BIOLOGIC ANTI-RHEUMATIC DRUG TREATMENT EFFECTS ON TELOMERE LENGTH IN COLLAGEN - INDUCED ARTHRITIS

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DECLARATION

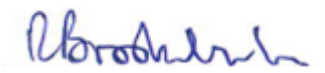
I, Rixile Allot Mahlaule, declare that this dissertation is my own, except to the extent indicated in the contribution and acknowledgments sections. It is being submitted for the degree of Master of Science in Medicine in the School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this dissertation has not been submitted for any degree or examination in this or any other University.

I hereby certify that the studies contained in this dissertation have been approved by the Animal Ethics Screening Committee, University of the Witwatersrand, Johannesburg. The ethics approval number is 2017/03/21/C.

Rixile Allot Mahlaule

Signed on the 20th day of November 2020

Dr Sulé Gunter



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ABSTRACT

Background. Telomere length is considered a marker of biological ageing. Several disease states have been associated with an increased rate of telomere attrition. Shorter telomere lengths are considered a predictor of cardiovascular disease risk. Chronic inflammation increases cell replication, hence increases telomere attrition. Chronic inflammation also increases cardiovascular disease risk. Recent studies in chronic high-grade inflammatory conditions in humans have shown controversial results when comparing telomere lengths of patients to healthy controls. Furthermore, paradoxical relationships between telomere length and sub-clinical cardiovascular disease markers have also been reported in inflammatory conditions. Therefore, this study aimed to determine the effect of high-grade inflammation on leukocyte telomere length and whether inflammation induced changes in telomere lengths are affected by biologic anti-inflammatory treatment.

Methods. Three-month-old, male and female Sprague-Dawley rats were randomly divided into four groups. Three of the groups were exposed to an arthritis inducing protocol where rats received a subcutaneous injection of bovine type-II collagen emulsified in incomplete Freund's adjuvant at the base of the tail. The other group served as the control (n = 25) that received a saline injection. After inoculation with collagen and upon signs of inflammation, one group received no treatment (Inflammation group, n = 22), one group received Etanercept, a TNF-α inhibitor (TNF-α blocker group, n = 11) every three days for six weeks and one group received Tocilizumab, an IL-6 receptor blocker (IL-6 blocker group, n = 12) once a week for six weeks. Body weight, blood pressure and arthritis scores in the hind paws were measured every two weeks. After six weeks of

treatment, cardiac function was measured by echocardiography and vascular reactivity to vasodilators and vasoconstrictors in renal arteries were measured using a wire myograph. Blood was obtained and serum concentrations of TNF-α, IL-6 and C-reactive protein (CRP) were measured by ELISA. Serum vascular adhesion molecule-1 (VCAM-1), a marker of endothelial activation was measured using ELISA. DNA was extracted from whole blood and from visceral adipose tissue. Quantitative real-time PCR (qRT-PCR) was used to determine relative telomere length of leukocyte DNA and visceral adipose tissue DNA. Group differences in body weight, blood pressure and arthritis scores were measured by repeated measures analysis of variance (ANOVA). Group differences in inflammatory cytokine concentrations and relative leukocyte telomere length were determined by two-way ANOVA with a Tukey post-hoc test. Group differences in tissue telomere length was determined by a Kruskal-Wallis test. Associations between relative leukocyte telomere length and inflammatory markers, vascular function markers and cardiac function markers were determined by Pearson's correlations.

Results. Body weight and blood pressure did not change over the course of the study and did not differ between the groups (all $p > 0.05$). Arthritis scores were higher in all groups inoculated with collagen from six weeks onwards, compared to baseline and to controls. Circulating inflammatory marker concentrations were higher in all rats inoculated with collagen compared to the control group (all $p < 0.05$). Serum VCAM-1 concentrations were significantly higher in the inflammation group compared to the control ($p = 0.0008$) and TNF-α blocker groups ($p = 0.02$). Leukocyte telomere lengths were significantly longer in the inflammation group compared to the control group ($p = 0.05$). Leukocyte

telomere length in the TNF-α blocker and IL-6 blocker groups were not different from controls ($p > 0.05$). Adipose tissue telomere length did not differ between the groups ($p = 0.32$) and was not associated with leukocyte telomere length ($r = -0.10$, $p = 0.44$). Higher circulating CRP concentrations were paradoxically associated with longer leukocyte telomere lengths ($r = 0.47$, $p = 0.001$). Controlling for body mass, sex and anti-inflammatory treatment did not materially alter this association. Adipose tissue telomere lengths were not associated with circulating inflammatory markers. Longer leukocyte telomere lengths were paradoxically associated with higher systolic blood pressure ($r = 0.29$, $p = 0.05$), higher pulse pressure ($r = 0.31$, $p = 0.04$), reduced maximal vascular relaxation ($r = -0.55$, $p = 0.006$) and reduced maximal vascular contractile ($r = -0.37$, $p = 0.04$) responses. Upon adjusting for body weight and sex, the results were materially unaltered, however, treatment with anti-inflammatory drugs impacted the relationship between telomere length and blood pressure, but not the relationship between telomere length and vascular reactivity. In all groups, longer leukocyte telomere lengths were associated with increased VCAM-1 concentrations ($r = 0.29$; $p = 0.05$). Longer telomere lengths were associated with increased left ventricular filling pressure ($r = 0.35$, $p = 0.04$) in control and inflammation groups. When adjusting for body weight and sex, leukocyte telomere length was associated with impaired left ventricular relaxation ($r = -0.37$, $p = 0.03$) and increased left ventricular filling pressure ($r = 0.39$, $p = 0.02$). When including the treatment groups in the model, the associations between leukocyte telomere length and cardiac function markers were no longer significant. Adipose tissue telomere lengths were not associated with any marker of vascular function, but shorter adipose tissue

telomere lengths were associated with impaired cardiac systolic function, regardless of drug treatment.

Conclusion. Exposure to high grade inflammation paradoxically resulted in longer leukocyte telomere length but did not affect adipose tissue telomere length. Treatment with anti-inflammatory drugs impacted the inflammation induced changes in leukocyte telomere length. In control and inflammation groups only, longer leukocyte telomere length was paradoxically associated with impaired vascular function and cardiac function. When including all groups in the analysis, longer leukocyte telomere length remained associated with impaired vascular function, but not with cardiac function markers. Adipose tissue telomere length was not associated with vascular function, but shorter adipose tissue telomere length was associated with impaired cardiac systolic function. These results suggest that exposure to high grade inflammation may impact leukocyte physiology and that leukocyte telomere length may not be an accurate marker of biological ageing or cardiovascular disease risk in patients exposed to high-grade inflammation or those using anti-inflammatory treatments.

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STATEMENT OF CONTRIBUTION TO DATA COLLECTION AND ANALYSIS

I declare that I designed this study in conjunction with my supervisors Professors AME Millen, R Brooksbank and Dr S Gunter. I was part of a team of researchers (including Prof Millen, Prof Michel, Dr Mokotedi, Dr Gunter, Ms Manilall) and postgraduate students responsible for collecting data for this study. I was responsible for collecting data for the main outcome variable in this thesis, namely telomere length. I performed the data analysis for this thesis with the help of Dr S Gunter and Prof AME Millen. I wrote this thesis which was reviewed by Prof Millen, Dr Gunter and Prof Brooksbank.

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ABBREVIATIONS

15-F2t-IsoP	15-F2-isoprostanes
8-epi-$PGF_{2\alpha}$	8-epi-prostaglandin F_2 alpha
8-oxodG	8-oxo-7,8-dihydrodeoxyguanosine
α	alpha
β	beta
Δ	delta
ΔCt	delta Ct
A	trans-mitral blood flow velocity in the late (atrial-A) period of left ventricular diastolic filling
a’	peak velocity of tissue lengthening at the lateral mitral annulus during late (atrial) diastole
ALT	alternative lengthening of telomeres
ANOVA	analysis of variance
AS	ankylosing spondylitis
BMI	body mass index
BW	body weight
CAD	coronary artery disease

CDK	cyclin-dependent kinase
CI	confidence interval
CIA	collagen induced arthritis
CRP	C-reactive protein
Ct	cycle threshold
CVD	cardiovascular disease
d	diastole
DBP	diastolic blood pressure
DCFH	dichloro-dihydro-fluorescein
DDR	DNA damage response
DM	diabetes mellitus
DMARDs	disease modifying anti-rheumatic drugs
DNA	deoxyribonucleic acid
E	trans-mitral flow velocity in early diastole
E/A	ratio of early to late diastolic filling velocity
E/e’	left ventricular filling pressures
e’	peak velocity of tissue lengthening at the lateral mitral annulus during early diastole

e’/a’	ratio of early to late mitral annular tissue lengthening
EDCFs	endothelium-derived contracting factors
EDHF	endothelium-derived hyperpolarising factor
EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay
eNOS	nitric oxide synthase
FISH	fluorescent in situ hybridization
FSmid	midwall fractional shortening
GM-CSF	granulocyte-macrophage colony stimulating factor
hTERT	human telomerase reverse transcriptase
ICAM-1	intercellular adhesion molecule 1
IHD	ischaemic heart disease
IL	interleukin
IVST	interventricular septal thickness
kbp	kilo base pair
KCL	potassium chloride
kg	kilogram

LTL	leukocyte telomere length
LVID	left ventricular internal diameter
mg	milligram
MI	myocardial infarction
mIL-6	membrane bound interleukin 6 receptor
ml	millilitre
mm	millimetre
mmHg	millimetres of mercury
n	sample size
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NO	nitric oxide
OA	osteoarthritis
OP	osteoporosis
PA	psoriatic arthritis
pg	picogram
POT-1	protection of telomeres 1

PWT	left ventricular posterior wall thickness
qRT- PCR	quantitative real time polymerase chain reaction
r	correlation coefficient
RA	rheumatoid arthritis
RAP1	repressor activator protein 1
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROS	reactive oxygen species
RWT	relative wall thickness
s	systole
SBP	systolic blood pressure
SEM	standard error of the mean
SLE	systemic lupus erythematosus
SSc	Systemic sclerosis
TAOS	total antioxidant status
TDI	Tissue Doppler Imaging
TERC	telomerase reverse transcriptase

TERRA	telomeric repeat-containing RNAs
TERT	telomerase RNA component
Th17	T helper 17 cells
TIN2	TRF1 interacting nuclear protein 2
TNFR	tumour necrosis factor receptor
TNF	tumour necrosis factor
Treg	regulatory T cells
TRF	telomeric repeat binding factor
TTP	telomere protection protein
T/S ratio	telomere repeat copy to single copy gene ratio
µg	micrograms
VCAM-1	vascular adhesion molecule 1

CHAPTER 1

INTRODUCTION

Medical advances have resulted in an increase in life expectancy of the world population (Christensen *et al*., 2009). Prolonged exposure to several environmental factors will result in a variety of clinical and health complications. As a result, the number of age-related diseases is expected to increase (Adhikari *et al*., 2010). Non-communicable diseases (NCDs) including diabetes mellitus, cardiovascular disease (CVD), cancer and respiratory diseases, are estimated to contribute to 70% of the total deaths worldwide (Noncommunicable diseases country profiles 2018, 2018). According to a World Health Organisation report (Noncommunicable diseases country profiles 2018, 2018), among non-communicable diseases, CVD accounts for the highest number of the total deaths. This report further stated that in South Africa, CVD is responsible for 19% of the total mortality. More than 75% of the deaths related to NCDs are recorded in people over the age of 60 years (Adhikari *et al*., 2010; Noncommunicable diseases country profiles 2018, 2018). Considering the change in the world population distribution, the burden of NCDs are expected to increase over the next decade, especially in low- and middle-income countries (Abegunde *et al*., 2007). In light of the increasing burden of NCDs, the pathophysiological mechanisms underlying CVD have been under investigation for some time.

Although traditional risk factors contribute largely to increased CVD rates, other non-traditional risk factors have also been associated with CVD (Harald *et al*., 2008; Shisana *et al*., 2013; Martinez & White, 2018). Chronic systemic inflammation is one non-traditional risk factor that has received considerable attention. Inflammatory markers and cytokines have been related to cardiovascular disease risk (Burke *et al*., 2002; Martinez & White, 2018) and cardiovascular events (Kaptoge *et al*., 2010). In addition, patients with

conditions characterised by high-grade systemic inflammation, such as rheumatoid arthritis (RA), have a much higher incidence of CVD compared to the general population. RA is a highly prevalent autoimmune disease affecting approximately 1% of the world population and up to 2.5% of the South African population (Engler & Skosana, 2016). RA is characterised by inflammation in the synovial joints; however, inflammatory cytokines are not only confined to the synovial joint (Cojocaru *et al*., 2010). Systemic inflammation in RA have been implicated in the development of CVD (Bongartz *et al*., 2006; Cojocaru *et al*., 2010). Treatment of patients with biologic anti-inflammatory drugs, known for their effect on disease progression, has shown controversial results on CVD risk. As a result, the mechanisms whereby systemic inflammation augments CVD risk in RA are being investigated (Cojocaru *et al*., 2010; Castaneda *et al*., 2016).

In an attempt to improve risk stratification in RA patients, researchers continuously search for biomarkers of CVD and inflammation. Given the role that inflammation plays on cellular replication and biological ageing, telomere length has received some attention as a possible biomarker for CVD risk stratification (Fitzpatrick *et al*., 2007, Masi *et al*., 2014, Wong *et al*., 2014). Telomeres are non-coding, double-stranded, deoxyribose nucleic acid (DNA) repeats of the sequence TTGGG that cap and protect the end of the chromosomes from damage during replication (De Lange, 2005). Telomeres are 4-20 kilobase pairs (kbp) in length and are not replicated by DNA polymerase. Telomeres become progressively shorter with proliferation. This process of progressive telomere shortening during cell replication is known as telomere attrition (Victorelli & Passos, 2017). When the telomeres reach a critical length, cell division is halted, leading to an irreversible cell cycle arrest known as cellular senescence (Victorelli & Passos, 2017). Telomere lengths have

been reported to predict the development of age-related diseases, including CVD (Cawthon *et al*., 2003; Willeit *et al*., 2010). Indeed, shorter telomere lengths are associated with CVD, independent of conventional risk factors (Fyhrquist *et al*., 2013; Haycock *et al*., 2014).

Systemic inflammation is a risk factor known for increasing the rate of telomere attrition (Steer *et al*., 2007; Aubert & Lansdorp, 2007; Barnes *et al*., 2019). Indeed, inflammation and oxidative stress, which play a central role in RA, are also associated with increased cell replication and accelerated telomere shortening (Wong *et al*., 2014; Correia-Melo *et al*., 2014; Barnes *et al*., 2019). In patients exposed to chronic inflammatory disorders, however, shorter telomeres are not consistently reported compared to healthy controls (Lee & Bae, 2018). In addition, the relationship between telomere length and CVD risk in patients exposed to high-grade inflammation are controversial (Haque *et al*., 2013; Raymond *et al*., 2016b).

Taken together, chronic inflammation increases CVD risk in RA, however, the effects of anti-inflammatory treatment on cardiac function is controversial. Inflammation induces telomere attrition in the general population, however, the relationship between inflammation and telomere shortening in inflammatory conditions is inconsistent. Whether leukocyte telomere length provides an index of the cumulative inflammatory load, and whether this relationship is impacted by anti-inflammatory treatments requires investigation. Moreover, whether telomere length is a potential biomarker of CVD in inflammatory conditions also requires elucidation.

This thesis will be structured as follows: Chapter 2 will provide a succinct view of the current literature and will explain the reasons for conducting this study. Thereafter Chapter 3 will describe the methods used and Chapter 4 will present the results obtained. Chapter 5 will discuss the results and highlight the main findings of this study in the context of previous literature.

CHAPTER 2

LITERATURE REVIEW

2.1 Rheumatoid arthritis

Rheumatoid Arthritis (RA) is an autoimmune disease characterised by chronic, high-grade inflammation within synovial joints (Ma & Xu, 2013). RA autoantibodies attack healthy joint tissue, and therefore disease progression is associated with irreversible tissue damage and reduced joint function (Nanke *et al*., 2002). RA affects approximately 1% of the world population (Kim *et al*., 2015). In South Africa, the prevalence of RA ranges between 0.9% and 2.5% (Engler & Skosana, 2016). RA patients experience a substantially reduced quality of life and a decreased life expectancy (Nanke *et al*., 2002; Listing *et al*., 2015).

Whilst the exact aetiology of RA remains unknown, some of the risk factors for developing RA include genetic predisposition, smoking, age and female gender (Dowman *et al*., 2012). Of these factors, genetic predisposition is believed to be the main reason for developing RA (Barton & Worthington, 2009). Emerging data suggests that genetically induced RA-related autoimmunity and inflammation may be initiated at a mucosal site years before the onset of detectable joint symptoms (Demoruelle *et al.,* 2014). Therefore, subtle genetic dysregulations may be present prior to the presence of clinical RA.

Following the initial onset, the inflammation in established RA is not confined to the synovial joints. The cytokines produced in the synovium spill into the systemic circulation, which causes high-grade, chronic systemic inflammation (Cojocaru *et al*., 2010). The systemic inflammation leads to several pathological processes (Cojocaru *et al*., 2010) known as extra-articular complications that may affect multiple organ systems (Turesson *et al*., 1999; Bongartz *et al*., 2006; Cojocaru *et al*., 2010; McInnes & Schett, 2011). Extra-

articular manifestations occur in roughly 40% of patients with RA (Cimmino *et al*., 2000) and reflects the level of disease activity and severity (Turesson *et al*., 1999).

2.2 Risk of cardiovascular disease in rheumatoid arthritis

The cardiovascular system is the most commonly affected system by extra-articular manifestations of RA. Indeed, RA patients have a significantly higher risk of cardiovascular events and CVD-related mortality compared with the general population (Avina-Zubieta *et al*., 2008; Avina-Zubieta *et al*., 2012; Zhang *et al*., 2014; Mackey *et al*., 2018; Blum & Adawi, 2019). RA patients have a higher incidence of endothelial dysfunction, atherosclerosis, arterial stiffness, impaired cardiac function and heart failure compared to the general population (Avina-Zubieta *et al*., 2008; Wållberg-Jonsson *et al*., 2008; Aslam *et al*., 2013; Ambrosino *et al*., 2015). Although traditional risk factors including age, hypertension, dyslipidaemia and diabetes mellitus contribute to the increased incidence of cardiovascular disease risk in RA, it is believed that chronic systemic inflammation plays a major role in the increased CVD risk (Del Rincón *et al*., 2001; López-Mejías *et al*., 2016). Circulating inflammatory markers are independently associated with cardiovascular mortality rates (Maradit-Kremers *et al*., 2005) and with sub-clinical CVD such as endothelial dysfunction, atherosclerosis, arterial stiffness and heart failure (Gonzalez-Gay *et al*., 2005; Dessein *et al*., 2007; Liang *et al*., 2010; Sharma *et al*., 2015; Gunter *et al*., 2017). Currently the mechanisms whereby exposure to chronic inflammation in RA results in various diseases, including CVD are under investigation (Cojocaru *et al*., 2010; Castaneda *et al*., 2016).

2.3 Pharmacological treatment in rheumatoid arthritis

Early diagnosis of RA and adequate treatment is important to prevent rapid disease progression and disease activity (Engler & Skosana, 2016). Conventionally, synthetic disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate, sulfasalazine and leflunomide have been prescribed to reduce disease activity (Weinblatt *et al*., 1994; O'Dell, 2002). Synthetic DMARDs are, however, unable to control disease activity in all patients and they have several adverse side effects (Fridlington *et al*., 2011; Aronoff & Aronoff, 2014; Bhatnagar *et al*., 2015; Engler & Skosana, 2016). Biologic DMARDs have recently been introduced, and have shown greater disease suppression, particularly in patients who did not respond to synthetic treatments. (Ma & Xu, 2013; Engler & Skosana, 2016; Benjamin & Lappin, 2019). Biologic DMARDs exert their action by inhibiting specific inflammatory molecules, rather than supressing the entire immune system as with synthetic DMARDs (Raychaudhuri & Raychaudhuri, 2009; Benjamin & Lappin, 2019). Studies investigating biologic DMARD treatment have demonstrated its effectiveness in improving quality of life, while decreasing disability and joint damage (Burke *et al*., 2014).

Biologic DMARDs prevent the damaging effects of inflammation at target receptors, by either competing for receptor binding or by high affinity binding of antibodies to circulating cytokines (Hamilton & Clair, 2000; Ma & Xu, 2013; Benjamin & Lappin, 2019). Although there are several biologic DMARDs commercially available, the most commonly used biologic DMARDs in RA patients are TNF-α inhibitors (Okuda, 2008). TNF-α is a key pro-inflammatory cytokine that is involved in the acute phase reaction. It is produced mainly by activated leukocytes, monocytes, macrophages, fibroblasts, and T and B lymphocytes. TNF-α stimulates the release of other inflammatory cytokines such as interleukins (IL-1,

IL-6, and IL-8) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Vasanthi *et al*., 2007). It also stimulates the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) from the fibroblast, endothelium and leukocytes (Golias *et al*., 2007; Vasanthi *et al*., 2007). TNF-α plays a vital role in progression and severity of RA, as it is responsible for a diverse range of signalling events within the synovial lining, endothelial cells, activated T and B lymphocytes and macrophages, which leads to necrosis or apoptosis (Idriss & Naismith, 2000; Vasanthi *et al*., 2007). Elevated synovial and circulating levels of TNF-α have been reported in RA patients (Manicourt *et al*., 1993; Thilagar *et al*., 2018). Hence it is not surprising that TNF-α inhibitors have been successfully used as therapeutic agents in reducing disease progression and severity (Roberts & McColl, 2004; Vasanthi *et al*., 2007).

More recently, another biologic DMARD that has been successful in the management of RA is the IL-6 receptor blockers. IL-6 is a pleiotropic inflammatory cytokine that has many biologic effects on liver hepatocytes, T and B lymphocytes, neutrophils, monocytes and platelets (Srirangan & Choy, 2010). IL-6 is well known for mediating the development and progression of autoimmunity (Navarro-Millan *et al*., 2012). High levels of IL-6 have been reported in the systemic circulation and the synovium of RA patients (Wong *et al*., 2006). In RA, IL-6 stimulates osteoclasts and increases bone resorption, hence leading to joint destruction (Srirangan & Choy, 2010). As IL-6 mediates a key step in the pathogenesis of RA, these IL-6 receptor blockers have proven therapeutic potential in the RA population (Kim *et al*., 2015). IL-6 receptor blocker therapy has shown sustained, rapid

improvements in RA disease severity and reducing the progression of joint damage (Nishimoto *et al*., 2004; Srirangan & Choy, 2010).

Despite improvements in RA disease severity and slowing disease progression with biologic DMARD treatment, the effects of these biologic DMARDs on CVD risk in RA patients are controversial (Baniaamam *et al*., 2018). Some have shown that biologic DMARDs reduce overall CVD risk (Westlake *et al.*, 2011) or risk of CV events (Greenberg *et al.*, 2011; Bili *et al.*, 2014; Lee *et al.*, 2018). Others have shown no change in CVD risk or CV events when receiving biologic DMARDs (Solomon *et al.*, 2006; Rao *et al.*, 2015). Similarly, some (Wolfe & Michaud, 2004; Morgan *et al.*, 2014), but not others (Bernatsky *et al*., 2005; Cole *et al*., 2007; Listing *et al*., 2008; Solomon *et al*., 2013; Schau *et al*., 2015) have shown a decreased risk of developing congestive heart failure when receiving biologic DMARD treatment. Interestingly, several studies have reported a paradoxical increased risk of developing heart failure when receiving biologic DMARDs (Curtis *et al.*, 2007; Setoguchi *et al.*, 2008; Al-Aly *et al.*, 2011). Specifically, with TNF-α inhibitors, there is a large body of evidence that supports the theoretic potential of these drugs in reducing the overall CVD risk (Dixon *et al*., 2007). One placebo controlled, double blind study conducted on the safety of TNF-α inhibitors reported a higher number of cardiovascular events in the TNF-α inhibitor group, although this observation was not statistically significant (Weisman *et al*., 2007). In contrast, one large (n=6393) study found that cardiovascular mortality following long-term TNF-α inhibition is reduced, when compared to methotrexate (Morgan *et al*., 2014). With regard to IL-6 receptor blocker therapy, multiple studies have found that, although IL-6 inhibition induced unfavourable alterations

to the lipid profile, the risk for cardiovascular events associated with IL-6 receptor blocker therapy was comparable to TNF-α inhibitors (Kim *et al.,* 2017; Xie *et al*., 2019).

In summary, RA is a chronic inflammatory disorder that severely impacts quality of life. Besides its role in joint destruction, inflammation has also been linked to several extra-articular manifestations, of which CVD is one of the most critical and widespread. CVD contributes to the increased mortality in RA patients. Considering that biologic DMARDs are effective in reducing disease severity and joint destruction, they could potentially exert similar protective effects on CVD risk factors and cardiovascular events. However, the evidence in this regard is controversial. As a result, the possible physiological mechanisms that underlie the association between inflammation and CVD are currently under investigation. Furthermore, potential biomarkers that may assist in the risk stratification of RA patients have received considerable attention in the recent literature, however, the use of biologic DMARDs on several of these biomarkers are currently unknown.

2.4 Telomere length

DNA replication is an important cell division process. During DNA replication, the double helix strands unwinds, RNA primers are synthesised, and the DNA template is then replicated in a 5’ to 3’ direction by DNA polymerase. The RNA primers are then removed and replaced with DNA. At the 5’-end of the newly synthesised DNA strand, the RNA primer cannot be replaced; hence every new strand of DNA will have an incomplete 5’-end with an overhang at the 3’ end (Correia-Melo *et al*., 2014). DNA repair machinery will recognise the incomplete 5’ end and the overhang on the 3’ as damaged or double

stranded breaks and will delete them (Blackburn, 2001). This shortening of the ends of chromosomes will eventually result in the loss of critical genetic information.

In order to prevent a critical loss of genetic information, specialised protein-deoxyribonucleic acid (DNA) complexes called telomeres are responsible for capping the ends of eukaryotic linear chromosomes (Weng, 2012). Telomeres consist of non-coding, guanine rich, hexamer-nucleotide repeats of the six base pair sequence 5'-TTAGGG-3'/5'-CCCTAA-3' (De Lange, 2005). They present a 3' single stranded G- overhang of 50-500 nucleotides which is able to fold back and invade the double stranded side of the telomere, creating what is known as the T loop (Griffith *et al*., 1999; De Lange, 2005). The T loop serves as a protective mechanism as it in essence hides the overhang on the 3' end, hence prevents the DNA ends from being recognised as areas of damage or double stranded breaks by DNA repair machinery (De Lange, 2005; Wang *et al*., 2007).

The T loop is stabilised and protected by a multi-protein complex, called shelterin (De lange, 2005). Shelterin consist of 6 proteins namely protection of telomeres 1(POT-1), telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), telomere protection protein 1 (TTP1), repressor activator protein 1 (RAP1) and TRF1 interacting nuclear protein 2 (TIN2) (Wu *et al*., 2006). The double stranded region of the telomeres is directly bound by TRF1 and TRF2 together with their interaction proteins RAP1 and TIN2 (Wu *et al*., 2006). The single stranded 3' overhang is bound by POT1 in complex with TTP1 forming the most important stable heterodimer (Wang *et al*., 2007). The heterodimer protects the chromosome ends from fusing and together with telomerase (see below) is responsible for the regulation of telomere length (Wang *et al*., 2007).

Telomere length is highly variable in eukaryotes and gets progressively shorter with cell division (Victorelli & Passos, 2017). With each cell division, a small fraction of approximately 20 to 200 base pairs (bp) of telomeric DNA are lost in a process called telomere attrition (Blackburn, 2001; Weng, 2012). As new DNA is synthesised only in the 5' to 3' direction, the leading strand (5' to 3') can be replicated until the end of the chromosome. However, for the lagging strand (3' to 5'), short RNA primers are required to allow synthesis of new DNA by the addition of short fragments (Blackburn, 2001). Consequently, DNA polymerase is unable to synthesise the complementary sequence of the lagging strand at the very end of the 3' chromosome region once the RNA primer at that location is removed (Blackburn, 2001). This results in the loss of some telomeric DNA sequences during each replication cycle at the 5' end of the new DNA strand, which causes a progressive shortening of telomere length with each cell division (Blackburn, 2001). As these telomere base-pairs do not code for any particular gene, their loss does not adversely affect the critical genetic information of the cell (Aubert & Lansdorp, 2008).

Although telomere shortening is a normal physiological process during cell division, critically short telomeres are recognised as areas of DNA damage, thus leading to persistent DNA damage response (DDR) activation (Chan & Blackburn, 2004). DDR ultimately leads to cellular senescence and apoptosis, through tumour suppressor protein p53 as well as the cell cycle inhibitor p16 ^{Ink4a} pathways (Blackburn, 2001). The activation of p53 results in the overexpression of p21, a cyclin-dependent kinase (CDK) inhibitor (Blackburn, 2001). To prevent genetic instability, both p21 and p16 ^{Ink4a} prevent the recurrence of cell proliferation (Victorelli & Passos, 2017). Hence, the preservation of the integrity of telomere length is critical to prevent cellular senescence and apoptosis.

The ribonucleoprotein enzyme, telomerase, provides a compensatory mechanism for telomere shortening. Telomerase consists of a telomerase reverse transcriptase subunit (TERT) and a telomerase-associated RNA component (TERC) that promotes the elongation, repair and stabilisation of the terminal regions of telomeric DNA (Chan & Blackburn, 2004). In humans, telomerase is expressed at high quantities pre-birth and decreases with age (Flores *et al*., 2008). Indeed, ageing is associated with telomere shortening (Steer *et al.,* 2007; Müezzinler *et al*., 2012; Hermann *et al*., 2018; Liu *et al*., 2019). Despite decreasing telomerase activity with age, together with the shelterin complex proteins discussed above, telomerase is an important enzyme that regulates telomere length, by addition of telomere nucleotide sequences. Considering telomeres shorten with every cycle of cell replication and their role in preserving genomic integrity (Chan & Blackburn, 2004), telomere lengths are considered an index of biological ageing, rather than just chronological aging (Aviv *et al*., 2006). Hence it is not surprising that telomere length has been considered a biomarker for the prediction of age-related disease, and all-cause and disease specific mortality (Jeanclos *et al*., 2000; Aviv *et al*., 2006; Willeit *et al*., 2010; Hermann *et al*., 2018).

With cell replication, telomere shortening occurs in all tissues. In clinical studies, measuring telomere length in tissues poses a practical limitation, as invasive biopsies are often required to obtain a sample. Therefore, telomere length measured in blood leukocytes has been used as a surrogate marker of the entire body's telomere shortening (Herrmann *et al*., 2018). The use of leukocyte telomere length (LTL) as a surrogate marker for overall telomere shortening is considered accepted practice and is often used in clinical studies, as the acquisition of blood samples is more feasible than obtaining

tissue specific samples (Herrmann *et al*., 2018). However, whether LTL is indeed an accurate marker of telomere shortening in all tissues remains uncertain. In this regard, leukocyte telomere length has been correlated with tissue telomere length in several tissues including aortic cells (Richard *et al*., 2008), buccal cells (Finnicum *et al.,* 2017), skeletal muscle (Ahmad *et al.,* 2012) and skin (Friedrich *et al*., 2000). In skin cells, leukocyte telomere length was significantly shorter than tissue telomeres (Friedrich *et al*., 2000). Dlouha *et al.* (2014) reported that leukocyte telomere length was poorly related to telomere length in 11 tissue samples including the liver, heart, spleen, brain, skin, triceps, tongue mucosa, intercostal skeletal muscles, kidney and subcutaneous and abdominal fat from 12 cadavers (tissues obtained less than 12 hours after death). They showed that leukocyte telomeres were more variable compared to tissues telomere length. Leukocyte telomere lengths were significantly longer compared to some of the tissue sample telomeres, including the liver, brain, muscles, skin, spleen and tongue mucosa, but was not different from adipose tissue and renal tissue telomere lengths (Dlouha *et al*., 2014). Similarly, others have reported that leukocyte telomere shortening does not reflect changes in tissue telomere length from follicular cells (Lara-Molina *et al*., 2020), other blood cells (Olsson *et al*., 2020) and lung tissue (Everaerts *et al*., 2018). In this regard, it has been suggested that mechanisms controlling telomere length are different in various tissues (Friedrich *et al.*, 2000; Lara-Molina *et al*., 2020). Despite the lack of conclusive evidence, several studies have suggested that leukocyte telomere length is a sufficiently accurate surrogate for telomere shortening (Mather *et al*., 2011; Müezzinler *et al*., 2012; Sanders & Newman, 2013; Dlouha *et al.,* 2014; Goglin *et al*., 2016; Hermann *et al.,* 2018).

2.5 Factors affecting telomere length

Although it is well established that telomeres become progressively shorter with cell division and that telomere attrition is considered a normal ageing process, it has been shown that telomere attrition can be accelerated in some individuals (Sanders & Newman, 2013). Several risk factors for increased rate of telomere attrition have been identified. Besides age, other non-modifiable risk factors include sex and genetics. (Hermann *et al*., 2018). Men have a significantly higher rate of telomere attrition compared to women (Blasco, 2007). This rate of telomere attrition is believed to be mediated by the effects of oestrogen. Increased oestrogen concentrations increase telomerase activity, and has antioxidative effects (Kyo *et al*., 1999; Barrett & Richardson, 2011). With regard to genetic influences, Blasco (2007) showed that telomere length is influenced by parents in which parental telomere length can be used to predict up to 80% of offspring's telomere length. Besides non-modifiable risk factors, modifiable risk factor exposure also influences telomere attrition. Several risk factors affect telomere length including smoking, physical activity, obesity, alcohol consumption, several drugs, dietary intake of antioxidants and vitamins, chronic inflammation and stress. Although an in-depth discussion of these risk factors are beyond the scope of this thesis, it is noteworthy that the most likely proposed mechanism whereby risk factor exposure increased telomere attrition is via increased inflammation and oxidative stress (Balaban *et al*., 2005).

2.6 Oxidative stress and leukocyte telomere length

An imbalance between reactive oxygen species (ROS) production and antioxidative effects is known as oxidative stress (Balaban *et al*., 2005). The increased production of

ROS is often the result of risk factor exposure including smoking and obesity, among others (Vassalle *et al*., 2009). In addition, ultraviolet radiation exposure, certain disease states and specifically exposure to increased systemic inflammation can also increase ROS production (Oikawa *et al*., 2001; Balaban *et al*., 2005). The DNA sequence of telomeres consist of high quantity of guanine residues which is more prone to oxidative damage (Kawanishi & Oikawa, 2004). In this regard, with oxidative stress, there is an increase in DNA damage and cleavage of the poly-guanosine sequence on telomeres (Oikawa *et al*., 2001). This will result in a large portion of un-replicated ends due to less efficient nucleotide damage repair responses, hence accelerating telomere attrition (von Zglinicki *et al*., 2000; von Zglinicki, 2002).

Table 2.1 shows a summary of animal and human studies that determined the relationship between oxidative stress and telomere length. In animal studies, in two different models of oxidative stress exposure, results show progressive shortening of telomeres with oxidative stress (Cattan *et al*., 2008; Martínez-González *et al*., 2019). In human studies the results are controversial. Although some studies showed associations between oxidative stress markers and shorter leukocyte telomere length (Demissie *et al*., 2006; Starr *et al*., 2008; Salpea *et al*., 2010), this was not consistent in all studies (Shen *et al.,* 2009; Watfa *et al*., 2011). The different methods of inducing and measuring oxidative stress in these studies may limit the comparison between studies. Majority of the aforementioned studies did not report inflammatory cytokines. In this regard, chronic exposure to inflammation is one the main sources of increased oxidative stress (Balaban *et al*., 2005). The section below will highlight the role of inflammation on leukocyte telomere length.

Table 2.1 The relationship between oxidative stress and leukocyte telomere length in animal and human studies

Author	Study design	LTL method	Measure of oxidative stress	Main findings
Animal studies				
Cattan *et al*., 2008	CAST/EI mice: n = 7 control n = 7 experimental	Southern blot	L-buthionine sulfoximine, an inhibitor of γ-glutamyl cysteine synthase	Telomere shortening based on exposure to increased oxidative stress stimuli is tissue dependent
Martínez-González *et al*., 2019	n = 12 control Alzheimer's transgenic mice: n = 7 (5 months) n = 9 (9 months) n = 8 (13 months)	qRT- PCR	DCFH	With progression of Alzheimer's disease, there is an increase in oxidative stress and a decrease in telomere length. No direct relationship between LTL and oxidative stress
Human Studies				
Demissie *et al*., 2006	n = 156 control n = 171 hypertension	Southern blot	Urinary 8-epi-$PGF_{2\alpha}$	LTL negatively associated with oxidative stress
Salpea *et al*., 2010	n = 448 healthy controls n = 742 Type 2 DM	qRT- PCR	Plasma TAOS (inhibitor of peroxidase-mediated formation of the 2,2-azino-bis-3-ethylbensthiazoline-6-sulfonic acid radical)	Negative relationship between plasma TAOS and LTL
Starr *et al*., 2008	n = 190 general population	qRT-PCR	Oxidative stress gene polymorphisms	Oxidative stress genes associated with LTL
Shen *et al*., 2009	n = 1110 control n = 1061 breast cancer	qRT-PCR	urinary 15-F_{2t}-IsoP & 8-oxodG	No relationship between LTL and oxidative stress
Watfa *et al*., 2011	n = 15 control n = 20 Parkinson's disease	qRT-PCR	Carbonyl protein	No relationship between LTL and oxidative stress

n, sample size; qRT- PCR, quantitative real time polymerase chain reaction; DCFH, Dichloro-dihydro-fluorescein; LTL, Leukocyte telomere length; 8-epi-PGF 2α, 8-epi-prostaglandin F2alpha; DM diabetes mellitus; TAOS, total antioxidant status; 8-oxodG, 8-oxo-7,8-dihydrodeoxyguanosine; 15-F_{2t}-IsoP, 15-F_2-isoprostanes

2.7 Effects of inflammation on leukocyte telomere length

In an inflammatory state, in order to restore homeostasis, there is an increased production of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) by tissue resident macrophages (Gordon & Taylor, 2005). Increased circulating cytokines increase the peripheral leukocyte count by stimulating differentiation and proliferation of new inflammatory cells from hematopoietic stem cell in the bone marrow (Gordon & Taylor, 2005; Chmielewski & Strzelec, 2018). Inflammation reduces the lifespan of leukocytes, as such, they die through cellular senescence and are removed from the circulation by the liver and the spleen (Chmielewski & Strzelec, 2018). In this regard, inflammation has been reported to increase leukocyte turnover, which further increases the proliferation of hematopoietic stem cell to compensate for the leukocyte loss from the circulation (Aviv & Levy, 2012; Majno & Joris, 2004; Aviv *et al*., 2015; Chmielewski *et al*., 2016). As inflammation increases, the numbers of white blood cells may increase or there may be a shift in white blood cell subtypes, leading to further increases in pro-inflammatory cytokines, as well as reactive oxygen species and other molecules that can perpetuate the inflammatory response (Libby, 2007).

Despite the effects of inflammation on leukocyte physiology, the effects of chronic inflammation on telomere shortening are controversial (Steer *et al.,* 2007; Aubert & Lansdorp, 2007). Table 2.2 provides a summary of the studies where the effects of inflammation on leukocyte telomere length were investigated. In an animal model of low-grade inflammation, shorter telomeres in rats exposed to inflammation compared to control were reported (Raymond *et al.*, 2014). In contrast, another animal study that induced an inflammatory state by knocking out the NF-κB1 subunit, showed no

differences in telomere lengths between control and knockout mice (Jurk *et al*., 2014). In case-control studies, although several different models of inflammatory conditions and auto-immune diseases were used, majority reported shorter telomeres in patients compared to controls (Artlett *et al*., 1996; Samani *et al*., 2001; Steer *et al*., 2007; Tamayo *et al*., 2010; Masi *et al*., 2011; Haque *et al*., 2013). In contrast, some studies reported no difference in leukocyte telomere length between patients and controls (Klapper *et al*., 2004; Beier *et al*., 2007). One study reported longer telomere lengths in patients exposed to inflammation compared to controls (MacIntyre *et al*., 2008). A recent meta-analysis further supported the lack of evidence that telomeres are shorter in RA patients compared to controls (Lee & Bae, 2018).

Chronically activated T-cells, as typically reported in patients with high-grade inflammation such as RA, are unable to stimulate increased telomerase activity (Valenzuela & Effros, 2002). As previously discussed, telomerase is a key enzyme responsible for the prevention of telomere attrition (Weng, 2012). It has been suggested that the pro-inflammatory cytokine, TNF-α, inhibits telomerase activity by affecting the expression of CD28 in $CD8^+$ T lymphocytes (Parish *et al*., 2009). Therefore, in RA patients, telomerase activity in CD4 T-cells has been reported insufficient to protect against telomere shortening by one (Fujii *et al*., 2009), but not other studies (Dehbi *et al*., 2013). In contrast, one study in systemic lupus erythematosus patients reported increased telomerase activity in patients compared to controls (Klapper *et al*., 2004). In the general population, some studies have shown that shorter telomeres are associated with markers of inflammation, including CRP and IL-6 (Bekaert *et al*., 2007; Carrero *et al*., 2007; O'Donovan *et al.*, 2011; Masi *et al*., 2014). Not all studies in the general population

support these findings (Steer *et al*., 2007; Fitzpatrick *et al*., 2007; Wong *et al*., 2014). In inflammatory disorders, several studies failed to show an inverse relationship between telomere length and inflammation and/or disease activity (Steer *et al*., 2007; Tamayo *et al*., 2010; Antoniou *et al*., 2012; Blinova *et al*., 2016). These contrasting findings in inflammatory disorders necessitate further investigations.

Despite the controversial results regarding the effects of inflammation and leukocyte telomere length, this relationship seems to be bidirectional. Although inflammation may increase telomere attrition, when telomeres reach a critical length cellular senescence and apoptosis pathways are activated. Senescent cells are a potent source of pro-inflammatory mediators and reactive oxygen species (ROS) (Correia-Melo *et al*., 2014; Campisi*,* 2013). This may result in a cycle of aberrant regulation and ensuing disease (Campisi, 2013). In this regard, inflammation is strongly related to biological aging (Carrero *et al*., 2007; Chung *et al*., 2009). Evidence suggests a causal role for inflammation in the pathogenesis of multiple age-related diseases, including endothelial dysfunction, atherosclerosis, cancer, autoimmune disorders, neurodegenerative diseases and diabetes mellitus (Lynch *et al*., 2017; Murata, 2018; Walker, 2019; Liberale *et al*., 2020). Considering the link between inflammation and telomere length, and the relationship between inflammation and several disease states, it is not surprising that telomere length has been considered a biomarker for age-related diseases.

Table 2.2 The Effects of inflammation on telomere length in human and animal studies

Author	Study design	Inflammation model	LTL method	Main findings
Animal studies				
Jurk *et al*., 2014	n = 6 control n = 6 NF-κB knockout	NF-κB gene knockout	qRT-PCR	No differences in LTL between control and inflammation group
Raymond, 2014	n = 30 control n = 30 experimental	*Staphylococcus aureus*	qRT-PCR	LTL significantly shorter in the experimental group when compared to control
Case control human studies				
Artlett *et al*., 1996	n = 96 control n = 43 patients	Systemic sclerosis	RFLP	LTL significantly shorter in SSc patients
Samani *et al*., 2001	n = 20 control n = 10 CAD patients	CAD	Southern blot	CAD patients had significantly shorter LTL when compared to control
Steer *et al*., 2007	n = 1151 control n = 1987 patients	Rheumatoid arthritis	Southern blot	LTL shorter in RA patients. No relationship between CRP and LTL
MacIntyre *et al*., 2008	n = 107 control n = 43 patients	Limited cutaneous systemic sclerosis	Southern blot	Patients had longer LTL and increased telomerase activity.
Tamayo *et al*., 2010	n = 130 control n = 86 RA, n = 56 PA, n = 59 AS, n = 35 OP, n = 34 OA	RA, PA, AS, OP, OA	qRT-PCR	RA, AS and PA patients had longer telomeres while OP patients had shorter telomeres when compared to control.
Masi *et al*., 2011	n = 207 controls n = 356 patients	Periodontitis	qRT-PCR	Shorter LTL in patients compared to control. LTL negatively associated with CRP
Haque *et al*., 2013	n = 63 control n = 63 patients	Systemic lupus erythematosus	qRT- PCR	SLE patients shorter LTL compared to control
Klapper *et al*., 2004	n = 9 control n = 9 patients	Systemic lupus erythematosus	Southern blot	No differences in LTL between patients and controls. High telomerase activity in SLE
Beier *et al*., 2007	n = 20 control n = 22 patients	Systemic lupus erythematosus	FISH	No differences in LTL between patients and controls
Antoniou *et al*., 2012	n = 6 controls n = 7 IPF	IPF & RA-IUP	qRT-PCR	No significant difference in telomere length between case and control

	n = 6 RA-IUP		
Cross-sectional human studies			
Bekaert *et al*., 2007	n = 2509 general population	Southern blot	LTL negatively associated with IL-6 and CRP
Carrero *et al*., 2007	n = 195 hemodialysis patients	qRT-PCR	LTL negatively associated with CRP in hemodialysis patients.
Fitzpatrick *et al*., 2007	n = 419 general population	Southern blot	No relationship between CRP and LTL
O Donovan *et al*., 2011	n =1962 general population	qRT-PCR	Short LTL associated with high IL-6 & TNF- α but not CRP
Weischer *et al*., 2012	n = 19838	qRT-PCR	LTL was negatively associated with CRP, BMI, Smoking, Hypertension, increased development of MI & IHD
Masi *et al*., 2014	n = 1080 between the age 13 and 16	qRT-PCR	LTL negatively associated with CRP and Fibrinogen
Wong *et al*., 2014	n = 87 general population	qRT- PCR	LTL negatively associated with CRP but not with IL-6 and TNF-α

n, sample size; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells ; qRT-PCR, quantitative real time PCR; LTL, leukocyte telomere length; RFLP, restriction fragment length polymorphism; CAD, coronary artery disease; SSc, Systemic sclerosis; CRP, c- reactive protein; RA, Rheumatoid arthritis; PA, Psoriatic arthritis; AS, ankylosis spondylitis; OA, osteoarthritis; OP, osteoporosis ; SLE, systemic lupus erythematosus; FISH, fluorescent in situ hybridization; IPF, Idiopathic pulmonary fibrosis; RA-UIP, Rheumatoid arthritis-usual interstitial pneumonia; IL-6, Interleukin- 6; TNF-α, Tumor necrosis factor alpha; BMI, body mass index ;MI, myocardial infarction; IHD, ischemic heart disease

2.8 Leukocyte telomere length as a biomarker for cardiovascular disease

Leukocyte telomere length has been associated with several age-related diseases, including neurodegenerative disorders (Thomas *et al.*, 2008), cancer (Odigari *et al*., 1994; Nakamura et *al*., 2000; Heaphy *et al*., 2010), osteoporosis (Valdes *et al*., 2007) and CVD (Fitzpatrick *et al*., 2007; Fyhrquist *et al*., 2013; Haycock *et al*., 2014). CVD is one of the leading causes of disability and mortality worldwide (Noncommunicable diseases country profiles 2018, 2018). Ageing and inflammation are both considered major risk factors for the development of CVD (Avina-Zubieta *et al*., 2008; Samani & van der Harst, 2008; Avina-Zubieta *et al*., 2012; Lynch *et al*., 2017; Liberale *et al*., 2020). Biomarkers that can be used in CVD risk stratification have received considerable attention in the literature over the past decade. Telomere length has also been suggested as a biomarker for CVD prediction and risk stratification (Fitzpatrick *et al*., 2007; Fyhrquist *et al*., 2013; Haycock *et al*., 2014). In this regard, in a meta-analysis including 27 observational studies, it was reported that with a one standard deviation decrease in leukocyte telomere length, there is up to 24% increase in CVD risk (D'mello *et al*., 2015). It has also been suggested that leukocyte telomere length can be used to predict mortality in CVD patients (Carty *et al*., 2015).

In human studies, decreased telomere lengths have been related to cardiovascular mortality (Cawthon *et al*., 2003; Epel *et al*., 2009). Shorter telomeres have been linked to increased risk for cardiovascular events including stroke, heart failure and myocardial infarction (Brouilette *et al*., 2003; Fitzpatrick *et al*., 2007; Zee *et al*., 2009; Willeit *et al*., 2010; Ding *et al*., 2012; Weischer *et al*., 2012; D'mello *et al*., 2015). The association between telomere length and CVD is independent of age, sex, and traditional CVD risk

factors (Fitzpatrick *et al*., 2006; Brouilette *et al*., 2003). In mechanistic studies, shorter telomeres have been linked to cellular senescence in cardiomyocytes (Chimenti *et al*., 2003; Leri *et al*., 2003).

In the vasculature, shorter telomere length has been associated with the presence of atherosclerosis (Benetos *et al*., 2004; Matthews *et al*., 2006; Mainous *et al*., 2010; Khan *et al*., 2012) endothelial dysfunction (Minamino *et al*., 2002) and arterial stiffness (Benetos *et al*., 2001, Tentolouris *et al*., 2007; Nawrot *et al*., 2010; Wang *et al*., 2011; Raymond, 2014). Some have suggested that senescent endothelial cells, induced by telomere shortening, produce molecules that are involved in the process of atherogenesis (Minamino *et al*., 2002; Libby, 2007). In contrast, others have posited that local factors related to altered haemodynamics, may control telomere length in the arterial wall (Fyhrquist *et al*., 2013). Nevertheless, telomere length has been associated with vascular ageing in the general population (Nilsson, 2012).

In inflammatory disorders, the relationship between leukocyte telomere length and traditional CVD risk factors, atherosclerosis and arterial function have been controversial (Haque *et al*., 2013; Raymond, 2014; Zidan, 2020). In the aforementioned studies, paradoxically longer telomere lengths were associated with a worse CVD risk profile. These authors were unable to provide mechanistic links between these paradoxical relationships and suggested further investigations to confirm their results. Several confounding factors, including anti-inflammatory treatments, were mentioned, which may affect the association between telomere length and CVD in inflammatory disorders. Therefore, mechanistic and basic studies to elucidate the role of high-grade inflammation

and anti-inflammatory drug treatment on telomere length and its association with CVD are warranted.

2.9 The collagen induced arthritis model

Human RA studies investigating telomere shortening have thus far been hindered by several confounding factors. Previous human studies examining telomere shortening in RA had small sample sizes and largely used cross-sectional designs (Costenbader *et al*., 2011). The majority of these studies did not account for smoking, obesity, drug treatment and other confounding factors (Costenbader *et al*., 2011; Lee & Bae, 2018). Indeed, drug treatments such as methotrexate, immunosuppressants or corticosteroids undoubtedly affect telomere length (Koetz *et al.,* 2000; Weyand *et al.,* 2002; Colmegna *et al.,* 2008). Further to this, patients show large heterogeneity due to differences in RA disease characteristics. Therefore, the use of animal models of high-grade inflammation may provide greater insight into the association between inflammation and telomere length and the impact that anti-inflammatory agents have on this relationship, without the influence of confounding or mediating factors. Although several animal models have been proposed, the structural and immunological changes of the collagen-induced arthritis (CIA) rat model best resemble that of RA (Larsson *et al*., 1990; Bolon *et al*., 2010). As male rats were shown to have a greater inflammatory response to collagen injections, previous CIA models mostly included male rats (Wooley, 2004; Wilson-Gerwing *et al*., 2013), however, evidence from human studies show that women are at a higher risk of developing RA (Ma & Xu, 2013). Therefore, the effect of high-grade inflammation on telomere length in both male and female rats require investigation.

2.10 Problem statement

RA is a chronic inflammatory disorder that severely impacts quality of life. Besides the destructive effects on synovial joints, inflammation has also been linked to several extra-articular manifestations, including CVD. Indeed, chronic systemic inflammation significantly increases the risk of CVD in patients exposed to high grade chronic inflammation. Anti-inflammatory biologic DMARDs have shown to be effective in reducing disease activity, however, its effectiveness in managing CVD risk is controversial.

Inflammation accelerates telomere shortening. Telomere length is considered a marker of biological ageing, cellular senescence and CVD risk. In chronic inflammatory disorders the effects of inflammation on telomere attrition and the relationship between telomere length and subclinical CVD are controversial. Whether biologic DMARDs influence telomere length in conditions of high-grade inflammation and whether biologic DMARDs influences the relationship between leukocyte telomere length and CVD risk is uncertain. An improved understanding of biologic DMARDs on the regulation of leukocyte telomere lengths will provide knowledge on the far-reaching effect of chronic inflammation on CVD.

2.11 Aim

This study aims to determine the effect of chronic inflammation on leukocyte telomere length and whether treatment with biologic disease modifying anti-rheumatic drugs has an impact on the inflammation-induced effects on leukocyte telomere length in a type II collagen induced arthritis (CIA) model in Sprague-Dawley rats.

2.12 Objectives

1. To determine the effects of collagen induced high-grade inflammation on leukocyte telomere length and adipose tissue telomere length.

2. To determine the effect of treatment with biological anti-inflammatory drugs (TNF-α inhibitors and IL-6 antagonists) on leukocyte and adipose tissue telomere length, in a model of collagen induced high-grade inflammation.

3. To determine whether relative telomere length is a potential biomarker of vascular and cardiac function in a model of collagen induced high-grade inflammation.

CHAPTER 3

METHODS

3.1 Animals

Three-month-old, male and female Sprague Dawley rats were obtained from the Central Animal services (CAS) of the University of the Witwatersrand as part of a larger study. From this larger study, high quality DNA could be extracted from 70 rats (male: n = 37 female n = 33) that were included in the current study. Rats were housed in individual cages in temperature-controlled rooms on a 12-hour light-dark cycle, to monitor individual sickness responses and food intake. Male and female rats were housed in separate rooms. All the procedures in this study were approved by the Animal Research Ethics Committee of the University of the Witwatersrand (approval number 2017/03/21C, Appendix A).

3.2 Study design

Rats were habituated for two weeks where they were weighed, and arthritis scores were measured weekly. Rats were habituated to restrainers, and blood pressure was measured twice a week. Rats had free access to water and standard rat chow during the habituation period and the rest of the study. After the habituation period, rats were randomly divided into four groups. The first group, the control group (n = 25, male = 14, female = 11), received a subcutaneous saline injection (0.2 ml) at the base of the tail. The other three groups, namely the inflammation (n = 22, male = 12, female = 10), the TNF-α blocker (n = 11, male = 6, female = 5) and IL-6 blocker groups (n = 12, male = 5, female = 7) were exposed to an arthritis-inducing protocol. In the three groups exposed to the arthritis protocol, upon signs of inflammation the inflammation groups received no treatment, the TNF-α blocker group received intraperitoneal injections of a TNF-α blocker

and the IL-6 blocker group received intraperitoneal injections of an IL-6 receptor blocker for six weeks. Over the course of the study body weight, blood pressure and arthritis scores were measured. At the end of the six-week treatment period, rats were anaesthetised by intramuscular injections of ketamine (100 mg/kg) and xylazine (5 mg/kg), during which time cardiac function was measured by echocardiography. Thereafter rats were killed by thoracotomy, hearts were removed and weighed and indexed to body weight. Vascular reactivity in response to vasodilators and vasoconstrictors were assessed in renal arteries using a wire myograph. Blood samples were obtained to measure circulating inflammatory markers and to extract DNA for determining leukocyte telomere length. Visceral adipose tissue was removed to extract DNA for determining adipose tissue telomere length.

3.3 Arthritis inducing protocol and drug treatment intervention

High grade systemic inflammation was induced using an arthritis inducing protocol as previously described (Mokotedi *et al*., 2019). To induce arthritis, rats were injected subcutaneously with 0.2 ml (200 µg) Bovine type II collagen (BCII) (Chondrex cat. #20021, Redmond, WA, USA) emulsified into incomplete Freud's adjuvant (Chondrex cat. #7002, Redmond, WA, USA) at the base of the tail. To ensure a high incidence and severity of arthritis a 0.1ml booster injection of the same emulsion was administered seven and 21 days after the first immunisation. The control group received a 0.2 ml subcutaneous injection of buffered saline solution. All injections were administered under general anaesthesia (isoflurane). At the first sign of inflammation, approximately three to four weeks after the primary immunisation, the TNF-α blocker and IL-6 blocker groups received biologic anti-inflammatory drugs in line with previously reported studies

(Kobayashi *et al*., 2014; Totoson *et al*., 2016; Hançerli *et al*., 2017). The IL-6 blocker group received intraperitoneal injections of Tocilizumab, an IL-6 receptor blocker, at a dose of 8mg/kg once a week for six weeks. The TNF-α blocker group received intraperitoneal injection of Etanercept, a TNF-α antagonist at a dose of 10 mg/kg every three days for six weeks. All rats exposed to the arthritis inducing protocol (inflammation, TNF-α blocker and IL-6 blocker groups) received Tramazac (1-4mg/kg) for pain management.

3.4 Arthritis scores and paw thickness

To measure the severity of arthritis, signs of inflammation in the hind paws of the rats was scored. To reduce intra-observer variability, paw scoring was performed by a single, trained individual. To reduce intra-observer variability, paw scoring was performed by a single, trained individual. The severity of inflammation (with a maximum of eight) for the hind paws were scored on a scale of zero to four as previously described (Mokotedi *et al*., 2019), The scoring was as follows: 0 = no swelling or focal redness in the paws; 1 = slight swelling of focal redness in the paws; 2 = low to moderate oedema in the paws; 3 = pronounced oedema in the paws with limited joint use and 4 = excessive oedema with rigidity and deformity of the paws. To provide a more objective measure of arthritis severity, paw thickness was measured at the ankle joint and the tarsometatarsal joints of the hind paws of the rats every two weeks, using a digital calliper.

3.5 Blood pressure

The blood pressure of the rats were measured with a tail cuff, non-invasive blood pressure amplifier (Biopac Systems, Santa Barbara, CA, USA). To measure the blood pressure,

rats were placed in restrainers with adjustable restriction panels. The tail was placed on a heating pad for ten minutes prior to measuring blood pressure. An average of three blood pressure measurements were taken at midday, once every two weeks.

3.6 Echocardiography

Rats were anaesthetised by intramuscular injections of ketamine (100 mg/kg) and xylazine (5 mg/kg) where after cardiac geometry and function were measured by echocardiography (Acuson SC 2000, Siemens Medical Solutions, USA). To determine cardiac geometry, the left ventricle was imaged with a high resolution (10MHz) ultrasound probe in the parasternal long axis view. Using 2D echocardiography, M-mode imaging was obtained to measure the interventricular septal thickness (IVST), left ventricular internal diameter (LVID) and left ventricular posterior wall thickness (PWT) in systole (s) and diastole (d) according to standard conventions (Lang *et al.*, 2015). The Teichholz formula was used to determine left ventricular end diastolic and end systolic volume from which stroke volume and ejection fraction were determined (Teichholz *et al.*, 1976). Midwall fractional shortening (FSmid), a marker of systolic function was calculated as [(LVIDd + PWTd/2 + IVSTd/2) - (LVIDs + Hs/2)]/[LVIDd + PWTd/2 + IVSTd/2)] x 100, where H represents the combined septal and posterior wall thickness (De Simone *et al.*, 1994). Relative wall thickness, a marker of left ventricular hypertrophy, was calculated as (IVSTd + PWTd) / LVIDd (Ganau *et al.*, 1992).

Diastolic function was measured using pulsed wave Doppler and Tissue Doppler in the apical four chamber view. Early (E) and late (A) mitral inflow velocity was measured by pulsed Doppler by placing the cursor at the tips of the mitral valve leaflet tips. Data were

expressed as the E/A ratio as a marker of relaxation. The rate of tissue lengthening (peak relaxation velocity) at the mitral annulus was measured using Tissue Doppler Imaging (TDI) during early (e') and late (a') diastole. A reduction in e' is considered one of the earliest markers of impaired relaxation. The e'/a' ratio was calculated and is considered a marker of ventricular stiffness. Because the mitral inflow velocity (E) is influenced by ventricular relaxation and atrial pressure and e' is considered a marker of relaxation, independent of pressure, the E/e' ratio was calculated as marker of left ventricular filling pressure.

3.7 Vascular reactivity

After echocardiography, rats were killed via thoracotomy, and the heart and kidneys were removed. The arteries from the kidney were isolated and the artery rings were cleaned of connective tissue and fat. Renal artery rings were suspended in a wire myograph system (model 610M, Danish Myo Technology, Aahrus, Denmark), designed to measure the reactivity of blood vessels. Renal artery rings were threaded over two parallel stainless-steel wires which were secured to support jaws, one of which were attached to a precision micrometer and the other to a force transducer for measuring force development. Isometric force measurements were obtained using a PowerLab (4SP, ADInstrument) data acquisition system. The renal artery rings were mounted at an optimal resting tension and was kept at 37 °C in physiological saline solution in an oxygenated chamber for 60 minutes. Thereafter renal artery rings were exposed to 80 mM potassium chloride (KCL) to induce a maximal, reference contraction response. Arteries were then exposed to half-log increases in phenylephrine concentrations between 0.1 nM and 0.1 mM. Renal artery contraction was expressed as a percentage of the maximal, reference contraction.

Relaxation induced by acetylcholine was determined during phenylephrine-induced contraction and was expressed as the percentage of baseline tension during phenylephrine-induced contraction.

3.8 Circulating inflammatory and endothelial activation markers

After thoracotomy, blood samples were obtained and centrifuged before being stored at -80°C for further analysis. To determine circulating inflammatory marker concentrations, blood samples were used to measure serum IL-6, TNF- α and CRP concentrations using solid-phase sandwich enzyme linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology Co. Ltd, Wuhan, China) in duplicate. The coefficients of variation were <10% for all kits. The lower detection limit for IL-6, TNF-α and CRP was 62.50 pg/ml, 78.13 pg/ml and 0.31 ng/ml respectively. To determine endothelial activation, an early risk marker of endothelial dysfunction and atherosclerosis, vascular adhesion molecule 1 (VCAM-1) concentrations were measured in a sub sample (n = 44) using ELISA (Elabscience Biotechnology Co. Ltd, Wuhan, China). The lower detection limit for VCAM-1 was 12.5 pg/ml.

3.9 Telomere length

Telomere length was measured via real time quantitative polymerase chain reaction (qRT-PCR). DNA was extracted from whole blood and visceral adipose tissue samples, where after the respective telomere lengths were determined as described below.

DNA extraction

Leukocyte DNA were isolated from whole blood samples using a NucleoSpin® DNA, extraction kit (Macherey-nagel GmbH & Co. KG, D-52355, Duren, Germany) according manufacturer guidelines. Visceral adipose tissue was homogenised, and DNA was extracted using an Accuprep® Genomic DNA extraction kit (Bioneer, K-3032, Daejeon, South Korea). The quality and quantity of the extracted DNA was determined by NanoDrop™ One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Fischer Scientific™, 840274200, Waltham, USA). The samples used in this study had a DNA yield of approximately ≥ 100 µg/ml. The DNA was transferred to cryotubes and stored at -20°C until further analysis.

Real-time quantitative PCR (qPCR)

A quantitative real-time PCR (qRT-PCR) method was used to determine relative telomere length of leukocytes and adipose tissue DNA (Gil & Coetzer, 2004, Cawthon, 2002). This method determines the factor by which telomere repeat copy number (T) differs from the copy number of a single copy gene (S). The single copy gene used in this protocol was the rat pyruvate kinase gene. Samples were amplified in duplicate in two different polymerase chain reaction (PCR) amplifications; one to determine telomere repeat copy number and the other to determine pyruvate kinase copy number. The PCR reactions were carried out using a StepOneplus real-time PCR machine (Applied Biosystems, USA) as per manufacturer's instructions. Each PCR reaction mix had a final volume of 20 µl consisting of approximately 50 ng genomic DNA, 10 µl SYBR Green Real-Time PCR Master Mix (ThermoFisher, USA), appropriate forward and reverse primers for either the telomere or pyruvate kinase reactions (5 pmol of each primer/reaction) (Inqaba Biotec, Pretoria, South Africa) and the volume was made up to 20 µl with nuclease free water.

Primer sequence and thermal profiles for the qRT-PCR reactions are shown in Tables 3.1 and 3.2 respectively.

Table 3.1 The telomere and pyruvate primer sequences for PCR amplifications

	Primer sequence
Telomere Forward (5'-3')	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGG TTTGGGTT
Telomer Reverse (5'-3')	GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT
Pyruvate Kinase Forward (5'-3')	TGTGGGTGATCTGGTGATTGTGGT
Pyruvate Kinase Reverse (5'-3')	AGGCATTTCAGGATACGCTCAGCA

Table 3.2 The LightCyler thermal profiles for the telomere and the pyruvate kinase PCR amplifications

	Number of cycles	Temperature (°C)	Duration
Thermal profile telomere			
Activation	1	95, 50	2 min, 2 min
Amplification	25	95, 54	15 seconds, 2 min
Cooling	1	30	30 seconds
Thermal profile pyruvate kinase			
Activation	1	95,50	2 mins, 2 mins
Amplification	30	95,58	15 seconds, 1 min
Cooling	1	30	30 seconds

The telomere primers used in this method were designed to ensure that in the first PCR cycle the forward and reverse telomere primers hybridized to the telomere sequence, but with a mismatch in the last six base pairs at the 5'-end. This mismatching ensures that the primers favour hybridization to the products formed at the end of the first PCR cycle over binding to the DNA template in subsequent cycles, thus the amount of telomere product at the end of the PCR is proportional to the product produced after the first PCR cycle. The amount of product produced after the first round of PCR cycle is determined by telomere length, hence the amount of telomere product produced in this method is directly proportional to telomere length.

Analysis of PCR amplification curves and construction of standard curves was carried out using the StepOne software v2.3 (Applied Biosystems, USA). The qRT-PCR method used in this study, includes a SYBR® Green dye, which is a double-stranded DNA intercalating dye that only fluoresces when binding to double stranded DNA. As the number of PCR cycles increases, the amount of double-stranded DNA increases and consequently the amount of fluorescence eventually reaches a threshold value (Ct), which is the point where fluorescence levels are statistically significant, hence the greater the amount of starting template there is, the fewer the number of PCR cycles required to reach Ct. With regards to telomere length, the telomere Ct is proportional to telomere length, with a smaller Ct representing a greater telomere length. The telomere Ct of each sample was compared to its respective pyruvate kinase Ct. Pyruvate kinase has only one copy per rat genome and allows for the correction of different sample DNA concentrations, thus

allowing for inter-individual comparisons. The relative telomere length of each sample was calculated using the following formula:

T/S ratio = $2^{(-\Delta^{Ct(sample)} - \Delta^{Ct(standard)})}$ where ΔCt is Ct (telomere) - Ct (pyruvate kinase)

Standard curve

To standardise the T/S ratios, one sample outside of our study, was diluted 5 times to produce a range from 100ng/ml to 3.125ng/ml of DNA concentration. The telomere and pyruvate kinase Ct of these concentrations were measured. The results produced a standard curve as seen in Figure 3.1, which indicate a strong negative correlation between DNA concentration and telomere Ct ($r = -0.998$, $p < 0.0001$) and pyruvate kinase ($r = 0.994$, $p < 0.0001$) and the slope of the linear relationship was -2.92 and -3.41 respectively. Samples that fell outside of the range of the linear correlation were diluted and re-run to ensure that they fell within the linear range of the standard curve.

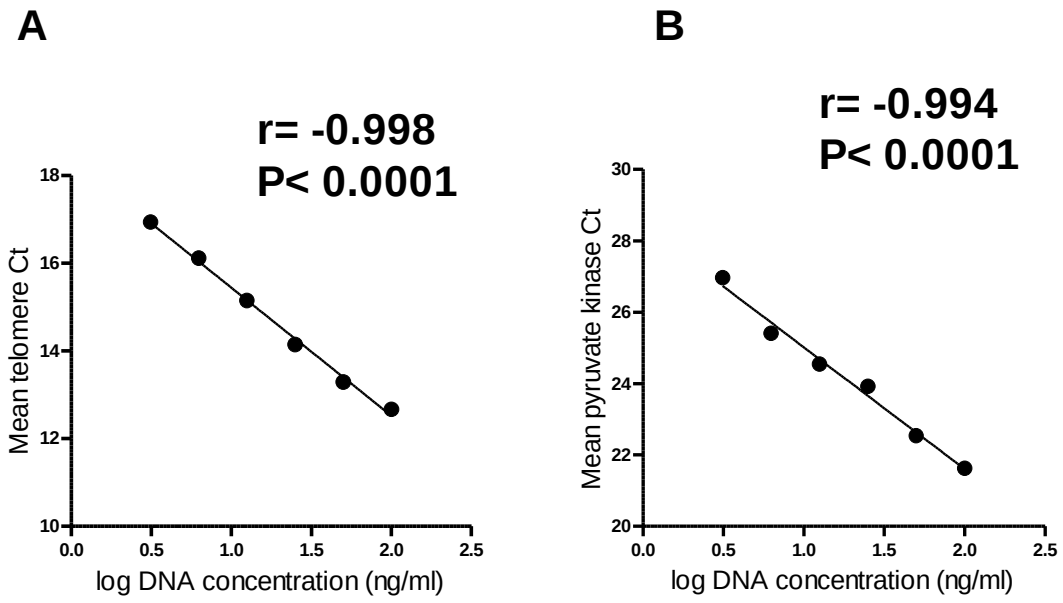


Figure 3.1 The relationship between DNA concentration (plotted as log) and telomere Ct (A) and pyruvate Ct (B) measured in duplicates from the standard sample. There was a strong negative linear relationship between the variables

3.10 Data analysis

The data were analysed using SAS Software, version 9.4 (SAS Institute Inc., Cary, NC). Data are represented as means ± standard error of the mean (SEM) for normally distributed data and as median (interquartile range; IQR) for non-normally distributed data. Changes in body weight, blood pressure, arthritis scores and paw thickness over time and between groups were determined by repeated measures analysis of variance (ANOVA), followed by Tukey *post-hoc* tests. Differences in leukocyte telomere length, inflammatory markers and VCAM-1 concentrations were determined by a two-way ANOVA, with group and sex as main effects. Differences in adipose tissue telomere length between the groups were determined by a Kruskal-Wallis test. There were no differences in the telomere lengths between males and females in the total study sample or in each group, hence the results for males and females were pooled. Associations between telomere lengths, circulating inflammatory markers, vascular function and cardiac function markers were determined using Pearson's correlation coefficients. Because body weight differed between males and females, body weight and sex were included as potential confounders in the correlation analysis. As drug treatment seemed to influence leukocyte telomere lengths, correlation analyses were performed in the control and inflammation groups alone and in all groups in separate models. Non-normally distributed variables were log transformed to improve normality, before including in regression analysis. A p value ≤ 0.05 was considered statically significant.

CHAPTER 4

RESULTS

4.1 Body weight, blood pressure, arthritis scores and paw thickness

Figure 4.1 shows the body weights in all rats (A) in males (B) and females (C) and systolic and diastolic blood pressure of control (n = 25), inflammation (n = 22), tumour necrosis factor alpha (TNF-α) blocker (n = 11) and interleukin 6 (IL-6) blocker (n = 12) groups over the study period. The mean body weight did not change significantly over time compared to baseline for any of the groups (all $p > 0.05$, Figure 4.1A). At week 10, the (mean ± SEM) body weights of the control group (487.2 ± 150.2g) were significantly higher than the IL-6 blocker group (363.5 ± 90g, $p = 0.04$). No further differences were noted between groups for body weight, at termination (Figure 4.1A). The body weights of the males were higher than females in all groups (all $p < 0.05$). In sensitivity analysis, between the groups, the body weights of the males (Figure 4.1B) and females (Figure 4.1C) were similar at week 0 ($p > 0.05$). At week eight and week 10, the (mean ± SEM) body weight of the male rats in the IL-6 blocker group (week 8: 454.8 ± 26.9g; week 10: 457.1 ± 24.1g) were lower compared to the male control rats (week 8: 590.3 ± 14.4g, $p = 0.004$; week 10: 603.5 ± 14.4g, $p = 0.001$, Figure 4.1B). No other differences were noted between the body weights of the male rats across the groups at any other time points. In the females, there were no differences in the body weights across the groups at any time point (all $p > 0.05$; Figure 4.1C).

Neither systolic (Figure 4.1D) nor diastolic (Figure 4.1E) blood pressure changed over the course of the study (all $p > 0.05$). There were no differences in systolic or diastolic blood pressure between the groups at any time point (all $p > 0.05$).

Figure 4.2 shows ths arthritis scores and paw thickness across the intervention. The arthritis scores of the control group did not change significantly over time ($p > 0.05$, Figure 4.2). The arthritis scores increased compared to baseline from week six onwards in all groups exposed to the arthritis-inducing protocol (all $p < 0.05$). The arthritis scores of the inflammation, TNF-α blocker, and IL-6 blocker groups were significantly higher than the control group at week six, eight and 10 ($p < 0.05$ for all). The arthritis scores in the IL-6 blocker group were significantly higher compared to the inflammation group at week six ($p = 0.02$) and week eight ($p = 0.002$).

Figure 4.2 shows that the paw thickness at the ankle joint and tarsometatarsal joints were increased in the inflammation group at week 8 and 10 compared to baseline (week 0) (all $p < 0.05$). The paw thickness at the ankle joint and tarsometatarsal joints were increased in the IL-6 blocker group at 10 weeks compared to baseline (week 0) (both $p <0.05$). At week 10, the paw thickness at the ankle ($p = 0.04$) and tarsometatarsal ($p = 0.05$) joints were increased in the inflammation group compared to the control group. The ankle joint thickness of the inflammation group at week 10 were increased compared to the TNF-α blocker group ($p = 0.04$). The paw thickness at the ankle and tarsometatarsal joints did not change in the control or TNF-α blocker groups over the duration of the study (all $p > 0.05$, Figure 4.2).

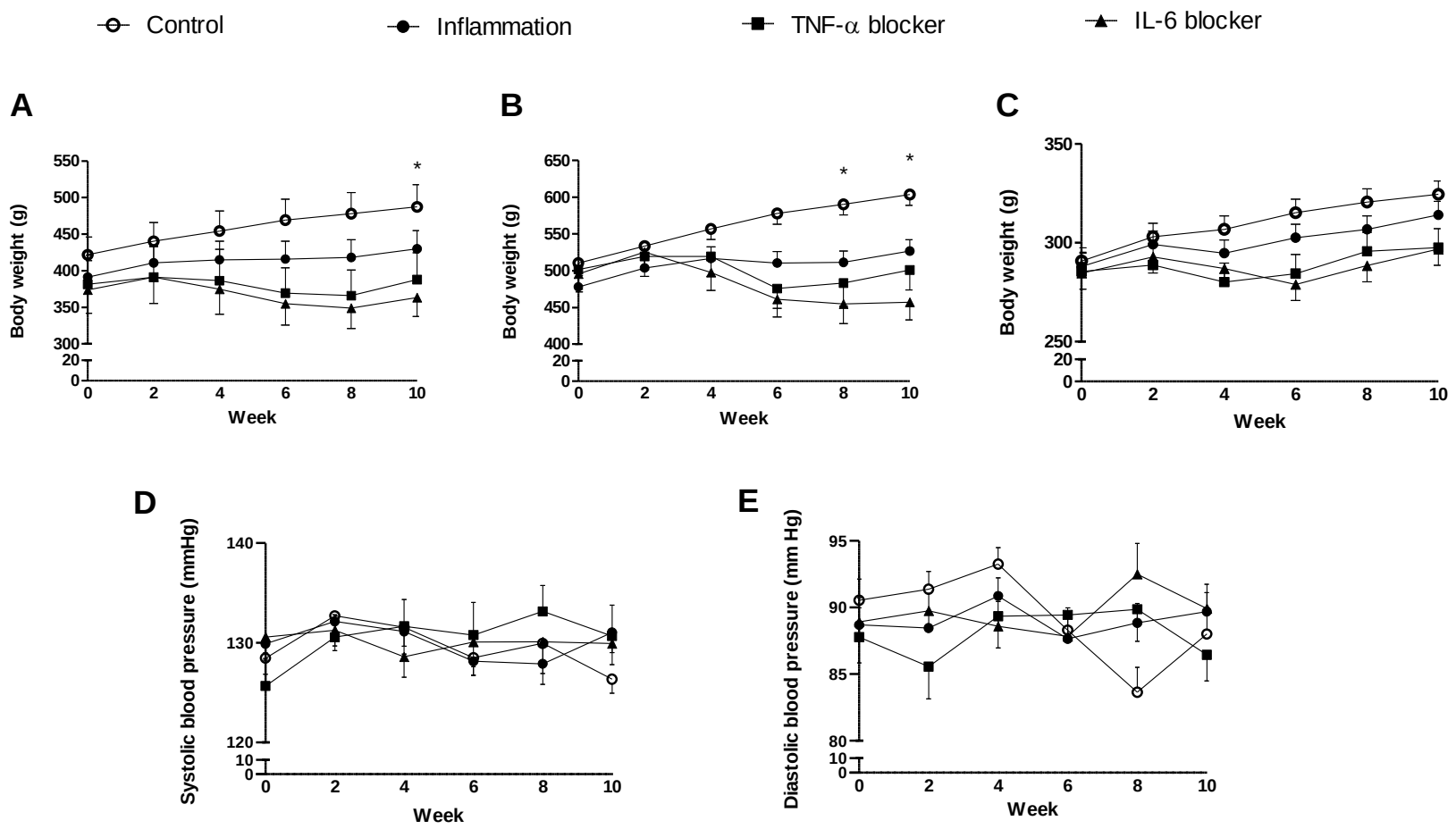


Figure 4.1 Bodyweight in all rats (A), males (B) and females (C) and systolic (D) and diastolic (E) blood pressure in control, inflammation, TNF-α blocker and IL-6 blocker groups over 10 weeks after the primary immunization (week 0). *p < 0.05 IL-6 blocker versus control.

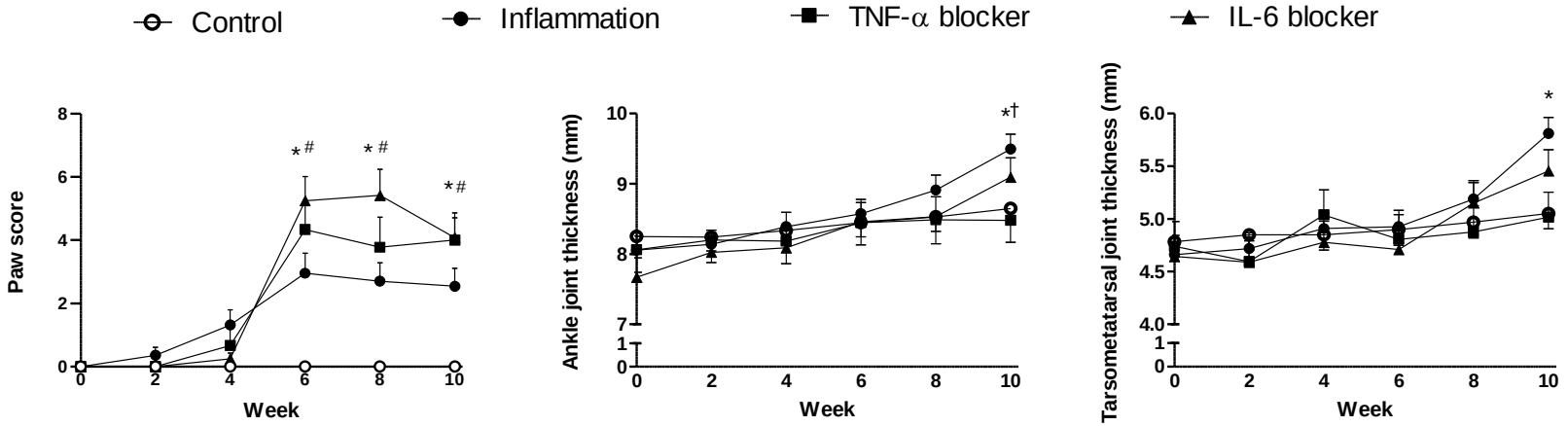


Figure 4.2 Arthritis scores and paw thickness and the ankle and tarsometatarsal joints in the control, inflammation, TNF-α blocker and IL-6 blocker groups over 10 weeks after the primary immunization (week 0). *$p < 0.05$ inflammation versus control, $^{\#}p < 0.05$ inflammation versus IL-6 blocker group, † $p < 0.05$ inflammation versus TNF-α blocker group.

4.2 Serum concentrations of inflammatory markers and endothelial activation

Compared to controls, circulating TNF-α concentrations were significantly higher in the inflammation, TNF-α blocker and IL-6 blocker groups (all $p < 0.001$, Table 4.1). There were no differences in the circulating TNF-α concentrations between the rats exposed to the inflammatory protocol. Circulating IL-6 concentrations were significantly higher in the inflammation, TNF-α blocker and IL-6 blocker groups compared to controls ($p < 0.001$, $p = 0.003$ and $p = 0.01$, respectively, Table 4.1). Circulating IL-6 concentrations did not differ between inflammation, TNF-α blocker and IL-6 blocker groups. Circulating CRP concentrations were significantly higher in the inflammation, TNF-α blocker and IL-6 blocker groups, compared to the control group ($p < 0.001$ for all, Table 4.1). No differences in CRP concentrations were observed between any of the groups exposed to inflammation. In the subsample ($n = 44$), serum VCAM-1 concentrations were significantly higher in the inflammation group ($n=14$) compared to the control group ($n = 16$; $p = 0.0008$) and compared to the TNF-α blocker group ($n = 7$; $p = 0.02$, Table 4.1). There were no differences in VCAM-1 concentrations between the control, TNF-α blocker and IL-6 blocker ($n = 7$) groups (all $p > 0.05$, Table 4.1).

Table 4.1 Circulating inflammatory and endothelial activation marker concentrations at termination, in control, inflammation, TNF-α blocker and IL-6 blocker groups

	Control	**Inflammation**	**TNF-α blocker**	**IL-6 blocker**
n	25	22	11	12
TNF-α (pg/ml)	96.1±6.2	172.4±10.2*	161.3±9.3*	165.3±8.2*
IL-6 (pg/ml)	18.1±1.9	31.8±2.4*	32.0±2.6*	30.5±3.5*
CRP (ng/ml)	0.13±0.03	0.58±0.05*	0.55±0.08*	0.63±0.05*
VCAM-1 (pg/ml)	75.7±5.5	109.9±5.9*#	78.4±8.1	87.9±9.2

Data expressed as means ± SEM. TNF-α, tumour necrosis factor alpha; IL-6, interleukin 6; CRP, C-reactive protein; VCAM-1, vascular adhesion molecule 1. *$p < 0.01$ versus control; # $p < 0.05$ versus TNF-α blocker.

4.3 Telomere lengths

The average (±SEM) relative leukocyte telomere length was significantly longer in the inflammation group (2.32 ± 0.19) compared to the control group (1.61 ± 0.19; $p = 0.05$, Figure 4.3). The average (±SEM) relative leukocyte telomere length of the TNF-α blocker group (1.55 ± 0.28) and the IL-6 blocker group (1.96 ± 0.27) did not differ significantly from the control group (both $p > 0.05$, Figure 4.3). The relative adipose tissue telomere length was not different between the groups ($p = 0.36$, Figure 4.3). There were no significant associations between relative leukocyte telomere length and relative adipose tissue telomere length in all groups ($n = 68$; $r = -0.10$, $p = 0.44$) or in control and inflammation groups ($n = 46$; $r = -0.13$, $p = 0.39$). There were no differences in either leukocyte or adipose tissue telomere lengths between males and females in the total sample or in any of the groups (all $p > 0.05$).

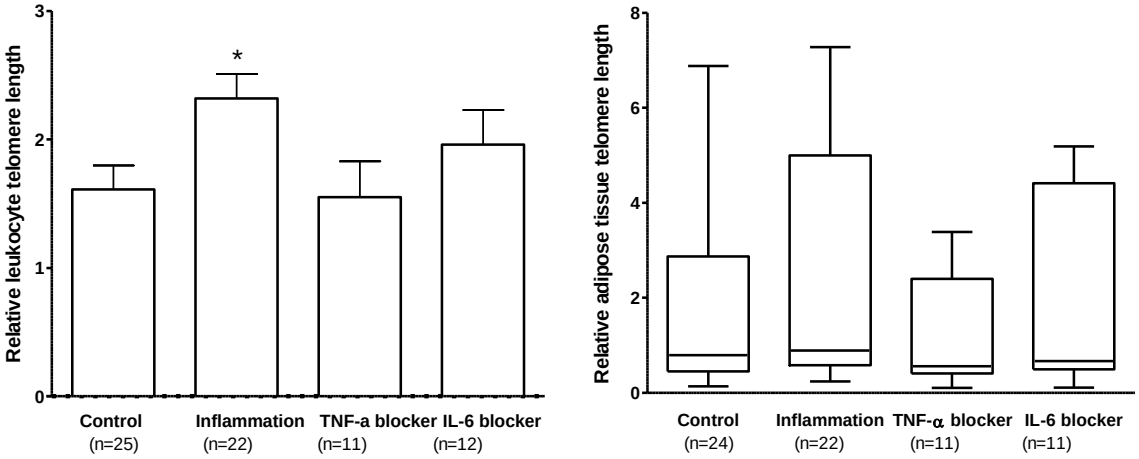


Figure 4.3 Relative leukocyte and adipose tissue telomere lengths in control, inflammation, TNF-α blocker and IL-6 blocker groups. Data presented as mean ± SEM for leukocyte telomere length and median (IQR) for adipose tissue telomere length. *p < 0.05 versus control

4.4 Associations between telomere length and circulating cytokine concentrations

In the control and inflammation groups only, longer relative leukocyte telomere length was associated with increased circulating CRP concentrations (n = 47, r = 0.47; p = 0.001, Figure 4.4A). These associations remained significant when adjusting for body weight and sex (r = 0.44; p = 0.01, Figure 4.4B). In all groups, longer relative leukocyte telomere length was associated with increased CRP concentrations (n = 70; r = 0.27; p = 0.03, Figure 4.4C) and remained significantly associated when adjusting for body weight and sex (r = 0.24, p = 0.05, Figure 4.4D).

Longer leukocyte telomere length was related to increased circulating IL-6 concentrations in the control and inflammation groups only (n = 47, r = 0.35; p = 0.02, Figure 4.4A) and remained significant upon adjustment for body weight and sex (r = 0.34; p = 0.03, Figure 4.4B). Leukocyte telomere length was not associated with circulating IL-6 concentrations across all groups (n = 70) in univariate (Figure 4.3B) or multivariate adjusted (Figure 4.4D) analysis. Circulating TNF-α concentrations were not associated with relative telomere length in either the control and inflammation group alone (Figure 4.4A), or across all groups (Figure 4.4B).

Relative adipose tissue telomere length was not associated with any marker of inflammation in control and inflammation groups (n = 46; Figure 4.4E) or in all groups (n = 68; Figure 4.4F). The results were materially unaltered when adjusting for body weight and sex (Figure 4.4G and Figure 4.4H).

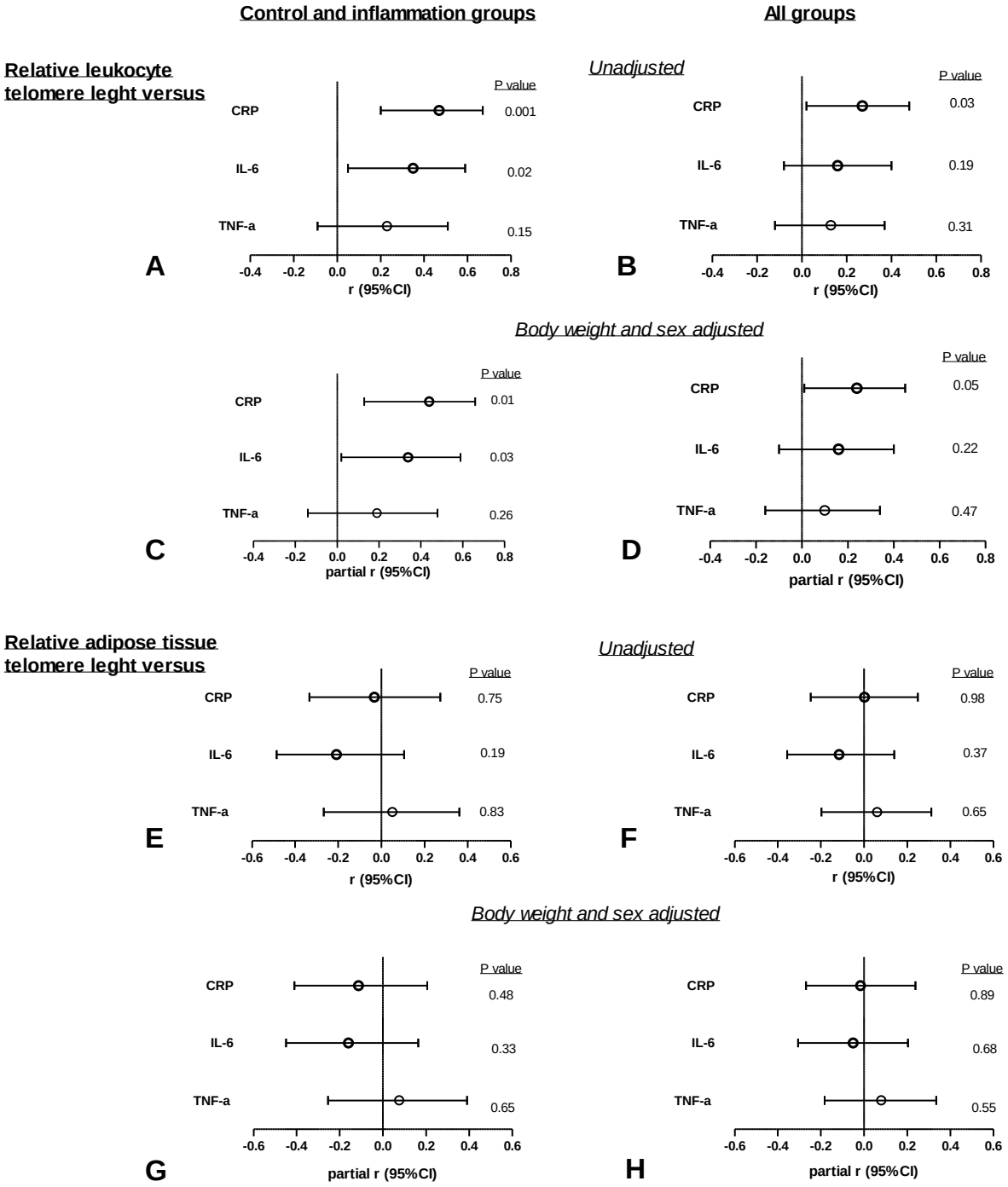


Figure 4.4 Associations between leukocyte (A - D) and adipose tissue (E – G) telomere length and circulating cytokine concentrations in control and inflammation groups only (left panel), and in all groups (right panel). Open circles represent the correlation coefficient (r) and horizontal lines represent the 95% confidence intervals (CI)

4.5 Associations between telomere length and markers of vascular function

Table 4.2 show the associations between relative leukocyte and adipose tissue telomere length and measures of vascular function, in control and inflammation groups with and without adjustment for body weight and sex, as well as in all groups. In the control and inflammation groups only, leukocyte telomere length was directly related to systolic blood pressure ($n = 47$, $r = 0.29$; $p = 0.05$) and pulse pressure ($r = 0.31$, $p = 0.04$). These associations were materially unaltered upon adjustment for bodyweight and sex ($r = 0.31$; $p = 0.04$ and $r = 0.38$, $p = 0.01$, respectively). In all groups ($n = 70$) leukocyte telomere length was not associated with systolic blood pressure ($r = 0.19$, $p = 0.12$) or pulse pressure ($r = -0.21$, $p = 0.09$). Leukocyte telomere length was not associated with diastolic blood pressure ($p > 0.05$). In all groups longer leukocyte telomere length was associated with increased concentrations of VCAM-1 ($n = 44$; $r = 0.29$; $p = 0.05$), but not in control and inflammation groups ($n = 30$; $r = 0.22$; $p = 0.11$).

In control and inflammation groups only, as well as in all groups, leukocyte telomere length was not associated with vascular half-maximal relaxation ($p = 0.35$ and $p = 0.99$), however, leukocyte telomere length was inversely associated with maximal vascular relaxation ($r = -0.55$; $p = 0.01$). The association between leukocyte telomere length and maximal vascular relaxation remained significant upon adjustment for bodyweight and sex ($r = -0.50$; $p = 0.02$) and when including all groups in the analysis ($r = -0.35$, $p = 0.03$).

In control and inflammation groups only, as well as across all groups, leukocyte telomere length was not associated with vascular half maximal contraction ($p = 0.69$ and $p = 0.53$, respectively). Longer leukocyte telomere length was associated with lower maximal

vascular contraction (r = -0.37; p = 0.04). This association was no longer significant following adjustment for body weight and sex (p = 0.13). In all groups, leukocyte telomere length remained negatively associated with maximal vascular contraction (r = -0.37, p = 0.01). Relative adipose tissue telomere length was not associated with any marker of vascular function (Table 4.2).

4.6 Associations between telomere length and markers of cardiac function

Table 4.3 shows the association between leukocyte telomere length and markers of cardiac function, in control and inflammation groups only with and without adjustment for bodyweight and sex, as well as across all groups. Leukocyte telomere length was not associated with heart weight indexed to body weight in either the control and inflammation groups alone or in all groups (p = 0.98 and p = 0.92). Leukocyte telomere length was not related to relative wall thickness in either the control and inflammation groups alone (r = 0.14, p = 0.39), or across all groups (r = 0.16, p = 0.23). Leukocyte telomere length was not associated with markers of systolic function as indexed by LV ejection fraction (r = 0.28, p = 0.10) and mid-wall fractional shortening (r = 0.21 p = 0.23). There were no associations between leukocyte telomere length and E/A (r = 0.02, p = 0.92) or left ventricular lateral wall e' (r = -0.24 p = 0.09). Longer leukocyte telomere length was related to E/e', a marker of increased LV filling pressures (r = 0.35, p = 0.03). However, this association was no longer significant following adjustment for body weight and sex (r = 0.14, p = 0.30) and when including all groups in the model (r = 0.14, p = 0.31).

Shorter relative adipose tissue telomere length was associated with increased heart weight indexed to body weight in all groups (n = 68, r = -0.28, p=0.03), but not in the

control and inflammation groups (n = 46, r = -0.20, p =0.19). Shorter relativeadipose tissue telomere length was associated with impaired systolic function (reduced ejection fraction and fractional shortening) in the control and inflammation groups, in univariate and multivariate adjusted analysis. The association between adipose tissue telomere length and midwall fractional shortening remained significant in all group (r = -0.27, p = 0.04). Adipose tissue telomere length was not associated with any marker of diastolic function (E/A, lateral e' or E/e').

Table 4.2 Associations between telomere length and markers of vascular function

	Unadjusted			BW and sex adjusted			Unadjusted		
	r	95 % CI	p	Partial r	95 % CI	p	r	95% CI	p
Relative leukocyte telomere length versus									
	Control and inflammation groups (n=47)						All groups (n=70)		
Systolic BP	**0.29**	**0.00-0.54**	**0.05**	**0.31**	**0.01-0.55**	**0.04**	0.19	-0.05-0.41	0.12
Diastolic BP	-0.10	-0.38-0.20	0.51	-0.17	-0.45-0.14	0.27	-0.07	-0.31-0.16	0.52
Pulse pressure	**0.31**	**0.02-0.55**	**0.04**	**0.38**	**0.09-0.61**	**0.01**	-0.21	-0.03-0.43	0.09
VCAM-1	0.22	-0.16-0.54	0.25	0.11	-0.28-0.47	0.59	**0.29**	**0.01-0.55**	**0.05**
Half-maximal relaxation*	0.21	0.24-0.58	0.35	0.13	-0.34-0.55	0.58	0.00	-0.34-0.34	0.99
Maximal relaxation	**-0.55**	**-0.79- -0.17**	**0.01**	**-0.50**	**-0.78- -0.06**	**0.02**	**-0.35**	**-0.62- -0.02**	**0.03**
Half-maximal contraction*	0.08	-0.30-0.43	0.69	0.14	-0.26-0.49	0.49	-0.09	-0.38-0.21	0.53
Maximal contraction	**-0.37**	**-0.65- -0.01**	**0.04**	-0.30	-0.61-0.01	0.13	**-0.37**	**-0.60--0.09**	**0.01**
Relative adipose tissue telomere length* versus									
	Control and inflammation groups (n=46)						All groups (n=68)		
Systolic BP	-0.15	-0.43-0.15	0.33	-0.19	-0.46-0.12	0.23	-0.13	-0.36-0.12	0.29
Diastolic BP	-0.18	-0.45-0.13	0.25	-0.24	-0.50-0.07	0.13	-0.16	-0.39-0.09	0.19
Pulse pressure	0.00	-0.29-0.29	0.99	0.01	-0.30-0.31	0.98	0.00	-0.25-0.24	0.98
VCAM-1	-0.02	-0.39-0.25	0.90	0.13	-0.26-0.49	0.49	-0.03	-0.33-0.28	0.86
Half-maximal relaxation*	0.32	-0.12-0.65	0.14	0.27	-0.02-0.76	0.13	0.12	-0.23-0.45	0.49
Maximal relaxation	0.04	-0.3-0.45	0.87	0.11	-0.36-0.54	0.63	-0.05	-0.39-0.29	0.78
Half-maximal contraction*	0.01	-0.37-0.38	0.98	0.29	-0.12-0.62	0.15	0.15	-0.17-0.43	0.36
Maximal contraction	0.05	-0.33-0.42	0.79	0.13	-0.28-0.50	0.52	0.14	-0.17-0.43	0.37

*Logarithmically transformed. Significant values shown in bold. BW, body weight; BP, blood pressure; VCAM1, vascular adhesion molecule 1.

Table 4.3 Associations between telomere length and markers of cardiac function

	Unadjusted			BW & sex adjusted			Unadjusted		
	r	95 % CI	p	partial r	95% CI	p	r	95% CI	p
Relative leukocyte telomere length versus									
	Control and inflammation groups (n = 47)						All groups (n = 70)		
HW/BW	0.00	-0.29-0.30	0.98	-0.02	-0.26-0.22	0.87	0.01	-0.23-0.25	0.92
Relative wall thickness	0.14	-0.20-0.46	0.39	0.15	-0.12-0.40	0.27	0.16	-0.10-0.40	0.23
Ejection fraction	0.28	-0.05-0.56	0.10	0.24	-0.03-0.47	0.08	0.21	-0.05-0.45	0.12
Midwall fractional shortening	0.21	-0.14-0.51	0.23	0.16	-0.11-0.41	0.25	0.14	-0.12-0.39	0.29
E/A	0.02	-0.32-0.35	0.92	-0.09	-0.34-0.18	0.53	-0.07	-0.32-0.20	0.61
Lateral e’	-0.24	-0.50-0.05	0.09	-0.14	-0.37-0.11	0.27	-0.14	-0.36-0.10	0.26
E/e’	**0.35**	**0.01-0.61**	**0.03**	0.14	-0.13-0.39	0.30	0.14	-0.13-0.38	0.31
Relative adipose tissue telomere length* versus									
	Control and inflammation groups (n = 46)						All groups (n = 68)		
HW/BW	-0.20	-0.47-0.10	0.19	0.27	-0.03-0.53	0.08	**-0.28**	**-0.49—0.03**	**0.03**
Relative wall thickness	0.25	-0.10-0.54	0.15	0.19	-0.17-0.51	0.29	0.09	-0.18-0.35	0.50
Ejection fraction	**-0.40**	**-0.65--0.07**	**0.02**	**-0.36**	**-0.63—0.01**	**0.04**	-0.18	-0.43-0.09	0.18
Midwall fractional shortening	**-0.50**	**0.71—0.19**	**0.002**	**-0.52**	**-0.74—0.21**	**0.002**	**-0.27**	**-0.50—0.009**	**0.04**
E/A	0.05	-0.29-0.38	0.79	0.03	-0.32-0.37	0.87	0.10	-0.17-0.35	0.47
Lateral e’	-0.03	-0.32-0.27	0.86	-0.15	-0.43-0.15	0.32	0.03	-0.22-0.27	0.82
E/e’	-0.04	-0.37-0.31	0.84	0.25	-0.11-0.55	0.16	-0.19	-0.43-0.08	0.17

*Logarithmically transformed. Significant values shown in bold. HW, heart weight; BW, body weight; E/A, ratio of early to late diastolic filling; Lateral e’, lateral wall mitral annular tissue relaxation in early diastole; E/e’, ratio of early diastolic filling to peak velocity during early diastole.

CHAPTER 5

DISCUSSION

The main aim of this study was to determine whether exposure to chronic inflammation affects leukocyte and adipose tissue telomere lengths. We also aimed to determine whether treatment with biologic DMARDs influenced the inflammation-induced changes in telomere length. Lastly, we aimed to determine whether telomere length is a biomarker of vascular and cardiac function in a model of high-grade inflammation. The main findings of the current study are that rats exposed to inflammation, without anti-inflammatory treatments, paradoxically had longer relative leukocyte telomere lengths compared to the control group. Rats exposed to inflammation and treated with biologic anti-inflammatory drugs had leukocyte telomere lengths similar to those of the control group. Higher circulating CRP concentrations were associated with longer relative leukocyte telomere lengths, however, there were no associations between circulating TNF-α concentrations and telomere length. Adipose tissue telomere lengths were not affected by inflammation or biologic DMARDs. Longer leukocyte telomere lengths were associated with higher systolic blood pressure and pulse pressure in the groups not treated with anti-inflammatory drugs, however, the drug treatments impacted the relationship between leukocyte telomere length and blood pressure. Longer leukocyte telomere lengths were associated with impaired endothelial dependent vascular relaxation and contraction, irrespective of body weight, sex or anti-inflammatory drug treatments. Longer leukocyte telomere lengths were also associated with increased concentrations of VCAM-1, a marker of endothelial activation. Leukocyte telomere lengths were not consistently associated with cardiac geometry, systolic function or diastolic function measures. Adipose tissue telomere lengths were not associated with any marker of vascular

function. Shorter adipose tissue telomere lengths were associated with impaired cardiac systolic, but not diastolic function.

5.1 The effects of inflammation on leukocyte and adipose tissue telomere length

Our results showed longer leukocyte telomere lengths in rats exposed to chronic inflammation, without anti-inflammatory treatments, compared to control rats. Longer leukocyte telomere lengths were related to higher circulating CRP concentrations, irrespective of body weight or sex. In contrast, previous animal studies reported shorter leukocyte telomere lengths after exposure to chronic low-grade inflammation (Raymond, 2014; Raymond *et al*., 2016a), which were correlated with circulating CRP concentrations (Raymond, 2014). Several cross-sectional human studies have also reported shorter leukocyte telomeres in patients exposed to low-grade inflammation, compared to healthy controls (Valdes *et al*., 2005; Buxton *et al*., 2011) and that CRP concentrations were associated with shorter leukocyte telomere length (Bekaert *et al*., 2007; Carrero *et al*., 2007; Masi, 2014; Wong *et al*., 2014). Despite these associations in low-grade inflammatory studies, it is well known that in conditions of high-grade inflammation, such as RA, several physiological processes are altered (McInnes & Schett, 2011). In this regard, two studies including patients with systemic lupus erythematosus, showed no differences in leukocyte telomere length between patients and controls (Beier *et al*., 2007; Klapper *et al*., 2004). Several studies included in a recent meta-analysis, especially larger studies, failed to show shorter telomeres in RA patients compared to controls (Lee & Bae, 2018).

Similar to our findings, Tamayo and colleagues (2010) reported significantly longer leukocyte telomere lengths in patients with rheumatological diseases compared to controls. Longer telomere lengths have also been reported compared to controls in patients with Parkinson's disease (Wang *et al*., 2008) and in patients with limited cutaneous systemic sclerosis (MacIntyre *et al*., 2008). Fibroblasts exposed to ionizing radiation, *in vitro,* also showed increased telomere length, possibly due to increased telomerase activity following an insult to the DNA (Sgura *et al*., 2006). To our knowledge, there are currently no animal studies reporting increases in telomere length upon exposure to high-grade inflammation. Animal studies of increased oxidative stress have, however, reported paradoxically increased telomere lengths (Brown *et al*., 2007; Shoeb *et al*., 2017).

Despite the inflammation-induced increases in leukocyte telomere length, adipose tissue telomere length in the current study was not affected by inflammation. The lack of any observed effect of inflammation on adipose tissue telomere length could potentially be attributed to the very low replicative rate of mature adipocytes (Spalding *et al*., 2008; Ali *et al*., 2013). Other animal models have also reported that despite exposure to inflammation and/or oxidative stress, tissue telomere lengths were not different from controls (Takahashi *et al*., 2014; Raymond *et al*., 2016a). When considering the results from the current study in context with previous literature that reported paradoxical longer telomere lengths in diseases states (MacIntyre *et al*., 2008; Wang *et al*., 2008; Tamayo *et al*., 2010) and the well-known reduced life expectancy in patients with RA (Nanke *et al*., 2002), it is unlikely that longer leukocyte telomeres indicate slower biological ageing. It is more likely that there is dysregulation in telomere length physiology as measured in

leukocytes of peripheral blood. In this regard, longer telomeres in high-grade inflammation has only been reported in studies where peripheral blood was used to measure telomere length. Indeed, the results on tissue specific telomere length in response to inflammation have been controversial. In two animal studies of chronic inflammation, despite both studies reporting shorter leukocyte telomere length, one study reported no change in liver or spleen tissue telomere length after exposure to chronic *Salmonella enterica* (Ilmonen *et al*., 2008), while the other study reported shortened cardiac tissue telomeres in response to low dose *Staphylococcus aureus* exposure (Raymond *et al*., 2014). In models of oxidative stress, disparate effects of oxidative stress on leukocyte and tissue telomere shortening have also been reported (Tajbakhsh *et al.,* 2015). Indeed, one study has shown that the effects of oxidative stress on telomere shortening is tissue specific (Cattan *et al*., 2008).

In addition to the tissue specific differences in telomere shortening, several possibilities why telomere physiology may be altered in leukocytes exposed to high-grade inflammation need to be considered. First, during high grade inflammation leukocyte proliferation is upregulated (Chmielewski & Strzelec, 2018). This increased proliferation will result in increased number of cell division, which has been the basis for the argument that inflammation increases telomere attrition (Kimura *et al*., 2010; Aviv & Levy, 2012). It is also possible that because of the increased demand for leukocytes in circulation in high-grade inflammation and the increased rate at which leukocytes reach senescence (Kimura *et al*., 2010), their half-life may be reduced (Chmielewski *et al*., 2016). This means that greater amounts of precursors or immature leukocytes will be released, while senescent cells will be removed from the circulation (Brümmendorf *et al*., 2001; Pavlaki

et al., 2012; Barden *et al*., 2016; Soehnlein *et al*., 2017). Although the T-lymphocyte pool and the addition of naïve T-lymphocytes to the circulation are tightly regulated, it has been suggested that the survival of naïve T-lymphocytes and the total pool can be disrupted as their regulation are dependent on certain cytokines (Moro-García *et al*., 2018). Indeed, Dieudonné *et al.,* (2019) recently showed increased immature B cells in patients with SLE compared to controls. The increased newly released white blood cells in circulation will arguably have longer telomeres, which may skew the telomere attrition rate of the overall blood sample, hence it may appear that telomeres are longer than controls. Indeed, a recent study performed in acutely ill patients admitted to intensive care, showed that in some patients, telomeres acutely shortened, while in others it lengthened (Zribi *et al*., 2019). Although CRP concentrations were elevated in all patients, telomere length was not associated with CRP concentrations. However, an increasing white blood cell count over seven days was associated with increasing leukocyte telomere lengths, independent of age (Zribi *et al*., 2019). In addition, in an older population, higher lymphocyte counts were associated with both increased IL-6 concentrations and with leukocyte telomere length (Compté *et al*., 2015). Although we did not measure white blood cell count in the current study, the increased circulating inflammatory marker concentrations in the rats exposed to the arthritis inducing protocol suggest white blood cell count may have been elevated in the inflammation group in the current study.

Second, the increased telomere length in high-grade inflammation may be as a result of a compensatory upregulation in telomerase expression. It has been suggested that telomerase expression decreases after birth to negligible levels later in life in humans (Flores *et al*., 2008). In humans, leukocytes have very low telomerase activity that does

not influence telomere attrition (Flores *et al.,* 2008). However, stimulation of primitive haematopoietic cells and leukocytes by antigens can increased telomerase activity (Chiu *et al*., 1996; Greider, 1996; Lansdorp, 1998; Takagi *et al*., 2003; Van Ziffle *et al*., 2003; Hills *et al*., 2009). Indeed, higher telomerase activity in leukocytes have been reported in several studies of high-grade inflammatory disorders and connective tissue diseases (Klapper *et al*., 2004; Kurosaka *et al*., 2003; Maclntyre *et al*., 2008; Katayama & Kohriyama, 2001; Tarhan *et al*., 2008; Dehbi *et al*., 2013). Despite the evidence of increased telomerase activity in chronic inflammation in humans, several studies have suggested differences in telomerase activity between humans and rodents (Martin-Rivera et al., 1998; Yamaguchi *et al*., 1998; Liu *et al.,* 1999; Brown *et al*., 2007). Indeed, telomerase activity is upregulated in rodents, following a DNA insult (Brown *et al*., 2007). This suggests that although the high-grade inflammation will increase the rate of leukocyte cell replication, the rate of telomere attrition may be slowed by the upregulation of telomerase (Honda *et al*., 2001). In this regard, the differences in telomerase activity between humans and rats may impact the generalisability of these result.

Third, besides telomerase, other associated proteins in the shelterin complex also regulate telomere length. Dysfunction in some of these associated proteins may contribute to increased telomere length. In this regard, TRF1 is a protein that has been identified to be important in the regulation of telomere length by regulating the access of telomerase to telomeres (Okamoto *et al*., 2008). A loss of TRF1 can result in increased telomere length (Donigian & De Lange, 2007). In a recent study in obesity, upregulation of TRF1 has been implicated in the increased rate of telomere shortening (Grun *et al*., 2018). However, the effects of chronic high-grade inflammation on the expression of

shelterin complex proteins and its effects on telomere length is currently not known. Considering the complexity of telomere physiology, it may be that inflammatory markers may directly or indirectly affect telomere shortening in white blood cells by affecting either telomerase or the associated proteins and cellular pathways. The impact of inflammation on the molecular pathways involved in telomere shortening should be investigated in future studies.

Fourth, another possible mechanism that could explain the lengthening of telomeres in the current study, is the alternative lengthening of telomeres (ALT) pathway. The ALT pathway is a process whereby telomeres are lengthened by a homologous recombination-based mechanism, often described in cancer (Sobinoff & Pickett, 2017). In this regard, chronic inflammation has been associated with cancer in RA (De Cock & Hyrich, 2018). Also, the accelerated T-cell aging, and the associated telomere attrition, in RA have been implicated in the risk for developing cancer (Li *et al.,* 2018). Despite these associations, currently no studies have investigated ALT in chronic inflammatory disorders. In organic pollutant exposure, which also induces a state of inflammation, it has been proposed that ALT pathways are increased (Yuan *et al*., 2018). Therefore, whether chronic inflammation affect ALT requires further investigation.

Lastly, several physiological and methodological factors seem to influence the outcome of telomere length studies. In this regard, besides the cell type where telomeres are measured, the techniques used to measure telomere length, the population included and their genetic predisposition to telomere shortening as well as other potential confounding factors such as body weight, sex and smoking status all influence telomere length (Lee & Bae, 2018; Tomayo *et al*., 2010; Jeanclos *et al*., 2000; Ormseth *et al*., 2016).

Methodological differences between the current study and prior studies may have resulted in contrasting findings. Results in the current study agree with some, but differ from several human RA studies, where shorter telomeres were reported in RA compared to controls (Lee & Bae, 2018). This discrepancy may be explained by the short duration of the inflammatory protocol in this study. Rats in the current study were exposed to inflammation for 10 weeks, which is far shorter than the disease duration reported in human RA studies. Although previous RA studies failed to show an association between disease duration and leukocyte telomere length (Steer *et al*., 2007; Raymond *et al*., 2016b; Kordinas *et al*., 2016), it may be that leukocyte counts are reduced in long term exposure to inflammation, compared to increased white blood cell count in early stages of the disease (Symmons *et al*., 1989). Moreover, it has been shown that in chronic immune activation, $CD8^+$ T-cells reduce the expression of CD28, which is required for optimal T-lymphocyte activation (Parish *et al*., 2009). Reduced CD28 expression have been linked to decreased telomerase activity and cellular senescence (Vallejo *et al*., 2004), which may explain the reduced telomere lengths reported in RA patients with prolonged disease duration. Also, differences in telomere biology between rats and humans may further confound the results (Brown *et al*., 2007). Nevertheless, future mechanistic and longitudinal studies should elucidate the mechanisms and cellular pathways whereby leukocyte telomeres are altered in the early and late stages of high grade-inflammatory conditions.

5.2 The effects of biologic DMARD treatment on telomere length in chronic inflammation

Based on our results and previous reports, inflammatory markers have a significant effect on telomere physiology and attrition (Correia-Melo *et al*., 2014; Kordinas *et al*., 2016). Targeting inflammation in an attempt to ameliorate telomere attrition has certainly been suggested (Kordinas *et al*., 2016). Besides the association between CRP and telomere length discussed above, cross sectional studies showed that TNF-α and IL-6 are associated with telomere length (Bekaert *et al*., 2007; O'Donovan *et al*., 2011), however, these results are not reported in all studies (Fitzpatrick *et al*., 2007; Wong *et al*., 2014). In the current study we showed that in the control and inflammation groups, both CRP and IL-6 were associated with leukocyte telomere length. When the drug treated groups were included in the analysis, the relationships between leukocyte telomere length and IL-6 were no longer significant, but the relationships between leukocyte telomere length and CRP persisted. Despite a lack of reduction in circulating inflammatory marker concentrations after biologic DMARD treatment, TNF-α inhibitors and IL-6 blockers prevented the inflammation induced alterations in leukocyte telomere length. There was no effect of the biologic DMARD treatments on adipose tissue telomere length. The sections below will highlight the effects of TNF-α and IL-6 on telomere physiology, in context of the current results.

5.2.1 The role of TNF-α in telomere physiology

In the current study, TNF-α inhibitors prevented the inflammation induced changes in leukocyte telomere length. TNF-α has been implicated in several mechanisms associated

with telomere physiology. Higher concentrations of TNF-α increase telomere shortening by inhibiting telomerase activity (Parish *et al*., 2009). More specifically, TNF-α is implicated in the reduction of CD28 expression in $CD8^+$ T lymphocytes, which are important for the prevention of cellular senescence (Parish *et al*., 2009). In RA, the inhibition of TNF-α reduces the proportion of T cells lacking CD28, which extends their proliferative protentional and the production of cytokines (Parish *et al*., 2009). It is believed that blocking of TNF-α increases telomerase activity (Parish *et al*., 2009). It has been suggested that TNF-α controls telomerase activity by inducing translocation from the cytoplasm to the nucleus of hTERT protein bound to NF-κB p65 (Akiyama *et al*., 2003). Others have supported the role of TNF-α in the recruitment of hTERT, the catalytic subunit of telomerase, to the telomeres (Maekawa *et al*., 2018). A recent study suggested that TNF-α increases the expression of telomeric repeat-containing RNAs (TERRA), which are implicated in inhibition of telomerase activity and telomere shortening (Liu *et al*., 2019). Taken together, our results and previous studies show that TNF-α is involved in several mechanisms and cellular pathways that impact telomere biology, by increasing the proliferation of T-lymphocytes and by inhibiting telomerase activity. Despite this role of circulating TNF-α in telomere physiology, we showed that TNF-α inhibitors impacted leukocyte telomere physiology, despite unchanged circulating TNF-α concentrations in the current study. This could possibly be explained by the mechanisms of TNF-α inhibitors used in the current study.

Circulating TNF-α can bind to either membrane bound or soluble TNF-α receptors (TNFR). Membrane bound TNFR are expressed in cells and respond to stimulation by circulating TNF-α, by releasing pro- and anti-inflammatory cytokines. Soluble TNFR

inactivate circulating TNF-α and blunts the immune response. The TNF-α inhibitor used in the current study, Etanercept is a fusion protein that inhibits the action of TNF-α by mimicking the soluble form of the TNF-α receptor, which binds to TNF-α in circulation and blunts its immune response (Reddy *et al*., 2016). Because etanercept is a fusion protein, it has a longer half-life in circulation. This results in a more potent biologic effect than naturally occurring soluble TNFR (Madhusudan *et al*., 2005). Also, circulating TNF-α can bind to one of two kinds of membrane TNFR (Chadwick *et al*., 2008). TNFR1 is extensively expressed, while TNFR2 is only expressed on cardiomyocytes, T-lymphocytes, thymocytes and stem cells (Wajant & Siegmund, 2019). Binding of TNF-α with the TNFR1 stimulates apoptotic pathways (Hanson, 2016). TNFR2 signalling is responsible for the regulation of cell proliferation and activation of regulatory T cells (Yang *et al*., 2018).

TNF-α drives inflammation in a receptor-mediated manner, by exerting pleiotropic effects on a number of different cell types, which in turn produce acute phase proteins like CRP, vascular adhesion molecules (VCAM), collagenases, matrix metalloproteinases, growth factors and other cytokines (Nishimoto *et al*., 2008; Ma & Xu 2013; Pironti *et al*., 2018). The circulating concentrations of TNF-α in the TNF-α blocker rats were increased compared to the control rats. Importantly, although etanercept binds to and inactivates TNF-α, it also prolongs its half-life (Goldenberg, 1999; Moreland *et al.,* 1999). Indeed, receptor inhibition is usually accompanied by increased serum levels of the cognate ligand, in this instance circulating concentrations of TNF-α (Mann *et al.,* 2008). Despite the high concentrations of circulating TNF-α, CRP concentrations were not reduced. This was indeed a surprising finding. The lack of effect of thee drugs on CRP concentrations

is likely because the humanised TNF-α binder may not be specific for receptors in rats. Future studies should measure the white cell count to confirm the drug effects. In contrast, despite high circulating TNF-α concentrations, the circulating VCAM-1 concentrations were significantly reduced in the TNF-α blocker group compared to the inflammation group. Although the arthritis scores in the TNF-α blocker group were similar to that of the inflammation group, the paw thickness, a more objective reflection of arthritis phenotype, was reduced in the TNF-α blocker group compared to the inflammation group.

Taken together, the physiological action of Etanercept, results in the deactivation of circulating TNF-α, hence the circulating concentration of TNF-α may not be reduced, but its ability to bind to membrane receptors is inhibited (Madhusudan *et al.,* 2004; Sato *et al.,* 2011). Indeed, the VCAM-1 concentrations in the TNF-α group were reduced compared to the inflammation group. The lack of activation of TNFR2, specifically located on T-lymphocytes, may have prohibited the proliferation and activation of these lymphocytes, hence prevented the inflammation induced increase in cell proliferation. Hence, the increase in telomere length as was seen in the inflammation group was not observed in the group treated with TNF-α receptor blockers. However, the exact mechanisms whereby TNF-α receptor blockers affect telomere length requires further study.

5.2.2 The role of IL-6 in telomere physiology

In the current study, IL-6 receptor blockers prevented the inflammation induced changes in leukocyte telomere length, despite unchanged circulating IL-6 concentrations. As previously mentioned, receptor inhibition is mostly accompanied by increased rather than

decreased serum levels of the cognate ligand, which explains the lack of reduction in circulating IL-6 concentrations (Mann *et al*., 2008; Nishimoto *et al*., 2008; Gibiansky & Frey, 2012). Tocilizumab, the IL-6 blocker used in this study, is a genetically engineered monoclonal antibody, which blocks IL-6 binding to the IL-6 receptor. Tocilizumab inhibits IL-6 binding to either membrane bound IL-6 receptors (mIL-6R) or soluble IL-6 receptors (Mihara *et al*., 2011). Following treatment with Tocilizumab, IL-6 and sIL-6 is inactivated by the formation of IL-6/Tocilizumab and sIL-6/Tocilizumab complexes; however, these tocilizumab/IL-6 complexes are still detected by ELISA (Gibiansky & Frey, 2012). This may explain the increased IL-6 concentrations in the IL-6 blocker group in the current study.

The signalling of IL-6 is quite complex and the activated pathways are intricately linked to the functions of other members of the IL-6 cytokine family (Gearing *et al*., 1987; Malik *et al*., 1989; Stockli *et al.,* 1989; Paul *et al*., 1990; Pennica *et al*., 1995; Senaldi *et al*., 1999). Signalling of the IL-6 cytokine family occurs through the ubiquitously expressed transmembrane receptor subunit glycoprotein130 (gp130). Signal specificity is achieved by either homo- or heterodimerization of gp130 with a ligand-specific α-receptor subunit that is specific for the different cytokines in the family (Kishimoto *et al*., 1995; Wollert & Drexler, 2001). Hence the physiological effect of IL-6 is largely dependent on the expression of its corresponding α receptor (Wollert & Drexler, 2001). Considering its function, IL-6 blockers may not necessarily reduce circulating levels of IL-6, but it may block the effects of IL-6 on target tissue. Indeed, previously it has been reported that treatment with IL-6 blockers fail to reduce circulating IL-6 concentrations (Kasama *et al*.,

2016). Nevertheless, the use of a humanized monoclonal antibody may have limited the drug effectiveness in rats.

Although circulating IL-6 concentrations have been associated with telomere length in cross-sectional studies, the direct role of IL-6 in telomere attrition has not been clearly defined. Elevated levels of IL-6 are responsible for many biologic effects on several organ systems, including in peripheral blood cells such as T and B lymphocytes, neutrophils, monocytes and platelets (Srirangan & Choy, 2010; Schinnerling *et al.*, 2017). In RA, IL-6 is involved in stimulating apoptosis in leukocytes and in the phenotypic differentiation of T cells, where the proportions of different CD4+ subsets, which are responsible for a normal immune response, are altered to a more pro-inflammatory course (Kasama *et al*., 2016; Schinnerling *et al.*, 2017). This is not limited to memory T-cells but also to naïve T cells, where the diversity of cells is reduced (Wagner *et al*., 1998). In this regard, the proportion of regulatory T cells (Treg), that have a protective effect, are reduced while T helper 17 cells (Th17) that are pro-inflammatory, are increased (Kasama *et al*., 2016). Besides the alterations in the diversity of T cells, there is also an increase in the rate of turnover of T cells in RA (Wagner *et al*., 1998). Alterations in these T cells are not only implicated in the pathogenesis of RA (Kasama *et al*., 2016), but also in changes in telomere physiology and the early senescence of T lymphocytes in patients with high grade inflammation (Wagner *et al*., 1998; Fessler *et al*., 2016; Fessler *et al*., 2017).

With regards to IL-6 blocker effects on peripheral blood, several studies have shown that Tocilizumab alters the regulation of peripheral blood cells in RA, however, some results were contradicting. Some showed that Tocilizumab administration in RA patients, among CD4+ T cells, induced a significant decrease in the proportion of Th17 and an increase

in naïve or activated Treg (Samson *et al*., 2012; Kasama *et al*., 2016). Others showed an expansion in the Treg population (Kikuchi *et al*., 2015; Schinnerling *et al*., 2017), with either no change or an increase in Th17 population (Dulic *et al*., 2017). Despite the contradictions, Tocilizumab treatment seem to restore the balance between the Treg and Th17 cells, which prevent downstream inflammatory effects (Schinnerling *et al*., 2017). Tocilizumab also changed the proportion of B-cell subsets and monocytes (Nakayamad *et al*., 2018).

Besides the role in T-cell phenotype differentiation, IL-6 is also the stimulus for increased production of reactive oxygen species (Win *et al*., 2018). Inflammation is intricately linked to oxidative stress. With increased oxidative stress, nuclear factor (NF-κB) pathways are activated which consequently enhances the production of cytokines and chemokines. This fuels the inflammation that stimulates ROS in a vicious cycle (Costa *et al*., 2018). Oxidative stress is a well-known mechanism implicated in accelerated telomere attrition and cellular senescence (von Zglinicki, 2002; Correia-Melo *et al*., 2014; Win *et al*., 2018). In a model of colorectal cancer, Wang *et al*. (2019), reported that IL-6/STAT3 signalling increased the expression of mitochondrial single-strand DNA binding protein (mtSSB), a vital molecule regulating mitochondrial DNA replication and TERT expressions. IL-6/STAT3 signalling also increase the production of ROS, which increased telomerase activity. In a micro-environment of inflammation, IL-6, via mtSSB, accelerated cell replication and the upregulation of telomerase activity, hence increase the longevity of the cancer cells. Although the regulation of telomere physiology may be altered in cancer, some insight into the effects of IL-6 on telomere attrition in chronic inflammation may be helpful.

In patients with RA, treatment with Tocilizumab decreases oxidative stress (Ruiz-Limón *et al*., 2017; Costa *et al*., 2018; Kizaki *et al*., 2018). There is currently no evidence that IL-6 blocker therapy impacts telomere length in RA. One study reported that abatacept therapy, a biologic therapy that also alters T cell function, suppressed the telomerase activity of both T and B lymphocytes (Otani & Kurosaka, 2019). Taken together, IL-6 blocker therapy may have indirectly altered telomere physiology in the current study by either changing T cell proliferation or by altering the oxidative stress. Although both TNF-α blocker and IL-6 blocker therapy likely altered telomere physiology, the mechanisms whereby these drugs acted are likely not the same. Future studies should include measures of oxidative stress and white blood cell count to elucidate the mechanisms whereby these DMARDs affect telomere biology.

5.3 Leukocyte telomere length as a biomarker for vascular function in inflammatory conditions

In the current study we investigated whether telomere length is associated with markers of vascular function. We showed that leukocyte telomere length was associated with systolic blood pressure and with pulse pressure in the control and inflammation groups. The relationship between leukocyte telomere length, systolic blood pressure and pulse pressure were unaltered after adjusting for body weight and sex. When the drug-treated groups were included in the model, these associations no longer persisted. Adipose tissue telomere length was not associated with blood pressure or pulse pressure. It is well known that persistent elevation in blood pressures is one of the main risk factors for cardiovascular events such as stroke, myocardial infarction, coronary artery disease and kidney damage (Wang *et al*., 2005). Pulse pressure, calculated as the difference between

systolic and diastolic blood pressure, is frequently considered a marker of arterial stiffness and predicts the development of CVD, independent of blood pressure (Millar & Lever, 2000; Blacher *et al*., 2000).

Despite several studies investigating the relationship between leukocyte telomere length and blood pressure, the results are contrasting. Numerous studies have reported inverse associations between telomere length and blood pressure and pulse pressure in the general population (Benetos *et al*., 2001; Jeanclos *et al*., 2000; Yang *et al*., 2009; Peng *et al*., 2017), while others reported no associations (Tentolouris *et al*., 2007; Aydos & Tükün 2007; Raymond *et al*., 2015; Koriath *et al*., 2019). The association between telomere length and blood pressure in high-grade inflammation is controversial (Haque *et al*., 2013; Rehkopf *et al*., 2016; Raymond *et al*., 2016b). Although most studies reported inverse associations between blood pressure and telomere length, our results showed a paradoxical direct relationship between leukocyte telomere length and blood pressure and pulse pressure.

We reported that independent of drug treatment, leukocyte telomere length, but not adipose tissue telomere length was associated with endothelial function. In the current study we showed that leukocyte telomere length was not associated with the sensitivity (half-maximal responses) of the renal arteries to vasodilators or vasoconstrictors, but that it was related to the maximal contraction and relaxation responses in renal arteries. The associations with endothelial dependent vasodilation and vasoconstriction were independent of drug treatments, as these associations remained significant when drug treated groups were included in the analysis. We further showed that in all groups,

leukocyte telomere length, but not adipose tissue telomere length, was associated with increased concentration of VCAM-1.

Blood vessel reactivity to vasodilators and vasoconstrictors is a marker of vascular function (Hassan, 2006). Particular in this study, the vasodilators and constrictors used involve endothelial dependent pathways of vessel dilation. Impaired vessel reactivity indicates impaired endothelial function (Palma Zochio Tozzato *et al.,* 2016). Endothelial dysfunction is associated with virtually all known risk factors for CVD (Sagara *et al*., 2016) and independently predicts the risk of future cardiovascular events in the general population (Bonetti *et al*., 2003). Endothelial dysfunction is increased in RA compared to the general population and is considered one of the earliest markers of CVD in RA (Bordy *et al*., 2018). Indeed, we have previously shown that exposure to inflammation in a model of CIA impairs endothelial dependent vessel reactivity (Mokotedi *et al*., 2019). VCAM-1 is a marker of endothelial activation and is considered one of the earliest markers of the development of endothelial dysfunction and atherosclerosis (Liao, 2013). Increased VCAM-1 concentrations are frequently reported in RA and is considered a risk factor for CVD in RA (Dessein *et al.,* 2005; Wållberg-Jonsson *et al.,* 2008). In this study we showed increased VCAM-1 concentrations in the inflammation group, which were reduced in the groups treated with DMARDs. Indeed, previous human studies have shown reduced VCAM-1 concentrations following treatment with biologic DMARDs (Verschueren *et al*., 1999; Ruiz-Limón *et al.,* 2017; Jawahar *et al*., 2018). A previous animal study has also shown improved endothelial function in rats treated with etanercept (Totoson *et al*., 2016). Taken together, endothelial dysfunction and VCAM-1 are important risk predictors for

atherosclerosis, arterial stiffness, increased blood pressure and heart failure (Ryan *et al*., 1995; Ceravolo *et al*., 2003; Bordy *et al*., 2018;).

Chronic inflammation and oxidative stress are central in the process of endothelial dysfunction and atherosclerosis. They cause accelerated telomere loss per cell replication and premature cellular senescence in endothelial cells, vascular smooth muscle cells and blood leukocytes (Yeh & Wang, 2016). Hence it is not surprising that leukocyte telomere length was associated with endothelial dysfunction markers in the current study. A seminal study, published in Circulation, reported that inhibition of telomere function in endothelial cells leads to cellular senescence that enhances endothelial cell activation and endothelial dysfunction (Minamino *et al*., 2002). In RA, Raymond *et al.* (2016b) also reported associations between leukocyte telomere length and markers of endothelial activation. The relationships between endothelial activation and telomere length is believed to be as a result of decreased nitric oxide synthase (eNOS) activity in endothelial cells (Sato *et al*., 1993). In agreement with previous studies, in the current study we showed associations between leukocyte telomere length and endothelial dependent vasoconstriction and vasodilation responses in renal arteries and with VCAM-1. Similar to the associations with blood pressure, the relationship between telomere length and endothelial function were paradoxically direct. Similarly, previous reports in high-grade inflammatory studies showed paradoxical relationships between telomere length and other traditional markers of CVD, including blood pressure, BMI, atherogenic index and arterial stiffness (Haque *et al*., 2013; Raymond *et al*., 2016b; Zidan, 2020).

In this regard, it is well known that cumulative inflammation uniquely affects traditional CVD risk factors in RA (Kitas & Gabriel, 2011; Gabriel & Crowson, 2012). For instance, RA patients with lower BMI, lower lipid concentrations and lower body weight have a higher risk of CVD and mortality (Maradit-Kremers *et al*., 2004; Escalante *et al*., 2005; Myasoedova *et al*., 2011). Furthermore, longer telomere lengths have also been paradoxically directly related to increased atherosclerosis in RA (Raymond *et al*., 2016b) and lupus (Haque *et al*., 2013). These findings are supported by evidence that inflammatory stimuli increase telomerase activity in macrophages to prevent cellular senescence during atherosclerosis (Gizard *et al*., 2011). It has been suggested that patients exposed to high-grade inflammation may have compensatory adaptive changes in metabolic pathways, that result in longer telomere lengths, in an attempt to reduce enhanced CVD risk (Raymond *et al*., 2016b). Therefore, rats in the current study with higher inflammatory load and hence increased risk may recruit compensatory mechanisms or may have increased the rate of leukocyte turnover which may explain the increase in telomere length to prevent cellular senescence. This may be plausible considering the controversial relationship between RA and telomerase activity (Dehbi *et al*., 2013) and the various paradoxical relationships reported between telomere length and obesity (Haque *et al*., 2013), lipid levels (Haque *et al*., 2013; Raymond *et al*., 2016b) arterial stiffness (Zidan, 2020) and atherosclerosis (Haque *et al*., 2013; Raymond *et al*., 2016b). Nevertheless, these controversial findings between telomere length and endothelial function in rats exposed to chronic inflammation warrant further investigation.

5.4 Leukocyte telomere length as a biomarker for cardiac structure and function in high-grade inflammation

In the current study we showed that leukocyte telomere length was not associated with any marker of cardiac remodelling or systolic function. In contrast, shorter adipose tissue telomere length was consistently associated with adverse left ventricular systolic, but not diastolic function. In the general population, patients with heart failure reportedly have shorter leukocyte telomere lengths (Van der Harst *et al*., 2007; Weischer *et al*., 2012). Telomere attrition in cardiac cells may contribute to the development of heart failure by stimulating apoptotic and cellular senescence pathways (Chimenti *et al*., 2003). Considering the strong associations between inflammation and heart failure (Yndestad *et al*., 2006) and between inflammation and telomere attrition (Bekaert *et al*., 2007), it is not surprising that telomere length has been considered a biomarker for the prediction of heart failure in inflammatory conditions.

Similar to the finding of the current study, in an animal study where rats were exposed to low-grade inflammation, despite reduced telomere length in leukocytes and in cardiac cells, neither leukocyte not cardiac tissue telomere length were associated with cardiac structural remodelling (Raymond *et al*., 2014). Similarly, Raymond *et al.* (2016a) reported in a model of alcohol induced cardiomyopathy, that telomere lengths were not associated with cardiac geometry changes. Although hypertrophic cardiac remodelling has been reported in chronic inflammation (Westermann *et al*., 2011; Mokotedi *et al*., 2020), previous studies have suggested that telomere length is not a suitable biomarker for changes in cardiac geometry(Akasheva *et al*., 2015; Raymond *et al*., 2016a;).

Exposure to inflammation was previously shown to impair systolic function (Torre-Amione *et al*., 1996) and patients with reduced ejection fraction have been noted to have shorter cardiomyocyte telomere length (Sharifi-Sanjani *et al*., 2017). Similar to previous studies, we showed no association between leukocyte telomere length and cardiac systolic function in animal models of inflammation and oxidative stress (Raymond *et al*., 2014; Denil *et al.,* 2014; Akasheva *et al.,* 2015; Raymond *et al*., 2016a). One previous study found shorter leukocyte telomere lengths to be related to impaired systolic function in the elderly (Collerton *et al*., 2007). In contrast, we showed an association between shorter adipose tissue telomere length and impaired cardiac systolic function. The discrepancies between our results and previous studies may be explained by different animal models of inflammation and the differences in tissues used to measure telomere length.

Several studies have reported a higher likelihood of diastolic dysfunction in RA (Liang *et al*., 2010; Davis *et al*., 2008). Diastolic function measures represent the ability of the ventricle to relax which in turn promotes ventricular filling (Henein & Lindqvist, 2015). Measures of diastolic function, including E/A and E/e' ratios are independent predictors of heart failure (Mitter *et al*., 2017). It has been indicated that inflammation is one of the risk factors for diastolic dysfunction in RA (Liang *et al*., 2010). In a recent study, our lab reported that rats exposed to collagen-induced inflammation developed impaired diastolic, but not systolic function, in the early stages of exposure to inflammation (Mokotedi *et al*., 2020).

Leukocyte telomere length was not associated with impaired relaxation, but it was associated with E/e', a marker of increased left ventricular filling pressures in diastole in the control and inflammation groups. When the drug treated groups were included in the

analysis this association was no longer significant. Adipose tissue telomere length was not associated with any marker of diastolic function. Previous studies investigating the association between leukocyte telomere length and markers of diastolic function have shown contradicting results in the general population (Farzaneh-Far *et al*., 2008; Collerton *et al*., 2007; Denil *et al*., 2014; Zhang *et al*., 2014; Akasheva *et al*., 2015). To our knowledge no previous studies have reported the relationship between telomere length and cardiac function in high-grade inflammation, hence further investigations are required to explore this relationship.

Taken together, leukocyte telomere length is not consistently associated with diastolic dysfunction markers, despite a high likelihood of adversely affected diastolic function in high grade inflammation. Leukocyte telomere length was also not associated with changes in cardiac geometry or left ventricular systolic function in high grade inflammation. In contrast, shorter adipose tissue telomere length was associated with impaired cardiac systolic function. These results suggest that the effects of inflammation on telomere attrition is different across tissues. Also, leukocyte and adipose tissue telomere length have disparate associations with markers of endothelial and cardiac function. The short duration of exposure to inflammation in the current study may have impacted cardiac apoptosis and cellular senescence, and hence the relationship between telomere length and cardiac function (Chimenti *et al*., 2003). Exposure to inflammation for longer periods and measuring telomere length in various different tissues as well as leukocytes should be investigated in future studies.

5.5 Limitations

This study had several further limitations. First, the DNA was extracted from whole blood and tissue samples were stored at -80°C. Although DNA is relative stable for extended periods of time, the storage may have affected the measurement of telomere length. Second, the qRT-PCR method measures the average telomere length across all leukocytes. Although relative telomere length measured by real time PCR is a validated approach, measures of telomerase activity, oxidative stress and white blood cell count may have strengthened our findings and elucidated the mechanisms involved in the findings. We found disparate results for tissue and leukocyte telomere lengths. This suggest that measures of leukocyte telomere length may not reflect telomere length in specific tissues. Although evidence suggests that telomere lengths in different tissues are strongly correlated (Takubo *et al*., 2002) leukocyte telomere length may not be an exact indication of telomere attrition in specific cells (Dlouha *et al*., 2014). Also, although we chose to measure telomere length in adipose tissue because of its relative stability, adipose tissue telomere length is not necessarily representative of telomere length in the vasculature or the heart. Future studies should consider measuring telomere length in the vasculature. However, this approach is complicated by the heterogenous composition of the vasculature, such as connective tissue, smooth muscle and endothelium. Hence the interpretation of such result could be difficult.

Including both male and female rats may have impacted our results. However, there were no differences in telomere lengths between males and females. We further included sex and body weight as potential confounders in all correlation analysis, which did not materially alter our results. This does not exclude the possibility that sex hormones may

have impacted the results, and hence should be measured in future studies. Lastly, we used humanised TNF-α dimer and a humanised anti-IL-6 monoclonal antibody in the biologic DMARD treated groups. As previously discussed in sections 5.2.1 and 5.2.2, it is possible that these treatments did not bind effectively to the rat cytokines and the receptors. The lack of white blood cell measures prohibited the confirmation of effective drug effect. Besides the use of human monoclonal antibodies in these studies which may have impacted the drug effectiveness in rats, in human studies, it is well known that not all patients respond equally well to DMARDs (Backhaus *et al*., 2015). Failure of DMARDs have largely been ascribed to genetic differences and the confounding effects of other drug treatments and environmental influences (Chen *et al*., 2015; Tarnowski *et al*., 2016; Smolen *et al*., 2017). The use of an animal study that is free of these confounding effects may limit the number of non-responders in the current study. Future studies should nevertheless determine whether biologic DMARD failure is associated with specific genetic polymorphisms. Despite these concerns, the TNF-α blocker group showed paw thickness measures similar to that of the control group. Although the arthritis scores were increased in the TNF-α blocker group, it may be that the subjective nature of arthritis scoring increases the risk of errors. Using paw thickness at the ankle and tarsometatarsal joints may be more reliable, as they are more objective methods of scoring paw swelling. There were also significant reductions in VCAM-1 concentrations in both drug-treated groups, which further support the drug effectiveness. Previously, etanercept has shown benefits in endothelial function in rats exposed to high grade inflammation (Totoson *et al*., 2016). Tocilizumab administration in rats have previously shown significant effects on disease progression, however, the mode of administration and the dosage were different

from the current study (Abdel-Maged *et al*., 2018). Hence, the frequency of the IL-6 blocker administration in the current study may explain the lack of effect on the paw scores and CRP concentrations. Indeed, a recent study suggested more frequent administration of IL-6 receptor blockers in the acute phase of disease (Ogata *et al*., 2019). Future studies should consider using a rat specific TNF-α inhibitor and a rat specific IL-6 receptor blocker.

5.6 Conclusion

In conclusion, this study indicates that exposure to chronic inflammation as induced by type II collagen in Sprague-Dawley rats resulted in a paradoxical longer leukocyte telomere length, but not adipose tissue telomere length, compared to control rats. Higher circulating CRP concentrations, but not TNF-α concentrations were associated with increased leukocyte telomere length. Administering biologic DMARDS targeting TNF-α and IL-6 receptors, prevented the inflammation induced changes in telomere length. Leukocyte telomere length was paradoxically associated with impaired endothelial dependent renal artery relaxation and contraction, independent of body weight, sex or drug treatment. Longer leukocyte telomere length was also associated with increased concentrations of VCAM-1. Although telomere length was associated with systolic blood pressure, pulse pressure and increased left ventricular filling pressure, this association was impacted by drug treatment. Leukocyte telomere length was not associated with markers of left ventricular remodelling or left ventricular systolic function. In contrast, shorter adipose tissue telomere length was associated with impaired cardiac systolic, but not diastolic function.

These results suggest that leukocyte and adipose tissue telomere length are not similarly affected by exposure to inflammation and that leukocyte telomere length may not be a surrogate marker for tissue specific telomere shortening in high grade inflammation. The longer leukocyte telomere length may therefore not be an indication of younger biological age in those exposed to inflammation, but rather that in the early stages of inflammatory conditions, cellular pathways and mechanisms which alter telomere physiology are upregulated as a protective mechanism for those at high risk. Biological DMARDs may impact the inflammation induced changes in leukocyte telomere physiology, but the mechanisms whereby the drugs act may be different. Future studies should investigate the exact mechanisms whereby biologic DMARDs affect telomere attrition in specific tissues. Our results and previous reports of paradoxical relationships between longer leukocyte telomeres and a worse CVD risk profile highlight the complex interaction between chronic exposure to high grade inflammation, cellular protection mechanisms and CVD risk. Our study adds to the mounting evidence suggesting that in chronic inflammation, compensatory protective mechanisms are employed in those with adverse CVD risk profiles. The exact mechanisms that underpin these intricate interactions require further investigation. Based on the current results, telomere length may not be a valid biomarker of vascular and cardiac function in high grade inflammation. The exact role of leukocyte and tissue telomere length in CVD risk stratification in chronic inflammation warrants further studies.

REFERENCES

Abdel-Maged AE, Gad AM, Abdel-Aziz AK, Aboulwafa MM & Azab SS (2018). Comparative study of anti-VEGF Ranibizumab and Interleukin-6 receptor antagonist Tocilizumab in Adjuvant-induced Arthritis. *Toxicol Appl Pharmacol* **356**, 65–75.

Abegunde DO, Mathers CD, Adam T, Ortegon M & Strong K (2007). The burden and costs of chronic diseases in low-income and middle-income countries. *Lancet* **370**, 1929–1938.

Adhikari NK, Fowler RA, Bhagwanjee S & Rubenfeld GD (2010). Critical care and the global burden of critical illness in adults. *Lancet* **376**, 1339-1346.

Ahmad S, Heraclides A, Sun Q, Elgzyri T, Rönn T, Ling C, Isomaa B, Eriksson KF, Groop L, Franks PW & Hansson O (2012). Telomere length in blood and skeletal muscle in relation to measures of glycaemia and insulinaemia. *Diabet Med* **29**, e377-e381.

Akasheva DU, Plokhova EV, Tkacheva ON, Strazhesko ID, Dudinskaya EN, Kruglikova AS, Pykhtina VS, Brailova NV, Pokshubina IA, Sharashkina NV & Agaltsov MV (2015). Age-related left ventricular changes and their association with leukocyte telomere length in healthy people. *PloS one* **10**.

Akiyama M, Hideshima T, Hayashi T, Tai YT, Mitsiades CS, Mitsiades N, Chauhan D, Richardson P, Munshi NC & Anderson KC (2003). Nuclear factor-κB p65 mediates tumor necrosis factor α-induced nuclear translocation of telomerase reverse transcriptase protein. *Cancer Res* **63**, 18-21.

Al-Aly Z, Pan H, Zeringue A, Xian H, McDonald JR, El-Achkar TM & Eisen S (2011). Tumor necrosis factor-α blockade, cardiovascular outcomes, and survival in rheumatoid arthritis. *Transl Res* **157**, 10-18.

Ali AT, Hochfeld WE, Myburgh R & Pepper MS (2013). Adipocytes and adipogenesis. *Eur J Cell Biol* 92, 229-236

Ambrosino P, Lupoli R, Di Minno A, Tasso M, Peluso R & Di Minno MN (2015). Subclinical atherosclerosis in patients with rheumatoid arthritis. *Thromb Haemost* **113**, 916-930.

Antoniou KM, Margaritopoulos GA, Proklou A, Karagiannis K, Lasithiotaki I, Soufla G, Kastrinaki MC, Spandidos DA, Papadaki HA & Siafakas NM (2012). Investigation of telomerase/telomeres system in bone marrow mesenchymal stem cells derived from IPF and RA-UIP. *J Inflamm* **9**, 27.

Aronoff GR & Aronoff JR (2014). Drug prescribing in kidney disease: can't we do better. *Am J Kidney Dis* **63**, 382-383.

Artlett CM, Black CM, Briggs DC, Stevens CO & Welsh KI (1996). Telomere reduction in scleroderma patients: a possible cause for chromosomal instability. *Rheumatol* **35**, 732-737.

Aslam F, Bandeali SJ, Khan NA & Alam M (2013). Diastolic dysfunction in rheumatoid arthritis: a meta-analysis and systematic review. *Arthritis Care Res* **65**, 534-543.

Aubert G & Lansdorp PM (2007). Telomerase and aging. *Physiol Rev* **88**, 557-579.

Avina-Zubieta JA, Choi HK, Sadatsafavi M, Etminan M, Esdaile JM & Lacaille D (2008). Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies. *Arthritis Care Res* **59**, 1690-1697.

Avina-Zubieta JA, Thomas J, Sadatsafavi M, Lehman AJ & Lacaille D (2012). Risk of incident cardiovascular events in patients with rheumatoid arthritis: a meta-analysis of observational studies. *Ann Rheum Dis* **71**, 1524-1529.

Aviv A & Levy D (2012). Telomeres, atherosclerosis, and the hemothelium: the longer view. *Annu Rev Med* **63**, 293-301.

Aviv A, Kark JD & Susser E (2015). Telomeres, atherosclerosis, and human longevity: a causal hypothesis. *Epidemiol* **26**, 295-299.

Aviv A, Valdes AM & Spector TD (2006). Human telomere biology: pitfalls of moving from the laboratory to epidemiology*. Int J Epidemiol* **35**,1424-1429.

Aydos SE & Tükün A (2007). Does telomere length affect blood pressure. *Adv Ther* **24**, 269-272.

Backhaus M, Kaufmann J, Richter C, Wassenberg S, Roske AE, Hellmann P & Gaubitz M (2015). Comparison of tocilizumab and tumour necrosis factor inhibitors in rheumatoid arthritis: a retrospective analysis of 1603 patients managed in routine clinical practice. *Clin Rheumatol* **34**, 673-681.

Balaban RS, Nemoto S & Finkel T (2005). Mitochondria,oxidants and aging. *Cell* **120**, 483- 495.

Baniaamam M, Paulus WJ, Blanken AB, Nurmohamed MT (2018). The effect of biological DMARDs on the risk of congestive heart failure in rheumatoid arthritis: a systematic review. *Expert Opin Biol Ther* **18**, 585-94.

Barden A, O'Callaghan N, Burke V, Mas E, Beilin LJ, Fenech M, Irish AB, Watts GF, Puddey IB, Huang RC & Mori TA (2006). n-3 fatty acid supplementation and leukocyte telomere length in patients with chronic kidney disease. *Nutrients* **8**, 175.

Barnes RP, Fouquerel E & Opresko PL (2019). The impact of oxidative DNA damage and stress on telomere homeostasis. *Mech Ageing Dev* **177**, 37-45.

Barrett EL & Richardson DS (2011). Sex differences in telomeres and lifespan. *Aging cell* **10**, 913-921.

Barton A & Worthington J (2009). Genetic susceptibility to rheumatoid arthritis: an emerging picture. *Arthritis Care Res* **61**, 1441-1446.

Beier F, Balabanov S, Amberger CC, Hartmann U, Manger K, Dietz K & Brümmendorf TH (2007). Telomere length analysis in monocytes and lymphocytes from patients with systemic lupus erythematosus using multi-color flow-FISH. *Lupus* **16**, 955–962.

Bekaert S, De Meyer T, Rietzschel ER, De Buyzere ML, De Bacquer D, Langlois M, Segers P, Cooman L, Van Damme P, Cassiman P & Van Criekinge W (2007). Telomere length and cardiovascular risk factors in a middle-aged population free of overt cardiovascular disease. *Aging cell* **6**, 639-647.

Benetos A, Gardner JP, Zureik M, Labat C, Xiaobin L, Adamopoulos C, Temmar M, Bean KE, Thomas F & Aviv A (2004). Short telomeres are associated with increased carotid atherosclerosis in hypertensive subjects. *Hypertension* **43**, 182-185.

Benetos A, Okuda K, Lajemi M, Kimura M, Thomas F, Skurnick J, Labat C, Bean K & Aviv A (2001). Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. *Hypertension* **37**, 381–385.

Benjamin O & Lappin SL (2019). Disease modifying anti-rheumatic drugs (DMARD). *InStatPearls [Internet] StatPearls Publishing*.

Bernatsky S, Hudson M & Suissa S (2005). Anti-rheumatic drug use and risk of hospitalization for congestive heart failure in rheumatoid arthritis. *Rheumatol* **44**, 677–680.

Bhatnagar A, Verma R, Vasudevan B & Saraswat N (2015). Acute methotrexate toxicity presenting as ulcers in plaques of psoriasis vulgaris. *Indian Dermatol online J* **6**, 232.

Bili A, Tang X, Pranesh S, Bozaite R, Morris SJ, Antohe JL, Kirchner HL & Wasko MC (2014). Tumor necrosis factor α inhibitor use and decreased risk for incident coronary events in rheumatoid arthritis. *Arthritis care Res* **66**, 355-363.

Blacher J, Staessen JA, Girerd X, Gasowski J, Thijs L, Liu L, Wang JG, Fagard RH & Safar ME (2000). "Pulse pressures not mean pressure determines cardiovascular risk in older hypertensive patients". *Arch Intern Med*. **160**, 1085–1089.

Blackburn EH (2001). Switching and signalling at the telomeres. *Cell* **106**, 661-673.

Blasco MA (2007). Telomere length, stem cells and aging. *Nat Chem Biol* **3**, 640-649.

Blinova EA, Zinnatova EV, Barkovskaya MS, Borisov VI, Sizikov AE, Kozhevnikov VS, Rubtsov NB & Kozlov VA (2016). Telomere length of individual chromosomes in patients with rheumatoid arthritis. *B Exp Biol Med* **160**, 779-782.

Blum A & Adawi M (2019). Rheumatoid arthritis (RA) and cardiovascular disease. *Autoimmunity Rev* **18**, 679-690.

Bolon B, Stolina M, King C, Middleton S, Gasser J, Zack D & Feige U (2010). Rodent Preclinical Models for Developing Novel Antiarthritic Molecules: Comparative Biology and Preferred Methods for Evaluating Efficacy. *J Biomed Biotechnol* **2011**, 569086.

Bonetti PO, Lerman LO & Lerman A (2003). Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol* **23**, 168-175.

Bongartz T, Cantaert T, Atkins SR, Harle P, Myers JL, Turesson C, Ryu JH, Baeten D & Matteson EL (2006). Citrullination in extra-articular manifestations of rheumatoid arthritis. *Rheumatol* **46**, 70-75.

Bordy R, Totoson P, Prati C, Marie C, Wendling D & Demougeot C (2018). Microvascular endothelial dysfunction in rheumatoid arthritis. *Nat Rev Rheumatol* **14**, 404-420.

Brouilette SW, Singh RK, Thompson JR, Goodall AH & Samani NJ (2003). Telomere length, risk of coronary heart disease and statin treatment in the west of scotland primary prevention study: a nested case-control study. *Lancet* **369**, 107-114.

Brown KE, Mathahs MM, Broadhurst KA, Coleman MC, Ridnour LA, Schmidt WN & Spitz DR (2007). Increased hepatic telomerase activity in a rat model of iron overload: a role for altered thiol redox state. *Free Radic Bio Med* **42**, 228–235.

Brümmendorf TH, Maciejewski JP, Mak J, Young NS & Lansdorp PM (2001). Telomere length in leukocyte subpopulations of patients with aplastic anemia. *Blood* **97**, 895-900.

Burke AP, Tracy RP, Kolodgie F, Malcom GT, Zieske A, Kutys R, Pestaner J, Smialek J & Virmani R (2002). Elevated C-reactive protein values and atherosclerosis in sudden coronary death: association with different pathologies. *Circulation* **105**, 2019-2023.

Burke R,White N, Farrell JF, Galloway EB & Minger H (2014). Biologic disease-modifying antirheumatic drugs. Pharmacotherapy self-assessment program. *Chronic illnesses* 9-31.

Buxton JL, Walters RG, Visvikis-Siest S, Meyre D, Froguel P & Blakemore AI (2011). Childhood Obesity Is Associated with Shorter Leukocyte Telomere Length. *J Clin Endocrinol Metabol* **96**, 1500-1505.

Campisi J (2013). Aging, cellular senescence, and cancer. *Ann Rev Physiol* **75**, 685-705.

Carty CL, Kooperberg C, Liu J, Herndon M, Assimes T, Hou L, Kroenke CH, LaCroix AZ, Kimura M, Aviv A & Reiner AP (2015). Leukocyte telomere length and risks of incident coronary heart disease and mortality in a racially diverse population of postmenopausal women. *Arterioscler Thromb Vasc Biol* **35**, 225-231.

Castaneda S, Nurmohamed MT & Gonzalez-Gay MA (2016). Cardiovascular disease in inflammatory rheumatic diseases. *Best Pract Res Clin Rheumatol* **30,** 851-869.

Cattan V, Mercier N, Gardner JP, Regnault V, Labat C, Mäki-Jouppila J & Lacolley P (2008). Chronic oxidative stress induces a tissue-specific reduction in telomere length in CAST/Ei mice. *Free Radic Biol Med* **44**, 1592–1598.

Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA (2003). Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet* **361**, 393-395.

Ceravolo R, Maio R, Pujia A, Sciacqua A, Ventura G, Costa MC, Sesti G & Perticone F (2003). Pulse pressure and endothelial dysfunction in never-treated hypertensive patients. *J Am Coll Cardiol* **41**, 1753-1758.

Chadwick W, Magnus T, Martin B, Keselman A, Mattson MP & Maudsley S (2008). Targeting TNF-α receptors for neurotherapeutics. *Trends Neurosci* **31**, 504-511.

Chan SR & Blackburn EH (2004). Telomeres and telomerase. *Philos Trans R Soc Lond B Biol Sci* **359**, 109-121.

Chimenti C, Kajstura J, Torella D, Urbanek K, Heleniak H, Colussi C, Di Meglio F, Nadal-Ginard B, Frustaci A, Leri A, Maseri A & Anversa P (2003). Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure. *Circ Res* **93**, 604– 613.

Chiu CP, Dragowska W, Kim NW, Vaziri H, Yui J, Thomas TE, Harley CB & Lansdorp PM (1996). Differential expression of telomerase activity in hematopoietic progenitors from adult bone marrow cells. *Stem Cells* **14**, 239–248.

Chmielewski PP & Strzelec B (2018). Elevated leukocyte count as a harbinger of systemic inflammation, disease progression, and poor prognosis: a review. *Folia Morphol* **77**(2), 171-178.

Chmielewski PP, Borysławski K, Chmielowiec K, Chmielowiec J & Strzelec B (2016). The association between total leukocyte count and longevity: Evidence from longitudinal and cross-sectional data. *Ann Anat* **204**, 1-10.

Christensen K, Doblhammer G, Rau R & Vaupel JW (2009). Ageing populations: the challenges ahead. *Lancet* **374**,1196-1208.

Chung HY, Cesari M, Anton S, Marzetti E, Giovannini S, Seo AY, Carter C, Yu BP & Leeuwenburgh C (2009). Molecular inflammation: underpinnings of aging and age-related diseases. *Ageing Res Rev* **8**, 18-30.

Cimmino MA, Salvarani C, Macchioni P, Montecucco C, Fossaluzza V, Mascia MT, Punzi L, Davoli C, Filippini D & Numo R (2000). Extra-articular manifestations in 587 Italian patients with rheumatoid arthritis*. Rheumatol Int* **19**, 213-217.

Cojocaru M, Cojocaru IM, Silosi I, Vrabie CD & Tanasescu R (2010). Extra-articular manifestations in rheumatoid arthritis. *Maedica* **5**, 286.

Cole J, Busti A & Kazi S (2007). The incidence of new onset congestive heart failure and heart failure exacerbation in Veteran's Affairs patients receiving tumor necrosis factor alpha antagonists. *Rheumatol Int* **27**, 369–373.

Collerton J, Barrass K, Bond J, Eccles M, Jagger C, James O, Martin-Ruiz C, Robinson L, von Zglinicki T & Kirkwood T (2007). The Newcastle 85+ study: biological, clinical

and psychosocial factors associated with healthy ageing: study protocol. *BMC geriatrics* **7**, 14.

Colmegna I, Diaz-Borjon A, Fujii H, Schaefer L, Goronzy JJ, Weyand CM (2008). Defective proliferative capacity and accelerated telomeric loss of hematopoietic progenitor cells in rheumatoid arthritis. *Arthritis Rheum* **58**, 990–1000.

Compté N, Bailly B, De Breucker S, Goriely S & Pepersack T (2015). Study of the association of total and differential white blood cell counts with geriatric conditions, cardio-vascular diseases, seric IL-6 levels and telomere length. *Exp Gerontol* **61**, 105-112.

Correia-Melo C, Hewitt G & Passos JF (2014). Telomeres, oxidative stress and inflammatory factors: partners in cellular senescence. *Longev Healthspan* **3**, 1.

Costa NT, Iriyoda TMV, Alfieri DF & Dichi I (2018). Influence of disease-modifying antirheumatic drugs on oxidative and nitrosative stress in patients with rheumatoid arthritis*. Inflammopharmacol* **26**, 1151-1164.

Costenbader KH, Prescott J, Zee R Y, & De Vivo I (2011). Immunosenescence and rheumatoid arthritis: does telomere shortening predict impending disease. *Autoimmunity Rev* **10**, 569–573.

Curtis JR, Kramer JM, Martin C, Saag KG, Patkar N, Shatin D, Burgess M, Xie A & Braun MM (2007). Heart failure among younger rheumatoid arthritis and Crohn's patients exposed to TNF-α antagonists. *Rheumatol* **46**, 1688-1693.

D'Mello MJ, Ross SA, Briel M, Anand SS, Gerstein H & Pare G (2015). Association between shortened leukocyte telomere length and cardiometabolic outcomes: systematic review and meta-analysis. *Circulation* **8**, 82-90.

Davis JM, Roger VL, Crowson CS, Kremers HM, Therneau TM & Gabriel SE (2008). The presentation and outcome of heart failure in patients with rheumatoid arthritis differs from that in the general population. *Arthritis Rheum* **58**, 2603-2611.

De Cock D & Hyrich K (2018). Malignancy and rheumatoid arthritis: Epidemiology, risk factors and management. *Best Pract Res Clin Rheumatol* **32**, 869-886.

De Lange T (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* **19**, 2100-2110.

De Simone GD, Devereux RB & Wallerson DC (1994). Echocardiographic assessment of left ventricular hypertrophy in rats using a simplified approach. *Am J Hypertens* **7,** 555-558.

Dehbi AZ, Radstake TR & Broen JC (2013). Accelerated telomere shortening in rheumatic diseases: cause or consequence. *Clin Immunol* **9**, 1193-1204.

Del Rincón I, Williams K, Stern MP, Freeman GL & Escalante A (2001). High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. *Arthritis Rheum* **44**, 2737-2745.

Demissie S, Levy D, Benjamin EJ, Cupples LA, Gardner JP, Herbert A, Kimura M, Larson MG, Meigs JB, Keaney JF & Aviv A (2006). Insulin resistance, oxidative stress,

hypertension, and leukocyte telomere length in men from the Framingham Heart Study. *Aging cell* **5**, 325-330.

Demoruelle MK, Deane KD & Holers VM (2014). When and where does inflammation begin in rheumatoid arthritis. *Curr Opin Rheumatol* **26**, 64.

Denil SL, Rietzschel ER, De Buyzere ML, Segers P, De Bacquer D, Van Criekinge W, Bekaert S, Gillebert TC, De Meyer T, Asklepios Investigators (2014). On cross-sectional associations of leukocyte telomere length with cardiac systolic, diastolic and vascular function: the Asklepios study. *PloS one* **9**, e115071.

Dessein PH, Joffe BI & Singh S (2005). Biomarkers of endothelial dysfunction, cardiovascular risk factors and atherosclerosis in rheumatoid arthritis. *Arthritis Res Ther* **7**, R634-R643.

Dessein PH, Norton GR, Woodiwiss AJ, Joffe BI & Solomon A (2007). Independent role of conventional cardiovascular risk factors as predictors of C-reactive protein concentrations in rheumatoid arthritis. *J Rheumatol* **34**, 681-688.

Dieudonné Y, Gies V, Guffroy A, Keime C, Bird AK, Liesveld J, Barnas JL, Poindron V, Douiri N, Soulas-Sprauel P & Martin T (2019). Transitional B cells in quiescent SLE: An early checkpoint imprinted by IFN. *J Autoimmun* **102**, 150–158.

Ding H, Chen C, Shaffer JR, Liu L, Xu Y, Wang X, Hui R & Wang DW (2012). Telomere length and risk of stroke in Chinese. *Stroke* **43**, 658-663.

Dixon WG & Symmons DP (2007). What effects might anti-TNFα treatment be expected to have on cardiovascular morbidity and mortality in rheumatoid arthritis? A review of the role of TNFα in cardiovascular pathophysiology. *Ann Rheum Dis* **66**, 1132-1136.

Dlouha D, Maluskova J, Kralova Lesna I, Lanska V & Habacek JA (2014). Comparison of the relative telomere length measured in Leukocytes and eleven different human tissues. *Physiol Res* **63**, 343-350.

Donigian JR & de Lange T (2007). The role of the poly (ADP-ribose) polymerase tankyrase1 in telomere length control by the TRF1 component of the shelterin complex. *J Biol Chem* **282**, 22662–22667.

Dowman B, Campbell RM, Zgaga L, Adeloye D &Chan KY (2012). Estimating the burden of rheumatoid arthritis in Africa: A systematic analysis. *J Glob Health* **2**, 020406.

Dulic S, Vásárhelyi Z, Sava F, Berta L, Szalay B, Toldi G & Balog A (2017). T-cell subsets in rheumatoid arthritis patients on long-term anti-TNF or IL-6 receptor blocker therapy. *Mediators inflamm* **2017**.

Engler D & Skosana P (2016). Rheumatoid Arthritis. *S Afr Pharm J* **83**, 34-40.

Epel ES, Merkin SS, Cawthon R, Blackburn EH, Adler NE, Pletcher MJ & Seeman TE (2009). The rate of leukocyte telomere shortening predicts mortality from cardiovascular disease in elderly men. *Aging* 2009 **1**, 81.

Escalante A, Haas RW & Del Rincon I (2005). Paradoxical effect of body mass index on survival in rheumatoid arthritis: role of comorbidity and systemic inflammation. *Arch Intern Med* **165**, 1624-1629.

Everaerts S, Lammertyn EJ, Martens DS, De Sadeleer LJ, Maes K, van Batenburg AA, Goldschmeding R, van Moorsel CH, Dupont LJ, Wuyts WA & Vos R (2018). The aging lung: tissue telomere shortening in health and disease. *Respir Res* **19**, 95.

Farzaneh-Far R, Cawthon RM, Na B, Browner WS, Schiller NB & Whooley MA (2008). Prognostic value of leukocyte telomere length in patients with stable coronary artery disease: data from the Heart and Soul Study. *Arterioscler Thromb Vasc Biol* **28**, 1379-1384.

Fessler J, Raicht A, Husic R, Ficjan A, Duftner C, Schwinger W & Schirmer M (2016). Premature senescence of T-cell subsets in axial spondyloarthritis. *Ann Rheum Dis* **75**, 748-754.

Fessler J, Raicht A, Husic R, Ficjan A, Schwarz C, Duftner C, Schwinger W, Graninger WB, Stradner MH & Dejaco C (2017). Novel senescent regulatory T-cell subset with impaired suppressive function in rheumatoid arthritis. *Front immunol* **8**, 300.

Finnicum CT, Dolan CV, Willemsen G, Weber ZM, Petersen JL, Beck JJ, Codd V, Boomsma DI, Davies GE & Ehli EA (2017). Relative telomere repeat mass in buccal and leukocyte-derived DNA. *PLoS One* **12**, e0170765

Fitzpatrick AL, Kronmal RA, Gardner JP, Psaty BM, Jenny NS, Tracy RP, Walston J, Kimura M & Aviv A (2007). Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. *Am J epidemiol* **165**, 14-21.

Flores I, Canela A, Vera E, Tejera A, Cotsarelis G & Blasco AM (2008). The longest telomeres: a general signature of adult stem cell compartments. *Genes Dev* **28**, 654-667.

Fridlington JL, Tripple JW, Reichenberg JS, Hall CS & Diven DG (2011). Acute methotrexate toxicity seen as plaque psoriasis ulceration and necrosis: A diagnostic clue. *Dermatol online J* **17**, 2.

Friedrich U, Griese E, Schwab M, Fritz P, Thon K & Klotz U (2000). Telomere length in different tissues of elderly patients. *Mech Ageing Dev* **119**, 89-99.

Fujii H, Shao L, Colmegna I, Goronzy JJ & Weyand CM (2009). Telomerase insufficiency in rheumatoid arthritis. *Proc NatI Acad Sci* **106**, 4360-4365.

Fyhrquist F, Saijonmaa O & Strandberg T (2013). The roles of senescence and telomere shortening in cardiovascular disease. *Nat Rev Cardiol* **10**, 274.

Gabriel SE & Crowson CS (2012). Risk factors for cardiovascular disease in rheumatoid arthritis. *Curr opin rheumatol* **24**, 171-176.

Ganau A, Devereux RB, Roman MJ, De Simone G, Pickering TG, Saba PS, Vargiu P, Simongini I & Laragh JH (1992). Patterns of left ventricular hypertrophy and geometric remodeling in essential hypertension. *J Am Coll Cardiol* **19**, 1550-1558.

Gearing AJH, Bird CR, Bristow A, Polle S &Thorpe R (1987). A simple and sensitive bioassay for interleukin-1 which is unresponsive to 103 U/ml of interleukin-2. *J Immunol Methods* **99**, 7-11.

Gibiansky L & Frey N (2012). Linking interleukin-6 receptor blockade with tocilizumab and its hematological effects using a modeling approach. *J Pharmacokinet Pharmacodyn* **39**, 5-16.

Gil ME & Coetzer TL (2004). Real-time quantitative PCR of telomere length. *Mol Biotechnol* **27**, 169-172.

Gizard F, Heywood EB, Findeisen HM, Zhao Y, Jones KL, Cudejko C, Post GR, Staels B & Bruemmer D (2011). Telomerase activation in atherosclerosis and induction of telomerase reverse transcriptase expression by inflammatory stimuli in macrophages. *Arterioscler thromb vasc biol* **31**, 245-252.

Goglin SE, Farzaneh-Far R, Epel ES, Lin J, Blackburn EH & Whooley MA (2016). Change in leukocyte telomere length predicts mortality in patients with stable coronary heart disease from the heart and soul study. *PloS One* **11**, e0160748.

Goldenberg MM (1999). Etanercept, a novel drug for the treatment of patients with severe, active rheumatoid arthritis. *Clin Ther* ***21***, 75-87.

Golias CH, Tsoutsi E, Matziridis A, Makridis P, Batistatou A & Charalabopoulos K (2007). Leukocyte and endothelial cell adhesion molecules in inflammation focusing on inflammatory heart disease. *In Vivo* **21**, 757-769.

Gonzalez-Gay MA, Gonzalez-Juanatey C, Piñeiro A, Garcia-Porrua C, Testa A & Llorca J (2005). High-grade C-reactive protein elevation correlates with accelerated atherogenesis in patients with rheumatoid arthritis*. J Rheumatol* **32**, 1219-1223.

Gordon S &Taylor PR (2005). Monocyte and macrophage heterogeneity. *Nat Rev Immunol* **5**, 953-964.

Greenberg JD, Kremer JM, Curtis JR, Hochberg MC, Reed G, Tsao P, Farkouh ME, Nasir A, Setoguchi S & Solomon DH (2011). Tumour necrosis factor antagonist use and associated risk reduction of cardiovascular events among patients with rheumatoid arthritis. *Ann Rheum Dis* **70**, 576-582.

Greider CW (1996). Telomere length regulation. *Annu Rev Biochem* **65**, 337–365.

Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H & De Lange T (1999). Mammalian telomeres end in a large duplex loop. *Cell* **97**, 503514.

Grun LK, da Rosa Teixeira Jr N, von Mengden L, De Bastiani MA, Parisi MM, Bortolin R, Lavandoski P, Pierdoná V, Alves LB, Moreira JC & Mottin CC (2018). TRF1 as a major contributor for telomeres' shortening in the context of obesity. *Free Radic Biol Med* **129**, 286-295.

Gunter S, Robinson C, Norton GR, Woodiwiss AJ, Tsang L, Dessein PH & Millen AM (2017). Cardiovascular risk factors and disease characteristics are consistently associated with arterial function in rheumatoid arthritis. *J Rheumatol* **44**,1125-1133.

Hamilton K & Clair EW (2000). Tumour necrosis factor-α blockade: a new era for effective management of rheumatoid arthritis. *Expert Opin Pharmacother* **1**, 1041-1052.

Hançerli Y, Kaplan M, Tanoğlu A, Yeşilbaş S, Küçükodacı Z, Yıldırım M, Narlı G & Sakin YS (2017). Efficacy of tocilizumab treatment in cerulein-induced experimental acute pancreatitis model in rats. *Turk J Gastroenterol* **28**, 485-491.

Hanson B (2016). Necroptosis: A new way of dying. *Cancer Biol Ther* **17**, 899.

Haque S, Rakieh C, Marriage F, Ho P, Gorodkin R, Teh LS, Snowden N, Day PJ & Bruce IN (2013). Brief report: shortened telomere length in patients with systemic lupus erythematosus. *Arthritis Rheum* **65**, 1319-1323.

Harald K, Koskinen S, Jousilahti P, Torppa J, Vartiainen E, Salomaa V (2008). Changes in traditional risk factors no longer explain time trends in cardiovascular mortality and its socioeconomic differences. *J Epidemiol Comm Health* **62**, 251-257.

Hassan W (2006). The endothelium and endothelin: beyond vascular reactivity. *Ann Saudi Med* **26**, 343–345.

Haycock PC, Heydon EE, Kaptoge S, Butterworth AS, Thompson A & Willeit P (2014). Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis. *BMJ* **349**, g4227.

Heaphy CM, Fleet TM, Treat EG, Lee SJ, Smith AY, Davis MS, Griffith JK, Fischer EG & Bisoffi M (2010). Organ wide telomeric status in diseased and disease-free prostatic tissue. *Prostate* **70**,1471-1479.

Henein MY & Lindqvist P (2015). Assessment of left ventricular diastolic function by Doppler echocardiography. *Cardiac failure Rev* **1**, 87.

Herrmann M, Pusceddu I, März W & Herrmann W (2018). Telomere biology and age-related diseases. *Clin Chem Lab Med* **56**, 1210-1222.

Hills M, Lücke K, Chavez EA, Eaves CJ & Lansdorp PM (2009). Probing the mitotic history and developmental stage of hematopoietic cells using single telomere length analysis (STELA). *J Am Soc Hematol* **113**, 5765-5775.

Honda M, Mengesha E, Albano S, Nichols WS, Wallace DJ, Metzger A, Klinenberg JR & Linker-Israeli M (2001).Telomere shortening and decreased replicative potential, contrasted by continued proliferation of telomerase-positive CD8+ CD28(Io) T cells in patients with systemic lupus erythematosus. *Clin Immunol* **99**, 211–221.

Idriss HT & Naismith JH (2000). TNFα and the TNF receptor superfamily: Structure-function relationship (s). *Microsc Res Techniq* **50**, 184-195.

Ilmonen P, Kotrschal A & Penn DJ (2008). Telomere attrition due to infection. *PLoS one* **3**, e2143.

Jawahar SV, and Valentinovich YA (2018). Biological therapy of rheumatoid arthritis: is it real prediction of efficacy?.*Медицинский вестник Северного Кавказа*, *13*(3).

Jeanclos E, Schork NJ, Kyvik KO, Kimura M, Skurnick J & Aviv A (2000). Telomere length inversely correlates with pulse pressure and is highly familial. *Hypertension* **36**, 195–200.

Jurk D, Wilson C, Passos JF, Oakley F, Correia-Melo C, Greaves L, Saretzki G, Fox C, Lawless C, Anderson R & Hewitt G (2014). Chronic inflammation induces telomere dysfunction and accelerates ageing in mice. *Nat Commun* **5**, 4172.

Kaptoge S, Di Angelantonio E, Lowe G (2010). C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet* **375**, 132–140.

Kasama T, Isozaki T, Takahashi R & Miwa Y (2016). Clinical effects of tocilizumab on cytokines and immunological factors in patients with rheumatoid arthritis. *Int Immunopharmacol* **35**, 301-306.

Katayama Y & Kohriyama K (2001) Telomerase activity in peripheral blood mononuclear cells of systemic connective tissue diseases. *J Rheumatol* **28**, 288–291.

Kawanishi S & Oikawa S (2004). Mechanism of telomere shortening by oxidative stress. *Ann NY Acad Sci* **1019**, 278-284.

Khan S, Naidoo DP & Chuturgoon AA (2012). Telomeres and atherosclerosis. *Cardiovasc J Afr* **23**, 563.

Kikuchi J, Hashizume M, Kaneko Y, Yoshimoto K, Nishina N & Takeuchi T (2015). Peripheral blood CD4+ CD25+ CD127 low regulatory T cells are significantly increased by tocilizumab treatment in patients with rheumatoid arthritis: increase in regulatory T cells correlates with clinical response. *Arthritis Res Ther* **17**, 10.

Kim GW, Lee NR, Pi RH, Lim YS, Lee YM, Lee JM, Jeong HS & Chung SH (2015). IL-6 inhibitors for treatment of rheumatoid arthritis: past, present, and future. *Arch Pharm Res* **38**, 575-584.

Kim SC, Solomon DH, Rogers JR, Gale S, Klearman M, Sarsour K & Schneeweiss S (2017). Cardiovascular safety of tocilizumab versus tumor necrosis factor inhibitors

in patients with rheumatoid arthritis: a multi-database cohort study. *Arthritis Rheumatol* **69**, 1154-1164.

Kimura M, Gazitt Y, Cao X, Zhao X, Lansdorp PM & Aviv A (2010). Synchrony of telomere length among hematopoietic cells. *Exp Hematol* **38**, 854-859.

Kishimoto T, Akira S, Narazaki M & Taga T (1995). Interleukin-6 family of cytokines and gp130. *Blood* **86**, 1243-1254.

Kitas GD & Gabriel SE (2011). Cardiovascular disease in rheumatoid arthritis: state of the art and future perspectives*. Ann Rheum Dis* **70**, 8-14.

Kizaki K, Yamashita F, Hayashi T & Funakoshi N (2018). Infliximab equivalently suppresses oxidative stress compared to tocilizumab among well-controlled patients with rheumatoid arthritis. *Int J Rheum Dis* **21**, 1815-1821.

Klapper W, Moosig F, Sotnikova A, Qian W, Schro¨der JO & Parwaresch R (2004). Telomerase activity in B and T lymphocytes of patients with systemic lupus erythematosus. *Ann Rheum Dis* **63**, 1681–1683.

Kobayashi H, Kobayashi Y, Giles JT, Yoneyama K, Nakajima Y & Takei M (2014). Tocilizumab treatment increases left ventricular ejection fraction and decreases left ventricular mass index in patients with rheumatoid arthritis without cardiac symptoms: assessed using 3.0 tesla cardiac magnetic resonance imaging. *J Rheumatol* **41**, 1916-1921.

Koetz K, Bryl E, Spickschen K, O'Fallon WM, Goronzy JJ, Weyand CM (2000). T cell homeostasis in patients with rheumatoid arthritis. *Proc Natl Acad Sci USA* **97**, 9203–9208.

Kordinas V, Ioannidis A & Chatzipanagiotou S (2016). The telomere/telomerase system in chronic inflammatory diseases, Cause or effect. *Genes* **7**, 60.

Koriath M, Müller C, Pfeiffer N, Nickels S, Beutel M, Schmidtmann I & Wild PS (2019). Relative Telomere Length and Cardiovascular Risk Factors. *Biomolecules* **9**, 192.

Kurosaka D, Yasuda J, Yoshida K, Yokoyama T, Ozawa Y, Obayashi Y, Kingetsu I, Saito S & Yamada A (2003). Telomerase activity and telomere length of peripheral blood mononuclear cells in SLE patients, *Lupus* **12**, 591–599.

Kyo S, Takakura M, Kanaya T, Zhou W, Fujimoto K, Nishio Y, Orimo A & Inoue M (1999). Estrogen activates telomerase. *Cancer Res* **59**, 5917-5921.

Lang RM, Badano LP, Mor-Avi V, Afilalo J, Armstrong A, Ernande L, Flachskampf FA, Foster E, Goldstein SA, Kuznetsova T & Lancellotti P (2015). Recommendations for cardiac chamber quantification by echocardiography in adults: an update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *Eur Heart J Cardiovasc Imaging* **16**, 233-271.

Lansdorp PM (1998). Stem cell biology for the transfusionist. *Vox Sang* **74**, 91–94.

Lara-Molina EE, Franasiak JM, Marin D, Tao X, Díaz-Gimeno P, Florensa M, Martin M, Seli E & Pellicer A (2020). Cumulus cells have longer telomeres than leukocytes in reproductive-age women. *Fertil Steril* **113**, 217-223.

Larsson P, Kleinau S, Holmdahl R & Klareskog L (1990). Homologous type II collagen-induced arthritis in rats. Characterization of the disease and demonstration of clinically distinct forms of arthritis in two strains of rats after immunization with the same collagen preparation. *Arthritis Rheum* **33**, 693-701.

Lee JL, Sinnathurai P, Buchbinder R, Hill C, Lassere M & March L (2018). Biologics and cardiovascular events in inflammatory arthritis: a prospective national cohort study. *Arthritis Res Ther* **20**, 171.

Lee Y H & Bae S C (2018). Association between shortened telomere length and rheumatoid arthritis. *Zeitschrift für Rheumatologie* **77**, 160-167.

Leri A, Franco S, Zacheo A, Barlucchi L, Chimenti S, Limana F, Nadal-Ginard B, Kajstura J, Anversa P & Blasco MA (2003). Ablation of telomerase and telomere loss leads to cardiac dilatation and heart failure associated with p53 upregulation. *EMBO J* **22**,131-139.

Li Y, Goronzy JJ & Weyand CM (2018). DNA damage, metabolism and aging in pro-inflammatory T cells: Rheumatoid arthritis as a model system. *Exp Gerontol* **105**, 118-127.

Liang KP, Myasoedova E, Crowson CS, Davis JM, Roger VL, Karon BL, Borgeson DD, Therneau TM, Rodeheffer RJ & Gabriel SE (2010). Increased prevalence of diastolic dysfunction in rheumatoid arthritis. *Ann Rheum Dis* **69**,1665-1670.

Liao JK (2013). Linking endothelial dysfunction with endothelial cell activation. *J Clin Investig* **123**, 540-541.

Liberale L, Montecucco F, Tardif JC, Libby P & Camici GG (2020). Inflamm-ageing: the role of inflammation in age-dependent cardiovascular disease. *Eur Heart J* **41**, 2974-2982.

Listing J, Kekow J, Manger B, Burmester GR, Pattloch D, Zink A & Strangfeld A (2015). Mortality in rheumatoid arthritis: the impact of disease activity, treatment with glucocorticoids, TNFα inhibitors and rituximab*. Ann Rheum Dis* **74**, 415-421.

Listing J, Strangfeld A, Kekow J, Schneider M, Kapelle A, Wassenberg S & Zink A (2008). Does tumor necrosis factor α inhibition promote or prevent heart failure in patients with rheumatoid arthritis. Arthritis & Rheumatism. *J Am Coll Rheumatol* **58**, 667-677.

Liu B, Maekawa T, Yoshida K, Ly NH, Inoue K, Hasegawa A & Ishii S (2019). Telomere shortening by transgenerational transmission of TNF-α-induced TERRA via ATF7. *Nucleic acids Res* **47**, 283-298.

Liu J, Wang L, Wang Z & Liu JP (2019). Roles of telomere biology in cell senescence, replicative and chronological ageing. *Cells* **8**, 54.

Liu JP (1999). Studies of the molecular mechanisms in the regulation of telomerase activity. *FASEB J* **13**, 2091–2104.

López-Mejías R, Castañeda S, González-Juanatey C, Corrales A, Ferraz-Amaro I, Genre F, Remuzgo-Martínez S, Rodriguez-Rodriguez L, Blanco R, Llorca J & Martín J (2016). Cardiovascular risk assessment in patients with rheumatoid arthritis: The relevance of clinical, genetic and serological markers. *Autoimmunity Rev* **15**,1013-1030.

Lynch M, Ahern T, Sweeney CM, Malara A, Tobin AM, O'Shea D & Kirby B (2017). Adipokines, psoriasis, systemic inflammation, and endothelial dysfunction. *Int J Dermatol* **56**, 1103-1118.

Ma X & Xu S (2013). TNF inhibitor therapy for rheumatoid arthritis. *Biomed Rep* **1**, 177-184.

MacIntyre A, Brouilette SW, Lamb K, Radhakrishnan K, McGlynn L, Chee MM & Shiels PG (2008). Association of increased telomere lengths in limited scleroderma, with a lack of age-related telomere erosion. *Ann Rheum Dis* **67**, 1780–1782.

Mackey RH, Kuller LH & Moreland LW (2018). Update on cardiovascular disease risk in patients with rheumatic diseases. *Rheum Dis Clin* **44**, 475-487.

Madhusudan S, Foster M, Muthuramalingam SR, Braybrooke JP, Wilner S, Kaur K, Han C, Hoare S, Balkwill F, Talbot DC & Ganesan TS (2004). A phase II study of etanercept (Enbrel), a tumor necrosis factor α inhibitor in patients with metastatic breast cancer. *Clin Cancer Res* **10**, 6528-6534.

Madhusudan S, Muthuramalingam SR, Braybrooke JP, Wilner S, Kaur K, Han C, Hoare S, Balkwill F & Ganesan TS (2005). Study of etanercept, a tumor necrosis factor-alpha inhibitor, in recurrent ovarian cancer. *J Clin Oncol* **23**, 5950-5959.

Maekawa T, Liu B, Nakai D, Yoshida K, Nakamura K I, Yasukawa M & Masutomi K (2018). ATF7 mediates TNF-α–induced telomere shortening. *Nucleic acids Res* **46**, 4487-4504.

Mainous AG, Codd V, Diaz VA, Schoepf UJ, Everett CJ, Player MS & Samani NJ (2010). Leukocyte telomere length and coronary artery calcification. *Atherosclerosis* **210**, 262-267.

Majno G & Joris I (2004). *Cells, Tissues, and Disease*. Second Edition ed. Oxford: Oxford University Press.

Malik N, Kallestad JC, Gunderson NL, Austin SD, Neubauer MG, Ochs V, Marquardt H, Zarling JM, Shoyab MO & Wei CM (1989). Molecular cloning, sequence analysis, and functional expression of a novel growth regulator, oncostatin M. *Mol Cell Biol* **9**, 2847-2853.

Manicourt DH, Triki R, Fukuda K, Devogelaer JP, Deuxchaisnes CN & Thonar EJ (1993). Levels of circulating tumor necrosis factor α and interleukin-6 in patients with rheumatoid arthritis. relationship to serum levels of hyaluronan and antigenic keratan sulfate. Arthritis & Rheumatism. *J Am Coll Rheumatol* **36**, 490-499.

Mann DL, Bozkurt B, Torre-Amione G, Soran OZ & Sivasubramanian N (2008). Effect of the soluble TNF-antagonist etanercept on tumor necrosis factor bioactivity and stability. *Clin Transl Sci* **1**, 142-145.

Maradit-Kremers H, Crowson CS, Nicola PJ, Ballman KV, Roger VL, Jacobsen SJ & Gabriel SE (2004). Increased unrecognized coronary heart disease in rheumatoid arthritis: a population-based cohort study. *Arthritis Rheum* **50**, S688-S688.

Maradit-Kremers H, Crowson CS, Nicola PJ, Ballman KV, Roger VL, Jacobsen SJ & Gabriel SE (2005). Increased unrecognized coronary heart disease and sudden

deaths in rheumatoid arthritis: a population-based cohort study. *Arthritis Rheum* **52**, 402-411.

Martinez BK & White CM (2018). The emerging role of inflammation in cardiovascular disease. *Ann Pharmacother* **52**, 801-809.

Martínez-González K, Islas-Hernández A, Martínez-Ezquerro JD, Bermúdez-Rattoni F & Garcia-delaTorre P (2019). Telomere length and oxidative stress in the progression of Alzheimer's disease*. BioRxiv* 684365.

Masi S, D'Aiuto F, Martin-Ruiz C, Kahn T, Wong A, Ghosh AK, Whincup P, Kuh D, Hughes A, Von Zglinicki T & Hardy R (2014). Rate of telomere shortening and cardiovascular damage: a longitudinal study in the 1946 British Birth Cohort. *Eur Heart J* **35**, 3296-3303.

Masi S, Salpea KD, Li K, Parkar M, Nibali L, Donos N, Patel K, Taddei S, Deanfield JE, D'Aiuto F & Humphries SE (2011). Oxidative stress, chronic inflammation, and telomere length in patients with periodontitis. *Free Radic Biol Med* **50**, 730-735.

Mather KA, Jorm AF, Parslow RA & Christensen H (2011). Is telomere length a biomarker of aging, A review. *J Gerontol* **66**, 202-213.

Matthews C, Gorenne I, Scott S, Figg N, Kirkpatrick P, Ritchie A, Goddard M & Bennett M (2006). Vascular smooth muscle cells undergo telomere-based senescence in human atherosclerosis: effects of telomerase and oxidative stress. *Circ Res* **99**,156-164.

McInnes IB & Schett G (2011). The pathogenesis of rheumatoid arthritis. *New Eng J Med* **365**, 2205-2219.

Mihara M, Ohsugi Y& Kishimoto T (2011). Tocilizumab, a humanized anti-interleukin-6 receptor antibody, for treatment of rheumatoid arthritis. *Open access Rheumatol Res Rev* **3**, 19.

Millar JA & Lever AF (2000). Implications of pulse pressure as a predictor of cardiac risk in patients with hypertension. *Hypertension* **36,** 907–911.

Minamino T, Miyauchi H, Yoshida T, Ishida Y, Yoshida H & Komuro I (2002). Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation* **105**,1541-1544.

Mitter SS, Shah, SJ & Thomas JD (2017). A Test in Context: E/A and E/e′ to Assess Diastolic Dysfunction and LV Filling Pressure. *J Am Coll Cardiol* **69**, 1451–1464.

Mokotedi L, Michel FS, Mogane C, Gomes M, Woodiwiss AJ, Norton GR, Dessein PH, Millen AME (2020). Associations of inflammatory markers with impaired left ventricular diastolic and systolic function in collagen-induced arthritis. *PLoS one* **15**, e0230657.

Mokotedi L, Millen AM, Mogane C, Gomes M, Woodiwiss AJ, Norton GR & Michel FS (2019). Associations of inflammatory markers and vascular cell adhesion molecule-1 with endothelial dysfunction in collagen-induced arthritis. *Eur J pharmacol* **865**, 172786.

Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, Weaver AL, Keystone EC, Furst DE, Mease PJ & Ruderman EM (1999). Etanercept therapy in rheumatoid arthritis: a randomized, controlled trial. *Ann Intern Med* **130**, 478-486.

Morgan CL, Emery P, Porter D, Reynolds A, Young A, Boyd H, Poole CD & Currie CJ (2014). Treatment of rheumatoid arthritis with etanercept with reference to disease-modifying anti-rheumatic drugs: long-term safety and survival using prospective, observational data. *Rheumatol* **53**, 186-194.

Moro-García M A, Mayo J C, Sainz R M, & Alonso-Arias R (2018). Influence of inflammation in the process of T lymphocyte differentiation: proliferative, metabolic, and oxidative changes. *Front Immunol* **9**, 339.

Müezzinler A, Zaineddin AK & Brenner H (2012). A systematic review of leukocyte telomere length and age in adults. *Ageing Res Rev* **12**, 509-519.

Muira M, Karasaki Y, Abe T, Higashi K, Ikemura K & Gotoh S (1998). Prompt activation of telomerase by chemical carcinogens in rats detected with a modified TRAP assay. *Biochem Biophys Res Comm* **246**, 13–19.

Murata M (2018). Inflammation and cancer. *Environ health prev med* **23**, 1-8.

Myasoedova E, Crowson CS, Kremers HM, Roger VL, Fitz-Gibbon PD, Therneau TM & Gabriel SE (2011). Lipid paradox in rheumatoid arthritis: the impact of serum lipid measures and systemic inflammation on the risk of cardiovascular disease. *Ann Rheum Dis* **70**, 482-487.

Nakamura KI, Furugori E, Esaki Y, Arai T, Sawabe M, Okayasu I, Fujiwara M, Kammori M, Mafune KI, Kato M & Oshimura M (2000). Correlation of telomere length in normal and cancer tissue in the large bowel. *Cancer Lett* **158**, 179-184.

Nakayamada S, Kubo S, Yoshikawa M, Miyazaki Y, Yunoue N, Iwata S, Miyagawa I, Hirata S, Nakano K, Saito K & Tanaka Y (2018). Differential effects of biological DMARDs on peripheral immune cell phenotypes in patients with rheumatoid arthritis. *Rheumatol* **57**, 164-174.

Nanke Y, Kotake S, Akama H & Kamatani N (2002). Alkaline phosphatase in rheumatoid arthritis patients: possible contribution of bone-type ALP to the raised activities of ALP in rheumatoid arthritis patients. *Clin Rheum*, **21**, 198-202.

Navarro-Millan I, Singh J, & Curtis JR (2012). Systematic review of Tocilizumab for rheumatoid arthritis: A new biologic agent targeting the interleukin-6 receptor. *Clin Ther* **34**, 788–802.

Nawrot TS, Staessen JA, Holvoet P, Struijker-Boudier HA, Schiffers P, Van Bortel LM, Fagard R, Gardner JP, Kimura M & Aviv A (2010). Telomere length and its associations with oxidized-LDL, carotid artery distensibility and smoking. *Front Biosci* **2**, 1164-1168.

Nilsson PM (2012). Impact of vascular aging on cardiovascular disease: the role of telomere biology. *J Hypertens* **30**, S9-S12.

Nishimoto N, Terao K, Mima T, Nakahara H, Takagi N & Kakehi T. (2008). Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-

6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. *Blood* **112**, 3959-3964.

Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Hashimoto J, Azuma J & Kishimoto T (2004). Treatment of Rheumatoid arthritis with humanised anti-interleukin-6 receptor antibody: a multicenter double-blind, placebo- controlled trial. *Arthritis Rheum* **50**,1761-1796.

Noncommunicable diseases country profiles 2018 (2018). Geneva: World Health Organization. Licence: CC BY-NC-SA 3.0 IGO. Retrieved from https://www.who.int/nmh/publications/ncd-profiles-2018/en/

O'Donovan A, Pantell MS, Puterman E, Dhabhar FS, Blackburn EH, Yaffe K, Cawthon RM, Opresko PL, Hsueh WC, Satterfield S & Newman AB (2011). Cumulative inflammatory load is associated with short leukocyte telomere length in the Health, Aging and Body Composition Study. *PLoS one* **6**, 19687.

O'Dell JR (2002). Treating rheumatoid arthritis early: a window of opportunity. *Arthritis & Rheum* **46**, 283-285.

Odigari E, Kanada N, Jibiki K, Demura R, Aikawa E & Demura H (1994). Reduction of telomeric length and c-erbB-2 gene amplification in human breast cancer, Fibroadenoma and Gynecomastia. Relationship to histologic grade and clinical parameters. *Cancer* **73**, 2978-2984.

Ogata A, Kato Y, Higa S & Yoshizaki K (2019). IL-6 inhibitor for the treatment of rheumatoid arthritis: a comprehensive review. *Modern Rheumatol* **29**, 258-267.

Oikawa S, Tada-Oikawa S & Kawanishi S (2001). Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening. *Biochem* **40**, 4763-4768.

Okamoto K, Iwano T, Tachibana M & Shinkai Y (2008). Distinct roles of TRF1 in the regulation of telomere structure and lengthening. *J Biol Chem* **283**, 23981–23988.

Okuda Y (2008). Review of Tocilizumab in the treatment of Rheumatoid Arthritis. *Biologics* **2**, 75-82.

Olsson M, Geraghty NJ, Wapstra E & Wilson M (2020). Telomere length varies substantially between blood cell types in a reptile. *R Soc Open Sci* **7**, 192136.

Ormseth MJ, Solus JF, Oeser AM, Bian A, Gebretsadik T, Shintani A, Raggi P & Stein CM (2016). Telomere length and coronary atherosclerosis in rheumatoid arthritis. *J Rheumatol* **43**, 1469-1474.

Otani K & Kurosaka D (2019). Abatacept suppresses the telomerase activity of lymphocytes in patients with rheumatoid arthritis. *Int J Rheum Dis* **22**, 1138-1144.

Palma Zochio Tozzato G, Taipeiro E F, Spadella MA, Marabini Filho P, de Assis MR, Carlos CP & Chies AB (2016). Collagen-induced arthritis increases inducible nitric oxide synthase not only in aorta but also in the cardiac and renal microcirculation of mice. *Clin Exp Immunol* ***183***, 341-349.

Parish ST, Wu JE, Effros RB (2009). Modulation of T lymphocyte replicative senescence via TNF-α inhibition: role of caspase-3. *J Immunol* **182**, 4237–4243.

Passos JF, Saretzki G &von Zglinicki T (2002). DNA damage in telomeres and mitochondria during cellular senescence: is there a connection. *Nucleic Acids Res* **35**, 7505–7513.

Paul SR, Bennett F, Calvetti JA, Kelleher K, Wood CR, O'hara RM, Leary AC, Sibley B, Clark SC & Williams DA (1990). Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proc Nat Acad Sci* **87**, 7512-7516.

Pavlaki KI, Kastrinaki MC, Klontzas M, Velegraki M, Mavroudi I & Papadaki HA (2012) Abnormal telomere shortening of peripheral blood mononuclear cells and granulocytes in patients with chronic idiopathic neutropenia. *Haematologica* **97**, 743-750.

Peng H, Mete M, Desale S, Fretts AM, Cole SA, Best LG & Zhao J (2017). Leukocyte telomere length and ideal cardiovascular health in American Indians: The Strong Heart Family Study. *Eur J Epidemiol* **32**, 67-75.

Pennica D, King KL, SHAw KJ, Luis E, Rullamas J, LuOH SM, Darbonne WC, Knutzon DS, Yen R & Chien KR (1995). Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy *Proc Nat Acad Sci* **92**, 1142-1146.

Pironti G, Bersellini-Farinotti A, Agalave NM, Sandor K, Fernandez-Zafra T, Jurczak A, Lund LH, Svensson CI & Andersson DC (2018). Cardiomyopathy, oxidative stress and impaired contractility in a rheumatoid arthritis mouse model. *Heart* **104**, 2026-2034.

Rao VU, Pavlov A, Klearman M, Musselman D, Giles JT, Bathon JM, Sattar N & Lee JS (2015). An evaluation of risk factors for major adverse cardiovascular events during tocilizumab therapy. *Arthritis Rheumatol* **67**, 372-380.

Raychaudhuri SP & Raychaudhuri SK (2009). Biologics: Target - specific treatment of systemic and cutaneous autoimmune diseases. *Indian J Dermatol* **54**, 100.

Raymond AR, Becker J, Woodiwiss AJ, Booysen HL, Norton GR & Brooksbank RL (2016a). Ethanol-associated cardiomyocyte apoptosis and left ventricular dilation are unrelated to changes in myocardial telomere length in rats. *J Cardiac Failure* **22**, 294-302.

Raymond AR, Brooksbank RL, Millen AM, Norton GR, Solomon A, Woodiwiss AJ, Tsang L, Dessein PH & Gonzalez-Gay MA (2016b). Telomere length, endothelial activation and carotid atherosclerosis in black and white African patients with rheumatoid arthritis. *Clin Exp Rheumatol* **34**, 864-871.

Raymond AR, Norton GR, Harden LM, Woodiwiss AJ, Brooksbank RL (2014). Chronic inflammation reduces cardiac relative telomere length without altering left ventricular chamber function. *Int J Cardiol* **175**, 367-9.

Raymond AR, Norton GR, Woodiwiss AJ & Brooksbank RL (2015). Impact of gender and menopausal status on relationships between biological aging, as indexed by telomere length, and aortic stiffness. *Am J Hypertens* **28**, 623-630.

Reddy SP, Shah VV & Lin EJ (2016). Etanercept, Therapy for Severe Psoriasis. *Elsevier* 83–96.

Rehkopf DH, Needham BL, Lin J, Blackburn EH, Zota AR, Wojcicki JM & Epel ES (2016). Leukocyte telomere length in relation to 17 biomarkers of cardiovascular disease risk: a cross-sectional study of US adults*. PLoS Med* **13**.

Roberts L & McColl GJ (2004). Tumour necrosis factor inhibitors: risks and benefits in patients with rheumatoid arthritis. *Internal Med J* **34**, 687-693.

Ruiz-Limón P, Ortega R, de la Rosa IA, del Carmen Abalos-Aguilera M, Perez-Sanchez C, Jimenez-Gomez Y, Peralbo-Santaella E, Font P, Ruiz-Vilches D, Ferrin G & Collantes-Estevez E (2017). Tocilizumab improves the proatherothrombotic profile of rheumatoid arthritis patients modulating endothelial dysfunction, NETosis, and inflammation. *Transl Res* **183**, 87-103.

Ryan SM, Waack BJ, Weno BL & Heistad DD (1995). Increases in pulse pressure impair acetylcholine-induced vascular relaxation. *Am J Physiol-Heart C* **268**, H359-H363.

Sagara M, Suzuki K, Aoki C, Tanaka S, Taguchi I, Inoue T & Aso Y (2016). Impact of teneligliptin on oxidative stress and endothelial function in type 2 diabetes patients with chronic kidney disease: a case–control study. *Cardiovasc Diabetol* **15**, 76.

Salpea KD, Talmud PJ, Cooper JA, Maubaret CG, Stephens JW, Abelak K & Humphries SE (2010). Association of telomere length with type 2 diabetes, oxidative stress and UCP2 gene variation. *Atherosclerosis* **209**, 42-50.

Samani NJ & van der Harst P (2008). Biological ageing and cardiovascular disease. *Heart* **94**, 537-539.

Samani NJ, Boultby R, Butler R, Thompson JR & Goodall AH (2001). Telomere shortening in atherosclerosis. *Lancet* **358**, 472-473.

Samson M, Audia S,Janikashvili N,Ciudad M, Trad Malika, Jennifer Fraszczak J, Paul Ornetti P, Jean-Francis Maillefert JF, Miossec P & Bonnotte B (2012). Inhibition of IL-6 function corrects Th17/Treg imbalance in rheumatoid arthritis patients. *Arthritis Rheum* **64**, 2499–2503.

Sanders JL & Newman AB (2013). Telomere length in epidemiology: a biomarker of aging, age-related disease, both or neither. *Epidemiol Rev* **35**, 112-131.

Sato I, Morita I, Kaji KA, Ikeda M, Nagao M & Murota S (1993). Reduction of nitric oxide producing activity associated with in vitro aging in cultured human umbilical vein endothelial cell. *Biochem Biophy Res Co* **195**, 1070-1076.

Sato M, Takemura M, Shinohe R & Shimizu K (2011). Serum cytokine concentrations in a patient with rheumatoid arthritis on etanercept therapy who subsequently developed pneumocystis pneumonia: a case report. *Case Reports Rheumatol* **2011**, 185657.

Schau T, Gottwald M, Arbach O, Seifert M, Schöpp M, Neuß M, Butter C & Zänker M (2015). Increased prevalence of diastolic heart failure in patients with rheumatoid arthritis correlates with active disease, but not with treatment type. *J Rheumatol* **42**, 2029-2037.

Schinnerling K, Aguillon JC, Catalan D & Soto L (2017). The role of interleukin-6 signalling and its therapeutic blockage in skewing the T cell balance in rheumatoid arthritis. *Clin Exp Immunol* **189**, 12-20.

Senaldi G, Varnum BC, Sarmiento U, Starnes C, Lile J, Scully S, Guo J, Elliott G, McNinch J, Shaklee CL & Freeman D (1999). Novel neurotrophin-1/B cell-stimulating factor-3: a cytokine of the IL-6 family. *Proc Nat Acad Sci* **96**, 11458-11463.

Setoguchi S, Schneeweiss S, Avorn J, Katz JN, Weinblatt ME, Levin R & Solomon DH (2008). Tumor necrosis factor-α antagonist use and heart failure in elderly patients with rheumatoid arthritis. *Am Heart J* **156**, 336-341.

Sgura A, Antoccia A, Berardinelli F, Cherubini R, Gerardi S, Zilio C & Tanzarella C (2006). Telomere length in mammalian cells exposed to low- and high-LET radiations. *Radiat Prot Dosim* **122**, 176–179.

Sharifi-Sanjani M, Oyster NM, Tichy ED, Bedi Jr KC, Harel O, Margulies KB & Mourkioti F (2017). Cardiomyocyte-Specific Telomere Shortening is a Distinct Signature of Heart Failure in Humans. *J Am Heart Assoc* **6**, e005086.

Sharma A, Kaushik R, Kaushik RM & Kakkar R (2015). Echocardiographic evaluation of diastolic dysfunction in rheumatoid arthritis–a case–control study. *Modern Rheumatol* **25**, 552-557.

Shen J, Gammon MD, Terry MB, Wang Q, Bradshaw P, Teitelbaum SL, Neugut AI & Santella RM (2009). Telomere length, oxidative damage, antioxidants and breast cancer risk. *Int J cancer* **124**, 1637-1643.

Shisana O, Labadarios D, Rehle T, Simbiya L, Zuma K, Dhansay A, Reddy P, Parker W, Hoosain E, Naidoo P, Hongoro C, Mchiza Z, Steyn NP, Dwane N, Makoae M, Maluleke T, Ramlagan S, Zungu N, Evans MG, Jacobs L & Faber M (2013). *South*

African national health and nutrition examination survey (SANHANES-1). Capetown, South Africa.

Shoeb M, Kodali VK, Farris BY, Bishop LM, Meighan TG, Salmen R, Eye T, Friend S, Schwegler-Berry D, Roberts JR & Zeidler-Erdely PC (2017). Oxidative stress, DNA methylation, and telomere length changes in peripheral blood mononuclear cells after pulmonary exposure to metal-rich welding nanoparticles. *NanoImpact* **5**, 61-69.

Smolen JS, Landewé R, Bijlsma J, Burmester G, Chatzidionysiou K, Dougados M, Nam J, Ramiro S, Voshaar M, Van Vollenhoven R & Aletaha D (2017). EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2016 update. *Ann Rheum Dis* **76**, 960-977.

Sobinoff AP & Pickett HA (2017). Alternative lengthening of telomeres: DNA repair pathways converge. Trends in Genetics **33**, 921-932.

Soehnlein O, Steffens S, Hidalgo A & Weber C (2017). Neutrophils as protagonists and targets in chronic inflammation. *Nat Rev Immunol* **17,** 248–261.

Solomon DH, Avorn J, Katz JN, Weinblatt ME, Setoguchi S, Levin R & Schneeweiss S (2006). Immunosuppressive medications and hospitalization for cardiovascular events in patients with rheumatoid arthritis. Arthritis & Rheumatism. *J Am Coll Rheumatol* **54**, 3790-3798.

Solomon DH, Curtis JR, Saag KG, Lii J, Chen L, Harrold LR, Herrinton LJ, Graham DJ, Kowal MK, Kuriya B & Liu L (2013). Cardiovascular risk in rheumatoid arthritis: comparing TNF-α blockade with nonbiologic DMARDs. *Am J Med* **126**, 730-739.

Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Näslund E, Britton T & Concha H (2008). Dynamics of fat cell turnover in humans. *Nature* **453**, 783-787.

Srirangan S & Choy EH (2010). The role of interleukin 6 in the pathophysiology of rheumatoid arthritis. *Ther Adv Musculoskelet Dis* **2**, 247-256.

Starr JM, Shiels PG, Harris SE, Pattie A, Pearce MS, Relton CL, & Deary IJ (2008). Oxidative stress, telomere length and biomarkers of physical aging in a cohort aged 79 years from the 1932 Scottish Mental Survey. *Mech Ageing Dev* **129**, 745–751.

Steer SE, Williams FM, Kato B, Gardner JP, Norman PJ, Hall MA, Kimura M, Vaughan R, Aviv A & Spector TD (2007). Reduced telomere length in rheumatoid arthritis is independent of disease activity and duration. *Ann Rheum Dis* **66**, 476–480.

Stockli KA Lottspeich E, Sendtner M, Masiakowski P, Carroll P, Gotz R, Lindholm D & Thoenen H (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature* **342**, 920-923.

Symmons DP, Farr M, Salmon M & Bacon PA (1989). Lymphopenia in rheumatoid arthritis. *J Roy Soc Med* **82**, 462-463.

Tajbakhsh S, Aliakbari K, Hussey DJ, Lower KM, Donato AJ & Sokoya EM (2015). Differential telomere shortening in blood versus arteries in an animal model of type 2 diabetes. *J Diabetes Res* **2015**, 153829.

Takagi S, Kinouchi Y, Chida M, Hiwatashi N, Noguchi M, Takahashi S & Shimosegawa T (2003). Strong telomerase activity of B lymphocyte from mesenteric lymph nodes of patients with inflammatory bowel disease. *Dig Dis Sci* **48**, 2091–2094.

Takahashi K, Takatsu M, Hattori T, Murase T, Ohura S, Takeshita Y, Watanabe S, Murohara T & Nagata K (2014). Premature cardiac senescence in DahlS.Z-Lepr(fa)/Lepr(fa) rats as a new animal model of metabolic syndrome. *Nagoya J Med Sci* **76**, 35-49.

Takubo K, Izumiyama-Shimomura N, Honma N, Sawabe M, Arai T, Kato M, Oshimura M & Nakamura KI (2002). Telomere lengths are characteristic in each human individual. *Exp Gerontol* **37**, 523-531.

Tamayo M, Mosquera A, Rego JI, Fernández-Sueiro JL, Blanco FJ & Fernández JL (2010). Differing patterns of peripheral blood leukocyte telomere length in rheumatologic diseases. *Mutat Res* **683**, 68-73.

Tarhan F, Vural F, Kosova B, Aksu K, Cogulu O, Keser G, Gündüz C, Tombuloglu M, Oder G, Karaca E & Doganavsargil E (2008).Telomerase activity in connective tissue diseases: elevated in rheumatoid arthritis, but markedly decreased in systemic sclerosis. *Rheumatol Int* **28**, 579–583.

Tarnowski M, Paradowska-Gorycka A, Dąbrowska-Zamojcin E, Czerewaty M, Słuczanowska-Głąbowska S & Pawlik A (2016). The effect of gene polymorphisms on patient responses to rheumatoid arthritis therapy. *Expert Opin Drug Metabol Toxicol* **12**, 41-55.

Teichholz LE, Kreulen T, Herman MV & Gorlin R (1976). Problems in echocardiographic volume determinations: echocardiographic-angiographic correlations in the presence of absence of asynergy. *Am J Cardiol* **37**, 7–11.

Tentolouris N, Nzietchueng R, Cattan V, Poitevin G, Lacolley P, Papazafiropoulou A, Perrea D, Katsilambros N & Benetos A (2007). White blood cells telomere length is shorter in males with type 2 diabetes and microalbuminuria. *Diabetes care* **30**, 2909-2915.

Thilagar S, Theyagarajan R, Sudhakar U, Suresh S, Saketharaman P & Ahamed Nizar (2018). Comparison of serum tumor necrosis factor-alpha levels in Rheumatoid Arthritis individuals with and without chronic periodentotis: a biochemical study. *J Indian Soc Periodontol* **22**, 116-121.

Thomas P, O'callaghan NJ & Fenech M (2008). Telomere length in white blood cells, buccal cells and brain tissue and its variation with ageing and Alzheimer's Disease. *Mech Ageing Dev* **129**,183-190.

Torre-Amione G, Kapadia S, Benedict C, Oral H, Young JB & Mann DL (1996). Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD). *J Am Coll Cardiol* **27**, 1201-1206.

Totoson P, Maguin-Gate K, Prigent-Tessier A, Monnier A, Verhoeven F, Marie C, Wendling D & Demougeot C (2016). Etanercept improves endothelial function via pleiotropic effects in rat adjuvant-induced arthritis. *Rheumatol* **55**, 1308-1317.

Turesson C, Jacobsson L & Bergström U (1999). Extra-articular rheumatoid arthritis: prevalence and mortality. *Rheumatol* **38**, 668-674.

Valdes AM, Andrew T, Gardner J P, Kimura M, Oelsner E, Cherkas LF & Spector TD (2005). Obesity, cigarette smoking, and telomere length in women. *Lancet* **366**, 662-664.

Valdes AM, Richards JB, Gardner JP, Swaminathan R, Kimura M, Xiaobin L, Aviv A & Spector TD (2007). Telomere length in leukocytes correlates with bone mineral density and is shorter in women with osteoporosis. *Osteoporosis Int* **18**, 1203-1210.

Valenzuela HF & Effros RB (2002). Divergent telomerase and CD28 expression patterns in human CD4 and CD8 T cells following repeated encounters with the same antigenic stimulus. *Clin Immunol* **105**, 117–125.

Vallejo AN, Weyand CM & Goronzy JJ (2004). T-cell senescence: a culprit of immune abnormalities in chronic inflammation and persistent infection. *Trends Mol Med* **10**, 119–124.

Van Der Harst P, Van Der Steege G, De Boer RA, Voors AA, Hall AS, Mulder MJ, van Gilst WH, van Veldhuisen DJ, Merit-Hf Study Group (2007). Telomere length of circulating leukocytes is decreased in patients with chronic heart failure. *J Am Coll Cardiol* **49**,1459–1464.

Van Ziffle JA, Baerlocher GM & Lansdorp PM (2003). Telomere length in subpopulations of human hematopoietic cells. *Stem Cells* **21**, 654-660.

Vasanthi P, Nalini G & Rajesekhar G (2007). Role of tumor necrosis factor in rheumatoid Arthritis: a review. *APLAR J Rheumatol* **10**, 270-274.

Vassalle C, Maffei S, Ndreu R & Mercuri A (2009). Age-related oxidative stress modulation by smoking habit and obesity. *Clin Biochem* **42**, 739-741.

Verschueren PC, Markusse H, Smeets TJM, Kraan M, Breedveld F & Tak PP (1999). Reduced cellularity and expression of adhesion molecules and cytokines after treatment with soluble human recombinant TNF receptor (p75) in RA patients. *Arthritis Rheum* **42**, S197.

Victorelli S & Passos JF (2017). Telomeres and Cell senescence-size matters not. *EbioMedicine* **21**, 14-20.

Von Zglinicki T (2002). Oxidative stress shortens telomeres. *Trends Biochem Sci* **27**, 339-344.

Von Zglinicki T, Pilger R & Sie N (2000). Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radical Biol Med* **28**, 64-74.

Wagner UG, Koetz K, Weyand C M & Goronzy JJ (1998). Perturbation of the T cell repertoire in rheumatoid arthritis. *Proc Nat Acad Sci* **95**, 14447-14452.

Wajant H & Siegmund D (2019). TNFR1 and TNFR2 in the control of the life and death balance of macrophages. *Front cell Dev Biol* **7**.

Walker KA (2019). Inflammation and neurodegeneration: chronicity matters. *Ageing* **11**, 3.

Wållberg-Jonsson S, Caidahl K, Klintland N, Nyberg G & Rantapää-Dahlqvist S (2008). Increased arterial stiffness and indication of endothelial dysfunction in long-standing rheumatoid arthritis. *Scand J Rheumatol* **37**, 1-5.

Wang F, Podell ER, Zaug AJ, Yang Y, Baciu O, Cech TR & Lei M (2007). The POT1-TTP1 telomere complex is a telomerase Processivity factor. *Nature* **445**, 506.

Wang G, Wang Q, Huang Q, Chen Y, Sun X, He L & He X (2019). Upregulation of mtSSB by interleukin-6 promotes cell growth through mitochondrial biogenesis-mediated telomerase activation in colorectal cancer. *Int J cancer* **144**, 2516-2528.

Wang H, Chen H, Gao X, McGrath M, Deer D, De Vivo I, Schwarzschild MA & Ascherio A (2008). Telomere length and risk of Parkinson's disease. *Mov Disord* **23**, 302-305.

Wang J, Staessen JA, Franklin SS, Fagard R & Gueyffier F (2005). Systolic and Diastolic Blood Pressure Lowering as Determinants of Cardiovascular Outcome. *Hypertension* **45**, 907-913.

Wang YY, Chen AF, Wang HZ, Xie LY, Sui KX & Zhang QY (2011). Association of shorter mean telomere length with large artery stiffness in patients with coronary heart disease. *Aging Male* **14**, 27-32.

Watfa G, Dragonas C, Brosche T, Dittrich R, Sieber CC, Alecu C, Benetos A & Nzietchueng R (2011). Study of telomere length and different markers of oxidative stress in patients with Parkinson's disease. *J Nutr, Health Aging* **15**, 277-281.

Weinblatt ME, Kaplan H, Germain BF, Block S, Solomon SD, Merriman RC, Wolfe F, Wall B, Anderson L, Gall E & Torretti D (1994). Methotrexate in rheumatoid arthritis. A five-year prospective multicenter study. *Arthritis Rheum* **37**,1492-1498.

Weischer M, Bojesen SE, Cawthon RM, Freiberg JJ, Tybjærg-Hansen A & Nordestgaard BG (2012). Short telomere length, myocardial infarction, ischemic heart disease, and early death. *Arterioscler Thromb Vasc Biol* **32**, 822-829.

Weisman MH, Paulus HE, Burch FX, Kivitz AJ, Fierer J, Dunn M, Kerr DR, Tsuji W & Baumgartner SW (2007). A placebo-controlled, randomized, double-blinded study evaluating the safety of etanercept in patients with rheumatoid arthritis and concomitant comorbid diseases. *Rheumatol* **46**, 1122–1125.

Weng N (2012). Telomeres and immune competency. *Curr Opin Immunol* **24**, 470-475.

Westermann D, Lindner D, Kasner M, Zietsch C, Savvatis K, Escher F, Von Schlippenbach J, Skurk C, Steendijk P, Riad A & Poller W (2011). Cardiac inflammation contributes to changes in the extracellular matrix in patients with heart failure and normal ejection fraction. *Circ Heart Failure* **4**, 44-52.

Westlake SL, Colebatch AN, Baird J, Curzen N, Kiely P, Quinn M, Choy E, Ostor AJ & Edwards CJ (2011). Tumour necrosis factor antagonists and the risk of cardiovascular disease in patients with rheumatoid arthritis: a systematic literature review. *Rheumatol* **50**, 518-531.

Weyand CM & Goronzy JJ (2002). Premature immunosenescence in rheumatoid arthritis. *J Rheumatol* **29**, 1141–1146.

Willeit P, Willeit J, Brandstätter A, Ehrlenbach S, Mayr A, Gasperi A, Weger S, Oberhollenzer F, Reindl M, Kronenberg F & Kiechl S (2010). Cellular ageing reflected by leukocyte telomere length predicts advanced atherosclerosis and cardiovascular disease risk. *Arterioscler Thromb Vasc Biol* **30**,1649-1656.

Willeit P, Willeit J, Mayr A, Weger S, Oberhollenzer F, Brandstätter A, Kronenberg F & Kiechl S (2010). Telomere length and risk of incident cancer and cancer mortality. *Jama* **304**, 69-75.

Wilson-Gerwing TD, Pratt IV, Cooper DM, Silver TI & Rosenberg AM (2013). Age-related differences in collagen-induced arthritis: clinical and imaging correlations. *Comp Med* **63**, 498-502.

Win S, Than TA, Zhang J, Oo C, Min RW & Kaplowitz N (2018). New insights into the role and mechanism of c-Jun-N-terminal kinase signaling in the pathobiology of liver diseases. *Hepatol* **67**, 2013-2024.

Wolfe F & Michaud K (2004). Heart failure in rheumatoid arthritis: rates, predictors, and the effect of anti–tumor necrosis factor therapy. *Am J Med* **116**, 305–311.

Wollert KC & Drexler H (2001). The role of interleukin-6 in the failing heart. *Heart failure Res* **6**, 95-103.

Wong JY, De Vivo I, Lin X, Fang SC & Christiani DC (2014). The relationship between inflammatory biomarkers and telomere length in an occupational prospective cohort study. *PloS one* **9**.

Wong PK, Quinn JM, Sims NA, Van Nieuwenhuijze A, Campbell IK & Wicks IP (2006). Interleukin-6 modulates production of T lymphocytes- derived cytokines in antigen-induced arthritis and drives inflammation -induces osteoclastogenesis. *Arthritis Rheum* **54**, 158-168.

Wooley PH (2004). The usefulness and the limitations of animal models in identifying targets for therapy in arthritis. *Best Pract Res Clin Rheumatol* **18**, 47-58.

Wu L, Multani AS, He H, Cosme-Blanco W, Deng Y, Deng JM, Bachilo O, Pathak S, Tahara H, Bailey SM & Deng Y (2006). POT1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. *Cell* **126**, 49-62

Xie F, Yun H, Levitan EB, Muntner P & Curtis JR (2019). Tocilizumab and the risk of cardiovascular disease: direct comparison among biologic disease-modifying antirheumatic drugs for rheumatoid arthritis patients. *Arthritis Care Res* **71**, 1004-1018.

Yamaguchi Y, Nozawa K, Savoysky E, Hayakawa N, Nimura Y & Yoshida S (1998). Change in telomerase activity of rat organs during growth and aging. *Exp Cell Res* **242**, 120–127.

Yang S, Wang J, Brand DD & Zheng SG (2018). Role of TNF–TNF receptor 2 signal in regulatory T cells and its therapeutic implications. *Front Immunol* **9**, 784.

Yang Z, Huang X, Jiang H, Zhang Y, Liu H, Qin C, Eisner GM, Jose P, Rudolph L & Ju Z (2009). Short telomeres and prognosis of hypertension in a Chinese population. *Hypertension* ***53***, 639-645.

Yeh JK & Wang CY (2016). Telomeres and telomerase in cardiovascular diseases. *Genes* **7**, 58.

Yndestad A, Damås JK, Oie E, Ueland T, Gullestad L & Aukrust P (2006). Systemic inflammation in heart failure-the whys and wherefores. *Heart Fail Rev* **11**, 83–92.

Yuan J, Liu Y, Wang J, Zhao Y, Li K, Jing Y & Wang F (2018). Long-term persistent organic pollutants exposure induced telomere dysfunction and senescence-associated secretary phenotype. *J Gerontol* **73**, 1027-1035.

Zee RY, Michaud SE, Germer S & Ridker PM (2009). Association of shorter mean telomere length with risk of incident myocardial infarction: a prospective, nested case–control approach. *Clinica Chimica Acta* **403**, 139-141.

Zhang J, Chen L, Delzell E, Muntner P, Hillegass WB, Safford MM, Millan IYN, Crowson CS & Curtis JR (2014). The association between inflammatory markers, serum lipids and the risk of cardiovascular events in patients with rheumatoid arthritis. *Ann Rheum Dis* **73**, 1301-1308.

Zhang WG, Zhu SY, Zhao DL, Jiang SM, Li J, Li Z X & Cai GY (2014). The correlation between peripheral leukocyte telomere length and indicators of cardiovascular aging. *Heart Lung Circ* **23**, 883-890.

Zidan ES (2020). *Telomere length and arterial stiffness in patients with Rheumatoid Arthritis* (unpublished Msc thesis). University of the Witwatersrand.

Zribi B, Uziel O, Lahav M, Mesilati Stahy R & Singer P (2019). Telomere length changes during critical illness: a prospective, observational study. *Genes* **10**, 761.

APPENDIX

AESC 2012 M&E

Please note that only type written applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Lebogang Mokotedi

b. Department: School of Physiology

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2017	03	21C
Other M&Es			1

d. Project Title: Cardiovascular morphology and function in a rat model of rheumatoid arthritis

		No.	Species
e.	Number and species of animals originally approved:	30	SD
f.	Number of additional animals previously allocated on M&Es:	5	SD
g.	Total number of animals allocated to the experiment to date:	35	SD
h.	Number of animals used to date:	35	SD

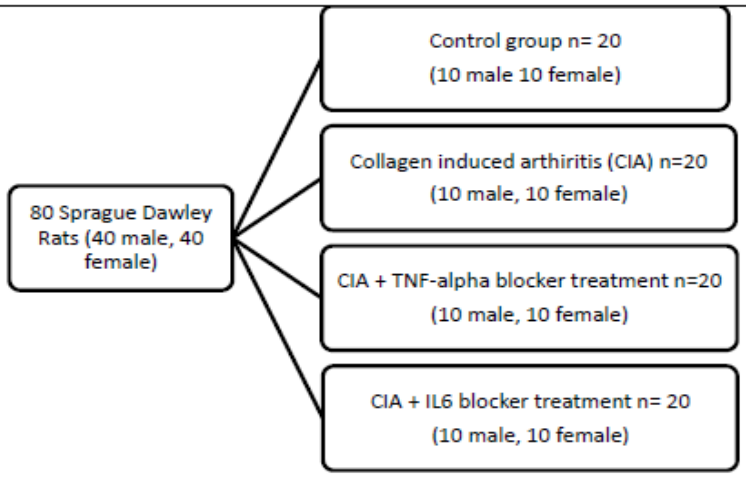
i. Specific modification / extension requested:

- **Addition of co-workers**

1. Ashmeetha Manilal (technical staff and PhD student) Staff number: A0029214, Tel: 0824617340; email: Ashmeeha.Manilal@wits.ac.za
2. Sulé Gunter (post-doctoral fellow). Staff number: A0045488; Tel: 0828141066; email: suledreyer@gmail.com
3. Serena Fourie, (MSc student) student number: 804802; Tel: 0741548402; email: fourie.serena@gmail.com
4. Regina le Roux, (MSc student) student number: 1588322; Tel: 0718719596; email: reginaleroux4@gmail.com

- **Additional rats required**

As per the original application, arthritis will be induced with Bovine Collagen and Incomplete Freund's Adjuvent and compared to a control group. In addition we would like to add two groups where the groups will also have collagen induced arthritis but they are given treatment to reduce inflammation. A flow diagram of the groups is presented below.



- **Addition of drug treatment regimen to reduce inflammation**
- **Use of pulse-pen to measure pulse wave velocity at termination.**

j. **Motivation for modification / extension:**

The addition of co-workers:

These added students and co-workers will contribute to all aspects of the projects. Two masters and one PhD students will also obtain higher degrees from work performed as a part of the modification of this project.

Additional rats required:

The original project was largely a pilot study in order to determine whether the inflammatory model will be successful. All the rats were used for this first part of the study and we have successfully established that the collagen induced inflammatory model is indeed successful. None of the animals died during the experiment due to adverse effects. In the first part of this study we have shown substantial differences in those rats that were exposed to inflammation. We would like to request an additional 80 rats for further study. The 80 (40 male and 40 female) rats will be divided into 4 groups (control group, inflammation group, inflammation + TNFα inhibitor group, and inflammation + IL6 inhibitor group) as indicated in the flow diagram. In essence the **intervention to the animals and measures obtained will not change** as per the original application, hence the rationale for applying for a modification to the original application. The addition of extra groups will not change the intervention to the animals, except for the addition of drugs to **reduced inflammation** and therefore it will not be harmful to the animal, but rather beneficial.

Addition of drug treatment regimen to reduce inflammation:

In order to reduce inflammation in persons with rheumatoid arthritis drugs that have shown good outcomes in these patients are biological disease modifying agents (DMARDS) (1). Two of these biological DMARDS that have shown to be effective for **reducing inflammation** as well as **decreasing cardiovascular disease risk** markers in persons with rheumatoid arthritis are TNFα

inhibitors and Interleukin-6 inhibitors (1). However the mechanisms whereby these drugs improve cardiac function is currently unknown. These drugs have also shown benefits in previous animal models of rheumatoid (2) and osteoarthritis (3), with no reported side-effects. The drugs will be administered intraperitoneally upon the first sign of arthritis (roughly 12 days after immunization) and given as indicated (below) until the termination of the study. The other two groups will receive intraperitoneal injections of phosphate buffered saline solution. The TNFα inhibitor (Enbrel) will be given at a dosage of 10 mg/kg (2) three time a week and the IL-6 inhibitor (Actemra (tocilizumab)) will be given at a dosage of 8 mg/kg once a week as previously described (4,5). The use of these drugs will provide us with a further understanding of the effect of inflammation on vascular and cardiac function and possibly identify novel targets for the treatment of diastolic dysfunction in inflammation induced heart disease. The protocol of inducing inflammation in the rats will not change from the previous application. The addition of these drugs will in all likelihood reduce inflammation and hence limit the discomfort of the animals.

Use of pulse-pen to measure pulse wave velocity at termination:

At termination we would like to measure aortic pulse wave velocity (PWV) by applanation tonometry (PulsePen device, DiaTecne, Milan, Italy). The measurement of PWV is non-invasive and will provide a further understanding to the arterial function of the animals and the effect of inflammation on aortic stiffness. This procedure will happen during the period when the rats are anesthetized for echocardiography (as per the initial application). In order to obtain the measurement the neck and inner right thigh will be shaved and animals will be placed on a heating pad in the supine position. Briefly, the carotid and femoral pulse wave velocities will be recorded simultaneously by placing one probe on the common carotid artery and one probe on the femoral artery. The two probes will be positioned and fixed on the arteries by means of mechanical arms, equipped with micro-regulators. After that, pressure signals will be transmitted to a computer by means of an optical fiber that ensures the electromagnetic isolation for the rat undergoing the test. After the tonometric pulse wave acquisition, the sites where probes will be placed will be marked on the skin and the distance from the carotid to the femoral will be measured. PWV will be calculated as the distance between the two recording sites divided by the time delay between the two arterial waveforms at each site (6).

References

1. Baniaamam M, Paulus WJ, Blanken AB, Nurmohamed MT (2018): The effect of biological DMARDs on the risk of congestive heart failure in rheumatoid arthritis: a systematic review. Exp Opin Biol Ther 2018; DOI:10.1080/14712598.2018.1462794.

2. Totoson P, Maguin-Gate K, Prigent-Tessier A et al. Etanercept improves endothelial function via pleiotropic effects in rat adjuvant-induced arthritis. Rheumatol 2016;55:1308-17.

3. Lin Y, Liu L, Jiang H, Zhou J, Tang Y. Inhibition of interleukin-6 function attenuates the central sensitization and pain behavior induced by osteoarthritis. Eur J Pharmacol. 2017;811:260-7.

4. Kobayashi Y, Takei M, Giles JT et al. Association of tocilizumab treatment with changes in measures of regional left ventricular function in rheumatoid arthritis, as assessed by cardiac magnetic resonance imaging. Int J Rheum Dis 2016;19:1169–1174.

5. Hançerli Y, Kaplan M, Tanoğlu A et al. Efficacy of tocilizumab treatment in cerulein-induced experimental acute pancreatitis model in rats. Turk J Gastroenterol. 2017;28:485-91.

6. Salvi P, Lio G, Labat C, Ricci E, Pannier B, Benetos A. Validation of a new non-invasive portable

tonometer for determining arterial pressure wave and pulse wave velocity: the PulsePen device. J Hypertens 2004; 22:2285-2293.

Date:07/05/2018

Signature:

RECOMMENDATIONS

Date: 22/5/2018

Signature:

Chairman, AESC

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 6 May 2019

Certificate reference: 2017/03/21C
Category: O
Applicant: Lebogang Mokotedi
Department: Physiology
Tel: 011 717 2262; **Email**: Lebogang.Mokotedi@wits.ac.za

RE: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that **Rixile Mahlaule (1063830),** Department of **Physiology** (WITS), does not require full Animal Ethics Research Committee clearance to undertake the work titled **"The impact of inflammation on telomere length in treated and untreated rats with collagen induced rheumatoid arthritis".**

Reason for waiver
Data from the project entitled "Cardiovascular morphology and function in a rat model of rheumatoid arthritis" has been collected from 2017 to 2018 on 125 rats. From these experiments, specimen samples were collected for future analysis. Rixile Mahlaule will be using these specimen samples (blood samples) as part of her MSC degree.

Details of the study
The aim of the study is to determine the impact of chronic inflammation on telomere length and the effect of biologic anti-rheumatic treatment drugs in Sprague Dawley rats exposed to collagen induced arthritis. Blood samples collected from the previous approved ethics application will be used for this study. Blood samples will be used to measure the differences in telomere length between the treatment and control groups.

Please contact me should you require further information.

Yours sincerely

Geoffrey Candy
Chair : Animal Ethics Research Committee, University of the Witwatersrand

Geoffrey P Candy PhD
Professor, Department of Surgery, Faculty of Health Sciences, University of the Witwatersrand, *7 York Road Parktown 2193, Johannesburg; Tel: 27-11-717-2574; Email: geoffrey.candy@wits.ac.za*