The Acute Effects of Aerobic Exercise on Leukocyte Telomere Biology

A thesis submitted in fulfilment of the requirements for the Doctorate of Philosophy

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Declaration

This thesis is being submitted for the Degree of Doctor of Philosophy at Federation University Australia, Ballarat. It has not been previously submitted for any other degree or academic award at any other university. I certify that, with the exception of specific examples listed in the acknowledgements, this thesis is the sole work of the author carried out as part of an approved research program.

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Lastly, I would like to issue a heartfelt apology to my own telomeres. Due to a persistent and protracted mix of stress and enforced inactivity they have almost certainly (and somewhat ironically) borne the deleterious brunt of my attempt to better understand them. If nothing else, the findings here within provide me with hope that I can affect a positive change.

Dedication

For the opportunities selflessly afforded to me by my parents, I am eternally appreciative and indebted.

To those closest to me, who with strained patience have waited unwaveringly - my undivided attention is now and for evermore yours.

List of Abbreviations

bp	Base pair
CAD	Coronary artery disease
CD	Clusters of differentiation
CPET	Cardiopulmonary exercise testing
CST	CTC1, STN1, and TEN1
CVD	Cardiovascular disease
$^{\Delta\Delta}$Ct	Delta-delta cycle threshold
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double strand break
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FSC-A	Forward scatter area
FSC-H	Forward scatter height
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HSP	Heat shock protein
hTER	Human telomerase RNA
hTERT	Human reverse transcriptase catalytic subunit
IEG	Immediate early gene
kb	Kilo base
KLRG1	Killer cell lectin-like receptor G1
LTL	Leukocyte telomere length
MHC	Major histocompatibility complex
mAb	Monoclonal antibody

miRNA	MicroRNA
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa beta
ng	Nanogram
NK	Natural killer
nm	Nano-metre
nt	Nucleotides
PBMC	Peripheral blood mononuclear cells
Pre-miRNA	Precursor microRNA
PRG	Primary response gene
Pri-miRNA	Primary microRNA
POT1	Protection of telomere 1
qPCR	Quantitative real-time polymerase chain reaction
RAD50	RAD50 double strand break repair protein
RAP1	Repressor/activator 1
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SIRT6	Sirtuin 6
SNPs	Single nucleotide polymorphisms
SRG	Secondary response gene
SSC-A	Side scatter area
TERRA	Telomeric repeat-containing RNA
T_H1	Type 1 T cell

T_H2	Type 2 T cell
TIN2	TRF1-interacting nuclear factor 2
TRF	Terminal restriction fragment
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
T/S	Telomere to single nuclear gene copy number
UTR	Untranslated region
$\dot{V}O_{2max}$	Maximal volume of oxygen
$\dot{V}O_{2peak}$	Peak volume of oxygen
µl	Microliter

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Abstract

Habitual exercise is unequivocally associated with decreased all-cause mortality and morbidity. Despite the strength of the association, a large part of the decreased risk is physiologically unaccounted for. Accumulating evidence indicates that leukocyte telomere length (LTL) may be one such explanatory mechanism. Telomeres are specialized deoxyribonucleic acid (DNA) sequences located at chromosomal ends where they protect the genomic DNA from enzymatic degradation. Excessive and/or premature telomere shortening in leukocytes is associated with a host of chronic diseases and impaired immune function. Observational associations exist between LTL and habitual physical activity/exercise in multiple cohorts. However, correlation does not imply causal story and the underpinning mechanisms behind the association are unclear. The current consensus is that long-term exercise-induced reductions in oxidative stress and inflammation mediate the association. The acute dynamics of telomere biology are poorly understood; however, a growing body of evidence suggests that telomeres may be amenable to acute modulation via expression of telomere-associated genes and microRNAs. Accordingly, the overarching aim of this thesis was to characterize the acute effects of aerobic exercise on leukocyte telomere biology.

Study One

Telomere biology underpins effective immune function, chronicling replicative history and defining replicative potential. The various immune cell subsets exhibit disparate exercise responses and heterogeneous telomere lengths. Therefore, study one began by quantifying exercise-induced changes in immune cell subsets.

Study one had two key aims: (i) to characterise the exercise-induced immune response and (ii) to analyse and sort T cell subsets for subsequent gene expression analysis. Twenty-two participants undertook a 30 min bout of treadmill running at precisely 80% of peak volume of oxygen intake ($\dot{V}O_{2peak}$). Blood samples were taken pre-, immediately post- and 60 min post-exercise. Flow cytometry analysis identified significant post-exercise decreases in total CD3+ T cells (P<0.001) and CD4+ T cells (P<0.001). This was followed by a return to resting levels 60 min post-exercise in CD3+ T cells and above resting levels in CD4+ T cells (P<0.001). The novel post-exercise decrease in CD3+ T cells represents an inversion of the archetypal CD3+ exercise response. A significant decrease in CD8+ T cells occurred 60 min post-exercise (P<0.01). Relative proportions of CD4+ naïve T cells decreased 60 min post-exercise (P=0.05) whilst CD8+ naïve T cells decreased post-exercise (P<0.05) before returning to resting levels 60 min post-exercise. In conclusion, 30 min of treadmill running at a constant 80% of $\dot{V}O_{2peak}$ was sufficient to elicit novel changes CD3+ T cells and the relative proportions of specific T cell subsets. The diverse functions within immune subsets and their capacity to be modulated by exercise warrants a clearer understanding of the molecular consequences. Importantly, given the heterogeneity of telomere lengths within immune subsets, relative changes in subset proportions should be accounted for when reporting mean LTL. Whilst conventional wisdom posits that LTL determines the immune environment, exercise also alters the immune environment and therefore mean LTL.

Study Two

In study two, acute exercise-induced epigenetic modification was assessed in leukocytes and T cell subsets. Study one identified the acute changes in microRNA

(miRNA) in leukocytes and immune cell subsets. Given the heterogeneous functions and gene expression signatures of each subset, even transient changes in the relative proportions of these cells will likely elicit physiological changes. An initial genome wide microarray (n=10) identified 56 differentially regulated miRNAs in leukocytes in response to exercise. Subsequent *in silico* analyses predicted miRNA/mRNA interactions between the following: miR-181b and *hTERT*, miR-186 and repressor/activator protein-1 (*RAP1*), RAD50 homolog (S. cerevisiae) (*RAD50)* and silent mating type information regulation 2 *(SIRT6)*, miR-96 and *RAP1,* and miR-15a and TATA box binding protein (*TBP*).

Subsequent quantitative polymerase chain reaction (qPCR) validations (n=18) identified significant upregulation between pre- and 60 min post-exercise in miR-186 ($P<0.001$), miR-15a ($P<0.001$) and miR-96 ($P<0.001$). Significant upregulation also occurred between post- and 60 min post-exercise in miR-186 ($P<0.01$), miR-15a ($P<0.001$), and miR-96 ($P<0.01$). Only miR-181b and miR-186 were detected in the sorted T cell subsets.

The findings of this study highlight the transcriptional responsiveness of leukocyte miRNAs to exercise. Four novel miRNAs with potential telomere biology involvement were identified. Additionally, the findings highlight discrepant miRNA expression profiles between whole leukocytes and T cell subsets, suggesting the composite signature is at least in part driven by other immune cell subsets.

Study Three

Study three characterized the expression of gene transcripts predicted to be targeted by miRNAs identified in study two. Study two identified miRNAs with *in silico* binding potential for several telomere-associated genes. In study three, a

telomere extension array was initially used to identify exercise-induced changes in several telomere-associated genes including predicted miRNA targets: *hTERT*, *SIRT6*, *RAP1* and *RAD50*. Subsequent qPCR validations (n=17) confirmed significant 60 min post-exercise upregulation of *hTERT* (*P*=0.001) and *SIRT6* mRNA (*P*=0.017) but discounted any interaction with the potential binding miRNAs. Decreased *RAP1* mRNA expression at 60 min post-exercise (*P*=0.002) was paralleled by concomitant increases in potential binding miRNAs (miR-186 and miR-96). This supports the notion of miRNA/mRNA interaction. A 60 min post-exercise decrease in *RAD50* mRNA coincided with increased expression of potential target miR-186 expression. The exercise-induced upregulation of *hTERT* mRNA was broadly confirmed in CD4+CD45RA+, CD4+CD45RO+ and CD8+CD45RA+ T cell pools.

The novel findings of this study are the exercise-induced upregulation of *hTERT* and *SIRT6* mRNA in leukocytes. hTERT plays critical roles in telomere biology and is the rate limiting component of telomerase activity. Additionally, this study validated the down-regulation of *RAP1* mRNA paralleled by increases in miR-186 and miR-96. Importantly, the findings of this study suggest that some telomere-associated genes have an immediate, early transcriptional response.

Study Four

The final study of this thesis utilized next generation RNA sequencing to characterize acute changes in the exercise-induced leukocyte transcriptome in healthy males (n=10). The exercise transcriptome represents the sum of all mRNA, non-coding RNA, and small RNA molecules expressed in a specific tissue in response to exercise. A total of 182 transcripts were differentially regulated between Pre-Ex to Post-Ex and Post-Ex to 24 h Post-Ex (FDR<0.01). Amongst the

differentially regulated transcripts were members of the heat shock protein families (HSP90 and HSP70), which showed significant upregulation post-exercise before returning to resting levels within 24 hours. HSP90 and HSP70 both have established roles in telomere biology. A total of 12 non-coding RNAs were also differentially regulated across the three time points, including miR-23a and miR-27a, both of which are associated with telomere biology. The results of this study both confirm and extend the results of the previous chapters by showing the exercise responsiveness of pro-telomere transcripts. The results show that exercise acutely elicits multiple pathways and that telomere maintenance may be one of them.

In summary, the above studies indicate that telomere biology within the immune system is acutely labile. Aerobic exercise differentially regulates many microRNAs some of which have potential telomeric involvement. Additionally, several key telomere-associated genes respond to aerobic exercise within 60 min of exercise cessation. The acute upregulation of *hTERT,* the rate-limiting component of telomerase, may provide a mechanistic insight into the observed positive association between exercise and telomere length. Additionally, with mounting evidence of the critical role of epigenetic chromatin modifications in telomere length regulation, the identification of miRNAs with potential telomeric involvement may help improve the understanding of the role of epigenetics and telomere homeostasis.

Chapter 1 – Review of the Literature

1.1 Overview

Aging and physical inactivity underpin two of the most significant and current health imperatives; cardiovascular disease (CVD) and obesity. The global population of persons aged 80 years or older is indexed to triple by 2050 (United-Nations, 2015). Disease free years have not increased proportionately despite increases in total life expectancy, creating a compression of chronic disease burden in old age (Murray et al., 2015). Aging is a complex physiological construct of outward macroscopic effects underpinned by complex molecular processes. In addition to being a strong independent and non-modifiable risk factor for chronic disease (Dillin, Gottschling, & Nyström, 2014; Niccoli & Partridge, 2012; Reeve, Simcox, & Turnbull, 2014; Shane Anderson & Loeser, 2010), chronological age is a measure of risk factor exposure (Kannel & Vasan, 2009; Sniderman & Furberg, 2008).

Biological aging is a distinct construct referring to processes that proceed independently of chronological aging. These changes collectively reduce organismal viability and increase disease vulnerability. Telomeres are widely viewed as both biomarkers and instigators of biological age. Consensus on this point has arisen from widely demonstrated associations with exercise, disease, and longevity. Telomeres are repetitive tandem DNA sequences located at chromosomal terminals where they preserve genomic integrity. Progressively shortening with replicative age, telomeres simultaneously record cellular replicative history and impose a finite replicative lifespan.

The complex network of molecular events that underpin aging phenotypes is the subject of considerable research focus. Two critical aging processes are

cellular senescence (Bhatia-Dey, Kanherkar, Stair, Makarev, & Csoka, 2016) and telomere attrition (Blackburn, Greider, & Szostak, 2006; von Zglinicki, Pilger, & Sitte, 2000). Cellular senescence is the irreversible exhaustion of cellular replicative capacity that contributes to tissue aging and dysfunction. Telomere shortening and destabilization are central components of this replicative exhaustion.

It has been proposed that human biology was forged during the period when humans were nomadic hunter gatherers (Trevathan, Smith, & McKenna, 1999). Genes that endowed survival benefit during this Late Paleolithic era have become antagonistically pleiotropic in the modern environment (Gerber & Crews, 1999). Two critical differences between the two epochs are nutritional excesses and physical inactivity. Physical inactivity is one of the 10 leading global mortality risk factors, accounting for approximately 3.2 million global deaths annually (Lim et al., 2012). Physical inactivity affects multiple physiological systems and is known to increase the risk of colon cancer (Wolin, Yan, Colditz, & Lee, 2009), postmenopausal breast cancer (Monninkhof et al., 2007), type 2 diabetes (Tuomilehto et al., 2001), CVD (Nocon et al., 2008), depression (Paffenbarger, Lee, & Leung, 1994) and dementia (Rovio et al., 2005).

Physical exercise represents an acute disruption to homeostasis that elicits a complex cascade of compensatory mechanisms at the systemic, cellular, and molecular level. Advances in molecular biology provide an unprecedented snapshot of the genetic and epigenetic influences on exercise-mediated phenotypes. Genome-wide association studies (GWAS) have identified gene variants that influence the health benefits of exercise (Sarzynski, Ghosh, & Bouchard, 2016; Tanaka, Wang, & Pitsiladis, 2016). Bioinformatics and

computational analytical technologies have ushered in data-rich fields of physiology including genomics, epigenomics, metabolomics, and proteomics. Microarray, and more recently next generation ribonucleic acid sequencing (RNA-seq) can provide a complete transcriptional footprint of exercise and help interpret the complex transcriptional networks.

Epigenetics has emerged as a critical and complex field in exercise physiology, highlighting the intimate relationship between environment and phenotype. Epigenetic modification refers to heritable alterations in gene function that occur without changes in nucleotide sequences (Bird, 2007). These changes can take the form of DNA methylation, histone modifications, and transcriptional regulation by small non-coding RNA molecules called microRNAs (miRNAs) (He & Hannon, 2004). Accumulating evidence indicates that exercise can alter the trajectory of biological aging and exert transgenerational influence on metabolic phenotypes and disease propensity (Barros & Offenbacher, 2009; Rodenhiser & Mann, 2006; van Dijk, Tellam, Morrison, Muhlhausler, & Molloy, 2015).

Telomere biology appears sensitive to a host of both negative and positive stimuli and lifestyle interventions. A rapidly growing body of evidence attests to a positive association between habitual physical activity/exercise and telomere length in various tissue types. Such an association may provide a direct means to influence the biological age of the responsive tissues. It may also provide novel and exciting opportunities to enhance the understanding of telomere dynamics and potentially increase healthy years of life through inexpensive behavioural interventions such as exercise.

1.2 Physical Activity and All-Cause Mortality

Physical activity (PA) and cardiorespiratory fitness (CRF) are stronger, independent predictors of CVD and all-cause mortality than smoking, hyperlipidaemia, hypertension, and diabetes (Blair et al., 1989; Gulati et al., 2005; Kokkinos, 2008; Mora et al., 2003; Myers et al., 2004; Myers et al., 2002; Sandvik et al., 1993). The landmark Harvard Alumni Study identified a 25-33% decrease in all-cause death rates (Paffenbarger, Hyde, Wing, & Hsieh, 1986), a 45% decrease in CVD risk, and a lifespan increase of 2.15 years in physically active individuals (Paffenbarger, Hyde, Wing, & Steinmetz, 1984). A 2014 analysis of 55,137 adults identified a 30% and 45% lower adjusted risks of all-cause and cardiovascular mortality respectively, giving rise to a 3 year increase in life expectancy (Lee et al., 2014).

CRF demonstrates a stronger negative association with CVD events and a steeper dose response gradient than PA alone (Blair & Jackson, 2001; Myers et al., 2004; Williams, 2001). The seminal Aerobics Centre Longitudinal Study (ACLS) established that the most fit men and women had 43% and 53% lower all-cause mortality and 47% and 70% lower CVD mortality rate respectively (Blair et al., 1989).

There is incontrovertible evidence that PA and CRF decrease all-cause mortality (Blair, Cheng, & Scott Holder, 2001 2001; Blair, Kohl, & Barlow, 1993; Kampert, Blair, Barlow, & Kohl Iii, 1996; Kodama et al., 2009; Lakka et al., 1994; Myers et al., 2004; Park, Chung, Chang, & Kim, 2009; Sui et al., 2007; Villeneuve, Morrison, Craig, & Schaubel, 1998; Wei, Gibbons, Kampert, Nichaman, & Blair, 2000). Despite the established benefits, the underpinning molecular mechanisms,

their targets, specificity, and time course are poorly understood. Additionally, the relatively modest exercise-induced changes seen in individual disease risk factors rarely scale with the comparatively large disease risk reduction (Mora, Cook, Buring, Ridker, & Lee, 2007).

1.2.1 Established Physiological Mechanisms of Exercise Adaptation

The molecular response to exercise consists of complex cell signalling pathways driven by specific primary and secondary messengers. The result is regulation of gene expression, increased production of requisite proteins, and ultimate modulation of phenotype (Williams & Neufer, 2011). A given metabolic phenotype reflects the transient and cumulative perturbations to multiple discrete bouts of exercise (Thompson et al., 2001). Considerable heterogeneity exists between the respective time courses and half-lives of such acute perturbations. The following section provides a brief overview of the major physiological adaptive mechanisms.

Cardio-metabolic Factors

Regular exercise elicits a host of positive myocardial (Kemi & Wisløff, 2010) and vascular adaptations (Haram, Kemi, & Wisloff, 2008; Kojda & Hambrecht, 2005). Habitual exercise training increases left ventricular (LV) end-diastolic diameter (Pelliccia, Culasso, Di Paolo, & Maron, 1999; Pelliccia , Maron , Spataro , Proschan , & Spirito 1991; Roeske, O'Rourke, Klein, Leopold, & Karliner, 1976), LV wall thickness (Pelliccia et al., 1991; Roeske et al., 1976), and LV mass (Pelliccia et al., 1991; Pressler et al., 2012). Exercise training also causes right ventricular enlargement (Scharf et al., 2010; Scharhag et al., 2002), reduced

arterial wall thickness, and increased lumen diameter (Green, Spence, Rowley, Thijssen, & Naylor, 2012; Mulvany, Hansen, & Aalkjaer, 1978).

Flow-mediated shear stress-induced increase in the vasodilator vascular nitric oxide (NO) is a critical adaptation to aerobic exercise (Schuler, Adams, & Goto, 2013). Increased NO enhances blood pressure regulation (Cornelissen & Fagard, 2005; Cornelissen, Fagard, Coeckelberghs, & Vanhees, 2011; Pescatello et al., 2004), coronary blood flow (Hambrecht et al., 2000), endothelial function (DeSouza et al., 2000; Green, Maiorana, O'Driscoll, & Taylor, 2004; Moyna & Thompson, 2004; Walsh et al., 2003), and haemostatic function (Rauramaa et al., 1986). Aerobic exercise also mobilizes endothelial progenitor cells and mesenchymal stem cells (Lenk, Uhlemann, Schuler, & Adams, 2011); potentially influencing vascular regulation and endothelial repair (Asahara et al., 1997; Huang & Li, 2008).

Habitual cardiorespiratory exercise also reduces CVD biomarkers including systemic inflammation (Adamopoulos et al., 2001; Mora et al., 2007; Petersen & Pedersen, 2005), enhances glucose homeostasis and insulin sensitivity (Alcazar, Ho, & Goodyear, 2007; Harris, Hadden, Knowler, & Bennett, 1987) and improves lipoprotein profiles (Tambalis, Panagiotakos, Kavouras, & Sidossis, 2009).

Skeletal Muscle Factors

Skeletal muscle makes up 40-50% of the human body's mass and plays critical roles in metabolism and exercise adaptation (Egan & Zierath, 2013; Izumiya et al., 2008; Lee et al., 2000). Skeletal muscle demonstrates extensive adaptive plasticity, undergoing biochemical, structural, and transcriptional changes in response to exercise (Egan & Zierath, 2013). The adaptive responses to exercise

are largely specific to the stimulus imparted. The peroxisome-proliferator-activated receptor gamma, coactivator 1 (PGC)-1α plays a critical role in signalling pathways activated by endurance exercise (Chan & Arany, 2014). PGC-1α and its family of transcriptional coactivators collectively regulate mitochondrial biogenesis and capillarity (Rowe et al., 2014). A single bout of endurance exercise has been shown to cause morphological and biochemical changes in skeletal muscle mitochondria (Picard et al., 2013). Habitual endurance training has been shown to increase mitochondrial density by approximately 40% (Montero et al., 2015). Endurance exercise also enhances skeletal muscle oxidative capacity (Booth & Baldwin, 2010) and anti-oxidant defence enzymes (Geng et al., 2010; Leick, Plomgaard, et al., 2010).

The conversion of mechanical signals into biochemical and molecular processes in skeletal muscle is known as mechanotransduction. Mechanical stress is transferred to the extracellular matrix via focal adhesions. Sufficient mechanical stress increases protein synthesis leading to mechanical load-induced hypertrophy; the reverse is true for skeletal muscle atrophy (Adams & Bamman, 2012). Resistance training predominantly activates hypertrophy pathways via the critical protein synthesis regulator mammalian target of rapamycin complex 1 (mTORC1).

Skeletal muscle also secretes a complex cascade of growth factors, cytokines, and myokines following exercise (Bortoluzzi, Scannapieco, Cestaro, Danieli, & Schiaffino, 2006; Henningsen, Rigbolt, Blagoev, Pedersen, & Kratchmarova, 2010; Norheim et al., 2011; Roca-Rivada et al., 2012; Yoon et al., 2009). This diverse family of secretory factors mediate anti-inflammatory pathways (Pedersen & Febbraio, 2008), modulate visceral fat deposition (Nielsen et al.,

2008; Quinn & Anderson, 2011), improve fat oxidation (Plomgaard, Fischer, Ibfelt, Pedersen, & Van Hall, 2007; van Hall et al., 2003), improve glucose utilization (Fischer et al., 2004), and enhance signalling pathways (Pedersen & Febbraio, 2008).

Oxidative Stress Factors

High intensity or long duration exercise enhances production of reactive oxygen species (ROS). Whilst ROS production is crucial for cellular communication and pathway activation, excessive ROS production overwhelms antioxidant defence capacity resulting in damage to lipids, nucleic acids, and proteins (Ray, Huang, & Tsuji, 2012). The accumulation of oxidative stress-induced DNA damage is causally linked with organismal aging and is a key factor in cellular dysfunction (Haigis & Yankner, 2010), neurodegeneration (Andersen, 2004; Shukla, Mishra, & Pant, 2011), atherosclerosis and diabetes (Paravicini & Touyz, 2006), and carcinogenesis (Trachootham, Alexandre, & Huang, 2009). In adaptive response, habitual exercise increases anti-oxidant defence systems (Ji, Gomez-Cabrera, & Vina, 2006; McArdle & Jackson, 2000; Radak, Chung, & Goto, 2005; Shin, Lee, Song, & Jun, 2008; Urso & Clarkson, 2003) and enhances oxidative damage repair systems (Radák et al., 2003; Sato, Nanri, Ohta, Kasai, & Ikeda, 2003). This decreases cellular and genomic damage caused by ROS.

Immunological Factors

Overview of the Immune System

The immune system consists of leukocytes (also known as white blood cells) distributed over the innate and the adaptive immune systems. The innate immune system is the non-specific (antigen independent) frontline defence whilst the adaptive immune system is antigen specific, mounting highly specific responses

guided by antigen-specific memory (Simpson, 2013). Leukocytes are a complex immunological conglomeration consisting of variable frequencies of natural killer cells, monocytes, granulocytes, and T and B cell lymphocytes (table 1). The relative subset proportions are dynamic, rapidly changing in response to a wide range of stimuli.

The lymphocyte subset is subdivided into T cells, B cells, and NK cells with each cell type identified by specific cell surface markers called clusters of differentiation (CD). T cells express the cell surface marker CD3 (CD3+) whilst B cells express CD19 but not CD3 (CD3-CD19+); NK cells express CD16 and CD56 and are therefore designated as CD16+CD56+. The relative expression of these cell surface markers reflects their cytokine profile, telomere length, subsequent replicative capacity, and antigen specificity (Appay, van Lier, Sallusto, & Roederer, 2008).

T cells

T cells are a diverse group of cells with wide ranging proliferative capacities, surface marker expressions, functions, and cytokine-secretion profiles (Simpson, 2013). T cells are broadly categorised as either CD4+ helper T cells (T_H) or CD8+ cytotoxic T cells (T_C) (figure 1). The CD4+ T_H cells exhibit both the CD3 and CD4 surface markers and facilitate immune responses by secreting an array of growth factors and cytokines. The primary function of CD8+ T_C cells is to destroy virally infected cells via toxic granules containing potent digestive enzymes.

T cells are phenotypically categorized according to their antigenic history. Circulating CD4+ and CD8+ T cells unexposed to foreign antigens are referred to as naïve and minimally express the cell surface marker CD45RA (Akbar, Terry,

Timms, Beverley, & Janossy, 1988). Prior to antigen exposure, naïve (CD45RA+) T cells circulate in a quiescent, non-proliferating state. T cells with previous antigen exposure are referred to as memory cells (CD45RO+) and express the cell surface marker CD45RO (Figure 1).

The CD4+ T cell subset is further divided into type 1 (T_H1) or type 2 (T_H2) according to their cytokine profile (figure 1). Type 1 T cells produce interleukin-2 (IL-2), interleukin-10 (IL-10), and interferon-γ (IFN-γ). Type 1 T cells elicit the cell-mediated immune response (activation of macrophages and CD8+ cytotoxic T cell proliferation) that defends against bacterial and intracellular viral pathogens. Type 2 T cells produce interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 9 (IL-9), interleukin 13 (IL-13) and interleukin 25 (IL-25) (Walsh et al., 2011). Type 2 T cells primarily activate and maintain the humoral immune response against extracellular bacteria or parasites.

Habitually active individuals have more effective immune function than inactive individuals, independent of age (Kohut & Senchina, 2004; Simpson et al., 2012). Moderate intensity exercise is associated with decreased circulating inflammatory cytokines (Pedersen & Bruunsgaard, 2003), improved vaccine responses (Kohut et al., 2004; Woods et al., 2009), enhanced immune cell phagocytic and cytotoxic activity (Nieman, Henson, et al., 1993; Woods et al., 1999; Yan et al., 2001), increased immune cell proliferation (Nieman, Henson, et al., 1993; Shinkai et al., 1995), decreased numbers of clonally exhausted T-cells (Spielmann et al., 2011), and increased interleukin-2 (IL-2) production (Drela, Kozdron, & Szczypiorski, 2004).

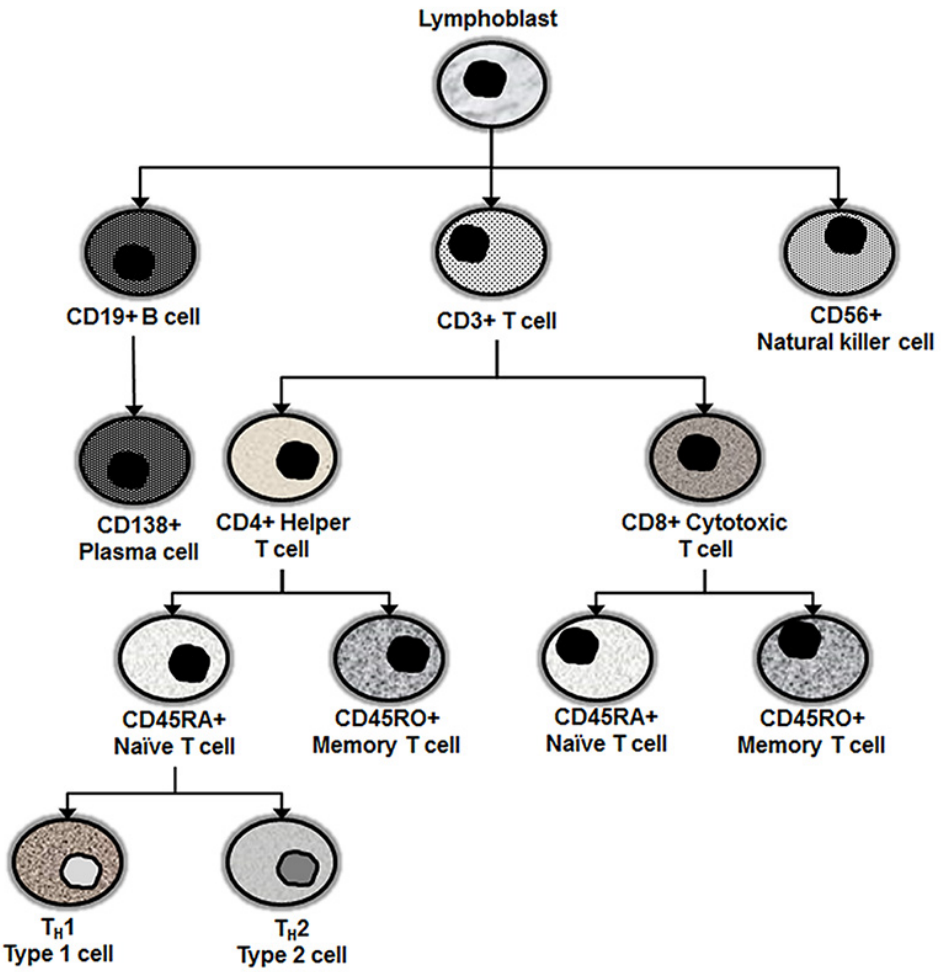


Figure 1. T cell Subsets: CD3+ T cells are broadly divided into CD4+ helper T cells and CD8+ cytotoxic T cells. Each subset consists of CD45RA+ and CD45RO+ populations. Naïve CD4+ T cells differentiate into Type I (TH1) and Type II (TH2) cells.

Table 1. Lymphocyte distribution

Subset	Percentages	Key Functions/Characteristics
T cells (CD3+):	**60-80% of lymphocytes**	
T_H (CD3+CD4+)	60-70% of T cells	Helper T cells Antigen recognition and co-ordination of immune response via stimulation of T and B cell proliferation and differentiation
T_C (CD3+CD8+)	30-40% of T cells	Cytotoxic T cells - destruction of various foreign targets and some tumour cells Suppression of the immune response
Memory/recently activated T cells (CD3+CD45RO+)	Variable – dependent upon health and age	Divided into: Central memory T cells – mounts recall response to antigen, rapidly proliferating and differentiating into effector T cells Effector memory T cells – rapid production of effector cytokines upon antigenic challenge

Table 1. continued

Subset	Percentages	Key Functions/Characteristics
Naïve and inactivated T cells (CD3+CD45RA+)	Variable – dependent upon health and age	Reside in secondary lymphoid organs such as lymph nodes and spleen Express L-selectin (CD62L), CC chemokine receptor 7 (CCR7), and leukocyte function antigen-1 (the αLβ2 integrin LFA-1)
B cells (CD19+CD20+CD22+)	5-15% of lymphocytes	Production and secretion of antigen-specific antibodies- antigen-specific memory
NK cells (CD3-CD16+CD56+)	5-20% of lymphocytes	Acute cytolytic activity against infected cells

CD = clusters of differentiation; **MHC** = major histocompatibility complex; **NK** = natural killer; **T_H** = helper T cell; **T_C** = cytotoxic T cell. Adapted from (Gleeson & Bosch, 2013)

The immunological benefits of exercise exist along an inverted 'U' response. Extended bouts of sedentary behaviour increase morbidity and mortality rates (Arem, Moore, Patel, & et al., 2015; Kanneganti & Dixit, 2012; Kraus et al., 2015; Sedentary Behaviour Research, 2012). At the other extreme, long duration and/or prolonged high-intensity exercise training represents a point of diminished returns, decreased immune function, and increased inflammation (Bonini et al., 2015; Gleeson & Walsh, 2012; Meeusen et al., 2013; Turner et al., 2013; Turner, Bosch, & Aldred, 2011).

The magnitude and direction of the exercise immune response is influenced by exercise type, intensity, duration, participant age, fitness level, and nutritional status (Woods, Vieira, & Keylock, 2006). The resultant physiological mechanisms that modulate the immune response include body temperature changes, increased blood flow, changes in fluid balance, lymphocyte apoptosis (Nieman, 2007), altered glutamine metabolism (Parry-Billings et al., 1992; Parry-Billings, Leighton, Dimitriadis, Bond, & Newsholme, 1990), and changes in stress hormones (Nieman, 2007; Ortega, 2003).

1.2.2 Exercise and Molecular Mechanisms of Adaptation

Exercise and Gene Expression

Changes in physiological phenotypes are mediated by adaptive changes in the activity and abundance of key associated proteins (Neufer et al., 2015). This in turn is a function of differential regulation of gene transcription, protein translation, and post-translational modifications. Exercise transiently disrupts metabolic homeostasis, activating transcription factors which in turn bind to regulatory sequences within target gene promotor regions. This signals increased

messenger RNA (mRNA) expression. Exercise-induced phenotypic changes are due to the cumulative and overlapping effects of acute mRNA modulation (Neufer & Dohm, 1993; Perry et al., 2010).

Exercise-induced increases in protein reflect a dynamic balance between the half-life of the protein, mRNA stability, and the transient change in gene expression between exercise bouts (Neufer et al., 2015). Proteins with a rapid turnover rate typically have low basal expression but increase significantly and acutely in response to exercise before rapidly returning to basal levels post-exercise. Slow turnover proteins typically display higher basal expression and are only minimally influenced by an exercise bout; however, typically remain elevated between exercise bouts (Booth & Neufer, 2012).

Genes can be temporally categorized as either primary response genes (PRGs), immediate-early gene (IEG) expression, delayed PRGs or secondary response genes (SRGs) (Bahrami & Drabløs, 2016). PRGs rapidly respond to cellular signals and are associated with a wide range of signalling pathways. Many PRGs encode transcription factors which in turn modulate secondary response genes (Winkles, 1997). PRGs can be further classified as either immediate early genes (IEGs) or delayed PRGs (Bahrami & Drabløs, 2016).

Immediate Early Gene Expression

Immediate early genes (IEGs) exhibit rapid and transient transcriptional responses shortly after stimulation (Simon, Fehrenbach, & Niess, 2006). The mRNA of IEGs is transcribed rapidly, even in the presence of protein synthesis inhibitors, indicating that the requisite proteins already exist within the cell (Herschman, 1991; Morgan & Curran, 1991). IEGs are shorter in length than most

other genes, measuring on average 19 kilobases (kb) versus 58 kb. They also possess fewer exons; however, they typically have more CpG islands and TATA boxes. IEGs also exhibit increased prevalence of specific transcription factor binding sites including nuclear factor kappa B (NF-κB), serum response factor (SRF) and cyclic AMP response element-binding protein (CREB) (Healy, Khan, & Davie, 2013). Expression of the critical immediate early FOS gene peaks 30-60 min after stimulation, resolving to basal concentration after 90 min (Greenberg & Ziff, 1984). The nature and amplitude of IEG expression varies according to the intensity, type, and duration of the exercise stimulus. IEG expression is required to elicit protein neo-synthesis and the downstream activation of late-response genes (Watson & Clements, 1980). A role for microRNAs in the rapid post-stimulation downregulation of IEGs has recently been posited (Aitken et al., 2015; Avraham et al., 2010).

Delayed primary response genes are similar to primary response genes in that many do not require *de novo* protein synthesis; however, their induction can be delayed and many exhibit different structure and function (Tullai et al., 2007). Secondary response genes are also responsive to signalling; however, they require *de novo* protein synthesis and therefore exhibit a slower transcriptional time course (Herschman, 1991; Serrat et al., 2014).

FOS and JUN are two established IEGs which collectively influence cellular differentiation, proliferation, and survival (Healy et al., 2013; O'Donnell, Odrowaz, & Sharrocks, 2012). The post-exercise increases in leukocyte heat shock proteins (HSPs) is another example of an IEG response (Pirkkala, Nykanen, & Sistonen, 2001). HSPs influence pro-inflammatory cytokine release, protection from DNA damage, and stimulation of innate and adaptive immune responses (Simon et al.,

2006). There are also numerous signalling pathways that regulate IEG protein expression via phosphorylation and activation, including RhoA-actiin, p38 MAPK, P13K, and ERK (Bahrami & Drabløs, 2016).

Connolly et al. investigated the peripheral blood mononuclear cell (PBMC) gene expression immediately after, and 1 hour after a 30 min treadmill run at 80% of $\dot{V}O_{2max}$ (Connolly et al., 2004). The 30 min exercise intervention was well suited to characterize IEG expression without potentially confounding expression of later responding genes. Additionally, most IEGs would have resolved to basal expression by the 1 hour post-exercise measurement. A total 311 genes were differentially regulated immediately after 30 min of exercise and 552 were differentially regulated 1 hour post-exercise.

Exercise-induced gene expression has been investigated in a range of tissues including skeletal muscle (Febbraio & Koukoulas, 2000; Kraniou, Cameron-Smith, Misso, Collier, & Hargreaves, 2000; Louis, Raue, Yang, Jemiolo, & Trappe, 2007; Pilegaard, Ordway, Saltin, & Neufer, 2000; Pilegaard, Saltin, & Neufer, 2003; Tunstall et al., 2002), and white blood cells (Booth, Chakravarthy, & Spangenburg, 2002; Büttner, Mosig, Lechtermann, Funke, & Mooren, 2007; Connolly et al., 2004; Nakamura et al., 2010; Radom-Aizik, Zaldivar, Leu, & Cooper, 2009; Radom-Aizik, Zaldivar, Leu, Galassetti, & Cooper, 2008; Whistler, Jones, Unger, & Vernon, 2005; Zieker et al., 2005). Exercise-induced modulation has been observed in genes associated with lipid and glucose metabolism (Arkinstall, Tunstall, Cameron-Smith, & Hawley, 2004; Christensen et al., 2013; Jeppesen et al., 2012), skeletal muscle growth and function (Holloway et al., 2009; Keller et al., 2011; McGee, Sparling, Olson, & Hargreaves, 2005; Pilegaard et al., 2003; Timmons et al., 2005), and

mitochondrial function (Leick, Lyngby, Wojtasewski, & Pilegaard, 2010; Tienen et al., 2012).

Advances in transcriptomic technologies such as next generation RNA-seq allows for the characterization of all RNA transcripts produced by the genome (known as the transcriptome) in a specific cell type under a given set of conditions. Specific exercise-transcriptomic networks have been associated with aging (Raue et al., 2012), frailty (Hangelbroek et al., 2016), immune activation (Gordon et al., 2012), aerobic training modes (Lundberg, Fernandez-Gonzalo, Tesch, Rullman, & Gustafsson, 2016), skeletal muscle remodelling (Neubauer et al., 2013) and metabolic responsiveness (Böhm et al., 2016). Whilst the exercise response has been widely studied in tissues with metabolic and biomechanical roles, comparatively little is known about the acute responses of tissues that are not primary targets of exercise such as immune cells (Neufer et al., 2015).

Exercise and epigenetics

Gene expression is subject to additional post-genomic or epigenetic regulatory processes. The term *epigenetics* broadly describes the transient modifications, occurring independently of nucleotide sequences that determine the extent of gene expression. Acute and chronic alterations in physiological environment, such as exercise, can induce changes in chromosomal regions and the subsequent expression of genes there within. The three major epigenetic mechanisms are: (i) methylation of DNA cytosine residues, (ii) histone post-translational modifications (e.g. phosphorylation, methylation, acetylation), and (iii) transcriptional regulation by microRNAs (miRNAs) (Bernstein, Meissner, & Lander, 2007; Goldberg, Allis, & Bernstein, 2007; Jenuwein & Allis, 2001).

Histone modification and DNA methylation determine the conformational state of the DNA as either opened (euchromatin) or closed (heterochromatin). The conformation determines access to the DNA for repair, transcription or replication. Histone post-translational modifications are largely reversible alterations on the lysine-rich tail region histones, particularly H3 and H4 histones. Acetyl groups are added to and removed from DNA by histone acetyltransferases (HATs) and histone deacetylases (HDACs) respectively, thereby regulating the level of transcriptional activation (McGee & Hargreaves, 2011; McKinsey, Zhang, & Olson, 2001). The methylation of DNA cytosine involves the addition of a methyl group at the 5' position of the cytosine base (Irizarry et al., 2009; Jones & Baylin, 2007).

1.2.3 MicroRNA

MicroRNAs are short non-coding RNA molecules ~21 nucleotides (nt) in length (He & Hannon, 2004). They are found in skeletal muscle, circulating in plasma/serum, urine, milk, and saliva (Kosaka et al., 2010). Approximately 2000 miR sequences have been identified in the human genome resulting in more than 50,000 miR/mRNA interactions (Santulli, 2015). They play diverse roles in cell proliferation, differentiation, development, apoptosis, and metabolic pathways (Kozomara & Griffiths-Jones, 2013).

MicroRNA Biogenesis

MiRNAs can be transcribed from protein coding genes (intragenic) or non-coding regions (intergenic) (Bartel, 2004). Mature miRNAs are produced from long primary miRNA transcripts (pri-miRNAs) transcribed from the genome by RNA polymerase II (figure 2). Pre-miRNAs can encode individual miRNAs (monocistronic) or multiple miRNAs (polycistronic) (Snyder, Ahmed, & Steel,

2009). The pri-miRNAs are processed by the RNAse III enzyme Drosha and its RNA-binding protein co-factor pasha DiGeorge Syndrome Critical Region 8 (DGCR8, referred to as Pasha in invertebrates) into shorter hairpin structures (60-100 bases) known as precursor miRNAs (pre-miRNAs) (Bartel, 2004). The pre-miRNAs are transported into the cytoplasm by the Ran-GTP-dependent shuttle nuclear transport receptor Exportin-5 (XP05) and cleaved by the RNase III enzyme Dicer into miRNA: miRNA* duplexes. From this duplex, a mature miRNA of approximately 15-22 bases is incorporated into the argonaute protein within the RNA-induced silencing complex (RISC). One of the double strands of the mature miRNA is then selected on the basis of thermodynamic stability of the 5' end (Siomi & Siomi, 2009). This guide strand is integrated into the RISC complex providing a template to locate the complementary motifs in the 3' – UTR of the target mRNA (Fabian & Sonenberg, 2012; von Brandenstein, Richter, & Fries, 2012). The subsequent miR/mRNA interaction either inhibits translation of the target mRNA protein or promotes target mRNA degradation (Kallen, Ma, & Huang, 2012; Papait, Kunderfranco, Stirparo, Latronico, & Condorelli, 2013). The remaining 'passenger' miRNA (denoted by miRNA*) was initially considered transcriptionally inert however recent research suggests functional roles for miRNA* strands (Okamura et al., 2008; Yang et al., 2011).

Gene Regulation by miRNA

It is estimated that miRNAs control the transcriptional activity of ~50% of human protein-coding genes (Friedman, Farh, Burge, & Bartel, 2009). This is achieved via translational repression or total degradation of the target mRNA (Lytle, Yario, & Steitz, 2007). MiRNAs regulate gene expression by sequence-specific binding to the 3'UTR and occasionally within the 5'UTR of target mRNA

sequences (Lee et al., 2009; Lytle et al., 2007; Ørom, Nielsen, & Lund, 2008). The complementarity between the mature miRNA and the putative target determines the mechanism of miRNA-mediated gene silencing. Partial complementarity can elicit miRNA-mediated inhibition of mRNA translation (Huntzinger & Izaurralde, 2011). The partial complementarity with mRNA and relative short length of the miRNA creates hundreds of potential mRNA targets (Bartel, 2009). Therefore, suppression of a single miRNA does not always affect the target protein. Additionally, each miRNA can utilize numerous binding sites within the same mRNA (Friedman et al., 2009). MiRNA-mediated gene regulation can also occur via target mRNA degradation whereby targets are deadenylated and de-capped by specific deadenylases and de-capping enzymes (Fabian & Sonenberg, 2012; Huntzinger & Izaurralde, 2011). The de-capped mRNAs and then degraded by cytoplasmic 5'-3' exoribonuclease (Fabian & Sonenberg, 2012).

MiRNAs can also play key roles in upregulation of mRNA translation (Henke et al., 2008; Vasudevan, Tong, & Steitz, 2007), signal transduction (Fabbri, Paone, Calore, Galli, & Croce, 2013; Fabbri et al., 2012; Lehmann et al., 2012), and silencing of transcriptional genes (Benhamed, Herbig, Ye, Dejean, & Bischof, 2012; Kim, Sætrom, Snøve, & Rossi, 2008). The quantitative effects on individual target protein expression are usually small, typically less than 2-fold (Baek et al., 2008). Despite that, miRNAs promote transcriptional rigour by buffering against variations in gene expression and extrinsic noise such as variations caused by differences such as ribosome concentration or transcription factor, correcting splicing errors and spatial control of mRNA expression (Ebert & Sharp, 2012).

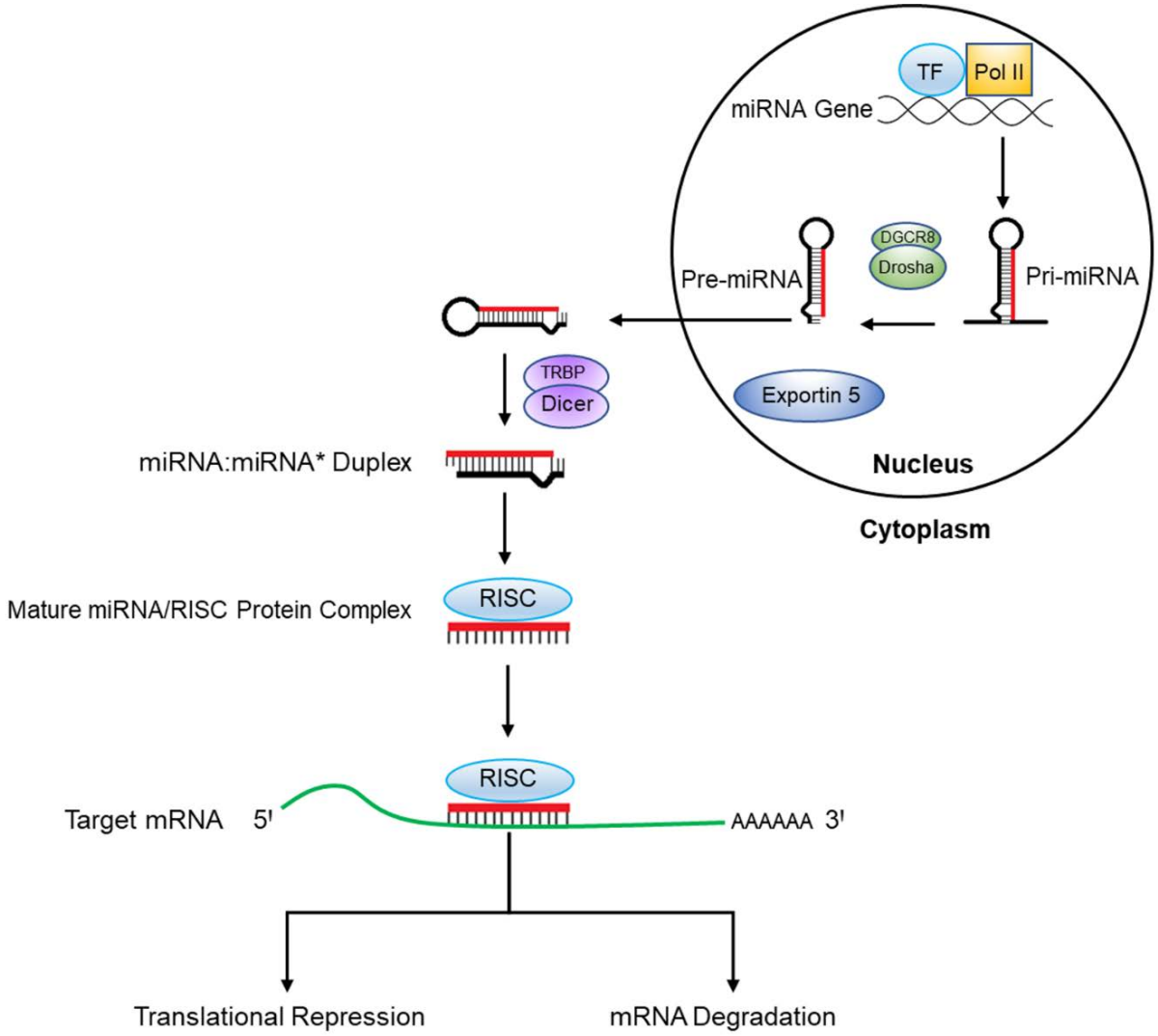


Figure 2. MicroRNA biogenesis: RNA polymerase II and specific transcription factors transcribe miRNA genes. The resultant long primary transcripts (pri-miRNA) are processed by the Drosha/DGCR8 complex into pre-miRNAs and exported by Exportin 5 into the cytoplasm. The pre-miRNAs are processed by the Dicer/TRBP complex into a guide strand (miRNA) and a passenger strand (miRNA*). The mature miRNA is assembled into the RISC complex where it uses sequence complementarity to recognize target mRNAs. The RISC complex can modulate gene expression via inhibition of translation or destabilization of target mRNAs. Adapted from (Gurtan & Sharp, 2013).

Exercise and miRNA

MiRNA expression plays important roles in acute exercise (Baggish et al., 2011; Cui et al., 2015; Cui et al., 2016; Radom-Aizik, Zaldivar, Oliver, Galassetti, & Cooper, 2010; Russell et al., 2013; Safdar, Abadi, Akhtar, Hettinga, & Tarnopolsky, 2009) and chronic endurance exercise (Aoi et al., 2013; Nielsen et al., 2014; Nielsen et al., 2010). MiRNAs are critical mediators of exercise-induced adaptive processes including inflammation (Davidson-Moncada, Papavasiliou, & Tam, 2010), cardiac and skeletal muscle contraction and hypertrophy (Davidsen et al., 2011; Williams, Liu, Van Rooij, & Olson, 2009), mitochondrial metabolism (Chan et al., 2009; Dang, 2010), and angiogenesis (Zhang, 2010).

Exercise-induced miRNA expression appears sensitive to exercise modality including resistance training (Davidsen et al., 2011; Mueller et al., 2011; Sawada et al., 2013), rowing (Baggish et al., 2011), running (Baggish et al., 2014; Clauss et al., 2016; de Gonzalo-Calvo et al., 2015; Gomes et al., 2014; Mooren, Viereck, Krüger, & Thum, 2014), walking (Banzet et al., 2013) treadmill exercise (Guescini et al., 2015; Tonevitsky et al., 2013), cycling (Aoi et al., 2013; Cui et al., 2015; Cui et al., 2016), and swimming (Fernandes et al., 2011; Melo et al., 2014). Specific miRNAs are altered by sustained aerobic exercise (miR-20a), acute exhaustive exercise (miR-21 and miR-221) or both (miR-146a and miR-222). Other miRNAs are largely unaffected by aerobic exercise (miR-133a, miR-210, miR-328) but respond to resistance training (miR-133) (Baggish et al., 2011).

Exercise-induced miRNA expression also demonstrates specificity for tissue type and has been investigated in skeletal muscle (Allen et al., 2009; Aoi & Sakuma, 2014; Drummond, McCarthy, Fry, Esser, & Rasmussen, 2008; Güller & Russell, 2010; Jeng et al., 2009; Keller et al., 2011; McCarthy & Esser, 2007;

McCarthy, Esser, Peterson, & Dupont-Versteegden, 2009; Mueller et al., 2011; Nielsen et al., 2010; Ringholm et al., 2011; Safdar et al., 2009), plasma (Baggish et al., 2011; Boon & Vickers, 2013; Bye et al., 2013; Da Silva et al., 2012; Nielsen et al., 2014; Sawada et al., 2013; Uhlemann et al., 2014), and various immune cell populations (Radom-Aizik, Zaldivar, Haddad, & Cooper, 2013; Radom-Aizik et al., 2010; Radom-Aizik et al., 2012; Radom-Aizik, Zaldivar Jr, Haddad, & Cooper, 2014; Tonevitsky et al., 2013).

Summary of Established Mechanisms

There is substantive evidence that habitual exercise elicits a myriad of positive multi-system effects with few, if any, negative side effects. Evolving technology has identified complex molecular pathways via which exercise exerts its prophylactic and potentially regenerative effects on human systems. Habitual exercise upregulates hundreds of genes in skeletal muscle and other tissues and can simultaneously exert epigenetic influence over their expression.

Whilst the immune system is not typically viewed as a first/primary target of the exercise stimulus, it has strong and multidirectional interactions with many other physiological systems. Immune cells can secrete and respond to cytokines, they have hormone receptors and can metabolise glucose and amino acids (Rosa Neto, Lira, de Mello, & Santos, 2011). Additionally, immune cells are dynamic and permeate every tissue in the body and can therefore influence and reflect the variant environments within the body.

Telomeres, the focus of the second part of this review, are increasingly viewed as critical biomarkers and mediators in biological aging and disease. There is a growing body of observational evidence associating habitual PA with longer

leukocyte telomere length (LTL) (Cherkas et al., 2008; Du et al., 2012; Kim, Ko, Lee, Lim, & Bang, 2012; Ludlow et al., 2008; Mirabello et al., 2009). There are however, considerable inconsistencies regarding the effects of long-term exercise on telomere length (Bekaert et al., 2007; Denham, 2016; Farzaneh-Far, Lin, Epel, Lapham, et al., 2010; Garcia-Calzon et al., 2014; Kadi et al., 2008; Laye et al., 2012; Mathur et al., 2013; Ponsot, Lexell, & Kadi, 2008; Rae et al., 2010; Song et al., 2010; Sun et al., 2012; Woo, Tang, & Leung, 2008). To unequivocally determine causation, physiological mechanisms must be characterized.

The protective effect of habitual PA is almost twice that predicted by traditional risk factor reduction; leaving approximately 50% of the protective effect unexplained (Joyner & Green, 2009; Neufer et al., 2015). It is here that exercise-induced telomere maintenance may offer an explanatory mechanism through influence on cellular senescence, preservation of immune function and reductions in inflammation.

1.3 Telomeres

Telomeres are specialized DNA sequences (5'-TTAGGG_n-3') located at chromosomal ends where they protect the gene-coding regions of DNA from enzymatic degradation (Blackburn, 2000). Telomeres progressively shorten by 30 to 200 base pairs (bps) with each round of mitotic division due to a phenomenon called the end replication problem. The DNA polymerase, which synthesises in the 5' to 3' direction, is unable to completely replicate the G-rich telomere ends due to the removal of the last 5' RNA primer (Harley, Futcher, & Greider, 1990; Levy, Allsopp, Futcher, Greider, & Harley, 1992; Vaziri et al., 1993). Accordingly, telomeres progressively shorten to a threshold known as the Hayflick limit after

approximately 50 population doublings. Beyond this limit, critically shortened telomeres are identified as double strand breaks (DSBs) (Fagagna et al., 2003; Takai, Smogorzewska, & de Lange, 2003). The progressive erosion of telomere repeats imposes a replicative limit upon primary cells and simultaneously chronicles their replicative histories. By enforcing a replicative limit on cells, telomeres limit the proliferation and malignant transformation of cells that have sustained DNA damage. However, in instances of accelerated shortening, senescence cells can accumulate within various tissues resulting in dysfunction.

Structure and Function

Vertebrate telomeres contain a double stranded component bound by histone and non-histone protein complexes. A guanine-rich (G-rich), single strand overhang of 75-200 bps is located at the 3' end of both chromosome ends (Rhodes, Fairall, Simonsson, Court, & Chapman, 2002). The double stranded and single-stranded components fold back upon themselves binding internally with telomere-associated proteins forming two complex three dimensional loops, the D-loop and the T-loop (Griffith et al., 1999) (Figure 1).The D- and T-loops form a cap that: (i) prevents chromosomal ends from being misidentified as DSBs, (ii) protects gene-coding regions of DNA from enzymatic degradation, and (iii) prevents chromosomal end to end fusion during DNA replication (Deng & Chang, 2007).

The Shelterin Complex

Telomeric DNA is arrayed upon a scaffold of six interacting telomere-specific proteins known as the shelterin complex. Shelterin internally binds and protects telomeric DNA as subsequently regulates telomerase access to it (de Lange, 2005). Modifications in the composition and/or structure of this complex can significantly impact upon telomere length, structure, and function (Chan &

Blackburn, 2004; de Lange, 2005). The six key proteins are telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), protection of telomere 1 (POT1), repressor/activator protein 1 (RAP1, also referred to as TERF2 interacting protein - TERF2IP), adrenocortical dysplasia homolog (ACD, also referred to as TINT1/PTOP/PIP1) (TPP1), and TRF1-interacting nuclear factor 2 (TIN2) (de Lange, 2005) (Figure 4).

Telomere length is negatively regulated by TRF1, TRF2, and POT1 which modulate telomerase access to telomeric ends (Loayza & de Lange, 2003; Shore & Bianchi, 2009; Smith & de Lange, 2000; van Steensel & de Lange, 1997). TRF1 and TRF2 interact directly with telomeric DNA to determine telomere conformation, telomerase access, and regulate telomere length (Court, Chapman, Fairall, & Rhodes, 2005; Sfeir & de Lange, 2012; Sfeir et al., 2009; Smogorzewska et al., 2000). POT1 prevents inappropriate recognition as DNA damage by binding to the single-stranded telomere overhang (Nandakumar & Cech, 2013).

RAP1 is recruited to the telomere via TRF2 where it regulates telomere length (Li, Oestreich, & de Lange, 2000; O'Connor, Safari, Liu, Qin, & Songyang, 2004). It achieves this by preventing non-homologous end joining (Sarthy, Bae, Scrafford, & Baumann, 2009), telomere fragility (Martínez et al., 2009; Sfeir et al., 2009), and recombination (Martinez et al., 2010). RAP1 also plays a role in obesity protection via regulation of several key metabolic genes (Martínez et al., 2013). RAP1 also participates in cellular senescence regulation (Platt et al., 2013) and suppression of DNA damage responses (DDRs) (Palm & de Lange, 2008).

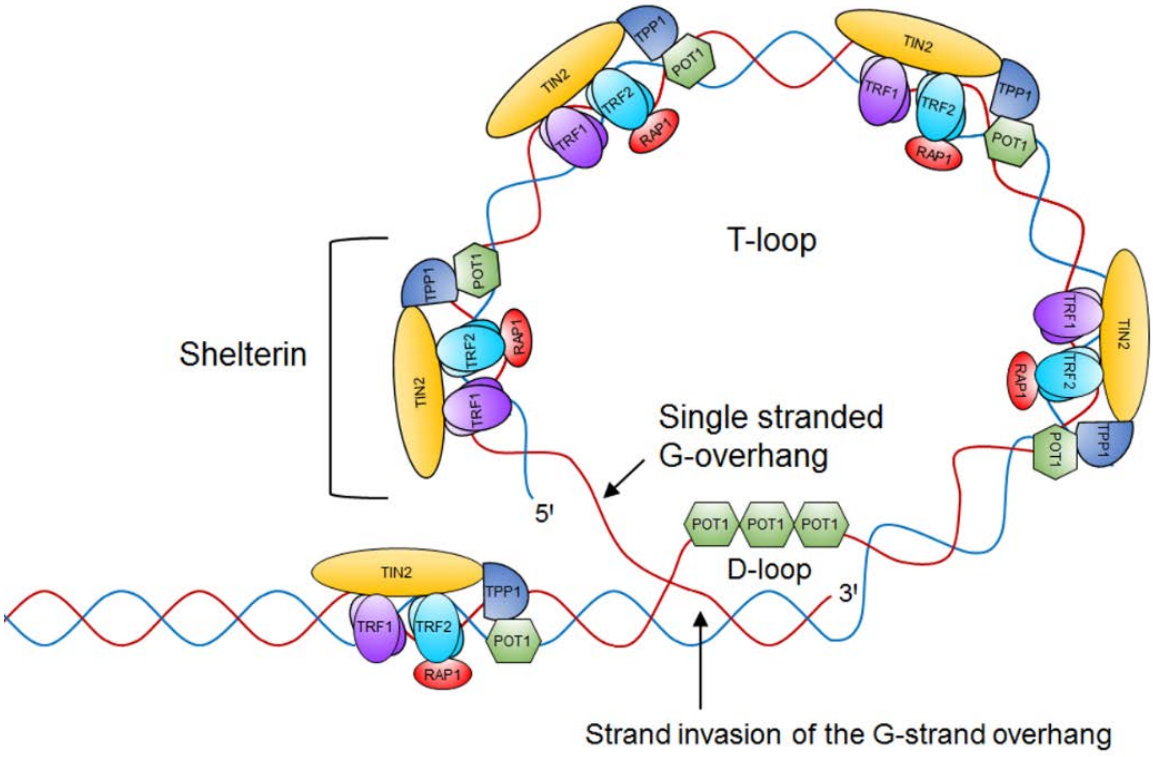


Figure 3. Vertebrate telomere structure: Telomeres terminate in a 75-200 nt single-stranded G-rich overhang which invades the double-stranded DNA, forming the T-loop and D-loop. Telomeric DNA is bound by the shelterin complex consisting of TRF1, TRF2, TIN2, RAP1, TPP1 and POT1. Adapted from (de Lange, 2005).

The CST Complex

The CST complex is a multi-protein complex consisting of CTC1, STN1, and TEN1 (figure 5). Localized to the single-stranded telomeric overhang (Miyake et al., 2009), the CST complex is critical for regulation of chromosomal end-capping and telomere length (Chen, Redon, & Lingner, 2012; Lin & Zakian, 1996; Wellinger, 2009). The critical pro-telomeric role is evidenced by the observation that CTC1 and STN1 mutations result in Coats Plus syndrome and dyskeratosis congenita (Anderson et al., 2012; Keller et al., 2012; Polvi et al., 2012; Simon et al., 2016; Walne et al., 2013). Accordingly, suppression of CST components results in telomere loss and increased frequency of telomere fragility (Gu et al., 2012; Huang, Dai, & Chai, 2012; Stewart et al., 2012).The CST complex also plays a key role in late S/G2-specific synthesis of telomeric C-strands, known as C-strand fill in (Gu et al., 2012; Huang et al., 2012; Stewart et al., 2012; Wang, Stewart, et al., 2012). Additionally, CST also appears to influence telomerase access to telomeric DNA by competing with the POT1/TPP1 complex (Chen et al., 2012).

Telomerase

Highly proliferative cell populations (sperm cells, stem cells, basal epidermal cell, and lymphocytes) maintain telomere length via the enzyme telomerase (Blackburn et al., 1989; Greider & Blackburn, 1987). Telomerase is an RNA-dependent DNA polymerase that reverse transcribes 6 bp telomeric repeats to the 3' end of genomic DNA during replication (figure 6). This compensation slows but does not prevent eventual telomere erosion (Blackburn et al., 1989; Greider & Blackburn, 1987). Telomerase is highly expressed in human somatic cells during embryonic development but then suppressed in most cells within a few weeks of birth (Oeseburg, de Boer, van Gilst, & van der Harst, 2009).

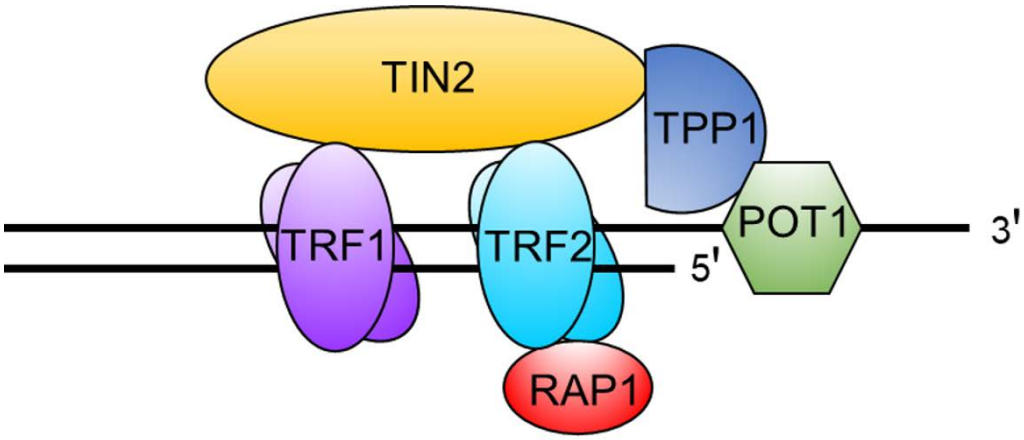


Figure 4. The shelterin complex*:* TRF1 and TRF2 directly interact with the double-stranded telomeric DNA. TPP1 and POT1 are incorporated into telomere via protein-protein interactions. POT1 binds to the single-stranded G-tail. Adapted from (Ishikawa, 2013).

Telomerase Structure

Telomerase consists minimally of two core components; a reverse transcriptase catalytic subunit (TERT) and an antisense RNA template (TER). Human TER (hTER) contains a complimentary sequence to telomeric DNA whilst human TERT (hTERT) is a catalytic reverse transcriptase. Whilst *hTER* expression is ubiquitous, transcriptional regulation of *hTERT* expression is the rate limiting component in telomerase activity (Cong, Wen, & Bacchetti, 1999; Horikawa, Cable, Afshari, & Barrett, 1999; Meyerson et al., 1997; Takakura et al., 1999). Expression of *hTERT* is sufficient to restore telomerase activity in telomerase-negative cells (Artandi et al., 2002; González-Suárez, Flores, & Blasco, 2002; González-Suárez et al., 2001; Qi et al., 2011; Stewart et al., 2002). Ectopic *hTERT* expression results in telomere elongation in endothelial cells, fibroblasts, and retinal pigment epithelial cells (Bodnar et al., 1998; Vaziri & Benchimol, 1998).

In addition to *hTERT* and *hTER*, telomerase also consists of the accessory dyskerin complex comprised of Dyskerin Pseudouridine Synthase 1 (DKC1), NHP2 ribonucleoprotein (NHP2), NOP10 Ribonucleoprotein (NOP10), and GAR1 Ribonucleoprotein (GAR1 proteins) (figure 7) (Mitchell, Wood, & Collins, 1999; Podlevsky & Chen, 2012).

Telomerase activity decreases with age but is upregulated in response to injury (Poss, Wilson, & Keating, 2002). A recent study demonstrated a significant increase in cardiomyocyte, endothelial cell, and fibroblast telomerase in injured murine heart tissue, positing a role for telomerase in tissue repair (Richardson et al., 2012). Inhibited or insufficient telomerase activity is associated with several telomere-mediated disorders including dyskeratosis congenita, idiopathic

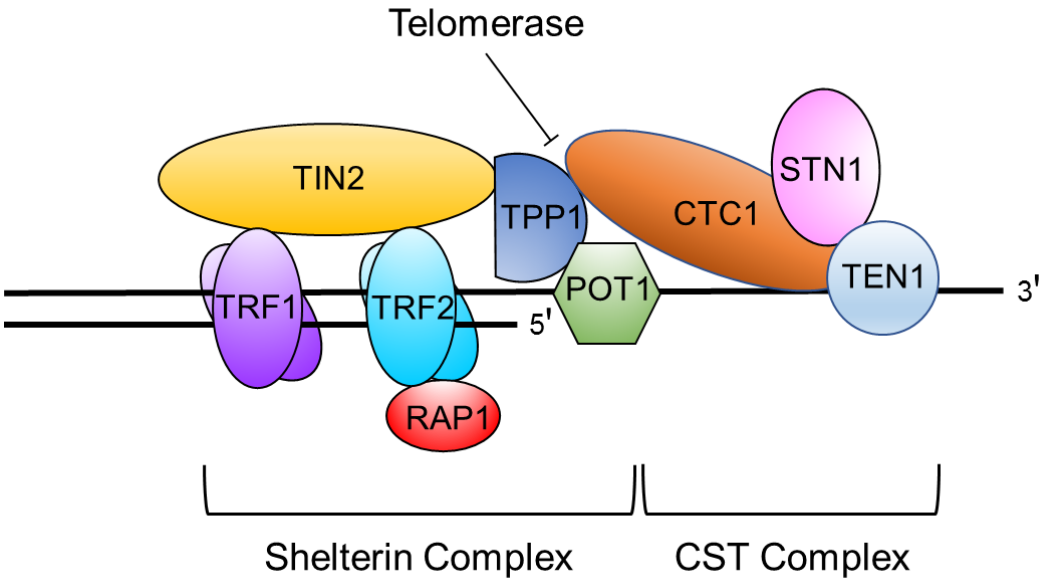


Figure 5. The CST complex: The CST complex binds with TPP1 of the shelterin complex, competitively blocking telomerase access to the 3′ end of the telomeric DNA. Adapted from (Rice & Skordalakes, 2016).

pulmonary fibrosis, and aplastic anaemia (Armanios & Blackburn, 2012; Armanios et al., 2007; Kirwan & Dokal, 2009; Nelson & Bertuch, 2012; Vulliamy, Marrone, Dokal, & Mason, 2002). Telomerase deficiency also precedes pathology in quiescent tissues, including insulin resistance, cardiomyopathy, and lung and liver fibrosis (Basel-Vanagaite et al., 2008; Leri et al., 2003).

Telomerase Regulation

Telomerase regulation consists of multiple levels of molecular control including transcription, mRNA splicing, maturation and modifications of *hTERT* and *hTER*, subcellular localization, and translocation of each core component (Cong, Wright, & Shay, 2002; Cukusić, Skrobot Vidacek, Sopta, & Rubelj, 2008). Various extra- and intracellular signals such as estrogen (Kyo et al., 1999), UV irradiation (Hande, Balajee, & Natarajan, 1997), and alpha interferon (Xu et al., 2000) are also known to regulate telomerase expression. Increases in the number of genes encoding for *hTERT* and *hTER* have been observed, resulting in increased enzyme activity in some circumstances (Bryce, Morrison, Hoare, Muir, & Keith, 2000; Saretzki, Petersen, Petersen, Kölble, & von Zglinicki, 2002; Soder et al., 1997; Yokoi et al., 2003).

Telomerase accesses telomeres via a negative feedback loop that preferences shorter telomeres. Longer telomeres integrate proportionally more shelterin making them less accessible to telomerase. Shorter telomeres are structurally more exposed and are therefore more likely to undergo telomerase-mediated lengthening (Teixeira, Arneric, Sperisen, & Lingner, 2004). This negative feedback loop promotes a telomere length equilibrium point within cells. Despite that, mitotic senescence is elicited *in vitro* by one (or a few) short telomeres, not the average length (Gilson & Londoño-Vallejo, 2007).

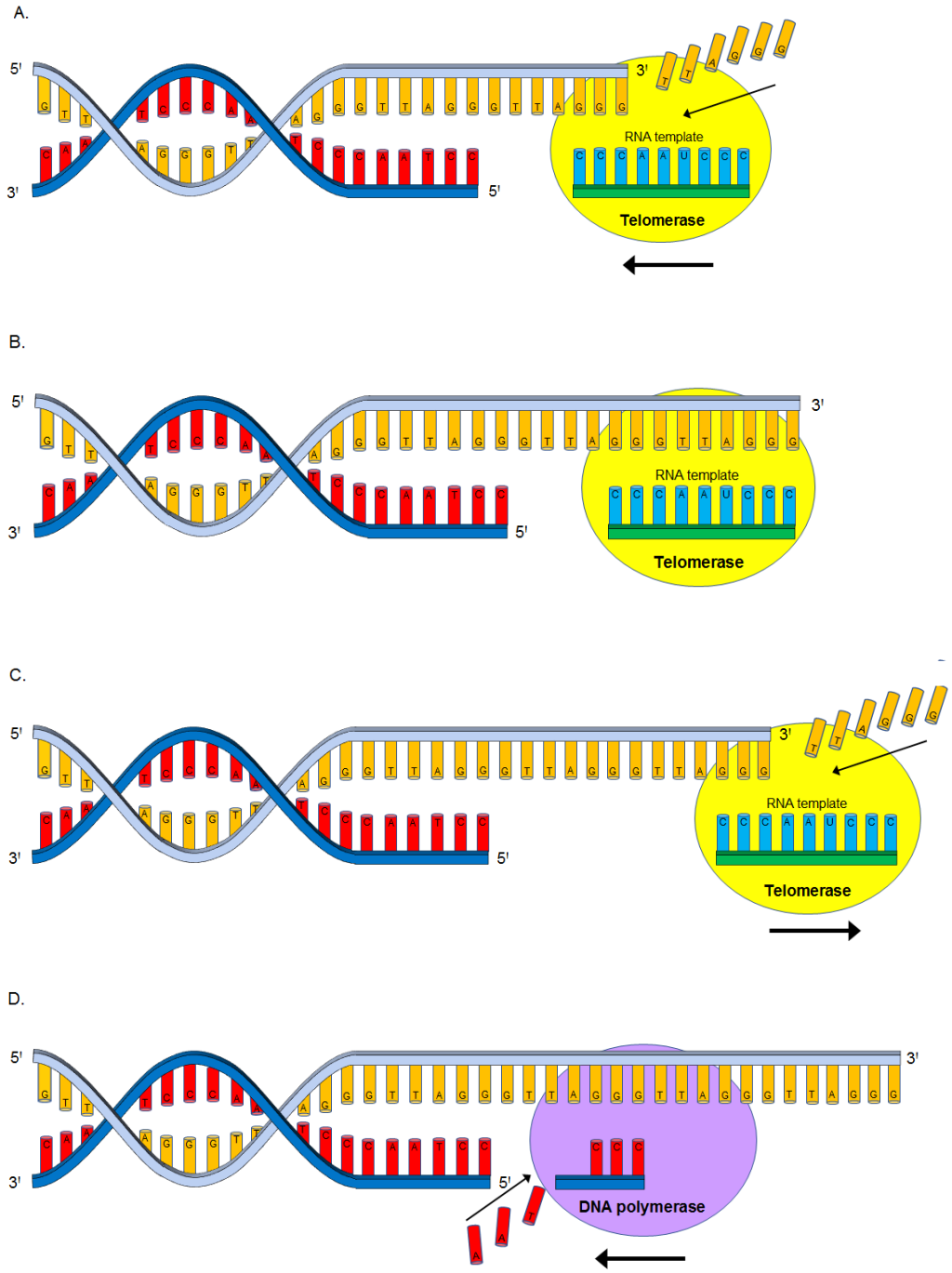


Figure 6. Telomerase-mediated telomere lengthening: The RNA template (TERC) component of telomerase binds to the 3 overhang **(A)**, complementary bases are added **(B)**, the telomerase complex moves further along the newly added bases and re-attaches **(C)**, and DNA polymerase extends an RNA primer to synthesize the complementary strand **(D)**.

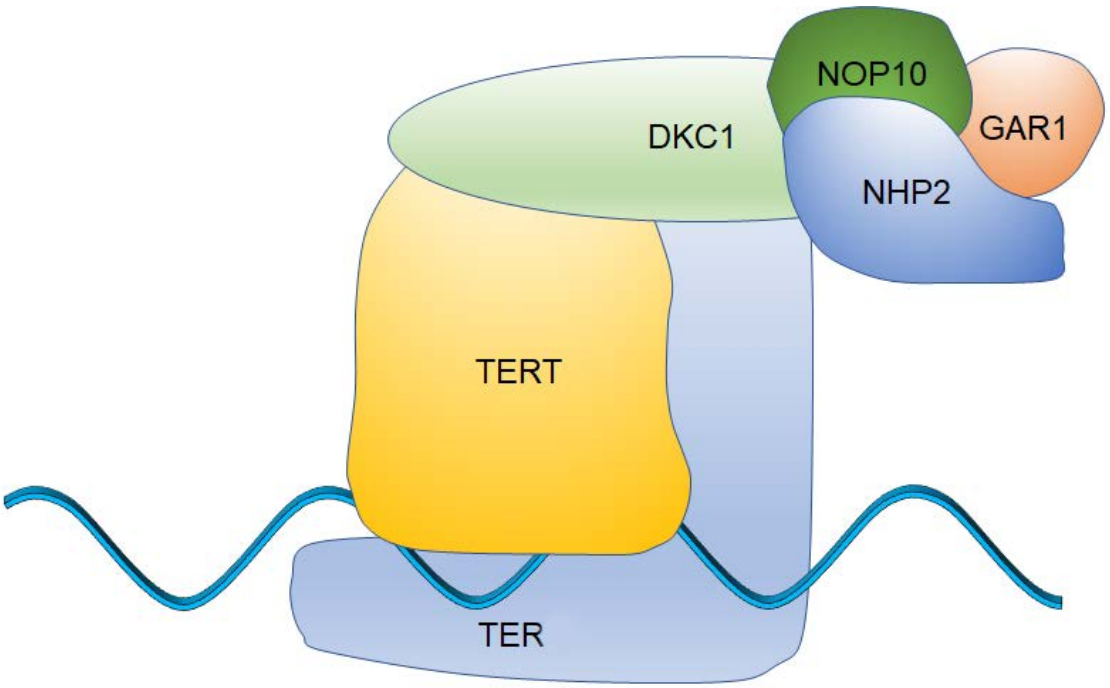


Figure 7. Telomerase structure: Human telomerase consists of the catalytic sub-unit hTERT, the RNA component (hTER), and accessory proteins DKC1, GAR1, NOP10, and NHP2. hTER binds directly to the telomeric G-rich overhang.

1.3.1 Telomere Dynamics

Telomere length is routinely measured in circulating blood leukocytes, a high yielding source of quality DNA that is easily accessed for epidemiological purposes. Mean LTL is highly variable at birth and throughout adult life, even within the same age group (Akkad et al., 2006; Factor-Litvak et al., 2016; Jeanclos et al., 2000; Nawrot, Staessen, Gardner, & Aviv, 2004; Vasan et al., 2008). Longest at birth, LTL rapidly shortens until adolescence and continues shortening (albeit at a slower rate) until old age (Sidorov, Kimura, Yashin, & Aviv, 2009).

Heritability estimates for telomere length range from 30%-80% (Blackburn, Epel, & Lin, 2015; Broer et al., 2013). Monozygotic twins exhibit very similar telomere length whereas dizygotic twins differ significantly (Slagboom, Droog, & Boomsma, 1994). This observation posits a potential genetic basis for telomere length; however, only a small group of putative candidate loci have been investigated (Andrew et al., 2006; Vasa-Nicotera et al., 2005). Genome-wide association studies have identified loci associated with LTL on chromosomes 18q12.2 and 3q26 (Levy et al., 2010; Mangino et al., 2012; Mangino et al., 2009); however, these findings only explain approximately 1.6% of LTL variation (Aviv, 2012; Codd et al., 2010; Mangino et al., 2009). Gene or promoter polymorphisms associated with telomerase complex proteins (Cohen et al., 2007) or the shelterin complex (de Lange, 2005) have also been proposed as a genetic determinants.

Telomere Attrition

Telomere attrition rates vary between individuals (Steenstrup et al., 2013), and cell types (Son, Murray, Yanovski, Hodes, & Weng, 2000). It is most pronounced from newborn to 4 years of age before gradually declining between

ages 4 to 39 years and remaining at a low and stable rate between the ages of 40 to 95 years (Frenck, Blackburn, & Shannon, 1998; Rufer et al., 1999). Within the lymphocyte subset, telomeres shorten by 35 ± 8 $bp^{-1} \cdot y^{-1}$ in CD4+ T cells, 26 ± 7 $bp^{-1} \cdot y^{-1}$ in CD8+ T cells and 19 ± 7 bp^{-1}/y^{-1} in CD19+ B cells (Son et al., 2000). Given an average starting length of 10-15 kb, even a yearly loss of approximately 50 bp should still sustain lymphocyte function well beyond 100 years of age. This supports the theory that the length of the shortest telomere, as opposed to the average telomere length, triggers cell cycle arrest, genomic instability, and senescence (Hemann, Strong, Hao, & Greider, 2001).

Endogenous Factors

The inter-individual variability in telomere length is partially due to differential exposure to deleterious instigators such as oxidative stress and inflammation (Aviv, 2004; De Meyer, Rietzschel, De Buyzere, Van Criekinge, & Bekaert, 2008; Houben, Moonen, van Schooten, & Hageman, 2008; Oikawa & Kawanishi, 1999; von Zglinicki, 2002). Telomeric DNA is particularly susceptible to oxidative damage due to the telomeric G triplet (Hewitt et al., 2012; Oikawa & Kawanishi, 1999; Von Zglinicki, 2000, 2002). This damage is largely irreparable (Fumagalli et al., 2012); therefore telomeres chronicle the cumulative exposure to oxidative stress (Woo, Suen, & Tang, 2010).

Chronic inflammation contributes to telomere shortening via induction of accelerated cell turnover, replicative senescence, induction of oxidative stress, and modulation of telomerase activity (Akiyama et al., 2004; Aviv, 2004; Jaiswal, LaRusso, Burgart, & Gores, 2000; Parish, Wu, & Effros, 2009; Xu et al., 2000). Shorter average telomere length may reflect an increased burden of senescent

cells which are known to create a pro-inflammatory phenotype (Coppé et al., 2008; Rodier et al., 2009).

Environmental Factors

Augmented inflammation and oxidative stress plausibly explain the inverse associations between telomere length and lifestyle factors such as smoking (Carnevali et al., 2003; Strandberg, Saijonmaa, Tilvis, Pitkälä, Strandberg, Miettinen, et al., 2011; Valdes et al., 2005), obesity (Strandberg, Saijonmaa, Tilvis, Pitkälä, Strandberg, Miettinen, et al., 2011; Valdes et al., 2005) , and alcohol intake (Comporti et al., 2010; Strandberg et al., 2012). Cumulative exposure to psychological stress throughout the lifespan has also been positively associated with increased oxidative stress and inflammation (Wolkowitz et al., 2011) and inversely associated with LTL (Epel et al., 2004; Kananen et al., 2010). Various psychological states are also associated with shortened telomeres (Lindqvist et al., 2015; O'Donovan et al., 2009) including depressive symptoms (Schutte & Malouff, 2015), schizophrenic symptoms (Polho, De-Paula, Cardillo, dos Santos, & Kerr, 2015), and dispositional traits such as pessimism (O'Donovan et al., 2009). Given the association between psychological stress and shortened telomeres, it is unsurprising that stress management techniques such as yoga and meditation are associated with increased telomerase expression in PBMCs (Schutte & Malouff, 2014).

Telomere Maintenance

Whilst it is unclear whether or not exercise can physically lengthen telomeres, habitual exercise is associated with longer LTL (Cherkas et al., 2008). In addition to self-reported PA and exercise, objectively measured aerobic fitness is associated with longer LTL in healthy populations (LaRocca, Seals, & Pierce,

2010) and pathological states (Krauss et al., 2011). The role of PA, exercise, and aerobic fitness in telomere maintenance is discussed at length elsewhere in this chapter.

Dietary factors such as increased marine ω-3 fatty acid intake (Farzaneh-Far, Lin, Epel, Harris, et al., 2010), increased blood concentrations of vitamin E, 25-hydroxyvitamin D , and vitamin C are associated with reduced LTL shortening (Richards et al., 2007; Xu et al., 2009). The underlying mechanisms are believed to be the modulation of oxidative stress (vitamins C and E) and inflammation (vitamin D) (Fyhrquist, Saijonmaa, & Strandberg, 2013). In a cohort of middle-aged men (aged 45-64 years), statin-mediated LDL-cholesterol reduction decreased the association between short LTL and increased CAD risk (Brouilette et al., 2007). Estrogen activates telomerase and therefore has a telomere-sparing effect (Kyo et al., 1999). This plausibly explains the longer telomeres observed in women.

Telomeric repeat-containing RNA - TERRA

Telomeres were traditionally considered transcriptionally silence; however, recent evidence indicates that sub-telomeric regions produce a class of long noncoding RNAs containing telomeric repeats called TERRA (Azzalin, Reichenbach, Khoriauli, Giulotto, & Lingner, 2007; Schoeftner & Blasco, 2010). TERRA molecules are transcribed from the sub-telomeric regions of chromosomes by RNA polymerase II (Azzalin et al., 2007; Schoeftner & Blasco, 2008, 2010). TERRA have been identified in several organisms and assist in telomere function and homeostasis via telomerase regulation, heterochromatin formation, and chromosomal capping (Cusanelli & Chartrand, 2015; Luke et al., 2008; Schoeftner & Blasco, 2008). TERRA associates with telomeres via several mechanisms including interactions with TRF1 and TRF2 (Deng et al., 2012).

TERRA molecules can also form RNA:DNA hybrid structures known as R-loops by base-pairing with their template DNA strands (Arora et al., 2014; Balk et al., 2013; Pfeiffer & Lingner, 2012; Yu, Kao, & Lin, 2014). The R-loops associated with G-rich sequences assist in gene expression (Ginno, Lott, Christensen, Korf, & Chédin, 2012) and transcription termination (Skourti-Stathaki, Proudfoot, & Gromak, 2011). Dysfunctional TERRA expression is associated with centromere instability, immunodeficiency, and the rare autosomal recessive immune disorder facial anomalies (ICF) syndrome (Xu et al., 1999).

TERRA plays a unique role in telomeric length regulation. TERRA is typically inversely associated with telomere length via mechanisms such as inhibition of telomerase activity (Schoeftner & Blasco, 2008), increasing euchromatin formation, and decreasing heterochromatin formation (Deng, Norseen, Wiedmer, Riethman, & Lieberman, 2009). Paradoxically, TERRA is also associated with protection of the telomere ends via the interaction with TRF2 (Poulet et al., 2012; Wang, Smogorzewska, & de Lange, 2004).

Telomeres and Cellular Senescence

Cellular senescence typically manifests in three different contexts in humans: normal aging, age-related disease, and therapeutic interventions (Childs, Durik, Baker, & van Deursen, 2015). Senescence can be classified as telomere-dependent (replicative senescence) or telomere-independent (stress-induced or cellular) senescence. Replicative senescence is the exhaustion of replicative capacity, characterized by telomere shortening (Fyhrquist et al., 2013). Stress-induced senescence is independent of telomeric shortening; triggered by external stimuli such as irradiation, oxidative stress, and mitogenic oncogenes (Blasco, 2005; Calado & Young, 2009).

A senescence response is typically triggered when the telomeric terminal restriction fragment (TRF) reaches a mean length of 4-7 kb (Campisi, 2001; Harley et al., 1990). At this critical threshold, the protective shelterin complex is disrupted and a DDR is triggered. This occurs via the phosphorylation of proteins H2A.X and NBS1 and kinases CHK1 and CHK2 by the phosphatidylinositol 3-kinase-like protein kinases ATM and ATR resulting in the downstream activation of p53 and p21 proteins (Rodier et al., 2009). If the DNA damage exceeds a repairable threshold, the cell undergoes apoptosis or senescence.

Cellular senescence serves several key functions, namely it prevents ongoing cellular replication after oncogene activation or critical telomere erosion (Campisi, Kim, Lim, & Rubio, 2001; Wright & Shay, 2001). Senescence also assists in tissue healing by preventing excessive cell proliferation and fibrotic matrix deposition (Jun & Lau, 2010; Krizhanovsky et al., 2008). However, this is thought to decrease the regenerative capacity of tissues in later life leading to an accumulation of senescent cells. In addition to lost replicative capacity, senescent cells exhibit a senescence-associated secretory phenotype (SASP). The SASP impacts tissue integrity by secreting a host of active peptides inflammatory cytokines, intercellular adhesion molecules, growth factors, and monocyte attractants (Campisi, 2011; Erusalimsky & Kurz, 2005).

Senescent cells also undergo changes in morphology, altered protein processing, metabolism, apoptosis resistance, nuclear structure, and gene expression (Bayreuther et al., 1988; Campisi, 2000; Narita et al., 2003; Sitte, Merker, von Zglinicki, & Grune, 2000; Von Zglinicki, 2000). Accumulating senescent cells contribute to proliferative and degenerative age-related changes by causing a chronic inflammation, remodelling, and tissue repair (Fyhrquist et al.,

2013). Accordingly, senescent cells are associated with age-related diseases such as atherosclerosis (Minamino & Komuro, 2007), diabetes (Sone & Kagawa, 2005), and appear to influence mammalian lifespan (Choudhury et al., 2007; Rudolph et al., 1999; Tyner et al., 2002).

Repeated antigenic challenges *in vivo* can cause telomere shortening and precipitate immune cell senescence. Critical telomere shortening in immune cells such as memory lymphocytes (CD45RO+) could negatively impact upon their ability to mount repeated immune responses (Reed et al., 2004). The accumulation of senescent immune cells can *crowd* the immune cell pool, restricting the development of new cells and impairing immune function (Pawelec, Adibzadeh, Pohla, & Schaudt, 1995).

1.3.2 Telomeres and Telomerase in the Immune System

Many human somatic cells lack detectable telomerase levels (Kim et al., 1994); however, immune cells are unique in their capacity to upregulate telomerase expression thereby reducing telomere attrition during periods of clonal expansion (Weng, 2002; Weng, Levine, June, & Hodes, 1997). Telomerase expression and telomere maintenance are critical to this proliferative capacity. Cell-specific telomere shortening has been observed in CD4+, CD8+ T lymphocytes, B lymphocytes, monocytes, granulocytes, and NK cell subsets (Kaszubowska, 2008). Telomere length has been rank-ordered as longest in B cells, followed by CD4+ and CD8+CD28+ T cells (similar lengths), and shortest in senescent CD8+CD28- T cells (Lin et al., 2010).

Accelerated telomere loss in lymphocytes contributes to the accelerated aging of the T cell pool and may predispose to autoimmune responses; potentially

explaining increased susceptibility to inflammatory diseases in the elderly (Kaszubowska, 2008). The loss of telomerase activity in senescent immune cells also parallels the loss of the major signalling molecule CD28, a hallmark of immune cell replicative senescence (Valenzuela & Effros, 2002).

Despite the heritability of telomere length and the innumerable lifestyle factors that influence it, telomerase expression independently modulates immune cell telomere stability, shortening trajectory, and lifespan (Boccardi & Paolisso, 2014). Telomerase expression is also heterogeneous within the immune system, being highest in B cells, followed by CD4+ T cells, CD8+CD28+ T cells and lowest in CD8+CD28- T cells (Lin et al., 2010). This mirrors age-associated telomere shortening, which is slower in B cells than T cells (Son et al., 2000). The physiological significance of telomerase is evidenced by the fact that ectopic *hTERT* expression extends the replicative lifespan of CD4+ and CD8+ T cells (Dagarag, Evazyan, Rao, & Effros, 2004; Luiten, Pene, Yssel, & Spits, 2003; Roth et al., 2003; Rufer et al., 2001).

Telomerase activity is very low or absent in unstimulated human T cells; however, activation induces high levels of telomerase activity but only a low to moderate increase in hTERT expression (Bodnar, Kim, Effros, & Chiu, 1996; Hiyama et al., 1995; Weng, Levine, June, & Hodes, 1996). In nonactivated CD4+ T cells, telomerase appears localized to the cytoplasm; however, hTERT protein is found in the nucleus of activated T cells (Liu, Hodes, & Weng, 2001). This activation-dependent nuclear localization of hTERT occurs in response to phosphorylation of hTERT via the PI3K/Akt pathway (Chung, Khadka, & Chung, 2012; Kawagoe et al., 2003; Kimura et al., 2004). The nuclear localization of hTERT results in the higher telomerase activity observed in activated T cells (Liu

et al., 2001). The nucleus is the site of telomerase activity as it contains the critical molecular chaperones heat shock protein (Hsp) 90 and p23, both of which are required for telomerase assembly (Forsythe, Jarvis, Turner, Elmore, & Holt, 2001; Holt et al., 1999). Importantly, binding of the molecular chaperone Hsp70 and C terminus of Hsc70-interacting protein (CHIP) to hTERT inhibits the nuclear translocation of hTERT by dissociating p23 (Lee, Khadka, Baek, & Chung, 2010).

Expression of hTERT is tightly regulated in human T cells by cytokines, intracellular or extracellular signalling and transcription factors (Barsov, 2011). IL-2 is a T cell growth factor that increases *hTERT* transcription in human T cells (Matsumura-Arioka, Ohtani, Hara, Iwanaga, & Nakamura, 2005) and post-translationally affects hTERT activity via the PI3K/Akt pathway (Kawauchi, Ihjima, & Yamada, 2005). IL-7 induces increased hTERT levels in naïve and memory T cells (Yang, An, & Weng, 2008), whilst IL-15 induces hTERT expression in memory CD8+ T cells via the Jak3 and PI3K signalling pathways (Li, Zhi, Wareski, & Weng, 2005).

Upregulation of hTERT can also be facilitated by specific signal transduction pathways such as the NF-κB pathway or the PI3K/Akt pathway. Protein kinase C (PKC) within the NF-κB pathway is essential in hTERT upregulation and post-transcriptional control of its enzymatic activity within stimulated human T cells (Sheng, Chien, & Wang, 2003). Akt kinase enhances telomerase activity through the phosphorylation and subsequent nuclear translocation of hTERT (Kawagoe et al., 2003; Kimura et al., 2004). More recent work identified serine residue 227 as the Akt phosphorylation site (Chung et al., 2012).

Transcription factors are thought to tightly regulate hTERT promoter activity. Nuclear factor of activated T cells (NFAT1) transcriptionally activates hTERT expression by binding to one of the NFAT1 binding sites within the hTERT promoter (Chebel et al., 2009). The hTERT promoter is also bound by Interferon regulatory factors (IRF)-4 and -8, resulting in transcriptional activation (Hrdličková, Nehyba, & Bose, 2009).

1.3.3 Telomeres in Health and Disease

Telomere Length and All-Cause Mortality

The link between telomere length and all-cause mortality is complex and at times equivocal. Most studies linking telomere length and all-cause mortality use circulating leukocytes as a physiological proxy for the target tissue. The conclusions drawn are predicated upon a supposed correlation between LTL and the target tissue. Correlations have been identified between LTL and vascular tissue telomere length (Wilson et al., 2008), muscle, skin and fat (Daniali et al., 2013) and synovial tissue (Friedrich et al., 2000). A more recent study found correlations with only two (intercostal skeletal muscle and liver) out of twelve human tissues assessed (Dlouha, Maluskova, Kralova Lesna, Lanska, & Hubacek, 2014). Differences in telomere length between intra-individual tissue types were thought to reflect tissue-specific attrition rates (Takubo et al., 2002); however, a more recent study correlated age-dependent telomere shortening rates between leukocytes, skeletal muscle, skin and subcutaneous fat (Daniali et al., 2013).

Age-adjusted inverse associations have been established between mean telomere length and all-cause mortality (Astrup et al., 2009; Bakaysa et al., 2007; Cawthon, Smith, O'Brien, Sivatchenko, & Kerber, 2003; Deelen et al., 2014;

Ehrlenbach et al., 2009; Fitzpatrick et al., 2011; Glei, Goldman, Weinstein, & Risques, 2014; Honig, Kang, Schupf, Lee, & Mayeux, 2012; Kim et al., 2012; Lee et al., 2012; Martin-Ruiz et al., 2006; Rehkopf et al., 2013; Rode, Nordestgaard, & Bojesen, 2015; Strandberg, Saijonmaa, Tilvis, Pitkälä, Strandberg, Miettinen, et al., 2011; Weischer et al., 2012). However, other studies have failed to replicate the association (Arai et al., 2015; Bendix et al., 2014; Bischoff et al., 2006; Epel et al., 2009; Fitzpatrick et al., 2007; Harris et al., 2006; Houben, Giltay, Rius-Ottenheim, Hageman, & Kromhout, 2010; Kimura et al., 2008; Martin-Ruiz, Gussekloo, van Heemst, von Zglinicki, & Westendorp, 2005; Njajou et al., 2009; Svensson et al., 2014).

Telomere Length and CVD

A host of CVD risk factors have been observationally associated with shortened LTL including smoking (Valdes et al., 2005), diabetes (Sampson, Winterbone, Hughes, Dozio, & Hughes, 2006), hypercholesterolemia (Strandberg, Saijonmaa, Tilvis, Pitkälä, Strandberg, Salomaa, et al., 2011), hypertension (Benetos et al., 2001), obesity (Müezzinler, Zaineddin, & Brenner, 2014), physical inactivity (Cherkas et al., 2008), alcohol consumption (Strandberg et al., 2012), and psychological issues (Cherkas et al., 2006). However, several other studies have since failed to replicate the association with key CVD risk factors (Bekaert et al., 2007; Bischoff et al., 2006; Fitzpatrick et al., 2007; Martin-Ruiz et al., 2005; Neuner et al., 2015).

Shorter LTL was initially associated with coronary artery disease (CAD) in 2001 (Samani, Boultby, Butler, Thompson, & Goodall, 2001); with similar CVD associations to follow (Brouilette, Singh, Thompson, Goodall, & Samani, 2003; Brouilette et al., 2007; Brouilette et al., 2008; Carty et al., 2015; D'Mello et al., 2015;

Ellehoj, Bendix, & Osler, 2016; Fitzpatrick et al., 2007; Haycock et al., 2014; Matthews et al., 2006; Willeit et al., 2010). However, three key studies have since failed to find any association between LTL and early atherosclerosis (De Meyer et al., 2009; Fernández-Alvira et al., 2016; Willeit et al., 2010). Furthermore, a recent study associated long, as opposed to short LTL with a nearly three-fold higher risk of developing myocardial infarction (Østhus, Lydersen, Dalen, Nauman, & Wisløff, 2017).

Further support for a plausible causal role for telomere shortening and CVD onset came from a series of genetic and observational prospective studies (Brouilette et al., 2007) (Broer et al., 2013; Hamad, Walter, & Rehkopf, 2016; Haycock et al., 2014; Madrid, Rode, Nordestgaard, & Bojesen, 2016). The West of Scotland Primary Prevention Study (WOSCOPS) showed that the lowest LTL tertile had a 44% increased CAD risk at a 5.5 year follow-up period (Brouilette et al., 2007). A recent genome wide association study identified seven single nucleotide polymorphisms (SNPs) associated with telomere length dysfunction, telomerase reduction, and replicative senescence (Blackburn et al., 2015). One such SNP is associated with decreased telomere length and increased CAD risk. One standard deviation decrease in telomere length was found to increase CAD risk by 21% (Codd et al., 2013).

Despite an abundance of conflicting evidence, the broader scientific consensus is that shortened LTL represents an increased risk of CVD and likely reflects accelerated leukocyte turnover due to oxidative stress and inflammation.

Telomere Length and Cancer

The relationship between telomere length and cancer is complex. A detailed review of the role of telomeres in cancer is beyond the scope of this review; what follows is a brief overview. Telomere shortening, uncapping (loss of shelterin integrity) and subsequent senescence are believed to play an anticancer role (Artandi & DePinho, 2010). Several studies have associated long LTL with increased risk of several cancer types (Anic et al., 2013; Iles et al., 2014; Julin et al., 2015; Lynch et al., 2013; Machiela et al., 2015; Nan et al., 2011; Pellatt et al., 2013; Qu et al., 2013; Sanchez-Espiridion et al., 2014; Seow et al., 2014). Paradoxically, shortened telomeres can potentiate cancer in some instances by fusing with other uncapped telomeres, thereby creating genome destabilizing fusion-bridge-breakage cycles (Artandi & DePinho, 2010). It is estimated that ~15% of human cancers maintain telomere length through one or more mechanisms referred to as Alternative Lengthening of Telomeres (ALT) (Novak, 2003). Telomerase activity is a critical component in malignant transformation with 85%–90% of all malignant tumours being telomerase positive (Kim et al., 1994; Shay & Bacchetti, 1997).

A cluster of seven alleles associated with LTL homeostasis, TER, TERT, oligonucleotide/oligosaccharide-binding fold containing one gene (OBFC1), zinc finger protein 208 (ZNF208), regulator of telomere elongation helicase 1 (RTEL1), acylphosphatase 2 (ACYP2) and nuclear assembly factor 1 ribonucleoprotein (NAF1) are simultaneously associated with CAD (Codd et al., 2013) and cancer (Iles et al., 2014; Machiela et al., 2015). If the alleles result in comparatively long telomeres, the cancer risk is elevated, and the CAD risk is reduced; the reverse is also true. It has been proposed that the cancer protection conferred by short

telomeres represents an evolutionary trade-off resulting in decreased proliferative and regenerative capacity (Williams, 1957). Non-linear U-shaped relationships have been observed between telomere length and several cancer risk profiles (Wang et al., 2014). This may in part be explained by the destabilizing and potentially oncogenic effects of shortened telomeres and the increased replicative capacity and potential accumulation of abnormalities associated with longer telomere length (Cesare & Reddel, 2010; Wu et al., 2003).

One proposed explanation for the discrepant associations is that telomere uncapping has an anti-cancer effect in the young but a potentially pro-cancer effect in the elderly (Yang, Song, & Johnson, 2016). Hypothetically, the more robust telomere dysfunction-based mechanism of the young would prevent tumorigenesis whilst the shortened, uncapped and depleted telomeres of the elderly may allow bypass of aberrant cells (Yang et al., 2016).

Correlations between LTL and other tissue types coupled with chronic disease associations strongly imply a causal story. Whilst some protective benefit of LTL can be inferred from the numerous associations, the extent to which restoration of telomere length restores tissue capacity and reduces disease burden is largely unknown. The significance of telomere biology in maintaining immune function is well established; however, it is unclear whether positive adaptations in LTL would reflect or promote similar changes in other tissue types. Whether dysfunctional expression of shelterin genes, hTERT or telomerase activity are causal or consequential in diseased or aging states are critical and largely unanswered questions.

The Epigenetics of Telomere Homeostasis

An accumulating body of evidence indicates that telomeric chromatin is subject to modifications. Murine studies show that telomeric and sub-telomeric chromatin exhibit histone modifications common to heterochromatin (García-Cao, O'Sullivan, Peters, Jenuwein, & Blasco, 2003; Gonzalo et al., 2005; Gonzalo et al., 2006). Whilst mammalian telomere repeats lack the CpG sequences (the substrates for mammalian methyltransferases) needed for methylation, sub-telomeric DNA can be methylated (Fraga et al., 2005; Tommerup, Dousmanis, & de Lange, 1994; van Overveld et al., 2003).

Histone Modifications

Histone and DNA modifications at pericentric heterochromatin result in chromosome segregation defects, potentially playing a role in tumour development (Peters et al., 2001). Telomeric DNA is subject to trimethylation of lysine 9 on histone 3 (H3K9) and of lysine 20 on histone 4 (H4K20) (Blasco, 2007). Heterochromatic disturbances appear to affect telomere length homeostasis by interacting with telomere regulators and/or by opening telomere configuration (Londoño-Vallejo, 2010). Cells exhibiting decreased levels of H3K9 trimethylation at telomeres also display abnormal telomere lengthening (García-Cao et al., 2003). Such heterochromatin disruption may alter telomere length via disruption of telomere-length regulators (Blasco, 2007).

Sub-telomeric DNA Methylation

Sub-telomeric DNA methylation appears to regulate telomere length independently of histone methylations (Blasco, 2007). Reductions in DNA methylation at sub-telomeric regions result in dramatically elongated telomeres (Gonzalo et al., 2006). Additionally, methylation of sub-telomeric DNA functions as

a repressor of homologous recombination at telomeres, plausibly regulating ALT (Blasco, 2007). No definitive link between sub-telomeric methylation and telomere binding proteins has been established. However, it remains a plausible hypothesis given altered expression of some telomere-binding proteins causes dysregulated telomere recombination (Blanco, Muñoz, Flores, Klatt, & Blasco, 2007; Wu et al., 2006).

Shelterin and Epigenetics

The shelterin components are subject to posttranslational modifications that modulate their localization, binding capacity, and stability (Peuscher & Jacobs, 2012; Walker & Zhu, 2012). If such posttranslational changes are present throughout mitosis or meiosis, they could be considered an epigenetic (heritable) change (Londoño-Vallejo, 2010). The best understood modifications are those occurring in *TRF1* and *TRF2*, specifically the poly-ADP-ribosylation of *TRF1* (Rippmann, Damm, & Schnapp, 2002). The poly-ADP-ribosylation of *TRF1* by tankyrase prevents the accumulation of TRF1 along the telomere length and subsequent blockade of telomerase access (Loayza & de Lange, 2003; Smith, Giriat, Schmitt, & de Lange, 1998). Phosphorylation of *TRF2* is another observed posttranslational modification however comparatively little is known about the underpinning mechanisms (Tanaka et al., 2005).

Little is known about exercise-induced changes in shelterin gene mRNA expression regulation, expression time course, and possible miRNA-mediated regulation. The association between regular physical activity and telomere length may in part be mediated by shelterin gene regulation, which itself may be partially subject to additional levels of regulation such as miRNA. A clearer understanding of the adaptive plasticity of shelterin and other telomeric genes is needed. Such an

understanding may lead to the development of therapeutic targets within telomere biology and may also inform exercise guidelines for healthy and pathological populations.

MicroRNA-dependent Regulation of Telomere Homeostasis

MicroRNAs represent a plausible regulator of telomere length and the list of putative candidates is steadily growing (table 2). A potential role for miRNA in telomere homeostasis was first observed in a 2008 study that associated miR-138 expression with telomerase activity in anaplastic thyroid carcinoma (ATC) (Mitomo et al., 2008). *In silico* analysis initially predicted *hTERT* as a regulatory target of miR-138 and a subsequent luciferase reporter assay in human embryonic kidney (HEK)-293 cells and measurement of hTERT protein confirmed the interaction. A potential role for miR-138 in post-transcriptional regulation has been proposed given that *hTERT* mRNA levels were not affected by miR-138 overexpression (Mitomo et al., 2008). Depletion of *hTERT* led to increased expression of miR-138 in human malignant neuroblastoma cells (Chakrabarti, Banik, & Ray, 2013).

A 2014 study identified 14 differentially specifically expressed miRNAs in gastric tumour tissue (Chen et al., 2014). Five of the fourteen miRNAs identified (miR-138, miR-491-5p, miR-1182, miR-1207-5p, and miR-126) reduced *hTERT* expression when their expression levels were restored via a miRNA mimic (Chen et al., 2014). *In silico* analyses and subsequent luciferase reporter assays confirmed let-7g*, miR-133a, miR-138-5p, and miR-491-5p as *hTERT* regulators (Hrdličková, Nehyba, Bargmann, & Bose, 2014).

Overexpression of miR-150 in T- and B-cell lymphoma cell lines inhibited telomerase activity and promoted telomere shortening (Watanabe et al., 2011).

Subsequent experimentation identified that miR-150 may regulate lymphoma cell telomerase activity via direct impairment of Dyskerin expression and interference with the phosphorylation of AKT/PKB at Ser473/4 (Watanabe et al., 2011). Induced downregulation of the oncomiR miR-21 resulted in inhibited cell proliferation, apoptosis induction, reduced Signal Transducer and Activator of Transcription 3 (STAT3) expression and phosphorylation, and decreased *hTERT* mRNA and protein (Wang, Sun, et al., 2012). Associations have also been established between miR-498 expression and telomerase expression in ovarian cancer cells (Kasiappan et al., 2012). A similar association exists between miR-143 and *hTERT* expression in human foreskin BJ fibroblasts (Bonifacio & Jarstfer, 2010).

MicroRNA regulation also modulates cellular senescence (Liu, Wen, & Liu, 2012). A recent study identified 33 differentially expressed miRNAs directly associated with telomere length (Slattery, Herrick, Pellatt, Wolff, & Mullany, 2016). The observed association was positive, with increased miRNA expression associated with longer telomeres. Additionally, a genetic variation of TERT (rs2736118) was associated with differential expression of 75 miRNAs between carcinoma and normal colonic mucosa (Slattery et al., 2016). Several genes associated with non-telomeric functions were regulated by the mRNA/miRNA interactions including the PTEN and PI3k/AKT signalling pathways (Slattery et al., 2016). The direction of the mRNA/miRNA regulation appears bi-directional with a recent study demonstrating TERT-based positive regulation of miRNAs in human cells. The expression levels of mature miRNAs were downregulated following TERT suppression in THP-1 and HeLa cells (Lassmann et al., 2015).

1.3.4 Telomere Length and Exercise

The widely touted relationship between PA and LTL is replete with inconsistencies. The positive association between PA and LTL has come from a range of cross-sectional studies (Bendix et al., 2011; Cherkas et al., 2006; Cherkas et al., 2008; Denham et al., 2013; Du et al., 2012; Garland et al., 2014; Kim et al., 2012; Kingma, de Jonge, van der Harst, Ormel, & Rosmalen, 2012; Krauss et al., 2011; LaRocca et al., 2010; Loprinzi, 2015; Ludlow et al., 2008; Østhus et al., 2012; Puterman, Lin, Krauss, Blackburn, & Epel, 2015; Savela et al., 2012; Silva et al., 2016; Venturelli et al., 2014; Werner et al., 2009; Williams et al., 2017; Zhu, Wang, et al., 2011). A summary of human studies that significantly associated PA with telomere length is contained in table 3.

Telomere length has been assessed in skeletal muscle cells and leukocytes in response to aerobic training, resistance training, and self-reported PA. The observed associations appear hormetic, with low and excessive levels of activity associated with shorter telomeres (Ludlow et al., 2008; Savela et al., 2012) and moderate levels more commonly associated with longer LTL (Kim et al., 2012).A 2016 study of 6474 males and females positively associated running with LTL yet found no associations with other PA domains including aerobics, basketball, bicycling, dancing, running, stair climbing, swimming, walking, and weight-lifting (Loprinzi & Sng, 2016). The authors speculate the sustained weight-bearing status of running may preferentially activate signaling pathways, citing other studies that demonstrated associations in ultra-endurance runners (Denham et al., 2013).

Table 2. A summary of miRNAs with putative telomeric involvement.

miRNAs	Physiological Function	Reference
miR-138	Post-transcriptional regulation of hTERT in anaplastic thyroid cancer cell lines.	(Mitomo et al., 2008)
miR-138	Expression levels associated with hTERT in neuroblastoma.	(Chakrabarti et al., 2013)
miR-1207-5p miR-1266	Differentially expressed in hTERT-positive gastric tumour compared to hTERT-negative tissues. Restoration of miRNA expression associated with decreased hTERT protein.	(Chen et al., 2014)
let-7g* miR-133a miR-138-5p miR-491-5p	Inhibits telomerase activity in HeLa cells. They interact with the hTERT 3'UTR in a luciferase reporter assay.	(Hrdličková et al., 2014)
RGM249	A potential miRNA precursor coding gene involved with hTERT expression in hepatocellular carcinoma.	(Miura et al., 2009)
miR-92	Positively correlated with telomerase activity in hepatocellular carcinoma.	(Romilda et al., 2012)

Table 2. continued

miRNAs	Physiological Function	Reference
miR-150	Inhibits lymphoma cell telomerase activity via Dyskerin down-regulation.	(Watanabe et al., 2011)
miR-21	Regulates STAT3-dependent expression of hTERT in glioblastoma cells.	(Wang, Sun, et al., 2012)
miR-21	Reduces lifespan of human endothelial cells.	(Dellago et al., 2013)
miR-498	Regulates hTERT expression levels in ovarian cancer.	(Kasiappan et al., 2012)
miR-138 miR-143 let-7 miR-17-92	Differentially expressed in cancer cells undergoing GRN163-mediated telomere shortening.	(Uziel et al., 2010)
miR-633 miR-638	Upregulated in three different senescent states; quiescence, stress-induced senescence and replicative senescence.	(Maes, Sarojini, & Wang, 2009)

Table 2. continued

miRNAs	Physiological Function	Reference
miR-143 miR-146a miR-155	miR-143 upregulated in senescent BJ fibroblasts; downregulated in late passage BJ fibroblasts expressing hTERT. Ectopic TERT expression elicits pro-inflammatory signalling, resulting in upregulation of miR-146a and miR-155.	(Bonifacio & Jarstfer, 2010)
miR-296	Regulates telomerase reactivation during tumorigenesis via impairment of the p53-p21^{WAF1} pathway.	(Yoon et al., 2011)
miR-290 family	Reduced expression in Dicer1-null mice cells leading to increased expression of Rbl-2, a transcriptional repressor of DNA methyltransferases. This in turn affects the status of sub-telomeric regions.	(Benetti et al., 2008)
miR-200 family	Downregulated in normal kidney cells undergoing telomere-mediated chromosomal instability.	(Castro-Vega et al., 2013)
33 differentially expressed miRNAs	Positively associated with telomere length. A TERT variant (rs2736118) was associated with differential expression of 75 miRNAs. PTEN and PI3k/AKT signalling pathways were regulated by mRNA/miRNA interactions.	(Slattery et al., 2016)

Insufficient statistical power may explain the lack of association found in other studies employing running as the independent variable (Mathur et al., 2013; Rae et al., 2010). Self-reported PA was positively associated with LTL in a cohort of 5823 men and women from the National Health and Nutrition Examination Survey (NHANES) (Tucker, 2017). Objectively measured cardiorespiratory fitness has also been positively associated with LTL in a cohort of obese women (Mason et al., 2013) and older exercise-trained participants (LaRocca et al., 2010).

The positive associations have been refuted by several observational and interventional studies (Bekaert et al., 2007; Cassidy et al., 2010; D'Mello et al., 2015; Denham, 2016; Farzaneh-Far, Lin, Epel, Lapham, et al., 2010; Fujishiro, Diez-Roux, Landsbergis, Jenny, & Seeman, 2013; Garcia-Calzon et al., 2014; Hovatta et al., 2012; Kadi et al., 2008; Laye et al., 2012; Mason et al., 2013; Mathur et al., 2013; Ponsot et al., 2008; Rae et al., 2010; Shin et al., 2008; Song et al., 2010; Sun et al., 2012; Svenson et al., 2011; Tiainen et al., 2012; Woo et al., 2008). A summary of human studies that found no significant association between PA and telomere length is contained in table 4. A rigorous 2015 systematic review and meta-analysis concluded that insufficient quality evidence exists to conclusively associate PA with LTL (Mundstock et al., 2015). Approximately 54% of studies reviewed found no relationship between PA and LTL; 41% found a positive association and 5% identified a curvilinear relationship (Mundstock et al., 2015). Most of the positive associations were weak to moderate with only two studies reporting strong associations (Denham et al., 2013; Kim et al., 2012). The analysis cited methodological issues such as weak correlations, assessment of varied tissue types, arbitrary cuff-off points, inadequate blinding of researchers, selective inclusion of other potentially confounding lifestyle factors and discrepant

measurement techniques as possible confounders (Mundstock et al., 2015). Discrepant modes of PA and the wide-spread use of self-reported PA with its inherent biases may also explain the lack of association in several of the studies. A similar review conducted in 2013 assessed the effect of exercise in animals and humans and identified three general association types: positive association, inverted 'U' response and no association (Ludlow, Ludlow, & Roth, 2013).

A study of 2006 Chinese participants found no significant difference in LTL across quartiles of PA (Woo et al., 2008). Crucially, the author cited possible decreased role of PA in the seventh decade given potential selection bias of recruiting healthy elderly participants. A common criticism of studies failing to find associations with PA is the lack of statistical power; however, a range of studies with sample sizes ranging from 1942–5862 participants have failed to find an association between PA and LTL (Bekaert et al., 2007; Cassidy et al., 2010; Sun et al., 2012; Tiainen et al., 2012; Weischer, Bojesen, & Nordestgaard, 2014; Woo et al., 2008). In a cohort of 4576 Danish men and women, PA was not associated with LTL change over 10 years (Weischer et al., 2014). A study by Sun et al. (2012) found no association between PA and LTL in a cohort of 5862 middle-aged women; however, the addition of five low-risk factors (smoking status, PA, adiposity, alcohol use and diet) to the analysis established a significant association (Sun et al., 2012).

Potential Mechanisms

The consensus is that the positive association between PA and telomere length is mediated by reductions in chronic oxidative stress and inflammatory processes (Campos et al., 2014; Gomes, Silva, & de Oliveira, 2012; Nimmo, Leggate, Viana, & King, 2013). Increases in antioxidant gene expression (Gomez-Cabrera, Domenech, & Viña, 2008), increased activity of DNA-repairing enzymes

(Radák et al., 2003), and decreased ROS (Bjork, Jenkins, Witkowski, & Hagberg, 2012) have also been implicated as likely mediators of the response. Exercise-induced changes in signalling mechanisms such as TERT, IGF-1, eNOS, and AKT associated with telomere biology have also been proposed (Ludlow et al., 2013; Ludlow & Roth, 2011; Werner et al., 2009; Werner et al., 2008). More recently, a single 45-min bout of cycle ergometry was shown to increase skeletal muscle TERRA in a small cohort (Diman et al., 2016).

Physical Activity and the Shelterin Complex

The shelterin complex appears to be sensitive to environmental stimuli such as exercise. Interventional animal studies have demonstrated exercise-induced regulation of shelterin components in skeletal muscle tissue (Ludlow et al., 2012; Werner et al., 2008). Increased expression of shelterin component mRNA and protein have been observed in well trained athletes when compared to controls (Denham, O'Brien, Prestes, Brown, & Charchar, 2016; Werner et al., 2009). Most human studies are limited by their observational design, which precludes conclusions about causality.

Telomerase and Physical Activity

The tacit assumption that longer telomeres reflect increased telomerase activity is not reliably supported by the evidence (Zalli et al., 2014). In a study of 124 healthy individuals, telomerase activity progressively decreased in concert with telomere length from ages 4 to 39 years. However, 65% of individuals aged 40 years or older exhibited low yet stable telomerase expression despite continued telomere shortening (Iwama et al., 1998).

Table 3. A summary of studies showing a positive association between physical activity and telomere length.

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Puterman et al., 2010)	Healthy post-menopausal women (n=63); sedentary group, active group	Leukocytes	T/S qPCR	Sedentary: one unit increase in the Perceived Stress Scale = 15-fold increase in odds of having short LTL (*P*<0.05). Active: Perceived stress unrelated to telomere length (*P*=0.45).
(Cherkas et al., 2008)	White twins 2401: females (n=2152), males (n=249)	Leukocytes	Southern blot TRF	Leisure time PA positively associated with LTL (*P*<0.001). LTLs of the most active subjects were 200 nucleotides longer than least active (*P*=0.01).
(Tucker, 2017)	5823 adult participants; males (n=2766), females (n=3057)	Leukocytes	T/S qPCR	Relative PA (*P*<0.001) and absolute PA (*P*= 0.005) associated with longer LTL after adjustment for demographic variables. Prevalence of short telomeres associated with relative PA (*P*<0.001).

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Cherkas et al., 2006)	1552 Caucasian female twins: dizygotic twins (n=749), mono-zygotic twins (n=27)	Leukocytes	Southern blot TRF	PA positively associated with TRFL (*P*< 0.005). Overall decreasing trend in TRFL with lower SES (*P*<0.024). Significant difference in TRFL between non-manual and manual workers (*P*<0.01).
(Kim et al., 2012)	Healthy, post-menopausal women (n=44)	Leukocytes	T/S qPCR	LTL significantly higher in habitual exercise group compared to sedentary group (*P*< 0.01).
(Bendix et al., 2011)	274 pairs same sex twins: dizygotic pairs (n=153), mono-zygotic pairs (n=121)	Leukocytes	Southern blot TRF	LTL is positively associated with self-reported physical ability in all pairs combined (*P*=0.006). Positive association between PA and LTL in all pairs (*P*=0.034).

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Denham et al., 2013)	Male ultra-marathon runners (n=67), controls (n=63)	Leukocytes	T/S qPCR	LTL 11% longer in ultra-marathon runners compared to controls (*P*<0.001).
(Du et al., 2012)	Nurse's health study - 7813 females	Leukocytes	T/S qPCR	Increased LTL in moderately and highly active women (*P*=0.02). Moderate or vigorous activity associated with longer LTL (*P*=0.02).
(Garland et al., 2014)	Post-menopausal women with Stage I–III breast cancer (n=392)	PBMCs	Southern blot TRF	No PA significantly associated with shorter LTL (*P*=0.03).
(Kingma et al., 2012)	895 participants: males (n=419), females (n=476)	Leukocytes	T/S qPCR	Low frequency PA an independent predictor of short LTL (*P*<0.001).

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Krauss et al., 2011)	944 CHD patients: low (n=299), mod (n=334), high (n=381) exercise capacity	Leukocytes	T/S qPCR	LTL significantly longer in subjects with high exercise capacity (*P*<0.001). Association remained after adjustment for CVD severity and physical inactivity (*P*=0.005)
(LaRocca et al., 2010)	57 participants: sedentary (n=5), young exercising (n=10), older sedentary (n=15), and older exercising (n=17)	Leukocytes	Southern blot TRF	LTL of Older exerciser had longer LTL compared to controls (*P*<0.001). Older exerciser LTL not significantly different from young exercisers (*P*=0.12). LTL positively associated with $\dot{V}O_{2max}$ (*P*<0.01).

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Loprinzi, 2015)	1764 adults: 51% males, 49% females, 73% non-Hispanic whites	Leukocytes	T/S qPCR	LTL longer in upper (*P*=0.04) and middle fitness tertiles (*P*=0.02) compared to lowest tertile.
(Ludlow et al., 2008)	69 healthy participants: males (n=34), females (n=35)	PBMCs	T/S qPCR	Significantly longer telomeres in second exercise energy expenditure quartile compared to first (*P*=0.001) and fourth (*P*=0.04) quartiles.
(Østhus et al., 2012)	20 male participants: young athletes (n=5), young non-athletes (n=5), older athletes (n=5), older non-athletes (n=5)	Skeletal muscle	T/S qPCR	Longer telomeres in older athletes compared to older non-athletes (*P*=0.04). Young athletes not different to young non-athletes (*P*=0.12). Strong correlation between $\dot{V}O_{2max}$ and T/S ratio in athletes (*P*=0.02).

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Puterman et al., 2015)	Post-menopausal women (n=239)	Leukocytes	T/S qPCR	One SD below mean PA levels, major life stressors were associated with LTL shortening (*P*=0.01). One SD above mean PA level, major life stressors were not associated with LTL shortening (*P*=0.48).
(Savela et al., 2012)	782 males: low PA (n=148), moderate PA (n=398), high PA (n=236)	Leukocytes	Southern blot TRF	Inverted “U” response. Moderate PA positively associated with longest LTL (*P*=0.03). LTL the same in low and high PA groups. Moderate PA group had lowest proportion of short LTL (*P*=0.02).

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Silva et al., 2016)	46 participants: never (n=15), moderately (n=16), and intensively trained (n=15)	PBMCs	Flow-FISH	T cell TL longer in moderately trained and intensively trained compared to never trained (P<0.05). Significantly longer telomeres in CD8+ T cells in IT group (P<0.05).
(Venturelli et al., 2014)	36 participants: young (n=12), old mobile (n=12), old immobile (n=12)	Skeletal muscle	T/S qPCR	Mean TL from leg muscle of the old immobile group significantly shorter than old mobile group (P<0.05), young group (P<0.05), arm muscle (P<0.05).
(Werner et al., 2009)	Young sedentary (n=26), athletes (n=32), middle-aged sedentary (n=21), athletes (n=25)	Leukocytes	FlowFISH and T/S qPCR	Shorter telomeres in older sedentary controls (P<0.001). Age-dependent telomere loss attenuated in lymphocytes (P<0.001) and granulocytes (P<0.001) of older athletes.

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Zhu, Wang, et al., 2011)	667 adolescents, white males (n=169), white females (n=179), black males (n=155), black females (n=164)	Leukocytes	T/S qPCR	Vigorous PA positively associated with telomere length (*P*=0.009)
(Loprinzi & Sng, 2016)	6474 participants: 49.6% males, 50.4% females	Leukocytes	T/S qPCR	LTL positively associated with running (*P*=0.03)
(Borghini et al., 2015)	Endurance athletes (n=20), age- and gender-matched controls (n=42)	Buccal cells	T/S qPCR	Baseline TL preserved in endurance athletes (*P*=0.003). Intermediate TL reduced in endurance athletes (*P*=0.002). Final time point TL reduced in endurance athletes (*P*<0.001).

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Latifovic, Peacock, Massey, & King, 2016)	Healthy males and females (n=477)	Leukocytes	T/S qPCR	Vigorous PA positively associated with LTL (*P*<0.01).
(Saßenroth et al., 2015)	814 participants: 397 males (n=397), females (n=417)	Leukocytes	T/S qPCR	LTL longer in participants: currently exercising compared to inactive (*P*=0.013), engaged in intensive activity (*P*=0.011), participating in sport for 10 years prior to assessment (*P*=0.017).
(Shadyab et al., 2017)	Older aged white and African American women (n=1476)	Leukocyte	Southern blot TRF	Longer LTL associated with highest PA (*P*=0.02), higher levels of moderate-to-vigorous PA (*P*=0.04), and faster walking speed (*P*=0.03).

Table 3. continued

Reference	Subjects (n)	Tissue	Measurement	Key Findings
(Soares-Miranda et al., 2015)	Older adults (n=582)	Leukocytes	Southern blot TRF	Longer LTL associated with walking distance (*P*=0.007), chair test performance (*P*=0.04). Change in chair test associated with less LTL shortening (*P*=0.04).
(Williams et al., 2017)	Healthy adults (n=4952)	Leukocytes	qPCR	Longer LTL associated with aerobic fitness (*P*=0.03), trunk muscle endurance (*P*=0.02).

CHD: coronary heart disease. **CVD:** cardiovascular disease. **FlowFISH**: fluorescence in situ hybridization combined with flow cytometry. **TL:** telomere length. **LTL:** leukocyte telomere length. **PA:** physical activity. **PBMCs:** Peripheral blood mononuclear cells. **TRF:** terminal restriction fragment analysis. **TRFL:** terminal restriction fragment length. **T/S qPCR:** the ratio of telomere PCR value to single-copy gene value derived from quantitative PCR. **SD:** standard deviation. **SES:** socio-economic status. **$\dot{V}O_{2max}$:** maximal volume of oxygen uptake.

Table 4. A summary of studies showing no association of physical activity with telomere length.

Ref.	Subjects (n)	Tissue	Measurement	Key Findings
(Bekaert et al., 2007)	2509 participants: males (n=1218), females (n=1291)	Leukocytes	Southern blot TRF	Self-reported PA not associated with LTL (*P*=0.806).
(Farzaneh-Far, Lin, Epel, Lapham, et al., 2010)	608 individuals with CAD: males (n=244), females (n=364)	Leukocytes	T/S qPCR	PA did not independently modulate LTL (*P*=0.59).
(Weischer et al., 2014)	Male and female Danish participants (n=4576)	Leukocytes	T/S qPCR	Change in LTL over 10 years not associated with PA (*P*=0.85)
(Mirabello et al., 2009)	Advanced prostate cancer (n=612), age-matched controls (n=1049)	Leukocytes	T/S qPCR	No association between PA and LTL (*P*=0.262). LTL positively associated with healthy lifestyle factors (e.g. diet, PA, smoking) (*P*=0.004).

Table 4. continued

Ref.	Subjects (n)	Tissue	Measurement	Key Findings
(Mathur et al., 2013)	Marathon runners (n=17), sedentary controls (n=15)	Lymphocytes/ granulocytes	T/S qPCR	No difference between marathon runner lymphocyte (*P*=0.6) and granulocyte TL (*P*=0.9) compared to controls.
(Rae et al., 2010)	Experienced runners (n=18), sedentary individuals (n=19)	Skeletal muscle	Southern blot TRF	No difference in minimum TRF between groups (*P*=0.805). Minimum TRF inversely related to years of running (*P*=0.007) and time spent training (*P*=0.035).
(Mason et al., 2013)	439 overweight women: dietary weight loss (n=118), aerobic exercise (n=117), diet/ exercise (n=117), control (n=87)	Leukocytes	T/S qPCR	No significant difference in LTL in exercise group (*P*=0.51), diet and exercise group (*P*=0.14). Baseline LTL positively associated with VO_{2max} (*P*=0.03).

Table 4. continued

Ref.	Subjects (*n*)	Tissue	Measurement	Key Findings
(Cassidy et al., 2010)	Nurses' Health Study: women (n=2284)	Leukocyte	T/S qPCR	No association between LTL and PA (*P*=0.69).
(Fujishiro et al., 2013)	981 individuals: males (n=467), females (n=514)	Leukocytes	T/S qPCR	Work related PA not associated with LTL (*P*=0.933)
(Garcia-Calzon et al., 2014)	Obese individuals (n=521) randomized into: 2 diet groups, 1 low-fat control group	Leukocytes	T/S qPCR	Self-reported PA not associated with telomere length (*P*=0.186).
(Hovatta et al., 2012)	Subjects with impaired glucose tolerance (n=190), controls (n=188)	Leukocytes	T/S qPCR	No significant difference in LTL following multi-faceted lifestyle intervention at 4.5 year follow up (*P*=0.76).

Table 4. continued

Ref.	Subjects (*n*)	Tissue	Measurement	Key Findings
(Kadi et al., 2008)	14 participants: powerlifters (n=7), controls (n=7)	Skeletal muscle	Southern blot TRF	TL not significantly associated with 8 ± 3 years of powerlifting experience (*P*=0.07).
(Laine et al., 2015)	599 males: former athletes (n=392), controls (n=207)	Leukocytes	T/S qPCR	No association between self-reported volume of leisure time PA and LTL in later life (*P*=0.845).
(Ponsot et al., 2008)	42 participants: young males (n=10), young females (n=6), old males (n=13), old females (n=13)	Skeletal muscle	Southern blot TRF	Tibialis anterior telomere length not significantly influenced by self-reported PA (*P* value not available).

Table 4. continued

Ref.	Subjects (*n*)	Tissue	Measurement	Key Findings
(Shin et al., 2008)	16 obese middle-aged women: exercise group, control group	Leukocytes	T/S qPCR	No significant change in LTL after 6 months of aerobic training.
(Song et al., 2010)	136 participants: males (n=50), females (n=86)	Lymphocytes	T/S qPCR	Self-reported PA not associated with longer TL (*P*=0.46).
(Sun et al., 2012)	5862 women	Leukocytes	T/S qPCR	No association between PA and LTL. Presence of five low-risk healthy lifestyle factors associated with longer LTL (*P*=0.02).
(Tiainen et al., 2012)	1942 male and female participants	Leukocytes	T/S qPCR	PA not significantly associated with LTL in males or females (*P* value not available)

Table 4. continued

Ref.	Subjects (*n*)	Tissue	Measurement	Key Findings
(Woo et al., 2008)	Male (n=976), female (n=1030)	Leukocytes	T/S qPCR	No significant difference in TL across quartiles of self-reported PA (*P*=0.32)
(Denham et al., 2016)	Exercise trained (n=44), recreationally active controls (n=40)	PBMCs	T/S qPCR	No significant difference in PBMC telomere length between exercising group and controls (*P*=0.72).
(von Kanel, Bruwer, Hamer, de Ridder, & Malan, 2017)	203 healthy participants; African (n=96), Caucasian (n=107)	Leukocytes	T/S qPCR	Habitual PA not associated with LTL.

CAD: coronary artery disease. **LTL:** leukocyte telomere length. **PA:** physical activity. **PBMCs:** peripheral blood mononuclear cells. **TRF:** terminal restriction fragment analysis. **T/S qPCR:** the ratio of telomere PCR value to single-copy gene value derived from quantitative PCR. **$\dot{V}O_{2max}$:** maximal volume of oxygen uptake.

Telomerase preferentially targets short telomeres (Britt-Compton, Capper, Rowson, & Baird, 2009), therefore acute increases in telomerase may actually be an attempt to stabilize critically shortened telomeres (Epel et al., 2006). This may explain the increased telomerase activity and shortened telomeres that accompany subclinical coronary atherosclerosis (Kroenke et al., 2012).

A range of studies have assessed exercise-induced changes in telomerase expression in human and animal models (table 5). Mouse models have demonstrated exercise-induced telomerase increases (Ludlow et al., 2012; Werner et al., 2009; Werner et al., 2008; Wolf, Melnik, & Kempermann, 2011); however, human results are conflicting. In one study, PBMC telomerase expression did not differ between physical fitness categories (Ludlow et al., 2008). However, a later study demonstrated increased leukocyte telomerase in endurance-trained athletes compared to controls (Werner et al., 2009). A 3 month multifaceted intervention consisting of diet, exercise, and stress management techniques increased PBMC telomerase expression in males; however, direct causation cannot be assigned to one specific modality (Ornish et al., 2008). Acute exercise studies have produced conflicting results, with some showing no change in leukocyte telomerase expression (Laye et al., 2012), and others showing increased PBMC telomerase expression after a single bout of treadmill running (Zietzer et al., 2016).

Changes in telomerase expression have more consistently been associated with dietary interventions (Balcerczyk et al., 2014; Boccardi et al., 2013; Zhu et al., 2012) and alternative lifestyle interventions (typically underpinned by some form of relaxation practice) (Daubenmier et al., 2012; Ho et al., 2012; Jacobs et al., 2011; Kumar, Yadav, Yadav, Tolahunase, & Dada, 2015; Lavretsky et al., 2013; Lengacher et al., 2014; Ornish et al., 2008).

1.3.5 Areas for Investigation

Exercise, miRNAs, and Telomeres

MicroRNAs post-transcriptionally modulate gene expression and mediate several exercise-induced adaptations (Chan et al., 2009; Davidsen et al., 2011; Davidson-Moncada et al., 2010; Williams et al., 2009; Zhang, 2010). Despite established roles in cellular aging and senescence pathways, little is known about the epigenetic role miRNAs play in telomere homeostasis. Additionally, the effect of exercise on telomere-associated genes and their potential regulation via miRNAs is unclear. Similarly, little is known about the immune cell subset-specific expression of miRNAs in response to exercise.

Many of the reported miRNA-telomere associations have been established in cancer cell lines, wherein dysregulated telomere maintenance mechanisms may have corrupted the observed associations. There are far fewer established *in vivo* associations between miRNAs and telomere maintenance in healthy cells.

The current study will further investigate the exercise-induced miRNA signature and potentially identify expression patterns of miRNAs with potential telomeric involvement. The widely observed associations between PA and LTL may be underpinned by upregulation of telomere genes; a phenomenon that may in part be regulated by miRNA epigenetic modulation.

Exercise and Telomere-Associated Genes

Despite the positive associations between PA and LTL, a clear understanding of how telomeres adapt to exercise is lacking. It is unknown if exercise-induced changes in shelterin activity are necessary and/or sufficient for changes in telomere

Table 5. A summary of studies investigating the association between physical activity and telomerase expression.

Ref.	Subjects (n)	Tissue	Measurement	Key Finding
(Laye et al., 2012)	Professional ultra-marathon runners (44 ± 2 years): males (n=7), female (n=1)	Leukocytes	TRAPeze qPCR	No change in leukocyte telomerase activity pre- to post-intervention (running 7 marathons in 7 days)
(Ludlow et al., 2008)	69 participants (60.33±4.9 y): males (n=34), females (n=35)	PBMCs	Gel-based TRAP	No significant difference in PBMC telomerase expression between different exercise level quartiles (*P*=0.84).
(Ludlow et al., 2012)	30 AST/Ei J mice; exercising (n=15), sedentary (n=15)	Mouse cardiac, skeletal and liver tissue.	TRAPeze qPCR	Significant difference between exercising skeletal muscle telomerase compared to sedentary mice after 12 months (*P*=0.02). No significant difference in cardiac and liver tissue telomerase between groups.

Table 5. continued

Ref.	Subjects (n)	Tissue	Measurement	Key Finding
(Melk et al., 2014)	Healthy middle-aged males (53 ± 6 years) (n=59)	PBMC	Not stated	Significant increase in PBMC telomerase activity after 6-month intervention (*P*<0.001)
(Ornish et al., 2008)	Biopsy-diagnosed prostate cancer (62.2 ± 7.5 years): (n=30)	PBMC	Gel-based TRAP	Significant increase in PBMC telomerase activity after 3-month intervention (*P*=0.031).
(Radak et al., 2001)	Rat	Liver and solid sarcoma cells	PCR ELISA	No significant change in telomerase activity in rat liver or sarcoma cells after 8 weeks of swimming exercise.
(Werner et al., 2008)	Male C57/B16 (eNOS)-/- mice TERT-/- mice, strain-matched controls	Mouse	TRAPeze qPCR	Increased telomerase activity in myocardial cells after short-term (21 days) and long-term (6 months) of voluntary wheel running (*P*=0.01)

Table 5. continued

Ref.	Subjects (n)	Tissue	Measurement	Key Finding
(Werner et al., 2009)	Young: controls (n=26), athletes (n=32), middle-aged: controls (n=21), athletes (n=25)	MNCs	TRAPeze qPCR	2.5-fold increase in young athlete MNC telomerase (P<0.001) and 1.8-fold increase in middle-aged athletes compared to controls (P=0.006)
(Wolf et al., 2011)	10, C57B1/6 mice (rodent model of schizophrenia)	Brain neural precursor cells from C57B1/6 mice	TRAP-based ELISA kit	Significant increase in murine neural precursor cell telomerase activity after 10 days of wheel running (P<0.01)
(Zietzer et al., 2016)	Young participants (n=26), elderly patients (n=14)	PMBCs	TRAPeze qPCR	Significant increase in PBMC telomerase activity after single treadmill exercise session (P≤0.05)

ELISA: enzyme-linked immunosorbent assay. **eNOS**: endothelial nitric oxide synthase. **MNCs:** mononuclear cells. **PA:** physical activity. **PBMCs**: peripheral blood mononuclear cells. **qPCR:** quantitative (real-time) polymerase chain reaction. **SE**: standard error. **TERT:** telomerase reverse transcriptase catalytic subunit. **TRAP:** telomeric repeat amplification protocol. **TRAPeze:** telomeric repeat amplification protocol utilizing qPCR. **$\dot{V}O_{2max}$:** maximal volume of oxygen uptake.

length to occur. It is plausible that shelterin changes may be epiphenomena, occurring simultaneously yet independently of telomere length changes. The time course and duration of adaptive responses are also unclear. It is unclear which (if any) of the shelterin genes undergo IEG expression or mount a more delayed response to exercise in humans. Such IEG is commonplace within the immune system however any such role in telomere homeostasis is unsubstantiated. This may have important exercise programming implications, guiding exercise frequency recommendations to capitalize on the cumulative yet potentially transient alterations in shelterin function.

The current study will assess the acute responsiveness of leukocyte shelterin genes to 30 min of aerobic exercise at 80% of $\dot{V}O_{2peak}$. Additionally, potentially regulatory miRNA will also be assessed. In doing so, the current study will improve the understanding of acute telomeric exercise adaptation within the immune system.

1.4 Thesis Overview

This thesis will examine the acute effects on intense cardiorespiratory exercise on immune cell distribution and telomere-associated genes, non-coding RNAs, and associated pathways. Cardiorespiratory exercise was chosen as the exercise intervention as most physical activity and mortality associations have utilized a measure of cardiorespiratory exercise. Additionally, most of the established associations between physical activity and LTL, and more than 50% of studies assessing physical activity and telomerase, utilized this exercise modality (see tables 3, 4 and 5 section 1.3.4). Many of the studies that utilized resistance training assessed skeletal muscle as the target tissue. Whilst skeletal muscle

satellite cells are undifferentiated myogenic precursors capable of replication (Kadi et al., 2005), adult skeletal muscle cells are multinucleated post-mitotic cells that do not divide and therefore do not impact upon telomere length.

Firstly, the effect of 30 min of intense exercise on the relative proportions of circulating immune cells will be investigated. Secondly, the differential expression of miRNAs in response to intense exercise will be investigated. Thirdly, the exercise-induced abundance of telomeric gene mRNA will be investigated. Lastly, changes in the exercise-induced transcriptome will be measured to assess regulation of potential pro-telomere transcripts and/or pathways in leukocytes.

Aims

The overall aim of this thesis is to investigate the acute exercise-induced immunological and molecular adaptations that may underpin the observed relationship between habitual exercise and increased leukocyte telomere length.

The specific aims of this thesis are to:

1. Characterize the acute leukocyte and T cell response to 30 min of intense cardiorespiratory exercise.
2. Characterize the acute effects of 30 min of intense cardiorespiratory exercise on the expression patterns of leukocyte miRNAs with potential involvement in telomere biology.
3. Characterize the acute effects of 30 min of intense cardiorespiratory exercise on the expression of telomere-associated gene transcripts.
4. To characterize the exercise-induced transcriptome in leukocytes to identify telomere-associated gene and/or pathway responsiveness.

Hypotheses

The research here within is premised upon the broad hypothesis that the observed chronic alterations in immune cell telomere dynamics reflect the cumulative acute perturbations in either telomeric genes, telomere-associated miRNAs, and/or telomerase activity. The specific hypotheses are:

1. An acute 30 min bout of continuously monitored, intense cardiorespiratory exercise will differentially regulate T cell subsets. Specifically, it is hypothesised that the strict maintenance of 80% of $\dot{V}O_{2peak}$ throughout the 30 min bout of running could alter the magnitude and time course of the routinely reported lymphocytosis and lymphopenia.
2. A 30 min bout of treadmill running at 80% $\dot{V}O_{2peak}$ will differentially regulate miRNAs in leukocytes and immune cell subsets. Specifically, aerobic exercise will differentially regulate miRNAs that potentially interact with telomere-associated transcripts.
3. A 30 min bout of aerobic exercise at 80% of $\dot{V}O_{2peak}$ will differentially regulate several telomere-associated such as those associated with telomerase function or shelterin structure.
4. A 30 min bout of aerobic exercise will significantly influence the leukocyte transcriptome. Specifically, exercise will regulate several genes and non-coding RNAs, as measured by next generation sequencing, which may have interactions with telomere biology.

1.4.1 Significance

Aging

The global population of older persons (aged 60 years or older) is projected to more than double by 2050, reaching 2.1 billion. The oldest-old persons (aged 80

years or older) are projected to more than triple in population, reaching 434 million (United-Nations, 2015). Advancing age is associated with increased susceptibility to a range of chronic conditions such as infectious diseases, Alzheimer's disease, CVD, cancer, and osteoporosis. This increased susceptibility is in part attributed to a dysregulated and dysfunctional immune system (De Martinis, Di Benedetto, Mengoli, & Ginaldi, 2006; Franceschi et al., 2007; Giunta et al., 2008; Malaguarnera, Cristaldi, & Malaguarnera, 2010; McElhaney & Effros, 2009; Weinberger, Herndler-Brandstetter, Schwanninger, Weiskopf, & Grubeck-Loebenstein, 2008; Weiskopf, Weinberger, & Grubeck-Loebenstein, 2009).

Telomeres are major determinants of biological age and are significantly influenced by lifestyle factors such as exercise (Zhang, Rane, et al., 2016). The trajectory of telomere shortening reflects cellular aging and is considered a marker of health status in the aging population (Samani & van der Harst, 2008). Leukocytes permeate every tissue and plausibly exert and simultaneously reflect a systemic exercise response. A better understanding of the homeostatic mechanisms regulating the telomere/telomerase system may inform exercise-based interventions for specific populations with impaired telomere maintenance.

Immune System

Immunosenescence and dysregulation can contribute to an array of age-related diseases (Blasko et al., 2004; Chinetti-Gbaguidi, Colin, & Staels, 2015; Dunn, Old, & Schreiber, 2004; Frostegård, 2013; Giunta et al., 2008; Kim, Emi, & Tanabe, 2007; Kolbus et al., 2013; Meng et al., 2016; Shi, Bot, & Kovanen, 2015; Yu, Park, Shin, & Lee, 2016; Zhang & Grizzle, 2003). The hallmark characteristic of a dysregulated immune system is telomere-dependent senescence. Cellular

senescence can influence immune function and mammalian lifespan (Choudhury et al., 2007; Rudolph et al., 1999; Tyner et al., 2002).

Maintenance of telomere length and telomerase activity are critical for effective functioning of the immune system. Modulation of telomerase activity and telomere length can mitigate senescence and the associated phenotypes (Jaskelioff et al., 2011; Minamino et al., 2002; Oh, Kyo, & Laimins, 2001). This effect has been observed in circulating leukocytes and vascular cells (Werner et al., 2009). Habitual PA and/or exercise have been shown to positively influence many indices of the aging immune system (Chin et al., 2000; Drela et al., 2004; Gano et al., 2011; Grant et al., 2008; Kadoglou et al., 2007; Nicklas et al., 2008; Nieman & Henson, 1994; Petersen & Pedersen, 2005; Shinkai et al., 1995; Simpson, 2011; Simpson & Guy, 2009; Smith, Dykes, Douglas, Krishnaswamy, & Berk, 1999; Smith, Kennedy, & Fleshner, 2004; Spielmann et al., 2011). Crucially, the effects of exercise on immune cell aging exhibit a 'U' shaped response, suggesting a point of diminishing returns and possible maladaptation (Turner, 2016). A better understanding of the factors contributing to this response may identify the physiological thresholds and mechanisms driving the hormetic response. This in turn may directly inform clinical exercise prescription for at-risk or pathological populations.

Cardiovascular Disease

Observational data show an inverse association between telomere length and CVD independent of conventional risk factors (Haycock et al., 2014). Despite the prevailing theory that LTL is a non-causal biomarker of atherosclerosis, recent studies provide considerable support for a causal role (Aviv, 2012; Codd et al., 2013; Haycock et al., 2014). A recent meta-analysis identified that one standard

deviation decrease in LTL was associated with a 21%, 24%, and 37% increased risk of stroke, myocardial infarction, and type 2 diabetes mellitus respectively (D'Mello et al., 2015).

Most telomere-modulating factors are also established CVD risk factors. Telomere-dependent senescence evokes a chronic state of oxidative stress, inflammation, increased expression of adhesion molecules, proteases, cytokines, and growth factors (Fyhrquist et al., 2013). This sequela can promote and propagate atherosclerotic processes and plaque instability (Yeh & Wang, 2016). Exercise-induced telomere homeostasis and epigenetic modifications may account for some of this unexplained protection against CVD afforded by habitual exercise.

Summary

Telomere length represents a central cog around which inflammation, cellular senescence, and biological aging are set in motion. Whilst a substantial body of knowledge exists regarding negative telomere instigators in health and disease, comparatively little is known about positive telomeric adaptations. In the face of equivocal correlational and associative findings, clarity must be sought at the mechanistic level. More accurate mapping of the immunological, epigenetic, and telomeric responses to exercise will enhance the understanding of the requisite systems and processes.

Establishing an epigenetic and/or pro-telomeric *adaptive signature* to various modes of exercise may help determine optimal exercise combinations and personalized approaches for specific physiological outcomes. The findings of this thesis may enhance the understanding of telomere homeostasis in health and disease and improve the understanding of the telomeric adaptive response to PA.

Chapter 2 - The T Cell Response to Intense Cardiorespiratory Exercise

2.1 Abstract

Introduction: Intense exercise results in transient changes in leukocyte numbers in a duration- and intensity-dependent manner. This phenomenon partially explains the improved health seen in the active and immune suppression exhibited by the overtrained. Importantly, the magnitude and duration of this effect appears to be intensity and duration dependent. ***Aims:*** The aim of this study is to characterize the T cell response to 30 min of intense cardiorespiratory exercise. ***Methods:*** Twenty-two healthy males (24.1 ± 1.55 years) undertook 30 min of treadmill running at 80% of $\dot{V}O_{2peak}$. Blood samples were taken before exercise, immediately post-exercise, and 60 min post-exercise. Isolated leukocytes were labelled with the monoclonal antibodies and flow cytometry was used to identify relative proportions of: CD3+, CD3+CD4+, CD4+CD45RA+, CD4+CD45RO+, CD3+CD8+, CD8+, CD8+CD45RA+ and CD8+CD45RO+ T cells. ***Results:*** Significant post-exercise decreases were observed in the percentage of total CD3+ T cells (P<0.001) and CD4+ T cells (P<0.001) followed by a return to resting levels 60 min post-exercise in CD3+ T cells and above resting levels in CD4+ T cells (P<0.001). A significant decrease in CD8+ T cells occurred 60 min post-exercise (P<0.01). Relative proportions of CD4+ naïve T cells decreased 60 min post-exercise (P=0.05) whilst CD8+ naïve T cells decreased post-exercise (P<0.05) before returning to resting levels 60 min post-exercise. ***Conclusion:*** Thirty min of treadmill running at a constant 80% of $\dot{V}O_{2peak}$ was sufficient to elicit novels changes in the relative proportions of specific T cell subsets.

2.2 Introduction

The Exercise Response

Leukocytes permeate every tissue and plausibly exert and reflect a systemic exercise response. The total number of circulating leukocytes increases during acute exercise (leukocytosis) due to the ingress of lymphocytes, neutrophils, and monocytes. Lymphocyte numbers typically reduce (lymphocytopenia) following exercise (30-50% of pre-exercise levels) paralleled by a sustained increase in neutrophils (Gleeson, Nieman, & Pedersen, 2004). These changes are largely due to the transient redistribution of immune cells throughout the body. Prolonged bouts of endurance exercise (164 ± 23 min at 55% of $\dot{V}O_{2max}$) elicit more pronounced leukocytosis than shorter bouts of high-intensity exercise (37 ± 19 min at 80% of $\dot{V}O_{2max}$) (Robson, Blannin, Walsh, Castell, & Gleeson, 1999). Shorter bouts of intensive exercise can elicit a delayed leukocytosis, peaking a few hours after exercise (Karsten, Frank-Christoph, & Christian, 2016). For exercise bouts longer than 60 min, duration has a greater modulatory effect on leukocyte numbers whilst exercise intensity has a greater effect within shorter bouts of exercise (Simpson, 2013).

Lymphocytes

Lymphocytes undergo a distinct biphasic response to acute exercise; increasing (lymphocytosis) during and immediately after exercise before rapidly decreasing (lymphocytopenia) below pre-exercise levels during recovery (Shek, Sabiston, Buguet, & Radomski, 1995). Despite some exercise-induced increase in leukocyte apoptosis (Kruger et al., 2009; Mooren, Blöming, Lechtermann, Lerch, & Völker, 2002), most of the change in lymphocyte numbers is attributed to cellular redistribution (Kruger & Mooren, 2007). The magnitude of this biphasic change is

proportional to the exercise intensity and to a lesser extent, duration (McCarthy & Dale, 1988; Shek et al., 1995).

The transient state of lymphocytosis is primarily due to two distinct yet parallel physiological processes; (i) increased haemodynamic shear forces that flush immune cells from marginal pools to the periphery (Shephard, 2003), and (ii) stimulation of β_2-adrenergic receptors expressed on cytotoxic T cells (Landmann, 1992; Maisel, Harris, Rearden, & Michel, 1990; Murray et al., 1992). The two processes result in increased migration of CD3+ T cells, CD8+ T cells, CD16+ NK cells, CD19+ B cells, and CD56+ NK cells into the periphery.

CD4+ and CD8+ T cells

T cells are acutely sensitive to exercise duration and intensity. A greater absolute number of CD4+ T cells are mobilized in response to an acute bout of exercise; however, the relative contribution (expressed as percentage change) is approximately 1.5-2.0 times greater in CD8+ T cells (Shek et al., 1995). The magnitude of exercise-induced egress is also three times greater in CD8+ T cells resulting in an immediate post-exercise reduction in the CD4:CD8+ T cell ratio (Simpson, 2013).

CD45RA+ and CD45RO+ T cells

Peripheral migration of T cells is also influenced by the stage of differentiation. Cells with greater antigenic history, tissue migratory, and effector functions are preferentially mobilized into the periphery in response to acute exercise (Simpson, 2011). Additionally, effector memory and CD8+CD45RA+ effector-memory (EMRA) T cells are preferentially mobilized over central memory and naïve CD8+ T cells in response to 20 min of cycling in an intensity dependent manner (Campbell

et al., 2009). More recently, a 60 min bout of intense cycling resulted in an immediate increase in CD27-CD8+ T cells which fell below resting concentration 60 min after the exercise bout (LaVoy, Bosch, Lowder, & Simpson, 2013). The observation that older memory cells are preferentially trafficked to the periphery is supported by the observation of shorter telomere lengths in activated CD8+ T cells after a maximal bicycle test (Bruunsgaard et al., 1999).

T cells and Telomeres

The proliferative capacity of lymphocytes is central to an effective immune response, facilitating the *en masse* production of antigen specific effector T cells. Crucially this clonal expansion is finite and largely dependent upon the length of T cell telomere length. The progressive erosion simultaneously provides an index of replicative history and imposes a replicative limit (Allsopp et al., 1992). In a typical immune response, a single naïve cell can undergo 15-20 divisions, resulting in approximately one million identical progeny and subsequent loss of telomeric DNA (Weng, 2008).

Critically shortened telomeres trigger cell cycle arrest and a subsequent state of senescence (Spaulding, Guo, & Effros, 1999). In this state, cells lose replicative capacity and undergo a host of morphological and functional changes (Effros, Dagarag, Spaulding, & Man, 2005). Increased proportions of these senescent T cells form part of an immune risk profile (IRP), a cluster of deleterious immune parameters known to predict mortality in the elderly (Ferguson, Wikby, Maxson, Olsson, & Johansson, 1995; Pawelec, Derhovanessian, Larbi, Strindhall, & Wikby, 2009). Other IRP parameters include: inverted CD4:CD8 T cell ratio, impaired T cell proliferative responses, decreased interleukin-2 (IL-2) production, a high ratio

of late stage (CD27-/CD28-) to early stage (CD27+/CD28+) differentiated CD8+ T cells, and latent cytomegalovirus seropositivity.

The constituent subsets of immune cells exhibit heterogeneous gene expression profiles (Palmer, Diehn, Alizadeh, & Brown, 2006), telomere length, telomerase activity (Lin et al., 2010), and telomere dynamics (Son et al., 2000). Microarray analysis has identified unique gene expression profiles in B cells (Ehrhardt et al., 2008), T cells (Wang, Windgassen, & Papoutsakis, 2008), dendritic cells (Lindstedt, Lundberg, & Borrebaeck, 2005), and monocytes (Zhao et al., 2009). Unique microRNA expression profiles are also exhibited in T cells (Neilson, Zheng, Burge, & Sharp, 2007; Wu, Neilson, et al., 2007), B cells (Basso et al., 2009), NK cells (Bezman et al., 2010), and dendritic cells (Kuipers, Schnorfeil, & Brocker, 2010). Consequently, slight changes in the relative proportions of immune subsets, as seen during exercise, could significantly alter the composite LTL and gene expression signature. The highly variable telomere lengths and shortening trajectories suggest unique replicative histories and distinctly divergent futures. It is therefore plausible that expression profiles of telomeric genes may also differ between immune cell subsets.

Areas for Investigation

Many similar studies have used low sample sizes, highly trained participants (Nieman et al., 1994), discrepant intervention durations (Nieman & Henson, 1994), and/or intensities (Shek et al., 1995). Additionally, many of the studies in the field of exercise immunology use exercise work rates equivalent to percentages of $\dot{V}O_{2peak}$ or heart rate, such as treadmill velocity achieved at a given percentage of $\dot{V}O_{2peak}$. This is likely to result in inaccuracies in intensity reporting due to the

inconsistent linearity between the two indices. Other studies report exercise intensities at specific intensities yet fail to report how or if the intensity was monitored throughout. The variable exercise intensity used across studies is of particular importance as the nature and magnitude of the exercise-induced immune response closely scales with the exercise intensity (Simpson, 2013). There is little known about the precise immune response to 30 min of treadmill running at a constant and accurately measured 80% of $\dot{V}O_{2peak}$. Although not measured directly in this study, the data will be used in a later assessment of exercise-induced changes in telomere-associated genes and pathways. Little is known about the acute mechanisms that may underpin the cross-sectional associations between PA and LTL. Given the heterogeneous telomere lengths and gene expression profiles of immune cell subsets, an accurate characterization of the immune response to exercise is a logical starting point.

2.3 Aims

The aims of this study were to: (i) characterize the acute T cell response to 30 min of intense cardiorespiratory exercise in males and, (ii) sort CD4+ and CD8+ T cell subsets for the assessment of telomeric gene expression and subsequent comparison with leukocyte composite gene expression signatures.

Hypothesis

The hypothesis for this study is that a 30 min bout of precisely and continuously monitored aerobic exercise at 80% of $\dot{V}O_{2peak}$ would differentially regulate T cell subsets.

2.4 Methods

Ethics Statement

All eligible participants read a plain language information statement outlining all aspects of the project in lay terminology. Informed consent documents explaining the purpose, potential risk and benefits of the project were then signed in the presence of a witness. The study, recruitment and consent procedures were approved by the Human Research Ethics Committee from Federation University Australia (HREC approval #: A10-119).

Participants

Twenty-two healthy, non-smoking males (mean age = 24.1 ± 1.5 years) were recruited to participate in this study (Table 6).

Physiological Measures

Resting blood pressure and pulse were taken using an Omron® HEM-7203 automated blood pressure monitor (Table 6). Participants were seated comfortably for 10-15 min prior to measurements to minimize sympathetic distortion. Each measure was taken 2-3 times throughout the assessment and average values were obtained and used.

Anthropometric Measurements

Body mass was determined using standard, calibrated electronic scales; height was determined using a standard free-standing stadiometer (table 6). Waist and hip measurements were taken at standardized sites using a 2 m metal anthropometry tape measure. Waist measurements were taken at the narrowest point between the iliac crest and bottom ribs. Hip circumference was taken at the point of maximum posterior extension of the buttocks, as viewed laterally.

Determination of Fitness Standard

Participants undertook a treadmill test with an incremental step protocol until exhaustion (ramp test to exhaustion, RTE) to determine the peak rate of oxygen consumption ($\dot{V}O_{2peak}$), measured as millilitres of oxygen per kilogram of body mass, per minute ($mL \cdot kg^{-1} \cdot min^{-1}$). $\dot{V}O_{2peak}$ is the highest rate of oxygen consumption attained during a given test and is viewed as a surrogate marker for $\dot{V}O_{2max}$ when definitive maximal values have not been achieved.

Physiological testing was conducted using a Metalyser® metabolic system (Cortex Biophysic, Leipzig, Germany). Inspiratory and expiratory air flow was measured by a mouth piece-mounted flow transducer which was calibrated using a 3 litre Hans Rudolph calibration syringe. Atmospheric pressure was determined using a digital barometer. The Metalyser® was initially calibrated using bottled commercial gas (O_2:16%, CO_2 :4%, balance in N_2) and again using ambient air. The Metalyser® recorded and displayed oxygen consumption data at 10 second averages and transferred the data to a computer for analysis using Metasoft II® software.

Prior to the test, participants were advised the following:

- Wear comfortable clothing and athletic shoes.
- Drink adequate fluids (water) during the 24 hour period before the test.
- Refrain from eating, smoking, and drinking alcohol or caffeine for at least 3 hours prior to the test.
- Do not engage in strenuous physical activity the day before the test.
- Get adequate sleep (6-8 h) the night before the test.

Table 6. Physical characteristics of the 22 male participants

Characteristic	Mean	SD
Age (years)	24.0	± 7.3
Height (cm)	180.7	± 4.3
Body Mass (kg)	78.5	± 9.0
BMI (kg/m^2)	24.0	± 2.5
Waist (cm)	81.5	± 5.6
Hip (cm)	98.6	± 5.1
Waist:hip ratio	0.8	± 0.03
Systolic BP (mmHg)	130.8	± 11.7
Diastolic BP (mmHg)	72.0	± 8.3
Resting HR ($b \cdot min^{-1}$)	64.1	± 11.6

SD (standard deviation); **BMI** (body mass index); **BP** (blood pressure); **HR** (heart rate); **mmHg** (millimetres of mercury); **$b \cdot min^{-1}$** (beats per minute).

Participants were fitted with a safety harness attached to an automatic shutdown switch to ensure immediate treadmill cessation if the participant stumbled. The test protocol started with a 5 min warm up period performed at 10km·h^{-1} after which the speed increased by 1km·h^{-1} each minute. The speed of work-rate imposition was chosen to decrease the likelihood of premature mechanical fatigue before metabolic exhaustion was achieved. The treadmill remained at a constant 0% gradient throughout the test. Breath by breath gas exchange and heart rate were continually monitored throughout.

The test was terminated upon participant volitional failure; however, all participants were vigorously encouraged to continue until an obvious a plateauing of O_2 intake was achieved. All testing was conducted between 7:30am and 10:30am to minimize potential circadian influence. A summary of exercise test data is contained in table 7.

Exercise Intervention

Participants were scheduled into an exercise intervention session a minimum of 5 days and no more than 7 days after the initial fitness test. This period was allowed to minimize the impact of any discomfort and inflammation resulting from the maximal treadmill test. All participants provided prior written informed consent and appropriate first aid trained staff and first aid equipment were at hand throughout all stages of the testing process. Participants were asked to refrain from vigorous physical activity during the preceding 48 hours. All exercise interventions were conducted between 7:30am to 10:30am to minimize potential circadian influence.

Participants arrived 10 min before their scheduled time and were seated quietly to ensure that baseline blood pressure and heart rate measures were not elevated.

A pre-exercise (Pre-Ex) 10 ml blood sample was taken from the median cubital vein using 10 ml K2E EDTA Vacutainer blood collection tubes with Eclipse® Blood Collection Needles and pre-attached holders (21G x 1-1/4") (BD Biosciences, Australia) (figure 8). All blood samples were kept on ice until white blood cell isolation and storage. Participants remained seated for an additional 30 min to ensure no adverse reaction to the blood sample during which time participants were briefed on the treadmill safety protocol.

Participants were then fitted with a heart rate monitor (Polar Electro®, Australia), safety harness and the respiratory mask head assembly. A 5 min warm-up period was undertaken at $10km \cdot h^{-1}$ before commencing a 30 min continuous bout of treadmill running at 80% of previously determined $\dot{V}O_{2peak}$. An exercise intensity of 80% of $\dot{V}O_{2peak}$ was selected as it had been used in other similar studies and shown to elicit a vigorous cardiorespiratory and metabolic response (Connolly et al., 2004; Cooper, Barstow, Bergner, & Lee, 1989). Other studies have demonstrated significant transcriptional responses to a 30 min bout of exercise at 80% $\dot{V}O_{2peak}$ (Radom-Aizik et al., 2008; Sakharov et al., 2012). Additionally, exercise-induced changes in T cell numbers are closely correlated with exercise intensity (McCarthy & Dale, 1988; Shek et al., 1995). A range of exercise immunology studies have elicited significant immune cell number responses using intensities of 80% of $\dot{V}O_{2peak}$ and durations of 30-45 min (Nieman et al., 2003; Nieman, Miller, et al., 1993; Robson, Blannin, Walsh, Bishop, & Gleeson, 1999).

Table 7. Treadmill ramp test exercise data.

Characteristic	Mean	SD
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	49.3	± 4.8
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	3.1	± 0.5
Maximal heart rate ($b.min^{-1}$)	178	± 8.9
Maximum V'E ($L \cdot min^{-1}$)	125.9	± 12.4
Maximum METs	13.9	± 1.5
Maximum RER	1.2	± 0.1
Test duration (min:s)	12.5	± 1.6
Velocity at $\dot{V}O_{2peak}$ ($km \cdot h^{-1}$)	17.1	± 1.6
Maximum velocity ($km \cdot h^{-1}$)	17.6	± 1.7
Ambient temp (°C)	16.8	± 2.7
Ambient pressure (mbar)	957.8	± 8.7

$\dot{V}O_{2peak}$ (highest oxygen consumption achieved in test); **$b \cdot min^{-1}$** (beats per minute); **V'E** (minute ventilation); **METs** (metabolic equivalents); **RER** (respiratory exchange ratio); **mbar** (millibar);

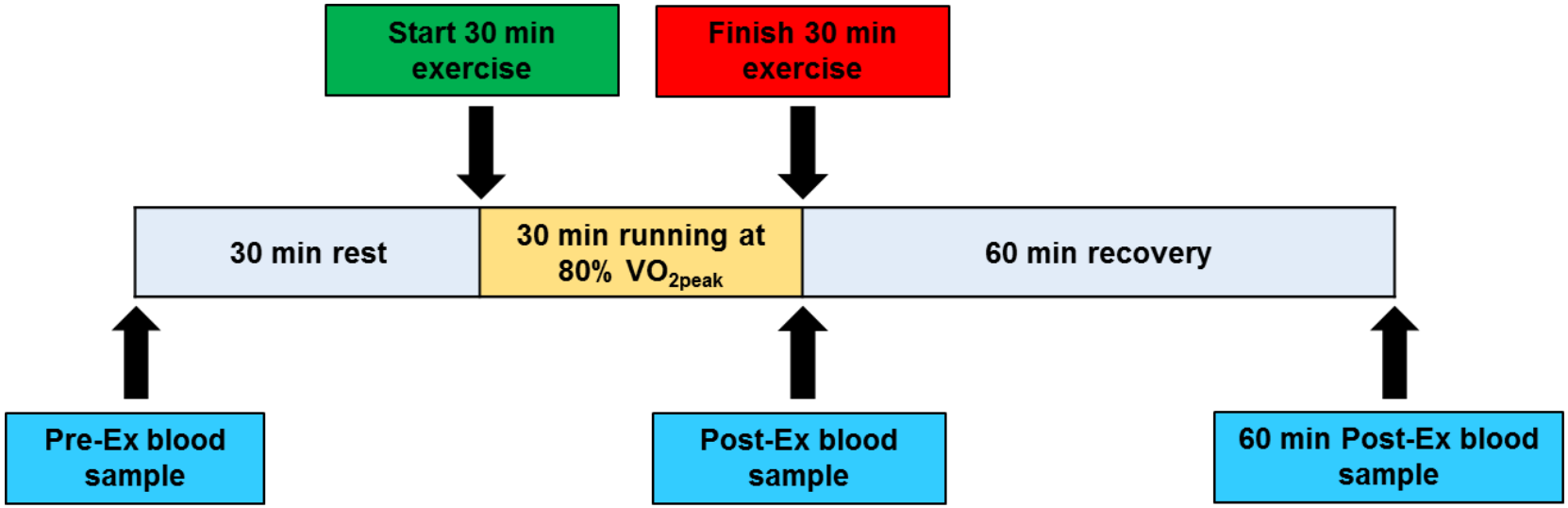


Figure 8. A schematic overview of study design. A baseline blood sample was taken 30 min before the onset of exercise. Participants then completed a 30 min bout of treadmill running at 80% of previously determined $\dot{V}O_{2peak}$. Additional blood samples were taken Post-Ex and 60 min Post-Ex.

Exercise intensity was calculated accordingly; $\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$) x 0.8 = target intensity. Percentages of heart rate reserve (%HRR) or oxygen uptake reserve (%$\dot{V}O_2R$) were not used to prescribe exercise intensity due to the methodological limitations and instability of the purported relationship between the two values (da Cunha, Farinatti, & Midgley, 2011). Heart rate and breath-by-breath gas exchange were continually monitored over the 30 min period and slight changes in treadmill speed were made to maintain the correct intensity.

Participants undertook a second blood test immediately after the exercise intervention (Post-Ex) before commencing a gentle walking cool down. A third blood sample (60 min Post-EX) was taken 60 min after the exercise intervention. Participants were instructed to passively rest and refrain from consuming caffeine, alcohol or nicotine in between the second and final blood tests. Once filled, the EDTA treated vacutainers were immediately inverted several times to ensure adequate mixing. The mean time from blood draw to white blood cell isolation did not exceed 90 min. A summary of participant exercise intervention data is contained in Table 8. The mean percentage of $\dot{V}O_{2peak}$ maintained throughout the 30 min intervention was 80.8% (SD ± 7.1). The SD of ± 7.1 represents 7.1% and reflects the spread of mean exercise intensities over the 30 min intervention period. Despite adjustments to treadmill speed throughout the intervention, some participants' $\dot{V}O_{2peak}$ transiently spiked for short periods of time before settling back into the desired range.

Table 8. Thirty minute treadmill intervention exercise data.

Characteristic	Mean	SD
% of $\dot{V}O_{2peak}$ during 30 min run	80.8	± 7.1
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	3.1	± 0.3
Average heart rate ($b \cdot min^{-1}$)	163.7	± 12.9
Average V'E ($L \cdot min^{-1}$)	94.6	± 14.3
Average METs	11.3	± 0.9
Average RER	1.0	± 0.1

$\dot{V}O_{2peak}$ (highest oxygen consumption achieved in test); **$b \cdot min^{-1}$** (beats per minute); **V'E** (minute ventilation); **METs** (metabolic equivalents); **RER** (respiratory exchange ratio).

Cell Preparation

Whole blood samples were spun at 1000 x g (2250rpm) (4°C) for 15 min to separate the plasma and haematocrit sub-fractions. The isolated buffy coats were treated with red blood cell lysis buffer, spun at 300 x g for 10 min, and washed twice in sterile phosphate buffered saline (PBS). This technique isolated leukocytes, a heterogeneous mix of neutrophils, basophils, eosinophils, lymphocytes, and monocytes. The leukocytes were re-suspended in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies®) containing 10% Fetal Bovine Serum (PBS/FBS) (Life Technologies®) and 200µl (10% of total end volume) of Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich®). Samples were stored at -80°C for 24 hour before being transferred to liquid nitrogen storage until analysis.

Labelling of Cell Surface Antigens

Frozen cells were rapidly thawed and resuspended in 15 ml of IMDM + 10% PBS/FBS to dilute the DMSO, which is toxic to thawed cells. Cells were then spun at 300 x g for 10 min, the supernatant was removed, and the cells were resuspended in 15 ml of IMDM prior to analysis. The thawed cells were incubated for 30 min at 4°C with the following fluorochrome-conjugated monoclonal antibodies (mAbs): fluorescein (FITC)-conjugated anti-CD3, allophycocyanin (APC)-conjugated anti-CD8, phycoerythrin (PE)-conjugated anti-CD45RO, V450-conjugated anti-CD4, and phycoerythrin cyanine dye Cy7 (PECy7)-conjugated anti-CD45RA (BD Biosciences®) (table 9). The fluorochrome-conjugated mAbs used in the antibody panel were a combination of single and tandem dyes. Fluorochromes are chemical labels that emit absorbed light at different wavelengths and are conjugated to specific monoclonal antibodies (mAbs).

Table 9. Selected fluorochrome-conjugated mAbs and respective emission spectra.

mAb	Fluorochrome	Laser excitation line (nm)	Maximal excitation (nm)	Maximal emission (nm)
CD3	FITC	488	494	520
CD45RO	PE	488, 532, 561	496	578
CD45RA	PECy7	488, 532, 561	496	785
CD8	APC	633, 635, 640	650	660
CD4	V450	405	404	448

Each tube was resuspended in 200µl of PBS/FBS and 5µl of Propidium iodide (PI) prior to analysis to identify live cells. Dead cells in suspension can confound results by generating nonspecific antibody binding and/or inappropriate assimilation of fluorescent probes. The membrane of a viable cell is impermeable; however, PI can infiltrate a damaged cell and bind to double stranded DNA. PI can be excited at of 488nm and emits at a wavelength of 617nm, making it compatible with fluorochromes such as PE and FITC.

Titrations

Titrations were performed to determine the most effective mAb concentration to achieve clear, disparate signals from both positive and negative cell populations. Excessive mAb concentration can contribute to background fluorescence and misinterpretation of data. Effective working concentrations had been determined for CD45RO-PE, CD45RA-PECy7, CD8-APC, and CD4-V450 prior to the experiments. A titration was conducted for CD3-FITC as this fluorochrome-conjugated mAb combination had not been used before. The final working volumes of each mAb in the antibody panel are summarized in table 10.

Single Antibody Stain Compensations

The broad spectral range of many fluorochrome emissions may result in spectral overlap into the detection threshold of another detector. This is known as spectral *spill over* or *overlap* and can be mitigated by spectral compensation. This is performed by subtracting a percentage of fluorescence present in the primary detector from the secondary detector; leaving the desired signal in the primary detector only. Pre-acquisition electronic compensations were conducted using single antibody stains.

Table 10. Working volumes of each mAb used in the antibody panel

mAb	Volume (µl) in a 120 µl reaction
CD3-FITC	10 µl
CD45RO-PE	10 µl
CD45RA-PECy7	2.5 µl
CD8-APC	5 µl
CD4-V450	2.5 µl

Unstained samples

The excitation sources used in flow cytometry can create auto-fluorescence by exciting cellular components such as flavins and nicotinamide adenine dinucleotide phosphate (NADPH). The extent of auto-fluorescence can also be influenced by cell type and physiological condition (Monici, 2005). Fully processed, unstained tubes for each sample of interest were analysed with the same settings used for the stained samples. Auto-fluorescence was then compared to values obtained from standardised calibration beads.

Fluorescence minus one

Florescence minus one (FMO) controls were also conducted as part of the compensation process. FMO controls contain every stain in the panel except the one of interest and are used to accurately identify gating boundaries. By omitting the stain of interest from the panel, the difference between intrinsic auto-fluorescence and bound fluorochrome fluorescence can be determined. This allows accurate determination of positive and negative cells within a particular subset (Tung et al., 2007). A summary of each FMO control used appears in table 11.

Flow Cytometry

Flow cytometry was performed using a FACSARIA™ II Flow Cytometer (BD Biosciences®), utilizing a red laser emitting at 633-nm, a blue laser emitting at 488-nm, and a violet laser emitting at 405-nm. The flow cytometer was configured with three detector arrays; an octagon and two trigons. The octagon array houses six photomultiplier tubes (PMTs) to detect light from the 488-nm blue laser and each trigon houses two PMTs to detect light from the red (633-nm) and violet (405-nm) lasers respectively (Table 12).

Table 11. Florescence minus one controls used in the pre-acquisition compensation.

Tube	FITC	V450	APC	PEcy7	PE
Full stain	CD3	CD4	CD8	CD45RA	CD45RO
FMO 1		CD4	CD8	CD45RA	CD45RO
FMO 2	CD3		CD8	CD45RA	CD45RO
FMO 3	CD3	CD4		CD45RA	CD45RO
FMO 4	CD3	CD4	CD8		CD45RO
FMO 5	CD3	CD4	CD8	CD45RA	

Table 12. The optical configuration of the FACSARIA™ II Flow cytometer.

Detector array	PMT	LP Mirror	BP Filter	Intended Fluorochrome
Octagon	A	735	780/60	PE-Cy™7
(488-nm blue laser)	B	655	695/40	PerCP-Cy5.5™ or PI
			675/20	PerCP, PE-Cy5
	C	610	616/23	PE-Texas Red®
	D	556	585/42	PE or PI
	E	502	530/30	FITC, GFP, Alexa 488
	F	-	488/10	Side scatter (SSC)
Trigon	A	502	530/30	Alexa Fluor® 430/Am Cyan
(405-nm violet laser)				
	B	-	450/40	Cascade Blue®, Pacific Blue™, DAPI, Hoechst, Alexa Fluor® 405
Trigon	A	735	780/60	APC-Cy7, APC-H7
(633-nm red laser)	B	-	660/20	APC

Identification of T Cell Subsets

Total CD3+ cells were electronically gated based on forward scatter (FSC-A)/side scatter (SSC-A) distribution and gated into subsets according to antigen expression (figure 9). Singlets were then electronically gated based on (FSC-A) and forward scatter height (FSC-H), and then SSC-A and side scatter height (SSC-H). The two singlet gates reduced the likelihood of including cells stuck vertically or horizontally together. Live lymphocytes were gated according to their expression of PI against FSC-A. The CD3+ T cell population was identified by FSC-A against FITC expression and then further separated into CD4+ and CD8+ subsets based on positive antigen expression of V450 and APC respectively.

The CD45RA+CD45RO- (naïve) and CD45RA-CD45RO+ (memory) subsets were broadly identified by differential CD45RA and CD45RO expression. Cells exclusively gated on positive expression of CD45RA will encompass naïve and CD45RA+ effector memory phenotypes in both CD4+ and CD8+ subsets. Similarly, exclusively gating on positive expression of CD45RO will encompass central memory and effector memory phenotypes (Sallusto, Geginat, & Lanzavecchia, 2004). All borderline populations were routinely back-gated to confirm their phenotype. Sorted cells were sorted into PBS and stored at -80°C until analysis.

Data Collection and Analysis

Cell numbers were collected in linear mode and fluorescent signals were collected in logarithmic mode. A minimum of 10, 000 events per sample were collected for analysis. All flow cytometry analysis was performed using FlowJo® flow cytometry analysis software.

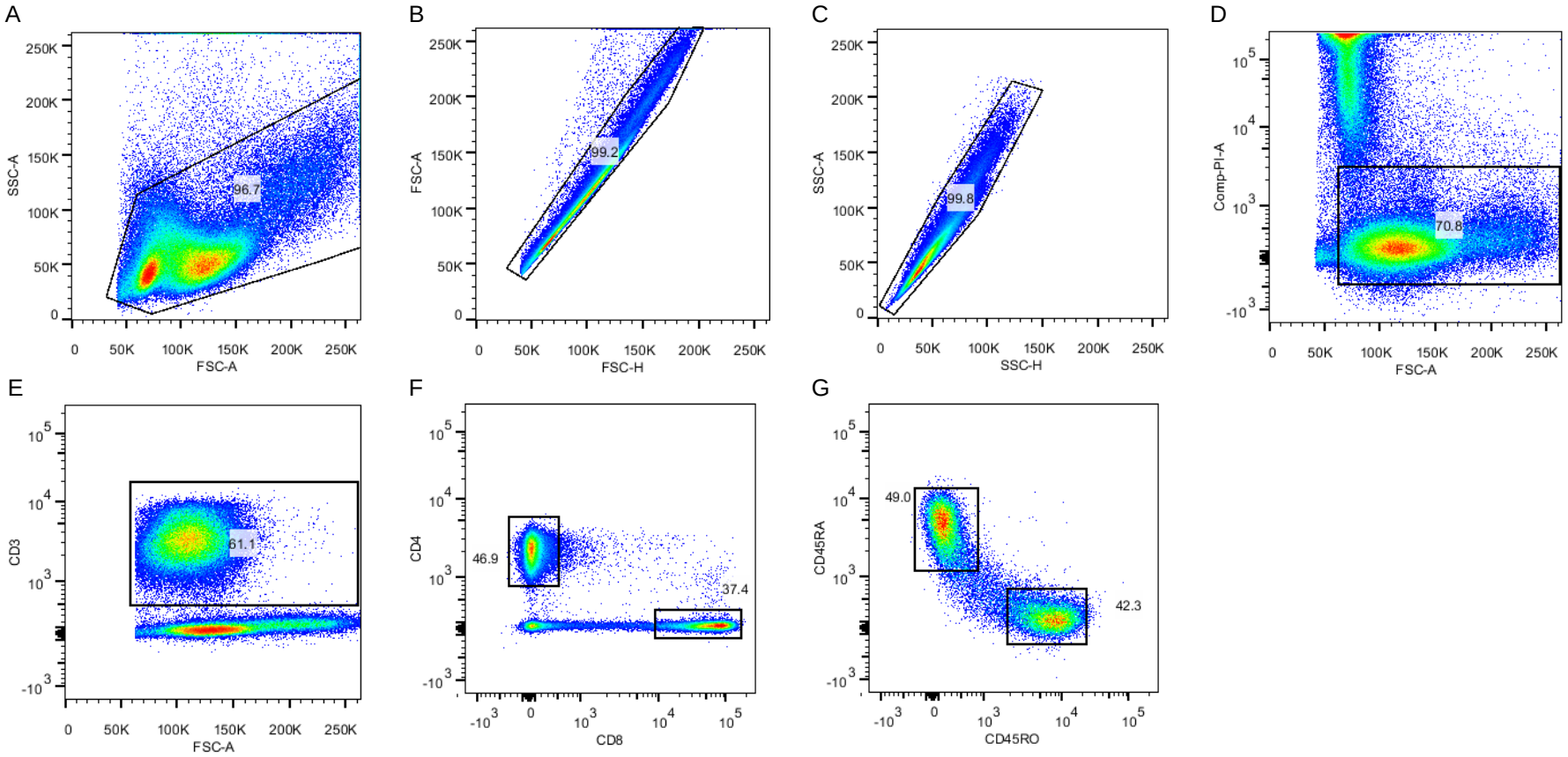


Figure 9. Flow cytometry gating strategy: T cells were gated on distribution against: SSC-A and FSC-A **(A)**. Singlet gates **(B),** and **(C).** Negative expression of PI against FSC-A **(D).** FITC fluorescence against FSC-A **(E).** Fluorescent expression of V450 and APC respectively **(F).** The CD45RA+CD45RO- and CD45RA-CD45RO+ subsets were gated according to their fluorescent expression of PECy7 and PE **(G).**

Statistical Analysis

All results are expressed as means ± standard error of the mean (SEM). Shapiro-Wilk normality tests were conducted on all data sets. Normally distributed (parametric) data were assessed using a repeated measures ANOVA. Post hoc pairwise comparisons with Bonferroni correction were also used to determine the significantly different time points. Non-normally distributed (non-parametric) data were assessed using a non-parametric Friedman's repeated measures ANOVA. Pairwise comparisons were performed where appropriate using a non-parametric Wilcoxon test. Statistical significance was set at $P<0.05$. All statistical analysis was performed using SPSS® (version 17).

2.5 Results

Immune response to exercise

A summary of exercise-induced subset changes appears in table 13. Values are presented as percentages of parent subsets.

Live cells (total leukocytes)

Normality checks (Shapiro-Wilk) determined the data was approximately normally distributed. Mauchly's test indicated the assumption of sphericity was met ($\chi^2(2) = 5.86$, $P=0.05$). A repeated measures ANOVA showed that mean relative leukocyte frequency differed significantly between time points [$F(2, 42) = 10.36$, $P<0.001$]. Post hoc pairwise comparisons using the Bonferroni correction revealed the significant effect of time between Pre-Ex and 60 min Post-Ex time points ($P<0.001$) (figure 10).

Table 13. A summary of exercise-induced changes in lymphocyte frequencies.

T cell Subset	Pre-Ex	Post-Ex	60 min Post-Ex
% of live cells (total leukocytes)	68.7 ± 2.4	64.5 ± 3.1	58.5 ± 2.5
CD3+ % of live cells	58 ± 2.3	50 ± 2.3	58.5 ± 2.5
CD4+ % of total CD3+	43.7 ± 2.8	36.7 ± 2.9	48.2 ± 2.5
CD4+CD45RA+CD45RO- % of total CD4+	50.5 ± 2.8	49.5 ± 2.6	46.8 ± 2.5
CD4+CD45RA-CD45RO+ % of total CD4+	36.2 ± 3.3	35.2 ± 2.9	38.4 ± 3.3
CD8+ % of total CD3+	41.9 ± 2.7	44.8 ± 2.9	39.9 ± 2.4
CD8+CD45RA+CD45RO- % of total CD8+	56.3 ± 2.8	52.1 ± 2.6	55.6 ± 2.4
CD8+CD45RA-CD45RO+ % of total CD8+	24.1 ± 2.3	26 ± 2.3	24.9 ± 2.3
CD4+/CD8+ ratio	1.2 ± 0.1	0.9 ± 0.1	1.4 ± 0.1

± standard deviation

CD3+ T cells

The data was determined to be normally distributed and the assumption of sphericity was met, sphericity met $\chi^2(2) = 2.76$, $P=0.25$. A repeated measures ANOVA determined that mean relative frequency of CD3+ T cells differed significantly between time points [$F(2, 42) = 12.95$, $P<0.001$]. Post hoc pairwise comparisons revealed a significant effect of time between Pre-Ex and 60 min Post-Ex ($P=0.001$), and Post-Ex and 60 min Post-Ex time points ($P=0.002$) (figure 10).

CD4+ T cells

Normally distributed data (sphericity met, $\chi^2(2) = 0.135$, $P=0.94$) was assessed using a repeated measures ANOVA. A significant effect of time on the relative frequency of CD4+ T cells (expressed as a percentage of CD3+ T cells) was observed across time points [$F(2, 42) = 34.4$, $P<0.001$]. Post hoc pairwise comparisons revealed a significant effect of time between Pre-Ex and Post-Ex ($P<0.001$), Pre-Ex and 60 min Post-Ex ($P=0.010$), and Post-Ex and 60 min Post-Ex time points ($P<0.001$) (figure 10). The relative frequency of CD4+CD45RA+ T cells (expressed as a percentage of CD4+ T cells) also demonstrated a significant effect of time across time points [$F(2, 42) = 4.48$, $P=0.017$]. Post hoc pairwise comparisons identified significant differences in means between Pre-Ex and 60 min Post-Ex time points ($P=0.05$). There were no significant changes in the relative percentage of CD4+CD45RO+ T cells across time points ($P=0.06$) (figure 11).

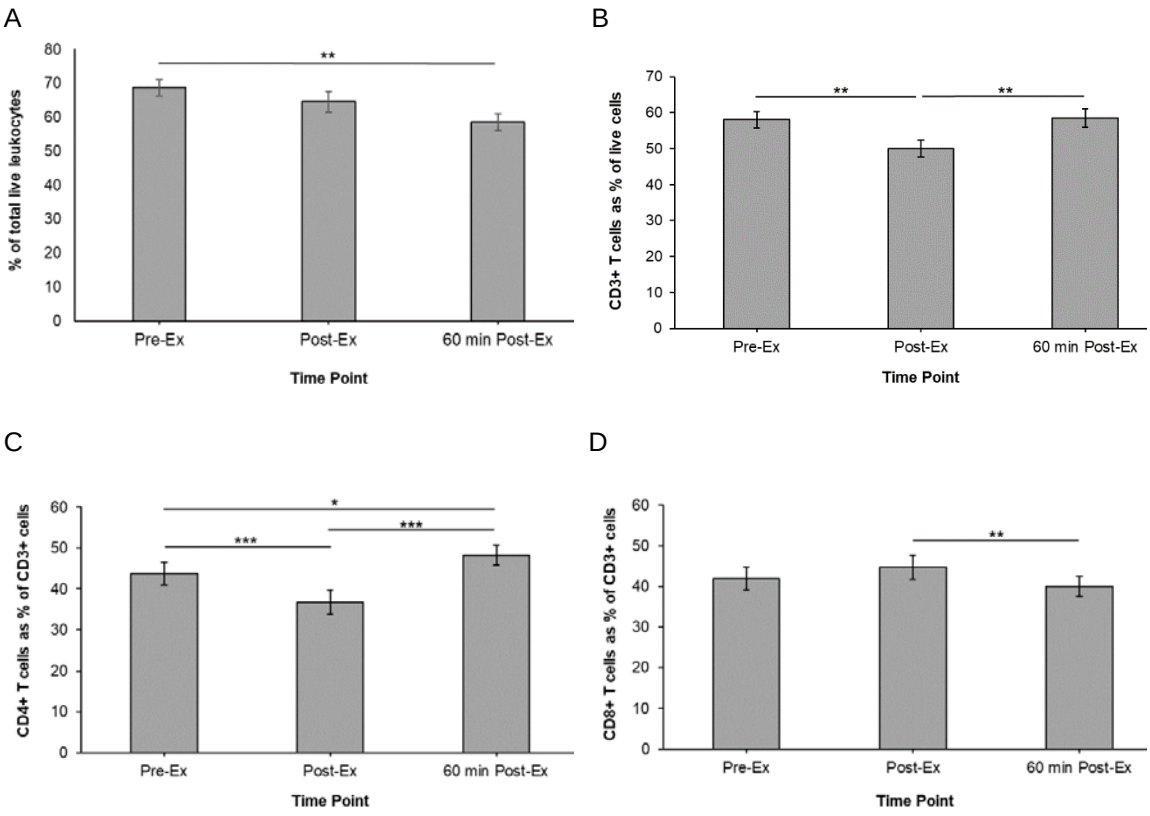


Figure 10. Exercise-induced changes in leukocyte and T cell populations: Leukocyte and T cell populations were measured at each time point and expressed as relative percentages (n = 22). The relative change in subset percentages were assessed in total live leukocytes **(A)**, CD3+ T cells **(B)** CD4+ T cells **(C)** and CD8+ T cells **(D)**. Error bars indicate SEM. † indicates *P*=0.05, * indicates *P*<0.05, ** indicates *P*<0.01 and *** indicates *P*<0.001.

CD8+ T cells

The normally distributed data (sphericity met, $\chi^2(2) = 1.18$, $P=0.56$) was assessed using a repeated measures ANOVA. There was a significant effect of time on the relative frequency of CD8+ T cells (expressed as a percentage of CD3+ T cells) [F (2, 42) = 10.30, $P<0.001$]. A post hoc pairwise comparison identified a significant difference in means between Post-Ex and 60 min Post-Ex time points ($P=0.001$). The relative frequency of CD8+ T cells underwent a non-significant increase between Pre-Ex to Post-Ex time points ($P=0.06$) (figure 10). The relative frequency of CD8+CD45RA+ T cells demonstrated a significant effect of time across time points [F (2, 42) = 4.56, $P=0.016$]. A significant difference in means was identified between Post-Ex and 60 min Post-Ex time points ($P=0.030$). There was no significant change in the relative percentage of CD8+CD45RO+ T cells (figure 11)

2.6 Discussion

The major finding of this study was that 30 min of treadmill running at a consistent 80% of $\dot{V}O_{2peak}$ elicited a Post-Ex percentage decrease in CD3+ T cells. The exercise-induced decrease in CD3+ cell numbers reverted to Pre-Ex concentration within 1 hour of exercise cessation. This finding runs orthogonal to the archetypal CD3+ T cell exercise response. What follows is a discussion of plausible hypotheses that may account for the novel results obtained. Whilst the current study was not designed to test explanatory mechanisms, it is likely that the results reflect the cumulative influence of more than one mechanism.

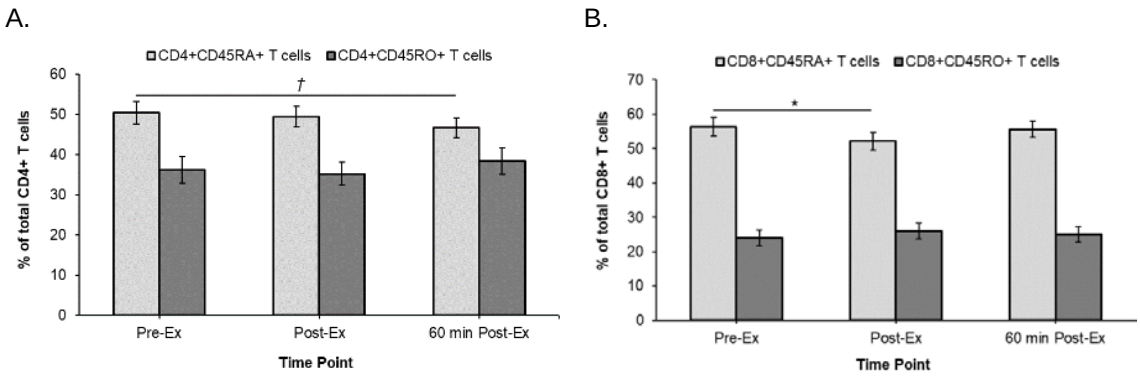


Figure 11. Exercise-induced changes in CD45RA+ and CD45RO+ T cell populations: Relative changes in CD45RA+ and CD45RO+ phenotypes were assessed in CD4+ T cells **(A)** and CD8+ T cells **(B)** respectively. Error bars indicate SEM. *†* indicates P=0.05, * indicates *P*<0.05.

The concomitant Post-Ex decrease in CD3+ and CD3+CD4+ T cells are likely linked. The CD3+ T cell population encompasses all lymphocytes expressing the cell maker CD3+ (CD4+ and CD8+ T cells). A significant decrease in CD3+CD4+ T cells would be reflected in total CD3+ T cells numbers. Despite the significant decrease in CD3+ T cells from Pre- to Post-EX, the CD3+CD8+ subset underwent a post-exercise increase (non-significant). Similarly, despite the significant Post- to 60 min Post-Ex increase in CD3+ T cells, the CD3+CD8+ subset significantly decreased at the same time point. This strongly implies that the CD3+CD4+ T cell subset underpinned the exercise-induced changes in CD3+ T cells.

Discrepant reporting conventions can obscure the broader consensus on exercise-induced changes in immune cells. Many research articles express cell changes as the number of cells per given unit of blood (litre); *cells x* $10^{9 \cdot l^{-1}}$ (Nieman, 1994; Robson, Blannin, Walsh, Castell, et al., 1999). Others express changes in a subordinate cell subset as a percentage of a parent subset, e.g. CD4+ T cells expressed as a percentage of all CD3+ T cells (Anane et al., 2009; Bruunsgaard & Pedersen, 2000; Haq, al-Hussein, Lee, & al-Sedairy, 1993; Steensberg et al., 2001). Some studies represent cell changes at multiple time-points as a percentage of the basal concentration (McCarthy et al., 1991). These subtle differences can at first glance provide confusing conclusions. Expressed in absolute terms (cells x $10^{9 \cdot l^{-1}}$), CD4+ T cell numbers often increase immediately post-exercise (Nieman et al., 1994). However, when expressed as a percentage of all CD3+ lymphocytes (a parent subset that is itself in exercise-induced flux), CD4+ T cells demonstrate a post-exercise decrease. The specific reporting method is often chosen to highlight a given aspect of the results i.e. changes in a specific cell type or changes in the distribution of one population relative to another. The results

in this thesis are reported as percentages of a parent subset to highlight the significant changes in cell proportions relative to each other.

The counter-intuitive CD3+ T cell profile in the present study may have been due to the high exercise intensity used, given that changes in T cells closely correlate with exercise intensity (McCarthy & Dale, 1988; Shek et al., 1995). A similar post-exercise decrease in the percentage of CD3+ T cells was demonstrated in marathon runners; however, mean participant exercise intensities were not available (Haq et al., 1993). In the present study, the intensity of the 30 min exercise bout was continuously monitored via heart rate and expired gas analysis. This allowed the treadmill speed to effectively be *titrated* to continuously elicit the correct intensity.

A constant exercise work rate performed for periods of 15 min or greater will typically elicit a gradual increase in heart rate and oxygen consumption. This is due to decreases in blood volume, cutaneous redistribution of blood, the subsequent reduction in stroke volume, and compensatory increase in heart rate. The continuous analysis of expired gas in the present study also allowed for a maximum time spent at 80% of $\dot{V}O_{2peak}$ and minimized potential lag time associated with a consistent workload.

The relative frequency of CD4+ T cells (expressed as a percentage of total CD3+ T cells) demonstrated an immediate Post-Ex decrease followed by a 60 min Post-Ex increase beyond resting concentration. This finding broadly concurs with previous studies (Nielsen, Secher, Kappel, & Pedersen, 1998; Nieman, 1994). The CD4+ biphasic response may be due to a reflexive extravasation of highly

differentiated CD4+ T cells such as Th1 and Th2 cells, Th17 cells, follicular helper (Tfh) cells, and induced regulatory T (iTreg) cells (Zhou, Chong, & Littman, 2009).

Changes in CD8+ T cells were also biphasic, increasing immediately post-exercise before returning to resting concentration. This concurs with the heightened exercise response typically observed in CD8+ T cells (Nielsen et al., 1998; Nieman, 1994). The CD8+ T cell subset exhibits high β_2-adrenergic receptor density which regulates migration into peripheral blood by facilitating cellular detachment from vascular endothelium (Anane et al., 2009; Benschop, Nijkamp, Ballieux, & Heijnen, 1994; Dimitrov, Lange, & Born, 2010). Whilst not statistically significant, the CD8+ T cell subset in the present study increased from Pre- to Post-Ex. This mild lymphocytosis may be attributed to preferential catecholamine-induced migration to the periphery. Exercise-induced lymphocytosis is transient and is typically followed by a reflexive egress of the same lymphocyte subsets. This extravasation, often greater in magnitude than the initial increase, causes a temporary state of lymphocytopenia (Simpson et al., 2010; Simpson et al., 2007). The CD8+ T cell subset in the present study demonstrated a similar reflexive egress that was observed at 60 min Post-Ex.

Exercise preferentially redistributes memory T cells (CD45RO+) into the periphery (Gabriel, Schmitt, Urhausen, & Kindermann, 1993; Gannon, Rhind, Shek, & Shephard, 2002; Lancaster et al., 2005); however, the present study only identified changes within the naïve subset. Elderly individuals have increased proportions of memory (CD45RO+) T cells and decreased proportion of naïve (CD45RA+) T cells in CD4+ and CD8+ subsets (De Paoli, Battistin, & Santini, 1988; Pilarski, Yacyshyn, Jensen, Pruski, & Pabst, 1991; Utsuyama et al., 1992). The lack of significant CD45RO+ T cell modulation in the present study may be due to

the relatively low mean age of the participants (24.1 ± 1.6 years) and the likely low basal number of CD45RO+ T cells. Another factor may have been the selective use CD45RA as the sole identifier of naïve T cell status. Expression of CD45RA is a necessary but not sufficient characteristic of naïve T cells. Whilst still viewed as a legitimate marker of naïve T cell status, CD45RA can be re-expressed on a subset of effector-memory T cells with significant replicative history (Sallusto, Lenig, Förster, Lipp, & Lanzavecchia, 1999). Combinatorial staining with CD45RA, CCR7 and CD62L would have provided finer discriminatory analysis of the naïve subset (Sallusto et al., 2004). Cells gated on expression of CD45RA+ alone will likely encompassed naïve and CD45RA+ effector memory phenotypes in both CD4+ and CD8+ subsets; potentially distorting naïve subset changes. Similarly, gating on expression of CD45RO as a marker of memory phenotype, likely captured central memory and effector memory phenotypes.

The CD3+ response in the present study may have been influenced by catecholaminergic signalling. Animal and human studies have demonstrated that plasma epinephrine and cortisol suppress IFN-γ and IL-2 producing type 1 T cells (Elenkov & Chrousos, 1999; Franchimont et al., 2000). Plasma epinephrine and cortisol increase during and immediately after exercise due to activation of the hypothalamic-pituitary-adrenal axis (HPA) and sympathetic nervous system (SNS) activation (Galbo, 1983). Steensberg et al. (2001) observed an increase in plasma epinephrine (significant) and cortisol (non-significant) after 30 min of treadmill running at 75% of $\dot{V}O_{2max}$. Although not measured in the present study, it is plausible that post-exercise cortisol and epinephrine concentrations were elevated and influenced immediate post-exercise T cell numbers via suppression of specific type 1 T cell subsets.

The effect of participant training status on leukocyte migration is unclear. Exercise-induced changes in peripheral lymphocyte percentages are not correlated with fitness standard (Kendall, Hoffman-Goetz, Houston, MacNeil, & Arumugam, 1990; Moyna et al., 1996). However, exercise-induced increases in absolute numbers of CD4+ T cells, CD8+ T cells, and NK cells have been observed in lower fitness standards (Kendall et al., 1990). Additionally, a six week aerobic training intervention blunted the effect of acute exercise on lymphocyte mobilisation (Soppi, Varjo, Eskola, & Laitinen, 1982). This blunting effect was caused by reduced β_2-adrenergic receptor sensitivity and density (Butler, O'Brien, O'Malley, & Kelly, 1982) and a reduction in glucocorticoid receptor sensitivity (Duclos, Gouarne, & Bonnemaison, 2003). The mean $\dot{V}O_{2peak}$ in the present study (49.3 ± 4.8 $mL \cdot kg^{-1} \cdot min^{-1}$) falls in the upper limit of the *good* cardiorespiratory fitness classification (46-50 $mL \cdot kg^{-1} \cdot min^{-1}$) (Cooper Institute for Aerobics, 2005). This $\dot{V}O_{2peak}$ value suggests a significant exercise history in the present cohort and may provide a plausible explanation for the absent post-exercise lymphocytosis.

The infection history or antigenic exposure of T cells also appears to modulate the migratory response to acute exercise. CD4+ and CD8+ T cells with previous antigen exposure express killer cell lectin-like receptor G1 (KLRG1) are more responsive to an acute bout of exercise than antigen naïve T cells (KLRG1-) (Simpson et al., 2008; Simpson et al., 2007). Moreover, KLRG1+/CD8+ T cells migrate in and out of the periphery two to three times greater than KLRG1-/CD8+ T cells (Simpson et al., 2008; Simpson et al., 2007). This preferential responsiveness of CD8+ T cells may have contributed to the non-significant Post-Ex increase and significant 60 min Post-Ex increase in the present study. The cell staining panel used in the present study lacked the discriminatory fidelity to

differentiate between KLRG1+ and KLRG1- negative cells; therefore, the CD8+ T cell population identified likely included both KLRG1+/CD8+ and KLRG1-/CD8+ T cells.

Latent infection with cytomegalovirus (CMV), a type of persistent and ubiquitous β-herpes virus has a significant confounding effect on lymphocyte responses to acute exercise (Bigley et al., 2012; Campbell et al., 2009).The CMV sero-prevalance rate in Australian males aged 20-24 years is 50% (Seale et al., 2006). It is therefore entirely plausible that some participants in the present study were seropositive for CMV. Financial and logistical constraints precluded the individual testing for CMV. Potential participants were excluded if they had been diagnosed with glandular fever, chronic fatigue syndrome or other viral infections lasting longer than 3 months. Given the asymptomatic nature of CMV infection, it is still possible that some participants were unknowingly seropositive and therefore confounded the lymphocyte response in the CD3+/CD8+ T cell subset.

Immune cells are also subject to circadian influence, with circulating T cell numbers reduced by 40% during circadian nadir compared to circadian peak numbers (Born, Lange, Hansen, Molle, & Fehm, 1997; Dimitrov, Lange, Nohroudi, & Born, 2007). Circadian influence on the immune system is mediated by the hypothalamic-pituitary-adrenal axis and sympathetic nervous system, and the respective changes in plasma cortisol and catecholamine concentrations (Abo, Kawate, Itoh, & Kumagai, 1981; Ottaway & Husband, 1994). Plasma concentrations of catecholamines and cortisol are at their lowest at night, rising to maximal concentrations at and during the beginning of the day (Born et al., 1997; Dodt, Breckling, Derad, Fehm, & Born, 1997).

Cortisol decreases circulating T cell numbers, redistributing them to the bone marrow (Fauci, 1975) whilst catecholamines such as epinephrine recruit T cells to the circulation by reducing cellular adhesive properties (Benschop, Rodriguez-Feuerhahn, & Schedlowski, 1996). Naïve, central memory, and effector memory T cells are negatively correlated with plasma cortisol concentrations, peaking during the night time when cortisol levels are lowest (Dimitrov et al., 2009). Effector CD8+ T cells are preferentially sensitive to catecholamine influence and cell numbers peak during daytime when catecholamine concentrations are highest (Dimitrov et al., 2009). The present study did not measure plasma cortisol or catecholamine concentrations; however, all testing was conducted between 7:30 am to 10:30 am to reduce the potential effect of circadian fluctuations.

The inverted CD3+ T cell response observed in the present study may be partially explained by exercise-induced lymphopenia resulting from two distinct processes; (i) redistribution of lymphocytes into various tissues and organs (Kruger & Mooren, 2007) and/or (ii) lymphocyte apoptosis (Mars, Govender, Weston, Naicker, & Chuturgoon, 1998; Mooren et al., 2002). These physiological processes appear to run concurrently and are sensitive to exercise mode and intensity (Hsu et al., 2002; Mooren et al., 2002).

Initially regarded as a maladaptive response precipitating immunosuppression in athletes, exercise-induced apoptosis appears to serve a positive function. Exercise preferentially mobilizes senescent lymphocytes into peripheral blood, potentially making space for emerging naïve cells (Simpson, 2011). In addition to aerobic exercise, long-term Tai Chi exercise has been shown to increase lymphocyte apoptosis and proliferation (Goon, Noor Aini, Musalmah, Yasmin

Anum, & Wan Ngah, 2008). To this end, exercise appears to serve a dual role in immune cell death and production (Kruger & Mooren, 2014).

Several theories have been proffered to explain exercise-induced lymphocyte apoptosis. Among them is cell surface receptor theory. Intensive treadmill running, intensive resistance training, and marathon running upregulate expression of CD95 receptors and CD95 ligands (Kruger et al., 2009; Mooren et al., 2002; Mooren, Lechtermann, & Volker, 2004). Exercise appears to induce a preferential shift to lymphocyte subpopulations characterised by a higher density of CD95 surface receptors (Mooren et al., 2002). Such a shift could theoretically predispose the re-distributed T cells to increased apoptosis.

Exercise-induced reactive oxygen species (ROS) is believed to partially mediate exercise-induced apoptosis. Convincing evidence of causal involvement has come from studies that have inhibited thymocyte and lymphocyte apoptosis via administration of antioxidants (Lin et al., 1999; Quadrilatero & Hoffman-Goetz, 2004). Repeated exposure to exercise leads to a protective upregulation of antioxidant enzymes (Fehrenbach & Northoff, 2001). The decreased apoptosis seen in fitter individuals may be due to increased antioxidant defences (Kruger & Mooren, 2014; Mooren et al., 2004; Peake et al., 2005).

Glucocorticoids (GCs) are a varied class of steroid hormones with distinct immunomodulatory functions and induce apoptosis in macrophages, monocytes, and T cells via binding to intracellular glucocorticoid receptors (Distelhorst, 2002; Tuckermann, Kleiman, McPherson, & Reichardt, 2005). As plasma cortisol concentrations were not measured in the present study, it is possible that during-

and post-exercise cortisol concentrations may have influenced lymphocyte apoptosis.

Intense exercise increases total numbers and relative percentages of apoptotic circulating lymphocytes (Kruger et al., 2009; Mooren et al., 2002). Lymphocyte apoptosis increases beyond an exercise intensity threshold of 40-60% $\dot{V}O_{2max}$ (Mooren et al., 2012). Increased apoptosis has been observed after triathlon (Levada-Pires et al., 2009), intensive treadmill running (Mooren et al., 2004), intensive cycle ergometer (Steensberg, Morrow, Toft, Bruunsgaard, & Pedersen, 2002), marathon running (Mooren et al., 2002; Mooren et al., 2004), and ultra-marathon (Atamaniuk et al., 2008). The prevailing hypothesis is that apoptosis mediators increase in an intensity and duration specific manner (Navalta, Sedlock, & Park, 2007; Steensberg et al., 2002). The intensity of the exercise intervention used in the present study (80% of $\dot{V}O_{2peak}$) may have increased lymphocyte and therefore T cell apoptosis.

Lymphocyte apoptosis is also sensitive to individual training status (Mooren et al., 2004; Peake et al., 2005), occurring to a greater extent in lesser trained individuals (Mooren et al., 2004). Mooren et al. (2004) divided their cohort into highly-trained (66.6 ± 8.0 $mL \cdot kg^{-1} \cdot min^{-1}$) and low-trained (50.1 ± 3 $mL \cdot kg^{-1} \cdot min^{-1}$) subgroups. Given that a mean $\dot{V}O_{2peak}$ of 50.1 ± 3 $mL \cdot kg^{-1} \cdot min^{-1}$ was associated with increased apoptosis by Mooren et al, it is physiologically plausible that the present study cohort (49.3 ± 4.8 $mL \cdot kg^{-1} \cdot min^{-1}$) experienced a similar increased apoptotic response. This may partially account for the inverted CD3+ T cell response observed in the present study.

The total immune cell pool can best be viewed as a malleable landscape, with the constituent cell types represented by changing peaks and troughs that have corresponding functional consequences. Any measure of LTL, telomerase expression, and/or telomere-associated gene expression represents the heterogeneous contributions of the constituent subpopulations.

Any observed increase in LTL is either due to *actual* telomerase-mediated elongation or *apparent* or *pseudo-lengthening* due to the redistribution of heterogeneous cells into the periphery (Epel, 2012). To better understand the mechanisms underpinning both actual lengthening and apparent lengthening, an enhanced understanding of exercise-induced cell population redistribution is required. Whilst no cell population functions in isolation *in vivo*, specific cell populations differentially influence mean LTL and telomerase expression. Such subpopulation-specific variances reflect the proliferative demands and replicative histories of the constituent cells. Significant differences in telomere length exist within the lymphocyte population; B cells have longest mean telomere length, followed in descending order of length, by CD4+, CD8+CD28+, CD8+CD28- T cells (Lin et al., 2010). PBMC telomere length is weakly correlated with B cells ($r = 0.35$) and CD4+ T cells ($r = 0.25$); whilst a stronger association exists for CD8+ T cells; CD8+CD28+ T cells ($r = 0.63$) and CD8+CD28- ($r = 0.40$) (Lin et al., 2010).

Telomerase expression intuitively follows a similar pattern of variability with PBMCs expressing the highest level of enzyme activity, followed in descending order of enzymatic activity by B cells, CD4+ T cells, CD8+CD28+ T cells, and CD8+CD28- T cells (Lin et al., 2010). The inter-subset heterogeneity in telomere lengths and telomerase expression means that any perturbation in immune cell demography could acutely influence proliferative capacity and apoptosis

resistance. Importantly, these functions are subject to age-related downregulation contributing to decreased immune competence seen in later life, collectively referred to as immunosenescence (Pawelec, 2014).

The results of the present study indicate that acute bouts of exercise conducted at 80% of $\dot{V}O_{2peak}$ create a transient extravasation of CD3+ T cells in healthy young males. Given the telomere length and telomerase expression exhibited by this subset, acute exercise may transiently lead to *apparent* post-exercise telomere shortening and telomerase down regulation. In the 60 min post-exercise period, the total CD3+ population returned to basal levels whilst the CD3+CD4+ T cells exceeded basal levels. Whilst the precise time course and duration of this super-compensatory increase is unknown, it may in part explain the increased telomere length and telomerase expression cross-sectionally observed in habitually active individuals.

Limitations

This study does have some limitations. As previously mentioned the use of CD45RA as the sole identifier of the naïve phenotype may falsely exclude some naïve phenotypes whilst incorrectly including others. In addition to CD45RA being a necessary but not sufficient marker of naïve status, I cannot exclude the possibility that unknown portion of the CD45RA+ T cells were a specific subset of effector-memory T cells re-expressing the CD45RA marker.

The design of this study precluded the testing of potentially explanatory mechanisms such as catecholaminergic signalling, increased reactive oxygen species, and lymphocyte apoptosis. Studies with greater discriminatory fidelity are needed to directly capture such mechanisms.

The inevitably large degree of inter-individual variances in both total immune cell and specific immune cell subset numbers may have affected the number of significant differences found between measurement time points. Additionally, the relatively low sample size cannot be discounted as a contributing factor to the inverted post-exercise profile observed in some subsets.

2.7 Conclusion

T cells are the corner stone of the adaptive immune response and appear to be exquisitely sensitive to exercise intensity and duration. This study identified a novel exercise-induced change in T cell distributions. Several plausible mechanisms were proposed for the novel findings, including: exercise intensity, immune cell migration, catecholaminergic signalling, participant training status, circadian influence, and lymphocyte apoptosis.

The findings of this study support the tenet that exercise acutely alters the immune landscape by redistributing specific immune cell subpopulations into the periphery. The capacity of exercise to acutely induce changes in the immune landscape may result in a range of immunological epiphenomena including increases in mean LTL, telomerase expression, proliferative capacity, apoptosis resistance, and immune function. In the context of habitual exercise participation, these changes may become cumulative, overlapping to form chronic alterations. In doing so, exercise may provide a relatively cheap and easy means of partially reversing or slowing the trajectory of age-associated changes in immune function.

The findings of this study have important ramifications for the design of future studies, specifically: (i) acutely redistributed cell populations should be accounted for when measuring acute changes in telomere homeostasis, (ii) the timing of blood

sampling in interventional studies is influential, and (iii) vague and approximate estimations of exercise intensity may not sufficiently elicit the appropriate nuanced immune response. In a clinical context, acute and transient immunological changes may cumulatively overlap in highly active or over-trained individuals creating windows of immune compromise. Whilst exercise-induced changes in T cell demographics provide an overview of the immune response, subset specific gene expression analysis could reveal the molecular consequences of these changes. In turn this may provide an insight into the functioning of specific immune cell subsets and their individual contributions to composite LTL and gene expression patterns.

Chapter 3 - The Acute MicroRNA Response to Intense Cardiorespiratory Exercise

3.1 Abstract

Introduction: Telomeres are specialized nucleoprotein structures that protect chromosomal ends from degradation. These structures progressively shorten during cellular division and can signal replicative senescence below a critical length. Habitual physical activity is associated with longer leukocyte telomere length; however, this does not imply causal story and the mechanisms are unclear. Research findings are now highlighting the critical roles of epigenetic modification in telomere length homeostasis. Despite this, little is known about the role of miRNA-mediated regulation of pro-telomeric genes. ***Aims:*** The acute exercise-induced response of miRNAs was investigated in 18 healthy males (mean age = 23.4 ± 7.1 years). ***Methods:*** Participants undertook 30 min of treadmill running at 80% of $\dot{V}O_{2peak}$; the highest value of oxygen of uptake achieved in a maximal test. Blood samples were taken before exercise, immediately post-exercise and 60 min post-exercise. RNA from leukocytes was submitted to miRNA arrays and results were individually validated using quantitative real-time PCR. ***Results:*** Fifty-six miRNAs were differentially regulated post-exercise (FDR<0.05). *In silico* analysis identified four miRNAs (miR-186, miR-181, miR-15a and miR-96) with potential interaction with telomere gene transcripts. Significant post-exercise upregulation was observed in miR-186, miR-96, and miR-15a (P<0.001). ***Conclusion:*** These results may provide a mechanistic insight into the epigenetic maintenance of immune cell telomere length.

3.2 Introduction

Physical activity (PA) is irrefutably associated with decreased cardiovascular and all-cause morbidity and mortality (Blair et al., 2001 2001; Blair et al., 1993; Kampert et al., 1996; Kodama et al., 2009; Lakka et al., 1994; Myers et al., 2004; Park et al., 2009; Sui et al., 2007; Villeneuve et al., 1998; Wei et al., 2000). Chronic phenotypic adaptations to exercise manifest across physiological systems including skeletal muscle hypertrophy (McDonagh & Davies, 1984), vascular angiogenesis (Laufs et al., 2004), myocardial remodelling (Sipola, Heikkinen, Laaksonen, & Kettunen, 2009), glucose metabolism (Sato, Nagasaki, Nakai, & Fushimi, 2003), and immune function (Nieman, 1994).

Differential gene expression underpins phenotypic adaptations; however, an expanding understanding of epigenetics reveals levels of regulation beyond canonical gene expression. Epigenetic modifications refer to changes in the DNA or chromatin structure that can influence gene transcription independent of the primary sequence (Bird, 2007). Such epigenetic modifications include histone methylation and acetylation, DNA methylation, and regulation of microRNAs (miRNAs) (Ntanasis-Stathopoulos, Tzanninis, Philippou, & Koutsilieris, 2013). MicroRNAs are a class of short (20-25 nucleotides), non-coding RNA molecules that post-transcriptionally regulate gene expression and RNA silencing by binding to the 3’ or 5’ untranslated regions (UTR) of messenger RNA (mRNA) (Bentwich et al., 2005).

Leukocyte miRNAs and Exercise

The expression profiles of miRNAs are sensitive to external stimuli such as exercise. Acute exercise-induced miRNA expression profiles have been observed

in several immune cell subsets (Radom-Aizik et al., 2013; Radom-Aizik et al., 2010; Radom-Aizik et al., 2012; Radom-Aizik et al., 2014; Tonevitsky et al., 2013) (Table 1). Post-exercise analysis of neutrophils identified 38 differentially regulated miRNAs influencing genes involved in several inflammatory response pathways including Jak-STAT signalling, ubiquitin-mediated proteolysis, and Hedgehog signalling pathways (Radom-Aizik et al., 2010). Exercise has also been shown to differentially regulate 23 miRNAs in natural killer cells (NK cells) influencing pathways associated with cell communication (adherens junction and focal adhesion) and cancer (p53 signalling, glioma, melanoma, and prostate cancer) (Radom-Aizik et al., 2013).

Analysis of PBMC miRNA revealed 34 significantly regulated miRNAs influencing genes associated with 12 signalling pathways (Radom-Aizik et al., 2013; Radom-Aizik et al., 2010; Radom-Aizik et al., 2012). These included MAPK signalling and TGF-β signalling (Radom-Aizik et al., 2012) which in turn regulate pro- and anti-inflammatory cytokine regulation (Hoene & Weigert, 2010; Kjær et al., 2009; Matsakas & Patel, 2009)(Hoene & Weigert, 2010; Kjær et al., 2009; Matsakas & Patel, 2009), lymphocyte activation and differentiation (Oh-hora, 2009), cell communication (Bopp, Radsak, Schmitt, & Schild, 2010), and cancer (Walsh et al., 2011). Analysis of exercise-induced monocyte miRNA expression identified 19 acutely regulated miRNAs, ten of which were associated with inflammatory processes (Radom-Aizik et al., 2014). Additional analysis identified involvement of Jak-STAT, p53 signalling, and endocytosis pathways.

Tonevitsky and colleagues implemented a 30 minute treadmill test at 80% of $\dot{V}O_{2max}$ and assessed leukocyte miRNA and mRNA expression pre-, immediately-post, 30 min post-, and 60 min post-exercise (Tonevitsky et al., 2013). Four

differentially expressed miRNAs and their respective targets were identified; miR-21 targeting TGFBR3, PDGFD, and PPM1L; miR-24-2 targeting MYC and KCNJ2; miR-27a targeting ST3GAL6 and miR-181a targeting ROPN1L and SLC37A3. Several of the target genes were associated with apoptosis, membrane traffic, transcription regulation and immune function. Some of the miRNA-mRNA networks, specifically miR-24-2-5p-MYC and miR-21-5p-TGFBR3 are associated with cancer development and progression (Lippi, Danese, & Sanchis-Gomar, 2016). Ten weeks of cardiac rehabilitation increased leukocyte expression of miR-92a and miR-92b and decreased expression of two predicted targets NDUFA1 (respiratory electron transport) and CASP3 (apoptosis and cell survival) respectively (Taurino et al., 2010). A recent study identified increased whole blood miR-1, miR-486, and miR-494 expression in endurance-trained athletes compared to healthy controls. Furthermore, miR-1, miR-486, and miR-494 were all downregulated in 19 healthy males after a single bout of maximal aerobic exercise (Denham & Prestes, 2016).

Exercise, Telomeres, and MicroRNAs

Epigenetic modifications such as chromatin modification (García-Cao et al., 2003; Gonzalo et al., 2005; Gonzalo et al., 2006) and histone modification (Blasco, 2007; García-Cao et al., 2003) are known to play critical roles in telomere homeostasis and hTERT/telomerase regulation (Gigek et al., 2009; Iliopoulos, Satra, Drakaki, Poultsides, & Tsezou, 2009; Wang, Hu, & Zhu, 2010). Despite that, comparatively little is known about miRNA-mediated epigenetic regulation of telomeres and telomeric genes. Telomeric repeat factor 1 (TRF1) is translationally repressed by miR-155 resulting in chromosome alterations and telomere fragility (Dinami et al., 2014). Mir-155 is involved in skeletal muscle regeneration following

injury balances pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages during skeletal muscle regeneration (Nie et al., 2016). Direct evidence of the role of miR-155 in leukocytes or the *in vivo* effect on telomere homeostasis is lacking. MiR-498 is known to target the 3' UTR of *hTERT* mRNA (Kasiappan et al., 2012); however, little is known about the exercise responsiveness of miR-498 in leukocytes. Mouse models have also demonstrated exercise-induced modulation of miR-138 (Miao et al., 2015), a miRNA previously associated with *hTERT* regulation in neuroblastoma and anaplastic thyroid cancer cells (Chakrabarti et al., 2013; Mitomo et al., 2008). A review of putative miRNA involvement in telomere homeostasis appears in Chapter 1 – Introduction.

The association between habitual PA and LTL is widely accepted despite a lack of mechanistic clarity. The broader scientific consensus is that the association is largely mediated by exercise-induced reductions in oxidative stress and inflammation. Unambiguous evidence that telomeres and their requisite components are directly amenable to exercise is lacking. Several miRNAs have been associated with specific telomere components; however, their phenotypic impact is unclear. Furthermore, the responsiveness of potential pro-telomere miRNAs to exercise is unknown. The established links between PA and miRNA regulation, and PA and LTL may plausibly converge upon exercise-induced miRNA regulation of telomeric genes.

Table 14. Reported expression profiles of human immune cell miRNAs after aerobic exercise.

Reference	MiRNA Upregulated		MiRNA Downregulated		Tissue	Exercise Type
(Dias et al., 2015)	hsa-miR-let-7f-1 hsa-miR-21	hsa-miR-29c hsa-miR-223	hsa-miR-let-7f-1 hsa-miR-21	hsa-miR-29c hsa-miR-223	PBMCs	Chronic exercise training (running, 3x/wk., 60-min, 18 wks.)
(Denham & Prestes, 2016)	*hsa-miR-1 *hsa-miR-486	*hsa-miR-494	†hsa-miR-1 †hsa-miR-486	†has-miR-494	Whole blood	*Elevated in endurance athletes. † Decreased immediately post-maximal aerobic exercise.
(Radom-Aizik et al., 2010)	hsa-miR-125a hsa-miR-145 hsa-miR-181b hsa-miR-193a hsa-miR-197 hsa-miR-212 hsa-miR-520d hsa-miR-629 hsa-miR-638	hsa-miR-223 hsa-miR-340 hsa-miR-365 hsa-miR-485 hsa-miR-505 hsa-miR-939 hsa-miR-940 hsa-miR-1225 hsa-miR-1238	hsa-let-7i hsa-miR-16 hsa-miR-17 hsa-miR-18a hsa-miR-18b hsa-miR-20a hsa-miR-107 hsa-miR-126 hsa-miR-130a hsa-miR-130b	hsa-miR-20b hsa-miR-22 hsa-miR-93 hsa-miR-96 hsa-miR-106a hsa-miR-185 hsa-miR-194 hsa-miR-363 hsa-miR-660 hsa-miR-151	Neutrophils	Acute exercise bout: (cycle ergometer, 10 x 2-min bouts, 1-min rest interval between bouts – 76% $\dot{V}O_{2peak}$)

Table 14. continued

Reference	MiRNA Upregulated		MiRNA Downregulated		Tissue	Exercise Type
(Radom-Aizik et al., 2012)	hsa-miR-7 hsa-miR-15a hsa-miR-21 hsa-miR-26b hsa-miR-132 hsa-miR-140 hsa-miR-181a	hsa-miR-181b hsa-miR-181c hsa-miR-338 hsa-miR-363 hsa-miR-939 hsa-miR-940 hsa-miR-1225	hsa-let-7e hsa-miR-23b hsa-miR-31 hsa-miR-99a hsa-miR-125a hsa-miR-125b hsa-miR-126 hsa-miR-130a hsa-miR-145	hsa-miR-151 hsa-miR-199a hsa-miR-199b hsa-miR-221 hsa-miR-320 hsa-miR-451 hsa-miR-486 hsa-miR-584 hsa-miR-652	PBMCs	Acute exercise bout: (cycle ergometer, 10 x 2-min bouts, 1-min rest interval between bouts – 76% $\dot{V}O_{2peak}$)
(Radom-Aizik et al., 2013)	hsa-miR-7 hsa-miR-29a hsa-miR-29b hsa-miR-29c hsa-miR-30e	hsa-miR-142 hsa-miR-192 hsa-miR-338 hsa-miR-363 hsa-miR-590	hsa-let-7e hsa-miR-126 hsa-miR-130a hsa-miR-151 hsa-miR-199a	hsa-miR-221 hsa-miR-223 hsa-miR-326 hsa-miR-328 hsa-miR-652	NK cells	Acute exercise bout: (cycle ergometer, 10 x 2-min bouts, 1-min rest interval between each bout, 77% $\dot{V}O_{2peak}$)

Table 14. continued

Reference	MiRNA Upregulated		MiRNA Downregulated		Tissue	Exercise Type
(Radom-Aizik et al., 2014)	hsa-miR-15a hsa-miR-29b hsa-miR-29c hsa-miR-30e hsa-miR-140 hsa-miR-324	hsa-miR-338 hsa-miR-362 hsa-miR-532 hsa-miR-660 hsa-miR-1202 hsa-miR-1305	hsa-miR-23b hsa-miR-130a hsa-miR-151	hsa-miR-199a hsa-miR-221	Monocytes	Acute exercise bout: (10 x 2-min bouts of cycle ergometer exercise, 82% $\dot{V}O_{2max}$)
(Taurino et al., 2010)	hsa-miR-92a hsa-miR-92b	-	-	-	Whole blood	Cardiac rehab program (60 min, 2/wk., 10 wks.)
(Tonevitsky et al., 2013)	hsa-miR-21-5p hsa-miR-24-2-5p	has-miR-27a-5p hsa-miR-181a-5p	-	-	Whole blood	Acute exercise bout: 30-min treadmill run at 80% $\dot{V}O_{2peak}$

3.3 Aims

The overarching aim of this study was to investigate potential mechanisms underpinning the positive association between PA and LTL. The specific aims were to investigate the acute effects of 30 min of intense cardiorespiratory exercise on the expression patterns of leukocyte miRNAs with potential involvement in telomere biology.

Hypothesis

The hypothesis for this study is that 30 min of treadmill running at 80% $\dot{V}O_{2peak}$ will differentially regulate miRNAs in leukocytes and immune cell subsets. Specifically, aerobic exercise will differentially regulate miRNAs that potentially interact with pro-telomere transcripts.

3.4 Methods

Ethics Statement

All eligible participants read a plain language information statement outlining all aspects of the project in lay terminology. Informed consent documents explaining the purpose, potential risk and benefits of the project were then signed in the presence of a witness. The study, recruitment and consent procedures were approved by the Human Research Ethics Committee from Federation University Australia (HREC approval #: A10-119).

Participants

A subset of 18 healthy, non-smoking males (mean age = 23.4 ± 7.1 years) were selected from the initial cohort of 22 (see chapter 2). General health and lifestyle information was also obtained via a questionnaire.

Physiological Measurements

Participant physiological measurements were taken in accordance with the methodology laid out in chapter 2. A summary of the participant physiological and exercise intervention data is contained in table 15.

Determination of Fitness Standard

Participants undertook a treadmill-based peak oxygen uptake ($\dot{V}O_{2peak}$) test using a Metalyser® metabolic system (Cortex Biophysic, Leipzig, Germany). The specifics of the protocol and associated cardiopulmonary exercise testing (CPET) data are contained in chapter 2.

Exercise protocol

Participants then undertook a 30 min bout of continuous treadmill running at 80% of previously determined $\dot{V}O_{2peak}$. Blood samples were taken pre-exercise (Pre-Ex), post-exercise (Post-Ex) and 60 min post-exercise (60 min Post-Ex). All CPET data and specific blood sampling procedures are contained in chapter 2.

Preparation of cells

The preparation and staining of leukocytes and all associated flow cytometry procedures are explained in detail in chapter 2.

RNA extraction

Total RNA for miRNA microarrays was extracted using TRIzol® (Life Technologies) according manufacturer's instructions. All RNA samples were quantified by spectrophotometry using a Nanodrop™ (Thermo Fisher).

miRNA expression microarrays

Agilent Human miRNA Microarrays (Release 19.0 – Agilent Technologies) were performed on Pre-Ex, Post-Ex, and 60 min Post-Ex samples from a subset of

10 male participants matched for age, BMI, and $\dot{V}O_{2peak}$. A subset of n=10 was chosen from the initial subset of 18 largely due to the high cost of analysis. The microarrays were performed at the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia), as previously described (Marques et al., 2011). The data set obtained has been deposited in the NCBI Gene Expression Omnibus database according to the *Minimum Information About a Microarray Experiment (MIAME)* guidelines (Brazma et al., 2001) with series accession number GSE45041.

miRNA target gene prediction

Differentially expressed miRNAs were further analysed for potential binding to telomere gene transcripts using TargetScan (Release 6.2) and microRNA.org (August 2010 release).

miRNA target gene prediction

TaqMan® assays (Life Technologies) were used to validate the target miRNAs in 18 male participants at the Pre-Ex and Post-Ex time-points in accordance with the miRNA expression arrays (table 16). The target miRNAs were also assessed in the same 18 males at the 60-min Post-Ex time-point. Total RNA (250 ng) was reverse transcribed for primers using Taqman® MicroRNA Reverse Transcription kit (Life Technologies) according to manufacturer's instructions. All reactions were performed in duplicate in a Viia™7 Real-Time PCR System (Applied Biosystems) using the following cycling conditions: 1 cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s. All reactions were normalized to the average of miRNA controls RNU44 and RNU48; both of which have been used extensively as endogenous controls in exercise and immunological studies (Radom-Aizik et al., 2013; Radom-Aizik et al., 2010; Radom-Aizik et al., 2012).

Table 15. Physiological characteristics and exercise intervention data of the 18 male participants.

Characteristic	Mean	SEM
Age (years)	23.4	± 7.1
Height (cm)	181.9	± 5.6
Body Mass (kg)	80.9	± 9.5
BMI (kg/m^2)	24.4	± 2.2
Waist:hip ratio	0.8	± 0.03
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	49.7	± 4.4
% $\dot{V}O_{2peak}$ during 30 min run	80.01	± 6.83

SEM: (standard error of mean); **BMI**: (body mass index); **$\dot{V}O_{2peak}$**: (highest oxygen consumption achieved in test)

Table 16. Quantitative real-time PCR TaqMan® microRNA assays

miRNA	**Assay identification #**
RNU44 (control)	001094
RNU48 (control)	001006
miR-181b	001098
miR-186	000486
miR-96	000434
miR-15a	000389

Pooled T cell Subpopulations

To differentiate transcriptionally responsive subsets, CD45RA+ and CD45RO+ subsets in CD4+ and CD8+ T cells were assessed at each time point. A total of 100ng of total RNA was pooled from each cell population at each time point from the 22 male participants recruited in chapter 2. The full n=22 were used in the pooled T cell samples compared to n=18 for the miRNA target gene validations due to limited RNA yield from four participants. As only a small RNA input was required from each participant for the pooled samples compared to the larger input for individual analyses, more participants were included. The smaller than expected RNA yield from the sorted cell subpopulations precluded large numbers of individual samples. Sample pooling identifies the transcriptional characteristics of specific cell populations as opposed to individuals and reduces the effects of biological variation. Whilst this technique restricts the scope of stringent statistical analysis, it does provide a transcriptional profile that can be compared to unsorted leukocytes. Reverse transcription and qPCR reactions were both performed according to previously outlined protocols.

Statistical Analysis

MiRNA microarray samples were between-array normalized using the quantile method in Partek Genomics Suite (version 6.6). Differentially expressed miRNAs were identified using a paired t-test and a false discovery rate (FDR)<0.05. Normally distributed qPCR data was assessed using a repeated measures ANOVA; significance was further assessed using paired t-tests with Bonferroni correction. Non-normally distributed qPCR data was assessed using a non-parametric Friedman's repeated measures ANOVA; where appropriate, a non-parametric pairwise comparison was performed (Wilcoxon Signed-Rank test).

Statistical significance was set at $P<0.05$. All statistical analysis was performed using SPSS® (version 17).

3.5 Results

The acute effect of cardiorespiratory exercise on genome wide miRNA expression

Fifty-six miRNAs were significantly differentially regulated in ten healthy males after 30 min of intense cardiorespiratory exercise (Table 17).

miRNA target gene prediction

Four miRNAs: miR-181b, miR-186, miR-15a, and miR-96 were selected for individual qPCR validations based on predicted *in silico* interactions (table 18). The predicted following miRNA/mRNA interactions were: miR-181b and *hTERT*, miR-186 and *RAP1, RAD50* and *SIRT6*, miR-96 and *RAP1*, and miR-15a and TATA box binding protein (*TBP*).

qPCR Validations of selected miRNAs

Due to the non-normal distribution of the miRNA data, Friedman tests were conducted to assess differences in mean miRNA expression (figure 12). Post hoc analysis using Wilcoxon signed-rank tests were performed using a Bonferroni correction. There was no statistically significant difference in miR-181b expression across the time points ($\chi^2(2) = 1.44$, $P=0.486$). There was a significant effect of time for miR-186 expression across time points ($\chi^2(2) = 19.44$, $P<0.001$). Post hoc analysis identified a significant difference in means between Pre-Ex and 60 min Post-Ex time points ($Z = -3.72$, $P<0.001$), and Post-Ex and 60 min Post-Ex time points ($Z = -2.68$, $P=0.007$). There was a significant effect of time for miR-15a across time points ($\chi^2(2) = 28.44$, $P<0.001$), with significant differences in means

Table 17. Significantly regulated miRNAs in Pre-Ex and Post-Ex samples detected via genome-wide microarray (Agilent Human miRNA Microarray, Release 19.0).

Probeset ID	Fold Change	FDR†	Probeset ID	Fold Change	FDR†
hsa-miR-1270	-11.45	0.03	hsa-miR-320a	-1.20	0.03
hsa-miR-96	-1.99	0.03	hsa-miR-320b	-1.19	0.03
hsa-miR-675*	-1.53	0.03	hsa-miR-139-3p	-1.19	0.03
hsa-miR-574-3p	-1.47	0.03	hsa-miR-320e	-1.18	0.03
hsa-miR-576-5p	-1.47	0.04	hsa-miR-4323	-1.17	0.03
hsa-miR-934	-1.43	0.03	hsa-miR-320d	-1.16	0.03
hsa-let-7d*	-1.41	0.04	hsa-miR-146a	-1.11	0.03
hsa-miR-2115	-1.39	0.03	hsa-miR-186	1.16	0.04
hsa-miR-193b	-1.38	0.03	hsa-miR-15a	1.17	0.03
hsa-miR-636	-1.35	0.03	hsv2-miR-H6	1.18	0.03
hsa-miR-1229	-1.35	0.03	hsa-miR-28-5p	1.21	0.04
hsa-miR-129*	-1.35	0.03	hsa-miR-4322	1.26	0.03
hsa-miR-129-3p	-1.34	0.03	hsa-miR-3648	1.27	0.04
hsa-miR-877*	-1.34	0.03	hsa-miR-23a*	1.33	0.04
hsa-miR-1227	-1.32	0.03	hsa-miR-181b	1.36	0.04
hsa-miR-125b	-1.31	0.03	hsa-miR-454*	1.42	0.03
hsv2-miR-H20	-1.31	0.04	hsa-miR-363	1.46	0.03
kshv-miR-K12-8*	-1.30	0.03	hsa-miR-583	1.66	0.03
hsa-miR-933	-1.29	0.03	kshv-miR-K12-6-5p	1.66	0.03
hsv2-miR-H7-3p	-1.29	0.03	hsa-miR-518c*	1.67	0.04

Table 17. continued

Probeset ID	Fold Change	FDR†	Probeset ID	Fold Change	FDR†
hsa-miR-3613-3p	-1.27	0.03	hsa-miR-1276	1.73	0.04
hsa-miR-3940	-1.26	0.04	hsa-miR-200b*	1.86	0.04
hsa-miR-378	-1.25	0.03	hsa-miR-3677	1.98	0.04
hsa-miR-1225-3p	-1.25	0.04	hsa-miR-1250	2.02	0.04
hsa-miR-92a	-1.22	0.04	hsa-miR-873	2.19	0.03
hsa-let-7f-1*	-1.22	0.04	hsa-miR-4316	2.37	0.03
hsv1-miR-H1*	-1.21	0.03	hsa-miR-764	3.26	0.03
hsa-miR-550a*	-1.21	0.04	hsa-miR-3146	4.35	0.03

The miRNA prefix ‘hsa’ denotes human origin, ‘hsv’ denotes herpes simplex virus, and ‘kshv’ denotes Kaposi’s sarcoma-associated herpes virus. † FDR: false discovery rate.

Table 18. Selected miRNAs and their potential mRNA interactions.

miRNA	Potential gene target	Gene symbol	Prediction database
miR-181b	Telomerase reverse transcriptase	*hTERT*	microRNA.org (release August 2010)
miR-186	RAD50 homolog	*RAD50*	microRNA.org (release August 2010)
	Repressor/activator protein 1	*RAP1*	TargetScan (release 6.2)
	Sirtuin-6	*SIRT6*	microRNA.org (release August 2010)
miR-96	Repressor/activator protein 1	*RAP1*	microRNA.org (release August 2010)
miR-15a	TATA-box binding-protein	*TBP*	microRNA.org (release August 2010)

between Pre-Ex and Post-Ex (Z = -2.77, *P*=0.006), Pre-Ex and 60 min Post-Ex (Z = -3.72, *P*<0.001), and Post-Ex and 60 min Post-Ex time points (Z = -3.55, *P*<0.001).

There was also a significant effect of time for miR-96a across time points ($\chi^2(2)$ = 16.93, *P*<0.001). Post hoc analysis identified significant differences in mean expression between Pre-Ex and 60 min Post-Ex time points (Z = -2.99, *P*=0.003) and Post-Ex and 60 min Post-Ex time points (Z = -2.84, *P*=0.004) (figure 12).

The effect of exercise on T cell subset miRNA expression

Only miR-181b and miR-186 were detected in the sorted T cell subsets. The expression profile of miR-181b demonstrated a biphasic Post-Ex down regulation in CD4+CD45RA+ T cells and a marginal Post-Ex increase in CD8+CD45RA+ T cells (figure 13). There was no appreciable regulation in miR-181b in CD4+CD45RO+ T cells whilst CD8+CD45RO+ T cells exhibited a stepwise increase. MiR-186 expression increased 60 min Post-Ex in CD4+CD45RA+ T cells and CD8+CD45RO+ T cells showed a stepwise increase in expression.

3.6 Discussion

The findings of this study demonstrate that exercise acutely regulates leukocyte miRNAs with potential epigenetic influence on telomere homeostasis. Exercise-induced regulation of pro-telomeric miRNAs may in part mechanistically contribute to the observed association between PA and LTL. This is a significant finding given the purported link between accelerated telomere shortening and chronic diseases (Calado & Young, 2009). Telomere homeostasis underpins the function of highly replicative cells such as immune cells (Weng, 2008), with accelerated shortening

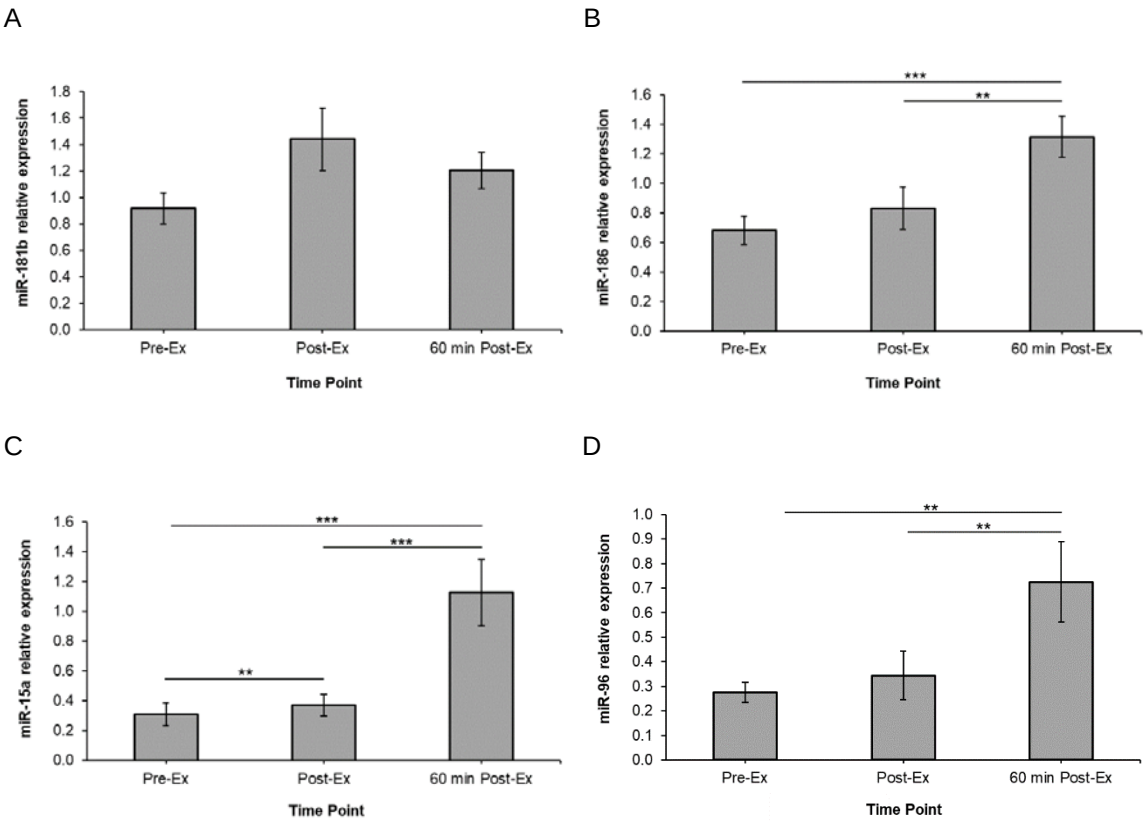


Figure 12. Differential regulation of selected miRNAs in unsorted leukocytes: Relative expression of each target miRNA was assessed at Pre-Ex, Post-Ex, and 60 min Post-Ex (n=18). Whilst only a (non-significant) strong trend was observed for miR-181b **(A)**, significant changes in regulation were observed for miR-186 **(B)**, miR-15a **(C)**, and miR-96 **(D)**. All data is expressed relative to an average of RNU44 and RNU48. Error bars indicate SEM. * indicates *P*<0.05 and ** indicates *P*<0.01, and *** indicates *P*<0.001.

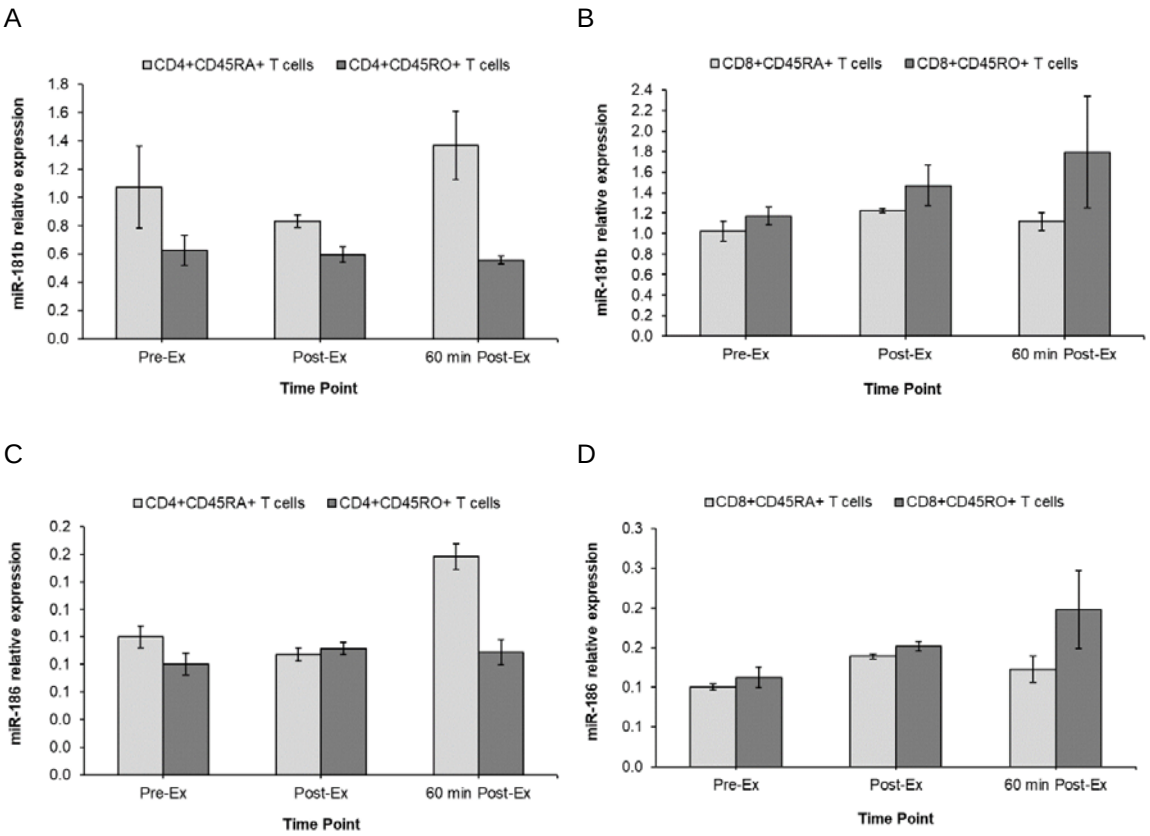


Figure 13. **Differential regulation of selected miRNAs in sorted T cell subset pools** (n = 22)**:** Each miRNA was assessed in T cell subset pools. miR-181b was expressed in CD4+ **(A)** and CD8+ T cells **(B)**. miR-186 was also expressed in CD4+ **(C)** and CD8+ T cells (**D)**.

leading to replicative senescence. The SASP acquired by senescent cells propagates chronic inflammation, age-related disease, carcinogenesis, and metastasis formation (Rodier & Campisi, 2011).

MiRNAs serve key regulatory roles in cellular senescence (Feliciano, Sánchez-Sendra, Kondoh, & LLeonart, 2011; Lafferty-Whyte, Cairney, Jamieson, Oien, & Keith, 2009), influencing major senescent pathways including p53/$p21^{Cip1}$ and $p16^{INK4A}$-pRB tumour suppressor pathways (Borgdorff et al., 2010; Gómez-Cabello et al., 2013; Overhoff et al., 2014; Ugalde et al., 2011; Yamakuchi, Ferlito, & Lowenstein, 2008). MiRNA dysregulation is associated with a host of diseases independently of telomere homeostasis, including CVD (Jovanović, Živković, Jovanović, Djurić, & Stanković, 2014; Menghini, Stöhr, & Federici, 2014), cancer (Iorio et al., 2005; Peng et al., 2011), viral diseases (Huang et al., 2007; Li et al., 2010; Randall et al., 2007), immune-related diseases (Karolina et al., 2011; Keller et al., 2009), and neurodegenerative conditions (Martins et al., 2011; Nunez-Iglesias, Liu, Morgan, Finch, & Zhou, 2010).

The current study provides a unique snap shot of 56 exercise-induced leukocyte miRNAs. *In silico* analyses predicted miRNA/mRNA interactions between: miR-181b and *hTERT*, miR186 and *RAP1, RAD50* and *SIRT6*, miR-96 and *RAP1,* and miR-15a and TATA box binding protein (*TBP*).

Although not achieving statistical significant across three timepoints, miR-181b trended towards increased abundance immediately post-exercise ($P<0.05$), before trending down to a level marginally higher than pre-exercise. The 60 min post-exercise decrease in miR-181b paralleled an increase in one of its putative targets (*hTERT*); however, the two events were not significantly associated. Importantly,

the magnitude of miRNA expression needed to elicit detectable decreases in target mRNA is unknown; however, it is likely to be small (Baek et al., 2008). Research continues to shed light on the complex and often divergent roles played by the miR-181 family (mir-181a, -181b, -181c, and -181d). The up regulation of miR-181 plays a critical role in the anti-inflammatory effect elicited by exercise and suppresses the inflammatory response within dendritic cells induced by low-density lipoprotein (Wu et al., 2012). The miR-181 family targets genes within the cytoplasm and mitochondria of the cardiovascular system (Das et al., 2017). Specifically, miR-181b plays a significant role in vascular stiffness (Hori et al., 2017), vascular inflammation and atherosclerosis (Sun, He, et al., 2014), and diabetic cardiomyopathy (Copier, Leon, Fernandez, Contador, & Calligaris, 2017). Exercise-induced upregulation of the miR-181 family within leukocytes has previously been demonstrated (Radom-Aizik et al., 2010; Radom-Aizik et al., 2012).

Exercise-induced upregulation of leukocyte miR-186 was observed at 60 min post-exercise compared to pre-exercise ($P<0.001$) and immediately post-exercise ($P<0.01$). The differential expression of miR-186 has been associated with various forms of cancer and their progression (He, Ping, & Wen, 2017; Niu et al., 2017; Sun, Jiao, Chen, Liu, & Zhao, 2015; Sun, Hu, Xiong, & Mi, 2014; Zhang, Wang, et al., 2016). Cardiomyocyte-enriched miR-186 has been associated with atherosclerosis progression (Boštjančič, Zidar, & Glavač, 2009; Das & Halushka, 2015; Min & Chan, 2015), cardiac injury, and myocardial infarction (Zeller et al., 2014).

In a recent study, overexpression of miR-186 via a miR-186 mimic increased macrophage lipid concentration (Yao et al., 2016). Additionally, inhibition of miR-

186 increased expression of the pro-inflammatory cytokine cystathionine-γ-lyase (CSE) in macrophages (Yao et al., 2016). Despite this, little is known about the role of miR-186 in leukocytes and its acute responsiveness to exercise. Whilst speculative, the exercise-induced upregulation of leukocyte miR-186 observed in the present study may be an acute attempt to mitigate inflammation within immune cell subsets. To the author's knowledge, the present study is the first to propose a potential pro-telomeric function for miR-186. Given that the 60 min post-exercise expression profile of miR-186 mirrored a decrease in one of its putative targets (*RAP1*), a role in acute telomere homeostasis is plausible.

The role of *RAP1* in telomere homeostasis and the potential significance of its regulation are addressed in detail within chapter 4. Briefly, *RAP1* is part of the shelterin complex and is recruited to telomeres via interaction with *TRF2* (also known as *TERF2*)(Li et al., 2000). The resultant complex formed with *TRF2* is essential to prevent the homologous recombination-mediated deletions and fusions of telomeres (Rai, Chen, Lei, & Chang, 2016). Despite discrepant findings, *RAP1* is widely considered a negative regulator of telomere length (O'Connor et al., 2004). Additional roles for *RAP1* include prevention of non-homologous end joining (Sarthy et al., 2009) and homology-directed repair (Sfeir, Kabir, van Overbeek, Celli, & de Lange, 2010), protection from obesity via regulation of metabolic genes (Martínez et al., 2013), and regulation of senescence (Platt et al., 2013).

Exercise-induced upregulation of leukocyte miR-96 was also observed at 60 min post-exercise compared to pre-exercise ($P<0.01$) and immediately post-exercise ($P<0.01$). *In silico* analysis also predicted *RAP1* as a regulatory target of miR-96. Established interactions exist between miR-96 and *FOXO1* mRNA

(Guttilla & White, 2009; La Rocca et al., 2009; Li et al., 2011; Myatt et al., 2010). Age-associated increased expression profiles of miR-96 and miR-145 have been proposed as potential contributors to the concomitant age-related decrease in *IGF-1R* and *FOXO1* mRNAs (Budzinska et al., 2016). The age-associated decrease in *IGF-1R* and *FOXO1* is thought to contribute to altered natural killer cell function, altered antibody production, and dysregulated production of pro- and anti-inflammatory agents. These factors are individually and collectively associated with age-related diseases (Franceschi & Campisi, 2014; Puzianowska-Kuźnicka et al., 2016). Mir-96 has also been implicated in several oncogenic processes including a likely onco-miR role via suppression of CDKN1A protein expression in bladder cancer cells (Wu et al., 2015). Increased expression of miR-96 promotes cell proliferation, migration, and invasion via targeting of PTPN9 in breast cancer (Hong et al., 2016), and post-transcriptional suppression of Anaplastic Lymphoma Kinase (ALK) expression (Vishwamitra et al., 2012).

T cell subset analysis identified more dynamic regulation of miR-181b and miR-186 in CD4+CD45RA+ T cells compared to CD4+CD45RO+ T cells. The reverse was true within the CD8+ T cell subset with higher and more differential expression observed in CD8+CD45RA+ T cells. Whilst the reasons for such differential expression are unclear, T cell subset-specific expression profiles of miRNAs have previously been reported throughout differentiation (Grigoryev et al., 2011; Rossi et al., 2011) and in response to exercise (Radom-Aizik et al., 2012).

The interactions between miRNAs and mRNA targets are complex. Significant functional redundancy means that a single miRNA can target hundreds of mRNAs. It is possible that miRNAs not identified via *in silico* analysis in the present study still exerted transcriptional influence on telomeric genes via seemingly unrelated

pathways such as inflammation and/or oxidative stress. Additional validation experiments would be required to confirm the legitimacy of the miRNA/mRNA interactions proposed in this study. Additionally, the direction of the miRNA/mRNA interaction within a telomere context is currently unclear. Chromosomal instability resulting from telomere shortening alters the cellular miRNA expression profile (Castro-Vega et al., 2013). Comparison between cells with normal and shortened telomeres identified 47 differentially expressed miRNAs (Uziel et al., 2015).

Mature miRNAs can remain viable for several hours to days after transcriptional shutdown or depletion of by miRNA processing enzymes (Baccarini et al., 2011; Gantier et al., 2011; Lee et al., 2003; Van Rooij & Olson, 2007). Consequently, miRNAs were initially considered to be inherently stable molecules; however, a complex picture of miRNA regulation and turnover is emerging. Several miRNAs demonstrate dynamic expression patterns throughout development, including rapid downregulation (Avril-Sassen et al., 2009; Kato, de Lencastre, Pincus, & Slack, 2009; Okamura et al., 2008; Wang et al., 2011). Additionally, various mature miRNAs demonstrate stage- or tissue-specific expression independent of their precursor pri- and pre-miRNAs (Lee et al., 2008; Martinez et al., 2008). There is also evidence that highly complementary targets can induce miRNA instability and degradation in animals (Ameres et al., 2010; Xie et al., 2012); however, the biological consequences of this are currently unclear.

MiRNA turnover may also be influenced by miRNA-degrading enzymes (collectively referred to as miRNases) including XRN1 (Bail et al., 2010), RRP41 (Bail et al., 2010), and PNPase$^{old\text{-}35}$ (Das et al., 2010). Selective loss of Eri1 was found to impair murine natural killer cell development, function, and maturation as

well as significantly increase several miRNAs in natural killer cells and T-cells (Thomas et al., 2012).

Limitations

There are some limitations to this study. Without *a priori* knowledge of the precise time course of miRNA expression, it is possible that the measurement time course used may have missed the greatest magnitude of change. Additionally, the study did not differentiate between the potential pro-telomeric functions and unrelated functions of the genes and miRNAs of interest. The low sample yield from sorted T cell populations necessitated pooling into cell/time point specific pools for analysis. Whilst this provided interesting, subpopulation-wide overviews, it precluded additional individual validations and robust statistical analysis.

The present study used computational prediction followed by candidate gene mRNA validation to propose putative miRNA/mRNA interactions. However; to more accurately confirm the legitimacy of the proposed miRNA/mRNA interactions, additional luciferase reporter assay and gain/loss of function experiments would be required. Downstream target gene protein detection using Western blotting was not performed in the present study; therefore, conclusions about mRNA stability and subsequent functional protein synthesis cannot be made. The functional outcomes of miRNA-mediated protein changes can be further assessed via the use of miRNA knockout/overexpression models as well as modified antisense oligonucleotides known as antimiRs. Whilst the present study was not designed to answer such questions, the addition of complex gain or loss of function studies in cultured cells would have provided definitive proof of miRNA/mRNA interaction.

Another consideration is that analysis of whole leukocytes may obscure population-specific miRNA expression profiles via exercise-induced changes in cellular subset proportions (Tonevitsky et al., 2013). White blood cells represent a heterogeneous conglomeration of lymphocytes, monocytes, natural killer cells and granulocytes of varying proportions. The relative contribution of the other cellular subsets to the composite miRNA signature is unknown. Work by Radom-Aizik (2012) has demonstrated the discrete exercise-induced miRNA expression profiles between neutrophils and PBMCs. Despite best attempts to minimize the time between blood draw and miRNA extraction, it is possible that miRNA expression profiles were altered post-draw due to turnover or degradation.

3.7 Conclusion

In conclusion this study has shown that 30 min of cardiorespiratory exercise is sufficient to elicit the differential regulation of 56 microRNAs in leukocytes. The regulated miRNAs included miR-186 and miR-96 which have potential transcriptional influence on telomeric genes, specifically *RAP1*. These results add to the developing understanding of epigenetic regulation of telomeres and may provide a mechanistic insight into the observed association between PA and telomere length.

An enhanced understanding of exercise-induced miRNA expression and the subsequent phenotypic effects may inform exercise guidelines (i.e. intensity, duration, and modality) for specific populations. A clearer understanding of exercise-induced miRNAs may also provide viable biomarkers via which to assess individual beneficial and/or detrimental exercise responses. This in turn may allow more targeted use of exercise as a first line treatment in conditions characterized

by aberrant miRNA regulation. Despite the growing body of evidence showing the exercise-responsiveness of miRNAs, the specific phenotypic effects on specific tissues (e.g. leukocytes) are poorly understood. Future studies should work to further establish the origin, target tissues, associated networks, and phenotypic implications of exercise-induced miRNAs.

Chapter 4 - The Acute Response of Telomere-Associated Genes to Intense Cardiorespiratory Exercise

4.1 Abstract

Telomeres are specialized nucleoprotein structures that protect chromosomal ends from degradation. These structures progressively shorten during cellular division and can signal replicative senescence below a critical length. Immune cell telomere length is associated with a host of chronic diseases and underpins the function of the adaptive immune system. Habitual physical activity is associated with longer leukocyte telomere length; however, this does not imply causal story and the mechanisms are unclear. Potential hypotheses include the regulation of telomere-associated genes and/or microRNAs (miRNAs). The acute exercise-induced response of telomere-associated genes was investigated in 17 healthy males (mean age = 23.8 ± 8.17 years). Participants undertook 30 min of treadmill running at 80% of $\dot{V}O_{2peak}$; the highest value of oxygen of uptake achieved in a maximal test. Blood samples were taken before exercise (Pre-Ex), immediately post-exercise (Post-Ex) and 60 min post-exercise (60 min Post-Ex). Total RNA from leukocytes was submitted to a telomere extension mRNA array. Results were individually validated in leukocytes and sorted T cell subsets using quantitative real-time PCR. Human telomerase reverse transcriptase (*hTERT*) mRNA (P=0.001) and sirtuin-6 (*SIRT6*) mRNA (P<0.05) expression were upregulated in leukocytes after exercise. Repressor/activator protein 1 (*RAP1*) mRNA was upregulated post-exercise (P=0.001) returning to below-basal concentration 60 min post-exercise (P=0.002). *RAD50* mRNA was significantly downregulated 60 min post-exercise (P=0.05). Intense cardiorespiratory exercise was sufficient to differentially regulate key telomere-associated genes in leukocytes. These results may provide a mechanistic insight into telomere homeostasis and improved immune function and physical health.

4.2 Introduction

There is mounting evidence of an association between habitual PA and longer LTL (Bendix et al., 2011; Cherkas et al., 2006; Cherkas et al., 2008; Denham et al., 2013; Du et al., 2012; Garland et al., 2014; Kim et al., 2012; Kingma et al., 2012; Krauss et al., 2011; LaRocca et al., 2010; Loprinzi, 2015; Ludlow et al., 2008; Østhus et al., 2012; Puterman et al., 2015; Savela et al., 2012; Silva et al., 2016; Venturelli et al., 2014; Werner et al., 2009; Zhu, Belcher, & van der Harst, 2011). This association with PA is disputed by a similar number of observational and interventional studies (Bekaert et al., 2007; Cassidy et al., 2010; Denham et al., 2016; Farzaneh-Far, Lin, Epel, Lapham, et al., 2010; Fujishiro et al., 2013; Garcia-Calzon et al., 2014; Hovatta et al., 2012; Kadi et al., 2008; Laine et al., 2015; Laye et al., 2012; Mason et al., 2013; Mathur et al., 2013; Ponsot et al., 2008; Rae et al., 2010; Shin et al., 2008; Song et al., 2010; Sun et al., 2012; Svenson et al., 2011; Tiainen et al., 2012; Woo et al., 2008). A clear understanding of the physiological mechanisms underpinning the putative association is lacking.

Telomeres bookend linear chromosomes protecting them false recognition as DNA damage and enzymatic degradation. The human telomere complex consists of three distinct yet inextricably linked functional components; the telomeric DNA, the telomerase complex, and the shelterin complex. It is also directly and indirectly influenced by numerous signalling pathways (Ludlow et al., 2013). Suppression or insult to any individual component or pathway can negatively influence telomere homeostasis. The corollary follows that upregulation (potentially exercise-induced) or attenuation of age-associated decrease in any negative components or pathways may positively influence telomere homeostasis. The influence of lifestyle-mediated epigenetic changes upon the telomere trajectory is only just beginning to

be understood. At present, the broad consensus is that exercise-induced amelioration of oxidative stress and inflammation likely underpins the association between PA and LTL. Accumulating evidence suggests that the association between PA and LTL may represent a confluence of several pro-telomeric adaptations.

Exercise and the Shelterin Complex

Mouse models have demonstrated exercise-induced plasticity of various shelterin components. Cardiac*TRF2* mRNA and protein increased after 21 days of voluntary running (Werner et al., 2008), whilst skeletal muscle *TRF1* mRNA increased after 44 weeks of voluntary running (Ludlow et al., 2012). Recently, acute exercise upregulated mouse cardiac *TRF1* and *TRF2* mRNA and protein, increased expression of DNA-repair genes Ku70 and Ku80, and increased mitogen-activated protein kinase (MAPK) signalling (Ludlow, Gratidão, Ludlow, Spangenburg, & Roth, 2017).

Human studies have identified increased mononuclear cell (MNC) *TRF2* mRNA and protein in young and middle-aged athletes compared to controls (Werner et al., 2009). Increased leukocyte *TPP1* mRNA expression was observed in chronically trained athletes compared to controls (Denham et al., 2016). Athletes who performed seven marathons in one week demonstrated upregulated DDR enzymes Ku70 and Ku80 in addition to increased *TRF1*, *TRF2*, and *Pot-1* mRNA expression. This occurred without concomitant changes in telomere length or telomerase activity (Laye et al., 2012). A significant 60 min post-exercise increases in PBMC *TRF2* mRNA were seen in older men after a 30 min bout of high-intensity cycling; expression levels of did not change in the young cohort (Cluckey, Nieto, Rodoni, & Traustadóttir, 2017).

Exercise and *hTERT* Regulation

Despite accumulating evidence of exercise-induced shelterin regulation, there is comparatively little evidence of exercise-induced *hTERT* regulation. Preliminary evidence has come from mouse models showing increases in telomerase and TERT protein after 21 days of voluntary running (Werner et al., 2008). Human athletes who performed seven marathons in a week did not exhibit significantly increase *hTERT* mRNA levels despite increased expression of shelterin components (Laye et al., 2012). Zietzer and colleagues demonstrated increased PBMC telomerase activity after a 30 min treadmill run at 65% of predicted maximal heart rate despite no increase in *hTERT* mRNA (Zietzer et al., 2016). It is worth noting that *hTERT* mRNA was only assessed in a subset of the exercising cohort (n=5) and was likely underpowered to detect meaningful change. In a recent observational study, chronically trained athletes exhibited increased expression of *hTERT* mRNA when compared to healthy controls (Denham et al., 2016).

A cohort of 11 young (5 men, 6 women) and 8 older participant (4 men, 4 women) undertook a 30 min bout of high-intensity cycling with blood samples taken at 30, 60, and 90 min post-exercise (Cluckey et al., 2017). The entire cohort demonstrated post-exercise increases in *hTERT* mRNA; however, the increases were greater in the young cohort. Expression levels of *TRF2* mRNA did not change in the young cohort but did increase in the old cohort at 60 min post-exercise. A significant effect for gender was seen across the entire cohort, regardless of age, with men demonstrating greater *hTERT* and *TRF2* responses compared to women (Cluckey et al., 2017). Such discordant findings may be due to variant exercise modes and intensities, sampling timeframes, and/or small sample sizes used in the various studies.

Exercise and DNA Damage Repair

Telomeric DNA is particularly susceptible to a range of insults including replication fork stalling (León-Ortiz, Svendsen, & Boulton, 2014; Martínez & Blasco, 2015), bulky lesion formation due to UV exposure (Douki & Cadet, 2001), base damage, and single strand breaks (Petersen, Saretzki, & Zglinicki, 1998). Accordingly, telomere homeostasis is supported by a host of DDR proteins such as Mre11/Rad50/Nsb1 (MRN complex) (Williams, Williams, & Tainer, 2007; Zhu, Küster, Mann, Petrini, & Lange, 2000), Ku70 and Ku80 (Hsu, Gilley, Blackburn, & Chen, 1999), replication protein A1 (RPA1) (Wold, 1997; Zou, Liu, Wu, & Shell, 2006), and RecQ helicases (Multani & Chang, 2007; Opresko, 2008). Habitual exercise enhances DDR capacity in animal models (Nakamoto et al., 2007; Radak et al., 2002; Schneider, Willis, & Parkhouse, 1995; Werner et al., 2009). Despite often being inferred by decreased oxidative stress and DNA damage in the physically active, comparatively little evidence of exercise-enhanced DDR protein exists (Cash et al., 2014).

Exercise and Sirtuins

Sirtuin 6 (SIRT6) is a mammalian homologue of yeast silent information regulator two (Sir2). In addition to DNA repair, SIRT6 regulates lifespan via key aging processes such as metabolism and telomere maintenance (Mao et al., 2011; Masri, 2015; Toiber et al., 2013; Xu et al., 2015). SIRT6 appears to play a multifaceted role in telomere maintenance including modulation of telomeric chromatin (Michishita et al., 2008; Michishita et al., 2009), maintenance of telomere position effect (TPE) (Tennen, Bua, Wright, & Chua, 2011), and replicative stress-induced interaction with TRF2 (Rizzo et al., 2017). Exercise attenuates the age-associated increase in skeletal muscle SIRT6 in aged rats (Koltai et al., 2010);

however little is known about the exercise responsiveness of SIRT6 within the human immune system.

4.3 Aims

The aim of this study was to investigate potential mechanisms underpinning the positive association between PA and LTL. The specific aims were to investigate the acute effects of 30 min of intense cardiorespiratory exercise on the expression of telomere-associated gene transcripts involved in leukocyte telomere regulation.

Hypothesis

The hypothesis for this study is that 30 min of aerobic exercise at 80% of $\dot{V}O_{2peak}$ will differentially regulate several genes associated with telomere biology i.e. those associated with telomerase function or shelterin structure.

4.4 Methods

Ethics Statement

All eligible participants read a plain language information statement outlining all aspects of the project in lay terminology. Informed consent documents explaining the purpose, potential risk and benefits of the project were then signed in the presence of a witness. The study, recruitment and consent procedures were approved by the Human Research Ethics Committee from Federation University Australia (HREC approval #: A10-119).

Participant Information

A subset of 17 healthy, non-smoking males (mean age = 23.8 ± 8.2 years) was selected from the initial cohort of 22 (see chapter 2). All participants provided

written, informed consent prior to participation. General health and lifestyle information was also obtained via a questionnaire.

Physiological Measurements

Participant physiological measurements were taken in accordance with the methodology laid out in chapter 2. A summary of the participant physiological and exercise intervention data is contained in table 19.

Determination of Fitness Standard

Participants undertook a treadmill-based peak oxygen uptake ($\dot{V}O_{2peak}$) test using a Metalyser® metabolic system (Cortex Biophysic, Leipzig, Germany). The specifics of the protocol and associated CPET data are contained in chapter 2.

Exercise protocol

Participants then undertook a 30 min bout of continuous treadmill running at 80% of previously determined $\dot{V}O_{2peak}$. Blood samples were taken Pre-Ex, Post-Ex and 60 min Post-Ex. All CPET data and specific blood sampling procedures are contained in chapter 2.

Preparation of cells

The preparation and staining of T cells and all associated flow cytometry procedures are explained in detail in chapter 2.

RNA extraction

Total RNA for the TaqMan® Array Human – telomere Extension by Telomerase (Life Technologies) and individual gene validations was extracted using TRIzol® (Life Technologies) according manufacturer's instructions. All RNA samples were quantified by spectrophotometry using a Nanodrop™ (Thermo Fisher).

Table 19. Physiological characteristics and exercise intervention data from the 17 male participants.

Characteristic	**Mean**	**SEM**
Age (years)	23.8	± 8.2
Height (cm)	182.1	± 5.77
Body Mass (kg)	78.6	± 10.8
BMI (kg/m^2)	23.7	± 2.7
Waist:hip ratio	0.8	± 0.03
$\dot{V}O_{2peak}$ ($mL{\cdot}kg^{-1}{\cdot}min^{-1}$)	50.03	± 5.10
% of $\dot{V}O_{2peak}$ during 30 min run	80.26	± 7.93

SEM (standard error of mean); **BMI** (body mass index); **$\dot{V}O_{2peak}$** (highest oxygen consumption achieved in test)

Global expression of telomere extension genes

To assess the acute effects of exercise on a wide range of telomeric genes, pooled leukocyte RNA from each time point was analysed using a TaqMan® Array Human - Telomere Extension by Telomerase (Life Technologies). Each plate contained 28 assays specific to telomere extension by telomerase associated genes and four assays to candidate endogenous control genes; all reactions were performed in triplicate in a Viia™7 Real-Time PCR System (Applied Biosystems) using the following cycling conditions: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 20 s, 40 cycles of 95°C for 3 s, 60°C for 30 s. Genes were selected for individual sample validation based on fold difference between the three time-points.

Validation of candidate telomere genes

Individual validations of expression levels of target genes were assessed at three time points via qPCR. Total RNA was reverse transcribed using the Applied Biosystems High Capacity Reverse Transcription Kit (Life Technologies). The qPCR reactions were performed for *hTERT*, *SIRT6*, *RAD50*, and *RAP1* in a Viia7™ PCR System (Life Technologies) using the following cycling conditions: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s. Details of qPCR primers are listed in Tables 20 and 21. Target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and analysed using the $^{\Delta\Delta}$Ct method (Livak & Schmittgen, 2001).

Statistical Analysis

All qPCR data was assessed using either Friedman repeated measures for non-parametric data or repeated measures ANOVA with appropriate post hoc analysis. Statistical significance was set at $P<0.05$. All statistical analysis was performed using SPSS® (version 17).

Table 20. Quantitative real-time PCR primers or assays and associated conditions

Gene	Primer Sequence	Concentration (nM)
GAPDH	*Fwd 5'* CTTTTGCGTCGCCAGCCGAG 3' *Rev 5'* GCGCCCAATACGACCAAATCCG 3'	200
TERT	Fwd 5' TACGGCGACATGGAGAACAAG 3' Rev 5' GGGCATAGCTGAGGAAGGTTT 3'	500
SIRT6	Fwd 5' CCACCAAGCACGACCGCCAT 3' Rev 5' CGCCCTCTCCAGCACACGG 3'	200

Fwd 5' (forward primer sequence), **Rev 3'** (reverse primer sequence), **nM** (nanomole)

Table 21. Quantitative real-time PCR TaqMan® gene expression assays

Gene symbol	GenBank Accession #	Assay Identification #
RAD50	NM_005732.3	Hs00990023_m1
RAP1	NM_018975.3	Hs00430292_m1
GAPDH	NM_002046.3	Hs02758991_g1

Fwd 5' (forward primer sequence), **Rev 3'** (reverse primer sequence)

4.5 Results

Global expression of telomere extension genes in pooled samples

Fold changes in relative expression appear in table 22. The TaqMan® Human Telomere Extension array was only performed once due to limited sample; therefore, results are limited to fold change without corresponding measures of statistical significance. In response to acute exercise, 16 of the 28 telomeric genes were upregulated from Pre-Ex to Post-Ex and 15 from Pre-Ex to 60 min Post-Ex. A total of 10 genes trended towards downregulation between Pre-Ex to Post-Ex and 13 trended towards downregulation from Pre-Ex to 60 min Post-Ex.

Validation of gene expression in individual subjects

hTERT

Expression levels of *hTERT* mRNA were assessed by qPCR based on the fold change observed in the TaqMan® Human Telomere Extension array and it's established role in telomere homeostasis. Results demonstrated non-normal distribution and were therefore assessed using a Friedman test. A significant effect of time for *hTERT* mRNA expression was observed across time points ($\chi^2(2) = 11.36$, $P=0.003$). Post hoc analysis using Wilcoxon Signed Ranks Test with Bonferroni correction identified significant upregulation between Pre-Ex and 60 min Post-Ex time points ($Z = -3.21$, $P=0.001$) and Post-Ex and 60 min Post-Ex time points ($Z = -2.50$, $P=0.12$) (figure 14).

To determine if a particular T cell subset was driving the observed changes, population specific pools were assessed over the three time points. The stepwise upregulation trend was broadly confirmed in CD4+CD45RA+, CD4+CD45RO+, and CD8+CD45RA+ T cell subsets (figure 14).

Table 22. Differential regulation obtained using the TaqMan® Telomere extension array.

	Fold Change		
Gene Symbol	**Pre-Ex to Post-Ex**	**Pre-Ex to 60-min Post**	**Post-Ex to 60-min Post-Ex**
ACTB	1.00	1.15	1.15
B2M	1.10	-1.27	-1.40
RPLPO	-1.03	-2.09	-2.04
HMBS	-1.08	1.07	1.15
TBP	1.04	-1.39	-1.44
PGK1	1.00	1.10	1.10
UBC	-1.05	-1.03	1.02
PPIA	1.01	-1.46	-1.48
TFRC	1.03	-1.13	-1.16
HNRNPA1	1.17	1.31	1.12
HNRNPA2B1	1.26	1.50	1.19
HNRNPAB	1.13	1.06	-1.07

Table 22. continued

	Fold Change		
Gene Symbol	**Pre-Ex to Post-Ex**	**Pre-Ex to 60-min Post**	**Post-Ex to 60-min Post-Ex**
HNRNPC	1.07	1.05	-1.02
HNRNPD	-1.26	-1.19	1.06
HNRNPF	1.18	-1.12	-1.33
MRE11A	1.13	1.14	1.01
NBN	-1.03	1.07	1.10
POT1	-1.08	-1.12	-1.04
RAD50	1.05	-1.17	-1.23
TERF1	-1.37	1.38	1.90
TERF2	-1.19	1.19	1.41
RAP1	1.02	-1.41	-1.43
TERT	-1.14	1.93	2.20
TINF2	1.24	1.31	1.06

Table 22. continued

	Fold Change		
Gene Symbol	**Pre-Ex to Post-Ex**	**Pre-Ex to 60-min Post**	**Post-Ex to 60-min Post-Ex**
TNKS	1.02	1.52	1.49
TNKS2	1.11	1.31	1.18
XRCC5	1.13	-1.09	-1.24
XRCC6	-1.02	-1.02	1.00

All gene expression data is expressed relative to the average of four endogenous controls: *18S, GAPDH, HPRT1,* and *GUSB.* The TaqMan® Human Telomere Extension array was only performed once due to limited sample. Results are limited to fold change without corresponding statistics

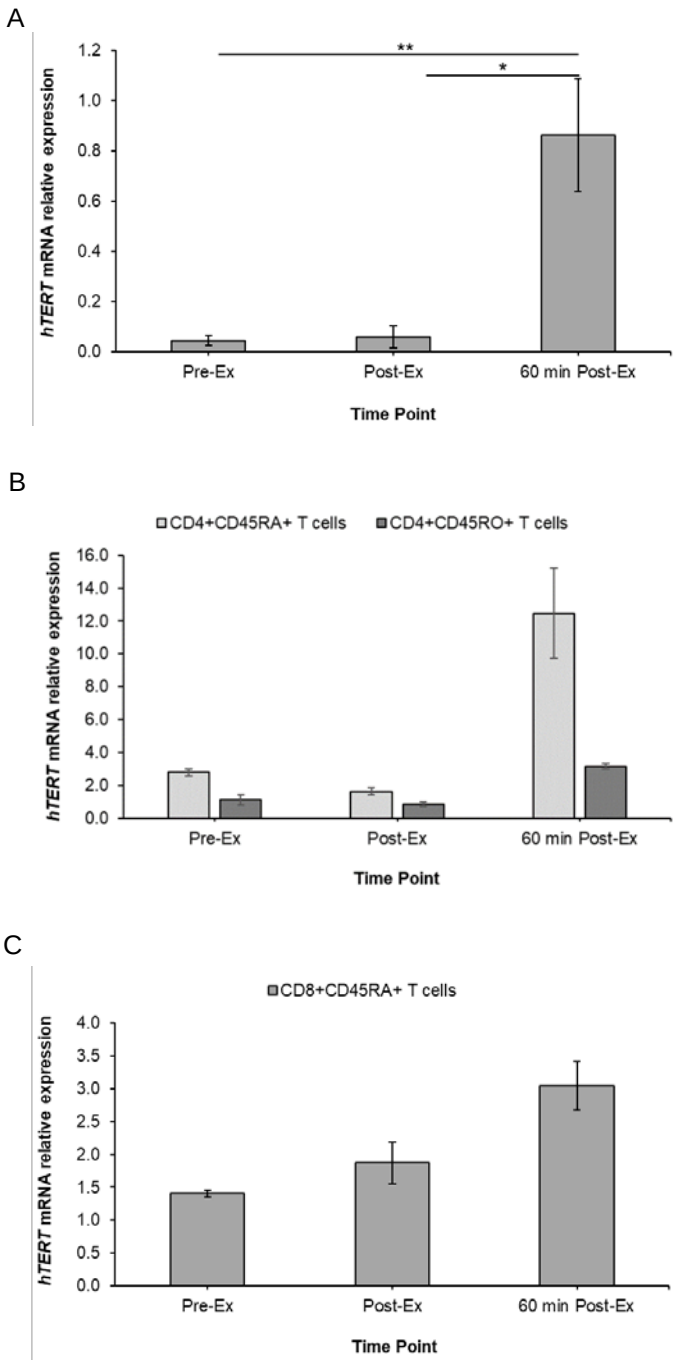


Figure 14. Differential regulation of *hTERT* mRNA expression*:*** *hTERT mRNA* expression was assessed in unsorted leukocytes (n = 17) **(A)**, CD4+CD45RA+ and CD4+CD45RO+ T cells (pool of n = 22) **(B)**, and CD8+CD45RA+ T cells (pool of n = 22) **(C)**. Gene expression data is expressed relative to endogenous reference gene (GAPDH). * indicates $P<0.05$, ** indicates $P<0.01$.

SIRT6

SIRT6 mRNA (not featured on the TaqMan® Human Telomere Extension array) was assessed as this gene plays a role in telomeric chromatin maintenance (Michishita et al., 2008). A Friedman test identified a significant effect of time for *SIRT6* mRNA expression across time points ($\chi^2(2) = 8.4$, $P=0.015$). Post hoc analysis identified significant upregulation between Pre-Ex and 60 min Post-Ex time points (Z = -2.38, $P=0.017$) and Post-Ex and 60 min Post-Ex time points (Z = -2.38, $P=0.017$). *SIRT6* was down-regulated Post-Ex in CD4+CD45RA+, CD8+CD45RA+ and CD4+CD45RO+ T cells (Figure 15).

RAP1

A repeated measures ANOVA revealed a significant effect of time for *RAP1* mRNA expression across time points [F (2, 24) = 15.48, $P<0.001$]. Post hoc pairwise comparisons using the Bonferroni correction revealed significant upregulation between Pre-Ex and Post-Ex time points ($P=0.001$) and down regulation between Post-Ex and 60 min Post-Ex time points ($P=0.002$). *RAP1* mRNA expression demonstrated a stepwise decrease from Pre-Ex to 60 min Post-Ex in CD4+CD45RA+ and CD8+CD45RA+ T cells (Figure 16).

RAD50

A repeated measures ANOVA revealed a significant effect of time for *RAD50* mRNA expression across time points [F (2, 28) = 4.19, $P=0.026$]. Post hoc pairwise comparisons with Bonferroni correction revealed significant downregulation between Post-Ex and 60 min Post-Ex time points ($P=0.05$). A stepwise decrease from Pre-Ex to 60 min Post-Ex was observed in CD4+CD45RA+ and CD8+CD45RA+ T cells (Figure 17).

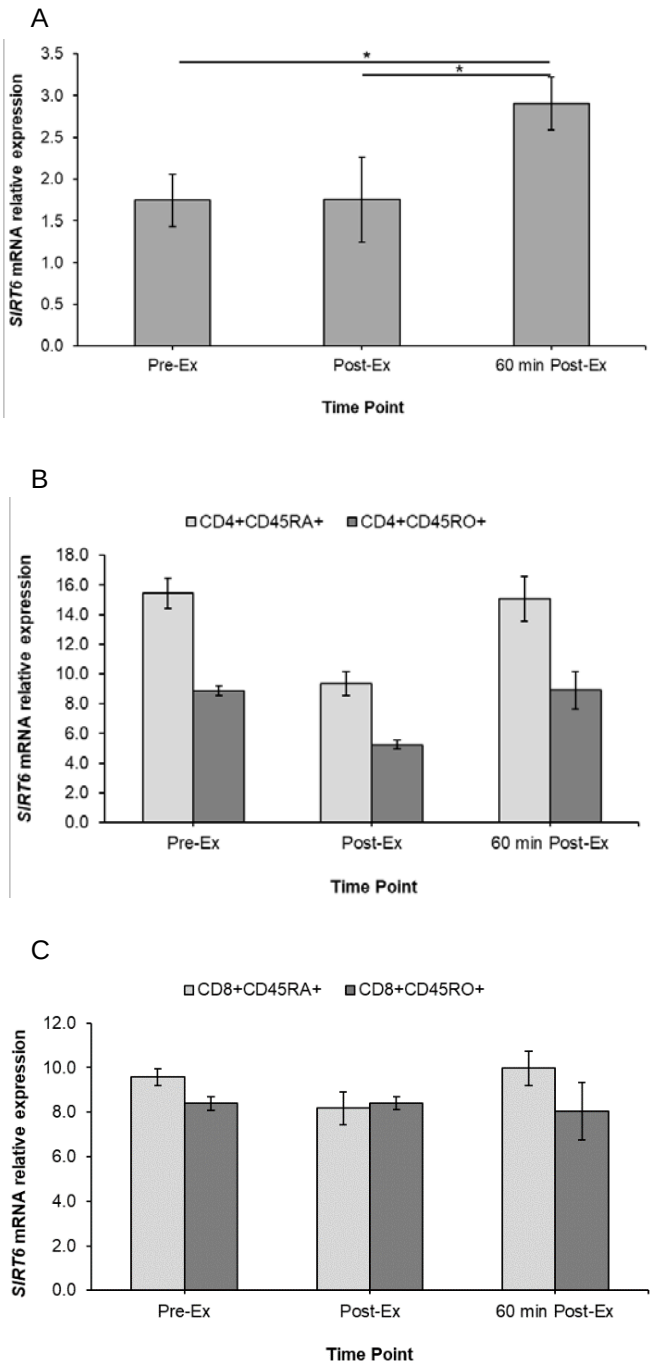


Figure 15. **Differential regulation of *SIRT6* mRNA expression:** Unsorted leukocytes (n = 17) (A), CD4+CD45RA+ and CD4+CD45RO+ T cells (pool of n = 22) (B), and CD8+CD45RA+ and CD8+CD45RO+ T cells (pool of n = 22) (C). Gene expression data is expressed relative to endogenous reference gene (GAPDH). * indicates $P<0.05$.

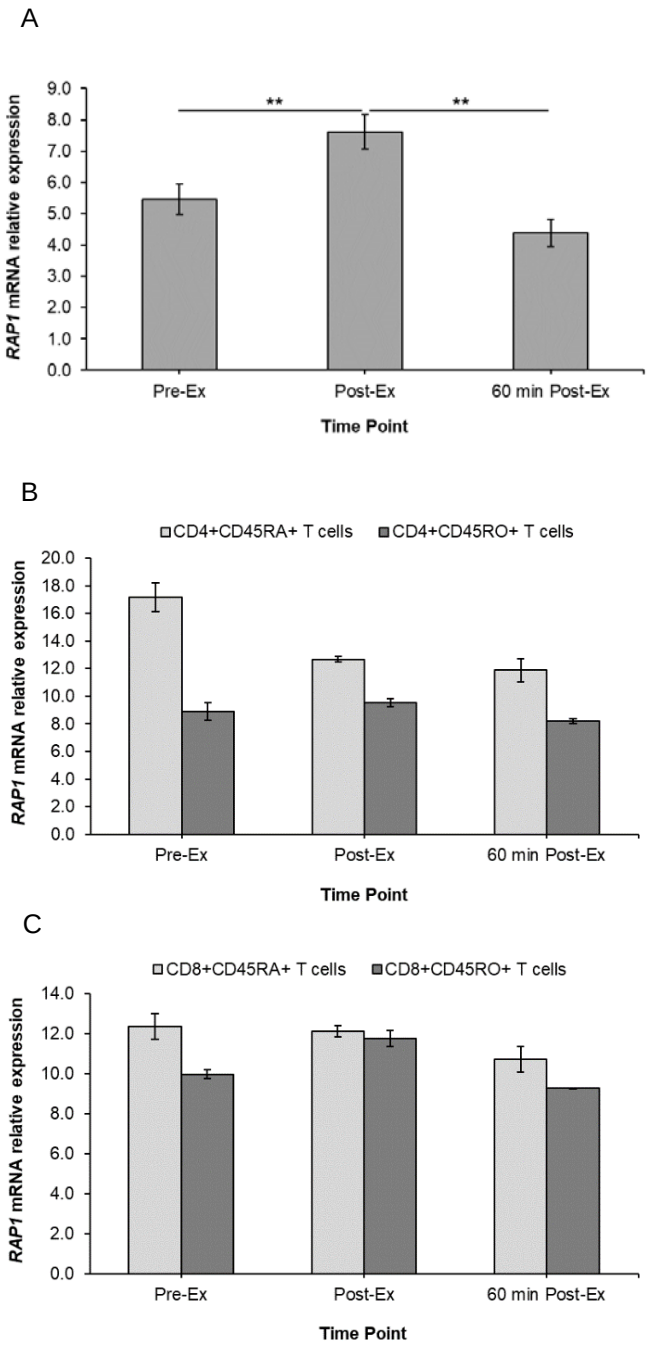


Figure 16. Differential regulation of *RAP1* mRNA expression: Relative expression in leukocytes (n = 16) **(A)**, in CD4+CD45RA+ and CD4+CD45RO+ T cell pools (n = 22) **(B),** and in CD8+CD45RA+ and CD8+CD45RO+ T cells pools (n = 22) **(C).** Gene expression data is expressed relative to endogenous reference gene (GAPDH). ** indicates *P*<0.01.

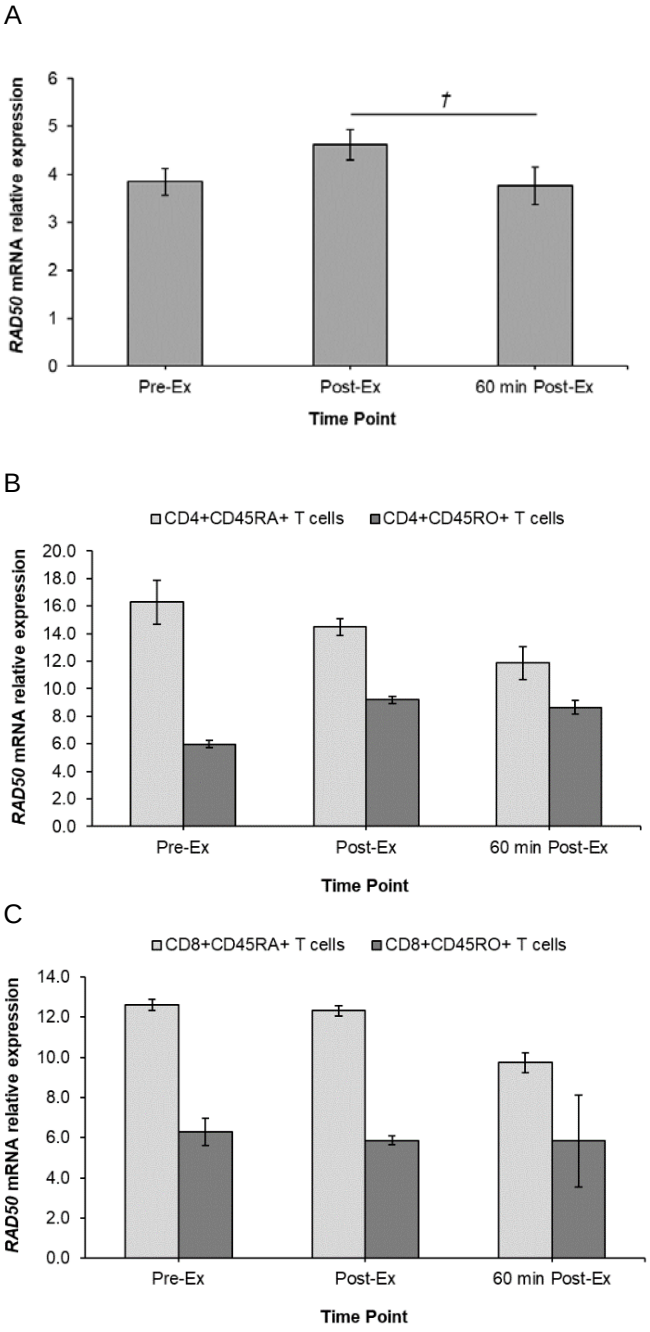


Figure 17. Differential regulation of *RAD50* mRNA expression: Relative expression in leukocytes (n = 16) **(A)**, in CD4+CD45RA+ and CD4+CD45RO+ T cell pools (n = 22) **(B),** and in CD8+CD45RA+ and CD8+CD45RO+ T cells pools relative expression (n = 22) **(C).** Gene expression data is expressed relative to endogenous reference gene (GAPDH). *†* indicates *P*=0.05

4.6 Discussion

Here I report for the first time that acute exercise leads to the transcriptional regulation of several key telomere-associated genes in leukocytes. These findings may provide an important mechanistic link between PA and telomere biology. An enhanced understanding of telomere homeostasis is critical given the strong association between telomere shortening and chronic diseases (Calado & Young, 2009). Telomere homeostasis underpins the function of several adaptive immune cell subsets (Weng, 2008), which in turn play critical roles in age-related diseases (Weiskopf et al., 2009), atherosclerosis (Hansson & Hermansson, 2011), and metabolic diseases (Osborn & Olefsky, 2012).

To the author's knowledge, this was the first study to report the upregulation of *hTERT* mRNA after acute aerobic exercise in leukocytes and T cell subsets. Other research groups have since made similar investigations producing disparate results (Cluckey et al., 2017; Laye et al., 2012; Zietzer et al., 2016). Transcription of *hTERT* is highly regulated and is considered the primary step in telomerase regulation (Aisner, Wright, & Shay, 2002). The positive correlations between *hTERT* mRNA and telomerase activity confirm this mutual inclusiveness (Cong et al., 1999; Li, Wu, Liang, & Wu, 2003; Yi, Tesmer, Savre-Train, Shay, & Wright, 1999). Despite established telomere length heritability estimates of 0.70 (Broer et al., 2013), telomerase activity, and by implication *hTERT* expression, significantly influence telomere shortening trajectory and lifespan potential (Boccardi & Paolisso, 2014). Immune cells are unique in their capacity to upregulate telomerase expression and thereby reduce telomere attrition during periods of clonal expansion. Ectopic expression of *hTERT* in CD4+ and CD8+ T cells extends

their replicative lifespan and enhances their resistance to oxidative stress and apoptosis (Dagarag et al., 2004; Luiten et al., 2003; Rufer et al., 2001).

DNA methylation, histone methylation, histone acetylation, and non-coding RNAs are all known epigenetic modifiers of *hTERT* expression (Lewis & Tollefsbol, 2016). Despite a current lack of evidence in lymphocytes, hTERT is known to serves several extra-telomeric functions critical for metabolic and mitochondrial homeostasis in human mammary epithelial cells (Stampfer et al., 2001), human fibroblasts (Lindvall et al., 2003) , murine cells (Geserick, Tejera, Gonzalez-Suarez, Klatt, & Blasco, 2006) and cardiovascular cells (Ahmed et al., 2008; Haendeler et al., 2009; Santos, Meyer, Skorvaga, Annab, & Van Houten, 2004; Santos, Meyer, & Van Houten, 2006). hTERT appears to play a role in protecting mitochondrial DNA from oxidative damage (Ahmed et al., 2008). In conditions of oxidative stress, nuclear export of hTERT and subsequent increase in mitochondrial hTERT expression have been observed, suggesting a possible *on-demand* redeployment (Zurek, Altschmied, Kohlgrüber, Ale-Agha, & Haendeler, 2016).

hTERT may also regulate glucose utilization pathways (Shaheen, Grammatopoulos, Müller, Zammit, & Lehnert, 2014). Inhibition of hTERT reduces glucose uptake whilst overexpression increases it (Shaheen et al., 2014). hTERT has also been shown to interact with glucose transporter proteins (GLUT) 1, 3, and 12 through insulin insensitive pathways and independent of PI3K and mechanistic target of rapamycin (mTOR) (Shaheen et al., 2014).

The discrepant results in the literature may be due to confounding lifestyle factors, variable blood collection timelines, and/or differing exercise intervention intensity or duration. Collection timelines are critical given that the half-life of

hTERT mRNA is approximately 24 hours (Chai et al., 2011). Exercise intensity is an important variable as it closely scales with the post-exercise inflammatory response (Nieman et al., 2012); an association driven in part by nuclear factor kappa B (NF- κB). A highly conserved NF- κB response element is located with the *hTERT* promotor. Inflammatory stimuli results in rapid recruitment of NF- κB to the site and subsequent *hTERT* transcription (Gizard et al., 2011). Additionally, the magnitude of the immune response and subsequent redistribution of immune cell subsets closely scales with exercise intensity (Simpson, 2013). The resultant immune pool composition in turn determines mean telomere length and telomerase activity (Lin et al., 2010).

The current study is unique in that it used an acute measurement timeline; providing a snapshot of transcriptional changes immediately after and 60 min after exercise. Whilst pro-telomeric functions can plausibly be inferred by increased *hTERT* mRNA, the present study is not methodologically equipped to detect extra-telomeric functions. The observed increases in leukocyte *hTERT* mRNA may be due to similar proinflammatory signalling as seen in macrophages (Gizard et al., 2011). Alternatively, the interaction between glucose transporter proteins and hTERT may explain the observed upregulation. Whilst the present study investigated the role of potential pro-telomeric miRNAs, it did not investigate the potential impact of DNA and histone methylation or histone acetylation; any or all of which may have affected *hTERT* mRNA expression.

An enhanced understanding of *hTERT* expression is important. In addition to underpinning the effective functioning of the adaptive immune system, *hTERT* regulation plays a key role in the pathogenesis of many cancers. Despite cancer cells exhibiting shorter telomeres than normal somatic cells, telomerase and

hTERT are upregulated in approximately 90% of all human cancers (Lewis & Tollefsbol, 2016).

To the author's knowledge, the present study is the first to identify a post-exercise increase in human leukocyte *SIRT6* mRNA expression. SIRT6 is a chromatin binding protein existing predominantly in the nucleus (Haigis & Sinclair, 2010). During exercise, internal stimuli such as mitochondriogenesis, lactate threshold shift, altered substrate utilization, oxygen consumption, and fibre-type transition impose significant functional and structural perturbations (Pucci et al., 2013).

Sirtuins have been proposed as critical regulators of exercise-induced changes and most demonstrate some degree of acute exercise responsiveness (Pucci et al., 2013; Suwa & Sakuma, 2013; Villanova et al., 2013). SIRT1 enhances PGC-1α activity and increased SIRT1 activity is present in rat skeletal muscle following endurance training (Koltai et al., 2010). SIRT3 also increases in response to endurance training in human skeletal muscle (Lanza et al., 2008). Exhaustive exercise has been shown to increase human PBMC expression of *SIRT1*, and decrease *SIRT3,* and *SIRT4* mRNA expression (Marfe et al., 2010).

SIRT6 plays several key roles in telomere maintenance. Functioning as a NAD^+ dependent histone H3 lysine 9 (H3K9) deacetylase, SIRT6 binds directly to telomeric chromatin and influences its structure and accessibility by deacetylating H3K9 and H3K56 (Michishita et al., 2008; Michishita et al., 2009). Experimental knockdown of *SIRT6* results in telomere abnormalities, telomere sequence loss, chromosomal end-to-end-fusions, genomic instability, and premature cellular senescence (Baur, Zou, Shay, & Wright, 2001). These pathologies are like those

characterised by Werner syndrome (Cheng, Muftuoglu, & Bohr, 2007; Michishita et al., 2008; Multani & Chang, 2007). During replicative stress, SIRT6 directly binds to shelterin component TRF2 in a DNA independent manner (Rizzo et al., 2017). SIRT6 also maintains telomere position effect (TPE) in human cells, dynamically regulating the silencing of a telomere-proximal transgene and repressing an endogenous telomere-proximal gene (Tennen et al., 2011).

SIRT6 also plays an important role in DNA repair mechanisms by modulating base excision repair (BER) (Mostoslavsky et al., 2006) and stimulating double strand break (DSB) repair (Kaidi, Weinert, Choudhary, & Jackson, 2010; McCord et al., 2009; Toiber et al., 2013). DSBs are repaired via one of two methods; homologous recombination (HR) and non-homologous end joining (NHEJ), both of which are stimulated by SIRT6 in response to oxidative stress (Mao et al., 2011). SIRT6 is recruited to DNA breaks via the phosphorylation by JNK on Serine 10 which in turn stimulates SIRT6 mono-ADP ribosylation of PARP1 and subsequent PARP1 recruitment to DNA breaks (Van Meter et al., 2016).

In addition to its telomeric functions, SIRT6 is implicated in a wide array of extra-telomeric roles including enhanced mitochondrial respiration (Mauro et al., 2011), transcriptional regulation of gene expression (Kawahara et al., 2009), regulation of cellular reprogramming in aging (Sharma et al., 2013), and positive regulation of pro-inflammatory cytokines (Van Gool et al., 2009). SIRT6 also plays key roles in various elements of systemic metabolism including glucose metabolism (Mostoslavsky et al., 2006; Xiao et al., 2010; Zhong & Mostoslavsky, 2010). Overexpression of *SIRT6* in high-fat or high-calorie fed mice protects against insulin insensitivity, defective glucose tolerance, and glucose-stimulated insulin secretion (Anderson et al., 2015; Kanfi et al., 2010). SIRT6 also regulates

lipid metabolism via repression of fatty-acid uptake and triglyceride synthesis, regulation of LDL cholesterol, and increased β-oxidation of fatty-acids (Elhanati et al., 2013; Kanfi et al., 2010; Kim et al., 2010; Tao, Xiong, DePinho, Deng, & Dong, 2013).

The increased production of ROS and/or pro-inflammatory cytokines resulting from exercise may in part explain the observed increase in *SIRT6* mRNA expression. The current study did not assess SIRT6-mediated chromatin modifications or enhanced DNA repair proteins. Given that many of the DNA repair functions of SIRT6 are initiated by oxidative stress, it is plausible that post-exercise mRNA upregulation reflects DNA repair functions.

DNA repair protein RAD50 is encoded by the *RAD50* gene and associates with meiotic recombination 11 (*MRE11*) and Nijmegen breakage syndrome protein (*NBS1*) to form the MRN complex. The MRN complex is a conserved multifunctional DNA DSB repair factor involved in the three requisite facets of DSB repair; namely damage detection, appropriate cell cycle responses to the damage, and capacity to catalyse lesion repair (Lamarche, Orazio, & Weitzman, 2010). The MRN complex also positively regulates telomerase-dependent telomere elongation via an interaction with TRF1 and ataxia telangiectasia mutated (*ATM*) DNA (Wu, Xiao, & Zhu, 2007). The complex formed by RAD50 and MRE11 is thought to help stabilize telomere t-loop formation (Zhong et al., 2007).

The 60 min Post-Ex decrease in *RAD50* mRNA appears counterintuitive. Possible hypotheses include transiently compromised stability of the shelterin complex and suppression of DNA damage signalling at the telomere by the heavily fortified shelterin complex (Fumagalli et al., 2012). Telomeric DNA is preferentially

damaged by oxidative stress (von Zglinicki, 2002); however, the conformation of the shelterin complex and T-loop may preclude access to sites of DNA damage. Chapter 3 of this thesis identified *RAD50* mRNA as a potential binding target for miR-186. The 60 min Post-Ex decrease in *RAD50* mRNA expression paralleled an increase in miR-186 expression. Given the repressive action of miRNAs on their targets, this may also account for the decrease in *RAD50* mRNA abundance.

RAP1 is a highly conserved telomere-interacting protein forming part of the shelterin complex. Equivocal results have identified human RAP1 as both a negative regulator (O'Connor et al., 2004) and a positive regulator of telomere length (Li et al., 2000). RAP1 forms a multifunctional complex with TRF2, subsequently suppressing homology-directed repair of chromosome ends (Kabir, Sfeir, & de Lange, 2010) and NHEJ (Bae & Baumann, 2007). The RAP1-TRF2 complex represses PARP1 and SLX4 localization to telomeres thereby preventing telomere resection, telomere loss, and formation of telomere-free chromosome ends (Rai et al., 2016). The RAP1-TRF2 complex is therefore considered a key telomere regulatory factor. Additional roles for RAP1 include prevention of non-homologous end joining (Sarthy et al., 2009) and homology-directed repair (Sfeir et al., 2010), protection from obesity via regulation of metabolic genes (Martínez et al., 2013), and regulation of senescence (Platt et al., 2013).

The immediate post-exercise increase in *RAP1* mRNA may represent an immediate damage control or DDR mechanism. There is no immediately intuitive reason for the 60-min Post-Ex down regulation of leukocyte *RAP1* mRNA as little is known about its transcriptional time course. It is plausible the intensity of the exercise was sufficient to transiently de-stabilize the shelterin complex. Intense aerobic exercise is known to generate increased lymphocyte ROS (Wang & Huang,

2005) and telomeric DNA is preferentially damaged by oxidative stress (von Zglinicki, 2002). The observational associations between habitual exercise and telomere length may be due to chronic anti-oxidant enhancement resulting from acute increases in oxidative stress. Alternatively, *RAP1* mRNA may exhibit a biphasic response with concentrations reflexively increasing again outside the time course assessed in this study.

Mass spectrometry and affinity purification identified an association between RAD50 and the RAP1-TRF2 complex (O'Connor et al., 2004). The post-exercise expression profiles of *RAD50* and *RAP1* mRNAs in the present study were both upregulated immediately post-exercise before returning to near resting concentrations 60 min post-exercise. The expression profiles of *RAD50* and *RAP1* mRNAs were also very similar in CD4+CD45RA+ and CD8+CD45RA+ T cells.

This study is not without limitations. Determining the optimal time frame to measure gene expression presents a challenge as little is known about the transcriptional timeline or half-life of telomere-associated gene transcripts. Additionally, the telomere-associated and extra-telomeric roles of the genes were not differentiated between in this study. The low sample yield from sorted T cell populations necessitated pooling into cell/time point specific pools for analysis. Whilst this provided interesting, subpopulation-wide overviews, it precluded additional individual validations and robust statistical analysis. The analysis was conducted in leukocytes and T cell subsets; analysis of other leukocyte subsets such as B cells may have provided a more complete picture of telomere homeostasis within the immune system.

4.7 Conclusion

In conclusion, 30 min of vigorous cardiorespiratory exercise differentially regulated the telomere-associated genes *hTERT*, *SIRT6, RAP1*, and *RAD50*. This information provides potentially mechanistic insights into the observed relationship between telomere homeostasis and PA. This is in turn may inform optimal exercise prescription that elicits pro-telomeric adaptations.

Understanding the environmental stimuli that elicit pro-telomeric gene responses and the epigenetic modifications that can influence them are essential steps in understanding the role of telomeres in health and disease. Whilst telomere dynamics have traditionally been portrayed as the slow shifting sands of cellular physiology, the results of this study indicate that leukocyte telomere homeostasis may be acutely responsive to physiological stressors. Such acute plasticity in pro-telomeric factors likely represents a double-edged sword, with telomeric factors similarly adaptive to negative instigators.

There likely exists an exercise dosage (intensity, duration, frequency or all) that represents a point of diminishing returns with regards to cellular stress. Beyond such a theoretical point, telomere stability could be compromised potentially leading to pathological cellular adaptations. Broadly establishing this point may be beneficial for healthy and pathological populations.

Chapter 5 – Acute Genome-Wide Transcriptional Changes in Response to Exercise

5.1 Abstract

Introduction: Telomeres are specialized nucleoprotein structures that protect chromosomal ends from degradation. Telomeres progressively shorten during cellular division and can induce replicative senescence below a critical length. Habitual physical activity is associated with longer leukocyte telomere and reductions in chronic oxidative stress and inflammation are widely accepted explanations for this association. Chapters 3 and 4 of this thesis demonstrate acute, exercise-induced regulation of individual telomere-associated genes and miRNAs with potential telomere interaction. ***Aims:*** To use next generation RNA sequencing to characterize exercise-induced changes in leukocyte transcriptome with a specific focus on telomere-associated genes and/or pathways over a 24 hour post-exercise period. ***Methods:*** Ten healthy males (27.3 ± 7.9 years) undertook 30 min of treadmill running at 80% of $\dot{V}O_{2peak}$; the highest value of oxygen of uptake achieved in a maximal test. Blood samples were taken before exercise, immediately post-exercise, and 24 hour post-exercise. Transcriptomic changes were studied using Next Generation RNA sequencing. ***Results:*** One hundred and eight-two transcripts were differentially expressed immediately post-exercise and 24 hour post-exercise (FDR<0.01). Several of the regulated transcripts have established roles in telomere biology. *In silico* analysis identified two miRNAs (miR-23a and miR-27a) that potentially target telomere gene transcripts. ***Conclusion:*** These results may provide an insight into the acute exercise-induced regulation of pro-telomeric components within human leukocytes. This in turn may contribute to the mechanistic understanding of the association between leukocyte telomere length and physical activity.

5.2 Introduction

The human transcriptome is the sum of all mRNA, non-coding RNA, and small RNA molecules expressed in a specific tissue under a given condition or time course. Characterization of the transcriptome is critical in that it represents the physiological nexus between environmental stimulus and resultant phenotype. The study of transcriptomics aims to elucidate gene transcriptional structure including splicing patterns, post-transcriptional modifications, start sites, and 5' and 3' ends (Wang, Gerstein, & Snyder, 2009). RNA transcription is subject to several possible levels of modulation including alternative polyadenylation (Di Giammartino, Nishida, & Manley, 2011; Lutz, 2008) , alternative splicing (Cooper, Wan, & Dreyfuss, 2009; Maniatis & Tasic, 2002), alternative transcription initiation (Davuluri, Suzuki, Sugano, Plass, & Huang, 2008; Moore & Proudfoot, 2009), RNA editing (Gott & Emeson, 2000; Knoop, 2011), post-transcriptional modifications (Karijolich & Yu, 2011; Martin & Keller, 2007; Rottman, Bokar, Narayan, Shambaugh, & Ludwiczak, 1994), and RNA interference (Huntzinger & Izaurralde, 2011; Lee et al., 2009; Lytle et al., 2007; Ørom et al., 2008).

RNA sequencing (RNA-seq) uses Next Generation deep sequencing technology to: (i) characterise the structures of all transcribed genes including their 5' and 3' ends and their splice junctions (Denoeud et al., 2008; Wilhelm et al., 2008; Yassour et al., 2009), (ii) quantify transcript expression (Marioni, Mason, Mane, Stephens, & Gilad, 2008; Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008), and (iii) quantify the extent of alternative splicing (Guttman et al., 2010; Pan, Shai, Lee, Frey, & Blencowe, 2008; Sultan et al., 2008; Trapnell et al., 2010; Wang, Sandberg, et al., 2008). The digital nature of RNA-Seq affords an almost unlimited dynamic range of detection and provides higher resolution of differentially

expressed genes and a lower detection limit than its microarray predecessor (Zhao, Fung-Leung, Bittner, Ngo, & Liu, 2014). Whilst microarrays can reliably detect a 2-fold change in transcript expression, RNA-Seq can accurately detect a 1.25-fold change (Mantione et al., 2014). Given that longer transcripts allow more fragments for sequencing, RNA-seq demonstrates an inherent bias towards longer transcripts (Oshlack & Wakefield, 2009; Young, Wakefield, Smyth, & Oshlack, 2010).

The progressive molecular characterization of exercise adaptation will eventually culminate in a comprehensive *exercise responsome* (Neufer et al., 2015). In one of the seminal studies in exercise-induced gene regulation, Connolly et al used microarray analysis to characterize expression profiles in PBMCs pre-exercise (Pre), immediately post-exercise (End-Ex) and 1 hour post-exercise (recovery) after a 30 min run at 80% of $\dot{V}O_{2max}$ (Connolly et al., 2004). The authors identified 311 differentially regulated genes from Pre- to End-Ex, 552 genes between End-Ex to Recovery, and 293 genes from Pre- to Recovery. The regulated genes were related to stress response, inflammatory response, growth factors, and transcription. The predominantly stress and inflammatory genes upregulated Pre- to End-Ex had returned to baseline levels by Recovery whilst anti-inflammatory genes were upregulated between End-Ex and Recovery. A range of other studies have since contributed to the understanding of exercise-induced gene expression (Büttner et al., 2007; Gjevestad, Holven, & Ulven, 2015; Nakamura et al., 2010; Palmer et al., 2006; Radom-Aizik et al., 2009; Radom-Aizik et al., 2008).

Habitual PA is widely associated with longer mean LTL (Bendix et al., 2011; Cherkas et al., 2006; Cherkas et al., 2008; Denham et al., 2013; Du et al., 2012; Garland et al., 2014; Kim et al., 2012; Kingma et al., 2012; Krauss et al., 2011;

LaRocca et al., 2010; Loprinzi, 2015; Ludlow et al., 2008; Østhus et al., 2012; Puterman et al., 2015; Savela et al., 2012; Silva et al., 2016; Venturelli et al., 2014; Werner et al., 2009; Zhu, Wang, et al., 2011). The dominant explanatory framework for the PA/telomere length association is the lifestyle-induced amelioration of chronic oxidative stress and inflammation. The acute responsiveness of telomere biology to physiological stress is important for the potential management of conditions characterised by accelerated telomere shortening. Just as important are the situations in which chronic oxidative stress or inflammatory burden are less amenable to modulation due to an underlying medical cause. Appropriately dosed exercise may be effective in positively influencing telomere-associated genes and pathways.

Importantly, chronic oxidative stress and inflammation can be both causal and consequential to accelerated telomere shortening. Excessive telomere shortening and the resultant inflammatory SASP can perpetuate telomere shortening via ROS positive feedback loops (Acosta et al., 2013; Correia-Melo, Hewitt, & Passos, 2014; Kuilman & Peeper, 2009; Passos et al., 2010; Passos & von Zglinicki, 2005; Wiley et al., 2016). Exercise-mediated telomere regulation may provide a means of interceding the recursive pathophysiological processes.

A small number of human studies have investigated the effect of acute and long-term exercise on telomere-associated genes such as those of the shelterin complex (Denham et al., 2016; Uhlemann et al., 2014; Werner et al., 2009) and telomerase enzyme (Cluckey et al., 2017; Denham et al., 2016). Most have been observational in design or used highly discrepant exercise interventions.

At the time of writing, only one other study has utilized RNA-seq technology to identify telomere-associated non-coding RNAs (Fujita, Yuno, Okuzaki, Ohki, & Fujii, 2015). The authors identified a host of RNAs associated with telomeres including: telomerase components (*Ter and Rmrp*), telomeric RNAs (TERRAs), small Cajal body-specific RNAs (scaRNAs) (*Scarna6, Scarna10, Scarna13, Scarna2*), H/ACA small nucleolar RNAs (snoRNAs) (*Snora23, Snora74a, Snora73b, Snora73a*), C/D snoRNAs (*Snord17, Snord15a, Snord118*) and long non-coding RNAs (lncRNA) (*Neat1*). Whilst providing a valuable insight into telomere regulation, the study contained no exercise intervention and therefore the results cannot be generalised to an exercise context.

The influence of lifestyle factors; such as PA, on telomere homeostasis is replete with observational findings and correlations. The underpinning molecular mechanisms must be identified to establish causation. A growing body of evidence, both observational and interventional, demonstrates exercise-induced regulation of shelterin and other telomere-associated genes (Cluckey et al., 2017; Denham et al., 2016; Laye et al., 2012; Werner et al., 2009). Whilst providing valuable insights, many of these studies have investigated small numbers of genes in relative isolation. RNA-seq provides a transcriptome-wide insight into exercise-induced regulation of transcripts, providing a deeper, more accurate characterization of involved pathways and networks.

Immune cells permeate every tissue in the body and can illicit far reaching physiological effects as evidenced in many chronic inflammatory diseases (Neufer et al., 2015). The responsiveness of many inflammatory diseases to PA strongly implies significant physiological plasticity within immune cells (Walsh et al., 2011). The replicative and functional lifespan of immune cells is inextricably linked to

telomere length and telomerase expression (Roth et al., 2003; Weng, Levine, June, & Hodes, 1995); however, the molecular processes underpinning these two factors within the context of exercise adaptation are poorly understood. As a systemic physiological stressor influencing innumerable pathways, PA can function as a lens through which these networks can be better understood. A key component of characterizing the molecular mechanisms of PA is understanding the cell types, structures, and networks impacted.

5.3 Aims

The aim of this study was to characterize the exercise-induced transcriptome in leukocytes to identify the responsiveness of telomere-associated genes and/or pathways. This would potentially further the understanding of mechanisms underlying the widely reported positive association between PA levels and LTL.

The specific aims of this study were:

1. To characterize the magnitude of exercise-induced differential expression of telomere-associated genes and non-coding RNAs within leukocytes.
2. To characterize the exercise-induced transcriptional time course of pro-telomere genes in leukocytes.
3. To conduct an enrichment analysis on differentially expressed gene sets to identify represented gene ontology terms.

Hypothesis

The hypothesis for this study is that 30 min of aerobic exercise will differentially regulate several genes and non-coding RNAs which may have interactions with telomere biology.

5.4 Methods

Ethics Statement

All eligible participants read a plain language information statement outlining all aspects of the project in lay terminology. Informed consent documents explaining the purpose, potential risk and benefits of the project were then signed in the presence of a witness. The study, recruitment and consent procedures were approved by the Human Research Ethics Committee (HREC) from Federation University Australia (HREC approval # A13-082).

Participants

Ten healthy, non-smoking males (27.3 ± 7.9 years) were recruited to participate in this study.

Physiological Measurements

Participant physiological measurements were taken in accordance with the methodology laid out in chapter 2. A summary of the participant physiological data is contained in table 23.

Determination of Fitness Standard

Participants undertook a treadmill-based peak oxygen uptake ($\dot{V}O_{2peak}$) test using a Metalyser® metabolic system (Cortex Biophysic, Leipzig, Germany). The specifics of the protocol and associated CPET data are contained in chapter 2.

Exercise protocol

Participants then undertook a 30 min bout of continuous treadmill running at 80% of previously determined $\dot{V}O_{2peak}$. Blood samples were taken before (Pre-Ex), immediately after (Post-Ex), and 24 hours post-exercise (24 h Post-Ex) (figure 18). All CPET data and specific blood sampling procedures were outlined in chapter 2.

Table 23. Physiological characteristics of the 10 male participants

Characteristic	**Mean**	**SD**
Age (years)	27.3	± 7.9
Height (cm)	180.0	± 0.1
Body Mass (kg)	74.4	± 11.2
BMI (kg/m^2)	22.8	± 2.0
Waist:hip ratio	0.8	± 0.03
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	56.2	± 6.4

SD (standard deviation); **BMI** (body mass index); **$\dot{V}O_{2peak}$** (highest oxygen consumption achieved in test)

RNA extraction

Total RNA was extracted using *mir*Vana™ miRNA Isolation Kit (Life Technologies™). Briefly, 1 mL of whole blood was lysed and the resulting pellet was processed using the column-based extraction in accordance with manufacturer's instructions. All RNA samples were quantified using a Qubit Fluorometer (ThermoFisher Scientific®).

RNA-seq - Library Preparation

The RNA library preparation was completed using the TruSeq Stranded Total RNA Sample Preparation Guide (Illumina®) in accordance with manufacturer's instructions. This kit was chosen as it has been widely published and it specifically depletes ribosomal RNA (rRNA) via biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal beads. The RNA-seq analysis was performed by NovoGene Bioinformatics Technology® – Hong Kong.

A graphical overview of the library preparation appears in figure 19. Briefly, 500ng of sample RNA was inputted and the pool of the desired RNA targets (mRNA in this case) was enriched by selection of polyadenylated molecules using magnetic beads. The larger RNA molecules were then fragmented into shorter strands of approximately 200 to 500 bps. The fragmented RNA strands were then primed with random hexamers and reverse transcribed into first strand cDNA using random primers and reverse transcriptase. The resulting cDNA strand contained a hairpin loop at the 3' end which served as a primer for the second strand synthesis. The library of double-stranded cDNA fragments was then phosphorylated and A-tailed in preparation for the ligation of specific adaptors with imbedded barcode indexes. The library of indexed reads was then amplified via PCR in preparation for clustering and sequencing.

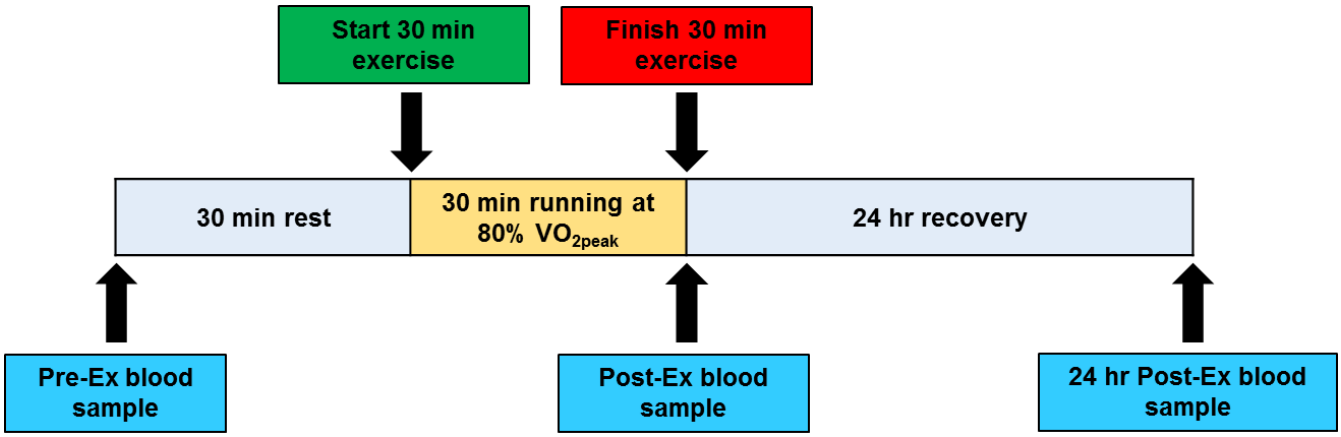


Figure 18. A schematic overview of participant blood sampling and exercise intervention. A baseline blood sample was taken 30 min before the onset of exercise. Participants then completed a 30 min bout of treadmill running at 80% of previously determined $\dot{V}O_{2peak}$. Additional blood samples were taken Post-Ex and 24 h Post-Ex.

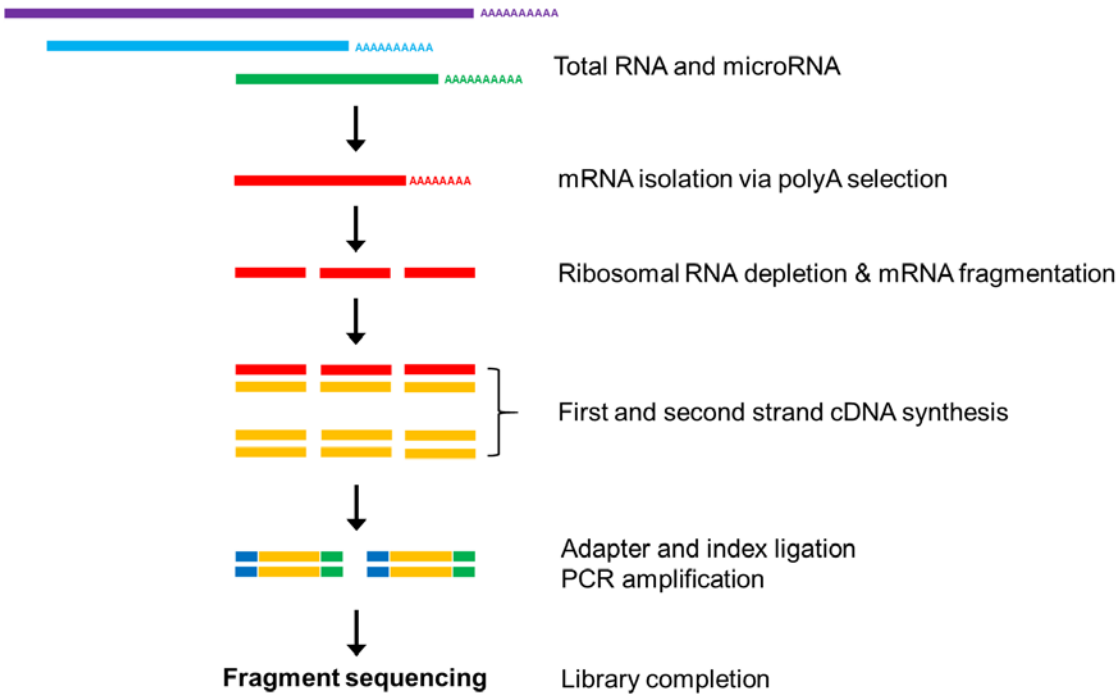


Figure 19. Overview of RNA-seq library construction. Enriched mRNA is fragmented before first and second strand cDNA synthesis is conducted. Adapters and indexes are ligated before PCR amplification is performed.

Sequencing

An analysis of mRNA, miRNA, lncRNA, and snoRNA abundance was performed on the indexed library using the HiSeq 150bp paired end reads (NovoGene Bioinformatics Technology® - Hong Kong).

Functional Analysis

Functional analysis of differentially expressed genes was conducted using the Database for Annotation, Visualization and Integrated Discovery (*DAVID*, version 6.8). The functional analysis was conducted on three different gene sets; (i) genes uniquely differentially expressed between Pre-Ex and Post-Ex time points, (ii) genes differentially expressed between Post-Ex and 24 h Post-Ex time points, and (iii) genes differentially expressed across both time points. Subsequent pathway analysis was performed to identify enriched upstream regulators, disease or biological functions, and biological pathways.

In Silico Binding Predictions

Reads were aligned and annotated using NCBI's latest human genome (GRCh38) with Rsubread version 1.28.1. Differentially expressed miRNAs were analysed for potential interaction with pro-telomeric elements using: TargetScan, microRNA.org, miRTarBase, RNA22-HAS, PicTar, Diana-Microtot, MirDB, and Target Miner.

Statistical Analysis

The libraries were then filtered, normalised, and paired-samples ANOVA carried out using edgeR (version 3.20.9.). The paired-samples ANOVA accounted for individual differences in gene expression at baseline. The ANOVA allowed investigation of differential expression across Pre-Ex, Post-Ex, and 24 h Post-Ex

time points simultaneously. All reported *P* values were adjusted to control the false discovery rate (FDR) ≤ 0.01 to reduce the likelihood of false positives. All analyses were adjusted for age, height, BMI, waist-hip ratio, hip circumference, blood pressure, and $\dot{V}O_{2peak}$.

Production of Heat Maps

The gene expression heat map representing the 182 differentially expressed genes across all time points (figure 21) was produced using the heatmap.2 function in gplots version 3.0.1 (R package). The values used were counts-per-million (CPM) gene expression values (adjusted by library size), which were scaled before plotting. The second heat map depicting interactions between non-coding RNAs and coding genes (figure 22) was produced using the clustered image map (CIM) function in mixOmics (R package). This function performed a canonical correlation analysis. Counts-per-million were scaled before being plotted.

Production of Circular Plot

Circular plots were generated using RCircos (version 1.2.0), as part of the R package.

5.5 Results

Differential Expression Analysis

All three time points were included for each participant except for one 24 h Post-Ex sample that did not pass quality control. Transcripts with a significant log2 fold change (logFC) (FDR ≤ 0.01) were reported as differentially expressed. A total of 182 genes were differentially expressed across Pre-Ex, Post-Ex, and 24 h Post-Ex time points (figure 20). A subset of 89 genes were uniquely expressed between

Pre-Ex to Post-Ex time points and 276 between Post-Ex to 24 h Post-Ex (FDR≤0.01).

Functional Analysis

A total of 178 of the 182 differentially expressed genes (FDR≤0.01) were matched via pathway analysis in *DAVID* (version 6.8). Six gene ontology (GO) terms were significantly enriched (FDR≤0.01) (table 24). The three most enriched terms were; stress response (FDR<0.001), chaperone protein (FDR<0.001), and inflammatory response (FDR<0.001).

Heat Map Analysis

Figure 21 depicts the 182 differentially regulated genes across all time points. Specific genes are represented in columns and individual participant time point samples are represented in each row. Red colouration depicts upregulation and blue depicts downregulation of the specific gene. The outer dendrograms cluster the genes according to similarity of differential expression. The heat map displays the clustering of genes/samples and highlights temporal similarities in differential expression. Figure 21 also highlights the considerable inter-individual variation in differentially expressed genes. An obvious clustering of differentially expressed genes around the Post-Ex time point and a significant reversal in expression by 24 h Post-Ex can be seen.

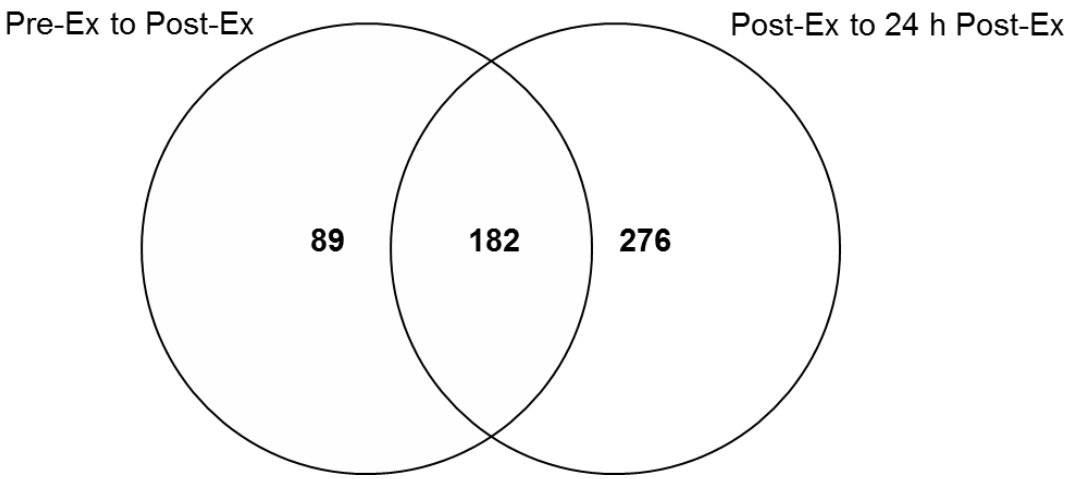


Figure 20. Overlapping gene expression between Pre-Ex to Post-Ex and Post-Ex to 24 h Post Ex time points. A subset of 89 genes were uniquely expressed between Pre-Ex to Post-Ex time points and 276 between Post-Ex to 24 h Post-Ex. A total of 182 genes were differentially regulated across both Pre-Ex to Post-Ex and Post-Ex to 24 h Post-Ex time points (FDR≤0.01).

Table 24. Significantly enriched gene ontology (GO) terms.

Enriched GO Term	Listed Genes	FDR
Stress response	HSPH1, HSP90AA1, DUSP1, HSPE1, HSPA1A, HSPA1B, DNAJB1, DNAJB4, PPP1R15A, AHSA1, HSPA8	0.000
Chaperone	TCP1, HSP90AA1, FKBP4, HSPA1A, HSPA1B, DNAJC30, DNAJA1, HSPE1, DNAJB1, DNAJB4, AHSA1, HSPA8, DNAJB6	0.000
Inflammatory response	PTGER2, IL18RAP, ANXA1, CHST2, CCL4, CD180, FOS, TNFAIP6, KLRG1, CXCR4, CCR2, RIPK2, VNN1, NFE2L2, TNFAIP3, THEMIS2, F2R	0.000
Unfolded protein binding	TCP1, HSP90AA1, DNAJA1, HSPE1, HSPA1A, HSPA1B, DNAJB1, DNAJB4, DNAJB6, HSPA8	0.000
Response to unfolded protein	HSPH1, HSP90AA1, DNAJA1, HSPE1, DNAJB1, DNAJB4, HSPA8	0.004
Regulation of cellular response to heat	HSPH1, HSP90AA1, FKBP4, HSPA1A, HSPA1B, DNAJB1, DNAJB6, HSPA8	0.010

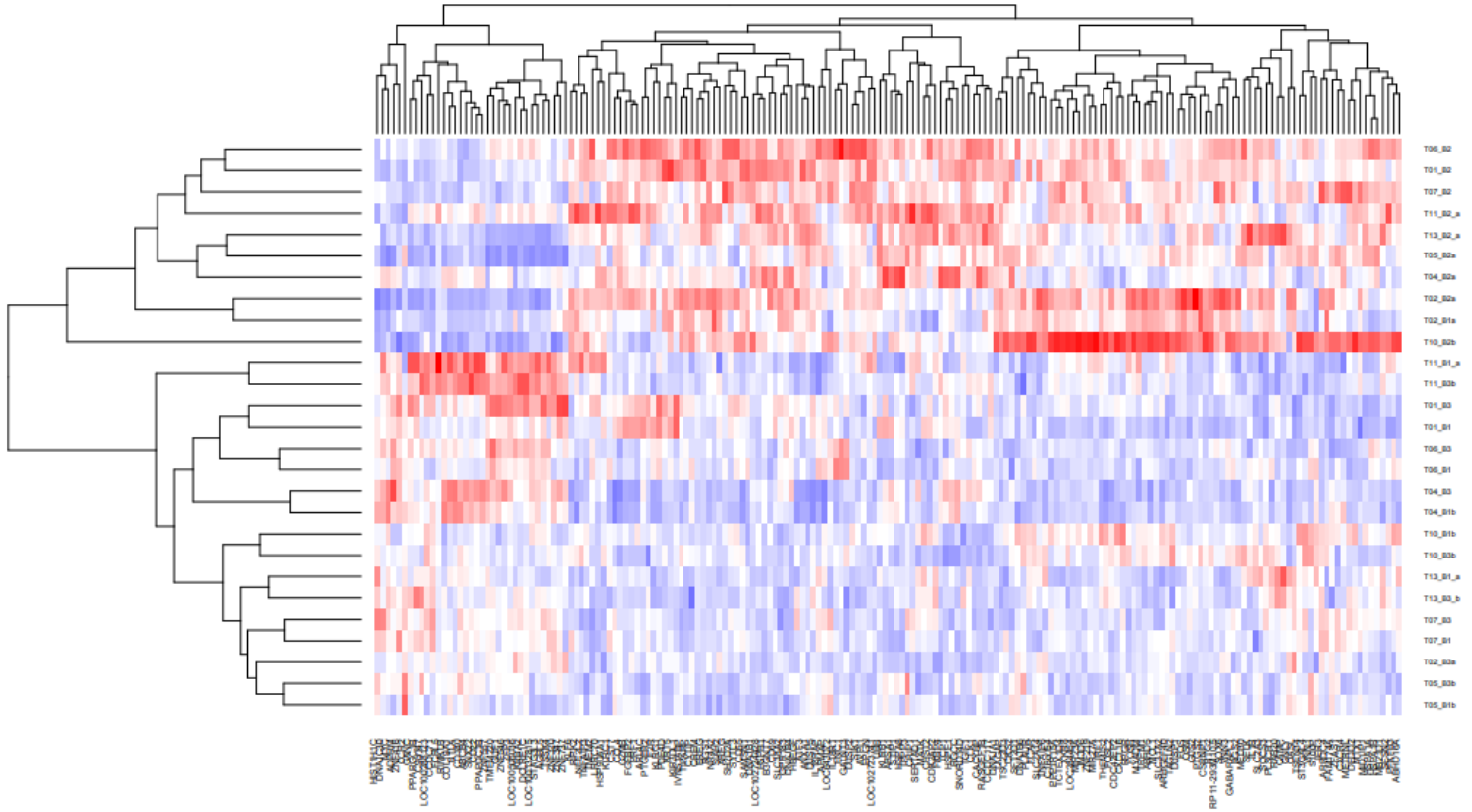


Figure 21. A heat map of 182 differentially expressed genes across Pre-Ex, Post-Ex, and 24 h Post-Ex time points. Columns represent data from specific genes; each row represents an individual participant time point sample. The outermost dendrogram cluster the genes according to similarity in expression changes. The plotted values represent scaled counts-per-million (CPM) gene expression values (adjusted by library size).

Stress Response Genes

Gene ontology analysis using *DAVID* (version 6.8) identified 11 of the differentially regulated genes (FDR≤0.01) to be associated with stress response pathways (table 25). Most of the differentially regulated genes belonged to the heat shock protein (HSP) family. HSP70 family representatives *HSPA1A* (FDR<0.001), *HSPA8* (FDR<0.001), and *HSPA1B* (FDR<0.001) were differentially expressed (figure 22). Within the HSP90 family, *HSP90AA1* (FDR<0.001), *HSPE1* (FDR<0.001) and *HSP90AB1* (FDR=0.027, not significant at ≤0.01 level) were differentially expressed (figure 23). Most of the significantly regulated genes demonstrated Pre-Ex to Post-Ex upregulation followed by Post-Ex to 24 h Post-Ex downregulation.

Chaperone Genes

Thirteen of the differentially regulated genes (FDR≤0.01) were associated with chaperone pathways (table 26). Many of these genes were duplicated in the stress response gene list, showing a similar Pre-Ex to Post-Ex upregulation followed by downregulation Post-Ex to 24 h Post-Ex.

Genes involved with inflammation

Seventeen genes were associated with inflammatory processes (FDR≤0.01) (table 27). Most of the differentially regulated genes demonstrated Pre-Ex to Post-Ex upregulation and subsequent Post-Ex to 24 h Post-Ex downregulation.

Table 25. Differentially regulated stress response genes.

Gene Symbol	Gene Name	Log2 Fold Change (FC) Pre-Ex to Post-Ex	Post-Ex to 24 h Post-Ex	Pre-Ex to 24 h Post-Ex	FDR
HSPH1	Heat shock protein family H (Hsp110) member 1	0.7	-0.7	0.0	0.000
HSP90AA1	Heat shock protein 90 alpha family class A member 1	0.4	-0.6	-0.2	0.005
DUSP1	Dual specificity phosphatase 1	0.9	-0.9	0.0	0.000
HSPE1	Heat shock protein family E (Hsp10) member 1	0.4	-0.6	-0.2	0.004
HSPA1A	Heat shock protein family A (Hsp70) member 1A	0.7	-0.6	0.0	0.000
HSPA1B	Heat shock protein family A (Hsp70) member 1B	0.9	-0.9	0.0	0.000
DNAJB1	DnaJ heat shock protein family (Hsp40) member B1	0.9	-0.9	0.0	0.000
DNAJB4	DnaJ heat shock protein family (Hsp40) member B4	0.5	-0.6	0.0	0.005
PPP1R15A	Protein phosphatase 1 regulatory subunit 15A	0.7	-0.6	0.1	0.002
AHSA1	Activator of HSP90 ATPase activity 1	0.3	-0.4	-0.1	0.001
HSPA8	Heat shock protein family A (Hsp70) member 8	0.5	-0.4	0.0	0.000

Table 26. Differentially regulated chaperone genes.

Gene Symbol	Gene Name	Log2 Fold Change (FC) Pre-Ex to Post-Ex	Post-Ex to 24 h Post-Ex	Pre-Ex to 24 h Post-Ex	FDR
TCP1	T-complex 1	0.2	-0.3	0.0	0.006
HSP90AA1	Heat shock protein 90 alpha family class A member 1	0.4	-0.6	-0.2	0.005
FKBP4	FK506 binding protein 4	0.4	-0.4	-0.1	0.000
HSPA1A	Heat shock protein family A (Hsp70) member 1A	0.7	-0.6	0.0	0.000
HSPA1B	Heat shock protein family A (Hsp70) member 1B	0.9	-0.9	0.0	0.000
DNAJC30	DnaJ heat shock protein family (Hsp40) member C30	-0.4	0.5	0.1	0.007
DNAJA1	DnaJ heat shock protein family (Hsp40) member A1	0.6	-0.7	-0.1	0.000
HSPE1	Heat shock protein family E (Hsp10) member 1	0.4	-0.6	-0.2	0.004
DNAJB1	DnaJ heat shock protein family (Hsp40) member B1	0.9	-0.9	0.0	0.000
DNAJB4	DnaJ heat shock protein family (Hsp40) member B4	0.5	-0.6	0.0	0.005
AHSA1	Activator of HSP90 ATPase activity 1	0.3	-0.4	-0.1	0.001
HSPA8	Heat shock protein family A (Hsp70) member 8	0.5	-0.4	0.0	0.000
DNAJB6	DnaJ heat shock protein family (Hsp40) member B6	0.2	-0.2	0.0	0.003

Table 27. Differentially regulated genes involved in inflammation. **Log2 Fold Change (FC)**

Gene Symbol	Gene Name	Pre-Ex to Post-Ex	Post-Ex to 24 h Post-Ex	Pre-Ex to 24 h Post-Ex	FDR
PTGER2	Prostaglandin E receptor 2	0.3	-0.3	-0.1	0.004
IL18RAP	Interleukin 18 receptor accessory protein	0.4	-0.6	-0.2	0.000
ANXA1	Annexin A1	0.5	-0.6	-0.1	0.000
CHST2	Carbohydrate sulfotransferase 2	0.4	-0.6	-0.1	0.010
CCL4	C-C motif chemokine ligand 4	0.7	-0.9	-0.2	0.000
CD180	CD180 molecule	-0.5	0.7	0.2	0.000
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	1.4	-1.3	0.1	0.001
TNFAIP6	TNF alpha induced protein 6	0.3	-0.5	-0.2	0.007
KLRG1	Killer cell lectin like receptor G1	0.3	-0.4	-0.1	0.002

Table 27. continued

Gene Symbol	Gene Name	Log2 Fold Change (FC) Pre-Ex to Post-Ex	Post-Ex to 24 h Post-Ex	Pre-Ex to 24 h Post-Ex	FDR
CXCR4	C-X-C motif chemokine receptor 4	0.3	-0.3	-0.1	0.007
CCR2	C-C motif chemokine receptor 2	-0.3	0.4	0.1	0.007
RIPK2	Receptor interacting serine/threonine kinase 2	0.2	-0.3	-0.1	0.007
VNN1	Vanin 1	0.2	-0.3	-0.1	0.007
NFE2L2	Nuclear factor, erythroid 2 like 2	0.2	-0.3	-0.1	0.004
TNFAIP3	TNF alpha induced protein 3	0.5	-0.6	-0.1	0.000
THEMIS2	Thymocyte selection associated family member 2	0.2	-0.3	0.0	0.008
F2R	Coagulation factor II thrombin receptor	0.4	-0.6	-0.1	0.008

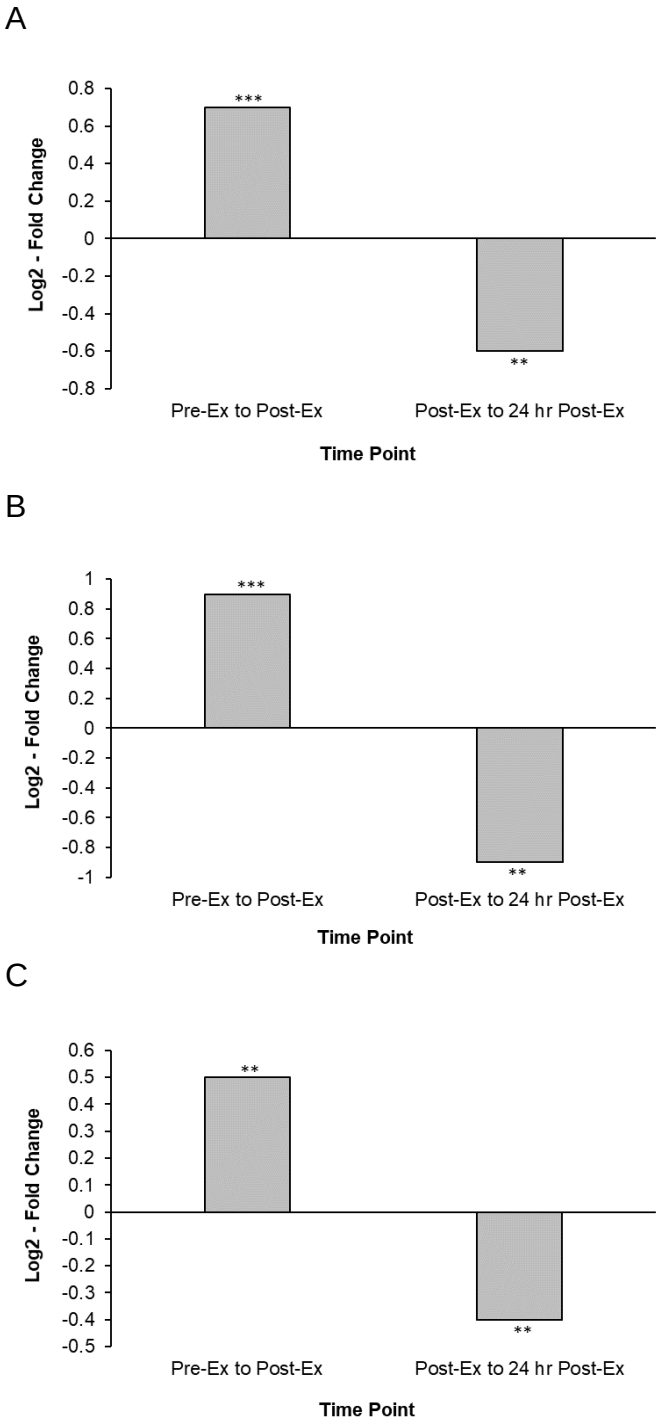
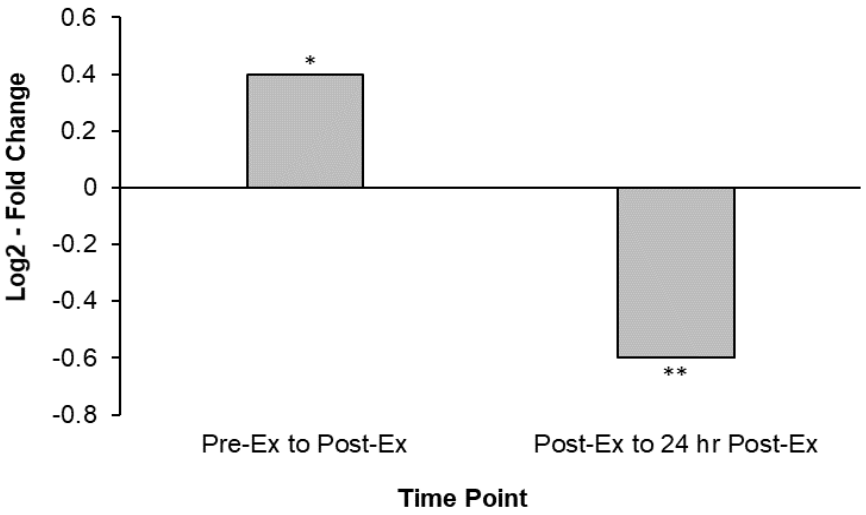


Figure 22. Differential regulation of selected HSP70 representatives in unsorted leukocytes: Differential transcript expression occurred in leukocytes (n=10) Pre-Ex to Post-Ex and Post-Ex to 24 h Post-Ex for *HSPA1A* mRNA **(A)**, *HSPA1B* mRNA **(B)**, and *HSPA8* mRNA **(C)**. ** indicates FDR<0.01.

A.



B.

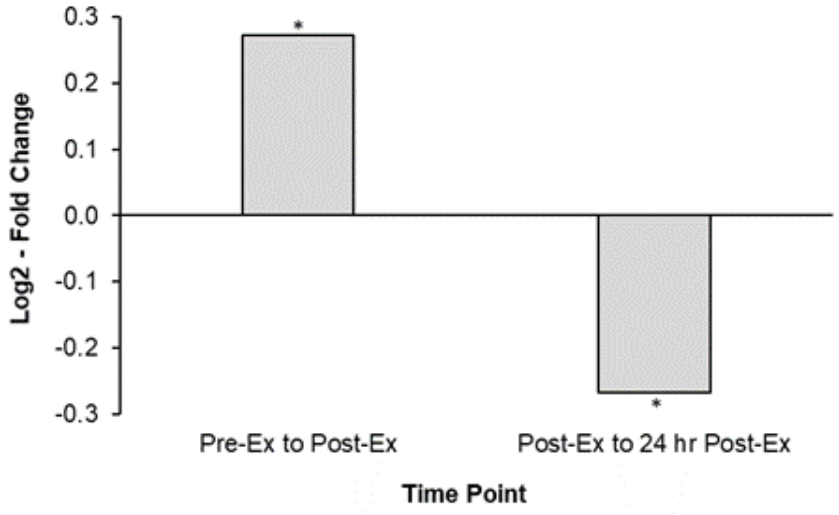


Figure 23. Differential regulation of selected HSP90 representatives: Transcript expression was assessed in leukocytes (n=10). Significant differential expression was observed from Pre-Ex to Post-Ex and from Post-Ex to 24 h Post-Ex for *HSP90AA1* mRNA **(A)** and *HSP90AB1* mRNA **(B).** * indicates FDR<0.05 and ** indicates FDR<0.01.

Non-coding RNAs

A total of 12 non-coding RNAs were detected in the RNA-seq data (table 28). Network analysis identified considerable interaction between the 12 non-coding RNAs and the differentially expressed protein-coding genes (figure 24). The heat map in figure 24 depicts the interactions between the 12 differentially expressed non-coding RNAs and differentially expressed coding-genes. The specific coding-genes are represented in columns and the non-coding genes are represented in rows. The outer dendrograms cluster the genes according to similarity in differential expression. The heat map identifies considerable interaction between non-coding RNAs (SNORD14D, LOC102723741, LOC643072, RP11-293M10.5, ZFAS1, LOC284454, miR-23a, and miR-27a) and a large subset of differentially expressed coding-genes. The above mentioned non-coding RNAs share a very similar interaction profile with the coding-genes (figure 24).

In silico analysis identified miR-23a and miR-27a as having potential interaction with telomere-associated genes (table 29). Expression of miR-23a increased 2.6-fold Pre-Ex to Post-Ex and decreased 3-fold Post-Ex to 24 h Post-Ex (FDR<0.001) (figure 25). Expression of miR-27a increased 2.1-fold Pre-Ex to Post-Ex and decreased 3-fold Post-Ex to 24 h Post-Ex (FDR=0.004) in 10 healthy males.

Circular Plot Analysis

The circular plots in figures 26 and 27 depict the interactions between miR-23a, miR-27a, and the differentially regulated coding-genes. The links between the miRNA (in red) and the genes represent interactions. All genes are ordered by chromosome number noted on the outside of the circular plot. The outermost concentric histogram

ring displays the log2 fold change in gene expression between Pre-Ex and Post-Ex time points (red bars indicate upregulation, blue indicates downregulation).

Both miR-23a (figure 26) and miR-27a (figure 27) interact with the same 46 differentially expressed coding-genes. An obvious clustering of upregulated genes can be seen in the Pre-Ex and Post-Ex histogram ring whilst the Post-Ex to 24 h Post-Ex histogram ring shows a commensurate downregulation during the 24 hours after exercise.

5.6 Discussion

In the present study, next generation RNA-seq was used to identify acute exercise-responsive leukocyte RNA transcripts with potential telomeric involvement. To the author's knowledge, there are no published studies that have attempted this. No significant differential expression of primary telomeric genes (*hTERT*, *hTER* or shelterin components) was observed within the assessed time course. Several genes associated with stress, inflammatory, and chaperone responses (namely heat shock proteins) were differentially regulated. It is now established that heat shock proteins (HSPs) play a significant role in telomere biology (Forsythe et al., 2001; Holt et al., 1999; Hunt et al., 2004; Pandita, 2005; Toogun , DeZwaan, & Freeman, 2008). Additionally, 12 non-coding RNAs were differentially expressed, two of which were predicted to have putative telomeric involvement. The exercise-induced regulation of these transcripts may represent an acute telomeric response to exercise.

Table 28. Differentially regulated non-coding RNAs.

miR Symbol	miR Name	Chromosome	Log2 Fold Change (FC) Pre-Ex to Post-Ex	Post-Ex to 24h Post-Ex	Pre-Ex to 24h Post-Ex	FDR
STAG3L3	Stromal antigen 3-like 3 (pseudogene)	7	-0.3	0.4	0.1	0.008
LOC100132815	Importin 5 pseudogene 1	19	-0.3	0.3	0.0	0.008
LOC100506036	Uncharacterised	2	-0.5	0.8	0.3	0.007
LOC100288123	Uncharacterized	19	-0.3	0.6	0.3	0.006
SNORD14D	Small nucleolar RNA, C/D box 14D	11	0.7	-0.6	0.1	0.001
LOC102723741	Uncharacterised	5	0.4	-0.6	-0.2	0.002
LOC643072	Uncharacterized	2	0.4	-0.4	0.0	0.005

Table 28. continued

miR Symbol	miR Name	Chromosome	Log2 Fold Change (FC) Pre-Ex to Post-Ex	Post-Ex to 24h Post-Ex	Pre-Ex to 24h Post-Ex	FDR
RP11-293M10.5	Long intergenic non-protein coding RNA 1220	14	0.8	-0.8	0.0	0.007
ZFAS1	ZNFX1 antisense RNA 1	20	0.3	-0.4	-0.1	0.004
MIR23A	MicroRNA 23a	19	1.4	-1.6	-0.2	0.000
MIR27A	MicroRNA 27a	19	1.1	-1.6	-0.5	0.004
LOC284454	Uncharacterised	19	1.2	-1.4	-0.2	0.000

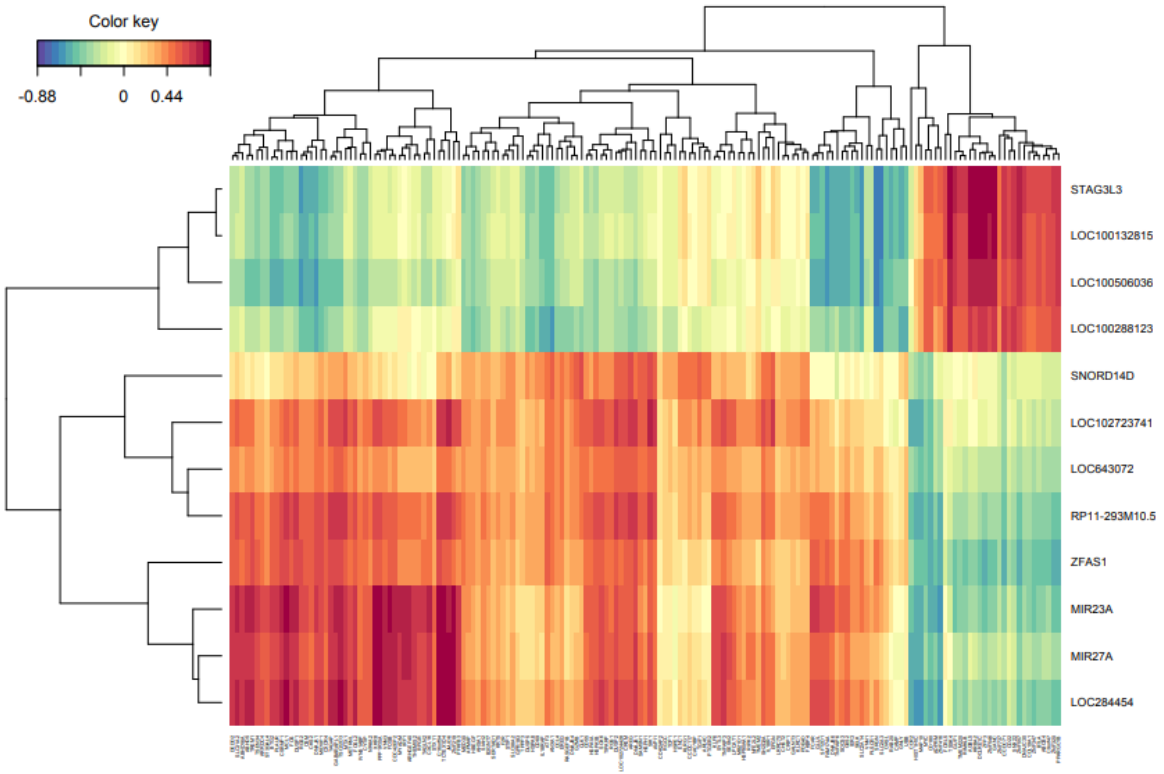


Figure 24. A heat map representing 12 non-coding RNAs and their interactions with differentially expressed coding-genes. Columns represent specific genes and rows represent specific non-coding RNAs. Expressed values represent counts-per-million (CPM) which were scaled before being plotted. The outermost dendrogram clusters genes according to similarity in expression changes.

Table 29. *In silico* binding predictions and target transcripts for miR-23a and miR-27a.

miRNA	Potential gene target	Target mRNA symbol	Prediction database
miR-23a	Telomeric repeat-binding factor 2	*TRF2*	TargetScan (version 7.1)
			microRNA.org (2010 release)
			miRTarBase (version 7.0)
			RNA22-HSA (version 2.0)
	Telomeric repeat binding factor 2, interacting protein	*TERF2IP*	TargetScan (version 7.1)
	Regulator of telomere elongation helicase	*RTEL1*	TargetScan (version 7.1)
	Protection of telomere	*POT1*	TargetScan (version 7.1)
miR-27a	Telomeric repeat-binding factor 2, interacting protein	*TERF2IP*	miRTarBase (version 7.0)
	Protection of telomere	*POT1*	TargetScan (version 7.1)
	PIN2/TERF1 interacting, telomerase inhibitor 1	*PINX1*	TargetScan (version 7.1)

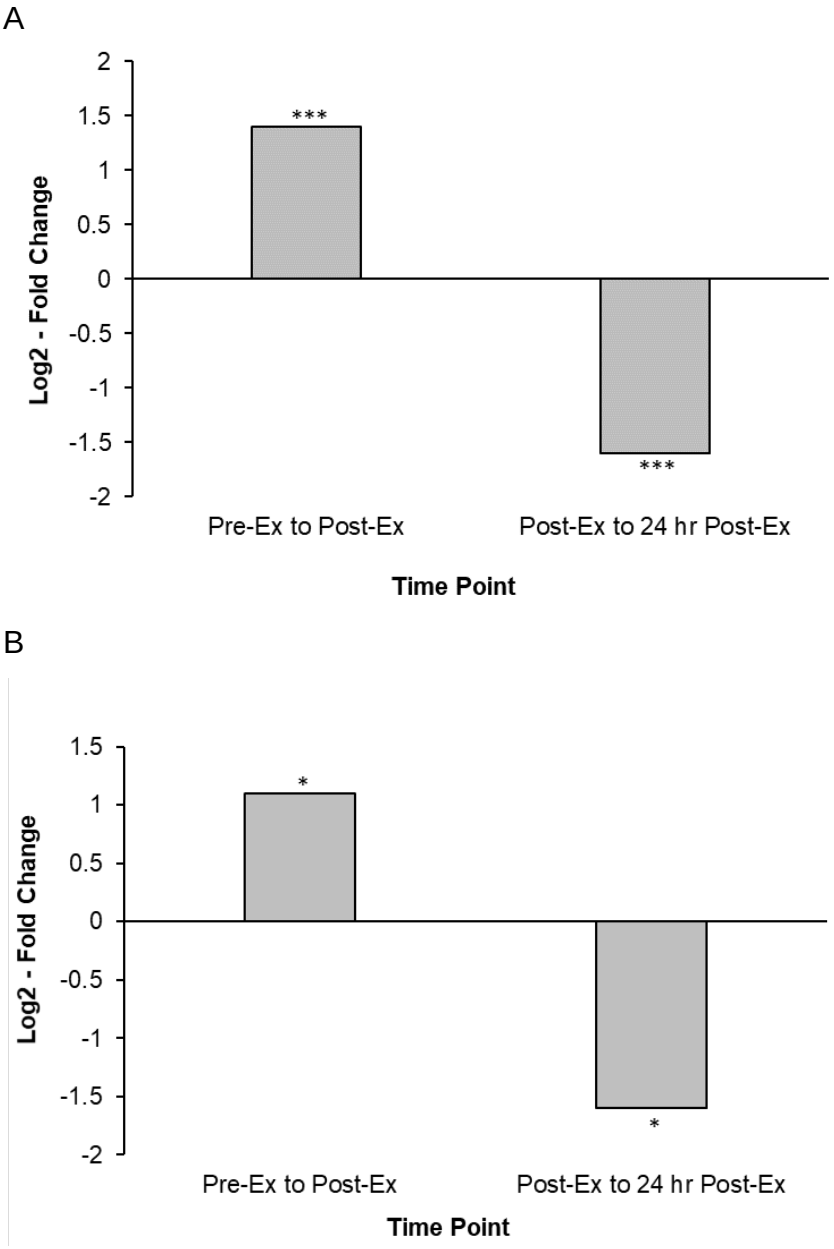


Figure 25. Differential regulation of miRNAs in unsorted leukocytes: Transcript expression was assessed in leukocytes (n=10). Significant differential expression was observed from Pre-Ex to Post-Ex and from Post-Ex to 24 h Post-Ex for miR-23a **(A)** and miR-27a **(B).** * indicates FDR<0.05, and *** indicates FDR<0.01.

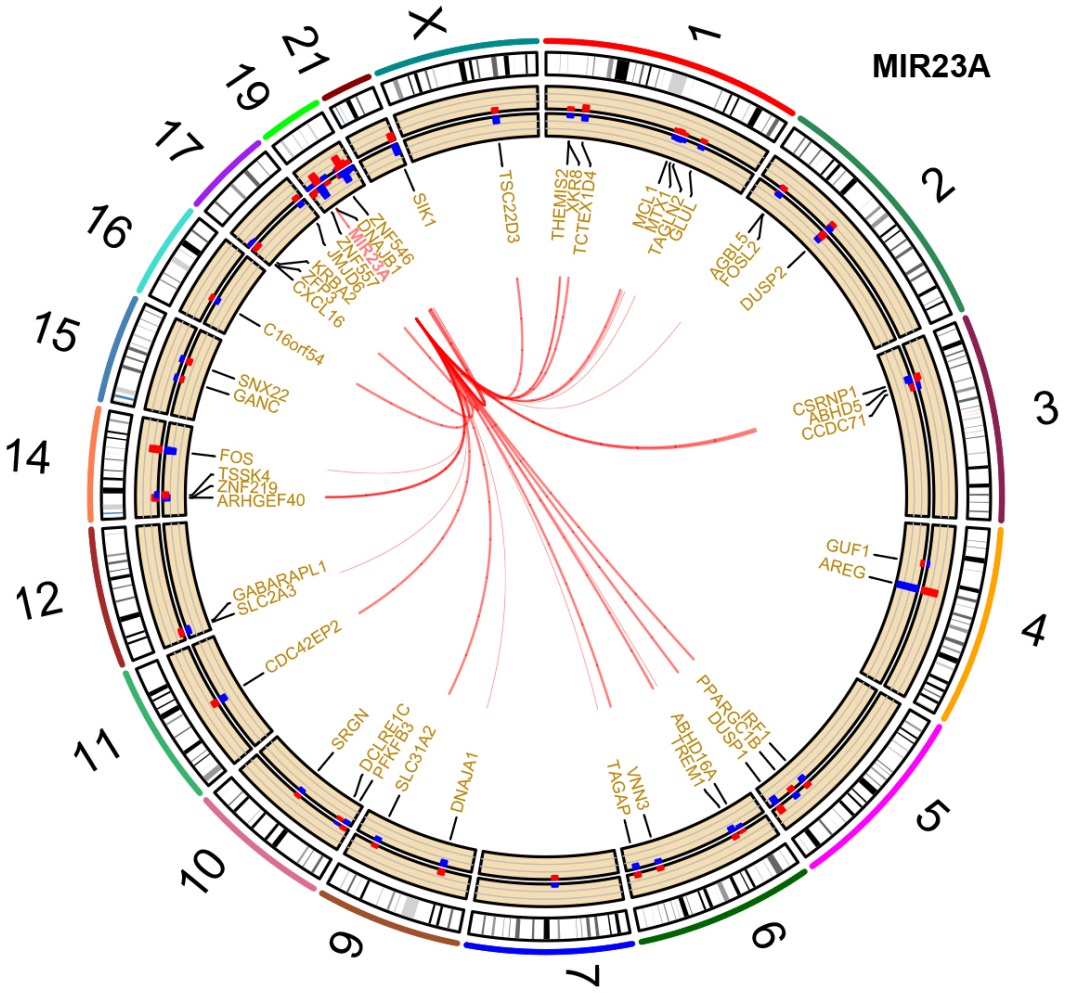


Figure 26. A circular plot representing the interactions between miR-23a and 46 differentially expressed protein-coding genes. Links between miR-23a and genes represent interactions. The outermost concentric histogram ring displays the log2 fold change in gene expression between Pre-Ex and Post-Ex time points (red bars indicate upregulation, blue indicates downregulation). The innermost histogram ring displays the log2 fold change in gene expression between Post-Ex and 24 h Post-Ex time points.

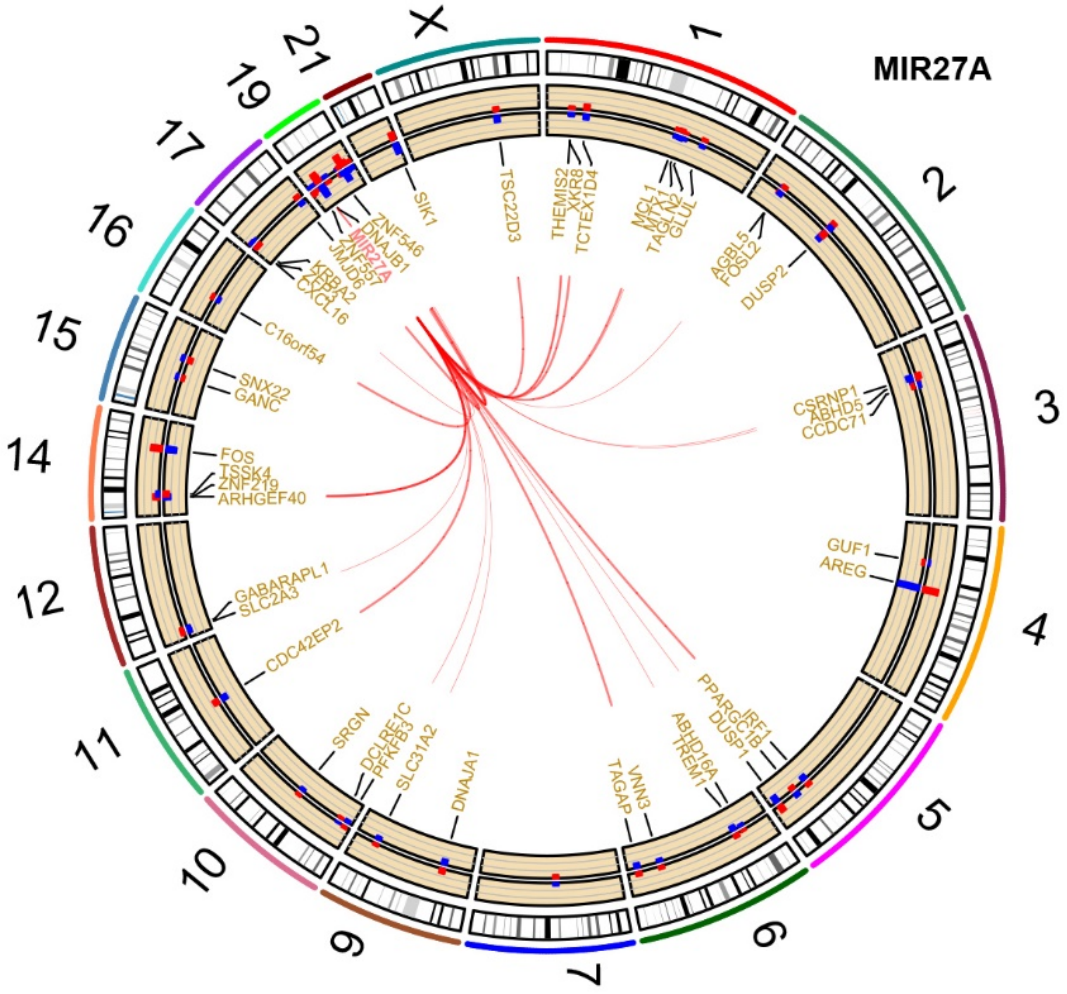


Figure 27. A circular plot representing the interactions between miR-27a and 46 differentially expressed protein-coding genes. Links between miR-27a and genes represent interactions. The outermost concentric histogram ring displays the log2 fold change in gene expression between Pre-Ex and Post-Ex time points (red bars indicate upregulation, blue indicates downregulation). The innermost histogram ring displays the log2 fold change in gene expression between Post-Ex and 24 h Post-Ex time points.

Most of the significantly regulated transcripts were upregulated between Pre-Ex and Post-Ex and downregulated to resting levels between Post-Ex and 24 h Post-Ex. These findings are broadly concurrent with earlier microarray studies that demonstrated acute upregulation of stress and inflammatory genes and a return to baseline within 1 hour of cessation (Büttner et al., 2007; Connolly et al., 2004). Acute exercise typically initiates a sequela of inflammatory events specific to the mode, duration and intensity of the exercise bout. High intensity exercise transiently modulates the production of metabolic enzymes, interleukins, HSPs and metabolic enzymes (Buchheit & Laursen, 2013; Gjevestad et al., 2015; Rutkowski, Pancewicz, Skrzydlewska, & Hermanowska-Szpakowicz, 2005; Szołtysek, Janus, & Widłak, 2011).

The stress response is an adaptive protective mechanism that strives to achieve allostasis in response to environmental stimuli such as oxidative damage, hypoxia, and heat shock (Kültz, 2005). A principal component of the stress response is the modulation of HSPs. Increases in intramuscular temperature activate expression of HSPs (Fehrenbach & Niess, 1999; Fehrenbach & Northoff, 2001). The ubiquitous family of HSPs facilitate polypeptide chain folding, protein transport, assembly of multi-protein complexes, and the prevention of protein aggregation (Hartl, 1996; Saibil, 2013). HSPs are also critical for cell structure maintenance; exerting influence over the formation and function of cytoskeleton elements (Liang & MacRae, 1997). The HSP family also plays diverse and critical roles within the immune system including pro-inflammatory cytokine release, protection from DNA damage, direct activation of NK cells, and stimulation of both adaptive and innate immune responses (Simon et al., 2006).

Significant exercise-induced expression of *HSPA1A*, *HSPA1B,* and *HSPA8* mRNA was observed in the present study. Exercise-induced elevation of HSP70 family is known to be intensity dependent (Milne & Noble, 2002). The HSPA1A isoform is highly responsive to cellular stress and can be triggered by a host of exercise-induced cellular stressors including oxidative stress/free radical formation, hypoxia/ischemia, increased calcium concentration, temperature alterations, glucose depletion, and altered pH (Kregel, 2002). A single bout of exercise has been shown to acutely upregulate *HSP70* mRNA in skeletal muscle (Febbraio & Koukoulas, 2000; Khassaf et al., 2001; Puntschart, Vogt, Widmer, Hoppeler, & Billeter, 1996).

The HSP70 family has been implicated in genomic instability and telomere biology. Cells with inactivated Hsp70 protein demonstrate spontaneous chromosomal aberrations and telomere instability in mouse bone marrow cells and embryonic cells (Hunt et al., 2004). Additionally, Hsp70 interacts with the catalytic unit of telomerase (TERT) (Forsythe et al., 2001). Inactivation of HspA1A affects telomerase activity and telomere length in a near identical fashion to TERT inactivation (Hunt et al., 2004). Whilst upregulation of *TERT* mRNA was not detected in this study, it was detected via qPCR immediately post- and 60 min post-exercise in chapter 4 of this thesis. The discrepant findings may be due to the different time courses and/or measurement techniques used. Additionally, the smaller sample size may have underpowered the present study.

It is biologically plausible that the acute exercise-responsiveness of *HSPA1A*, *HSPA8,* and *HSPA1B* mRNA may represent a transient pro-telomeric response. The increased expression may represent increased demand for Hsp70 protein to maintain genomic stability in an environment of acute metabolic and oxidative

stress. Given the sensitivity of telomeric DNA to oxidative damage, the observed increase in HSP mRNA may represent an attempt to fortify telomeric DNA against increases in ROS. The HSP response is sensitive to ROS (Dimauro, Mercatelli, & Caporossi, 2016) and upregulation of Hsp70 is known to form part of the adaptive response to oxidative stress (Milne & Noble, 2002; Puntschart et al., 1996; Salo, Donovan, & Davies, 1991; Skidmore, Gutierrez, V. Guerriero, & Kregel, 1995; Suzuki et al., 2006). The ROS-induced HSP response has been observed in leukocytes after half marathon and marathon running (Fehrenbach et al., 2000), treadmill running (Niess et al., 2002; Simar, Malatesta, Badiou, Dupuy, & Caillaud, 2007; Simar et al., 2004), and cycle ergometry (Brunelli et al., 2012; Lengacher et al., 2014). Whilst oxidative stress was not measured in the present study, it is plausible that the Post-Ex increase in HSPs may have been due to exercise-induced ROS.

The HSP increase may also reflect HSP70's putative role in telomerase stabilization. Hsp70 and telomerase are both expressed constitutively in autonomous cells yet only transiently in non-autonomous cells (Barker et al., 2002). Whilst Hsp70 is not rate-limiting for telomerase activity, it appears to be required for stability of the telomerase complex (Pandita, 2005). Exercise-induced mobilization of lymphocytes with shorter telomeres (Bruunsgaard et al., 1999) and senescent phenotypes (Simpson et al., 2010; Simpson et al., 2007) into the periphery may be accompanied by a concomitant increase in genome stabilizing HSP70 transcripts and proteins.

A similar expression profile was observed in heat shock protein 90 (HSP90) family representatives *HSP90AA1* and *HSP90AB1* (FDR = 0.027 – *HSP90AB1* not significant at FDR ≤ 0.01). Hsp90 is a highly conserved eukaryotic protein that

mediates a host of cellular processes including hormone signalling, cell cycle control, cell survival, and cellular stress response (Borkovich, Farrelly, Finkelstein, Taulien, & Lindquist, 1989; Richter, Haslbeck, & Buchner, 2010; Wandinger, Richter, & Buchner, 2008; Young, Moarefi, & Hartl, 2001; Zhao et al., 2005). Molecular chaperones Hsp70, Hsp90, and p23 facilitate protein-DNA dynamics within processes such as RNA transcription, DNA repair, and DNA replication (Hager, McNally, & Misteli, 2009; Konieczny & Zylicz, 1999; Richter, Hendershot, & Freeman, 2007).

The Hsp90/telomere connection was initially established after the observation that chaperones Hsp90, Hsp40, Hsp-organising protein (HOP), and p23 directly associated with the holoenzyme telomerase (Forsythe et al., 2001; Holt et al., 1999). The putative connection was reinforced by the observation that pharmacological inhibition of Hsp90 shortened telomeres by approximately 2.2kb over a 2-month period (Compton, Elmore, Haydu, Jackson-Cook, & Holt, 2006). Additionally, impairment of Hsp90 was shown to decrease telomerase activity (Toogun et al., 2008).

It was initially thought that the Hsp90 machinery aided assemblage of the reverse transcriptase telomerase subunits and RNA template (Forsythe et al., 2001; Holt et al., 1999). More recent findings suggest that Hsp90 facilitates DNA binding via telomerase (Keppler, Grady, & Jarstfer, 2006; Toogun et al., 2008). HSP90 is required for *in vitro* and *in vivo* telomerase assembly (Holt et al., 1999); deactivation of Hsp90 results in a decrease in *hTERT* expression (Kim & Jobin, 2005). Paradoxically, both overexpression and impairment of Hsp90 can elicit decreases in telomeric DNA length (Grandin, Damon, & Charbonneau, 2001; Toogun et al., 2008). HSP90 has also been shown to associate with the hTERT

promoter complex, enhancing promoter activity and *hTERT* expression in telomerase positive oral cancer cell lines (Kim, Kim, et al., 2008).

It has been proposed that the HSP90 network modulates the DNA-bound state of telomeric proteins thereby facilitating a shift between functional states (DeZwaan & Freeman, 2010). Telomere homeostasis is a dynamic process requiring transition between different conformations, appropriate shelterin assemblage, and telomerase repositioning during DNA extension (DeZwaan & Freeman, 2008). Within yeast, Hsp82 (the alias for yeast Hsp90) displaces bound proteins, primarily cell division cycle 13 (Cdc13), at the 3' DNA end effectively establishing an extendable state. This in turn allows telomerase access to telomeric DNA and subsequent extension (DeZwaan, Toogun, Echtenkamp, & Freeman, 2009). Hsp82 also appears to mitigate competitive binding at the single-stranded G-rich DNA binding proteins (DeZwaan & Freeman, 2008; DeZwaan et al., 2009). Such competitive binding could prevent DNA extension by telomerase.

The Pre-Ex to Post-Ex increase in *HSP90AA1* and *HSP90AB1* mRNAs in the present study may reflect an acute change in telomeric conformational state. This change may allow telomerase, DNA repair enzymes, pro-telomeric proteins or associated helicases access to the telomeric DNA. Alternatively, the increased transcript expression of *HSP90AA1* and *HSP90AB1* may be due to an exercise-induced shift in the relative distribution of immune cell subsets. As previously stated, exercise acutely redistributes leukocytes with shortened telomeres (Bruunsgaard et al., 1999) and senescent phenotypes (Simpson et al., 2010; Simpson et al., 2007) into the periphery. Cells containing shorter telomeres may express higher levels of *HSP90AA1* and *HSP90AB1* mRNAs given that telomerase

preferentially lengthens the shortest telomeres each cell cycle (Teixeira et al., 2004).

The understanding of epigenetic modifications in telomere regulation is in its infancy. At present there are only a small number of putative associations between miRNAs and telomere biology (Bonifacio & Jarstfer, 2010; Chakrabarti et al., 2013; Chen et al., 2014; Hrdličková et al., 2014; Kasiappan et al., 2012; Lassmann et al., 2015; Mitomo et al., 2008; Slattery et al., 2016; Wang, Sun, et al., 2012; Watanabe et al., 2011). There is however, a growing body of evidence indicating a role for miR-23a in telomere regulation via an interaction with the 3' untranslated region (3' UTR) of telomeric repeat binding factor 2 (TRF2) (Luo et al., 2015). This interaction reduces telomere-bound Trf2 and increases telomere dysfunction-induced foci (Luo et al., 2015). Trf2 is a double-stranded DNA binding protein that forms part of the shelterin complex and is essential for telomere end protection and T-loop formation (Griffith, Bianchi, & de Lange, 1998; Takai et al., 2003; van Steensel, Smogorzewska, & de Lange, 1998). Trf2 also prevents telomeric DNA being falsely recognized as double-strand breaks via repression of ataxia-telangiectasia-mutated (ATM) signalling (Denchi & de Lange, 2007; Karlseder, Broccoli, Dai, Hardy, & de Lange, 1999).

In vitro findings demonstrate that miR-23a increases oxidative stress-induced apoptosis and subsequently induces cell senescence in retinal pigment epithelium cells (Li et al., 2016). Inhibition of *TRF2* elicits telomere dysfunction and subsequently promotes cellular senescence in human fibroblast cells (Takai, Hooper, Blackwood, Gandhi, & de Lange, 2010). More recent findings indicate that miR-23a also strongly predicts cardiovascular events, particularly coronary

atherosclerosis, via *TRF2* downregulation (Satoh et al., 2017). These results strongly imply a role for miR-23a in telomere homeostasis and cellular senescence.

Exercise-induced upregulation of miR-23a has been observed in mouse skeletal muscle after resistance exercise (Wang et al., 2017) and down regulation in human skeletal muscle after both resistance exercise (D'Souza et al., 2017; Ringholm et al., 2011) and aerobic exercise (Russell et al., 2013). At present, no published studies have reported significant regulation of miR-23a in human leukocytes in response to aerobic exercise.

In the present study, miR-23a increased 2.6-fold Post-Ex before decreasing 3-fold over the following 24 hours. Whilst the present study was not designed to characterize the physiological rationale for differential miR-23a expression, a plausible (yet speculative) model presents itself. Trf1 and Trf2 protein are both considered negative regulators of telomere length (Smogorzewska et al., 2000). The interaction involves Trf2 in the formation of telomeric T-loops (Stansel, de Lange, & Griffith, 2001), the lariat-like structures formed when the 3' single-stranded telomeric overhand folds back upon itself invading the duplex component of the telomeric tract. The t loop segregates the telomeric end, protecting it from DNA repair enzymes and cellular checkpoint proteins (Griffith et al., 1999). It is widely believed that telomerase requires an accessible 3' overhang to bind to and extend telomeric DNA (Lee & Blackburn, 1993; Lingner & Cech, 1996; Wang & Blackburn, 1997), and is therefore unable to act upon the telomere in its closed conformation. Increased expression of miR-23a may be a critical trigger in changing the conformational state of the telomere. The down regulation of Trf2 may allow access to DNA repair mechanisms in the case of oxidative damage or telomerase-mediated extension. No differential regulation of *TRF2* mRNA was

detected in the present study. It is worth noting the transcriptional time course of *TRF2* mRNA may differ from that of miR-23a and therefore may not have been detected within the time points assessed.

The increased Post-Ex expression of miR-23a may have also been due to the exercise-induced modulation of immune cell subsets. It is conceivable that miR-23a is more highly expressed in leukocytes with shorter telomeres. Therefore, the exercise-induced migration of leukocytes with shortened telomeres and senescent phenotypes may explain the increase.

In silico predictions indicate a potential interaction between miR-27a and PIN2/TERF1 interacting telomerase Inhibitor (*PINX1*), a significant telomerase inhibitor (Zhou & Lu, 2001). PinX1 has the unique ability to directly interact with Tert and inhibit telomerase catalytic activity (Soohoo et al., 2011). Overexpression of PinX1 in human cancer cells inhibits tumorigenicity, elicits crisis and shortens telomeres (Soohoo et al., 2011). Despite a trend toward a Post-Ex increase in miR-27a, there was no significant differential regulation of *PINX1* mRNA in the present study. This may simply be due to miR-27a being functionally irrelevant to *PINX1* mRNA in this context. MiR-27a has numerous putative mRNA targets involved in disparate pathways that may not have been activated by the exercise stimulus. Additionally, the expression time course of *PINX1* mRNA may not have been detected in the present study.

Limitations

The present study has limitations. The relatively small sample (n=10), reflects the current cost of analysis. Whilst the cost has decreased over the last few years, it was still outside the project budget to assess a larger sample.

Studies using time course analysis face the challenge of establishing the optimal time course. Without *a priori* knowledge of the expression time course and transcript half-lives, it is possible that the greatest magnitude of change was missed in the present study. Whilst the time course used was effective at capturing early gene expression, it is possible that intermediate- and late-responding gene expression was missed. Further research utilizing multiple time points (e.g. 1 hour, 2 hours, 4 hours, 6 hours and 8 hour) will assist in determining expression kinetics.

As discussed in earlier chapters, the leukocytes collected for analysis are a heterogeneous mixture of cell sub-sets (e.g. lymphocytes, natural killer cells and granulocytes). The relative contributions of each subset to the transcriptional signature cannot be determined in the present study. Additionally, exercise-induced changes in immune cell subsets have not been accounted for in the present study.

Without extensive gain/loss of function studies with cultured cell lines, differentiating between potential pro-telomeric and extra-telomeric functions of the key transcripts is not possible. Genes such as the HSP family serve several cellular functions ranging from the acute stress response through to genomic stability.

Another limitation to the study is that RNA-seq data was not validated. Validation of RNA-seq data is still widely performed, largely at the behest of journal reviewers. In many cases it is a largely perfunctory practice, stemming more from tradition than clinical necessity. The typical means of validation is qPCR owing to its relative convenience, sensitivity and dynamic range (SEQC/MAQC-III Consortium, 2014). Quantitative PCR is not without its limitations; in addition to GC bias (Aird et al., 2011), the specifics of protocol calibration and primer selection

present a challenge that can significantly influence the outcome (VanGuilder, Vrana, & Freeman, 2008). Additionally, the different PCR based assays can produce significantly different measurements, prompting the Sequencing Quality Control (SEQC) project to reject qPCR's designation as the *gold standard* for validation (SEQC/MAQC-III Consortium, 2014). The customary practice of conducting qPCR validations on fresh aliquots from the same samples used in the RNA-seq merely validates the technology, not the differentially expressed transcripts (Allison, Cui, Page, & Sabripour, 2006; Fang & Cui, 2011). To validate RNA-seq experimental conclusions, different replicates from the same population should be validated (Allison et al., 2006).

There are many publications with qPCR validation of microarray data with established correlations and several good reviews outlining the precise technical requirement of effective validation (Allison et al., 2006; Rockett & Hellmann, 2004). Published validation studies show high consistency between RNA-seq and qPCR data (Core, Waterfall, & Lis, 2008; Feng et al., 2010; Nagalakshmi et al., 2008). Measures of relative expression have also been shown to correlate well across RNA-seq, qPCR, and microarrays (SEQC/MAQC-III Consortium, 2014).

The over-arching aim of the study was to characterize the overall pattern of differentially expressed genes and conduct functional and network analysis, not to conduct functional assays on specific genes. The intention was to capture exercise-responsive pathways and then use existing literature to support a potential role in telomere biology.

5.7 Conclusion

In conclusion, 30 min of intense aerobic exercise was sufficient to differentially regulate 182 transcripts over a 24 hour post-exercise period. The most heavily regulated pathways were those associated with inflammation, stress response, and chaperone proteins. In addition to their canonical exercise adaptive roles, several of the differentially expressed genes, particularly the HSP70 and HSP90 families, have established pro-telomeric roles. Twelve non-coding RNAs were differentially expressed, one of which (miR-23a) has a *bone fide* role in telomere biology. Several of the differentially expressed genes appear to influence telomere biology either by interacting with telomerase or by modulating telomere conformation, thereby influencing telomerase access to telomeric DNA.

RNA-seq can provide a whole transcriptome view of the exercise response may reveal disparate and/or contiguous pathways involved in telomere biology in a way that isolated, or reductionist approaches may not. This in turn may inform the current understanding of the observed positive association between habitual PA and LTL.

Chapter 6 - Conclusions and Summary

The aim of this thesis was to characterize the acute effects of aerobic exercise on immune cell redistribution and telomere biology. The studies here within utilized methodologies from the fields of exercise physiology, immunology, and molecular biology to assess acutely regulated indices involved with telomere biology.

Telomere biology sits astride the paired paradoxes of accelerated aging and longevity, health and cancer. Accordingly, an enhanced understanding of telomere homeostasis holds significant clinical utility in fields as diverse as gerontology, immunology, oncology, and exercise physiology. Telomere biology is central to cellular aging, simultaneously chronicling replicative history and dictating remaining replicative capacity. Telomere biology is at the core of most molecular aging pathways including cellular senescence, stem cell exhaustion, genomic instability, mitochondrial dysfunction, and epigenetic alterations. A body of evidence indicates a positive relationship between habitual PA and LTL; however, much of the current evidence is correlational and observational in nature.

By way of a broad summary (figure 28), 30 min of aerobic exercise regulated T cell subsets and several miRNAs with potential (and established) interaction with telomere-associated gene transcripts. Exercise also directly upregulated the key telomerase component hTERT and the telomere-associated SIRT6. Several components of the HSP family with established interactions with telomere biology (hTERT regulation, telomere stability, and telomerase assemblage) were differentially regulated by exercise. The above findings collectively suggest that despite telomere biology typically being viewed as a chronic biomarker, it is acutely amenable to physiological stresses.

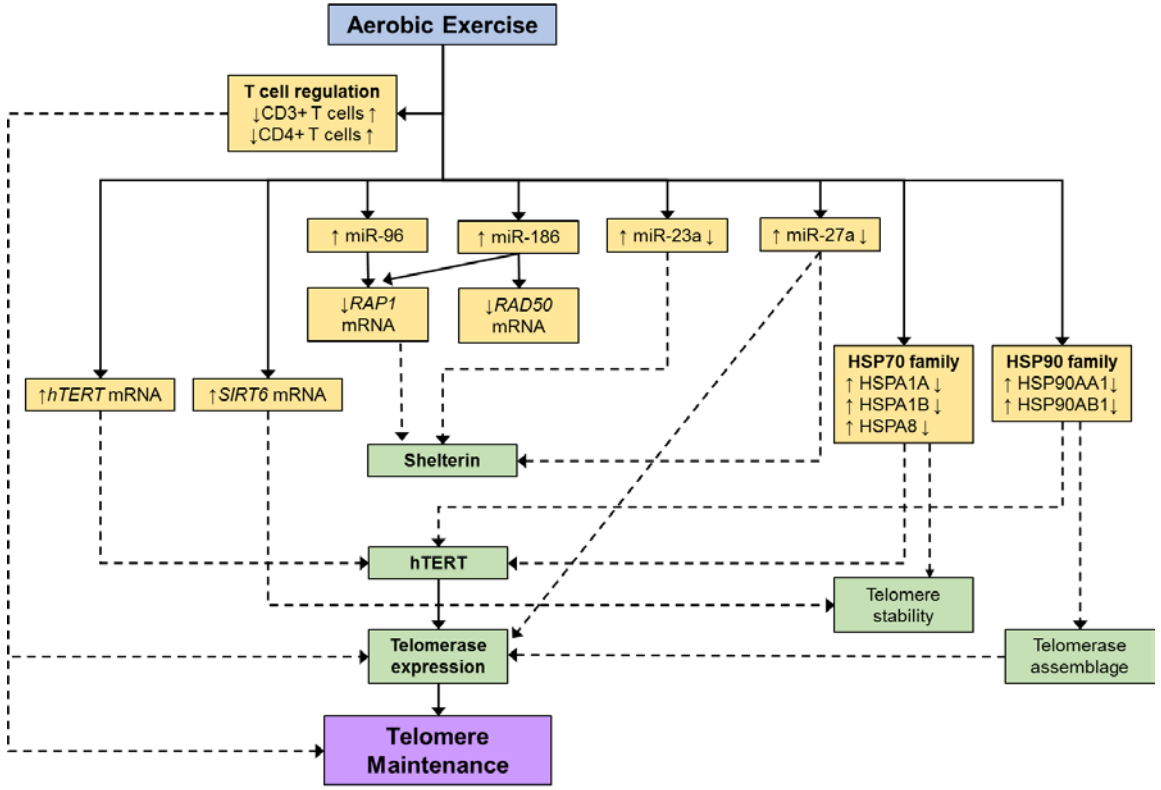


Figure 28. A diagrammatic summary of the findings from this thesis and their potential interaction with telomere biology: ↑ ↓ indicates upregulation at Post-Ex, downregulation at 24 h Post-Ex; ↑ indicates upregulation at 60 min at Post-Ex. Solid arrows and orange boxes indicate elements tested in this thesis; dashed arrows and green boxes indicate speculative interactions or established relationships not directly tested in this thesis.

In the first study (chapter two), the acute immune response to exercise was characterized using flow cytometry. Specifically, the acute T cell response to a 30 min bout of treadmill running at 80% of $\dot{V}O_{2max}$ was assessed in a cohort of males. Immune cell subsets were sorted for downstream gene and microRNA analysis. The immune response to exercise closely reflects the exercise intensity, duration, and type. Accordingly, the system can either positively or negatively adapt to the stimulus.

The novel finding of this study was that 30 min of treadmill running at a constant 80% of $\dot{V}O_{2peak}$ was sufficient to elicit novel changes in the relative proportions of specific T cell subsets. In the present study, the typical immediate post-exercise increase in T cell numbers was inverted; decreasing in number before reverting to resting levels within 1 hour of exercise cessation. Potential reasons for the novel findings include the high intensity of the exercise intervention (80% of $\dot{V}O_{2peak}$), catecholaminergic signalling, cortisol secretion, participant fitness standard, tissue and organ redistribution of lymphocytes, and lymphocyte apoptosis.

Much of the existing literature on the immune response to aerobic exercise utilizes highly variable exercise interventions. Several studies measure participants' responses after competitive events such as marathons whilst others use estimated $\dot{V}O_{2max}$ or a treadmill speed *equivalent* to a given intensity. Such interventions allow considerable variability in the exercise intensity and likely influence the magnitude of the immune response. In the present study, $\dot{V}O_{2peak}$ was assessed and expired gas analysis was used to maintain a continuous intensity of 80% of established $\dot{V}O_{2peak}$.

The findings of this study have implications for telomere biology as each of the differentially regulated subsets are known to exhibit unique telomere lengths, telomerase expression, and cytokine profile. There is also evidence that many genes are expressed in a subset-specific manner. The timing of participant blood sampling will influence the representation of constituent cells and will likely influence subsequent conclusions regarding telomere-associated indices. Given the diverse functions and physiologies of immune cell subsets, the findings of this study underscore the importance of accounting for the relative changes in subsets when analysing composite transcriptional signatures. An enhanced understanding of subset specific responses to exercise may inform clinical exercise guidelines for specific populations such as those with immune-suppression.

Future research will need to characterize the physiological consequences of transient immune cell redistribution. In the field of telomere biology, the effect of immune cell redistribution on telomere-associated factors such as gene expression, epigenetic regulation, and telomerase activity will need to be explored further.

Study two (chapter three) investigated exercise-induced miRNAs within leukocytes and subsequently investigated those with potential involvement in telomere biology. A genome-wide microarray, *in silico* prediction software, and subsequent qPCR validations were used to characterize the exercise-induced leukocyte miRNA expression profile. MiRNA expression is sensitive to exercise and is viewed as a critical mediator of exercise adaptations, showing expression specificity for tissue type, exercise modality, and intensity.

A total of 56 miRNAs were significantly differentially regulated between Pre-Ex and Post-Ex time points. Four miRNA/mRNA interactions with potential

involvement in telomere biology were further investigated with qPCR. MiR-186 and miR-96 were identified as having potential interaction with the shelterin component *RAP1*. RAP1 plays a critical role in telomere biology and can function as both a negative and positive regulator of telomere length via an interaction with TRF2. Additionally, the findings highlight discrepant miRNA expression profiles between unsorted leukocytes and sorted T cell subsets, suggesting the composite signature is in part driven by other immune cell subsets.

The roles of histone modifications and sub-telomeric DNA methylation are comparatively well established in telomere biology; however, the direct influence of miRNAs is an area of active and ongoing investigation. Whilst several miRNAs have been associated with telomeres in specific cancer cell lines, little is known about exercise-induced expression in healthy immune cell subsets. The findings of this study provide novel evidence of exercise-induced miRNA expression with potential involvement in telomere biology.

In addition to adding to the knowledge base on exercise-mediated miRNA expression, the results of this study add to the developing understanding of epigenetic regulation of telomere biology. This in turn may provide a mechanistic insight into the observed association between PA and LTL. The results from this study suggest that exercise-induced miRNA may play an acute role in telomere biology. The widely held consensus is that the relationship between PA and LTL is mediated by chronic adaptations; however, the results from this study suggest acute regulatory factors.

Telomere biology and miRNA regulation jointly converge upon multiple physiological aging processes including DNA damage, stem cell exhaustion,

cellular senescence, and telomere shortening. An enhanced understanding of exercise-induced miRNA expression and the subsequent phenotypic effects may inform exercise guidelines for specific populations. A clearer understanding of exercise-induced miRNAs, specifically those associated with telomere biology, may provide viable biomarkers via which to assess exercise adaptive responses. This in turn may allow more targeted use of exercise as a first line treatment in conditions characterized by aberrant miRNA and/or telomere regulation.

Future research needs to establish the origin, target tissues, associated networks, and phenotypic implications of exercise-induced miRNAs. Gain- and loss-of-function experiments are needed to characterize the direct phenotypic effects of putative pro-telomere miRNA expression and repression on specific tissues. The expression time course, transcriptional half-life, and degradation rate of exercise-induced miRNAs also need to be more clearly characterized to better inform optimal stimulus imposition. The precise direction of the miRNA/mRNA relationship must also be established. Whilst conventional wisdom posits that mRNA expression is influenced by miRNA repression, recent research has shown that modulation of a target transcript can itself determine miRNA expression levels.

Having identified the transcriptional plasticity of selected miRNAs, the aims of study three (chapter four) were to directly validate the predicted miRNA-mRNA targets and assess the exercise-induced regulation of other pro-telomeric gene transcripts. An initial telomere extension mRNA array identified exercise-induced changes in several pro-telomeric genes including predicted miRNA targets: *hTERT*, *SIRT6*, *RAP1,* and *RAD50*. Subsequent validations revealed significantly decreased *RAP1* mRNA paralleled by concomitant increases in potential binding miRNAs (miR-186 and miR-96).

Another novel finding of this study was the exercise-induced upregulation of *hTERT* and *SIRT6* mRNA expression in leukocytes. The post-exercise increase in *hTERT* mRNA expression is of considerable significance to the field of telomere biology. In addition to being the rate limiting component of telomerase, hTERT plays several extra-telomeric functions. Telomere dynamics are often considered the slow shifting sands of cellular physiology, chronicling replication and DNA damage over extended periods of time. The finding that several key pro-telomere genes are acutely transcribed significantly informs the current understanding of telomere dynamics. The difference between mere amelioration of negative telomere factors and the actual enhancement of positive telomere factors is subtle yet important. The ability to accurately prescribe exercise to enhance telomere homeostasis in clinical populations characterized by accelerated telomere shortening may have significant clinical utility.

The results of this study suggest that several pro-telomere genes exhibit IEG behaviour. IEGs have important and established roles within the immune system, most of which centres around activation of immune cells such as T and B cells. Exercise-based studies may help to characterize acutely labile signature gene sets or pathways associated with telomere biology. This in turn may assist the understanding of potential telomeric therapeutic targeting.

Future research will need to determine not only the expression time course of exercise-induced telomere genes but the mRNA stability and subsequent translation into functional proteins. Characterization of associated signalling pathways and their respective time courses i.e. transient or sustained, should also be the focus of future telomere research.

The aim of study four (chapter five) was to characterize the exercise-induced transcriptome in leukocytes and to identify any telomere-associated genes and/or associated pathways. This study assessed expression changes before, immediately after, and 24 hours after exercise. Next generation RNA-seq was used to capture both protein-coding and non-coding RNAs. Subsequent pathway enrichment analysis was conducted on differentially expressed gene sets.

Thirty minutes of aerobic exercise at 80% of $\dot{V}O_{2peak}$ differentially regulated 182 transcripts in leukocytes over a 24 hour post-exercise time course. Inflammatory, stress, and chaperone protein pathways were significantly regulated immediately post-exercise highlighting the multi-faceted IEG response to exercise. The results of this study identified significant regulation of the HSP70 and HSP90 families, both of which have established exercise adaptive and pro-telomere roles. Several of the differentially regulated genes influence telomere biology either via modulation of telomere conformation or by directly interacting with telomerase. The study also identified 12 non-coding RNAs including miR-23a and miR-27a, both of which have established roles in telomere biology. The results of this study confirm and extend the preliminary findings from chapter four that some telomere-associated genes and miRNAs demonstrate IEG transcriptional behaviour.

The entire transcriptome view provided by next generation RNA-seq may reveal key exercise-induced telomere pathways. The identification of contiguous and/or disparate telomere pathways will not only enhance the understanding of the relationship between PA and telomere length, it will extend the potential therapeutic capacity of telomere biology. Future research efforts are likely to centre around complex bioinformatics. The data output provided by RNA-seq is substantial and

can be subjected to multiple levels of pathway, network, and functional analysis that can identify complex coding and non-coding transcript interactions.

Telomeres hold significant promise as both a biomarker of cellular stress exposure and as a therapeutic target. In time, telomere and telomerase research will make truly significant contributions to human health and disease management. However, a clearer understanding of the underpinning mechanisms is needed to move from the current correlations to causation. The precise association between PA and LTL is currently unclear and investigations into plausible mechanisms have returned promising yet inconsistent findings. The novel findings from this thesis suggest possible IEG transcriptional behaviour from several key telomere-associated genes, potential miRNAs, and pathways. In addition to being an empowering public health message, such acute influence over genomic stability may provide tantalizing future therapeutic targets.

The extent to which LTL can be meaningfully lengthened, not merely offset from the age-associated shortening trajectory, is still largely unknown. If exercise-induced telomere lengthening can be clearly and consistently demonstrated in a healthy, young population, it must then be assessed in aged and diseased cohorts. It must then be determined if positive changes in telomere length reflect differential regulation of pro-telomeric genes or simply the amelioration of negative telomere instigators.

The possibility that LTL is a physiological epiphenomenon cannot be excluded. Telomere length may be more of a cellular description than explanation, with changes in length reflecting but not directly influenced by the primary mechanism. Future research must longitudinally monitor phenotypic consequences of telomere

modulation to determine the direction of the causal arrow. Determining whether an individual can move from the lowest to the highest quartile of LTL through lifestyle interventions such as exercise remains an open and essential question. The effective use of mediation analysis models would also help to reveal whether changes in telomere length can occur independently of factors like oxidative stress and inflammation.

If an exercise/telomere-protective effect can be unequivocally established, determining the optimal exercise dose will be an important next step. The far-reaching physiological effects of exercise demonstrate a degree of overall risk factor and all-cause mortality reduction greater than many pharmacological agents. Exercise programming variables such as modality, intensity, and duration are analogous to drug type, concentration, and dosage. Accordingly, there will be optimal doses, ineffectively low doses, and potentially dangerous overdoses that negatively impact upon telomere length. For this reason alone, a clearer understanding of the molecular effects of exercise is warranted.

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