure the complete removal of any residual cells. The resultant serum was removed and aliquoted into vials prior to storage at −80°C. Tempus blood RNA tubes (Applied Biosystems, Massachusetts, USA) were utilised for the stabilisation and isolation of RNA and were immediately inverted several times to mix upon collection. Tubes were then kept at room temperature for approximately 15 minutes before transfer to −80°C, where they were stored until analysis. Differential cell count analysis was performed with EDTA anticoagulated blood within 30 minutes of withdrawal using a Yumizen H500 (Horiba Medical, Montpellier, France). After processing procedures, all tubes and aliquoted samples were immediately stored at −80°C until analysis.

2.3.2 Buccal swab collection and processing

Following a visual demonstration and verbal instructions, all participants collected buccal samples themselves using Isohelix DNA buccal swabs (Cell Projects Ltd, Maidstone, UK). Samples were immediately placed in a 1.5ml microcentrifuge tube and kept at room temperature for no longer than 2 hours, before being stored at −80°C until DNA extraction.

2.3.3 DNA extraction and analysis

DNA was extracted from whole blood and buccal swabs using the commercial ReliaPrep™ Blood gDNA Miniprep System (Promega, Wisconsin, USA) and ReliaPrep™ Tissue gDNA Miniprep System (Promega, Wisconsin, USA), respectively, according to the manufacturer's instructions. The quantity and quality of extracted genomic DNA were assessed by spectrophotometry using a NanoDrop 2000 (ThermoFisher, Massachusetts, USA), with an absorbance ratio $1.7 < A260/280 < 2.0$ considered acceptable. DNA was diluted to a final concentration of 10ng/µl using RNAse/DNAse free water prior to analysis for TL.

2.3.4 RNA isolation, analysis of content and cDNA synthesis

RNA was extracted from whole blood collected in Tempus Blood RNA tubes using the commercially available Tempus™ Spin RNA Isolation Kit (Applied Biosystems, Massachusetts, USA) according to the manufacturer's instructions. The quantity and quality of extracted genomic RNA were assessed by spectrophotometry using a NanoDrop 2000 (ThermoFisher, Wilmington, USA), with an absorbance ratio $2.1 < A260/280 < 2.2$ considered acceptable. RNA was reverse-transcribed to cDNA using the High-Capacity Reverse Transcription kit (Applied Biosystems, Massachusetts, USA) according to the manufacturer's instructions. cDNA was diluted to a final concentration of 5ng/µl using RNAse/DNAse free water prior to gene expression analysis.

2.3.5 Gene expression analysis

Relative mRNA expression levels for genes of interest (Table 2.1.) were normalised to the geometric mean of the housekeeper genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cytochrome C1 (CYC1) and ATP synthase F1 subunit beta (ATP5B) (Chapter III). GAPDH was utilised as the sole housekeeper gene in Chapters IV and V since it is stably expressed in response to both endurance and resistance exercise (Mahoney et al., 2004). Each

PCR reaction mixture contained 5µl of SybrGreen PrecisionPlus qPCR Master Mix (Primerdesign, Southampton, UK), 0.5µl of primer mix and 4µl of 5ng/µl cDNA, with the total volume made up to 10µl using RNAse/DNAse free water. All samples were run in duplicate on a Viia7 Real-Time PCR system (Applied Biosystems, Massachusetts USA) using the following cycling conditions: denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60s (Chapter III). Alternatively, samples were run in duplicate using the following cycling conditions: denaturation at 95°C for 2 min, 40 cycles of 95°C for 10s and 60°C for 30s (Chapters IV and V). Melt curves were visually inspected for a single peak indicating the generation of a single product. The relative mRNA expression for the genes of interest was calculated using the $2^{-(\Delta\Delta Ct)}$ formula. Values $2-(^{\Delta\Delta Ct})$ were log-transformed prior to statistical analysis to avoid any abnormal distribution.

Table 2.1. List of primers used for qPCR analyses

Primer	Primer sequence/Assay ID/Accession Number	Supplier
TelF (1b)	5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'	IDT
TelR (2b)	5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'	IDT
36B4F	5′-CAGCAAGTGGGAAGGTGTAATCC-3′	IDT
36B4R	5′-CCCATTCTATCATCAACGGGTACAA-3'	IDT
GAPDH	NM_002046	Primer Design
CYC1	NM_001916	Primer Design
ATP5B	NM_001686	Primer Design
ACE F	5′-CTGGAGACCACTCCCATCCTTTCT-3′	Merck
ACE R	5′-GATGTGGCCATCACATTCGTCAGAT-3′	Merck
TERT	qHsaCID0009247	Bio-Rad
TRF1	qHsaCED0045382	Bio-Rad
SOD2	qHsaCED0036418	Bio-Rad
GPX1	qHsaCED0037003	Bio-Rad

2.3.6 Telomere length analysis

TL was measured by a modified protocol based on the qPCR method previously described by Cawthon (Cawthon, 2002). This method was utilised since it is high-throughput, simple, cost effective, and requires small quantities of DNA. Primer sequences for telomeres (Tel1b and Tel2b - Table 2.1.) were both diluted to a final concentration of 300nM. Primer sequences for the single-copy gene (36B4F and 36B4R - Table 2.1.) were both diluted to a final concentration of 500nM. Final volumes per qPCR reaction for telomere (T) and single-copy gene (S) are outlined in Table 2.2 and Table 2.3., respectively.

Table 2.2. Components per qPCR reaction for telomere (T)

Reagent	**Volume (µl)**
SensiMix™ SYBR® No-ROX Kit	12.5
Telomere primers (Tel1b + Tel2b)	1.5
Template DNA (10ng/µl)	3
Distilled water	8
TOTAL	**25**

Table 2.3. Components per qPCR reaction for single-copy gene (S)

Reagent	**Volume (µl)**
SensiMix™ SYBR® No-ROX Kit	12.5
Single-copy gene primers (36B4F + 36B4R)	2.5
Template DNA (10ng/µl)	3
Distilled water	7
TOTAL	**25**

To achieve a high level of accuracy and reproducibility, all reactions were set up using a Qiagility robotic liquid handling system (Qiagen, Hilden, Germany) in a rotordisc 100. 48 samples, plus negative test control (nuclease-free water) and a calibrator sample (pooled DNA), were run on a Rotorgene Q (Qiagen, Hilden, Germany). To ensure intra-run reliability each sample was run in duplicate. The telomere PCR cycling conditions consisted of 95°C for 10 min, 20 cycles of 95°C for 15 sec and 58°C for 1 min. The single-copy gene PCR cycling conditions consisted of 95°C for 10 min, 30 cycles of 95°C for 15 sec and 58°C for 1 min. Telomere and single copy gene runs were performed separately, starting with former and followed immediately by the latter to keep conditions uniform. To ensure inter-run reliability PCR reactions were performed twice on two separate days. Inter-assay CV for calculated T/S ratio was expected to be <5%.

Analysis was performed using the Qiagen rotorgene comparative quantitation software (Qiagen, Hilden, Germany). The point 80% back from the peak of the amplification plot was defined as the take-off point (Ct) for each sample. The reaction efficiency was calculated from the slope between take-off and the point where maximum amplification stops for each sample. The mean amplification of all samples in the run (MAE) was also calculated. The concentration of each sample relative to the calibrator sample was calculated using the equation:

$$\text{Sample concentration} = \text{MAE}^{(\text{calibrator take-off} - \text{sample take-off})}$$

This calculation was performed for both the T run and S run using the average Ct of each sample duplicate. TL was expressed as T relative concentration/S relative concentration (T/S) relative to the calibrator sample (pooled DNA). To ensure that data were highly reproducible, any duplicate samples which had take-off values exceeding 0.2 of a cycle difference were excluded and re-run. The expected mean intra-assay CV for the T/S ratios was expected to be <5% and the T/S ratios were required to correlate (R^2>0.9).

2.3.7 ACE genotype determination

ACE genotype was determined using the primers (ACE F and ACE R) detailed in Table 2.1., which were designed to amplify the polymorphic region from human genomic DNA. PCR reaction mixture contained 10µl of SybrGreen PrecisionPlus qPCR Master Mix (Primerdesign, Southampton, UK), 0.5µl of ACE forward primer mix, 0.5µl of ACE reverse primer mix and 4µl of 5ng/µl DNA, with the total volume made up to 20µl using RNAse/DNAse free

water. PCR cycling conditions were as follows: 35 cycles of 30s at 95˚C for denaturation, 90s at 60˚C for annealing, 30s at 72˚C for extension, followed by a final cycle of 10 min at 66˚C. PCR products were electrophoresed on 2% w/v agarose gel detected as 190-bp fragments in the absence of the insertion (D allele) and 490-bp fragments in the presence of the insertion (I allele). 15% of samples were randomly repeated to check the validity of the genotyping.

2.3.8 Inflammatory marker quantification

Serum concentrations of IL-6, interleukin-8 (IL-8) and TNF-α were measured in duplicate by commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, USA). The intra-assay CV of duplicate samples had to be ≤ 10%, whilst inter-assay CV's were to be ≤ 15%. Serum CRP was determined using enzymatic, colorimetric methods on a Pentra 400 semiautomated benchtop analyser (Horiba Medical, Montpellier, France). Samples collected for each participant were analysed during the same run to eliminate any inter-assay variation. The mean within-batch CV for assays performed in each study can be found in the individual 'Methods' section for each chapter.

2.4 Statistical analysis

The study-specific statistical analysis methodology is described in detail in each relevant chapter. Data were assessed for normality using the Shapiro-Wilk test. Non-normally distributed data were either log transformed prior to evaluation, or analysed utilising non-parametric tests (e.g., Chapter III due to small sample size). Within- and between-group differences were investigated utilising a variety of statistical tests, including the student's *t*-test (paired and/or unpaired), Wilcoxon signed-rank test, analysis of variance (ANOVA), analysis of covariance (ANCOVA), repeated-measures ANOVA with Bonferroni correction or two way repeated-measures ANOVA. Correlations between continuous variables were investigated with Pearson's correlation coefficient or Spearman's rank correlation. Effect sizes were calculated using Cohen's D; 0.3 was considered a small effect, 0.5 a medium effect and 0.8 a large effect. All analyses were performed using SPSS 23.0 (IBM Corporation, USA) with p-values <0.05 considered significant unless stated otherwise.

CHAPTER III - THE EFFECT OF A 12-WEEK RESISTANCE TRAINING INTERVENTION ON LEUKOCYTE TELOMERE LENGTH

The study presented in this chapter has been published with the following citation:

Nickels, M, Mastana, S, Hunter, D, Denniff, M, Codd, V, & Akam, E (2020). The effect of a 12-week resistance training intervention on leukocyte telomere length. *Heliyon*, 6(6), e04151.

3.1 Abstract

The aim of this study was to investigate the effect of a low-resistance, high-repetition RT intervention on LTL and associated health parameters. 23 sedentary middle-aged adults volunteered for this study (16 female/7 male; age = 51.5 ± 4.9 years) and performed two one-hour sessions of Les Mills BODYPUMP™ per week for 12 weeks. Outcome measures were taken at baseline, after the training intervention, and at 12-month follow-up. LTL remained unchanged following the training intervention (pre median T/S ratio = 0.809, range = 0.609 - 1.079 vs post median T/S ratio = 0.799, range = 0.577 - 1.049, p = 0.420), despite a borderline significant increase in hTERT expression (p = 0.05). Circulating levels of TNF-α were reduced after the intervention (p = 0.001). At 12-month follow-up, participants who returned to a sedentary lifestyle (n = 10) displayed shorter telomeres compared to their pre intervention values (p = 0.036). However, participants who continued to exercise after the RT intervention (n = 6) did not show any telomere attrition at their 12-month follow-up visit (p > 0.05). In conclusion, no changes were observed in LTL following the 12-week training intervention, despite improvements in molecular parameters associated with telomere dynamics. It appears continued long-term exercise (>12 months) is necessary to preserve LTL in previously sedentary individuals.

3.2 Introduction

Telomeres are repeat, non-coding DNA sequences located at chromosome ends. They function as protective caps that confer genomic stability, protecting internal regions from deterioration, end-to-end fusion and recombination (Neidle and Parkinson, 2003). TL declines with age and is considered a key biomarker of biological ageing. Short telomeres are associated with numerous age-related diseases, such as CVD, diabetes, and several types of cancer (Rizvi et al., 2015). When a cell's telomeres become critically short it ceases to divide and undergoes replicative senescence (Harley et al., 1990).

Inflammation and oxidative stress are two primary factors identified to accelerate telomere attrition (Correia-Melo et al., 2014). Psychosocial, behavioural, and environmental factors that promote inflammation and/or oxidative stress are associated with shorter telomeres, such as obesity, smoking, and psychological stress (Starkweather, 2014). Conversely, lifestyle behaviours that reduce inflammation and oxidative stress are positively associated with TL, such as habitual exercise. Most studies to date have investigated the effects of aerobic endurance exercise on TL, primarily reporting a positive association (Borghini et al., 2015; Denham et al., 2013; LaRocca et al., 2010). Several cross-sectional studies have also shown a positive relationship between self-reported PA and TL (Bendix et al., 2011; Cherkas et al., 2008; Latifovic et al., 2016). However, research is currently lacking regarding the effects of different exercise modalities on TL, particularly RT.

Long-term RT ameliorates systemic inflammation and age-related decline in muscle strength (Calle and Fernandez, 2010; Mayer et al., 2011). Furthermore, greater lower extremity muscle strength has been linked with longer LTL (Dimauro et al., 2016; Loprinzi and Loenneke, 2016), highlighting the potential of RT to influence telomere dynamics. In support of the possible association between RT and TL, 12 weeks of low frequency, moderate intensity, explosive-type RT prevented telomere shortening (Dimauro et al., 2016). Conversely, TL was unchanged in both sedentary and elderly individuals following separate 6-month RT interventions (Tosevska et al., 2016; Werner et al., 2018).

The present study aimed to investigate the effects of a 12-week low-resistance, high-repetition RT intervention on LTL and inflammatory markers in sedentary middle-aged individuals.

3.3 Methods

Ethical approval

Ethical approval for this study was provided by the Loughborough University ethical advisory committee (R18-P075). Written informed consent was obtained from all participants and the study conformed to the standards detailed by the latest revision of the Declaration of Helsinki.

Participants

38 untrained healthy participants were recruited to the study. There was a 39% dropout rate, leaving 23 participants to complete RT intervention period and testing (16 female/7 male: age = 51.5 ± 4.9 years, height = 166.3 ± 9.3 cm, body mass = 78.4 ± 14.7 kg, BMI = 28.0 ± 4.6 kg/m^2, body fat = 34.6 ± 9.2 %). The health status (Appendix E), physical activity level (International Physical Activity Questionnaire - Short Form (IPAQ-SF)) (Appendix H), and mental wellbeing (The Warwick-Edinburgh Mental Wellbeing Scale (WEMWBS)) (Appendix I) of each participant were assessed by questionnaires prior to testing. Exclusion criteria included known metabolic disorders, cardiovascular disease, cancer, use of anti-inflammatory medication or steroids, and any chronic age-related condition that could influence outcome measurements. All participants were considered sedentary prior to the study (based on a low score on the IPAQ-SF (<600 MET minutes/ week)) and had no or minimal previous RT experience. At the 12-month follow-up visit, participants were considered recreationally active if they scored moderate (600-3000 MET minutes/ week) or high (>3000 MET minutes/ week) on the IPAQ-SF. Participants who scored low on the IPAQ-SF were considered to have returned to a sedentary lifestyle. An additional question required participants to confirm that they had maintained their calculated IPAQ-SF score for at least 11 months of the subsequent 12-month period to accurately reflect their PA level for the previous year.

Experimental design and training protocol

All participants were required to attend a laboratory testing session the week before (pre) and a week after (post) the 12-week RT intervention. The order of the tests was the same for all participants and both testing sessions were performed at the same time of the day to minimise

any impact of diurnal variation. The testing protocol for all participants was as follows: anthropometric measurements, blood sample collection, and physical performance measures (CMJ, isometric leg strength, grip strength, chair stand test).

Participants attended two supervised 1-hour sessions of Les Mills BODYPUMP™ per week for 12 weeks. Sessions were held at the same time of day and conducted on two non-consecutive days by a Les Mills licensed instructor. Les Mills BODYPUMP™ is a RT class demonstrated to improve muscular strength and decrease metabolic stress, but has no influence on running aerobic fitness (Greco et al., 2011). Training sessions involved participants using a self-selected light to moderate weight as part of a low-resistance, high-repetition total body workout that was continually adjusted according to the perception of the participants and instructor. Each session was performed using the pre-defined BODYPUMP™ exercise programme which consists of 10 exercise-music selections, each lasting 4-6 minutes, for approximately 60 minutes. Detailed information regarding exercise order and duration is presented elsewhere (Greco et al., 2011).

Participants were recalled for anthropometric measurements, questionnaires, and blood sample collection a year after their 'post' visit as part of a 12-month follow-up.

Anthropometric measurements

Height, body mass, BF%, FM, FFM, TBW, BMI, and visceral fat rating were quantified as outlined in section '2.1.1 Height, body mass, BMI, and body composition measurements'.

Physical performance measures

All participants performed a standardised warm-up prior to physical testing; stepping up and down on a 30cm high step, one foot at a time, to a metronome beat at 15 steps/minute for 3 minutes. The step rate then increased to 20 steps/minute for another 3 minutes, for a total time of 6 minutes.

All physical performance tests (CMJ, isometric leg strength, grip strength, chair stand test) were performed as detailed in section '2.2 Physical performance measures'.

Blood sample collection and processing

A trained phlebotomist collected blood the week before and after the 12-week RT intervention. An additional blood sample was taken a year after participants 'post' visit as part of the 12-month follow-up. All bloods were taken at least 24 hours post-exercise. Whole blood was collected and processed for DNA extraction, serum isolation and RNA analysis as detailed in section '2.3 Biochemical analyses'.

DNA extraction and telomere length analysis

DNA was extracted and assessed following the methodology in section '2.3.3 DNA extraction and analysis'. DNA purity reported acceptable A260/280 values (1.93 ± 0.02). TL was quantified using the methodology described in section '2.3.6 Telomere length analysis'. Intra-assay CV for calculated T/S ratio was 4.9%. Inter-assay CV for calculated T/S ratio was 5.1%.

RNA extraction and gene expression analysis

RNA was extracted from whole blood, analysed, reverse transcribed to cDNA, and diluted as detailed in section '2.3.4 RNA isolation, analysis of content and cDNA synthesis'. RNA purity reported acceptable A260/280 values (2.12 ± 0.01). The relative mRNA expression level for hTERT was quantified using the protocol ($2^{-(\Delta\Delta Ct)}$) outlined in section '2.3.5 Gene expression analysis'. The pooled mean pre-intervention Ct from all participants was used as the control.

Measurement of inflammatory markers

Inflammatory markers IL-6, IL-8, TNF-α and CRP were all measured in serum as described in section '2.3.8 Inflammatory marker quantification'. Average intra- and inter-assay CV was 5.3 ± 1.5% and 3.4 ± 2.1% for the different ELISA assays, respectively. All CRP samples were analysed on a single run and the average intra-assay CV was 1.5 ± 0.1%.

Statistical analysis

The Wilcoxon signed-rank test was used to compare pre- and post-intervention data, circumventing the requirement of making assumptions about the distributions of variables in a small sample size. Spearman's test was used to verify correlations. Differences within groups at 12-month follow-up were determined using a repeated-measures ANOVA and a Bonferroni correction. If data violated the assumption of sphericity, a repeated-measures ANOVA with a Greenhouse-Geisser correction was used. Total sample size (n = 23) conferred a statistical power of 73% ($\beta = 0.73$) with a significance level of 5% ($\alpha = .05$) and moderate effect size (d= 0.5) for the primary outcome. All analyses were performed using SPSS 23.0 (IBM Corporation, USA) with p-values < 0.05 considered significant.

3.4 Results

Telomere length

There was no difference between pre- (median = 0.809, range = 0.609 - 1.079) and post-intervention (median = 0.799, range = 0.577 - 1.049) LTL (Z = -0.806, p = 0.420) (Figure 3.1. (A)). Inter-individual responses revealed telomere shortening in 52% of participants and lengthening in 48% following the RT intervention (Figure 3.1. (B)). Age was not associated with LTL at pre (r = -0.135, p = 0.539) or post (r = -0.210, p = 0.337) timepoints.

Inflammatory markers

Participants displayed significantly reduced levels of serum TNF-α following the 12-week intervention. No other inflammatory markers showed any significant change (Table 3.1.). Significant inverse associations were found between CRP level and LTL at pre and post timepoints. LTL was not associated with any other inflammatory marker (Table 3.2.).

Gene expression

There was no change in the relative mRNA expression level of hTERT following the RT intervention (median pre $2^{-(\Delta\Delta Ct)}$ values = 0.9, range = 0.4 - 2.6 vs median post $2^{-(\Delta\Delta Ct)}$ values = 1.4, range = 0.4 - 2.3) (p = 0.050), although it was bordering a significant increase (Figure 3.2.).

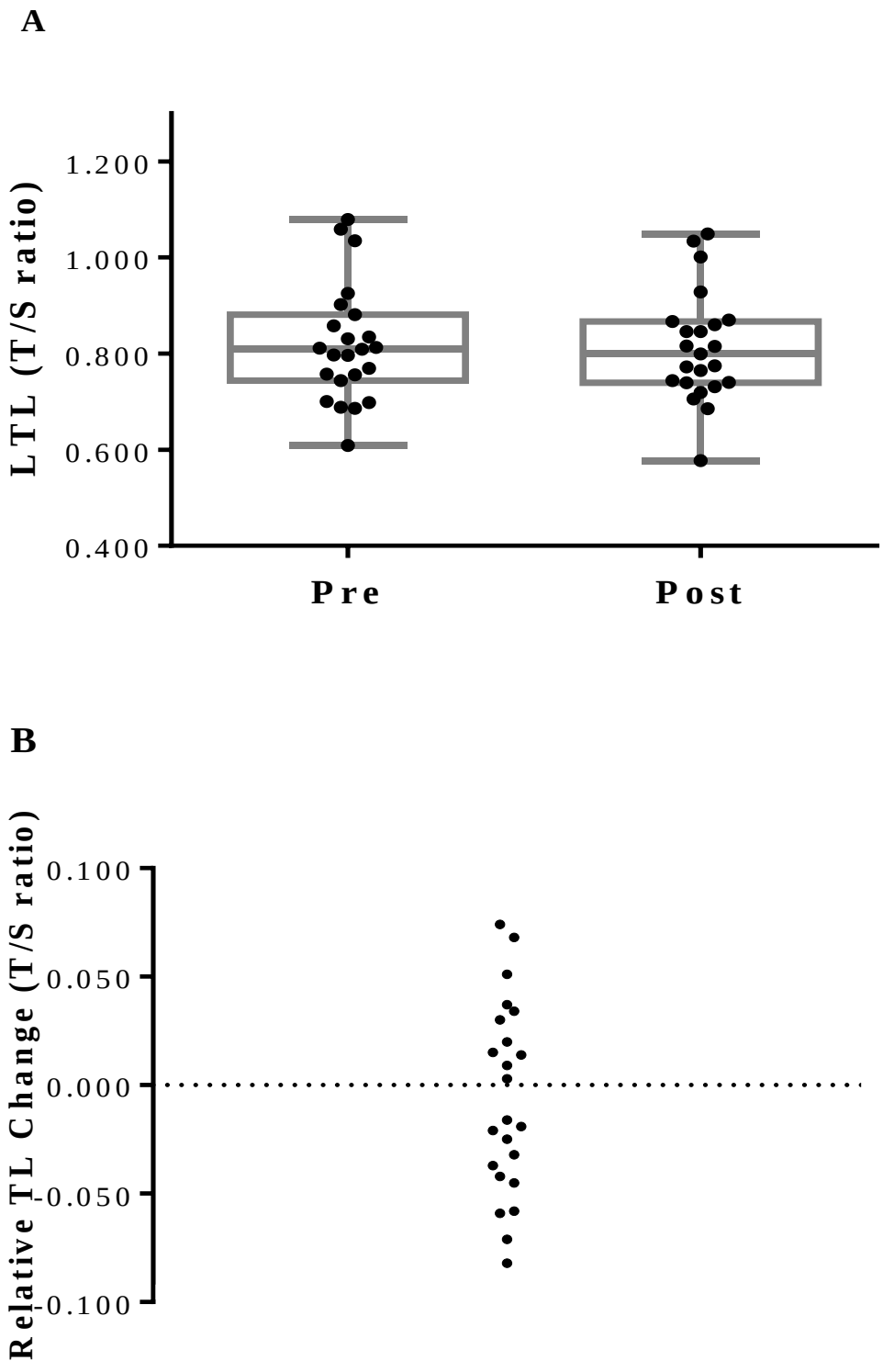

Figure 3.1. (A) Pre and post LTL (median and range) and (B) inter-individual relative TL change following a 12-week RT intervention. Data are presented as T/S ratio.

Table 3.1. Levels of inflammatory markers at baseline (pre) and following (post) 12-weeks low-resistance, high-repetition RT

	pre	post	p
TNF-α (pg/ml)	0.85 (0.50 - 1.89)	0.76 (0.40 - 2.12)	**0.001***
IL-6 (ng/ml)	2.86 (0.66 - 7.75)	2.93 (0.45 - 14.20)	0.761
IL-8 (ng/l)	9.88 (5.71 - 18.54)	9.42 (6.23 - 18.23)	0.211
CRP (mg/l)	1.14 (0.15 - 18.20)	0.86 (0.16 - 17.71)	0.637

Data presented as median (range). *Statistical significance defined as p-values <0.05, Wilcoxon signed-rank test and expressed to three decimal places.

Table 3.2. Spearman correlation coefficients for inflammatory markers and telomere lengths observed before (pre) and after (post) the 12-week RT intervention.

	pre		post	
	r	p	*r*	p
TNF-α (pg/ml)	-0.309	0.151	-0.269	0.214
IL-6 (ng/ml)	-0.277	0.200	-0.025	0.911
IL-8 (ng/l)	-0.105	0.634	-0.051	0.816
CRP (mg/l)	-0.437	**0.037***	-0.439	**0.036***

r, Spearman correlation coefficient. *Statistical significance defined as p-values <0.05

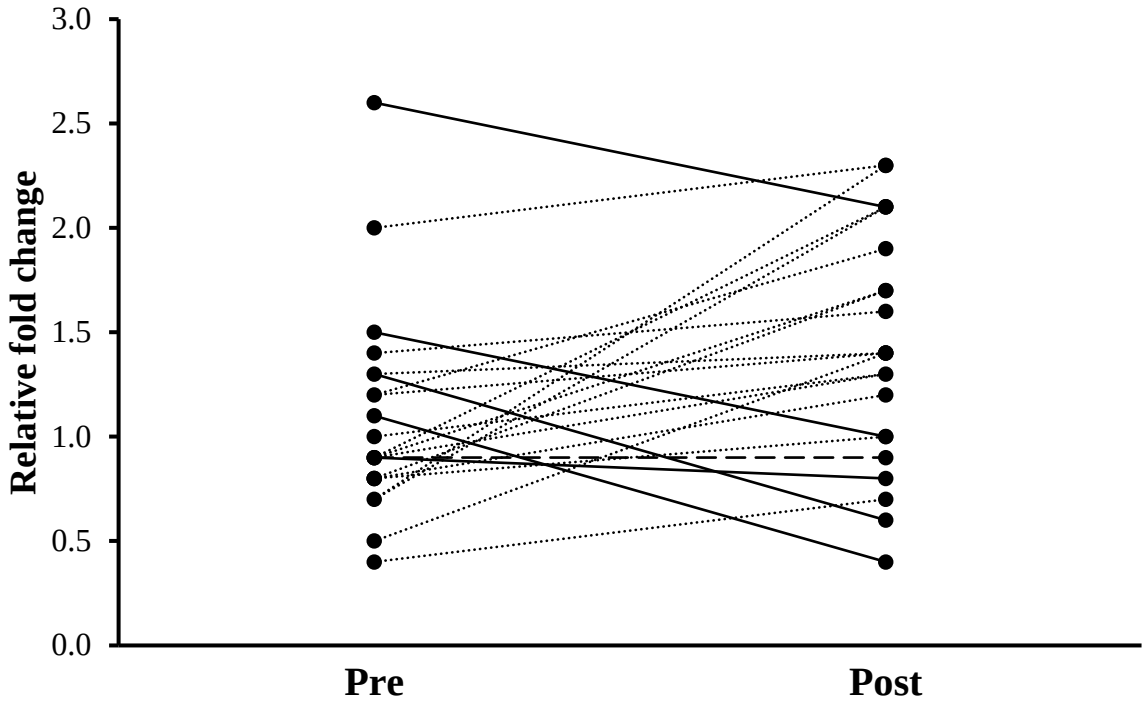

Figure 3.2. Inter-individual responses of hTERT mRNA relative fold change following a 12-week RT intervention. Gene expression data is expressed relative to the geometric mean of housekeeper genes CYC1, ATP5B and GAPDH. Solid line indicates decrease (n = 5), dotted line indicates increase (n = 16), and dashed line indicates no change (n = 1). Data was lost for one participant during sample preparation.

Body composition

There was a significant decrease in participant body mass following the 12-week RT intervention. However, all other measures of body composition remained unchanged (Table 3.3). No measures of body composition were associated with LTL at any timepoint ($p > 0.05$), so no further adjustments were required.

Physical performance measures

There was a significant increase in mean physical performance scores for CMJ, grip strength (right), isometric leg strength and chair stand test following the RT intervention (Table 3.3). No physical performance measures were associated with LTL at any timepoint ($p > 0.05$).

Table 3.3. Body composition and physical performance measures at baseline (pre) and after (post)12 weeks RT

	pre	post	p value
Body composition			
Body mass (kg)	77.6 (46.6 - 102.5)	76.8 (44.9 - 101.6)	**0.002***
BMI (kg/m^2)	29.2 (18.3 - 37.3)	28.1 (18.0 - 36.9)	0.158
Body fat (%)	35.4 (20.5 - 49.3)	34.8 (16.7 - 50.0)	0.079
FM (kg)	26.2 (12.0 - 50.2)	24.8 (10.3 - 50.8)	0.070
FFM (kg)	48.8 (33.7 - 73.3)	49.1 (34.6 - 73.0)	0.807
TBW (kg)	35.7 (24.7 - 53.7)	35.9 (25.3 - 53.4)	0.794
Visceral fat rating	9.0 (4.0 - 14.0)	9.0 (4.0 - 14.0)	0.564
Physical performance			
CMJ (cm)	15.5 (7.9 - 34.5)	20.4 (9.6 - 38.0)	**0.001***
Grip strength (kg) (right)	29.0 (5.0 - 47.5)	32.0 (14.0 - 46.5)	**0.012***
Grip strength (kg) (left)	28.0 (3.5 - 46.5)	30.0 (17.0 - 43.0)	0.097
Isometric leg strength (kg)	70.0 (14.0 - 176.0)	85.0 (52.0 - 169.0)	**0.009***
Sit-to-stand test (s)	22.0 (15.0 - 43.0)	19.0 (10.0 - 29.0)	**<0.001***

Data presented as median (range). ; BMI, body mass index. FM, fat mass. FFM, fat free mass. TBW, total body water. CMJ, countermovement jump. *Statistical significance defined as p-values <0.05, Wilcoxon signed-rank test, and expressed to three decimal places.

Mental wellbeing

Participants mental wellbeing significantly improved following the 12-week RT intervention (pre = 48.0, range = 38.0 - 59.0 vs post = 52.0, range = 41.0 - 65.0) ($p = 0.014$), but was not associated with TL at any timepoint and required no further adjustments ($p > 0.05$).

12-month follow-up visit

Individuals who returned to a sedentary lifestyle (n = 10) displayed significantly shorter telomeres at 12-month follow-up (0.734 ± 0.087) compared to their pre intervention measurements (0.819 ± 0.108, $p = 0.036$), but not their post values (0.817 ± 0.112, $p = 0.050$), although this was approaching significance (Figure 3.3.). There was no difference in CRP (pre 3.02 ± 5.81, post 3.29 ± 5.63, 12-month follow-up 2.20 ± 2.93) or TNF-α (pre 0.844 ± 0.353, post 0.746 ± 0.292, 12-month follow-up 0.871 ± 0.284) at any time point in sedentary individuals ($p > 0.05$). Circulatory CRP levels were not associated with LTL at any time point ($p > 0.05$). However, LTL was negatively associated with pre ($r = -0.758$, $p = 0.011$) and post ($r = -0.748$, $p = 0.013$) circulatory TNF-α levels in individuals who returned to a sedentary lifestyle, but not 12-month follow-up ($p > 0.05$).

Individuals who continued to exercise (n = 6) following the 12-week RT intervention showed no difference in TL (pre 0.874 ± 0.124, post 0.849 ± 0.110, 12-month follow-up 0.831 ± 0.154) (Figure 3.3.), CRP (pre 0.81 ± 0.50, post 0.59 ± 0.31, 12-month follow-up 0.52 ± 0.22) or TNF-α (pre 1.21 ± 0.40, post 0.84 ± 0.19, 12-month follow-up 0.91 ± 0.09) levels between pre, post or 12-month follow-up ($p > 0.05$). Furthermore, LTL did not correlate with CRP at any time point ($p > 0.05$) but was surprisingly positively associated with TNF-α at 12-month follow-up ($r = 0.900$, $p = 0.037$). Pre and post TNF-α levels were not associated with LTL in individuals who remained active ($p > 0.05$).

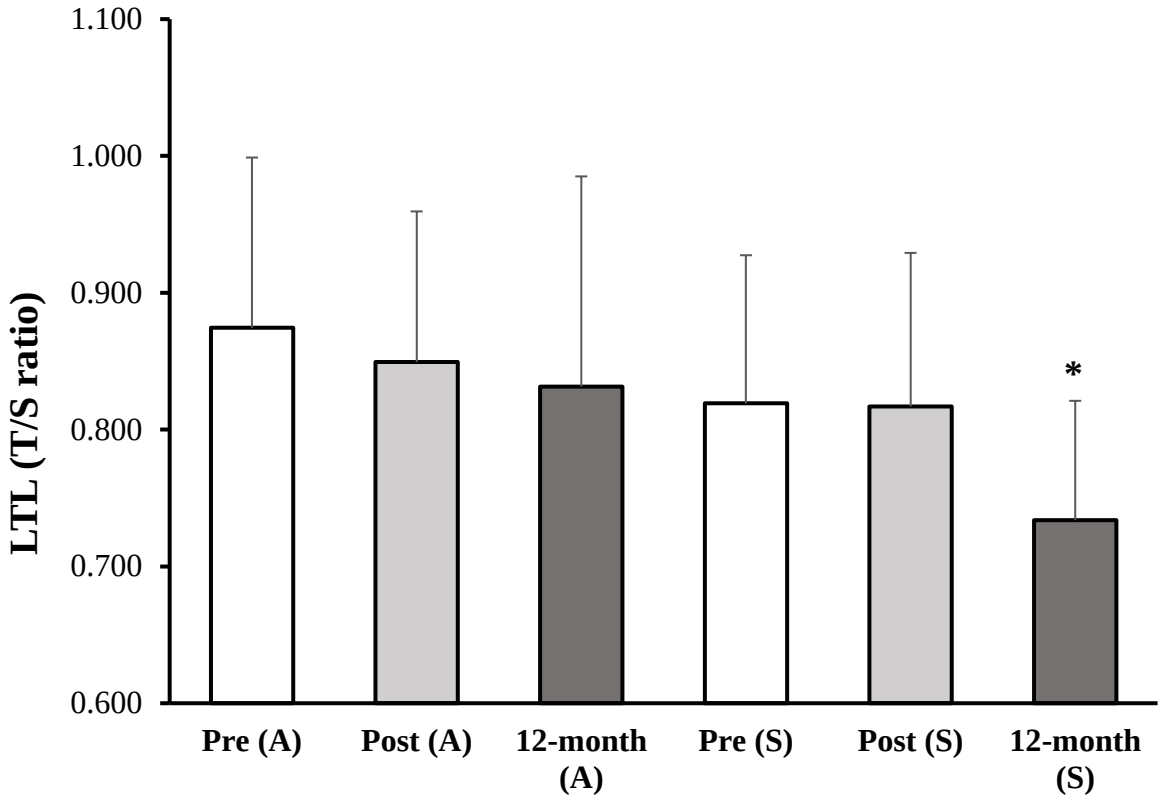

Figure 3.3. Pre, post and 12-month LTL for active (A) and sedentary (S) groups. (mean + SD). Data are presented as T/S ratio. *: different from Pre (S).

3.5 Discussion

From pre and post analysis of a 12-week low-resistance, high-repetition RT intervention in healthy sedentary middle-aged participants we provide evidence that 1) no changes in TL were observed; 2) circulating TNF-α levels significantly decreased; 3) circulating CRP levels were inversely associated with LTL. In addition, a 12-month follow-up showed that long-term (>12 months) regular exercise may act to preserve TL in previously sedentary middle-aged individuals. However, the present study only comprised a small sample size and the data should be interpreted with caution.

The results presented in Chapter III lend support to the null hypothesis that no observable changes in TL occurred following the 12-week RT intervention. This study is the first to longitudinally examine the effect of low-resistance, high-repetition RT on TL. There is a paucity of intervention studies investigating the relationship between TL and exercise, with most of the present research employing a cross-sectional design. Moreover, the current literature principally reports the effects of endurance aerobic exercise on TL. Among the few

longitudinal studies that have assessed the influence of a RT intervention on telomere dynamics the results are inconclusive, with reported relationships of TL maintenance (Dimauro et al., 2016), no effect (Hagstrom, 2018; Tosevska et al., 2016; Werner et al., 2018), and telomere shortening (Miranda-Furtado et al., 2015). The discrepant findings may be a consequence of variations in the frequency, volume, intensity, and duration of the individual study RT protocols. It may be that 'explosive-type' RT , employed by Dimauro *et al*., is a more influential form of RT regarding LTL maintenance (Dimauro et al., 2016). However, this study shows that low-resistance, high-repetition RT does not appear to have any effect on LTL over a 12-week intervention period. Thus, our results support previous findings that short-term RT interventions do not elicit any observable changes in TL (Hagstrom, 2018; Tosevska et al., 2016; Werner et al., 2018).

Although it appears that the low-resistance, high-repetition RT intervention employed in the present study does not influence LTL, it would be unreasonable to completely dismiss the potential benefits of this modality of exercise on telomere dynamics. Firstly, participants who continued to exercise following the RT intervention did not display significant LTL loss at their 12-month follow-up visit, whereas participants who reverted to a sedentary lifestyle showed significant telomere attrition from their pre-RT intervention values. However, the absence of significant telomere attrition in the exercise group cannot be solely attributed to RT, as participants who continued to perform PA reported utilising various modalities in addition to RT (e.g., cycling, running, tennis). Nonetheless, inflammatory marker levels can be reduced by long-term RT (Calle and Fernandez, 2010), which is one of the main factors proposed to influence telomere attrition (Correia-Melo et al., 2014). Present results report that circulating levels of TNF-α were significantly reduced following the 12-week RT intervention. This finding is in line with data from Hagstrom *et al.* who found a significant reduction in natural killer and killer T-cell expression of TNF-α following a 16-week RT intervention (Hagstrom et al., 2016). Higher plasma concentrations of TNF-α have been previously reported to be inversely associated with TL (O'Donovan et al., 2011), a finding replicated in the sedentary participants of the present study at 12-month follow-up. Thus, a decline in circulating TNF-α concentration suggests a potential mechanism by which low-resistance, high-repetition RT could positively influence telomere dynamics. We speculate that a longer intervention period may have therefore been required to reveal a significant effect of RT on telomere dynamics.

The pro-inflammatory cytokine TNF-α usually acts as part of an inflammatory response. Initially secreted by a limited cell population, consisting of macrophages and T-cells, TNF-α exerts its effects via cognate receptors (TNF-RI and TNF-RII) which display a much wider cellular expression. This ubiquitous expression, in conjunction with cell-specific effector molecules that are triggered by receptor binding, may explain the variety of effects of TNF-α which include apoptosis, the synthesis of protein and lipid inflammatory molecules, and transcription factors. TNF-α-mediated inflammation contributes to chronic low-grade inflammation associated with advanced age (Franceschi et al., 2006) and is connected with many age-related diseases, such as CVD and rheumatoid arthritis (Rea et al., 2018). Linked to its pivotal role in the generation of inflammation, anti-TNF-α drug therapy has been successful in ameliorating several inflammatory conditions (Lopetuso et al., 2017). However, primary non-response, or loss of response, and negative side effects arise in certain individuals (Lopetuso et al., 2017). Thus, reducing TNF-α via low-resistance, high-repetition RT could be a viable method to delay inflammaging and associated diseases without the negative side effects of anti-TNF-α drugs.

Higher plasma concentrations of CRP have also been previously linked with shorter telomeres (O'Donovan et al., 2011; Rode et al., 2014), which our findings support. Furthermore, TNF-α and CRP levels are inversely associated with muscle mass (Visser et al., 2002) and strength (Westbury et al., 2018). To highlight a potential interconnected relationship between the factors, TL has been positively associated with measures of physical function, such as faster walking speed (Dimauro et al., 2016), CMJ height (Dimauro et al., 2016), and lower body muscle strength (Dimauro et al., 2016; Loprinzi and Loenneke, 2016), although the current literature is inconsistent regarding these relationships (Baylis et al., 2015; Mather et al., 2010). In the present study, only LTL was inversely associated with circulating TNF-α and CRP levels at various timepoints. Thus, our data agree with previous studies that report physical function is not associated with TL, despite increases in performance measures following the RT intervention. Nevertheless, since long-term RT significantly reduces TNF-α and CRP levels (Santiago et al., 2018; Sardeli et al., 2018), this may provide a mechanistic explanation as to how RT delays age-related declines in LTL, muscle mass and strength, and improves functional performance.

TL has been associated with multiple behavioural and psychosocial factors, including diet, body composition, psychological stress, and sleep (Starkweather, 2014). Thus, it is problematic isolating the effects of exercise on telomere dynamics without controlling for

these variables. In the present study, we only measured body composition and mental wellbeing, neither of which were associated with LTL. Nevertheless, we cannot be sure that discrepancies in participants' diet and sleep patterns may have influenced our results. In addition to not controlling for these potential confounding factors, another major limitation of the present study is that it lacks a control group. Although we report that the RT intervention had no observable change on LTL, we cannot be certain that a sedentary control group would have also produced comparable results over a 12-week period. For example, Dimauro *et al*. have previously reported that whilst TL remained unchanged in participants performing a 3-month RT intervention, their telomeres were significantly longer than a sedentary control group after the study (Dimauro et al., 2016). However, this scenario is unlikely as it appears that sedentary individuals do not typically show any observable telomere attrition over such short intervention periods (Hagstrom, 2018; Tosevska et al., 2016; Werner et al., 2018). The appropriateness of the current intervention period is further questioned by the lack of differences witnessed in FFM and FM despite 12 weeks of RT. It is also possible that the small sample size may have affected the present results. Thus, longer intervention periods may be required to reveal the full potential benefits of RT on LTL and this study may serve to act as a pilot to further investigations that utilise a control group, larger sample size and longer intervention periods (> 12 months).

In support of a longer intervention period, there was a borderline significant increase in relative mRNA expression level of hTERT. Expression of hTERT mRNA has been reported to correlate with telomerase activity (Kirkpatrick et al., 2003), congruent with the notion that synthesis and expression of this subunit represents the rate-limiting determinant of telomerase activity (Counter et al., 1998). The mRNA expression level of hTERT may have become significant had a 12-month RT intervention been employed, potentially reflecting increased telomerase activity, which could have had a positive influence on TL. Although we did not measure telomerase activity directly our findings support those of Werner *et al.*, who recently demonstrated that telomerase activity was unchanged following a 6-month RT intervention (Werner et al., 2018). However, this conclusion should be interpreted with caution, as it assumes that hTERT mRNA expression level accurately represents telomerase activity.

In conclusion, the results of this chapter demonstrate that a 12-week low-resistance, high-repetition RT intervention does not elicit any observable changes in LTL in healthy sedentary middle-aged participants, despite improved molecular and physical parameters associated with telomere dynamics. It appears that long-term, regular exercise is necessary to preserve

TL (minimum of a moderate score on IPAQ-SF for >12-months), which may be mediated by molecular pathways that are influenced by low-resistance, high-repetition RT.

CHAPTER IV - PILATES AND TELOMERE DYNAMICS: A 12-MONTH LONGITUDINAL STUDY

The study presented in this chapter has been published with the following citation:

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4.1 Abstract

Pilates is an increasingly popular exercise modality that is reported to exert beneficial physiological effects in the body, although the cellular mechanisms are poorly understood. The aim of the present study was to investigate the influence of Pilates exercise on LTL. This longitudinal study followed experienced female Pilates practitioners (n = 11, 50.8 ± 7.5 years) and healthy age- and sex-matched sedentary controls (n = 11, 49.3 ± 6.1 years) over a 12-month period. LTL was quantified using qPCR. Circulatory inflammatory markers, mRNA gene expression, body composition, physical performance, and mental well-being were also assessed. LTL was comparable between Pilates practitioners and controls at baseline (Pre) and 12-months (Post) ($p > 0.0125$). Pilates practitioners displayed enhanced mRNA gene expression of antioxidant enzymes (SOD2 and GPX1), and lower body fat percentage and visceral fat rating, compared with sedentary controls ($p < 0.0125$). Over the 12-month longitudinal period, Pilates participants significantly increased dynamic balance in their right ($p = 0.044$) and left ($p = 0.018$) legs. In conclusion, long-term Pilates participation does not appear to influence LTL. Nonetheless, Pilates exercise may increase antioxidant enzyme gene expression, effectively manage body composition, and improve dynamic balance.

4.2 Introduction

Chronic inflammation and oxidative stress are proposed to accelerate telomere attrition (Correia-Melo et al., 2014) and contribute to the onset of age-related diseases (Khansari et al., 2009) (Section 1.1.3 Telomere attrition and replicative senescence). Better lifestyle choices can reduce inflammation and oxidative stress, and subsequently have a positive impact on telomere dynamics. For example, participation in exercise (Cherkas et al., 2008) and meditation (Alda et al., 2016) are both associated with longer telomeres. As such, involvement in these practices may delay the initiation of age-related diseases and therefore increase lifespan.

There is an increasing awareness of Pilates exercise and a growing acceptance of its scientific value. Several studies have revealed the physiological and psychological benefits of Pilates, reporting improvements in physical fitness (Campos et al., 2016; Cancela et al., 2014), psychological wellbeing, quality of life, mood, depression, and anxiety (Fleming and Herring, 2018; Vancini et al., 2017). Pilates has long been compared with Yoga and induces comparable increases in flexibility (Mangal and Abhilash, 2012) and balance (Irandoust and Taheri, 2016). Conversely, differences have also been observed where Pilates appears to promote greater enhancements in functional movement and individual health level than Yoga (Lim and Park 2019). Nonetheless, there is a larger body of research investigating the influence of Yoga on physiological and biochemical parameters in humans. Only two studies have however studied the specific association between Yoga and TL. Cross-sectional analysis of experienced Yoga practitioners revealed that they possess longer telomeres and a greater total antioxidant status than their sedentary counterparts (Krishna et al., 2015). TL was positively correlated with this greater antioxidant status, highlighting the potential mechanistic relationship between oxidative stress and telomere maintenance. Conversely, TL was unchanged following a 12-week Yoga intervention, despite significant reductions in oxidative stress and inflammation (Tolahunase et al., 2017). No study has explored the relationship between TL and Pilates exercise to date. Thus, this study aims to investigate the impact of Pilates on telomere dynamics and a range of associated cellular markers.

4.3 Methods

Ethical approval

Ethical approval for this study was provided by the Loughborough University ethical advisory committee (R18-P150). Written informed consent was obtained from all participants and the study conformed to the standards detailed by the latest revision of the Declaration of Helsinki.

Participants

All participants in the study were considered healthy, non-smokers, who were not using any medication and were recruited via email advertisement, posters, and word of mouth. Exclusion criteria included known metabolic disorders, cardiovascular disease, cancer, use of anti-inflammatory medication or steroids, and any chronic age-related condition that could influence outcome measurements. The health status of each participant was also evaluated by a questionnaire preceding any testing (Appendix E). 19 Pilates practitioners were originally recruited to the study and there was a 42% dropout rate. Pilates practitioners (n = 11) were middle-aged females (50.8 ± 7.5 years old) who possessed 5.7 ± 1.5 years of training experience and attended a minimum of two Pilates sessions per week (minimum session length one hour). Control participants (n = 11) were age-matched (49.3 ± 6.1 years old) females considered to be sedentary, but clinically healthy (no engagement in any recreational exercise for the previous 12 months).

Experimental design and training protocol

All participants attended the laboratory at baseline (Pre) and 12 months after their initial visit date (Post) (± one week). The sequence of tests was identical for all participants and data were collected at an equivalent time of the day (± one hour) to prevent diurnal contamination. The testing protocol for all participants was: anthropometrics, blood sample, physical performance tests (forearm plank, Y-balance test, sit and reach test). Physical performance parameters were only measured in Pilates practitioners since it is well documented that individuals who perform Pilates display greater performance in Pilates specific physical performance tests than controls (Johnson et al., 2007; Lim and Park, 2019; Mangal and Abhilash, 2012). Pilates practitioners were required to continue their usual Pilates exercise

routine, attending a minimum of two 1-hour sessions per week for the subsequent 12 months. All Pilates sessions were performed by the same instructor, with a focus on lumbopelvic flexibility, balance, and core strengthening. Briefly, a typical Pilates session consisted of an initial warm up focused on light breathing (5 minutes), main exercises (~50 minutes), and a cool down (5 minutes). Control participants were required to continue with their sedentary lifestyle and did not perform any recreational exercise. The Warwick-Edinburgh Mental Wellbeing Scale (WEMWBS) (Appendix I) was used to assess the mental wellbeing of each participant as an outcome measure.

Anthropometric measurements

Height, body mass, BF%, FFM, BMI, and visceral fat rating were quantified as outlined in section '2.1.1 Height, body mass, BMI, and body composition measurements'.

Physical performance measures

All participants performed a standardised warm of 5 minutes on a cycle ergometer (60 rpm) prior to physical testing. Physical performance measures followed the same standardised pattern (forearm plank, Y-balance test, sit and reach test) and were performed as detailed in section '2.2.2 Physical performance measures'.

Blood sample collection and processing

A resting blood sample was drawn from the antecubital vein, a minimum of 24 hours post-exercise, at baseline and 12 months. Whole blood was collected and processed for DNA extraction, serum isolation and RNA analysis as detailed in section '2.3 Biochemical analyses'.

DNA extraction and telomere length analysis

DNA was extracted and assessed following the methodology in section '2.3.3 DNA extraction and analysis'. DNA purity reported acceptable A260/280 values (1.91 ± 0.02). TL was quantified using the methodology described in section '2.3.6 Telomere length analysis'.

Intra- assay CV for calculated T/S ratio was 4.6%. Inter-assay CV for calculated T/S ratio was 2.8%.

RNA extraction and gene expression analysis

RNA was extracted from whole blood, analysed, reverse transcribed to cDNA, and diluted as detailed in section '2.3.4 RNA isolation, analysis of content and cDNA synthesis'. RNA purity reported acceptable A260/280 values (2.11 ± 0.02). Relative gene expression of target genes TERT, SOD2, GPX1 and TRF1 were normalised to the expression of the housekeeper gene GAPDH using the protocol ($2^{-(\Delta\Delta Ct)}$) outlined in section '2.3.5 Gene expression analysis'. All data is expressed relative to the pooled mean Pre Ct value from the control group for each target gene.

Measurement of inflammatory markers

Inflammatory markers IL-8, TNF-α, and CRP were all measured in serum as described in section '2.3.8 Inflammatory marker quantification'. Average intra- and inter-assay CV was 6.3 ± 0.7% and 4.6 ± 0.2% for the different ELISA assays, respectively. All CRP samples were analysed on a single run and the average intra-assay CV was 0.4 ± 0.1%.

Statistical analysis

The Shapiro-Wilk test was used to assess the normality of data for all parameters analysed. Nonparametric data were log transformed prior to analysis. Data were evaluated using a two-way repeated measures ANOVA. Based on the two-way repeated measures ANOVA results, further paired *t*-tests or unpaired *t*-tests were used to investigate for significant differences within and between individual groups, respectively. Statistical significance was only accepted if its significance was less than α/number of comparisons. Total sample size enlisted in this study (n = 22) conferred a statistical power of 99% (β = 0.99) with a significance level of 5% (α = .05) and moderate effect size (d= 0.5) for the primary outcome. Physical performance measures were tested by a paired *t*-test. Correlations were tested using the two-tailed Pearson correlation coefficient. All data were analysed using SPSS 23.0. Statistical significance was set at $p < 0.05$ unless stated otherwise.

4.4. Results

Telomere length

There was no difference in TL within or between groups at any timepoint (Pilates (Pre) (0.712 ± 0.816), Pilates (Post) (0.699 ± 0.169), controls (Pre) (0.792 ± 0.132), controls (Post) (0.810 ± 0.138)) ($p > 0.05$) (see Figure 4.1.). As expected, age was negatively correlated with TL at baseline across the whole cohort ($r = -.513$, $p = <0.001$).

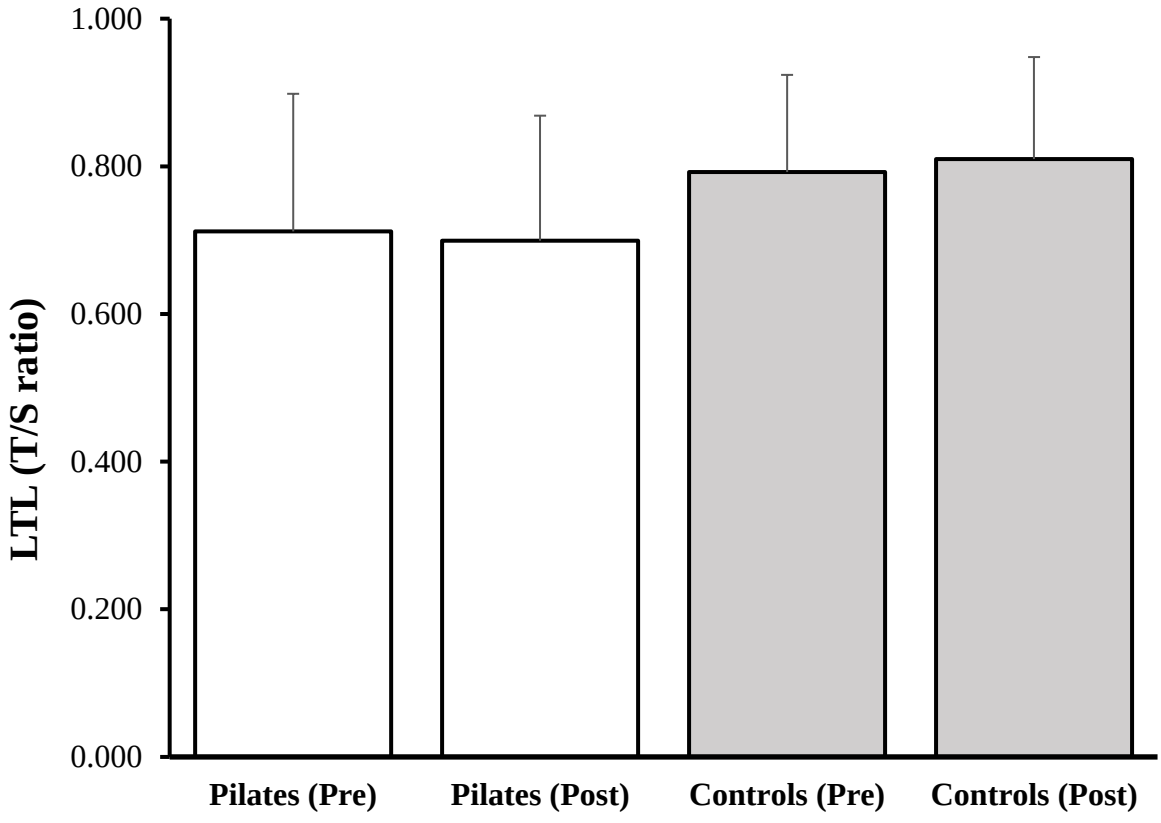

Figure 4.1. Leukocyte telomere length (LTL) for Pilates practitioners and controls at baseline (Pre) and 12-months (Post). Data presented as T/S ratio (mean + SD).

Inflammatory markers

Pilates practitioners showed a significant reduction in IL-8 levels over the 12-month period (see Table 4.1.). Additionally, Pilates practitioners showed significantly reduced CRP levels at baseline compared with controls ($p = 0.006$). No further differences were found within or between groups for circulatory inflammatory markers ($p > 0.0125$). TNF-α serum levels were

negatively correlated with TL across the whole cohort (all timepoints) (r = -0.310, p = 0.040) and between TNF-α and control TL at 12-months (r = -.619, p = 0.042). TL was not associated with any other inflammatory markers at any timepoint (p > 0.05).

Table 4.1. Circulatory levels of inflammatory markers at baseline (pre) and 12-months (post) for Pilates practitioners and controls

	pre	post	Δ post vs. pre	p
Pilates				
TNF-α (pg/ml)	0.68 ± 0.23	0.70 ± 0.27	0.02 ± 0.16	0.660
IL-8 (ng/l)	12.18 ± 3.62	9.96 ± 3.76	-2.22 ± 2.24	**0.008***
CRP (mg/l)	**0.48 ± 0.40†**	0.68 ± 0.54	0.20 ± 0.33	0.085
Controls				
TNF-α (pg/ml)	0.74 ± 0.30	0.84 ± 0.30	0.10 ± 0.21	0.173
IL-8 (ng/l)	8.72 ± 1.32	10.06 ± 4.42	1.34 ± 5.09	0.553
CRP (mg/l)	3.55 ± 5.20	2.70 ± 2.78	- 0.85 ± 3.00	0.224

Data are means ± SD. *: within-group difference; †: between-group difference with controls (pre). Significance level set at p < 0.0125.

Telomere-regulating protein gene expression

There was no difference in TERT or TRF1 mRNA gene expression within or between the Pilates and control group at any timepoint (p > 0.0125) (see Figure 4.2.). However, TRF1 was positively associated with TL in the control group at 12 months (r = 0.629, p = 0.038). TL was not associated with gene expression of TERT or TRF1 at any other timepoint (p > 0.05).

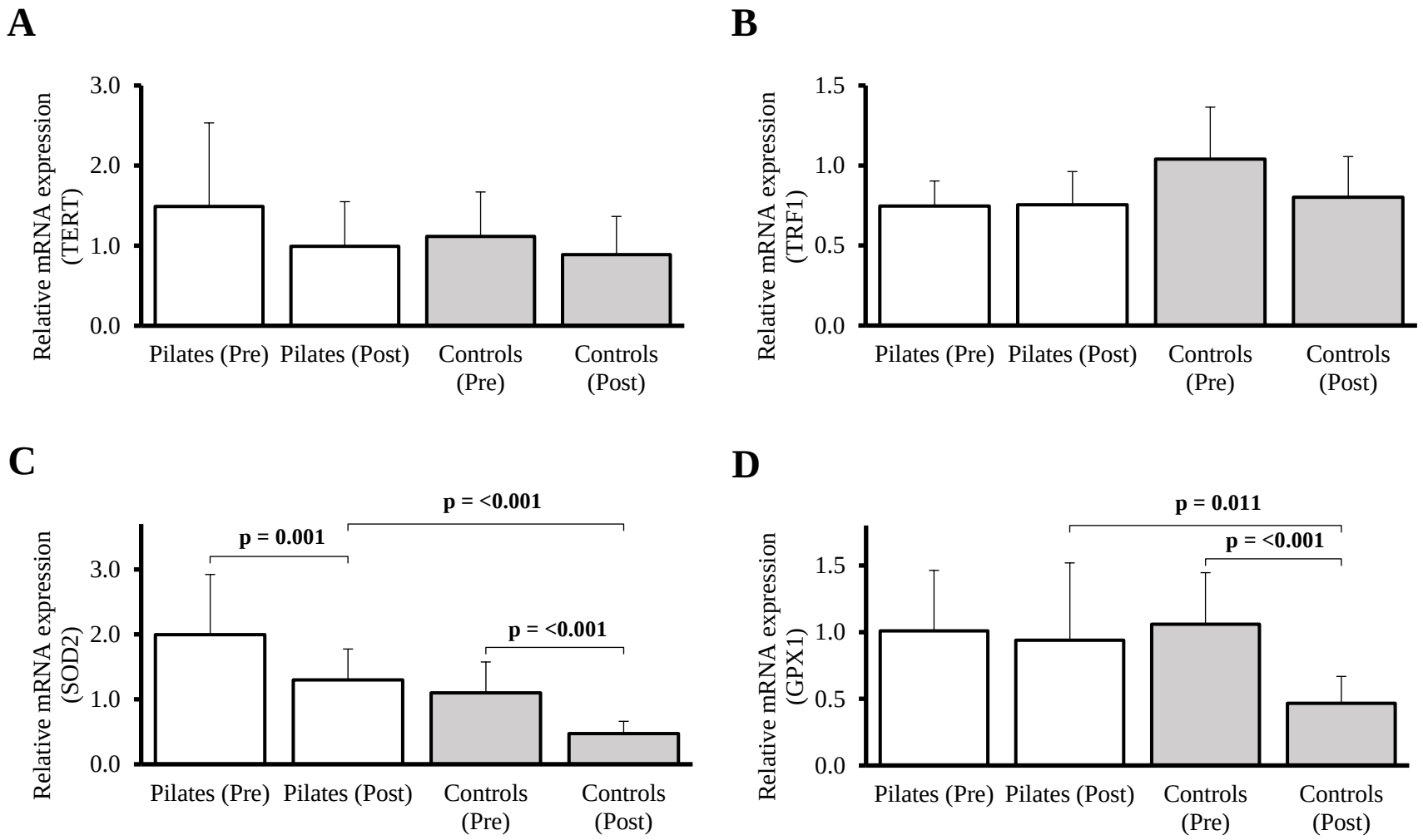

Figure 4.2. Relative mRNA gene expression of TERT (A), TRF1 (B), SOD2 (C) and GPX1 (D) for all groups. Data are means + SD. Significant difference set at $p < 0.0125$.

Antioxidant enzyme gene expression

Both the Pilates ($p = 0.001$) and control group ($p = <0.001$) displayed a significant reduction in SOD2 mRNA expression over the 12-month longitudinal period (see Figure 4.2.). The Pilates group had higher SOD2 mRNA expression values than controls at the post timepoint ($p = <0.001$). Pilates practitioners showed no change in GPX1 mRNA gene expression, whereas control participants showed a significant reduction ($p = <0.001$). Additionally, the Pilates group showed higher GPX1 mRNA expression than controls at the post timepoint ($p = 0.011$). Baseline GPX1 mRNA gene expression was negatively associated with TL across the whole cohort ($r = -.426$, $p = 0.048$). When analysed by individual groups, TL was negatively associated with GPX1 mRNA gene in Pilates practitioners at baseline expression ($r = -.682$, $p = 0.021$). TL was not associated with gene expression of SOD2 or GPX1 at any other timepoint across the whole cohort ($p > 0.05$).

Body composition

Pilates participants had lower body fat than controls at both Pre ($p = 0.009$) and Post ($p = 0.004$) timepoints (see Table 4.2.). Pilates participants also had a lower visceral fat rating at the post timepoint ($p = 0.004$). There were no within group changes for any body composition measurement over the 12-month period ($p > 0.0125$). TL was positively associated with body mass ($r = 0.379$, $p = 0.011$) and FFM ($r = 0.482$, $p = 0.001$) across the whole cohort. TL was not associated with any other body composition parameters at any timepoint ($p > 0.0125$).

Physical performance measures

Pilates practitioners showed a significant increase in their dynamic balance (right and left leg) over the 12-month period ($p > 0.05$) (see Table 4.3.). Furthermore, sit and reach performance of Pilates practitioners was negatively associated with TL at baseline ($r = -.620$, $p = 0.042$). No other performance measure changed over the 12-month period or was associated with TL at any timepoint ($p > 0.05$).

Table 4.2. Body composition measures at baseline (pre) and 12 months (post) for Pilates practitioners and controls

	pre	post	Δ post vs. pre	p
Pilates				
Body mass (kg)	64.0 ± 12.3	64.7 ± 13.6	0.7 ± 2.1	0.279
BMI (kg/m^2)	23.0 ± 3.4	23.3 ± 3.7	0.3 ± 0.7	0.239
Body fat (%)	**30.9 ± 7.8†**	**31.8 ± 7.3#**	0.9 ± 2.8	0.326
FFM (kg)	43.5 ± 4.9	43.4 ± 5.2	-0.1 ± 1.9	0.855
Visceral fat rating	5.8 ± 2.0	**6.1 ± 2.1#**	0.3 ± 0.8	0.279
Controls				
Body mass (kg)	76.1 ± 13.0	78.5 ± 13.4	2.4 ± 3.7	0.056
BMI (kg/m^2)	28.7 ± 4.1	29.6 ± 4.6	0.9 ± 1.4	0.075
Body fat (%)	39.4 ± 6.1	41.1 ± 6.2	1.7 ± 2.5	0.049
FFM (kg)	45.5 ± 5.0	45.7 ± 4.9	0.2 ± 1.1	0.716
Visceral fat rating	8.5 ± 2.6	9.2 ± 2.6	0.7 ± 0.9	0.046

Data are means ± SD; BMI, body mass index. FFM, fat free mass. †: between-group difference with controls (pre), #: between-group difference with controls (post). Significance level set at $p < 0.0125$.

Table 4.3. Physical performance measures at baseline (pre) and 12 months (post) for Pilates practitioners

	pre	post	Δ post vs. pre	p
Balance (right) (cm)	82.1 ± 12.7	86.1 ± 10.2	4.0 ± 5.7	**0.044***
Balance (left) (cm)	83.1 ± 13.3	86.9 ± 11.0	3.8 ± 4.6	**0.018***
Plank (s)	121.8 ± 83.2	145.5 ± 70.4	23.7 ± 44.0	0.105
Sit and Reach (cm)	37.7 ± 11.0	39.7 ± 11.7	2.0 ± 5.1	0.235

Data are means ± SD. *: within-group difference. Significance level set at $p < 0.05$.

Mental wellbeing

There were no changes in the mental wellbeing (WEMWBS score) of Pilates practitioners (pre = 51.5 ± 5.0 vs post = 53.9 ± 5.2) or control participants (pre = 51.0 ± 7.0 vs post = 49.4 ± 8.1) ($p > 0.0125$) over the 12-month period.

4.5 Discussion

Although there is an increasing recognition of Pilates as a viable training modality (Johnson et al., 2007; Lim and Yoon, 2017) and rehabilitation tool (Donzelli et al., 2006; Rydeard et al., 2006) in the scientific community, there is a paucity of data clarifying its impact on the body at a molecular level. To our knowledge, this study is the first to explore the association between Pilates and TL. Thus, the findings can only be best compared to previous research exploring the association between TL and Yoga, as the two training modalities feature several comparable elements (Kloubec, 2011).

The principal finding of this chapter is that long-term Pilates exercise (> 5 years) does not appear to regulate telomere dynamics, with experienced Pilates practitioners and sedentary controls displaying comparable TL at baseline. The lack of any significant observable difference between the two groups may be explained by a combination of several different factors that have been shown to influence TL. For example, it has been suggested that at least 10 years of regular PA is required to achieve a prolonged effect on LTL in older individuals (Saßenroth et al., 2015). However, it is unlikely that the lack of training years accumulated by

Pilates practitioners is responsible for the absence of influence on TL in the present study, as Krishna *et al*. reported that Yoga practitioners with only a minimum of two years of practice displayed longer telomeres compared to sedentary controls (Krishna et al., 2015). Cross-sectional studies have also demonstrated that ultra-endurance runners (minimum 2 years training) (Denham et al., 2013) and individuals participating in various sporting activities (minimum 5 years training) (Silva et al., 2016) possess longer telomeres than sedentary controls.

Another key finding of this study is that TL remained stable in both Pilates practitioners and control participants over the 12-month longitudinal period. Our results are in agreement with that of Tolahunase *et al*., who showed that a 12-week Yoga and meditation based lifestyle intervention (YMLI) had no effect on mean TL in adults (Tolahunase et al., 2017). It must be noted, however, that our study utilised a much greater longitudinal period. Nevertheless, numerous studies have shown that aerobic exercise (Puterman et al., 2018), interval training (Werner et al., 2018), and concurrent training (strength and aerobic exercise) (Brandao et al., 2020), can all positively influence TL over much shorter intervention periods (≤6 months) than the one employed in the present study. It is again therefore unlikely that the longitudinal period we employed was too short to observe any positive effect that Pilates exercise may have on telomere dynamics.

We report that telomere-regulating protein expression (TERT and TRF1) was comparable between Pilates practitioners and controls at both pre and post timepoints. TERT is the essential catalytic subunit of the telomerase enzyme and its expression has been demonstrated to increase TL and extend cellular life span (Vaziri and Benchimol, 1998). TERT mRNA is upregulated in white blood cells in response to an acute session of aerobic exercise (Chilton et al., 2014). To signify the potential importance of TERT mRNA expression on TL, endurance athletes reported to possess longer telomeres had 2.0-fold upregulated expression of this reverse transcriptase compared with sedentary controls (Denham et al., 2015). Additionally, exercise has been shown to both increase (Laye et al., 2012; Saki et al., 2016; Werner et al., 2009) and decrease (De Carvalho Cunha et al., 2018; Ludlow et al., 2012a, 2012b) the quantity of TRF1 protein. TRF1 is a negative regulator of TL (Smogorzewska et al., 2000) and overexpression induces telomere shortening as a result of end-to-end fusions, chromosomal aberrations, and telomere recombination (Munoz et al., 2009), which may influence the ageing process. The present results do however show that TRF1 was positively associated with TL in the control group at 12 months, which contrasts with the stated

previous findings. Nonetheless, as we demonstrate that TERT and TRF1 gene expression is comparable between Pilates practitioners and controls, these findings may partly explain the absence of any significant difference in LTL between the two groups.

Based on our findings and those of previous studies, we speculate that the inability of Pilates exercise to impact telomere dynamics is predominantly explained by a lack of exercise intensity. Pilates exercise has been successfully utilised as a therapeutic exercise to reduce chronic low back pain (Donzelli et al., 2006; Rydeard et al., 2006) and increase functional capacity in heart failure patients (Guimarães et al., 2012). As Pilates exercise has been regarded as safe to perform in these specific vulnerable populations, it can be assumed that the exercise modality is broadly considered low intensity. Among 1,476 older women, a lack of association has been reported between LTL and LPA, but a significant positive association with MVPA was reported (Shadyab et al., 2017b). Edwards *et al*. also disclosed MVPA (METs $\geq$ 3.0) to be the only independent factor positively associated with LTL (Edwards and Loprinzi, 2017). To highlight the potential importance of VPA on telomere dynamics, total PA was not associated with LTL in a cohort of 667 adolescents. However, the average minutes of VPA per day, but not MPA, was positively associated with LTL (Zhu et al., 2011). We, therefore, propose that the intensity of Pilates exercise may be inadequate to impact telomere dynamics. Nonetheless, it is reported that Yoga practitioners exhibit greater TL than their sedentary counterparts (Krishna et al., 2015), suggesting that further research is required comparing the influence of Pilates and Yoga on telomere dynamics.

Numerous studies indicate that oxidative stress enhances telomere attrition (Masi et al., 2011; Richter and Zglinicki, 2007). The present findings show that gene expression of the antioxidant enzyme, SOD2, was reduced over time in Pilates practitioners and controls. This finding supports previous data that SOD2 mRNA expression shows an age-related decline and is significantly reduced in women older than 38 years (Tatone et al., 2006). However, Pilates exercise appears to assist in the maintenance of a higher baseline expression of SOD2. To highlight the importance of SOD on TL, overexpression of this enzyme in human fibroblasts with reduced antioxidant capacity elevates total cellular SOD activity, attenuating telomere attrition rate and extending cellular life span (Serra et al., 2003). Pilates exercise also maintained the mRNA expression of another key enzymatic antioxidant, GPX1, compared with sedentary controls. Our findings are in agreement with previous data that report trained individuals display upregulated activity of SOD2 and GPX in blood at rest (Urso and Clarkson, 2003). Gene expression of SOD2 and GPX1 has been reported to be

significantly reduced in oligodendrocytes of individuals diagnosed with major depressive disorders, along with a reduction of TL (Szebeni et al., 2014). Thus, elevating endogenous levels of SOD2 and GPX1 via exercise may serve as an effective therapeutic strategy to combat oxidative stress-induced pathology and ageing. Interestingly, the present study demonstrates a negative correlation between LTL and GPX1 gene expression. LTL was also comparable between Pilates practitioners and sedentary controls in the present study, highlighting that further research is required investigating the relationship between the gene expression of antioxidant enzymes and telomere dynamics.

Intimately aligned with oxidative stress, inflammation is also associated with telomere driven cellular senescence and is a major component of both pathological and normal ageing (Zhang et al., 2016). Our results show that Pilates practitioners displayed lower CRP levels than controls at baseline, suggesting long-term participation may reduce levels of this acute inflammatory marker. This finding supports previous data that Pilates training can significantly reduce serum CRP levels (Khajehlandi et al., 2020). On the contrary, it has been reported that a 20-week Pilates intervention does not affect serum CRP levels in elderly individuals (Pestana et al., 2016). Additionally, circulatory CRP levels remained unchanged in both the Pilates and control groups over the 12-month study period in the present study and were not different at the post timepoint. We also report that circulatory TNF-α did not change over the 12-month period for both groups and that their levels were comparable at all timepoints. This finding is in agreement with those of Khajehlandi *et al*. who reported serum levels of TNF-α were comparable between the Pilates and control group after a 12-week Pilates intervention (Khajehlandi et al., 2020). Nevertheless, our findings indicate that serum TNF-α concentration was negatively associated with LTL, as previously described (Nickels et al., 2020; O'Donovan et al., 2011). The only inflammatory marker to show any significant change between timepoints in the present study was the reduction of circulatory IL-8 in Pilates practitioners. Although Pilates practitioners displayed reduced serum IL-8 levels over the 12-month period, their absolute values were no different to controls at any timepoint. When our data is considered with that of previous studies, the relationship between Pilates exercise and the level of circulatory inflammatory markers (e.g., CRP, TNF-α, IL-8) appears ambiguous and warrants further investigation.

The most explored area of Pilates research to date describes its impact on physical performance. Pilates-based exercise programmes have been shown to increase dynamic balance, (Johnson et al., 2007), static balance (Lim and Yoon, 2017), abdominal endurance,

abdominal and lower back muscular strength, hamstring flexibility, and upper-body muscular endurance (Kloubec, 2010; Sekendiz et al., 2007) in participants with no prior substantive Pilates experience. To our knowledge, we are the first to show that Pilates exercise can continue to stimulate improvements in dynamic balance in experienced long-term practitioners. However, our results suggest improvements in physical performance in experienced Pilates practitioners may be limited to dynamic balance, as we did not observe any change in abdominal endurance (plank) or hamstring flexibility (sit and reach test). Furthermore, our findings suggest that Pilates can be utilised as a workable strategy to enhance body composition, as Pilates practitioners displayed lower body fat percentage (pre and post) and visceral fat rating (post) than controls. This is in agreement with previous data that Pilates participation can reduce relative body fat percentage (Rogers and Gibson, 2009).

Even though this study is the first to investigate the association between Pilates exercise and TL, it is important to state that we were unable to control for several confounding variables, such as nutrition and sleep (Starkweather, 2014). We recommend that further research is required that compares the effects of Pilates and Yoga on telomere dynamics, whilst controlling for as many confounding variables as possible.

To conclude, long-term Pilates practitioners (> 5 years) display comparable TL to sedentary age-matched controls. Nonetheless, Pilates exercise may still be utilised to enhance antioxidant enzyme gene expression, effectively manage body composition, and improve dynamic balance in experienced practitioners.

CHAPTER V - COMPARISON OF TELOMERE LENGTH IN YOUNG AND MASTER ENDURANCE RUNNERS AND SPRINTERS

The study presented in this chapter has been published with the following citation:

Nickels, M., Mastana, S., Codd, V., Denniff, M., & Akam, E. (2021). Comparison of Telomere Length in Young and Master Endurance Runners and Sprinters, *Journal of Aging and Physical Activity* (Epub ahead of print).

5.1 Abstract

It is unclear how running modality influences TL. The aim of this single laboratory visit study was to compare the TL of master endurance runners and master sprinters with their young counterparts. The correlation between LTL and buccal cell TL in athletes was also explored. Participants consisted of 11 young controls, 11 young sprinters, 12 young endurance runners, 12 middle-aged controls, 11 master sprinters and 12 master endurance runners. Blood and buccal samples were collected and randomised for analysis of TL by qPCR. Young endurance athletes displayed longer telomeres than master athletes ($p < 0.05$), however, these differences were not significant when controlled for covariates ($p > 0.05$). A positive correlation existed between leukocyte and buccal cell TL in athletes ($r = 0.567$, $p < 0.001$). In conclusion, young endurance athletes possess longer telomeres than master endurance runners and master sprinters, which appears to be a consequence of lower BMI and visceral fat.

5.2 Introduction

There is an ever-increasing body of evidence that demonstrates various modalities of exercise (e.g., running) can positively influence telomere dynamics (Dimauro et al., 2016; Krishna et al., 2015; Werner et al., 2018). To date, most studies exploring the relationship between TL and exercise are centred on self-reported PA (Dankel et al., 2017; Krauss et al., 2011; Latifovic et al., 2016) and endurance athletes (Hernando et al., 2020; Mathur et al., 2013; Rae et al., 2010). Loprinzi and Sng have however reported 'running-specific' exercise to be the only evaluated mode of exercise positively associated with TL out of 9 distinct physical activities (Loprinzi and Sng, 2016), although the precise type of running (e.g., endurance or sprinting) was not defined. Previous studies on TL influenced by running evidence diverse results. Some studies have shown that both endurance runners (Borghini et al., 2015; Denham et al., 2013; Hernando et al., 2020) and sprinters (Simoes et al., 2017) display longer telomeres than sedentary age-matched controls. In other studies, middle-aged endurance runners have been reported to show comparable TL to young sedentary individuals (Sousa et al., 2018) and even young endurance runners (Werner et al., 2009). However, there is also evidence that both young (Denham, 2016; Werner et al., 2009) and middle-aged (Mathur et al., 2013; Rae et al., 2010) endurance runners have comparable TL to their sedentary age-matched counterparts. The only study to directly compare the influence of differing running types on TL (e.g., endurance runners and sprinters) was performed in a small cohort of master athletes (n = 31) (Rosa et al., 2020) that revealed they possessed comparable TL to both young and middle-aged sedentary controls. Thus, the influence of distinct running modalities on TL cannot be clearly defined by the current literature. No study has directly compared the TL of both young and master endurance runners and sprinters, warranting the current study to address a significant gap in research.

Biochemical testing of athletes in the field can present numerous logistical issues (e.g., invasiveness of test, sample processing, storage conditions). Thus, it may be advantageous to use minimally invasive techniques that require less immediate sample processing in the field when aiming to quantify TL, such as the buccal swab. Previous studies have demonstrated buccal cell TL is positively correlated with LTL in a twin cohort (Finnicum et al., 2017) and patients with dyskeratosis congenita (Gadalla et al., 2010), despite variation in their absolute values. No studies have specifically compared TL across leukocytes and buccal cells in an athlete cohort. Thus, it is important to explore whether leukocyte and buccal cell telomeres respond to exercise in synchrony.

This study aims to compare TL between sprinters and endurance runners in both young and middle-aged individuals. The secondary aim is to examine the correlation between leukocyte and buccal cell TL in an athlete cohort. We hypothesise that middle-aged athletes will demonstrate longer telomeres than sedentary aged-matched controls, comparable to their younger active counterparts.

5.3 Methods

Ethical approval

Ethical approval for this study was provided by the Loughborough University ethical advisory committee (R19-P056). Written informed consent was obtained from all participants and the study conformed to the standards detailed by the latest revision of the Declaration of Helsinki.

Participants

The health status (Appendix E) and mental wellbeing (Warwick-Edinburgh Mental Wellbeing Scale (WEMWBS)) (Appendix I) of each participant was assessed by questionnaire prior to testing. Participants were recruited via internet and word of mouth advertisements. Exclusion criteria included known metabolic disorders, cardiovascular disease, cancer, use of anti-inflammatory medication or steroids, and any chronic age-related condition that could influence outcome measurements. Participants attended a single laboratory visit, at least 24 hours after exercise, which involved anthropometric measurements followed by a blood sample for biomolecular analysis. Athletes were also required to complete an additional buccal swab and questionnaire detailing their training and competition history (Appendix F and G).

Young athletes were required to be 18-34 years old and master athletes were 35-60 years old. The rationale for this categorisation was centered on the fact that athletes are typically considered masters once they are older than 35 years (Tayrose et al., 2015), and seniors once they pass 60 years (Trappe, 2001). Athletes were considered sprinters if they competed in events 60-400 metres and endurance runners if they competed in distances >3000m. All athletes possessed a minimum of 5 years of regular competitive practice.

Young endurance athletes had an average of 12.1 ± 6.8 years of training or competitive practice, covered an average training distance of 47.5 ± 19.0 miles per week, spent an average of 7.8 ± 3.0 hours training per week and had a maximal time of inactivity (e.g., injury) of 6.3 ± 8.6 months. Master endurance athletes had an average of 19.3 ± 13.3 years of training or competitive practice, covered an average training distance of 39.0 ± 16.7 miles per week, spent an average of 6.5 ± 2.3 hours training per week and had a maximal time of inactivity of 9.9 ± 14.3 months.

Young sprint athletes had an average of 11.1 ± 3.8 years of training or competitive practice, spent an average of 10.5 ± 2.4 hours training per week (average three sprint and two weight training sessions) and had a maximal time of inactivity of 4.6 ± 3.6 months. Master sprinters had an average of 30.9 ± 16.8 years of training or competitive practice, spent an average of 6.9 ± 2.1 hours training per week (average two sprint and two weight training sessions) and had a maximal time of inactivity of 11.7 ± 12.2 months.

All young (18-34 years) and middle-aged controls (35-60 years) were required to be untrained and have no engagement in any routine exercise. The final sample size (n = 69) consisted of 12 young endurance runners (YE), 11 young sprinters (YS), 11 young controls (YC), 12 master endurance runners (ME), 11 master sprinters (MS) and 12 middle-aged controls (MC) (Table 5.1.).

Table 5.1. Body composition of athletes and age-matched controls

	YE	YS	YC	ME	MS	MC
n	12 (5 f, 7 m)	11 (5 f, 6 m)	11 (5 f, 6 m)	12 (5 f, 7 m)	11 (6 f, 5 m)	12 (7 f, 5 m)
Age (yr)	25.6 ± 3.7	24.4 ± 4.4	24.1 ± 1.6	**45.5 ± 4.9*¥#**	**50.0 ± 9.1*¥#**	**49.2 ± 7.0*¥#**
Height (cm)	175.9 ± 8.4	175.4 ± 8.2	171.5 ± 9.7	171.1 ± 10.0	174.2 ± 8.1	167.4 ± 11.6
Body mass (kg)	62.4 ± 9.0	69.2 ± 12.2	66.2 ± 11.0	64.5 ± 8.9	73.8 ± 12.8	**82.2 ± 15.4*#†**
BMI (kg/m^2)	20.0 ± 1.5	22.3 ± 2.1	22.5 ± 3.4	21.9 ± 1.8	**24.4 ± 2.7***	**29.3 ± 2.8*¥#†‡**
Body fat (%)	13.0 ± 4.6	13.8 ± 2.6	19.4 ± 8.0	15.4 ± 5.0	**20.3 ± 4.7***	**34.6 ± 7.2*¥#†‡**
FFM (kg)	54.5 ± 9.2	59.8 ± 11.5	53.5 ± 11.2	54.8 ± 9.6	59.0 ± 11.9	54.0 ± 13.3
Visceral fat	1.3 ± 0.6	1.4 ± 0.7	1.9 ± 1.3	**4.2 ± 1.5*¥#**	**5.7 ± 3.2*¥#**	**10.6 ± 3.2*¥#†‡**

Data are means ± SD; f, female. m, male. BMI, body mass index. FFM, fat free mass. *: different from YE; ¥: different from YS; #: different from YC; †: different from ME; ‡: different from MS. Statistical significance defined as p-values <0.003.

Anthropometric measurements

Height, body mass, BF%, FFM, BMI, and visceral fat rating were quantified as outlined in section '2.1.1 Height, body mass, BMI, and body composition measurements'.

Blood and buccal sample collection and processing

Whole blood and buccal swabs were collected and processed as detailed in sections '2.3.1 Blood sample collection and processing' and '2.3.2 Buccal swab collection and processing', respectively.

DNA extraction and telomere length analysis

DNA was extracted and assessed following the methodology in section '2.3.3 DNA extraction and analysis'. DNA purity reported acceptable A260/280 values (1.98 ± 0.10). TL was quantified using the methodology described in section '2.3.6 Telomere length analysis'. Intra-assay CV for calculated T/S ratio was 2.5%. Inter-assay CV for calculated T/S ratio was 4.0%.

RNA extraction and gene expression analysis

RNA was extracted from whole blood, analysed, reverse transcribed to cDNA, and diluted as detailed in section '2.3.4 RNA isolation, analysis of content and cDNA synthesis'. RNA purity reported acceptable A260/280 values (2.10 ± 0.02). Relative gene expression of target genes TERT, SOD2, GPX1 and TRF1 were normalised to the expression of the housekeeper GAPDH using the protocol ($2^{-(\Delta\Delta Ct)}$) outlined in section '2.3.5 Gene expression analysis'. All data is expressed relative to the pooled mean Ct from the middle-aged control group for each target gene.

Measurement of inflammatory markers

Inflammatory markers IL-8, TNF-α and CRP were all measured in serum as described in section '2.3.8 Inflammatory marker quantification'. Average intra- and inter-assay CV was 8.1 ± 0.9% and 6.0 ± 0.5% for the different ELISA assays, respectively. All CRP samples were analysed on a single run and the average intra-assay CV was 1.7 ± 0.1%.

Statistical analysis

The Shapiro-Wilk and Levene tests were used to test the normality and homogeneity of data, respectively. Non-normally distributed data were log transformed preceding analysis. ANOVA with Tukey's post hoc and ANCOVA were applied to evaluate all continuous variables. The paired samples *t*-test test was employed to analyse TL between leukocytes and buccal cells. Pearson's correlation was utilised to assess relationships with TL. Total sample size recruited in this study (n = 69) bestowed a statistical power of 89 % ($\beta = 0.89$) with a significance level of 5% ($\alpha = 0.05$) and moderate effect size (d = 0.5) for the primary outcome. Physical performance level for athletes was calculated as their career personal best time (PB) or season's best time (SB) (in the last year) for their primary event distance as a percentage of the current world record. SPSS 23.0 (IBM Corporation, USA) was used for the analysis of the data with significance accepted at $p < 0.05$, unless stated otherwise.

5.4 Results

Telomere Length

LTL was inversely correlated with chronological age across all participants ($r = -.511$, p = <0.001). YE (0.944 ± 0.159) displayed significantly longer LTL than ME (0.783 ± 0.135, p = 0.023), MS (0.765 ± 0.111, p = 0.010) and MC (0.790 ± 0.110, p = 0.045) (Figure 5.1.). LTL was comparable between all other groups (YS, 0.881 ± 0.109; YC, 0.899 ± 0.098) ($p > 0.05$). After adjusting for BMI and visceral fat rating as covariates, all differences in LTL were no longer significant ($p > 0.05$).

Buccal cell TL was measured in athletes only (n = 39) (7 buccal samples not analysed further owing to poor DNA quality post-extraction) and showed a significant positive correlation ($r = 0.567$, $p < 0.001$) with LTL (Figure 5.2.). However, absolute values of LTL (0.832 ± 0.149) and buccal cell TL (0.951 ± 0.185) were significantly different (p = <0.001). Again, chronological age was negatively correlated with buccal cell TL in athletes ($r = -.359$, p = 0.025).

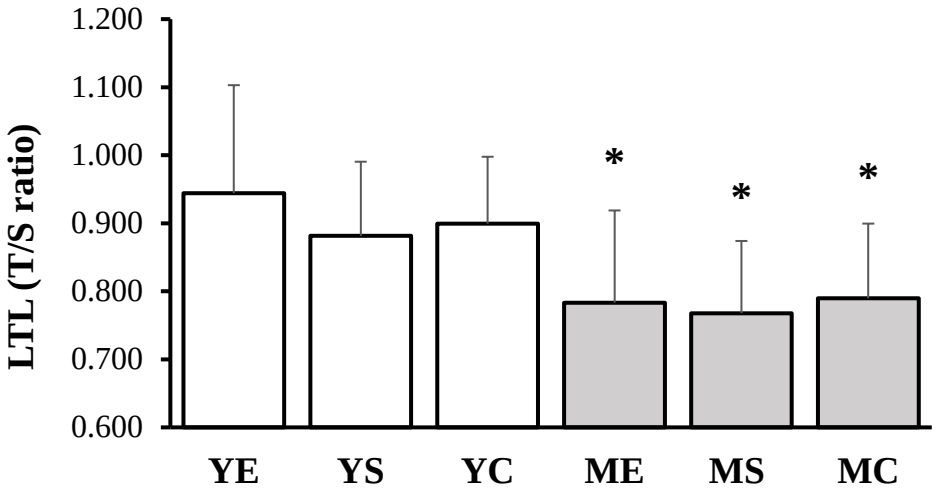

Figure 5.1. Leukocyte telomere length (LTL) of young endurance runners (YE), young sprinters (YS), young controls (YC), master endurance runners (ME), master sprinters (MS) and middle-aged controls (MC). *: different from YE. Statistical significance defined as $p < 0.05$. Data expressed as mean + standard deviation.

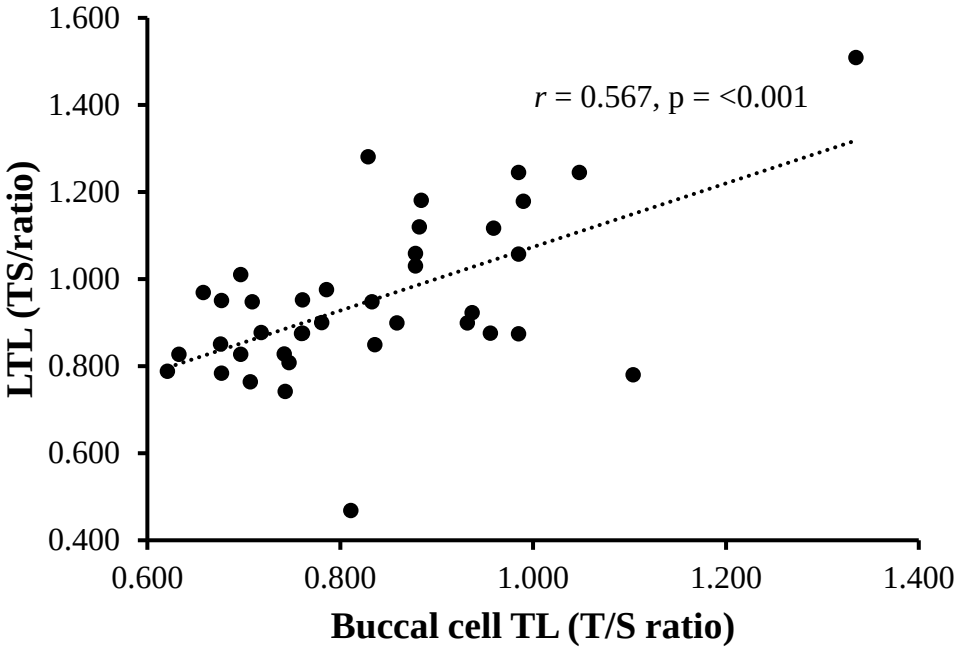

Figure 5.2. Pearson's correlation analysis between LTL and buccal cell TL for athletes (n = 39).

Inflammatory markers

MC had higher CRP than YE ($p = 0.010$), ME ($p = 0.007$) and MS ($p = 0.002$). There was no difference between groups for any other inflammatory markers ($p > 0.05$) (Table 5.2.). Additionally, no inflammatory markers were associated with LTL ($p > 0.05$).

Telomere-regulating gene expression

There was no difference in TERT gene expression between groups ($p > 0.05$) (Figure 5.3.), although it was positively associated with LTL across all participants ($r = 0.297$, $p = 0.014$). TRF1 gene expression was upregulated in YE compared with YC ($p = 0.016$), ME ($p = 0.001$) and MS ($p = 0.014$) (Figure 5.3.). The YS group exhibited similar results and possessed higher TRF1 gene expression levels than YC ($p = 0.015$), ME ($p = 0.001$) and MS ($p = 0.014$). TRF1 expression was greater in MC compared with ME ($p = 0.029$). TRF1 mRNA expression was not associated with LTL across all participants ($p > 0.05$).

Antioxidant enzyme gene expression

MC had elevated SOD2 mRNA expression compared with YE ($p = <0.001$), YS ($p = <0.001$), YC ($p = 0.019$) and ME ($p = 0.001$) (Figure 5.3). GPX1 gene expression was markedly reduced in the MS group compared with YE ($p = 0.018$), YC ($p = <0.001$) and ME ($p = <0.001$) (Figure 5.3.). SOD2 and GPX1 mRNA expression were not correlated with LTL across the whole cohort ($p > 0.05$).

Body composition

The MC group weighed significantly more than the YE ($p = 0.002$), YC ($p = 0.040$), and ME ($p = 0.012$) groups (see Table 5.1.). Furthermore, the MC group had a greater BMI than all other groups ($p = <0.001$). MS also displayed greater BMI than YE ($p = <0.001$). Similarly, body fat percentage in the MC group was substantially greater than all other groups ($p = \leq 0.002$) and MS had a higher body fat percentage than YE ($p = 0.007$). MC also had a higher visceral fat rating all other groups than ($p = \leq 0.003$). Body mass ($r = -.270$, $p = 0.025$), BMI ($r = -.298$, $p = 0.013$) and visceral fat ($r = -.473$, $p = <0.001$) rating were also significantly inversely related to LTL. Body fat and FFM were not correlated with LTL ($p > 0.05$).

Physical performance measures

Seasons best time (r = -.618, p = 0.014) and total years training (r = -.341, p = 0.020) were inversely associated with LTL in MS athletes and all athletes, respectively. Seasons best time, all-time PB, miles run per week, total hours training per week, and total years training were not correlated with LTL in any other individual group, or the whole athlete cohort ($p > 0.05$).

Mental wellbeing

WEMWBS scores were not different between groups (YC, 51 ± 5; YS, 52 ± 3; YE, 52 ± 5; MC, 49 ± 8; MS, 54 ± 11; ME, 53 ± 9) and did not correlate with LTL ($p > 0.05$).

Table 5.2. Inflammatory markers of athletes and controls

	YE	YS	YC	ME	MS	MC
TNF-α (pg/ml)	0.73 ± 0.28	0.92 ± 0.47	0.74 ± 0.30	0.65 ± 0.23	0.88 ± 0.48	0.93 ± 0.49
IL-8 (ng/l)	10.63 ± 3.83	10.06 ± 3.63	7.30 ± 2.30	11.42 ± 3.68	10.66 ± 2.86	9.87 ± 4.77
CRP (mg/l)	0.46 ± 0.42	0.95 ± 1.16	0.71 ± 0.72	0.46 ± 0.50	0.31 ± 0.17	**4.69 ± 7.30*†‡**

Data are means ± SD. *: different from YE; †: different from ME; ‡: different from MS. Statistical significance defined as p-values <0.05.

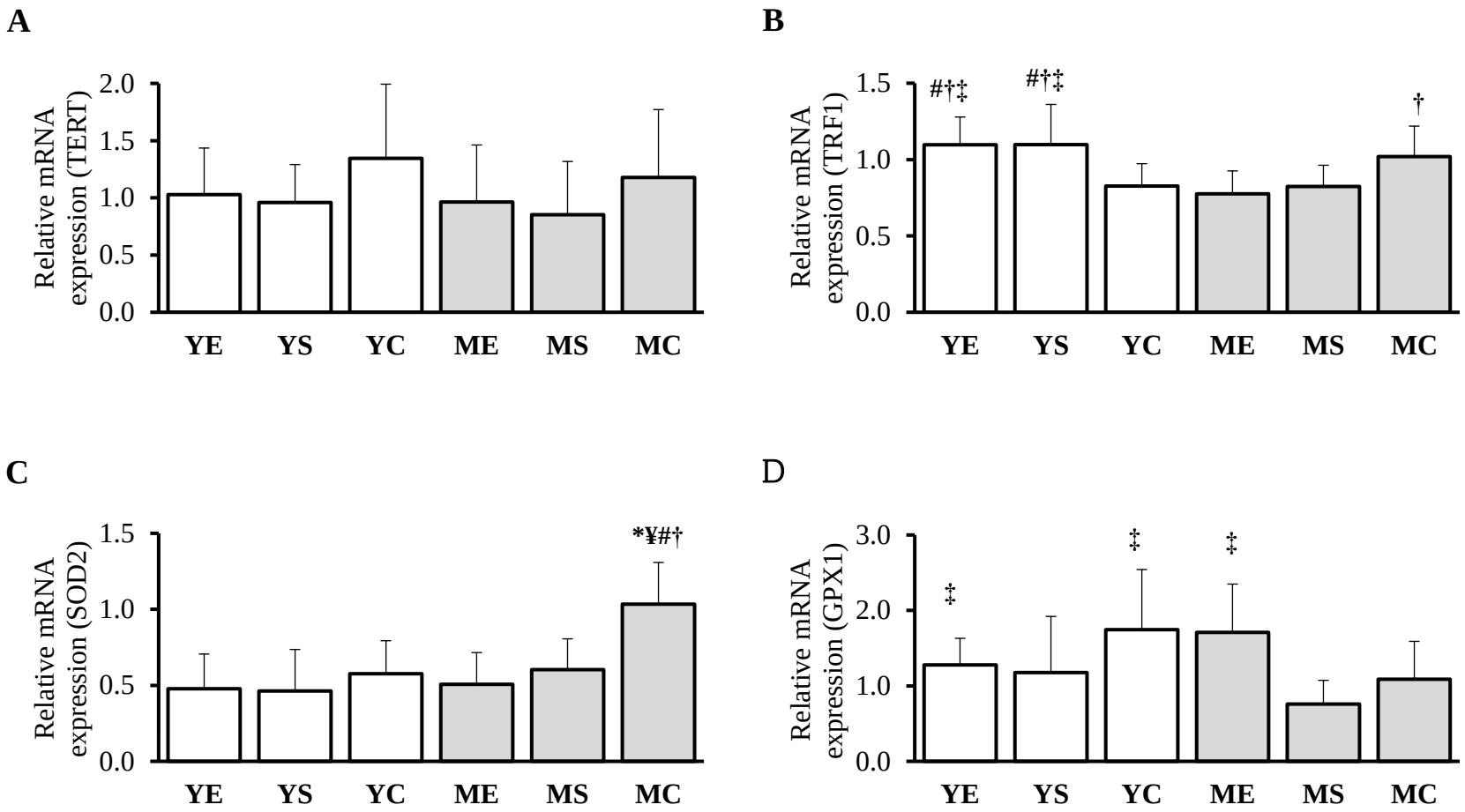

Figure 5.3. Relative fold expression of TERT (A), TRF1 (B), SOD2 (C) and GPX1 (D) for all groups. Data are means + SD. *: different from YE; ¥: different from YS; #: different from YC; †: different from ME; ‡: different from MS. Statistical significance defined as $p < 0.0125$.

5.5 Discussion

This study offers the first direct comparison of TL between both young and master endurance runners and sprinters. The key finding of this chapter is that YE possess longer telomeres than ME, MS, and MC groups. Our data is congruent with that of previous studies reporting middle-aged endurance runners display similar LTL to both age-matched (Mathur et al., 2013; Rae et al., 2010) and young sedentary controls (Rosa et al., 2020; Sousa et al., 2018; Werner et al., 2009). However, our results are discordant with that of Werner *et al*., who revealed middle-aged endurance runners display comparable TL to young endurance runners (Werner et al., 2009). Additionally, prior studies have described middle-aged endurance runners (Borghini et al., 2015; Denham et al., 2013; Sousa et al., 2018; Werner et al., 2009) and master sprinters (Simoes et al., 2017) display longer telomeres than sedentary age-matched controls, which is not supported by our results. To date, only one previous study had directly compared TL in sprinters and endurance runners, revealing that master sprinters and endurance runners display comparable TL to both young and age-matched sedentary controls (Rosa et al., 2020), a finding that our study supports. However, the study by Rosa *et al*. also demonstrated that middle-aged controls had shorter telomeres than young controls. The discrepancy between our findings and those of previous studies may be the result of differences in study design, tissue type measured (e.g., skeletal muscle, lymphocytes, granulocytes, saliva), telomere assay methodology (e.g., qPCR, Southern Blot) and/or athlete population utilised. For example, Rae *et al.* measured TL in skeletal muscle (Rae et al., 2010), a tissue type that may uniquely respond to exercise due to its satellite cell content (Kadi and Ponsot, 2010).

When BMI and visceral fat were controlled for as covariates in the present study, the greater LTL displayed by YE, compared with ME, MS, and MC, was no longer significant. Visceral fat content and BMI are positively associated with oxidative stress (Fujita et al., 2006; Furukawa et al., 2004) and systemic inflammation (Coelho et al., 2013; Ellulu et al., 2016); two processes which are proposed to initiate telomere attrition (Correia-Melo et al., 2014). We speculate that the longer LTL exhibited by YE may therefore be explained by reduced oxidative stress and systemic inflammation as a product of their lower visceral fat rating and BMI, although our results only partially support this hypothesis at the molecular level. Our divergent findings may however be a consequence of the limited profile of cytokine and oxidative markers undertaken in this study. Nonetheless, a lower BMI and body fat

percentage have been negatively associated with TL in master sprinters, highlighting the potential influence of body adiposity on TL regulation in athletes (Simoes et al., 2017).

Endurance and sprint training programs are vastly divergent. The former is characterised by low-intensity, high-volume sessions that rely on aerobic metabolism, whilst the latter incorporates high-intensity, low-volume sessions that depend on anaerobic pathways (Patel et al., 2017). Although we did not observe significant differences in antioxidant gene expression and inflammatory markers in the present study, master endurance runners have been shown to have higher SOD activity and IL-15 concentration than master sprinters, but lower IL-10 concentrations and F_2-Isoprostane levels (Rosa et al., 2020). Thus, distinct running modalities would appear to provide different adaptations in redox balance and cytokine expression, highlighting their potential to induce divergent effects on telomere dynamics.

It has been suggested that the shortening of TL with advanced age may be the result of low levels of telomerase activity (e.g. the telomere-telomerase hypothesis) (Holt et al., 1996). Our findings show that TERT gene expression, which is closely correlated with telomerase enzymatic activity (Kirkpatrick et al., 2003), was positively associated with TL across the whole cohort. Endurance athletes have previously displayed longer telomeres and upregulated TERT gene expression compared with controls (Denham et al., 2015). Our results are in opposition to this finding, and we speculate that the absence of any significant difference in TERT expression between groups in the present study may partly explain their comparable TL, once controlled for covariates.

The second notable finding of this study is that buccal cell TL is strongly correlated with LTL in athletes. Buccal cell TL was only quantified in athletes since it is already reported to be positively associated with LTL in non-athlete cohorts (Finnicum et al., 2017; Gadalla et al., 2010). We also observed that absolute values of TL were considerably greater in buccal cells compared with leukocytes, once again reaffirming preceding data (Gadalla et al., 2010). Our findings reinforce the idea that buccal cell samples can be used as an alternative non-invasive measure of TL and provide the first evidence that this tissue type can be used to reliably investigate the influence of exercise in athlete cohorts. Our findings also indicate that leukocyte and buccal cell telomere dynamics may be impacted via shared exercise-induced mechanisms. However, additional research is necessary to fully elucidate and compare the exercise-induced mechanisms that regulate telomere dynamics in buccal cells and leukocytes.

The present study provides two key novel aspects; 1) it is the first to compare TL between endurance runners and sprinters in both young and master athletes; 2) it is also the first to compare TL across leukocytes and buccal cell tissue types in an athlete cohort. Nonetheless, it is essential to note that the present study has limitations. For example, confounding variables of TL, such as sleep and diet (Starkweather, 2014), were not controlled for. The limited sample size of the present study also dictates that our results ought to be viewed with vigilance. Moreover, the study design does not permit us to infer biological causality for our findings and rules them observational. As such, further research is required investigating the influence of endurance running and sprinting on TL in young and master athletes over longitudinal periods, incorporating a broader range of inflammatory and oxidative stress markers.

To conclude, young endurance runners possess longer telomeres than master endurance runners, master sprinters, and middle-aged controls, which appears to be the consequence of their reduced BMI and visceral fat rating. Buccal cell TL is also positively correlated with LTL in athletes, suggesting this non-invasive measure of TL can be reliably utilised to investigate exercise-induced changes in telomere dynamics.

CHAPTER VI - ELITE SWIMMERS POSSESS SHORTER TELOMERES THAN RECREATIONALLY ACTIVE CONTROLS

The study presented in this chapter has been published with the following citation:

Nickels, M, Mastana, S, Denniff, M, Codd, V, & Akam, E (2021). Elite swimmers possess shorter telomeres than recreationally active controls. *Gene*, 769:145242.

6.1 Abstract

Elite athletes are reported to possess longer telomeres than their less active counterparts. Angiotensin converting enzyme (ACE) gene (Insertion/Deletion) polymorphism has been previously associated with elite athletic performance, with the deletion (D) variant appearing more frequently in short distance swimmers. Additionally, the D allele has been reported to have a negative effect on TL. The aim of this study was to investigate the TL of elite swimmers compared with recreationally active controls. Additionally, the potential association between TL and ACE genotype was explored in elite swimmers. TL was measured by qPCR and ACE I/D genotypes analysed by standard PCR and electrophoresis in 51 young elite swimmers and 56 recreationally active controls. Elite swimmers displayed shorter telomeres than control participants (1.043 ± 0.127 vs 1.128 ± 0.177, $p = 0.006$). When split by sex, only elite female swimmers showed significantly shorter telomeres than their recreationally active counterparts ($p = 0.019$). ACE genotype distribution and allelic frequency did not differ between elite swimmers and controls, or by event distance among elite swimmers only. No association was observed between TL and ACE genotype in the whole cohort. Our findings suggest a negative effect of high-level swimming competition and/or training on TL and subsequent biological ageing, particularly in females. However, this significant difference in TL does not appear to be attributed to the D allele as we report a lack of association between TL and ACE genotype frequency in elite swimmers and controls.

6.2 Introduction

Lifestyle factors including nutrition, sleep, stress management, and exercise have all been proposed to influence TL (Starkweather, 2014). For example, middle-aged and older individuals with higher levels of PA typically display longer telomeres than their sedentary counterparts (Cherkas et al., 2008; Saßenroth et al., 2015). Still, how exercise influences TL in young individuals is less clear. The majority of literature reports no association between PA and TL in young individuals (LaRocca et al., 2010; Østhus et al., 2012), which may partly be explained by an absence of significant age-related telomere attrition and/or insufficient exposure to PA levels (e.g., activity years). Nevertheless, recent studies provide evidence that potential exercise-mediated effects on telomere maintenance may commence in adolescence/young adults (Mofrad and Ebrahim, 2018; Zhu et al., 2011). A cohort of young elite athletes (27.2 ± 4.9 y) have also been reported to possess longer telomeres than sedentary age-matched controls (Muniesa et al., 2017), suggesting that extremely high levels of exercise training, such as those performed by elite athletes, may be required to influence TL in young individuals.

ACE converts the hormone angiotensin I to the active vasoconstrictor angiotensin II. Angiotensin II has numerous actions, including promotion of a pro-inflammatory environment and increased oxidative stress (Fyhrquist and Saijonmaa, 2008), both of which are proposed to shorten TL (Correia-Melo et al., 2014). A 287-base *AluI* polymorphism in intron 16 has been described (Insertion (I) / Deletion (D)) within the ACE gene, resulting in three possible genotypes (II, ID and DD) (Rigat et al., 1990). The presence of the D allele has been linked with increased circulating ACE activity levels compared with II homozygotes (Rigat et al., 1990), which may lead to enhanced inflammation and oxidative stress. Interestingly, hypertensive patients with left ventricular hypertrophy with ID or DD genotypes display shortened leukocyte telomeres, suggesting a potential negative effect of the D allele on TL (Fyhrquist et al., 2013). It is well documented that the ACE D allele is associated with elite short distance swimming, and the ACE I allele with elite middle- and long-distance events (Costa et al., 2009; Nazarov et al., 2001; Tsianos et al., 2004; Woods et al., 2001). However, no study has investigated the notion that elite short distance swimmers may possess shortened telomeres compared with their middle- and long-distance counterparts due to the potential negative effect of the ACE gene D allele.

The primary aim of the present study is to investigate TL and ACE genotype in elite swimmers and recreationally active controls.

6.3 Methods

Ethical approval

Ethical approval for this study was provided by the Loughborough University ethical advisory committee (R18-P175). Written informed consent was obtained from all participants and the study conformed to the standards detailed by the latest revision of the Declaration of Helsinki.

Participants

A complete health assessment was completed for each participant using a structured questionnaire (Appendix E). Exclusion criteria for the study were known metabolic disorders, smoking, CVD, use of anti-inflammatory medication or steroids, any condition or disease that may interfere with the primary outcome measurement.

The study population comprised 51 elite young swimmers (characteristics in Table 6.1.), reporting an average training distance of 43,196 ± 9,408 m/week, reaction time of 0.69 ± 0.07s and FINA point score of 805 ± 82. All swimmers were currently competing at a minimum of national level, ranging up to Olympic standard (66% National, 2% European Championships, 8% Commonwealth Games, 14% World Championships, 10% Olympics). Swimmers were further categorised into three distinct categories based on their main event distance (41% sprint (≤ 100m), 39% middle (≤ 200-800m), and 20% long (≥ 800m)).

A cohort of 56 healthy, age-matched control participants was recruited. Control participants were recreationally active and participated in a minimum 150 minutes of moderate PA, or 75 minutes of vigorous PA, per week. However, they were not involved in any structured training programme or competitive sports.

Table 6.1. Characteristics of elite swimmers and controls

	Female controls *n* = 29	Female swimmers *n* = 22	Male controls *n* = 27	Male swimmers *n* = 29
Age (years)	**19.6 ± 2.2***	20.5 ± 1.4	20.3 ± 4.7	20.6 ± 1.7
Height (cm)	**165.1 ± 6.3***	171.4 ± 5.9	**178.4 ± 6.0#**	183.5 ± 5.3
Body mass (kg)	61.7 ± 10.8	65.5 ± 6.3	**72.4 ± 12.2#**	77.7 ± 6.8
BMI (kg/m^2)	22.7 ± 3.4	22.3 ± 1.8	22.7 ± 3.5	23.0 ± 1.4
Body fat (%)	**27.5 ± 6.0***	20.4 ± 3.9	**14.0 ± 4.1#**	11.1 ± 2.8
FFM (kg)	**44.4 ± 4.5***	52.1 ± 5.0	**62.0 ± 8.8#**	69.0 ± 5.6

Data are means ± SD; *n*, no. of participants. BMI, body mass index. FFM, fat free mass. *: different from female swimmers; #: different from male swimmers.

Anthropometric measurements

Height, body mass, BF%, FFM and BMI were quantified as outlined in section '2.1.1 Height, body mass, BMI, and body composition measurements'.

Buccal swab collection and processing

Buccal swabs were collected and processed for DNA extraction as detailed in section '2.3.2 Buccal swab collection and processing'.

DNA extraction and telomere length analysis

DNA was extracted and assessed following the methodology in section '2.3.3 DNA extraction and analysis'. DNA purity reported acceptable A260/280 values (1.88 ± 0.162). TL was quantified using the methodology described in section '2.3.6 Telomere length analysis'. Intra- assay CV for calculated T/S ratio was 3.3%. Inter-assay CV for calculated T/S ratio was 4.1%.

ACE genotyping

ACE genotype was established using the methodology in section '2.3.8 ACE genotype determination'.

Statistical analysis

The normality and homogeneity of data were verified by the Shapiro-Wilk and Levene tests, respectively. The independent *t*-test or ANOVA was used to examine differences in mean TL, anthropometrics, genotype, and performance data between groups. An ANCOVA was also used to compare differences between groups when necessary. All associations were tested by two-tailed Pearson correlation. Allele frequencies were estimated by allele-counting method. Genotype distribution and allele frequencies between groups were compared by the Chi-Square test, as were estimates of Hardy–Weinberg equilibrium. Total sample size enlisted in this study ($n = 22$) conferred a statistical power of 99% ($\beta = 0.99$) with a significance level of 5% ($\alpha = .05$) and moderate effect size ($d = 0.5$) for the primary outcome. All analyses were performed using SPSS 23.0 (IBM Corporation, USA) with p-values < 0.05 considered significant, unless stated otherwise.

6.4 Results

Telomere length

Data analysis revealed control participants possessed longer telomeres (1.128 ± 0.177) than elite swimmers (1.043 ± 0.127) ($p = 0.006$, calculated effect size = 0.55) (Figure 6.1.). This group effect remained significant after adjusting for all covariates ($p = 0.031$). When analysed by sex, female swimmers displayed significantly shorter telomeres (1.049 ± 0.115) than female controls (1.154 ± 0.176) ($p = 0.019$, calculated effect size = 0.70), which remained significant after adjusting for all covariates ($p = 0.012$). There was no difference in TL between male swimmers (1.039 ± 0.136) and male controls (1.099 ± 0.176) ($p > 0.05$). There was no significant sex difference in TL for all participants after adjusting for covariates (males: 1.068 ± 0.158, females: 1.109 ± 0.160, $p = 0.114$).

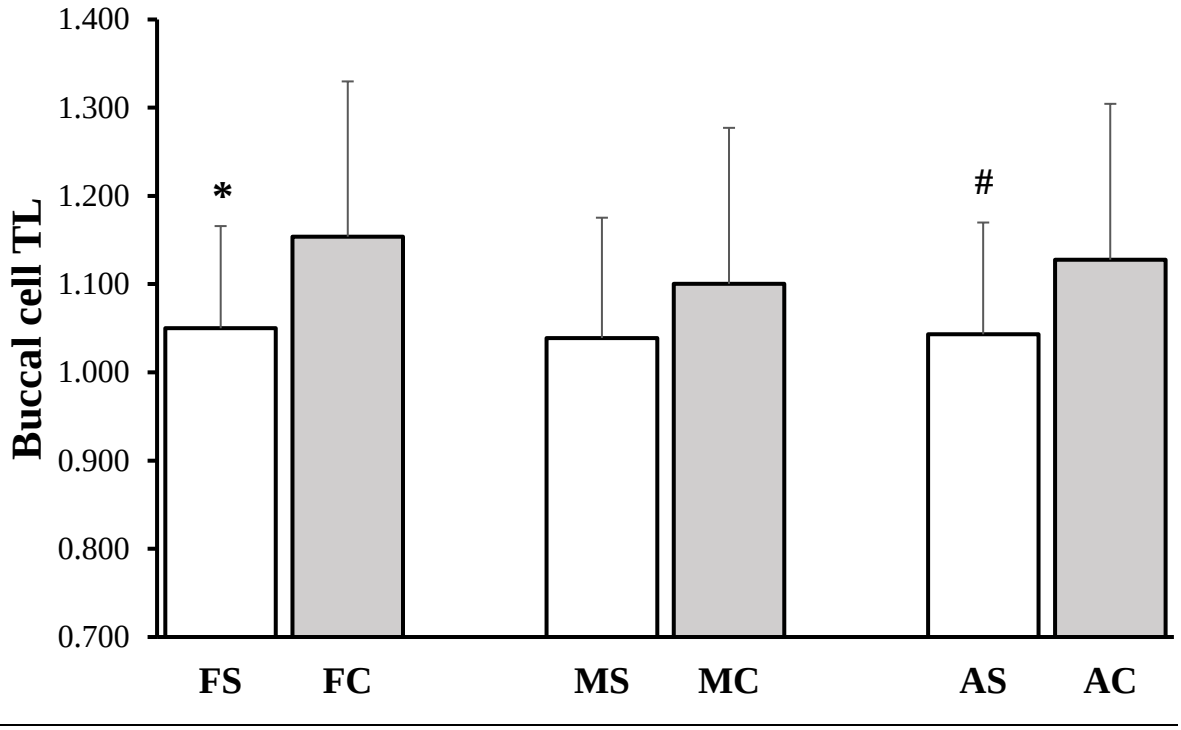

Figure 6.1. Buccal cell TL for female swimmers (FS), female controls (FC), male swimmers (MS), male controls (MC), all swimmers (AS) and all controls (AC) (mean ± SD). *: different from FC; #: different from AC. Statistical significance defined as p-values <0.05.

Lack of association between telomere length and swimming performance

TL was not associated with swimming performance (FINA points), reaction time, average weekly training distance, main event distance, or highest level of competition in swimmers overall ($p > 0.05$). The lack of associations remained when swimmers were analysed by separate groups based on their sex ($p > 0.05$).

ACE I/D genotype and allele frequency

Genotypic frequencies in both swimmers and controls were consistent with Hardy–Weinberg equilibrium. There were no statistical differences for genotype and allele distributions between swimmers and controls overall (Table 6.2.) or when analysed by distinct groups based on sex ($p > 0.05$). Furthermore, no statistical differences were observed for genotype distributions and allelic frequencies when the swimmers were stratified into three groups based on main event distance, or when the control group was compared with each individual swimming distance ($p > 0.05$).

Table 6.2. ACE genotype distribution and allele frequencies of swimmers and controls

Participants	*n*	ACE genotype			Allele frequency	
		DD	ID	II	D	I
Swimmers overall	50	13 (0.26)	25 (0.50)	12 (0.24)	0.51	0.49
Sprint	21	6 (0.29)	11 (0.52)	4 (0.19)	0.55	0.45
Middle	19	7 (0.37)	7 (0.37)	5 (0.26)	0.55	0.45
Long	10	3 (0.3)	7 (0.7)	0 (0.0)	0.65	0.35
Controls	54	12 (0.22)	29 (0.54)	13 (0.24)	0.49	0.51

Telomere length and ACE I/D genotype

In the whole cohort, TL was not associated with ACE I/D genotype ($p > 0.05$). Furthermore, TL was not associated with ACE I/D genotype when the cohort was stratified into distinct groups of swimmers, controls or split by sex ($p > 0.05$). There was also no association between TL and ACE I/D genotype when swimming groups were individually analysed by main event distance ($p > 0.05$).

6.5 Discussion

The main findings of this chapter are that: 1) elite swimmers display shorter TL compared with their recreationally active peers, which appears specific to females; 2) buccal cell TL is not associated with ACE I/D genotype.

There is a progressive loss of telomeres with each round of cell division and they eventually reach a critically short length, culminating in replicative senescence (Olovnikov, 1973). However, engagement in moderate levels of PA is associated with longer telomeres and may preserve their length (Cherkas et al., 2008; Saßenroth et al., 2015). Conversely, middle-aged and older individuals with extremely low or high PA levels appear to exhibit shorter telomeres than their moderately active counterparts (Cherkas et al., 2006; Savela et al., 2012). To highlight, individuals with extremely high levels of PA have been reported to display telomeres

comparable in length to their low PA level counterparts (Ludlow et al., 2008). As such, an 'inverted U' relationship between PA level and TL has been proposed, in which low and extremely high levels of PA may be detrimental to TL and lead to accelerated shortening (Ludlow et al., 2008).

In the present study, the relative TL of young recreationally active control participants was 8.1% greater than the elite swimmers. When that data was analysed split by sex, female controls displayed telomeres 9.9% longer than elite female swimmers. The lack of difference in TL observed between male controls and elite swimmers indicates that participation in elite-level swimming training/competition may only be detrimental to telomere maintenance and subsequent biological ageing in females. We cannot be certain that these findings are biologically relevant at such as young age, but short buccal cell telomeres have been associated with age-related disease (Broberg et al., 2005; Thomas et al., 2008). These major findings are in contrast with previous data that reported young high-level international athletes possess longer LTL than healthy controls (Muniesa et al., 2017). The discrepancy in these findings may be explained by the fact that the elite athletes used in the study by Muniesa *et al*. were recruited from a wide variety of sport specialities (running events >1500 m, triathlons, weightlifting, judo, gymnastics, canoeing, basketball, soccer, taekwondo), each of which generates different metabolic demands (Muniesa et al., 2017). Several other studies investigating TL and PA in young individuals have involved non-elite athletes and have reported no difference in TL between active and sedentary individuals (LaRocca et al., 2010; Østhus et al., 2012; Werner et al., 2009). This may be because young individuals have not accumulated enough training years/volume, or they are yet to experience any significant age-related telomere attrition. Nonetheless, we are the first to demonstrate that extremely high training loads can be detrimental to TL in elite young athletes, specifically females, a finding which may offer support to the hypothesis of an 'inverted U' relationship between PA and TL. In combination with the results reported by Muniesa *et al*., it appears that the physical demands of elite level sports competition/training may provide a novel stimulus that is able to both positively, and negatively, influence telomere dynamics in young individuals.

Rae *et al*. have previously reported that skeletal muscle TL was inversely correlated with the number of hours and years spent training in older endurance runners (Rae et al., 2010). Additionally, it has previously been reported that individuals with high PA (exercising 10–14 hours/week) possess shortened TL compared with moderately active individuals (Ludlow et al., 2008). At the time of data collection, swimmers were in competition season and training

~14 hours/week in the pool alone (excluding any additional training). Thus, despite a lack of association between the average weekly training distance and buccal cell TL amongst swimmers in the present study, the high number of training hours they performed may potentially explain their shortened telomeres compared with the recreationally active controls. Furthermore, highly trained middle-aged endurance athletes experiencing 'exercise-associated chronic fatigue' or 'overtraining' report significantly shorter skeletal muscle TL compared with age- and training-matched athletes free from these symptoms (Collins et al., 2003). Although we did not specifically diagnose whether the swimmers were 'overtrained' in the present study, we can only speculate that some of them may have been in such a physical state based on their reportedly high weekly training hours and average swimming distance. If this was the case, then the elevated resting levels of ROS and inflammation associated with overtraining (Margonis et al., 2007) and shortened TL (Correia-Melo et al., 2014) may explain the results of the present study. It is important to reiterate that whilst this is biologically plausible, we cannot deduce such a conclusion from our findings and that further research is required.

Due to time constraints and the nature of the study cohort, we were only permitted to collect DNA from the swimmers using non-invasive procedures. TL is typically measured in leukocytes extracted from venous blood, which would have permitted us to measure inflammatory and oxidative stress markers and potentially enable us to deduce biological causality for our findings. The rationale for using buccal cell-derived DNA in the present study was based on evidence that although absolute leukocyte and buccal cell TL values vary, there is a significant intra-individual correlation across the two tissue types, and they appear to respond to exercise in synchrony (as detailed in Chapter V). Buccal cell TL measurements also show a significant association with both sex and age (Finnicum et al., 2017), as seen with LTL, providing further evidence of similarities in telomere dynamics between the tissues.

TL and shortening rate is tissue-specific (Aubert and Lansdorp, 2008), with the variability in attrition across tissue types likely the result of divergent proliferative capacities; highly proliferative tissues (e.g. leukocytes and epithelial cells) typically display greater age-related telomere shortening than minimally proliferative tissues (e.g. skeletal muscle) (Kadi and Ponsot, 2010). Many of the studies referenced in the present chapter have measured TL across a variety of tissues, which may lead to different associations with TL and exercise performance. However, Daniali *et al*. revealed that intra-individual age-related telomere attrition is similar across somatic tissues with divergent replicative status (e.g., leukocytes,

muscle, skin and fat), despite variations in their absolute length (Daniali et al., 2013). This finding suggests that there may be a shared molecular mechanism regulating TL across somatic tissue types. Several other studies have also reported significant correlations in TL between tissue types with different proliferative demands (Friedrich et al., 2000; Hiam et al., 2020). As we previously revealed that TL is strongly correlated across leukocytes and buccal cells in a group of young and master runners (Chapter V), we are inclined to believe that telomeres respond to exercise in synchrony in these two tissue types and are impacted via the same exercise-induced mechanisms (e.g., inflammation and oxidative stress). More recently, genomic DNA isolated from buccal swabs revealed elderly ultra-trail runners display longer telomeres compared to age-matched sedentary controls (Hernando et al., 2020). Similar studies have also previously shown that ultra-endurance athletes have better preserved telomeres in both skeletal muscle (Østhus et al., 2012) and leukocytes (Denham et al., 2013), suggesting a synchrony in TL across tissue types in response to exercise. Thus, we speculate that the present results would have been replicated in our cohort if TL had been measured in leukocytes (although absolute values would be different). However, it must be stated that there is evidence to suggest that the response of TL to exercise may be unique in skeletal muscle, which is likely caused by their satellite cell content. As exercise promotes muscle damage and stimulates the proliferation of satellite cells (Yin et al., 2013), and telomere attrition occurs with cell division, exercise may cause telomere loss in skeletal muscle tissue of highly active individuals. Chronic endurance running has therefore been reported to shorten skeletal muscle TL due to the increased demand on satellite cells to proliferate and repair damaged tissue (Rae et al., 2010). Long-term exercise also accelerates TL attrition in skeletal muscle in mice, but attenuates age-related decreases in TL in cardiac and liver tissue (Ludlow et al., 2012c). Thus, the difference in TL between elite swimmers and controls in the present study may be even more significant when measured in skeletal muscle. Additionally, no study has investigated how exercise specifically impacts replicative stress in the mouth of humans and further research is required to determine whether TL responds to exercise in a synchronous or tissue-specific fashion.

Another key finding of the present study is the lack of association between ACE genotype and TL in elite swimmers or controls. It has been previously reported that the presence of the D allele is associated with shortened TL in hypertensive patients with left ventricular hypertrophy (Fyhrquist et al., 2013). Additionally, several previous studies have reported an excess of the D allele in elite short distance swimming (Costa et al., 2009; Nazarov et al., 2001; Tsianos et

al., 2004; Woods et al., 2001). However, our results reveal that TL is not associated with ACE I/D genotype distribution in swimmers or controls. As such, the accelerated telomere attrition displayed by the swimmers in the present study cannot be attributed to a negative effect of the D allele and/or variations in ACE activity. We also observed a lack of association between swimming event distance and ACE I/D genotype. Although this finding is not novel, as the association between ACE I/D genotype and athletic performance is well studied amongst elite swimmers, the duplicability of such an association is contentious. For instance, studies have shown an increased frequency of the I allele with middle- (Nazarov et al., 2001) and long-distance swimming (Tsianos et al., 2004). However, our results reinforce previous reports that show a lack of association between elite long-distance swimming and ACE genotype (Nazarov et al., 2001; Woods et al., 2001) and also between controls and elite swimmers competing in events >400m (Costa et al., 2009). Further research is required to clarify our findings due to the ambiguity of the current literature and based on the limited sample size, the present data should be interpreted with caution.

The novel aspects of our study are that it is the first to specifically investigate the TL of elite swimmers, and to report that elite athletes display shortened telomeres compared to their recreationally active counterparts. Additionally, no study has previously explored the association between ACE genotype and TL in elite athletes. However, the study is not without limitations. For example, we did not control for known certain confounders of TL, such as psychological stress or nutrition. The work discussed in this chapter also lacked an additional group of young sedentary controls, which would have permitted us to further explore the 'inverted U' hypothesis and determine whether the elite swimmers had comparable TL to their sedentary peers. As such, further studies are required to investigate the associations between TL, oxidative stress and inflammation in young elite athletes compared with both recreationally active and sedentary participants.

To conclude, Chapter VI reveals that elite female swimmers, who routinely engage in high levels of competition and/or training, possess shorter telomeres than their recreationally active counterparts. Participation in elite level swimming training/competition may therefore be detrimental to telomere maintenance and subsequent biological ageing in females, which may be significant in later life as short buccal cell telomeres are associated with age-related disease (Broberg et al., 2005; Thomas et al., 2008). However, as we did not observe an association between ACE I/D genotype distribution and TL in elite swimmers and/or controls, the

accelerated telomere attrition detected in elite swimmers does not appear to be influenced by a potential negative effect of the D allele and/or variations in ACE activity.

CHAPTER VII - GENERAL DISCUSSION

The primary aims of this thesis were to:

1. explore how exercise modality, intensity, and dosage influence TL
2. investigate potential mechanisms of exercise-induced telomere dynamics
3. compare TL across tissue types in athletes

7.1 The influence of exercise modality, intensity, and dosage on telomere length

Results from the opening study of this thesis (Chapter III) indicated that a 12-week low-resistance, high-repetition RT intervention does not impact TL in sedentary middle-aged participants and agreed with the null hypothesis. Only a few studies have investigated the influence of RT interventions on telomere dynamics, producing divergent outcomes. There have been reported relationships of TL maintenance (Dimauro et al., 2016), no effect (Hagstrom, 2018; Tosevska et al., 2016; Werner et al., 2018), and telomere shortening (Miranda-Furtado et al., 2015) in response to RT interventions. These discrepant findings may be explained by the variations in the frequency, volume, intensity, and duration of the RT protocols employed by each study. Nonetheless, the findings in Chapter III support studies reporting that short-term RT interventions do not influence TL (Hagstrom, 2018; Tosevska et al., 2016; Werner et al., 2018). Although we report that the RT intervention had no notable influence on LTL, we cannot be certain that a sedentary control group would have also produced comparable results over a 12-week period. Dimauro *et al*. previously reported that although TL remained unchanged in participants following a 3-month RT intervention, their telomeres were significantly longer than a sedentary control group after the study (Dimauro et al., 2016). This situation is unlikely, however, as sedentary individuals do not typically experience any substantial telomere attrition over such short intervention periods (Hagstrom, 2018; Tosevska et al., 2016; Werner et al., 2018). Nevertheless, a 12-month follow-up of participants revealed significant telomere attrition in those who reverted to a sedentary lifestyle after the RT intervention, compared with their pre-RT intervention values. Conversely, TL was preserved in participants who continued to exercise for the 12-month period following the RT intervention. The maintenance of TL in the exercise group cannot be directly attributed to RT, however, as participants who continued to exercise described utilising various modalities (e.g.,

cycling, running, tennis, RT). These finding suggests that either a longer intervention period (e.g., 12 months), or a variety of exercise modalities, may be required to elicit a meaningful influence on TL.

Based on the findings in Chapter III, the next study (Chapter IV) employed a 12-month longitudinal period and investigated the unexplored relationship between TL and Pilates. The principal finding was that experienced Pilates practitioners and sedentary controls displayed comparable LTL at baseline, suggesting long-term Pilates exercise (> 5 years) does not appear to influence telomere dynamics. Another notable finding was that LTL remained unchanged in Pilates practitioners and sedentary controls over the 12-month longitudinal period. We speculate that the inability of Pilates exercise to impact telomere dynamics is likely explained by its lower intensity nature, since LPA does not appear to be associated with TL (Shadyab et al., 2017b). Nonetheless, Yoga practitioners (30-40 years) with at least two years of practice have been shown to possess longer telomeres than sedentary controls (Krishna et al., 2015), suggesting that further research is required comparing the influence of Pilates and Yoga on telomere dynamics.

Running exercise was selected as the exercise modality for Chapter V, since it has been reported to be the only evaluated physical activity associated with LTL out of nine separate exercise modalities (Loprinzi and Sng, 2016). The primary finding of Chapter V is that young endurance runners possess longer telomeres than master endurance runners, master sprinters and middle-aged sedentary controls. However, all significant differences were lost when controlled for covariates, suggesting that running-specific exercise does not influence TL when considered in isolation. A secondary finding in Chapter V was that LTL is negatively associated with the number of training years across all athlete groups, in agreement with previous data that studied a population of endurance runners (Rae et al., 2010). However, although this finding suggests that the accumulation of training years could be detrimental to telomere preservation in endurance runners and sprinters, our results still revealed no observable difference in TL between groups.

For the final experiment of this thesis (Chapter VI), TL was investigated in an elite athlete cohort undertaking high dose, high intensity exercise. The findings of Chapter VI disclose that elite swimmers display shorter TL compared with their recreationally active peers, which appears specific to females. To highlight, TL of control participants was 8.1% greater than elite swimmers when considered as a whole sample. However, when the data was analysed

split by sex, female controls possessed telomeres 9.9% longer than elite female swimmers. Additionally, there was no difference in TL between male swimmers and their sedentary counterparts, indicating that participation in elite level swimming training/competition may only be detrimental to telomere maintenance and subsequent biological ageing in females. These results do not agree with data that young elite athletes possess longer telomeres than healthy controls (Muniesa et al., 2017). The discrepancy in these findings may be explained by the fact that elite athletes in the study by Muniesa *et al*. were recruited from a wide variety of sport specialties (running events > 1500 m, triathlons, weightlifting, judo, gymnastics, canoeing, basketball, soccer, taekwondo), each of which generates different metabolic demands (Muniesa et al., 2017). Nonetheless, we demonstrate that extremely high training loads/competition levels can be detrimental to TL in elite young athletes, specifically females, a finding which may offer support to the hypothesis of an 'inverted U' relationship between PA and TL. However, as this study lacked a young sedentary control group, we can only speculate as to whether they would have shown comparable TL to the elite swimmers.

In brief, the results of this thesis typically agree with the associations between PA and TL reported in the wider literature: a positive association, an inverted U relationship, and no association at all. For instance, Chapter III indicates that utilising a variety of exercise modalities can preserve TL, although a minimum of 12 months participation may be required. Low intensity exercise modalities (e.g., low-load, high-repetition RT and Pilates) (Chapters III and IV) and running-specific exercise (Chapter V) do not appear to be a sufficient stimulus to regulate telomere dynamics, and extremely high intensities and/or doses of swimming exercise appear detrimental to TL in females (Chapter VI). Therefore, a sustained engagement in various exercise modalities, at moderate intensities and dosages, may be most beneficial for telomere dynamics.

7.2 Potential exercise-induced mechanisms regulating telomere dynamics

Telomere-regulating proteins

The transcriptional expression of TERT is closely correlated with telomerase enzymatic activity and is considered to be the rate-limiting determinant (Kirkpatrick et al., 2003). To highlight the importance of telomerase, low levels of enzymatic activity are proposed to cause age-related telomere attrition (e.g. the telomere-telomerase hypothesis) (Holt et al., 1996). Results from Chapter V show that hTERT gene expression was positively associated with LTL

across the whole cohort and offer limited support to the telomere-telomerase hypothesis. However, hTERT gene expression was not associated with TL in Chapters III and IV. The initial study of this thesis revealed that a 12-week of low-resistance, high-repetition RT intervention had no influence on TL or hTERT gene expression, although we report a near borderline significant increase in the latter. This finding tenuously supports previous data that a 6-month RT intervention had no effect on telomerase activity (Werner et al., 2018). Due to the trend of a reported increase in hTERT in Chapter III, we continued to investigate hTERT in Chapters IV and V and report that long-term Pilates practitioners, endurance runners, and sprinters exhibit similar gene expression of this enzymatic subunit compared with sedentary age-matched controls. The results in Chapter V are in opposition to previous data that report endurance athletes display upregulated TERT mRNA expression compared with controls (Denham et al., 2015). Similarly, endurance and interval training have been reported to increase TL and telomerase activity (upregulated by two- to three-fold) in blood mononuclear cells (Werner et al., 2018). The absence of a significant difference in hTERT expression between any exercise and control group in this thesis may partly explain their comparable TL. Additionally, the experimental results also suggest that the exercise modalities employed in this thesis do not influence hTERT gene expression, in disagreement with a recent systematic review and meta-analysis (Denham and Sellami, 2021).

The shelterin protein complex is understood to protect telomeres and regulate telomerase activity (De Lange, 2005). The overexpression of TRF1 has been shown to stimulate telomere shortening (Munoz et al., 2009) as a result of end-to-end fusions, chromosomal aberrations, and telomere recombination (Munoz et al., 2009). It has been suggested that chronic exercise is a negative regulator of TRF1 gene expression (Ludlow et al., 2012b). Nevertheless, several studies have demonstrated that various modalities and intensities of exercise can either increase (Laye et al., 2012; Saki et al., 2016; Werner et al., 2009) or decrease (De Carvalho Cunha et al., 2018; Ludlow et al., 2012a) TRF1 gene expression. The results of Chapter IV demonstrate that TRF1 was positively associated with TL in the control group at 12 months, which disputes the notion that TRF1 is a negative regulator of TL. Additionally, young endurance runners and young sprinters had higher TRF1 gene expression than young controls, master endurance runners, and master sprinters in Chapter V, which would in theory increase their susceptibility to telomere attrition compared with the other groups. However, young endurance runners displayed the longest telomeres. Thus, the

findings of this thesis imply that the effect of exercise on TRF1 gene expression, and its subsequent impact on telomere dynamics, remains unclear.

Inflammatory markers

Inflammation is associated with telomere driven cellular senescence and is a major component of both pathological and normal ageing (Zhang et al., 2016). The initial study of this thesis (Chapter III) revealed TNF-α was significantly reduced following a 12-week RT intervention, although TL remained unchanged. The former finding is in agreement with previous data showing that an 8-week RT intervention can significantly reduce serum levels of TNF-α in elderly women (Santiago et al., 2018). Additionally, TNF-α was negatively associated with TL in participants who returned to a sedentary lifestyle. We also report a negative association between TNF-α and TL across the whole cohort (all timepoints) in Chapter IV, supporting previous data that reports individuals with elevated levels of TNF-α had significantly higher odds for short telomeres (O'Donovan et al., 2011).

Higher plasma concentrations of CRP have been previously linked with shorter telomeres (Rode et al., 2014; Wong, 2004). The findings of Chapter III support this data, as TL was negatively associated with circulatory CRP across all participants at pre- and post-time points. CRP levels were surprisingly unchanged in Chapter III, as Santiago *et al.* have previously demonstrated significantly reduced levels following an 8-week RT intervention (Santiago et al., 2018). However, in Chapter IV we report that Pilates practitioners display lower baseline CRP levels than controls. This finding indicates long-term Pilates participation may reduce levels of this acute-phase protein and supports previous data (Khajehlandi et al., 2020). On the contrary, a 20-week Pilates intervention did not affect serum CRP levels in elderly individuals (Pestana et al., 2016). Nonetheless, TL was still comparable between groups in Chapter IV and was not associated with CRP levels. We also report that circulatory CRP levels showed no change in both the Pilates and control groups over the 12-month longitudinal period and that the significant between-group difference was lost at the post timepoint. In Chapter V, the middle-aged control group did, however, display higher circulatory CRP levels than young endurance runners, master endurance runners, and master sprinters. Even so, CRP was not associated with TL in Chapter V and only young endurance runners displayed longer telomeres than middle-aged controls, emphasising the ambiguous relationship between CRP levels and TL.

Although the present results revealed several significant associations between TL and both TNF-α (Chapters III and IV) and CRP (Chapter III), it is important to state that we also report no association between these markers in Chapters IV (CRP) and V (TNF-α and CRP). Moreover, IL-6 (Chapter III) and IL-8 (Chapters III, IV and V) were not associated with TL in any study contained in this thesis and support previous findings (Lustig et al., 2017; Wong, 2004). In summary, our results suggest that the broader relationship between exercise, inflammation, and telomere biology requires further exploration.

Antioxidant gene expression

Oxidative stress is intimately aligned with inflammation and also enhances telomere attrition (Masi et al., 2011; Richter and Zglinicki, 2007). It has been proposed that oxidative stress may even influence telomere attrition to a greater extent than the end-replication problem (Von Zglinicki et al., 2000). In Chapters IV and V, SOD2 gene expression was not associated with TL, although Pilates exercise appears to assist in the maintenance of a higher baseline expression of SOD2 (Chapter IV). This finding supports previous data that trained individuals typically display upregulated activity of SOD2 in blood at rest (Urso and Clarkson, 2003). Nonetheless, SOD2 gene expression was reduced in both Pilates practitioners and controls over 12 months and is likely a reflection of the typical age-related decline observed in women older than 38 years (Tatone et al., 2006). Interestingly, the middle-aged control group displayed significantly elevated SOD2 gene expression compared with all other groups in Chapter V (excluding MS). This finding was unexpected given that endurance exercise upregulates SOD mRNA levels (García-López et al., 2007).

Pilates exercise maintained higher mRNA levels of GPX1 compared with a sedentary lifestyle in Chapter IV, despite gene expression of this antioxidant enzyme showing a negative association with TL across the whole cohort at baseline. This deleterious relationship was not to be expected, as decreased GPX1 levels have been implicated in the shortening of telomeres in patients suffering from major depressive disorders (Szebeni et al., 2014). TL was, however, comparable between Pilates practitioners and sedentary controls across all timepoints, indicating that this negative association does not appear to influence telomere dynamics. Conversely, GPX1 gene expression was not associated with TL in Chapter V. It was also unanticipated that the master sprinters showed significantly lower GPX1 gene expression than master endurance runners given that strength training provides a

more optimal stimulus to upregulate mRNA GPX1 levels compared with endurance training (García-López et al., 2007).

It is plausible to suggest that exercise-induced increases in the endogenous levels of SOD2 and GPX1 may have the potential to serve as an effective therapeutic strategy to combat oxidative stress-induced telomere attrition and ageing. However, the results of this thesis indicate the relationship between exercise, antioxidant gene expression, and TL demands additional investigation.

7.3 Leukocyte and buccal cell telomere length are highly correlated in athletes

The biochemical testing of athletes in the field can present numerous logistical issues (e.g., invasiveness of test, sample processing, storage conditions). Thus, it may be advantageous to use minimally invasive techniques that require less immediate sample processing when aiming to quantify TL in the field (e.g., buccal swab). Previous studies have demonstrated buccal cell TL is positively correlated with leukocyte TL in a twin cohort (Finnicum et al., 2017) and patients with dyskeratosis congenita (Gadalla et al., 2010), despite variation in their absolute values. More recently, buccal cells have been utilised to demonstrate that elderly ultra-trail runners display longer telomeres than age-matched sedentary controls (Hernando et al., 2020). Although intra-individual age-related telomere attrition is similar across somatic tissues with divergent replicative status (Daniali et al., 2013), no study had specifically compared TL across leukocytes and buccal cells in an athlete cohort. The results of Chapter V demonstrate that buccal cell TL is strongly correlated with LTL, despite the former displaying greater absolute values (T/S ratio). Our findings reinforce the idea that buccal cell samples can be used as an accurate, alternative, non-invasive measure of TL, but also provide the first evidence that this tissue type can be used to reliably investigate the influence of exercise on telomeres in athlete cohorts. These results also indicate that leukocyte and buccal cell telomeres appear to respond to exercise in synchrony and suggest that the telomere dynamics of both tissue types are impacted via the same exercise-induced mechanisms, although further longitudinal studies are warranted to elucidate and confirm these mechanisms. However, the sole use of buccal swabs to determine TL, such as in Chapter VI, severely limits the capacity to deduce biological causality for any findings due to the lack of blood and serum samples that are typically used to quantify telomerase activity, gene expression, and inflammatory markers.

7.4 Novel contributions to knowledge and implications to the wider field

This thesis contributes several exclusive findings to the field of telomere and exercise research. The studies in this thesis are the first to:

1. examine the effect of low-resistance, high-repetition RT on TL
2. investigate the association between Pilates exercise and TL
3. compare TL between young and middle-aged endurance runners and sprinters
4. compare leukocyte and buccal cell TL in an athlete cohort
5. demonstrate that elite young swimmers possess shorter telomeres than their recreationally active counterparts

Several systematic reviews and meta-analyses have investigated the influence of exercise on TL (Aguiar et al., 2021; Chilton et al., 2017; Lin et al., 2019; Marques et al., 2020; Mundstock et al., 2015; Semeraro et al., 2020). These reviews, and the wider literature, typically indicate that regular and continued exercise over an extended time induces a positive impact on TL and cellular ageing. Nonetheless, there are a lack of consistent findings in the current literature, particularly regarding the limited available studies that provide mechanistic insights. To highlight, a systematic review and meta-analysis that included thirty-seven original studies and 41,230 participants revealed that ~54% of the included studies failed to detect a significant association between TL and PA, 41% noticed a positive association, and 5% identified an inverted 'U' relationship (Mundstock et al., 2015).

Broadly, the results of this thesis tend to support the conclusions of Mundstock *et al*., reporting a variety of relationships exist between exercise and TL; positive (Chapters III), negative (Chapter VI), and no association (Chapters III, IV and V). This thesis also provides further evidence that inconsistent findings in the current literature may be the consequence of divergent exercise modalities, intensities, and dosages. In terms of real-world impact, the findings of this thesis provide the first evidence that buccal cell samples can be used as a reliable, alternative non-invasive measure of TL in athlete cohorts. Additionally, the present results suggest that any individual who aims to utilise exercise to positively impact telomere biology should likely focus on a continuous engagement (>12 months) in a variety of exercise modalities, at moderate intensities and dosages.

It is essential to note that the studies in this thesis were not without limitations (as stated in each chapter), which should be considered in future investigations. TL has been associated with

multiple behavioural and psychosocial factors (Starkweather, 2014), which increases the difficulty to fully segregate the effects of PA on telomere dynamics. For example, as the studies in this thesis did not control for factors such as diet and sleep, discrepancies in these factors between participants may have influenced the present results. Thus, future studies should aim to control for as many variables as logistically possible to isolate the effects of exercise on telomere biology.

In combination with previous studies (Hagstrom, 2018; Tosevska et al., 2016; Werner et al., 2018), the findings of Chapter III suggest that low-resistance, high-repetition RT is unlikely to regulate TL, especially over short intervention periods. However, no study to date has investigated TL in response to RT over a longitudinal intervention period greater than one year. It may be that longer intervention periods are required to reveal the full potential benefits of RT on TL. As such, the study in Chapter III may serve to act as a pilot to a further investigation that utilises a control group, larger sample size, and longer intervention period (> 12 months).

The results of Chapter IV suggest that long-term Pilates exercise does not influence TL. We speculate this is due to a low exercise intensity, which is purported to exert no influence on TL (Shadyab et al., 2017a). Nonetheless, Yoga practitioners have been shown to display longer telomeres than their sedentary counterparts (Krishna et al., 2015). A future study that employs a larger sample size and compares the effects of Pilates and Yoga on telomere dynamics is required.

Owing to the cross-sectional design, the findings in Chapter V dictates that our findings can only be considered observational. Thus, additional research is warranted examining the effects of endurance running and sprinting on TL in young and middle-aged athletes over longitudinal periods, with the aim of elucidating the relationship and mechanistic pathways between running-specific exercise and telomere biology.

The use of buccal cells in Chapter VI did not allow us to deduce biological causality for our findings. The lack of a sedentary control group also restricted our ability to confirm an ‘inverted U’ relationship between TL and PA. Further investigations are therefore required to explore the association between TL, oxidative stress, and inflammation in young elite swimmers compared with both recreationally active and sedentary controls.

7.5 Concluding remarks

Telomere research has increased in popularity over the last decade, since they offer enormous potential as therapeutic targets in disease management. The relationship between exercise and TL appears complex, but several studies provide promising results. Although the primary findings of this thesis suggest that the influence of exercise on telomeres may not be as positive as previously reported, they still provide evidence of three primary associations: positive, an inverted U relationship, and no association at all. Validation of how different modalities, intensities, and dosages of exercise influence telomere biology appears to hold substantial promise. To emphasise, it is biologically plausible to propose that specific exercise protocols may one day be prescribed in clinical settings with the aim of preserving TL and delaying the onset of age-related diseases. However, conflicting findings and a lack of mechanistic studies dictate that there is considerably more to discover and understand prior to the measurement of TL being clinically adopted.

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APPENDICES

Appendix A: Telomere length assay transfer and validation

All TL measurements reported in the experimental Chapters of this thesis were performed at the University of Leicester according to the methodology described in 'Chapter II - Materials and Methods'. Following the completion of all TL analysis, 10 samples utilised in the experimental Chapters of this thesis were randomly selected for use in the inception and validation of a telomere assay at Loughborough University.

Aim

To establish and validate a protocol for the determination of TL in the School of Sport, Exercise and Health Sciences at Loughborough University.

Methods

All samples (n = 10) were analysed on two separate days (Run 1 and Run 2) according to an optimised step-by-step protocol for measuring relative TL (Joglekar et al., 2020). Brief details of the basic materials and procedure for this methodology are outlined below.

Primer sequences for telomere and human β-globin (hbg) (single-copy gene) (Integrated DNA Technologies, USA) are detailed in Table 1. Primer sequences for TelA and TelB were diluted to final concentrations of 100nm and 300nm, respectively. The primers for the single-copy gene (hbg1 and hbg2) were diluted to final concentrations of 300nm and 700nm, respectively.

9 µl of master-mix (either for telomere or human β-globin - Table 2.) was pipetted into each well of a 384-well reaction plate.1 µl of either Nuclease-free water (for no template control/NTC), DNA sample (25 ng/µl) or Control DNA (25 ng/µl), was then added to make a final per reaction volume of 10µl.

Table 1. *Telomere and human β-globin primer sequences for PCR*

Primer	Primer sequence
Tel (A)	5'- CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT -3'
Tel (B)	5'- GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT -3'
hbg1	5′- GCTTCTGACACAACTGTGTTCACTAGC -3′
hbg2	5′- CACCAACTTCATCCACGTTCACC -3'

Table 2. *Master-mix reagents and their volumes per PCR reaction.*

Reagent	Volume/Reaction (µl)
2X Fast SYBR® Green Master Mix	5
Telomere A OR hbg1 primer (working concentration)	1
Telomere B OR hbg2 primer (working concentration)	1
Nuclease-free water	2
TOTAL	9

All samples were plated in triplicate and run on a Viia7 thermal cycler with 384-well blocks (ThermoFisher Scientific, USA). T and S runs were performed separately, starting with T and followed immediately by S run to keep conditions uniform. The telomere PCR cycling conditions consisted of 95°C for 3 min, 40 cycles of 95°C for 15 sec and 56°C for 1 min. The single-copy gene PCR cycling conditions consisted of 95°C for 3 min, 40 cycles of 95°C for 15 sec and 58°C for 1 min.

Results

Dilution results were obtained for telomere and single-copy (human β-globin) genes, as shown in Figures 1 and 2, respectively. Three samples at three different dilutions (100 ng/µl, 50 ng/µl, 25 ng/µl) were used in the telomere PCR reaction, and four samples at four different dilutions (25 ng/µl, 12.5 ng/µl, 6.25 ng/µl, 3.125 ng/µl) were used in the hgb PCR reaction, to obtain Ct values for telomere and the hgb expression. Amplification curves were

exponential and set in the linear range of the curves for telomeres (delta Rn = 12500, Figure 1) and human β-globin (delta Rn = 42290, Figure 2).

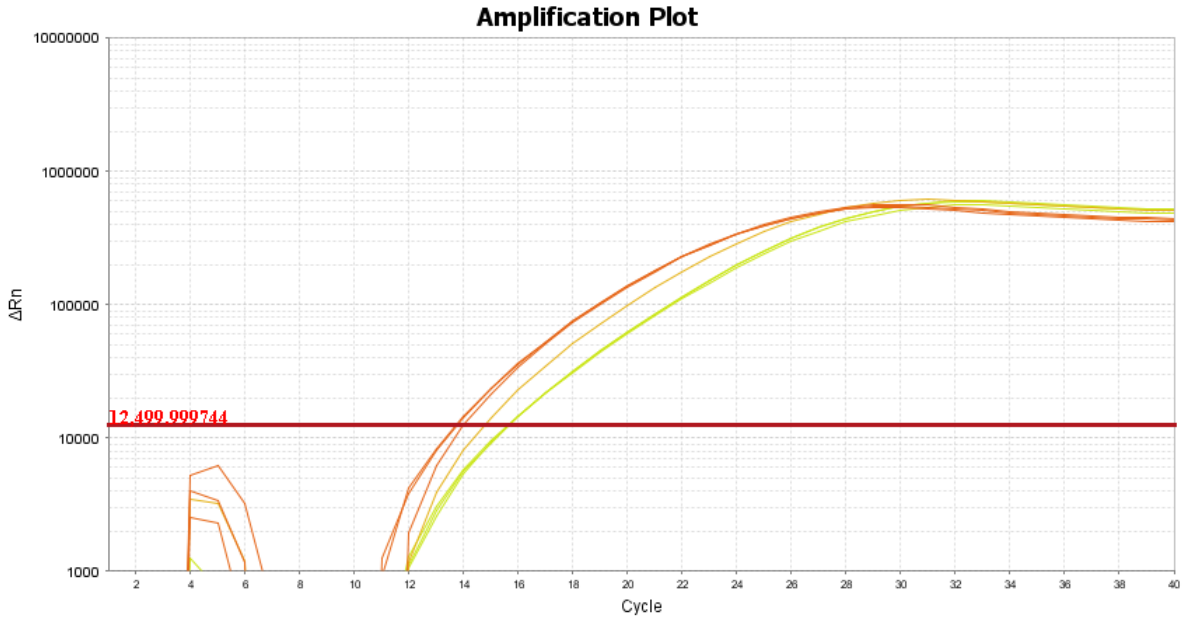

Figure 1. Representative telomere primer amplification plot for three DNA concentrations performed in triplicate (100 ng/µl, 50 ng/µl and 25 ng/µl).

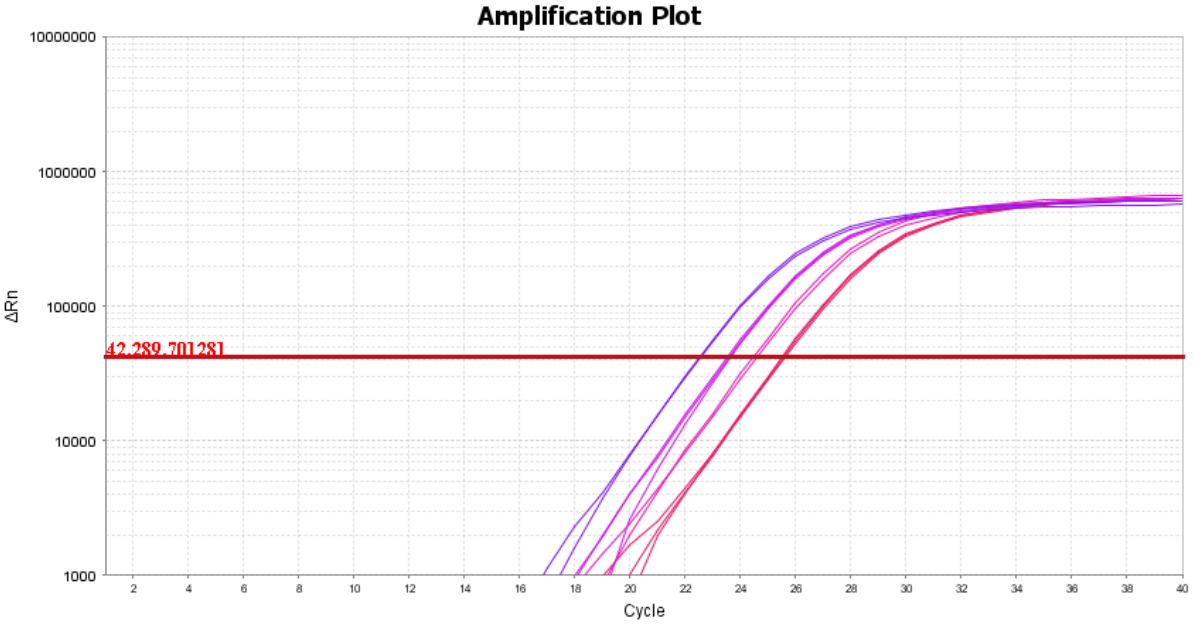

Figure 2. Representative human β-globin primer amplification plot for four DNA concentrations performed in triplicate (25 ng/µl, 12.5 ng/µl, 6.25 ng/µl, 3.125 ng/µl).

Melt curve analysis displayed a single peak for telomere (Figure 3) and human β-globin (Figure 4) reactions, demonstrating the existence of a single amplicon.

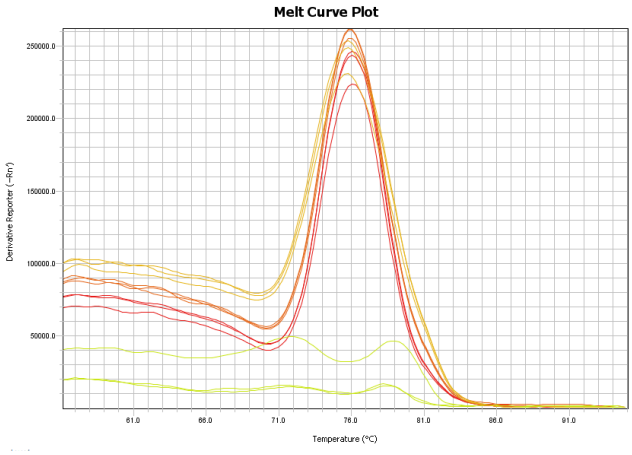

Figure 3. Representative image of the melt curve for telomere PCR products.

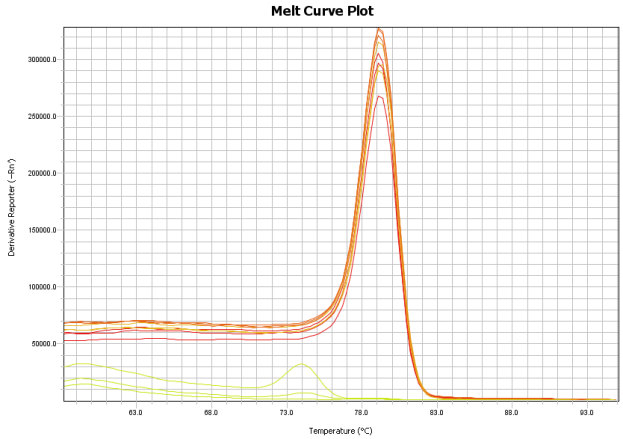

Figure 4. Representative image of the melt curve for human β-globin PCR products.

Pearson correlation values were very high for both the telomere (r = 0.95, Figure 5) and human β-globin genes (r = 0.99, Figure 6). PCR efficiencies were observed to be 114% for telomeres (Figure 5) and 104% for the human β-globin gene (Figure 6).

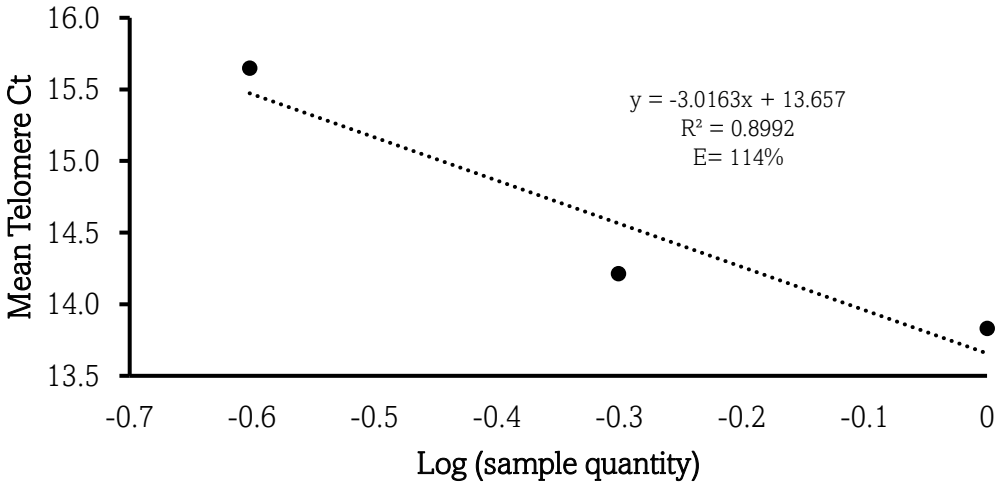

Figure 5. Efficiency of telomere primers.

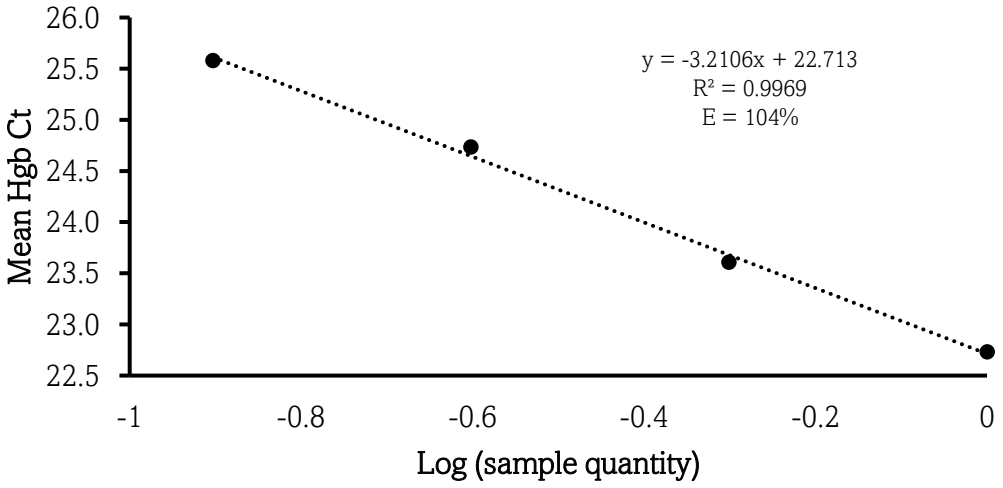

Figure 6. Efficiency of human β-globin primers.

We observed high reproducibility in qPCR data for telomere (Figure 7) and human β-globin (Figure 8) expression, with correlation coefficients >0.9. Typical amplification plots for telomeres and human β-globin PCR data are shown in Figures 9 and 10, respectively.

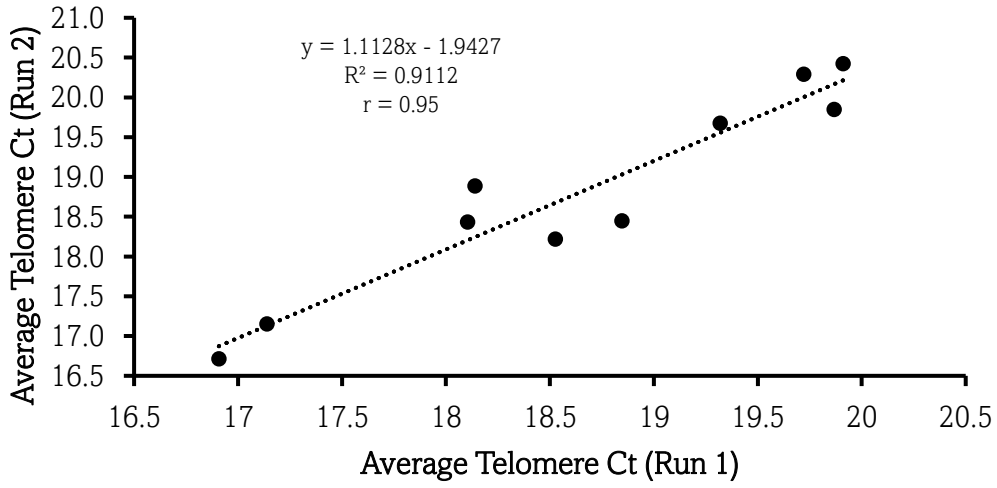

Figure 7. Correlation between results for telomere PCR data from the same sample analysed on separate days.

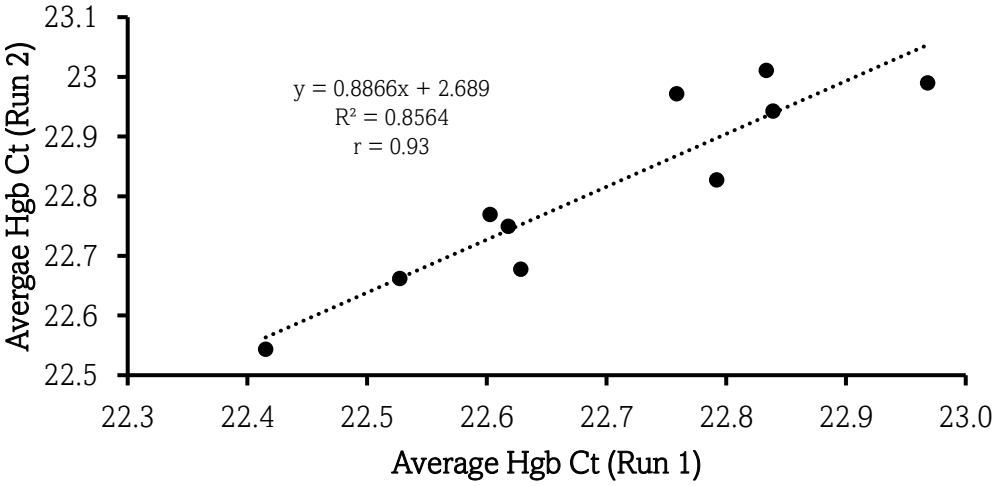

Figure 8. Correlation between results for human β-globin PCR data from the same sample analysed on separate days.

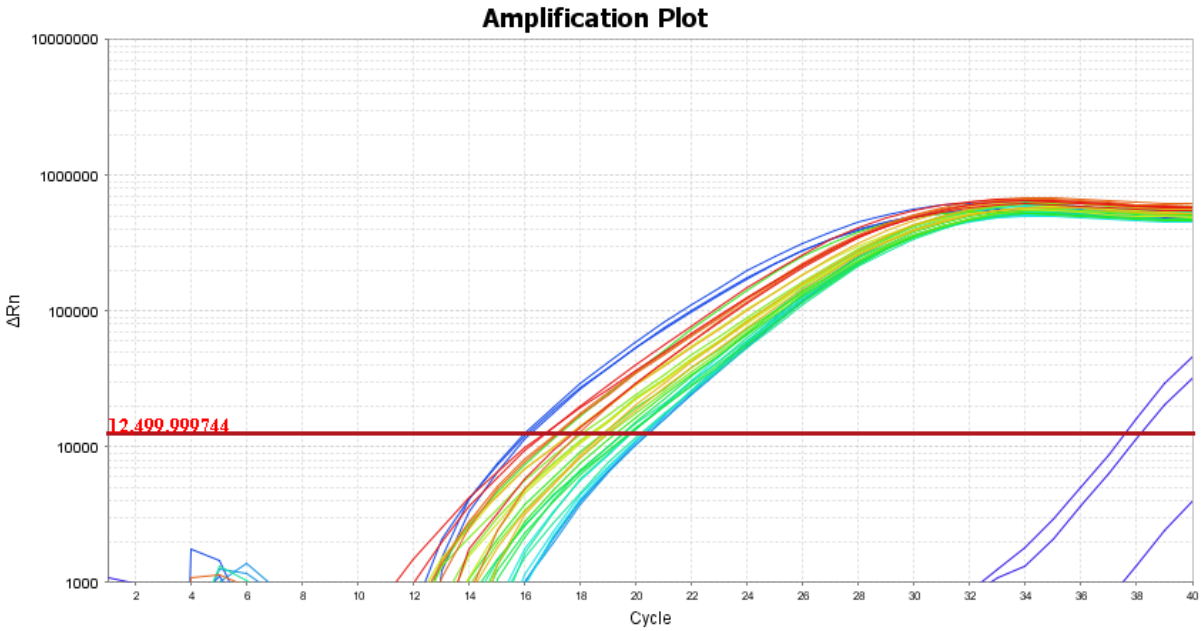

Figure 9. Typical amplification plot for telomere PCR data

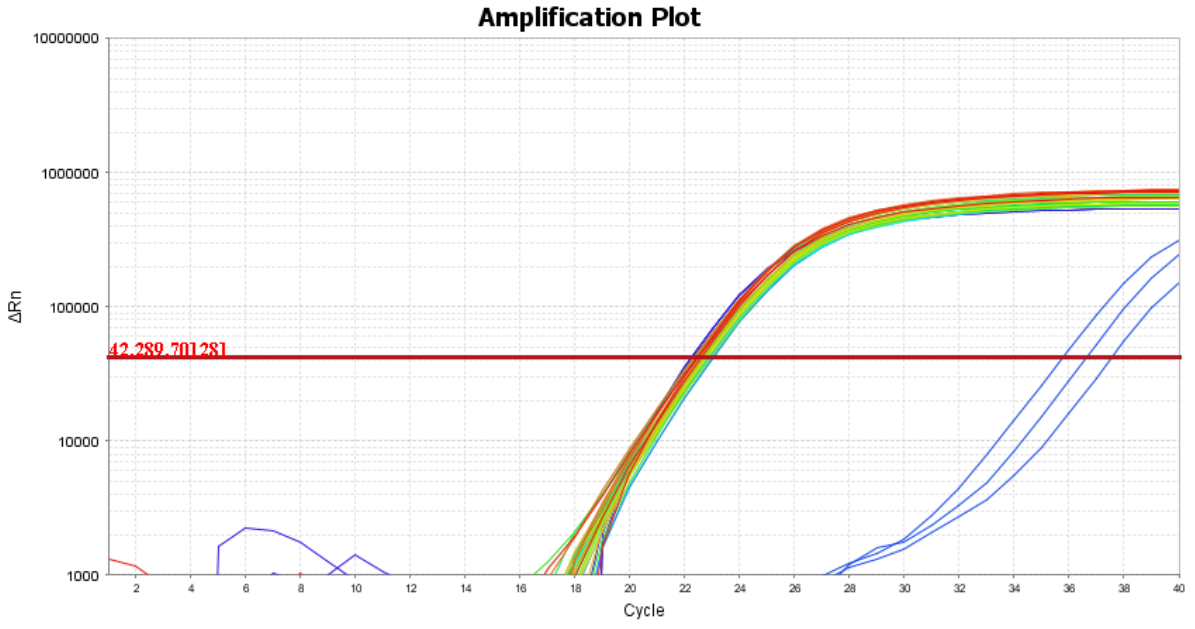

Figure 10. Typical amplification plot for human β-globin PCR data

Relative telomere length (T/S ratio) was calculated for each run using the following formula:

Relative telomere length = (Sample Average Telomere Ct - Sample Average hbg Ct) / (Control DNA Average Telomere - Ct Control DNA Average hbg Ct).

Calculated T/S ratios from Run 1 and Run 2 were highly correlated (r = 0.94, Figure 11) and illustrates the reproducibility of the method.

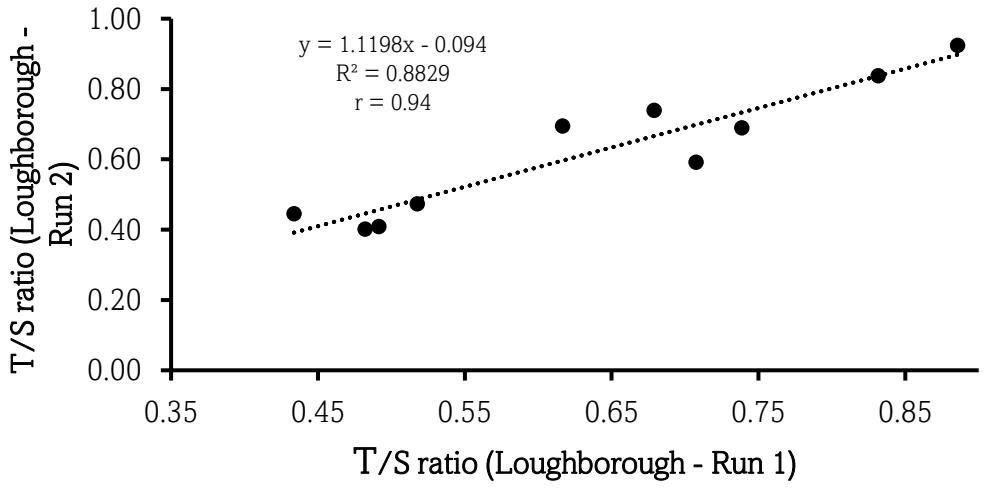

Figure 11. Correlation plot for T/S ratio data performed on the same sample analysed on separate days.

Additionally, T/S ratio data for Run 1 (r = 0.93, Figure 12) and Run 2 (r = 0.88, Figure 13) were highly correlated with their respective values obtained at the University of Leicester.

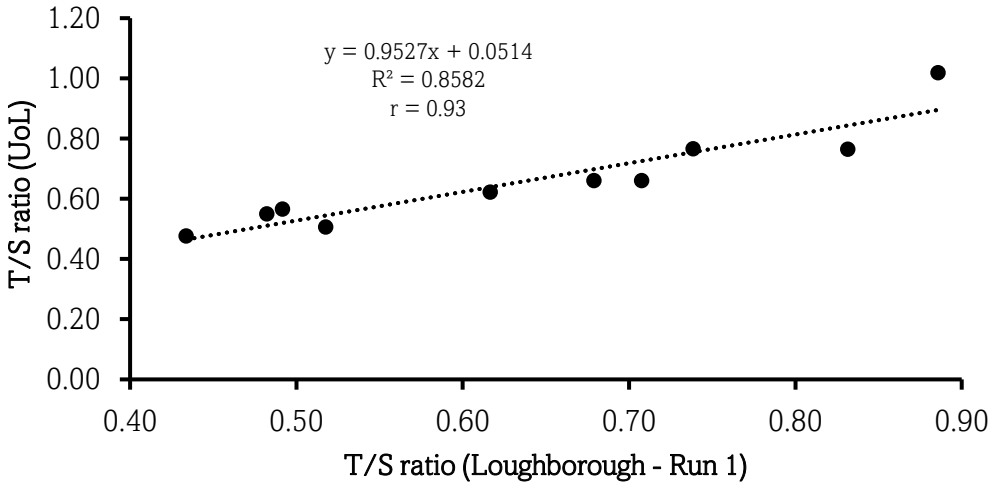

Figure 12. Correlation plot for T/S ratio data performed at the University of Leicester (UoL) and Loughborough (Run 1).

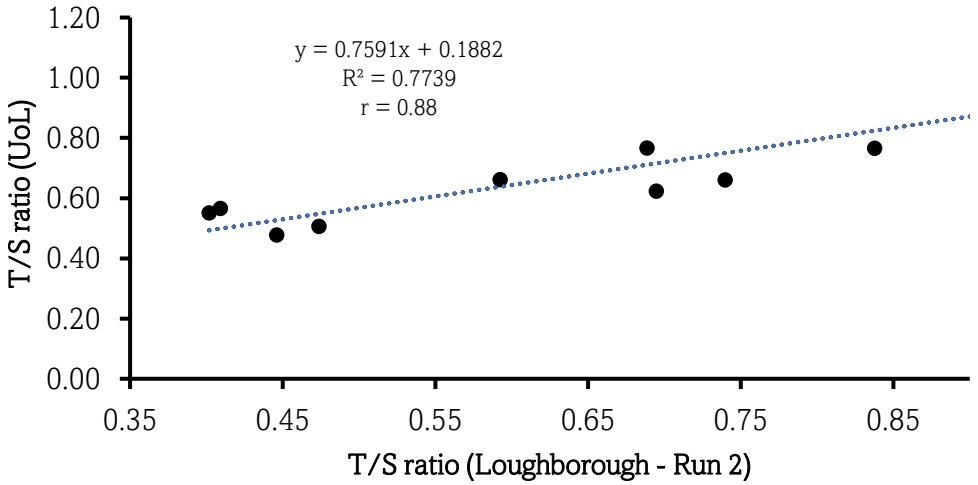

Figure 13. Correlation plot for T/S ratio data performed at the University of Leicester (UoL) and Loughborough (Run 2).

Conclusion

In summary, we observed very low assay CVs, with intra-assay CV <2.3% (CV between the replicate values of Control DNA in the same plate), inter-assay CV <1.6% (CV between the replicate values of Control DNA on different plates), similar PCR efficiencies for telomeres (114%) and single-copy genes (104%) (within acceptable range), linear dynamic range of amplification between 7–34 cycles, and almost undetectable signal in NTC (Figures 9 and 10). T/S ratio data for Run 1 and Run 2 were also highly correlated with their respective values obtained at the University of Leicester. As such, this protocol can be considered an accurate and reliable measure of relative TL.

Appendix B: Example research proposal

Ethics Approvals (Human Participants) Sub-Committee

Loughborough University

Research Proposal for Studies Involving Human Participants

PROJECT DETAILS

1. Project Title: The relationship of Pilates on telomere length and telomerase activity in both the immune cells of older men and women.

2. Aims and objectives of the study (200 words maximum)

To assess the physical activity (PA) of Pilates with telomere length and telomerase activity in immune cells. Assessing the effects of Pilates exercise intervention in a control matched longitudinal study.

Aims: 1 - To assess the effect of Pilates training on telomere length and telomerase activity in immune cells as a key identifier of anti-ageing benefits.

2 - Identification of individuals with different hTERT genotypes and assess the changes in telomere length and responses to Pilates.

3 - To measure PA changes and align data sets with telomere length and telomerase activity in immune cells with other PA parameters and health measurements.

4 – To determine the influence of Pilates on inflammatory balance and telomere dynamics, and to determine the association between training type and frequency, instructor-defined exercise performance parameters and telomere biology.

3. Lay summary of the study (500 words maximum)
Note: *This should be understandable to a non-expert and should not be a copy of the research proposal. It should include the reasons for the research, the background to it and why the area is important to investigate.*

Telomeres are the functional caps on the end of chromosomes. They serve as the boundary between essential genetic material and the cellular environment and as such act as guardians to protect DNA against cellular stress. The length of these units is vital for health, but inevitably telomere length shortens with age. Progressive shortening of telomeres leads to cell death, affecting the health and lifespan of an individual. Both physical inactivity and stress levels display an inverse relationship with telomere length, with better lifestyle choices (e.g. exercise, diet, meditation) having immense potential to reduce the rate of

telomere shortening. Consequently, the onset of age-associated diseases wold be delayed and lifespan increased.

Pilates is a physically demanding mode of exercise that improves psychological wellbeing, mood and perceived stress. Thus, participation in Pilates may negate the effects of two major culprits associated with telomere shortening (physical inactivity and stress). Of the nearly 400 articles found on Pubmed when using Pilates as a search term, over half have been generated in the last 4 years. This is suggestive of a growing awareness of Pilates and a deepening acceptance of its scientific value. Within these articles there are a number related to positive gains in healthy individuals and even more relating to the benefits in different disease populations. Despite the scientific communities deepening interest, there is a paucity of data relating to the overall strength gains and any concomitant biochemical changes, with no studies investigating the relationship between Pilates and telomere length. This study would aim to address the gap by measuring a range of output parameters at both a physical and a biochemical level.

4. Start date of study: 1/11/18

5. End date of study: 31/12/21

6. Start date for data-collection: 1/11/18
Note: *Data collection should not commence before final ethical approval is confirmed.*

7. Location of the study: Loughborough University and The Pilates Studio (Kegworth)

8. Reasons for undertaking the study (e.g. contract, student research): Student research

	Yes	No
9. Do any of the researchers stand to gain from a <u>particular</u> conclusion of the research study?	☐	☒

If Yes, how do the researchers stand to gain?

APPLICANT DETAILS

10. Name of Applicant: Matt Nickels

Position (Undergraduate, Postgraduate, Staff): Postgraduate student

School/Department: SSEHS

Email address: M.Nickels@lboro.ac.uk

11. Name of Responsible Investigator (if different to above): Dr Elizabeth Akam
Academic member of staff responsible for conduct of the study (supervisor for student studies)

School/Department: SSEHS

Email address: E.C.Akam@lboro.ac.uk

12. Additional Investigators – (add rows if required)

Name	School	Position	Email
Dr Sarabjit Mastana	SSEHS	Staff	S.S.Mastana@lboro.ac.uk
Kathy Riley Smith	External	External	info@thepilatesstudiokrs.co.uk
		Choose an item.	

13. Experience of all investigators in the methods to be used in this study
Note: *Please ensure the experience of all investigators which is relevant to this study is included in this section.*

PARTICIPANT INFORMATION

14. Number of participants to be recruited: 15-20 active participants, 15-20 control subjects

15. Details of participants (age, gender, special interests etc):

Participants – middle-aged males and females already participating in Pilates (or new)

Controls - sedentary middle-aged males and females

16. How will participants be selected?
Note: *Include the inclusion/exclusion criteria to be used.*

Participants must be middle-aged males and females already participating in Pilates or are signing up to participation.

17. How will participants be recruited and approached?
Note: *If an advertisement or forum post is to be used, please include this in your application to the Sub-Committee.*

Participants will be recruited and approached by Kathy Riley Smith who runs The Pilates Studio in Kegworth. Any new members to her classes will be approached to see if they are interested or willing to partake in the study.

18. Please state the demand on participants' time:

Note: *Where possible, include a breakdown of how long each part of the study will take, as well as a total time demand.*

Pre-tests (1 hr) (Loughborough University)
Inter-test visit after one year (1 hr)
Participants – continue Pilates classes (2 years)
Post-tests (1hr) (Loughborough University)

	Yes	No
19. Will control participants be used?	☒	☐

If **No**, please go to Question 20.
If **Yes**, please answer the questions 19a-19c below.

19a. How will control participants be selected?
Note: *Include the inclusion/exclusion criteria to be used.*

Controls will be healthy sedentary middle-aged males or females who will continue their usual routine and exercise levels.

19b. How will control participants be recruited and approached?
Note: *If an advertisement or forum post is to be used, please include this in your application to the Sub-Committee.*

Control participants will be approached through email, word of mouth and advertisement poster.

19c. Please state the demand on control participants' time:
Note: *Where possible, include a breakdown of how long each part of the study will take, as well as a total time demand.*

Pre-tests (1 hr) (Loughborough University)
Inter-test visit after one year (1 hr)
Post-tests (1hr) (Loughborough University)

20. Please provide procedures for the chaperoning and supervision of the participants during the study. *(required for vulnerable participants or invasive procedures/physical activity)*.

Kathy Riley Smith, a trained Pilates professional, will supervise all classes.
A trained phlebotomist will draw blood from participants.
Two individuals will supervise the pre-, inter- and post-test measurements.

21. Please give details of possible risks, discomforts and/or distress to participants. Please include details of cultural issues for participants.

Risks and discomfort associated with drawing blood.

22. Please provide details of any incentives, reimbursements or payments being offered to the participants.

RESEARCHER SAFETY

	Yes	No
23. Are there any potential risks to the researchers in this study?	☐	☒

If **No**, please go to Question 24.
If **Yes**, please answer the questions 23a-23b below.

23a. What are the potential risks to the researchers?

23b. What measures have been put in place to address these risks?

STUDY DETAILS

24. Description of study design and methodology:
Note: *It should be clear what each participant will have to do, how many times, and in what order. All of this information should also be included on the Participant Information Sheet.*

Participants and controls will be required to give a blood sample at the beginning of each visit (pre-, inter- and post-tests) to measure the following markers:

Biochemistry markers - Glucose; IL1-beta, IL-8; CRP and TNF alpha

Genetic markers - Telomere length; Telomerase activity; TNF alpha methylation; CRP methylation

Participants and controls will also be required to complete the following as part of their pre-, inter- and post-tests:

Participant self-assessment - IPAQ (short form); Wellbeing (WEMWBS); RPE

Physical activity (PA) markers - Body composition; Countermovement jump test; Grip strength; Lower limb strength - Isometric Strength; Core strength test; Dominant leg balance test; Sit and Reach test.

During the 2-year study period, participants will be required to attend weekly Pilates classes.

25. Measurements to be taken:
Note: *All measurements and samples to be taken from participants should be included here. Measurements can include questionnaire, observations and photographic data.*

Participants and controls will be required to give a blood sample at each lab visit (pre-, inter- and post-tests) so that the following markers can be measured:

Blood biochemistry - Glucose; IL1-beta, IL-8; CRP and TNF alpha

Genetic markers - Telomere length; Telomerase activity; TNF alpha methylation; CRP methylation

Participants and controls will also be required to complete the following as part of their pre-, inter- and post-tests:

Participant self-assessment - IPAQ (short form); Wellbeing (WEMWBS); RPE

Physical activity (PA) markers - Body composition; Countermovement jump test; Grip strength; Lower limb strength - Isometric Strength; Core strength test; Dominant leg balance test; Sit and Reach test.

26. What Personal Information or Sensitive Personal Information are you collecting:
Note: See ICO guidance on personal data at: https://ico.org.uk/for-organisations/guide-to-the-general-data-protection-regulation-gdpr/key-definitions/what-is-personal-data/

27. Does the proposed study involve any of the following? Select those that apply:

27a. Involves taking bodily samples	☒
27b. Involves procedures which are physically invasive (including the collection of body secretions by physically invasive methods)	☒
27c. Is designed to be challenging:	
Physically (includes any study involving physical activity)	☒
Psychologically	☐
27d. Involves procedures that are likely to cause:	
Physical distress to participants	☐

Psychological distress to participants	☐
Social distress to participants	☐
Emotional distress to participants	☐
27e. Involves intake of compounds additional to daily diet, or other dietary manipulation/supplementation	☐
27f. Involves pharmaceutical drugs (Please refer to published guidelines)	☐
27g. Involves testing new equipment	☐
27h. Involves procedures which may cause embarrassment to participants	☐
27i. Involves use of radiation (Please refer to published guidelines and contact the University's Radiological Protection Officer before beginning any study which exposes participants to ionising radiation)	☐
27j. Involves use of hazardous materials (Please refer to published guidelines)	☐
27k. Assists/alters the process of conception in any way	☐
27l. Involves methods of contraception	☐
27m. Involves genetic engineering	☐

If you have selected any of the above, please give specific details of each of the procedures to be used and arrangements to deal with adverse effects:

Blood will be taken during pre-, inter- and post-testing from all participants and controls by a trained phlebotomist who will also have a chaperone with them.

Participants will be required to perform the physically demanding Pilates for 2 years. All classes will be supervised by Kathy Riley Smith, an accredited Pilates professional.

CONSENT

	Yes	No
28. Will written informed consent be obtained from participants?	☒	☐

If **Yes**, please attach a copy of the consent form to be used.
If **No**, please explain why written consent is not being obtained:

29. Will any of the participants be from the following vulnerable groups? <u>Select those that apply:</u>

Children under 18 years of age	☐
Persons incapable of making an informed decision for themselves	☐
Pregnant Women	☐
Prisoners/other detained persons	☐

Adults who are vulnerable because of their social, psychological or medical circumstances	☐
Other vulnerable groups Please specify:	☐

If **you have selected any of the above**, please answer the following questions:

29a. What special arrangements have been made to deal with the issues of consent?

29b. Have the researchers obtained necessary police registration/DBS clearance?
Note: *Please provide details, including date clearance obtained, or indicate why this is not applicable to your study.*

WITHDRAWAL

30. How will participants be informed of their right to withdraw from the study?

Participants will be informed of their right to withdraw from the study in the participant information sheet and on their first visit to the laboratory for the pre-tests.

31. How will participants be informed of the timeframe for withdrawing their data before this has been aggregated in the study or published?

Participants will be informed of the timeframe to withdraw their data from the study before it is published in the participant information sheet and on their first visit to the laboratory for the pre-tests.

STORAGE AND SECURITY OF DATA

32. Will the study include the use of any of the following? Select those that apply:

Observation of participants	☐
Audio recording	☐
Video recording	☐

		Yes	No	N/A
33.	**Will the collection and storage of the <u>personal data</u> comply with current Data Protection legislation? See ICO Guidance on personal data:** https://ico.org.uk/for-organisations/guide-to-the-general-data-protection-regulation-gdpr/key-definitions/what-is-personal-data/	☒	☐	☐

If **Yes**, please give details of legal basis for processing personal data:
If **No**, please give details:

		Yes	No
34.	**Will participants <u>personal data</u> be stored securely, in line with participants informed consent, and deleted when no longer required for this study**	☒	☐

If **No**, please give details:

		Yes	No
35.	**Will <u>study data/results</u> be anonymised at the earliest opportunity (see Guidance** at https://www.ukdataservice.ac.uk/manage-data/legal-ethical/anonymisation**).**	☒	☐

If **Yes**, please explain how data will be anonymised:
If **No**, please give details:

		Yes	No	N/A
36.	**Will <u>original hardcopies</u> of study documents (interview notes/transcripts/questionnaires) be stored securely for <u>ten years</u> from completion of the project within a locked office at the University or scanned, encrypted and securely stored on the University's IT system**	☒	☐	☐

If **No**, please give details:

Yes	No	N/A

37.	**Will original recordings (audio/video) and photographs be anonymised, encrypted and retained securely for six years from completion of the project on the University's IT system.**	☐	☐	☒

If **No**, please give details:

		Yes	No
38.	**Will data/results be encrypted and securely stored for six years from completion of the project on the University's IT system**	☒	☐

If **No**, please give details:

		Yes	No
39.	**Will data/results be permanently archived to the University's Data Repository or another repository (*If yes, details should be included on the Participant Information Sheet and Informed Consent Form.)***	☒	☐

If **Yes**, give details including whether the deposit will be open or confidential:
If **No**, please give details:

40. **If bodily samples which are Human Tissue Act Relevant Material are to be taken, please give details of, and the timeframe for, the disposal of the tissue.** (See: the University's HTA Quality Manual and https://www.hta.gov.uk/policies/relevant-material-under-human-tissue-act-2004)
Note: *Please also ensure that this information is included on the Participant Information Sheet.*

41. **If Human Tissue Act Relevant Material is being transferred to or from Loughborough University please give details, including whether a Materials Transfer Agreement has been approved and signed.**

SPONSORSHIP AND INSURANCE

Note: It is the responsibility of investigators to ensure that there is appropriate insurance cover for the study.

	Yes	No
42. Is the study being sponsored/funded?	☐	☒

If **Yes**, please state source of funds including a contact name and address for the sponsor:
If **No**, please go to 37.

	Yes	No
36a. Is the study to be covered by the sponsors/funders insurance?	☐	☒

If **No**, please confirm who will be insuring the study: University

	Yes	No
43. Is the study to be covered by the University's insurance?	☒	☐

If **No**, please confirm who will be insuring the study:

The University maintains in force a Public Liability Policy, which indemnifies it against its legal liability for **accidental** injury to persons (other than its employees) and for accidental damage to the property of others. Any **unavoidable** injury or damage therefore falls outside the scope of the policy.

	Yes	No
44. Will any part of the study result in <u>unavoidable</u> injury or damage to participants or property?	☐	☒

If **Yes**, please detail the alternative or additional insurance cover arrangements and include the supporting documentation in this application.

The University Insurance relates to claims arising out of all **normal** activities of the University, see Appendix 2, but Insurers require to be notified of anything of an unusual nature or taking place outside of the UK.

	Yes	No
45. Is the study classed as <u>normal</u> activity?	☒	☐

If **No**, or your study is taking place outside of the UK, please complete the **Insurance Questionnaire** and submit with a copy of the research proposal to the University Insurance Officer to confirm that the policy will cover the activity. If the activity falls outside the scope of the policy, please detail the alternative or additional insurance cover arrangements and include the supporting documentation in this application.

DECLARATION

I have read the University's Code of Practice on Investigations on Human Participants and have completed this application. I confirm that the above named investigation complies with published codes of conduct, ethical principles and guidelines of professional bodies associated with my research discipline.

I agree to provide the Ethics Approvals (Human Participants) Sub-Committee with appropriate feedback upon completion of my study.

Signature of applicant:

Signature of Supervisor (if applicable):

Signature of Dean of School/Head of Department:

(or their nominee)

Date:

Appendix 1: Application Checklist

Please ensure that you have attached copies of the following documentation to your application:

For all applications:

- ☐ Participant Information Sheet
- ☐ Informed Consent Form
- ☐ Risk Assessment

Where applicable:

- ☐ Willingness to Participate/Assent forms (for studies involving participants under 18)
- ☐ Parental/Guardian Information Sheet
- ☐ Children's Information Sheet
- ☐ Letter of Approval(s) from Head Teacher(s)
- ☐ Opt-Out Letters
- ☐ Health Screen Questionnaire
- ☐ Questionnaires
- ☐ Interview Questions
- ☐ Advertisement/Recruitment material
- ☐ Evidence of approval from other Committees (including International organisations)
- ☐ Additional Insurance Cover

Appendix 2: Insurance – Normal Activities

Cover is automatic if the research is within the UK and limited to the following activities (e.g. normal activities):

i. Questionnaires, interviews, focus groups, physical activity/exercise, psychological activity including Cognitive behavioural therapy;
ii. Venepuncture (withdrawal of blood);
iii. Muscle biopsy;
iv. Measurements or monitoring of physiological processes including scanning;
v. Collections of body secretions by non invasive methods;
vi. Intake of foods or nutrients or variation of diet (other than administration of drugs).

All other Research involving human participants, including studies outside of the UK, should be referred to the Insurance Officer along with the completed **Insurance Questionnaire** to arrange cover - which may incur a charge. Early submission is recommended.

Appendix C: Example participant information sheet

The relationship of Pilates on telomere length and telomerase activity in the immune cells of both older men and women

Adult Participant Information Sheet

Investigators Details:

Elizabeth Akam (Staff/Study Lead), School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, Leicestershire, LE11 3TU. Tel: 01509 222759.

Kathy Riley-Smith (Instructor), The Pilates Studio KRS, 36A Upper Floor, Barton House, Ashby Road, Kegworth, DE74 2DH. Tel: 07980 752888

Dr S Mastana, (Staff) School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, Leicestershire, LE11 3TU. Tel: 01509 223041.

Mr M Nickels (Staff), School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, Leicestershire, LE11 3TU. Tel: 01509 226444.

We would like to invite you to take part in our study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. Talk to others about the study before deciding if you wish.

What is the purpose of the study?
This research aims to explore the health benefits of participating in Pilates in a community-based or one to one exercise setting. The information gained from this study can be used to inform adults on the utility of Pilates as a significant form of physical exercise and training for health.

Who is doing this research and why?
This work is being completed by Kathy Riley-Smith, Matt Nickels and Elizabeth Akam as part of ongoing sports and exercise-based research projects. The supervisor and responsible investigator will be Dr E. C. Akam.

Are there any exclusion criteria?
- Chest pains or history of chest pain
- Habitual use of anti-inflammatory drugs
- Serious allergy
- Known to have blood-borne virus
- Symptoms of general illness

What will I be asked to do?

There will be three testing sessions in total which are detailed below. Once testing session 1 is complete (pre-assessment) you will be asked to attend Pilates once a week for 52 weeks, you will then complete testing session 2 (inter-assessment). Then after a further commitment

to Pilates once a week for 52 weeks, you will then complete testing session 3 (post-assessment).

Visit 1 Pre-Assessment (Loughborough University approx. 1 hour)

Height, mass and body composition (muscle mass, body fat %, fat free mass etc.) will be measured using scales (bio-impedance scales). Your overall physical readiness will be assessed by a series of questions in a questionnaire format and you will be asked to give a blood sample from a vein in your arm. Some simple strength tests will then be performed, these will be Countermovement jump test; Grip strength; Lower limb strength - Isometric Strength; Core strength test; Dominant and non-dominant leg balance test; Sit and Reach test. (more detail below).

With the jump test we will ask you to stand on a thin mat with your hands on your hips. Then when instructed by the test administrator, you should jump as high as possible and attempt to land in the same location on the mat as you took-off from (you will need to keep your hands on your hips as you jump).

For grip strength we will ask you to squeeze a hand-held device as hard as you can, three times in each hand. You will be seated for this test with your elbow at 90 degrees.

Leg strength testing will be performed using a Leg and back dynamometer (strength testing machine). Basically, we will ask you to pull as hard as you can on a cable that will not move.

The core strength tests consist of a one-legged hip bridge hold and both the plank and side-plank for as long as possible.

For the single leg balance test, you will be assessed under eyes open and closed conditions. You will have one practice trial and 2 trials on each leg for each condition, with a rest period of 30 seconds between trials. The test will be performed bare foot and you will have to maintain balance on one leg for as long as possible. The Y-balance test will measure dynamic balance you will be required to perform three consecutive trials of reaching in anterior, posteromedial, and posterolateral directions for each lower extremity. You will be pushing a box with the end of your toes as you reach to measure distance.

With a sit and reach testing box you will be asked to sit on the floor with your legs stretched out in front of you with knees straight and feet flat against the front end of the test box. Then you will be asked to lean forward at the hips, keeping your knees straight and slide your hand up the ruler on the box as far as you can go. The furthest distance reached along the scale will be recorded to the nearest 0.5 cm.

When helping us with these tests (thank you) we want you to know there is no expectation and no competition associated with them. They are simple standardised tests, so we can assess where you were when you started and where you end up.

Exercise class participation (approx. 2 years)

Visit 2: Inter-test visit after one year (1 hr) and Visit 3: Post-tests (1hr) (Loughborough University)

These sessions will consist of the same measurements as the pre-assessment and requires you to still be active in a Pilates programme.

Once I take part, can I change my mind?

Yes. After you have read this information and asked any questions you may have if you are happy to participate we will ask you to complete an Informed Consent Form, however if at any time, before, during or after the sessions you wish to withdraw from the study please just contact the main investigator. You can withdraw at any time, for any reason and you will not be asked to explain your reasons for withdrawing. However, once the results of the study are aggregated (expected to be by December 2020), it will not be possible to withdraw your individual data from the research.

Will I be required to attend any sessions and where will these be?
You will be required to attend three testing sessions. Three sessions (pre, inter and post) to measure your anthropometrics, balance, flexibility and strength and which will take place in the School of Sports exercise and Health Sciences at Loughborough University (blood sampling required).

How long will it take?
Anthropometrics, blood sampling, strength, balance and flexibility testing = 3 x 60min = 180mins. Total demand time in university testing = 180 min.
Also requires the attendance of Pilates class for a minimum of 1h x week for 104 weeks = 104hrs

What type of clothing should I wear?
You will need to wear clothes that you are comfortable to exercise in.

Is there anything I need to do before the sessions?
No nothing specific but it is advisable that you eat and drink prior to arriving to the lab on the morning of the biological tests.

Are there any disadvantages or risks in participating?
With venepuncture there is a risk of fainting or feeling light-headed, haematoma and infection. Venepuncture can also lead to local thrombophlebitis (inflammation) in a superficial vein, but the absolute level of risk is very low.

Data Protection Privacy Notice

Loughborough University will be using information/data from you in order to undertake this study and will act as the data controller for this study. This means that the University is responsible for looking after your information and using it properly. Loughborough University will keep identifiable information about you for 10 years after the study has finished (2030). The University's Data Protection Officer can be contacted at: dp@lboro.ac.uk

What personal information will be required from me?
In a master file we would collate your name, contact details, gender, and general health status including general health questionnaire including a physical readiness and "wellness" questionnaire, blood pressure measurement, height and weight.

Why is this personal information being collected?
This data is being collated so that we can best identify a "control" subject. That is someone like you in terms of age, height, weight but not currently undertaking any Pilates training.

How long will my personal data be retained?
All primary information will be anonymised and securely retained for 10 years.

Will my taking part in this study be kept confidential?
All information will be treated with strict confidentiality and participants will be assigned a reference code so that a set of data, blood samples cannot be related back to any individual. Personal data will be processed under General Data Protection Regulation (GDPR updated May 2018), based on 'public task' and the data gathered may be used for scientific/educational purposes, publications, reports, web pages, and other research outputs. Investigators will ensure that no information is published that would allow individuals to be identified. All samples and primary information will be securely locked away until either 01/12/2030 or withdrawal from study, following this it will be securely destroyed.

How will the data collected from me be used?
The data will be used to inform the wider public on the utility of Pilates training it will be used for scientific/educational purposes and will possibly be disseminated via reports, journal publications, conference papers, web pages, and other research outputs.

What is the legal basis for processing the data?
Personal data will be processed on the public task basis. Individuals' rights to erasure and data portability do not apply if you are processing on the basis of public task. However, individuals do have a right to object and under GDPR (updated May 2018), some of the personal data which will be collected from you is categorised as "sensitive data". The processing of this data is necessary for scientific research in accordance with safeguards. This means that study has gone through an ethical committee to ensure that the appropriate safeguards are put in place with respect to the use of your personal data.

I have some more questions; who should I contact?
Elizabeth Akam (Staff/Study Lead), School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, Leicestershire, LE11 3TU. Tel: 01509 222759. and/or Matt Nickels, (Staff) School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, Leicestershire, LE11 3TU. Tel: 01509 226444.

What will happen to the results of the study?
Results of the study will be analysed and published as part of the investigators PhD thesis. Results may also be published in a peer reviewed journal and/or as a conference proceeding. Investigators will ensure that no information is published that would allow individuals to be identified.

What if I am not happy with how the research was conducted?
If you are not happy with how the research was conducted, please contact the Secretary of the Ethics Approvals (Human Participants) Sub-Committee, Research Office, Hazlerigg Building, Loughborough University, Epinal Way, Loughborough, LE11 3TU. Tel: 01509 222423. Email: researchpolicy@lboro.ac.uk

The University also has policies relating to Research Misconduct and Whistle Blowing which are available online at http://www.lboro.ac.uk/committees/ethics-approvals-human-participants/additionalinformation/codesofpractice/ .
If you have taken steps to have a concern or complaint about Loughborough University's handling of data resolved but are still not satisfied you have a right to lodge a complaint with the Information Commissioner's Office (ico), who are the relevant regulator for data privacy and protection matters. The ico can be contacted at Wycliffe House, Water Lane, Wilmslow, SK9 5AF and your will find more information at https://ico.org.uk.

Appendix D: Example informed consent form

Investigating the effects of running on telomere length and telomerase activity in immune cells.

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

Taking Part	**Please initial to confirm agreement**
The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethics Approvals (Human Participants) Sub-Committee.	----------
I have read and understood the information sheet and this consent form.	----------
I have had an opportunity to ask questions about my participation.	----------
I understand that taking part in the project will involve physiological measurements and venepuncture.	----------
I understand that sensitive personal information will be collected during this study.	----------
I understand that my DNA will be extracted from the blood sample I provide and that genetic analyses on the resulting DNA will be performed	----------
I understand that the personal information collected will be name, date of birth, height, weight etc.	----------
I understand that I am under no obligation to take part in the study, have the right to withdraw from this study at any stage for any reason, and will not be required to explain my reasons for withdrawing.	----------

Use of information

I understand that all the personal information I provide will be processed in accordance with data protection legislation on the public task basis and will be treated in strict confidence unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others or for audit by regulatory authorities.	----------
I understand that information I provide will be used for publications, reports and research outputs.	----------
I understand that personal information collected about me that can identify me, such as my name or where I live, will not be shared beyond the study team.	----------
I agree that information I provide can be quoted anonymously in research outputs.	----------

Bodily Samples [please only initial ONE of the following two statements]

I agree that the bodily samples taken during this study can be stored until 01/4/2029 for **future research** in the same research theme as this project.	----------

[Or]

I agree that the bodily samples taken during this study can **only be used for this study** and will be disposed of within **10 years** (01/4/2029) [or] upon completion of the research**.** ----------

Consent to participate

I voluntarily agree to take part in this study. ----------

_____		_____	_____
Name of participant	[printed]	Signature	Date
_____		_____	_____
Researcher	[printed]	Signature	Date

Appendix E: Example Health Screen Questionnaire

Loughborough University

Name/Number .....…….
Male/Female .....…….
Date of Birth .....…….
BMI .....…….

Health Screen Questionnaire for Study Volunteers

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research.

Please complete this brief questionnaire to confirm your fitness to participate:

1. At present, do you have any health problem for which you are:

(a)	on medication, prescribed or otherwise	Yes	☐	No	☐
(b)	attending your general practitioner	Yes	☐	No	☐
(c)	on a hospital waiting list	Yes	☐	No	☐

2. In the past two years, have you had any illness or injury which required you to:

(a)	consult your GP.....	Yes	☐	No	☐
(b)	attend a hospital outpatient department	Yes	☐	No	☐
(c)	be admitted to hospital	Yes	☐	No	☐

3. Have you ever had any of the following:

(a)	Convulsions/epilepsy	Yes	☐	No	☐
(b)	Asthma	Yes	☐	No	☐
(c)	Eczema	Yes	☐	No	☐
(d)	Diabetes	Yes	☐	No	☐
(e)	A blood disorder	Yes	☐	No	☐
(f)	Head injury	Yes	☐	No	☐
(g)	Digestive problems	Yes	☐	No	☐
(h)	Heart problems/chest pains..……	Yes	☐	No	☐
(i)	Problems with muscles, bones or joints	Yes	☐	No	☐
(j)	Disturbance of balance/coordination	Yes	☐	No	☐

(k)	Numbness in hands or feet	Yes		No	
(l)	Disturbance of vision	Yes		No	
(m)	Ear/hearing problems	Yes		No	
(n)	Thyroid problems	Yes		No	
(o)	Kidney or liver problems	Yes		No	
(p)	Problems with blood pressure	Yes		No	

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....
....

4. Smoking, physical activity and family history

(a)	Are you a current or recent (within the last six months) smoker?	Yes		No	
(b)	Are you physically active (30 minutes of moderate intensity, physical activity on at least 3 days each week for at least 3 months)?	Yes		No	
(c)	Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise?	Yes		No	

5. Allergy Information

(a)	Are you allergic to any food products?	Yes		No	
(b)	Are you allergic to any medicines?	Yes		No	
(c)	Are you allergic to plasters?	Yes		No	
(d)	Are you allergic to latex?	Yes		No	

If YES to any of the above, please provide additional information on the allergy

……
……..

6. Additional questions for female participants

(a)	Are your periods normal/regular?	Yes		No	
(b)	Are you on hormonal contraception	Yes		No	
(c)	Could you be pregnant?	Yes		No	
(d)	Are you taking hormone replacement therapy (HRT)?	Yes		No	

7. Are you currently involved in any other research studies at the University or elsewhere?

Yes ☐ No ☐

If yes, please provide details.

……
……

8. Have you recently given blood or been involved with research involving blood samples?

Yes ☐ No ☐

If yes, please provide details.

……
……

9. Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name
……

Telephone Number
……

Work ☐ Home ☐ Mobile ☐

Relationship to Participant
…….....

10. Please enter your height in metres (m) and weight in kilograms (kg).

Height (M) ……..

Weight (Kg)…….

Appendix F: Endurance Running Activity Questionnaire

Endurance Running Activity Questionnaire

Date : _____

Name: _____

Date of birth: _____

1. What year did you start training?

   Year started: _____

2. What year did you start competing? If applicable, what year did you stop competing?

   Year started: _____ Year stopped: _____

3. What is your maximal time of inactivity (injury, personal reasons, etc.) since you first started training or competing? Please include details of any inactivity breaks >2 months.

   _____
   _____
   _____

4. Indicate your primary competitive event/distance and all-time personal best (PB) record (circle the event from the list below).
   Note – please include PB times for any other distances you have also competed in and the year it was achieved (can be multiple). If you are still competing and know your PB for a certain event in the last year, then please include this time.

Event	All-time PB Record		PB record in the last year
	Time	Year	
800m			
1500m			
3000m			
5000m			
10000m			
5k			
10k			
5 miles			
10 miles			
Half marathon			
Full marathon			
Other			

5. On average, how many events were you competing in a year at your peak? If you are still competing, then also include the number of events for the last year.

   Peak: _____ Last year: _____

6. What was the highest level of competition/event you competed in (Olympic games, Commonwealth games, national, local etc.)?

   _____

7. Please give details of a typical training week during your peak and over the last year.

	Average no. runs/week	Average miles/week	Hour's training/week	Average no. of weeks training/year
Peak				
Last year				

Appendix G: Sprinter Physical Activity Questionnaire

Sprinter Physical Activity Questionnaire

Date today: _____

Name: _____

Date of birth: _____

1. What year did you start training?

   Year started: _____

2. What year did you start competing? If applicable, what year did you stop competing?

   Year started: _____ Year stopped: _____

3. What is your maximal time of inactivity (injury, personal reasons, etc.) since you first started training or competing? Please include details of any inactivity breaks >2 months.

   _____
   _____
   _____

4. Indicate your primary competitive event/distance and all-time personal best (PB) record (circle the event from the list below).
   Note – please include PB times for any other distances you have also competed in and the year it was achieved (can be multiple). If you are still competing and know your PB for a certain event in the last year, then please include this time.

Event	All-time PB Record		PB record in the last year
	Time	Year	
60m			
100m			
200m			
400m			
110m hurdles			
400m hurdles			
Other			

5. On average, how many events were you competing in a year at your peak? If you are still competing, then also include the number of events for the last year.

   Peak: _____ Last year: _____

6. What was the highest level of competition/event you competed in (Olympic games, Commonwealth games, national, local etc.)?

   _____

7. Please give details of a typical training week during your peak and over the last year.

	Average no. sprint sessions/week	Average weight sessions/ week	Hours training/week	Average no. of weeks training/year
Peak				
Last year				

Appendix H: International Physical Activity Questionnaire - Short Form

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your sparetime for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathemuch harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physicalactivities like heavy lifting, digging, aerobics, or fast bicycling?

_____ days per week

☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on oneof those days?

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you didfor at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis?Do not include walking.

_____ days per week

☐ No moderate physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on oneof those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and athome, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutesat a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting orlying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

Appendix I: The Warwick-Edinburgh Mental Well-being Scale Questionnaire

The Warwick-Edinburgh Mental Well-being Scale (WEMWBS)

Below are some statements about feelings and thoughts.

Please tick the box that best describes your experience of each over the last 2 weeks

STATEMENTS	None of the time	Rarely	Some of the time	Often	All of the time
I've been feeling optimistic about the future	1	2	3	4	5
I've been feeling useful	1	2	3	4	5
I've been feeling relaxed	1	2	3	4	5
I've been feeling interested in other people	1	2	3	4	5
I've had energy to spare	1	2	3	4	5
I've been dealing with problems well	1	2	3	4	5
I've been thinking clearly	1	2	3	4	5
I've been feeling good about myself	1	2	3	4	5
I've been feeling close to other people	1	2	3	4	5
I've been feeling confident	1	2	3	4	5
I've been able to make up my own mind about things	1	2	3	4	5
I've been feeling loved	1	2	3	4	5
I've been interested in new things	1	2	3	4	5
I've been feeling cheerful	1	2	3	4	5