AN INVESTIGATION INTO THE RELATIONSHIPS BETWEEN TELOMERE LENGTH AND CARDIAC FUNCTION AND GEOMETRY

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DECLARATION

This thesis is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

I declare that this thesis is my own. Data that was not obtained by me includes the multiple gated equilibrium cardiac blood pool scintigraphic scans to determine left ventricular ejection fraction, as this had to be performed by a qualified professional. Furthermore, echocardiography data was obtained by a single experienced observer to negate inter-observer variation and ensure blinding to the designation of control and experimental groups during echocardiography measurements.

I certify that all human and animal studies in this thesis had approval from the Committee for Research in Human Studies and the Animal Ethics Screening Committee of the University of the Witwatersrand respectively.

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ABSTRACT

Telomere length is inversely associated with the presence of heart failure. However, it is uncertain whether a reduced telomere length has pathophysiological consequences in the heart or is merely an epiphenomenon. The relationship between telomere length and heart failure may be causal, or it may reflect an association with inflammation or with risk factors for coronary artery disease, in which case it would be specific to ischaemic heart disease. Therefore, the main aim of this thesis was to determine whether telomere attrition is a cause of heart failure or a consequence of ongoing processes (eg. cardiomyocyte apoptosis, senescence, or mitochondrial dysfunction) in heart failure. Hence, I investigated the relationships between telomere length and a non-ischaemic form of heart disease (excludes oxidative stress and inflammation associated with coronary artery disease and its risk factors) and between telomere length and measurements of cardiac remodelling and systolic function prior to the development of heart failure.

Hence, in my first study I evaluated associations between leukocyte telomere length and the presence or severity of a non-ischaemic form of heart failure, namely idiopathic dilated cardiomyopathy (IDC). Relative telomere length (log T/S), measured using a quantitative real-time polymerase chain reaction assay, was correlated with age in patients with IDC ($p=0.002$, $n=223$) and in control participants ($p=0.023$, $n=227$). However, no differences in leukocyte log T/S were noted between IDC patients and healthy controls both prior to ($p=0.16$) and after (log T/S: IDC=-0.22±0.022 vs. control=-0.22±0.024, $p=0.114$) adjustments for confounders. Moreover, with adjustments for age, sex, regular alcohol consumption and smoking, log T/S was not associated with echocardiographic left ventricular (LV) ejection fraction (EF) or LV end diastolic diameter (EDD) in patients with IDC. In conclusion, relative leukocyte telomere length is not associated with the presence of IDC or the severity of heart failure in IDC (as indexed by LVEF and LVEDD). These data suggest

that telomere attrition is unlikely to be a cause of IDC (non-ischaemic heart disease) or a consequence of heart failure in general.

To further explore whether telomere shortening is a cause of cardiac remodelling and systolic dysfunction, I investigated the relationships between telomere length and an animal model of alcoholic cardiomyopathy (a form of dilated cardiomyopathy). Sprague-Dawley (SD) rats received either drinking water with (ethanol: n=19) or without (control: n=19) 5% (v/v) ethanol *ad libitum*, for four months. Echocardiography was performed to determine LV dimensions and function *in vivo* and isolated perfused heart preparations (ethanol n=10, control n=10) were performed to obtain *ex vivo* load-independent measurements of LV chamber geometry and function. Ethanol consumption resulted in LV dilatation (LVEDD: ethanol=8.20±0.14mm vs. control=7.56±0.11mm, p=0.0014; the volume intercept at 0mmHg (V_0) of the LV end diastolic pressure-volume relationship: ethanol=0.40±0.03ml vs. control=0.31±0.02ml, p=0.020), and a reduced LV relative wall thickness (ethanol=0.50±0.08mm vs. control=0.60±0.09mm, p=0.0011); but no changes in LV systolic chamber function as indexed by endocardial fractional shortening (FS_{end}) or the slope of the LV systolic pressure-volume relation (end systolic elastance-E_{es}). Ethanol administration resulted in a marked increase in cardiomyocyte apoptosis (ethanol=0.85±0.13% vs. control=0.36±0.06%, p=0.0021), and % apoptosis was correlated with LVEDD (r=0.39, n=38, p=0.021) and V_0 (r=0.44, n=19, p=0.046). However, no differences in leukocyte, cardiac, liver, or kidney telomere length were noted between ethanol and control groups; and cardiac telomere length was not associated with indices of LV dilatation or apoptosis. In conclusion, chronic alcohol-induced cardiac remodelling and apoptosis in the absence of systolic dysfunction are independent of changes in telomere length.

Having shown in two studies that telomere attrition is unlikely to be a pathophysiological cause of heart failure, I then explored whether telomere shortening in

heart failure is due to processes such as β-adrenergic receptor activation and inflammation, which occur in heart failure. I first evaluated whether LV dilatation and systolic chamber dysfunction produced by chronic β-adrenergic receptor (β-AR) activation are associated with leukocyte or cardiac telomere shortening. Six months of daily injections of the β-AR agonist, isoproterenol (0.02 $mg.kg^{-1}.day^{-1}$) (ISO: n=16), or the saline vehicle (control: n=15) to SD rats, resulted in LV dilatation (LVEDD: ISO=9.18±0.60mm vs. control=8.41±0.90mm, p=0.010; V_0: ISO=0.45±0.06ml vs. control=0.37±0.08ml, p=0.03) and LV systolic chamber dysfunction (FS_{end}: ISO=39.88±5.18% vs. control=45.14±5.64%, p=0.01; E_{es}: ISO=608.60±175.40 vs. control=901.10±229.60, p=0.01). Although leukocyte log T/S decreased over six months in rats receiving either the β-AR agonist or the saline vehicle ($p<0.05$); neither cardiac (ISO=-0.10±0.14 vs. control=-0.15±0.12, p=0.35) nor leukocyte log T/S (ISO=-0.11±0.19 vs. control=-0.15±0.18, p=0.52) were altered by chronic β-AR activation. Moreover, cardiac telomere length was not associated with indices of LV dilatation or systolic chamber function. In conclusion, chronic β-AR activation that is sufficient to produce LV dilatation and systolic chamber dysfunction was not associated with alterations in leukocyte or cardiac telomere length. Hence, telomere shortening in chronic heart failure is unlikely to be attributed to chronic β-AR activation.

I then evaluated the impact of chronic inflammation on telomere length, LV remodelling and systolic function in an animal model. After 12 months of fortnightly intraperitoneal injections of $2.5x10^7$ *Staphylococcus aureus* cell walls (*S. aureus*: n=30) or saline vehicle (control: n=30) in SD rats, there were no differences in LV dimensions (EDD, V_0) or systolic chamber function (FS_{end}, E_{es}) between the two groups. In the control group no differences in plasma C-reactive protein concentrations (a marker of inflammation) were noted at 3 or 11 months compared to the baseline concentrations at 1 month. However, in the *S. aureus* group there was an increase in CRP concentrations at both 3 months (*S. aureus*: 3

months=435.40±89.95µl.ml^{-1} vs. 1 month=347.80±101.90µl.ml^{-1}, p<0.0001) and 11 months (*S. aureus*: 11 months=511.40±93.34µl.ml^{-1} vs. 1 month=347.80±101.90µl.ml^{-1}, p<0.0001), and CRP concentrations were increased in the *S. aureus* group compared to the control group at 11 months (*S. aureus*=511.40±93.30µl.ml^{-1} vs. control=391.20±122.10µl.ml^{-1}, p<0.01). Leukocyte log T/S (at 11 months) (*S. aureus* =-0.21±0.15 vs. control=-0.053±0.11, p = 0.002) and cardiac log T/S (*S. aureus*=-0.20±0.10 vs. control=-0.11±0.084, p= 0.0021) were reduced in the *S. aureus* group. However cardiac log T/S was not associated with LVEDD, V_0, FS_{end}, or E_{es}. In conclusion, a chronic inflammatory stimulus that produced reductions in leukocyte and cardiac telomere length did not induce LV dilatation and a reduction in systolic chamber function. Hence, the negative association between telomere length and heart failure may be due to the impact of inflammatory processes on telomere length rather than signifying a cause of heart failure.

Having shown that telomere length may be reduced as an epiphenomenon due to ongoing inflammatory processes with no pathophysiological consequences on cardiac geometry or function in rats, I attempted to validate these findings in humans. Hence, I evaluated the relationship between leukocyte telomere length, indices of inflammation, LV remodelling, and systolic function in a cross-sectional human study. Leukocyte telomere length was determined in 622 participants from a randomly selected community based cohort. There was a negative bivariate relationship between T/S ratio and age in the group (p<0.0001) and telomere length was reduced in participants that regularly consumed alcohol compared to those who did not (p=0.01). No relationships between log T/S and smoking, sex, BMI, or blood pressure were noted. However, there was a negative relationship between leukocyte telomere length and C-reactive protein concentrations after multivariate adjustments for confounders (r=-0.11; p=0.0039; n=662). There were no associations between log T/S and echocardiographic indices of LV dilatation (LVEDD) or systolic

dysfunction (FS_{end}, FS_{mid}) in the participants. In conclusion, leukocyte telomere length was inversely associated with indices of inflammation. However, leukocyte telomere length was not associated with indices of LV remodelling and systolic function. These findings are consistent with the previous findings that chronic inflammation in rats capable of producing a marked decrease in cardiac telomere length has no pathophysiological consequence on cardiac dimensions or systolic function. Hence, inflammation may be an important determinant of leukocyte telomere length in humans, but leukocyte telomere length is not a bio-marker of adverse cardiac geometry or function.

The main findings from my thesis are as follows; a reduced telomere length was not associated with IDC (a non-ischaemic form of heart disease) nor with a model of alcoholic cardiomyopathy, which demonstrates that reduced telomere length may not be associated with all types of cardiomyopathy. Although chronic β-AR activation and ethanol consumption induced cardiac remodelling and β-AR activation induced systolic dysfunction, cardiac telomere length was not altered. Moreover, in IDC, alcoholic cardiomyopathy, chronic β-AR stimulation, chronic inflammation, and in a random, community-based population a reduced telomere length was not associated with features of heart failure such as LV dilatation (LVEDD and V_0), reduced systolic chamber function (FS_{end} and E_{es}), reduced intrinsic myocardial function (FS_{mid} and E_{en}), and myocardial apoptosis. However, chronic inflammation resulted in reductions in leukocyte and cardiac telomere length in the absence of changes in cardiac geometry and systolic function. These data indicate that the pathophysiological processes of heart failure, namely LV dilatation and reduced systolic function, may be independent of changes in telomere length and that telomere length may only be reduced as an epiphenomenon due to inflammatory processes in heart failure.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

Publication - Raymond AR, Norton GR, Sareli P, Woodiwiss AJ, Brooksbank RL. Relationship between average leukocyte telomere length and the presence or severity of idiopathic dilated cardiomyopathy in black Africans. European Journal of Heart Failure 2013; 15:54-60.

Publication - Raymond AR, Hodson B, Woodiwiss AJ, Norton GR, Brooksbank RL.Telomere length and adrenergic-induced left ventricular dilatation and systolic chamber dysfunction in rats. European Journal of Applied Physiology 2013 (in press).

Publication under review (International Journal of Cardiology) - Raymond AR, Becker J, Woodiwiss AJ, Booysen HL, Norton GR, Brooksbank RL. Ethanol induced left ventricular dilatation and cardiomyocyte apoptosis precedes intrinsic myocardial systolic dysfunction.

Poster presentation **-** No association between leukocyte telomere length and left ventricular cavity size or function in idiopathic dilated cardiomyopathy. Wits Health Sciences Research Day and Postgraduate Expo (2010), shortlisted for best overall student poster.

Oral presentation **-** Chronic alcohol consumption causes cardiac dilation in rats. Wits Health Sciences Research Day and Postgraduate Expo (2012).

Oral presentation **-** Chronic alcohol consumption causes cardiac dilation in rats. Physiology Society of Southern Africa (2012), Second place in the Wyndham competition (competition for best overall oral presentation).

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7,8-Dihydro-8-oxogaunine	8-oxodG
β-adrenergic receptor	β-AR
Aortic constriction	AOC
Ataxia-telangiectasia- and Rad3-related	ATR
Ataxia-telangiectasia mutated	ATM
Butylated hydroxytoluene	BHT
Cell division cycle 25	Cdc25
Checkpoint kinase 1	Chk 1
Checkpoint kinase 2	Chk 2
Confidence interval	CI
Correlation coefficient	r
C-reactive protein	CRP
Developed pressure-volume	dP-V
Distrene xylene/dibutyl phthalate xylene	DPX
Echocardiography	ECHO
End systolic elastance	E_{es}
Endocardial fractional shortening	FS_{end}
Enzyme linked immuno-adsorbent assay	ELISA
glycated haemoglobin	HbA1c
Idiopathic dilated cardiomyopathy	IDC
Interleukin-1β	IL-1β
Interleukin-6	IL-6
Intraperitoneal	i.p.
Isoproterenol	ISO
Left ventricular ejection fraction	LVEF
Left ventricular end diastolic diameter	LVEDD
Left ventricular mass	V_m
Left ventricular end systolic diameter	LV ESD
Left ventricular	LV
Midwall fractional shortening	FS_{mid}
New York Heart Association	NYHA

Occlusion cuff	O-cuff
Peroxisome proliferator-activated receptor gamma	PPAR-γ
Phosphate buffered saline	PBS
Posterior wall thickness at end-diastole	PWTED
Posterior wall thickness at end-systole	PWTES
Pressure	P
Pressure-volume	P-V
Qualitative fluorescence *in situ* hybridisation	Q-FISH
Quantitative real-time polymerase chain reaction	qRT-PCR
Radionuclide multiple-gated acquisition	MUGA
Reactive oxygen species	ROS
Recombinant terminal deoxynucleotidyl transferase	rTdT
Relative telomere length	T/S
Relative wall thickness	RWT
Reverse transcriptase component	Tert
RNA Component	Terc
rTdT mediated dUTP Nick-End Labeling	TUNEL
Standard error of mean	SEM
Single copy gene repeat number	S
Solid phase extraction	SPE
Sprague-Dawley	SD
Standard deviation	SD
Systolic myocardial elastance	E_{en}
Telomerase reverse transcriptase knockout	Terc $^{-/-}$
Telomere repeat binding factor 1	TRF1
Telomere repeat binding factor 2	TRF2
Telomere repeat copy number	T
Telomere-loop	t-loop
Terminal restriction fragment	TRF
Threshold crossing point	Ct
Tumour necrosis factor-alpha	TNF-α
Volume	V
Volume intercept at 0 mmHg	V_0
Volume pressure recording	VPR

CHAPTER 1 - INTRODUCTION

1.1 OVERVIEW

It is predicted that by the year 2020 cardiovascular disease will be responsible for approximately 25 million deaths per year and will be a leading cause of death worldwide (Yusuf *et al.*, 2001). One of the reasons for the increased prevalence in cardiovascular disease is the ongoing epidemiological shifts, especially in developing countries (Levenson *et al.*, 2002). These epidemiological shifts affect improvements in healthcare, which subsequently decrease the number of deaths due to infectious diseases and malnutrition and increase the prevalence of age and lifestyle associated diseases such as cardiovascular diseases (Levenson *et al.*, 2002). In order to treat and manage cardiovascular diseases a comprehensive understanding of the risk factors associated with cardiovascular disease is required.

A prominent risk factor for heart failure is advancing age. Heart failure can be defined as an inability of the heart to pump sufficient blood to meet the body's metabolic requirements (Mann, 1999). Despite a good characterisation of risk factors for cardiovascular diseases, the age of onset and disease progression are still highly variable between individuals (Wong[b] *et al.*, 2010). The variations in the onset and progression of heart failure may be due to discrepancies between chronological and biological age (Huzen *et al.*, 2010). Chronological age is merely the number of years from birth, whereas biological age represents the cumulative effect of various stresses on tissues throughout life. It has been proposed that telomere length could serve as a possible marker of biological age (Zglinicki and Martin-Ruiz, 2005).

Telomeres are specialized structures that cap the end of chromosomes whose main function is to maintain chromosomal integrity. As telomere length decreases progressively with each cell division; telomere length is thought to represent the replication history of the cell (Aubert and Lansdorp, 2008), and by inference the age of the cell. Moreover, telomere

length is also influenced by important features associated with the pathophysiology of diseases, such as oxidative stress and inflammation (Houben *et al.*, 2008; Zglinicki and Martin-Ruiz, 2005). Hence, telomere length is a possible biomarker of aging. Further credence to the tenet of telomere length as a marker of biological age can be found in human disorders of premature aging. In this regard, telomere length has been associated with disorders such as Werner syndrome (Schulz *et al.*, 1996), Bloom syndrome (Yankiwski *et al.*, 2000), Hutchinson-Gilford progeria (Allsopp *et al.*, 1992), Down syndrome (Vaziri *et al.*, 1993), and dyskeratosis congenita (Mitchell *et al.*, 1999) in which age related pathologies such as; atherosclerosis, osteoporosis, cataracts, and immune deficiency occur. The association between telomere length and human disorders of aging demonstrates a link between telomere length and the biological changes associated with the aging phenotype. This is consistent with the notion of telomere length as a bio-marker of aging that provides a surrogate marker of the cumulative effects of various stresses on the body throughout life.

Given the importance of advancing age in cardiovascular disease, it has emerged that telomere biology could play a role as a risk factor in the pathophysiology of cardiovascular diseases. In this regard, cross-sectional studies have shown that telomere length is inversely associated with heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012). These associations may indicate that reduced telomere length is either a causal factor or alternatively an epiphenomenon of heart failure. Hence, the main aim of my thesis was to further investigate the relationships between telomere length and cardiac remodelling, systolic function, and heart failure to determine whether telomere attrition is a cause of heart failure or a consequence of ongoing processes in heart failure. As an introduction to my thesis the sections to follow will elaborate on the structure and function of telomeres and outline the evidence demonstrating a link between telomere biology and cardiovascular disease, which

encompasses; diseases of the heart and blood vessels, adverse cardiac remodelling that precedes heart failure, and heart failure itself.

1.2 WHAT ARE TELOMERES?

Telomeres consist of non-coding repeats of 5'-TTAGGG-3'/5'-CCCTAA-3' located at the end of chromosomes. In humans the telomere sequence can extend from 5 to 15 000 base pairs (Aubert and Lansdorp, 2008). Telomeres function to protect against chromosomal degradation, fusion and the recognition of chromosomal ends as double-stranded breaks (Aubert and Lansdorp, 2008). Telomere length shortens progressively with each cell division (Aubert and Lansdorp, 2008), and when telomere length reaches a threshold level, cellular senescence and apoptotic pathways are activated (Aubert and Lansdorp, 2008; Artandi andAttardi, 2005). Hence, telomere length is important in maintaining normal cellular activity.

Telomere length is maintained by the enzyme telomerase, which consists of an RNA component (Terc) and a reverse transcriptase component (Tert). The Terc component of telomerase provides an RNA template for the Tert enzyme, which then facilitates the addition of new telomeric sequences from the RNA template and hence the elongation of telomeres (Aubert and Lansdorp, 2008). In addition to telomerase, two important proteins associated with telomeres are telomere repeat binding factors (TRF) 1 and 2 (Aubert and Lansdorp, 2008; Artandi and Attardi, 2005). TRF1 regulates access of telomerase to the telomere complex and thus is crucial in the regulation of telomere length (Artandi and Attardi, 2005). TRF2 is responsible for maintaining the protective ends of telomeres and is important in the suppression of DNA damage pathways (Artandi and Attardi, 2005). In this regard, telomeres

are characterised by a single stranded guanine rich 3' overhang that folds back on itself to form a protective structure known as the telomere-loop (t-loop) (FIGURE1.1).

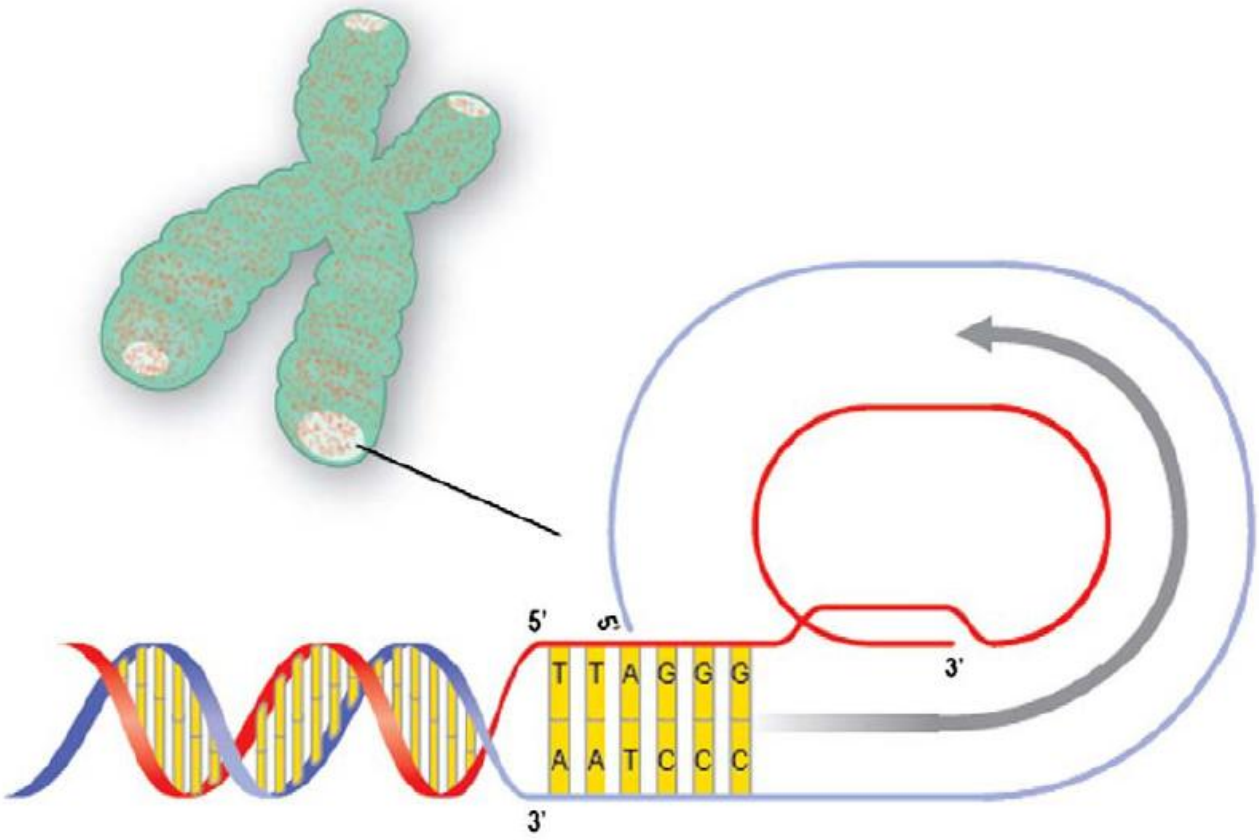

FIGURE 1.1 Representation of the telomere sequence found at the end of the chromosome with the guanine rich overhang at the 3' end, which forms the protective telomere-loop structure (Wong *et al.*, 2008).

This protective t-loop, which is maintained by TRF2, prevents the recognition of DNA ends as breaks and in doing so prevents the activation of cellular senescence and apoptotic pathways (Aubert and Lansdorp, 2008; Artandi and Attardi, 2005).

The maintenance of telomere length is crucial in the prevention of cellular senescence and activation of apoptotic pathways (FIGURE1.2) (Artandi and Attardi, 2005; Wong *et al.*, 2009). Senescence is characterised by full cessation of normal cellular activity and apoptosis is a mechanism of regulated cellular death. The activation of these pathways is associated with ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia- and Rad3-related (ATR) kinases. These kinases (ATM/ATR) phosphorylate DNA checkpoint kinase (Chk) 1 and 2 (Artandi and Attardi, 2005; Wong *et al.*, 2009). Chk 1 and 2 activation lead to senescence via the inhibition cell division cycle 25 (Cdc25). Furthermore, Chk 1 and 2 phosphorylate p53, which induces senescence via increased p21 expression, which is linked to p16 whose mechanism of increased expression during DNA damage signalling is currently unknown (Artandi and Attardi, 2005; Wong *et al.*, 2009). In terms of apoptosis, p53 activation results in the increased expression of genes involved in apoptosis such as; *bax*, *noxa*, *Perp*, and *puma*. The driving factors that result in either senescence or apoptosis once telomere induced DNA damage signaling has been activated are currently unknown (Artandi and Attardi, 2005; Wong *et al.*, 2009).

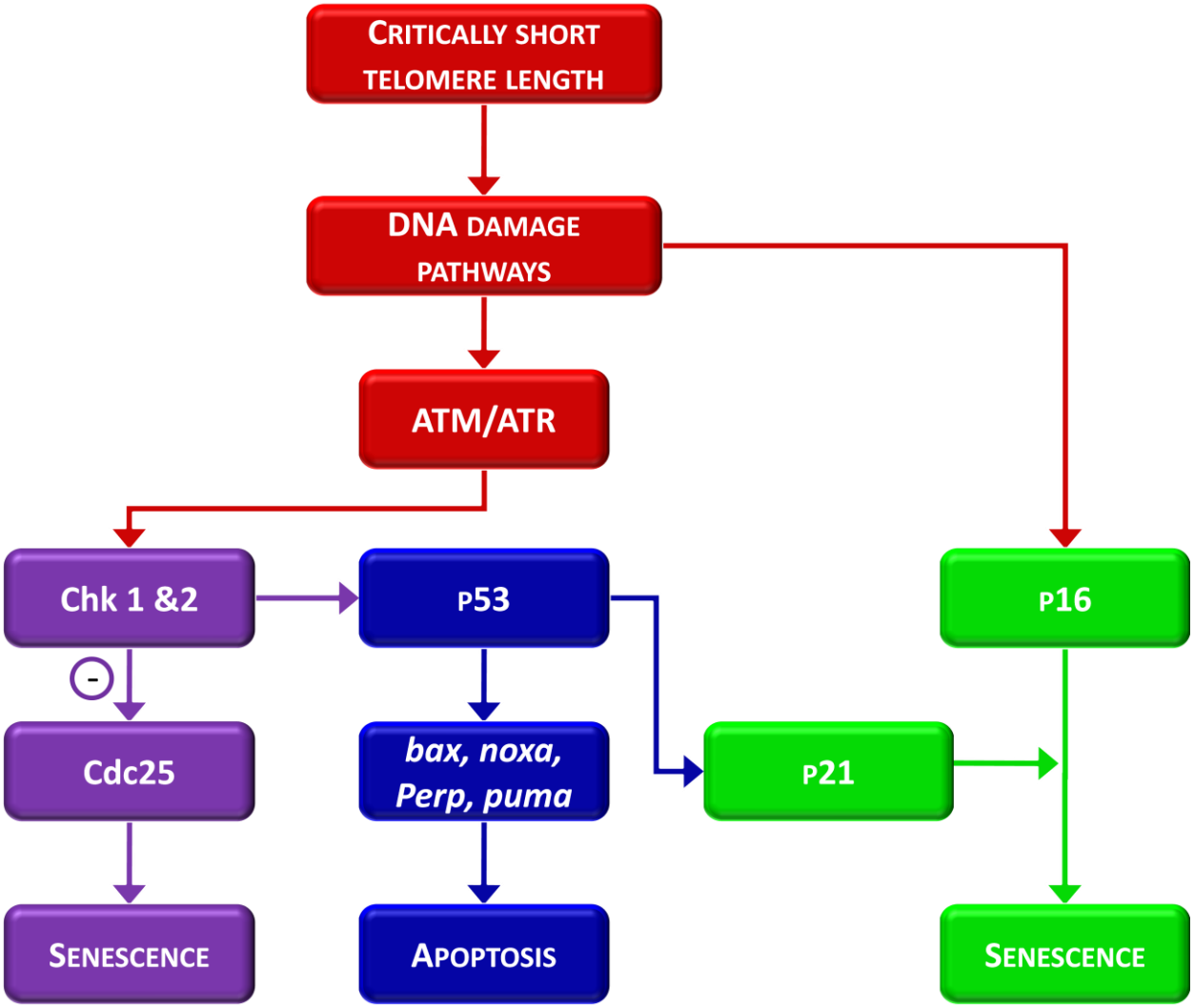

FIGURE 1.2 Diagram representing the pathway of telomere shortening resulting in the activation of cellular senescence and apoptosis. ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia - and Rad3 - related kinases; Chk, DNA checkpoint kinase; Cdc25, cell division cycle 25; ⊖ denotes an inhibitory pathway. (Figure modified from Artandi and Attardi, 2005 and Wong *et al.*, 2009).

As telomere biology plays a crucial role in maintaining genomic stability via the regulation of apoptotic pathways and cellular senescence, it is reasonable to expect that telomeres may have an important role in the pathophysiology of diseases associated with aging such as cardiovascular diseases. In this regard, the evidence demonstrating a relationship between telomere biology and cardiovascular disease, which includes diseases of the heart and blood vessels, adverse cardiac remodelling, and heart failure, will be discussed in the sections to follow.

1.3 WHAT IS THE EVIDENCE TO INDICATE A RELATIONSHIP BETWEEN TELOMERE LENGTH AND CARDIOVASCULAR DISEASE?

Studies conducted on cardiac tissue have provided evidence that telomere length may be associated with cardiovascular disease (TABLE 1.1). It has been demonstrated in the 5th generation offspring of Terc knockout mice that telomere length was reduced in cardiomyocytes. Later generations of these Terc knockout mice presented with cardiac characteristics similar to that of a dilated cardiomyopathy in humans (Leri *et al*., 2003). Moreover, cardiomyocyte replication was reduced and accompanied by an increased expression of p53 and apoptosis in these animals. The data from Terc knockout mice suggest that critically short telomere length could have a role in the pathophysiological processes present in heart failure. In this regard, in a model of cardiac hypertrophy caused by aortic constriction in rats, telomere length was reduced, apoptosis was increased, p53 expression was up-regulated, and TRF2 was down-regulated (Sheng *et al.*, 2013). In diseased human hearts, it was found that telomere length in cardiomyocytes was reduced and was associated with an increased expression of p16 and senescent cardiomyocytes.

TABLE 1.1 Studies demonstrating a link between telomere length and cardiovascular disease.

Main findings	Cell type	Human/animal	Methodology	Reference
TL was reduced and resulted in a dilated cardiomyopathy	Cardiomyocyte	Terc ¯/¯ mice	Q-FISH	Leri *et al.*,2003
Pressure overload increased TL attrition	Cardiomyocyte	AOC rat	TRF	Sheng *et al.*, 2013
TL was reduced in diseased hearts	Cardiomyocyte	Human	Q-FISH	Chimenti *et al.*, 2003
TL reduced in heart failure (with reduced TRF2 and Chk2 activation)	Cardiomyocyte	Human	TRF	Oh *et al.*, 2003
Severe cardiac loading reduced TL and TRF2	Cardiomyocyte	Mice	TRF	Oh *et al.*, 2003
TL reduced in degenerative aortic valve stenosis	Leukocyte	Human	qRT-PCR	Kurz *et al.*, 2006
TL is reduced in patients with heart failure	Leukocyte	Human	qRT-PCR	Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012
Reduced TL in participants with coronary artery disease	Endothelial, leukocyte	Human	Slot blot, qRT-PCR	Ogami *et al.*, 2004; Brouilette *et al.*, 2007; Brouilette *et al.*, 2003
Reduced TL associated with increased risk for myocardial infarction	Leukocyte	Human	TRF, qRT-PCR	Zee *et al.*, 2009; Weischer *et al.*, 2012 Brouilette *et al.*, 2008; Fitzpatrick *et al.*, 2007
Offspring from parents who suffered cardiac events inherited shorter TL	Leukocyte	Human	TRF, qRT-PCR	Wong *et al.*, 2011

TL, telomere length; Terc ¯/¯, telomerase reverse transcriptase knockout; TRF2, telomere repeat binding factor 2; Chk 2, DNA checkpoint kinase 2; AOC, aortic constriction; Q-FISH, qualitative fluorescence *in situ* hybridisation; TRF, terminal restriction fragment; qRT-PCR, quantitative real time polymerase reverse chain reaction.

Furthermore, the cardiomyocytes with reduced telomere length were also associated with apoptosis (Chimenti *et al*., 2003). Similarly, Oh *et al.* (2003) demonstrated that reduced telomere length and apoptosis in cardiomyocytes was present in heart failure. Oh *et al.* (2003) discovered that this reduction in telomere length was also accompanied by a decreased expression of TRF2, which is consistent with the results from the aortic constriction rat model conducted by Sheng *et al.* (2013). Further investigations by Oh *et al.* (2003) showed that reductions in TRF2 expression could be a determining factor behind telomere attrition and cardiomyocyte apoptosis in diseased hearts.

In summary, these data suggest that there is an association between reduced telomere length and dysfunctional cardiac tissue. Importantly, the reductions in cardiomyocyte telomere length are associated with adverse changes in cardiomyocytes such as senescence (Chimenti *et al*., 2003) and apoptosis (Chimenti *et al*., 2003; Oh *et al.*, 2003; Leri *et al*., 2003; Sheng *et al.*, 2011), which are pathways known to be regulated by telomere length (Artandi and Attardi, 2005). Furthermore, the Terc knockout model (Leri *et al*., 2003) provides evidence to suggest that reduced telomere length has a causal role in heart failure. In this regard, later generations of these mice with the shortest cardiomyocyte telomeres developed heart failure. However, the studies linking cardiomyocyte telomere length to diseased heart tissue are limited. Moreover, the two investigations conducted by Chimenti *et al*. (2003) and Oh *et al.* (2003) that have investigated human heart tissue samples have limited sample sizes of 19 and 14 respectively. Thus, in order to firmly establish a link between telomere length and cardiovascular disease in humans, further evidence is required and is discussed in the sections to follow.

1.3.1 IS TELOMERE LENGTH ASSOCIATED WITH THE PRESENCE OF CARDIOVASCULAR DISEASE IN HUMANS?

Supporting evidence for a negative relationship between telomere length and cardiovascular disease in humans has been demonstrated by numerous large cross-sectional studies (TABLE 1.1), that have found an association between reduced leukocyte telomere length and cardiovascular disease. In this regard, leukocyte telomere length is negatively associated with ventricular function in the elderly (Collerton *et al.*, 2007); is reduced in patients with degenerative aortic valve stenosis compared to healthy participants (Kurz *et al.*, 2006); is associated with increased risk for myocardial infarctions (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Weischer *et al.*, 2012; Zee *et al.*, 2009); and is reduced in patients with heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012). Moreover, it has been demonstrated that leukocyte telomere length is associated with the severity of heart failure (van der Harst *et al.*, 2007).

There is therefore significant evidence demonstrating that reduced leukocyte telomere length is associated with the presence of cardiovascular disease (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Kurz *et al.*, 2006; Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012; Zee *et al.*, 2009). However, these studies fail to elucidate the cause and effect relationships between reduced leukocyte telomere length and heart failure. From the available data it is uncertain if reduced telomere length is a causal factor heralding the onset of heart failure. In this regard, the relationships between leukocyte telomere length and cardiovascular disease have only been determined using outcome based studies and not using measurements of cardiac function. Although Collerton *et al.* (2007) did show that leukocyte telomere length was negatively associated with left ventricular ejection fraction, an important consideration is that this study consisted of individuals older than 85 years. Given the strong negative relationship between telomere length and age and between ejection fraction and age

the association between telomere length and ejection fraction in this study is to be expected. Hence, telomere length may have no impact on cardiac structure and function and may merely be reduced as a consequence of the pathophysiological processes of cardiovascular diseases. Furthermore, there are numerous risk factors for cardiovascular disease that could also contribute to the increased telomere attrition evident in cardiovascular disease.

1.3.2 IS TELOMERE LENGTH ASSOCIATED WITH RISK FACTORS FOR CARDIOVASCULAR DISEASE IN HUMANS?

Negative associations between leukocyte telomere length and numerous risk factors for cardiovascular disease have been found. These factors include; coronary artery disease (Brouilette *et al.*, 2007; Ogami *et al.*, 2004), homocysteine concentrations (Richards *et al.*, 2008), intima-media thickness (O'Donnell *et al.*, 2008), physical fitness (Krauss *et al.*, 2011), hypertension (Fuster *et al.*, 2007; Weischer *et al.*, 2012), atherosclerosis (Huzen *et al.*, 2011; Samani *et al.*, 2001), anaemia (Wong *et al.*, 2010), left ventricular mass (Kuznetsova *et al.*, 2010; Vasan *et al.*, 2009), type 2 diabetes mellitus (Salpea *et al.*, 2010; Weischer *et al.*, 2012), obesity (Valdes *et al.*, 2005; Weischer *et al.*, 2012), smoking (Valdes *et al.*, 2005; Weischer *et al.*, 2012), total cholesterol, high-density lipoprotein cholesterol, and C-reactive protein concentrations (Weischer *et al.*, 2012). Moreover, reduced leukocyte telomere length has also been associated with a family history of ischaemic heart disease (Brouilette *et al.*, 2008; Wong *et al.*, 2011). It is well established that a previous family history of cardiovascular disease is a risk factor for heart failure. In this regard, the healthy offspring of individuals with cardiovascular disease had reduced telomere length compared to age-matched controls whose parents did not suffer from cardiovascular disease (Brouilette *et al.*, 2008; Wong *et al.*, 2011). These results demonstrate an inheritance of shorter telomere length

from parents with ischaemic heart disease and by inference a possible inherited risk for ischaemic heart disease in these individuals (Brouilette *et al.*, 2008; Wong *et al.*, 2011).

It should be noted that there are numerous inconsistencies within the literature regarding associations between leukocyte telomere length and risk factors for cardiovascular disease (Bekaert *et al.*, 2007). Some studies have demonstrated associations whereas others have not. In this regard, differences in methodologies and study populations need to be taken into consideration (Bekaert *et al.*, 2007). The measurement of mean telomere length is traditionally determined via Southern blot analysis (Cawthon, 2002; O'Donnell *et al.*, 2008; Richards *et al.*, 2008; Samani *et al.*, 2001; Valdes *et al.*, 2005; Vasan *et al.*, 2009). However, this protocol is labour intensive and has a low throughput and hence Southern blotting is not an ideal methodolgy for large-scale epidemiological studies (Cawthon, 2002). In this regard, Cawthon (2002) developed a high throughput quantitative real-time polymerase chain reaction (qRT-PCR) protocol that determines relative telomere length. This real-time 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 (Brouilette *et al.*, 2007; Cawthon, 2002). Modified versions of the qRT-PCR protocol developed by Cawthon have been adopted in many investigations between telomere length and risk factors for cardiovascular disease (Brouilette *et al.*, 2007; Brouilette *et al.*, 2008; Huzen *et al.*, 2011; Krauss *et al.*, 2011; Kuznetsova *et al.*, 2010; Salpea *et al.*, 2010; Weischer *et al.*, 2012; Wong *et al.*, 2010; Wong *et al.*, 2011). Hence, differences in the approach taken to measure leukocyte telomere length could account for the inconsistencies within the literature regarding associations between leukocyte telomere length and risk factors for cardiovascular disease. However, it is more likely that differences in the age range and the characteristics of the cohorts under investigation that could account for these inconsistencies.

The association between telomere length and both cardiovascular disease and risk factors for cardiovascular diseases further complicates the cause and effect relationships between reduced telomere length and cardiovascular disease. In this regard, it is uncertain if reduced telomere length is a causal factor heralding the onset of heart failure or if telomere length is reduced as a consequence of the pathophysiological processes of heart failure. Furthermore, the relationships between cardiovascular disease and reduced leukocyte telomere length may represent an epiphenomenon attributed to the relationship between risk factors for cardiovascular disease and increased leukocyte telomere attrition. Hence, currently, the relative importance of risk factors for cardiovascular disease with regards to the relationship between telomere and heart failure is uncertain and requires further investigation. It is possible that reduced telomere length is independent of the pathophysiological processes of heart failure and is merely a consequence of the risk factors for heart failure.

1.3.3 IS REDUCED TELOMERE LENGTH AN INDEPENDENT RISK FACTOR FOR HEART FAILURE?

With regards to the cause and effect relationship between telomere length and heart failure, prospective data indicate that reduced leukocyte telomere length may be an independent risk factor for heart failure. In prospective and case-control studies, leukocyte telomere length was determined in clinically stable participants and cardiovascular outcomes were determined through follow up or hospital records (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Weischer *et al.*, 2012; Zee *et al.*, 2009). These studies found that individuals with reduced leukocyte telomere length had a greater risk for developing a myocardial infarction (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Weischer *et al.*, 2012; Zee *et al.*, 2009). Furthermore, these data demonstrate that reduced telomere length infers risk for developing

ischaemic heart disease and that a reduced telomere length is not merely an epiphenomenon attributed to the pathophysiological processes of heart failure.

However, it has to be noted that in the case-control studies (Brouilette *et al.*, 2003; Zee *et al.*, 2009) the cases had a greater prevalence of risk factors for cardiovascular disease compared to the control groups. Even though these risk factors were adjusted for, in the respective multivariate regression analyses, it follows that individuals with a higher prevalence of risk factors for cardiovascular disease subsequently suffer cardiac events. Hence, these data are insufficient to conclusively determine if reduced telomere length is a cause of heart failure and is independent of other risk factors for cardiovascular disease. Nevertheless, these data do indicate that reduced leukocyte telomere length is associated with an increased risk for ischaemic heart disease.

1.3.4 IS TELOMERE LENGTH ASSOCIATED WITH NON-ISCHAEMIC FORMS OF HEART FAILURE?

The association between reduced telomere length and increased risk for myocardial infarction raises the question of whether telomere length is also associated with non-ischaemic forms of heart failure. Although reduced leukocyte telomere length has been found in patients in heart failure compared to healthy controls (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012), this data is primarily in ischaemic forms of heart failure. Van der Harst *et al.* (2007) did however demonstrated that leukocyte telomere length was reduced in patients with non-ischaemic heart disease. However, a high proportion of these patients with non-ischaemic heart disease had a history of stroke, peripheral arterial disease, angina pectoris, and the cause of heart failure in the remaining patients was not described (Van der Harst *et al.*, 2007). Furthermore, Starr *et al.*(2007) demonstrated that reduced

telomere length was associated with ischaemic changes on electrocardiograms while the same associations were absent with non-ischaemic disease manifestations.

There is currently no data available to suggest that reductions in telomere length are associated with non-ischaemic forms of heart failure. A possible explanation for the strong association between leukocyte telomere length and ischaemic forms of heart failure may be due to the confounding effects of risk factors that predispose individuals to ischaemic heart disease. In this regard, reduced telomere length has been associated with many risk factors for ischaemic heart disease, which include; increased intima-media thickness (O'Donnell *et al.*, 2008), hypertension (Fuster*et al.*, 2007; Weischer *et al.*, 2012), atherosclerosis (Huzen *et al.*, 2011; Samani *et al.*, 2001), increased left ventricular mass (Kuznetsova *et al.*, 2010; Vasan *et al.*, 2009), type 2 diabetes mellitus (Salpea *et al.*, 2010; Weischer *et al.*, 2012), obesity (Valdes *et al.*, 2005; Weischer *et al.*, 2012), smoking (Valdes *et al.*, 2005; Weischer *et al.*, 2012), increased total cholesterol, and decreased high-density lipoprotein cholesterol (Weischer *et al.*, 2012). Hence, reduced leukocyte telomere length evident inischaemic heart disease may represent an epiphenomenon due to the presence of risk factors for cardiovascular disease. Therefore, it is important that non-ischaemic forms of heart failure are also investigated to determine if the relationship between telomere length and heart failure holds true for all types of heart failure. Furthermore, this will allow for the elucidation of whether the relationship between reduced leukocyte telomere length and heart failure is due to the associations between telomere length and risk factors for ischaemic heart disease or is an independent risk factor for heart failure.

1.3.5 THE IMPLICATIONS OF LONGITUDINAL LEUKOCYTE DATA ON TELOMERE LENGTH RESEARCH IN CARDIOVASCULAR DISEASE.

A major assumption with regards to telomere research to date is that leukocyte telomere length decreases progressively with age. This notion is supported by all of the cross-sectional studies cited previously, which demonstrate leukocyte telomere length is negatively associated with age. If this assumption holds true, then leukocyte telomere length could then be utilised, as marker of biological age representing the cumulative effects of stress on the body, to identify individuals with increased risk for future cardiac events. This hypothesis is supported by prospective data, which have demonstrated that reduced leukocyte telomere length is associated with an increased risk for ischaemic heart disease, namely myocardial infarction (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Weischer *et al.*, 2012; Zee *et al.*, 2009).

However, whether or not telomere length in an individual decreases in a predictable manner over time can only be determined using longitudinal data. In this regard, in a study consisting of 959 participants with a follow up interval of approximately ten years, although most participants showed a decline in leukocyte telomere length (Nordfjäll *et al.*, 2009), one third of this cohort showed either no change or increased leukocyte telomere length over time. Similarly, Aviv *et al.* (2009) found that, in 635 individuals with a mean follow up period of four and a half years, leukocyte telomere length either decreased or increased in individuals. These longitudinal data indicate that leukocyte telomere length is variable and may increase, remain the same or decrease in length over time. The high temporal variation in leukocyte telomere length (Aviv *et al.*, 2009; Nordfjäll *et al.*, 2009), suggests that the use of leukocyte telomere length as a possible predictor of risk for heart failure is unlikely as leukocyte telomere attrition may be specific to each individual (Aviv *et al.*, 2009; Nordfjäll *et al.*, 2009).

These data obtained from Nordfjäll*et al.*(2009) and Aviv *et al.* (2009) highlight the importance of investigating telomere length longitudinally. Currently, with regards to heart failure, there is no investigation that has assessed telomere length longitudinally.

In addition to the high temporal variation in leukocyte telomere length, telomere length of leukocytes has been used as a surrogate marker of telomere length of cardiovascular tissues in all of the cross-sectional human studies investigating telomere length and cardiovascular disease to date. Support for the use of leukocyte telomere as surrogate marker of telomere length in other tissues emanates from a study conducted by Friedrich *et al.* (2000). This investigation found that leukocyte telomere length was correlated with telomere length in skin and synovial fluid samples. On the basis of these findings it was concluded that leukocyte telomere length could serve as a surrogate marker of telomere length in other tissues (Friedrich *et al.*, 2000). The major limitation of this study relates to the small sample size (only nine participants). Furthermore, the age range of the participants was narrow (22 years) and represented only an elderly cohort (individuals between the ages of 73 to 95 years). The bias of this sample towards an elderly population and the small sample size raise doubts as to whether these relationships between leukocyte telomere length and telomere length in other tissues would be present in the general population. Moreover, with regards to the implications of research involving telomere length and heart failure, this study conducted by Friedrich *et al.* (2000) did not correlate leukocyte telomere length to any tissue type related to the cardiovascular system. This raises concerns for the validity of using leukocyte telomere length as a surrogate marker in epidemiological studies investigating telomere length and cardiovascular disease.

Nevertheless, subsequent supporting evidence for the use of leukocyte telomere length as a surrogate marker of telomere length in cardiovascular tissue has been demonstrated. Wilson *et al.* (2008) showed that leukocyte telomere length was correlated

with telomere length in samples obtained from the abdominal aorta. However, as with the study conducted by Friedrich *et al.*(2000), this study is also hindered by a small sample size (n=32) and an older cohort with ages ranging from 56 to 78 years. However, despite the small sample size, a very strong positive association between leukocyte and aortic telomere length was found ($r=0.62$, $p<0.001$). Furthermore, Huzen *et al.* (2011) found a positive association between leukocyte telomere length and telomere length of carotid artery atherosclerotic plaques. The sample size of this study was large and therefore the issues of statistical power with previous studies were not a factor. However, an important finding from the study of Huzen *et al*. (2011) was that leukocyte telomere length and plaque telomere length were correlated with different aspects of plaque histology. Importantly, these findings highlight that leukocyte telomere length may correlate with telomere length in cardiovascular tissues but may not represent the overall diseased state of the tissue. Moreover, plaque telomere length was not correlated with age suggesting that plaque telomere length may not decrease at the same rate as leukocyte telomere length, which was associated with age in this study. Further evidence that telomere attrition may proceed at different rates over time in different cell types is demonstrated by O'Sullivan *et al.* (2006), who showed that telomere length in leukocytes decreased with age but the same association was absent in stromal cells of colonocytes. These data indicate that attrition of telomere length may proceed at different rates in different tissues.

Hence, although there is significant evidence demonstrating that reduced leukocyte telomere length is associated with heart failure and some evidence that leukocyte telomere length is correlated with telomere length in tissues related to the cardiovascular system; leukocyte telomere length may not be representative of the diseased state of the tissue in question. The measurement of leukocyte telomere length, or another easily accessible tissue, is however fundamental for future epidemiological studies investigating telomere length and

cardiovascular disease. Nevertheless, whether leukocyte telomere length can be used as a surrogate marker of telomere length in heart tissue in healthy or diseased states has yet to be established.

1.3.6 Overview of evidence investigating telomere length and heart failure.

The main findings with regards to telomere length and cardiovascular disease are as follows: reduced telomere length has been demonstrated in cardiomyocytes of failing hearts and is associated with cardiomyocyte apoptosis and senescence (Chimenti *et al.*, 2003; Leri *et al*., 2003; Oh *et al.*, 2003; Sheng *et al.*, 2013). These data suggest that reduced telomere length may be associated with adverse cardiomyocyte changes in diseased hearts. These findings, have been supported by large cross-sectional studies in humans. In this regard, reduced leukocyte telomere length is associated with degenerative aortic valve stenosis (Kurz *et al.*, 2006), increased risk for myocardial infarctions (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Weischer *et al.*, 2012; Zee *et al.*, 2009); and is reduced in patients with heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012). Importantly, leukocyte telomere length is also associated with the severity of heart failure (van der Harst *et al.*, 2007) and indicates that telomere length may be associated with the pathophysiological processes that worsen heart failure. However, the major caveat with regards to the literature regarding telomere length and human heart failure is the inherit inability of cross-sectional data to elucidate cause and effect relationships. Nevertheless, there is significant data demonstrating that reduced telomere length is associated with cardiovascular disease and the sections to follow outline potential mechanisms that may explain the relationship between telomere length and heart failure specifically.

1.4 MECHANISMS LINKING TELOMERE LENGTH TO HEART FAILURE

The following sections will discuss the potential biological mechanisms that provide explanations for the relationship between reduced telomere length and heart failure. These features include; oxidative stress, inflammation, apoptosis, and mitochondrial dysfunction.

1.4.1 OXIDATIVE STRESS, HEART FAILURE AND TELOMERE LENGTH.

Heart failure is associated with increased oxidative stress (Ahmed and Tang, 2012; Giordano, 2005; Papaharalambus and Griendling, 2007; Singal *et al.*, 1998), which is the result of an imbalance between the production of reactive oxygen species (ROS) and the body's ability to eliminate ROS, resulting in a net increase in ROS and oxidative stress. In heart failure, ROS production has been shown to be derived from NADPH oxidases, xanthine oxidase, and uncoupling of the mitochondrial respiratory chain (Ahmed and Tang, 2012; Papaharalambus and Griendling, 2007). The most prominent of the oxidases, in terms of ROS production during heart failure, is NADPH oxidase. In the myocardium of failing hearts, NADPH oxidase-derived ROS are increased (Maack *et al.*, 2003). Moreover, NADPH oxidases have also been associated with increased angiotensin II, endothelin-1, oxidized low density lipoproteins, and adrenaline, which are all factors related to the pathophysiology of heart failure (Lassègue *et al.*, 2001; Seddon *et al.*, 2007; Wingler *et al.*, 2001). Furthermore, animal studies have demonstrated that NADPH oxidase causes adverse changes in the heart including angiotensin II-induced cardiac hypertrophy, cardiac fibrosis (Johar *et al.*, 2006), cardiomyocyte apoptosis, contractile dysfunction, andventricular remodelling (Bell *et al.*, 2005; Looi *et al.*, 2008).

Increases in oxidative stress are undoubtedly an important feature in the pathophysiology of heart failure (Ahmed and Tang, 2012; Giordano, 2005; Papaharalambus and Griendling, 2007; Singal *et al.*, 1998). Oxidative stress has also been found to have a negative influence on telomere length. Indeed, *in vitro* studies have demonstrated that oxidative stress results in increased telomere attrition (Harboa *et al.*, 2012; von Zglinicki *et al.*, 1995; von Zglinicki *et al.*, 2000). Cross-sectional data in humans are consistent with *in vitro* findings, with negative associations found between leukocyte telomere length and markers of oxidative stress (Bekaert *et al.*, 2007; Demissie *et al.*, 2006; Masi *et al.*, 2011; Watfa *et al.*, 2011; Wolkowitz *et al.*, 2011). In terms of *in vivo* data, Cattan *et al.* (2008) inhibited the production of glutathione (an important antioxidant) in mice and found that telomere length was reduced in some but not all tissues as a result of increased oxidative stress. In this regard, telomere length was reduced in the testes, brown fat, tail, white fat and skin (Cattan *et al.*, 2008). Importantly however, telomere length of heart tissue in the experimental group was not significantly different to that of the control group (Cattan *et al.*, 2008). While *in vitro* studies have illustrated that increased oxidative stress is associated with a decrease in telomere length, the data from Cattan *et al.* (2008) provide evidence to suggest that tissues may differ in their susceptibility to telomere length attrition caused by oxidative stress.

In terms of the mechanisms explaining oxidative stress induced telomere attrition, the predominant factor rendering telomeres susceptible to damage via oxidative stress is the high guanine content of telomeres (Houben *et al.*, 2008). The guanine content of telomeres is high in both the telomeric sequence itself (5'-TTAGGG-3') and in the single stranded guanine overhang on the 3' end that forms the protective t-loop structure (Oikawa and Kawanishi, 1999; Oikawa *et al.*, 2001; Sitte *et al.*, 1998). ROS interact with the guanine nucleotides to form 7,8-Dihydro-8-oxogaunine (8-oxodG) (Rhee *et al.*, 2011). The presence of 8-oxodG in

telomeric DNA has been found to disrupt telomerase function (Szalai *et al.*, 2002), which has a negative influence on the maintenance of telomere length. ROS have also been shown to promote the translocation of telomerase out of the nucleus and into the cytosol (Haendeler *et al.*, 2003). Furthermore, ROS also result in single strand breaks in DNA, and telomeres are less efficient, compared to other regions of DNA, in the repair of these single strand breaks (Petersen *et al.*, 1998). The inability of telomere regions to repair single strand breaks is possibly due to TRF2 inhibiting the access of DNA repair enzymes to telomeric regions (Ohki *et al.*, 2004; Richter *et al.*, 2007). Indeed, TRF2 has been implicated in impaired DNA repair responses (Fotiadou *et al.*, 2004; Karlseder *et al.*, 2004).

Hence, together these data indicate that oxidative stress-induced reductions in telomere length may explain the associations between reduced leukocyte telomere length in heart failure.

1.4.2 INFLAMMATION, HEART FAILURE AND TELOMERE LENGTH.

It has been established that inflammation plays a key role in the pathophysiology of heart failure (Yndestad *et al.*, 2006). Patients in heart failure have increased concentrations of inflammatory cytokines, such as tumour necrosis factor (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) (Adamopoulos *et al.*, 2001; Aukrust *et al.*, 1999; Levine *et al.*, 1990; Testa *et al.*, 1996; Yndestad *et al.*, 2006). Moreover, increases in these cytokines have been correlated with the severity of heart failure (Aukrust *et al.*, 1999; Testa *et al.*, 1996) and worse clinical outcomes (Torre-Amione *et al.*, 1996). The increase in concentrations of inflammatory cytokines in heart failure is possibly due to; mechanical stress in the heart (Shioi *et al.*, 1997), ischaemia (Singal *et al.*, 1998), toll-like receptor activation (Satoh *et al.*, 2005), and sustained white cell activation (Brooksbank[a] *et al.*, 2005; Brooksbank[b] *et al.*,

2005). The importance of inflammation as a possible causal factor of heart failure is highlighted by animal studies. In mice, the over expression of TNF-α resulted in heart failure, which was characterised by ventricular hypertrophy, dilatation and fibrosis (Kubota *et al.*, 1997). Similarly, in rats injected with TNF-α adverse left ventricular function and dilatation were noted (Bozkurt *et al.*, 1998).

In addition to heart failure, inflammation has also been associated with telomere length. Compared to healthy participants a reduced leukocyte telomere length has been associated with a number of inflammatory diseases (Houben *et al.*, 2008) such as; atherosclerosis (Huzen *et al.*, 2011; Samani *et al.*, 2001), diabetes mellitus (Salpea *et al.*, 2010; Weischer *et al.*, 2012), inflammatory bowel disease (Kinouchi *et al.*, 1998), chronic hepatitis (Aikata *et al.*, 2000), and obesity (Valdes *et al.*, 2005). Furthermore, leukocyte telomere length has been negatively correlated with markers of inflammation including; IL-6 (Carrero *et al.*, 2008; Shiels *et al.*, 2011; Wolkowitz *et al.*, 2011), C-reactive protein (CRP) (Bekaert *et al.*, 2007; Carrero *et al.*, 2008; Masi *et al.*, 2012), and fibrinogen (Bekaert *et al.*, 2007). Moreover, the probability of having shorter leukocyte telomere length was increased in patients with high concentrations of TNF-α and IL-6 (O'Donovan *et al.*, 2011). In two large cross-sectional studies in adolescent and middle-aged participants that had no signs of cardiovascular disease it was found that telomere length was not associated with traditional risk factors for cardiovascular disease such as; dyslipidaemia, hypertension, insulin resistance, and obesity (Bekaert *et al.*, 2007; Masi *et al.*, 2012). However, there was a negative association between telomere length and markers of inflammation in these two studies. These data indicate that there is a strong negative relationship between telomere length and inflammation. This tenet is reinforced by animal studies where mice injected with *Salmonella enterica* bacteria had reduced leukocyte telomere length compared to their sham-

injected siblings (Ilmonen *et al.*, 2008). These data indicate that inflammation is capable of inducing telomere attrition.

With regards to the cellular mechanisms of inflammatory induced telomere attrition, Beyne-Rauzy *et al.* (2004) showed that in leukaemic cells TNF-α resulted in a decreased telomere length and reduced expression of Tert. The same investigator (Beyne-Rauzy *et al.*, 2005), treated leukaemic cells and normal bone marrow CD34 hematopoietic progenitor cells with TNF-α and found that Tert expression was reduced in both cell types. These studies suggest that inflammation could reduce the expression of Tert, which would then result in a decreased ability to maintain telomere length. However, there are conflicting data with regards to the negative effects of TNF-α on telomere length. Akiyama *et al.* (2003) demonstrated that TNF-α mediates the nuclear translocation of Tert. The translocation of Tert into the nucleus could possibly have a positive effect on telomere length by increasing the availability of Tert for telomere elongation. The exact mechanisms behind TNF-α having both positive and negative effects on Tert are currently unknown. In this regard, the mechanisms behind inflammatory induced reductions in telomere length require further elucidation.

Hence, these data indicate that inflammatory induced reductions in telomere length could provide an explanation for the associations between telomere length and heart failure.

1.4.3 INFLAMMATION, OXIDATIVE STRESS, AND HEART FAILURE

It is well established that oxidative stress (Giordano, 2005; Singal *et al.*, 1998) and inflammation (Yndestad *et al.*, 2006) are key features of both heart failure itself and risk factors for heart failure (Russel, 1999; Tardif, 2000). Furthermore, oxidative stress and

inflammation are known to occur concomitantly. In this regard, oxidative stress can illicit inflammation (Knuefermann *et al.*, 2004; Frantz *et al.*, 2000) and conversely inflammatory mediators can increase oxidative stress (Floyd *et al.*, 1999; Jaiswal *et al.*, 2000).

As discussed in Sections 1.4.1 and 1.4.2 above, telomere attrition is increased by inflammation as well as oxidative stress (Houben *et al.*, 2008). Hence, the relationship between oxidative stress, inflammation and heart failure has implications regarding investigations between telomere length and heart failure. Firstly, the mechanisms whereby oxidative stress and inflammation increase telomere length attrition are not mutually exclusive. Secondly, to determine independent relationships between telomere length and heart failure, inflammation and oxidative stress have to be measured in order to account for their influences on telomere length. Hence, in order to establish cause and effect relationships between telomere length and heart failure, measurements of inflammation and oxidative stress are required to determine whether changes in telomere length during heart failure are independent of inflammation and oxidative stress.

1.4.4 APOPTOSIS, HEART FAILURE AND TELOMERE LENGTH

There is significant evidence to suggest that apoptosis is associated with heart failure. In this regard, apoptotic death of cardiomyocytes has been found to be increased in patients with heart failure compared to healthy controls (Guerra *et al.*, 1999; Olivetti *et al.*, 1997; Saraste *et al.*, 1999), with the incidence of apoptosis being between 0.08-0.25% in these patients. Moreover, animal models have illustrated that apoptosis itself is sufficient to herald the onset of heart failure. Wencker *et al.* (2003), using transgenic mice with a cardiac specific over expression of caspase enzymes, found that after several weeks these mice developed a dilated cardiomyopathy. Moreover, the rate of apoptosis was low (0.023%) and comparable

to levels seen in human studies. These data demonstrate that low levels of apoptosis are pathologically relevant and could be an important pathophysiological process in heart failure.

With regards to the consequences of apoptosis in the heart, there has been research into adverse ventricular remodelling as a result of apoptosis (Abbate *et al.*, 2002). Olivetti *et al.*(1990) showed that apoptosis potentially results in side-to-side slippage of cardiomyocytes, which then results in architectural rearrangements of the myocardium contributing towards ventricular dilatation. However, these findings were noted in a myocardial infarction model of heart failure in rats. In this regard, myocardial infarction results in acute changes in the heart, however these findings could still be applicable to other types of heart failure where the rate of cardiomyocyte apoptosis is reduced compared to acute myocardial infarction (Guerra *et al.*, 1999; Olivetti *et al.*, 1997; Saraste *et al.*, 1999; Wencker *et al.*, 2003), but is continuous over a number of years. In addition to architectural rearrangements of the myocardium, apoptosis may have an impact on intrinsic myocardial function. In this regard, intrinsic myocardial function is determined by the number of functional cardiomyocytes and their ability to contract. Therefore, a significant increase in apoptosis may result in the reduction of intrinsic myocardial function by decreasing the available number of functional cardiomyocytes.

As discussed previously in Section 1.3, telomere length has been associated with apoptosis in cardiomyocytes in heart failure (Chimenti *et al.*, 2003; Oh *et al.*, 2003). Moreover, telomere length has a major role in the regulation of apoptosis (Section 1.2). Therefore, reductions in telomere length could potentially mediate the activation of apoptosis in heart failure resulting in adverse left ventricular remodelling and reduced intrinsic myocardial function.

1.4.5 MITOCHONDRIAL DYSFUNCTION, HEART FAILURE AND TELOMERE LENGTH.

Mitochondrial dysfunction is another key feature of heart failure (Bayeva *et al.*, 2012; Marin-Garcia and Goldenthal, 2008). The pathophysiological consequences of mitochondrial dysfunction in heart failure include; decreased energy production, ROS generation, apoptosis, and disruptions of Ca^{2+} transients with subsequent alterations in cardiac contractility (Marin-Garcia and Goldenthal, 2008). Definitive evidence for the adverse effects of mitochondrial dysfunction on the heart are found with genetic mutations that effect mitochondrial function and produce cardiac dysfunction (Bayeva *et al.*, 2012).

In terms of the link between mitochondrial function and telomere length, Sahin *et al*. (2011) noted that telomere dysfunction in mice (Tert and Terc knockout mice) resulted in suppression of pathways linked to peroxisome proliferator-activated receptor gamma (PPAR-γ) coactivators 1 α and β. These PPAR-γ co-activators play a crucial role in the biogenesis and functioning of mitochondria. Hence, these mice displayed characteristics associated with abnormal mitochondrial functioning, which included; disruptions in oxidative phosphorylation, increased oxidative stress and reduced gluconeogenesis (Sahin *et al*., 2011). Furthermore, Sahin *et al*. (2011) showed in p53 knockout mice that the adverse effects caused by telomere induced mitochondrial dysfunction were ameliorated. These data suggest that the link between reduced telomere length and the suppression of PPAR-γ co-activators are directly associated with p53. This study demonstrated that when critically short telomeres activate p53, PPAR-γ coactivators 1 α and β are suppressed and these changes are associated with mitochondrial dysfunction.

In terms of heart failure, these data suggest that reductions in telomere length could be an important factor causing mitochondrial dysfunction in heart failure. Therefore, telomere length attrition may have a role in mitochondrial-induced decreased energy production, ROS

generation, apoptosis, and disruptions of Ca^{2+} transients with subsequent alterations in cardiac contractility (Marin-Garcia and Goldenthal, 2008). Hence, telomere attrition may play a causal role in the development of heart failure.

Although mechanism of inflammation, oxidative stress, and apoptosis were explored in my thesis, thorough investigations of the relationship between telomere length and mitochondrial function would have required me to conduct a multitude of additional assays (relating to metabolic function) which were outside the scope of my current thesis and hence were not done. Although it may well be interesting as part of a thesis on metabolic function.

1.4.6 Overview of potential mechanisms linking telomere length to heart failure.

The discussions in earlier sections provide the framework for a potential mechanistic links between reduced telomere length and heart failure (FIGURE 1.3). Currently a number of possibilities exist. Firstly, telomere length could be a causal factor in heart failure. In this regard, individuals with a higher prevalence of risk factors for heart failure have increased oxidative stress and inflammation (Russel, 1999; Tardif, 2000). Increases in oxidative stress and inflammation could precipitate an increase in telomere attrition (Beyne-Rauzy *et al.*, 2005; Houben *et al.*, 2008) and when telomere length of cardiomyocytes reaches a threshold level, cellular senescence and apoptotic pathways are activated (Artandi andAttardi, 2005). Furthermore, critical telomere shortening is also associated with mitochondrial dysfunction (Sahin *et al*., 2011). Hence, reductions in cardiomyocyte telomere length could herald the activation of senescence (Chimenti *et al*., 2003), apoptosis (Chimenti *et al*., 2003; Oh *et al*., 2003; Leri *et al*., 2003; Sheng *et al*., 2011), and mitochondrial dysfunction (Sahin *et al*., 2011) and potentially result in the onset of heart failure (Leri *et al*., 2003). Alternatively,

telomere length may not be a causative factor but could mediate the changes in heart failure. In this regard, increased oxidative stress (Singal *et al.*, 1998; Giordano, 2005) and inflammation (Yndestad *et al.*, 2006) present in heart failure may cause a decrease in cardiomyocyte telomere length with the subsequent activation of apoptosis (Artandi and Attardi, 2005), senescence, and mitochondrial dysfunction (Sahin *et al*., 2011). The activation of these telomere regulated pathways may not be the underlying cause of heart failure, but could mediate the adverse changes in cardiac function and structure evident in heart failure (Abbate *et al.*, 2002; Bayeva *et al.*, 2012; Marin-Garcia and Goldenthal, 2008). Lastly, reduced telomere length may have no pathophysiological consequences in heart failure and may merely be an epiphenomenon due to the increased oxidative stress and inflammation caused by heart failure or risk factors associated with the development of heart failure.

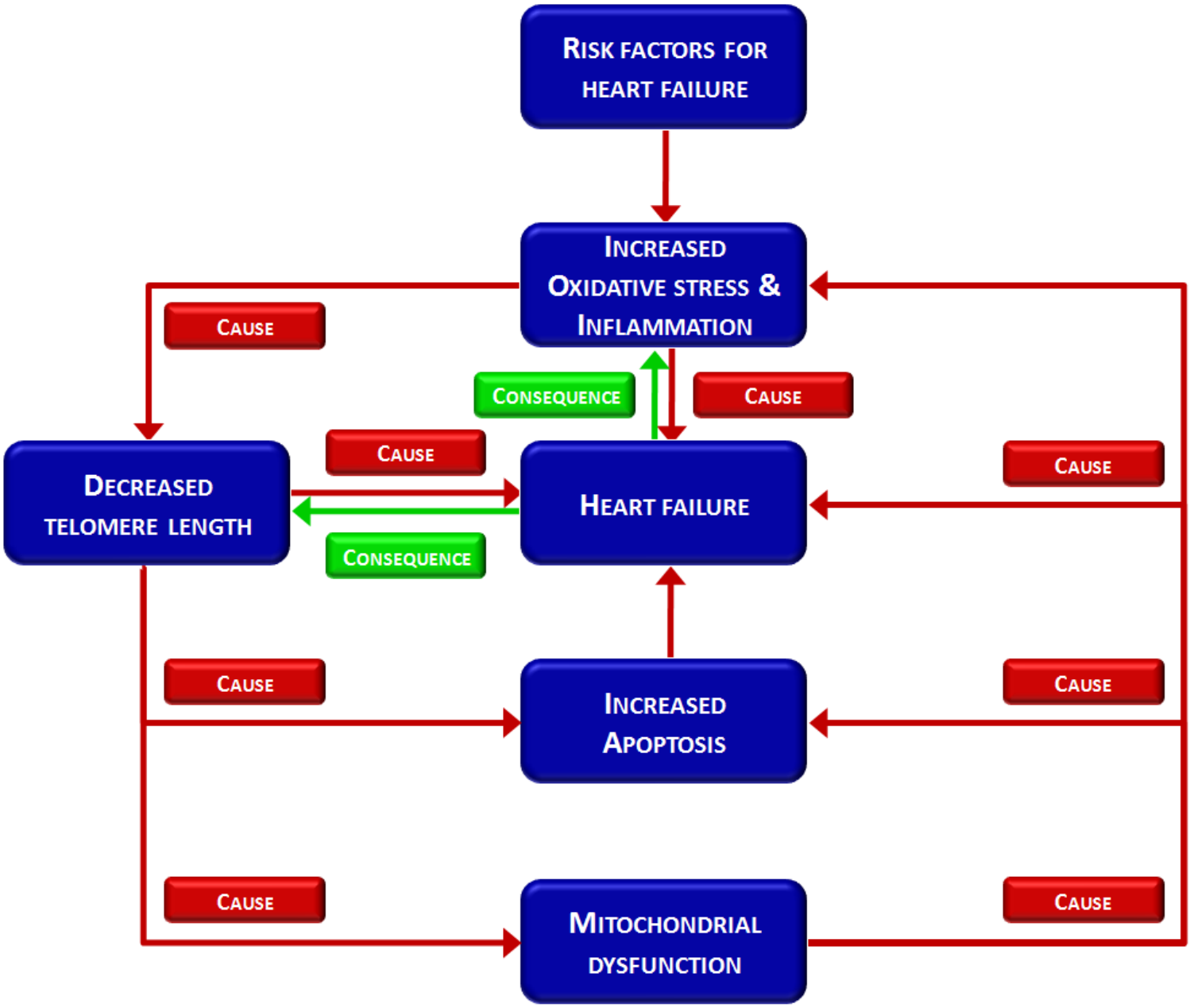

FIGURE 1.3 Potential pathway of telomere-induced activation of cellular senescence, apoptosis, and mitochondrial dysfunction in heart failure. Red arrows indicate possible causal pathways and green arrows indicate possible consequential pathways linking decreased telomere length and heart failure.

1.5 PROBLEM STATEMENTS AND AIMS

There is a clear negative association between telomere length and heart failure. However, the cause and effect relationships between telomere length and heart failure are uncertain. In this regard, whether reduced telomere length plays a role in the pathophysiological processes of heart failure or if telomere length is reduced in heart failure merely due to the pathophysiological processes involving oxidative stress and inflammation is unknown. The caveats within the literature regarding the data investigating the relationship between telomere length and heart failure have been discussed in detail in previous sections. A summary of the problems are: the utilisation of leukocyte telomere length as a surrogate marker for cardiovascular telomere length; the relationship between telomere length and non-ischaemic forms of heart failure have not been investigated (non-ischaemic forms of heart failure do not have atherosclerosis associated oxidative stress and inflammation); the relationship between telomere length and heart failure has not been investigated longitudinally; and associations between telomere length and cardiac geometry and function have not been adequately investigated.

The main aim of this thesis was to therefore further investigate the relationship between telomere length and heart failure in light of the deficiencies within the literature. In this regard, I investigated the relationships between telomere length and a non-ischaemic form of heart failure, and between telomere length and measurements of cardiac remodelling and systolic function prior to the development of heart failure. The specific aims for each of my studies were as follows:

- To investigate the association of telomere length with the severity and presence of a non-ischaemic form of heart failure (Chapter 2).

- To investigate whether telomere shortening is a cause of adverse left ventricular remodelling and systolic dysfunction in an animal model of dilated cardiomyopathy (chronic ethanol consumption) (Chapter 3).
- To determine whether adverse left ventricular remodelling and systolic chamber dysfunction produced by chronic β-adrenergic receptor activation are associated with telomere shortening (Chapter 4)
- To determine the impact of chronic inflammation on telomere length, left ventricular remodelling, and systolic dysfunction (Chapter 5).
- To determine the relationships between telomere length and inflammation, left ventricular geomtery, and systolic function in a cross-sectional community study (Chapter 6)

CHAPTER 2 - IDIOPATHIC DILATED CARDIOMYOPATHY AND TELOMERE LENGTH

The data in this chapter has been published in the European Journal of Heart Failure: Raymond AR, Norton GR, Sareli P, Woodiwiss AJ, Brooksbank RL. Relationship between average leukocyte telomere length and the presence or severity of idiopathic dilated cardiomyopathy in black Africans. Eur J Heart Fail 2013; 15:56-60.

2.1 ABSTRACT

Aims: A reduced average leukocyte telomere length is associated with ischaemic heart disease. Whether this relationship represents a cause or consequence of heart failure or is attributed to associated risk factors and coronary artery disease is uncertain. We evaluated if average leukocyte telomere length is associated with idiopathic dilated cardiomyopathy (IDC) or its severity. ***Methods and Results:*** We compared average leukocyte telomere length in 223 patients with heart failure due to IDC and 227 healthy controls of black African ancestry. We also evaluated the relationship between average leukocyte telomere length and left ventricular (LV) ejection fraction (EF). LVEF was determined using echocardiography and radionuclide multiple-gated acquisition (MUGA) scans in patients with IDC. Relative leukocyte telomere length (T/S) was measured using a quantitative real-time polymerase chain reaction assay. Log T/S was negatively correlated with age in patients with IDC ($p=0.0007$) and in controls ($p=0.030$), and with alcohol consumption ($p=0.032$) and regular smoking ($p=0.021$) in patients with IDC. Log T/S did not differ between IDC and control groups either before ($p=0.11$) or after (IDC=0.071±0.19 vs. Control=0.071±0.19, $p=0.99$) adjustments for confounders. Log T/S was not associated with echocardiographic ($p=0.47$) or MUGA ($p=0.99$) LVEF or LV end diastolic diameter (LVEDD) ($p=0.34$) in patients with IDC. With adjustments for age, sex, alcohol consumption and smoking, log T/S was similarly not associated with echocardiographic ($p=0.60$) or MUGA ($p=0.91$) LVEF or LVEDD ($p=0.53$) in patients with IDC. ***Conclusions:*** Average relative leukocyte telomere length is not associated with IDC or its severity in groups of black African ancestry.

2.2 INTRODUCTION

Recent evidence suggests that patients with chronic heart failure have a reduced average leukocyte telomere length (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) and that telomere length is associated with the severity of heart failure (van der Harst *et al.*, 2010). A decreased average leukocyte telomere length also predicts deaths and hospitalisations in patients with New York Heart Association (NYHA) functional class II-IV heart failure (van der Harst*et al.*, 2010). Moreover, in the very elderly, a shorter leukocyte telomere length is related to a reduced left ventricular (LV) ejection fraction (EF) (Collerton *et al.*, 2007). A number of possibilities may explain these relationships including cardiomyocyte apoptosis and premature senescence caused by telomere attrition (Chimenti *et al.*, 2003; Leri *et al.*, 2001; Leri *et al.*, 2003; Oh *et al.*, 2003), or repression of mitochondrial biogenesis and performance caused by telomere dysfunction (Sahin *et al.*, 2011). In addition heart failure-induced chronic inflammation, oxidative stress or renal dysfunction may result in telomere attrition (Beyne-Rauzy *et al.*, 2004; Houben *et al.*, 2008; van der Harst *et al.*, 2008). Moreover, relationships between heart failure and a reduced average leukocyte telomere length (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) may represent an epiphenomenon attributed to the relationship between telomere length and coronary artery disease (Brouilette *et al.*, 2003; Ogami *et al.*, 2004) or its risk factors (Fitzpatrick *et al.*, 2007; Valdes *et al.*, 2005). Currently, the relative importance of each of these potential explanations is uncertain.

If the relationship between average telomere length and heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) or its outcomes (van der Harst *et al.*, 2010) are an index of the deleterious consequences of heart failure (Beyne-Rauzy *et al.*, 2004; Houben *et al.*, 2008; van der Harst *et al.*, 2008), then one would expect most forms of heart failure, depending on their severity, to be associated with telomere length. Similarly, if

a reduced average leukocyte telomere length reflects the impact of telomere attrition or dysfunction on heart failure through apoptosis (Chimenti *et al.*, 2003; Leri *et al.*, 2001; Leri *et al.*, 2003; Oh *et al.*, 2003), and/or repression of mitochondrial biogenesis and function (Sahin*et al.*, 2011), then one would anticipate this relationship to exist in all forms of heart failure associated with excessive apoptosis or impaired mitochondrial biogenesis and function. In contrast, if the relationship between average leukocyte telomere length and heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) reflects an association with coronary artery disease (Brouilette *et al.*, 2003; Ogami *et al.*, 2004) and/or its risk factors (Fitzpatrick *et al.*, 2007; Valdes *et al.*, 2005) then it is more likely that this relationship will be specific to ischaemic heart disease. In this regard, to-date only one study has reported on the relationship between average leukocyte telomere length and heart failure of non-ischaemic as opposed to ischaemic origins (van der Harst *et al.*, 2007). Although both forms of heart failure were associated with a reduced average leukocyte telomere length, a high proportion of patients with non-ischaemic heart disease had a history of stroke, peripheral arterial disease and angina pectoris (van der Harst *et al.*, 2007). Furthermore, the causes of heart failure in the remaining patients in this study were not described (van der Harst *et al.*, 2007). Consequently, there is a need to establish whether average leukocyte telomere length is associated with non-ischaemic heart disease and its severity. In the present study we therefore evaluated the relationship between average relative leukocyte telomere length and either the presence or the severity of idiopathic dilated cardiomyopathy (IDC), a form of heart failure associated with excess inflammation (Brooksbank[a] *et al.*, 2005; Brooksbank[b] *et al.*, 2005; Sliwa *et al.*, 1998), oxidative stress (Yücel *et al.*, 1998), and cardiomyocyte apoptosis (Kostin *et al.*, 2003).

2.3 METHODOLOGY

2.3.1 Study groups: The present study was conducted according to the principles outlined in the Helsinki declaration. The Committee for Research on Human Subjects of the University of the Witwatersrand approved the protocol (approval number: M951122). Participants provided informed, written consent. 223 patients of black African ancestry with IDC and 227controls randomly recruited from the same community and who had no history of cardiovascular disease, were normotensive (not taking medication and had a normal seated blood pressure) and had no diabetes mellitus (no glucose-lowering therapy and a random glucose<7.0mmol.l^{-1}), participated in this study.

A diagnosis of IDC was established by the presence of NYHA class II-IV heart failure despite stable medical therapy for at least 1 month before entry into the study and an LVEF<45% determined from echocardiography. Exclusion criteria have previously been reported on (Candy *et al.*, 1999) and included previous cardiac ischaemic events, the use of nitrates, the presence of angina pectoris or peripheral arterial disease or a history of stroke. In addition, patients were excluded if they had wall motion abnormalities on echocardiography or pathologic Q waves on electrocardiography. Furthermore, patients with primary rheumatic valve disease, congenital heart disease, active myocarditis or a history of myocarditis, clinical and echocardiographic features consistent with an obstructive, hypertrophic, or restrictive cardiomyopathy, or pericardial disease were excluded. Moreover, patients with obvious hypertensive or alcohol-induced dilated cardiomyopathy, a history of hypertension, or systolic or diastolic blood pressure of greater than 170 and 105 mmHg respectively were excluded. Patients with liver and renal function changes that could only be attributed to primary hepatic and renal disease as opposed to changes that may occur subsequent to heart failure were also excluded. To avoid the impact of arrhythmias on systolic and diastolic left ventricular performance, patients with significant arrhythmias were similarly excluded. To

ensure that co-morbidities did not contribute toward cardiac performance, patients with significant pulmonary, neurologic, or endocrine disease were excluded.

Coronary angiography was performed in 28 patients in whom, on history, a potential ischaemic contribution was uncertain. Angiography was not routinely performed in the rest of the cohort as we have previously shown that patients from this population labelled as IDC according to the above criteria, have a very low incidence of coronary artery pathology on angiography (Sliwa *et al.*, 1998; Wisenbaugh *et al.*, 1993). In all of the 28 patients who may have had ischaemic pathology the coronary anatomy was noted to be normal on angiography.

2.3.2 Left ventricular performance: A multiple gated equilibrium cardiac blood pool scintigraphic (MUGA) technique was used to measure LVEF. This procedure was performed by an experienced cardiologist. The MUGA scans were performed using a Elscint Apex 409M mobile scintillation camera equipped with a collimator (Elscint APC-3, Haifa, Israel). The scans were performed at rest, in participants who consented, after a 2 ml intravenous injection of stannous pyrophosphate and an injection of 1,100 MBq (20 mCi) technetium-99m 20 minutes later. In addition to MUGA scans, LVEF was determined using two-dimensional echocardiography. Hewlett Packard 1000 or 2000 echocardiographs coupled to 2.5- or 3.5-MHz transducers were used to determine LV dimensions (Hewlett Packard, Boeblingen, Germany). LV dimensions were measured according to the American Society of Echocardiography guidelines and LVEF was determined using a standard formula: LVEF = (LVEDD-LV end systolic diameter (ESD))/ LVEDD (Sahn *et al.*, 1978). Moreover, left ventricular dilation was assessed using the LV internal dimensions during end diastole and systole.

2.3.3 Average relative telomere length: Leukocyte DNA for each participant was extracted from whole blood samples using the salting out method of Higuchi *et al.*(1989) 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 (Cawthon, 2002; Gil and Coetzer, 2004). 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 PCR amplifications; one to determine telomere repeat copy number and the other to determine 36B4 copy number. The PCR reactions were carried out using LightCycler FastStart DNA Master SYBR I Green kits (Roche Diagnostics GmbH, Mannheim, Germany) as per manufacturer's instructions. Each reaction mix contained FastStart Taq Polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye, dNTP mix, and the appropriate primers for either the telomere or 36B4 reaction (TABLE 2.1).

TABLE 2.1 The primer sequences and LightCycler 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	95°C	10 min
Amplification	20	95, 56, 72°C	5, 10, 60 sec
Cooling	1	30°C	30 sec

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	95°C	10 min
Amplification	30	95, 58, 72°C	5, 10, 40 sec
Cooling	1	30°C	30 sec

All temperature transition rates were 20°C per second, except for the telomere annealing transition rate, which was 4°C per second.

The telomere primers 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' prime 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.

PCR amplifications and their analysis were carried out using a Roche LightCycler v1.5 and LightCycler software v1.4 respectively (Roche Diagnostics GmbH, Mannheim, Germany). The qRT-PCR method used in this study, includes a SYBR® Green I 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, Ct is the number of PCR cycles required to reach threshold levels of fluorescence. Hence, the greater the amount of starting template there is, the less number of PCR cycles are required to reach Ct. In 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:

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

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 2.1) from the calibrator that was serially diluted to produce a range of DNA quantities from 200ng to 5ng. The reaction efficiencies of 2.1 and 1.9, and the tube-to-tube variations of 0.09 and 0.001 for the 36B4 and telomere standard curves, respectively, were well within accepted limits (Gil and Coetzer, 2004). For the telomere and 36B4 standard curves, the slopes of the fitted regressions were –3.18±0.3 and –3.75±0.3 ($p<0.001$, FIGURE 2.1), respectively. The regression coefficients were 0.998 and 1.00 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 (FIGURE 2.2). 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 and Coetzer, 2004).

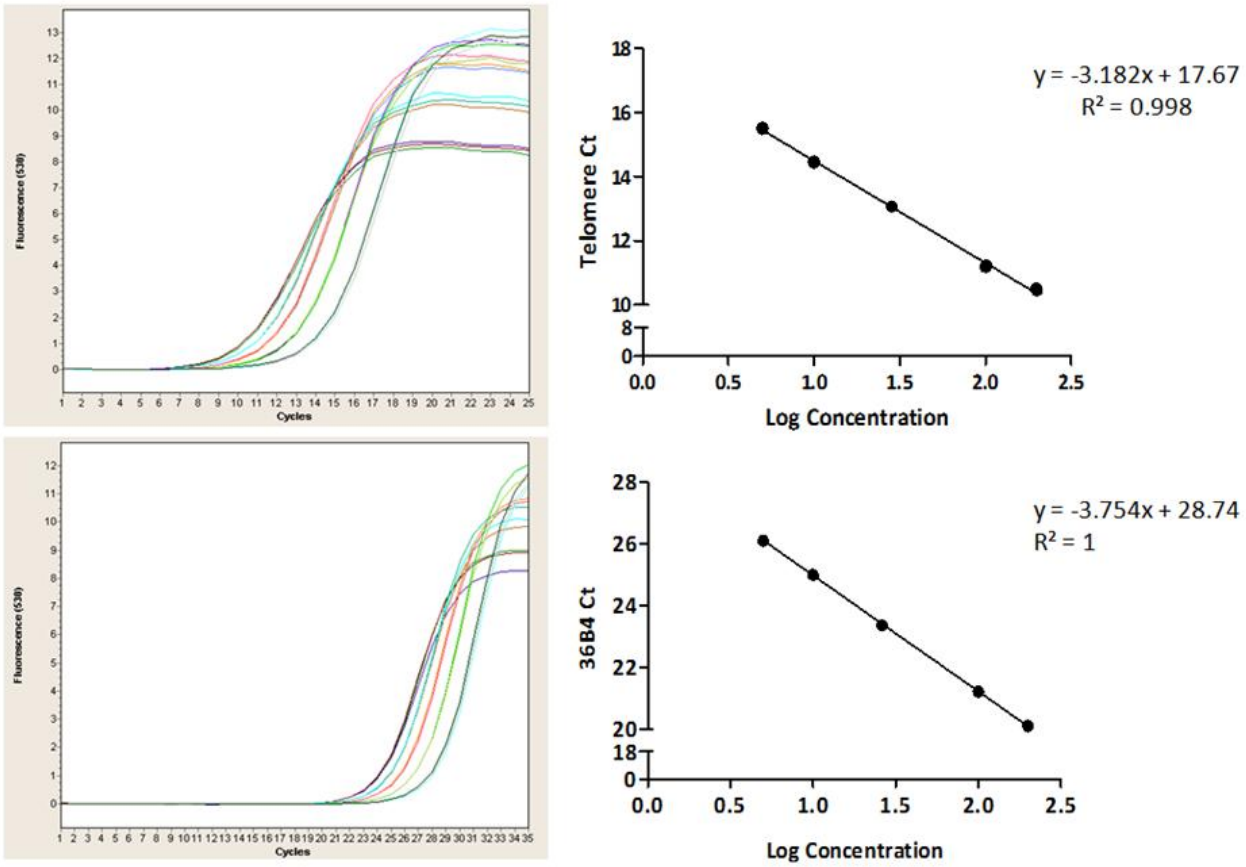

FIGURE 2.1 Figures on the left show the amplification curves generated from the Lightcycler software for the telomere and 36B4 standard curves respectively. The quantity of DNA used to generate the curves ranged from 200ng to 5ng. The amplification curve on the far left corresponds to 200ng and the curve on the far right corresponds to 5ng of DNA in the telomere and 36B4 amplification curves respectively. Figures on the right are a plot of Ct versus log concentration for the telomere and 36B4 dilution series respectively. These figures indicate that there is a strong linear relationship between DNA concentration and the PCR cycles required to reach threshold levels of fluorescence (Ct).

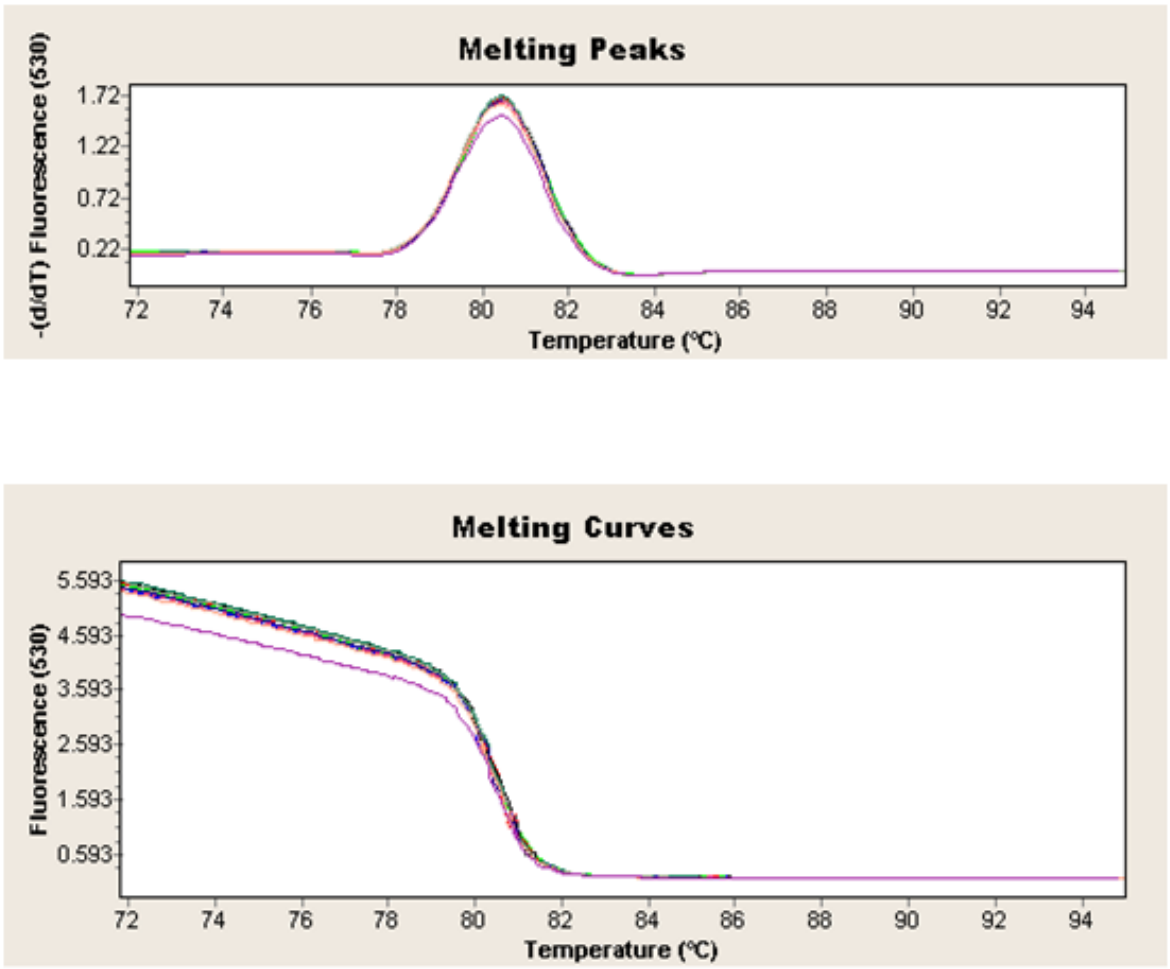

FIGURE 2.2 Melting curves and peaks for the 36B4 PCR reaction.

2.3.4 Statistical analysis: All tests were carried out on log-transformed T/S crossing point ratios, as they were not normally distributed. A Student's unpaired t-test was used for unadjusted comparisons and bivariate relationships were identified from a Pearson's correlation. Multivariate regression analysis was performed to assess independent relationships. Statistical analysis was conducted using SAS software, version 9.1 (SAS Institute Inc., Cary, NC, USA). Data are represented as mean±standard deviation (SD).

2.4 RESULTS

2.4.1 Participant characteristics: TABLE 2.2 shows the characteristics of the IDC and control groups. The control group was younger than the IDC group. The proportion of females, the proportion that smoked regularly and the proportion that consumed alcohol was greater in the control group as compared to the IDC group. The mean BMI and blood pressures were similar between the groups.

2.4.2 Relationships between average relative leukocyte telomere length and participant characteristics: There was a negative bivariate relationship between T/S ratio and age in both the IDC and control groups (TABLE 2.3). Although the T/S ratio was not affected by smoking or alcohol consumption in control participants, the T/S ratio was decreased in patients with IDC who regularly smoked or consumed alcohol. No relationships between the T/S ratio and sex, BMI, or blood pressure were noted.

2.4.3 Relationships between average relative leukocyte telomere length and IDC: The median (interquartile range) for the non log-transformed T/S ratios were 1.18 (0.95 to 1.50) for the control group and 1.12 (0.89 to 1.40) for the IDC group. TABLE 2.4 shows the unadjusted and multivariate adjusted average relative leukocyte telomere lengths in participants with IDC as compared to controls. The mean T/S ratio in the IDC group did not differ from that of the control group either before or after adjustments.

TABLE 2.2 Characteristics of patients with idiopathic dilated cardiomyopathy (IDC) and control participants.

	Controls (n=227)	IDC (n=223)	p value
Age (years)	39.9 ± 15.4	51.1 ± 11.3	<0.0001*
% Female	49	37	0.007*
Regular alcohol consumption (%)	35	14	<0.0001*
Regular smoking (%)	32	17	<0.0001*
Body mass index ($kg.m^{-2}$)	24.3±4.9	25.0±4.4	0.13
Systolic blood pressure (mm Hg)	121±15	118±20	0.17
Diastolic blood pressure (mm Hg)	78±8	77±13	0.28
LVEF (echocardiography) (%)	-	24.8±7.5	
LVEF (MUGA) (%)	-	26.1±10.6 (n = 219)	
LVEDD (mm)	-	66.7±7.1	

LVEF, left ventricular ejection fraction determined using echocardiography or multiple gated equilibrium cardiac blood pool scintigraphic techniques (MUGA); LVEDD, left ventricular end-diastolic diameter. Values are represented as mean±SD or percentage; *p<0.05 versus IDC and control groups.

TABLE 2.3 Relationship between relative leukocyte telomere length (log T/S) and risk factors for cardiovascular disease in control participants and in patients with idiopathic dilated cardiomyopathy.

	Controls (n=227)			**Idiopathic dilated cardiomyopathy (n=223)**		
	Pearson's r	95% CI	p value	Pearson's r	95% CI	p value
Age	-0.14	-0.27 to -0.01	0.03*	-0.23	-0.35 to -0.10	0.0007*
Body mass index	-0.06	-0.19 to 0.08	0.41	-0.03	-0.18 to 0.12	0.71
Systolic blood pressure	-0.08	-0.20 to 0.06	0.26	-0.11	-0.23 to 0.03	0.11
Diastolic blood pressure	-0.06	-0.19 to 0.08	0.41	-0.07	-0.20 to 0.06	0.29
	Mean±SD log T/S		p value**	Mean±SD log T/S		p value**
Gender	M= 0.074±0.196	F= 0.093±0.173	0.43	M= 0.043±0.158	F= 0.083±0.165	0.07
Regular alcohol	Yes= 0.079±0.211	No= 0.086±0.173	0.82	Yes= 0.005±0.139	No= 0.068±0.164	0.032*
Regular smoking	Yes= 0.085±0.187	No= 0.083±0.185	0.93	Yes=-0.014±0.131	No= 0.066±0.163	0.021*

r, correlation coefficient; CI, confidence interval; M, male; F, female. *p<0.05;** Student's t-test.

TABLE 2.4 Comparison of unadjusted and multivariate adjusted average relative leukocyte telomere length (log T/S, mean±SD) between patients with idiopathic dilated cardiomyopathy and control participants.

	Controls (n=227)	IDC (n=223)	p value
Unadjusted	0.084±0.173	0.058±0.173	0.11
Age- and sex-adjusted	0.070±0.179	0.071±0.179	0.95
Age-, sex- and risk factor*-adjusted	0.071±0.187	0.071±0.187	0.99

* regular alcohol consumption and regular smoking.

2.4.4 Relationships between average relative leukocyte telomere length and the severity of IDC: FIGURE 2.3 shows the bivariate relations between T/S ratios and LV dimensions or pump function. TABLE 2.5 gives the multivariate adjusted correlation coefficients between T/S ratios and LVEDD (echocardiography) and LVEF (echocardiography and MUGA) in the IDC group. There was no association between T/S and indices of LV dilatation or pump dysfunction in patients with IDC and heart failure.

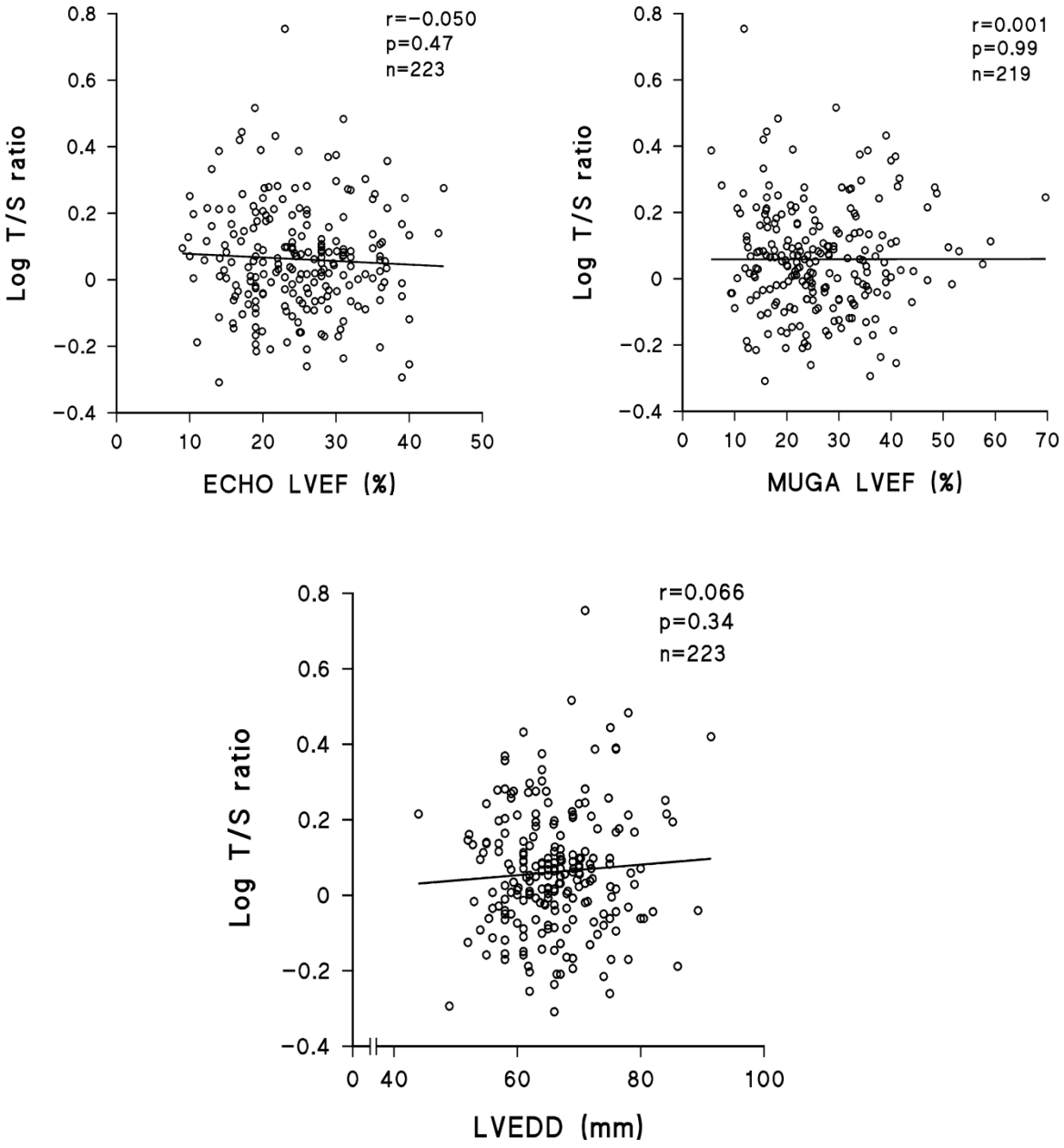

FIGURE 2.3 Bivariate relationships between average relative leukocyte telomere length (log T/S) and left ventricular end-diastolic diameter (LVEDD) (*lower panel*) or left ventricular ejection fraction (LVEF) (echocardiography (ECHO) (*upper left panel*) and multiple gated acquisition scan (MUGA) (*upper right panel*)) in patients with idiopathic dilated cardiomyopathy.

TABLE 2.5 Multivariate adjusted relations between average relative leukocyte telomere length (log T/S) and left ventricular (LV) end diastolic diameter and ejection fraction (EF) in patients with idiopathic dilated cardiomyopathy (n=223). Bivariate relationships are shown in FIGURE 2.3.

Log T/S versus	Partial r	95% CI	p value
LV end diastolic diameter:			
Age- and sex-adjusted	0.052	-0.084 to 0.185	0.46
Age-, sex- and risk factor*-adjusted	0.043	-0.093 to 0.178	0.53
LVEF (echocardiography):			
Age- and sex-adjusted	-0.048	-0.182 to 0.087	0.49
Age-, sex- and risk factor*-adjusted	-0.036	-0.171 to 0.099	0.60
LVEF (MUGA): (n=219)			
Age- and sex-adjusted	0.010	-0.123 to 0.143	0.88
Age-, sex- and risk factor*-adjusted	-0.008	-0.141 to 0.126	0.91

r, correlation coefficient; CI, confidence interval; Multiple gated equilibrium cardiac blood pool scintigraphic scan (MUGA), left ventricular ejection fraction (LVEF).*regular alcohol consumption and regular smoking.

2.5 DISCUSSION

The main findings of this chapter of my thesis are as follows: In a group of black African ancestry, IDC is not associated with average relative leukocyte telomere length. Furthermore, average telomere length is not related to the severity of pump dysfunction (MUGA and echocardiographic EF) and chamber dilatation (LVEDD) in patients with mainly NYHA functional class II-III heart failure (86.5%) and IDC.

Whilst prior studies have largely been conducted in ischaemic heart disease (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012), the present study is the first to assess the relationship between average leukocyte telomere length and IDC or the severity of pump dysfunction and chamber dilatation in patients with IDC and heart failure. In this regard, the usual risk factors for atheroma formation, which may themselves be causally related to telomere shortening (Fitzpatrick *et al.*, 2007; Valdes *et al.*, 2005), and are associated with ischaemic heart disease, are not characteristic of IDC. Hence, by studying relationships between average telomere length and IDC or its severity, the impact of these risk factors on average telomere length can be separated from alternative potential determinants of telomere length and heart failure. As the present study indicates that average telomere shortening, at least in leukocytes, is not associated with IDC or its severity, these data therefore suggest that previously described associations between average leukocyte telomere length and ischaemic heart disease, its severity and its outcomes (Starr *et al.*, 2007; van der Harst *et al.*, 2007; van der Harst *et al.*, 2010; Weischer *et al.*, 2012), may be by virtue of associations with coronary artery disease (Brouilette *et al.*, 2003; Ogami *et al.*, 2004) or its risk factors (Fitzpatrick *et al.*, 2007; Valdes *et al.*, 2005).

Although a prior study has demonstrated a relationship between average leukocyte telomere length and non-ischaemic heart disease, in that study 22% of the non-ischaemic group of patients had advanced atheromatous disease as indexed by a history of stroke,

peripheral arterial disease or angina pectoris (van der Harst *et al.*, 2007). In that study telomere length was strongly associated with atherosclerotic disease (van der Harst *et al.*, 2007). Furthermore, the causes of heart failure in the remaining non-ischaemic patients were not stated (van der Harst *et al.*, 2007). In contrast, in the present study I evaluated relationships between average leukocyte telomere length and IDC, a specific cause of heart failure, where patients with co-morbid conditions consistent with advanced atheroma were excluded. It is therefore possible that inconsistencies between this prior study (van der Harst *et al.*, 2007), and the present study may be attributed to differences in the disease profiles of the patient groups. Importantly, a similar proportion of patients with NYHA functional class II-III heart failure of non-ischaemic aetiology was evaluated in the present (86.5%) as compared to this prior (99%) (van der Harst *et al.*, 2007) study. Thus, it is unlikely that differences in the severity of heart failure contributed toward the discrepant outcomes between the present and this prior study (van der Harst *et al.*, 2007). Nevertheless, the present study was conducted in participants of black African ancestry, whilst the prior study was conducted in Caucasians (van der Harst *et al.*, 2007). In this regard, telomere length has a high inter-individual variability; and hence a cross-sectional determination of telomere length at one specific time point may not accurately reflect long-term telomere length dynamics in individuals (Nordfjäll *et al.*, 2009). It is possible that in groups of African ancestry a greater inter-individual variability of telomere length exists thus reducing the chances of demonstrating relationships in cross-sectional studies. Whether the results of the present study can be reproduced in alternative population samples therefore warrants further investigation.

The results of this chapter do not discount an important contribution of cardiomyocyte telomere attrition or dysfunction to the pathogenesis of heart failure. In this regard, leukocyte telomere length may not closely represent the extent of telomere attrition that occurs in

cardiomyocytes. Indeed, cardiomyocyte telomere attrition or dysfunction is associated with cardiomyocyte apoptosis, cell death and premature senescence in human heart failure (Chimenti *et al.*, 2003; Oh *et al.*, 2003) and with repression of mitochondrial biogenesis and performance (Sahin *et al.*, 2011). Importantly, genetic knockout of the telomerase enzyme in mice is associated with telomere attrition and the development of overt heart failure in subsequent generations (Leri *et al.*, 2003). Moreover, the length of the shortest telomere in a particular population of cells rather than average telomere length of a population of leukocytes, as studied in the present and in prior (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012)studies, determines cellular function (Hemann *et al.*, 2001). Thus, average relative telomere length may not closely reflect the impact of telomeres on cellular performance.

The previously described relationships between average leukocyte telomere length and ischaemic heart disease (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) may also be attributed to the impact of heart failure on oxidative stress and inflammation, both of which are associated with shortened telomeres (Houben *et al.*, 2008; Beyne-Rauzy *et al.*, 2004). Although in the present study I did not assess relationships between leukocyte telomere length and either oxidative stress or inflammation, IDC has previously been shown to be associated with both of these pathophysiological processes (Sliwa *et al.*, 1998; Yücel *et al.*, 1998). Thus, I assume that in the present study, these two mechanisms for progressive heart failure are likely to play a role. The lack of relationship between average leukocyte telomere length and either IDC or its severity in the present study therefore suggests that the previously described relationships between average leukocyte telomere length and heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) are unlikely to be explained by an impact of oxidative stress and inflammation on telomere length. However, the possibility that the duration of heart failure in the patients in

the present study was too short for either of these mechanisms to cause significant telomere attrition should also be considered. Prospective intervention studies evaluating the impact of therapy in heart failure over a prolonged period are required to address this question.

In terms of study limitations, firstly, this is a cross-sectional study which does not allow for the elucidation of conclusions regarding cause and effect. In addition, the cross-sectional design may in-part explain the inability to show relationships between average leukocyte telomere length and left ventricular function and dimensions. In this regard, left ventricular function and dimensions were evaluated at different times in the development of the disease. Moreover, as highlighted in the aforementioned discussion, telomere length has a high inter-individual variability; and hence a cross-sectional determination of telomere length at one specific time point does not accurately reflect long-term telomere length dynamics of individuals (Nordfjäll *et al.*, 2009). These arguments nevertheless do not detract from the fact that if average leukocyte telomere length did herald a pathophysiological change that causes IDC, we should have been able to show a decreased average leukocyte telomere length in patients with IDC irrespective of the point in the progression of the disease at the time of recruitment. Second, it may be argued that the point at which a diagnosis of heart failure in IDC is made is the end-stage of the disease and hence the range of LV diameters or EF values would be too narrow to show relations between telomere length and LV function or dimensions. However, examination of the bivariate relations shown in the figure indicates that a wide range of LV dimensions and EF values were obtained. Third, as highlighted in the aforementioned discussion, we did not assess the relationships between average leukocyte telomere length and indexes of oxidative stress or inflammation. Hence, it is possible that leukocyte telomere length could be related to these parameters in heart failure, but that this does not translate into relationships with the severity of LV dysfunction or dilatation. Last, as also indicated in the aforementioned discussion we assessed the relationship between average

leukocyte telomere length and IDC in one ethnic group only (black African). Hence, further studies are required to determine whether a lack of relationship exists between average leukocyte telomere length and IDC in other ethnic groups.

In conclusion, the present study shows that average relative leukocyte telomere length is not associated with IDC or the severity of IDC in groups of black African ancestry. Thus, previously described relationships between average leukocyte telomere length and largely ischaemic heart disease (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012), may represent an epiphenomenon attributed to the co-existence of coronary artery disease and its risk factors (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Ogami *et al.*, 2004; Valdes *et al.*, 2005), which themselves are related to telomere attrition. Further prospective studies in IDC are required to validate the results of the present cross-sectional study.

CHAPTER 3 - CARDIAC REMODELLING, SYSTOLIC DYSFUNCTION, AND TELOMERE LENGTH

Data from this chapter have been submitted for review in: International Journal of Cardiology.

3.1 ABSTRACT

Aims: In order to further explore whether telomere shortening is a cause of cardiac remodelling and systolic dysfunction, the relationships between telomere length and an animal model of alcoholic cardiomyopathy (a form of dilated cardiomyopathy) were investigated. ***Methods and Results:*** Sprague-Dawley (SD) rats received either drinking water with (ethanol: n=19) or without (control: n=19) 5% (v/v) ethanol *ad libitum*, for four months. Echocardiography was performed to determine LV dimensions and function *in vivo* and isolated perfused heart preparations (ethanol: n=10; control: n=10) were performed to obtain *ex vivo* load independent measurements of LV chamber geometry and function. Ethanol consumption resulted in LV dilatation (LVEDD: ethanol=8.20±0.14mm vs. control=7.56±0.11mm, p=0.0014; the volume intercept at 0 mmHg (V_0) of the left ventricular end diastolic pressure-volume relationship: ethanol=0.40±0.03ml vs. control=0.31±0.02ml, p=0.020), and a reduced LV relative wall thickness (ethanol=0.50±0.08mm vs. control=0.60±0.092mm, p=0.0011). However, there were no changes in systolic chamber function as indexed by endocardial fractional shortening (FS_{end}) or the slope of the LV systolic pressure-volume relation (end systolic elastance-E_{es}). Ethanol administration resulted in a marked increase in cardiomyocyte apoptosis (ethanol=0.85±0.13% vs. control=0.36±0.06%, p=0.0021). Furthermore, % apoptosis was correlated with LVEDD (r=0.39, n=38, p=0.021) and V_0 (r=0.44, n=19, p=0.046). However, no significant differences in leukocyte, cardiac, liver, or kidney telomere length were noted between ethanol and control groups. In addition, cardiac telomere length was not associated with indices of LV dilatation (LVEDD and V_0) or apoptosis. ***Conclusions:*** In conclusion, chronic alcohol-induced cardiac remodelling and apoptosis in the absence of systolic dysfunction are independent of changes in telomere length.

3.2 INTRODUCTION

Having shown that average relative leukocyte telomere length was not associated with the presence or severity of a non-ischaemic cardiomyopathy (Raymond *et al.*, 2013), there is a need to establish the cause and effect relationships between reduced telomere length and heart failure. In this regard, in order to further explore whether telomere shortening is a cause of cardiac remodelling and systolic dysfunction, I investigated the relationships between telomere length and cardiac remodelling and systolic dysfunction in an animal model of alcoholic cardiomyopathy.

Chronic, excessive alcohol consumption is a leading cause of non-ischaemic, dilated cardiomyopathy (Piano, 2002). Ninety grams of ethanol per day for approximately five years is known to result in a alcoholic cardiomyopathy, which is characterised by LV dilatation, hypertrophy, myocardial wall thinning, and ventricular dysfunction (Capasso *et al.*, 1991; Capasso *et al.*, 1992; Doser *et al.*, 2009; Piano, 2002; Ren and Brown, 2000; Urbano-Marquez *et al.*, 1989; Zhang *et al.*, 2010). Importantly, all of these changes are produced in the absence of coronary artery disease (Piano, 2002). An alcoholic cardiomyopathy is characterised by two stages, the asymptomatic stage where LV dilatation, hypertrophy, and diastolic dysfunction are common features, which eventually lead to systolic dysfunction and heart failure in the symptomatic stage (Piano, 2002). Chronic alcohol consumption may produce reduced systolic function as a consequence of intracellular organelle dysfunction, alterations in contractile proteins, disruption of calcium homeostasis, and cardiomyocyte loss (George and Figueredo, 2011; Laonigro *et al.*, 2009). Importantly, with respect to cardiomyocyte loss, there is significant evidence demonstrating that cardiomyocyte apoptosis may be a fundamental change with chronic alcohol consumption (Fernández-Solà *et al.*, 2006; Guan *et al.*, 2004; Hajnóczky *et al.*, 2005; Jing *et al.*, 2012). Furthermore, an alcoholic cardiomyopathy is associated with mitochondrial dysfunction (Hajnóczky *et al.*, 2005) and

oxidative stress (Guo and Ren, 2010; Jing *et al.*, 2012; Kalaz *et al.*, 2012). Thus, chronic ethanol consumption is associated with prominent features of telomere attrition such as oxidative stress (Houben *et al.*, 2008), mitochondrial dysfunction (Sahin *et al*., 2011), and apoptosis (Artandi and Attardi, 2005). Hence, telomere attrition may be associated with the pathophysiological processes present in an alcoholic cardiomyopathy. Therefore, in the present study I investigated the relationship between telomere length and adverse LV remodelling in a animal model of chronic alcohol consumption.

3.3 METHODS

3.3.1 Animal model: This study was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (ethics number: 2011/18/05). Thirty eight male Sprague-Dawley (SD) rats at 3 months of age were studied. The experimental group (n=19) received drinking water with 5% (v/v) ethanol, whilst the control group (n=19) received normal water for a 4 month period. No pair-feeding was performed and no micro or macronutrient supplementation was provided as a previous study has demonstrated that 5% v/v ethanol in drinking water has no effect on food or fluid intake (Segel *et al.*, 1975), but is still capable of producing changes in the myocardium. Indeed, in the present study, as assessed over a 7 day period, neither food intake (ethanol group=24.6g.day^{-1} vs. control=23.9g.day^{-1}) nor fluid intake (ethanol group= 39.1ml.day^{-1} vs. control= 42.8ml.day^{-1}) differed between the groups. Importantly, in the present study the experimental rats consumed on average 2.4±0.3g ethanol.kg body weight^{-1}.day^{-1}. This is approximately the average ethanol intake known to produce an alcoholic cardiomyopathy in humans (>90g ethanol per day, or assuming an average body weight of 70 kg, 1.29 g ethanol.kg body weight^{-1}.day^{-1}) (Piano, 2002).

3.3.2 Echocardiography: To determine *in vivo* LV internal dimensions and function, echocardiography measurements were performed on all of the rats from the experimental and control groups (Woodiwiss *et al.*, 2001). Fifteen minutes after intraperitoneal injections of ketamine (75 $mg.kg^{-1}$) and xylazine (15$mg.kg^{-1}$) to anaesthetise animals, the chest area was shaved. A high resolution 7.5-MHz linear array transducer attached to an Acuson Cyprus echocardiograph machine (Siemens AG, Munich, Germany) was used to obtain two-dimensional guided M-mode images (FIGURE 3.1) of the left ventricle at the level of the papillary muscles. Measurements were taken from three consecutive beats. All left ventricular dimensions were determined using the American Society for Echocardiography's leading edge method (Sahn *et al.*, 1978). All echocardiographic measurements were performed by the same experienced investigator who was blinded to the designation of animals to experimental or control groups.

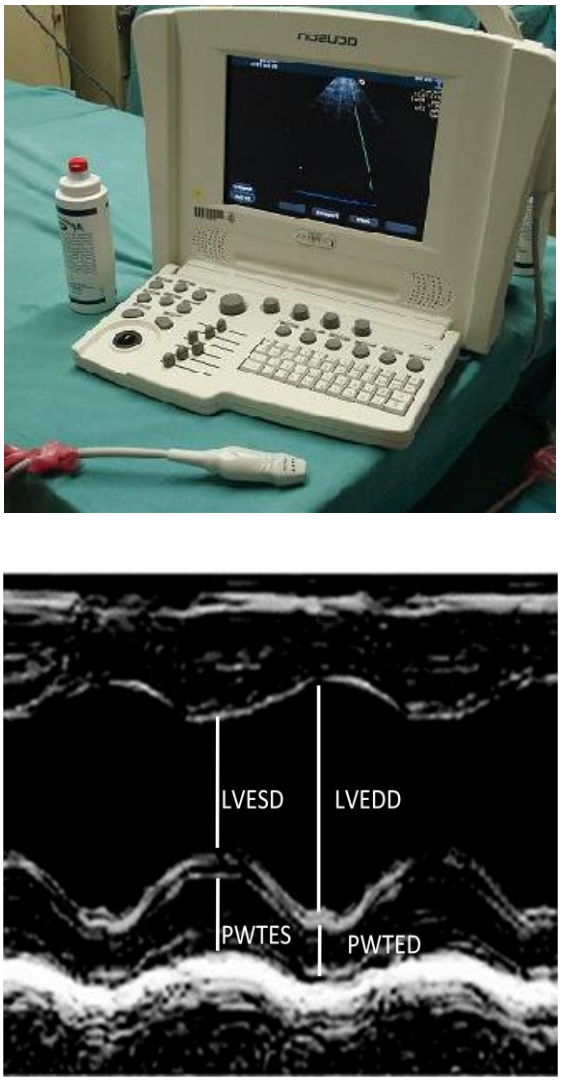

FIGURE 3.1 Accuson Cyprus echocardiograph machine and the high resolution 7.5-MHz linear array transducer (*upper panel*) employed to obtain echocardiography measurements from rats. A representative 2D M-mode image of the left ventricle obtained via echocardiography (*lower panel*). LVESD, left ventricular end systolic diameter; LVEDD, left ventricular end diastolic diameter; PWTES, posterior wall thickness at end-systole; PWTED, posterior wall thickness at end-diastole.

With respect to adverse cardiac remodelling, LV internal dimensions at end diastole were used as an index of LV dilatation. Moreover, relative wall thickness (RWT) was also calculated, which is an index of wall thinning where left ventricular wall thickness is expressed relative to internal chamber dimensions, and was calculated using the following formula:

RWT = (PWTED x 2)/ LVEDD

LVEDD, left ventricular end diastolic diameter; PWTED, posterior wall thickness at end-diastole

With respect to systolic LV function, midwall fractional shortening (FS_{mid}) and endocardial fractional shortening (FS_{end}) are load-dependant indices of left ventricular intrinsic myocardial function and chamber function respectively. FS_{end} is analogous to ejection fraction in that it represents the change in diameter in the left ventricle during a single heartbeat, thus representing the ability of the left ventricle to pump and hence chamber function. FS_{mid} is a measure of intrinsic myocardial function and differs from chamber function in that it refers specifically to the ability of cardiomyocytes to contract. FS_{mid} and FS_{end}, were calculated using the following formulae:

FS_{mid} = ((LVEDD + PWTED) - (LVESD +PWTES))/ (LVEDD + PWTED) *100

FS_{end} = (LVEDD-LVESD)/ LVEDD *100

LVESD, left ventricular end systolic diameter; LVEDD, left ventricular end diastolic diameter; PWTES, posterior wall thickness in end systole; PWTED, posterior wall thickness in end diastole.

3.3.3 Isolated, perfused heart preparations: Ten rats each from the experimental and control groups had e*x vivo* analysis of cardiac remodeling and function performed using Langendorff isolated, perfused heart preparations (Weber *et al.*, 1988; Woodiwiss *et al.*, 2001). Langendorff preparations always took place after echocardiography. Hearts were removed via thoracotomy and rinsed in ice cold physiological solution (118.0mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 10.0 mM glucose). The hearts were then immediately mounted onto a perfusion apparatus (FIGURE 3.2) and retrogradely perfused through the aorta at a constant rate with the same physiological solution, which was warmed to 37 °C and was saturated with 95% O_2 and 5% CO_2. The flow rate was determined volumetrically over thirty seconds and was adjusted to achieve a flow rate of 12ml.min^{-1}.g heart weight^{-1}. Platinum wire electrodes were placed on the right atrium and at the apex of the heart and were paced at a constant rate of 330 beats per minute.

Left ventricular developed pressure and diastolic pressures were determined using a water-filled balloon-tipped cannula coupled to a Gould P50 pressure transducer that was inserted into the left ventricular cavity via the left atrium. The volume of the balloon was known, was kept constant throughout each study, and was corrected for when determining pressure-volume relationships. The left ventricular pressures were recorded using a Hellige polygraph. A micromanipulator was used to increase the volume of the water-filled balloon and thus left ventricular volumes until there were no further increases in left ventricular developed pressures (difference between systolic and diastolic pressures). This technique was performed to determine cardiac function under controlled heart rates, loading conditions, and without any interference of anaesthetic drugs. All Langendorff heart preparations were performed by the same experienced investigator throughout, who was blinded to the designation of animals to experimental and control groups.

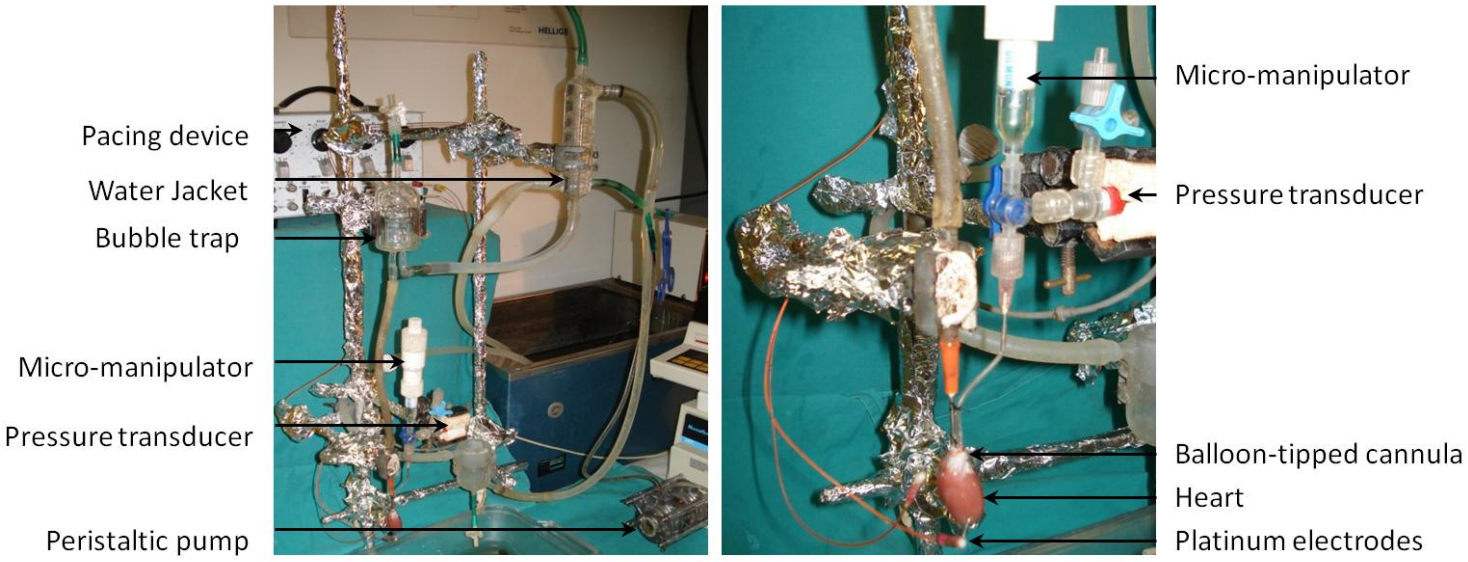

FIGURE 3.2 The perfusion apparatus used for all the Langendorff heart preparations (*left panel*). The water jacket, fed from the water bath, was used to warm the physiological solution to 37 °C. The bubble trap ensured that no air bubbles reached the coronary circulation. The peristaltic pump was use to perfuse the heart with the physiological solution. The figure in the *right panel* is a close up viewillustrating the heart; platinum electrodes that kept heart rates constant, the water-filled balloon tipped cannula, the micro-manipulator used to steadily increase the volume of the balloon, and the pressure transducer used to record pressures in the left ventricle.

Left ventricular remodelling was assessed from the LV diastolic pressure-volume (P-V) relationships, using the volume intercept at zero mmHg (V_0) for comparative purposes. A dilated ventricle requires a greater volume of blood to fill the ventricle and cause a subsequent increase in LV pressure during diastole. Thus, a greater V_0 is an index of a larger chamber cavity and by inference left ventricular dilatation. The V_0 value for each animal was determined from the Hellige polygraph data at the point of first increase in diastolic pressure as the volume of the water-filled balloon (left ventricular volume) was increased.

LV systolic chamber performance was determined from the left ventricular (LV) developed pressure-volume (dP-V) relationships. The LV dP-V relationship was determined using the Hellige polygraph data, where systolic and diastolic pressures for every increment in volume were measured. A load independent measure of LV systolic chamber function was determined from the slope of the linear portion of the LV systolic dP-V relationship (LV end systolic elastance-E_{es}). In addition, a load independent measure of intrinsic myocardial systolic function was assessed by constructing LV developed stress-strain relationships and comparing the slopes (LV systolic myocardial elastance-E_{en}) of these relationships (Weber *et al.*, 1988). LV developed stress and strain were calculated using the following equations:

$$\text{Stress} = (1.36\ P\ V_m)/((V + V_m)^{2/3} - V^{2/3})$$

$$\text{Strain} = ((V^{1/3} + (V + V_m)^{1/3})/(V_0^{1/3} + (V_0 + V_m)^{1/3})) - 1$$

P, pressure; V_m, left ventricular muscle weight; V_0, systolic volume intercept at zero mmHg (Weber *et al.*, 1988).

3.3.4 Blood sampling: Blood samples for DNA extraction were collected from the tail vein at monthly intervals. In order to collect blood samples, rats were placed in restrainers

and their tails were cleaned with a disinfectant alcohol spray. A 24 gauge JELCO intra-venous catheter (Smith's Medical, London, United Kingdom) was used to collect blood from the tail vein of the rats. 200 µl of blood was collected from the open end of the catheter and transferred to Eppendorf tubes containing 10 µl of heparin. The Eppendorf tubes were then centrifuged at 8 000xg for 10 minutes to collect plasma samples. Care was taken to ensure that no white or red blood cells were collected with the plasma samples.

Blood samples were also collected in heparinised tubes at the termination of the animals. These tubes were subsequently centrifuged in order to obtain plasma samples. All blood and plasma samples were stored at -80 °C until analysed. In addition, fasting blood samples were also collected in sodium fluoride tubes for the determination of fasting blood glucose concentrations using a glucose oxidase based assay, which was conducted by an external laboratory accredited for good laboratory procedures.

3.3.5 Urine collection: Urine samples were collected in the morning prior to each monthly collection of blood samples. The collection of urine and blood samples took place on the same day to ensure the variables measured in each of these respective body fluids were temporally matched. Metabolic chambers (FIGURE3.3) were employed for the collection of urine. These chambers are designed for the separation and collection of urine and faeces respectively.

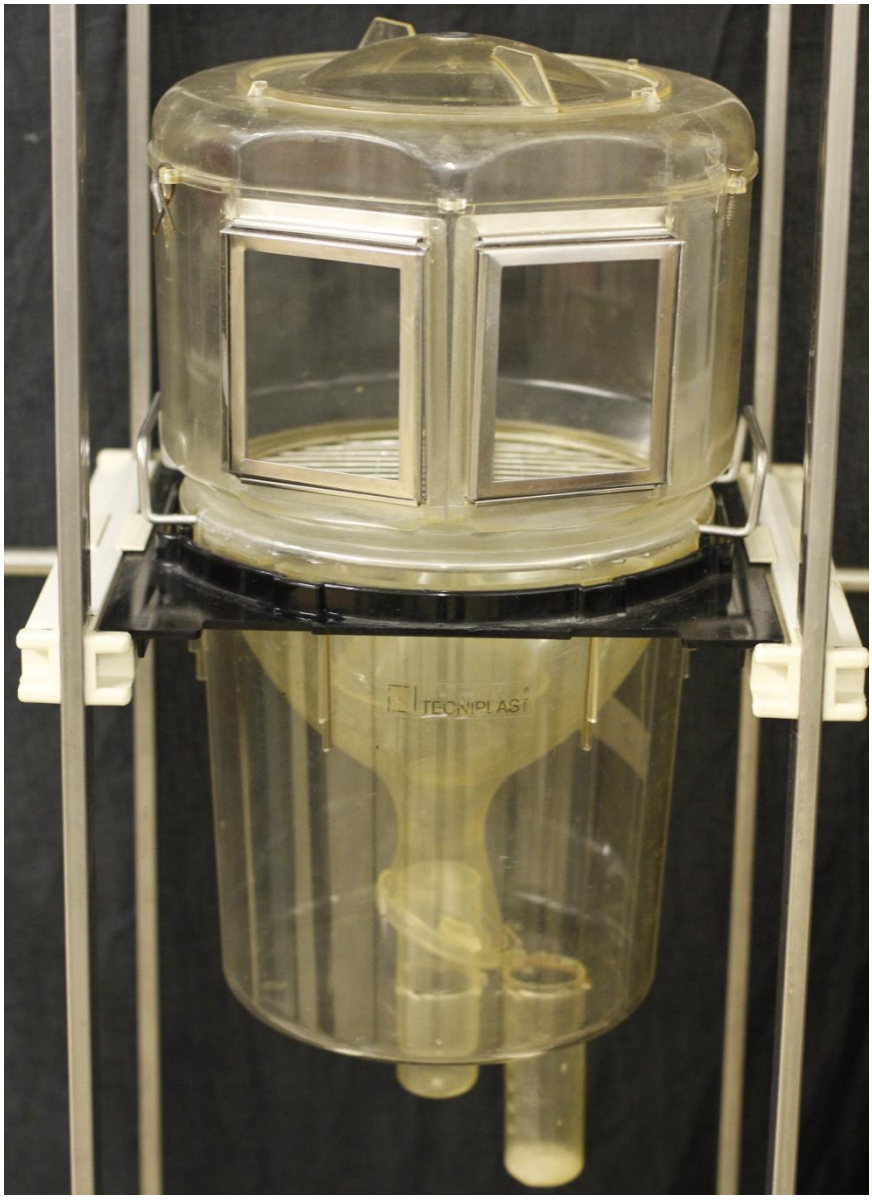

FIGURE 3.3 Metabolic chamber used to collect urine samples from animals.

Urine samples were also collected at termination. After a thoracotomy was performed to remove the heart, the incision for the thoracotomy was continued down the length of the abdomen until the bladder was exposed. Urine samples were collected directly from the bladder using a syringe.

0.05% (v/v) butylated hydroxytoluene (BHT) in ethanol was added to all urine samples collected. BHT is an organic anti-oxidant, which prevented oxidation of urine samples during storage. All urine samples were stored at -80°C until analysed.

3.3.6 DNA isolation: Genomic DNA from stored whole blood was extracted using NucleoSpin® Blood QuickPure kits (Macherey-Nagel, Dueren, Germany) as per manufacturer's instructions. DNA was released from leukocytes by incubating samples at 70 °C for one hour in the lysis and Proteinase K solutions provided in the kit. Ethanol was then added to each sample, which alters the binding conditions and allows DNA to bind to the silica membrane present in the NucleoSpin®columns. The columns were then centrifuged at 11 000xg for one minute to discard protein in the samples. The columns were then washed twice using the wash buffers provided in the kit. During the wash stages any protein that was present on the silica membrane was removed. The NucleoSpin® columns were then placed into sterile 1.5 ml Eppendorf tubes, elution buffer (warmed to 70 °C) was added to each column, samples were incubated at room temperature for three minutes and the tubes were then centrifuged at 11 000xg for one minute. The elution buffer modified the binding conditions of the DNA in the silica membrane and ensured that the pure DNA present in the NucleoSpin® columns was transferred to the Eppendorf tubes.

Genomic DNA from cardiac, liver and kidney tissue samples were extracted using NucleoSpin® Tissue kits (Macherey-Nagel, Dueren, Germany), as per manufacturer's instructions. Twenty five milligrams of tissue sample was homogenised using a TissueRuptor

homogeniser (Qiagen, Hilden, Germany) in phosphate buffered saline (PBS) (NaCL 137mM, KCl 2.7 mM, Na_2HPO_4 10mM, KH_2PO_4 1.8mM). Once the samples were homogenised, DNA was released from cells by incubating samples at 56 °C overnight in the lysis and Proteinase Ksolutions provided in the kit. The following day, the samples were vortexed and the samples were further lysed with another incubation in the lysis solution. Ethanol was then added to each sample to alter the binding conditions of the DNA. The samples were then loaded into the NucleoSpin®columns, which contain a silica membrane that DNA binds to. The columns were centrifuged at 11 000xg and all of the protein that was not bound to the silica membrane was discarded. The columns were then washed twice using the wash buffers provided in the kit to ensure that no protein was bound to the silica membrane. The NucleoSpin® columns were then placed into sterile 1.5 ml Eppendorf tubes, and the elution buffer (warmed to 70°C) provided in the kit was added to each column, samples were incubated at room temperature for three minutes and the NucleoSpin® columns were then centrifuged at 11 000xg for one minute. The elution buffer modified the binding conditions of the DNA in the silica membrane and ensured that the pure DNA present in the NucleoSpin® columns were transferred to the Eppendorf tubes.

3.3.7 Blood pressure measurement: Blood pressure in rats was determined one week prior to termination using a CODA non-invasive tail-cuff blood pressure system designed for laboratory animals (Kent Scientific Corporation, Connecticut, USA.) (FIGURE 3.4). This system usesvolume pressure recording (VPR). An occlusion cuff (O-cuff) inflates and restricts blood flow to the tail and then steadily deflates while the VPR cuff measures the changes in blood flow in the tail. The pressure of the O-cuff at the first instance of blood return to the tail corresponds to systolic blood pressure. The point at which the increase in blood volume to the tail ceases is the point at which diastolic blood pressure is defined.

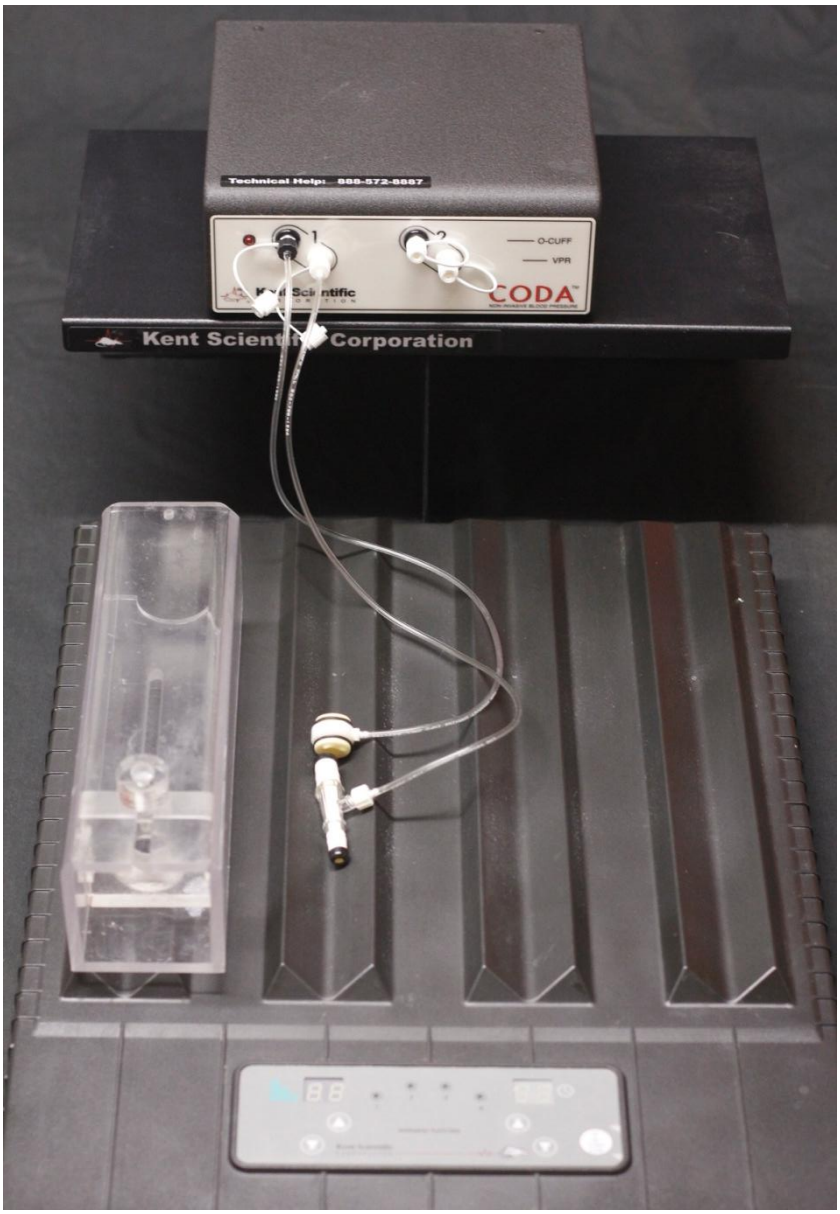

FIGURE 3.4 CODA (Kent Scientific Corporation, Connecticut, USA.)non-invasive blood pressure system employed to record blood pressure in rats.

An hour prior to blood pressure measurements, a heating pad was switched on and rat restrainers were placed on the pad in order to ensure they were at the correct temperatures for blood pressure measurements. The temperature in the animal's tails required for recordings was approximately 30°C. Once the restrainers and room were at the appropriate temperature, the rats were placed into the restrainers and the O-cuff was placed as close to the base of the tail as possible followed by the VPR cuff. The animals were then allowed to acclimatise to the restrainers and cuffs for 15 minutes prior to blood pressure measurements. A cloth was placed over the animals to reduce stress and ensure that the temperatures remained constant. It was not necessary to acclimatise the animals to the restrainers prior to blood pressure measurements as these animals were already acclimatised to the restrainers due to the blood sampling that took place once a month (see Section 3.3.4). Each animal had 20 blood pressure measurements recorded over 10 minutes. The first 10 recordings were acclimatisation recordings and were not included in the data analyses.

3.3.8 Telomere length measurement: Relative telomere length in leukocytes, and in the DNA of the LV lateral wall of the myocardium, liver, and kidney tissue was determined. The qRT-PCR methodology employed to determine average relative telomerelength in the IDC study was used to determine relative telomere length in this study (see Section 2.3.3), with the only modification being in the single copy gene employed. The single copy gene used for rat DNA was pyruvate kinase, as this gene has been used as a single copy gene in other studies conducted in rat DNA (Walters *et al.*, 2009; Ross-Inta *et al.*, 2009). The PCR reactions were carried out using LightCycler FastStart DNA Master SYBR I Green kits. The thermal profile and reaction mixes were the same described for 36B4 (see Section 2.3.3) except for the primer sequences for pyruvate kinase (Walters *et al.*, 2009), which were:

Pyruvate kinase forward primer: 5'-tgtgggtgatctggtgattgtggt-3'

Pyruvate kinase reverse primer: 5'-aggcatttcaggatacgctcagca-3'

As with the IDC study, standard curves were generated for both telomere and pyruvate kinase reactions (FIGURE 3.5) from a single calibrator that was serially diluted to produce a range of DNA quantities from 200 ng to 5 ng of DNA. The regression coefficients were 0.989 and 0.996 for the telomere and pyruvate kinase standard curves, respectively, and the coefficients of variation of the S and T assays were 0.86% and 2.09%, respectively. Similar to the IDC study, the standard curves for the animal studies illustrate that there was a strong negative linear relationship between DNA concentration and crossing point. 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.

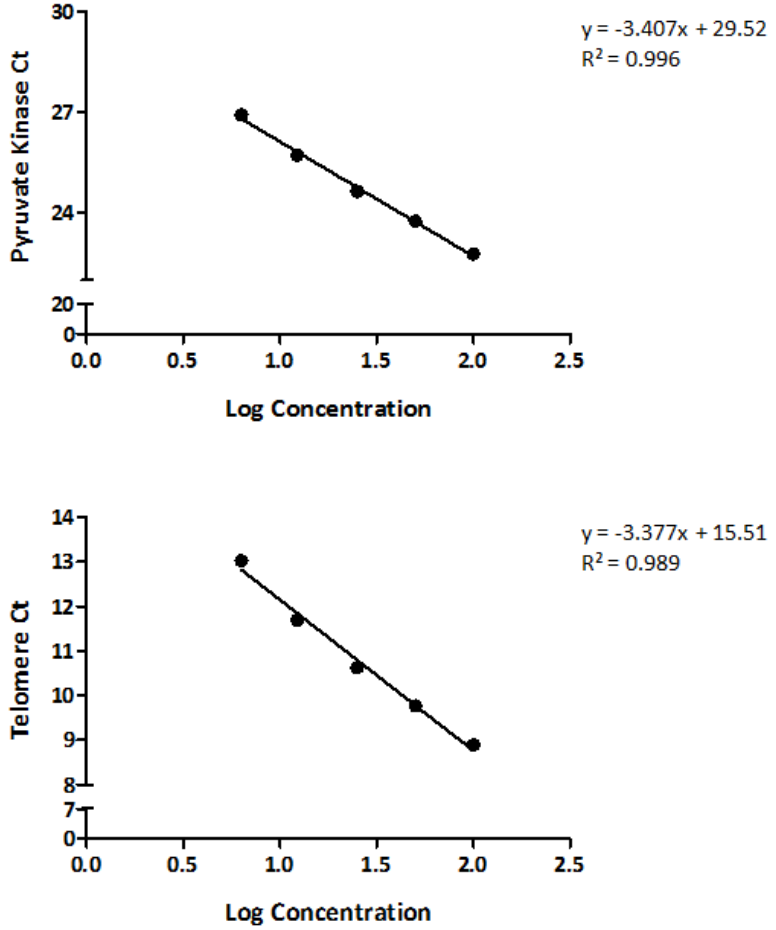

FIGURE 3.5 A plot of crossing point (Ct) versus log concentration for the pyruvate kinase (*upper panel*) and telomere (*lower panel*) standard curves respectively. The DNA dilution series was from 200ng to 5 ng of DNA.

3.3.9 Assay for C-reactive protein concentration: Plasma CRP concentrations were determined using a commercially available rat-specific CRP enzyme linked immuno-adsorbent assay (ELISA) as per manufacturer's instructions (eBioscience, San Diego, USA.). Samples were diluted 1:10 000 prior to loading in the pre-coated 96 microwell plate and incubated at room temperature for two hours. The CRP present in the rat plasma samples was bound to the walls of the microwell plate during this incubation period. The plate was washed using a Thermo Labsystems original wellwash 4 (Agilent Technologies Inc., Santa Clara, USA) ELISA plate washer during which all substrates not bound to the microwell plate were removed. A rat CRP antibody that was conjugated to horseradish peroxidase was added to each well and incubated for a further hour. During this incubation period the rat CRP antibody bound to the CRP present in the wells. The plate was then washed again, allowing for the removal all unbound rat CRP antibody, and a 3,3',5,5'-tetramethylbenzidine substrate solution was added. The tetramethylbenzidine solution reacts with the horseradish peroxidase, that is conjugated to the rat CRP antibody, to produce a blue colour. The intensity of the colour produced via thehorseradish peroxidase and tetramethylbenzidine interactions was proportional to the amount of CRP present in each well. This reaction was allowed to proceed for five to ten minutes before a stop solution provided in the kit was added. Absorbance readings were recorded using a Thermo Labsystems Multiskan Ascent ELISA plate reader (Agilent Technologies Inc., Santa Clara, USA) set at a wavelength of 570 nm. CRP concentrations were then determined from a standard curve, which was plotted from standard calibrators (66 $ng.ml^{-1}$ to 2 $ng.ml^{-1}$ of CRP), which were supplied with the kit.

3.3.10 Assay to assess oxidative stress: All urine samples were purified using a solid phase extraction (SPE) protocol previously described (Dahl and van Breemen, 2010).Urine samples were thawed, vortexed, and centrifuged for 3 min at 3500xg to separate particulates that may be present in the urine. Three and a half millilitres of 50 mMTris–HCl buffer (pH

6.00, using 50 mMTris base) was added to each 0.5 ml urine sample. Then, Strata X-AW (60 mg, 3 ml) (Phenomenex, Torrance, USA.) SPE cartridges were placed onto a vacuum manifold (FIGURE 3.6) and preconditioned with 2 ml of 2% formic acid in methanol followed by 2 ml of water. Urine samples were then added to the SPE columns, and the cartridges were washed sequentially with, 2 ml of water, 4 ml of 25% methanol in water, and 2 ml of 100% acetonitrile. After the washing sequence, the cartridges were dried under vacuum and samples were then eluted with three elutions of 100% methanol each of 1ml, with 15 seconds of vacuum drying between each elution. The methanol was then completely evaporated using nitrogen and 8-isoprostanes were reconstituted in Tris buffer ($(HOCH_2)_3CNH_2$).

Oxidative stress was then assessed by the measurement of 8-isoprostanes from urine samples using STAT-8-Isoprostane EIA kits (Cayman Chemicals, Ann Arbor, USA.) as per manufacturer's instructions. This assay is based on the competition between 8-iosprostane and 8-isoprostane-alkaline phosphatase conjugate (tracer) for 8-isoprostane specific binding sites on rabbit antiserum. The concentration of the tracer remains constant in each well while the concentration of 8-isoprostane in the sample varies. Therefore the greater the concentration of 8-isoprostane contained in the sample the greater the competition for binding sites on rabbit antiserum. Thus the concentration of tracer that is bound to the rabbit antiserum is inversely proportional to 8-isoprostane concentration that is contained in the sample.

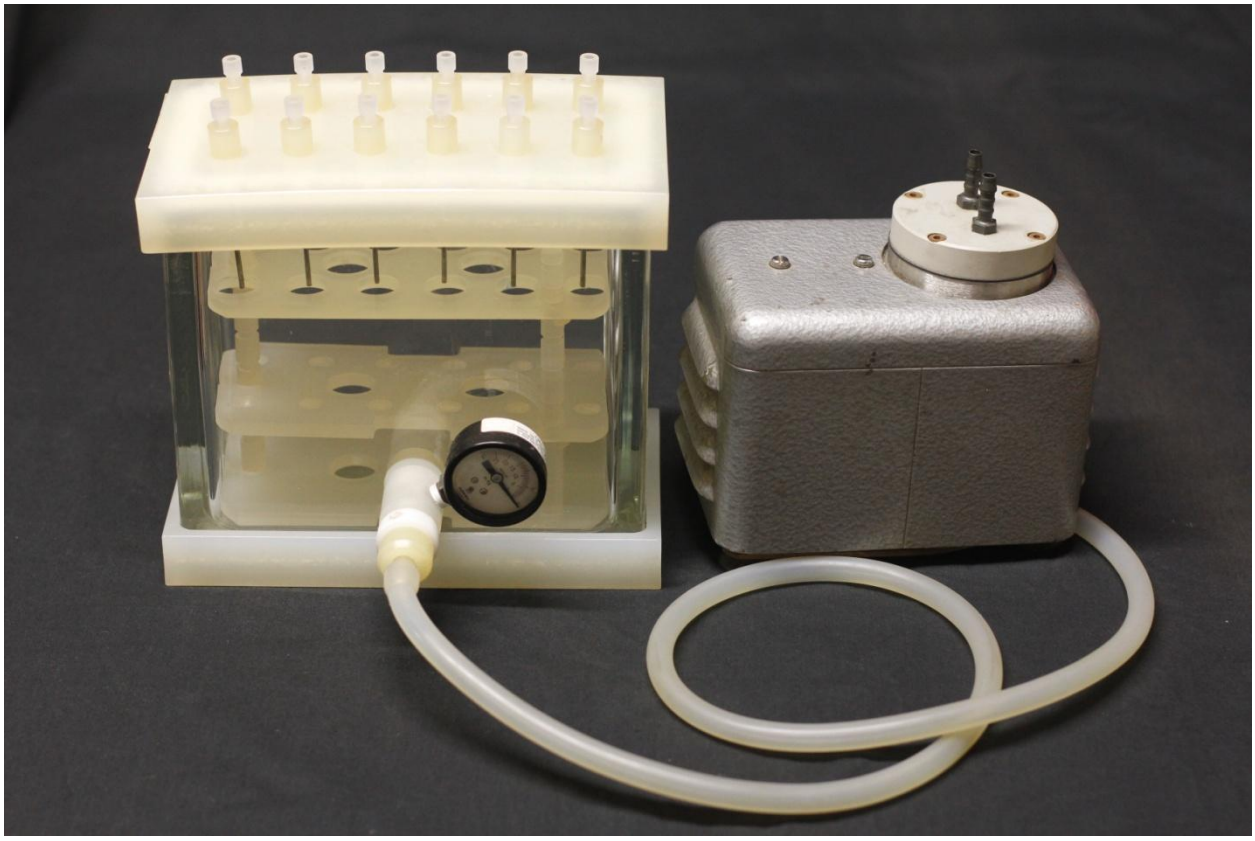

FIGURE 3.6 Vacuum manifold attached to a pump that allowed fluids to be drawn through the Strata X-AW (60 mg, 3 ml) solid phase extraction cartridges.

The 8-isoprostane calibrators provided with the kit were prepared to produce standards ranging from 3000 $pg.ml^{-1}$ to 23.4 $pg.ml^{-1}$. The 96 microwell plate was then loaded following the manufacturer's protocol. After all the samples and reagents were loaded, the plate was covered with a plastic film and incubated at room temperature for one hour on an orbital shaker. The plate was then washed five times using a Thermo Labsystems original wellwash 4 (Agilent Technologies Inc., Santa Clara, USA) ELISA plate washer and p-nitrophenol phosphate solution provided in the kit was added to each well and produced a yellow colour. The plate was subsequently covered with a plastic film and incubated at room temperature for an additional hour. The plate was periodically checked and when the absorbance values of the maximum binding wells were in the range of 0.3-1.0, the plate was read in a Thermo Labsystems Multiskan Ascent ELISA plate reader (Agilent Technologies Inc., Santa Clara, USA) at a wavelength of 417nm.

3.3.11 Determination of cardiomyocyte apoptosis: After termination of rats, LV tissue was stored in 10% phosphate buffered formaldehyde. Myocardial tissue samples were then embedded in paraffin wax and five µm tissue sections were subsequently cut and fixed to microscope slides. Apoptotic cell death was determined in LV sections prepared for light microscopy using a non-radioactive *in situ* apoptotic cell death detection kit (DeadEndTM Colorimetric TUNEL system, Promega, Madison,WI, USA). This assay labels the ends of fragmented DNA in apoptotic nuclei using terminal deoxynucleotidyl transferase (rTdT) mediated dUTP Nick-End Labeling (TUNEL). Biotinylated nucleotides are incorporated at the 3'-OH ends of DNA using the TdT enzyme. Horseradish peroxidase labeled streptavidin is then bound to these biotinylated nucleotides, which are visualised using peroxidase substrate, hydrogen peroxide and diaminobenzidine.

Briefly, the TUNEL staining procedure was as follows: slides were deparaffinised with two five minute incubations in 100% xylene. The tissue sections were then rehydrated

with immersion in graded ethanol of (100%-50%) for three minutes each. Tissue sections were then incubated in PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH = 7.4) for five minutes and then fixed to microscope slidesby immersion in 4% paraformaldehyde for 15 minutes. A 20 $\mu g.ml^{-1}$ solution of Proteinase K was then added to the tissue sections and incubated for 30 minutes at room temperature in order to break down cell walls. The sections were then washed in PBS for five minutes and refixed to slides in 4% paraformaldehyde for an additional five minutes. Subsequently, the tissue sections were washed again in PBS for five minutes and the sections were covered with an equilibration buffer and incubated for 10 minutes. The rTdT reaction mix was then added and the sections were covered with a plastic film and incubated at 37ºC for an hour inside a humidified chamber to allow for labeling of fragmented DNA to occur. Labeling was stopped with a 15 minute incubation in saline-sodium citrate (3 M NaCl, 300 mM $Na_3C_6H_5O_7$). The sections were then washed twice in PBS for 5 minutes and endogenous peroxidases were blocked with 0.3% hydrogen peroxide for five minutes followed by another wash in PBS for five minutes. Horseradish-peroxidase-labeled streptavidin was then added to the sections and incubated for 30 minutes. The sections were subsequently washed in PBS for five minutes and hydrogen peroxide and diaminobenzidine solutions were added to each section and developed until there was a light brown background in the tissue section. The slides were then rinsed in deionised water several times and dehydrated in graded ethanol (50%-100%). The sections were then incubated twice in 100% xylene for five minutes, covered in distrene xylene/dibutylphthalate xylene (DPX) fixative and coverslips were then placed over the tissue sections. Apoptotic nuclei were stained brown and visible under a microscope after TUNEL staining (FIGURE 3.7). Positive (DNase treated) and negative (no addition of rTdT enzyme) control sections were incorporated in each assay.

The number of apoptotic cardiomyocyte nuclei and total cardiomyocyte nuclei were then determined using a computer-based image acquisition system (Axiovision 3, Carl Zeiss, Gottingen, Germany). The total number of cardiomyocyte nuclei, visualised using haematoxylin and eosin staining (FIGURE 3.7), present in each slide were counted on ten evenly spaced fields from the apex to base using acomputer based image acquisition and analysis system (Osadchii *et al.*, 2007). The degree of apoptosis was then expressed as a percentage of total cardiomyocyte nuclei. All data were obtained by the same investigator who was blinded to the designation of histology slides to experimental and control groups.

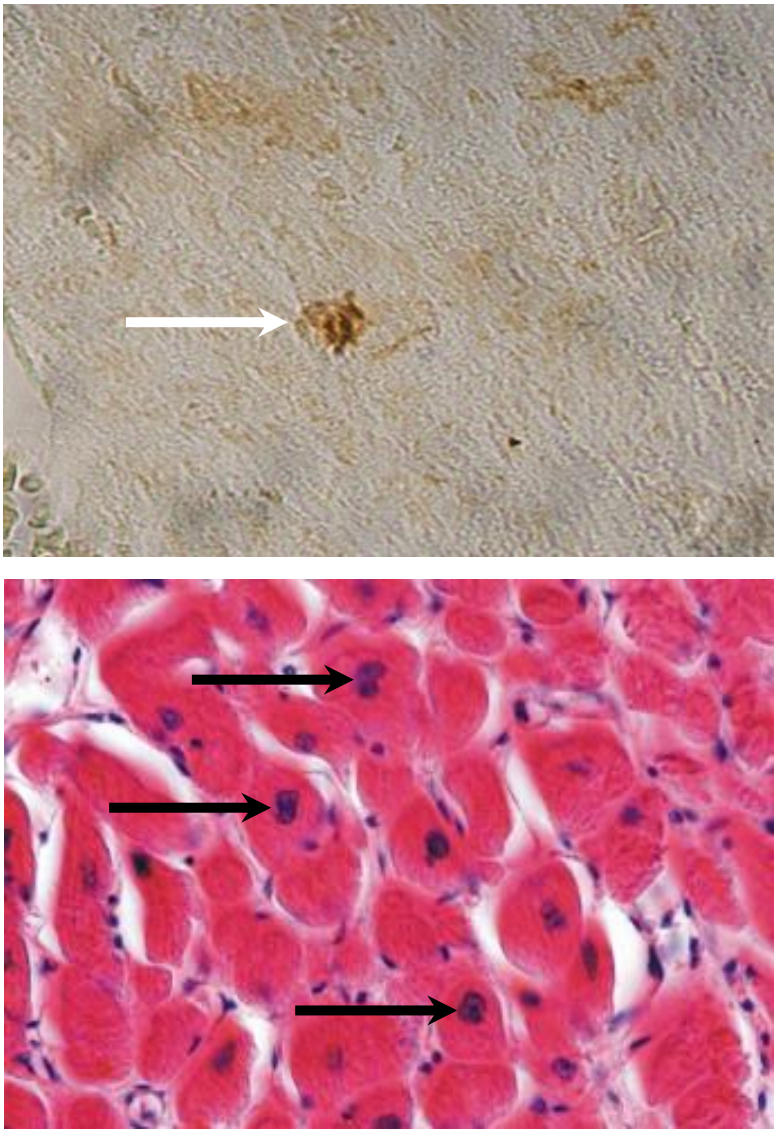

FIGURE 3.7 A representative image of an apoptotic nuclei stained using TUNEL staining (white arrow) (*upper panel*) and cardiomyocyte nuclei stained using haematoxylin and eosin staining (black arrows) (*lower panel*).

***3.3.12 Statistical analysis*:** All statistical analyses were performed on log transformed T/S ratios. All analyses were conducted using a Student's paired and unpaired t-test where appropriate. All associations between variables were conducted using Pearson's correlation coefficients. A $p<0.05$ was considered to be statistically significant. Data are represented as mean±SD or mean±SEM where indicated.

3.4 RESULTS

3.4.1 Heart weight, blood pressure, and blood parameters: TABLE 3.1 shows body and heart weights, blood pressures and blood parameters of rats receiving ethanol-containing or normal drinking water. There were no differences in body weight, whole heart weight, and LV weight between the rats receiving ethanol versus controls (TABLE 3.1). Similarly, no differences in systolic and diastolic blood pressures, plasma CRP concentrations, and fasting blood glucose concentrations were noted between the groups receiving ethanol versus controls (TABLE 3.1).

TABLE 3.1 The effects of 4 months of ethanol consumption on body weight, heart weight, blood pressure, plasma C-reactive protein concentrations, and fasting blood glucose concentrations in rats.

	Ethanol (n=19)	**Control (n=19)**	**p value**
Body weight (g)	645.6±52.4	658.5±51.2	0.45
Whole heart weight (g)	1.56±0.19	1.62±0.20	0.34
Left ventricular weight (g)	1.28±0.16	1.34±0.16	0.24
Systolic blood pressure (mmHg)	130±11	137±14	0.17
Diastolic blood pressure (mmHg)	101±12	97±10	0.23
Plasma C-reactive protein ($ng.ml^{-1}$)	451.7±138.1	443.7±110.2	0.85
Fasting blood glucose ($mmol.l^{-1}$)	13.9±1.7	13.8±2.0	0.91

Values are represented as mean±SD.

3.4.2 Telomere length: The T/S ratios of leukocyte DNA were greater, which indicates longer leukocyte telomere length, in blood samples obtained at 4 months as compared to 1 month of the study (TABLE 3.2). This relationship was present in both the rats receiving ethanol and those receiving water for 4 months (TABLE 3.2). However, no significant differences in the T/S ratios were noted between the rats receiving ethanol or normal water when assessed at either the first month, or at 4 months of the study (TABLE 3.2). Furthermore, there was no significant difference in the change in telomere length from the first to the fourth month of the study between the experimental and control groups (TABLE 3.2). Moreover, the T/S ratios in cardiac, kidney, and liver tissues, were not significantly different between the ethanol and control groups (TABLE 3.2). Leukocyte telomere length at termination (4 months) was correlated with telomere length in cardiac (Pearson's r=0.49, r^2=0.24, p=0.0021), and kidney (Pearson's r=0.52, r^2=0.27, p=0.0017), but not with telomere length in liver tissue (Pearson's r=0.032, r^2=0.0010, p=0.87). Furthermore, neither indices of LV dilatation (LVEDD, V_0), LV systolic chamber function (FS_{end}, LV E_{es}) nor inflammation (CRP) were correlated with cardiac T/S ratios (TABLE 3.3).

TABLE 3.2 The effects of 4 months of ethanol consumption on relative telomere length, as indexed by log T/S, in leukocyte, cardiac, liver, and kidney tissues in rats.

	Ethanol (n=19)	Control (n=19)	p value
Leukocyte T/S month 1	-0.76±0.58	-0.79±0.51	0.87
Leukocyte T/S month 4	-0.22±0.11*	-0.19±0.10*	0.33
Δ Leukocyte T/S from month 1	-0.38±0.85	-0.62±0.49	0.38
Cardiac T/S	-0.19±0.10	-0.15±0.089	0.29
Liver T/S	-0.21±0.11	-0.20±0.13	0.86
Kidney T/S	-0.20±0.20	-0.18±0.20	0.78

Δ, percentage change in leukocyte telomere length from one month to four months. Values are represented as mean±SD; *$p<0.05$, month 4 versus month 1 leukocyte T/S.

TABLE 3.3 Correlations between cardiac telomere length and indices of left ventricular (LV) dilatation (the volume intercept at 0 mmHg (V_0) in the left ventricular (LV) diastolic pressure-volume relations and LV end diastolic diameter (LVEDD)), systolic chamber function (slope of the systolic pressure-volume relations (LV E_{es}) and endocardial fractional shortening (FS_{end})), intrinsic myocardial function (slope of the systolic stress-strain relations (E_{en}) and midwall fractional shortening (FS_{mid})) and inflammation (C-reactive protein (CRP)).

	V_0	LVEDD	LV E_{es}	FS_{end}	LV E_{en}	FS_{mid}	CRP (month 4)
Ethanol							
Pearson's r	-0.11	0.041	0.11	-0.35	-0.076	-0.34	-0.26
95% CI	-0.75 to 0.66	-0.43 to 0.50	-0.64 to 0.76	-0.70 to 0.13	-0.70 to 0.62	-0.71 to 0.17	-0.68 to 0.29
p value	0.80	0.78	0.79	0.15	0.85	0.18	0.35
Control							
Pearson's r	0.16	-0.0056	-0.25	-0.36	0.098	-0.52	0.015
95% CI	-0.72 to 0.52	-0.46 to 0.45	-0.76 to 0.45	-0.70 to 0.12	-0.61 to 0.72	-0.70 to 0.07	-0.48 to 0.50
p value	0.66	0.98	0.48	0.14	0.80	0.26	0.95
Combined							
Pearson's r	-0.20	-0.098	-0.13	-0.31	0.044	-0.27	-0.011
95% CI	-0.61 to 0.32	-0.41 to 0.24	-0.57 to 0.35	-0.57 to 0.01	-0.43 to 0.50	-0.54 to 0.053	-0.35 to 0.33
p value	0.45	0.57	0.59	0.065	0.86	0.10	0.95

r, correlation coefficient; CI, confidence interval

3.4.3 LV remodelling: FIGURE 3.8 and TABLE 3.4 show the effect of chronic ethanol administration on indices of adverse LV remodelling. Chronic ethanol administration resulted in LV dilatation as illustrated by increases in LV end diastolic (FIGURE 3.8) and systolic diameters (TABLE 3.4), a right shift in the LV diastolic P-V relationship (FIGURE 3.8), and an increase in the LV V_0 (FIGURE 3.8) in rats receiving ethanol as compared to the control group. Furthermore, chronic ethanol administration resulted in LV wall thinning as indexed by a decrease in LV posterior wall thickness at end-diastole and a reduction in LV relative wall thickness in the group receiving ethanol (TABLE3.4).

3.4.4 LV systolic function: FIGURES 3.9 and 3.10 show the effect of chronic ethanol administration on LV systolic chamber (FIGURE 3.9) and intrinsic myocardial (FIGURE 3.10) function. Chronic ethanol administration failed to modify systolic LV chamber function as indexed by either the load and heart rate-dependent measure, FS_{end}, or the load- and heart rate-independent measure, E_{es} (slope of the linear portion of the LV systolic P-V relationship) (FIGURE 3.9). Moreover, chronic ethanol administration failed to modify LV intrinsic myocardial systolic function as indexed by either the load and heart rate-dependent measure, FS_{mid}, or the load- and heart rate-independent measure, E_n (slope of the LV systolic stress-strain relationship) (FIGURE 3.10).

3.4.5 Cardiomyocyte apoptosis: FIGURE 3.11 shows the effect of chronic ethanol administration on cardiomyocyte apoptosis as assessed using TUNEL staining and the relationships between cardiomyocyte apoptosis and indices of LV dilatation. Chronic ethanol administration resulted in an increase in the percentage of apoptotic nuclei present in cardiomyocytes (FIGURE 3.11). Furthermore, the percentage apoptosis was associated with both *ex vivo* (LV V_0) and *invivo* (LVEDD) indices of LV dilatation in the groups combined (FIGURE 3.11). Pearson's correlation coefficient between indices of LV dilatation and apoptosis was greater in the experimental group (r=0.42) compared to the control group (r=0.29). Importantly, neither leukocyte, cardiac, kidney, or liver telomere length was associated with percentage apoptosis (TABLE 3.5).

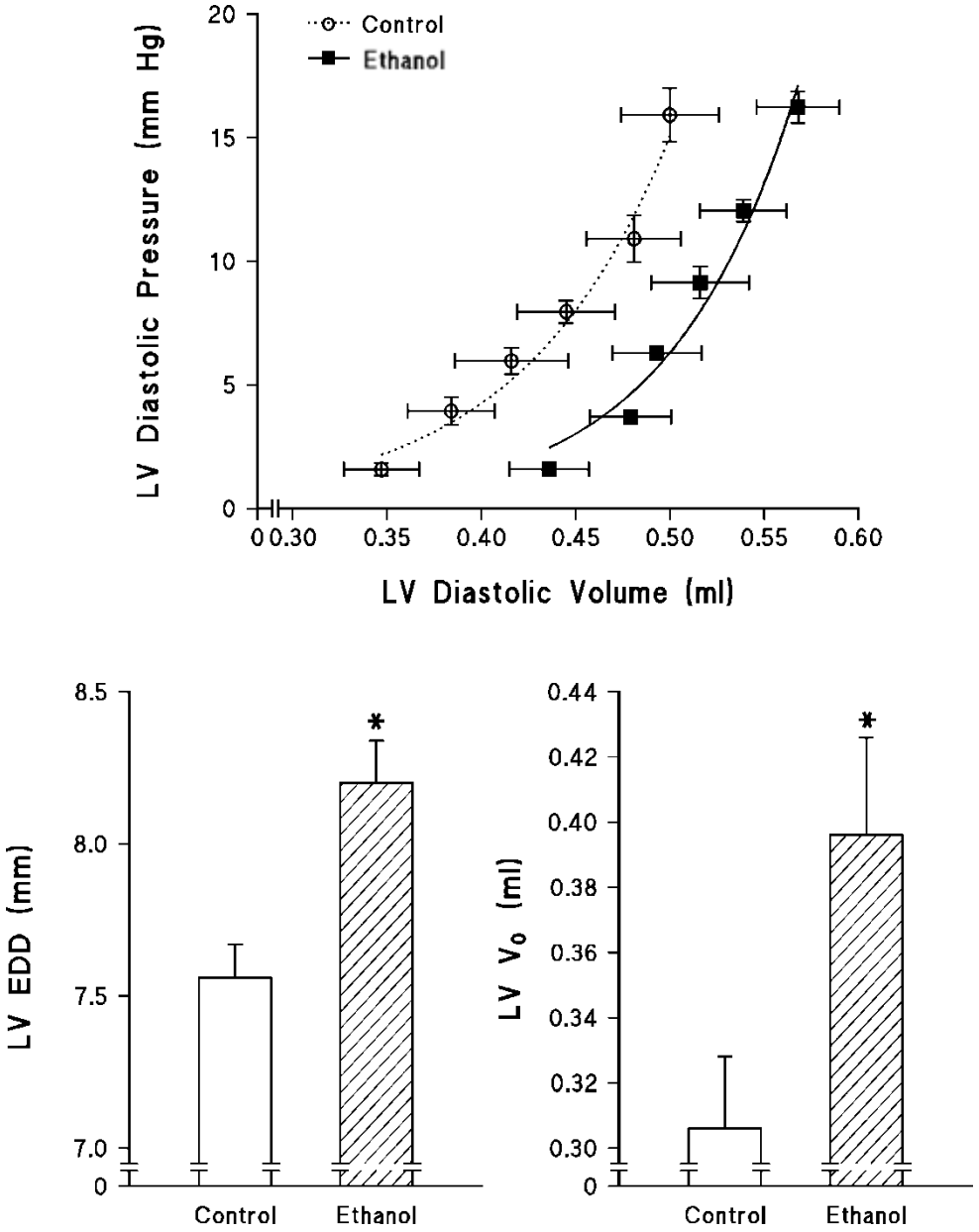

FIGURE 3.8 Effect of 4-months of ethanol consumption in rats on left ventricular (LV) chamber dimensions or volumes as indexed by LV end diastolic diameter (LVEDD) (*lower left panel*), LV diastolic pressure-volume relations (*upper panel*) and the volume intercept at 0 mmHg (V_0) (*lower right panel*) LV diastolic pressure. Data are represented as mean±SEM; *p<0.05 versus control.

TABLE 3.4 The effects of 4 months of ethanol consumption in rats on left ventricular (LV) dimensions.

	Ethanol (n=19)	Control (n=19)	p value
LVESD (mm)	4.6±0.8	4.2±0.4	0.034*
ESPWT (mm)	2.9±0.4	3.0±0.3	0.51
EDPWT (mm)	2.0±0.3	2.3±0.2	0.012*
RWT (mm)	0.5±0.08	0.6±0.09	0.0014*

LVESD, left ventricular end systolic diameter; EDPWT, end diastolic posterior wall thickness; ESPWT, end-systolic PWT; RWT, relative wall thickness. Values are represented as mean±SD;*$p<0.05$ versus ethanol and control groups.

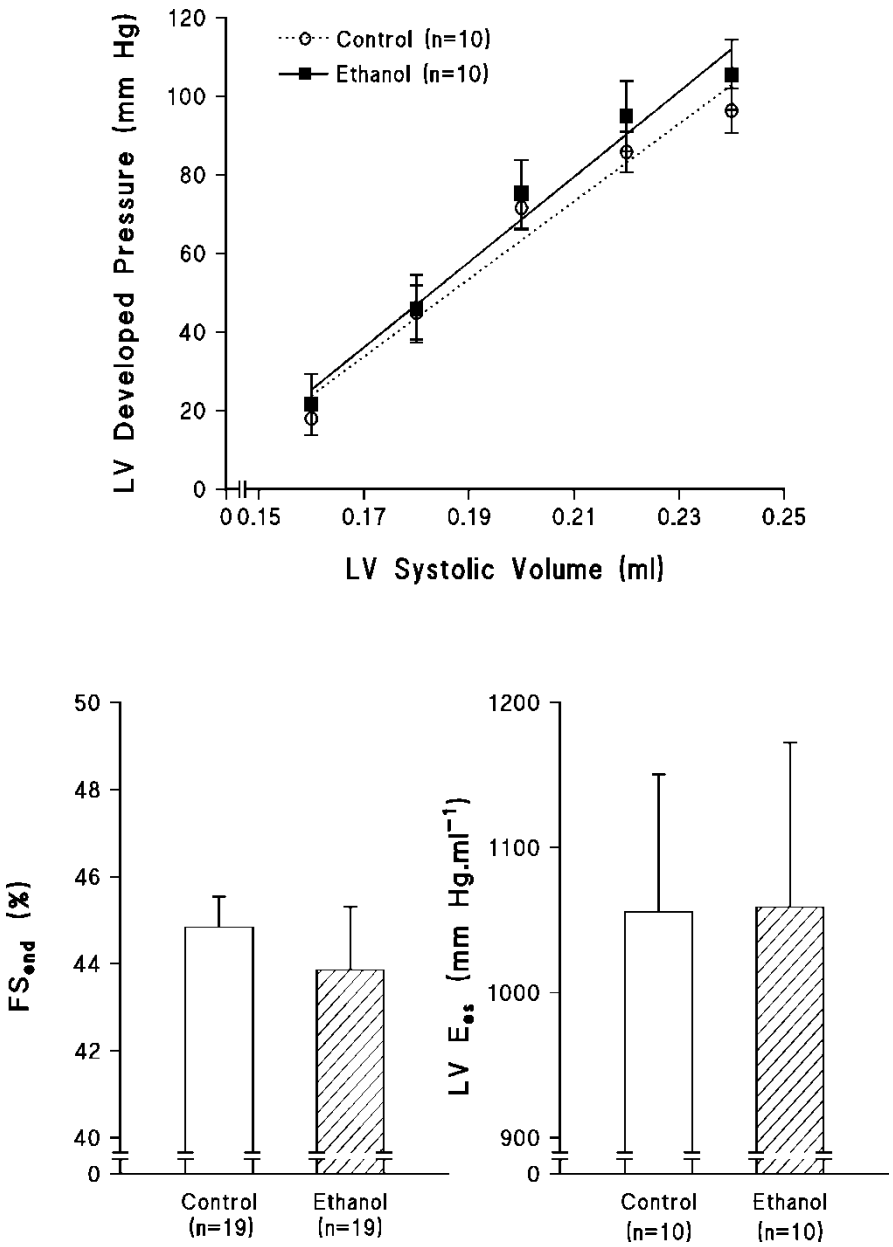

FIGURE 3.9 Effect of 4 months of ethanol consumption in rats on left ventricular (LV) systolic chamber function as indexed by LV endocardial fractional shortening (FS_{end}) (*lower left panel*), LV systolic pressure-volume relations (*upper panel*), and the slope of the systolic pressure-volume relations (LV E_{es}) (*lower right panel*). Data are represented as mean±SEM.

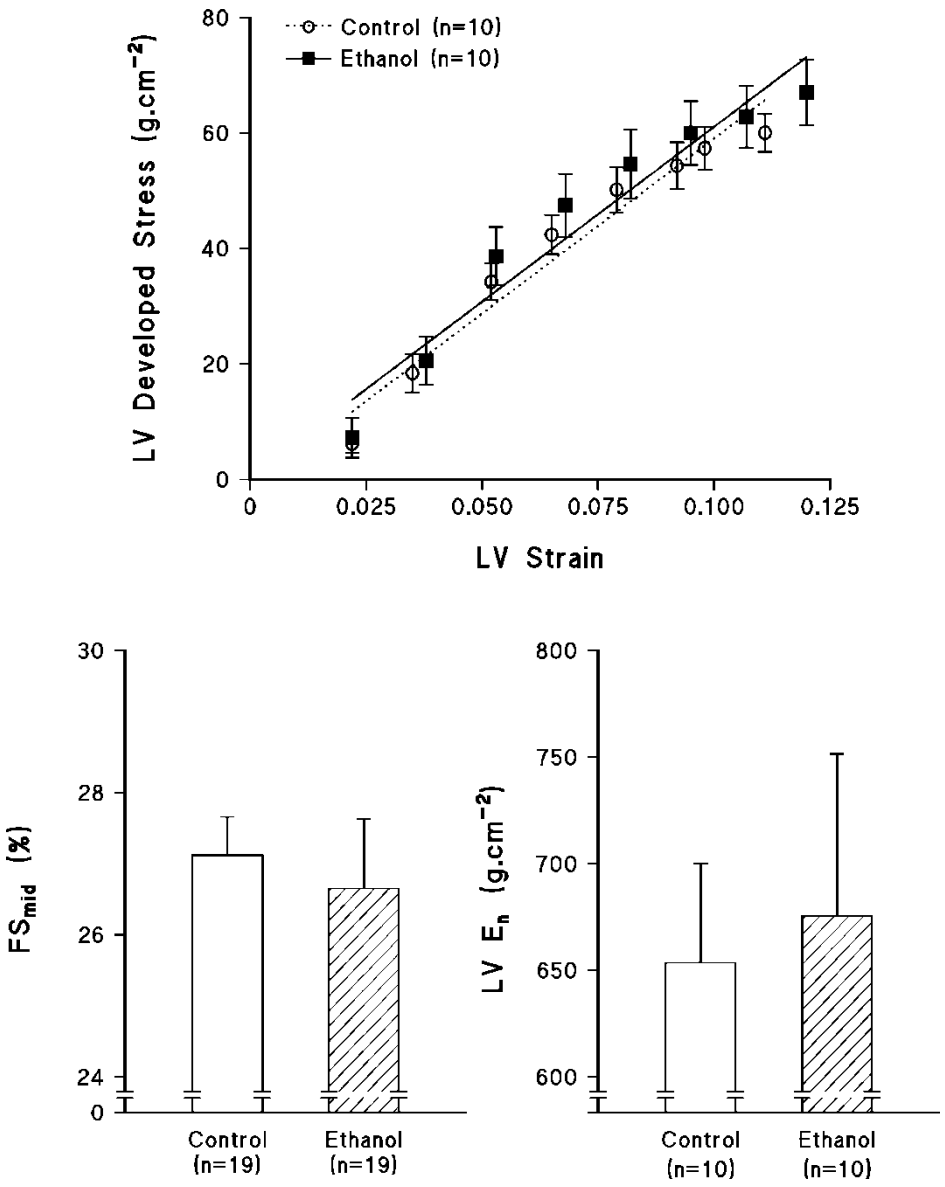

FIGURE 3.10 Effect of 4-months of ethanol consumption in rats on left ventricular (LV) intrinsic myocardial systolic function as indexed by LV midwall fractional shortening (FS_{mid}) (*lower left panel*), LV systolic stress-strain relations (*upper panel*), and the slope of the systolic stress-strain relations (LV E_n) (*lower right panel*). Data are represented as mean±SEM.

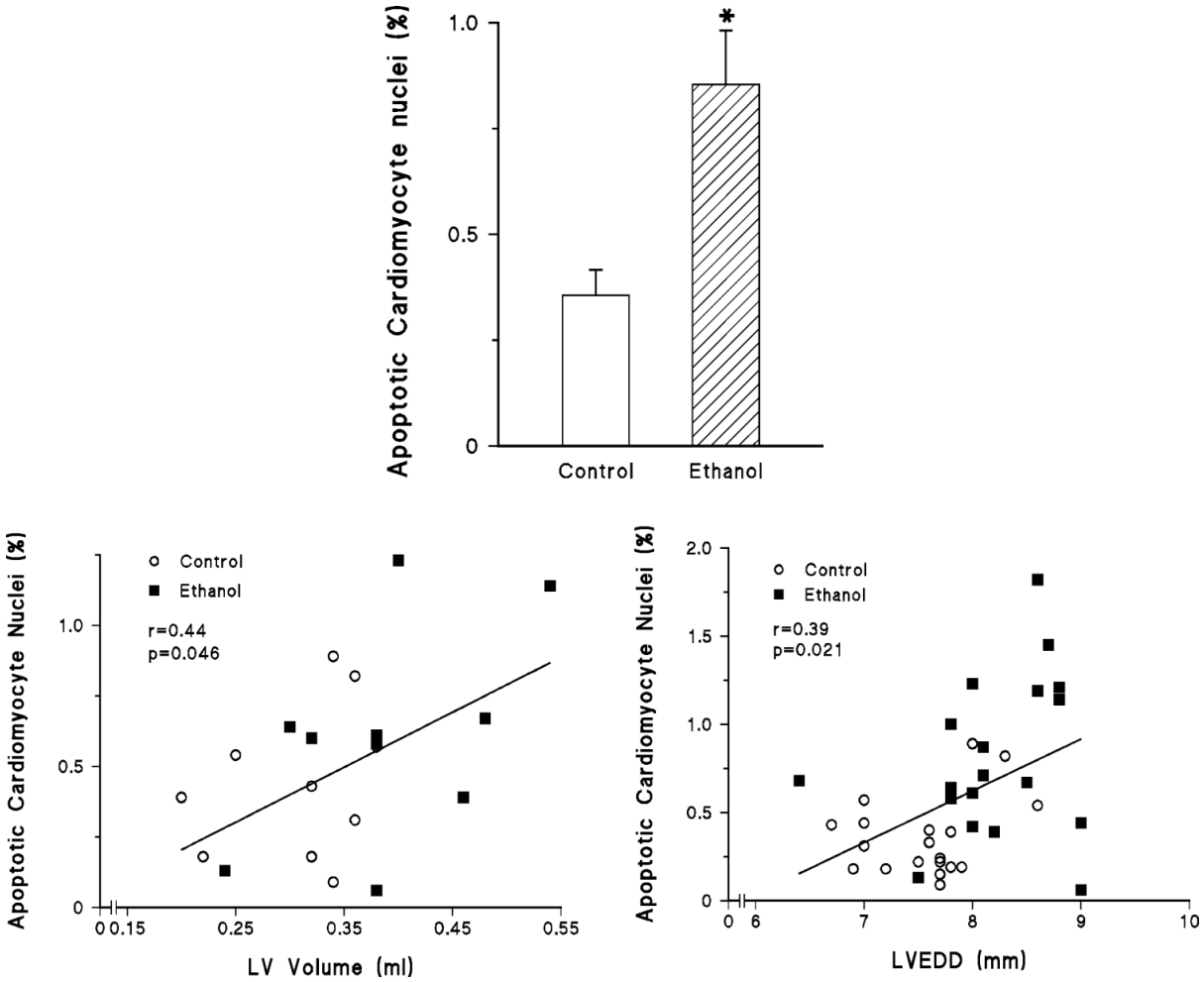

FIGURE 3.11 Effect of 4-months of ethanol consumption on the mean percentage apoptotic-to-total cardiomyocyte nuclei (% apoptosis) determined in the left ventricle (LV) (*upper panel*) and relationships between % LV apoptosis and indices of LV dilatation, namely the volume intercept at 0 mm Hg of the LV diastolic pressure-volume relationship (V_0) (*lower left panel*) and LV end diastolic diameter (LVEDD) (*lower right panel*). Data are represented as mean±SEM; *p<0.005 versus ethanol and control groups.

TABLE 3.5 The relationships between leukocyte, cardiac, kidney, and liver telomere length to cardiomyocyte apoptosis.

	Leukocyte	Cardiac	Kidney	Liver
Ethanol				
Pearson's r	-0.081	0.021	-0.19	0.42
95% CI	-0.51 to 0.39	-0.45 to 0.48	-0.61 to 0.32	-0.070 to 0.75
p value	0.74	0.93	0.47	0.089
Control				
Pearson's r	0.099	0.25	0.23	-0.048
95% CI	-0.46 to 0.60	-0.28 to 0.66	-0.34 to 0.68	-0.58 to 0.52
p value	0.73	0.35	0.43	0.88
Combined				
Pearson's r	-0.068	-0.13	-0.065	0.22
95% CI	-0.39 to 0.27	-0.45 to 0.22	-0.41 to 0.30	-0.15 to 0.53
p value	0.70	0.46	0.73	0.25

r, correlation coefficient; CI, confidence interval

3.4.6 Oxidative stress: TABLE 3.6 demonstrates the 8-isoprostane concentrations obtained using STAT-8-Isoprostane EIA kits (Cayman Chemicals, Ann Arbor, USA) as per manufacturer's instructions. Each sample was measured in duplicate at different dilutions. After the assay was performed and after correction for dilution factors the percentage difference between the samples should have been within 20%. However, after several attempts and after purification of all urine samples using the solid phase extraction purification protocol described above, the percentage difference of the samples obtained were not consistently within acceptable limits. The differences ranged from 280 to 0.4 % (TABLE 3.6) versus the acceptable limits of 20 % as per manufacturers guidelines. The same technical issues were experienced in subsequent animal studies (Chapters 4 and 5) and hence the measurement of urinary 8-isoprostane concentration as a marker of oxidative stress was abandoned.

TABLE 3.6 8-isoprostane absolute and corrected concentrations determined using STAT-8-Isoprostane EIA kits (Cayman Chemicals, Ann Arbor, USA).

Sample no.	Dilution 1	Corrected concentration for dilution 1 (pg.ml^{-1})	Dilution 2	Corrected concentration for dilution 2 (pg.ml^{-1})	% Difference
1	1163.3	1163.3	2007.5	4015.0	-245.14
2	333.5	333.5	293.1	586.2	-75.77
3	1246.8	1246.8	2368.8	4737.6	-279.98
4	2087.9	2087.9	3089	6178.0	-195.90
5	655.9	655.9	331.9	663.8	-1.20
6	346.2	346.2	175.8	351.6	-1.56
7	1134.5	1134.5	604.5	1209.0	-6.57
8	901.2	901.2	338.2	676.4	24.94
9	297.1	297.1	207	414.0	-39.35
10	955	955	648.2	1296.4	-35.75
11	434.6	434.6	259.3	518.6	-19.33
12	1225	1225	615	1230.0	-0.41
13	863.9	863.9	228.6	457.2	47.08
14	1735.7	1735.7	287.8	575.6	66.84
15	2.6	2.6	3.2	6.4	-146.15
16	1463.1	1463.1	1143.9	2287.8	-56.37
17	1328.6	1328.6	1080.2	2160.4	-62.61
18	611.5	611.5	327.3	654.6	-7.05
19	671.8	671.8	208	416.0	38.08
20	801.7	801.7	297.1	594.2	25.88
21	625.8	625.8	205.2	410.4	34.42
22	829.1	829.1	266.4	532.8	35.74
23	291.8	291.8	220.5	441.0	-51.13
24	512.5	512.5	233.8	467.6	8.76
25	407.5	407.5	213.7	427.4	-4.88

3.5 DISCUSSION

The main findings of the present study are as follows: Chronic (4 months) ethanol administration (5% v/v in the drinking water) to rats failed to reduce either leukocyte, cardiac, liver or kidney relative telomere length. However, ethanol induced LV dilatation as indexed by increases in LV end diastolic diameter (LVEDD) and an increased volume intercept of the LV diastolic P-V relationship (LV V_0), without associated changes in LV systolic intrinsic myocardial (FS_{mid} and LV E_{en}) or chamber (FS_{end} and LV E_{es}) function. Moreover, ethanol-induced LV dilatation was associated with marked increases in cardiomyocyte apoptosis as determined from TUNEL positive nuclei, with strong correlations noted between the extent of apoptosis and indices of LV dilatation (LVEDD and LV V_0). Cardiac telomere length was not correlated to indices of LV dilatation, systolic function or cardiomyocyte apoptosis.

The present investigation is the first to assess the relationship between telomere length in an animal model of alcoholic cardiomyopathy. In the present investigation after four months of chronic ethanol intake no significant differences in leukocyte, cardiac, liver or kidney relative telomere length were noted between the ethanol and control groups. Hence, in a animal model of chronic ethanol intake, that produced changes similar to those seen in an asymptomatic human alcoholic cardiomyopathy, reduced telomere length was not associated with the pathophysiological processes involved in LV dilatation and cardiomyocyte apoptosis. These results suggest that telomere length does not have a causal role in an alcoholic cardiomyopathy and further demonstrate that reduced telomere length may not be associated with all types of cardiomyopathies.

The failure of chronic ethanol consumption to produced changes in telomere length may be due to the absence of inflammatory processes in the current study. Inflammation is a prominent feature of heart failure (Yndestad *et al.*, 2006) and is also known to increase

telomere attrition (Beyne-Rauzy *et al.*, 2005; Houben *et al.*, 2008). With respect to inflammation in this study, no significant differences in CRP concentrations were noted between the ethanol and control groups. Hence, the failure of chronic ethanol consumption to increase telomere attrition, despite producing marked LV dilatation and increased cardiomyocyte apoptosis, may be due to the absence of inflammatory processes.

Consistent with the present findings, a number of prior studies have demonstrated LV dilatation (Kim *et al.*, 2001 ; Kim *et al.*, 2003; Law *et al.*, 2012; Lazarević *et al.*, 2000; Mathews *et al.*, 1981) and cardiomyocyte apoptosis (Fernández-Solà *et al.*, 2006; Guan *et al.*, 2004; Hajnóczky *et al.*, 2005; Jing *et al.*, 2012) following ethanol consumption. However, previous studies have not demonstrated that cardiomyocyte apoptosis is associated with indices of LV dilatation in a rat model of chronic ethanol intake. These data therefore suggest a possible causal role for cardiomyocyte apoptosis in the adverse chamber remodelling produced by chronic ethanol consumption. In this regard, ethanol consumption is known to result in increased oxidative stress (Guo and Ren, 2010; Jing *et al.*, 2012; Kalaz *et al.*, 2012) that is possibly responsible for ethanol-induced cardiomyocyte apoptosis (Jing *et al.*, 2012). Ethanol consumption may precipitate increases in oxidative stress through the production of the metabolite, acetaldehyde, which results in the formation of free radicals and reduced efficacy of the anti-oxidant defence system (Guo and Ren, 2010). Ethanol-induced oxidative stress may activate cardiomyocyte apoptosis through adverse actions on mitochondria (Guan *et al.*, 2004; Hajnóczky *et al.*, 2005). The increased apoptosis, oxidative stress and mitochondrial dysfunction produced by ethanol consumption may have an important causal role in initiating adverse LV remodelling present in the early stages of an alcoholic cardiomyopathy. Importantly, neither percent apoptosis nor indices of LV dilatation were associated with relative telomere length in any tissue type. These data indicate that

cardiomyocyte apoptosis possibly has a causal role in the LV dilatation produced by chronic ethanol intake, but that these changes are independent of telomere attrition.

An observation that requires further discussion is that leukocyte telomere length increased from one to four months in both experimental and control groups in this study. These findings have, however, been demonstrated previously. In this regard, longitudinal data in human and animal studies, noted that leukocyte telomere length is variable and may increase, remain the same, or decrease in length over time (Aviv *et al.*, 2009; Ilmonen *et al*., 2008; Nordfjäll *et al.*, 2009). Moreover, it has been noted that telomere attrition was highly associated with baseline telomere length (Aviv *et al.*, 2009). Thus, a major factor with respect to the maintenance of telomere length may be initial telomere length itself (Aviv *et al.*, 2009). In this regard, there was no significant difference in leukocyte telomere length at 1 month between the ethanol and control groups and subsequently both groups had an increase in leukocyte telomere length from 1 month to 4 months after their respective treatments. Hence, one can conclude that chronic ethanol intake did not increase leukocyte telomere attrition, but more importantly that ethanol did not alter normal leukocyte telomere length maintenance in the ethanol group when compared to the control group.

The results of the present study do not exclude a contribution of cardiomyocyte telomere dysfunction to the pathogenesis of ethanol-induced LV dilatation. In this regard, the length of the shortest telomere in a particular population of cells rather than average telomere length, as evaluated in this study, determines cellular function (Hemann *et al.*, 2001). Thus, average relative telomere length may not closely reflect the impact of telomeres on cellular performance. Further studies are required to evaluate whether ethanol reduces the length of the shortest telomere.

In terms of study limitations, firstly, cardiac telomere length may not have been reduced in the ethanol compared to the water group due to the fact that, at this four month time point, all of the cardiomyocytes with critically short telomere length may have already undergone apoptosis. However, there is evidence demonstrating that average cardiomyocyte telomere length is reduced in diseased heart tissue even where there is an increase in cardiomyocyte apoptosis (Chimenti *et al.*, 2003). Hence, the inability of ethanol to decrease telomere length in cardiac tissue is not due to the fact that all of the cardiomyocytes with critically short telomere length had already undergone apoptosis. Secondly, no measurements of myocardial oxidative stress were available and as such it is not known if the dose of ethanol employed in this study was sufficient to cause an increase in oxidative stress. In this regard, it remains uncertain whether the absence of a relationship between reduced telomere length,ethanol-induced dilatation and cardiomyocyte apoptosis in this model of alcoholic cardiomyopathy is independent of increased oxidative stress. Thirdly, TUNEL staining was employed to determine cardiomyocyte apoptosis and this approach has well recognised caveats (Kang and Izumo, 2000). In this regard, an increase in TUNEL stained nuclei may reflect either apoptotic cell death or DNA repair. However, either change would reflect excessive cardiomyocyte tissue damage produced by alcohol administration and hence indicates a pathological change. Fourthly, no markers for oxidative stress were measured. However, if oxidative stress was assumed to be increased in the present study, based on previous findings in an alcoholic cardiomyopathy (Jing *et al.*, 2012; Kalaz *et al.*, 2012; Guo and Ren, 2010), then one could argue that oxidative stress did not increase telomere attrition in leukocyte, cardiac, liver, and kidney tissues in a rat model of chronic ethanol excess. This possibility is supported by previous findings demonstrating that increased oxidative stress did not increase telomere attrition in heart, liver and kidney tissues (Cattan *et al.*, 2008). Hence, the failure of ethanol-induced oxidative stress to increase telomere attrition may be due to the

fact that not all tissue types are susceptible to oxidative stress-induced telomere attrition. Importantly, the inability to measure oxidative stress does not discount the findings that reduced telomere length is not associated with ethanol-induced LV dilatation and cardiomyocyte apoptosis.

In conclusion, ethanol intake in rats in amounts (2.4±0.3g ethanol.kg body weight^{-1}.day^{-1}) that correspond with the minimum daily intake known to produce an alcoholic cardiomyopathy (>90g.day^{-1} or >1.29 g.kg^{-1} body weight per day assuming an average 70 kg male) (Piano, 2002) resulted in LV dilatation and cardiomyocyte damage that was independent of changes in telomere length. Importantly, the extent of cardiomyocyte apoptosis was correlated with indices of LV dilatation but neither apoptosis nor LV dilatation were associated with telomere length. Hence, cardiomyocyte apoptosis may play a causal role in the ethanol-induced LV dilatation, but these changes are independent of changes in cardiac telomere length. Furthermore, these data provide further evidence to demonstrate that reduced telomere length may not be associated with all forms of heart failure, including an ethanol-induced cardiomyopathy.

CHAPTER 4 - CHRONIC β-ADRENERGIC RECEPTOR ACTIVATION AND TELOMERE LENGTH

The data in this chapter has been accepted for publication in the European Journal of Applied Physiology: Raymond AR, Hodson B, Woodiwiss AJ, Norton GR,Brooksbank RL. Telomere Length and Adrenergic-Induced Left Ventricular Dilatation and Systolic Chamber Decompensation in Rats. Eur J Appl Physiol 2013 (publication in press).

4.1ABSTRACT

Aims: The mechanisms responsible for telomere shortening in chronic heart failure, are uncertain. We evaluated whether left ventricular (LV) dilatation and systolic chamber decompensation produced by chronic β-adrenergic receptor (β-AR) stimulation is associated with leukocyte or cardiac telomere shortening. ***Methods and Results:*** Following 6 months of daily injections of the β-AR agonist, isoproterenol (0.02 $mg.kg^{-1}.day^{-1}$) (n=16), or the saline vehicle (n=15) to SD rats, the extent of LV dilatation and LV systolic chamber decompensation were determined using echocardiography and isolated perfused heart procedures, and relative telomere length of leukocyte and cardiac DNA was determined using a quantitative real-time polymerase chain reaction assay. Chronic β-AR stimulation resulted in LV dilatation as indexed by an increased LV diastolic diameter (9.2±0.6mm vs. 8.4±0.9mm, p=0.01), and an increased volume intercept at zero pressure of the LV diastolic pressure-volume relationship (isolated, perfused heart preparation) (0.40±0.06ml vs. 0.37±0.08ml, p=0.03). Moreover, chronic β-AR stimulation resulted in LV systolic chamber decompensation as indexed by reductions in LV endocardial fractional shortening (0.40±0.05 vs. 0.60±0.06, p=0.01) and the slope of the LV systolic pressure-volume relation (609±176 vs. 901±230, p=0.01). Although leukocyte telomere length (log T/S) decreased with age in rats receiving either the β-AR agonist or the saline vehicle ($p<0.05$), neither cardiac (-0.10±0.14 vs. -0.15±0.12, p=0.3) nor leukocyte telomere length (-0.11±0.19 vs. -0.15±0.18, p=0.5) were modified by chronic β-ARstimulation. ***Conclusions:*** In conclusion, chronic β-AR stimulation, sufficient to produce LV dilatation and systolic chamber decompensation, is not associated with alterations in leukocyte or cardiac telomere length. Telomere shortening in chronic heart failure is unlikely to be attributed to chronic β-AR stimulation.

4.2 INTRODUCTION

Studies have demonstrated that reduced average leukocyte telomere length is associated with chronic heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012). However, the cause and effect relationships between telomere length and heart failure are uncertain. In this regard, whether reduced telomere length plays a role in the pathophysiological processes of heart failure or if telomere length is reduced in heart failure merely due the pathophysiological processes involving oxidative stress and inflammation is unknown. Hence, the relationship between telomere length and prominent features of heart failure need to be further investigated in order to elucidate that cause and effect relationships between reduced telomere length and heart failure.

An important pathophysiological feature of progressive heart failure is stimulation of adrenergic receptors via activation of the sympathetic nervous system (neurohumoral activation) (Triposkiadis *et al.*, 2009), a change which results in oxidative stress (Xu *et al.*, 2011), and inflammation (Kumar *et al.*, 2009), and activates a cellular pathway (p53) (Zhou *et al.*, 2006) that is known to mediate apoptosis associated with short telomeres (Artandi and Attardi, 2005; Aubert and Lansdorp, 2008). Thus, it is possible that in heart failure, chronic adrenergic receptor stimulation contributes toward telomere attrition. In order to test this hypothesis, I investigated whether left ventricular dilatation and systolic chamber decompensation induced by chronic adrenergic receptor stimulation in rats (Woodiwiss *et al.*, 2001) is associated with cardiac and/or leukocyte telomere shortening.

4.3 METHODS

4.3.1 Animal model: This study was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (clearance number: 2010/25/04). Thirty one male SD rats at three months of age were assigned to receive either subcutaneous injections of 0.02 $mg.kg^{-1}$ of isoproterenol (n=16), or a 0.9% saline vehicle of 0.2 ml volume (n=15) daily for 6 months. In an attempt to reduce animal deaths as a result of initial excessive adrenergic stimulation, animals received incremental doses of isoproterenol. During the first week rats received a 0.005 $mg.kg^{-1}$ dose of isoproterenol in the morning and again in the evening. During the second week the rats received a single 0.01 $mg.kg^{-1}$ daily dose of isoproterenol. During the third week rats received a 0.01 $mg.kg^{-1}$ dose of isoproterenol in the morning and again in the evening. For the subsequent five months rats received once off daily injections of isoproterenol at a dose of 0.02 $mg.kg^{-1}$. This protocol utilising a chronic low dose of isoproterenol is a well established model that is considered to accurately replicate the pathogenesis of human heart failure (Carll *et al.*, 2011).

4.3.2 Echocardiography: Echocardiography was performed as previously described (see Section 3.3.2) just prior to termination to determine LV chamber dimensions and posterior wall thickness at end-diastole and end-systole.Furthermore,load-dependant indices ofintrinsic myocardial function (FS_{mid}) andleft ventricular systolic chamber function (FS_{end}) were determined using echocardiography. Rats were evaluated 24 hours after receiving the last dose of isoproterenol. Echocardiography measurements were performed by the same experienced investigator who was blinded to the designation of animals to experimental and control groups. Immediately after echocardiography a carotid catheter was inserted to measure carotid pressures (Norton *et al.*, 2002) and LV end-systolic wall stress was determined, assuming spherical LV geometry, from the formula:

LV end-systolic wall stress = LV end-systolic pressure x ((ESD + ESPWT)/ESPWT) x (1-(ESPWT/(2x(ESD + ESPWT))) - 0.5), where end-systolic pressure was assessed from the carotid incisura and where ESD is end-systolic diameter and ESPWT is end-systolic posterior wall thickness (Norton *et al.*, 2002; Shimizu *et al.*, 1991). The carotid incisura was sufficiently obvious to identify end-systolic pressure in five out of six rats in the group receiving isoproterenol and four out of five rats in the group receiving the vehicle.

4.3.3 Isolated, perfused heart preparations: Eight rats each from the experimental and control groups had LV dimension and function measurements determined using isolated, perfused heart preparations as previously described (see Section 3.3.3). Left ventricular diastolic remodelling was assessed from the LV diastolic pressure-volume relationships, using the volume intercept at zero pressure (V_0) for comparative purposes, with an increased V_0 representative of an increased LV chamber size in diastole. Left ventricular systolic chamber performance was determined from the slope of the linear portion of the left ventricular developed pressure-volume relationships (end systolic elastance-E_{es}). In addition, a load-independent measure of intrinsic myocardial systolic function was assessed by constructing LV developed stress-strain relations and comparing the slopes (LV end systolic myocardial elastance-E_n) of these relationships.

4.3.4 Telomere length: DNA was extracted from leukocytes, LV lateral wall of the myocardium and kidney tissue using NucleoSpin® Blood and Tissue kits (Macherey-Nagel, Dueren, Germany) respectively, as per manufacturer's instructions(see Section 3.3.6). Kidney telomere length was determined to evaluate whether changes in the myocardium were replicated in other tissues. The methodology employed to determine relative telomere length in leukocyte and tissue DNA was as described previously (see Sections 2.3.3 and 3.3.8). A standard sample was included in each amplification run and was used to standardise the entire

sample T/S ratios as well as to determine coefficients of variation. The coefficients of variation of the S and T assays were 2.10% and 4.1%, respectively.

4.3.5 C-reactive protein concentrations: Blood samples were obtained at 1 and 6 months of the study. CRP concentrations were determined, as previously described (see Section 3.3.9), using a commercially available rat-specific CRP enzyme-linked, immunoabsorbant kit (eBioscience, San Diego, USA.). This assay was performed without any modifications to manufacturer's instructions.

4.3.6 Statistical analysis: All statistical analyses performed on telomere length were conducted on log transformed T/S ratios. Student's unpaired and paired t-tests were used to determine differences between and within groups receiving isoproterenol and saline where appropriate. All associations between variables were carried out using Pearson's correlation coefficients. A p value of less than 0.05 was considered statistically significant. All data are represented as mean±SD and mean±SEM where indicated.

4.4 RESULTS

4.4.1 Body and heart weight: No significant differences in body weight, whole heart weight, or left ventricular weight were noted between the groups receiving isoproterenol and saline for 6 months (TABLE 4.1). Rats receiving daily isoproterenol had an increased left ventricular end systolic wall stress (TABLE 4.1).

TABLE 4.1 Effect of 6 months of isoproterenol (ISO) versus vehicle (Control) administration on body weight, heart weight, and echocardiographically determined left ventricular dimensions in rats.

	ISO (n=16)	**Control (n=15)**	**p value**
Body weight (g)	628.6±48.2	676.7±111.1	=0.16
Left ventricular weight (g)	1.24±0.40	1.2±0.1	=0.46
Whole heart weight (g)	1.53±0.44	1.4±0.2	=0.49
LVESD (mm)	5.5±0.8	4.7±0.9	=0.0079*
LVESPWT (mm)	2.9±0.4	3.0±0.3	=0.36
LVEDPWT (mm)	2.8±0.4	2.7±0.5	=0.61
LVRWT (mm)	0.6±0.1	0.6±0.1	=0.40
LVESS (g.cm^{-2})	135±14 (n=5)	98±24(n=4)	=0.023*

LV, left ventricular; ESD, end-systolic diameter; EDPWT, end-diastolic posterior wall thickness; ESPWT, end-systolic posterior wall thickness; RWT, relative wall thickness; ESS, end-systolic wall stress. Values are represented as mean±SD; *p<0.05 versus ISO andControl group.

4.4.2 Left ventricular remodelling: As assessed *in vivo*, chronic isoproterenol administration resulted in LV dilatation as indexed by increases in LV end diastolic (FIGURE 4.1) and systolic diameters (TABLE 4.1). There were no significant differences in the LV posterior wall thickness during either end-diastole or systole between the groups (TABLE 4.1). However, the increase in LVEDD and lack of wall thinning did not translate into decreases in RWT (TABLE 4.1). Furthermore, as assessed *ex vivo* at the same heart rate, afterload and at comparable coronary flow rates, a right shift in the LV diastolic P-V relationship and an increase in the mean V_0 in rats receiving chronic isoproterenol administration as compared to the saline-treated group were noted (FIGURE 4.1).

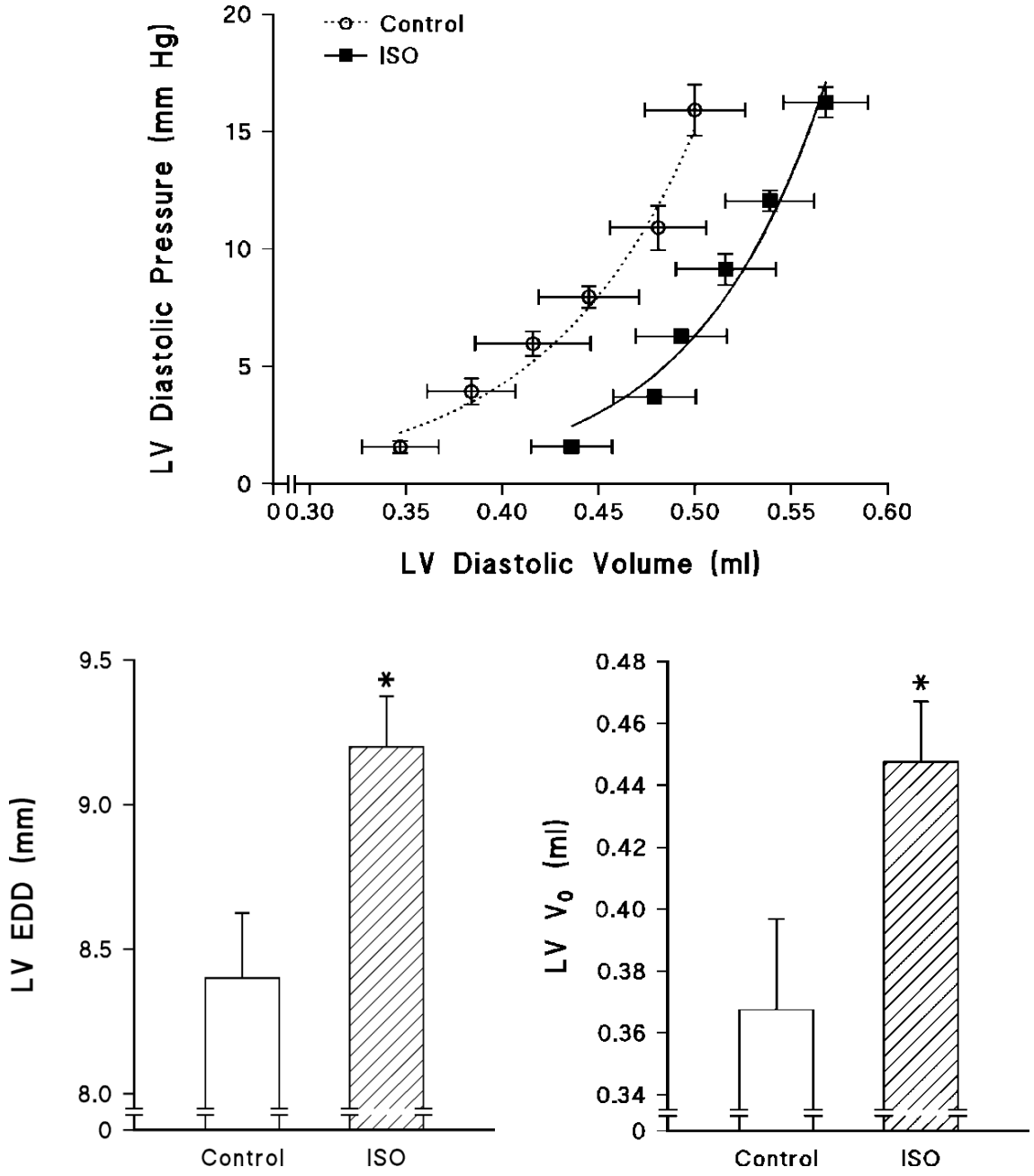

FIGURE 4.1 Effect of 6 months of isoproterenol (ISO) versus vehicle (Control) as indexed by LV end diastolic diameter (LVEDD) (*lower left panel*), LV diastolic pressure-volume relations (*upper panel*) and the volume intercept at 0 mm Hg (V_0) (*lower right panel*) LV diastolic pressure. Data are represented as mean±SEM; *p<0.05 versus ISO and Control groups.

***4.4.3 Left ventricular systolic chamber and myocardial function*:** As assessed *in vivo*, the group receiving isoproterenol had a reduced systolic chamber function as indexed by a reduced FS_{end} (FIGURE 4.2) compared to the control group, while there were no differences in the FS_{mid} (FIGURE 4.3) between the groups. Consistent with *in vivo* findings, *ex vivo* data at the same heart rate, afterload and at comparable coronary flow rates, illustrated the LV developed P-V relationship was right shifted and mean E_{es} was reduced in rats receiving isoproterenol for 6 months as compared to control rats (FIGURE 4.2), while there were no shifts in LV developed stress-strain relations and mean E_{en} (FIGURE 4.3).

4.4.4 Telomere length: The leukocyte T/S ratios were reduced, which indicates shorter leukocyte telomere length, in blood samples obtained at 6 months as compared to 1 month of the study (TABLE 4.2). This relationship was present in both the rats receiving isoproterenol and those receiving saline for 6 months (TABLE 4.2). However, no significant differences in the T/S ratios were noted between the rats receiving isoproterenol or saline when assessed at either the first month, or at 6 months of the study (TABLE 4.2). Furthermore, there was no significant difference in the percentage change in telomere length from the first to the sixth month of the study between groups receiving daily isoproterenol or saline (TABLE 4.2). The T/S ratios in heart and kidney tissues, were not significantly different between groups receiving isoproterenol and saline groups (TABLE 4.2). Leukocyte telomere length at termination was not correlated with telomere length in cardiac (Pearson's $r=0.21$, $r^2=0.044$, $p=0.30$) or kidney tissue (Pearson's $r=0.21$, $r^2=0.043$, $p=0.31$). Furthermore, neither indices of LV dilatation (LVEDD and V_0), chamber function (FS_{end}, and E_{es}), nor inflammation (CRP) were correlated with cardiac telomere length (TABLE 4.3).

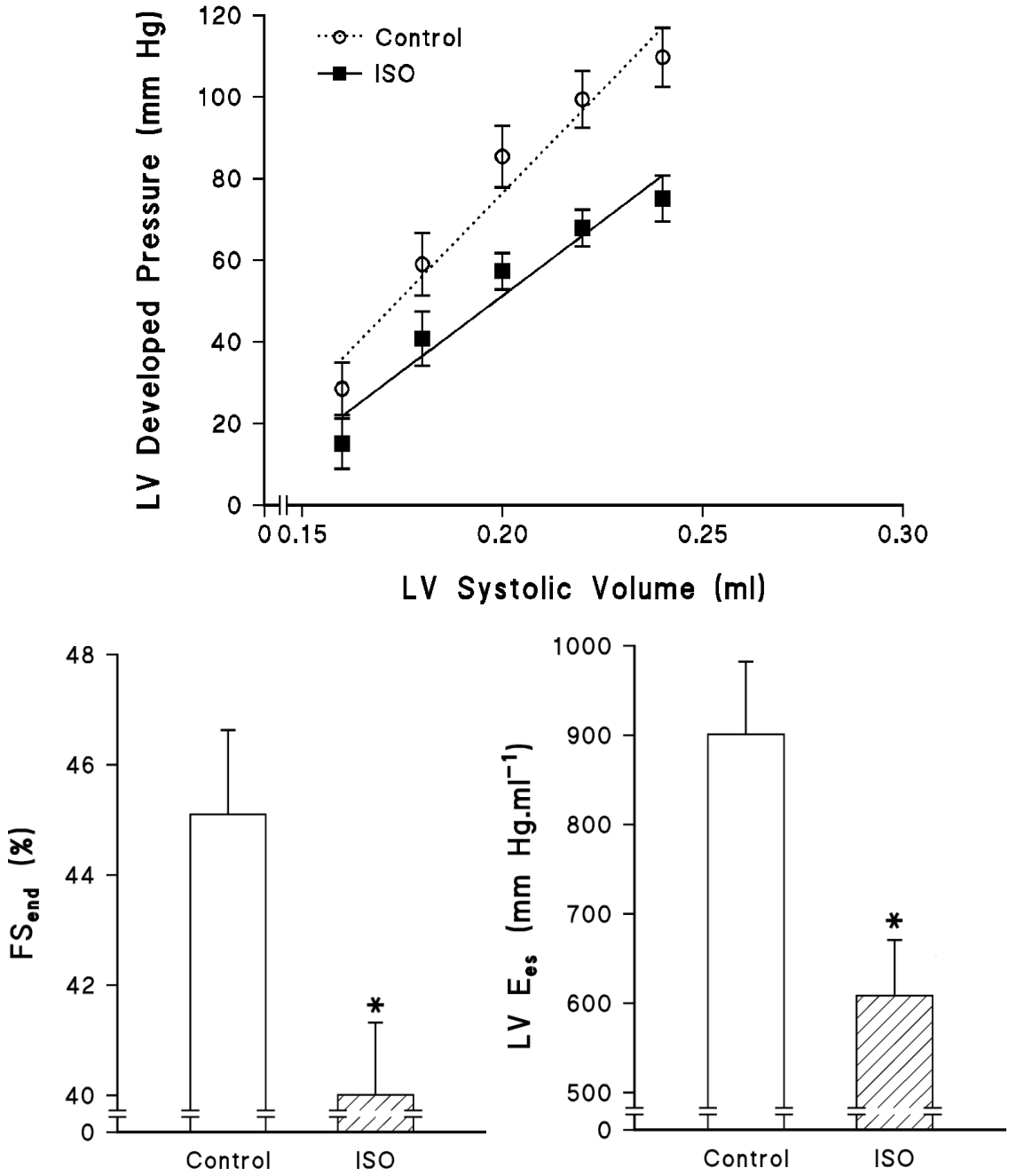

FIGURE 4.2 Effect of 6 months of isoproterenol (ISO) versus vehicle (Control) administration on left ventricular (LV) systolic chamber function as indexed by LV endocardial fractional shortening (FS_{end}) (*lower left panel*), LV systolic pressure-volume relations (*upper panel*), and the slope of the systolic pressure-volume relations (LV E_{es}) (*lower right panel*). Data are represented as mean±SEM; *p<0.05 versus ISO andControl groups.

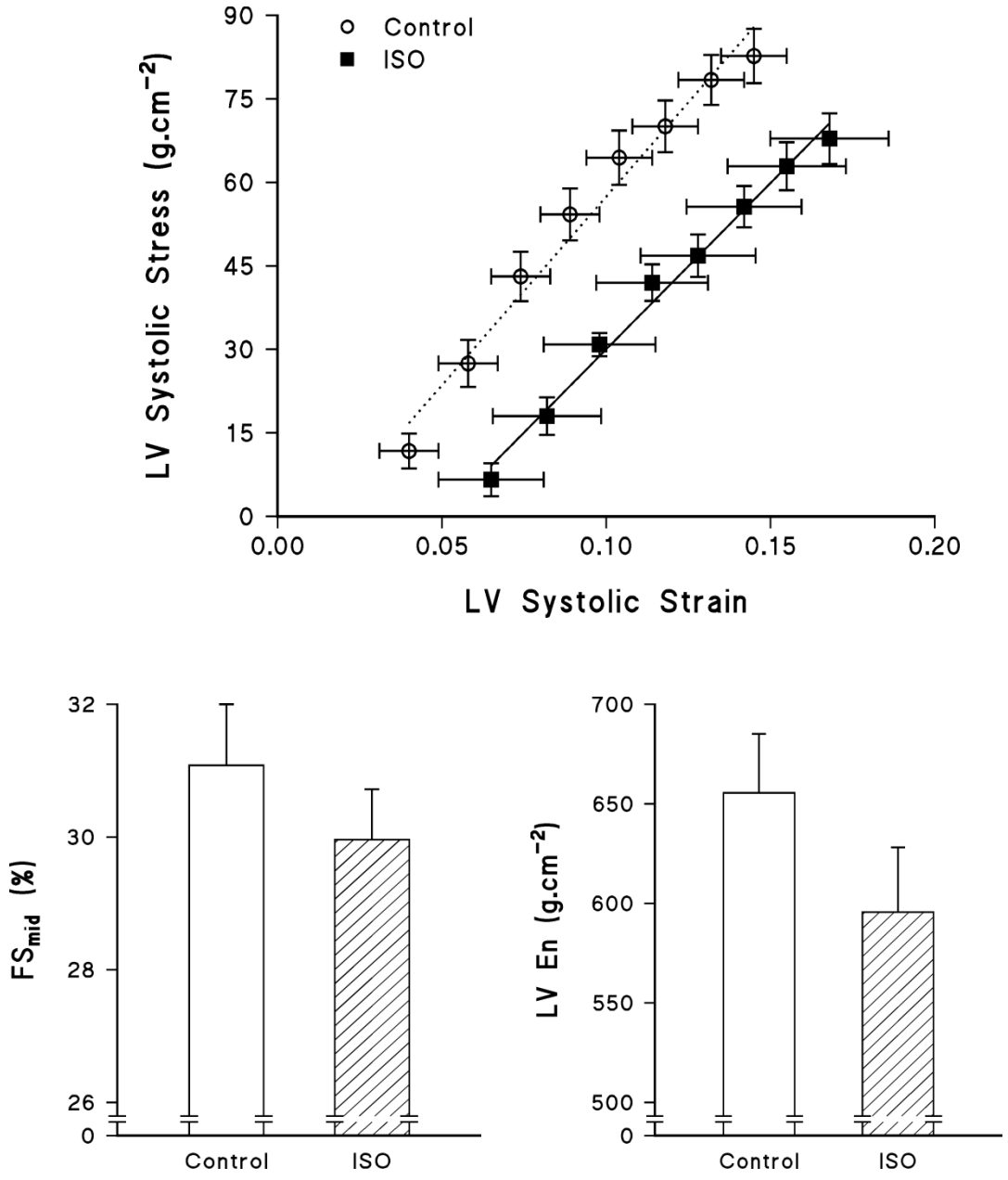

FIGURE 4.3 Effect of 6 months of isoproterenol (ISO) versus vehicle (Control) administration on left ventricular (LV) intrinsic myocardial systolic function as indexed by LV midwall fractional shortening (FS_{mid}) (*lower left panel*), LV systolic stress-strain relations (*upper panel*), and the slope of the systolic stress-strain relations (LV E_n) (*lower right panel*). Data are represented as mean±SEM.

TABLE 4.2 Effect of 6 months of isoproterenol (ISO) versus vehicle (Control) administration on relative telomere length as indexed by log T/S±SD in leukocytes, myocardium and kidneys in rats.

	ISO (n=16)	**Control (n=15)**	**p value**
Leukocyte T/S month 1	0.15±0.010	0.11±0.12	=0.47
Leukocyte T/S month 6	-0.11±0.19 *****	-0.15±0.18 *****	=0.50
Δ Leukocyte T/S from month 1	-1.54±1.69	-1.74±1.49	=0.78
Cardiac T/S	-0.10±0.14	-0.15±0.12	=0.32
Kidney T/S	-0.11±0.11	-0.12±0.15	=0.83

Δ, percentage change in leukocyte telomere length from 1 month to 6 months. Values are represented as mean±SD; *p<0.05 versus month 1leukocyte T/S.

TABLE 4.3 Correlations between cardiac telomere length and indices of left ventricular (LV) dilatation (the volume intercept at 0 mm Hg (V_0) in the left ventricular (LV) diastolic pressure-volume relations and LV end diastolic diameter (LVEDD)), systolic chamber function (slope of the systolic pressure-volume relations (LV E_{es}) and endocardial fractional shortening (FS_{end})), intrinsic myocardial function (slope of the systolic stress-strain relations (E_{en}) and midwall fractional shortening (FS_{mid})) and inflammation (C-reactive protein (CRP)).

	V_0	LVEDD	LV E_{es}	FS_{end}	E_{en}	FS_{mid}	CRP (6 months)
Isoproterenol							
Pearson's r	-0.29	0.14	-0.70	-0.32	-0.58	-0.059	0.0048
95% CI	-0.83 to 0.52	-0.38 to 0.60	-0.94 to 0.01	-0.71 to 0.24	-0.91 to 0.20	-0.54 to 0.45	-0.51 to 0.52
p value	0.49	0.60	0.054	0.25	0.13	0.93	0.98
Control							
Pearson's r	-0.09	0.43	-0.12	-0.31	0.28	0.27	-0.26
95% CI	-0.75 to 0.66	-0.13 to 0.78	-0.76 to 0.64	-0.75 to 0.32	-0.69 to 0.89	-0.30 to 0.70	-0.69 to 0.32
p value	0.83	0.13	0.78	0.32	0.59	0.34	0.38
Combined							
Pearson's r	-0.15	0.35	-0.38	-0.29	-0.37	0.058	-0.11
95% CI	-0.63 to 0.41	-0.01 to 0.63	-0.75 to 0.17	-0.60 to 0.10	-0.76 to 0.20	-0.31 to 0.41	-0.46 to 0.27
p value	0.60	0.06	0.16	0.14	0.19	0.76	0.57

r, correlation coefficient; CI, confidence interval

4.4.5 C-reactive protein: Plasma CRP concentrations were greater at 6 months as compared to at 1 month of the study in groups receiving either daily isoproterenol or saline (FIGURE 4.4). However, no significant differences in CRP concentrations between groups receiving isoproterenol and saline at either the first month or at termination were noted. Moreover, there was no difference in the percentage change in CRP concentrations from the first to the sixth month of the study between rats receiving daily isoproterenol and saline ($p=0.28$). The plasma CRP concentrations at either time point for both groups receiving isoproterenol or saline were within normal ranges for rats.

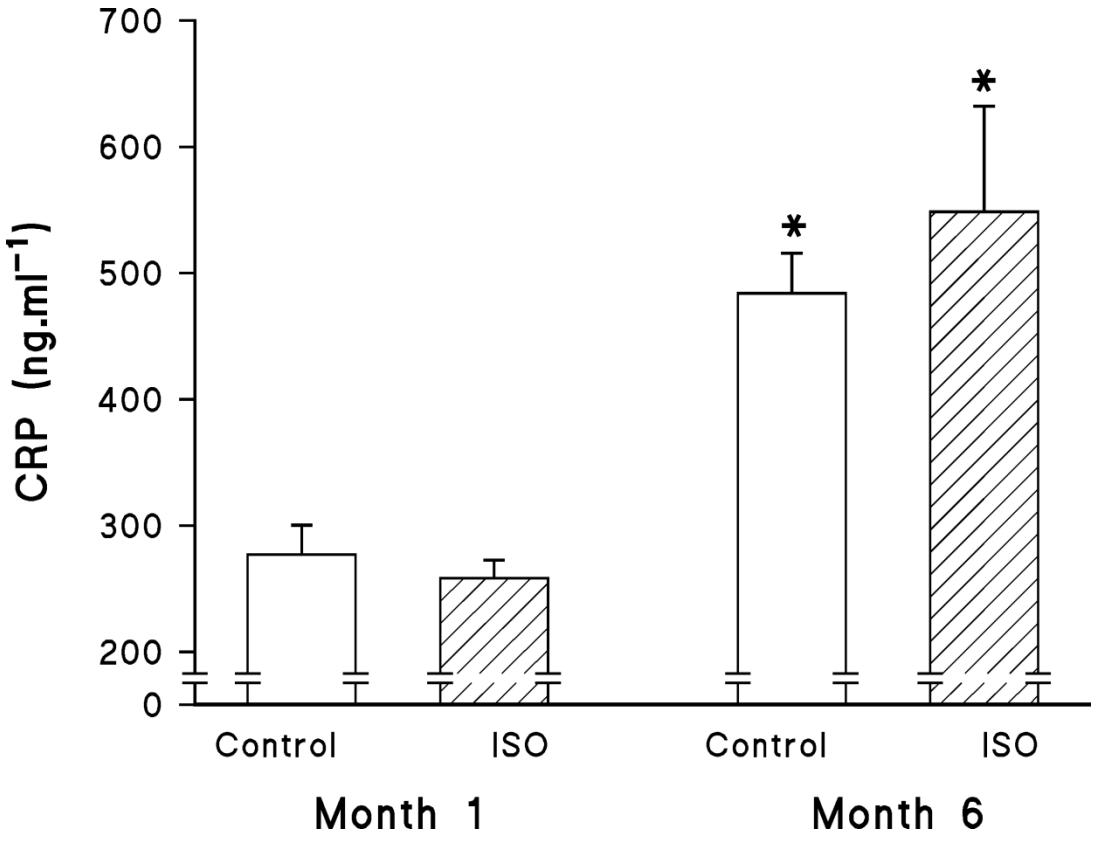

FIGURE 4.4 Effect of 6 months of isoproterenol (ISO) versus vehicle (Control) administration on plasma C-reactive protein (CRP) concentrations from 1 month to 6 months. Values are represented as mean±SD; *p<0.001 versus 1 month in ISO and Control groups.

4.5 DISCUSSION

Chronic (6 months) β-adrenergic receptor stimulation in rats, produced by daily administration of isoproterenol, despite producing LV dilatation (an increased LVEDD and LV V_0), and LV systolic chamber dysfunction (a reduced FS_{end} and E_{es}), did not increase either myocardial or leukocyte telomere attrition. Furthermore, no relationships between cardiac telomere length and indices of LV dilatation and function were noted.

To the best of my knowledge the results of the present study are the first to evaluate whether chronic β-adrenergic receptor stimulation, sufficient to produce structural and functional changes in the LV congruent with advanced cardiac pathology (LV systolic chamber dysfunction and chamber dilatation), promotes telomere shortening in either leukocyte or cardiac DNA. In this regard, chronic adrenergic stimulation is a well recognised change responsible for progressive heart failure (Triposkiadis *et al.*, 2009), and is associated with an increased oxidative stress (Xu *et al.*, 2011), and inflammation (Kumar *et al.*, 2009) in cardiomyopathies, and activates a cellular pathway (p53) (Zhou *et al.*, 2006) that is known to mediate apoptosis associated with short telomeres (Artandi and Attardi, 2005; Aubert and Lansdorp, 2008). The results of the present study therefore suggest that telomere shortening is not a sensitive biomarker of the adverse effects of adrenergic stimulation in heart failure.

The inability of chronic adrenergic stimulation to promote telomere shortening despite producing LV dilatation and systolic dysfunction may have several explanations. Inflammation may be caused by heart failure (Yndestad *et al.*, 2006) and inflammatory processes may be responsible for telomere shortening in heart failure (Beyne-Rauzy *et al.*, 2005; Houben *et al.*, 2008). In this regard, in the present study I was unable to show increased plasma CRP concentrations in rats receiving the β-adrenergic receptor stimulant compared to the control group. This may be explained by the absence of clinical features of heart failure, despite the presence of structural and functional changes in the heart. This may

be attributed to the dose of isoproterenol employed in this study. Previous studies have demonstrated that much higher doses of isoproterenol (5 $mg.kg^{-1}$) than that employed in the present study (0.02 $mg.kg^{-1}$) resulted in increased circulating concentrations of TNF-α and IL1-β (Kumar *et al.*, 2009). Hence, it is possible that with a higher dose of isoproterenol, telomere shortening may have occurred. Importantly, however, the dose of isoproterenol employed in the present study produced striking changes in cardiac structure and function after 6 months of administration as demonstrated by indices of LV dilatation and systolic dysfunction. Thus, an inability of the dose of isoproterenol to promote inflammatory changes in the present study does not preclude me from drawing a conclusion that telomere length is an insensitive biomarker of adrenergic-induced LV dilatation and systolic dysfunction.

Telomere attrition may also occur as a consequence of oxidative stress (Houben *et al.*, 2008) and adrenergic-induced cardiomyopathies are associated with increases in oxidative stress (Xu *et al.*, 2011). In this regard, an alternative explanation for the inability of chronic adrenergic stimulation to promote telomere length changes in the present study, may be due to the fact that the dose of isoproterenol may have been insufficient to induce oxidative stress. In this regard, a limitation of the present study is that we did not measure myocardial oxidative stress. However, in the present study, LV wall end systolic wall stress was increased, and this could translate into increases in myocardial oxidative stress (Giordano, 2005). If oxidative stress was assumed to be increased in the present study, then one could argue that oxidative stress did not promote telomere attrition in leukocyte, cardiac, and kidney tissues in a rat model of chronic β-adrenergic receptor stimulation. This possibility is supported by previous findings demonstrating that increased oxidative stress does not increase telomere attrition in heart and kidney tissue (Cattan *et al.*, 2008). Hence, the failure of adrenergic induced oxidative stress to increase telomere attrition may be due to the fact that not all tissue types are susceptible to oxidative stress-induced telomere attrition.

Therefore, my inability to measure myocardial oxidative stress does not preclude me from drawing a conclusion that telomere length is an insensitive biomarker of adrenergic-induced LV dilatation and systolic dysfunction.

The results of the present study have implications with respect to current evidence demonstrating telomere attrition in heart failure. In this regard, although a prior study (van der Harst *et al.*, 2007) has demonstrated a relationship between reduced leukocyte telomere length and both ischaemic and non-ischaemic heart disease, in that study (van der Harst *et al.*, 2007) 22% of the non-ischaemic group of patients had advanced atheromatous disease as indexed by a history of stroke, peripheral arterial disease or angina pectoris. Moreover, in that study telomere length was strongly associated with atherosclerotic disease (van der Harst *et al.*, 2007). Furthermore, the causes of heart failure in the remaining non-ischaemic patients were not stated (van der Harst *et al.*, 2007). In contrast, as described in Chapter 2 of this thesis, I have failed to demonstrate similar relationships between average leukocyte telomere length and idiopathic dilated cardiomyopathy, a non-ischaemic cause of heart failure (Raymond *et al.*, 2013). Moreover, Starr *et al.* (2007) demonstrated that reduced telomere length was associated with ischaemic changes on electrocardiograms, while the same associations were absent with non-ischaemic disease manifestations. Hence, the relationship between reduced leukocyte telomere length and ischaemic heart disease and the absence of this relationship between non-ischaemic heart disease, may be attributed to differences in the disease profiles of the patient groups rather than cause and effect relationships. In this regard, this study extends these previous studies, and suggests at least that neurohumoral activation (sympathetic nervous system activation), a major cause of progressive heart failure irrespective of the cause (Triposkiadis *et al.*, 2009), may not be responsible for average telomere shortening in heart failure.

Importantly, the results of the present study do not discount an important contribution of cardiomyocyte telomere dysfunction to the pathogenesis of adrenergic-induced LV dysfunction and dilatation. In this regard, the length of the shortest telomere in a particular population of cells rather than average telomere length of a population of leukocytes, as evaluated in the present study, determines cellular function (Hemann *et al.*, 2001). Thus, average relative telomere length may not closely reflect the impact of telomeres on cellular performance. Further studies are required to evaluate whether adrenergic stimulation reduces the length of the shortest telomere.

In conclusion, the results of the present study show that chronic β-adrenergic receptor stimulation in rats, despite producing LV dilatation and LV systolic chamber dysfunction, failed to modify either cardiac or leukocyte telomere length. These results suggest that average telomere length is not a sensitive biomarker of LV systolic dysfunction and dilatation produced by chronic β-adrenergic receptor stimulation and that previously described relationships between average telomere length and heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) are therefore unlikely to be attributed to chronic adrenergic stimulation.

CHAPTER 5 - CHRONIC INFLAMMATION AND TELOMERE LENGTH

5.1 ABSTRACT

Aims: A prominent feature that is associated with both telomere attrition and chronic heart failure is inflammation. Hence, I evaluated the impact of chronic inflammation on telomere length and LV remodelling and systolic function in an animal model. ***Methods and Results:*** After 12 months of fortnightly intraperitoneal injections of $2.5x10^7$ *Staphylococcus aureus* cell walls (*S. aureus*: n=30) or saline vehicle (control: n=30) in SD rats, there were no differences in LV dimension (LVEDD, V_0) or systolic chamber function (FS_{end}, E_{es}) between the two groups. In the control group no significant differences in plasma C-reactive protein concentrations (marker of inflammation) were noted at 3 or 11 months compared to the baseline concentrations at 1 month. However, in the *S. aureus* group there was a significant increase in CRP concentrations at both 3 months (*S. aureus*=3 months: 435.4±90.00µl.ml^{-1} vs. 1 month: 347.8±101.9µl.ml^{-1}, p<0.0001) and 11 months (*S. aureus*=11 months: 511.4±93.3µg.ml^{-1} vs. 1 month: 347.8±101.9µg.ml^{-1}, p<0.0001) compared to the baseline CRP concentrations at 1 month. Furthermore, CRP concentrations were significantly increased in the *S.aureus* group compared to the control group at 11 months (511.4±93.3µg.ml^{-1} vs. 391.2±122.1µg.ml^{-1}, p<0.01). Leukocyte log T/S was significantly reduced in the *S.aureus* compared to the control group at 11months (*S.aureus*: -0.21±0.15 vs.control: -0.053±0.11, p=0.002). Furthermore, cardiac log T/S was significantly reduced in the *S.aureus* compared to the saline group (*S. aureus*: -0.20±0.10 vs. control: -0.11±0.084, p=0.0021). However cardiac log T/S was not associated with LVEDD, V_0, FS_{end}, E_{es}, FS_{mid}, or E_{en}. ***Conclusions:*** In conclusion, a chronic inflammatory stimulus that produced reductions in leukocyte and cardiac telomere length did not induce LV dilatation and a reduction in systolic chamber function. Hence, the negative association between telomere length and heart failure may be due to the impact of inflammatory processes on telomere length rather than signifying a cause of heart failure.

5.2 INTRODUCTION

Studies have demonstrated that reduced average leukocyte telomere length is associated with chronic heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012). From these association studies it is unclear whether telomere attrition is caused by increased inflammation.

An important pathophysiological feature of heart failure that has been associated with reduced telomere length is inflammation (Beyne-Rauzy *et al.*, 2005; Yndestad *et al.*, 2006). Hence, it is possible that chronic inflammation may be responsible for the telomere attrition evident in heart failure. Indeed, leukocyte telomere length is inversely associated with markers of inflammation (Aikata *et al.*, 2000; Bekaert *et al.*, 2007; Huzen *et al.*, 2011; Ilmonen *et al.*, 2008; Kinouchi *et al.*, 1998; Masi *et al.*, 2012; Salpea *et al.*, 2010; Samani *et al.*, 2001; Valdes *et al.*, 2005; Weischer *et al.*, 2012) and patients in heart failure have increased concentrations of inflammatory cytokines (Adamopoulos *et al.*, 2001; Aukrust *et al.*, 1999; Levine *et al.*, 1990; Testa *et al.*, 1996; Yndestad *et al.*, 2006). In this regard, there is evidence demonstrating that inflammation alone is capable of heralding the onset of adverse cardiac remodelling (Bozkurt *et al.*, 1998; Kubota *et al.*, 1997).

Nevertheless, it is unclear whether telomere attrition in heart failure is caused by inflammation. Hence, we developed an animal model of chronic inflammation in order to evaluate the relationship between telomere length and chronic inflammation. This investigation allowed for the determination of whether chronic inflammation affects telomere dynamics and if changes in telomere length could then precipitate adverse cardiac remodelling and systolic dysfunction.

5.3 METHODS

5.3.1 Animal model: This study was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (ethics number: 2010/49/04). Sixty male SD rats at three months of age were used. The experimental group of rats (n=30) were injected intraperitoneally (i.p.) with 2.5 $x10^7$ cell walls of *Staphylococcus aureus* (Pansorbin; Calbiochem, California, USA) fortnightly for a 12 month period. Previous studies have demonstrated that injection of 2.5 $x10^9$ cell walls of *S. aureus* produced a febrile response in rats (Luker *et al.*, 2000). In this current investigation, a sub-pyrogenic dose was desirable, thus a hundredth of the pyrogenic dose (Luker *et al.*, 2000) was administered in the experimental group in an attempt to achieve this. Moreover, *S. aureus* was chosen for this study as there have been previous reports suggesting that animals do not develop diminished inflammatory responses after serial injections with gram positive bacterium (Goelst and Laburn, 1991). The control group (n=30) received 0.5ml of the saline vehicle every two weeks for the same 12 month treatment period. Great care was taken to avoid any possible contamination of the saline group with *S. aureus*. Such precautions included; housing of the *S. aureus* animals in a separate infectious area, handling of the saline rats was always conducted first on days requiring animal handling, and the *S. aureus* was not kept in the animal unit and was only brought into the animal unit on days of injection.

5.3.2 Echocardiography: Echocardiography was performed as previously described (see Section 3.3.2) to determine LV chamber dimensions and posterior wall thickness at end-diastole and end-systole in 15 rats each from the experimental and control groups. Furthermore, load-dependant indices of intrinsic myocardial function (FS_{mid}) and left ventricular systolic chamber function (FS_{end}) were also determined using echocardiography. Echocardiographic measurements were performed by a single experienced investigator who was blinded to the designation of animals to experimental and control groups.

5.3.3 Isolated, perfused heart preparations: 10 rats from each of the experimental and control groups had LV structural and functional measurements determined using isolated, perfused heart preparations as previously described (see Section 3.3.3). Left ventricular diastolic remodelling was assessed from the LV diastolic pressure-volume relationships, using the volume intercept at zero pressure (V_0) for comparative purposes, with an increased V_0 representative of an increased LV chamber size in diastole. Left ventricular systolic chamber performance was determined from the slope of the linear portion of the left ventricular developed pressure-volume relationships (E_{es}). In addition, a load-independent measure of intrinsic myocardial systolic function was assessed by constructing LV developed stress-strain relations and comparing the slopes (LV end systolic myocardial elastance-E_n) of these relationships.

5.3.4 Telomere length: DNA was extracted from leukocytes and the LV lateral wall of the myocardium using NucleoSpin® Blood and Tissue kits (Macherey-Nagel, Dueren, Germany) respectively, as per manufacturer's instructions (see Section 3.3.6). Leukocyte telomere length was determined at 1 month, 11 months, and at termination (13 months). The methodology employed to determine relative telomere length in leukocyte and tissue DNA is that described previously (see Sections 2.3.3 and 3.3.8). A standard sample was included in each amplification run and was used to standardise the entire sample T/S ratios as well as to determine coefficients of variation. The coefficients of variation of the S and T assays were 1.23% and 1.93%, respectively.

5.3.5 C-reactive protein: Plasma CRP concentrations at 1, 3 and 11 months were determined using a commercially available rat-specific CRP enzyme-linked, immunoabsorbant kit (eBioscience, San Diego, USA.). This assay was performed without any modifications to the manufacturer's instructions (see Section 3.3.9).

***5.3.6 Blood pressure*:** Prior to termination, tail-cuff blood pressures were determined using a CODA (Kent Scientific Corporation, Connecticut, USA.) non-invasive blood pressure system specifically designed for use on laboratory animals (see Section 3.3.7).

5.3.7 Running wheel activity: All of the animals in the experimental and control groups were housed in running wheel cages (FIGURE 5.1). The running wheels were equipped with bicycle ergometers and allowed for the measurement of voluntary activity. The activity levels were measured weekly, with a decrease in voluntary activity used as an indicator of illness-induced behavior (Grassi-Zucconi *et al.*, 1995). The percentage change in running wheel activity in non-injected weeks compared to injection weeks was also calculated: Δ= (non-injected weeks-injected weeks)/injected weeks).

5.3.8 Blood collection: For the first month both the experimental and control animals were not given any interventions and had no procedures performed on them. Blood and plasma samples were collected at the end of month one which then served as the baseline measurement for all subsequent months. For the subsequent 12 months the experimental and control groups received their respective treatments and blood samples were collected as described previously (see Section 3.3.4).

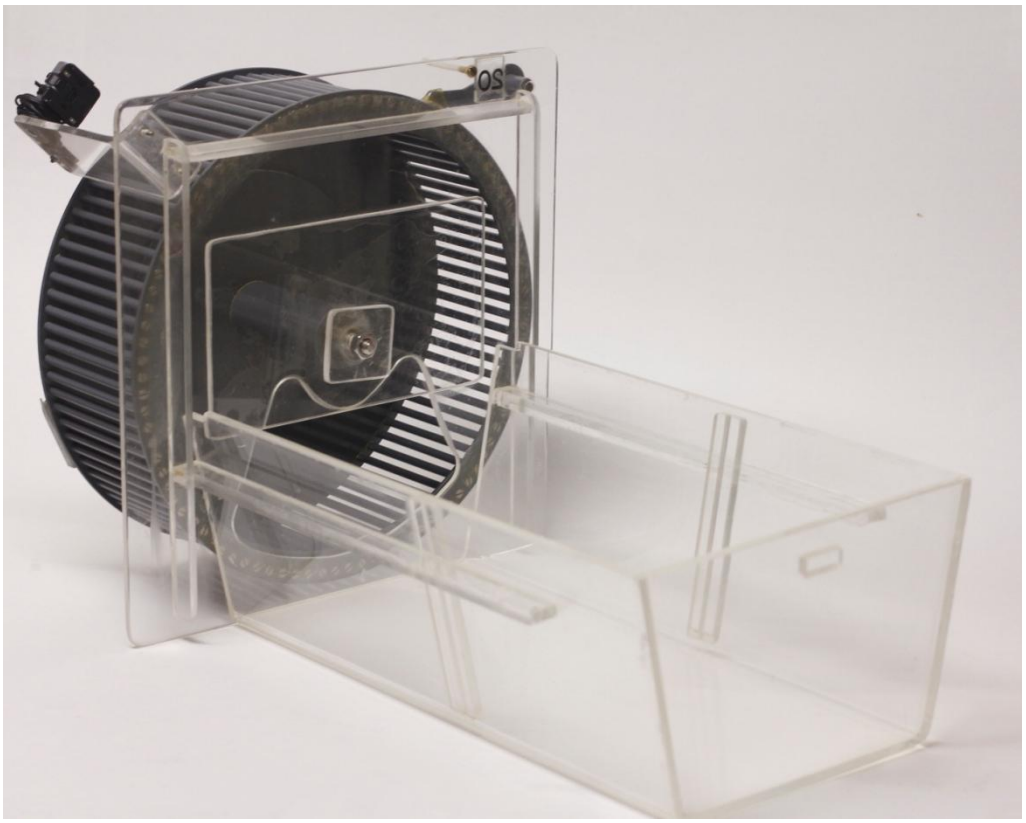

FIGURE 5.1 Running wheel cage employed to measure voluntary activity in the *S.aureus* and saline groups.

5.3.9 Statistical analysis: All statistical analyses were performed on log transformed T/S ratios. All inter-group analyses were conducted using a Student's t-test. All intra-group analyses were conducted using a repeated measures ANOVA with a Dunnett's Multiple Comparison post-hoc test, using 1 month as the control measurement. All associations between variables were conducted using Pearson's correlation coefficients. A $p<0.05$ was considered to be statistically significant. Data are represented as mean±SD and mean±SEM where indicated.

5.4 RESULTS

5.4.1 Animal deaths, weight data, and blood pressure: In total eight animals, four in the *S. aureus* and four the saline group respectively, died during the course of this investigation. The causes of death included; tooth malformations (n=2: 1 in the saline group and 1 in the *S. aureus* group), complications during i.p. injections (n=4: 3 in the saline group and 1 in the *S. aureus* group), growths (n=1), and one unknown cause (n=1).

TABLE 5.1 demonstrates that the *S. aureus* group had a greater mean body weight compared to the saline group. However, there were no differences in whole heart weight, LV weight, SBP, or DBP noted between the experimental and control groups. In light of the fact that body weight was significantly different between the *S. aureus* and saline groups, whole heart weight and LV weight were adjusted to tibia length. Even after adjustment, no differences between whole heart and left ventricular weight were noted (TABLE 5.1).

TABLE 5.1 The effects of 12 months of *S. aureus* on body weight, heart weight, and blood pressure.

	***S.aureus* (n=26)**	**Saline (n=26)**	**p value**
Body weight (g)	638.2±101.9	580.3±97.6	0.048*
Whole heart weight (g)	1.53±0.11	1.49±0.18	0.43
Whole heart weight/tibial length	0.33±0.028	0.33±0.041	0.49
Left ventricle weight (g)	1.30±0.10	1.27±0.15	0.43
Left ventricle weight/tibial length	0.28±0.025	0.28±0.035	0.52
DBP (mmHg)	94±15	85±14	0.054
SBP (mmHg)	133±18	128±15	0.34

DBP, diastolic blood pressure; SBP, systolic blood pressure. Values are represented as mean±SD;*$p<0.05$ versus *S.aureus* and saline groups.

5.4.2 LV remodelling: As assessed *in vivo*, chronic *S.aureus* injections did not result in LV dilatation as indexed by increases in LVEDD (FIGURE 5.2) and ESD (TABLE 5.2). Moreover, there were no significant differences in the LVPWED or PWES between the groups and as a consequence, there were no reductions in LVRWT at end-diastole (TABLE 5.2). As assessed *ex vivo* at the same heart rate, afterload and at comparable coronary flow rates, no right shift in the LV diastolic P-V relationship and no increase in mean V_0 in rats receiving *S. aureus* as compared to the saline treated group were noted (FIGURE 5.2).

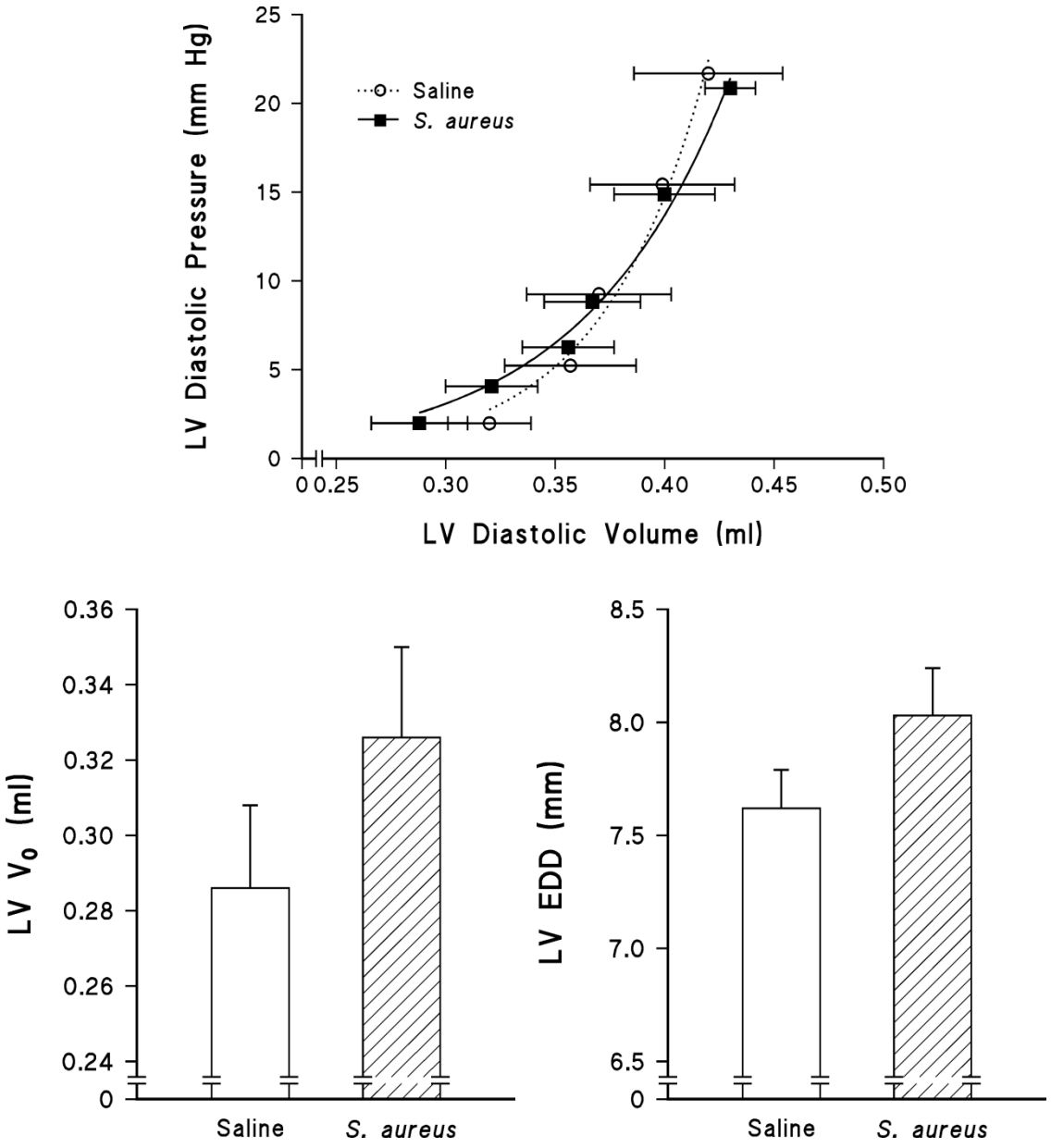

FIGURE 5.2 Effect of 12-months of *S.aureus* in rats on left ventricular (LV) chamber dimensions or volumes as indexed by LV end diastolic diameter (LVEDD) (*lower right panel*), LV diastolic pressure-volume relations (*upper panel*) and the volume intercept at 0 mm Hg(V_0) LV diastolic pressure (*lower left panel*). Data are represented as mean±SEM.

TABLE 5.2 The effects of 12 months of *S.aureus* injections in rats on echocardiography determined left ventricular dimensions.

	***S. aureus* (n=26)**	**Saline (n=26)**	**p value**
LVESD (mm)	4.3±0.5	4.0±0.1	0.15
PWED (mm)	2.2±0.2	2.0±0.2	0.53
PWES (mm)	2.9±0.2	2.8±0.2	0.18
RWT (mm)	0.6±0.1	0.6±0.1	0.66

LV, left ventricle; ESD, end systolic diameter; PWES, LV end systolic posterior wall thickness; PWED, LV end diastolic posterior wall thickness; RWT,relative wall thickness. Values are represented as mean±SD.

5.4.3 LV systolic function: FIGURE 5.3 shows the effect of chronic inflammation on LV systolic chamber function. *S.aureus* produced no effects on systolic LV chamber function as indexed by either the *in vivo* load and heart rate-dependent measure, FS_{end}, or the *ex vivo* load and heart rate independent measure, E_{es} (FIGURE 5.3). Furthermore, chronic *S.aureus* administration had no impact on intrinsic myocardial function as indexed by either the *in vivo* load and heart rate-dependent measure, FS_{mid}, or the *ex vivo* load and heart rate independent measure, E_{en} (FIGURE 5.4)

5.4.4 C-reactive protein: FIGURE 5.5 demonstrates the effects of chronic inflammation on mean plasma CRP concentrations between the experimental and control groups over the 12 month treatment protocol. No significant differences in CRP concentrations at 3 and 11 months compared to baseline measurements after 1 month of treatment were noted in the saline group. In contrast, the *S. aureus* group had a significant increase in CRP concentrations at both 3 and 11 months compared to the baseline CRP concentrations determined after 1 month. In terms of inter-group analysis, the saline group had a greater mean CRP concentration at month 1 compared to the *S.aureus* group (FIGURE 5.5). After 3 months no differences between CRP concentrations between the experimental and control groups were noted (FIGURE 5.5). The *S.aureus* group had increased plasma CRP concentrations after 11 months of treatment when compared to the saline group (FIGURE 5.5).

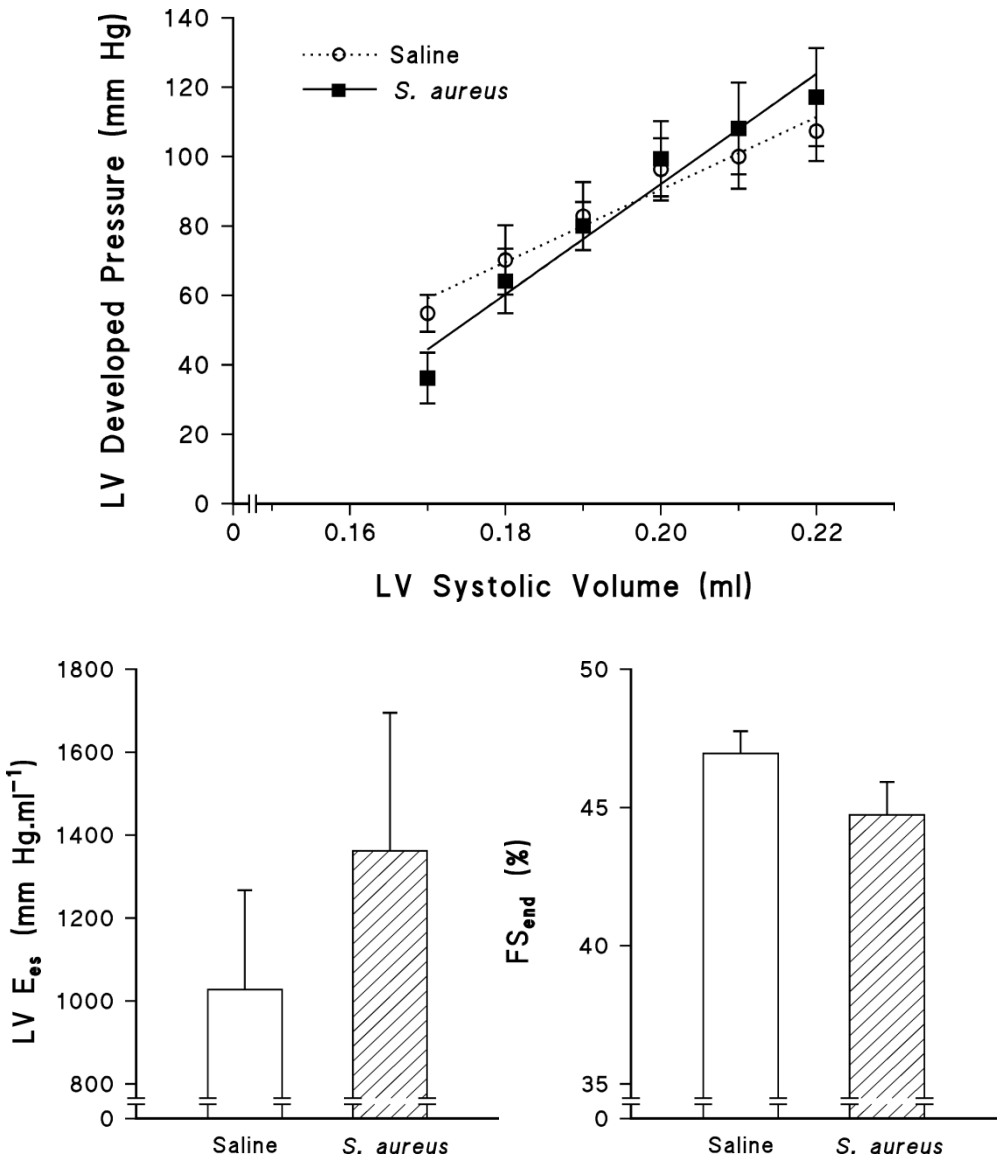

FIGURE 5.3 Effect of 12-months of *S.aureus* in rats on left ventricular (LV) systolic chamber function as indexed by LV endocardial fractional shortening (FS_{end}) (*lower right panel*), LV systolic pressure-volume relations (*upper panel*), and the slope of the systolic pressure-volume relations (LV E_{es}) (*lower left panel*). Data are represented as mean±SEM.

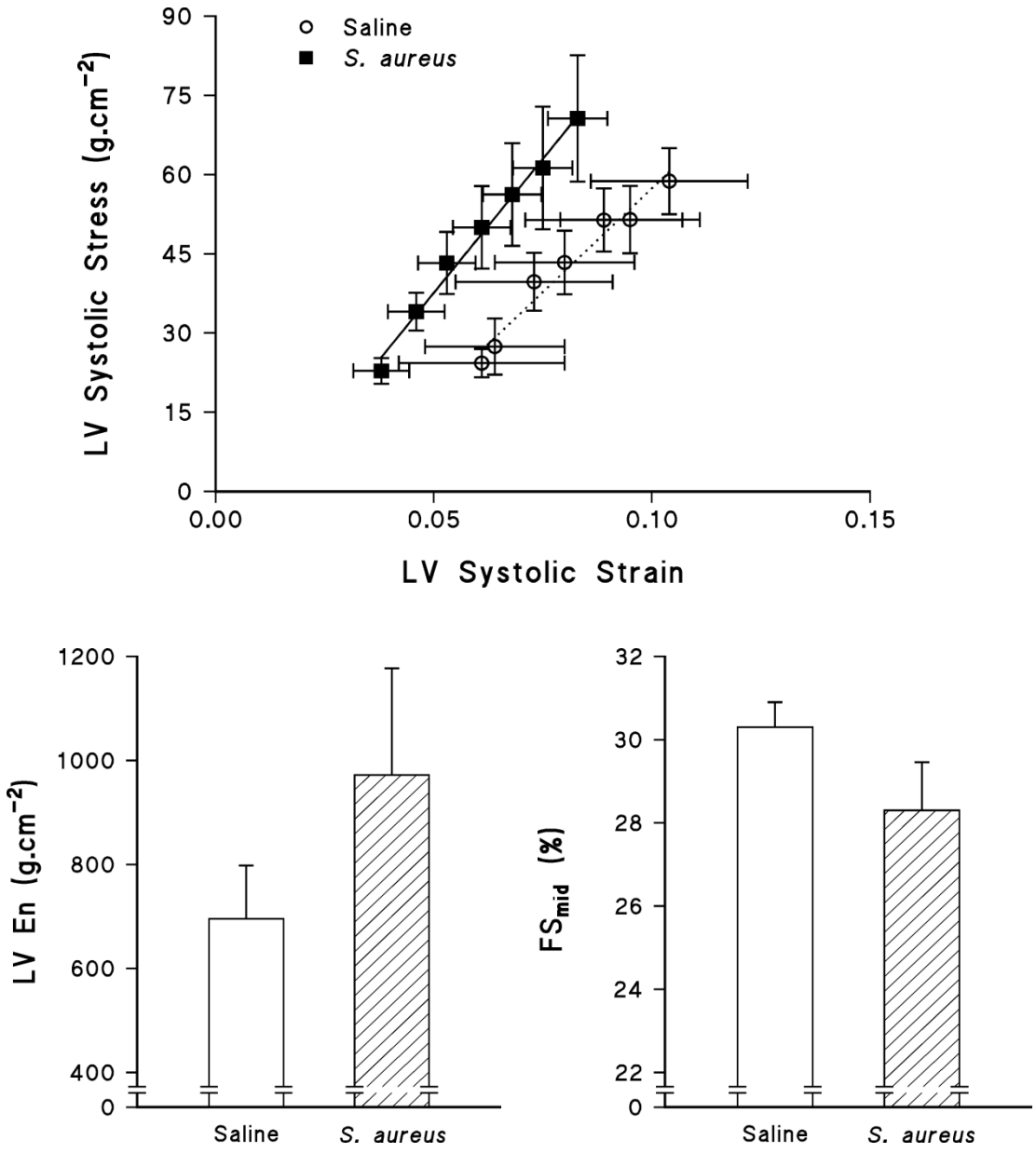

FIGURE 5.4 Effect of 12-months of *S.aureus* in rats on left ventricular (LV) intrinsic myocardial function as indexed by LV midwall fractional shortening (FS_{mid}) (*lower right panel*), LV systolic stress-strain relations (*upper panel*), and the slope of the systolic stress-strain relations (LV E_{en}) (*lower left panel*). Data are represented as mean±SEM.

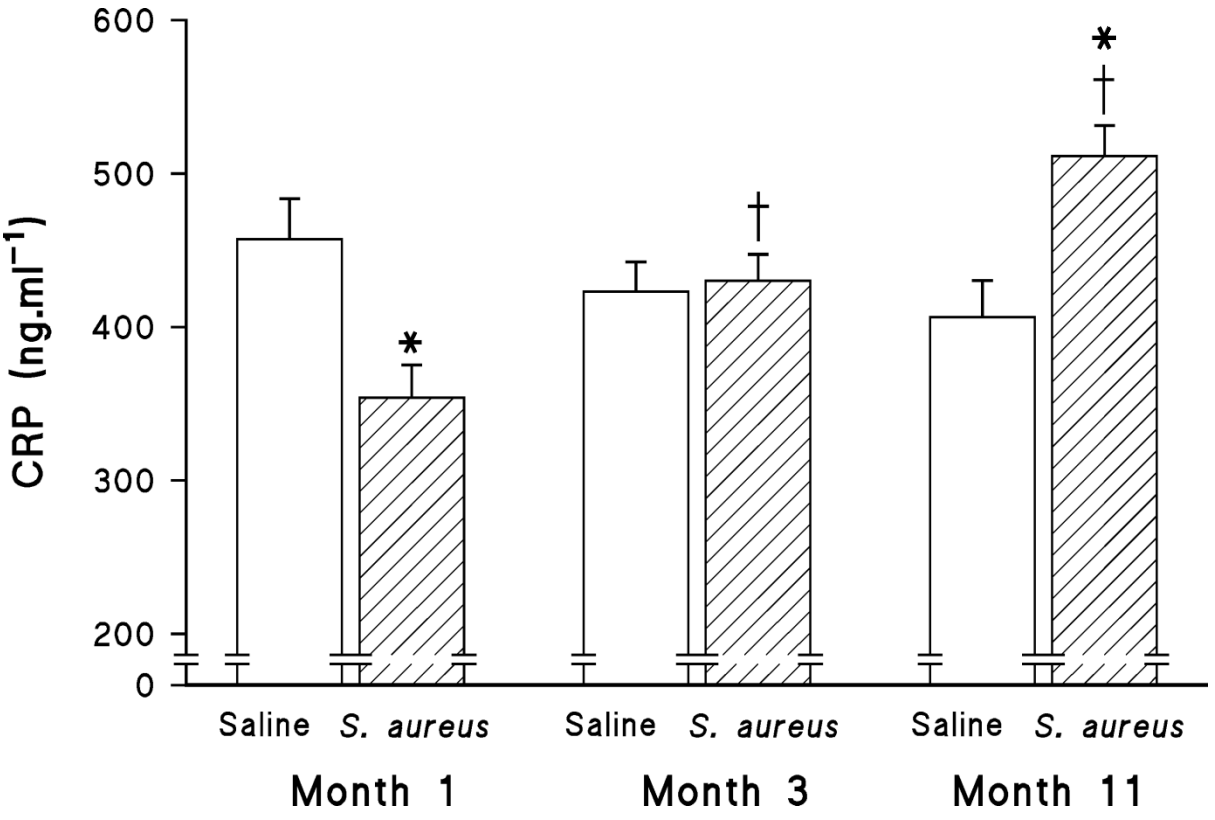

FIGURE 5.5 Mean plasma C-Reactive protein (CRP) concentrations for 1, 3, and 11 months in the *S.aureus* (n=20) and saline (n=17) groups respectively. Data are represented as mean±SD; *p<0.05 *S.aureus* versus saline group; †p<0.05 versus month 1 in the *S.aureus* and saline groups respectively.

5.4.5 Running wheel: With regards to running wheel activity, only animals that had a mean weekly running distance of greater than 1km.week^{-1} in the *S.aureus* (n=20) and saline (n=17) groups were included for analysis. This was done as the possible impact of inflammation on voluntary activity can not be determined in animals that were sedentry prior to the initiation of treatment. FIGURE 5.6 shows the mean weekly running distance over the 12 month treatment protocol in the *S.aureus* group was not significantly reduced compared to the saline group either in the injection or non-injection weeks. However intra-group analysis revealed that the experimental group had reduced weekly running distance on weeks that they were injected with *S.aureus* compared to weeks they received no injections. Importantly, this finding was not present in the control group when comparing weeks the animals were injected with saline compared to weeks they received no injections. These data indicate that the reduction in running wheel activity evident in the experimental group during injection weeks was due to *S. aureus* and not a result of the injections themselves.

5.4.6 Telomere length: FIGURE 5.7 shows the changes in leukocyte T/S ratios over time in the experimental and control groups. In the *S.aureus* group, there was a decline in leukocyte telomere length at 11 months compared to after 1 month. However, there was a subsequent increase in the leukocyte telomere length at termination when compared to log T/S ratios determined after 1 month in the *S.aureus* group. With respect to the saline group, there was a significant increase in leukocyte telomere length at both 11 months and termination when compared to log T/S ratios at 1 month. In terms of inter group analysis, no significant differences between leukocyte telomere length at month 1 and termination were noted between the experimental and control groups. However, the mean leukocyte T/S ratio at month 11 was significantly reduced in the *S.aureus* compared to the saline group. With respect to the telomere length of cardiac tissue, FIGURE 5.8 demonstrates that cardiac telomere length was significantly reduced in the *S.aureus* compared to the saline group.

FIGURE 5.6 Effect of 12-months of *S.aureus* in rats on weekly running distance. Data are represented as mean±SD (*upper panel*) and percentage change (*lower panel*); *p<0.05 injected versus non-injected weeks.

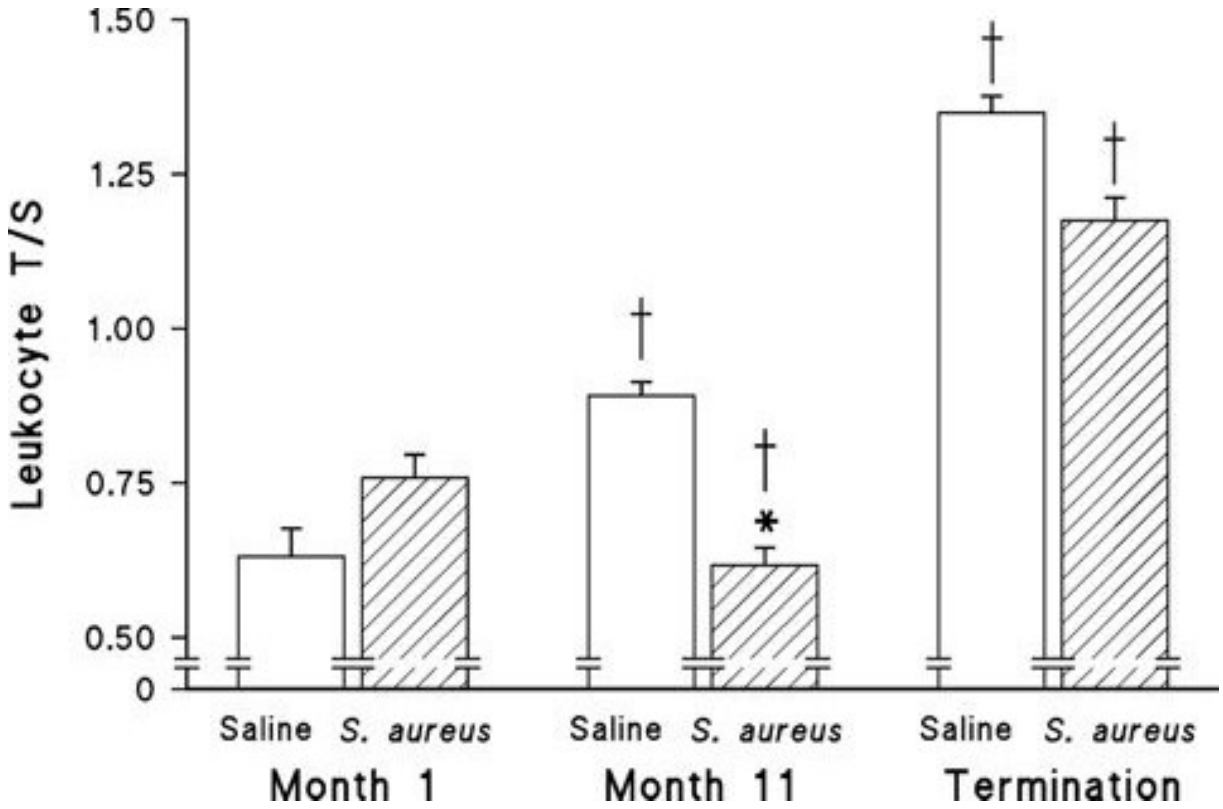

FIGURE 5.7 Relative leukocyte telomere lengths (as indexed by T/S) at 1 month, 11 months, and at termination in the *S.aureus* and saline groups. Data are represented as mean±SD; *p<0.05 *S.aureus* versus saline groups; †p<0.05 versus month 1 in the *S. aureus* and saline groups.

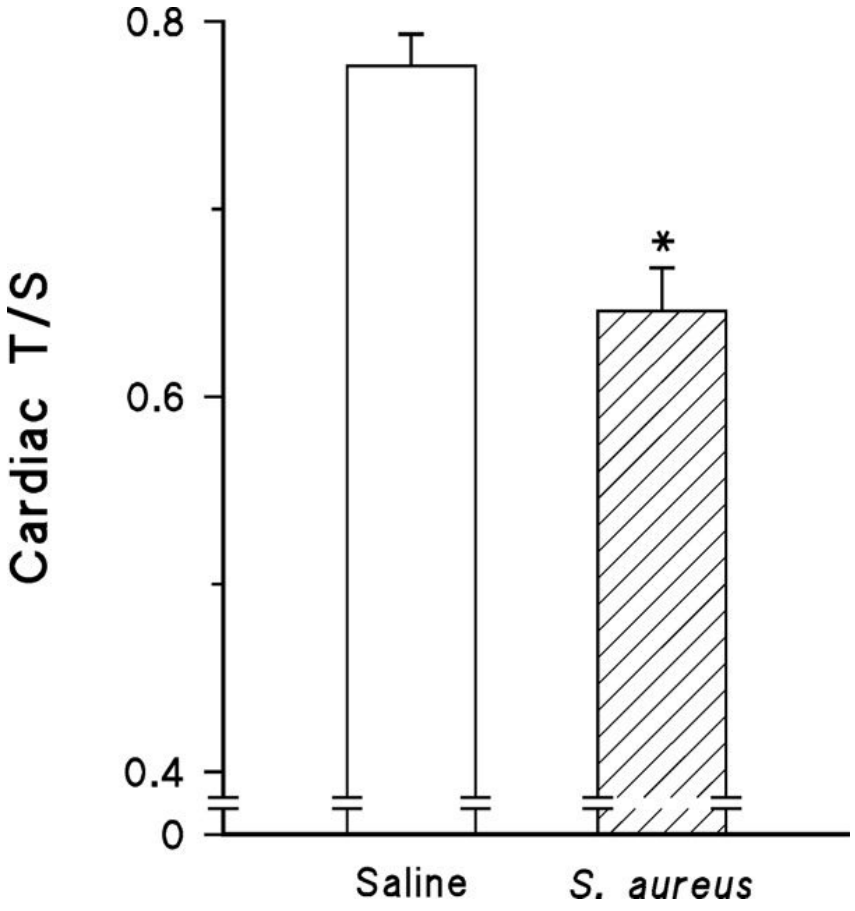

FIGURE 5.8 Effect of 12-months of *S.aureus* in rats on cardiac telomere length (as indexed by T/S). Data are represented as mean±SD; * $p<0.05$ versus saline group.

5.4.7 Telomere correlations: Neither indices of LV dilatation (LVEDD, V_0), systolic chamber function (FS_{end}, E_{es}), nor intrinsic myocardial function (FS_{mid}, E_{en}) were associated with cardiac telomere length in this study (TABLE 5.3). However, there was a significant negative association between leukocyte T/S ratio and CRP concentrations at month 11 but not month 1 in the combined groups (TABLE 5.3). Furthermore, there was a strong positive association between leukocyte telomere length at termination and cardiac telomere length (Pearson's $r = 0.7$, $p < 0.0001$, $R^2 = 0.4$).

TABLE 5.3 Correlations between cardiac telomere length and indices of left ventricular (LV) dilatation (the volume intercept at 0 mm Hg (V_0) in the left ventricular (LV) diastolic pressure-volume relations and LV end diastolic diameter (LVEDD)), systolic chamber function (slope of the systolic pressure-volume relations (LV E_{es}) and endocardial fractional shortening (FS_{end})), intrinsic myocardial function (slope of the systolic stress-strain relations (E_{en}) and midwall fractional shortening (FS_{mid})), and correlations between leukocyte telomere length and inflammation (C-reactive protein (CRP)).

	V_0	**LVEDD**	**LV E_{es}**	**FS_{end}**	**LV E_{en}**	**FS_{mid}**	**CRP 1 month**	**CRP 11 Months**
S. aureus								
Pearson's r	-0.13	-0.21	0.11	0.19	0.53	0.19	0.041	-0.30
95 %CI	-0.73 to 0.59	-0.67 to 0.36	-0.64 to 0.75	-0.38 to 0.66	-0.38 to 0.92	-0.38 to 0.66	-0.38 to 0.45	-0.68 to 0.21
p value	0.74	0.46	0.79	0.51	0.22	0.51	0.86	0.24
Saline								
Pearson's r	0.34	-0.39	0.30	0.064	0.31	-0.028	0.014	-0.22
95 %CI	-0.42 to 0.82	-0.76 to 0.18	-0.46 to 0.80	-0.48 to 0.57	-0.50 to 0.83	-0.55 to 0.51	-0.43 to 0.45	-0.58 to 0.21
p value	0.37	0.17	0.44	0.83	0.45	0.92	0.95	0.32
Combined								
Pearson's r	0.054	-0.26	0.22	0.23	0.054	0.22	-0.020	-0.46
95 %CI	-0.42 to 0.51	-0.58 to 0.14	-0.30 to 0.63	-0.15 to 0.56	-0.54 to 0.61	-0.17 to 0.54	-0.32 to 0.28	-0.68 to -0.17
p value	0.83	0.21	0.40	0.23	0.88	0.26	0.89	0.003*

r, correlation coefficient; CI, confidence interval. *p<0.05

5.5 DISCUSSION

The main findings of this study are as follows: chronic (12 months) inflammatory stimulus with *S.aureus* bacterial cell walls produced a significant reduction in cardiac and leukocyte telomere length in rats. However, neither the increase in cardiac telomere attrition nor chronic inflammation heralded the onset of LV dilatation and systolic dysfunction in this study. Furthermore, no associations between cardiac telomere length and LV structure or function were noted.

The main aim of the current investigation was to investigate whether inflammation causes telomere attrition which in turn results in cardiac remodelling and dysfunction. In this regard, there was clear evidence of immune activation following i.p. injections of *S. aureus* cell walls, demonstrated by plasma CRP concentrations that were both increased from baseline concentrations and were greater in the *S. aureus* animals compared to the control group after 11 months of treatment. Furthermore, the experimental group had reduced running wheel activity during weeks of *S. aureus* injections while this reduced activity was absent in the saline group. These data indicate a decrease in voluntary activity in the *S. aureus* group, which is an illness-induced behavioural response (Grassi-Zucconi *et al.*, 1995). Collectively, these data indicate that fortnightly i.p. injections of $2.5x10^7$ *S. aureus* cell walls did illicit immune responses that coincided with increased CRP concentrations and illness-induced behavioural responses and hence that this is a model of chronic inflammation.

The results from this study demonstrate that chronic inflammation increases telomere attrition in leukocyte and cardiac tissue. This association between increased telomere attrition and inflammation is consistent with previous studies. Indeed, there is significant evidence demonstrating that leukocyte telomere length is negatively associated with markers of inflammation (Bekaert *et al.*, 2007; Carrero *et al.*, 2008; Masi *et al.*, 2012; Shiels *et al.*, 2011; Wolkowitz *et al.*, 2011). Furthermore, Ilmonen *et al*. (2008) infected mice with bacteria and

noted that after 7 months leukocyte telomere length was reduced in the infected animals whilst there was a corresponding increase in leukocyte telomere length in the control group. Ilmonen *et al*. (2008), on the basis of these findings concluded that immune activation could increase telomere attrition. These findings are consistent with the current investigation, as leukocyte telomere length was reduced after 11 months in *S. aureus* group, but with a corresponding increase in leukocyte telomere length in the saline group. However, in this study there was an additional time point two months later where it was found that leukocyte telomere length subsequently increased in the *S. aureus* group as well. Hence, both telomere attrition and elongation were observed in the *S. aureus* group during this study. These findings have been demonstrated previously, longitudinal data in humans have indicated that leukocyte telomere length is variable and may increase, remain the same, or decrease in length over time (Aviv *et al.*, 2009; Nordfjäll *et al.*, 2009). Moreover, these studies have noted that telomere attrition is associated with baseline telomere length (Aviv *et al.*, 2009). Thus, a strong component of the rate of telomere attrition may be determined by initial telomere length itself. In this regard, there was no significant difference between leukocyte telomere length in the experimental and control groups after 1 month, thus one can still argue that inflammation increased leukocyte telomere attrition as leukocyte telomere decreased in the experimental group, but increased in the control group. However, when leukocyte telomere length reached a particular length in the *S. aureus* group telomerase (Nordfjäll*et al.*, 2009) and perhaps other factors involved in telomere length maintenance were activated to increase telomere length in the circulating population of leukocytes. Furthermore, it also possible that at any time point the circulating population of leukocytes had been replaced by new cells from bone marrow, with significantly different telomere lengths, and may also account for the fluctuations seen in leukocyte telomere length. This may explain the marked difference in telomere length measured over two months.

The results from this study provide insight into the cause and effect relationships between reduced telomere length evident in heart failure. In this regard, one of the possible explanations for reduced leukocyte telomere length in patients with chronic heart failure may be due to inflammation (Beyne-Rauzy *et al.*, 2005; Yndestad *et al.*, 2006). Indeed, the results from this investigation support this notion as both cardiac and leukocyte telomere lengths were reduced as a result of chronic inflammation. However, it is unknown if reduced cardiac telomere length has any pathophysiological consequences in the heart or if reduced telomere length represents an epiphenomenon due to the ongoing processes of heart failure. In this regard, this study demonstrated that marked reductions in cardiac telomere length, produced by 12 months of repeated *S. aureus* infections, did not herald the onset of any pathophysiological changes in the heart that are associated with heart failure measured in this study, namely LV dilatation and systolic dysfunction. Thus, previous studies demonstrating that reduced telomere length is associated with heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) and risk factors for cardiovascular disease (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Ogami *et al.*, 2004; Valdes *et al.*, 2005; Zee *et al.*, 2009) may represent an epiphenomenon due to ongoing inflammatory processes. Importantly, the results from this investigation indicate that increased cardiac telomere attrition as a consequence of chronic inflammation for 12 months in rats has no pathophysiological consequence on the heart in terms of LV structure and function. However, it has to be stated that if the animals received *S. aureus* infections for a longer period of time, whether or not these changes in cardiac telomere length coupled with continued inflammation would result in adverse cardiac remodelling and heart failure is uncertain. Nonetheless, these animals were 16 months of age at termination, which represents a significant proportion of the lifespan of a Sprague-Dawley rat.

There is evidence to suggest that inflammation is sufficient to cause adverse cardiac remodelling (Bozkurt *et al.*, 1998, 1998; Kubota *et al.*, 1997). In this regard, previous studies have demonstrated that TNF-α produces ventricular hypertrophy, dilatation, and fibrosis in mice (Kubota *et al.*, 1997) and rats (Bozkurt *et al.*, 1998). However, in the present study, after repeated infection with *S. aureus* there was no evidence of LV dilatation or systolic dysfunction. A possible explanation for differences between the current study and previous reports in terms of cardiac remodelling and dysfunction after immune activation may be due to the concentrations of pro-inflammatory cytokines. In this regard, high concentrations of TNF-α are known to produce septic shock (Meldrum, 1998). Moreover, septic shock has been demonstrated to produce LV dilatation and systolic dysfunction (Parker and Parrillo, 1983; Parker *et al.*, 1984; Parker *et al.*, 1990). Hence, the previous studies demonstrating adverse cardiac remodelling with TNF-α (Bozkurt *et al.*, 1998; Kubota *et al.*, 1997) may have produced TNF-α concentrations sufficiently high enough to illicit septic shock, which then resulted in LV dilatation and dysfunction (Parker and Parrillo, 1983; Parker *et al.*, 1984; Parker *et al.*, 1990). In contrast, the dose of *S. aureus* used in this study was a hundredth of a known pyrogenic dose and was intended to provide enough stimulus to illicit a immune response and is unlikely to have produced septic shock. This is demonstrated by the fact that no differences in SBP or DBP blood pressures were noted between the experimental and control groups, as shock is characterised by hypotension (Celes *et al.*, 2013). Moreover, there were no changes in cardiac function in the *S. aureus* group associated with septic shock (Celes *et al.*, 2013). Hence, one can conclude that the dose of *S. aureus* utilised in this study did not produce septic shock. Nonetheless, the dose of *S. aureus* was still sufficient to illicit both biological (increase CRP concentration) and behavioural (decreased voluntary activity) markers of immune activation. Thus, one could argue that a chronic infection with *S. aureus*

in rats, with concentrations of pro-inflammatory mediators that unequivocally did not result in septic shock, does not result in LV dilatation and systolic dysfunction.

Previously I have made statements that the absence of telomere attrition did not exclude a contribution of cardiomyocyte telomere dysfunction to the pathogenesis of ethanol and chronic β-AR induced LV dilatation. In this regard, the length of the shortest telomere in a particular population of cells rather than average telomere length determines cellular function (Hemann *et al.*, 2001). Thus, average relative telomere length may not closely reflect the impact of telomeres on cellular performance. However, the results of this study indicate that even a decrease in average cardiac telomere length was not sufficient to herald the onset of adverse cardiac remodelling and dysfunction in rats after 12 months of chronic *S. aureus*infection. These results further support the findings that ethanol and chronic β-AR induced LV dilatation are independent of telomere attrition despite the fact that the average, and not the shortest, telomere length was measured.

An observation of the current investigation that requires further explanation is that reduced leukocyte telomere length was not associated with plasma CRP concentrations determined at month 1, but was however correlated after 11 months. Telomere length is thought to represent the cumulative effects of inflammation throughout an individual's lifespan (Zglinicki and Martin-Ruiz, 2005), thus baseline measurements after 1 month in this study represented a time point early in the animal's life span where inflammation was possibly negligible. Hence, the lack of association between leukocyte telomere length and CRP at 1 month may be due to the absence of sufficient inflammation required to induce telomere attrition in the early lives in the experimental and control groups.

In terms of study limitations, firstly, there were no markers of oxidative stress available in this study. Inflammation is known to result in an increase in oxidative stress (Frantz *et al.*, 2000; Knuefermann *et al.*, 2004), which could potentially increase telomere

attrition. Thus, the changes in leukocyte and cardiac telomere length that are attributed to oxidative stress in this study cannot be determined. Nonetheless, even if there was an increase in oxidative stress in this study, the reductions in cardiac telomere length had no pathological consequences on the heart. Secondly, body temperatures were not measured in the animals. In the current study a sub-pyrogenic dose was desired in an attempt to model individuals with chronic inflammatory disorders. Importantly, these individuals have chronic immune activation but without fever. In the present study there were biological and behavioural indicators of immune activation, but whether these changes occurred without fever is uncertain.

In conclusion, chronic inflammation with *S. aureus* caused a decrease in cardiac telomere length but these changes in telomere length were not associated with adverse LV remodelling and systolic dysfunction. These data indicate that previous associations between reduced telomere length and heart failure and risk factors for heart failure may represent an epiphenomenon due to ongoing inflammatory processes.

CHAPTER 6 - IS TELOMERE LENGTH ASSOCIATED WITH INFLAMMATION, LEFT VENTRICULAR GEOMETRY AND FUNCTION, AND TELOMERE LENGTH IN A CROSS-SECTIONAL COMMUNITY STUDY?

6.1 ABSTRACT

Aims: To determine, in a cross-sectional human study, whether inflammation and telomere attrition are associated with cardiac geometry and function. ***Methods and Results:*** Leukocyte telomere length (log T/S) was determined in 662 participants from a randomly selected community based cohort. There was a negative bivariate relationship between T/S ratio and age in the group ($p<0.0001$) and telomere length was reduced in participants who regularly consumed alcohol compared to those who did not ($p=0.01$). No relationships between log T/S and smoking, sex, BMI, diabetes mellitus, or blood pressure were noted. However, there was a negative relationship between leukocyte telomere length and C-reactive protein concentrations after multivariate adjustments ($r=-0.11$; $p=0.0039$; $n=662$). There were no associations between log T/S and echocardiographic indices of LV geometry (LVEDD) or systolic function (FS_{end}, FS_{mid}) in the participants. ***Conclusions:*** In conclusion, leukocyte telomere length is inversely associated with indices of inflammation. However, leukocyte telomere length is not associated with indices of LV geometry or function. Hence, inflammation may be an important determinant of leukocyte telomere attrition, but leukocyte telomere length is not a marker of alterations in cardiac geometry or function.

6.2 INTRODUCTION

Studies have demonstrated that reduced average leukocyte telomere length is associated with chronic heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012). An important pathophysiological feature of heart failure that has been associated with reduced telomere length is inflammation (Beyne-Rauzy *et al.*, 2005; Yndestad *et al.*, 2006). In this regard, leukocyte telomere length has been demonstrated to be inversely associated with indices of inflammation (Aikata *et al.*, 2000; Bekaert *et al.*, 2007; Huzen *et al.*, 2011; Ilmonen *et al.*, 2008; Kinouchi *et al.*, 1998; Masi *et al.*, 2012; Salpea *et al.*, 2010; Samani *et al.*, 2001; Valdes *et al.*, 2005; Weischer *et al.*, 2012) and patients in heart failure have increased concentrations of inflammatory cytokines (Adamopoulos *et al.*, 2001; Aukrust *et al.*, 1999; Levine *et al.*, 1990; Testa *et al.*, 1996; Yndestad *et al.*, 2006).

Hence, it is possible that inflammation may be responsible for the telomere attrition evident in heart failure. In this regard, I have demonstrated in an animal model that chronic inflammation decreased cardiac and leukocyte telomere length (see Chapter 5). However, these changes in telomere length were not associated with adverse LV remodelling and systolic dysfunction. These data indicate that previous associations between reduced telomere length and heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012) and risk factorsfor heart failure (Brouilette *et al.*, 2007; Fuster *et al.*, 2007; Huzen *et al.*, 2011; Ogami *et al.*, 2004; Salpea *et al.*, 2010; Samani *et al.*, 2001;Valdes *et al.*, 2005; Valdes *et al.*, 2005; Weischer *et al.*, 2012) may represent an epiphenomenon due to ongoing inflammatory processes.

In terms of human data that has investigated the associations between telomere length, cardiac geometry and function, Collerton *et al.* (2007) did show that leukocyte telomere length was negatively associated with left ventricular ejection fraction. However, an

important consideration is that this study consisted of individuals older than 85 years. Given the strong negative relationship between telomere length and age and between ejection fraction and age the association between telomere length and ejection fraction in this study is to be expected. In addition, Starr *et al.* (2007) demonstrated that reduced telomere length was associated with ischaemic changes on electrocardiograms. However, it is still unclear whether telomere attrition in humans has a pathophysiological role in heralding the onset of LV remodelling and systolic dysfunction in heart failure. Hence, in order to confirm my findings demonstrated in the animal model of chronic inflammation, a cross-sectional investigation to assess the relationship between leukocyte telomere length and LV cardiac geomtery and function in a community based population was conducted.

6.3 METHODS

6.3.1 Study group: The present study was conducted according to the principles outlined in the Helsinki declaration. The Committee for Research on Human Subjects of the University of the Witwatersrand approved the protocol (approval number: M02-04-72 and renewed as M07-04-69). Participants provided informed, written consent. All participants in this study were a part of a large scale community based study, which has been described previously (Norton *et al.*, 2012). Families of black African descent, with siblings greater than 16 years of age were randomly recruited from the South West Township of Johannesburg, South Africa. The sample size consisted of 1179 participants in whom leukocyte telomere length and CRP concentrations were assessed in 662 individuals.

6.3.2 Clinical, demographic, and anthropometric measurements: A standard questionnaire was used to obtain demographic, clinical, smoking, and drinking history of the participants (Norton *et al.*, 2012). Height and weight, for the determination of BMI, were measured using standard methods, and participants were defined as obese if their BMI was greater than 30 $kg.m^{-2}$ (Norton *et al.*, 2012). Blood pressure measurements were obtained using a standard mercury sphygmomanometer (Norton *et al.*, 2012). Hypertension was defined as a mean systolic blood pressure greater than 140 mmHg or diastolic pressure greater than 90 mmHg. Standard laboratory blood tests of percentage glycated haemoglobin were performed and diabetes mellitus was defined as a percentage glycated haemoglobin (HbA1c) value greater than 6.1% (Norton *et al.*, 2012). CRP concentrations were determined using a immunoturbidimetric assay (Architect, Sentinel Diagnostics, USA) performed on Olympus OSR 6185 reagent (Olympus Diagnostics, Lismeehan, Ireland).

6.3.3 LV dimensions and function: In 409 of the 662 participants, 2D, M-mode echocardiography was performed using a Hewlett-Packard Sonos 2500 system with a 2.5-MHz transducer. LV internal dimensions were determined as previously described (Norton *et al.*, 2012; see Section 2.5.3).

6.3.4 Average telomere length: The methodology employed to determine relative telomere length in leukocyte and tissue DNA is that described previously (see Section 2.3.3). A standard sample was included in each amplification run and was used to standardise the entire sample T/S ratios as well as to determine coefficients of variation. The coefficients of variation of the S and T assays were 1.19% and 1.36%, respectively.

6.3.5 Statistical analysis: All tests were carried out on log-transformed T/S crossing point ratios and CRP concentrations, as they were not normally distributed. Multivariate regression analysis was performed to assess independent relationships. Adjustments were

made for age and sex as these are strong determinants of telomere length. For the relationship between telomere length, CRP, LVEDD, FS_{end}, and FS_{mid} adjustments for BMI, smoking, drinking, treatment for hypertension, and diabetes mellitus were also included as they may impact on CRP concentrations, cardiac function and geometry. Statistical analysis was conducted using SAS software, version 9.3 (SAS Institute Inc., Cary, NC, USA). Data are represented as mean±SD.

6.4 RESULTS

6.4.1 Participant characteristics: TABLE 6.1 shows the characteristics of the cross-sectional community based cohort. This population consisted of primarily female participants with a high proportion of obesity and hypertension. However, there was a low proportion of other prominent risk factors for cardiovascular disease such as diabetes mellitus, regular smoking, and regular alcohol consumption in this cohort.

TABLE 6.1 Demographics and prevalence of risk factors for cardiovascular diseasein the participants.

n	662
Age (years)	44±19
% Female	64
Regular alcohol consumption (%)	22
Regular smoking (%)	14
% Diabetes mellitus (HbA1c>6.1)	24
Body mass index ($kg.m^{-2}$)	29.5±9.1
% Obese	43
Systolic blood pressure (mm Hg)	121±23
Diastolic blood pressure (mm Hg)	84±13
% Hypertensive	40

Values are represented as mean±SD or percentage.

6.4.2 Relationships between average relative leukocyte telomere length and participant characteristics: There was a negative bivariate relationship between T/S ratio and age in the participants (FIGURE 6.1). The mean log T/S ratio of the participants who drank were reduced compared to those who did not regularly consume alcohol (TABLE 6.2). No relationships between T/S ratio and smoking, sex, BMI, diabetes mellitus, or blood pressure were noted (TABLE 6.2).

6.4.3 Relationships between average relative leukocyte telomere length and CRP: FIGURE 6.2 shows the negative bivariate relationship between leukocyte telomere length and log CRP concentrations in this cohort. The relationship persisted after multivariate adjustment for age, BMI, sex, smoking, drinking, treatment for hypertension and diabetes mellitus (r=-0.11; p=0.0039; n=662).

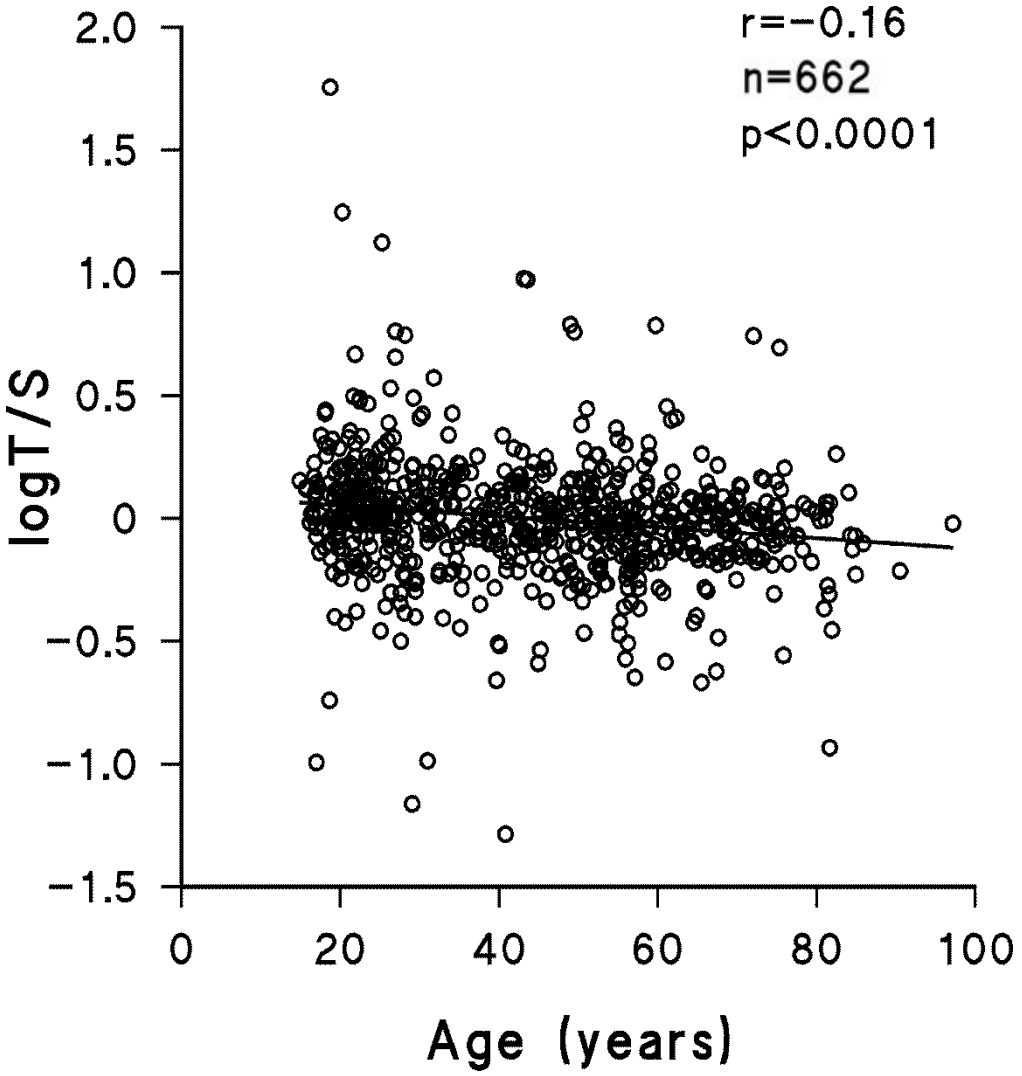

FIGURE 6.1 Bivariate relationships between leukocyte telomere repeat number/single copy gene repeat number ratio (log T/S) and age (years) in a randomly selected community based cohort.

TABLE 6.2 Relationship between relative leukocyte telomere length (log T/S) and risk factors for cardiovascular disease in participants (n=662).

	Pearson's r	**95% CI**	**p value**
Body mass index‡	0.015	-0.060 to 0.089	0.70
Systolic blood pressure‡	-0.024	-0.10 to 0.051	0.53
Diastolic blood pressure‡	-0.060	-0.13 to 0.016	0.12
	Mean±SD T/S		**pvalue****
Gender	M=1.19 ±1.07 (n=224)	F=1.31±2.9 (n=398)	0.77
Diabetes mellitus	Yes=1.08 (n=149)	No=1.33 (n=513)	0.48
Regular alcohol	Yes=1.03±0.62 (n=136)	No=1.33±2.7 (n=526)	0.012*
Regular smoking	Yes=1.31 (n=87)	No=1.26 (n=575)	0.43

r, correlation coefficient; CI, confidence interval; M, male; F, female. *p<0.05; ‡ age- and sex-adjusted; ** Student's t-test.

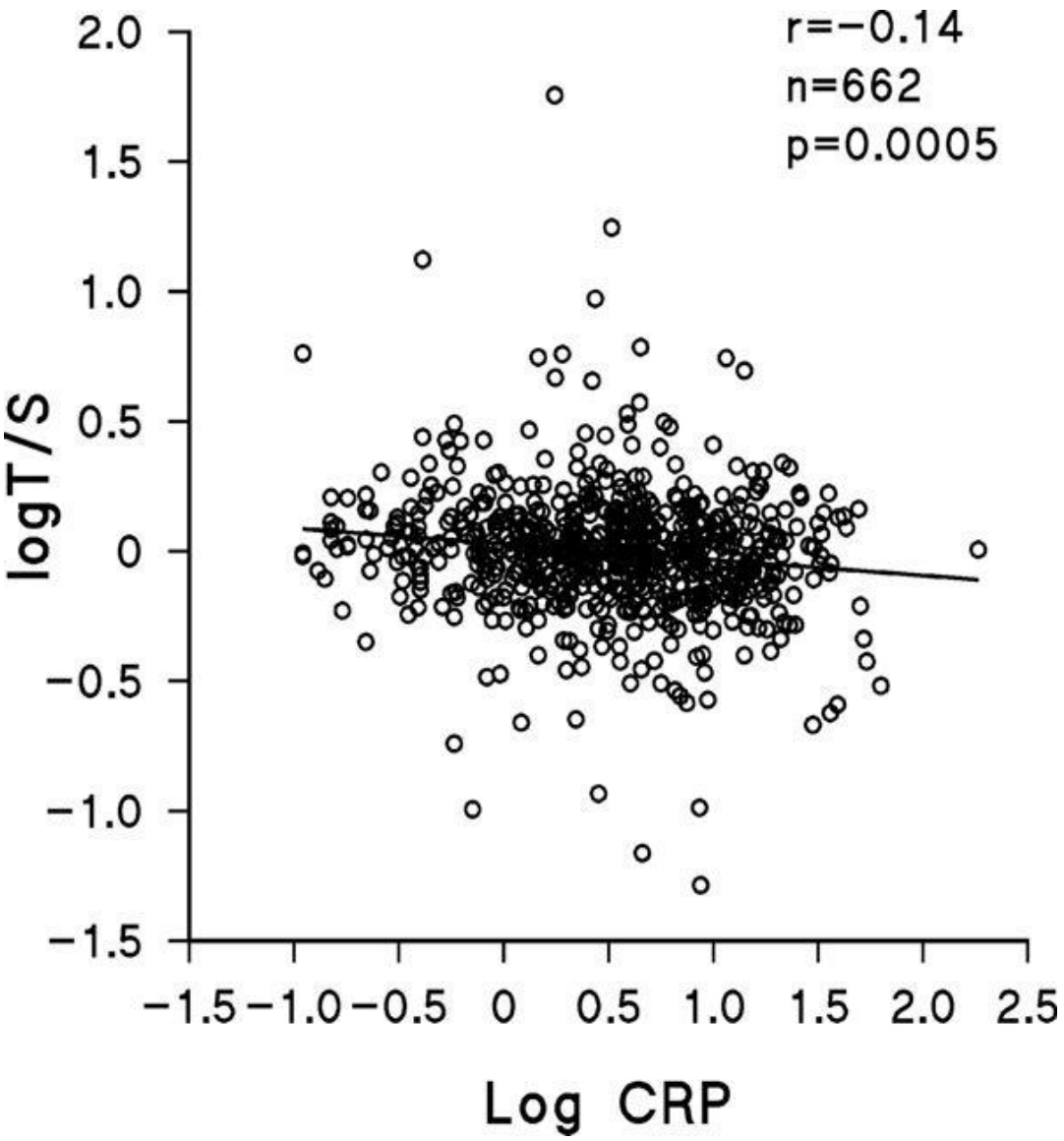

FIGURE 6.2 Bivariate relationships between leukocyte telomere repeat number/single copy gene repeat number ratio (log T/S) and log transformed C-reactive protein (CRP) concentrations in a randomly selected community based cohort.

6.4.4 Relationship between average relative leukocyte telomere length LV geometry and function: TABLE 6.3 gives the univariate and multivariate adjusted correlation coefficients between log T/S ratios and LVEDD, FS_{end}, and FS_{mid} in the participants. There were no association between log T/S ratios and indices of LV dilatation (LVEDD) or cardiac dysfunction (FS_{end}, FS_{mid}) in the participants.

TABLE 6.3 Multivariate adjusted relations between relative leukocyte telomere length (log T/S) and left ventricular end diastolic diameter (LVEDD), intrinsic myocardial function (FS_{mid}), and ejection fraction (FS_{end}) in participants.

Log T/S versus	Partial r	95% CI	p value
LVEDD (n=409):			
Unadjusted	0.0013	-0.095 to 0.098	0.98
Age- and sex-adjusted	0.000053	-0.097 to 0.097	0.99
Age-, sex- and risk factor*- adjusted	0.019	-0.80 to 0.12	0.70
FS_{end} (n=409):			
Unadjusted	-0.017	-0.11 to 0.08	0.73
Age- and sex-adjusted	-0.0054	-0.10 to 0.09	0.91
Age-, sex- and risk factor*-adjusted	-0.0013	-0.10 to 0.10	0.97
FS_{mid} (n=409):			
Unadjusted	-0.05	-0.14 to 0.05	0.35
Age- and sex-adjusted	-0.03	-0.13 to 0.06	0.48
Age-, sex- and risk factor*-adjusted	-0.03	-0.13 to 0.07	0.50

r, correlation coefficient; CI, confidence interval. *age, body mass index, sex, smoking, drinking, treatment for hypertension, diabetes mellitus, and CRP.

6.5 DISCUSSION

The main findings of this chapter in my thesis are that average leukocyte telomere length is inversely associated with CRP concentrations. However, leukocyte telomere length was not associated with indices of LV geometry (LVEDD) or systolic function (FS_{end}, FS_{mid}) in humans of black ancestry.

The results of this study are in keeping with previous published reports investigating leukocyte telomere length and markers of inflammation. In this regard, CRP concentrations have been demonstrated to be inversely associated with relative leukocyte telomere length (Bekaert *et al.*, 2007; Carrero *et al.*, 2008; Masi *et al.*, 2012). However, the results of this study extend previous findings by investigating the relationship between leukocyte and CRP over a wide age range (16-97 years of age). In this regard, previous large-scale cross-sectional studies have been conducted in samples with relatively narrow age ranges such as adolescent (13-16 years of age) (Masi *et al.*, 2012) and middle aged populations (35-55 years of age) (Bekaert *et al.*, 2007). In addition to CRP, leukocyte telomere length has been negatively correlated with other markers of inflammation including; IL-6 (Carrero *et al.*, 2008; Shiels *et al.*, 2011; Wolkowitz *et al.*, 2011), and fibrinogen (Bekaert *et al.*, 2007). Collectively these data indicate that inflammation could potentially increase telomere length attrition. Indeed, I have demonstrated that chronic inflammation with *S. aureus* resulted in a marked decrease in cardiac and leukocyte telomere length in rats and that leukocyte telomere length was inversely associated with CRP concentrations (see Chapter 5).

Hence, it is possible that chronic inflammation could explain the reduced average leukocyte telomere length associated with chronic heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012). However, the relationships between leukocyte telomere length and cardiovascular disease have mainly been determined using outcome

based studies and not using measurements of cardiac function. However, Collerton *et al.* (2007) did show that leukocyte telomere length was negatively associated with left ventricular ejection fraction. However, an important consideration is that this study consisted of individuals older than 85 years. Moreover, Starr *et al.* (2007) demonstrated that reduced telomere length was associated with ischaemic changes on electrocardiograms. However, from this data it is not conclusive whether telomere attrition in humans has a pathophysiological role in heralding the onset of LV remodelling and systolic dysfunction in heart failure. Hence, telomere length may have no impact on cardiac structure and function and may merely be reduced as a consequence of the pathophysiological processes of cardiovascular diseases, such as inflammation. In this regard, the results of this study demonstrate that leukocyte telomere length was not associated with indices of LV geometry and systolic function, despite negative associations with CRP concentrations. The results of this study are consistent with previous findings in my thesis (see Chapter 5) demonstrating that marked reductions in cardiac and leukocyte telomere length, produced by 12 months of chronic *S. aureus* infections, did not herald the onset of any pathophysiological changes in the heart that are associated with heart failure. Hence, the results from this study support the associations between inflammation, telomere length, and cardiac geometry and function that I have demonstrated in rats (see Chapter 5). Collectively, these data indicate that previous associations between reduced leukocyte telomere length, heart failure (Starr *et al.*, 2007; van der Harst *et al.*, 2007; Weischer *et al.*, 2012), and risk factors for cardiovascular disease (Brouilette *et al.*, 2003; Fitzpatrick *et al.*, 2007; Ogami *et al.*, 2004; Valdes *et al.*, 2005; Zee *et al.*, 2009) may represent an epiphenomenon due to ongoing inflammatory processes and that reductions in telomere length may have no impact on cardiac geometry and function.

In terms of study limitations, firstly, this is a cross-sectional study which does not allow for conclusions regarding cause and effect relationships. Nevertheless, the results of

this study, in conjunction with the longitudinal chronic inflammation study in rats (see Chapter 5), support the notion that telomere length is merely reduced as an epiphenomenon due to ongoing inflammatory processes in heart failure or risk factors for heart failure. Secondly, as highlighted in the aforementioned discussions, telomere length has a high inter-individual variability (Aviv *et al.*, 2009; Nordfjäll *et al.*, 2009); and hence a cross-sectional determination of telomere length at one specific time point does not accurately reflect long-term telomere length dynamics of individuals. Hence, this could potentially explain the inability to demonstrate associations between leukocyte telomere length and cardiac geometry and function. Nevertheless, if leukocyte telomere length is associated with LV dimension and function, my inability to demonstrate this relationship in a large cross-sectional study highlights that leukocyte telomere length may not be a sensitive marker of LV geometry and systolic function. Thirdly, this study only included particpants of African ancestry. Lastly, average leukocyte telomere length may not closely represent the extent of telomere attrition that occurs in cardiomyocytes. Indeed, cardiomyocyte telomere attrition or dysfunction has been associated with cardiomyocyte apoptosis, cell death and premature senescence in human heart failure (Chimenti *et al.*, 2003; Oh *et al.*, 2003) and with repression of mitochondrial biogenesis and performance (Sahin *et al.*, 2011). However, I have demonstrated that leukocyte telomere length is correlated with cardiac telomere length (see Chapters 3 and 5) in rats and hence leukocyte telomere may serve as a surrogate marker of cardiac telomere length. Furthermore, I have demonstrated that a decrease in average cardiac telomere length was not sufficient to herald the onset of adverse cardiac remodelling and dysfunction (see Chapter 5).

In conclusion, leukocyte telomere length is inversely associated with inflammation. However, leukocyte telomere length is not associated with indices of LV geometry or systolic

dysfunction. Hence, inflammation is a strong determinant of leukocyte telomere attrition, but leukocyte telomere length is not a marker of changes in cardiac geometry or function.

CHAPTER 7 - SUMMARY AND CONCLUSIONS

7.1 SUMMARY AND CONCLUSIONS

The main aims of this thesis were to investigate: the association of telomere length with the severity and presence of a non-ischaemic form of heart failure; whether telomere shortening is a cause of adverse left ventricular remodelling and systolic dysfunction in an animal model of dilated cardiomyopathy; whether adverse left ventricular remodelling and systolic chamber dysfunction produced by chronic β-adrenergic receptor activation are associated with telomere shortening; the impact of chronic inflammation on telomere length, left ventricular remodelling, and systolic dysfunction; and to the relationships between telomere length and inflammation, left ventricular geometry, and systolic function in a cross-sectional community study.

The main findings from my thesis are as follows (FIGURE 7.1); a reduced telomere length was not associated with a non-ischaemic form of heart disease nor with a model of alcoholic cardiomyopathy, which demonstrates that reduced telomere length may not be associated with all types of cardiomyopathy. Moreover, these data indicate that telomere length may not be a causal factor in heart failure. Although chronic β-AR activation and ethanol consumption induced cardiac remodelling and β-AR activation induced systolic dysfunction, cardiac telomere length was not altered. Moreover, in IDC, alcoholic cardiomyopathy, chronic β-AR stimulation, chronic inflammation, and in a random community based population study a reduced telomere length was not associated with pathological features of heart failure such as LV dilatation, systolic dysfunction, intrinsic myocardial function, and myocardial apoptosis. Furthermore, when the control groups of all the animal studies mentioned above were combined, I was still unable to show any associations between cardiac telomere length and indices of cardiac geometry and function (TABLE 7.1).

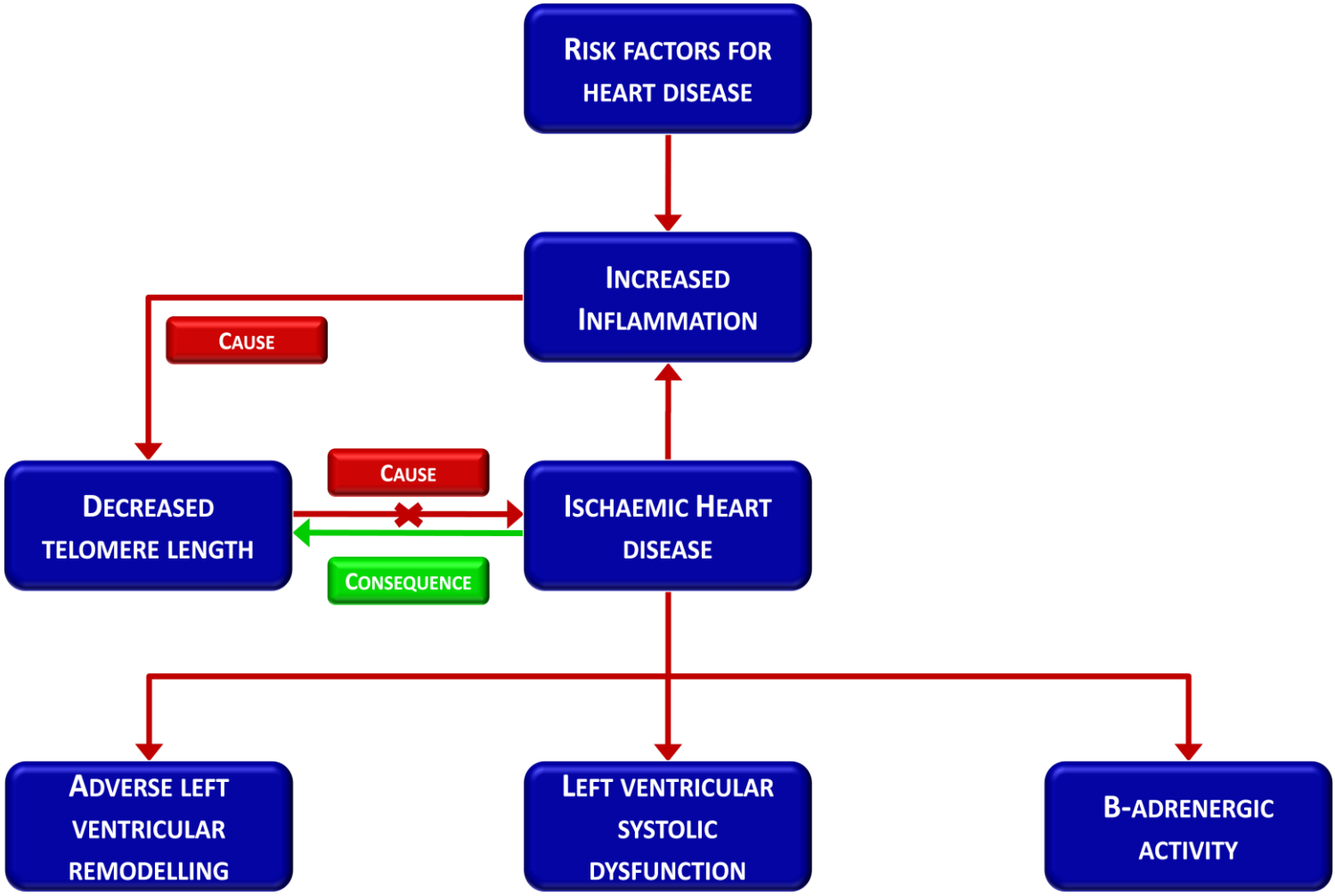

FIGURE 7.1 Diagram illustrating that reduced telomere length may be a consequence of increased inflammation in heart disease. Furthermore, telomere length is independent of adverse left ventricular remodelling, left ventricular systolic dysfunction, and β-adrenergic activity.

TABLE 7.1 Correlations between cardiac telomere length and indices of left ventricular (LV) dilatation (the volume intercept at 0 mm Hg (V_0) in the left ventricular (LV) diastolic pressure-volume relations and LV end diastolic diameter (LVEDD)), systolic chamber function (slope of the systolic pressure-volume relations (LV E_{es}) and endocardial fractional shortening (FS_{end})), and intrinsic myocardial function (slope of the systolic stress-strain relations (E_{en}) and midwall fractional shortening (FS_{mid})) in Sprague-Dawley rats (control groups from Chapters 3, 4, 5).

	V_0 (n=27)	**LVEDD (n=47)**	**LV E_{es} (27)**	**FS_{end} (46)**	**LV E_{en} (n=26)**	**FS_{mid} (n=47)**
Pearson's r	-0.15	0.047	0.068	-0.20	0.037	-0.023
95% CI	-0.50 to 0.24	-0.24 to 0.33	-0.32 to 0.43	-0.47 to 0.093	-0.36 to 0.47	-0.31 to 0.27
p value	0.44	0.75	0.73	0.18	0.86	0.88

r, correlation coefficient; CI, confidence interval

In terms of future research into telomere length, One potential animal model that could be utilised to further investigate the relationships between telomere length and heart failure may be the telomerase deficient mouse model (Terc knockout mice). In this regard, it has been demonstrated that the 5th generation of Terc knockout mice present with cardiac characteristics similar to that of a dilated cardiomyopathy in humans (Leri *et al*., 2003). Hence, the Terc knockout model provides an animal model that includes the heritability of both telomere length and heart failure.

The Terc knockout model provides a good model to further investigate the relationship between telomere length and heart failure using intervention studies. In this regard, based on the findings of thesis it would be of great interest to determine if animals on anti-inflammatory treatment would still progress to the heart failure phenotype demonstrated in the 5th generation offspring of these mice. Furthermore, one could combine the p53 deficient mice model utilised by Sahin *et al*. (2011) with the Terc knockout model. This would provide a model, whereby it is known that progressive telomere loss through several generation results in heart failure, but the mechanism (p53) through which telomere length regulates apoptosis and senescence would be inhibited. Such studies would provide valuable insight into the cause and effect relationship between telomere length and heart failure and also provide possible mechanisms that could explain this relationship.

One of the major caveats with human studies is the use of leukocyte telomere length as a surrogate marker of telomere length in cardiovascular tissues. In an attempt to address this issue I investigated if cardiac telomere length in the control groups of all the animal studies (Chapters 3, 4, 5) was correlated to leukocyte telomere length. These analyses demonstrated that both cardiac and kidney telomere length were strongly correlated to leukocyte telomere length in rats. Hence, leukocyte telomere length may serve as a potential surrogate marker of telomere length in cardiac tissue (FIGURE 7.2). Collectively, the data from the animal and human studies indicate that inflammation is potentially a major determinant of telomere attrition, but that telomere length is not a bio-marker of changes in cardiac dimension or function.

In conclusion, the data emanating from my thesis indicate that telomere length may not be associated with all types of heart failure, specifically non-ischaemic forms of heart failure. Moreover, the pathophysiological processes of heart failure, namely LV dilatation and reduced systolic chamber function, may be independent of changes in telomere length. Hence in heart failure, telomere length may only be reduced as an epiphenomenon due to ongoing inflammatory processes associated with the presence of heart failure and associated risk factors.

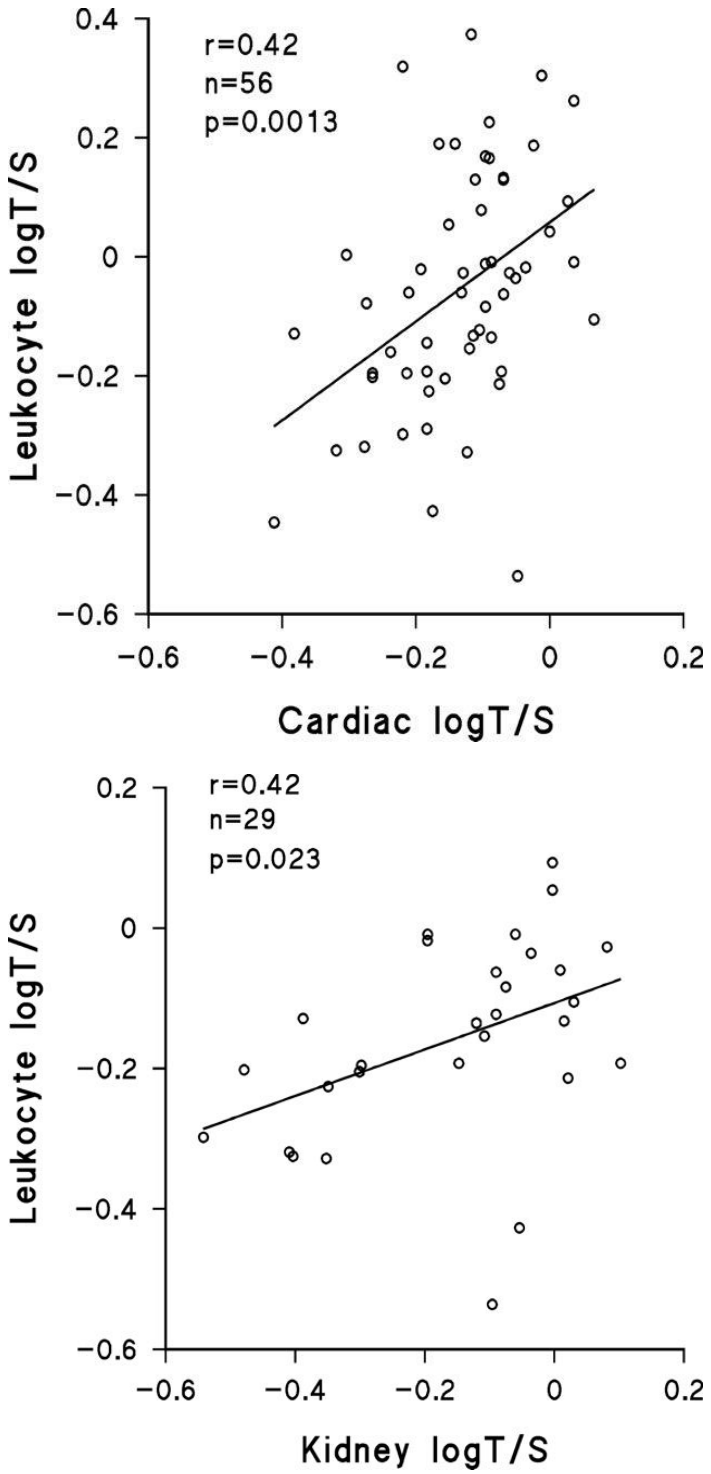

FIGURE 7.2 Bivariate relationships between average relative leukocyte telomere length (log T/S), cardiac (*upper panel*) and kidney telomere length (*lower panel*).

REFERENCES

Abbate A, Biondi-Zoccai GG, Baldi A. Myocardiocyte loss due to apoptosis. Eur Heart J 2002; 23:1889-90.

Adamopoulos S, Parissis JT, Kremastinos DT. A glossary of circulating cytokines in chronic heart failure. Eur J Heart Fail 2001; 3:517-26.

Ahmed Z, Tang WH. Pharmacologic strategies to target oxidative stress in heart failure. Curr Heart Fail Rep 2012; 9:14-22.

Aikata H, Takaishi H, Kawakami Y, Takahashi S, Kitamoto M, Nakanishi T, Nakamura Y, Shimamoto F, Kajiyama G, Ide T. Telomere reduction in human liver tissues with age and chronic inflammation. Exp Cell Res 2000; 256:578-82.

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

Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci USA 1992; 89:10114-8.

Artandi SE and Attardi LD. Pathways connecting telomeres and p53 in senescence, apoptosis, and cancer. Biochem Biophys Res Commun 2005; 331:881-90.

Aubert G and Lansdorp P. Telomeres and Aging. Physiol Rev 2008; 88:557-79.

Aukrust P, Ueland T, Lien E, Bendtzen K, Müller F, Andreassen AK, Nordøy I, Aass H, Espevik T, Simonsen S, Frøland SS, Gullestad L. Cytokine network in congestive

heart failure secondary to ischaemic or idiopathic dilated cardiomyopathy. Am J Cardiol 1999; 83:376-82.

Aviv A, Chen W, Gardner JP, Kimura M, Brimacombe M, Cao X, Srinivasan SR, Berenson GS. Leukocyte telomere dynamics: longitudinal findings among young adults in the Bogalusa Heart Study. Am J Epidemiol 2009; 169:323-9.

Bayeva M, Gheorghiade M, Ardehali H. Mitochondria as a therapeutic target in heart failure. J Am Coll Cardiol 2013; 61:599-610.

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, Verdonck P, De Backer GG, Gillebert TC, Van Oostveldt P; Asklepios investigators. Telomere length and cardiovascular risk factors in a middle-aged population free of overt cardiovascular disease. Aging Cell 2007; 6:639-47.

Bell RM, Cave AC, Johar S, Hearse DJ, Shah AM, Shattock MJ. Pivotal role of NOX-2-containing NADPH oxidase in early ischaemic preconditioning. FASEB J 2005; 19:2037-9.

Beyne-Rauzy O, Prade-Houdellier N, Demur C, Recher C, Ayel J, Laurent G, Mansat-De Mas V. Tumor necrosis factor-alpha inhibits hTERT gene expression in human myeloid normal and leukemic cells. Blood 2005; 106:3200-5.

Beyne-Rauzy O, Recher C, Dastugue N, Demur C, Pottier G, Laurent G, Sabatier L, Mansat-De Mas V. Tumor necrosis factor alpha induces senescence and chromosomal instability in human leukemic cells. Oncogene 2004; 23:7507-16.

Bozkurt B, Kribbs SB, Clubb FJ Jr, Michael LH, Didenko VV, Hornsby PJ, Seta Y, Oral H, Spinale FG, Mann DL. Pathophysiologically relevant concentrations of tumor

necrosis factor-alpha promote progressive left ventricular dysfunction and remodeling in rats. Circulation 1998; 97:1382-91.

Brooksbank[a] R, Woodiwiss A, Sliwa K, Deftereos D, Essop MR, Sareli P, Norton GR. Sustained white cell cytokine activation in idiopathic dilated cardiomyopathy despite haemodynamic improvement with medical therapy. Cardiovasc J S Afr 2005; 16:200-4.

Brooksbank[b] R, Woodiwiss AJ, Sliwa K, Badenhorst D, Deftereos D, Wadee AA, Essop MR, Sareli P, Norton GR. Endotoxin-independent white cell cytokine production in haemodynamically stable patients with idiopathic dilated cardiomyopathy. Cardiovasc J S Afr 2005; 16:260-5.

Brouilette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. Arterioscler Thromb Vasc Biol 2003; 23:842-6.

Brouilette SW, Moore JS, McMahon AD, Thompson JR, Ford I, Shepherd J, Packard CJ, Samani NJ; West of Scotland Coronary Prevention Study Group. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study. Lancet 2007; 369:107-14.

Brouilette SW, Whittaker A, Stevens SE, van der Harst P, Goodall AH, Samani NJ. Telomere length is shorter in healthy offspring of subjects with coronary artery disease: support for the telomere hypothesis. Heart 2008; 94:422-5.

Candy GP, Skudicky D, Mueller UK, Woodiwiss AJ, Sliwa K, Luker F, Esser J, Sareli P, Norton GR. Association of left ventricular systolic performance and cavity size with

angiotensin-converting enzyme genotype in idiopathic dilated cardiomyopathy. Am J Cardiol 1999; 83:740-4.

Capasso JM, Li P, Guideri G, Anversa P. Left ventricular dysfunction induced by chronic alcohol ingestion in rats. Am J Physiol 1991; 261:H212-9.

Capasso JM, Li P, Guideri G, Malhotra A, Cortese R, Anversa P. Myocardial mechanical, biochemical, and structural alterations induced by chronic ethanol ingestion in rats. Circ Res 1992; 71:346-56.

Carll AP, Willis MS, Lust RM, Costa DL, Farraj AK. Merits of non-invasive rat models of left ventricular heart failure. Cardiovasc Toxicol 2011; 11:91-112.

Carrero JJ, Stenvinkel P, Fellström B, Qureshi AR, Lamb K, Heimbürger O, Bárány P, Radhakrishnan K, Lindholm B, Soveri I, Nordfors L, Shiels PG. Telomere attrition is associated with inflammation, low fetuin-A levels and high mortality in prevalent haemodialysis patients. J Intern Med 2008; 263:302-12.

Cattan V, Mercier N, Gardner JP, Regnault V, Labat C, Mäki-Jouppila J, Nzietchueng R, Benetos A, Kimura M, Aviv A, Lacolley P. Chronic oxidative stress induces a tissue-specific reduction in telomere length in CAST/Ei mice. Free Radic Biol Med 2008; 44:1592-8.

Cawthon RM. Telomere measurement by quantitative PCR. Nucleic Acids Res 2002; 30:e47.

Celes MR, Prado CM, Rossi MA. Sepsis: going to the heart of the matter. Pathobiology 2013; 80:70-86.

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. Senescence and death of

primitive cells and myocytes lead to premature cardiac aging and heart failure. Circ Res 2003; 93:604-13.

Collerton J, Martin-Ruiz C, Kenny A, Barrass K, von Zglinicki T, Kirkwood T, Keavney B; Newcastle 85+ Core Study Team. Telomere length is associated with left ventricular function in the oldest old: the Newcastle 85+ study. Eur Heart J 2007; 28:172-6.

Dahl JH, van Breemen RB. Rapid quantitative analysis of 8-iso-prostaglandin-F(2alpha) using liquid chromatography-tandem mass spectrometry and comparison with an enzyme immunoassay method. Anal Biochem 2010; 404:211-6.

Demissie S, Levy D, Benjamin EJ, Cupples LA, Gardner JP, Herbert A, Kimura M, Larson MG, Meigs JB, Keaney JF, Aviv A. Insulin resistance, oxidative stress, hypertension, and leukocyte telomere length in men from the Framingham Heart Study. Aging Cell 2006; 5:325-30.

Doser TA, Turdi S, Thomas DP, Epstein PN, Li SY, Ren J. Transgenic overexpression of aldehyde dehydrogenase-2 rescues chronic alcohol intake-induced myocardial hypertrophy and contractile dysfunction. Circulation 2009; 119:1941-9.

Fernández-Solà J, Fatjó F, Sacanella E, Estruch R, Bosch X, Urbano-Márquez A, Nicolás JM. Evidence of apoptosis in alcoholic cardiomyopathy. Hum Pathol 2006; 37:1100-10.

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

Floyd RA, Hensley K, Jaffery F, Maidt L, Robinson K, Pye Q, Stewart C. Increased oxidative stress brought on by pro-inflammatory cytokines in neurodegenerative processes and the protective role of nitrone-based free radical traps. Life Sci 1999; 65:1893-9.

Fotiadou P, Henegariu O, Sweasy JB. DNA polymerase beta interacts with TRF2 and induces telomere dysfunction in a murine mammary cell line. Cancer Res 2004; 64:3830-7.

Frantz S, Kelly RA, Bourcier T. Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. J Biol Chem 2001; 276:5197-203.

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

Fuster JJ, Díez J, Andrés V. Telomere dysfunction in hypertension. J Hypertens 2007; 25:2185-92.

George A, Figueredo VM. Alcoholic cardiomyopathy: a review. J Card Fail 2011; 17:844-9.

Gil ME, Coetzer TL. Real-time quantitative PCR of telomere length. Mol Biotechnol 2004; 27:169-72.

Giordano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest 2005; 115:500-8.

Goelst K, Laburn H. Response of body temperature and serum iron concentration to repeated pyrogen injection in rabbits. Pflugers Arch 1991; 417:558-61.

Grassi-Zucconi G, Harris JA, Mohammed AH, Ambrosini MV, Kristensson K, Bentivoglio M. Sleep fragmentation, and changes in locomotor activity and body temperature in trypanosome-infected rats. Brain Res Bull 1995; 37:123-9.

Guan Z, Lui CY, Morkin E, Bahl JJ. Oxidative stress and apoptosis in cardiomyocyte induced by high-dose alcohol. J Cardiovasc Pharmacol 2004; 44:696-702.

Guerra S, Leri A, Wang X, Finato N, Di Loreto C, Beltrami CA, Kajstura J, Anversa P. Myocyte death in the failing human heart is gender dependent. Circ Res 1999; 85:856-66.

Guo R, Ren J. Alcohol and acetaldehyde in public health: from marvel to menace. Int J Environ Res Public Health 2010; 7:1285-1301.

Haendeler J, Hoffmann J, Brandes RP, Zeiher AM, Dimmeler S. Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Mol Cell Biol 2003; 23:4598-610.

Hajnóczky G, Buzas CJ, Pacher P, Hoek JB, Rubin E. Alcohol and mitochondria in cardiac apoptosis: mechanisms and visualization. Alcohol Clin Exp Res 2005; 29:693-701.

Harbo M, Koelvraa S, Serakinci N, Bendix L. Telomere dynamics in human mesenchymal stem cells after exposure to acute oxidative stress. DNA Repair 2012; 11:774-9.

Hemann MT, Strong MA, Hao LY, Greider CW. The shortest telomere, not average telomere length is critical for cell viability and chromosomal stability. Cell 2001; 107:67-77.

Higuchi R. Rapid and efficient DNA extraction for PCR from cells or blood. Amplifications 1989; 2:2-3.

Houben JM, Moonen HJ, van Schooten FJ, Hageman GJ. Telomere length assessment: biomarker of chronic oxidative stress? Free Radic Biol Med 2008; 44:235-46.

Huzen J, de Boer RA, van Veldhuisen DJ, van Gilst WH, van der Harst P. The emerging role of telomere biology in cardiovascular disease. Front Biosci 2010; 15:35-45.

Huzen J, Peeters W, de Boer RA, Moll FL, Wong LS, Codd V, de Kleijn DP, de Smet BJ, van Veldhuisen DJ, Samani NJ, van Gilst WH, Pasterkamp G, van der Harst P.

Circulating leukocyte and carotid atherosclerotic plaque telomere length: interrelation, association with plaque characteristics, and restenosis after endarterectomy. Arterioscler Thromb Vasc Biol 2011; 31:1219-25.

Ilmonen P, Kotrschal A, Penn DJ. Telomere attrition due to infection. PLoS One 2008; 3:e2143.

Jaiswal M, LaRusso NF, Burgart LJ, Gores GJ. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res 2000; 60:184-90.

Jing L, Jin CM, Li SS, Zhang FM, Yuan L, Li WM, Sang Y, Li S, Zhou LJ. Chronic alcohol intake-induced oxidative stress and apoptosis: role of CYP2E1 and calpain-1 in alcoholic cardiomyopathy. Mol Cell Biochem 2012; 359:283-92.

Johar S, Cave AC, Narayanapanicker A, Grieve DJ, Shah AM. Aldosterone mediates angiotensin II-induced interstitial cardiac fibrosis via a Nox2-containing NADPH oxidase. FASEB J 2006; 20:1546-8.

Kalaz EB, Evran B, Develi S, Erata GÖ, Uysal M, Koçak-Toker N. Effect of binge ethanol treatment on prooxidant-antioxidant balance in rat heart tissue. Pathophysiology 2012; 19:49-53.

Kang PM, Izumo S. Apoptosis and heart failure: A critical review of the literature. Circ Res 2000; 86:1107-13.

Karlseder J, Hoke K, Mirzoeva OK, Bakkenist C, Kastan MB, Petrini JH, de Lange T. The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. PLoS Biol 2004; 2:e240.

Kim SD, Beck J, Bieniarz T, Schumacher A, Piano MR. A rodent model of alcoholic heart muscle disease and its evaluation by echocardiography. Alcohol Clin Exp Res 2001; 25:457-63.

Kim SD, Bieniarz T, Esser KA, Piano MR. Cardiac structure and function after short-term ethanol consumption in rats. Alcohol 2003; 29:21-9.

Kinouchi Y, Hiwatashi N, Chida M, Nagashima F, Takagi S, Maekawa H, Toyota T. Telomere shortening in the colonic mucosa of patients with ulcerative colitis. J Gastroenterol 1998; 33:343-8.

Knuefermann P, Vallejo J, Mann DL. The role of innate immune responses in the heart in health and disease. Trends Cardiovasc Med 2004; 14:1-7.

Kostin S, Pool L, Elsässer A, Hein S, Drexler HC, Arnon E, Hayakawa Y, Zimmermann R, Bauer E, Klövekorn WP, Schaper J. Myocytes die by multiple mechanisms in failing human hearts. Circ Res 2003; 92:715-24.

Krauss J, Farzaneh-Far R, Puterman E, Na B, Lin J, Epel E, Blackburn E, Whooley MA. Physical fitness and telomere length in patients with coronary heart disease: findings from the Heart and Soul Study. PLoS One 2011; 6:e26983.

Kubota T, McTiernan CF, Frye CS, Slawson SE, Lemster BH, Koretsky AP, Demetris AJ, Feldman AM. Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-alpha. Circ Res 1997; 81:627-35.

Kumar S, Seth S, Jaiswal A, Enjamoori R, Dinda AK, Ray R, Maulik SK. Chronic beta-adrenergic activation-induced left ventricular systolic dysfunction is associated with systemic release of TNF-alpha and IL-1-beta in rats. Pharmacol Rep 2009; 61:870-6.

Kurz DJ, Kloeckener-Gruissem B, Akhmedov A, Eberli FR, Bühler I, Berger W, Bertel O, Lüscher TF. Degenerative aortic valve stenosis, but not coronary disease, is associated with shorter telomere length in the elderly. Arterioscler Thromb Vasc Biol 2006; 26:e114-7.

Kuznetsova T, Codd V, Brouilette S, Thijs L, González A, Jin Y, Richart T, van der Harst P, Díez J, Staessen JA, Samani NJ. Association between left ventricular mass and telomere length in a population study. Am J Epidemiol 2010; 172:440-50.

Laonigro I, Correale M, Di Biase M, Altomare E. Alcohol abuse and heart failure. Eur J Heart Fail 2009; 11:453-462.

Lassègue B, Sorescu D, Szöcs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK. Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. Circ Res 2001; 88:888-94.

Law BA, Levick SP, Carver WE. Alterations in cardiac structure and function in a murine model of chronic alcohol consumption. Microsc Microanal 2012; 18:453-61.

Lazarević AM, Nakatani S, Nesković AN, Marinković J, Yasumura Y, Stojicić D, Miyatake K, Bojić M, Popović AD. Early changes in left ventricular function in chronic asymptomatic alcoholics: relation to the duration of heavy drinking. J Am Coll Cardiol 2000; 35:1599-1606.

Leri A, Barlucchi L, Limana F, Deptala A, Darzynkiewicz Z, Hintze TH, Kajstura J, Nadal-Ginard B, Anversa P. Telomerase expression and activity are coupled with myocyte proliferation and preservation of telomeric length in the failing heart. Proc Natl Acad Sci USA 2001; 98:8626-31.

Leri A, Franco S, Zacheo A, Barlucchi L, Chimenti S, Limana F, Nadal-Ginard B, Kajstura J, Anversa P, Blasco MA. Ablation of telomerase and telomere loss leads to cardiac dilatation and heart failure associated with p53 upregulation. EMBO J 2003; 22:131-9.

Levenson JW, Skerrett PJ, Gaziano JM. Reducing the global burden of cardiovascular disease: the role of risk factors. Prev Cardiol 2002; 5:188-99.

Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. N Engl J Med 1990; 323:236-41.

Looi YH, Grieve DJ, Siva A, Walker SJ, Anilkumar N, Cave AC, Marber M, Monaghan MJ, Shah AM. Involvement of Nox2 NADPH oxidase in adverse cardiac remodeling after myocardial infarction. Hypertension 2008; 51:319-25.

Luker FI, Mitchell D, Laburn HP. Fever and motor activity in rats following day and night injections of Staphylococcus aureus cell walls. Am J Physiol Regul Integr Comp Physiol 2000; 279:R610-6.

Maack C, Kartes T, Kilter H, Schäfers HJ, Nickenig G, Böhm M, Laufs U. Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-GTPase and represents a target for statin treatment. Circulation 2003; 108:1567-74.

Mann DL. Mechanisms and models in heart failure: A combinatorial approach. Circulation 1999; 100:999-1008.

Marín-García J and Goldenthal MJ. Mitochondrial centrality in heart failure. Heart Fail Rev 2008; 13:137-50.

Masi S, Nightingale CM, Day IN, Guthrie P, Rumley A, Lowe GD, von Zglinicki T, D'Aiuto F, Taddei S, Klein N, Salpea K, Cook DG, Humphries SE, Whincup PH, Deanfield JE. Inflammation and not cardiovascular risk factors is associated with short leukocyte telomere length in 13- to 16-year-old adolescents. Arterioscler Thromb Vasc Biol 2012; 32:2029-34.

Masi S, Salpea KD, Li K, Parkar M, Nibali L, Donos N, Patel K, Taddei S, Deanfield JE, D'Aiuto F, Humphries SE. Oxidative stress, chronic inflammation, and telomere length in patients with periodontitis. Free Radic Biol Med 2011; 50:730-5.

Mathews EC Jr, Gardin JM, Henry WL, Del Negro AA, Fletcher RD, Snow JA, Epstein SE. Echocardiographic abnormalities in chronic alcoholics with and without overt congestive heart failure. Am J Cardiol 1981; 47:570-8.

Meldrum DR. Tumor necrosis factor in the heart. Am J Physiol 1998; 274:R577-95.

Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. Nature 1999; 402:551-5.

Nordfjäll K, Svenson U, Norrback KF, Adolfsson R, Lenner P, Roos G. The individual blood cell telomere attrition rate is telomere length dependent. PLoS Genet 2009; 5:e1000375.

Norton GR, Majane OH, Maseko MJ, Libhaber C, Redelinghuys M, Kruger D, Veller M, Sareli P, Woodiwiss AJ. Brachial blood pressure-independent relations between radial late systolic shoulder-derived aortic pressures and target organ changes. Hypertension 2012; 59:885-92.

Norton GR, Woodiwiss AJ, Gaasch WH, Mela T, Chung ES, Aurigemma GP, Meyer TE. Heart failure in pressure overload hypertrophy. The relative roles of ventricular remodeling and myocardial dysfunction. J Am Coll Cardiol 2002; 39:664-71.

O'Donnell CJ, Demissie S, Kimura M, Levy D, Gardner JP, White C, D'Agostino RB, Wolf PA, Polak J, Cupples LA, Aviv A. Leukocyte telomere length and carotid artery intimal medial thickness: the Framingham Heart Study. Arterioscler Thromb Vasc Biol 2008; 28:1165-71.

O'Donovan A, Pantell MS, Puterman E, Dhabhar FS, Blackburn EH, Yaffe K, Cawthon RM, Opresko PL, Hsueh WC, Satterfield S, Newman AB, Ayonayon HN, Rubin SM, Harris TB, Epel ES; Health Aging and Body Composition Study. Cumulative inflammatory load is associated with short leukocyte telomere length in the Health, Aging and Body Composition Study. PLoS One 2011; 6:e19687.

Ogami M, Ikura Y, Ohsawa M, Matsuo T, Kayo S, Yoshimi N, Hai E, Shirai N, Ehara S, Komatsu R, Naruko T, Ueda M. Telomere shortening in human coronary artery diseases. Arterioscler Thromb Vasc Biol 2004; 24:546-50.

Oh H, Wang SC, Prahash A, Sano M, Moravec CS, Taffet GE, Michael LH, Youker KA, Entman ML, Schneider MD. Telomere attrition and Chk2 activation in human heart failure. Proc Natl Acad Sci USA 2003; 100:5378-83.

Ohki R, Ishikawa F. Telomere-bound TRF1 and TRF2 stall the replication fork at telomeric repeats. Nucleic Acids Res 2004; 32:1627-37.

Oikawa S and Kawanishi S. Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening. FEBS Lett 1999; 453:365-8.

Oikawa S, Tada-Oikawa S, Kawanishi S. Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening. Biochemistry 2001; 40:4763-8.

Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA, Quaini E, Di Loreto C, Beltrami CA, Krajewski S, Reed JC, Anversa P. Apoptosis in the failing human heart. N Engl J Med 1997; 336:1131-41.

Olivetti G, Capasso JM, Sonnenblick EH, Anversa P. Side-to-side slippage of myocytes participates in ventricular wall remodeling acutely after myocardial infarction in rats. Circ Res 1990; 67:23-34.

Osadchii OE, Norton GR, McKechnie R, Deftereos D, Woodiwiss AJ. Cardiac dilatation and pump dysfunction without intrinsic myocardial systolic failure following chronic beta-adrenoreceptor activation. Am J Physiol Heart Circ Physiol 2007; 292:H1898-1905.

O'Sullivan J, Risques RA, Mandelson MT, Chen L, Brentnall TA, Bronner MP, Macmillan MP, Feng Z, Siebert JR, Potter JD, Rabinovitch PS. Telomere length in the colon declines with age: a relation to colorectal cancer? Cancer Epidemiol Biomarkers Prev 2006; 15:573-7.

Papaharalambus CA and Griendling KK. Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. Trends Cardiovasc Med 2007; 17:48-54.

Parker MM, McCarthy KE, Ognibene FP, Parrillo JE. Right ventricular dysfunction and dilatation, similar to left ventricular changes, characterize the cardiac depression of septic shock in humans. Chest 1990; 97:126-31.

Parker MM, Parrillo JE. Septic shock. Hemodynamics and pathogenesis. JAMA 1983; 250:3324-7.

Parker MM, Shelhamer JH, Bacharach SL, Green MV, Natanson C, Frederick TM, Damske BA, Parrillo JE. Profound but reversible myocardial depression in patients with septic shock. Ann Intern Med 1984; 100:483-90.

Petersen S, Saretzki G, von Zglinicki T. Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. Exp Cell Res 1998; 239:152-60.

Piano MR. Alcoholic cardiomyopathy: incidence, clinical characteristics, and pathophysiology. Chest 2002; 121:1638-50.

Raymond AR, Norton GR, Sareli P, Woodiwiss AJ, Brooksbank RL. Relationship between average leukocyte telomere length and the presence or severity of idiopathic dilated cardiomyopathy in black Africans. Eur J Heart Fail 2013; 15:54-60.

Ren J, Brown RA. Influence of chronic alcohol ingestion on acetaldehyde-induced depression of rat cardiac contractile function. Alcohol 2000; 35:554-560.

Rhee DB, Ghosh A, Lu J, Bohr VA, Liu Y. Factors that influence telomeric oxidative base damage and repair by DNA glycosylase OGG1. DNA Repair (Amst) 2011; 10:34-44.

Richards JB, Valdes AM, Gardner JP, Kato BS, Siva A, Kimura M, Lu X, Brown MJ, Aviv A, Spector TD. Homocysteine levels and leukocyte telomere length. Atherosclerosis 2008; 200:271-7.

Richter T, Saretzki G, Nelson G, Melcher M, Olijslagers S, von Zglinicki T. TRF2 overexpression diminishes repair of telomeric single-strand breaks and accelerates telomere shortening in human fibroblasts. Mech Ageing Dev 2007; 128:340-5.

Ross-Inta CM, Zhang YF, Almendares A, Giulivi C. Threonine-deficient diets induced changes in hepatic bioenergetics. Am J Physiol Gastrointest Liver Physiol 2009; 296:G1130-9.

Russel R. Atherosclerosis - an inflammatory disease. N Engl J Med 1999; 340**:**115-26.

Sahin E, Colla S, Liesa M, Moslehi J, Müller FL, Guo M, Cooper M, Kotton D, Fabian AJ, Walkey C, Maser RS, Tonon G, Foerster F, Xiong R, Wang YA, Shukla SA, Jaskelioff M, Martin ES, Heffernan TP, Protopopov A, Ivanova E, Mahoney JE, Kost-Alimova M, Perry SR, Bronson R, Liao R, Mulligan R, Shirihai OS, Chin L, DePinho RA. Telomere dysfunction induces metabolic and mitochondrial compromise. Nature 2011; 470:359-65.

Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. Circulation 1978; 58:1072-83.

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

Samani NJ, Boultby R, Butler R, Thompson JR, Goodall AH. Telomere shortening in atherosclerosis. Lancet 2001; 358:472-3.

Saraste A, Pulkki K, Kallajoki M, Heikkilä P, Laine P, Mattila S, Nieminen MS, Parvinen M, Voipio-Pulkki LM. Cardiomyocyte apoptosis and progression of heart failure to transplantation. Eur J Clin Invest 1999; 29:380-6.

Satoh M, Shimoda Y, Maesawa C, Akatsu T, Ishikawa Y, Minami Y, Hiramori K, Nakamura M. Activated toll-like receptor 4 in monocytes is associated with heart failure after acute myocardial infarction. Int J Cardiol 2006; 109:226-34.

Schulz VP, Zakian VA, Ogburn CE, McKay J, Jarzebowicz AA, Edland SD, Martin GM. Accelerated loss of telomeric repeats may not explain accelerated replicative decline of Werner syndrome cells. Hum Genet 1996; 97:750-4.

Seddon M, Looi YH, Shah AM. Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. Heart 2007; 93:903-7.

Segel LD, Rendig SV, Choquet Y, Chacko K, Amsterdam EA, Mason DT. Effects of chronic graded ethanol consumption on the metabolism, ultrastructure, and mechanical function of the rat heart. Cardiovasc Res 1975; 9:649-63.

Sheng R, Gu ZL, Xie ML. Epigallocatechin gallate, the major component of polyphenols in green tea, inhibits telomere attrition mediated cardiomyocyte apoptosis in cardiac hypertrophy. Int J Cardiol 2013; 162:199-209.

Shiels PG, McGlynn LM, MacIntyre A, Johnson PC, Batty GD, Burns H, Cavanagh J, Deans KA, Ford I, McConnachie A, McGinty A, McLean JS, Millar K, Sattar N, Tannahill C, Velupillai YN, Packard CJ. Accelerated telomere attrition is associated with relative household income, diet and inflammation in the pSoBid cohort. PLoS One 2011; 6:e22521.

Shimizu G, Hirota Y, Kita Y, Kawamura K, Saito T, Gaasch WH. Left ventricular midwall mechanics in systemic arterial hypertension. Myocardial function is depressed in pressure-overload hypertrophy. Circulation 1991; 83:1676-84.

Shioi T, Matsumori A, Kihara Y, Inoko M, Ono K, Iwanaga Y, Yamada T, Iwasaki A, Matsushima K, Sasayama S. Increased expression of interleukin-1 beta and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the hypertrophied and failing heart with pressure overload. Circ Res 1997; 81:664-71.

Singal PK, Khaper N, Palace V, Kumar D. The role of oxidative stress in the genesis of heart disease. Cardiovasc Res 1998; 40:426-32.

Sitte N, Saretzki G, von Zglinicki T. Accelerated telomere shortening in fibroblasts after extended periods of confluency. Free Radic Biol Med 1998; 24:885-93.

Sliwa K, Skudicky D, Candy G, Wisenbaugh T, Sareli P. Randomised investigation of effects of pentoxifylline on left-ventricular performance in idiopathic dilated cardiomyopathy. Lancet 1998; 351:1091-3.

Starr JM, McGurn B, Harris SE, Whalley LJ, Deary IJ, Shiels PG. Association between telomere length and heart disease in a narrow age cohort of older people. Exp Gerontol 2007; 42:571-3.

Szalai VA, Singer MJ, Thorp HH. Site-specific probing of oxidative reactivity and telomerase function using 7,8-dihydro-8-oxoguanine in telomeric DNA. J Am Chem Soc 2002; 124:1625-31.

Tardif JC. Insights into oxidative stress and atherosclerosis. Can J Cardiol 2000; 16:2D-4D.

Testa M, Yeh M, Lee P, Fanelli R, Loperfido F, Berman JW, LeJemtel TH. Circulating levels of cytokines and their endogenous modulators in patients with mild to severe congestive heart failure due to coronary artery disease or hypertension. J Am Coll Cardiol 1996; 28:964-71.

Torre-Amione G, Kapadia S, Benedict C, Oral H, Young JB, Mann DL. 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 1996; 27:1201-6.

Triposkiadis F, Karayannis G, Giamouzis G, Skoularigis J, Louridas G, Butler J. The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications. J Am Coll Cardiol 2009; 54:1747-62.

Urbano-Marquez A, Estruch R, Navarro-Lopez F, Grau JM, Mont L, Rubin E. The effects of alcoholism on skeletal and cardiac muscle. N Engl J Med 1989; 320:409-15.

Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. Lancet 2005; 366:662-4.

van der Harst P, de Boer RA, Samani NJ, Wong LS, Huzen J, Codd V, Hillege HL, Voors AA, van Gilst WH, Jaarsma T, van Veldhuisen DJ. Telomere length and outcome in heart failure. Ann Med 2010; 42:36-44.

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. Telomere length of circulating leukocytes is decreased in patients with chronic heart failure. J Am Coll Cardiol 2007; 49:1459-64.

van der Harst P, Wong LS, de Boer RA, Brouilette SW, van der Steege G, Voors AA, Hall AS, Samani NJ, Wikstrand J, van Gilst WH,van Veldhuisen DJ; MERIT-HF Study Group. Possible association between telomere length and renal dysfunction in patients with chronic heart failure. Am J Cardiol 2008; 102:207-10.

Vasan RS, Demissie S, Kimura M, Cupples LA, White C, Gardner JP, Cao X, Levy D, Benjamin EJ, Aviv A. Association of leukocyte telomere length with echocardiographic left ventricular mass: the Framingham heart study. Circulation 2009; 120:1195-202.

Vaziri H, Schächter F, Uchida I, Wei L, Zhu X, Effros R, Cohen D, Harley CB. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am J Hum Genet 1993; 52:661-7.

von Zglinicki T and Martin-Ruiz CM. Telomeres as biomarkers for ageing and age-related diseases. Curr Mol Med 2005; 5:197-203.

von Zglinicki T, Pilger R, Sitte N. Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. Free Radic Biol Med 2000; 28:64-74.

von Zglinicki T, Saretzki G, Döcke W, Lotze C. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? Exp Cell Res 1995; 220:186-93.

Walters MW, Bjork JA, Wallace KB. Perfluorooctanoic acid stimulated mitochondrial biogenesis and gene transcription in rats. Toxicology 2009; 264:10-5.

Watfa G, Dragonas C, Brosche T, Dittrich R, Sieber CC, Alecu C, Benetos A, Nzietchueng R. Study of telomere length and different markers of oxidative stress in patients with Parkinson's disease. J Nutr Health Aging 2011; 15:277-81.

Weber KT, Janicki JS, Shroff SG, Pick R, Chen RM, Bashey RI. Collagen remodeling of the pressure-overloaded, hypertrophied nonhuman primate myocardium. Circ Res 1988; 62:757-65.

Weischer M, Bojesen SE, Cawthon RM, Freiberg JJ, Tybjærg-Hansen A, Nordestgaard BG. Short telomere length, myocardial infarction, ischaemic heart disease, and early death. Arterioscler Thromb Vasc Biol 2012; 32:822-9.

Wencker D, Chandra M, Nguyen K, Miao W, Garantziotis S, Factor SM, Shirani J, Armstrong RC, Kitsis RN. A mechanistic role for cardiac myocyte apoptosis in heart failure. J Clin Invest 2003; 111:1497-504.

Wilson WR, Herbert KE, Mistry Y, Stevens SE, Patel HR, Hastings RA, Thompson MM, Williams B. Blood leukocyte telomere DNA content predicts vascular telomere DNA content in humans with and without vascular disease. Eur Heart J 2008; 29:2689-94.

Wingler K, Wünsch S, Kreutz R, Rothermund L, Paul M, Schmidt HH. Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system in vitro and in vivo. Free Radic Biol Med 2001; 31:1456-64.

Wisenbaugh T, Katz I, Davis J, Essop R, Skoularigis J, Middlemost S, Röthlisberger C, Skudicky D, Sareli P. Long-term (3 month) effects of a new beta-blocker (Nebivolol) on cardiac performance in dilated cardiomyopathy. J Am Coll Cardiol 1993; 21:1094-1100.

Wolkowitz OM, Mellon SH, Epel ES, Lin J, Dhabhar FS, Su Y, Reus VI, Rosser R, Burke HM, Kupferman E, Compagnone M, Nelson JC, Blackburn EH. Leukocyte telomere length in major depression: correlations with chronicity, inflammation and oxidative stress-preliminary findings. PLoS One 2011; 6:e17837.

Wong LS, de Boer RA, Samani NJ, van Veldhuisen DJ, van der Harst P. Telomere biology in heart failure. Eur J Heart Fail 2008; 10:1049-56.

Wong LS, Huzen J, de Boer RA, van Gilst WH, van Veldhuisen DJ, van der Harst P. Telomere length of circulating leukocyte subpopulations and buccal cells in patients with ischaemic heart failure and their offspring. PLoS One 2011; 6:e23118.

Wong[a] LS, Huzen J, van der Harst P, de Boer RA, Codd V, Westenbrink BD, Benus GF, Voors AA, van Gilst WH, Samani NJ, Jaarsma T, van Veldhuisen DJ. Anaemia is associated with shorter leukocyte telomere length in patients with chronic heart failure. Eur J Heart Fail 2010; 12:348-53.

Wong LS, Oeseburg H, de Boer RA, van Gilst WH, van Veldhuisen DJ, van der Harst P. Telomere biology in cardiovascular disease: the TERC-/- mouse as a model for heart failure and ageing. Cardiovasc Res 2009; 81:244-52.

Wong[b] LS, van der Harst P, de Boer RA, Huzen J, van Gilst WH, van Veldhuisen DJ. Aging, telomeres and heart failure. Heart Fail Rev 2010; 15:479-86.

Woodiwiss AJ, Tsotetsi OJ, Sprott S, Lancaster EJ, Mela T, Chung ES, Meyer TE, Norton GR. Reduction in myocardial collagen cross-linking parallels left ventricular dilatation in rat models of systolic chamber dysfunction. Circulation 2001; 103:155-60.

Xu Q, Dalic A, Fang L, Kiriazis H, Ritchie RH, Sim K, Gao XM, Drummond G, Sarwar M, Zhang YY, Dart AM, Du XJ. Myocardial oxidative stress contributes to transgenic β_2-adrenoceptor activation-induced cardiomyopathy and heart failure. Br J Pharmacol 2011 162:1012-28.

Yankiwski V, Marciniak RA, Guarente L, Neff NF. Nuclear structure in normal and Bloom syndrome cells. Proc Natl Acad Sci USA 2000; 97:5214-9.

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

Yücel D, Aydoğdu S, Cehreli S, Saydam G, Canatan H, Seneş M, Ciğdem Topkaya B, Nebioğlu S. Increased oxidative stress in dilated cardiomyopathic heart failure. Clin Chem 1998; 44:148-54.

Yusuf S, Reddy S, Ounpuu S, Anand S. Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. Circulation 2001; 104:2746-53.

Zee RY, Michaud SE, Germer S, Ridker PM. Association of shorter mean telomere length with risk of incident myocardial infarction: a prospective, nested case-control approach. Clin Chim Acta 2009; 403:139-41.

Zhang B, Turdi S, Li Q, Lopez FL, Eason AR, Anversa P, Ren J . Cardiac overexpression of insulin-like growth factor 1 attenuates chronic alcohol intake-induced myocardial contractile dysfunction but not hypertrophy: Roles of Akt, mTOR, GSK3beta, and PTEN. Free Radic Biol Med 2010; 49:1238-53.

Zhou B, Wu LJ, Tashiro S, Onodera S, Uchiumi F, Ikejima T. Silibinin protects rat cardiac myocyte from isoproterenol-induced DNA damage independent on regulation of cell cycle. Biol Pharm Bull2006; 29:1900-05.

ETHICS CLEARANCE CERTIFICATES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Candy

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 951122

PROJECT	Genetic Polymorphisms in the urban Black hypertensive population
INVESTIGATORS	Mr G P Candy
DEPARTMENT	Nuclear Medicine, Medical School
DATE CONSIDERED	951124
DECISION OF THE COMMITTEE *	Approved unconditionally
DATE	960223

CHAIRMAN. ..... (Professor P E Cleaton-Jones)

c c Supervisor: Dr G Norton
Dept of Physiology, Medical School

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE 4/3/1996 SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2011/18/05

APPLICANT: Mr A Raymond

SCHOOL: Physiology
DEPARTMENT:
LOCATION: Medical School

PROJECT TITLE: Effect of alcohol intake on rat leukocyte and cardiac telomere length

Number and Species

40 male Sprague-Dawley rats

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20110329. This approval remains valid until 20130328.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

None

Signed: _____ Date: 07/04/2011
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 08/04/2011
(Registered Veterinarian)

cc: Supervisor: Dr R Brooksbank
Director: CAS

Works 2000/lain0015/AESCCert.wps

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2010/25/04

APPLICANT: Mr B Hodson

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: **The effect castration or testosterone receptor blockade on β-adreneric induced left ventriclar pump dysfunction in male rats**

Number and Species

128 Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 04.05.2010. This approval remains valid until 04.05.2012

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

- Researcher lists himself as either the investigator or a co worker.
- The researcher has not indicated that flutamide is a testosterone receptor blocker. The researcher should clarify this for the committee.
- Saline needs to given for 2 weeks prior to starting the isoproterenol injections to habituate rats to the animal handling and the procedure.

Signed: _____ Date: 20/05/2010
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 20/05/2010
(Registered Veterinarian)

cc: Supervisor:
Director:

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2010/49/04

APPLICANT: Mr A Raymond

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: *An investigation into the relationship between telomere length and inflammation*

Number and Species

60 Sprague Dawley

Approval was given for the use of animals for the project described above at an AESC meeting held on 26.10.2010. This approval remains valid until 26.10.2012.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form, is subject to the establishment of a financial agreement with either the Research office or the Wits Health Consortium in the case that the research has potential commercial outputs, is subject to the arrangements negotiated with Central Animal Services personnel restricted to the applicant and or listed co-workers and is subject to the following additional conditions:

- That a signature for the drugs is provided.
- That the correct numbers of rats are indicated on page 7
- That at any time point, rats that are 15% of the predicted body weight will be euthanized.

Signed: _____ Date: 18/11/2010
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 18/11/2010
(Registered Veterinarian)

cc: Supervisor:
Director: CAS
Works 2000/Iain0015/AESCCert.wps

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Prof A/G Woodiwiss/Norton

CLEARANCE CERTIFICATE	**M1204108**
PROJECT	Gene Candidates as Determinants of Blood Pressure and Intermediary Phenotypes in Pathogenesis of Hypertension in Black South Africans (Previously M020472 and M070469)
INVESTIGATORS	Prof A/G Woodiwiss/Norton.
DEPARTMENT	School of Physiology
DATE CONSIDERED	Ad hoc
DECISION OF THE COMMITTEE*	Renewal Approved

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 2012/05/18 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof A Woodiwiss

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...