Biologic disease modifying anti-rheumatic treatment effects on cardiac geometry and function in collagen-induced arthritis

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Declaration

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

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

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Regina le Roux

Signed on .....8th..... day of .....April..... 2020.

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Dedication

In memory of my grandmother

Lea Marta Maria Oosthuizen

1926 - 2018

Abstract

Chronic systemic inflammation significantly increases the risk of heart failure. Diastolic dysfunction is a preclinical disorder that frequently develops into heart failure with a reduced ejection fraction and is particularly prevalent in persons exposed to high-grade inflammation, including rheumatoid arthritis (RA). Inflammation has been directly linked to the development of diastolic dysfunction. In the RA population, disease progression is dramatically reduced when treatment is aimed at inflammation, using biologic anti-rheumatic drugs, including tumor necrosis factor alpha (TNF-α) inhibitors and interleukin 6 (IL-6) receptor blockers. However, the effect of these drugs on cardiac geometry and function needs further elucidation. The aim of our study was to determine the effect of biologic disease modifying anti-rheumatic drugs (DMARDs) on cardiac geometry and function in a collagen induced arthritis (CIA) rat model.

Seventy-eight male and female Sprague Dawley rats were randomly divided into four groups, namely an untreated control group, an inflammation group, a TNF-α inhibitor group and an IL-6 receptor blocker group. Inflammation was induced by a 0.2 ml subcutaneous injection of Bovine Type II collagen blended into incomplete Freund's adjuvant at the base of the tail, in all groups except the control group. Following the onset of inflammation, the TNF-α group received 10 mg/kg of Enbrel every third day for six weeks and the IL-6 group received 8mg/kg of Actemra once a week for six weeks. Arthritis scores and body weights were measured weekly and blood pressure were measured every two weeks throughout the study. Following six weeks of drug treatment, cardiac geometry and function were measured using echocardiography and inflammatory cytokine concentrations (C-reactive protein, TNF-α, IL-6) were determined using enzyme-linked immunosorbent assays (ELISAs). Changes in body weight, arthritis scores and blood pressure were determined by repeated measures analysis of variance (ANOVA). Differences in inflammatory markers and echocardiography variables were determined by two-way ANOVA followed by Tukey post-hoc tests.

Body weight and blood pressure did not change during the intervention in any of the groups (all $p > 0.05$). The inflammation and IL-6 group had a significantly greater interventricular

septal thickness (IVST) and posterior wall thickness (PWT) in diastole, as well as an increased relative wall thickness (RWT) compared to controls ($p < 0.05$ for all). The TNF-α treatment group had significantly smaller IVST and PWT in diastole and RWT compared to the inflammation group ($p < 0.001$, $p = 0.004$ and $p = 0.001$, respectively). However, no differences were recorded in any systolic function markers (ejection fraction, stroke volume or fractional shortening) between groups (all $p > 0.05$). Diastolic function was significantly reduced in all groups exposed to inflammation. Lateral mitral annular velocity (e') was lower in the inflammation (3.8 ± 0.1 cm/s; $p = 0.001$), TNF-α (3.5 ± 0.2 cm/s; $p < 0.001$) and IL-6 (3.7 ± 0.2 cm/s; $p < 0.001$) groups, compared to the control group (4.5 ± 0.1 cm/s). Filling pressure (E/e') was higher in the inflammation (29.9 ± 1.2; $p = 0.02$), TNF-α (32.5 ± 1.5; $p = 0.001$) and IL-6 (29.9 ± 1.7; $p = 0.048$) groups compared to the control group (24.9 ± 1.2). The TNF-α group additionally showed an increased E/A, and decreased lateral wall e'/a', compared to the inflammation group ($p = 0.02$ and $p = 0.03$, respectively). Inflammatory cytokine concentrations were strongly associated with changes in RWT (CRP: $r = 0.48$; $p < 0.0001$, TNF-α: $r = 0.45$; $p < 0.0001$, IL-6: $r = 0.36$; $p = 0.001$), IVST in diastole (CRP: $r = 0.43$; $p = 0.001$, TNF-α: $r = 0.50$; $p < 0.001$, IL-6: $r = 0.32$; $p = 0.01$) and PWT in diastole (CRP: $r = 0.44$; $p < 0.001$, TNF-α: $r = 0.48$; $p < 0.001$, IL-6: $r = 0.34$; $p = 0.003$). These associations remained significant even after adjusting for body weight and sex. Changes in diastolic function was not consistently related to inflammatory markers.

In conclusion, exposure to inflammation adversely affected cardiac remodelling and diastolic function in a CIA rat model. TNF-α inhibition was protective against cardiac remodelling but not against changes in diastolic function. IL-6 receptor blockade did not protect against inflammation-induced cardiac geometry or function changes. Further investigation is required to underpin the exact mechanisms whereby inflammation affects cardiac geometry and function.

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Abbreviations

A	trans-mitral blood flow velocity in the late (atrial-A) period of left ventricular diastolic filling
a'	peak velocity of tissue lengthening at the lateral mitral annulus during late (atrial) diastole
ATP	adenosine triphosphate
Ca^{2+}	calcium
CI	confidence interval
CIA	collagen induced arthritis
cm	centimetre
CRP	C-reactive protein
CVD	cardiovascular disease
DMARD	disease modifying anti-rheumatic drug
DNA	deoxyribonucleic acid
E	trans-mitral blood flow velocity in the early period of left ventricular diastolic filling
E/A	ratio of early to late diastolic filling velocity
E/e'	index of left ventricular filling pressures
e'	peak velocity of tissue lengthening at the lateral mitral annulus during early diastole
e'/a'	ratio of early to late mitral annular tissue lengthening
ECM	extracellular matrix
EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay

FSend	endocardial fractional shortening
FSmid	midwall fractional shortening
gp130	glycoprotein 130
HFpEF	heart failure with a preserved ejection fraction
HFrEF	heart failure with a reduced ejection fraction
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-6	interleukin 6
IVRT	isovolumetric relaxation time
IVS_d	interventricular septum thickness in diastole
IVS_s	interventricular septum thickness in systole
kg	kilogram
LV	left ventricle
LVEDD	left ventricular end diastolic diameter
LVESD	left ventricular end systolic diameter
mg	milligram
mIL-6	membrane bound interleukin 6 receptor
ml	millilitre
mm	millimetre
mmHg	millimetres of mercury
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
Na^+	sodium

ng	nanogram
NO	nitric oxide
$ONOO^-$	peroxynitrate
pg	picogram
PKG	protein kinase G
PWT_d	posterior wall thickness in diastole
PWT_s	posterior wall thickness in systole
r	correlation coefficient
RA	rheumatoid arthritis
ROS	reactive oxygen species
RWT	relative wall thickness
SA	South Africa
SEM	standard error of the mean
SERCA	sarcoplasmic endoplasmic reticulum ATPase pump
sIL-6	soluble interleukin 6 receptor
SR	sarcoplasmic reticulum
TDI	Tissue Doppler imaging
TGF-β	transforming growth factor beta
TNFR	tumor necrosis factor receptor
TNF-α	tumor necrosis factor alpha
µg	micrograms

Chapter 1 : Introduction

Non-communicable diseases accounted for up 70% of total mortality in 2015, and are thus considered to be the leading cause of death (Wang *et al.*, 2016; Geneva World Health Organization, 2018). Amongst non-communicable diseases, cardiovascular disease (CVD) accounts for more deaths than cancer, respiratory diseases and diabetes (Roth *et al.*, 2015; Wang *et al.*, 2016; Geneva World Health Organization, 2018). It has been estimated that 51% of the total adult deaths in South Africa (SA) occur as a result of non-communicable diseases, with CVD accounting for 19% of the total mortality rates (World Health Organization - Noncommunicable Diseases (NCD) Country Profiles, 2018).

Hypertension, obesity, smoking, high- and low-density lipoprotein cholesterol concentrations and diabetes mellitus are amongst the various traditional CVD risk factors that have been widely accepted as major contributors to CVD risk (Hobbs, 2004). The incidence of CVD is strongly associated with these traditional risk factors (Yusuf *et al.*, 2004; O'Donnell *et al.*, 2010). Despite the strong evidence that traditional risk factors contribute to CVD incidence, these risk factors fail to fully explain incident CVD (Greenland *et al.*, 2003; McGill *et al.*, 2008). Indeed, all CVD events cannot be explained by these traditional risk factors, (Harald *et al.*, 2008; Olsen, 2010; Wallace *et al.*, 2014). and in approximately half of the coronary artery disease cases admitted to hospital, traditional risk factors are within normal range (Lawes *et al.*, 2006; Sachdeva *et al.*, 2009). Furthermore, the underlying mechanisms linking these risk factors to CVD have been under investigation for several years (Lloyd-Jones *et al.*, 2006).

Numerous novel risk factors have been considered in the pathogenesis of CVD (Lin *et al.*, 2018). One of these non-traditional risk factors that has received considerable attention is systemic inflammation (Martinez and White, 2018). Epidemiological studies demonstrated that elevated circulating cytokine concentrations are associated with a significantly increased risk for CV events, independent of traditional risk factors (Woodward *et al.*, 2007; Kaptoge *et al.*, 2012; Welsh *et al.*, 2016). In this regard, various inflammatory cytokines, namely, interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), C-reactive protein (CRP), interleukin 1 beta (IL-1β) and interleukin 2 (IL-2) have been associated with various cardiac events, including myocardial infarction, stroke and heart failure (Coma-Canella *et al.*, 2005; Danesh *et al.*, 2008; Sattar *et al.*, 2009; Kaptoge *et*

al., 2010). These cytokines have also been directly related to subclinical CVD including coronary artery disease, atherosclerosis and endothelial dysfunction (Zhang *et al.*, 1999; Upadhya *et al.*, 2004; Merhisoussi *et al.*, 2005; Bradley, 2008; Schuett *et al.*, 2012).

In addition to the direct association between inflammation and subclinical CVD and CV events, it has been suggested that inflammation may be the mechanistic link between traditional risk factors and CVD (Paulus and Tschöpe, 2013). Risk factor exposure as with obesity, hypertension and diabetes mellitus reportedly cause a state of chronic low-grade systemic inflammation (Lopez-Candales *et al.*, 2017). The inflammatory state induced by diseases of lifestyle have been related to subclinical CVD (Rocha and Libby, 2009; Franssen *et al.*, 2016). Taken together, chronically increased systemic inflammation has emerged as a critical risk factor in the prediction of CV events (Mason and Libby, 2015). Furthermore, evidence suggest that diseases characterised by chronically increased systemic inflammation, such as rheumatoid arthritis (RA), lupus and inflammatory bowel syndrome markedly increase the risk for CVD (Sattar *et al.*, 2003; Charles-Schoeman, 2012).

RA is the most prevalent high-grade inflammation disorder, affecting approximately 1-2% of people in the general population (McInnes and Schett, 2017). The estimated prevalence of RA in South Africa appears to be as high as 2.5% (Usenbo *et al.*, 2015). RA is characterised by synovial joint inflammation which lead to increased circulating inflammatory markers (Urman *et al.*, 2018). Several extra-articular manifestations are directly associated with chronic systemic inflammation (Young and Koduri, 2007). CVD is recognised as a critical extra-articular manifestation in RA (Avina-Zubieta *et al.*, 2012). The increased risk for CVD in RA cannot be fully explained by traditional cardiovascular risk factors (del Rincón *et al.*, 2001). Chronic, high grade systemic inflammation in RA has been suggested as the possible pathophysiological link for the observed increase in CVD risk (Sattar *et al.*, 2003; López-Mejías *et al.*, 2016). The mechanisms whereby systemic inflammation enhances CVD risk in RA are currently under investigation (Castañeda *et al.*, 2016).

RA pharmacological treatment traditionally involved the use of synthetic disease modifying anti-rheumatic drugs (DMARDs) (O'Dell, 2002). Although these synthetic treatments are effective in slowing disease progression, not all patients respond positively (Fridlington *et al.*, 2011; Bhatnagar *et al.*, 2015). More recently, biologic DMARDS has shown promising results in management of RA. Although biologic DMARDs may be a more effective treatment option for some, its clinical use is somewhat limited by its high cost. The use of biologic DMARDs in the treatment of RA have shown attenuated disease progression and a reduction in the devastating consequences of chronic systemic inflammation (Benjamin and Lappin, 2019). However, the effect of biologic DMARDs on cardiovascular disease risk in RA is uncertain, making its use controversial in this population (Baniaamam *et al.*, 2018). Hence the role of biologic DMARDs on subclinical cardiovascular disease risk markers in RA warrant investigation.

This thesis begins with a literature review in chapter 2, that summarises the current evidence of inflammation effects on cardiovascular disease, and specifically the risk for heart failure in RA. Chapter 2 will also highlight the current inconsistencies in the anti-inflammatory treatment effects on CVD in RA. Chapter 3 outlines the methods used, and chapter 4 the results obtained in the current study. Chapter 5 will discuss the findings in context of previous literature.

Chapter 2 : Literature

2.1 Cardiovascular disease in rheumatoid arthritis

The relatively high risk of cardiovascular-related mortality in RA compared to the general population is largely attributed to high-grade systemic inflammation (Gabriel, 2008). High cytokines concentrations alter the function of several tissues, including the vascular endothelium and may generate various pro-atherogenic changes, including oxidative stress and endothelial dysfunction (Sattar *et al.*, 2003). High concentrations of CRP relate to preclinical markers of CVD including endothelial dysfunction (Dessein *et al.*, 2005) and carotid intima-media thickness (Gonzalez-Gay *et al.*, 2005) in RA patients. Hence, it has been suggested that RA disease-related inflammation may contribute to accelerated atherosclerosis (Dessein *et al.*, 2005; Gonzalez-Gay *et al.*, 2005). Indeed, coronary heart disease and cerebrovascular events are the main causes of the increased cardiovascular mortality rates in RA (Avina-Zubieta *et al.*, 2012). Although coronary heart disease and the closely associated atherosclerosis contribute largely to the increase in CVD mortality, persons with RA also have twice the risk of developing heart failure, which accounts for approximately 13% of CVD mortality (Minaur *et al.*, 2004; Nicola *et al.*, 2005; Avina-Zubieta *et al.*, 2012).

2.2 Cardiac dysfunction in rheumatoid arthritis

The aetiology and progression of heart failure can differ substantially between patients (ElGuindy and Yacoub, 2012). Changes in either systolic function or diastolic function may lead to heart failure. Hence, heart failure can be classified into two main phenotypes, namely heart failure with a preserved ejection fraction (EF) (HFpEF; EF ≥ 50%) and heart failure with a reduced ejection fraction (HFrEF; EF < 50%) (ElGuindy and Yacoub, 2012). Although the characteristic symptoms of dyspnoea, fatigue, pulmonary and peripheral oedema, amongst others (Watson *et al.*, 2000) are similar between these two phenotypes, the pattern of ventricular remodelling in HFpEF and HFrEF differs considerably (Fukuta and Little, 2007).

Despite RA patients having twice the risk of developing heart failure compared to the general population (Nicola *et al.*, 2005), RA patients with heart failure less commonly

demonstrate the typical signs and symptoms of heart failure. In this regard, RA patients are more likely to have HFpEF compared to non-RA individuals with heart failure (Arslan *et al.*, 2006; Davis *et al.*, 2008; Liang *et al.*, 2010). Although chronic inflammation is related to both HFpEF and HFrEF in the general population (Torre-Amione *et al.*, 1996; Deswal *et al.*, 2001; Dick and Epelman, 2016), no association has been shown between inflammation and reductions in ejection fraction in RA (Rudominer *et al.*, 2009; Løgstrup *et al.*, 2014). However, levels of inflammation and disease severity are strongly associated with HFpEF in RA (Liang *et al.*, 2010). HFpEF is common in long-term treated RA patients, even when clinically evident CVD and other traditional CVD risk factors are absent (Gonzalez-Juanatey *et al.*, 2004). Given the association between HFpEF and RA, a recent study has suggested that rheumatology visits should be amalgamated with a cardiac function evaluation (Pascale *et al.*, 2018).

Extensive studies have been performed to improve the understanding of the underlying mechanisms of HFrEF; however, the pathogenesis of HFpEF is still poorly understood. With the increased prevalence of HFpEF over the last two decades, mostly as a result of the growing ageing population and greater risk factor exposure, it is important to better understand the mechanisms underlying HFpEF (Owan *et al.*, 2006). Furthermore, treatment strategies to manage HFrEF have been very successful in reducing the risk of death in these patients (Borlaug and Redfield, 2011). However, these treatment strategies for HFpEF have not matched the success of the treatment strategies for HFrEF (Borlaug and Redfield, 2011; Pitt *et al.*, 2014). Considering the escalating numbers of HFpEF, the lack of success of treatment strategies in these patients and the increased risk of HFpEF in patients exposed to chronic inflammation, the pathophysiology of HFpEF warrants further study.

2.3 Heart failure with a preserved ejection fraction

HFpEF is characterised by impaired myocardial relaxation that leads to inadequate left ventricular (LV) filling (Gladden *et al.*, 2014). The impaired diastolic relaxation causes increased filling pressures in the ventricle that leads to the typical presentation of heart failure symptoms. Diastolic dysfunction is a preclinical pathophysiological abnormality

that mostly explains the development of HFpEF (LeWinter and Meyer, 2013). Diastolic dysfunction is characterised by decreased compliance of the LV, resulting in asynchronous relaxation and passive stiffness of the LV myocardium, ultimately leading to impaired ventricular filling during diastole (Achong *et al.*, 2009).

2.3.1 Pathophysiology of diastolic dysfunction

Under physiological conditions, the myocardium relaxes during diastole to allow the ventricles to fill with blood. Myocardial relaxation is dependent on both active and passive processes (Duarte and Fernandez, 2010) which require adenosine triphosphate (ATP) (Biesiadecki *et al.*, 2014). During the active process, calcium (Ca^{2+}) is rapidly removed from the cytosol. The passive process requires adequate cardiac compliance to allow the ventricle to recoil (Biesiadecki *et al.*, 2014). Altered cellular processes during either the active or passive processes may contribute to impaired, inadequate or prolonged relaxation, that may give rise to diastolic dysfunction.

Active relaxation in a healthy heart entails the binding of ATP to α- and β- subunits of myosin heavy chains, which causes the detachment of myosin from actin filaments. The cross-bridge detachment relies on the removal of Ca^{2+} from the cytoplasm (Maharaj, 2012). Ca^{2+} removal from the cytosol also requires ATP and is achieved by reuptake of Ca^{2+} into the sarcoplasmic reticulum (SR) via the sarcoplasmic endoplasmic reticulum Ca^{2+} transport ATPase (SERCA) pump. Phosphorylation of phospholamban results in increased SERCA activity that increases the Ca^{2+} transport velocity (Katz *et al.*, 1985). In addition, Ca^{2+} is extruded from the cytosol to the extracellular space by various ATP dependent Ca^{2+} transporters (Bers, 2008). The removal of Ca^{2+} from the cytosol decreases the intracellular Ca^{2+} concentration which results in the detachment of Ca^{2+} from troponin C, allowing the cardiomyocytes to relax (Biesiadecki *et al.*, 2014).

Insufficient Ca^{2+} removal from the cytosol or cross-bridge detachment may cause perturbances in the relaxation process (Kass *et al.*, 2004). Impaired Ca^{2+} removal, through phospholamban-modulated uptake via SERCA have been implicated in altering the Ca^{2+} transient in the failing heart, which ultimately leads to delayed relaxation (MacLennan and

Kranias, 2003). When Ca^{2+} removal from the sarcoplasm is decreased, diastolic Ca^{2+} levels increase, resulting in increased end-diastolic pressures and abnormal ventricular filling. In diastolic dysfunction, decreased phospholamban phosphorylation, and hence SERCA activity results in a decreased capacity to recapture Ca^{2+} released during contraction (Hoshijima *et al.*, 2006). Ca^{2+} levels are elevated further by leaking of Ca^{2+} from the SR via the ryanodine receptor (Yano and Zarain-Herzberg, 1994). Human and animal studies have shown that hyperphosphorylation of the ryanodine receptor results in increased end-diastolic Ca^{2+}, which leads to incomplete cross-bridge detachment and delayed relaxation (Li *et al.*, 2002; Ginsburg and Bers, 2004; Lanner *et al.*, 2010).

Besides the effective removal of Ca^{2+}, passive ventricular filling during diastole is also dependent on the elastic properties of the myocardium. The passive relaxation component of normal diastolic function requires adequate LV compliance, which is determined by the properties of the extracellular matrix (ECM) and the structure of the sarcoplasmic molecule, titin (Tanaka *et al.*, 2018). The cellular and structural integrity of the myocardium is supported by the ECM, which is largely dependent on the three-dimensional, cross-linked collagen-network (Frangogiannis, 2017). ECM structural elements are dynamic and tightly regulated (Ma *et al.*, 2012). The most important component within the ECM, which is involved in the development of diastolic dysfunction, is the ratio of type I to type II collagen. Elevated levels of type I collagen is associated with a stiffer ventricular myocardium (Kato *et al.*, 1995). Collagen composition and ECM properties can be altered through several mechanisms, including transcriptional and post-transcriptional regulation, neurohormonal activation, growth factors and cross-linking (Schirone *et al.*, 2017). Excessive type I collagen deposition results from a dysregulation of its synthesis and the degradation by matrix metalloproteases (MMPs) (Weber *et al.*, 1993). Collagen content has been shown to be increased in HFpEF patients, when compared to the general population (Borbély *et al.*, 2005). In addition to collagen composition, enhanced cross-linking between collagen molecules also contributes to myocardial stiffness (López *et al.*, 2009; Herum *et al.*, 2015). Small changes in collagen composition and cross-linking can affect myocardial relaxation and hence lead to diastolic dysfunction (Eckhouse and Spinale, 2012).

Besides the collagen and ECM changes, previous studies have highlighted the importance of the sarcoplasmic molecule titin, as a determinant of cardiomyocyte passive stiffness (Sorop *et al.*, 2018). Titin is a cytoskeletal elastic protein within the cardiomyocyte and it is responsible for myocardial compliance (Kass *et al.*, 2004; Borbély *et al.*, 2009). During diastole, titin acts as a viscoelastic spring that is responsible for the recoil and distensibility of the cardiomyocyte (Wu *et al.*, 2000). Titin exists in two isoforms, namely N2A and N2BA, where the latter is a smaller and stiffer isoform (Fukuda *et al.*, 2005). The modified isotype expression affects the ability of titin to act as a spring and reduces the cardiac myocyte compliance and thus contributes to impaired relaxation and ultimately diastolic dysfunction (Krüger *et al.*, 2009).

2.3.2 The role of inflammation on diastolic dysfunction

Although it is well known that inflammation adversely effects the vasculature, it also confers detrimental effects directly on the heart (Colombo *et al.*, 2012). In this regard, markers of chronic inflammation are associated with LV diastolic dysfunction in human studies (Liang *et al.*, 2010; Masiha *et al.*, 2013). Based on the above discussion of the pathophysiology of diastolic dysfunction, inflammation has been shown to affect both active and passive prosses.

With regards to the active processes, chronic inflammation has been found to decrease the expression of SERCA messenger ribonucleic acid (mRNA), delaying diastolic Ca^{2+} uptake in cardiomyocytes (Wu *et al.*, 2011). Proinflammatory cytokines are involved in the transformation of nitric oxide (NO) to peroxynitrate ion ($ONOO^-$). $ONOO^-$ causes elevated protein phosphatase 2a activity, which reduces phospholamban phosphorylation and subsequently reduces Ca^{2+} uptake by SERCA, thus resulting in increased diastolic cytosolic Ca^{2+} (Kohr *et al.*, 2009). The increased cytosolic Ca^{2+} delays myocardial relaxation and increases end diastolic pressure.

Regarding the passive processes, proinflammatory cytokines have been implicated in altered ECM composition. Myocardial stiffness is promoted by pro-inflammatory cytokines due to an increase in the production of collagen and a decrease

in collagen degradation (Dinh *et al.*, 2009). A cross-linked and insoluble collagen network is formed, resulting from the activation of lysyl oxidase-1 by proinflammatory cytokines. Proinflammatory cytokines are associated with an increase in the ratio of MMP to tissue inhibitors of metalloproteinases (Dinh *et al.*, 2009). In animal studies, where high-grade inflammation was induced, MMP activity was significantly increased and collagen synthesis was accelerated (Van Nieuwenhoven and Turner, 2013). Chronic inflammation has been implicated in isoform switching of titin (Hutchinson *et al.*, 2015), by causing dephosphorylation of titin via the induction of oxidative stress (Tschöpe and Lam, 2012).

In addition to the direct effect that inflammation has on LV relaxation, it has recently been suggested that risk factor exposure produce a systemic proinflammatory state that leads to excessive production of reactive oxygen species (ROS) within the coronary microvasculature (Sorop *et al.*, 2018). The production of ROS by proinflammatory cytokines are induced by the activation of nicotinamide adenine dinucleotide phosphate oxidases (Griendling *et al.*, 2000). ROS inhibits NO bioavailability, which induces cardiomyocyte stiffness through the reduction in protein kinase G (PKG) activity (Paulus and Tschöpe, 2013). Increased levels of ROS also promote fibrosis by direct activation of the transforming growth factor-beta (TGF-β)/Smad3 pathway (Cucoranu *et al.*, 2005).

Taken together, the associations between elevated proinflammatory cytokine concentrations and diastolic dysfunction and the high prevalence of HFpEF in RA patients suggest that systemic inflammation may be the driving force underlying abnormalities in diastolic function. However, in human studies of RA, several confounding factors prohibit drawing conclusions about the direct effect of high-grade inflammation on diastolic function. Hence further studies in high-grade systemic inflammatory models, free of confounding factors are required to investigate the direct effect of chronic exposure to inflammation on diastolic dysfunction.

2.4 Measuring cardiac function

Cardiac function is commonly assessed non-invasively by echocardiography (Sahin *et al.*, 2012). A number of measures of cardiac function using echocardiography

has enabled the characterisation of impaired cardiac function. Echocardiography can be used to obtain measures of geometry, systolic and diastolic function.

Echocardiography is utilised to assess cardiac geometry, using two-dimensional M-mode imaging, which allows for measurements of the interventricular septum, left ventricular end diameters and posterior wall thickness, in systole and diastole. Based on the geometry measures, relative wall thickness (RWT) and LV mass can be determined in order to assess remodelling patterns of the LV (Gaasch, 1979; Levy *et al.*, 1987) The RWT and LV mass is used to assess remodelling patterns of the LV, be it physiological or pathophysiological (Gaasch, 1979; Levy *et al.*, 1987). Pathological remodelling may adapt a concentric pattern, where the RWT is increased without an increase in LV mass or eccentric pattern, where RWT is normal or reduced with an increase in LV mass (Opie *et al.*, 2006). LV concentric remodelling is a subtle and early change in cardiac geometry, and is associated with increased cardiovascular risk (Verdecchia *et al.*, 1995).

Systolic function can be measured by LV stroke volume and ejection fraction. Although ejection fraction is commonly used to measure systolic function, it is regarded as a measure of ventricular-arterial coupling rather than contractility because it is dependent on chamber size and loading conditions (Borlaug *et al.*, 2009). Other measures used to determine abnormalities in myocardial systolic function, include endocardial (FSend) and midwall fractional shortening (FSmid), or Tissue Doppler imaging (TDI) measurements of shortening within the septum of the lateral wall of the left ventricle (Lang *et al.*, 2015). Impaired regional systolic function in the wall of the ventricle has been reported despite normal ejection fraction (Yip *et al.*, 2002; Yu *et al.*, 2002). Ejection fraction, fractional shortening and TDI determined systolic function are all robust measures of systolic function, however they have shortcomings in their measurement of systolic function (Kadappu and Thomas, 2015). Two-dimensional speckle tracking echocardiography (STE) has emerged as a more sensitive index of myocardial systolic performance. STE is a relatively new technique which is angle-dependent and allows a more comprehensive assessment of myocardial function in both global and individual segments of the myocardium (Leitman *et al.*, 2004; Langeland *et al.*, 2005). STE enables the assessment of myocardial deformation (strain and strain rate) and motion (velocity

and displacement) on the longitudinal, circumferential and radial planes of the myocardium (Dandel *et al.*, 2009). However, the use of STE echocardiography is beyond the scope of this thesis.

Diastolic dysfunction often occurs prior to impaired systolic chamber function (de Simone *et al.*, 2000). Briefly, echocardiography allows the measure of the velocity of the mitral inflow to visualize the phases of ventricular filling, and diastolic function using pulsed wave Doppler (Dugo *et al.*, 2016). In this regard, mitral inflow velocity is measured in early diastole (E) and late diastole (A) (Henein and Lindqvist, 2015). In a normal ventricle, passive (early) filling constitutes the majority of the total diastolic filling, hence the E wave is larger than the A wave. However, in diastolic dysfunction altered pressures in the ventricle, causes early filling of the ventricle to be compromised and thus the A wave will increase compared to the E wave (Pasipoularides, 2013). Alternatively, when LV filling pressure or left atrial pressure is increased a restrictive filling or pseudonormal pattern may be observed (Nishimura and Tajik, 1997; Mottram and Marwick, 2005; Mitter *et al.*, 2017). With increased left atrial pressures early filling is increased which will present as a normal or increased E. This higher early filling in a stiff ventricle will cause a greater trans-mitral pressure gradient which will cause the E wave to be greater than the A wave (Nishimura and Tajik, 1997). Because a pseudonormal and restrictive filling pattern both appear normal, further echocardiographic measurements need to be considered.

TDI is used to measure tissue lengthening at the mitral annulus during early (e') and late (a') diastole (Mottram and Marwick, 2005). Similar to the pulsed wave Doppler, the e' should be greater than the a' in a normal ventricle. A reduced e' is considered to be one of the earliest features of diastolic dysfunction and is more sensitive to show changes in LV relaxation compared to trans-mitral flow (Sohn *et al.*, 1997), as it is less dependent on pre-load and heart rate. The E wave is dependent on relaxation alone. Hence the ratio of E/e' is regarded as an index of left ventricular filling pressures (Sharifov *et al.*, 2016). In diastolic dysfunction the E/e' is increased (Ommen *et al.*, 2000). The diagnosis of diastolic dysfunction relies mostly on the assessments of E/A and E/e' as these measurements are associated with the development of heart failure and adverse cardiovascular outcomes (Aurigemma *et al.*, 2001; Redfield *et al.*, 2003).

2.5 Biological anti-inflammatory treatment effects on cardiac function in rheumatoid arthritis

The lack of understanding of the pathophysiological mechanisms underlying diastolic dysfunction, has greatly limited its treatment options. The therapies proven to benefit HFrEF have consistently shown negative results in HFpEF (Gladden *et al.*, 2014; Shah *et al.*, 2014). Considering the strong influence of inflammation on the development of diastolic function (Paulus and Tschöpe, 2013), it has become increasingly recognised that treating chronic inflammation may be a more suitable alternative treatment strategy for HFpEF and for CVD as a whole (Tschöpe *et al.*, 2017). Indeed, a recent seminal study showed that treatment with a biologic anti-inflammatory drug reduced the risk for atherosclerotic CVD, independent of the lipid level (Ridker *et al.*, 2017). However, what is the evidence that treatment of inflammation in RA patients reduces the risk of CVD?

Disease modifying anti-rheumatic drugs have emerged as an effective treatment for RA patients. Early treatment with DMARDs resulted in more favourable clinical and radiological outcomes (O'Dell, 2002). The most commonly prescribed synthetic DMARD is methotrexate, a chemotherapeutic drug that is structurally similar to folic acid, and exerts its action by inhibiting dihydrofolate reductase and so interferes with DNA synthesis and mitosis (Madke and Singh, 2015). Although methotrexate is the most commonly used synthetic DMARD, physicians are cautious of prescribing this drug and limits its use, as its synthetic nature has been associated with several toxicities (Fridlington *et al.*, 2011; Bhatnagar *et al.*, 2015). Dose reduction is often required during methotrexate therapy in patients with liver disease and renal dysfunction (Aronoff and Aronoff, 2014).

Biologic DMARDs were introduced as an alternative treatment for patients that did not respond favourably to synthetic DMARD therapy and has since become increasingly popular (Rau and Herborn, 2004; Nam *et al.*, 2017). Biologic DMARDs exerts its action by inhibition of specific inflammatory mediators, and may be more effective in reducing inflammation compared to synthetic DMARDs that supresses the immune system as a whole (Raychaudhuri and Raychaudhuri, 2009; Benjamin and Lappin, 2019). Studies investigating biologic DMARD treatment has demonstrated its effectiveness in improving

quality of life, while decreasing disability and joint damage (Burke *et al.*, 2014). Nevertheless, the use of biologic DMARD treatment for CVD and specifically cardiac structure and function in RA have been contradictory (Baniaamam *et al.*, 2018).

Some have shown that treatment with biologic DMARDs have either reduced overall CVD risk (Westlake *et al.*, 2011) or reduced the risk of CV events (Greenberg *et al.*, 2011; Bili *et al.*, 2014; Lee *et al.*, 2018). However, others have shown no change in CVD or CV events with biologic DMARD treatment (Solomon *et al.*, 2006; Rao *et al.*, 2015). Moreover, some studies reported a decrease in the risk of developing congested heart failure when receiving biologic DMARD treatment (Wolfe and Michaud, 2004; Morgan *et al.*, 2014), while others showed no reduction in the risk of developing heart failure (Bernatsky *et al.*, 2005; Cole *et al.*, 2007; Listing *et al.*, 2008; Solomon *et al.*, 2013; Schau *et al.*, 2015). Some have even reported an increased risk for developing congested heart failure in patients using biologic DMARDs (Curtis *et al.*, 2007; Setoguchi *et al.*, 2008; Al-Aly *et al.*, 2011).

With regards to pre-clinical studies that evaluated the effects of biologic DMARDs on heart structure and function, the results are equally contradictory (Baniaamam *et al.*, 2018). Table 2.1 summarises studies that have investigated the effects of biologic DMARDs on cardiac function in RA. The majority of studies that measured cardiac geometry have shown that biologic DMARDs are successful in decreasing LV mass (Giles *et al.*, 2010; Kotyla *et al.*, 2012; Daïen *et al.*, 2013; Kobayashi *et al.*, 2014). However, only a small number of these studies that showed improved LV mass also showed improved systolic function (Kotyla *et al.*, 2012; Kobayashi *et al.*, 2014). Nevertheless, one study showed a decrease in systolic function (Giles *et al.*, 2010). Other studies that did not report LV structure, showed either that biologic DMARDs improved systolic function (Ikonomidis *et al.*, 2009, 2014; Kobayashi *et al.*, 2016), that it did not change systolic function (Senel *et al.*, 2012; Vizzardi *et al.*, 2016) or that it reduced systolic function (Santos *et al.*, 2012). With regards to diastolic function, some studies indicated improvement (Ikonomidis *et al.*, 2008, 2009, 2014; Çetin *et al.*, 2014), whereas other studies failed to report a beneficial effect of biologic DMARDs on diastolic function (Senel *et al.*, 2012; Daïen *et al.*, 2013).

Despite these conflicting results, findings should be interpreted with care. Firstly, in these clinical trials, patients receiving biologic DMARDs have usually been unresponsive to several first line therapies of synthetic DMARDs, and hence generally have greater disease activity and severity at baseline. Therefore, these patients potentially have a greater risk of developing systolic or diastolic dysfunction (Jacobsson *et al.*, 2005). Secondly, majority of studies used several combinations of drugs to treat RA and the risk of CVD, therefore the independent effect of the biologic DMARDs are difficult to discern. Lastly, these patients often have several traditional CVD risk factors, which amplifies their risk profile, and hence the confounding effect of traditional risk factors in the development of cardiac dysfunction cannot be excluded. Therefore, the effect of biologic DMARDs on cardiac geometry and function warrants further investigation. Animal models of high-grade inflammation may be more appropriate to investigate the effects of biologic DMARDs on cardiac structure and function, as confounding factors can be limited.

Table 2.1 Summary of studies investigating the effect of biologic DMARDs on cardiac function in RA.

Author	Study design	RA population	Cardiac measures	Main outcomes
Ikonomidis, 2008	N= 23 anakinra (single injection) N= 19 prednisone N= 23 controls	Age: 57 ± 17 years Disease duration: 1 - 27 years	s', e', a' and E	IL-1β inhibitory treatment reduced E/e' and increased e'/a' compared to prednisone and placebo controls
Ikonomidis, 2009	N= 23 anakinra (30 days) N= 23 prednisone N= 23 controls	Age: 57 ± 17 years Disease duration: 6.5 - 35 years	LVEDV, LVESV, fractional shortening and EF. Global longitudinal, circumferential and radial strain and strain rate	IL-1β inhibitory treatment improved longitudinal, radial, circumferential strain, and longitudinal and circumferential strain rate in systole and early diastole compared to prednisone
Giles, 2010	N= 36 various biologic DMARD treatments N= 225 controls	Age: 59.1 ± 9.1 years Disease duration: 7 - 14 months	LVEDV, LVESV, LV mass, stroke volume, cardiac output and ejection fraction	TNF-α inhibition associated with a reduction in LVEDV, stroke volume and LV mass in RA
Senel, 2012	N= 16 etanercept (6 months)	Age: 27 - 69 years.	LV EF, E, A, s', e' and a'	TNF-α inhibition did not change cardiac function in the RA group
Kotyla, 2012	N= 23 infliximab (one year)	Age: 51.3 ± 1.55 years Disease duration: 7.1 ± 1 years	LV mass, IVST_d, PWT_d, LVEDD, LV EF, E and A	TNF-α inhibition associated with an increase in EF and a decrease in LV mass
Santos, 2012	N= 14 infliximab N= 14 RA control	Age: 47.2 ± 8.8 years Disease duration: 10.9 ± 4.4 years	heart rate, stroke volume and cardiac output	Infliximab infusion decreased cardiac output and stoke volume compared to saline infusion
Daien, 2013	N= 28 etanercept (6 months) N= 20 synthetic DMARD	Age: 51 ± 17 years (etanercept); 57 ± 13 years (synthetic DMARD) Disease duration: 1-4 years	LV mass, LV mass index, E, A, s' and e'	Etanercept decreased in LV mass

Ikonomidis, 2014	N= 80 anakinra N= 80 placebo (own controls)	Age: 59.5 ± 18 years (coronary artery disease); 58 ± 17 years (without coronary artery disease) Disease duration: 5 - 23.5 years (coronary artery disease); 1 - 27.1 years (without coronary artery disease)	LVEDV, LVESV, EF, E, s', e' and a' Global longitudinal and circumferential strain and strain rate, peak twisting and untwisting velocities	IL-1β inhibition improved myocardial deformation and LV twisting. RA participants with coronary artery disease showed greater improvement in LV EF, E/e' and LV myocardial deformation compared to non-coronary artery disease
Cetin, 2014	N= 20 infliximab (three months) N= 30 prednisone	Age: 52.1 ± 11.1 years Disease duration: 8.2 ± 6.45 years	IVST, PWT, LVEDV, LVESV, EF, E, A, IVRT and LAVI, e' and a', Left atrial global strain, and strain rate	Infliximab improved E/e' and reduced left atrium volume index compared to prednisone
Kobayashi, 2014	N= 20 tocilizumab (one year) N= 20 control	Age: 56.5 years Disease duration: 2.5 years	LVEDV, LVESV, LV mass, LV mass index, stroke volume, EF and cardiac output	Tocilizumab decreased in LV mass index and LVEDV and increased in LV EF.
Vizzardi, 2016	N= 13 etanercept, infliximab and adalimumab (one year)	Age: 51 ± 13 years Disease duration: 11.4 ± 4.9 years.	LVEDV, LVESV and EF Global longitudinal peak systolic strain	TNF-α inhibition did not change strain
Kobayashi, 2016	N= 13 tocilizumab (one year) N= 10 controls	Age: 55 years Disease duration: 1.2 - 10.8 years	radial strain (err%) of the LV at rest	Tocilizumab improved LV regional function

RA, rheumatoid arthritis; DMARD, disease modifying anti-rheumatic drugs; E, early diastolic filling velocity; A, late diastolic filling velocity; e', peak velocity during early diastole; a', peak velocity during late diastole; s', peak systolic velocity; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume; IVST, interventricular septum thickness; PWT, posterior wall thickness; d, in diastole; EF, ejection fraction; IL-1β, interleukin 1 beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha, IVRT, isovolumetric relaxation time; LAVI, left atrial volume index.

2.6 Problem statement

Cardiovascular disease is the principle cause of death world-wide and is a major cause of mortality in South Africa. Although traditional risk factors, such as age, hypertension, diabetes mellitus, smoking and dyslipidaemia are involved in the pathogenesis of cardiovascular disease, whether they account for all cardiovascular events is controversial. One alternative potential mechanism that could in-part contribute toward cardiovascular disease onset is the presence of chronic inflammation. Evidence suggests that disorders associated with chronic high-grade inflammation, including RA, markedly exacerbate the risk for cardiovascular events. However, there is no direct evidence from pre-clinical studies that inflammation is directly related to cardiovascular disease.

Although the majority of research in RA in South Africa has been performed on the risk for atherosclerosis, persons with RA are at a two-fold risk for developing heart failure. More specifically in RA, heart failure with a preserved ejection fraction is more common. Heart failure with a preserved ejection fraction has been described as the ‘single largest unmet need in cardiovascular medicine’ and accounts for approximately half of all heart failure cases (Sharma and Kass, 2014). In this regard, diastolic dysfunction is a pre-clinical measure that often progresses to heart failure with a preserved ejection fraction. Although various traditional risk factors are related to the increased risk for diastolic dysfunction in RA, they fail to account for all the changes in cardiac function. Indeed, inflammatory processes have been suggested to significantly accelerate the diastolic function changes in the heart in various populations. Although there is some evidence to support a role of chronic inflammation in the pathogenesis of changes in diastolic function, the exact inflammatory mechanisms that mediate this role are uncertain.

There are currently no effective treatment strategies for the management of diastolic dysfunction and heart failure with a preserved ejection fraction, which further reflects the ongoing lack of understanding of this condition. From cross-sectional clinical studies, the strong association between inflammatory markers and diastolic dysfunction warrants the exploration of anti-inflammatory drugs as potential therapeutic target for

diastolic dysfunction and heart failure with a preserved ejection fraction. In RA studies, treatment with biologic anti-inflammatory drugs on cardiac function and the risk for heart failure has shown contrasting results. Even those studies where biologic anti-inflammatory drugs were associated with improved cardiac function, the mechanisms for theses associations are unclear. Hence an improved understanding of the role of inflammation and anti-inflammatory treatments on cardiac geometry and function will elucidate the possibility of novel treatment strategies for diastolic dysfunction and the prevention and management of heart failure with a preserved ejection fraction.

2.7 Aim

The aim of this study was to determine the effect of inflammation on cardiac function, and whether biologic anti-rheumatic drugs alter the outcomes of inflammatory-induced changes in cardiac structure and function.

Objectives

The specific objectives are to determine:

1. changes in left ventricular structure and diastolic function in Sprague Dawley rats exposed to collagen-induced arthritis (CIA) compared to controls
2. the association between inflammatory markers and left ventricular structure and function
3. whether treatment with anti-rheumatic drugs (TNF-α inhibitor and IL-6 receptor blocker) influence inflammation-induced changes in left ventricular structure and function.

Chapter 3 : Methods

3.1 Animals

One hundred, male (n=55) and female (n=45) Sprague Dawley rats, aged three months, were obtained from the Central Animal Services from the University of the Witwatersrand. Rats that did not respond to the collagen-induced arthritis protocol (n=17) or those that died (n=5) were not included in the final study sample. Rats were housed individually, and male and female rats were kept in separate, temperature-controlled rooms fitted with a 12-hour light-dark cycle. Rats were housed individually to monitor food intake of each rat and to easily identify rats that were on the different treatments. All experimental procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC approval: 2017/03/21C).

3.2 Experimental design

Rats underwent a two-week familiarisation period prior to undergoing experimental procedures. During this period rats had free access to standard rat chow (Nutritionhub, Stellenbosch, South Africa) and water. Blood pressure, body weight, arthritis scores and paw thicknesses were measured once a week. Following the two-week familiarisation period rats were randomly divided into four groups namely; the control group (n=22, males: n=13 and females: n=9), the inflammation group (n=26, males: n=18 and females: n=8), the TNF-α inhibitor group (TNF-α group) (n=15, males: n=8 and females: n=7) and the IL-6 receptor blocker group (IL-6 group) (n=15, males: n=8 and females: n=7). The control group was injected intraperitoneally with a phosphate buffer saline solution. The inflammation, TNF-α and the IL-6 groups were all exposed to an arthritis-inducing protocol. Following the onset of arthritis, rats in the inflammation, TNF-α and IL-6 groups received 1-4 mg/kg Tramazec daily, for pain relief. The TNF-α group received Enbrel, a TNF-α inhibitor, every three days for six weeks and the IL-6 groups received Actemra, an IL-6 receptor blocker, once a week for 6 weeks. The dosages of these treatments were based on previous studies (Kobayashi *et al.*, 2016; Totoson *et al.*, 2016; Lin *et al.*, 2017). The half-life of Etanercept and Tocilizumab were 1.9 days (Lon *et al.*, 2011) and 6 to 9 days, respectively (Ding and Jones, 2008).

During the intervention body weight and disease severity was measured weekly. Tail cuff blood pressure, arthritis scores and paw thickness as the ankle joint and metatarsal joint was measured before induction of inflammation and every second week throughout the study. After six weeks of drug treatment, rats were anaesthetised by intramuscular injections of ketamine (100 mg/kg) and xylazine (5 mg/kg). Cardiac function was assessed non-invasively using echocardiography after which rats were terminated by thoracotomy. At termination blood samples were collected and the heart was removed during dissection from the chest cavity. A summary of the experimental design is outlined in Figure 3.1.

3.2.1 Collagen induced arthritis

After the two-week habituation period, an arthritis inducing emulsion was administrated to rats under general anaesthesia (isoflurane), to expose them to systemic high-grade inflammation. For the arthritis inducing emulsion, type II collagen (Chondrex cat. #20021, Redmond, WA, USA) was dissolved in 0.05 M acetic acid by stirring it overnight at 4°C. The dissolved bovine collagen (2 mg/ml) were mixed with an electric homogeniser with an equal amount of incomplete Freund's adjuvant (Chondrex cat. #7002, Redmond, WA, USA). Immediately after the emulsion was prepared, rats in the inflammation, TNF-α and IL-6 groups were immunised by administrating a 0.2 ml (200 µg) subcutaneous injection of the arthritis-inducing emulsion at the base of the tail. To ensure a high incidence of arthritis, the rats in the inflammation, TNF-α and IL-6 groups received 0.1 ml (100 µg) booster injections of the emulsion seven and 21 days after the initial immunisation.

3.2.2 Drug intervention

Following the onset of arthritis, rats in the TNF-α group received a TNF-α inhibitor, Enbrel (etanercept) intraperitoneally, at a dosage of 10 mg/kg every three days for six weeks (Totoson *et al.*, 2016). The IL-6 group rats received an IL-6 receptor blocker Actemra (tocilizumab) intraperitoneally, at a dosage of 8 mg/kg once a week for six weeks

(Kobayashi *et al.*, 2016; Lin *et al.*, 2017). Following the onset of arthritis, rats in the inflammation, TNF-α and IL-6 groups received 1-4 mg/kg Tramazec daily, for pain relief.

3.3 Articular index score

To evaluate the severity of the arthritis, rat hind paws were scored using a 0-4 grading scale as previously reported (Winter *et al.*, 1962). Arthritis scores were obtained once every two weeks throughout the study. Arthritis severity was scored as follow, a score of 0 was given if there was not swelling or focal redness of the paws, a score of 1 was given if there was slight swelling with/without focal redness, a score of 2 was given if the paw displayed mild-to-moderate oedema, a score of 3 was given if pronounced oedema was present with limited use of the joint and 4 if excessive oedema was present with deformation and joint rigidity. The cumulative score for the hind paws of each rat (maximum score of 8) was used to represent the overall disease severity and progression. As the rat paw arthritis score provides a subjective quantification of the inflammation, the hind paw diameter at the tarsometatarsal and ankle joint were measured using a digital calliper every two weeks.

3.4 Tail cuff blood pressures

Blood pressure was assessed via the tail cuff technique which employs non-invasive blood pressure amplifiers (Biopac Systems, Santa Barbara, CA, USA). To measure the tail-cuff blood pressure, rats were placed in restrainers with adjustable restriction panels. Rats were habituated to restrainers for two weeks prior to obtaining any blood pressure measures. The tail of the rat was placed on heating pad for 10 minutes prior to measuring the blood pressure. Measures were made midday to avoid diurnal variation and was measured every two weeks. An average of three measures were taken as the blood pressure.

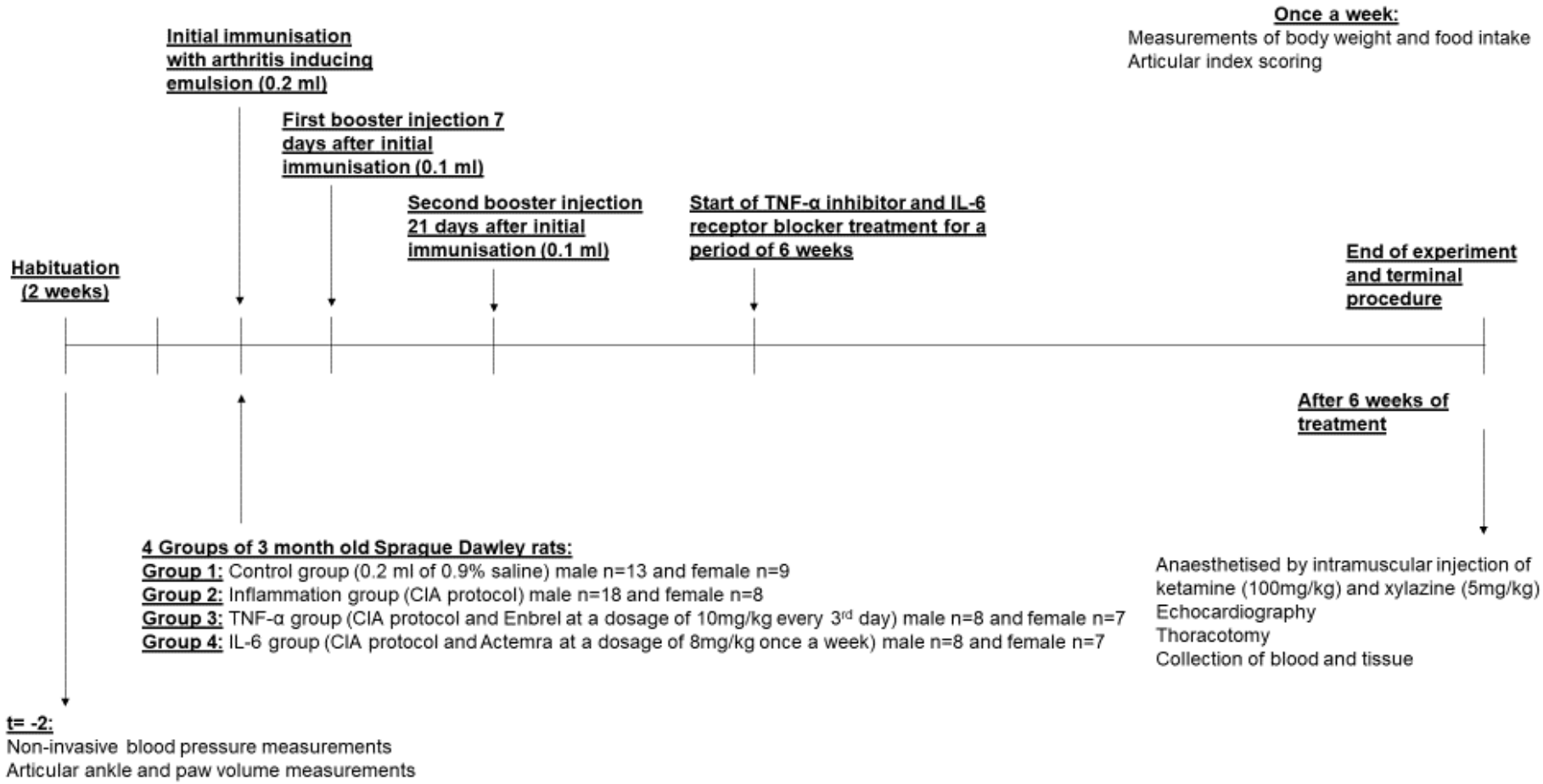

Figure 3.1 Experimental procedures for the experimental groups.

TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; ml, millilitres; mg/kg, milligram per kilogram.

3.5 Echocardiography

Prior to termination, rats were anaesthetised by intramuscular injections of ketamine (100 mg/kg) and xylazine (5 mg/kg) to measure cardiac function echocardiography (Acuson SC 2000 Diagnostic ultrasound system/Siemens Medical Solutions, USA, Inc., version 3.5). The chests of the rats were shaved, and rats were placed in a left lateral decubitus position. Using a high resolution (paediatric) ultrasound probe (10MHz) the long axis parasternal view of the left ventricle was obtained. Using 2-D directed, M-mode imaging, the left ventricular end diastolic diameter (LVEDD) and LV end systolic diameter (LVESD), the interventricular septal thickness (IVST) and posterior wall thickness (PWT) were measured in systole (s) and diastole (d) according to the American Society of Echocardiography convention (Figure 3.2) (Lang *et al.*, 2015). The LV dimensions were measured only when an appropriate visualisation of both the right and left septal surfaces was obtained and where the endocardial surfaces of both the septal and posterior walls were clearly visible.

LV end diastolic volume (LVEDV) and LV end systolic volume (LVESV) was determined using the Teichholz Formula, as LVEDV = [7/ (2.4 + LVEDD)] x $LVEDD^3$ and LVESV = [7/ (2.4 + LVESD)] x $LVESD^3$, where LVEDD = LV end diastolic diameter and LVESD = LV end systolic diameter (Teichholz *et al.*, 1976).

Ejection fraction (EF) was calculated as EF = (LVEDV-LVESV)/LVEDV x 100 (Teichholz *et al.*, 1976). Stroke volume (SV) was calculated using the standard formula, SV = LVEDV – LVESV. Endocardial fractional shortening (FSend) was calculated as FSend = LVEDD − LVESD/LVEDD × 100 (Wachtell *et al.*, 2010). Midwall fractional shortening (FSmid) was calculated as, FSmid = [(LVEDD + PWTd/2 + IVSTd/2) - (LVESD + Hs/2)]/[LVEDD + PWTd/2 + IVSTd/2)] x 100, where H represents the combined septal and posterior was thickness (de Simone *et al.*, 1994). LV relative wall thickness was calculated as (PWT in diastole x 2)/LVEDD) (Ganau *et al.*, 1992).

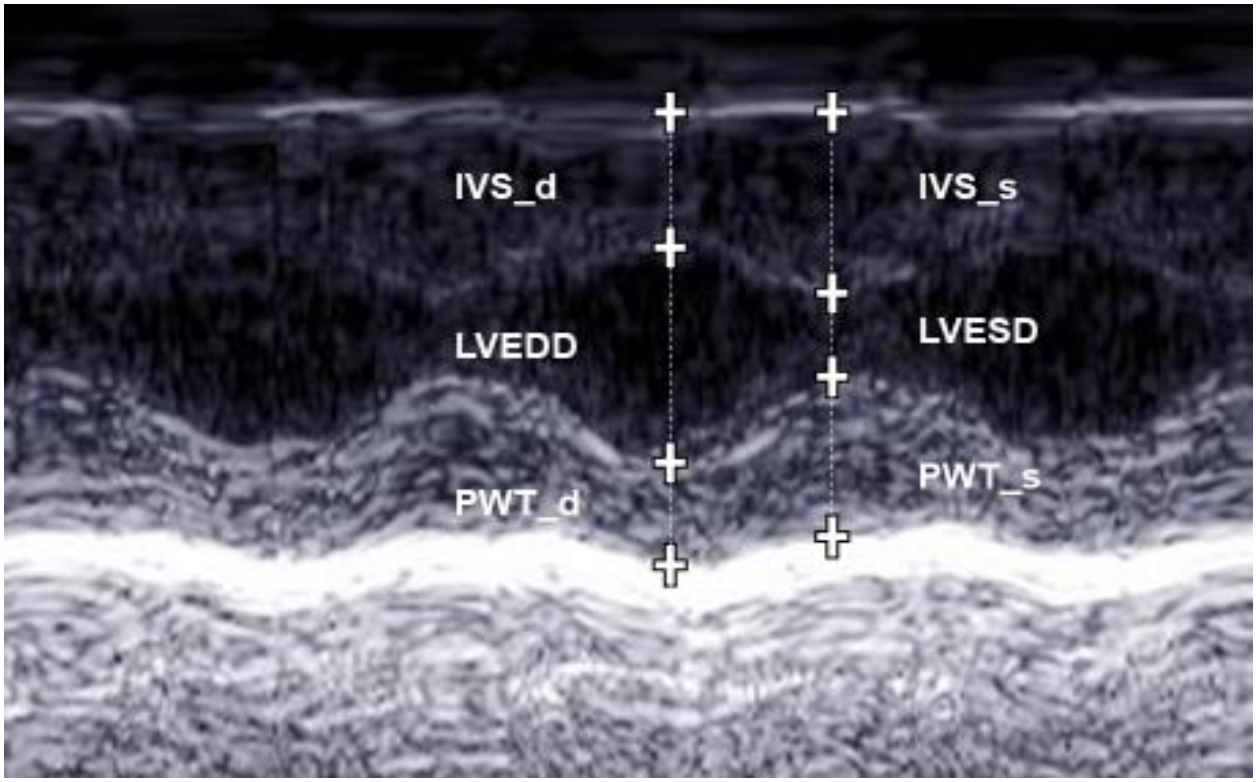

Figure 3.2 Echocardiographic M-mode image showing the left ventricular dimensions in the parasternal long axis view.

IVS_d, interventricular septum in diastole; LVEDD, left ventricular end diastolic diameter; PWT_d, posterior wall thickness in diastole; IVS_s, interventricular septum in systole; LVESD, left ventricular end systolic diameter; PWT_s, posterior wall thickness in systole.

LV diastolic function was assessed using pulse waved and Tissue Doppler. Mitral inflow velocity was determined in the apical four-chamber view, with the sample volume placed at the tip of the mitral valve. Trans-mitral velocity measurements were obtained during the early (E) and late (atrial, A) periods of the LV diastolic inflow and expressed as the E/A ratio (Figure 3.3). Tissue Doppler imaging (TDI) was used to measure the motion of the mitral annulus in the apical four-chamber view. The peak relaxation velocities during early (e') and late (a') diastole was obtained at the lateral mitral annulus (Figure 3.4). The E/e' is obtained by the early LV diastolic inflow (E) divided by the peak relaxation velocity during early diastole (e'). Early diastolic mitral inflow velocity (E) is influenced by ventricular relaxation and left atrial pressures, while e' is influenced by ventricular relaxation, hence E/e' is considered an index of LV filling pressures (Park and Marwick, 2011). The ratio of early to late peak tissue relaxation velocities (e'/a'), was also calculated and considered an index of LV stiffness.

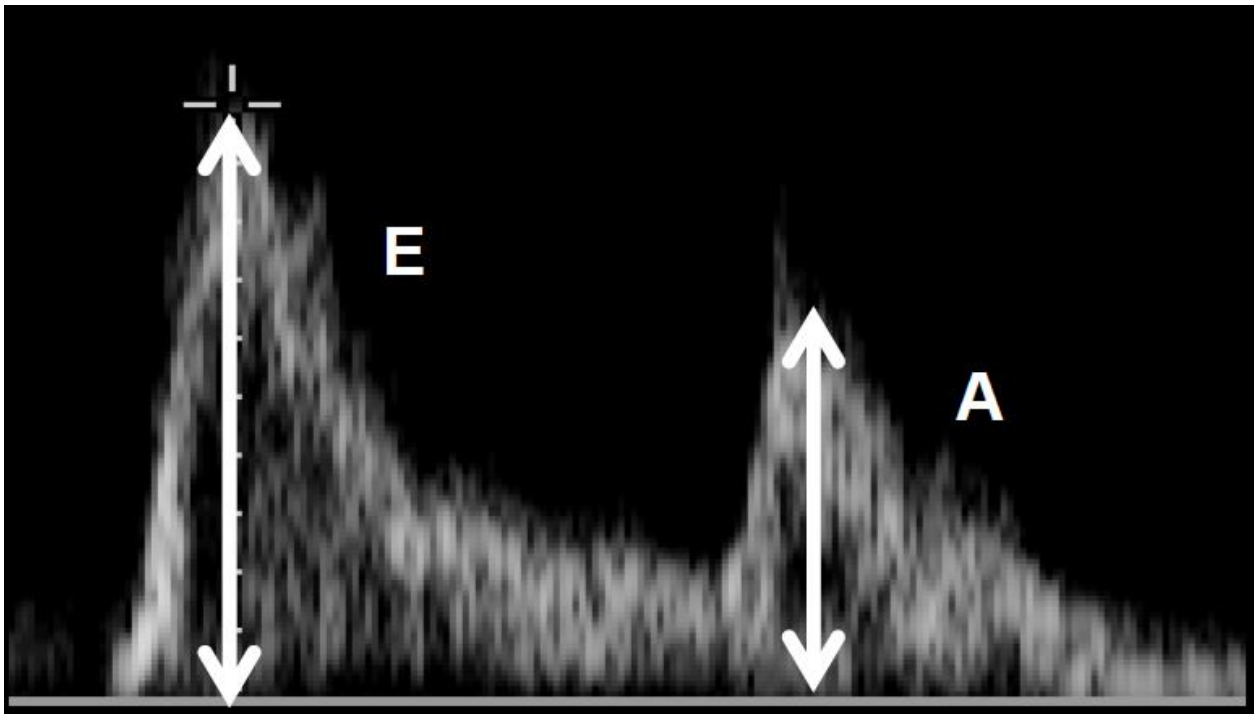

Figure 3.3 Echocardiographic image showing trans-mitral inflow velocity in early and late diastole using pulsed wave Doppler.

E, early diastolic filling velocity; A, late diastolic filling velocity.

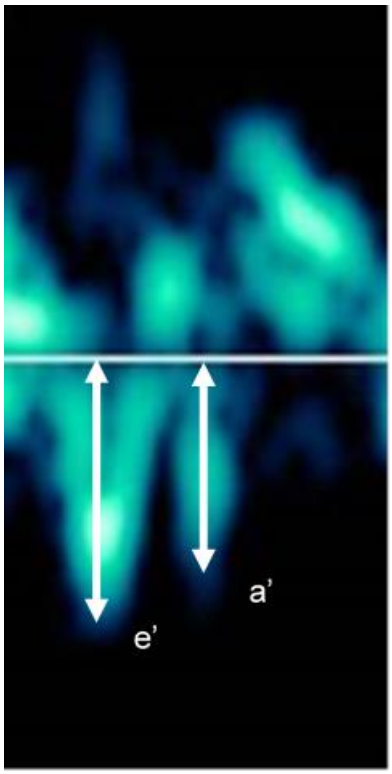

Figure 3.4 Echocardiographic image showing the rate of tissue relaxation at the mitral annulus using tissue Doppler imaging.

e', peak relaxation velocity during early diastole; a', peak relaxation velocity during late diastole.

3.6 Heart weight and circulating inflammatory markers

After echocardiography, the anaesthetised rats were terminated by thoracotomy. The hearts were removed from the thoracic cavity. The heart weight was obtained using an analytical balance (Precisa Gravimetrics AG, Lucerne, Switzerland). After weighing the whole hearts, the ventricles were separated from the atria and the total ventricle weight was obtained. Thereafter the right and left ventricles were separated and weighed.

Blood samples were collected into sterile serum clot activator tubes. After allowing to clot for 30 minutes on ice, blood was centrifuged and stored at -80°C for further analysis. Serum concentrations of IL-6, TNF-α and C-reactive protein (CRP) were measured by a solid phase sandwich enzyme-linked immunosorbent assays (ELISA) (Elabscience Biotechnology Co. Ltd, Wuhan, China). The lower detection limit for TNF-α, IL-6 and CRP were 78.13 pg/ml, 62.50 pg/ml and 0.31 ng/ml respectively, all with coefficients of variation of < 10%.

3.7 Statistical analysis

Statistical analysis was performed using SAS software, version 9.4 (SAS Institute Inc., USA). Continuous data are expressed as means ± standard error of mean (SEM). Differences in food intake, body weight, arthritis scores, paw thickness measurements and blood pressure between the groups were determined using repeated-measures two-way analysis of variance (ANOVA) followed by Tukey post-hoc tests. Because there were no group effects for blood pressure, arthritis scores and paw thickness measures, results were pooled for males and females. Differences in cardiac geometry and function between the groups were determined using a two-way ANOVA, where the main effects were group and sex. In case of a significant overall F-value, a Tukey post-hoc test was performed. Unless otherwise specified, echocardiographic and inflammatory markers were pooled for males and females when there were no sex differences. In case of sex effects in the two-way ANOVA, sensitivity analysis was performed in male and female rats to determine the influence of sex on the inflammation induced changed in cardiac function. Associations between inflammatory markers and echocardiographic measures were determined using Pearson's correlations. Because there were sex differences in

body mass, body weight and sex were included as potential confounders in correlation analysis. As blood pressure is one of the most important contributors to cardiac dysfunction, blood pressure was included as an additional confounder in the correlation analysis. Differences were considered statistically significant when $p < 0.05$.

Chapter 4 : Results

4.1 Experimental model characterisation

The occurrence of systemic inflammation was confirmed by the presence of erythema, oedema and joint rigidity in the joints of the inflammation, TNF-α and IL-6 groups as shown in Figure 4.1. The onset of arthritis was evident in the inflammation, TNF-α and IL-6 groups within 21-28 days of the primary immunisation (Figure 4.2 A).

There was no change in arthritis scores in the control group throughout the study. The arthritis scores of the inflammation group (1.8 ± 2.7) was significantly higher compared to the control group from 4 weeks after the primary immunisation and this increase persisted for the duration of the study (all $p < 0.0001$). The arthritis scores of the TNF-α and the IL-6 groups were significantly higher compared to the control group from 6 weeks after primary immunisation and remained significantly higher than the control group arthritis scores for the duration of the study (week 6: TNF-α 4.3 ± 2.4, IL-6 3.9 ± 1.7; week 8: TNF-α 4.3 ± 2.3, IL-6 4.1 ± 2.2; week 10: TNF-α 3.7 ± 2.4, IL-6 3.1 ± 1.4; all $p < 0.0001$). The arthritis scores did not differ between the inflammation, TNF-α or IL-6 groups at any time (all $p > 0.05$). Arthritis scores were significantly correlated to circulating concentrations of CRP ($r = 0.42$, $p < 0.0001$) and TNF-α ($r = 0.50$, $p = 0.0001$), independent of body weight and sex.

At baseline paw thicknesses measured at the ankle and tarsometatarsal joint did not differ across groups (all $p > 0.05$). Ten weeks after the primary immunisation the inflammation group had significantly greater paw thicknesses at the ankle joint (control: 8.59 ± 0.82 mm, inflammation: 9.59 ± 1.26 mm; $p = 0.001$) and the tarsometatarsal joint (control: 5.04 ± 0.56 mm, inflammation: 6.01 ± 1.28 mm; $p = 0.002$) compared to the control group (Figure 4.2 B and C). The paw thicknesses at the ankle joint and tarsometatarsal joint of the TNF-α and IL-6 groups were not different compared to the control or inflammation groups at any time (all $p > 0.05$).

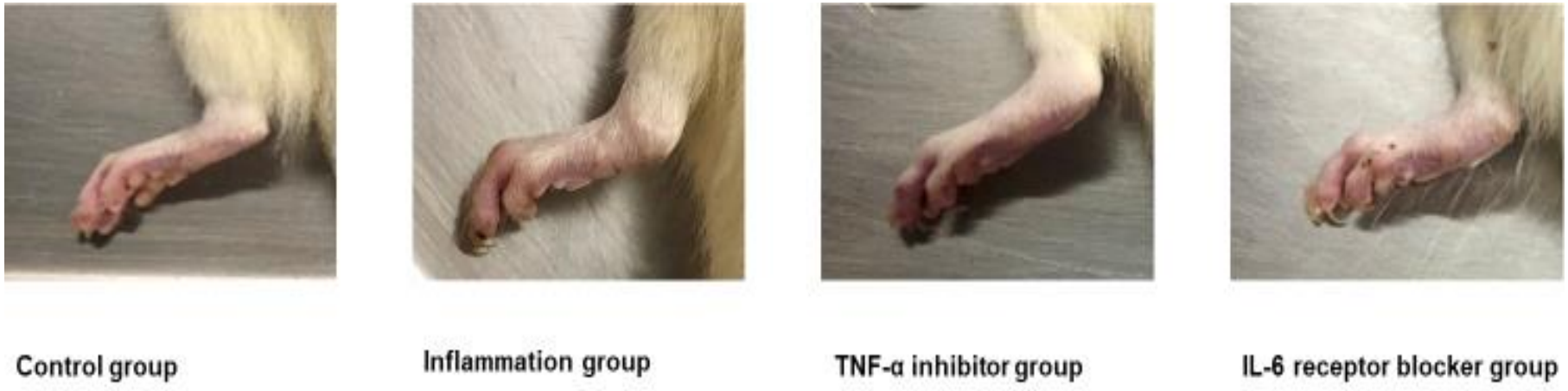

Figure 4.1 Photographs of hind paws of rats in the ventral view, at termination.

TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6.

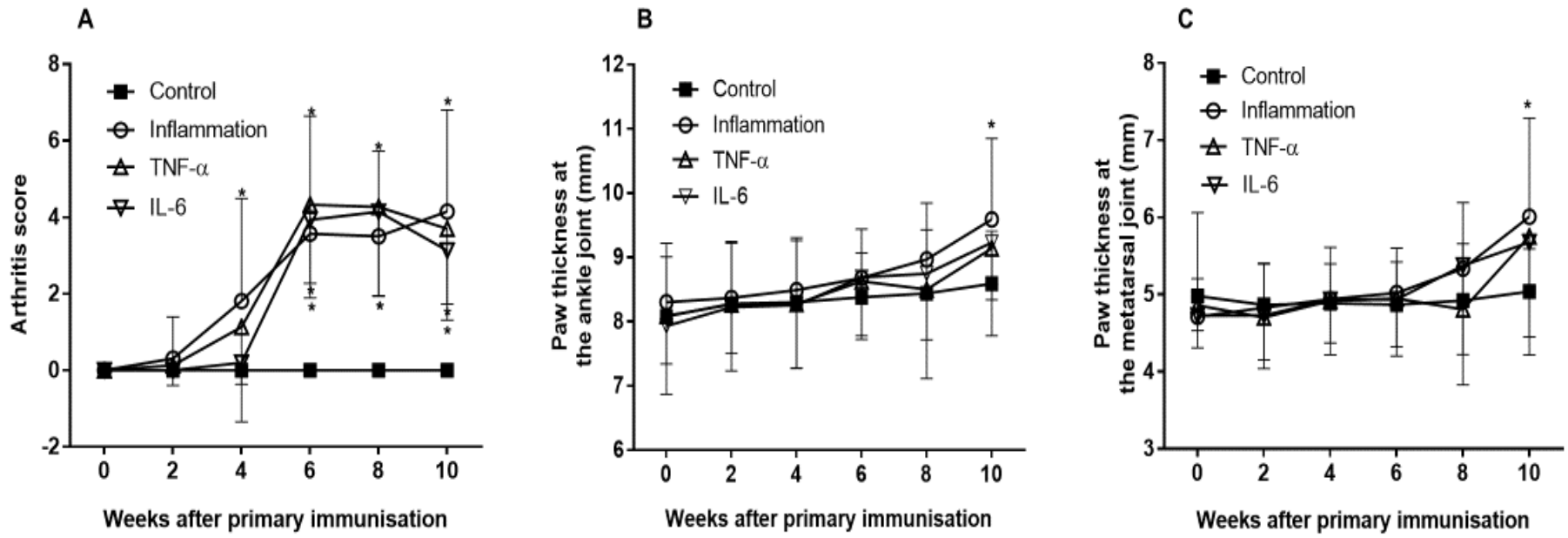

Figure 4.2 The degree of swelling and inflammation as measured by arthritis scores, paw thickness at the ankle joint and paw thickness at the tarsometatarsal joint in the control, inflammation, TNF-α and IL-6 rats over 10 weeks after the primary immunisation (week 0).

Solid squares represent control rats (n= 22), open circles present inflammation rats (n= 26), upright triangles present the TNF-α group (n= 15) and upside-down triangles represent the IL-6 group (n= 15). TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; mm, millimetres; * $p < 0.05$ compared to control group.

4.2 Body weight, food intake and blood pressure

Table 4.1 shows the body weight, food intake and blood pressure of the control, inflammation, TNF-α and IL-6 groups. Body weight did not change over time in any of the groups (all $p > 0.05$). There were also no differences in body weight between groups at baseline or at termination (all $p > 0.05$). Males had significantly higher body weights than females in all groups at baseline that remained significant throughout the study (all $p < 0.05$). Food intake did not differ between groups at baseline (all $p > 0.05$), however the IL-6 group (144.2 ± 25.4 g) consumed less food compared to the control group at 10 weeks (182.3 ± 72.3 g; $p = 0.04$). There were no differences in food intake between the groups for the rest of the study period. Systolic and diastolic blood pressure remained constant over the intervention in all groups (all $p > 0.05$). There were no differences in systolic or diastolic blood pressure between the groups at baseline or at termination (all $p > 0.05$). There were no differences in blood pressure between males and females in any of the groups (all $p > 0.05$).

Table 4.1 Body weights, food intake and tail cuff blood pressure at baseline and termination in the control, inflammation, TNF-α and IL-6 groups.

Baseline	**Controls (n=22)**	**Inflammation (n=26)**	**TNF- α (n=15)**	**IL- 6 (n=15)**
Body weight (g)	426.8 ± 25.3	430.7 ± 23.3	398.8 ± 30.7	407.0 ± 30.7
Food intake (g/week)	216.1 ± 55.2	196.9 ± 57.8	216.0 ± 57.2	226.1 ± 50.9
Blood pressure				
Systolic blood pressure (mm Hg)	128 ± 2	128 ± 2	129 ± 2	131 ± 2
Diastolic blood pressure (mm Hg)	90 ± 1	89 ± 1	87 ± 2	88 ± 2
Termination				
Body weight (g)	486.7 ± 25.3	454.4 ± 23.3	411.9 ± 30.6	387.6 ± 30.7
Food intake (g/week)	182.3 ± 72.3	151.6 ± 24.4	160.7 ± 50.4	144.2 ± 25.4*
Blood pressure				
Systolic blood pressure (mm Hg)	128 ± 2	129 ± 2	132 ± 2	129 ± 2
Diastolic blood pressure (mm Hg)	88 ± 2	89 ± 1	86 ± 2	90 ± 2

Data expressed as means ± SEM. TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; g, grams; mmHg, millimeters of mercury, * $p < 0.05$ compared to controls.

4.3 Serum inflammatory marker concentrations

Table 4.2 shows the serum inflammatory marker concentrations at termination for the four groups. Serum TNF-α concentrations were significantly higher in the inflammation (187.48 ± 9.44 pg/ml; $p < 0.001$), TNF-α (146.17 ± 11.17 pg/ml; $p = 0.02$) and IL-6 (162.78 ± 11.56 pg/ml; $p = 0.001$) groups compared to the control group (100.26 ± 9.92 pg/ml). Serum TNF-α concentrations were significantly lower in the TNF-α group, compared to the inflammation group ($p = 0.03$).

Serum IL-6 concentrations were significantly higher in the inflammation (32.17 ± 2.11 pg/ml; $p < 0.001$) and IL-6 (30.77 ± 2.69 pg/ml; $p = 0.001$) groups, compared to the control group (19.22 ± 2.26 pg/ml). The serum IL-6 concentrations of the TNF-α group (27.91 ± 2.61 pg/ml) did not differ significantly from the control group ($p > 0.05$).

Serum CRP concentrations were significantly higher in the inflammation (0.62 ± 0.04 ng/ml; $p < 0.001$), TNF-α (0.44 ± 0.06 ng/ml; $p= 0.001$) and IL-6 (0.65 ± 0.06 ng/ml; $p < 0.001$) groups compared to the control group (0.15 ± 0.05 ng/ml). Serum CRP concentration was significantly higher in the IL-6 group compared to the TNF-α group ($p= 0.04$).

Table 4.2 Inflammatory marker concentrations in the control, inflammation, TNF-α and IL-6 groups.

Inflammatory markers	**Control (n=21)**	**Inflammation (n=24)**	**TNF- α (n=15)**	**IL- 6 (n=14)**
TNF-α (pg/ml)	100.26 ± 9.92	187.48 ± 9.44*	146.17 ± 11.17*#	162.78 ± 11.56*
IL-6 (pg/ml)	19.22 ± 2.26	32.17 ± 2.11*	27.91 ± 2.61	30.77 ± 2.69*
CRP (ng/ml)	0.15 ± 0.05	0.62 ± 0.04*	0.44 ± 0.06*	0.65 ± 0.06* ǂ

Data expressed as means ± SEM. TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; CRP; C-reactive protein; pg, picogram; ng, nanogram; ml, millilitre;* $p < 0.05$ compared to controls; # $p < 0.05$ compared to inflammation; ǂ $p < 0.05$ compared to TNF-α.

4.4 Cardiac weight and geometry

Table 4.3 shows the heart weights and left ventricular geometry in the control, inflammation, TNF-α and IL-6 groups for all rats and in sensitivity analysis in males and females. In all rats, whole heart weights were significantly lower in the TNF-α group, compared to controls (control: 1.3 ± 0.1 g, TNF-α: 1.1 ± 0.1 g; $p = 0.04$). Heart weight indexed to body weight was significantly higher in the IL-6 group compared to controls (control: 2.7 ± 0.1, IL-6: 3.0 ± 0.1; $p = 0.05$). There were no differences in LV weight or LV weight indexed to body weight between the groups (all $p > 0.05$).

The interventricular septal thickness in diastole differed significantly across groups ($p < 0.001$; Table 4.3). The inflammation and the IL-6 groups had significantly greater IVST in diastole compared to the control group (control: 0.21 ± 0.01 mm, inflammation: 0.25 ± 0.00 mm, IL-6: 0.24 ± 0.01 mm; $p < 0.001$ and $p = 0.001$, respectively). The TNF-α group IVST in diastole (0.22 ± 0.01 mm) was significantly lower than that of the inflammation group ($p < 0.001$), but it was not different from the control group ($p = 0.53$).

The posterior wall thickness in diastole of the inflammation and IL-6 groups were significantly greater than the controls (control: 0.20 ± 0.01 mm; inflammation: 0.25 ± 0.01 mm; IL-6: 0.23 ± 0.01 mm; $p < 0.001$ and $p= 0.05$, respectively). There were no differences in PWT in diastole between the control and the TNF-α groups ($p = 0.56$). The PWT in diastole of the inflammation group was significantly greater than the TNF-α ($p < 0.001$) and IL-6 groups ($p = 0.05$). There were no differences in the LV internal diameter in systole or diastole between any of the groups (all $p > 0.05$). The LV septal and posterior wall thickness in systole was similar between the groups (all $p > 0.05$).

The relative wall thickness of the inflammation and the IL-6 groups were significantly higher than the control group (control: 0.61 ± 0.22 mm, inflammation: 0.78 ± 0.02 mm, IL-6: 0.71 ± 0.03 mm; $p < 0.001$ and $p = 0.02$, respectively). There was no difference in the RWT between the control (0.61 ± 0.22 mm) and TNF-α groups (0.65 ± 0.03; $p = 0.55$). The RWT of the TNF-α group was significantly lower compared to the inflammation group ($p = 0.001$).

There were significant sex effects for heart weights ($p < 0.001$), LV weights ($p < 0.001$), IVST ($p = 0.001$) and PWT ($p = 0.001$) in diastole. However, when adjusting for body weight, the sex effects for heart weight ($p = 0.12$) and LV weight ($p = 0.43$) were no longer significant. In sensitivity analysis in males, the inflammation, TNF-α and IL-6 rats had significantly lower heart weights compared to the controls (control: 1.49 ± 0.03 g, inflammation: 1.36 ± 0.03 g, TNF-α: 1.30 ± 0.04 g, IL-6: 1.33 ± 0.04 g; $p= 0.04$, $p = 0.03$ and $p = 0.02$, respectively) (Table 4.3). However, upon adjustment for body weight these differences were no longer significant (all $p > 0.05$). The LV weights of the male IL-6 group were significantly lower than the male control group (control: 0.75 ± 0.03 g, IL-6: 0.63 ± 0.03; $p= 0.02$) but were no longer significant when adjusting for body weight ($p = 0.42$). In the female rats, neither heart weights nor LV weights differed significantly across groups (all $p > 0.05$).

In sensitivity analysis in male rats (Table 4.3), the IVST in diastole was significantly higher in the inflammation group, compared to the control (control: 0.22 ± 0.01 mm; inflammation: 0.26 ± 0.01 mm; $p < 0.001$) and the TNF-α groups (0.23 ± 0.01 mm; $p = 0.006$). The PWT in diastole was significantly higher in the inflammation group compared to the control group (control: 0.22 ± 0.01 mm, inflammation: 0.25 ± 0.01 mm; $p = 0.02$).

In sensitivity analysis in the female rats, the inflammation and the IL-6 groups had significantly greater IVST in diastole compared to controls (control: 0.19 ± 0.01 mm, inflammation: 0.24 ± 0.01 mm, IL-6: 0.23 ± 0.01 mm; $p < 0.001$ and $p = 0.001$, respectively). The TNF-α group (0.21 ± 0.01 mm) had significantly lower IVST in diastole compared to the inflammation group ($p = 0.01$). The PWT in diastole was significantly higher in the inflammation group compared to the control, TNF-α and IL-6 groups (control: 0.18 ± 0.01 mm, inflammation: 0.25 ± 0.01 mm, TNF-α: 0.20 ± 0.01 mm, IL-6: 0.21 ± 0.01 mm; $p < 0.001$; $p = 0.004$ and $p = 0.01$, respectively).

Table 4.3 Heart weight and cardiac geometry in the control, inflammation, TNF-α and IL-6 groups.

	Control	Inflammation	TNF-α	IL-6
All rats	(n=22)	(n=26)	(n=15)	(n=15)
Heart weight (g)	1.27 ± 0.05	1.24 ± 0.05	1.13 ± 0.06*	1.14 ± 0.06
LV weight (g)	0.65 ± 0.03	0.62 ± 0.03	0.58 ± 0.03	0.56 ± 0.03
RV weight (g)	0.22 ± 0.02	0.22 ± 0.01	0.20 ± 0.02	0.20 ± 0.02
Heart weight/body weight	2.68 ± 0.08	2.82 ± 0.07	2.87 ± 0.10	2.98 ± 0.10*
LV weight/body weight	1.37 ± 0.06	1.41 ± 0.05	1.45 ± 0.07	1.40 ± 0.07
Heart weight/tibial length	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
IVS_d (mm)	0.21 ± 0.01	0.25 ± 0.00*	0.22 ± 0.01#	0.24 ± 0.01*
LVEDD (mm)	0.69 ± 0.02	0.64 ± 0.02	0.67 ± 0.00	0.66 ± 0.02
PWT_d (mm)	0.20 ± 0.02	0.25 ± 0.01*	0.22 ± 0.01#	0.23 ± 0.01* #
IVS_s (mm)	0.34 ± 0.01	0.36 ± 0.01	0.33 ± 0.01	0.35 ± 0.01
LVESD (mm)	0.37 ± 0.01	0.36 ± 0.01	0.37 ± 0.02	0.37 ± 0.02
PWT_s (mm)	0.30 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.30 ± 0.01
RWT (mm)	0.61 ± 0.22	0.78 ± 0.02*	0.65 ± 0.03#	0.71 ± 0.03*
Males	(n=13)	(n=18)	(n=8)	(n=8)
Heart weight (g)	1.49 ± 0.03	1.36 ± 0.03*	1.30 ± 0.04*	1.33 ± 0.04*
LV weight (g)	0.75 ± 0.03	0.67 ± 0.02	0.68 ± 0.03	0.63 ± 0.03*
RV weight (g)	0.21 ± 0.02	0.25 ± 0.01	0.23 ± 0.22	0.25 ± 0.02
Heart weight/body weight	2.51 ± 0.08	2.66 ± 0.07	2.63 ± 0.10	2.85 ± 0.10
LV weight/body weight	1.22 ± 0.05	1.29 ± 0.05	1.33 ± 0.07	1.35 ± 0.07
Heart weight/tibial length	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
IVS_d (mm)	0.22 ± 0.01	0.26 ± 0.01*	0.23 ± 0.01#	0.24 ± 0.01
LVEDD (mm)	0.72 ± 0.02	0.66 ± 0.02	0.67 ± 0.00	0.71 ± 0.03
PWT_d (mm)	0.22 ± 0.01	0.25 ± 0.01*	0.23 ± 0.00	0.24 ± 0.01
IVS_s (mm)	0.36 ± 0.01	0.37 ± 0.01	0.34 ± 0.01	0.38 ± 0.01
LVESD (mm)	0.41 ± 0.02	0.36 ± 0.01	0.37 ± 0.02	0.39 ± 0.02
PWT_s (mm)	0.31 ± 0.01	0.33 ± 0.01	0.34 ± 0.01	0.32 ± 0.01
RWT (mm)	0.61 ± 0.03	0.78 ± 0.02*	0.69 ± 0.04	0.69 ± 0.04
Females	(n=9)	(n=8)	(n=7)	(n=7)
Heart weight (g)	0.94 ± 0.03	0.96 ± 0.04	0.92 ± 0.04	0.93 ± 0.04
LV weight (g)	0.51 ± 0.03	0.50 ± 0.03	0.47 ± 0.03	0.48 ± 0.03
RV Weight (g)	0.16 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
Heart weight/body weight	2.91 ± 0.01	3.19 ± 0.12	3.13 ± 0.13	3.13 ± 0.13
LV weight/body weight	1.58 ± 0.09	1.67 ± 0.09	1.59 ± 0.10	1.62 ± 0.10
Heart weight/tibial length	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
IVS_d (mm)	0.19 ± 0.01	0.24 ± 0.01*	0.21 ± 0.01#	0.23 ± 0.01*
LVEDD (mm)	0.63 ± 0.02	0.60 ± 0.02	0.68 ± 0.03	0.61 ± 0.03
PWT_d (mm)	0.18 ± 0.01	0.25 ± 0.01*	0.20 ± 0.01#	0.21 ± 0.01#

IVS_s (mm)	0.32 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
LVESD (mm)	0.31 ± 0.02	0.34 ± 0.02	0.38 ± 0.02	0.33 ± 0.02
PWT_s (mm)	0.30 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.29 ± 0.01
RWT (mm)	0.60 ± 0.03	0.79 ± 0.00*	0.62 ± 0.04$^{\#}$	0.74 ± 0.04*

Data expressed as means ± SEM. TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; LV, left ventricular; RV, right ventricular; IVS, interventricular septum; LVEDD, left ventricular end diastolic diameter; PWT, posterior wall thickness; LVESD, left ventricular end systolic diameter; RWT, relative wall thickness; d, diastolic; s, systolic; g, grams; mm, millimetre; * $p < 0.05$ compared to control; $^{\#}$ $p < 0.05$ compared to inflammation.

4.5 Left ventricular systolic function

Table 4.4 shows the left ventricular systolic function measurements for all rats and in sensitivity analysis for male and female rats. There were no differences in ejection fraction, endocardial fractional shortening or midwall fractional shortening between any of the groups (all $p > 0.05$). However, there was a trend towards a significantly lower stroke volume in the inflammation group (0.49 ± 0.03 ml/beat) compared to the control group (0.60 ± 0.03 ml/beat; $p = 0.06$). Although there were no groups effects, there was a significant sex effect for stroke volume ($p < 0.001$). Following adjustment for body weight, these differences were no longer significant ($p = 0.16$). In sensitivity analysis in the males (Table 4.4), stroke volume was significantly lower in the inflammation group (0.52 ± 0.04 ml/beat) compared to the control (0.66 ± 0.05 ml/beat; $p = 0.04$) and IL-6 groups (0.65 ± 0.06 ml/beat; $p = 0.05$). There were no differences in ejection fraction or fractional shortening between groups (all $p > 0.05$). There were no differences in any of the systolic function variables between the groups in sensitivity analysis in female rats (all $p > 0.05$; Table 4.4).

Table 4.4 Left ventricular systolic function in the control, inflammation, TNF-α and IL-6 groups.

	Control	**Inflammation**	**TNF-α**	**IL-6**
All rats	**(n=22)**	**(n=26)**	**(n=15)**	**(n=15)**
Stroke volume (ml/beat)	0.60 ± 0.03	0.49 ± 0.03	0.53 ± 0.04	0.55 ± 0.04
Ejection fraction (%)	82.94 ± 1.41	81.18 ± 1.29	80.94 ± 1.70	81.12 ± 1.70
Endocardial fractional shortening (%)	47.29 ± 1.38	44.64 ± 1.27	44.91 ± 1.67	45.09 ± 1.67
Midwall fractional shortening (%)	22.93 ± 0.69	21.90 ± 0.63	21.15 ± 0.84	22.23 ± 0.87
Males	**(n=13)**	**(n=18)**	**(n=8)**	**(n=8)**
Stroke volume (ml/beat)	0.66 ± 0.05	0.52 ± 0.04	0.56 ± 0.06	0.65 ± 0.06[#]
Ejection fraction (%)	80.93 ± 1.53	81.33 ± 1.30	81.46 ± 1.95	80.62 ± 1.95
Endocardial fractional shortening (%)	43.96 ± 1.75	44.90 ± 1.45	45.19 ± 2.37	45.45 ± 1.94
Midwall fractional shortening (%)	22.15 ± 0.86	22.20 ± 0.73	20.92 ± 1.10	21.81 ± 1.91
Females	**(n=9)**	**(n=8)**	**(n=7)**	**(n=7)**
Stroke volume (ml/beat)	0.51 ± 0.04	0.42 ± 0.04	0.51 ± 0.04	0.43 ± 0.04
Ejection fraction (%)	85.84 ± 2.70	80.84 ± 2.86	80.40 ± 3.06	81.69 ± 3.06
Endocardial fractional shortening (%)	51.00 ± 2.48	43.01 ± 2.63	44.32 ± 2.81	45.54 ± 2.81
Midwall fractional shortening (%)	24.05 ± 1.17	21.22 ± 1.24	21.42 ± 1.30	22.72 ± 1.33

Data expressed as means ± SEM. TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; ml/beat, millilitre per beat; [#] $p < 0.05$ compared to inflammation.

4.6 Left ventricular diastolic function

Table 4.5 shows left ventricular diastolic function measures in the four groups. Early mitral inflow velocity (E wave) did not differ across the groups ($p = 0.68$). Late atrial mitral inflow velocity (A wave) was significantly lower in the TNF-α and IL-6 groups compared to the inflammation group (inflammation: 70.27 ± 2.64 cm/s, TNF-α: 59.42 ± 3.47 cm/s, IL-6: 57.99 ± 3.47 cm/s; $p = 0.05$ and $p = 0.02$, respectively).

The ratio of early to late mitral inflow velocity (E/A) was higher in the TNF-α group (1.95 ± 0.08) compared to the inflammation group (1.66 ± 0.06; $p = 0.02$). There were no significant differences in the E/A ratio between any of the other groups (all $p > 0.05$).

The e' differed significantly across groups (all $p < 0.001$). The e' of the inflammation, TNF-α and the IL-6 groups was significantly decreased, compared to the control group (mean ± SEM; control: 4.52 ± 0.13 cm/s; inflammation: 3.78 ± 0.12 cm/s, TNF-α: 3.50 ± 0.16 cm/s, IL-6: 3.66 ± 0.16 cm/s; $p= 0.001$, $p < 0.001$ and $p < 0.001$, respectively).

The a' did not differ significantly across groups ($p = 0.68$). The e'/a' ratio in the TNF-α group was significantly lower compared to the control group and the inflammation group (control: 1.48 ± 0.07, inflammation: 1.33 ± 0.06, TNF-α: 1.04 ± 0.08; $p < 0.001$ and $p = 0.03$, respectively).

The E/e' ratio of the inflammation, TNF-α and IL-6 groups were significantly higher compared to the control group (control: 24.92 ± 1.21; inflammation: 29.89 ± 1.15, TNF-α: 32.53 ± 1.48, IL-6: 29.92 ± 1.46; $p = 0.02$, $p = 0.001$ and $p = 0.04$, respectively). There were no sex effects in any of the diastolic function variables.

Table 4.5 Left ventricular diastolic function in the control, inflammation, TNF-α and IL-6 groups.

	Control (n=22)	**Inflammation (n=26)**	**TNF-α (n=15)**	**IL-6 (n=15)**
E (cm/s)	117.91 ± 3.42	115.01 ± 3.15	114.08 ± 4.15	111.44 ± 4.15
A (cm/s)	65.54 ± 2.87	70.72 ± 2.64	59.42 ± 3.47$^{\#}$	57.99 ± 3.47$^{\#}$
E/A	1.74 ± 0.06	1.66 ± 0.06	1.95 ± 0.08$^{\#}$	1.83 ± 0.08
Lateral e' (cm/s)	4.52 ± 0.13	3.79 ± 0.12*	3.50 ± 0.16*	3.66 ± 0.16*
Lateral a' (cm/s)	3.07 ± 0.13	3.02 ± 0.12	3.22 ± 0.15	2.99 ± 0.15
e'/a'	1.48 ± 0.07	1.33 ± 0.06	1.04 ± 0.08*$^{\#}$	1.29 ± 0.09
E/e'	24.92 ± 1.21	29.89 ± 1.15*	32.53 ± 1.48*	29.92 ± 1.46*

Data expressed as means ± SEM. TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; E, early diastolic filling velocity; A, late diastolic filling velocity; e', peak velocity during early diastole; E/A, ratio of early to late diastolic filling; a', peak velocity during late diastole; e'/a', ratio of early to late peak velocities during diastole ; E/e', ratio of early diastolic filling to peak velocity during early diastole; cm/s, centimetre per second; * $p < 0.05$ compared to control; $^{\#}$ $p < 0.05$ compared to inflammation.

4.7 Association between inflammatory markers and cardiac structure and function

4.7.1 Association between inflammatory markers and cardiac geometry

Figure 4.3 show the associations between inflammatory markers and measures of cardiac geometry. Neither heart weight indexed to body weight (Figure 4.3 A), nor LV weight indexed to body weight (Figure 4.3 B) was associated with any of the inflammatory markers. There was a significant association between IVST in diastole and serum TNF-α ($r = 0.50$; $p < 0.001$), IL-6 ($r = 0.32$; $p = 0.01$) and CRP ($r = 0.43$; $p= 0.001$) concentrations (Figure 4.3 C). PWT in diastole was associated with serum TNF-α ($r = 0.48$; $p < 0.001$), IL-6 ($r = 0.34$; $p = 0.003$) and CRP ($r = 0.44$; $p < 0.001$) concentrations (Figure 4.3 D). RWT was significantly associated with serum TNF-α ($r = 0.45$; $p < 0.0001$), IL-6 ($r = 0.36$; $p= 0.001$) and CRP ($r = 0.48$; $p < 0.001$) concentrations (Figure 4.3 E). Upon adjustment for body weight and sex these associations were materially unaltered (IVST in diastole vs TNF-α: partial $r = 0.47$; $p < 0.0001$, IL-6: partial $r = 0.37$; $p = 0.001$, CRP: partial $r = 0.38$; $p = 0.001$); (PWT in diastole vs TNF-α: partial $r = 0.45$; $p < 0.0001$, IL-6: partial $r = 0.39$; $p = 0.001$, CRP: partial $r = 0.39$; $p = 0.001$) (RWT vs TNF-α: partial $r = 0.42$; $p < 0.0001$, IL-6: partial $r = 0.36$; $p = 0.002$, CRP: partial $r = 0.45$; $p < 0.0001$). Left ventricular end diastolic diameter was inversely related to serum IL-6 concentrations ($r = -0.24$; $p= 0.04$) (Figure 4.3 F). However, this association was no longer significant when adjusting for sex (partial $r = -0.23$; $p= 0.06$). Inflammatory markers were not significantly associated with any other markers of LV geometry.

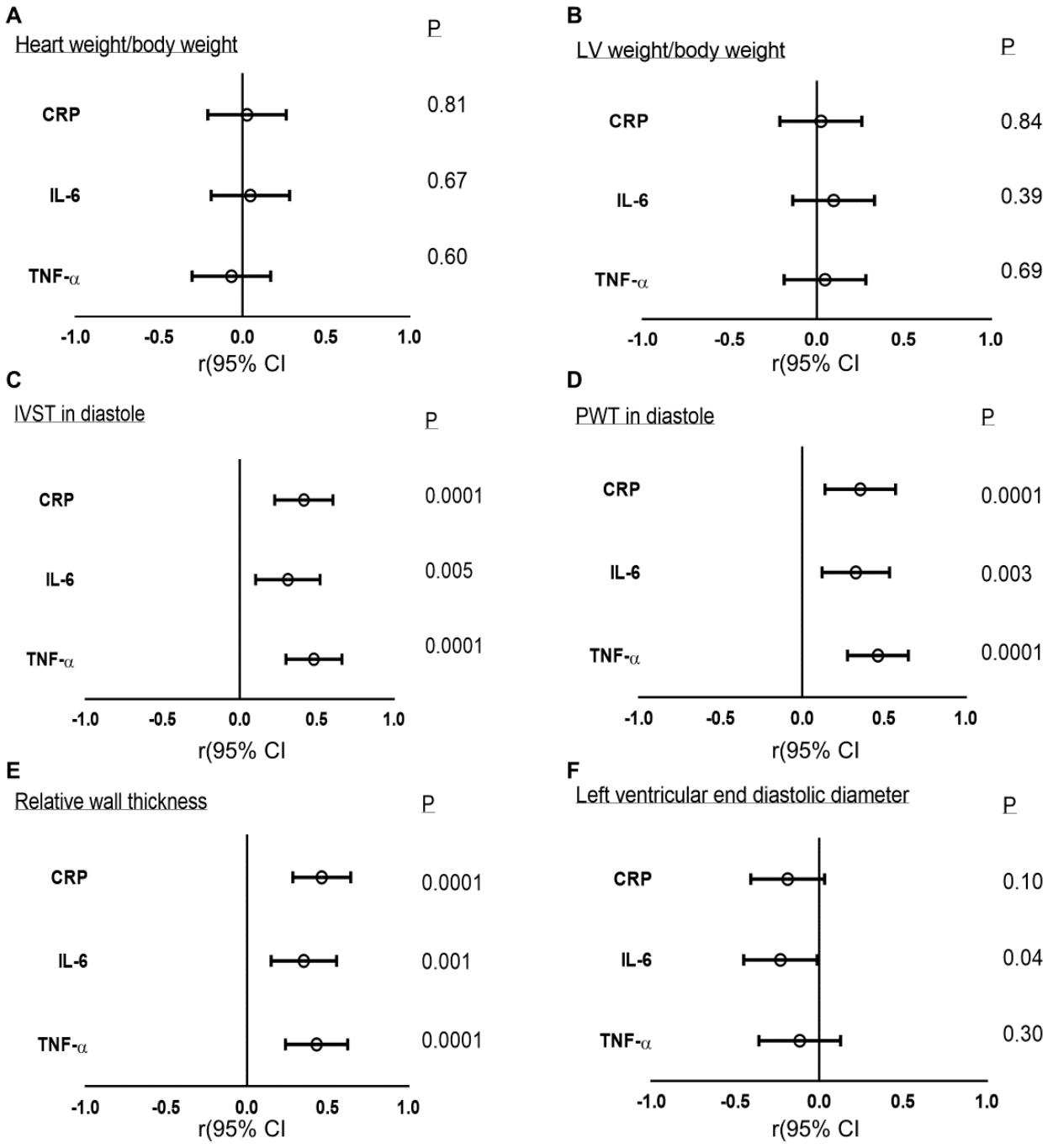

Figure 4.3 Associations between left ventricular geometry and inflammatory cytokines.

CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha, LV, left ventricle; IVST, interventricular septal thickness; PWT, posterior wall thickness. Open circles represent the correlation coefficient (r) and horizontal lines represent the 95% confidence intervals (CI).

4.7.2 Association between inflammatory markers and measures of systolic function

Associations between serum cytokine concentrations and systolic function are given in Figure 4.4. Stroke volume was inversely associated with serum IL-6 concentrations (r = -0.23; p = 0.05) (Figure 4.4 A). This association was no longer significant when adjusting for sex (partial r = -0.22; p = 0.07). Although no significant association was observed between stroke volume and serum CRP concentrations (r = -0.21; p = 0.08) in bivariate analysis, this relationship became significant when adjusting for sex (partial r = -0.30; p = 0.01). No further significant associations were noted between serum concentrations of inflammatory cytokines and measures of systolic function (Figure 4.4).

4.7.3 Association between inflammatory markers and measures of diastolic function

Association between serum cytokine concentrations and diastolic function are reported in Figure 4.5. The E wave was not related to serum cytokine concentrations (Figure 4.5 A). Serum TNF-α concentrations were significantly associated with the A wave (r = 0.44; p = 0.0001) (Figure 4.5 B) and E/A ratio (r = -0.35; p = 0.003) (Figure 4.5 C). These relationships remained significant when adjusting for sex and body weight (A wave: partial r = 0.48; p < 0.0001; E/A: partial r = -0.40; p = 0.0007). Lateral e' was inversely associated with serum CRP concentrations (r = -0.33; p = 0.005) (Figure 4.5 D). This association remained significant when adjusting for sex and body weight (partial r = -0.35; p = 0.003). E/e' was significantly associated with serum TNF-α (r = 0.23; p = 0.05) and CRP (r = 0.25; p = 0.03) concentrations (Figure 4.5 E). These associations were materially unaltered following adjustment for sex and body weight (TNF-α: partial r = 0.26, p = 0.03; CRP: partial r = 0.29, p = 0.02). Serum cytokine concentrations were not related to e'/a' ratio (Figure 4.5 F). No significant associations were observed between measures of diastolic function and serum IL-6 concentrations (Figure 4.5).

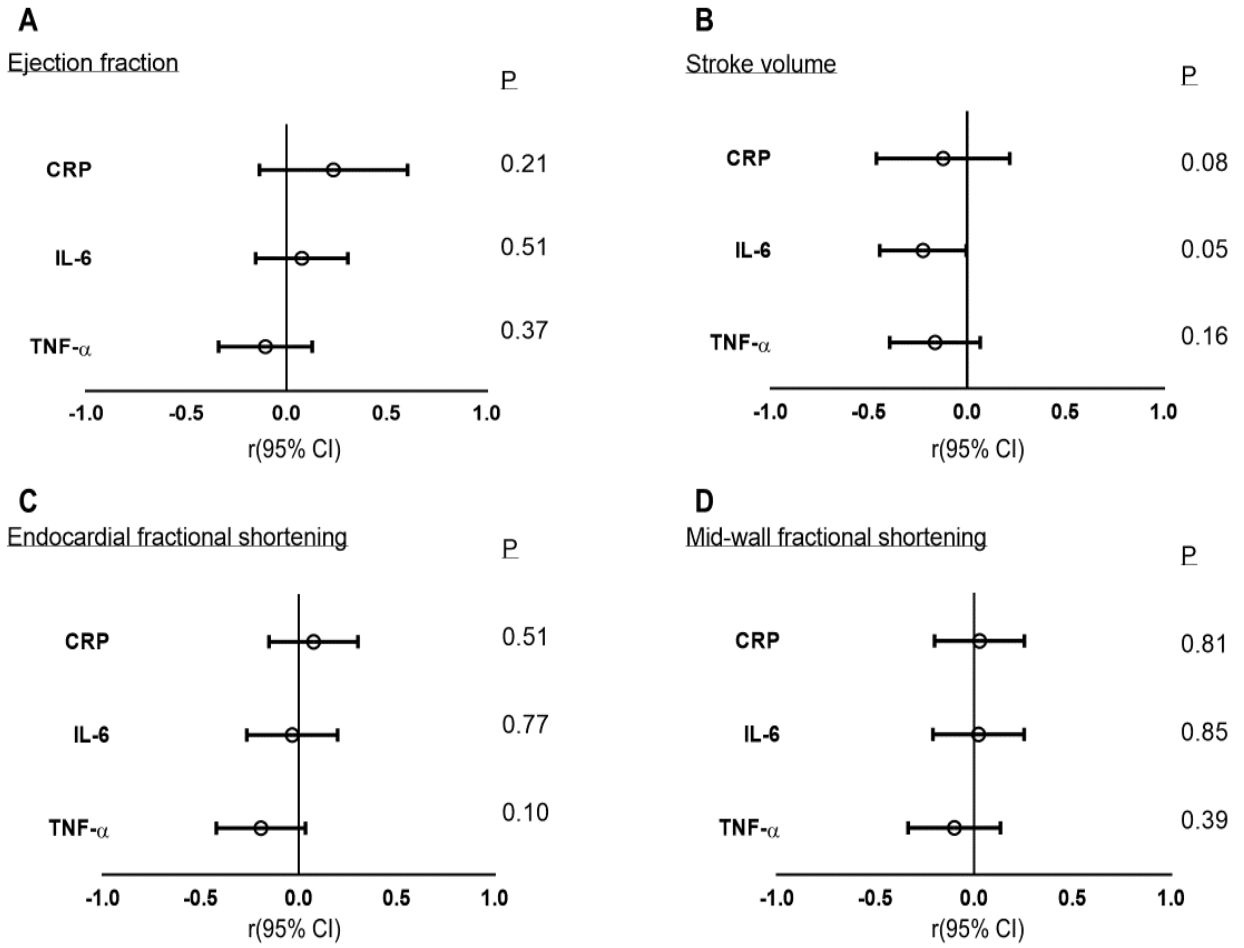

Figure 4.4 Associations between left ventricular systolic function measurements and inflammatory cytokines.

CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha. Open circles represent the correlation coefficient (r) and horizontal lines represent the 95% confidence intervals (CI).

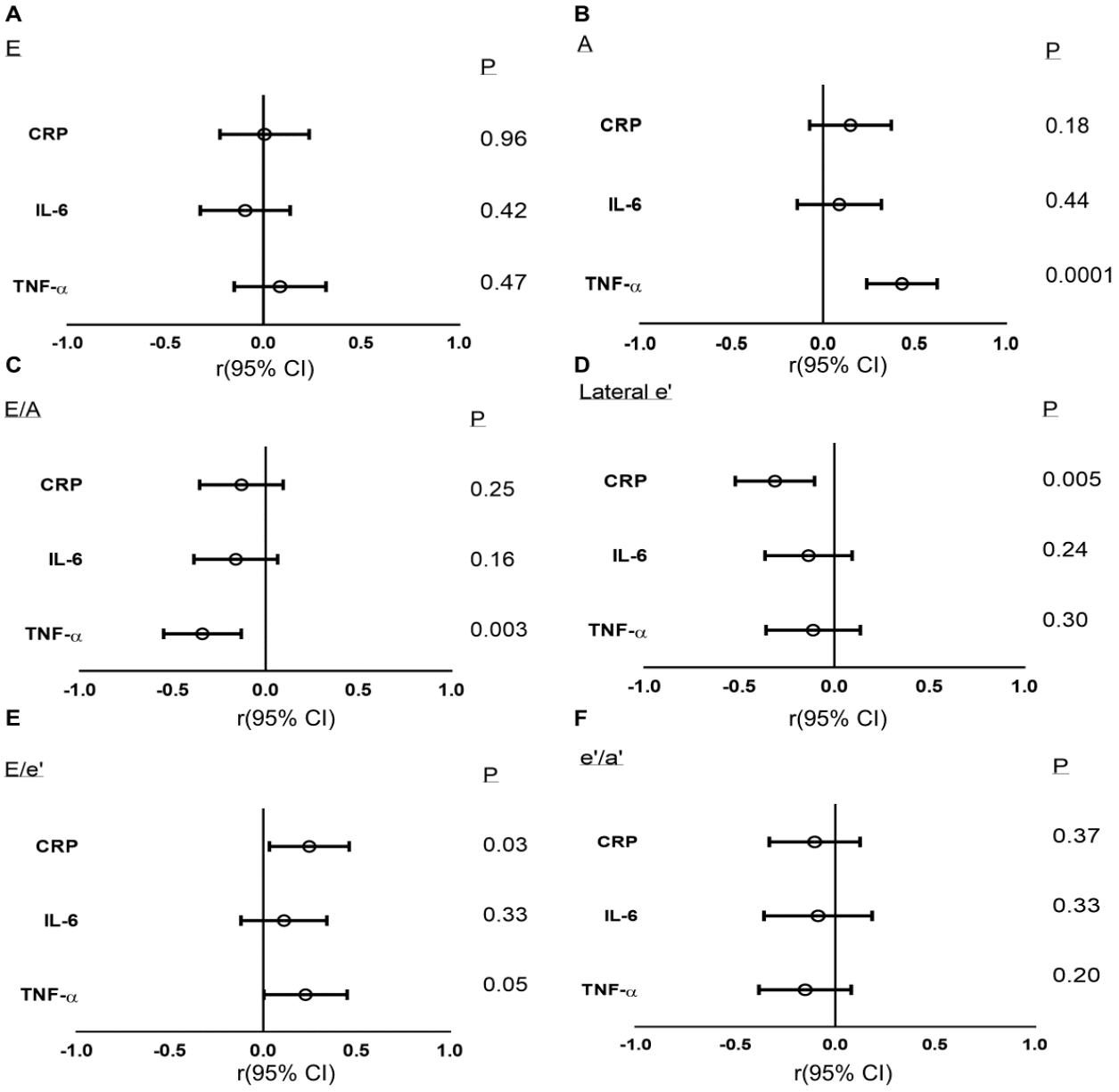

Figure 4.5 Associations between measures of left ventricular diastolic function and inflammatory cytokines.

E, early diastolic filling velocity; A, late diastolic filling velocity; E/A, ratio of early to late diastolic filling; e', peak velocity during early diastole; a', peak velocity during late diastole; E/e', ratio of early diastolic filling to peak velocity during early diastole; e'/a'; ratio of early to late peak velocities during diastole; CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha. Open circles represent the correlation coefficient (r) and horizontal lines represent the 95% confidence intervals (CI).

Chapter 5 : Discussion

In this study, we investigated the effects of high-grade systemic inflammation on cardiac geometry and function and determined whether treatment with DMARDs may ameliorate inflammation-induced changes in LV structure and function. The main findings of the current study are that inflammation was associated with LV concentric remodelling without significant changes in LV mass. TNF-α inhibitors protected against adverse LV remodelling, but IL-6 receptor blocker treatment did not alter the inflammation induced LV concentric remodelling. Exposure to inflammation caused significant reductions in diastolic function, but no changes in systolic function. Neither TNF-α inhibitors nor IL-6 receptor blockers were successful in ameliorating inflammation-induced diastolic dysfunction. Circulating inflammatory markers were strongly related to changes in LV geometry, but were not consistently related to changes in diastolic function.

5.1 Effects of inflammation on cardiac geometry

In the present study, CIA in Sprague Dawley rats resulted in significant cardiac remodelling, as indicated by the increased IVST in diastole, PWT in diastole and RWT compared to the control group. Changes in cardiac diameters occurred without an increase in heart weight or LV mass or changes in the LV internal diameter. This suggests that a concentric remodelling pattern was present in all groups exposed to inflammation. Our results are in line with previous clinical studies where RA was found to be associated with concentric LV remodelling, independent of cardiovascular disease risk factors and comorbidities (Myasoedova *et al.*, 2013). However, others have shown increases in LV mass indexed to body mass (Corrao, 1996; Rudominer *et al.*, 2009). LV remodelling may have a time-specific expression pattern, whereby increases in RWT are often seen before changes in LV mass (Gaasch and Zile, 2011). Hence it may be that a longer duration of exposure to inflammation may lead to more significant changes in LV mass (Myasoedova *et al.*, 2013).

Growing evidence suggests that inflammatory cytokines mediate cardiac remodelling and myocardial fibrosis by altering myocyte metabolism and stimulating sarcomeric protein synthesis (Krown *et al.*, 1996; Yokoyama *et al.*, 1997; Westermann *et al.*, 2011). TNF-α drives cardiac remodelling by directly activating MMPs, altering collagen

composition and increasing fibroblast proliferation (Sivasubramanian *et al.*, 2001; Siwik and Colucci, 2004; Sciarretta *et al.*, 2009; Frangogiannis, 2012). In this regard, cardiac-specific expression of TNF-α in mice is associated with both ventricular hypertrophy (Bryant *et al.*, 1998), and increased MMP-2 and MMP-9 activity (Li *et al.*, 2000). Theoretically, TNF-α induced upregulations in MMP activity should result in the degradation of collagen and decreased fibrosis (Cowan *et al.*, 1999; Dixon *et al.*, 2007). However, degraded products of matrix proteins stimulate collagen synthesis, as well as the subsequent deposition of unstructured fibrotic tissue (Li *et al.,* 2000). The interrelated nature of the two processes could then result in adverse LV myocardial remodelling, without changes in LV mass. IL-6 has also been strongly implicated in the pathological remodelling process and has been shown to induce cardiac fibrosis by strongly regulating fibroblast function in both human (Wollert and Drexler, 2001; Meléndez *et al.*, 2010) and animal models (Hirota *et al.*, 1995; Zhao *et al.*, 2016). Indeed, in the current study we found strong relations between circulating inflammatory markers and increased IVST, PWT and RWT.

Hypertension is a primary driver of concentric LV remodelling. In hypertension the myocardium attempts to maintain wall stress in the presence of chronically elevated pressures (Gaasch and Zile, 2011). Hypertension typically evolves from a phase of structural LV remodelling to LV decompensation (Bamaiyi *et al.*, 2019). However, in the current study we showed concentric remodelling patterns similar to those reported in hypertension, without any changes in blood pressure. In addition, pro-inflammatory cytokine concentrations were related to RWT and these associations remained significant even after adjusting for blood pressure. Indeed, previous studies are in agreement with our study, where infusion of TNF-α or IL-6 resulted in increased collagen content and LV stiffness, independent of blood pressure changes (Meléndez *et al.*, 2010). Clinical studies, have also shown that RA is independently associated with increases in RWT (Midtbø *et al.*, 2013). Taken together, our results show that inflammation independently affects the myocardial remodelling processes and that inflammation may be a potential therapeutic target to prevent cardiac remodelling. Future studies should identify the exact mechanisms whereby inflammation causes myocardial remodelling.

5.2 Effect of inflammation on diastolic function

Inflammation has several adverse effects on both active and passive myocardial relaxation processes (Paulus and Tschöpe, 2013). In the current study, CIA caused impaired LV relaxation (decreased lateral e') (Sohn *et al.*, 1997) as well as increased LV filling pressures (E/e') (Sharifov *et al.*, 2016). Changes in TDI measures of diastolic function was evident, despite no changes in the pulsed wave Doppler measured trans-mitral inflow patterns (E/A). Only one previous rat study, using Wistar female rats showed a reduced diastolic function (Dai *et al.*, 2017). However, the previous study showed a decrease in the pulsed waved Doppler measured early diastolic filling (E wave) but did not report TDI determined measures of diastolic function (Dai *et al.*, 2017). Although the assessment of trans-mitral inflow velocity using pulsed wave Doppler is validated, TDI determined markers of diastolic function are deemed to be more sensitive, as they are less pre-load and heart rate dependent and they relate more strongly to diastolic function using invasive measures of diastolic function, such as cardiac catheterisation (Sohn *et al.*, 1997; Kasner *et al.*, 2007).

In our study, TDI determined e', an index of myocardial relaxation was reduced in inflammation rats compared to controls. Impaired myocardial relaxation was also related to an increase in circulating inflammatory markers in the inflammation group. Our results are in agreement with previously published cross-sectional RA studies that found impaired diastolic function in patients with adverse inflammatory profiles (Torre-Amione *et al.*, 1996; Liang *et al.*, 2010; Aslam *et al.*, 2013; Tomáš *et al.*, 2013). Indeed, it has previously been shown that inflammation impairs active LV relaxation (Riehle and Bauersachs, 2019). Inflammation impairs SERCA and thus delays Ca^{2+} reuptake which leads to a delay in LV relaxation due to changes in the intracellular Ca^{2+} homeostasis.

Besides the impairment in active relaxation, our result showed that inflammation also impairs the processes of passive relaxation. In this regard rats exposed to inflammation had increased ventricular stiffness as indexed by a reduced e'/a'. Indeed, inflammatory cytokines mediate hypertrophic remodelling and myocardial fibrosis by promoting collagen synthesis and activating matrix metalloproteinases within cardiac

fibroblasts (Meléndez *et al.*, 2010; Westermann *et al.*, 2011). Moreover, recent findings suggest a role for systemic inflammation in the hypophosphorylation of the cytoskeletal protein titin, which contributes to passive myocardial stiffness (Franssen *et al.*, 2016). Passive myocardial stiffness is brought about by both collagen and titin phosphorylation (Zile *et al.*, 2015).

Both the impairment in active and passive relaxation may have contributed to the increased filling pressure reported in the rats exposed to inflammation. Previous studies showed that higher collagen content (Kasner *et al.*, 2011), greater myocardial stiffness (Kasner *et al.*, 2007), and impairments in relaxation (Selby *et al.*, 2011) all relate to increased LV filling pressures. This increase in filling pressure may have resulted in compensatory increases in early diastolic filling (Nishimura and Tajik, 1997; Mottram and Marwick, 2005; Mitter *et al.*, 2017). When filling pressure in the LV increases, left atrial pressure is increased and acts as a driving force for early diastolic filling (Nishimura and Tajik, 1997; Mottram and Marwick, 2005; Mitter *et al.*, 2017). This suggests that the impaired LV relaxation resulted in elevated LV filling pressures, which in the current study, may have led to a pseudonormal filling pattern (Nishimura and Tajik, 1997; Mottram and Marwick, 2005; Mitter *et al.*, 2017). A pseudonormal filling pattern is observed as the E/A ratio may appear normal, despite increased LV filling pressures.

Despite diastolic impairment in the groups exposed to inflammation, the associations between inflammatory cytokines and markers of diastolic function were not consistent. In this regard, in the current study we showed circulating TNF-α concentrations to be associated with an increased A wave and increased filling pressures (E/e'), while TNF-α concentrations were negatively correlated with E/A ratio. Serum CRP concentrations were negatively correlated to LV relaxation (e'), and directly related to LV filling pressures (E/e'). However, we did not find a correlation between serum IL-6 concentrations and any diastolic function measures in the current study.

TNF-α reportedly downregulates the expression of the SERCA gene, resulting in a decreased Ca^{2+} reuptake into the sarcoplasmic reticulum during diastole (Yokoyama *et al.*, 1997; Wu *et al.*, 2011). The decreased Ca^{2+} reuptake delays LV relaxation during early

diastole. CRP concentrations have previously been associated with impaired LV relaxation as well as elevated LV filling pressures (Shah *et al.*, 2006; Williams *et al.*, 2008). Exposure to IL-6 have resulted in diastolic dysfunction in a rat model, by causing cardiac fibrosis and increased LV stiffness (Meléndez *et al.*, 2010). Nonetheless, in the present study IL-6 was not related to changes in LV relaxation. However, all inflammatory markers were significantly related to concentric LV remodelling. This suggests that diastolic dysfunction may not be directly related to inflammation but rather that the impaired relaxation and increased filling pressure are mediated by the extracellular matrix remodelling induced by inflammation. Indeed, previous studies have shown that concentric remodelling may be an important risk factor for diastolic dysfunction (Fouad *et al.*, 1984; Ken *et al.*, 2017). Concentric remodelling has previously been associated with impaired diastolic function, increased LV stiffness, and impaired myocardial relaxation (Fouad *et al.*, 1984; Ken *et al.*, 2017). Hence inflammation may be indirectly causing the diastolic dysfunction via changes in the ECM composition.

Besides concentric remodelling, hypertension is a primary risk factor for diastolic dysfunction and a leading cause of heart failure (Levy *et al.*, 1996). However, even in the absence of hypertension, inflammation independently relates to diastolic dysfunction (Paulus and Tschöpe, 2013; Suthahar *et al.*, 2017). Indeed, hypertension has been linked to increased myocardial collagen content, collagen cross linking and myocardial fibrosis which leads to diastolic dysfunction (Harvey *et al.*, 2016; Suthahar *et al.*, 2017). However, recent studies indicate that hypertension cannot fully account for the development of diastolic dysfunction in RA patients (Ghaleb *et al.*, 2019; Targońska-Stępniak *et al.*, 2019). Similar to previous CIA studies conducted in rats (Reynolds *et al.*, 2012; Verhoeven *et al.*, 2017), we found that blood pressure was not different in the inflammation rats compared to control groups. Hence our results showed that inflammation may be associated with adverse cardiac remodelling and diastolic dysfunction, independent of load. Future studies will need to determine whether inflammation affects cellular processes, including Ca^{2+} handling, ECM maintenance and titin, which are responsible for maintaining adequate diastolic function or whether it is mediated via concentric remodelling alone.

5.3 Effect of inflammation on systolic function

In the current study, we found that exposure to inflammation did not change systolic function. Previous CIA rat models also demonstrated preserved LV EF and FS as measured by echocardiography (Dai *et al.*, 2017; Mokotedi *et al.*, 2020). In contrast, a previous *in vivo* collagen antibody-induced arthritis mouse model reported that LV contractility was impaired (Pironti *et al.*, 2018). Although collagen antibody-induced arthritis shares many characteristics with CIA, there are differences in the onset and severity of inflammation as well as the duration between the two studies. Similarly in another study where rats were exposed to human TNF-α via an osmotic pump, LV EF was reduced and rats developed LV dilation (Bozkurt *et al.*, 1998). In this regard, exogenous TNF-α has previous shown to activate apoptotic pathways in myocytes (Xu *et al.*, 2011). The different methods of inducing inflammation may therefore explain the differences seen in systolic function changes.

Chronic systemic inflammation has previously been related to HFrEF in the general population (Torre-Amione *et al.*, 1996). Higher levels of inflammatory cytokines adversely affect systolic function and heart failure severity (Torre-Amione *et al*., 1996), and independently predict mortality in HFrEF (Deswal *et al.*, 2001). However, in previous cross-sectional RA studies, inflammation was not related to reduced EF (Rudominer *et al.*, 2009; Løgstrup *et al.*, 2014). Indeed, it has previously been suggested that HFpEF occurs largely as a result of LV diastolic irregularities. However, several studies have reported myocardial systolic dysfunction despite a preserved EF in HFpEF (Yip *et al.*, 2002; Yu *et al.*, 2002; Brucks *et al.*, 2005; Fukuta and Little, 2007). Although LV EF is one the most commonly used indices of systolic function, it is dependent on chamber size and pressure conditions and is therefore more an indication of ventricular-arterial coupling rather than contractility (Borlaug *et al.*, 2009). Hence to determine myocardial systolic function beyond EF, several measures including FS and TDI measured s' can be used. These measures determine the extent of fiber shortening during systole (Lang *et al.*, 2015). Indeed, it has been shown that these measures can be impaired while EF is preserved in HFpEF (Yip *et al.*, 2002; Yu *et al.*, 2002; Brucks *et al.*, 2005; Fukuta & Little,

2007). Nevertheless, these measures still have limitation (Kadappu & Thomas, 2015) and we showed no changes in FS in the current study.

Subsequently, two-dimensional STE is now considered to be an even more sensitive index of myocardial systolic performance. STE is a relatively new technique which allows a more precise and thorough assessment of myocardial function in the global LV and in discrete segments of the myocardium (Leitman *et al.*, 2004; Langeland *et al.*, 2005). In more recent RA studies, despite a normal EF impaired myocardial deformation (early systolic function abnormalities using STE) have been reported (Sitia *et al.*, 2012; Fine *et al.*, 2014; Baktir *et al.*, 2015). Moreover, early myocardial deformation has been associated with inflammation (Hamdy *et al.*, 2017; Lo Gullo *et al.*, 2018).

Previous studies have suggested that myocardial systolic function and performance may be adversely affected prior to any changes in systolic chamber function (EF), as the presence of concentric remodelling may aid in maintaining chamber function. It has previously been shown that EF is preserved by hypertrophy of the myocardial wall in HFpEF (Adeniran *et al.*, 2015). Other studies have also suggested that concentric remodelling may mask impaired systolic function as measured by a reduced EF and FS (Aurigemma, Gaasch, *et al.*, 1995; Aurigemma, Silver, *et al.*, 1995; Palmieri *et al.*, 2001). Systolic function was reported to be preserved even in the presence of cardiac remodelling in an RA population (Rudominer *et al.*, 2009).

Nevertheless, in a prior study where EF was reduced in an inflammatory model, the authors reported dysregulation of intracellular calcium signalling pathways that resulted in reduced contractile capacity (Pironti *et al.*, 2018). Indeed, impaired calcium handling and ion channel remodelling affect both systole and diastole in diastolic dysfunction (Kasner *et al.*, 2011). In this regard, action potentials are prolonged, and calcium is elevated in diastole, which limit the availability of calcium in systole and thereby reduces contractile force (Pironti *et al.*, 2018). Although relaxation and contractility are impaired, EF is preserved because of the hypertrophied myocardial wall in HFpEF (Pironti *et al.*, 2018). However, concentric cardiac remodelling increases myocardial tissue stress and strain (Pironti *et al.*, 2018). Hence in the current study concentric remodelling

(increased relative wall thickness) may have ensured the maintenance of ejection fraction. However, it is not known whether myocardial strain was increased which may have reduced systolic performance. Future studies should include more sensitive markers of systolic function to elucidate the role of inflammation on early systolic performance.

5.4 Effect of TNF-α inhibitors on cardiac geometry and function

In the present study, the selective inhibition of TNF-α ameliorated the LV concentric remodelling that was evident in the inflammation group. The RWT was not increased in the TNF-α group compared to the control group and was significantly lower than the RWT of the inflammation group. In a previous rat model where volume overload was induced by a aortocaval fistula implant, demonstrated that myocardial remodelling was inhibited by TNF-α inhibitor treatment (Jobe *et al.*, 2009). Clinical studies have reported conflicting results regarding the effects of TNF-α inhibitors on cardiac geometry. In non RA patients with heart failure, TNF-α inhibitor treatment improved LV geometry (Bozkurt *et al.*, 2001). In RA patients, TNF-α inhibitor treatment also resulted in significant decreases in indices of LV mass (Giles *et al.*, 2010; Kotyla *et al.*, 2012; Daïen *et al.*, 2013). However, these studies should be interpreted with care as one study did not have a control group (Daïen *et al.*, 2013), another reported that LV mass was unexpectedly lower in the RA compared to the controls, with further decreases with TNF-α inhibitor treatment (Giles *et al.*, 2010). In contrast, one study found no changes in cardiac geometry following three months of infliximab treatment (Çetin *et al.*, 2014). However, in the previous study there were no differences in cardiac geometry between RA patients and controls at baseline (Çetin *et al.*, 2014).

Despite the limited cardiac remodelling in the rats receiving TNF-α inhibition, it failed to protect against inflammation-induced diastolic function impairment. The TNF-α group showed impaired relaxation (decreased e’), increased filling pressures (E/e’), increased LV stiffness (increased e’/a’) and an increased E/A. Although an increased E/A ratio may be indicative of improved diastolic function, as previously mentioned, it may also represent a pseudonormal filling pattern in the presence of increased LV filling pressure and left atrial pressure.

Clinical studies in RA and non-RA populations using TNF-α inhibitor treatment for diastolic dysfunction have yielded conflicting results. Some showed improvements in diastolic function following biologic DMARD therapy (Deswal *et al.*, 1999; Bozkurt *et al.*, 2001; Kotyla *et al.*, 2012; Daïen *et al.*, 2013; Çetin *et al.*, 2014) while others reported no changes in diastolic function (Senel *et al.*, 2012). The discrepant results among these clinical studies could be as a result of the large differences in methodological design between these studies. Furthermore, disparities between these studies may also be explained by the differing roles of TNF-α in the pathogenesis of heart failure in the different stages of heart failure (Wolfe and Michaud, 2004). Although TNF-α has immediate negative inotropic properties (Eichenholz *et al.*, 1992), it has also been shown to delay myocardial relaxation by depressing the Ca^{2+} reuptake by SERCA (Eichenholz *et al.*, 1992; Yokoyama *et al.*, 1993). Furthermore, although TNF-α leads to cell apoptosis and necrosis during ischemia, it also activates transcriptional factor (NF-κB) in the nucleus (Gordon *et al.*, 2011), which has a protective effect on surviving myocytes (Baetz *et al.*, 2005). Moreover, in severe heart failure, proinflammatory cytokines activate MMPs and proteases to eliminate the damaged cells and repair the injured cells (Mann, 2002; Hori and Yamaguchi, 2013). This biological response is required for the repair and survival of tissue, and the protective mechanism may be lost with the inhibition of TNF-α. TNF-α inhibitors may therefore induce different effects, depending on the severity and aetiology of the heart failure. In this regard, striking differences in pathophysiology, heart structure, function and cytokine concentrations are commonly noted in RA patients with heart failure compared to non-RA patients with heart failure (Kotyla, 2018). Hence further investigation is required to better understand the pathogenesis of diastolic dysfunction when blocking TNF-α.

Nevertheless, the question arises why TNF-α treatment prevented impairments in cardiac geometry but not cardiac function? Indeed, studies conducted on the efficacy of TNF-α treatment on myocardial remodelling and function in animal models have shown variable results (Bradham *et al.*, 2002; Berry *et al.*, 2004; Moe *et al.*, 2004; Gurantz *et al.*, 2005). Similar to our findings, a previous study using a rat model of volume overload, reported that although TNF-α inhibition prevented myocardial remodelling, LV stiffness

was increased (Jobe *et al.*, 2009). The authors suggest that the increased myocardial stiffness may be as a result of the changes in collagen types and increased cross-linking, irrespective of collagen volume (Jobe *et al.*, 2009). In myocardial infarction models, TNF-α inhibition was shown to decrease ventricular remodelling (Berry *et al.*, 2004; Gurantz *et al.*, 2005) and to preserve cardiac function (Berry *et al.*, 2004). However, in models of heart failure using TNF-α inhibition, some have reported benefits in LV remodelling (Bradham *et al.*, 2002), while others showed no change (Moe *et al.*, 2004). Neither of these previous studies in heart failure models showed any benefit in cardiac function following TNF-α inhibition (Moe *et al.*, 2004; Bradham *et al.*, 2002). Disparities between studies may be explained by differences in intervention timelines, or intrinsic differences in underlying mechanisms which may be model specific. It is also possible that the lack of benefit shown is not unexpected, as TNF-α and cytokine activation are likely not the sole mechanisms that mediate the developed congested heart failure in these models. Alternatively, down-regulation of phospholamban and SERCA have previously been reported in TNF-α inhibition, which may explain the impaired relaxation in the TNF-α group (Feldman *et al.*, 2000). Hence, the TNF-α inhibition effects on diastolic function may have been independent of its effect on ECM remodelling.

Another important factor worth considering, and which was not explored in the present study, is the divergent effects of the different TNF-α receptors (TNFR) on TNF-α mediated cardiac remodelling. The human body contains two types of TNF-α receptors (TNFR), namely the membrane bound TNFR and the soluble TNFR. Membrane bound TNFR are embedded in cells and respond to circulating TNF-α by releasing other cytokines. In contrast, soluble TNFR deactivate TNF-α and blunts the immune response. In this regard, Etanercept (Enbrel) is a soluble TNF-α receptor. Etanercept is dimeric fusion protein, that is made up of an extracellular ligand-binding portion of the TNFR, which is linked to the Fc portion of the IgG 1 (Frenzel *et al.*, 2016). Etanercept mimics the inhibitory effect of the naturally occurring soluble TNFR, by competitively binding to TNF-α, and thereby blocking the interaction of TNF-α with membrane bound receptors (Reddy *et al.*, 2016). Because Etanercept is a fusion protein, it has an extended half-life in circulation and therefore a more profound biologic effect than naturally occurring soluble

TNFR (Madhusudan *et al.*, 2005). Therefore, Enbrel is effective in modulating the inflammatory responses induced by TNF-α.

With regards to the differing effects of TNF-α inhibitors on the ECM remodelling and function, it has previous been shown that circulating TNF-α interact with two types of membrane TNFR, namely TNFR1 and TNFR2 (Chadwick *et al.*, 2008). TNFR1 is widely expressed, whereas TNFR2 expression if typically limited to T-lymphocytes, cardiomyocytes, stem cells and thymocytes (Wajant and Siegmund, 2019). Binding of TNFR1 activates apoptotic pathways resulting in cell death (Hanson, 2016). TNFR2 signalling plays an central role in the regulation of cell proliferation and survival, as well as the activation of regulatory T cells (Yang *et al.*, 2018). TNF-α may exert differing physiological effects, which is largely determined by the type of receptor it activates (Yang *et al.*, 2018). Inhibition of TNF-α in the current study may have removed the cardioprotective mechanism of TNFR2 signalling, leading to an increase in myocyte apoptosis and decreased proliferation, ultimately resulting in LV stiffness and impaired LV relaxation (Lakatta and Levy, 2003; Redfield *et al.*, 2005; Chantler and Lakatta, 2012).

Lastly, it must be acknowledged that besides TNF-α, various additional factors are capable of modulating myocardial remodelling; therefore, TNF-α inhibition may not consistently improve all aspects of myocardial remodelling and function (Aukrust *et al.*, 1999). Although the inhibition of TNF-α had a seemingly protective role, it may also confer a detrimental effect on cardiac function, as TNF-α has a protective role on cardiac function (Kurrelmeyer *et al.*, 2000). Future studies should determine the cellular mechanisms of cardiac remodelling and function that may be affected by TNF-α inhibition. Although TNF-α inhibition did not improve diastolic function, we showed that TNF-α inhibition prevented concentric remodelling in a CIA model. Hence, TNF-α may be a possible therapeutic target to prevent adverse cardiac remodelling in high-grade inflammation in early disease stages.

5.5 Effect of IL-6 receptor blockers on cardiac geometry and function

In the current study Tocilizumab did not have a protective effect on either LV remodelling or cardiac function, as evidenced similar responses in the IL-6 blocker group compared to the inflammation group in all echocardiographic markers. Studies conducted on the effect of IL-6 receptor blockers on cardiac structure and function are very limited. Two previous studies using cardiac magnetic resonance imaging in RA patients found significant improvements in LV mass index, EF, and regional LV function following one year of Tocilizumab therapy (Kobayashi *et al.*, 2014, 2016). However, this has never been shown in pre-clinical models of heart failure or inflammation.

Tocilizumab ameliorates autoimmune inflammation associated with IL-6 overproduction (Liu *et al.*, 2016). Tocilizumab is a genetically engineered monoclonal antibody, which competitively binds to the IL-6 binding site, resulting in IL-6 blockade. Tocilizumab inhibits binding of IL-6 to both the membrane bound IL-6R as well as the soluble IL-6 receptor (Mihara *et al.*, 2011). In this regard, IL-6 plays a primary role in the onset of all inflammatory processes. However, this cytokine's signalling is particularly complex, and its function is intricately related to the other members of the IL-6 cytokine family. Besides IL-6, this cytokine family encompasses interleukin 11, leukemia inhibitory factor , oncostatin M, ciliary neurotrophic factor, cardiotrophin-1, and novel neurotrophin-1/B cell stimulatory factor -3 (Gearing *et al.*, 1987; Malik *et al.*, 1989; Stöckli *et al.*, 1989; Paul *et al.*, 1990; Pennica *et al.*, 1995; Senaldi *et al.*, 1999). Signalling of all IL-6 related cytokines occurs through the ubiquitously expressed transmembrane receptor subunit glycoprotein130 (gp130). Signal specificity is achieved by either homo- or heterodimerisation of gp130 with a ligand-specific α-receptor subunit for the different cytokine members (eg. IL-6 receptor (IL-6R), interleukin 11 receptor, ciliary neurotrophic factor receptor, cardiotrophin-1 receptor, leukemia inhibitory factor receptor (for leukemia inhibitory factor and oncostatin M)) (Kishimoto *et al.*, 1995; Wollert and Drexler, 2001). As gp130 and leukemia inhibitory factor receptor is expressed ubiquitously, the physiological effect of IL-6, or any of the related cytokines is largely dependent on the expression of its corresponding α receptor (Wollert and Drexler, 2001).

Importantly, in the context of inflammation, where membrane bound IL-6R is absent, IL-6 can act on cells through the formation of a complex with the agonistic soluble IL-6R (Peters, 1996). Soluble IL-6R are produced either through alternative mRNA splicing (Muller-newen *et al.*, 1996), or through proteolytic cleavage of membrane bound IL-6R (Müllberg *et al.*, 1995).The alternative signalling pathway through the soluble IL-6R enables IL-6 to act on cells lacking the membrane bound IL-6R, and this feature notably widens the spectrum of cells responsive to IL-6 related cytokines (Tamura *et al.*, 2018). Given the functional pleiotropy and redundancy this family of cytokines is highly varied, yet well regulated.

Hypertrophic responses in cardiomyocytes are dependent on the presence of the soluble IL-6R (Wollert and Drexler, 2001). This suggests that inflammation induced remodelling is largely driven by soluble IL-6R signalling (Tamura *et al.*, 2018). In this regard, membrane bound IL-6 signalling in heart failure appears to be cardioprotective rather than detrimental (Hilfiker-Kleiner *et al.*, 2006). Therefore, it may be possible that IL-6 receptor blocking, may inhibit cardioprotective signalling mechanisms and ultimately cause more damage. Further investigation is required to determine specific IL-6 signalling during inflammation driven cardiac remodelling, as well as during IL-6 receptor blocker treatment.

In addition to the differences noted in membrane bound and soluble IL-6 signalling, alternative signalling mechanisms may also be produced via the IL-6 related cytokines. Leukemia inhibitory factor reportedly downregulated cardiac fibroblast remodelling by inhibiting MMP activation (Wang *et al.*, 2002), but cardiotrophin-1 stimulated collagen synthesis in cardiac fibroblasts in culture (Tsuruda *et al.*, 2002). Therefore, during IL-6 receptor blockade, leukemia inhibitory factor and cardiotrophin-1 may become upregulated, resulting in alterations in cardiac myocyte and fibroblast function (Fredj *et al.*, 2005). Further research on the interactions between the IL-6 related cytokines is required to elucidate the effect of IL-6 receptor blockade on cardiac function.

Alternatively, lack of effectiveness of the IL-6 receptor blocker may be because Tocilizumab is a humanised monoclonal antibody. Hence, this receptor blocker may not

have been able to effectively bind to rat IL-6 receptors. Future rat models should preferably make use of a rodent IL-6 antibody such as the rat anti-mouse IL-6R monoclonal antibody MR16-1 (Katsume *et al.*, 2002; Okazaki *et al.*, 2002).

5.6 Limitations

This study has further limitations. The inclusion of both male and female rats for the given sample size, may have reduced statistical power. The individual housing of the rats may have increased stress, but that the same housing conditions were used for all groups, hence the group differences are unlikely to be affected. Including females in the study may have impacted the results due to different physiological response in males and females. Although we did not determine the oestrus cycle of the female rats, it is unlikely that oestrous affected the inflammatory protocol, as the intervention lasted for 10 weeks. The female rat oestrous cycle lasts only 4-5 days, all females would have undergone several oestrous cycles during the intervention period. Moreover, males and females were housed separately.

Speckle Tracking Echocardiography is considered a more sensitive measure of systolic function, and subtle dysregulations may have been detected using this method. Further studies on the effects of biologic DMARDs on cardiac function should include STE in their measures of systolic function. To study the effect that inflammation has on various cellular processes involved in cardiac remodelling and changes in LV function require mechanistic studies. Future studies should also consider measuring the degree of TNF-α and IL-6 activity within the myocardial compartment, which was not directly measured in this this study. In addition, we did not measure the expression of the soluble IL-6 receptor, or any other members of the IL-6 related cytokine family. Characterisation of the signalling cascade may have been useful, to determine the exact effect of IL-6 receptor blockade on cardiac function. Furthermore, the current study made use of a humanised TNF-α dimer and a humanised anti-IL-6 monoclonal antibody. It is possible that these treatments did not bind effectively to the rat cytokines and the receptors. Future studies should consider using a rat specific TNF-α inhibitor and a rat specific IL-6 receptor blocker.

5.7 Conclusion

The result of the current study aids our understanding of the role of inflammation on the development of diastolic dysfunction, and the role of biologic DMARDs on cardiac function in the treatment of high-grade inflammatory disorders. This study demonstrated that high-grade systemic inflammation induced concentric remodelling and considerable impairments in LV diastolic function, independent of hypertension. Circulating inflammatory cytokines were consistently related to cardiac geometry and concentric remodelling. Although circulating inflammatory markers were related to diastolic function, these relationships were not always consistent. Considering that concentric remodelling is a common precursor for the development of diastolic dysfunction, our results suggest that inflammation may have indirectly induced diastolic dysfunction via changes in cardiac ECM remodelling. Although TNF-α inhibition was successful in preventing concentric remodelling, it did not prevent the inflammation-induced changes in diastolic function. Although TNF-α may be beneficial in preventing cardiac remodelling in patients with high-grade inflammatory diseases, is it possible that inhibiting TNF-α exert may also lose its cardioprotective function thus leading to impaired myocardial relaxation and stiffness. Although TNF-α inhibition did not uniformly improve all aspects of myocardial remodelling and function, it should be considered a therapeutic target to prevent adverse cardiac remodelling in high-grade inflammation. In contrast, IL-6 receptor blockers failed to improve cardiac geometry and cardiac function in rats exposed to high-grade inflammation. However, IL-6 receptor blocker treatment was not detrimental to cardiac function as the IL-6 blocker groups showed similar results to the inflammation group receiving no treatment. Nevertheless, our result does not support IL-6 blockade as a therapeutic target for managing cardiovascular disease risk in conditions of high-grade inflammation.

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Appendices

Appendix A: Animal ethics clearance certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/03/21/C

APPLICANT: Ms L Mokotedi

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Cardiovascular morphology and function in a rat model of rheumatoid arthritis

Number and Species

30 X 3 month old male Sprague-Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/03/28. This approval remains valid until 2019/04/12.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

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 is subject to any additional conditions listed below:

None

Signed: _____ (Chairperson, AESC) Date: 13/04/2017

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: _____ (Registered Veterinarian) Date: 10/4/17

cc: Supervisor: Drs A Millen and F Michel
Director: CAS

Works 2000/lain0015/AESCCert.wps

AESC 2012 M&E

Please note that only type written applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Lebogang Mokotedi

b. Department: School of Physiology

c. Experiment to be modified / extended

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

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

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

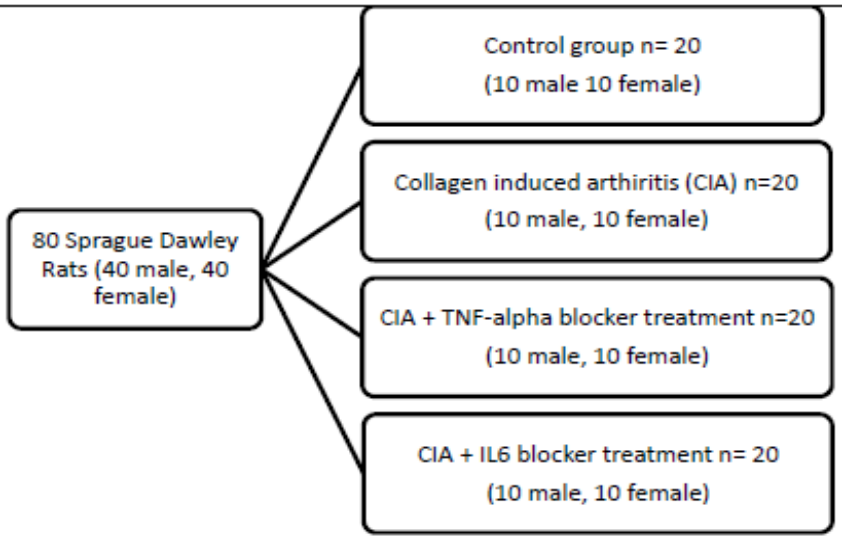
i. Specific modification / extension requested:

- Addition of co-workers

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

- Additional rats required

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



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

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

The addition of co-workers:

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

Additional rats required:

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

Addition of drug treatment regimen to reduce inflammation:

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

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

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

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

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Date:07/05/2018

Signature:

RECOMMENDATIONS
Date: 22/5/2018

Signature:

Chairman, AESC