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Telomere length and arterial stiffness in patients with rheumatoid arthritis

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TELOMERE LENGTH AND ARTERIAL STIFFNESS IN PATIENTS WITH RHEUMATOID ARTHRITIS

Eman Salem Zidan

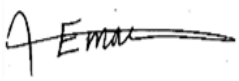
A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2020

DECLARATION

I, Eman Salem Zidan, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine, in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. The work contained in this thesis, has not been submitted for any degree or examination in this University, or any other University.

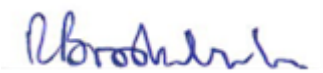
I hereby certify that the studies contained in this thesis have been approved by the Committee for Research in Human Subjects, University of the Witwatersrand, Johannesburg. The ethics approval number is M120562 renewed as M170592.



Eman Salem Zidan

Signed on the 15th day of April 2020





Assoc Prof Aletta Millen (supervisor) Assoc Prof Richard Brooksbank (supervisor)

ABSTRACT

Background. Chronic inflammation increases cell replication and thereby accelerates relative leukocyte telomere length shortening. Telomere attrition is associated with cardiovascular disease. However, a direct relationship between telomere length and atherosclerosis was recently reported in patients with rheumatoid arthritis (RA) and lupus. Whether telomere length is associated with other markers of sub-clinical cardiovascular disease in patients with rheumatic disease awaits elucidation.

Objective. This study aimed to determine whether relative leukocyte telomere length is associated with aortic function in RA.

Methods. Arterial function and relative leukocyte telomere length was assessed in 145 (99 white, 25 Asian, 17 black and 4 mixed race) patients with RA. Arterial function markers was determined by applanation tonometry using SphygmoCor software and included carotid femoral pulse wave velocity (PWV), central systolic and pulse pressure, central augmentation pressure, pulse pressure amplification and the magnitude and timing of the forward and reflected waves. Relative leukocyte telomere length was determined by quantitative real-time polymerase chain reaction (PCR). Relationships between telomere length and comprehensively evaluated arterial function markers were determined in confounder adjusted, multivariate regression models. Sensitivity analysis was performed in white patients with RA.

Results. In demographic characteristic adjusted analysis, relative telomere length was associated with abatacept use (partial r=0.19, p=0.02), but not with traditional cardiovascular disease risk factors in all patients (all p>0.05). In sensitivity analysis in white African patients, relative telomere length was associated with body mass index and waist circumference (both partial r=0.21; p=0.05), with disease activity score in 28 joints (partial r=-0.21, p=0.05), deformed joints (partial r=-0.20, p=0.05) and with

sulphasalazine (partial r=-0.24, p=0.02) and abatacept (partial r=0.24, p=0.02) use. In known confounder adjusted backward regression models, relative telomere length was associated with central pulse pressure (std β(SE)=0.13(0.07), p=0.05), central augmentation pressure (std β(SE)=0.13(0.07), p=0.05), backward wave pressure (std β(SE)=0.13(0.06), p=0.04) and reflection magnitude (std β(SE)=0.14(0.07), p=0.05) in all patients. In fully adjusted regression analysis, the results remained materially unaltered. In sensitivity analysis in white African patients, relative telomere length was associated only with central pulse pressure in known confounder (std β(SE)=0.18(0.09), p=0.04) and fully adjusted models (std β(SE)=0.18(0.08), p=0.04). Leukocyte telomere length was not associated with any marker of arterial function in sensitivity analysis in women.

Conclusions. This study documents a paradoxically direct relationship between relative telomere length and central pressure and wave reflection in RA. The role of relative telomere length in cardiovascular disease in RA requires further longitudinal and mechanistic investigation.

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CONTENTS

Declaration ii

Abstract iii

Acknowledgements v

Contents vi

List of tables viii

List of figures viii

Abbreviations ix

Chapter 1 Introduction 1

Chapter 2 Literature review 5

2.1 Leukocyte telomere length 6

2.2 Leukocyte telomere length and cardiovascular disease 7

2.3 Leukocyte telomere length and arterial function 9

2.4 Rheumatoid arthritis and cardiovascular disease 13

2.5 Inflammation and leukocyte telomere lengths 15

2.6 Problem statement 16

2.7 Aims 17

Chapter 3 Methods 18

3.1 Patients 19

3.2 Demographic, anthropometric and clinical variables 19

3.3 Large artery function 20

3.4 Average relative telomere length 22

3.5 Statistical analysis 27

Chapter 4 Results 28

4.1 Demographic characteristics 29
4.2 Associations of traditional risk factors and RA disease characteristics with relative leukocyte telomere length 32
4.3 Arterial function variables across tertiles of relative leukocyte telomere length 35
4.4 Independent associations of arterial function measures with relative leukocyte telomere length 37
Chapter 5 Discussion 40
5.1 Summary of main findings 41
5.2 Leukocyte telomere length and traditional cardiovascular disease risk factors 41
5.3 Leukocyte telomere length and RA disease characteristics 43
5.4 Leukocyte telomere length and arterial function 45
5.5 Limitations of study 49
5.6 Conclusion 49
References 51
Appendix A 73

LIST OF TABLES

Table 2.1. A summary of studies investigating the relationship between telomere length and arterial function measures 11

Table 3.1. The primers and Light-Cycler thermal profiles for the telomere and 36B4 PCR amplifications 23

Table 4.1. Recorded characteristics in 141 patients with RA 30

Table 4.2. Traditional cardiovascular disease risk factors and RA disease characteristics associated with relative leukocyte telomere length in all patients, white patients and women 33

Table 4.3. Relative contribution of leukocyte telomere length to arterial function variables in all patients and in white patients 38

LIST OF FIGURES

Figure 3.1 Standard curves for the telomere (upper panel) and 36B4 (lower panel) qRT-PCR reaction 26

Figure 4.1. Changes in arterial function variables across tertiles of relative leukocyte telomere length 36

ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
AIx	Augmentation index
AP	Augmentation pressure
BMI	Body mass index
β	Beta
CAD	Coronary artery disease
CDAI	Clinical disease activity index
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration equation
cf PWV	Carotid femoral pulse wave velocity
cr PWV	Carotid radial pulse wave velocity
cPP	Central pulse pressure
cSP	Central systolic pressure
CVD	Cardiovascular disease
CI	Confidence interval
DAS 28	Disease Activity Score in 28 joints
DBP	Diastolic blood pressure
DM	Diabetes mellitus
DMARD	Disease-modifying antirheumatic drugs
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism

GFR	Glomerular filtration rate
HAQ-DI	Stanford Health Assessment Questionnaire disability index
HDL	High density lipoprotein
HOMA-IR	Homeostatic Model Assessment of insulin resistance
IL-6	Interleukin-6
IQR	Interquartile range
LDL	Low density lipoprotein
mmHg	Millimeters of mercury
mmol/l	Millimole per litre
m/s	Meter per second
MTX	Methotrexate
NSAID	Non- steroidal anti-inflammatory drugs
Pb	Reflected wave magnitude
Pf	Forward wave pressure
pg/ml	Picogram per millilitre
PPamp	Pulse pressure amplification
pPP	Peripheral pulse pressure
PP	Pulse pressure
ROS	Reactive oxygen species
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RM	Reflection magnitude

SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
TL	Telomere length
TRF	Terminal restriction fragment
TNF-α	Tumour necrosis alpha
WCC	White cell (leukocyte) count
WHR	Waist to hip ratio

CHAPTER 1

INTRODUCTION

Non-communicable diseases, including cardiovascular disease (CVD), account for approximately 60% of global mortality (Yusuf et al., 2001). There is now considerable evidence that CVD is becoming increasingly more prevalent in the developing world (Yusuf et al., 2004). In South Africa, it is estimated that cerebrovascular events (strokes) represent the 5th, heart failure the 8th and ischaemic heart disease the 10th leading causes of death (Bradshaw et al., 2010).

Although it is well accepted that traditional risk factors for CVD, including age, obesity, increased low density lipoprotein cholesterol concentrations, decreased high density lipoprotein (HDL) concentrations, hypertension, diabetes mellitus (DM) and smoking predict the risk for cardiovascular events, there is still considerable controversy as to whether risk prediction using these traditional risk factors alone fully accounts for cardiovascular events. Indeed, in older studies conducted in developed countries, traditional risk factors were reported to account for less than half the risk of cardiovascular events (Gordon et al., 1974). In more recent studies, while some studies demonstrate a good correlation between these risk factors and cardiovascular events (Menotti et al., 2009), and suggest that traditional risk factors account for most cardiovascular events (Yusuf et al., 2004) other studies have reported that traditional risk factors do not account for changes in cardiovascular mortality across socio-economic groups (Harald et al., 2008). One possible non-traditional risk factor which has received considerable attention in the recent past is that of chronic systemic inflammation.

Indices of inflammation have been associated with sub-clinical cardiovascular disease risk markers (Burke et al., 2002; Rubin et al., 2011) and with cardiac events (Kaptoge et al., 2010). Furthermore, evidence suggests that disorders associated with chronic

high-grade inflammation, including rheumatoid arthritis (RA), markedly exacerbate the risk for cardiovascular events.

Cardiovascular disease is a major cause of morbidity and mortality in chronic conditions associated with high-grade inflammation. Rheumatoid arthritis (RA) is the most common chronic systemic inflammatory disease, affecting approximately 1% of the general adult population (McInnes & Schett 2011). In addition to the synovial inflammation and hyperplasia which underpins RA, it is also a disease of systemic inflammation and auto-antibody production which results in additional extra-articular complications (McInnes & Schett, 2011). RA patients have a 50% higher mortality rate (Avina-Zubeita et al., 2008) and risk for cardiovascular events (Avina-Zubeita et al., 2012) compared to the general population. The increased CVD risk in RA cannot be fully explained by traditional risk factors (Del Rincón et al., 2001). High-grade systemic inflammation in RA appears to provide a pathophysiological link for the increased CVD risk (Sattar et al., 2003; López-Mejías et al., 2016). The mechanisms whereby systemic inflammation enhances CVD risk in RA are currently under investigation (Castaneda et al., 2016).

Numerous biomarkers of cardiovascular disease and inflammation have been investigated in recent literature in an attempt to improve risk stratification in RA patients. Given the role that inflammation plays on cellular replication and biological ageing, telomere length has received some attention as a possible biomarker for cardiovascular disease risk stratification (Fitzpatrick et al., 2006, Masi et al., 2012, Wong et al., 2014). Telomeres are specialized DNA-protein structures that cap the end of chromosomes. Telomeres preserve the integrity and stability of the genome during duplication as they protect the DNA protein structure against recombination and

degradation, fusion and loss (Blackburn, 2001). Leukocyte telomere lengths are an index of biological aging, rather than chronological age, as they undergo shortening with every cell cycle of replication (Aviv, 2004).

Shorter telomere lengths have been associated with cardiovascular disease, independent of conventional risk factors (Fyhrquist et al., 2013; Haycock et al., 2014). Furthermore, persons with autoimmune diseases also have shorter telomeres compared to healthy counterparts (Lee & Bae 2018). 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). As chronic inflammation has been shown to increase cardiovascular disease risk in RA, and induce telomere attrition, whether leukocyte telomere length provides an index of the cumulative inflammatory load, that is associated with cardiovascular disease risk in RA, warrants investigation.

This thesis, in Chapter 2, begins with a literature review that summarises the current knowledge and incongruities in the field, which will highlight the reasons for conducting the study presented in this thesis. Chapter 3 describes the methods deployed in the study and in Chapter 4 the results obtained are presented. Chapter 5 discusses the findings in the context of the scientific literature.

CHAPTER 2

LITERATURE REVIEW

2.1 Leukocyte telomere length

During replication of DNA in cell division, the RNA primer synthesised is removed and replaced with DNA by DNA-polymerase. However, the replication machinery is unable to replace the RNA primer at the 5'-end with new DNA (Correia-Melo et al., 2014). As a result, with cell division, every new strand of DNA has an incomplete 5'- end with an overhang at the 3'-end. Without the necessary cellular structures, these single-strand 3' overhangs will be deleted, which means that DNA would become shorter with every replication (Blackburn, 2001). Ultimately, the terminal attrition of DNA would lead to the loss of critical genetic information and cells would be unable to multiply (Blackburn, 2001). Hence, chromosomes require additional cellular structures and mechanisms to complete the 5'-end of DNA strands during replication.

Telomeres are specialized DNA-protein structures that cap the end of chromosomes. Telomeres comprise several 1000 non-coding repeats of the six base pair sequence, 5'-TTAGGG-3'/5'-CCCTAA-3'. During DNA replication, some of the telomere base pairs are lost at the 5' end of the new DNA strand, which results in a progressive shortening of telomere lengths. Because these base-pairs are not coding for any particular gene, the loss of these base pairs does not adversely affect the genetic information of the cell (Aubert & Lansdorp, 2008). Nevertheless, when telomere length reaches a critical level, cellular senescence and apoptosis pathways are activated (Shammas, 2011). Therefore, telomeres preserve the integrity and stability of the genome during replication as they protect the DNA protein structure against recombination and degradation, fusion and apoptosis as well as harmful DNA signalling and mitochondrial dysfunction. (Blackburn, 2001).

In order to protect against the loss of loss of genetic information due to terminal chromosomal attrition, telomere length is maintained by the ribonucleoprotein enzyme telomerase. This enzyme lengthens, repairs and stabilises the terminal regions of telomeric DNA by RNA-templated addition of tandemly repeated, telomeric sequences (Greider & Blackburn, 1985). Ensuring the integrity of telomere length is critical in the prevention of cellular senescence and apoptosis.

As a result of the shortening of telomere length during cell replication, telomere lengths are considered to be an index of biological aging, rather than chronological age (Aviv, 2004). 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, 2004; Willeit et al., 2010).

2.2 Leukocyte telomere length and cardiovascular disease

Although ageing is strongly associated with telomere length, risk factor exposure and oxidative stress can also accelerate the rate of telomere attrition (Georgin-Lavialle et al., 2010). As previously mentioned, when telomeres reach a critical length, cellular senescence and apoptosis pathways are activated (Shammas, 2011). Senescent cells, with critically short or dysfunctional telomeres, contribute to cellular decline and aging, as these cells are a potent source of pro-inflammatory mediators and reactive oxygen species (ROS) (Correia-Melo et al., 2014). Both inflammation and ROS are involved in causing more damage to the cell via impaired cellular regeneration and accelerated age related dysfunction and disease (Jurk et al., 2014).

Indeed, several studies have shown that shorter leukocyte telomere lengths are associated with cardiovascular disease (Fitzpatrick et al., 2006; Fyhrquist et al., 2013;

Haycock et al., 2014). In cross-sectional, clinical human studies, shorter telomere lengths have been associated with cardiovascular mortality (Cawthon et al., 2003; Epel et al., 2008) and increased risk for cardiovascular events (Brouilette et al., 2003; Fitzpatrick et al., 2006; Zee et al., 2009; Ding et al., 2012; Weischer et al., 2012). The association of shorter telomere lengths and cardiovascular disease is reportedly independent of chronological age, sex, and conventional risk factors (Fitzpatrick et al., 2006; Brouilette et al., 2007).

In basic and mechanistic studies in animals and humans, shorter telomeres have been linked to cellular senescence in cardiomyocytes (Chimenti et al., 2003; Leri et al., 2003). These results are supported by associations between telomere length and cardiac dysfunction and/or heart failure (Starr et al., 2007; Weischer et al., 2012).

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) and with dysfunction in vascular endothelial cells (Minamino et al., 2002). 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). In contrast others have posited that local factors, related to altered hemodynamics, may control telomere length in the arterial wall (Fyhrquist et al., 2013). Nevertheless, telomere length has been associated with vascular ageing (Nilsson et al., 2012). Hence the association between telomere length and cardiovascular disease may be partly attributed to changes in arterial function.

2.3 Leukocyte telomere length and arterial function

Although various methods have been used to quantity arterial function and aortic stiffness, carotid-femoral pulse wave velocity (PWV) is considered the most reliable, non-invasive marker of aortic stiffness (Townsend et al., 2015). PWV is an independent predictor of cardiovascular disease (Vlachopoulos et al., 2010). Increases in arterial stiffness have been shown to alter aortic hemodynamics by increasing the impedance to aortic flow. This results in an increased pulse pressure and increased aortic systolic pressure which ultimately increases workload for the heart and decreased coronary flow (Dart & Kingwell, 2001; Mottram et al., 2005; Townsend et al., 2015). Indeed, central aortic pressure and pulse pressure are independently associated with cardiovascular disease (Safar et al., 2002; Roman et al., 2007).

Besides the workload on the heart, increased aortic stiffness may also accelerate the risk for cardiovascular disease by increased wave reflection. With ventricular contraction, a pressure wave is generated that travels along the arterial tree. When the pressure wave reaches the smaller arteries and branching points, the pressure wave is reflected back to the heart (Mitchell et al., 2010). As a result, the central, aortic wave form is comprised of the forward and reflected waves (Mitchell et al., 2010). With increased arterial stiffness, the reflected wave returns to the heart earlier, which results in augmenting the central systolic pressure, instead of diastolic pressure (Nichols & Edwards, 2001). Consequently, an earlier return of the reflected wave increases the workload of the heart and reduced coronary perfusion (Chirinos et al., 2012. Moreover, increased arterial stiffness and wave reflection also increase cardiovascular risk by increasing the pulsatile energy transmission in small vessels supplying target organs (Mitchell et al., 2011). In this regard an increased aortic wave reflection has been

considered an important risk marker for cardiovascular disease (Dart & Kingwell 2001). Although augmentation index is considered a marker of wave reflection, using wave separation analysis to determine reflected wave magnitude and timing is considered a more accurate measure of wave reflection (Wang et al., 2010; Chirinos et al., 2012). Wave reflection as determined by wave separation analysis has been associated with target organ damage and cardiovascular risk (Wang et al*.,* 2010, Weber et al., 2012; Booysen et al., 2015).

Table 2.1 shows a summary of the studies that have determined whether leukocyte telomere length is associated with markers of arterial function. Some studies (Benetos et al., 2001, Tentolouris et al., 2007, Nawrot et al., 2010, Wang et al., 2011, Raymond et al., 2014), but not all (Tentolouris et al., 2007; Eguchi et al., 2017; Picarelli et al., 2017) have shown that leukocyte telomere length is inversely associated with pulse wave velocity. Although one study showed an association between telomere length and augmentation index (Eguchi et al., 2017), this was not supported by others (Raymond et al., 2015). To date, there is no evidence whether telomere length is associated with wave reflection as determined by wave separation analysis. Lastly, some suggest that the relationship between telomere length and arterial function is only significant in men (Benetos et al., 2001; Eguchi et al., 2017; Wang et al., 2011). Despite discrepancies in the data, the evidence suggests that leukocyte telomere length may be a biomarker for the prediction of risk of cardiovascular disease.

Table 2.1. A summary of studies investigating the relationship between telomere length and arterial function measures

Study	Population	Measure of TL	Arterial function	Main outcome
Benetose et al., 2001	N=193, general population	TRF	cf PWV PP	TL inversely related to PP in men but not women TL inversely related to PWV in men and women
Denil et al., 2014	N=2509, general population	TRF	cf PWV, PP	No correlation between TL and PWV
Dudinskaya et al., 2015	N=99, 50 diabetes, 49 controls	qRT PCR	cf PWV	TL inversely related to PWV in diabetes and control groups
Eguchi et al., 2017	N=770 general population.	qRT PCR	ba PWV AIx	No association between TL and PWV. Inverse relationship between TL and AI only in men
Jeanclose et al., 2000	N=98, general population, twins	Southern blot	PP	TL inversely related to PP
McDonnell et al., 2017	N=904, general population	qRT PCR	cf PWV	TL related inversely to PWV in younger and directly to PWV in older participants
Nawrot et al., 2010	N=305, general population	TRF	Carotid distensibility	TL directly associated with carotid distensibility
Picarelli et al., 2017	N=45, 24 juvenile idiopathic arthritis, 21 controls	qRT PCR	PWV	No correlation between TL and PWV
Raymond et al., 2015	N=580 general population	qRT PCR	cf PWV, AIx, Pf, AP	TL inversely associated with PWV. No association between TL and any other measure of arterial function
Strazhesko et al., 2015	N=303, general population, free of CVD	qRT PCR	cf PWV	c-f PWV is inversely associated with TL
Tentolouris et al., 2007	N=84 men with type 2 diabetes	TRF	cf PWV, cr PWV, PP	No association between TL and cf PWV or PP. Inverse association between TL and cr PWV
Wang et al., 2011	N=275 men, 163 with CAD and 112 healthy controls.	qRT PCR	cf PWV	TL inversely associated with PWV in CAD and controls

TL, telomere length; qRT PCR, quantitative real time polymerase chain reaction; TRF, terminal restriction fragments; cf, carotid femoral; PWV, pulse wave velocity; ba, brachial ankle; cr, carotid radial; AIx, augmentation index; PP, pulse pressure, AP, augmentation pressure; Pf, forward wave pressure; CAD, coronary artery disease

2.4 Rheumatoid arthritis and cardiovascular disease

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune inflammatory condition affecting approximately 0.5 to 1% of the population (McInnes & Schett, 2011). RA is characterized by synovial inflammation and hyperplasia which leads to joint destruction, which is associated with significant disability and a poor quality of life (McInnes & Schett, 2011). Auto-antibody production including anti-citrullinated peptide antibody (ACPA) and rheumatoid factor (RF) are characteristic of RA (Rindfleisch & Muller, 2005, McInnes & Schett, 2011).

In addition to joint manifestations, the systemic inflammation in persons with RA causes extra-articular complications affecting multiple organ systems (Turesson et al., 1999; McInnes & Schett, 2011). Extra articular manifestations occur in approximately 40% of RA patients (Cimmino et al., 2000). Systemic features of RA are often a reflection of long-term inflammation and relate to increased disease activity and severity (Turesson et al., 1999; Hochberg et al., 2008).

It is well known that RA significantly increases the risk of cardiovascular mortality and cardiovascular event incidence compared to the general population (Avina-Zubeita et al., 2008; Avina-Zubeita et al., 2012). Cardiovascular events occur a decade earlier in RA patients compared to the general population (Bacon et al., 2002). Patients with RA also have a higher prevalence of sub-clinical cardiovascular disease including atherosclerosis, left ventricular dysfunction and heart failure (Avina-Zubeita et al., 2008; Aslam et al., 2013).

Although traditional risk factors are uniquely affected in patients with RA, the increased CVD risk in RA cannot be explained by traditional risk factors alone (Del

Rincón et al., 2001). Both genetic polymorphisms and chronic systemic inflammation are implicated in the increased CVD risk in RA (Gonzalez-Gay et al., 2008; López-Mejías et al., 2016). Markers of inflammation have been independently associated with cardiovascular related mortality in RA (Maradit Kremers et al., 2005). Systemic inflammatory markers are also related to sub-clinical cardiovascular disease including atherosclerosis, endothelial dysfunction, left ventricular dysfunction and heart failure (Gonzalez-Gay et al., 2005; Dessein et al., 2007; Liang et al., 2010; Sharma et al., 2015). The mechanisms underlying the increased cardiovascular disease risk in RA is currently under investigation.

One of the possible mechanisms whereby systemic inflammation may influence cardiovascular disease risk is by impaired arterial function. In this regard chronic systemic inflammation alter arterial function by causing vascular smooth muscle cell proliferation, increased collagen deposition and elastin degradation (Wang et al., 2001; Hattori et al., 2003; Hirsch & Ghigo, 2014). Indeed arterial stiffness (increased PWV), altered central hemodynamics and increased wave reflection have been reported in RA (Ambrosino et al., 2015). Both risk factor exposure and increased inflammation are associated with increased arterial stiffness in RA (Crilly et al., 2009; Jain et al., 2014; Gunter et al., 2017). Moreover, increased arterial stiffness in RA has recently been linked to the incidence of cardiovascular events (Ikdahl et al., 2016), atherosclerosis (Gunter et al., 2018) and left ventricular dysfunction (Mokotedi et al., 2019). Clearly there is a complex interplay between systemic inflammation, arterial function and the risk of developing cardiovascular disease in RA.

2.5 Inflammation and leukocyte telomere length

Besides the destructive effect of chronic inflammation on arterial function and structure, inflammation is also suggested to affect the rate of telomere attrition. In this regard, inflammation is strongly linked to biological aging (Chung et al., 2001; Carrero et al., 2008). Evidence suggests a causal role for inflammation in the pathogenesis of multiple age-related diseases including cancer, atherosclerosis, endothelial dysfunction, autoimmune disorders, diabetes, and neurodegenerative diseases (Pradhan et al., 2001; Moss et al., 2005; Wyss-Coray et al., 2006). Similarly, telomere attrition has been associated with several of these inflammatory diseases (Samani et al., 2001; Weischer et al., 2012; Lee & Bae, 2018). Shorter telomeres have been associated with markers of inflammation, including CRP and IL-6 (Bekaert et al., 2007; Carrero et al., 2008; Masi et al., 2012). A rat model of chronic inflammation showed that leukocyte telomere length is an index of cumulative exposure to inflammation (Raymond et al., 2014).

Furthermore, inflammation increases oxidative stress, via the release of reactive oxygen species (Jaiswal et al., 2000). Oxidative stress has also been found to have a negative impact on telomere length in *in vitro* (Harboa et al., 2012), and cross-sectional human studies (Masi et al., 2011). Since oxidative stress and inflammation are considered key mechanisms underlying the pathophysiology of CVD in RA and contribute to telomere shortening (Wong et al., 2014; Correia-Melo et al., 2014), it has been suggested that telomere attrition may well be accelerated in RA.

Although some studies have shown that persons with autoimmune diseases have shorter telomeres than their healthy counterparts (Steer et al., 2007; Georgin-Lavialle et al., 2010), a recent meta-analysis showed that a large number of studies do not

support this notion (Lee & Bae, 2018). Nevertheless, chronically activated T-cells, as seen typically in RA, lose the ability to up-regulate telomerase activity (Valenzuela et al., 2002). Indeed, Parish et al. (2009) showed that the pro-inflammatory cytokine, TNF-α, influences the expression of CD28 in $CD8^+$ T lymphocytes, which is linked to altered telomerase activity and cellular senescence. However, in clinical RA studies, telomerase activity in CD4 T-cells have been reported insufficient to protect against telomere shortening by one (Fujii et al., 2009), but not other studies (Dehbi et al., 2013). Furthermore, several studies in inflammatory disorders have failed to show that telomere length is inversely related to disease activity and/or inflammation (Steer et al., 2007; Tomayo et al., 2010; Antoniou et al., 2012; Blinova et al., 2016). These contrasting findings in RA necessitate further investigations to determine the role of telomere length as a marker of cardiovascular disease risk.

2.6 Problem statement

The discussion in the above sections provides the context for the potential association between telomere length and arterial function in rheumatoid arthritis. Briefly, chronic inflammation has been shown to increase cardiovascular disease risk in RA. Similarly, chronic inflammation and oxidative stress also induce telomere attrition. Yet, in patients with RA, the findings of telomere attrition compared to controls are controversial. As chronic inflammation has been shown to increase cardiovascular disease risk in RA, whether leukocyte telomere length provides an index of the cumulative inflammatory load, that is associated with cardiovascular disease risk in RA, warrants investigation.

Arterial stiffness is increased in RA, and has been related to atherosclerosis and left ventricular dysfunction in RA. Hence it may provide an early risk marker for the development of cardiovascular disease. Arterial stiffness has been related to telomere

length in the general population, however some of these results are contentious. In inflammatory disorders, the relationships between leukocyte telomere length and traditional cardiovascular disease risk factors and atherosclerosis are controversial (Haque et al., 2013; Raymond et al., 2016). Therefore, whether leukocyte telomere length is associated with markers of arterial function in RA needs elucidation.

2.7 Aims

The present study aimed to determine the relationship between relative leukocyte telomere length and comprehensively assessed markers of arterial function in patients with rheumatoid arthritis. In addition, the study aimed to determine the potential influence of population origin on the relationship between relative leukocyte telomere length and arterial function.

CHAPTER 3

METHODS

3.1 Patients

The current study forms part of ongoing investigations of cardiovascular risk in RA patients from an out-patient rheumatoid clinic at Milpark Hospital in Johannesburg South Africa (Gunter et al., 2017). One-hundred and eighty six men and women, that met the 1988 American College of Rheumatology (Arnett et al., 1988) and 2012 American College of Rheumatology/European League against Rheumatism criteria for rheumatoid arthritis (Singh et al., 2012) were included in the study. One-hundred-and-forty-one patients who had high quality arterial function measures and from whom relative telomere length measures could be obtained were included in the analysis for this study. The study was performed according the Helsinki declaration principles and was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (ethics clearance number M120562 renewed as M170592). Informed, written consent was obtained from all patients.

3.2 Demographic, anthropometric and clinical variables

Demographic, anthropometric and clinical variables were recorded as previously described (Gunter et al., 2017; Mokotedi et al., 2019). Briefly, at inclusion into the study, routine history and physical examination data were recorded by a physician. Demographic and anthropometric variables, including height, weight and waist and hip circumference were measured using standard approaches.

Resting systolic and diastolic blood pressure was measured according to standard approaches. Hypertension was defined as the current use of anti-hypertensive medication or an average systolic blood pressure of ≥140 mm Hg and/or a diastolic blood pressure of ≥ 90 mm Hg.

Standard laboratory blood test of renal and liver function, haematological parameter, lipid and glucose were performed. Dyslipidaemia was diagnosed when the atherogenic index, i.e. the cholesterol-HDL cholesterol ratio, was > 4, where the proatherogenic non-HDL cholesterol concentration were calculated by subtracting HDL cholesterol from total cholesterol concentration. Diabetes was classified as the use of glucose lowering medications or a fasting plasma glucose of ≥ 7 mmol/l. The homeostatic model assessment of insulin resistance was calculated as previously described (Wallace et al., 2004). The glomerular filtration rate was estimated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (Levey et al., 2009).

RA disease characteristics were determined. Disease activity was determined from the Disease Activity Score in 28 joints (DAS28) and the Clinical Disease Activity Index (CDAI). Disease severity was determined from deformed joint counts, rheumatoid factor (RF) status, anti–citrullinated peptide antibody (ACPA) status and extra-articular manifestations. Physical impairment was estimated from the Stanford Health Assessment Questionnaire disability index. C-reactive protein (CRP) was measured by immunoturbidimetry and erythrocyte sedimentation rate (ESR) was measured according to standard approaches. Interleukin (IL) -6 concentrations were measured using a solid-phase sandwich enzyme-linked immunosorbant assay (ELISA) (Quantikine HS, R&D Systems, Inc., Minneapolis, MN, USA).

3.3 Large artery function

Large artery function was measured as previously described (Gunter et al., 2017). Briefly, after patients rested for 15 minutes in the supine position, the radial waveform was recorded by applanation tonometry during an 8 second period. A highly-fidelity 301 micro manometer (Millar instrument, Inc, Houston, Texas), interfaced with a

computer utilizing SphygmoCor software, version 9.0 (AtCor Medical Pty. Ltd ,West Ryde, New South Wales, Australia) were employed. Recordings were discarded when the systolic or diastolic variability of consecutive waveforms exceeded 5%, or when the amplitude of the waveform signal was less than 80mV. The waveform was calibrated with auscultatory measures of brachial blood pressure, obtained immediately prior to the recordings. From the radial waveform the SphygmoCor software calculated the aortic pressure waveform by means of a validated generalized transfer function. Aortic pulse pressure (PPc) was calculated as aortic systolic minus diastolic BP. Pulse pressure amplification (PPamp) was calculated as the ratio of central to brachial pulse pressure. The magnitude and timing of the forward and reflected waves of the aortic pressure waveform were determined by wave separation analysis using a modified triangular waveform (SphygmoCor Software). The reflection magnitude was determined as reflected wave magnitude (derived from wave separation analysis) divided by the forward wave amplitude.

Aortic pulse wave velocity (PWV) was measured by sequential recording of the arterial pressure waveform at the carotid and femoral artery sites. The time delay in pulse waves between the carotid and femoral sites were determined using an electrocardiograph-derived R wave as a fiducially point. Distances from the suprasternal notch to the carotid sampling site (distance A) and from the suprasternal notch to the femoral artery (distance B) were measured. Pulse wave velocity distance was calculated as distance B minus distance A. Pulse transit time, calculated as the mean time difference between sites A and B was determined from the average of 10 consecutive beats. Aortic PWV was calculated as the ratio of the distance in meters to the transit time in seconds.

3.4 Average relative telomere length

Leukocyte DNA for each participant was extracted from whole blood samples using a Nucleospin® Whole Blood DNA extraction kit (Machery-Nagel, Germany) and samples were stored at -20 °C until analysed. A quantitative real-time PCR (qRT-PCR) method was used to determine relative telomere length of leukocyte 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 36B4 gene located on chromosome 12, which codes for acidic ribosomal phosphoprotein PO. 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 36B4 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 20µl consisting of approximately 50ng genomic DNA, 10µl SYBR Green Real-Time PCR Master Mix (ThermoFisher, USA), appropriate forward and reverse primers for either the telomere or 36B4 reactions (5pmol of each primer/reaction) and the volume was made up to 20µl with nuclease free water. Primer sequence and thermal profiles for the RT-PCR reactions are shown in Table 3.1.

Table 3.1. The primers and thermal profiles for the telomere and 36B4 PCR amplifications.

Telomere PCR primers and thermal profile			
Telomere forward primer: 5'-3'	CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT		
Telomere reverse primer: 5'-3'	GCC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT		
Thermal profile:	Number of cycles	Temperature	Duration
Activation	1	50°C, 95°C	2 minutes at each temperature
Amplification	25	95°C, 54°C	15s and 2 minutes
36B4 PCR primers and thermal profile			
36B4 forward primer 5'-3'	CAG CAA GTG GGA AGG TGT AAT CC		
36B4 reverse primer: 5'-3'	CCC ATT CTA TCA TCA ACG GGT ACA A		
Thermal profile:	Number of cycles	Temperature	Duration
Activation	1	50°C, 95°C	2 minutes at each temperature
Amplification	30	95°C, 58°C	15s and 1 minute

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 36B4 Ct. 36B4 has only one copy per 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:

$$\text{T/S ratio} = 2^{-(\Delta Ct(\text{sample}) - \Delta Ct(\text{standard}))} \text{ where } \Delta Ct = Ct(\text{telomere}) - Ct(36B4).$$

DNA from a single individual was used as the calibrator in this study to standardise all of the T/S ratios. Standard curves were generated for both telomere and 36B4 reactions (Figure 3.1) from the calibrator that was serially diluted to produce a range of DNA from 100ng to 1.56ng for telomeres or 3.25ng for 36B4. The reaction efficiencies of 100% and 99.8%, of the telomere and 36B4 standard curves, respectively, were within accepted limits (Gil & Coetzer, 2004). For the telomere and 36B4 standard curves, the slopes of the fitted regressions were –3.321 and –3.326 respectively. The regression coefficients were 0.983 and 0.975 for the telomere and 36B4 standard curves, respectively. The standard curves showed that there was a strong negative linear relationship between DNA concentration and Ct. Moreover, melting curve analysis were performed on the 36B4 reaction to ensure that the method was producing a single product. As the telomere reaction generates a range of product sizes, this approach would not be appropriate for demonstrating the specificity of the telomere reaction. Furthermore, the calibrator was included in each amplification run and was used to determine coefficients of variation of the S and T assays, which were 3.12% and 3.46% respectively. Samples that fell outside the linear concentration range of the standard curves were diluted and re-run in order to ensure they fell within the linear range of the standard curves. This real-time quantitative PCR method has been validated and shown to give a measure of relative telomere length, which is proportional to mean telomere length as determined by Southern blotting (Cawthon, 2002; Gil & Coetzer, 2004).

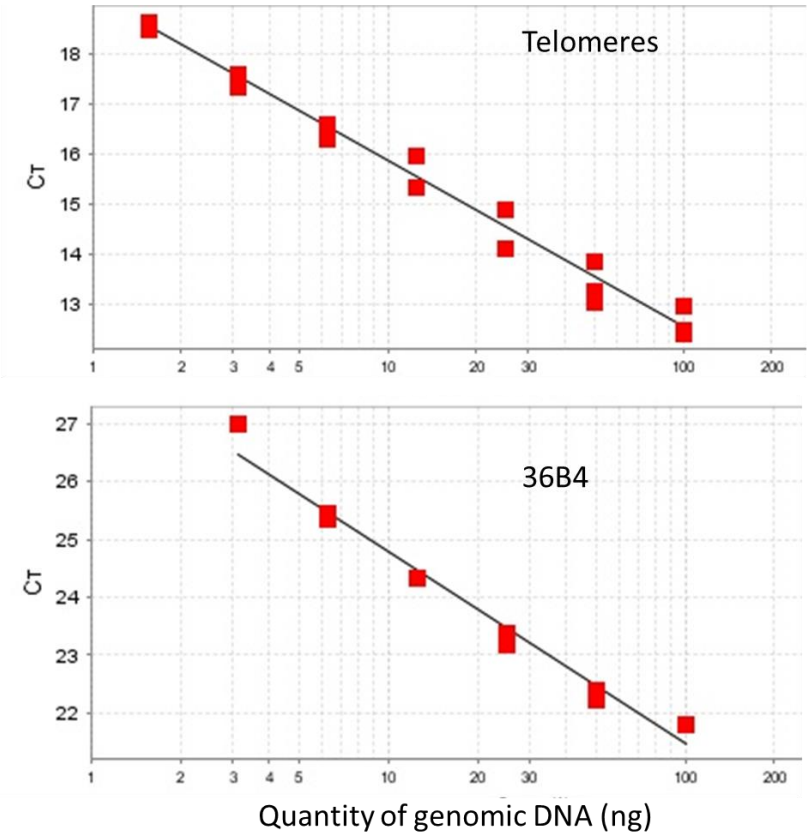


Figure 3.1 Standard curves for the telomere (upper panel) and 36B4 (lower panel) qRT-PCR reactions. The curves indicate that there is a strong linear relationship between DNA concentration and the PCR cycles required to reach threshold levels of fluorescence (Ct).

3.5 Statistical analysis

Statistical analysis was performed using SAS software version 9.4 (SAS institute Inc., Cary, NC). Data are expressed as mean (SD), median (interquartile range, IQR) or proportions. Non-normally distributed variables were logarithmically transformed prior to inclusion in regression analysis. Bivariate relationships between demographic characteristics and relative telomere length were assessed in age, sex and race adjusted linear regression models. Independent associations between relative telomere length and arterial function variables were assessed in multivariate linear and backward regression models with inclusion of potential confounding variables as determined in previous analysis (age, sex and race adjusted associations of patient characteristics with telomere length) and in known confounder adjusted analysis as per previous literature (Gunter et al., 2017). As it has been previously reported that the associations between telomere length and subclinical cardiovascular disease (atherosclerosis) is different among black and white patients (Raymond et al., 2016), and between men and women (Benetose et al., 2001; Eguchi et al., 2017), we performed sensitivity analysis in white patients and in women in separate models. Results were considered statistically significant if $p<0.05$.

CHAPTER 4

RESULTS

4.1 Demographic characteristics

The recorded patient characteristics are given in Table 4.1. Mean (SD) age at time of the study was 58.8 (11.9) years and median (IQR) RA duration was 14.5 (8.9–22.1) years. Majority of the participants (82%) were female. Of the 141 RA patients, 99 were white, 25 Asian, 17 black and 4 mixed race. The mean systolic and diastolic blood pressure was 128 (15) and 81 (8) mm Hg respectively. Of the total participants 39.7% were hypertensive, of which all were receiving anti-hypertensive treatment. Dyslipidaemia and diabetes were recorded in 48.2% and 6.4% of participants respectively. Rheumatoid factor (RF) and anticitrullinated protein antibody (ACPA) positivity were present in 71.6% and 65.3% of the participants, respectively. Disease activity was overall well controlled with a median (IQR) Clinical Disease Activity Index (CDAI) score of 5 (1–13) and a mean (SD) 28-joint Disease Activity Score (DAS28) of 2.9 (1.7). All patients were using synthetic disease modifying agents. The median (IQR) pulse wave velocity and relative leukocyte telomere length were 7.4 (6.2–9.4) m/sec and 0.96 (0.62-1.51) respectively.

Table 4.1. Recorded characteristics in 141 patients with RA

Demographic characteristics		DAS 28	2.9 (1.7)
Age at study time, years	58.8 (11.9)	ESR, mm/h	12 (4-25)
Age at disease onset, years	42.7 (14.3)	CRP, mg/l	3.2 (1.2-6.7)
Female gender, n (%)	115 (82)	Interleukin 6, pg/ml	8.0 (4.1-14.3)
Lifestyle factors		Leukocytes, n/nl	5.3 (4.4-6.9)
Exercise	33.3	Deformed joints, n	0 (0-10)
Alcohol use	31.9	Extra-articular manifestations	19.2
Current smoking	9.9	Stanford HAQ	0.4 (0-0.9)
Anthropometry		**Synthetic disease modifying agents**	
Body mass index, kg/m^2	27.3 (5.4)	Methotrexate	74.5
Waist circumference, cm	92.7 (13.4)	Chloroquine	48.9
Waist-to-hip ratio	0.88 (0.09)	Leflunomide	42.6
Metabolic risk factors		Sulphasalazine	17.0
Hypertension	39.7	Tetracycline	14.9
Systolic BP, mm Hg	128 (15)	Azathioprine	7.1
Diastolic BP, mm Hg	81 (8)	Current DMARDS, n	2.0 (1.1)
Total cholesterol, mmol/l	4.5 (1.0)	**Biological disease modifying agents**	
HDL cholesterol, mmol/l	1.63 (0.46)	TNF-α inhibitors	7.1
LDL cholesterol, mmol/l	2.40 (0.81)	Abatacept	2.8
Triglycerides, mmol/l	0.95 (0.71-1.30)	NSAID	31.9
Cholesterol-HDLratio	2.71 (2.32-3.24)	Prednisone use	2.8
Chol-HDL ratio >4, %	10.6	GFR, ml/min/1.73m^2	91.3 (23.7)
Dyslipidemia, %	48.2	Heart rate, beats per minute	71.5 (11.9)
Diabetes, %	6.4	Framingham score	1.13 (0.57-2.37)
Glucose, mmol/l	4.9 (4.5-5.1)	**Arterial function measures**	
HOMA-IR	1.5 (1.0-2.0)	PWV, m/s	7.4 (6.2-9.4)
Cardiovascular agents		Augmentation index, %	31.7 (11.0)
Antihypertensives	39.7	Central systolic BP, mm Hg	127 (15)
Statins	41.1	Central pulse pressure, mm Hg	43 (14)
Ezetimibe	8.5	Brachial pulse pressure, mm Hg	46 (14)
Oral glucose lowering agents	2.1	Pulse pressure amplification	1.13 (0.27)
Insulin	2.1	Forward wave pressure, mm Hg	28 (8)
RA characteristics		Backward wave pressure, mm Hg	21 (8)
RA duration, years	14.5 (8.9-22.1)	Reflection magnitude	0.76 (0.23)
Rheumatoid factor positive	71.6	Time to peak forward wave, msec	139.1 (40.5)
ACPA positive	65.3	Time to wave reflection, msec	254.8 (19.9)
CDAI	5 (1-13)	Relative leukocyte telomere length	0.96 (0.62-1.51)

Dichotomous variables are expressed as proportions or percentages and continuous variables as mean (SD) or median (interquartile range).

BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, homeostatic model of insulin resistance; ACPA, anti-citrullinated peptide antibody; CDAI, clinical disease activity index; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; HAQ-DI, health assessment questionnaire disability index; DMARD, disease modifying anti-rheumatic drugs; TNF, tumor necrosis factor; NSAID, non-steroidal anti-inflammatory drugs; GFR, glomerular filtration rate; PWV, pulse wave velocity

4.2 Associations of traditional risk factors and RA disease characteristics with relative leukocyte telomere length

The association of relative leukocyte telomere length with traditional risk factors in all patients, in white patients and in women only are shown in Table 4.2. No traditional cardiovascular disease risk factors were associated with relative leukocyte telomere length in all patients. In white patients, relative leukocyte telomere length was associated with body mass index (partial r = 0.20, p = 0.05) and with waist circumference (partial r = 0.21, p = 0.05). In women, relative leukocyte telomere length was associated with alcohol intake (partial r = -0.19, p = 0.04) body mass index (partial r = 0.20, p = 0.03) and with waist circumference (partial r = 0.20, p = 0.03). RA disease characteristics and inflammatory markers were not associated with relative leukocyte telomere length in all patients or in women. In white patients, relative leukocyte telomere length was associated with DAS28 (partial r = -0.20, p = 0.05) and number of deformed joints (partial r = -0.20, p = 0.05). Abatacept use was associated with relative leukocyte telomere length in all patients (partial r = 0.19, p = 0.02) and in white patients (partial r = 0.24, p = 0.02) and in women (partial r = 0.22, p = 0.02). Sulphasalazine was associated with relative leukocyte telomere length (partial r = -0.24, p = 0.02) in white patients.

Table 4.2. Traditional cardiovascular disease risk factors and RA disease characteristics associated with relative leukocyte telomere length* in all patients, white patients and women

`	All patients (n=141)			White patients (n=95)			Women (n=95)		
	partial r	**95% CI**	**P**	**partial r**	**95% CI**	**P**	**partial r**	**95% CI**	**P**
Age	0.05	-0.11-0.21	0.54	0.11	-0.10-0.30	0.30	0.03	-0.15-0.22	0.72
Age at disease onset	0.09	-0.08-0.25	0.29	0.16	-0.05-0.35	0.13	0.06	-0.12-0.24	0.51
Sex	0.07	-0.10-0.23	0.43	0.05	-0.15-0.25	0.61	-	-	-
Race	0.13	-0.03-0.29	0.12	-	-	-	0.13	-0.06-0.31	0.17
Exercise	-0.05	-0.21-0.12	0.56	-0.11	-0.31-0.09	0.29	-0.03	-0.21-0.16	0.79
Alcohol	-0.14	-0.30-0.02	0.09	-0.13	-0.33-0.07	0.21	**-0.19**	**-0.36--0.01**	**0.04**
Smoking	-0.12	-0.28-0.05	0.16	-0.08	-0.28-0.13	0.45	-0.08	-0.26-0.10	0.38
Heart rate	-0.02	-0.19-0.14	0.79	0.02	-0.18-0.22	0.84	0.02	-0.16-0.21	0.79
Systolic BP	0.13	-0.03-0.29	0.12	0.12	-0.09-0.31	0.27	0.09	-0.10-0.27	0.35
Diastolic BP	0.01	-0.15-0.18	0.88	0.00	-0.20-0.21	0.98	0.005	-0.18-0.19	0.96
Antihypertensive agents	0.12	-0.06-0.29	0.18	0.07	-0.14-0.28	0.50	0.11	-0.08-0.29	0.26
Body mass index	0.13	-0.04-0.30	0.14	**0.20**	**0.00-0.39**	**0.05**	**0.20**	**0.01-0.37**	**0.03**
Waist circumference	0.13	-0.05-0.29	0.16	**0.21**	**0.00-0.39**	**0.05**	**0.20**	**0.02-0.37**	**0.03**
Waist-hip ratio	0.04	0.13-0.21	0.65	0.07	-0.13-0.27	0.50	0.12	-0.07-0.29	0.23
Statin	0.06	-0.11-0.23	0.49	0.03	-0.18-0.23	0.79	0.08	-0.12-0.26	0.43
Ezetimibe	-0.05	-0.22-0.13	0.59	-0.07	-0.27-0.14	0.53	0.06	-0.14-0.24	0.57
Oral glucose lowering agents	-0.08	-0.25-0.10	0.39	-0.01	-0.22-0.20	0.94	-0.02	-0.20-0.18	0.88
Insulin	-0.05	-0.22-0.13	0.60	0.11	-0.10-0.30	0.30	0.10	-0.10-0.22	0.40
HOMA IR*	0.01	-0.16-0.19	0.89	0.05	-0.16-0.25	0.65	-0.05	-0.24-014	0.60
Disease duration*	-0.03	-0.20-0.13	0.70	-0.04	-0.24-0.16	0.70	-0.02	-0.21-0.16	0.81
Rheumatoid factor positive	-0.003	-0.17-0.16	0.97	-0.05	-0.26-0.15	0.62	0.03	-0.15-0.22	0.72
CDAI*	-0.06	-0.22-011	0.49	-0.17	-0.36-0.04	0.11	-0.07	-0.25-0.12	0.48
SDAI*	-0.01	-0.18-0.15	0.88	-0.16	-0.35-0.05	0.13	-0.05	-0.23-0.14	0.63
DAS28	-0.07	-0.23-0.10	0.44	**-0.21**	**-0.40--0.003**	**0.049**	-0.07	-0.26-0.12	0.45
DAS28-CRP	-0.04	-0.20-0.13	0.64	-0.17	-0.37-0.03	0.09	-0.02	-0.21-0.17	0.82
ESR*	-0.03	-0.19-0.14	0.73	-0.13	-0.33-0.08	0.21	-0.04	-0.22-0.15	0.7

CRP*	0.04	-0.12-0.21	0.61	-0.06	-0.26-0.15	0.59	0.11	-0.08-0.29	0.25
IL6*	-0.11	-0.27-0.06	0.20	-0.15	-0.35-0.06	0.15	-0.14	-0.32-0.05	0.15
White cell count*	-0.05	-0.21-0.12	0.57	-0.01	-0.22-0.19	0.90	-0.06	-025-0.13	0.52
ACPA positive	-0.10	-0.26-0.07	0.24	-0.11	-0.31-0.10	0.29	-0.16	-0.33-0.03	0.11
Deformed joints*	-0.10	-0.26-0.07	0.24	**-0.20**	**-0.39-0.00**	**0.05**	-0.09	-0.27-0.10	0.37
Extra articular manifestations	-0.06	-0.22-0.11	0.48	-0.13	-0.32-0.08	0.23	-0.09	-0.27-0.10	0.35
Methotrexate	0.02	-0.14-0.19	0.79	-0.07	-0.27-0.13	0.48	0.04	-0.15-0.220	0.71
Chloroquine	-0.04	-0.21-0.12	0.62	-0.02	-0.23-0.18	0.83	0.05	-0.13-0.24	0.58
Leflunomide	-0.08	-0.24-0.09	0.37	-0.03	-0.23-0.17	0.76	-0.06	-0.24-0.13	0.55
Sulphasalazine	-0.07	-0.23-0.10	0.42	**-0.24**	**-0.42--0.03**	**0.02**	-0.03	-0.21-0.16	0.77
Tetracycline	-0.05	-0.21-0.11	0.54	-0.01	-0.21-0.19	0.96	-0.08	-0.26-0.11	0.41
Azathioprine	-0.06	-0.22-0.11	0.51	-0.12	-0.31-0.09	0.27	-0.09	-0.27-0.09	0.33
Abatacept	**0.19**	**0.03-0.34**	**0.02**	**0.24**	**0.04-0.42**	**0.02**	**0.22**	**0.04-0.39**	**0.02**
Biologic use	0.08	-0.09-0.24	0.37	0.14	-0.06-0.34	0.17	0.08	-0.11-0.26	0.43
NSAID	-0.10	-0.26-0.07	0.25	-0.18	-0.37-0.02	0.08	-0.03	-0.21-0.16	0.78
Prednisone	-0.01	-0.17-0.16	0.91	-0.01	-0.21-0.19	0.93	-0.03	-0.21-0.16	0.76
TNF-α	-0.03	-0.20-0.13	0.70	0.02	-0.19-0.22	0.87	-0.05	-0.23-0.13	0.58
GFR	-0.10	-0.28-0.08	0.27	-0.02	-0.22-0.18	0.81	-0.06	-0.24-0.13	0.54
Total cholesterol	-0.02	-0.19-0.15	0.83	-0.03	-0.23-0.18	0.80	-0.05	-0.24-0.13	0.57
HDL cholesterol	-0.07	-0.23-0.10	0.45	-0.01	-0.22-0.20	0.93	-0.12	-0.30-0.07	0.22
LDL cholesterol	0.02	-0.15-0.19	0.81	-0.04	-0.25-0.17	0.70	0.005	-018-0.19	0.96
Triglycerides*	-0.03	-0.19-0.14	0.75	-0.001	-0.21-0.21	0.99	0.01	-0.18-0.19	0.93
Chol/HDL ratio*	0.04	-0.13-0.20	0.68	-0.02	-0.22-0.19	0.88	0.07	-0.12-0.25	0.47
Chol/HDL > 4	-0.02	-0.19-0.15	0.81	-0.03	-0.24-0.18	0.77	-0.01	-0.20-0.18	0.92
Diabetes	-0.04	-0.20-0.13	0.63	0.01	-0.20-0.22	0.91	0.03	-0.15-0.22	0.72
Glucose*	0.04	-0.13-0.20	0.66	0.02	-0.18-0.23	0.82	0.05	-0.14-0.24	0.6

Associations were adjusted for age, sex and race.

BP, blood pressure; HOMA IR, homeostatic model of insulin resistance; ACPA, anti-citrullinated peptide antibody; CDAI, clinical disease activity index; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; TNF, tumor necrosis factor; NSAID, non-steroidal anti-inflammatory drugs; GFR, glomerular filtration rate; HDL, high density lipoprotein; LDL, low density lipoprotein.

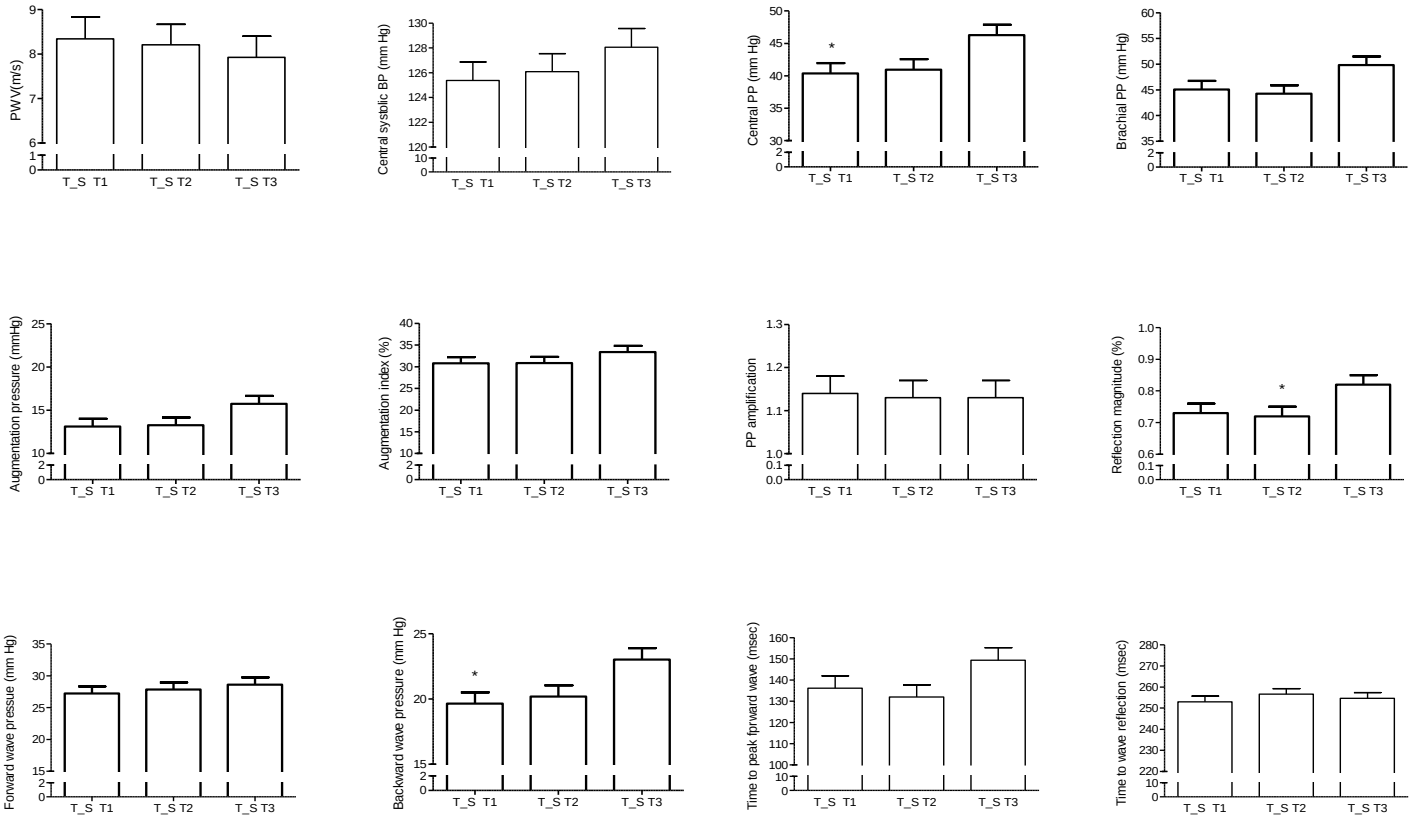
Significant associations are shown in bold.

*Logarithmically transformed variable

4.3 Arterial function variables across tertiles of relative leukocyte telomere length

Figure 4.1. shows the changes in arterial function variables across tertiles of relative leukocyte telomere length. Pulse wave velocity, central systolic pressure, brachial pulse pressure, augmentation pressure, augmentation index, pulse pressure amplification, forward wave pressure, time to the peak of the forward wave and time to wave reflection did not differ across tertiles of relative leukocyte telomere length. The adjusted mean central pulse pressure was higher in tertile 3 versus tertile 1 (mean (SE) tertile 1: 40.4(1.6) mm Hg; tertile 3: 46.3 (1.6) mm Hg, p=0.04). The mean (SD) reflected wave pressure was higher in tertile 3 (23.0 (0.9) mm Hg) compared to tertile 1 (19.7 (0.9) mm Hg; p=0.02). The mean (SD) reflection magnitude was higher in tertile 3 (0.82 (0.03)) compared to tertile 2 (0.72 (0.03), p=0.04).

Figure 4.1. Changes in arterial function variables across tertiles of relative leukocyte telomere length



Adjusted for age, sex, race, height, weight, heart rate, mean arterial pressure, abatacept use. P<0.05 versus tertile 3T_S, relative leukocyte telomere length; T, tertile; PWV, pulse wave velocity; BP, blood pressure; PP, pulse pressure.

4.4 Independent associations of arterial function measures with relative leukocyte telomere length

Table 4.3 shows the relative contribution of relative leukocyte telomere length to various arterial function measures. In age, sex, race and abatacept adjusted backward regression (model 1) no arterial function variables were associated with relative leukocyte telomere length in all patients. When known confounders of arterial function measures were additionally included in the model (model 2) relative leukocyte telomere length was associated with central pulse pressure (std β(SE) = 0.13(0.07), p = 0.05), central augmentation pressure (std β(SE) = 0.13(0.07), p = 0.05), backward wave pressure (std β(SE) = 0.13(0.06), p = 0.04) and reflection magnitude (std β(SE) = 0.14(0.07), p = 0.05) in all patients. When additional confounders for each of the arterial function variables, as previously published (Gunter et al., 2017), were introduced into the backward regression model (model 3) the result were materially unaltered in all patients. In sensitivity analysis in white patients, relative leukocyte telomere length was independently associated with central pulse pressure in model 1 (std β(SE) = 0.23(0.10), p = 0.03), model 2 (std β(SE) = 0.18(0.09), p = 0.04), and model 3 (std β(SE) = 0.18(0.08), p = 0.04). No other arterial function variables were associated relative leukocyte telomere length in white patients only. In sensitivity analysis in women only, relative leukocyte telomere length was not related to any arterial function variables (all p>0.05).

Table 4.3. Relative contribution of leukocyte telomere length to arterial function variables in all patients and in white patients

Arterial function variables	Std β	SE	P	Model R^2	Std β	SE	P	Model R^2	Std β	SE	P	Model R^2
In all patients		Model 1				Model 2				Model 3		
Central pulse pressure	-	-	-	0.28	0.13	0.07	0.05	0.41	0.15	0.07	0.03	0.44
Central augmentation pressure	-	-	-	0.18	0.13	0.07	0.05	0.42	0.13	0.07	0.05	0.42
Backward wave pressure	-	-	-	0.29	0.13	0.06	0.04	0.47	0.13	0.06	0.04	0.47
Reflection magnitude	-	-	-	0.15	0.14	0.07	0.05	0.38	0.13	0.07	0.05	0.41
In white patients		Model 1				Model 2				Model 3		
Central pulse pressure	0.23	0.1	0.03	0.33	0.18	0.09	0.04	0.45	0.18	0.08	0.04	0.45

Std β, standardised beta coefficient; SE, standard error.

*Model 1 adjustors for all patients included: age, sex, race, and abatacept use

#Model 1 adjustors for white patients included: age, sex, waist circumference, DAS 28, deformed joints, sulphasalazine and abatacept use

†Model 2 adjustors included: Model 1 + other known confounders - height, weight, mean arterial pressure and heart rate

‡ Model 3 adjustors included: Model 2 + each of arterial function variables additional confounders:

Pulse wave velocity - waist-hip ratio, total cholesterol-HDL cholesterol ratio, extra-articular manifestations, statin therapy, TNF-α inhibitor therapy, abatacept therapy

Augmentation index - total cholesterol concentration, HDL cholesterol concentrations, homeostatic model of insulin resistance, TNF-α inhibitor therapy

Central systolic blood pressure - total cholesterol-HDL cholesterol ratio, statin therapy, chloroquine therapy

Central pulse pressure - total cholesterol-HDL cholesterol ratio, statin therapy, chloroquine therapy

Central augmentation pressure - total cholesterol-HDL cholesterol ratio, statin therapy, chloroquine therapy
Peripheral pulse pressure - total cholesterol-HDL cholesterol ratio, C-reactive protein concentrations, statin therapy, tetracycline therapy, chloroquine therapy
Pulse pressure amplification - waist-hip ratio, total cholesterol concentrations, glucose concentrations, leflunomide therapy, TNF-α inhibitor therapy
Forward wave pressure- total cholesterol-HDL cholesterol ratio, statin therapy
Backward wave pressure- number of DMARD
Reflection magnitude - HDL cholesterol concentrations, TNF-α inhibitor therapy, leflunomide therapy
Time to wave reflection - waist-hip ratio, erythrocyte sedimentation rate, white cell count, ACPA positivity

CHAPTER 5

DISCUSSION

5.1 Summary of main findings

The main findings of the current study are in all patients, leukocyte telomere length was not associated with any traditional risk factors or RA characteristics. In white patients and in women, leukocyte telomere length was paradoxically directly associated with obesity. In white patients, leukocyte telomere length was inversely associated with RA characteristics. In all models, leukocyte telomere length was associated with abatacept use. With regards to arterial function measures, in all patients, leukocyte telomere length was paradoxically related to wave reflection, augmentation pressure and central pulse pressure, while in white patients the relationship was only significant for central pulse pressure. Leukocyte telomere length was not associated with pulse wave velocity in RA patients. Lastly, in women leukocyte telomere length was not associated with any marker of arterial function.

5.2 Leukocyte telomere length and traditional cardiovascular disease risk factors

Several non-RA studies have shown positive associations between traditional cardiovascular disease risk factors and leukocyte telomere length (Rehkopf et al., 2016; Haycock et al., 2014). However, this is not replicated in all studies (Bekaert et al., 2007; Masi et al., 2012; Neuner et al., 2015; Raymond et al., 2015; Raymond et al., 2016). Similarly, in the current study we have shown no associations between traditional cardiovascular disease risk factors and telomere length in the total group. Importantly, the associations between traditional risk factors and telomere length, is undoubtedly influenced by genetic polymorphisms, gender and race (Steer et al., 2007; Dessein et al., 2013a; Dessein & Solomon, 2013).

In this regard, in women we have shown that reduced leukocyte telomere length was associated with alcohol consumption. Although the literature is inconsistent about the association between leukocyte telomere length and alcohol consumption, Shin & Baik (2016), recently showed that this relationship is impacted by genetic factors. Genetic differences may therefore explain the relationship between leukocyte telomere length and alcohol in women only.

Several non-RA studies have shown inverse relationships between obesity and leukocyte telomere length (Fitzpatrick et al., 2006; Norfdfjall et al., 2008). Similarly, Raymond et al. (2016) showed an inverse relationship between abdominal adiposity and shorter relative telomere length in black African, but not white, subjects with RA. Contrary to expectations, the current study showed that in white patients and in women, there was a significant positive correlation between leukocyte telomere length and BMI and waist circumference. Similarly, Haque et al. (2013) also previously reported a paradoxical direct relationship between telomere length and BMI and higher triglyceride concentrations in lupus patients. Furthermore, Raymond et al. (2016) reported that longer leukocyte telomere lengths are associated with a higher atherogenic index in white, but not black RA patients.

It is well known that cumulative inflammation uniquely affects traditional cardiovascular disease risk factors in RA (Kitas & Gabriel, 2011; Gabriel & Crowson et al., 2012). Obesity and lipid concentrations are two of the traditional risk factors that have shown contrasting properties in RA compared to the general population. Indeed, the ‘obesity paradox’ is a well-known phenomenon in RA. In this regard it has been shown that RA patients with lower BMI have a higher risk of cardiovascular disease and mortality (Maradit Kremers et al., 2004; Escalante et al., 2005). Similarly, high lipid

concentrations have also been associated with reduced cardiovascular disease risk in RA (Myasoedova et al., 2011). In various RA studies, obesity has been shown to impact on the relationship between circulating inflammatory markers and adipokines (biomarkers of cardiovascular disease) and cardiovascular disease risk (Dessein et al., 2013b; Dessein & Solomon, 2013). Taken together, these results suggest that RA patients may have compensatory adaptive changes in metabolic pathways, that result in longer telomere lengths, in an attempt to reduce enhanced cardiovascular disease risk. Nevertheless, this paradoxical relationship between metabolic risk factors and telomere length in RA warrant further investigation in longitudinal studies.

5.3 Leukocyte telomere length and RA disease characteristics

The current study showed no association between relative telomere length and RA disease characteristics or markers of inflammation in all patients. In sensitivity analysis in white patients, leukocyte telomere length was inversely associated with the number of deformed joints and disease activity (DAS28). Although shortened telomere lengths have been observed in RA patients compared to controls (Lee & Bae 2018), some studies reported contrasting findings (Ormseth et al., 2016; Steer et al., 2007). Gamal et al. (2018) recently reported that shorter leukocyte telomere length is associated with RA disease markers. Others have shown no association between leukocyte telomere length and disease activity (Steer et al., 2007; Raymond et al., 2016). Despite shortened telomere lengths in RA patients, currently there is a lack of evidence that inflammation or disease severity is responsible for this attrition (Kordinas et al., 2016). Although one study posits that telomerase activity is reduced in RA (Fujii et al., 2009), others have shown accelerated telomerase activity, consistent with increased T-cell activation (Dehbi et al., 2013).

Regulation of telomerase activity in RA is more complex than meets the eye. CD28 expression in CD 8 T-cells is required for optimal T lymphocyte activation during a normal immune response (Parish et al., 2009). In chronic immune activation of T lymphocyte, as seen with RA, can result in the loss of CD28 expression (Schmidt et al., 1996). CD 28 expression has been related to upregulation of telomerase activity. A lack of expression of CD28 has been linked to cellular senescence (Vallejo et al., 2004). TNFα regulation has been shown to alter the expression of CD28 and hence telomerase activity (Parish et al., 2009). Nevertheless, CD28 expression is also involved in signal transduction for the production of cytokines (Parish et al., 2009). Considering previously reported normal telomerase activity in RA (Dehbi et al., 2013), clearly the regulation of CD28 expression in RA is more complex than merely a function of TNFα concentration. The expression of CD28 and activation of telomerase in RA warrants further investigation.

Various studies have previously shown that population origin affects the relationship between arthritis and cardiovascular disease risk, and that results in a specific population cannot be extrapolated or generalised to all population groups (Dessein & Solomon, 2013; Ormseth et al., 2016; Raymond et al., 2016). Our results confirm that population origin impacts on the relationship between disease markers and leukocyte telomere length.

In the current study, abatacept use was associated with telomere length in all patients, and in sensitivity analysis in sub-groups. Importantly, only 3% of the study population used abatacept therapy. Abatacept is a biologic, anti-rheumatic drug that is mostly given as a second-line therapy to patients in whom the disease is refractory. Abatacept selectively inhibits cellular signals necessary for T-cell activation (Blair & Deeks, 2017).

A recent study reported that telomerase activity in B and T lymphocytes are reduced after treatment with abatacept therapy in RA patients (Otani & Kurosaka 2019). Hence the contrasting direct relationship seen between abatacept therapy and longer telomere lengths in the current study needs further investigation.

5.4 Leukocyte telomere length and arterial function

A majority of previous, non-RA studies investigating the relationship between telomere length and arterial function used aortic (carotid femoral) pulse wave velocity as a measure of arterial stiffness. In contrast to some (Benetos et al., 2001, Tentolouris et al., 2007, Nawrot et al., 2010, Wang et al., 2011, Raymond et al., 2014), but in agreement with others (Tentolouris et al., 2007; Denil et al., 2014; Eguchi et al., 2017; Picarelli et al., 2017), we showed no association between leukocyte telomere length and pulse wave velocity. A possible reason for the discrepancies between the results of these studies could be the vast differences in the population groups studied. There is a large spread in the age ranges, health and disease and population origins of these studies.

Although most studies in the general population showed a relationship between telomere length and pulse wave velocity, these results were influenced by age (McDonnell et al., 2017) and sex (Benetose et al., 2011). Our population were largely women and most participants were older than 50 years, hence this may have influenced our results. Nevertheless, based on previous discussion, patients exposed to chronic inflammation have uniquely affected cellular (Correia-Melo et al., 2018; Jurk et al., 2014) and vascular function (Ambrosino et al., 2015; Gunter et al., 2017). In this regard, three previous studies have been published where patients may have been exposed to chronic inflammation. Two of these studies were performed in patients with

diabetes (Tentolouris et al., 2007; Dudinskaya et al., 2015). One study showed an inverse relationship between leukocyte telomere length and aortic pulse wave velocity (Dudinskaya et al., 2015), while the other showed no such association (Tentolouris et al., 2007). Another study in juvenile idiopathic arthritis patients reported no association between telomere length and pulse wave velocity (Picarelli et al., 2017). Taken together, previous studies and our results suggest that in chronic exposure to inflammation, leukocyte telomere length is not associated with pulse wave velocity.

Recent studies, in the general population and RA, have shown that various other markers of arterial function provide a broader perspective of vascular function and are associated with sub-clinical cardiovascular disease independent of pulse wave velocity (Gunter et al., 2018; Booysen et al., 2015). Particularly studies in which wave separation analysis was performed, have identified wave reflection as an independent contributor to cardiovascular outcomes (Wang et al., 2010; Weber et al., 2012).

Only a small number of previous studies have reported on a relationship between leukocyte telomere length and other markers of arterial function. In this regard two previous studies reported an inverse relationship between leukocyte telomere length and pulse pressure (Jeanclos et al., 2000; Benetose et al., 2001). Similar to our results, two previous studies reported no association between leukocyte telomere length and pulse pressure (Raymond et al., 2015; Tentolouris et al., 2007). Other than pulse pressure, Eguchi et al. (2017) reported an inverse relationship between leukocyte telomere length and augmentation index, while Raymond et al. (2015) reported no association between leukocyte telomere length and any other marker of arterial function (including augmentation index and augmentation pressure). Nevertheless, wave reflection was not reported in the prior study (Raymond et al., 2015).

In contrast to what we expected, our results showed a consistent, direct association between leukocyte telomere length and markers of wave reflection and augmented central pressure (augmentation pressure, central pulse pressure) in RA patients. These association remained significant, independent of traditional and arterial function specific potential confounders. This suggest that those with longer telomeres have a higher wave reflection and hence increased augmentation pressure and central pulse pressure. Previous studies in RA have shown that increased wave reflection is independently associated with atherosclerosis (Gunter et al., 2018). Furthermore, longer telomere lengths have also been paradoxically related to increased atherosclerosis in RA (Raymond et al., 2016) and lupus (Haque et al., 2013). Moreover, Gizard and colleagues (2011) reported that inflammatory stimuli increase telomerase activity in macrophages to prevent cellular senescence during atherosclerosis. Considering the strong relationship between wave reflection and atherosclerosis and the previous paradoxical relationship between telomere length and atherosclerosis, our findings of the association between longer telomere length and increased wave reflection support these previous paradoxical relationships between risk of sub-clinical cardiovascular disease.

These findings also support the paradoxical relationship between adiposity and telomere length as discussed in Section 5.3. Therefore, in line with previous suggestions, patients with increased conventional risk may recruit compensatory mechanisms that may increase 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 2016) and atherosclerosis (Haque et al., 2013; Raymond

et al., 2016). Alternative, it has previously been suggested that those patients with the most severe RA and possibly the shortest telomeres may not have visited our clinic, and hence were not included in the data set (Haque et al., 2013; Raymond et al., 2016). Indeed, our patients had only mild disease activity, as shown by a low median DAS28 and CDAI scores. Nevertheless, these controversial findings between telomere length and cardiovascular disease risk factors in patients exposed to chronic inflammation warrant further longitudinal studies.

In sensitivity analysis in white patients, the relationship between leukocyte telomere length and central pulse pressure remained significant, while wave reflection was no longer significant. Although these results further support the findings in all patients, previous studies in RA patients from black African descent have reported higher disease activity and comparable risk of target organ damage compared to white African patients (Solomon et al., 2010; Raymond et al., 2016). Indeed the paradoxical relationship between leukocyte telomere length and atherosclerosis was previously reported in black African, but not white African patients (Raymond et al., 2016). Taken together, these results support our previous findings that population origin strongly influence the relationship between leukocyte telomere length and sub-clinical cardiovascular disease risk in RA.

In sensitivity analysis in women, we found no association between leukocyte telomere length and any marker of arterial function. These results are in line with previous studies that have suggested that the relationship between leukocyte telomere length and arterial function are impacted by sex (Benetose et al., 2001 Eguchi et al., 2017). Similar to our results, Benetose et al. (2001) reported no relationship between leukocyte telomere length and pulse pressure in women, while Eguchi et al. (2017)

reported no association between leukocyte telomere length and augmentation index in women. Taken together these results confirm that the relationship between leukocyte telomere length and arterial function are impacted by sex.

5.5 Limitations of study

The present study has some limitations. The cross-sectional design of the study and the lack of a control group preclude the examination of causal relationships. Hence it may be possible that in fact the relationship between arterial function and telomere length may be spurious. Although relative leukocyte telomere length measured by real time PCR is a validated approach, measures of telomerase activity may have strengthened our findings. Furthermore, measures of leukocyte telomere length may not reflect telomere length in the aortic wall cells. However, evidence suggests that telomere lengths in different tissues are strongly correlated (Tabuko et al., 2002).

Sensitivity analysis was performed in white patients and females, which showed significantly different results from the overall cohort. Our study consists largely of white, female patients. As a result, we were not statistically powered to perform sensitivity analysis in men and other race groups.

5.6 Conclusion

In conclusion this study revealed a paradoxical, direct relationship between relative leukocyte telomere length and traditional risk factors for cardiovascular disease, specifically adiposity. This relationship was impacted by race and gender. Despite relatively low disease activity in the study population, increased disease activity was associated with shorter telomeres, but only in white African patients This relationship between disease activity and telomere length was not significant in black African

patients or in women. Longer leukocyte telomere length was also associated with increased wave reflection, central augmentation pressure and central pulse pressure. This suggests that longer telomeres are associated with increased risk for an increased workload on the heart and possible target organ damage. Our results and previous reports of paradoxical relationships between longer telomeres and a worse cardiovascular disease risk profile highlight the complex interaction between chronic exposure to high grade inflammation, cellular protection mechanisms and cardiovascular disease risk. Accumulating evidence suggests that in chronic inflammation, compensatory protective mechanisms are recruited in patients with adverse cardiovascular disease risk profiles. The exact mechanisms underlying these complex interactions require further investigation. The exact role of leukocyte telomere length in cardiovascular disease risk stratification in chronic inflammation should be investigated in longitudinal and mechanisms investigations.

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APPENDIX A

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Zidan Eman Salem et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1804101

NAME: (Principal Investigator)	Dr Zidan Eman Salem et al
DEPARTMENT:	Physiology Milpark Private Hospital and Rheumatology Division Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Telomere length and arterial stiffness in patients with rheumatoid arthritis
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	A Sub-study under Primary Study M170592 Prof P. Dessein et al
SUPERVISOR:	Prof A. Millen and Prof R. Brooksbank
APPROVED BY:	Professor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	08/06/2018

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **April** and will therefore be due in the month of **April** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

R14/49 Prof Patrick Dessein et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170592

NAME: (Principal Investigator)	Prof Patrick Dessein et al
DEPARTMENT:	School of Physiology Internal Medicine
PROJECT TITLE:	The Contribution of Interaction of Rhematoid Arthritis Diseas Activity with Metabolic Syndrome Features to Endothelial Dysfuntion, Arterial Stiffness and Athero-Sclerosis
DATE CONSIDERED:	31/05/2012 (Initial approval) 26/05/2017 (Recertified)
DECISION:	Approved unconditionally
CONDITIONS:	Renewal for the period May 2017 - May 2022 Previously M120562
SUPERVISOR:	Prof B Joffe
APPROVED BY:	Prof P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	12/06/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in May and will therefore be due in the month of May each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES