THE IMPACT OF CARDIOVASCULAR DISEASE RISK FACTORS, INFLAMMATION AND THE VASCULATURE ON DIASTOLIC FUNCTION IN RHEUMATOID ARTHRITIS

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Declaration

I, Lebogang Mokotedi, declare that this thesis is my own, except to the extent indicated in the contribution and acknowledgments sections. It is being submitted for the degree of Doctor of Philosophy in the School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this thesis has not been submitted for any degree or examination in this or any other University.

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Signed on .....day of..... 2019.

Abstract

Cardiovascular disease (CVD) is a major cause of mortality in patients with rheumatoid arthritis (RA). In RA, heart failure contributes to the increased CVD mortality rates and is most often characterised by diastolic dysfunction and a normal ejection fraction. High-grade systemic inflammation and its related adverse traditional cardiovascular risk factors contribute to CVD in RA. However, the relative contribution of traditional risk factors and inflammation to diastolic dysfunction in RA is uncertain. Furthermore, the mechanisms whereby chronic systemic inflammation impairs diastolic function are currently under investigation. This thesis compromise of a series of human and animal studies designed to advance our understanding of the direct and indirect role of systemic inflammation in the development of diastolic dysfunction.

Recently the criteria for the classification of diastolic dysfunction have been revised. In the first study, I compared the estimated prevalence and potential determinants of left ventricular (LV) diastolic dysfunction upon applying different (previous versus current) classification criteria in 176 RA patients who met the American College of Rheumatology/European League against Rheumatism criteria for RA. I found that the waist-to-hip ratio was independently associated with impaired LV relaxation as indexed by the early-to-late diastolic filling velocity ratio (E/A) and the lateral early diastolic mitral annular velocity (e'). Low diastolic blood pressure was independently associated with increased LV filling pressures as indexed by E/e'. The estimated prevalence of diastolic dysfunction was markedly lower upon application of the current criteria (22%) compared to previous (59%) criteria ($p<0.0001$). Moreover, one SD increase in waist-to-hip ratio was associated with diastolic dysfunction when applying the new criteria (OR=2.61 (95% CI=1.51–4.52), p=0.0006), whereas one SD increase in diastolic blood pressure was inversely associated with diastolic dysfunction when using previous criteria (OR=0.57 (95% CI=0.40–0.81), p=0.002). These data therefore suggest that central adiposity and low diastolic blood pressure are independently associated with diastolic function in patients with RA. Further, the application of different criteria markedly alters the prevalence and associated risk factors of diastolic dysfunction in RA.

Although there is an association between chronic inflammation and diastolic dysfunction in cross-sectional studies, there is currently no direct evidence to support the role of high-grade inflammation mediating changes in cardiac function in experimental models of RA. In the second study, I assessed the role of high-grade inflammation on LV diastolic and systolic function in collagen-induced arthritis (CIA) rats (an experimental model most similar to RA). Systolic function was determined using conventional, two-dimensional echocardiography and Speckle Tracking Echocardiography. Compared to controls (n=12), CIA (n=21) rats had impaired LV relaxation as indexed by reduced lateral e' and e'/a' (early-

to-late diastolic mitral annular myocardial velocity) and increased filling pressures as indexed by E/e'. Although there were no differences in ejection fraction and LV endocardial fractional shortening between the groups, LV global radial strain ($p<0.0001$), circumferential strain ($p=0.01$), global strain rate ($p=0.0003$) and circumferential strain rate ($p=0.001$) were reduced in CIA compared to controls. Higher concentrations of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β) and C-reactive protein (CRP) were associated with reduced lateral e', e'/a', LV global radial and circumferential strain and strain rate. This study showed that exposure to high-grade inflammation directly affects LV diastolic function and myocardial deformation without causing contractile changes.

In RA, endothelial dysfunction is central to the development of atherosclerosis. Recently, altered microvascular endothelial changes have been proposed as the link between exposure to inflammation and diastolic dysfunction. In the third study, I determined the effect of high-grade inflammation on vascular function in CIA rats. Vascular function was measured using vascular reactivity in the mesenteric and saphenous arteries and circulating biomarkers of vascular function including vascular adhesion molecules (VCAM-1) and asymmetric dimethylarginine (ADMA) of rats exposed to inflammation ($n=21$) for 10 weeks and controls ($n=12$). Circulating inflammatory markers including TNF-α, IL-6, IL-1β and CRP were elevated in CIA compared to controls (all $p<0.00001$). Acetylcholine-induced relaxation was impaired in mesenteric arteries of CIA compared to controls ($p=0.01$). VCAM-1 was higher in CIA compared to controls ($p=0.02$); ADMA was similar in both groups. TNF-α, IL-6, IL-1β and CRP were significantly related to VCAM-1. VCAM-1 was associated with impaired maximal relaxation (E_{max}) induced by acetylcholine in mesenteric arteries. Circulating ADMA was not associated with inflammatory markers or acetylcholine-induced relaxation in mesenteric arteries. This study showed that circulating VCAM-1 may be a key mediator of inflammation-induced endothelial dysfunction in arthritic rats.

Inflammation impairs vascular function and RA patients experience increased arterial stiffness. In non-RA populations, arterial function is related to diastolic function. Hence, I evaluated the potential impact of impaired arterial function on diastolic function in patients with RA. Arterial function was measured with applanation tonometry in 173 RA patients. The timing of the reflected wave was associated with E/A (partial $r=0.17$, $p=0.03$) and arterial stiffness as indexed by carotid-femoral pulse wave velocity (PWV) was related to increased E/e' (partial $r=0.19$, $p=0.02$). The timing of the reflected wave, forward wave magnitude and pulse pressure amplification were associated with impaired relaxation (E/A <0.8). PWV was related to increased left ventricular filling pressure (E/e' >12). The timing of the reflected wave (OR=0.66 (95% CI=0.47-0.92), $p=0.002$) and PWV (OR=1.86 (95% CI=1.20-2.90, $p=0.006$) were related to diastolic dysfunction according to previous criteria; timing of the forward wave (OR=1.71 (95% CI=1.11-2.66, $p=0.03$) was associated with diastolic

dysfunction according to recent criteria. Taken together, this study showed that early wave reflection and arterial stiffness are associated with LV impaired relaxation and increased filling pressure, respectively, in RA. In addition, different classification criteria set markedly impact the associations of arterial function measures with estimated diastolic dysfunction in RA.

In conclusion, the findings reported in this thesis advance our understanding of the mechanisms whereby CVD risk is increased in conditions characterised by high-grade inflammation. Evidence is provided that traditional risk factors are strongly related to diastolic dysfunction in RA. Additionally, this thesis provides evidence that exposure to systemic inflammation impairs left ventricular diastolic function and causes early alterations in myocardial systolic function, not reflected in changes in myocardial contraction. Furthermore, inflammation impairs vascular function via endothelial activation. Arterial function is independently related to diastolic function in patients with RA. Impaired arterial function may therefore link cardiovascular risk factor exposure to CVD outcomes in patients with RA.

Publications and presentations arising from this thesis

Publications

Mokotedi L, Gunter S, Robinson C, Norton GR, Woodiwiss AJ, Tsang L, Dessein PH, Millen AME (2017). The Impact of Different Classification Criteria Sets on the Estimated Prevalence and Associated Risk Factors of Diastolic Dysfunction in Rheumatoid Arthritis. *Int J Rheumatol* **2017,** 1-13.

Mokotedi L, Michel FS, Mogane C, Gomes M, Woodiwiss AJ, Norton GR, Dessein PH, Millen AME. Inflammation induced changes in left ventricular diastolic and systolic function in collagen-induced arthritis. *Cardiovasc Res* (under review).

Mokotedi L, Millen AME, Mogane C, Gomes M, Woodiwiss AJ, Norton GR, Dessein PH, Michel FS. Vascular Cell Adhesion Molecule-1 Mediates Inflammation Induced Endothelial Dysfunction in Collagen-Induced Arthritis. *Arthritis Rheum* (under review).

Mokotedi L, Gunter S, Robinson C, Michel FS, Solomon A, Norton GR, Woodiwiss AJ, Tsang L, Dessein PH, Millen AME. Early wave reflection and arterial stiffness are associated with diastolic dysfunction in rheumatoid arthritis. *J Cardiovasc Trans* (in press).

Oral presentation

Mokotedi L, Gunter S, Robinson C, Norton GR, Woodiwiss AJ, Tsang L, Dessein PH, Millen AME. The impact of different classification criteria on the prevalence of diastolic dysfunction and the associated risk factors in patients with rheumatoid arthritis. 45th Annual Conference of the Physiological Society of Southern Africa, Pretoria, South Africa, August 2017.

Poster presentations

Mokotedi L, Michel FS, Mogane C, Dessein PH, Millen AME. Impaired left ventricular relaxation and its association with inflammatory markers in collagen-induced arthritis. Faculty of health science Research day, Johannesburg, South Africa, September 2018.

Mokotedi L, Michel FS, Mogane C, Gomes M, Woodiwiss AJ, Norton GR, Dessein PH, Millen AME. Impaired left ventricular relaxation and its association with inflammatory markers in collagen-induced arthritis. European League Against Rheumatism (EULAR), Amsterdam, Netherlands, June 2018.

Millen AME, **Mokotedi L**, Gunter S, Michel F, Robinson C, Woodiwiss A, Tsang L, Norton GR, Dessein P. Aortic stiffness and time to wave reflection are associated with left ventricular diastolic dysfunction measures in rheumatoid arthritis. European League Against Rheumatism Conference (EULAR), Amsterdam, Netherlands, June 2018.

Millen AME, **Mokotedi L**, Gunter S, Robinson C, Norton GR, Woodiwiss AJ, Tsang L, Dessein PH. Independent associations of disease characteristics and cardiovascular risk factors with left ventricular diastolic function in rheumatoid arthritis. European League Against Rheumatism Conference (EULAR), Madrid, Spain, June 2017.

Statement of contribution to the research

As part of the declaration of this thesis, I acknowledge contributions by various individuals as detailed below.

The cross-sectional study was designed in conjunction with Prof Patrick Dessein and Prof Aletta Millen. As part of a team of researchers, including Dr. Sule Gunter, Mrs. Chanel Robinson and Prof Aletta Millen, we performed all echocardiographic and applanation tonometry assessments and Prof Patrick Dessein performed all the clinical assessments. The animal study was designed by myself with the guidance of my supervisors, Prof Aletta Millen and Dr Frederic Michel. All experimental data collection was performed by myself, with assistance from my supervisors, as well as from Mr. Conrad Mogane. I performed all data analysis, interpreted the data and wrote the manuscript for this thesis with the help of my supervisors. All the co-authors on the individual papers contributed to data interpretation, critically revising the manuscripts and provided expert opinions.

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Abbreviations

a’	peak velocity during late (atrial) diastole
A	trans-mitral blood flow velocity in the late (atrial-A) period of left ventricular diastolic filling
ACE	angiotensin converting enzyme inhibitors
ACh	acetylcholine
ACPA	anti-citrullinated peptide antibody
ADMA	asymmetric dimethylarginine
AIx	aortic augmentation index
AP	augmented pressure
ARB	angiotensin receptor blockers
ASE	American Society of Echocardiography
BB	beta blockers
BMI	body mass index
BP	blood pressure
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CCB	calcium channel blockers
CDAI	Clinical Disease Activity Index
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CI	confidence interval
CIA	collagen-induced arthritis
cm	centimetre
cm/s	centimetre per second
CO_2	carbon dioxide
cGMP	cyclic guanosine monophosphate
CVD	cardiovascular disease

cPP	central pulse pressure
cSP	central systolic pressure
DAS28	Disease Activity Score in 28 joints
DBP	diastolic blood pressure
DD	diastolic dysfunction
deg/s	degree per second
DMARD	Disease-modifying antirheumatic drugs
e’	peak velocity during early diastole at the mitral annulus
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’/a’	ratio of early to late mitral annulus flow velocity
ECG	electrocardiogram
E/e’	index of LV filling pressures
EF	ejection fraction
eGFR	estimated glomerular filtration rate
EC_{50}	half-maximal response
EDHF	endothelial derived hyperpolarizing factor
ELISA	enzyme-linked immunosorbent assay
E_{max}	maximal response
ESR	erythrocyte sedimentation rate
FSmid	midwall fractional shortening
Ft	time to the peak of the forward wave
HAQ-DI	Stanford Health Assessment Questionnaire disability index
HDL	high density lipoprotein
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction

HOMA-IR	Homoeostatic Model Assessment of insulin resistance
hs-CRP	high-sensitivity C-reactive protein
IL-1β	interleukin 1beta
IL 6	interleukin 6
IQR	interquartile range
IVST	interventricular septum thickness
KCl	potassium chloride
Kg	kilogram
kg/m^2	kg per $meter^2$
KH_2PO_4	potassium phosphate
LA	left atrial
LAVI	left atrial volume index
LV	left ventricular
LVEDD	left ventricular end diastolic diameter
LVESD	left ventricular end systolic diameter
LVM	left ventricular mass
LVMI	left ventricular mass indexed to body surface area
ml	millilitre
ml/m^2	millilitre per $meter^2$
mm Hg	millimetres of mercury
mmol/l	millimole per litre
MMP	matrix metalloproteinase
Na^+	sodium
NaCl	sodium chloride
$NaHCO_3$	sodium bicarnonate
ng	nanogram
NO	nitric oxide

Pb	backward wave pressure
Pf	forward wave pressure
pg	picogram
PP	pulse pressure
PPamp	pulse pressure amplification
PPc	central aortic pulse pressure
pPP	peripheral pulse pressure
$ONOO^-$	peroxynitrite
OR	Odds ratio
PKG	protein kinase G
PWV	pulse wave velocity
PWT	posterior wall thickness
RA	rheumatoid arthritis
RF	rheumatoid factor
Rm	reflected wave magnitude
RWT	relative wall thickness
r	correlation coefficient
SD	standard deviation
SEM	standard error of the mean
SERCA	sarcoplasmic reticulum ATPase pump
SNP	sodium nitroprusside
STE	speckle tracking echocardiography
TDI	tissue Doppler imaging
TNF-α	tumour necrosis factor alpha
VCAM-1	vascular adhesion molecule 1
WCC	white cell (leukocyte) count
WHR	waist to hip ratio

Preface

Heart failure with a preserved ejection fraction (HFpEF) contributes to approximately 50% of all cases of heart failure. Various comorbidities are associated with the development of HFpEF. Recently, it has been suggested that independent of aetiology, a systemic pro-inflammatory state underlies the mechanisms responsible for diastolic dysfunction, a preclinical condition that frequently precedes HFpEF. This is supported by evidence in patients exposed to chronic high-grade inflammation, such as rheumatoid arthritis (RA), where the prevalence of diastolic dysfunction and HFpEF is significantly greater than in the general population. As there are currently no effective treatment strategies for HFpEF, a better understanding of the pathophysiological mechanisms underlying diastolic dysfunction is needed.

The present thesis was prompted by a need to address some outstanding issues regarding the direct and indirect impact of inflammation on cardiac function. Firstly, it is well known that traditional risk factors such as obesity, hypertension and atherosclerotic cardiovascular disease contribute to the development of diastolic dysfunction. Yet traditional risk factors fail to account for all changes in diastolic function. Furthermore, it is well known that chronic inflammation alters traditional risk factor profiles of patients with RA. Although disease duration has been linked to diastolic dysfunction in RA, the contribution of other established traditional cardiovascular risk factors to diastolic dysfunction, independent of confounding variables warrant further investigation. Hence in the present thesis, I investigated the association of comprehensively assessed traditional risk factors and RA characteristics with diastolic dysfunction in a cohort of RA patients.

Secondly, various inflammatory cytokines have been associated with diastolic dysfunction in cross-sectional studies in RA and non-RA populations. Although some evidence supports a role of high-grade inflammation in the pathogenesis of diastolic dysfunction in RA, the exact inflammatory mechanisms mediating this role are uncertain. Furthermore, the cross-sectional nature of these studies make it hard to discern the contribution of inflammation on altered cardiac function, independent of confounding factors. Hence, in an animal model of collagen-induced arthritis (most closely related to human RA), I evaluated the effect of inflammation on systolic and diastolic function.

Thirdly, in addition to the direct effects of inflammation on myocardial remodelling which influence LV diastolic function, a new paradigm for the development of HFpEF has recently been proposed. In this regard, inflammation-induced microvascular endothelial dysfunction has been suggested to play a central role in the pathophysiology of diastolic dysfunction and HFpEF. Moreover, it is well established that endothelial dysfunction is central to the development of atherosclerosis, a major contributor to increased

cardiovascular mortality in RA. Currently, the direct evidence supporting the role of inflammation on endothelial dysfunction is limited. Therefore, I evaluated the effect of inflammation on endothelial function in an animal model of collagen-induced arthritis. Lastly, bearing in mind that inflammation impairs vascular function, and that altered vascular function has been proposed to affect cardiac function, the association between these variables need elucidation. In this regard, RA patients experience increased arterial stiffness and worse arterial function (wave reflection and pressure pulsatility) compared to the general population. Adverse arterial functional properties have been associated with atherosclerosis in this population. Strong associations have been reported between impaired arterial function and diastolic dysfunction in non-RA populations. Whether this association holds true in RA needs further investigation. Hence I investigated the association between comprehensively assessed arterial functional properties and diastolic function in patients with RA.

The present thesis is written as a series of semi-independent chapters, each with its own introduction, methods, results and discussion (chapters 2 to 5). The thesis begins with a literature review chapter that summarises the current knowledge in the literature and where I will highlight the reasons for conducting the various studies in this thesis. Chapter 6 summarises the main findings of each chapter and accentuates the relevance in context to the medical field. Chapter 7 provides the list of references cited in the thesis and Chapter 8 provides the list of appendices.

Chapter 1: Introduction

1.1. Risk factors for cardiovascular disease

Cardiovascular disease (CVD) is a major global disease burden and is one of the leading causes of death in South Africa (Bradshaw *et al.*, 2012; Benjamin *et al.*, 2018). Although traditional cardiovascular risk factors including aging, obesity, hypertension, diabetes mellitus and dyslipidaemia predict the risk for cardiovascular events, there is still considerable controversy as to whether these traditional risk factors alone fully account for cardiovascular events. While some suggest that traditional risk factors account for most cardiovascular events (Yusuf *et al.*, 2004; O'Donnell *et al.*, 2010), others have shown that traditional risk factors do not account for all cardiovascular events (Harald *et al.*, 2008; Menotti *et al.*, 2009; Goff *et al.*, 2014). The question thus arises, what are the possible non-traditional risk factors that could account for cardiovascular events? One possibility that has received considerable attention in the recent past is chronic systemic inflammation.

Emerging evidence suggests chronic systemic inflammation plays a crucial role in the pathogenesis of CVD (Martinez & White, 2018). Circulating high-sensitivity C-reactive protein (hs-CRP) concentration, an index of chronic systemic inflammation, is associated with an increased prevalence of thin-cap atheromas as well as a greater risk of sudden cardiac death (Burke *et al.*, 2002). Similarly, increased hs-CRP concentration is associated with an increased risk of stroke, myocardial infarction and cardiovascular deaths (Kaptoge *et al.*, 2010). However, not all studies support a role for hs-CRP in CVD (Hunt *et al.*, 2001; Folsom *et al.*, 2001). There is nevertheless evidence to demonstrate that the risk for CVD is increased in chronic inflammatory conditions resulting from high- rather than low-grade inflammation (Sattar *et al.*, 2003). High-grade inflammation is characterised by increased (≥ 10mg/l) CRP concentrations and several-fold (≥4) higher levels of inflammatory cytokines (Sattar *et al*., 2003). Indeed, the mechanisms underlying the increased CVD risk in persons exposed to high-grade inflammation is currently under investigation.

1.2. Cardiovascular diseases in chronic inflammatory disorders

Considerable evidence indicates that CVD is a major cause of morbidity and mortality in chronic inflammatory disorders characterised by high-grade inflammation including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) and chronic human immunodeficiency virus (HIV) infection (Butt *et al*., 2011; Nicola *et al.,* 2005; Schoenfeld *et al*., 2013). Patients with chronic inflammatory disorders have a heightened risk of developing myocardial infarction and congestive heart failure. In SLE patients, myocardial infarction is a major cause of premature mortality and has been reported to be 3-times higher in SLE patients in comparison to controls (Zeller & Appenzeller, 2008). In addition, the incidence of heart failure is 50% greater in SLE

patients compared to the general population (van der Laan-Baalbergen *et al*., 2009). Furthermore, HIV patients have a 2-fold increased risk of myocardial infarction and heart failure (Freiberg *et al*., 2013) which are associated with a 1.5 to 2 times greater risk of cardiovascular mortality (Butt *et al*., 2011; Freiberg *et al*., 2013). The underlying mechanisms causing the increased CVD risk in these conditions are not fully understood but a heightened immune response seems to play a key role (Varma *et al.*, 2012). In addition, chronic inflammatory disorders share common genetic polymorphisms relating to major histocompatibility complex molecule expression (Swanberg *et al*., 2005). Whether these shared genetic factors contribute to the increased prevalence of CVD in chronic inflammatory disorders is a thought-provoking possibility which merits further investigation.

Although a number of chronic inflammatory disorders contribute substantially to the CVD burden, the focus of this thesis is to determine the impact of CVD risk factors, inflammation and the vasculature on diastolic function in RA. Therefore, in the subsequent sections I will focus my discussion on RA. In this regards, the section below highlight what is currently know about CVD in RA.

1.3. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease affecting approximately 1% of the population worldwide (McInnes & Schett, 2011). Along with the synovial-based inflammatory events, RA is a disease of systemic inflammation which results in a number of extra-articular manifestations (McInnes & Schett, 2011). The presence of extra-articular manifestations in patients with RA is associated with severe disease activity and increased mortality (Cojocaru *et al.*, 2010). Persons with RA demonstrate increases of up to 50% in mortality rates (Aviña-Zubieta *et al.*, 2008) and cardiovascular events occur a decade earlier in RA compared to the general population (Bacon *et al.*, 2002; Avina-Zubieta *et al.*, 2012).

Both traditional cardiovascular risk factors and RA disease characteristics contribute to the increased cardiovascular mortality in patients with RA. In this regard hypertension (Naranjo *et al.*, 2008; Innala *et al.*, 2011), obesity (Kremers *et al.*, 2008; Stavropoulos-Kalinoglou *et al.*, 2009), a history of smoking, diabetes mellitus and physical inactivity (Maradit-Kremers *et al.*, 2005) contribute to cardiovascular events and mortality in RA. Although traditional risk factors are consistently associated with CVD in RA, these risk factors fail to account for all adverse cardiovascular outcomes in patients with RA (del Rincón *et al.*, 2001). Furthermore, even after controlling for traditional risk factors, RA is considered a risk factor for premature ischaemic heart disease (Naranjo *et al.*, 2008; Aviña-Zubieta *et al.*, 2008).

High-grade inflammation is a key driver of the increased cardiovascular risk in persons with RA (Sattar *et al.*, 2003; Gonzalez *et al.*, 2008; Rodríguez-Rodríguez *et al.*, 2011). Systemic inflammatory markers including erythrocyte sedimentation rate (ESR) and CRP have been associated with cardiovascular death in patients with RA, independent of traditional cardiovascular risk factors (Maradit-Kremers *et al.*, 2005). Further, high levels 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. Given this observation, it has been suggested that RA disease-related inflammation might contribute to accelerated atherosclerosis (Dessein *et al.*, 2005; Gonzalez-Gay *et al.*, 2005). Although ischaemic heart disease and atherosclerosis contribute largely to the increased CVD mortality, persons with RA also have twice the risk of developing heart failure, which accounts for about 13% of mortality in RA (Nicola *et al.*, 2005).

1.4. Heart failure in rheumatoid arthritis

Heart failure is a complex syndrome that can occur with either reduced or preserved ejection fraction (Savarese & Lund, 2017). Until recently, heart failure with a reduced ejection fraction (HFrEF), (ejection fraction <50%) which is associated with impaired systolic chamber function (Borlaug & Redfield, 2011), was recognised as the most common form of heart failure. Currently, more than 50% of all patients with heart failure have heart failure with a preserved ejection fraction (HFpEF; ejection fraction >50%) (Gottdiener *et al.*, 2002; Owan *et al.*, 2006). HFpEF is underscored by inadequate left ventricular (LV) filling due to impaired myocardial relaxation (Gladden *et al.*, 2014).

Compared to the general population, persons with RA experience a 2-fold increased risk of developing heart failure (Nicola *et al.*, 2005). Although chronic inflammation is associated with HFrEF in the general population (Torre-Amione *et al.*, 1996; Deswal *et al.*, 2001), no association has been shown between inflammation and reduced ejection fraction in patients with RA (Rudominer *et al.*, 2009; Løgstrup *et al.*, 2014). Indeed RA patients more frequently present with HFpEF compared to non-RA patients with heart failure (Davis *et al.*, 2008).

To date, the pathophysiology of HFpEF is poorly understood. The reported prevalence of HFpEF has substantially increased over the last two decades, largely as a result of an ageing population and the increasing prevalence of risk factors, such as hypertension and diabetes mellitus (Owan *et al.*, 2006). Treatment strategies have been extensively proven to reduce the risk of death and hospitalisation in patients with HFrEF (Borlaug & Redfield, 2011). However, these treatment strategies fail to benefit patients with HFpEF (Borlaug & Redfield, 2011; Pitt *et al.*, 2014). Considering the rising prevalence of HFpEF, the poor outcomes of treatment

strategies in patients with HFpEF and the increased risk of HFpEF in patients exposed to chronic inflammation, there is a critical need for a better understanding of the pathophysiology of HFpEF.

1.5. Diastolic dysfunction as a preclinical measure of heart failure with a preserved ejection fraction

The fundamental pathophysiological abnormality that explains the development of HFpEF is a decreased LV diastolic function (Borlaug & Paulus, 2011). The impaired relaxation during diastole leads to increased filling pressures in the ventricle which ultimately presents as the typical clinical pathological features of heart failure. LV diastolic dysfunction represents a preclinical cardiac alteration which is highly predictive of cardiac events and often progresses to HFpEF (Halley *et al.*, 2011).

In patients with RA, heart failure is more frequently associated with impaired LV diastolic function compared to the general population (Davis *et al.*, 2008; Aslam *et al.*, 2013). The overall prevalence of diastolic dysfunction ranges from 31% to 55% in persons with RA (Liang *et al.*, 2010; Abdul Muizz *et al.*, 2011; Davis *et al.*, 2011; Renjith *et al.*, 2017). Given the high prevalence of diastolic dysfunction and HFpEF in patients with RA, early identification of risk factors associated with LV diastolic dysfunction are important to possibly prevent the onset of HFpEF. Considering that conventional CVD risk stratification underestimates the CVD risk in RA (Dessein & Semb, 2013), the factors associated with the development of diastolic abnormalities warrant further investigation.

Before describing the factors associated with diastolic dysfunction in patients with RA, the section below briefly explains the pathophysiology of diastolic dysfunction and highlights the various echocardiographic techniques for assessing diastolic function.

1.5.1. Understanding the pathophysiology of diastolic dysfunction

Diastole is the period of the cardiac cycle during which the myocardium relaxes, allowing the ventricles to fill with blood (Kapila & Mahajan, 2009). The process of diastole is determined by the ability of the ventricle to relax effectively (Kapila & Mahajan, 2009). Diastolic dysfunction occurs when relaxation of the LV is prolonged, slowed, or incomplete which leads to impaired LV relaxation resulting in increased LV filling pressures (Biesiadecki *et al.*, 2014). Various cellular processes may contribute to diastolic dysfunction, alterations which influence either active or passive processes (Lewis *et al.*, 2017).

LV relaxation is an active process which requires adenosine triphosphate (ATP) for the detachment of the actin-myosin cross-bridges and calcium (Ca^{2+}) homeostasis in

cardiomyocytes (Biesiadecki *et al.*, 2014). Under normal physiological conditions, Ca^{2+} removal from the cytosol is regulated by sarcoplasmic reticulum ATPase (SERCA) pump and the sodium-calcium exchanger (Biesiadecki *et al.*, 2014). Removal of Ca^{2+} from the cytosol decreases the intracellular Ca^{2+} concentration which results in detachment of Ca^{2+} from troponin C allowing the cardiomyocytes to relax (Biesiadecki *et al.*, 2014). Abnormalities in the ion channels responsible for Ca^{2+} reuptake and decreased availability of energy for these processes impairs the ability of the sarcoplasmic reticulum to sequester Ca^{2+}, which increases diastolic tension, resulting in abnormal cardiomyocyte relaxation (Biesiadecki *et al.*, 2014; Lewis *et al.*, 2017).

Ventricular filling during diastole is also influenced by chamber stiffness, which represents the passive elastic capacity of the ventricle. The extracellular matrix and cardiomyocytes are the two main compartments that regulate myocardial stiffness (Zile & Brutsaert, 2002). The extracellular matrix is largely composed of collagen which is important for structural integrity of the heart (Borg & Caulfield, 1981; Weber, 1989). Collagen synthesis is influenced by load, neurohumoral activation and growth factors and is degraded by matrix metalloproteases (MMPs) (Nagatomo *et al.*, 2000; Spinale *et al.*, 2000). Because collagen is a relatively stiff material, small changes in the synthesis and degradation of collagen lead to alterations in the passive mechanical properties of the heart. Increased collagen synthesis leads to increased myocardial stiffness which impairs effective relaxation and hence leads to diastolic dysfunction (Zile & Brutsaert, 2002). In addition to changes in the extracellular matrix, intrinsic cardiomyocyte stiffness also contributes to myocardial passive stiffness. Titin is a cytoskeletal elastic protein which is a major component of the intrinsic cardiomyocyte, responsible for myocardial stiffness (Kass *et al.*, 2004; Borbély *et al.*, 2009). Titin functions like a viscoelastic spring which is responsible for cardiomyocyte diastolic recoil during early diastole and distensibility in late diastole (Zile & Brutsaert, 2002). Titin has two isoforms namely, N2B (more rigid form) and N2BA (more compliant). Changes in titin isotypes affects myocardial stiffness and contributes to diastolic dysfunction (Kass *et al.*, 2004; Borbély *et al.*, 2009; van Heerebeek *et al.*, 2012). Impaired LV relaxation and increased LV myocardial stiffness have both been related to increased LV filling pressure (Kasner *et al.*, 2007, 2011; Selby *et al.*, 2011), the main pathophysiological consequence of diastolic dysfunction (Cohen-Solal, 1998; Zile & Brutsaert, 2002). A number of techniques have been developed to assess LV relaxation, stiffness and filling pressures as indices of diastolic function.

1.5.2. Echocardiographic assessment of diastolic function

Initially, the identification of diastolic dysfunction was based on cardiac catheterization which allows for the assessment of LV filling pressures, relaxation, and stiffness (Maurer *et al.*,

2006). However, this diagnostic method is invasive and is not feasible for routine clinical practice. During the last two decades high-resolution ultrasound imaging of the heart (echocardiography), a non-invasive tool has gained a central role in the assessment of LV function. A number of echocardiographic measures have improved the ability to diagnose patients at risk for cardiovascular events (Swedberg *et al.*, 2005; Hunt *et al.*, 2005; Ciampi & Villari, 2007; Capotosto *et al.*, 2018). Using echocardiography, measures of geometry, systolic function and diastolic function can be obtained. Until relatively recently, reduced LV ejection fraction (a measure of systolic chamber function) was believed to indicate the onset of cardiovascular events (Borlaug & Paulus, 2011). It is not disputed that a reduced ejection fraction is an essential predictor of the development of heart failure (Ho *et al.*, 2016; Bloom *et al.*, 2017). However, a number of additional measures of cardiac function, including diastolic function, predict cardiovascular outcomes independent of traditional risk factors (Tsang *et al.*, 2002*b*; Kane *et al.*, 2011; Han *et al.*, 2015; Park *et al.*, 2017). Diastolic dysfunction is frequently noted well before systolic chamber function is impaired (de Simone *et al.*, 2000). Although an in-depth discussion of echocardiography measures are beyond the scope of this thesis, I will briefly highlight the better-established measures of diastolic dysfunction obtained with echocardiography in the section below. Although the main focus of the current thesis is on diastolic function, a brief discussion of the use of echocardiographically determined systolic function is presented in section 1.8.

Echocardiography enables imaging of the velocity of blood flow across the mitral valve using pulse wave Doppler and tissue lengthening at the mitral annulus using Tissue Doppler Imaging (TDI) (Thomas *et al.*, 1997; Redfield *et al.*, 2003; Nagueh *et al.*, 2016). Mitral inflow velocity is measured during early (E) and late (atrial) (A) period of diastole (Nishimura & Tajik, 1997; Mottram & Marwick, 2005; Mitter *et al.*, 2017). Similarly, tissue lengthening at the mitral annulus is measured during early (e') and late (a') diastole (Nishimura & Tajik, 1997; Mottram & Marwick, 2005; Mitter *et al.*, 2017). Because passive filling contributes approximately 70% of the total diastolic filling in a ventricle with normal function, mitral inflow velocity and myocardial tissue lengthening is greater during the early (E or e') compared to the late (A or a') diastolic period (Nishimura & Tajik, 1997; Mottram & Marwick, 2005; Mitter *et al.*, 2017). When LV function is compromised during diastole a number of echocardiographic changes could be observed. Firstly, impaired LV relaxation results in reduced driving force to fill the ventricle (Nishimura & Tajik, 1997). In this instance, transmitral blood flow in the early period (E) will be reduced which will be compensated by increased A as the ventricle relies more on atrial contraction to adequately fill the ventricle (Nishimura & Tajik, 1997; Mottram & Marwick, 2005; Mitter *et al.*, 2017). Alternatively when left atrial pressure or filling pressures in the ventricle is

increased a pseudonormal or restrictive filling pressure may be observed, respectively (Nishimura & Tajik, 1997; Mottram & Marwick, 2005; Mitter *et al.*, 2017). With increased left atrial pressures the driving forces for early filling are increased which will present as a normal or increased E. The increased early filling in a non-distensible ventricle causes a higher transmitral pressure gradient resulting in E being much higher than A (Nishimura & Tajik, 1997). Because a pseudonormal and restrictive filling pattern both appear normal, further echocardiographic measures need to be considered.

In this regard e' is a more sensitive indicator of LV relaxation than transmitral mitral flow variables (Sohn *et al.*, 1997), as it is less pre-load and heart rate dependent. A decreased e' is an earlymarker of diastolic dysfunction (Sohn *et al.*, 1997). Since E is reliant on ventricular relaxation and left atrial driving forces, whereas e' is reliant on relaxation alone, E/e' is considered to be an index of LV filling pressures (Sharifov *et al.*, 2016). In patients with impaired LV relaxation, because e' is reduced or stays unchanged, while E increases due to higher filling pressures, the E/e' is increased (Ommen *et al.*, 2000). Indeed LV diastolic dysfunction as assessed by both the E/A and E/e' is associated with the development of heart failure and predict adverse cardiovascular outcomes (Aurigemma *et al.*, 2001; Redfield *et al.*, 2003). Nevertheless, the criteria and thresholds that define the classification of diastolic dysfunction are controversial.

1.5.3. Classification criteria for diastolic dysfunction

Previous guidelines from the American Society of Echocardiography recommended grading diastolic dysfunction into three main categories namely: mild (impaired relaxation pattern), moderate (pseudonormal pattern) and severe (restrictive filling pattern) diastolic dysfunction (Redfield *et al.*, 2003) assessed from a combination of E/A and E/e'. Various methodological criticism of using the E/A ratio in classifying diastolic dysfunction in patients with an ejection fraction >50% have resulted in alternative criteria being proposed (Nagueh *et al.*, 2009, 2016). In the updated criteria from the European Association of Cardiovascular Imaging and the American Society of Echocardiography, the E/A ratio is no longer considered in the diagnosis of diastolic dysfunction in patients with an ejection fraction of ≥ 50% (Nagueh *et al.*, 2016). Furthermore, instead of an E/e' threshold of 10 as previously suggested (Redfield *et al.*, 2003), the current criteria considers diastolic dysfunction only when E/e' > 14. Additionally, the updated criteria also consider the lateral e', the septal e' and left atrial volume indexed to body surface area in the diagnosis of diastolic dysfunction. In this regard, left atrial volume index correlates closely to prolonged increases in filling pressure (Tsang *et al.*, 2002*a*) and is related to the severity and duration of diastolic dysfunction (Teo *et al.*, 2010). Left atrial volume index is

an independent predictor of cardiovascular events (Tsang *et al.*, 2002*a*; Barnes *et al.*, 2004; Takemoto *et al.*, 2005) and has been associated with poor cardiovascular outcomes in patients with HFpEF (Tsang *et al.*, 2002*a*; Zile *et al.*, 2011).

When applying different criteria sets there is poor agreement on the proportions of patients classified as having diastolic dysfunction (Rasmussen-Torvik *et al.*, 2017). With the application of the new criteria, a very large proportion of patients are considered to have "indeterminate diastolic function". This may result in a number of patients being undiagnosed and not adequately treated to prevent heart failure. Furthermore, the risk factors associated with diastolic dysfunction differ depending on the classification criteria used (Rasmussen-Torvik *et al.*, 2017). Clearly, the definition of diastolic dysfunction and the algorithms used to classify patients have a major impact on the interpretation of research results. Further research is needed to define the optimal classification criteria for diastolic dysfunction.

Nevertheless, as previously mentioned, a number of studies have demonstrated an increased prevalence of diastolic dysfunction in patients with RA (Aslam *et al.*, 2013). Majority of these studies used previously reported criteria (Redfield *et al.*, 2003) or based their classification of diastolic dysfunction on E/A alone. Classification of diastolic dysfunction using the new criteria has not been applied in RA patients. *Therefore I aimed to determine the estimated prevalence of diastolic dysfunction in RA patients upon applying different classification criteria sets.*

1.6. Factors associated with diastolic dysfunction in rheumatoid arthritis

It is well known that traditional CVD risk factors including older age, obesity, smoking, diabetes mellitus, hypercholesterolemia and hypertension contribute to the development of diastolic dysfunction in the general population (Masoudi *et al.*, 2003; Bhatia *et al.*, 2006; Owan *et al.*, 2006; Lee *et al.*, 2009; Borlaug & Paulus, 2011). Similar to other subclinical markers of CVD, neither traditional risk factors nor coronary artery disease account for all changes in diastolic function (Crowson *et al.*, 2005). However, it is well known that chronic inflammation alters the traditional cardiovascular disease risk profile in RA patients (Chung *et al.*, 2008; Libby, 2008; Myasoedova *et al.*, 2011; Innala *et al.*, 2011; Choy *et al.*, 2014). Chronic inflammation is also strongly related to atherosclerosis in RA (Skeoch & Bruce, 2015). Thus, it may be that the systemic inflammation may in part explain the changes in diastolic function in persons with RA. Considering that diastolic dysfunction plays a key role in the development of heart failure in RA patients and risk stratification in persons with RA is essential for CVD management (Dessein & Semb, 2013), the factors associated with the development of diastolic abnormalities warrants investigation. In the section below I will briefly highlight what is known about the risk factors that are associated with diastolic dysfunction in RA.

Only a limited number of studies have investigated the association between diastolic dysfunction and demographical and clinical characteristics in RA patients. A significant relationship between diastolic function / dysfunction and RA disease duration has been consistently shown (Di Franco *et al.*, 2000; Arslan *et al.*, 2006; Rexhepaj *et al.*, 2006; Canturk *et al.*, 2006; Udayakumar *et al.*, 2007; Wislowska *et al.*, 2008; Liang *et al.*, 2010; Garza-García *et al.*, 2013; Tomáš *et al.*, 2013; Sakthiswary & Das, 2015; Sharma *et al.*, 2015) even after adjustment for traditional CVD risk factors (Liang *et al.*, 2010). However, none of the aforementioned studies reported a relationship between traditional risk factors and diastolic function, except for associations between age and blood pressure with diastolic function (Montecucco *et al.*, 1999; Liang *et al.*, 2010). Other RA disease risk markers which have been associated with diastolic function and/or dysfunction include inflammatory markers such as interleukin 6 (IL-6) levels (Liang *et al.*, 2010), tumour necrosis factor-α (TNF-α) (Tomáš *et al.*, 2013) and markers of disease severity (Gonzalez-Juanatey *et al.*, 2004; Garza-García *et al.*, 2013; Sharma *et al.*, 2015).

However, these studies have some limitations. Majority of the aforementioned studies have a limited demographic to which the results can be applied and have small sample sizes (n<100) (Di Franco *et al.*, 2000; Arslan *et al.*, 2006; Rexhepaj *et al.*, 2006; Canturk *et al.*, 2006; Udayakumar *et al.*, 2007; Tomáš *et al.*, 2013). Furthermore, only one study adjusted for confounding variables (Liang *et al.*, 2010). Lastly the majority of these studies reported only on a reduced E/A ratio as a marker of diastolic dysfunction (Montecucco *et al.*, 1999; Di Franco *et al.*, 2000; Rexhepaj *et al.*, 2006; Canturk *et al.*, 2006; Udayakumar *et al.*, 2007; Tomáš *et al.*, 2013). Hence the factors associated with diastolic dysfunction and specifically tissue Doppler indices of diastolic function in RA warrant further investigation. Therefore to address the aforementioned concerns, in the present thesis, *I evaluated the association between comprehensively assessed traditional risk factors and RA disease characteristics and LV diastolic function parameters using pulsed Doppler and Tissue Doppler Indices of diastolic function.*

1.7. The role of inflammation in diastolic dysfunction

Given the existing evidence that inflammatory markers are associated with diastolic dysfunction in cross-sectional studies (Masiha *et al.*, 2013; Akin *et al.*, 2014), the question arises, what are the mechanisms explaining the associations between RA or associated inflammatory changes and measures of diastolic function? The section below briefly highlights the mechanisms whereby inflammation may mediate diastolic dysfunction.

1.7.1. Direct role of inflammation on altered diastolic function

Evidence suggests that inflammation affect various cellular processes which may alter both active and passive processes related to diastolic dysfunction (as outlined in section 1.4.1). The mechanisms whereby inflammation impairs active processes (relaxation) centers around myocardial intracellular Ca^{2+} homeostasis. Inflammatory cytokines such as TNF-α and interleukin 1β (IL-1β) downregulate the expression of the SERCA gene and protein expressions which result in a decrease Ca^{2+} re-uptake into the sarcoplasmic reticulum during diastole (Yokoyama *et al.*, 1997; McTiernan *et al.*, 1997; Lee *et al.*, 2007; Wu *et al.*, 2011). The resultant alterations in intracellular Ca^{2+} homeostasis impair myocardial relaxation. Inflammatory cytokines also directly inhibit the gene expression of the Ca^{2+} handling protein phospholamban in cultured cardiomyocytes (McTiernan *et al.*, 1997; Combes *et al.*, 2002). Changes in the expression levels and phosphorylation state of phospholamban directly influences the activity of SERCA (Tada, 1992; Simmerman & Jones, 1998). Decreased levels of phospholamban results in decreased SERCA activity and sarcoplasmic reticulum Ca^{2+} uptake (McTiernan *et al.*, 1997; Combes *et al.*, 2002) leading to increased diastolic tension and hence abnormal relaxation of the ventricle (Biesiadecki *et al.*, 2014; Lewis *et al.*, 2017).

Besides the impaired active relaxation, evidence also points to inflammation-induced structural remodelling of the extracellular matrix and cardiomyocyte. In this regard, inflammatory cytokines such as TNF-α and interleukin 6 (IL-6) promote cardiomyocyte hypertrophy (Yokoyama *et al.*, 1997; Meléndez *et al.*, 2010). Previous studies have shown that inflammatory cytokines activate fibroblasts to secrete transforming growth factor-β. The secretion of transforming growth factor-β increases collagen synthesis which results in fibrosis of the myocardium that impairs distensibility of the ventricle (Westermann *et al.*, 2011). Besides the increased collagen volume, inflammation also increases the expression of lysyl oxidase (Voloshenyuk *et al.*, 2011), a marker of collagen cross-linking (Marturano *et al.*, 2014) which further increases passive stiffness of the ventricle (Voloshenyuk *et al.*, 2011). *In vitro* studies have also shown that inflammation decreases the synthesis of MMPs which are responsible for degradation of collagen and thus increases myocardial stiffness (Liacini *et al.*, 2003; Stearns *et al.*, 2004; Kawamura *et al.*, 2005). IL-6 enhances cardiomyocyte stiffness by altering the phosphorylation of titin which limits the distensibility of the myocardium (Savvatis *et al.*, 2014).

Clearly, the presence of inflammatory cytokines alters the cellular processes involved in myocardial remodelling, active relaxation and passive stiffness. The resultant effects are increased myocardial stiffness and restricted LV filling which are well-known mechanisms that contribute to diastolic dysfunction. This suggests that in RA, chronic inflammation may promote

abnormalities in diastolic function. However, currently, there is no direct evidence to support the role of chronic exposure to inflammation in the development of diastolic dysfunction in an experimental model of RA, where the concentrations of inflammatory cytokines may be considerably modified. Hence, in this thesis, *I aimed to determine in a preclinical model of high-grade inflammation whether diastolic function is altered and whether there is a relationship between circulating inflammatory markers and LV diastolic function*.

1.7.2. Indirect role of inflammation on altered diastolic function

In addition to the direct contribution of inflammation to myocardial changes which influence LV diastolic function, indirect mechanisms have also been suggested. Paulus and Tschöpe (2013) recently postulated a new paradigm for HFpEF. They suggested that exposure to metabolic risk factors induce a systemic inflammatory state that ultimately drives the development of diastolic dysfunction.

In this regard, a systemic inflammatory state results in increased plasma levels of cytokines such as IL-6, TNF-α and Pentraxin 3 (Paulus & Tschöpe, 2013). Increased levels of these cytokines damage the endothelium which upregulates the expression of soluble intercellular adhesion molecules including vascular adhesion molecule-1 (VCAM-1) and E-selectin (Trepels *et al.*, 2006; Glezeva & Baugh, 2014). Endothelial inflammation increases the production of reactive oxygen species (ROS) which translates to low nitric oxide (NO) bioavailability and peroxynitrite ($ONOO^-$) production, thus reducing cyclic guanosine monophosphate (cGMP) production (Schulz *et al.*, 2008) and lowering protein kinase G (PKG) activity within the myocardium (van Heerebeek *et al.*, 2012). These altered signaling pathways and PKG activity are associated with increased diastolic stiffness and dysfunction through downstream hypophosphorylation of the titin protein (Paulus & Tschöpe, 2013). Indeed, patients with HFpEF have a myocardium with a higher resting tension compared to healthy controls which may be attributed to the hypophosphorylation of titin (Borbély *et al.*, 2009; van Heerebeek *et al.*, 2012). In a model of type II diabetes, the altered passive stiffness driven by inflammation and microvascular endothelial dysfunction have recently been reported (Franssen *et al.*, 2016). However, this model of type II diabetes is confounded by the glycosylating effects of raised plasma glucose concentrations. Hence the role of inflammation on endothelial function and the circulating biomarkers that represent endothelial dysfunction, independent of confounders needs investigation. Therefore, in the present thesis, *I aimed to determine the effect of high-grade inflammation on endothelial function and whether circulating biomarkers of endothelial dysfunction mediate these changes.*

1.8. The role of altered aortic function in diastolic dysfunction

As discussed previously (section 1.6.2), traditional CVD risk factors and comorbid conditions induce a state of systemic inflammation that may cause structural and functional changes in the endothelium and vascular wall of microvessels (Paulus & Tschöpe, 2013). Although the effects of inflammation in microvessels are suggested to be implicated in diastolic dysfunction (Paulus & Tschöpe, 2013), some studies have suggested that longer-term exposure to inflammation may not only affect microvessels but also larger arteries such as the aorta (Wållberg-Jonsson *et al.*, 2008). Indeed structural and functional changes in the vasculature result in decreased vascular distensibility and increase arterial stiffening (Dart & Kingwell, 2001; O'Rourke *et al.*, 2002; Mitchell *et al.*, 2004; Barodka *et al.*, 2011; Cecelja & Chowienczyk, 2012; Chirinos *et al.*, 2012*a*; Townsend *et al.*, 2015). A recent review article confirms that aortic function is impaired in persons with RA (Ambrosino *et al.*, 2015).

Although several methods have been employed to measure aortic stiffness, carotid-femoral pulse wave velocity (PWV) is considered the most accurate index of aortic stiffness and is an independent predictor of cardiovascular events (Laurent *et al.*, 2006; Townsend *et al.*, 2015). Increased arterial stiffness increases the impedance to aortic flow and hence enhances pulse pressure and LV afterload, an effect that is well-recognised as producing LV hypertrophy and several changes which influence both active and passive filling of the LV (Mottram *et al.*, 2005). Further, a stiff aorta, by decreasing the capacity to accommodate blood during systole, is unable to contribute to a normal flow during diastole which results in reduced aortic diastolic pressure and hence a decrease in coronary flow (which mainly occurs during diastole) (Dart & Kingwell, 2001; Nair *et al.*, 2005; Townsend *et al.*, 2015). Indeed, a number of studies have shown an association between markers of arterial stiffness with LV diastolic function in the general population (Abhayaratna et al., 2008; Cauwenberghs et al., 2016; Kim et al., 2017; Peterson et al., 2016), in clinical populations (Jaroch *et al.*, 2012; Roos *et al.*, 2013; Solovjova *et al.*, 2016) and in patients with cardiovascular disease (Kocabay *et al.*, 2014; Namba *et al.*, 2016).

In addition to aortic stiffness measurements, aortic wave reflection has been considered an important determinant of LV diastolic dysfunction. The aortic pressure waveform consists of the sum of the forward traveling wave generated by ventricular contraction and the reflected traveling wave generated by the reflection of the forward wave from downstream segments of the arterial tree (Nichols & Edwards, 2001; Mitchell *et al.*, 2010; Barodka *et al.*, 2011). Changes in the vessel composition and diameter of the peripheral vasculature at the major sites of reflection influence the reflected wave (Nichols & Edwards, 2001). Increased arterial stiffness

causes the reflected wave to travel more rapidly along the arterial tree (Nichols & Edwards, 2001). In this regard, the pressure gradient generated by the early return of the reflected wave adds to the pressure gradient of the forward wave and hence augments systolic aortic pressure and reduces diastolic pressure which results in decreased coronary flow (Chirinos *et al.*, 2012*a*). Augmentation index (Alx) is conventionally used to measure aortic wave reflection. However, studies suggest that AIx is an unreliable marker of wave reflection (Hughes *et al.*, 2013) as it underestimates the contribution of the reflected wave (Booysen *et al.*, 2015). Hence in order to account for the precise outcome of the reflected wave, wave separation analysis is required (Wang *et al.*, 2010; Chirinos *et al.*, 2012*a*). Indeed, measurement of the reflected wave using wave separation analysis is a better marker of wave reflection than Alx (Wang *et al.*, 2010). In this regard, only two studies have shown that the reflected wave using wave separation analysis contributes substantially to impaired diastolic function in the general population (Kaess et al., 2016; Peterson et al., 2016).

We have recently shown a consistent association between RA disease characteristics and arterial stiffness and wave reflection measures (Gunter *et al.*, 2017). Hence it is possible that associations between RA or the related inflammatory changes and LV diastolic function can be explained by arterial stiffness and aortic wave reflection. A recent study showed an association between aortic stiffness with diastolic dysfunction in a small group (n=50) of patients with Sjogren's syndrome (Çiçek *et al.*, 2014). However, the relationship between comprehensively assessed arterial function variables and diastolic function has not been investigated in RA. Hence in the present thesis, *I aimed to determine the relationship between comprehensively assessed arterial functional variables and diastolic function variables independent of traditional risk factors.*

1.9. Preclinical systolic dysfunction in rheumatoid arthritis patients with heart failure with a preserved ejection fraction

It has been previously believed that HFpEF is mainly due to diastolic abnormalities (as discussed in section 1.4). However, a number of recent studies reported preclinical myocardial systolic dysfunction despite preserved LV ejection fraction in patients with HFpEF (Yip *et al.*, 2002; Yu *et al.*, 2002; Brucks *et al.*, 2005; Fukuta & Little, 2007). LV ejection fraction is the most commonly used index of systolic function. Ejection fraction is dependent on chamber size and loading conditions and is regarded as a measure of ventricular-arterial coupling rather than contractility (Borlaug *et al.*, 2009). Midwall fractional shortening (FSmid) (a degree of systolic shortening in midwall fibres) or tissue Doppler measures of shortening can be used to identify deformities of myocardial systolic function in a concentrically remodelled heart (Lang *et al.*,

2015). In 2002, two seminal studies reported impaired regional systolic function as assessed by TDI despite normal ejection fraction (Yip *et al.*, 2002; Yu *et al.*, 2002). Subsequently, a number of studies have similarly shown impaired systolic function in HFpEF (Brucks *et al.*, 2005; Fukuta & Little, 2007). Although a number of studies have shown impaired systolic function using TDI in patients with HFpEF, TDI is angle-dependent and only measures mitral annular motion (Kadappu & 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-independent 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). Myocardial deformation analysis is more constant along the ventricular wall and allows discrimination between active and passive movement of the myocardium (Dandel *et al.*, 2009). In this regard, myocardial deformation is a more accurate marker for detection of regional myocardial dysfunction than wall motion analysis (Urheim *et al.*, 2000; Dandel *et al.*, 2009).

Although it is well established that RA patients frequently present with diastolic dysfunction and a preserved ejection fraction, recent evidence suggests the presence of preclinical systolic dysfunction in RA patients using STE (Sitia *et al.*, 2012; Fine *et al.*, 2014; Baktir *et al.*, 2015; Hamdy *et al.*, 2017; Midtbø *et al.*, 2017; Lo Gullo *et al.*, 2018). LV longitudinal and radial strain are significantly impaired in RA patients without coronary artery disease compared to non-RA patients (Sitia *et al.*, 2012). Further, in RA patients with a preserved ejection fraction disease activity is associated with impaired global longitudinal strain independent of traditional cardiovascular risk factors (Midtbø *et al.*, 2017). Higher levels of circulating inflammatory markers and more severe disease markers are associated with impaired global longitudinal strain in RA patients with preserved ejection fraction (Hamdy *et al.*, 2017; Lo Gullo *et al.*, 2018). Although these results suggest that systemic inflammation may promote preclinical systolic dysfunction despite normal ejection fraction in patients with RA, whether this relationship exists independent of confounding factors are not known. Therefore, in addition to evaluating the direct role of inflammation on diastolic function, in the present thesis, *I evaluated the role of inflammation on myocardial systolic function in a preclinical model of RA.*

1.10. Problem statement

In summary, the present thesis was intended to address some outstanding issues regarding the mechanisms of inflammation-associated CVD risk. In this regard, patients with RA are at increased risk to develop heart failure. More specifically in RA, HFpEF is more common and is often preceded by diastolic dysfunction. Although RA disease characteristics have been associated with increased prevalence of diastolic dysfunction, it is uncertain whether these associations exist beyond traditional cardiovascular risk factors. Although there is a relationship between inflammatory markers and accelerated diastolic dysfunction in cross-sectional RA studies, there is currently no direct evidence that exposure to chronic inflammation causes diastolic dysfunction independent of traditional cardiovascular risk factors. Although patients with RA more frequently present with diastolic dysfunction and a normal ejection fraction, evidence has suggested that preclinical myocardial systolic function may be impaired. Whether inflammation is associated with this impaired myocardial systolic function is not known. Besides the direct effect inflammation may have on myocardial remodelling, recently it has been proposed that systemic inflammation may drive the development of diastolic dysfunction via microvascular endothelial dysfunction. In this regard, previous evidence has suggested that endothelial dysfunction is central to the development of other subclinical CVD such as atherosclerosis in RA. Whether exposure to chronic inflammation directly impairs endothelial function needs to be confirmed. Endothelial dysfunction impairs arterial function. In non-RA populations impaired arterial function has been associated with diastolic function. Considering the unique situation that inflammation affects traditional risk factors and arterial function in RA, whether there is an independent association between arterial function and diastolic function in RA needs elucidation. The aims of the present thesis can therefore be summarised as follows:

1.11. Study aims

1. To determine the estimated prevalence and risk factors associated with echocardiographically determined measures of diastolic function and dysfunction upon applying different classification criteria sets in RA patients.
2. To determine the effect of high-grade inflammation on diastolic function and systolic function using pulsed Doppler, tissue Doppler and STE analysis in a model of collagen-induced arthritis.
3. To determine the impact of high-grade inflammation on endothelial function and its association with circulating biomarkers of endothelial dysfunction in a model of collagen-induced arthritis.

4. To determine large artery functional characteristics that are associated with alterations in left ventricular function, independent of traditional cardiovascular risk factors and disease markers in patients with RA.

Chapter 2: The impact of different classification criteria sets on the estimated prevalence and associated risk factors of diastolic dysfunction in rheumatoid arthritis

The data in this chapter have been published in the International Journal of Rheumatology:

Mokotedi L, Gunter S, Robinson C, Norton GR, Woodiwiss AJ, Tsang L, Dessein PH, Millen AME (2017). The Impact of Different Classification Criteria Sets on the Estimated Prevalence and Associated Risk Factors of Diastolic Dysfunction in Rheumatoid Arthritis. *Int J Rheumatol* **2017,** 1-13.

2.1. Abstract

Background. The criteria for the classification of diastolic dysfunction have recently been revised. This study compared the estimated prevalence and potential determinants of left ventricular (LV) diastolic dysfunction upon applying two different reported classification criteria sets in rheumatoid arthritis (RA).

Methods. LV diastolic function was assessed echocardiographically by pulsed Doppler (E/A ratio), tissue Doppler (E/e' ratio, lateral and septal e') and left atrial volume index in 176 patients with RA. Relationships of traditional cardiovascular risk factors and RA characteristics with LV diastolic function parameters and diastolic dysfunction according to previous and current criteria were determined in multivariate regression models.

Results. Waist-hip ratio was associated with E/A (standardised β(SE)=-0.28 ± 0.09, p=0.0002), lateral e' (standardised β(SE)=0.26 ± 0.09, p=0.01) and left atrial volume index (standardised β(SE)=0.27±0.11, p=0.01); low diastolic blood pressure related to E/e' (standardised β(SE)=-0.16 ± 0.08, p=0.04). The estimated prevalence of diastolic dysfunction differed upon applying previous (59%) compared to current (22%) classification criteria (p<0.0001). One SD increase in waist-hip ratio was directly associated with diastolic dysfunction when applying current criteria (OR=2.61 (95% CI=1.51-4.52), p=0.0006) whereas one SD increase in diastolic blood pressure related inversely to diastolic dysfunction upon using previous criteria (OR=0.57 (95% CI=0.40-0.81), p=0.002).

Conclusions. Application of current compared to previously reported diastolic dysfunction classification criteria sets markedly alters its estimated prevalence and associated risk factors in RA.

2.2. Introduction

Rheumatoid arthritis (RA) increases the risk of heart failure 2-fold (Nicola *et al.*, 2005). Heart failure accounts for 13% of the mortality in RA (Nicola *et al.*, 2006). Among heart failure patients, those with RA experience less frequently typical symptoms and signs of heart failure and are more likely to have a preserved ejection fraction (EF) (Davis *et al.*, 2008; Aslam *et al.*, 2013). Left ventricular (LV) diastolic dysfunction is the most common cause of heart failure with preserved ejection fraction and associates with adverse cardiovascular outcomes including incident heart failure (Aurigemma *et al.*, 2001; Schillaci *et al.*, 2002). Metabolic risk factor driven inflammation is strongly implicated in diastolic dysfunction (Shah *et al.*, 2016). RA patients experience interrelated high-grade inflammation and increased metabolic risk (Dessein *et al.*, 2016). In RA, traditional cardiovascular risk factors and coronary artery disease do not fully explain all changes in diastolic dysfunction (Crowson *et al.*, 2005). In this regard, disease duration (Di Franco *et al.*, 2000; Arslan *et al.*, 2006; Rexhepaj *et al.*, 2006; Canturk *et al.*, 2006; Birdane *et al.*, 2007; Udayakumar *et al.*, 2007; Wislowska *et al.*, 2008; Liang *et al.*, 2010; Davis *et al.*, 2011; Garza-García *et al.*, 2013; Tomáš *et al.*, 2013; Sharma *et al.*, 2015) and circulating concentrations of the inflammatory markers interleukin-6 (Liang *et al.*, 2010) and tumour necrosis factor-α (Tomáš *et al.*, 2013) have been associated with diastolic dysfunction in patients with RA. Patients with RA also experience increased left ventricular mass (Rudominer *et al.*, 2009), which is associated with diastolic dysfunction. Nevertheless, most previous studies on diastolic dysfunction in RA had small sample sizes (Montecucco *et al.*, 1999; Di Franco *et al.*, 2000; Arslan *et al.*, 2006; Rexhepaj *et al.*, 2006; Canturk *et al.*, 2006; Udayakumar *et al.*, 2007; Tomáš *et al.*, 2013) and traditional cardiovascular risk factors were consistently adjusted for in only two of these (Liang *et al.*, 2010; Davis *et al.*, 2011). The relative impact of traditional cardiovascular risk factors and disease characteristics beyond structural changes, on diastolic dysfunction and its different components in RA remains uncertain.

Diastolic dysfunction is most frequently assessed by echocardiography. Importantly in the present context, several RA investigations focused on the pulsed Doppler determined early filling velocity (E) to atrial contraction velocity (A) (E/A) ratio as a parameter of diastolic function (Gonzalez-Juanatey *et al.*, 2004; Rexhepaj *et al.*, 2006; Udayakumar *et al.*, 2007; Garza-García *et al.*, 2013; Tomáš *et al.*, 2013; Sharma *et al.*, 2015). However, measures that require tissue Doppler imaging including particularly the E/early peak velocity during diastole at the mitral annulus (e') (E/e') ratio, were proven to more reliably represent diastolic function as assessed by invasive pressure-volume analysis (Kasner *et al.*, 2007). In 2003, a criteria set for classification of diastolic dysfunction that included both pulsed and tissue Doppler determined parameters was reported by Redfield and colleagues (Redfield *et al.*, 2003). Upon applying the respective criteria

set, Liang and colleagues documented an increased prevalence of diastolic dysfunction among RA patients in a community-based study in 2010 (Liang *et al.*, 2010). In 2016, Nagueh and colleagues reported new recommendations for the evaluation of diastolic dysfunction. The E/A ratio was no longer considered in patients with an ejection fraction of ≥50% and a relatively high cut-off value for raised E/e' of 14 cm/s was proposed (Nagueh *et al.*, 2016). Whether this changed approach can influence the identification of RA patients who are at risk of developing heart failure with preserved ejection fraction is unknown.

In this study, I determined (1) independent relationships of traditional cardiovascular risk factors and disease characteristics with pulsed and tissue Doppler measures of diastolic function and (2) whether application of different diastolic dysfunction classification criteria sets impacts on its estimated prevalence and associated risk factors in patients with RA.

2.3. Materials and Methods

2.3.1. Patients

We recruited 186 patients that met the 2010 American College of Rheumatology /European League Against Rheumatism criteria for RA (Aletaha *et al.*, 2010) at the Milpark Hospital in Johannesburg, South Africa. Of these, 176 patients (117 white, 29 Asian, 22 black and 8 of mixed ancestry) in whom high-quality echocardiographic measures were obtained and that did not have heart failure were included in the study. This investigation was performed in line with the principles of the Helsinki declaration and approval was obtained from the University of Witwatersrand Human (Medical) Research Ethics Committee (approval number: M06-07-33; protocol number: M120562) in Johannesburg, South Africa. All participants gave written, informed consent.

2.3.2. Baseline characteristics

Baseline characteristics were assessed using standard approaches as previously reported (Dessein *et al.*, 2014). Demographic characteristics, lifestyle factors and anthropometric variables were recorded. Systolic blood pressure and diastolic blood pressure (DBP) were measured and hypertension was identified in the presence of systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg or/and use of anti-hypertensive agents. Standard laboratory blood tests of renal and liver function, lipids, glucose, haematological variables and erythrocyte sedimentation rate (ESR) were performed. Dyslipidemia was diagnosed when the cholesterol/high density lipoprotein (HDL) ratio was >4 (Peters *et al.*, 2010) or/and lipid lowering agents were employed. Diabetes was identified when the fasting plasma glucose was ≥7 mmol/l or/and glucose lowering agents were used.

RA disease duration, rheumatoid factor and anti-citrullinated peptide antibody (ACPA) status were assessed. Disease activity was estimated by the Disease Activity Score in 28 joints (DAS28) and the Clinical Disease Activity Index (CDAI) (Aletaha *et al.*, 2005). Current or previously identified (hospital record review) extra-articular manifestations (Dessein *et al.*, 2014) were recorded. Physical impairment was evaluated by the Stanford Health Assessment Questionnaire disability index (HAQ-DI). C-reactive protein (CRP) concentrations were determined using immunoturbidimetric methods. Renal function was assessed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) estimated glomerular filtration rate (eGFR) (Levey *et al.*, 2009). Insulin resistance was estimated by the Homeostasis Model Assessment for insulin resistance (HOMA-IR) (Wallace *et al.*, 2004).

2.3.3. Echocardiography

Echocardiography was performed using a Sonosite M-Turbo ultrasound (SonoSite® Inc., Bothell, WA, USA) with the patient in the partial left decubitus position according to the American Society of Echocardiography convention (Sahn *et al.*, 1978). LV dimensions were determined using two-dimensional directed M-mode echocardiography in the parasternal long axis view. LV end systolic (LVESD) and end diastolic (LVEDD) internal diameters and septal (IVST) and posterior wall thickness (PWT) were measured in systole and diastole (Figure 2.1). The LV dimensions were measured only when appropriate visualisation of both the right and the left septal surfaces occurred and where the endocardial surfaces of both the septal and posterior wall were clearly visible. Left ventricular end diastolic and systolic volumes were assessed using the Teichholz method (Teichholz *et al.*, 1976). LV ejection fraction was calculated as [(LVEDD-LV ESD) / LV end-diastolic volume] x 100. Left ventricular mass was determined using a standard formula (Lang *et al.*, 2015) and indexed to body surface area (LVMI). LV relative wall thickness (RWT) was calculated as (PWT in diastole x 2) / LV EDD) (Ganau *et al.*, 1992). Left ventricular hypertrophy was identified when left ventricular mass index was >95 g/m^2 for women and >115 g/m^2 for men (Lang *et al.*, 2015).

Left ventricular diastolic function was assessed using pulsed Doppler, tissue Doppler imaging and left atrial volume (Nagueh *et al.*, 2016). Transmitral flow patterns were recorded at the mitral valve leaflet tips using pulsed Doppler in the apical four chamber view.

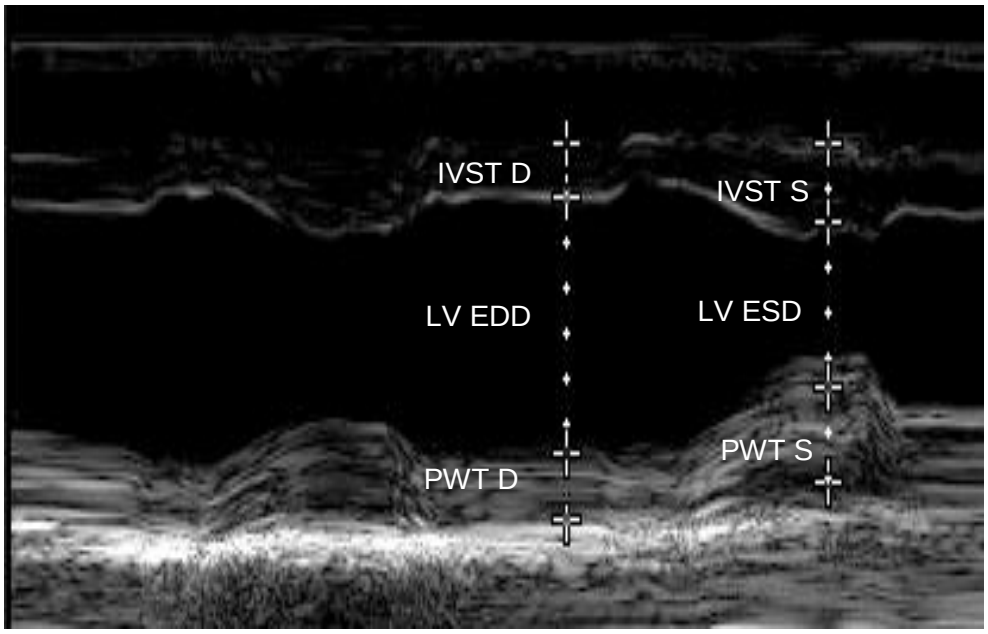


Figure 2.1 Example of an echocardiographic M-Mode image showing the measurement of left ventricular dimensions. Left ventricular end systolic internal diameter (LV ESD); Left ventricular end-diastolic internal diameter (LV EDD); Intraventricular septal wall thickness during systole (IVST S); Intraventricular septal wall thickness during diastole (IVST D); Posterior wall thickness during systole (PWT S); Posterior wall thickness during diastole (PWT D). These values were used for the calculation of stroke volume, ejection fraction and LV mass.

From the mitral valve inflow velocity curve, the early (E) and late (atrial-A) diastolic wave were measured and the ratio of early and late diastolic filling (E/A) was calculated (Figure 2.2).

To perform tissue Doppler imaging (TDI), the velocity of myocardial tissue lengthening was measured by placing the cursor at the septal and lateral corners of the mitral annulus in the apical four chamber view. To determine diastolic function using TDI, the peak velocities during early (e') and late (a') diastole were recorded (Figure 2.3). Because mitral E is dependent on ventricular relaxation as well as left atrial driving forces (pressures), while e' is dependent on relaxation alone, E/e' is considered to be an index of left ventricular filling pressures. The E/e' ratio was calculated as mitral E / the average of septal and lateral e'. Left atrial area was determined using planimetry at end-systole in the apical four chamber or two chamber views from which left atrial volume was calculated by the area-length method and was indexed to body surface area (LAVI) (Figure 2.4).

The presence of diastolic dysfunction was determined using previously (Redfield *et al.*, 2003) and currently reported approaches (Nagueh *et al.*, 2016). Briefly, according to previous approaches, mild diastolic dysfunction was diagnosed when E/A was <0.75 and E/e' <10, moderate diastolic dysfunction when 0.75 ≤ E/A <1.5 and E/e' ≥10, and severe diastolic dysfunction when E/A was ≥ 1.5 and E/e' ≥ 10 (Redfield *et al.*, 2003). According to the current recommendations, in patients with an ejection fraction >50%, diastolic dysfunction was identified when at least 2 of the following criteria were met: E/e' >14, lateral e' <10 cm/s or septal e' <7 cm/s, and left atrial volume index >34 ml/m^2; in those with an ejection fraction <50%, mild diastolic dysfunction was diagnosed when E/A was <0.8, severe diastolic dysfunction when E/A was >2, and moderate diastolic dysfunction when E/A was >0.8 and <2, E/e' >14 and left atrial volume index >34 ml/m^2 (Nagueh *et al.*, 2016).

Echocardiography was performed by two experienced operators that were blinded to the clinical data and cardiovascular disease risk factor profiles of the patients. All echocardiographic images were reviewed by a single experienced operator. Intra- and inter-observer studies were conducted on 20 individuals. For the inter-observer variability, the Pearson's correlation coefficients for LV end-diastolic diameter, septal wall thickness, posterior wall thickness, E, A and e' were 0.71, 0.84 and 0.81, 0.95, 0.92 and 0.92 respectively ($p<0.0001$ for all) and the coefficient of variation for LV end-diastolic diameter, septal wall thickness, posterior wall thickness, E, A and e' were 2.7%, 2.9%, 2.6%, 1.9%, 3.9% and 4.2% respectively. There were no significant differences between the measures on unpaired t-tests ($p>0.2$ for all).

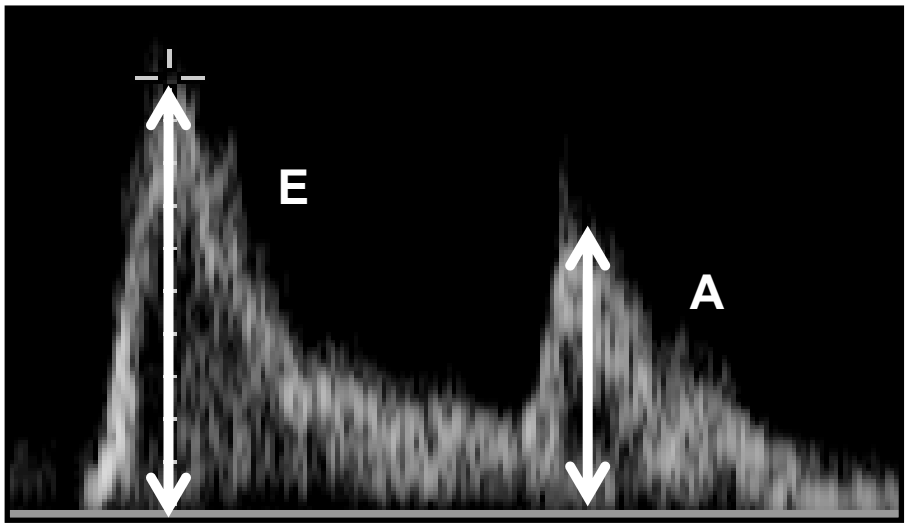


Figure 2.2 Example of an echocardiographic image showing pulse waved Doppler obtained from the mitral valve leaflet tips. Early diastolic filling velocity (E); Late diastolic filling velocity (A).

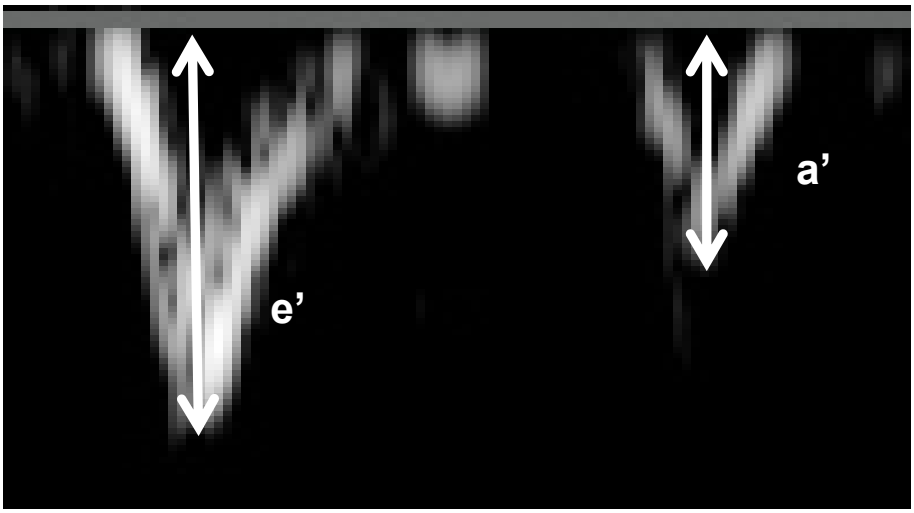


Figure 2.3 Example of an echocardiographic image showing tissue Doppler imaging obtained from the mitral annulus. Early diastolic mitral annulus velocity (e’); Late diastolic mitral annulus velocity (a’).

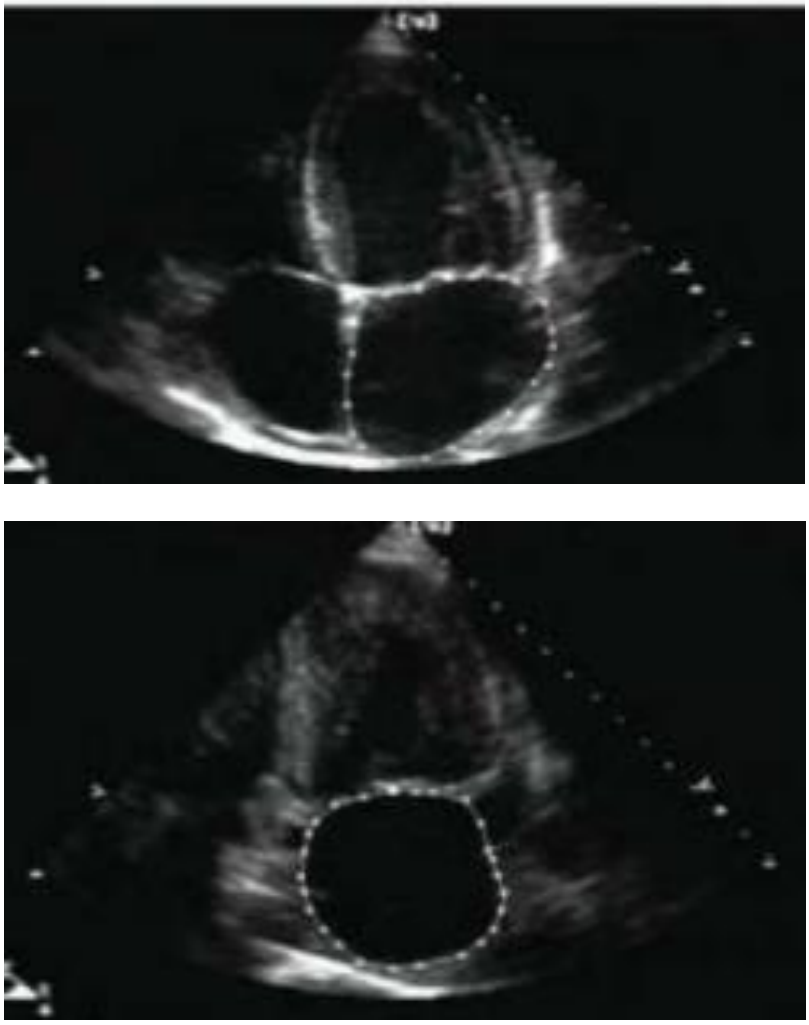


Figure 2.4 Example of echocardiographic images showing the measurement of left atrial area measured using planimetry in the apical four-chamber (upper panel) and two-chamber (lower panel) views at left ventricular end systole.

For the intra-observer variability, the Pearson's correlations for repeat measures ranged between 0.74 and 0.98 for the one and 0.82 and 0.94 for the other observer for all the aforementioned measures (all $p<0.001$). The respective coefficients of variation ranged between 1.4% and 4.7% for the one and 1.2% and 5.4% for the other observer for all the aforementioned measures. There were no differences in the repeat measures on paired t-tests for either observer ($p>0.2$ for all).

2.3.4. Statistical analysis

Database management and statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Descriptive data are presented as mean (SD), median (interquartile range (IQR)) or proportions for normally distributed, non-normally distributed and categorical variables, respectively. Non-normally distributed variables were logarithmically transformed in order to improve skewness and kurtosis for statistical analysis. Potential relationships of baseline characteristics including traditional cardiovascular risk factors and RA features with markers of diastolic function (as well as LV mass parameters) were first assessed in age, sex and race adjusted linear regression models. The independent associations of baseline characteristics with diastolic function parameters and diastolic dysfunction according to previous and current diastolic dysfunction classification criteria sets, were subsequently determined in linear and backward logistic regression models, respectively. As LV mass and concentric hypertrophy are strongly associated with diastolic function, the associations of LV mass index and relative wall thickness with diastolic function parameters were determined. LV mass index was associated with E/A ratio ($r=-0.30$, $p<0.0001$), lateral e' ($r=-0.30$, $p<0.0001$), septal e' ($r=-0.23$, $p=0.002$) and LAVI ($r=0.19$; $p=0.009$); relative wall thickness was not associated with any LV diastolic function parameters. In age, sex and race adjusted analysis, neither LV mass index nor relative wall thickness were associated with diastolic dysfunction parameters (data not shown). Nevertheless, as LV mass index was more strongly and consistently associated with diastolic function parameters than relative wall thickness in univariate analysis, LV mass index was forced in additional regression models with diastolic dysfunction as dependent variable. Dyslipidemia, smoking and diabetes were added as independent variables into final models on diastolic dysfunction among all RA patients. As the impact of risk factors on cardiovascular disease can differ in women compared to men, the independent associations of baseline characteristics with diastolic dysfunction were also assessed in a sensitivity analysis among women. Lastly, sensitivity analysis was performed in patients without established cardiovascular disease (n=9) and in those with an ejection fraction of ≥50% (isolated diastolic dysfunction). Significance was set at $p \leq 0.05$.

2.4. Results

2.4.1. Patient characteristics

Table 2.1 gives the recorded characteristics. Mean (SD) age and median (IRQ) disease duration were 58.4 (12.4) years and 14.5 (8.9-22) years, respectively. Of the participants, 40% had hypertension, 49% dyslipidemia and 5% diabetes. All patients with hypertension received anti-hypertensive agents. Disease activity was overall well controlled with a median (IQR) CDAI of 6 (1-13) and mean (SD) DAS28 of 2.8 (1.7); 73.7% of the patients tested rheumatoid factor positive and 69.5% ACPA positive; 22% experienced extra-articular manifestations.

The mean (SD) ejection fraction was 58.7 (13.2) %, 21% of participants had an ejection fraction of <50% and 19.3% left ventricular hypertrophy. The E/e' ratio was ≥10 and >14 in 58% and 20% of participants, respectively. Upon applying previously reported criteria (Redfield *et al.*, 2003), 58.6% of the patients were classified as having diastolic dysfunction and none had indeterminate diastolic function; when the recently reported criteria (Nagueh *et al.*, 2016) criteria were applied, 22% had diastolic dysfunction and 23% indeterminate diastolic function; 21.6% of the participants were classified with diastolic dysfunction according to both criteria sets, 37.4% met the previous but not recent criteria and 1.2% met the recent but not previous criteria. The estimated proportion of RA persons with diastolic dysfunction differed significantly when applying previous (Redfield *et al.*, 2003) compared to current criteria (Nagueh *et al.*, 2016) ($p<0.0001$).

2.4.2. Relationships of traditional risk factors and RA characteristics with indices of LV diastolic function and LV geometry

The significant age, sex and race adjusted associations of baseline characteristics with markers of diastolic function and LV geometry are shown in Table 2.2. Age was associated with all markers of diastolic function ($p<0.01$ for all) and LV mass index (partial $r=0.36$, $p<0.0001$) but not relative wall thickness ($p=0.4$). Female sex (partial $r=0.16$, $p=0.03$) and heart rate (partial $r=-0.29$, $p<0.001$) were associated with E/A. Estimated glomerular filtration rate was directly related to E/A (partial $r=0.22$, $p=0.01$), lateral e' (partial $r=0.27$, $p=0.002$) and septal e' (partial $r=0.27$, $p=0.001$) and inversely associated with left atrial volume index (partial $r=-0.17$, $p=0.04$), and LV mass index (partial $r=-0.24$, $p=0.005$). Waist-hip ratio (WHR) comprised the adiposity marker that was most consistently associated diastolic function markers and was therefore included in subsequent analysis.

Table 2.1 Recorded characteristics in 176 patients with RA

Demographic characteristics		CRP, mg/l	3.2 (1.2-8.0)
Age at study time, years	58.4 (12.4)	Leukocytes, n/nl	5.5 (4.6-7.1)
Age at disease onset, years	42.3 (14.1)	Deformed joints, n	0 (0 - 11)
Female sex, %	81.6	Extra-articular manifestations, %	22
Lifestyle factors		Stanford HAQ-DI	0.38 (0.0-0.88)
Exercise, %	32.4	Synthetic DMARD, %	
Alcohol use, %	31.8	Methotrexate	75.4
Current smoking, %	10.1	Chloroquine	48.0
Anthropometry		Leflunomide	40.2
Body mass index, kg/m^2	26.7 (5.3)	Sulphasalazine	15.6
Waist circumference, cm	91 (13)	Tetracycline	16.2
Waist-hip ratio	0.88 (0.09)	Azathioprine	6.7
Metabolic risk factors		Current DMARDS, n	2.0 (1.1)
Hypertension, %	40.2	Biological DMARD, %	
SBP, mm Hg	128 (15)	TNF-α inhibitors	8.4
DBP, mm Hg	81 (8)	Abatacept	2.2
Pulse pressure, mm Hg	47 (13)	NSAID, %	32.9
Total cholesterol, mmol/l	4.5 (1.1)	Prednisone use, %	2.2
HDL cholesterol, mmol/l	1.64 (0.47)	GFR, ml/min/1.73m^2	99.3 (87.7-107.6)
LDL cholesterol, mmol/l	2.42 (0.88)	Heart rate, beats per minute	72 (12)
Triglycerides, mmol/l	0.95 (0.7-1.3)	Framingham score	3.02 (5.38)
Cholesterol-HDL ratio	2.7 (2.3-3.3)	Echocardiographic measures	
Dyslipidemia, %	49.2	E/A	1.01 (0.82-1.32)
Diabetes, %	5.0	E/e'	11.10 (8.01-13.42)
Glucose, mmol/l	4.8 (4.5-5.1)	Lateral e', cm/s	9.60 (7.68-12.20)
HOMA-IR	1.4 (1.0-1.9)	Septal e', cm/s	7.94 (6.38-9.64)
Cardiovascular agents, %		Left ventricular mass index, g/m^2	78.19 (66.11-92.72)
Antihypertensives	40.2	Left ventricular hypertrophy, %	19.3
Statins	41.9	Left ventricular RWT	0.44 (0.08)
Ezetimibe	9.5	Concentric remodelling, %	55.1
Oral glucose lowering agents	1.7	Stroke volume, ml	56.10 (20.21)
Insulin	1.7	Ejection fraction, %	58.66 (13.20)
RA characteristics		Depressed ejection fraction, %	21
RA duration, years	14.5 (8.9-22.0)	Left atrial volume, ml	36.28 (11.51)
Rheumatoid factor positive, %	73.7	Left atrial volume index, ml/m^2	20.47 (5.92)
ACPA positive, %	69.5	Increased LAVI, %	2.8
CDAI	6 (1-13)	Classification of DD	
DAS 28	2.8 (1.7)	DD by previous criteria, %	58.6
ESR, mm/h	12 (4-26)	DD by new criteria, %	22.0

SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, homeostasis model of insulin resistance; ACPA, anti-citrullinated protein antibody, CDAI, clinical disease activity index; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; HAQ-DI, Health Assessment Questionnaire disability index, DMARD, disease modifying anti-rheumatic drugs, NSAID, non-steroidal anti-inflammatory drugs; GFR, glomerular filtration rate; RWT, relative wall thickness; LAVI, left atrial volume index; DD, diastolic dysfunction.

Waist-hip ratio was associated with low E/A (partial r=-0.24, p=0.003), decreased lateral e' (partial r=-0.34, p<0.001) and with increased LAVI (partial r=0.27, p=0.0005) and LV mass index (partial r=0.19, p=0.02). Diastolic blood pressure was related to E/e' (partial r=-0.16 p=0.04) and lateral e' (partial r=0.19 p=0.02) and hypertension was related to LAVI (partial r=0.27, p=0.0003). Triglycerides were inversely related to lateral e' (partial r=-0.2, p=0.01) and directly related to LAVI (partial r=0.25, p=0.001). HOMA-IR was inversely associated with E/A (partial r = -0.17, p=0.04) and lateral e' (partial r=-0.16, p=0.05) and directly with LVAI (partial r=0.18, p=0.03).

Among RA characteristics, disease duration was most consistently associated with diastolic function markers including E/A (partial r=-0.25 p=0.0001), lateral e' (partial r=-0.28 p=0.0002), left atrial volume index (partial r=0.21 p=0.007) and with LV mass index (partial r=0.20 p=0.01). DAS 28 (partial r=-0.17, p=0.03), erythrocyte sedimentation rate (partial r=-0.20, p=0.01) and C-reactive protein (partial r=-0.16, p=0.04) were related to E/A. ACPA status (partial r=0.18, p=0.02) and white cell count (partial r=0.19, p=0.01) related to relative wall thickness. The presence of extra-articular manifestations was associated with LAVI (partial r=0.16, p=0.04).

Baseline characteristics that were not significantly related to diastolic function parameters are given in Table 2.3.

Table 2.2 Associations of traditional risk factors and RA characteristics with markers of left ventricular diastolic function and left ventricular geometry

Data were analysed in age, sex and race adjusted linear regression models; age was replaced by age at disease onset in the model on disease duration, and age, sex and race were omitted in the models on GFR. Significant associations are shown in bold. DBP, diastolic blood pressure; HDL, high density lipoprotein cholesterol; HOMA-IR, homeostasis model of insulin resistance; ACPA, anti-citrullinated peptide antibody; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; WCC, white cell count; EAM, extra articular manifestations; GFR, glomerular filtration rate.* Logarithmically transformed

	Log E/A		Log E/e'		Log lateral e'		Log septal e'		LAVI		RWT		Log LVMI	
	partial r	P	partial r	P	partial r	P	partial r	P	partial r	P	partial r	P	partial r	P
Age	**-0.39**	**<0.001**	**0.19**	**0.01**	**-0.47**	**<0.001**	**-0.44**	**<0.001**	**0.28**	**0.0002**	0.06	0.43	**0.36**	**<0.001**
Sex	**0.16**	**0.03**	0.11	0.15	0.07	0.37	0.05	0.49	-0.07	0.33	0.15	0.06	**-0.28**	**0.0002**
Body mass index	-0.07	0.40	-0.02	0.79	-0.04	0.63	-0.13	0.10	0.09	0.28	-0.12	0.14	**0.22**	**0.007**
Waist	-0.14	0.07	0.03	0.67	**-0.21**	**0.009**	-0.13	0.09	**0.18**	**0.03**	-0.06	0.45	**0.24**	**0.002**
Waist-hip ratio	**-0.24**	**0.003**	0.04	0.59	**-0.34**	**<0.001**	-0.14	0.08	**0.27**	**0.0005**	0.11	0.16	**0.19**	**0.02**
Heart rate	**-0.29**	**0.0002**	-0.02	0.77	-0.15	0.06	-0.05	0.52	-0.03	0.70	0.13	0.09	-0.14	0.07
Hypertension	-0.09	0.24	-0.05	0.48	-0.04	0.60	-0.03	0.65	**0.27**	**0.0003**	-0.04	0.65	0.12	0.11
DBP	-0.03	0.67	**-0.16**	**0.04**	**0.19**	**0.02**	-0.05	0.55	-0.05	0.52	-0.03	0.73	0.03	0.73
HDL cholesterol	0.13	0.11	-0.12	0.12	**0.18**	**0.02**	-0.01	0.89	-0.10	0.21	-0.07	0.37	**-0.20**	**0.01**
Triglycerides*	-0.10	0.22	-0.001	0.99	**-0.20**	**0.01**	-0.001	0.99	**0.25**	**0.001**	0.01	0.86	0.05	0.57
Dyslipidaemia	-0.06	0.44	0.09	0.22	**-0.20**	**0.009**	-0.06	0.46	0.10	0.18	-0.03	0.66	0.12	0.11
HOMA_IR*	**-0.17**	**0.04**	-0.04	0.60	**-0.16**	**0.05**	-0.03	0.73	**0.18**	**0.03**	-0.03	0.71	0.01	0.90
Disease duration*	**-0.25**	**0.001**	0.08	0.29	**-0.28**	**0.0002**	**-0.35**	**<0.001**	**0.21**	**0.007**	0.06	0.43	**0.20**	**0.01**
ACPA	-0.11	0.16	0.13	0.09	-0.01	0.92	-0.01	0.88	0.06	0.43	**0.18**	**0.02**	0.08	0.29
DAS28	**-0.17**	**0.03**	-0.03	0.75	-0.03	0.72	-0.06	0.44	-0.02	0.79	0.07	0.35	-0.12	0.12
ESR*	**-0.20**	**0.01**	-0.07	0.35	0.01	0.93	-0.04	0.61	0.08	0.32	0.08	0.29	-0.11	0.18
C-reactive protein	**-0.16**	**0.04**	0.01	0.90	0.03	0.69	-0.10	0.20	-0.01	0.89	0.06	0.45	-0.07	0.40
WCC*	0.10	0.22	0.10	0.18	0.02	0.78	0.01	0.90	0.09	0.31	**0.19**	**0.01**	-0.09	0.25
EAM	-0.05	0.53	0.01	0.94	-0.09	0.24	-0.04	0.57	**0.16**	**0.04**	-0.02	0.81	-0.01	0.92
Sulphasalazine	-0.01	0.89	-0.01	0.87	0.009	0.90	0.08	0.32	0.01	0.89	0.02	0.80	**0.18**	**0.02**
Azathioprine	-0.09	0.32	0.003	0.97	0.02	0.82	**-0.17**	**0.02**	0.01	0.87	0.01	0.87	-0.09	0.27
GFR*	**0.22**	**0.01**	-0.13	0.12	**0.27**	**0.002**	**0.27**	**0.001**	**-0.17**	**0.04**	0.001	0.99	**-0.24**	**0.005**

Table 2.3 Non-significant associations of traditional risk factors and RA characteristics with markers of diastolic function and left ventricular geometry

	Log E/A		Log E/e'		Log lateral e'		Log septal e'		LAVI		RWT		LVMI	
	partial		partial		partial		partial		partial		partial		partial	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P
Age dis onset	0.02	0.77	0.07	0.36	-0.001	0.99	0.02	0.76	0.002	0.97	-0.10	0.19	0.10	0.20
Race	0.02	0.98	0.08	0.32	-0.04	0.56	-0.07	0.34	-0.14	0.07	0.09	0.20	-0.02	0.85
Exercise	-0.01	0.86	0.08	0.32	0.02	0.81	-0.11	0.14	-0.04	0.64	-0.05	0.49	0.02	0.81
Alcohol	-0.06	0.45	-0.05	0.53	0.07	0.36	-0.12	0.12	0.29	0.70	0.01	0.88	-0.01	0.88
SBP	0.002	0.98	-0.03	0.67	0.11	0.16	-0.003	0.97	0.08	0.27	-0.03	0.70	-0.03	0.66
Pulse pressure	0.03	0.75	0.07	0.35	0.00	0.99	0.03	0.71	0.14	0.08	-0.02	0.83	-0.05	0.45
Total cholesterol	0.06	0.46	-0.13	0.1	0.07	0.40	-0.01	0.94	0.03	0.71	-0.04	0.64	-0.13	0.10
LDL cholesterol	0.06	0.43	-0.09	0.26	0.06	0.45	0.05	0.50	0.01	0.88	-0.01	0.95	-0.05	0.56
Chol/HDL chol*	-0.09	0.28	0.03	0.66	-0.15	0.06	-0.01	0.93	0.15	0.05	0.05	0.56	0.07	0.39
Diabetes	-0.05	0.57	-0.03	0.73	0.01	0.91	-0.13	0.11	0.02	0.77	0.01	0.91	-0.08	0.28
Glucose*	-0.07	0.36	-0.04	0.64	-0.06	0.47	-0.06	0.48	0.12	0.14	-0.10	0.23	-0.02	0.76
OGLA	-0.11	0.16	-0.08	0.34	-0.002	0.98	-0.12	0.13	-0.05	0.54	-0.03	0.75	-0.13	0.10
Insulin treatment	-0.03	0.68	-0.09	0.28	-0.02	0.76	-0.04	0.62	0.04	0.61	-0.03	0.76	0.03	0.75
RF positive	-0.13	0.11	0.06	0.47	-0.001	0.99	-0.001	0.99	-0.02	0.83	0.03	0.71	0.06	0.41
CDAI*	-0.02	0.76	0.002	0.98	-0.06	0.41	-0.02	0.54	-0.03	0.72	0.05	0.49	-0.04	0.65
SDAI*	-0.03	0.72	-0.05	0.55	-0.02	0.83	0.02	0.80	-0.08	0.33	0.11	0.14	-0.04	0.60
DAS28-CRP	-0.13	0.09	0.02	0.85	-0.02	0.78	-0.08	0.30	-0.07	0.38	0.06	0.46	-0.10	0.19
Deformed joint*	-0.02	0.84	0.09	0.23	-0.13	0.10	-0.003	0.94	-0.01	0.95	0.12	0.12	-0.03	0.66
Methotrexate	-0.03	0.71	-0.03	0.69	0.03	0.69	0.02	0.78	0.07	0.36	-0.03	0.76	0.14	0.08
Chloroquine	-0.05	0.56	0.01	0.92	0.06	0.47	0.03	0.73	-0.05	0.53	-0.01	0.87	0.001	0.99
Leflunomide	-0.06	0.47	0.26	0.74	-0.10	0.19	-0.08	0.32	-0.01	0.93	0.03	0.65	0.01	0.91
Tetracycline	-0.08	0.34	-0.07	0.38	-0.03	0.65	0.03	0.73	0.02	0.78	0.01	0.89	0.01	0.91
Biologic agent	0.15	0.07	-0.003	0.97	-0.05	0.48	0.02	0.82	0.01	0.93	-0.004	0.96	-0.02	0.83

use														
TNF-α	0.08	0.33	-0.04	0.59	-0.04	0.60	0.01	0.85	-0.05	0.54	-0.08	0.28	0.03	0.72
Abatacept	0.16	0.06	0.07	0.36	-0.04	0.62	0.01	0.89	0.10	0.19	0.15	0.06	-0.09	0.27
NSAID	0.10	0.23	0.02	0.81	0.05	0.50	-0.07	0.35	-0.003	0.97	-0.03	0.69	-0.10	0.18
Prednisone	0.06	0.48	-0.03	0.67	0.08	0.29	0.11	0.16	-0.002	0.98	0.14	0.06	-0.01	0.86

Data were analysed in age, sex and race adjusted linear regression models. Dis, disease; SBP, systolic blood pressure; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein; chol, cholesterol; OGLA, oral glucose lowering agents; RF, rheumatoid factor; CDAI, clinical disease activity index; SDAI, simple disease activity index; DAS28, disease activity score in 28 joints; TNFα, tumor necrosis factor alpha inhibitors; NSAID, non-steroidal anti-inflammatory drugs. * Logarithmically transformed.

Clinical characteristics that were associated with diastolic function markers in the previous analysis were then entered into separate linear regression models with diastolic function markers as dependent variables. Table 2.4 gives the independent relationships of traditional cardiovascular risk factors and RA characteristics with diastolic function markers. The results are expressed as standardised β (SE) to allow for comparison of the contributions of the different baseline recorded characteristics to the variation in diastolic function. Age at disease onset (β (SE)=-0.31 (0.11), p=0.0006), race (β (SE)=0.19 (0.09), p=0.02), heart rate (β (SE)=-0.21 (0.08), p=0.01) and waist-hip ratio (β (SE)=-0.28 (0.09), p=0.002) remained significantly associated with E/A. Age (β (SE) = 0.19 (0.08), p-0.01) and diastolic blood pressure (β (SE)=-0.16 (0.08), p=0.04) were associated with E/e'. Age at disease onset (β (SE) = -0.34 (0.11), p=0.002) and waist-hip ratio (β (SE)=-0.26 (0.09), p=0.01) were associated with the lateral e'. Age at disease onset (β (SE)=-0.46 (0.10), p<0.0001), disease duration (β (SE) = -0.35 (0.09), p=0.0003) and azathioprine (β (SE)=-0.24 (0.08), p=0.005) use were associated with septal e'. Race (β (SE)=-0.22 (0.09), p=0.01), waist -hip ratio (β (SE)=0.27 (0.11), p=0.01) and triglyceride concentrations (β (SE)=0.20 (0.09), p=0.03) were associated with left atrial volume index.

2.4.3. Independent relationships of traditional risk factors and RA characteristics with LV diastolic dysfunction according to previous and current criteria sets

Baseline characteristics that remained associated with diastolic function markers in Table 2.4 were subsequently entered into backward logistic regression models with diastolic dysfunction according to previously (Redfield *et al.*, 2003) or currently reported (Nagueh *et al.*, 2016) criteria sets as dependent variables. As shown in Table 2.5, age at disease onset (OR= 2.28 (95% CI=1.22-4.25), p=0.01) and the waist- hip ratio (OR=2.61 (95% CI=1.51-4.52), p=0.0006) were associated with diastolic dysfunction upon using the current criteria; these relationships were materially unaltered after adding LV mass index (p=0.005 and p=0.0004, respectively) as an additional potential confounder to the model. Upon applying the previous criteria, only a low diastolic blood pressure (OR=0.57 (95% CI=0.40-0.81), p=0.002) was associated with diastolic dysfunction; low diastolic blood pressure remained independently associated with diastolic dysfunction (according to previous criteria) when LV mass index (p=0.003) was added to the model. When adding smoking, dyslipidemia and diabetes to the models, age at disease onset (OR=2.27 (95% CI=1.18-4.37), p=0.01) and waist-hip ratio (OR= 2.44 (95% CI=1.39-4.27), p=0.002) remained associated with diastolic dysfunction according to previous criteria and low diastolic blood pressure (OR=0.56 (95% CI=0.39-0.81), p=0.002) remained related to diastolic dysfunction according to recent criteria. After exclusion of patients

with established cardiovascular disease (n=9) the results were materially unaltered (data no shown).

As given in Table 2.6, in sensitivity analysis among women, waist-hip ratio (OR=3.49 (95% CI=1.79-6.82), p=0.0002) was associated with diastolic dysfunction according to current criteria and a low diastolic blood pressure (OR=0.62 (95% CI=0.42-0.92), p=0.02) was related to diastolic dysfunction according to previous criteria. When adding LV mass index to the models, these associations remained significant. Sensitivity analysis was not performed in men due to the small numbers involved (n=32).

In Table 2.7, a sensitivity analysis among patients with an ejection fraction of ≥50% was performed. Age at disease onset was associated with diastolic dysfunction according to the current criteria (OR=2.45 (95% CI=1.20-5.02), p=0.01). When LV mass index was added to the model, age at disease onset remained associated (p=0.01) with diastolic dysfunction according to the current criteria. Additionally, waist-hip ratio (OR=1.99 (95% CI=1.04-3.80), p=0.03) and disease duration (OR=1.99 (95% CI=1.01-3.93), p=0.05) were related to diastolic dysfunction according to the current criteria. A low diastolic blood pressure was significantly associated with diastolic dysfunction according to previous criteria before (OR=0.59 (95% CI=0.40-0.89), p=0.01) and after (OR=0.60 (95% CI=0.40-0.91), p=0.01) LV mass index was added to the model.

When azathioprine was omitted as independent variable from the models in Tables 2.5, 2.6 and 2.7, the results were unaltered (data not shown).

2.5. Discussion

This study examined the potential contribution of comprehensively assessed traditional cardiovascular risk factors and disease characteristics to LV diastolic function in patients with RA. The analysis revealed that waist-hip ratio was independently associated with the E/A ratio, lateral e' and left atrial volume index whereas low diastolic blood pressure related to the E/e' ratio. We also report for the first time that the estimated prevalence of diastolic dysfunction is markedly lower upon application of the recently reported classification criteria by Nagueh and colleagues (Nagueh *et al.*, 2016) compared those previously recommended by Redfield and colleagues (Redfield *et al.*, 2003) (22.0% versus 58.6%) in RA. Moreover, the risk factors associated with diastolic dysfunction differed consistently when previous compared to current classification criteria sets were used.

Table 2.4 Independent relationships of traditional risk factors and RA disease characteristics with markers of diastolic function

	Log E/A			Log E/e'			Log lateral e'			Log septal e'			LAVI		
	Std β	SE	P	Std β	SE	P	Std β	SE	P	Std β	SE	P	Std β	SE	P
Age				0.19	0.08	0.01									
Age at disease onset	-0.31	0.11	0.006				-0.34	0.11	0.002	-0.46	0.10	<.0001			
Race	0.19	0.09	0.02										-0.22	0.09	0.01
DBP				-0.16	0.08	0.04									
HR	-0.21	0.08	0.01												
WHR	-0.28	0.09	<0.0001				-0.26	0.09	0.01				0.27	0.11	0.01
Disease Duration										-0.35	0.09	0.0003			
Triglycerides													0.20	0.09	0.03
Azathioprine										-0.24	0.08	0.005			

DBP, diastolic blood pressure; HR, heart rate; WHR, waist-hip ratio.

Table 2.5 Independent relationships of 1 SD increase in traditional risk factors and RA disease characteristics with diastolic dysfunction based on new and previous criteria in all patients

	Diastolic dysfunction by new criteria				Diastolic dysfunction by previous criteria			
	Without LVMI in model		With LVMI in model		Without LVMI in model		With LVMI in model	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Age at disease onset	**2.28 (1.22-4.25)**	**0.01**	**2.65 (1.34-5.20)**	**0.005**	1.32 (0.84-2.06)	0.23	1.51 (0.95-2.42)	0.08
Sex	3.04 (0.95-9.75)	0.06	2.95 (0.90-9.66)	0.07	1.61 (0.59-4.40)	0.35	1.41 (0.50-3.96)	0.51
Race	1.01 (0.70-1.46)	0.96	0.98 (0.67-1.42)	0.89	1.01 (0.75-1.36)	0.95	1.02 (0.75-1.37)	0.92
Heart rate	0.95 (0.62-1.43)	0.79	0.94 (0.62-1.43)	0.77	1.13 (0.78-1.64)	0.51	1.07 (0.73-1.56)	0.74
Diastolic blood pressure	0.71 (0.46-1.08)	0.11	0.73 (0.48-1.11)	0.14	**0.57 (0.40-0.81)**	**0.002**	**0.57 (0.40-0.83)**	**0.003**
Waist-hip ratio	**2.61 (1.51-4.52)**	**0.0006**	**2.76 (1.58-4.85)**	**0.0004**	1.37 (0.89-2.12)	0.16	1.45 (0.93-2.26)	0.10
Disease duration	1.57 (0.90-2.74)	0.12	1.69 (0.95-3.01)	0.07	1.40 (0.93-2.12)	0.10	1.50 (0.98-2.29)	0.06
Azathioprine	1.02 (0.19-5.42)	0.98	0.90 (0.16-4.93)	0.90	1.11 (0.27-4.67)	0.88	0.74 (0.16-3.39)	0.70
Triglycerides	1.00 (0.87-1.16)	0.98	1.00 (0.87-1.16)	0.98	1.07 (0.95-1.21)	0.26	1.08 (0.95-1.22)	0.24
LVMI	-		0.77 (0.47-1.29)	0.32	-		0.73 (0.49-1.11)	0.14

LVMI, left ventricular mass indexed to body surface area.

Table 2.6 Independent relationships of 1 SD increase in traditional risk factors and RA disease characteristics with diastolic dysfunction based on new and previous criteria in women with RA

	Diastolic dysfunction by new criteria				Diastolic dysfunction by previous criteria			
	Without LVMI in model		With LVMI in model		Without LVMI in model		With LVMI in model	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Age at disease onset	1.80 (0.88-3.71)	0.11	2.01 (0.94-4.33)	0.07	1.26 (0.76-2.10)	0.37	1.44 (0.84-2.47)	0.18
Race	1.00 (0.65-1.56)	0.99	0.96 (0.62-1.49)	0.85	1.30 (0.90-1.88)	0.16	1.34 (0.92-1.97)	0.13
Heart rate	0.96 (0.60-1.55)	0.88	0.98 (0.61-1.58)	0.95	1.02 (0.67-1.56)	0.93	0.97 (0.63-1.50)	0.9
Diastolic blood pressure	0.81 (0.51-1.27)	0.35	0.82 (0.52-1.29)	0.39	**0.62 (0.42-0.92)**	**0.02**	**0.62 (0.42-0.93)**	**0.02**
Waist-hip ratio	**3.49 (1.79-6.82)**	**0.0002**	**3.56 (1.80-7.03)**	**0.0003**	1.30 (0.80-2.10)	0.29	1.41 (0.88-2.26)	0.24
Disease duration	1.30 (0.67-2.51)	0.43	1.36 (0.69-2.69)	0.37	1.32 (0.83-2.08)	0.24	1.41 (0.88-2.26)	0.15
Azathioprine	0.83 (0.08-8.23)	0.87	0.79 (0.08-8.25)	0.85	0.78 (0.15-4.12)	0.77	0.43 (0.07-2.59)	0.36
Triglycerides	1.05 (0.88-1.25)	0.61	1.04 (0.87-1.25)	0.64	1.16 (0.97-1.38)	0.11	1.16 (0.97-1.39)	0.1
LVMI	-		0.88 (0.50-1.55)	0.65	-		0.81 (0.51-1.29)	0.38

LVMI, left ventricular mass indexed to body surface area.

Table 2.7 Independent relationships of 1 SD increase in traditional risk factors and RA disease characteristics with diastolic dysfunction based on new and previous criteria in RA patients with isolated diastolic dysfunction (EF ≥50%)

	Diastolic dysfunction by new criteria				Diastolic dysfunction by previous criteria			
	Without LVMI in model		With LVMI in model		Without LVMI in model		With LVMI in model	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Age at disease onset	**2.45 (1.20-5.02)**	**0.01**	**3.04 (1.36-6.81)**	**0.01**	1.35 (0.83-2.20)	0.23	1.47 (0.88-2.45)	0.14
Sex	3.55 (0.74-17.02)	0.11	3.37 (0.70-16.23)	0.13	1.25 (0.37-1.24)	0.72	1.11 (0.32-3.83)	0.87
Race	1.08 (0.71-1.67)	0.71	1.09 (0.71-1.69)	0.70	1.07 (0.77-1.49)	0.69	1.05 (0.76-1.47)	0.76
Heart rate	0.97 (0.59-1.59)	0.90	0.94 (0.57-1.55)	0.82	1.02 (0.67-1.53)	0.94	0.96 (0.63-1.46)	0.84
Diastolic blood pressure	0.72 (0.43-1.20)	0.20	0.71 (0.42-1.20)	0.21	**0.59 (0.40-0.89)**	**0.01**	**0.60 (0.40-0.91)**	**0.01**
Waist-hip ratio	1.76 (0.94-3.30)	0.07	**1.99 (1.04-3.80)**	**0.03**	1.20 (0.73-1.97)	0.48	1.28 (0.77-2.14)	0.34
Disease duration	1.78 (0.93-3.39)	0.08	**1.99 (1.01-3.93)**	**0.05**	1.51 (0.95-2.41)	0.08	1.58 (0.98-2.55)	0.06
Azathioprine	0.63 (0.06-6.33)	0.70	0.57 (0.06-5.75)	0.64	0.49 (0.10-2.56)	0.40	0.45 (0.09-2.34)	0.34
Triglycerides	0.99 (0.81-1.22)	0.94	0.99 (0.81-1.23)	0.38	1.05 (0.92-1.20)	0.43	1.06 (0.92-1.21)	0.43
LVMI	-		0.65 (0.35-1.24)	0.19	-		0.73 (0.46-1.15)	0.18

EF, ejection fraction; LVMI, left ventricular mass indexed to body surface area.

We found that waist-hip ratio contributed to the variability of E/A ratio and e' to a similar extent as age at disease onset, and diastolic blood pressure was as strongly related to E/e' as age. Adequate LV diastolic function depends on active and passive processes. The active process comprises high energy requiring relaxation (Biesiadecki *et al.*, 2014). It involves rapid removal of cytosolic calcium into the sarcoplasmic reticulum, thin filament deactivation and cross bridging kinetics (Biesiadecki *et al.*, 2014). The passive process consists of cardiac chamber compliance that is reduced by the expression and cross-linking of collagen in the interstitium and alterations in titin expression within sarcomeres when the ventricle stiffens (Kass *et al.*, 2004). In the present study, waist-hip ratio was inversely related to the E/A ratio and e', and diastolic blood pressure was inversely associated with E/e'. In this regard, a low E/A ratio and e' represent impaired relaxation whereas a large E/e' ratio is an index of filling pressure and is more strongly associated with fibrosis as part of myocardial stiffness (Moreo *et al.*, 2009; Wu *et al.*, 2014). Taken together, these results indicate that excess central adiposity may impair LV relaxation whereas low diastolic blood pressure may contribute to myocardial fibrosis and a stiffened ventricle in RA.

The question arises as to why there is a 2 to 3 fold difference in estimated diastolic dysfunction prevalence as well as a consistent disparity in associated risk factors by applied classification criteria in RA. Both criteria sets include pulsed and tissue Doppler measurements. However, in contrast to previously reported criteria, the E/A ratio is not included as a criterion among patients with an ejection fraction $\geq$50% in the recent recommendations. Additionally, the E/e' ratio is considered as a criterion when its value in $\geq$10 cm/s in the previous compared to >14 cm/s in the recent recommendations. In the present study, waist-hip ratio was related to the E/A ratio and with diastolic dysfunction according to the recent but not previous criteria. On the other hand, diastolic blood pressure was associated with the E/e' ratio and diastolic dysfunction according to the previous, but not current criteria. Moreover, 20% of patients had an E/e' ratio of >14 and 58% experienced and E/e' ratio of ≥10. These findings suggest that the reduced sensitivity in classifying patients as having diastolic dysfunction by the recent criteria set is at least partly due to the high threshold that is used upon considering an increase in E/e' ratio. These results indicate the need for further studies aimed at determining adequate heart failure risk stratification in RA. Interestingly, a recent population study similarly reported marked differences in the prevalence and predictors of diastolic dysfunction according to different classification criteria (Rasmussen-Torvik *et al.*, 2017).

In the present study, 1 SD increase in diastolic blood pressure reduced the odds ratio for diastolic dysfunction according to previous criteria to 0.57 whereas 1 SD increase in waist-hip ratio increased the odds ratio for diastolic dysfunction according to current criteria 2.61 fold in

patients with RA. These relationships were independent of LV mass index and reproduced in sensitivity analyses among women and patients without established cardiovascular disease as well as those with isolated diastolic dysfunction. In aged subjects, low diastolic blood pressure forms part of an increased pulse pressure, which is mediated by increased arterial stiffness and wave reflection (Franklin *et al.*, 1997). Neither systolic blood pressure nor pulse pressure were associated with diastolic function markers or diastolic dysfunction classification in this study. In this regard, low diastolic blood pressure in aged subjects was found to enhance LV stiffening by reducing coronary perfusion during diastole (Franklin, 2007). In the non-RA population, excess central adiposity was shown to play an important role in the development of diastolic dysfunction (Ammar *et al.*, 2008; Libhaber *et al.*, 2009; Russo *et al.*, 2011; Aljaroudi *et al.*, 2012; Millen *et al.*, 2014). In patients with RA, central adiposity is associated with systemic inflammation and metabolic risk factors (Giles *et al.*, 2010). Also, the waist-hip ratio is related to atherosclerosis in RA and this association is explained by metabolic risk factors (Solomon *et al.*, 2012). The current findings suggest that low diastolic blood pressure may need to be considered in cardiovascular risk evaluation and further support the role of central obesity in cardiovascular disease risk among RA patients.

An association between RA duration and diastolic function parameters or/and dysfunction according to reported criteria was previously reported in some (Di Franco *et al.*, 2000; Arslan *et al.*, 2006; Rexhepaj *et al.*, 2006; Canturk *et al.*, 2006; Birdane *et al.*, 2007; Udayakumar *et al.*, 2007; Wislowska *et al.*, 2008) but not all studies (Montecucco *et al.*, 1999; Abdul Muizz *et al.*, 2011; Garza-García *et al.*, 2013; Sharma *et al.*, 2015). In the present investigation, RA duration was related to several diastolic function parameters in age, sex and race adjusted analysis. However, in fully adjusted models, RA duration remained associated with septal e' only. RA duration was associated with diastolic dysfunction according to the new criteria only in those with an ejection fraction $\geq$50% and after adjustment for LV mass index. DAS28, erythrocyte sedimentation rate and C-reactive protein concentrations were associated with the E/A ratio in age, sex and race adjusted analysis but not in fully adjusted models.

The study design was cross-sectional, thereby precluding drawing conclusions on cause and effect relationships. The present findings require further evaluation in future longitudinal studies. I did not record echocardiographically determined tricuspid regurgitation velocity. The left atrial volume index was remarkably low in this RA study. Upon application of diastolic classification criteria, we used left atrial volume index threshold values as given in recent recommendations that are largely based on data obtained in American populations (Nagueh *et al.*, 2016). In this regard, Chahal and colleagues (Chahal *et al.*, 2010) reported that the left atrial volume index differs substantially across population groups. Also, previous studies performed in

South Africa have reported similar low left atrial volume indices as in found in our present study (Meel et al., 2016; Peterson et al., 2016). We may therefore have underestimated the actual prevalence of diastolic dysfunction according to the new criteria. Further studies are needed to determine optimal left atrial volume index threshold values in the classification of diastolic dysfunction in the South African context. Lastly, the E/A ratio may be affected by pseudonormalisation (Redfield *et al.*, 2003), which could have impacted our results. Nevertheless, whereas the E/A ratio is not included among the new diastolic dysfunction classification criteria upon their application in persons without systolic dysfunction (Nagueh *et al.*, 2016), our results were materially unaltered in a sensitivity analysis among RA patients with isolated diastolic dysfunction.

In conclusion, in this study we showed that central adiposity and low diastolic blood pressure are independently associated with diastolic function in patients with RA. The application of different reported criteria markedly influences the estimated prevalence and associated risk factors of diastolic dysfunction in RA. As inflammation alters the traditional risk factor profile in RA, it is suggested that inflammation is associated with diastolic dysfunction. However, because of the confounding effect of traditional risk factors, the direct impact of inflammation on cardiac function needs to be investigated.

Chapter 3: Inflammation induced changes in left ventricular diastolic and systolic function in collagen-induced arthritis

The data in this chapter have been submitted for publication in the Cardiovascular Research Journal:

Mokotedi L, Michel FS, Mogane C, Gomes M, Woodiwiss AJ, Norton GR, Dessein PH, Millen AME. Inflammation induced changes in left ventricular diastolic and systolic function in collagen-induced arthritis. *Cardiovasc Res* (under review).

3.1. Abstract

Background. High-grade inflammation may play a pivotal role in the pathogenesis of left ventricular (LV) dysfunction. Direct evidence to support the role of systemic inflammation in mediating impaired LV function in experimental models of RA remains limited. The aim of the present study was to determine the effects of high-grade systemic inflammation on LV diastolic and systolic function in collagen-induced arthritis (CIA).

Methods. To induce arthritis, bovine type-II collagen emulsified in incomplete Freund's adjuvant was injected at the base of the tail into 21 three-month-old Sprague Dawley rats. LV function was assessed by pulsed Doppler, tissue Doppler imaging and Speckle tracking echocardiography nine weeks after the first immunisation. Cardiac collagen content was determined by picrosirius red staining; circulating inflammatory markers were measured using ELISA.

Results. Compared to controls (n=12), CIA rats had reduced myocardial relaxation as indexed by lateral e' (early diastolic mitral annular velocity) and e'/a' (early-to-late diastolic mitral annular velocity) ($p<0.0001$ and $p=0.0002$ respectively) and increased filling pressures as indexed by E/e' ($p=0.04$). No differences in ejection fraction and LV endocardial fractional shortening between the groups were recorded; LV global radial strain ($p<0.0001$), circumferential strain ($p=0.01$), radial strain rate ($p=0.0003$) and circumferential strain rate ($p=0.001$) were reduced in CIA rats compared to controls. Higher concentrations of circulating inflammatory markers were associated with reduced lateral e', e'/a', radial and circumferential strain and strain rate. Greater collagen content was associated with increased concentrations of circulating inflammatory markers and E/e'.

Conclusions. Exposure to high-grade inflammation impairs LV diastolic function and causes greater myocardial deformation without changes in LV contractility in CIA.

3.2. Introduction

Heart failure with a preserved ejection fraction (HFpEF) accounts for more than 50% of all heart failure cases and is associated with an increased morbidity and mortality (Owan *et al.*, 2006). There are currently no effective treatment strategies for HFpEF (Ilieșiu & Hodorogea, 2018), which highlights the need for a better understanding of its pathophysiology. Impaired left ventricular (LV) diastolic function has been proposed as a pre-clinical measure that underlies the pathophysiology of and frequently progresses to HFpEF (Wan *et al.*, 2014). The multiple aetiologies of diastolic dysfunction and the heterogeneity of HFpEF (Shah & Solomon, 2012; Lewis *et al.*, 2017) underscore the need for further investigations.

Hypertension, diabetes mellitus and obesity comprise traditional cardiovascular risk factors that are associated with diastolic dysfunction (Alsamara & Alharethi, 2014). However, these metabolic abnormalities fail to account for all changes in diastolic function (Crowson *et al.*, 2005). A systemic pro-inflammatory state has been proposed as a mediator for the causal molecular or biochemical mechanisms of diastolic dysfunction in persons exposed to metabolic risk factors (Paulus & Tschöpe, 2013). In this regard, diseases that are associated with chronic high-grade inflammation including rheumatoid arthritis (RA), markedly exacerbate the risk for developing diastolic dysfunction and HFpEF compared to the general population (Nicola *et al.*, 2005; Aslam *et al.*, 2013). In cross-sectional studies, inflammatory markers including interleukin 6 (IL-6) (Liang *et al.*, 2010) and tumour necrosis factor-α (TNF-α) (Tomáš *et al.*, 2013) levels were independently associated with impaired diastolic function in RA. Although there is some controversy, treatment with biological disease modifying anti-rheumatic agents aimed at reducing inflammation has shown improvements in cardiac function in patients with RA (Baniaamam *et al.*, 2018). However, there is currently no direct evidence to support the role of inflammation in mediating impaired diastolic function in experimental models of RA.

Chronic systemic inflammation is also associated with heart failure with a reduced ejection fraction (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). In cross-sectional RA studies, inflammation was not related to reduced ejection fraction (Rudominer *et al.*, 2009; Løgstrup *et al.*, 2014). Recently, velocity, displacement, and deformation imaging (strain and strain-rate) as estimated by speckle-tracking echocardiography (STE) were documented to represent valuable tools in the comprehensive and reliable assessment of myocardial systolic function (Langeland *et al.*, 2005; Bauer *et al.*, 2011; Kleijn *et al.*, 2012; Kadappu & Thomas, 2015). Interestingly, impaired myocardial deformation in the presence of a

normal ejection fraction was reported in RA (Sitia *et al.*, 2012; Fine *et al.*, 2014; Baktir *et al.*, 2015). Myocardial deformation is related to inflammation (Hamdy *et al.*, 2017; Lo Gullo *et al.*, 2018), disease activity and/or severity in RA (Løgstrup *et al.*, 2014; Fine *et al.*, 2014; Baktir *et al.*, 2015). Until recently, the lack of high-sensitive imaging has limited the use of STE in small animal models. Hence, the effects of high-grade inflammation on STE estimated myocardial function have not been studied in experimental models of high-grade inflammation. In the present study, we assessed the effects of high-grade inflammation on diastolic and systolic function and myocardial deformation and motion as estimated by pulsed and tissue Doppler indices, M-mode echocardiography and STE, respectively, in collagen-induced arthritis (CIA).

3.3. Methods

3.3.1. Animals and experimental design

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition, updated by the US National Research Council Committee in 2011 and were approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC number: 2017/03/21C). Thirty-three, three-month-old male Sprague Dawley rats (480-510g) were studied. Rats were housed individually in cages in a temperature-controlled room with a 12-hour light-dark cycle and allowed free access to food and water. During the two week acclimatisation period, blood pressure (BP) was measured twice a week and body weight, paw thickness and articular index scores were measured once a week. Following acclimatisation, rats were randomly assigned to the control group (CNTRL, n=12) that had no intervention, or the collagen-induced arthritis group (CIA, n=21) that were exposed to high-grade inflammation. Body weight, paw thickness, articular index scores and BP were measured once a week for nine weeks. Echocardiography was performed and blood samples were obtained at the end of the nine-week study period.

3.3.2. Arthritis induction and assessment

Experimental arthritis was induced in rats as previously described (Jasemian *et al.*, 2011). Briefly, bovine type II collagen (Chondrex cat. #20021, Redmond, WA, USA) was dissolved in 0.05M acetic acid by gently stirring overnight at 4°C. Equal amounts of dissolved bovine type II collagen (2mg/ml) and incomplete Freund's adjuvant (Chondrex cat. #7002, Redmond, WA, USA) were mixed using an electric homogenizer. The arthritis-inducing emulsion was prepared immediately before immunisation. Under general anaesthesia, rats were immunised with 0.2ml (200µg) of the emulsion by a subcutaneous injection at the base of the tail. To ensure a high incidence and severity of arthritis, a 0.1ml (100µg) booster injection was administered seven days after the first immunisation. Control rats received a subcutaneous

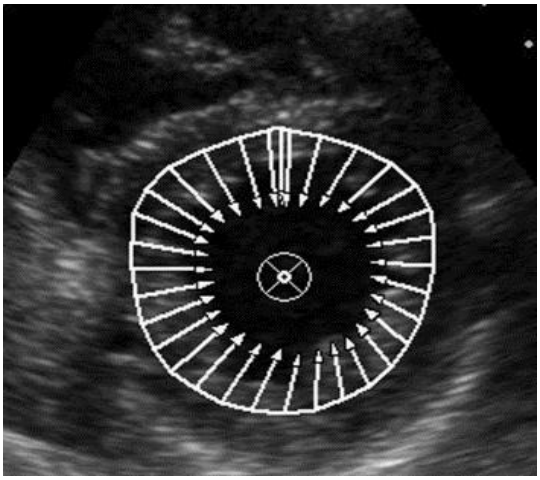
injection of 0.1ml (100 µg) of 0.05M acetic acid at the base of the tail. To quantitatively evaluate the severity of arthritis, rat paws were scored using a previously described five-point scoring system (Kim *et al.*, 2010) where 0 = no swelling or focal redness (normal); 1 = slight swelling and/or focal redness; 2 = low-to-moderate oedema, 3 = pronounced oedema with reduced paw function; 4= excessive oedema with deformity and joint rigidity. The cumulative score for the two hind paws of each rat (maximum score of 8) were used to represent the overall disease severity. As the rat paw arthritis score only provides a subjective quantification of inflammation, hind paw thickness at the ankle and tarsometatarsal joints were measured once every week using a digital caliper as another measure of arthritis severity.

3.3.3. Non-invasive blood pressure measurements

Blood pressure was measured weekly using the tail-cuff technique (Biopac Systems, Santa Barbara, CA, USA). Each rat was placed in a restrainer with a cuff attached to a heated tail. Measurements were taken at midday to avoid diurnal variation.

3.3.4. Echocardiography

Nine weeks after the first immunisation, rats were anaesthetised with ketamine ($100mg.kg^{-1}$) and xylazine ($5mg.kg^{-1}$). Echocardiography was performed by an experienced observer, according to the American Society of Echocardiography conventions, with the rats (anterior chest hair shaved) in the left lateral decubitus position using a high resolution ultrasound probe (10 MHz) coupled to an echocardiogram (Siemens, Acuson SC2000, Diagnostic ultrasound system; Siemens Medical Solutions, USA, Inc.). A description of the echocardiographic approach to the assessment of cardiac geometry, LV diastolic and systolic function is provided in chapter 2 page 21 of the present thesis. To further evaluate systolic function Speckle tracking echocardiography was used to obtain B-mode images to determine LV strain, strain rate, velocity and displacement in the parasternal short axis view (circumferential or rotational). B-mode video loops were selected based upon image quality and well-defined endocardial and epicardial borders with no substantial image artefacts. An average of at least five consecutive heartbeats was used to minimize beat-to-beat variability for all measurements. The endocardium and epicardium were traced semi-automatically using vendor's software (velocity vector imaging). The traces were manually adjusted to ensure adequate tracking of the endocardial and epicardial borders.



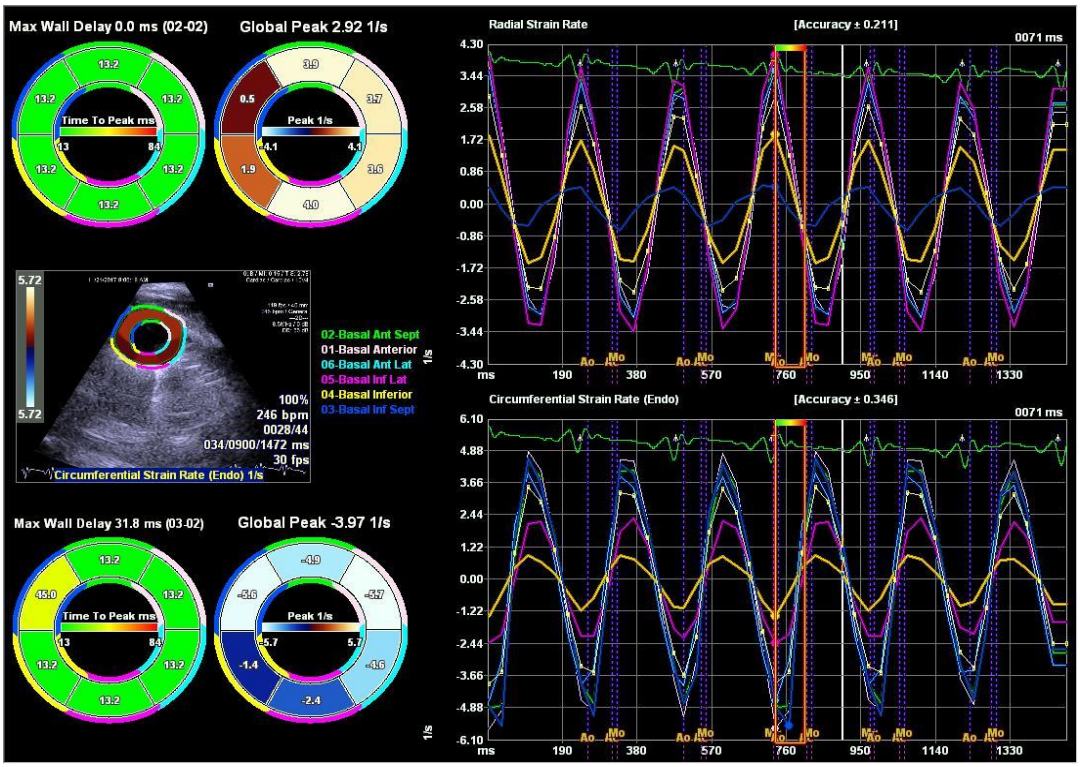


Figure 3.1 Example of speckle-tracking 2D-echocardiographic images obtained from the parasternal short-axis view using velocity vector imaging analysis. The upper panel depicts endocardial regions of interest in the short axis view. The lower panel shows the global peak radial and circumferential strain rate values and regional curves (distinctively coloured curves for 6 left ventricular wall segments).

Tracked images were processed in a frame-by-frame manner for strain measurements. Strain, strain rate, velocity and displacement were calculated in the radial and circumferential planes. Segmental analyses were performed on the short-axis images with the LV being split into the following regions: anterior septal, anterior, lateral, posterior, inferior and septal regions. The average global strain values were obtained from six independent anatomical segments of the LV. During systole, the myocardium shortens in the circumferential plane hence strain and strain rate values are negative whereas, the myocardium thickens/lengthens in the radial plane which results in positive deformation values.

3.3.5. Enzyme-linked immunosorbent assay

Rats were sacrificed with an overdose of anaesthesia (ketamine 200 $mg.kg^{-1}$ and xylazine 10 $mg.kg^{-1}$) followed by thoracotomy. After thoracotomy, blood was sampled and allowed to clot for 2 hours at room temperature. Blood was centrifuged and serum was collected and stored at -80°C until assayed. Enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology Co. Ltd, Wuhan, China) were used to measure serum levels of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β) and C-reactive protein (CRP) according to the instructions of the manufacturer (Elabscience Biotechnology Co. Ltd, Wuhan, China). Each sample was measured in duplicate. The lower detection limit for TNF-α, IL-6, IL-1β and CRP were 78.13 pg/ml, 62.50 pg/ml, 31.2 pg/ml and 0.31 ng/ml respectively, all with coefficients of variation of <10%.

3.3.6. Total collagen content

Cardiac tissue samples were fixed in 10% buffered formalin and routinely processed for paraffin embedding. Five µm thick tissue sections were deparaffinised, rehydrated and stained with a 0.1% Sirius Red solution dissolved in aqueous saturated picric acid for 60 min at room temperature. After washing in acidified water, slides were dehydrated and mounted with DPX mounting. The tissue sections were analysed using a Zeiss Axioskop 2 Plus microscope equipped with a Zeiss AxioCam (Zeiss, Peabody, MA, USA). Tissue sections viewed under bright-field and polarized light were obtained with a 10x objective lens (x100 magnification). Using ImageJ software, the collagen area fraction was calculated for each tissue section by dividing the collagen area by the total tissue area.

3.3.7. Statistical analysis

Data are expressed as means ± SD. Data analysis was performed using SAS software version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Unpaired, two-tailed t-tests were

performed to determine differences in echocardiographic measures and inflammatory markers between the CIA and control groups. Associations between diastolic and systolic function parameters and inflammatory markers were determined by Pearson's correlation coefficients. A P value < 0.05 was considered statistically significant.

3.4. Results

3.4.1. Characterisation of the experimental model

The onset of arthritis in the CIA group occurred within 21-28 days after the first immunisation. Clear signs of inflammation including erythema, oedema and joint rigidity were observed in the CIA group (Figure 3.2A). Compared to the control group, the CIA group showed a significantly higher arthritis score (Figure 3.2B) four weeks after immunisation that persisted for the duration of the study. CIA rats also showed increased swelling at the tarsometatarsal joint (Figure 3.2C) from week five to week nine and increased swelling at the ankle joint (Figure 3.2D) from week four until week nine after the primary immunisation.

3.4.2. Body weight, blood pressure and inflammatory markers

Table 3.1 shows the body weight, blood pressure and inflammatory markers of the control and CIA groups. Nine weeks after the primary immunisation, there were no significant differences in body weight, systolic or diastolic blood pressure between the groups (all $p>0.05$). Serum concentrations of TNF-α, IL-1β, IL-6 and CRP were significantly higher in the CIA group compared to the control group (all $p<0.0001$).

3.4.3. Cardiac weight and geometry

Table 3.2 shows the cardiac geometry of the control and CIA groups. Although the control group had similar heart weights as the CIA group ($p>0.05$) at termination, the (mean ± SD) heart weight indexed to body weight (control: 2.42 ± 0.20; CIA: 2.63 ± 0.26, $p=0.02$), LV weight (control: 0.95 ± 0.09 g; CIA: 1.03 ± 0.12 g, $p=0.04$) and LV weight/ indexed to body weight (control: 1.74 ± 0.12; CIA: 1.97 ± 0.26, $p=0.01$) were significantly higher in the CIA group compared to the control group.

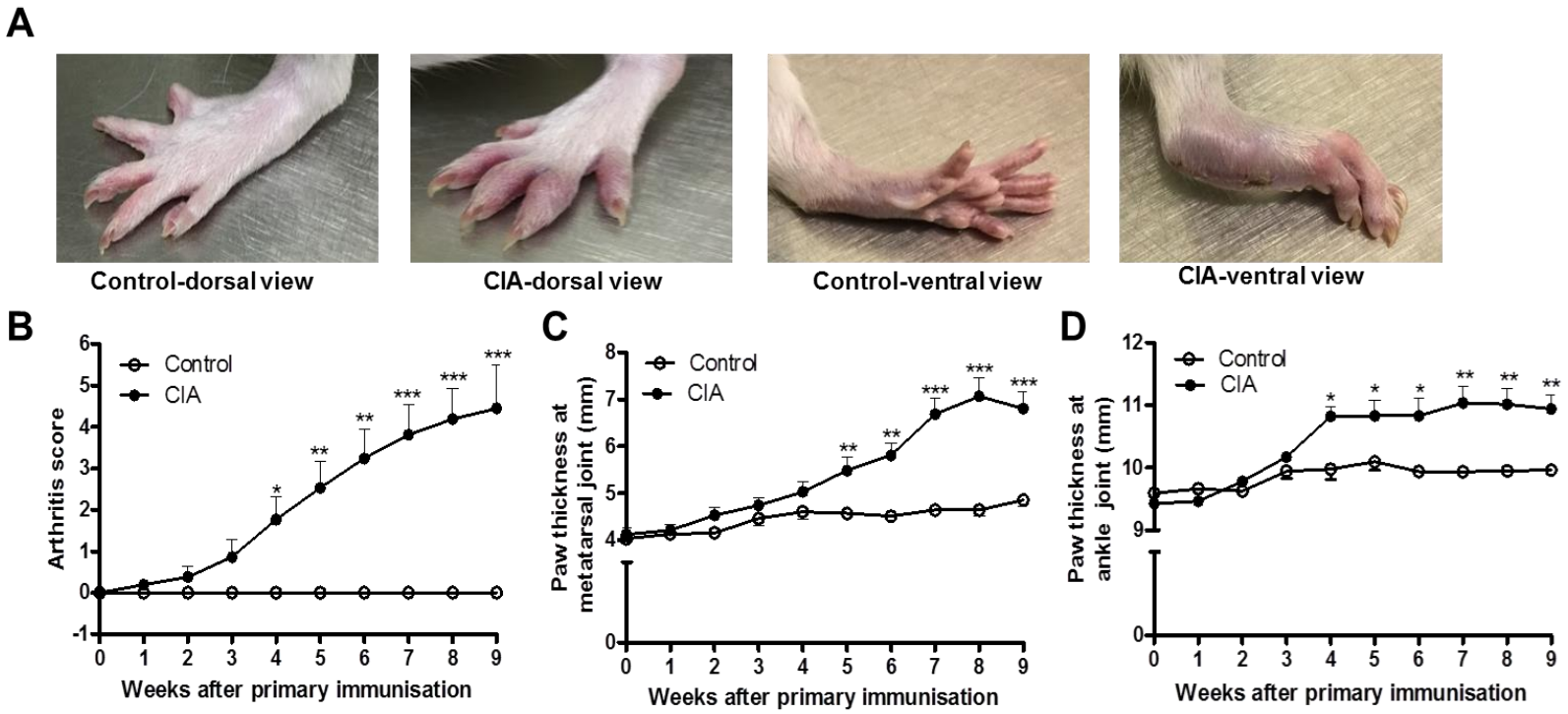


Figure 3.2 Macroscopic observation of joint swelling in collagen-induced arthritis (CIA) rats. (**A**) Photographs of hind paws, (**B**) arthritis scores, (**C**) paw thickness at the tarsometatarsal joint and (**D**) paw thickness at the ankle joint in control and CIA rats nine weeks after the primary immunisation. *P < 0.05 compared to control group. Open circles represent the control group (n=12) and closed circles represent the CIA (n=21) group. **P < 0.01 compared to control group. ***P < 0.0001 compared to control group.

Table 3.1 Body weights, tail cuff blood pressure and inflammatory markers in controls and collagen-induced arthritis rats

	Control (n=12)	**CIA(n=21)**	**P**
Body weight (g)	549 ± 56	529 ± 57	0.35
Blood pressure			
Systolic blood pressure (mm Hg)	133 ± 3	137 ± 13	0.40
Diastolic blood pressure (mm Hg)	92 ± 5	94 ± 6	0.38
Inflammatory markers			
Tumor necrosis factor-α (pg/ml)	277.9 ± 88.1	645.9 ± 128.8	<0.0001
Interleukin-6 (pg/ml)	100.4 ± 72.9	377.8 ± 92.6	<0.0001
Interleukin-1β (pg/ml)	104.5 ± 55.4	245.5 ± 51.3	<0.0001
C-reactive protein (ng/ml)	0.2 ± 0.3	1.0 ± 0.4	<0.0001

Data expressed as means ± SD. CIA, collagen-induced arthritis

Table 3.2 Left ventricular geometry, diastolic and systolic function in controls and collagen-induced arthritis rats

	Control (n=12)	CIA (n=21)	P
Cardiac geometry			
Heart weight (g)	1.32 ± 0.10	1.38 ± 0.13	0.17
Heart weight/body weight x 10^3	**2.42 ± 0.20**	**2.63 ± 0.26**	**0.02**
Right ventricular weight (g)	0.28 ± 0.05	0.27 ± 0.06	0.38
LV weight (g)	**0.95 ± 0.09**	**1.03 ± 0.12**	**0.04**
LV weight/body weight x 10^3	**1.74 ± 0.12**	**1.97 ± 0.26**	**0.01**
LV end diastolic diameter (mm)	6.95 ± 0.07	6.65 ± 0.07	0.25
LV end diastolic posterior wall thickness (mm)	**2.00 ± 0.02**	**2.30 ± 0.03**	**0.002**
LV end systolic diameter (mm)	4.15 ± 0.05	3.90 ± 0.07	0.28
LV end systolic posterior wall thickness (mm)	**2.90 ± 0.03**	**3.14 ± 0.03**	**0.03**
Relative wall thickness (mm)	**0.59 ± 0.10**	**0.69 ± 0.09**	**0.01**
Left ventricular diastolic function			
E (cm/s)	133 ± 17	134 ± 15	0.83
E/A	1.95 ± 0.35	1.75 ± 0.29	0.14
e' (cm/s)	**4.51 ± 0.81**	**3.45 ± 0.39**	**<0.0001**
e'/a'	**1.42 ± 0.22**	**1.04 ± 0.26**	**0.0002**
E/e'	**31.78 ± 10.42**	**39.45 ± 7.34**	**0.04**
Left ventricular systolic function			
Stroke volume (ml)	0.59 ± 0.19	0.53 ± 0.15	0.31
Ejection fraction (%)	75.71 ± 7.75	76.95 ± 9.61	0.71
Endocardial fractional shortening (%)	40.07 ± 6.41	41.52 ± 8.56	0.60
Global radial strain (%)	**11.82 ± 1.02**	**9.68 ± 1.15**	**<0.0001**
Global circumferential strain (%)	**-26.40 ± 1.38**	**-21.86 ± 4.74**	**0.01**
Global radial strain rate (1/s)	**2.10 ± 0.41**	**1.71 ± 0.19**	**0.003**
Global circumferential strain rate (1/s)	**-3.61 ± 0.47**	**-3.10 ± 0.20**	**0.001**
Global radial velocity (cm/s)	**1.37 ± 0.25**	**1.09 ± 0.25**	**0.01**
Global rotational velocity (degree/s)	**54.47 ± 9.05**	**44.66 ± 8.59**	**0.01**
Global radial displacement (mm)	0.70 ± 0.15	0.66 ± 0.17	0.55
Global circumferential displacement (degree)	1.28 ± 0.25	1.10 ± 0.31	0.16

Data expressed as means ± SD. CIA, collagen-induced arthritis; LV, left ventricular

The posterior wall thickness in systole (mean ± SD; control: 2.00 ± 0.02 mm; CIA: 2.30 ± 0.03 mm, p=0.03) and diastole (mean ± SD; control: 2.90 ± 0.03 mm; CIA: 3.14 ± 0.03 mm, p=0.002) were higher in the CIA group compared to controls, which resulted in an increased relative wall thickness in the CIA group (mean ± SD; control: 0.59 ± 0.10 mm; CIA: 0.69 ± 0.09 mm, p=0.01).

3.4.4. Left ventricular systolic function

There were no differences in stroke volume (p=0.31), ejection fraction (p=0.71) or endocardial fractional shortening (p=0.60) between the groups (Table 3.2). Global radial strain (mean ± SD; control: 11.82 ± 1.02 %; CIA: 9.68 ± 1.15 %, p<0.0001), global circumferential strain (mean ± SD; control: -26.40 ± 1.38 %; CIA: -21.86 ± 4.74 %, p=0.01), global radial strain rate (mean ± SD; control: 2.10 ± 0.41 1/s; CIA: 1.71 ± 0.19 1/s, p=0.003) and global circumferential strain rate (mean ± SD; control: -3.61 ± 0.47 1/s; CIA: -3.10 ± 0.20 1/s, p=0.001) were significantly reduced in the CIA group compared to the control group (Table 3.2). Global radial velocity (mean ± SD; control: 1.37 ± 0.25 cm/s; CIA: 1.09 ± 0.25 cm/s, p=0.01) and global rotational velocity (mean ± SD; control: 54.47 ± 9.05 degree/s; CIA: 44.66 ± 8.59 degree/s, p=0.01) were significantly reduced in the CIA group compared to the control group, however global radial displacement and global circumferential displacement were similar between the groups (both p>0.05; Table 3.2).

In segmental analysis in the short-axis radial strain, circumferential strain, radial strain rate and circumferential strain rate were predominantly impaired at the anterior-septal, anterior and septal LV segments in the CIA group compared to controls (all p<0.05; Table 3.3). In the lateral LV segment, only circumferential strain (p=0.02) and radial strain rate (p=0.05) were reduced in the in the CIA group compared to controls (Table 3.3). Strain or strain rate was not different between the groups in the posterior or inferior segments (all p>0.05; Table 3.3). Table 3.4 shows that radial velocity was significantly reduced at the anterior septal (p=0.02), anterior (p=0.05) and septal (p=0.03) LV segments in the CIA group compared to controls.

Rotational velocity was significantly reduced at the anterior septal (p=0.004) and anterior (p=0.04) LV segments. There were no differences in the radial or circumferential displacement in any of the segments between the groups (Table 3.4).

Table 3.3 Short- axis systolic segmental strain and strain rate in the CIA and control groups

	Control (n=12)	CIA (n=21)	P
Radial strain (1/s)			
Anterior Septal	**14.96 ± 3.13**	**11.61 ± 3.79**	**0.03**
Anterior	**15.89 ± 3.09**	**12.06 ± 4.78**	**0.04**
Lateral	13.29 ± 3.78	12.28 ± 6.44	0.66
Posterior	13.90 ± 3.79	12.77 ± 3.80	0.47
Inferior	12.71 ± 3.49	11.29 ± 4.21	0.49
Septal	**13.39 ± 6.28**	**8.31 ± 2.83**	**0.01**
Circumferential strain (1/s)			
Anterior Septal	**-22.85 ± 7.41**	**-16.95 ± 5.45**	**0.03**
Anterior	**-28.04 ± 6.63**	**-21.48 ± 6.66**	**0.02**
Lateral	**-27.95 ± 6.24**	**-21.36 ± 7.27**	**0.03**
Posterior	-25.99 ± 7.21	-22.13 ± 8.17	0.24
Inferior	-26.37 ± 6.42	-21.79 ± 7.61	0.28
Septal	**-26.68 ± 4.56**	**-22.34 ± 5.09**	**0.04**
Radial strain rate (%)			
Anterior Septal	**2.07 ± 0.18**	**1.79 ± 0.48**	**0.05**
Anterior	**2.09 ± 0.24**	**1.79 ± 0.35**	**0.03**
Lateral	**2.11 ± 0.23**	**1.80 ± 0.42**	**0.05**
Posterior	2.28 ± 0.38	2.05 ± 0.63	0.32
Inferior	2.03 ± 0.21	1.71± 0.34	0.21
Septal	**2.07 ± 0.47**	**1.59 ± 0.49**	**0.02**
Circumferential strain rate (%)			
Anterior Septal	**-3.28 ± 0.77**	**-2.47 ± 0.61**	**0.01**
Anterior	**-3.59 ± 0.57**	**-3.01 ± 0.68**	**0.05**
Lateral	-3.62 ± 0.36	-3.07 ± 0.81	0.06
Posterior	-3.62 ± 0.39	-3.56 ± 0.96	0.87
Inferior	-3.54 ± 0.45	-3.24 ± 0.72	0.61
Septal	**-3.99 ± 0.46**	**-3.16 ± 0.36**	**0.05**

Data expressed as means ± SD. CIA, collagen-induced arthritis

Table 3.4 Short-axis systolic segmental velocity and displacement in the CIA and control groups

	Control (n=12)	CIA (n=21)	P
Radial velocity (cm/s)			
Anterior Septal	**1.28 ± 0.24**	**1.08 ± 0.16**	**0.02**
Anterior	**1.24 ± 0.28**	**1.03 ± 0.23**	**0.05**
Lateral	1.19 ± 0.23	1.04 ± 0.27	0.15
Posterior	1.16 ± 0.23	1.12 ± 0.18	0.67
Inferior	1.18 ± 0.26	1.14 ± 0.21	0.68
Septal	**1.26 ± 0.27**	**1.04 ± 0.21**	**0.03**
Rotational velocity (degree/s)			
Anterior Septal	**54.27 ± 9.47**	**36.63 ± 6.79**	**0.004**
Anterior	**45.35 ± 12.05**	**35.46 ± 10.03**	**0.04**
Lateral	62.01 ± 28.30	53.66 ± 30.08	0.49
Posterior	61.49 ± 29.22	44.13 ± 21.28	0.10
Inferior	46.64 ± 17.45	48.58 ± 19.64	0.47
Septal	56.19 ± 19.80	54.96 ± 28.31	0.91
Radial displacement (mm)			
Anterior Septal	0.78 ± 0.10	0.68 ± 0.15	0.08
Anterior	0.68 ± 0.12	0.59 ± 0.24	0.31
Lateral	0.65 ± 0.10	0.69 ± 0.28	0.59
Posterior	0.75 ± 0.13	0.69 ± 0.19	0.36
Inferior	0.69 ± 0.15	0.65 ± 0.16	0.57
Septal	0.66 ± 0.07	0.67 ± 0.23	0.94
Circumferential displacement (degree)			
Anterior Septal	1.48 ± 0.80	1.01 ± 0.64	0.12
Anterior	1.37 ± 0.62	1.23 ± 0.63	0.58
Lateral	1.12 ± 0.42	0.89 ± 0.37	0.16
Posterior	1.28 ± 0.62	1.14 ± 0.91	0.67
Inferior	1.24 ± 0.54	1.05 ± 0.86	0.26
Septal	1.31 ± 0.55	0.94 ± 0.15	0.06

Data expressed as means ± SD. CIA, collagen-induced arthritis

3.4.5. Total collagen content

Figure 3.3A shows greater collagen accumulation in CIA rats compared to controls as evidenced by the increased red staining of cardiac tissue sections visualised under bright-filed microscopy. Figure 3.3B shows cardiac tissue sections under polarized light, where large collagen fibres appear orange or yellow and thin fibres appear green. The total collagen content was significantly greater in the CIA group compared to controls (mean ± SD; control: 2.45 ± 0.71 %; CIA: 8.62 ± 3.39 %, p=0.01; Figure 3.3C).

3.4.6. Associations between inflammatory markers, cardiac geometry and collagen content

Figure 3.4 shows that relative wall thickness was related to increased circulating inflammatory marker concentrations (TNF-α: r=0.45; p=0.009, IL6: r=0.44; p=0.01, IL-1β: r=0.39; p=0.03, CRP: r=0.40; p=0.02). LV weight indexed to body weight was related to increased TNF-α (r=0.44; p=0.01) and there was a trend toward significance with IL-6 (r=0.34; p=0.06). Increased collagen content was associated with higher serum concentrations of TNF-α (r=0.76; p=0.005) and IL-6 (r=0.70; p=0.01) and there was a trend towards significance with IL-1β (r=0.57; p=0.07) and CRP (r=0.58; p=0.06).

3.4.7. Associations between inflammatory markers and diastolic function

Figure 3.5 shows the associations between circulating inflammatory marker concentrations and markers of diastolic function. Reduced e' was associated with higher serum concentrations of TNF-α (r=-0.54; p=0.001), IL-6 (r=-0.57; p=0.0007), IL-1β (r=-0.53; p=0.002) and CRP (r=-0.48; p=0.006). Reduced e'/a' was associated with higher serum concentrations of TNF-α (r=-0.44; p=0.01), IL-6 (r=-0.49; p=0.005), IL-1β (r=-0.46; p=0.008) and CRP (r=-0.39; p=0.03, Figure 3.5). There was no association between E/A and serum concentrations of TNF-α (r=-0.22; p=0.28), IL-6 (r=-0.14; p=0.50), IL-1β (r=-0.17; p=0.42), or CRP (r=-0.09, p=0.67; Figure 3.5). There was a trend toward an association between E/e' and serum concentrations of TNF-α (r=0.36; p=0.07), and IL-6 (r=0.37; p=0.06) (Figure 3.5).

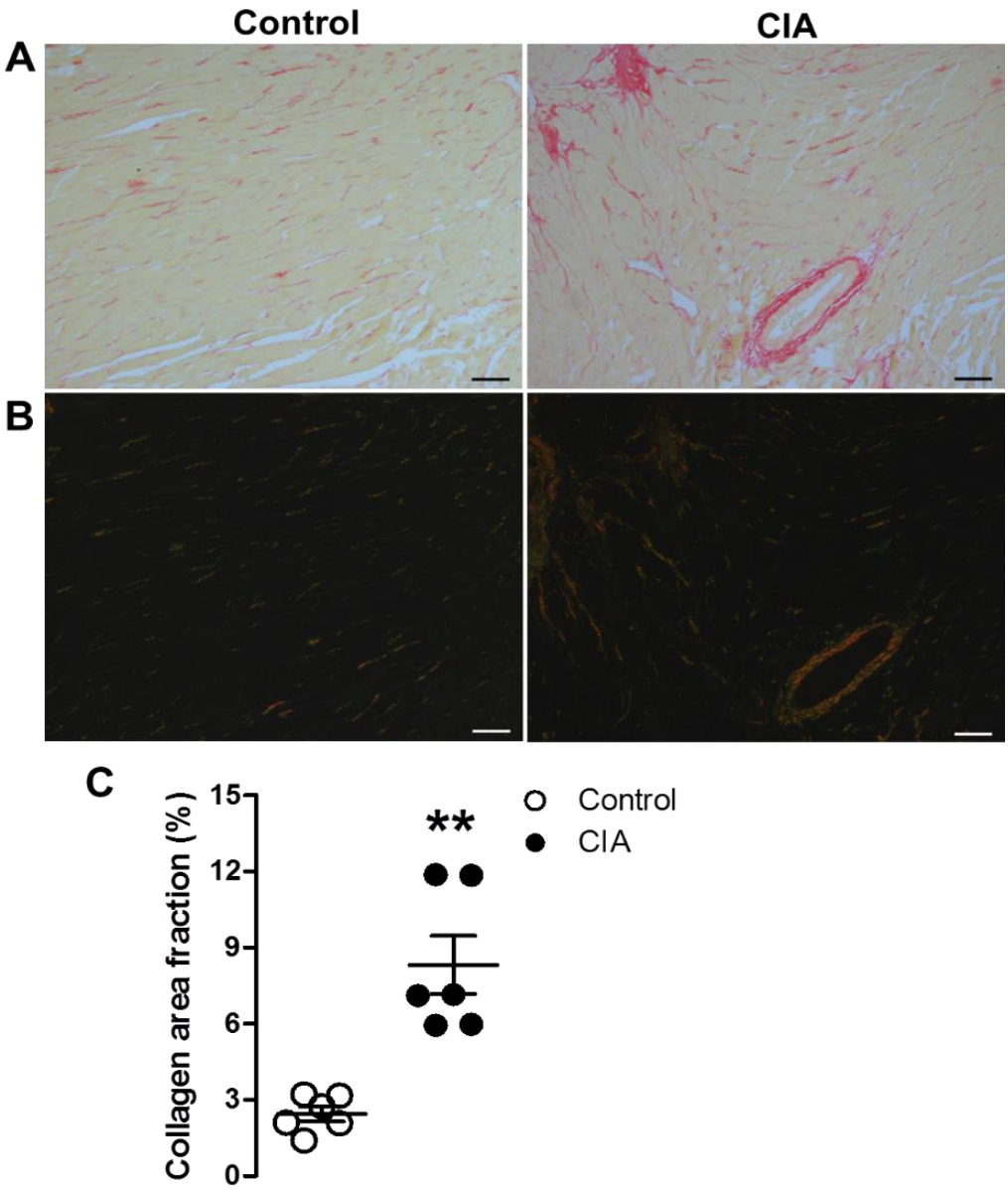


Figure 3.3 Total collagen content in cardiac tissue of control and collagen-induced arthritis (CIA) rats. Representative Picrosirius red stained micrographs imaged at x100 magnification viewed in (**A**) bright-field and under (**B**) polarized light. (**C**) Total collagen content (% area fraction) calculated from Picrosirius red stained sections (n=6 per group). **P < 0.01 CIA compared to control. Scar bar=200µm

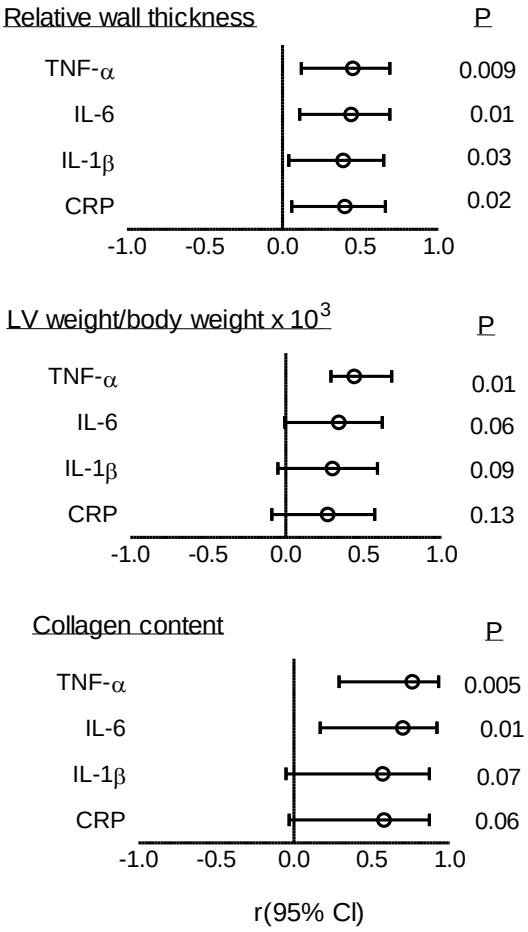


Figure 3.4 Associations of cardiac geometry and total collagen content with inflammatory cytokines. TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; IL-1β, interleukin 1 beta; CRP, C-reactive protein. Open circles represent the correlation coefficient (r) and horizontal lines represent the 95% confidence intervals (CI).

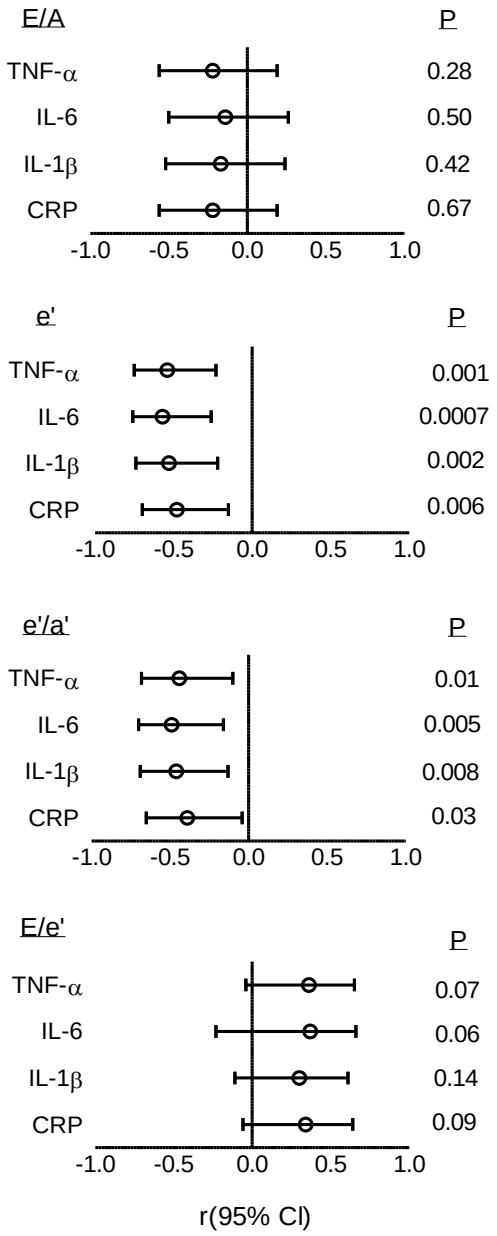


Figure 3.5 Associations between left ventricular diastolic function markers and circulating inflammatory markers. TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; IL-1β, interleukin 1 beta; CRP, C-reactive protein. Open circles represent the correlation coefficient (r) and horizontal lines represent the 95% confidence intervals (Cl).

3.4.8. Associations between inflammatory markers and systolic function

There were no significant associations between serum concentrations of inflammatory markers and ejection fraction (TNF-α: r=0.22; p=0.22, IL-1β: r=0.19; p=0.28, IL-6: r=0.20; p=0.26, CRP: r=0.17; p=0.37) or endocardial fractional shortening (TNF-α: r=-0.19; p=0.29, IL-β: r=-0.19; p=0.30, IL-6: r=-0.14; p=0.45, CRP: r=-0.08, p=0.65).

Figure 3.6 shows that greater myocardial deformation was associated with higher levels of circulating inflammatory markers. Reduced radial strain was associated with higher serum concentrations of TNF-α (r=-0.70; p<0.0001), IL-6 (r=-0.70; p<0.0001), IL-1β (r=-0.60; p=0.001) and CRP (r=-0.71; <0.0001; Figure 3.6). Reduced (less negative) circumferential strain was associated with higher serum concentrations of TNF-α (r=0.46; p=0.03) and IL-1β (r=0.40; p=0.05; Figure 3.6). No significant associations were shown between circumferential strain and IL-6 (r=0.35; p=0.08) or CRP (r=0.38; p=0.07) concentrations (Figure 3.6). Reduced radial strain rate was associated with higher TNF-α (r=-0.48; p=0.01), IL-6 (r=-0.51; p=0.008), IL-1β (r=-0.55; p=0.004) and CRP (r=-0.52; p=0.007) concentrations (Figure 3.6). Reduced (less negative) circumferential strain rate was associated with higher serum concentrations of TNF-α (r=0.59; p=0.002), IL-6 (r=0.63; p=0.007), IL-1β (r=0.69; p=0.0001) and CRP (r=0.61; p=0.001; Figure 3.6). There were no associations between markers of myocardial motion (velocity and displacement) and circulating inflammatory markers (data not shown).

3.5. Discussion

In the present study, CIA in adult, male Sprague Dawley rats caused impaired LV relaxation (decreased e') and increased LV stiffness (decreased e'/a') and LV filling pressures (increased E/e'). These diastolic abnormalities were noted despite a normal blood pressure and unaltered mitral inflow patterns (E/A). Whereas LV chamber pump function (ejection fraction) and contractility (fractional shortening) were preserved, arthritic rats demonstrated early myocardial dysfunction as indexed by myocardial deformation (radial and circumferential strain and strain rate). Elevated inflammatory cytokines were associated with impaired diastolic function (e' and e'/a'), increased collagen content and global radial and circumferential strain and strain rate. These findings indicate that LV diastolic function and myocardial deformation are adversely affected, independent of load when exposed to high-grade inflammation in rats.

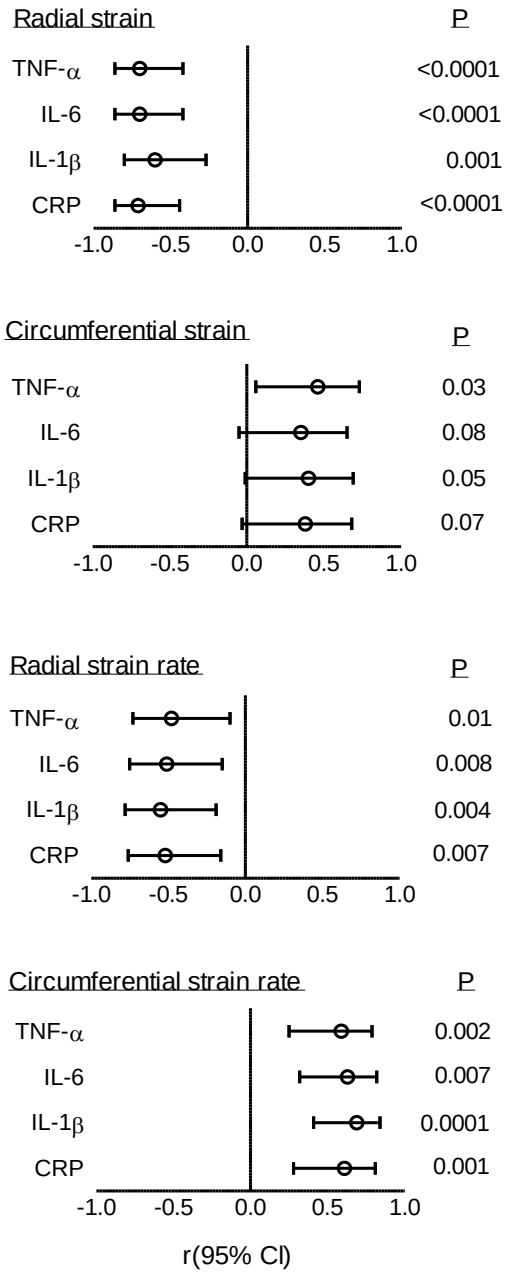


Figure 3.6 Associations between left ventricular systolic function and circulating inflammatory markers. TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; IL-1β, interleukin 1 beta; CRP, C-reactive protein. Open circles represent the correlation coefficient (r) and horizontal lines represent the 95% confidence intervals (Cl).

In the present study, TDI determined e' and e'/a' were reduced while E/e' was increased in CIA rats compared to controls. These results are consistent with the impaired diastolic function that was reported in clinical RA studies (Torre-Amione *et al.*, 1996; Liang *et al.*, 2010; Aslam *et al.*, 2013; Tomáš *et al.*, 2013). Only one previous study in rats showed impaired early diastolic filling velocity (E) in female CIA rats (Dai *et al.*, 2017). However, no other markers of diastolic function were assessed in the respective investigation (Dai *et al.*, 2017). In the current study, compared to controls, there were no changes in pulsed Doppler markers of diastolic function (E/A) in CIA rats. These discrepancies could possibly be explained by differences in rat strain and the onset, severity and duration of the different arthritic protocols used in the latter and current studies. Although pulsed Doppler is a valid tool to assess mitral inflow velocity, TDI determined markers of diastolic function are more strongly related to invasively assessed parameters of diastolic function (Kasner *et al.*, 2007). The changes in diastolic function markers that we observed in CIA rats are consistent with a pseudonormal filling pattern in moderate diastolic dysfunction (Redfield *et al.*, 2003).

Hypertension is reportedly strongly related to diastolic dysfunction and considered to be the leading cause of heart failure (Levy *et al.*, 1996). However, inflammation can drive the molecular and cellular myocardial remodelling in diastolic dysfunction, independent of its aetiology (Paulus & Tschöpe, 2013; Suthahar *et al.*, 2017). In various disease states associated with diastolic dysfunction, including hypertension, myocardial remodelling is effectuated by increases in myocardial collagen content and cross-linking, which leads to increased myocardial fibrosis and myocardial diastolic stiffness (Harvey *et al.*, 2016; Suthahar *et al.*, 2017). Elevated passive myocardial stiffness contributes to the pathogenesis of diastolic dysfunction (Shah & Solomon, 2012; Lewis *et al.*, 2017). Similar to previous studies on CIA in rats (Reynolds *et al.*, 2012; Verhoeven *et al.*, 2017*a*), there were no differences in blood pressure between the CIA and control groups in the current investigation. Yet, CIA rats had increased relative wall thickness, increased ventricular stiffness as indexed by reduced e'/a' and increased collagen content, each of which was associated with circulating inflammatory markers.

Indeed, growing evidence suggests that inflammatory cytokines mediate hypertrophic remodelling and myocardial fibrosis through regulation of collagen synthesis and matrix metalloproteinase activity of cardiac fibroblasts (Meléndez *et al.*, 2010; Westermann *et al.*, 2011). Additionally, recent findings support a role for systemic inflammation in the hypophosphorylation of the cytoskeletal protein titin, which contributes to passive myocardial stiffness (Franssen *et al.*, 2016). Both collagen and titin phosphorylation contribute to passive myocardial stiffness (Zile *et al.*, 2015). Herein, we report for the first time *in vitro* that systemic inflammation results in diastolic dysfunction, independent of load (blood pressure). Taken together, these findings support the paradigm in which inflammation forms an important part of

the pathophysiological mechanisms that give rise to myocardial remodelling and diastolic dysfunction (Paulus & Tschöpe, 2013).

Besides passive myocardial stiffness, impaired active relaxation also contributes to the pathogenesis of diastolic dysfunction (Shah & Solomon, 2012; Lewis *et al.*, 2017). Congruently, we found that myocardial relaxation (reduced e') was impaired in the CIA group compared to the control group. Impaired relaxation (reduced e') was associated with increased circulating inflammatory marker concentrations. Ion channel remodelling and lower resting energy reserve decrease the ability of the sarcoplasmic reticulum to sequester calcium, which increases diastolic tension and hence impairs cardiomyocyte relaxation (Selby *et al.*, 2011; Adeniran *et al.*, 2015). Inflammatory cytokines mediate a number of cellular changes that cause abnormalities in the active calcium-dependent processes involved in myocardial relaxation (McTiernan *et al.*, 1997; Combes *et al.*, 2002). These results show that exposure to high-grade inflammation impairs myocardial relaxation *in vitro*.

Rats in the CIA group had higher filling pressures compared to the control group. Previous studies documented that myocardial stiffness (Kasner *et al.*, 2007), collagen content (Kasner *et al.*, 2011) and impaired relaxation (Selby *et al.*, 2011) each relate to increased LV filling pressures. In the present study, inflammatory markers tended to relate to increased filling pressure but these associations were not significant ($p=0.06$ to 0.14). This suggests that increased filling pressure may not be directly related to inflammation but is rather mediated by the extra-cellular matrix driven passive stiffness and calcium-dependent relaxation abnormalities induced by inflammation.

Similar to described findings in RA patients (Kleijn *et al.*, 2012; Sitia *et al.*, 2012; Fine *et al.*, 2014; Baktir *et al.*, 2015), despite a normal pump function and contractility, LV systolic myocardial deformation (radial and circumferential strain and strain rate) and myocardial motion (circumferential rotation rate and radial velocity) were impaired in the CIA group. Myocardial deformation, but not contractility, was associated with increased circulating inflammatory cytokine concentrations. This contrast the findings of a previous *in vitro* mouse model of collagen antibody-induced arthritis (CAIA), where contractility as assessed by conventional approaches, was impaired (Pironti *et al.*, 2018). While CAIA shares many characteristics with CIA, the differences in onset and severity of inflammation, as well as duration of the studies, may explain the differences in contractility between these two studies. Although STE was not performed in the latter study, the authors reported dysregulation of intracellular calcium signal 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 (Adeniran *et al.*, 2015). In this regard, action potentials are prolonged and calcium is elevated in diastole, which limits the availability of calcium in systole and thereby reduce contractile force (Adeniran

et al., 2015). Although relaxation and contractility are impaired, ejection fraction is preserved because of the hypertrophied myocardial wall in HFpEF (Adeniran *et al.*, 2015). However, concentric cardiac remodelling, increases myocardial tissue stress and strain (Adeniran *et al.*, 2015). In our study, concentric remodelling (increased relative wall thickness) may have ensured the maintenance of ejection fraction while increasing myocardial strain.

In the present CIA investigation, myocardial deformation and motion were consistently reduced in the anterior septal, anterior and septal LV myocardial segments. The lateral LV segment was affected to a lesser extent whereas the inferior and posterior walls were unaltered. Indeed, the septal wall has a greater sensitivity to fibrosis (Bravo *et al.*, 2016) and increased septal myocardial stress has been associated with early structural remodelling in patients with HFpEF (Yalçin *et al.*, 2015). Previously reported findings together with our findings suggest that structural remodelling is most extensive in the septal and anterior walls during the early stages of inflammation-induced myocardial dysfunction, which results in greater strain in these areas.

Inflammation was associated with increased myocardial deformation in previous cross-sectional RA studies (Sitia *et al.*, 2012; Fine *et al.*, 2014; Baktir *et al.*, 2015; Hamdy *et al.*, 2017; Lo Gullo *et al.*, 2018). To my knowledge, this is the first study to show direct evidence of inflammation induced early myocardial deformation in a pre-clinical model of high-grade inflammation. Although conventional echocardiography and TDI are considered reliable methods in assessing pump function and contractility, these procedures lack sensitivity in detecting subtle myocardial changes (Bauer *et al.*, 2011; Kadappu & Thomas, 2015). STE may provide a more sensitive alternative to identify early myocardial contractile changes in RA.

The current study has limitations. Firstly, we used non-invasive approaches rather than catheter-based systems to assess diastolic function. However, tissue Doppler indices of diastolic function were previously shown to correlate well with invasively measured LV relaxation, stiffness and filling pressures (Kasner *et al.*, 2007). Secondly, the present study focused on short-axis circumferential and radial STE analyses. Although longitudinal STE analyses may have provided additional information on LV myocardial deformation and motion, circumferential STE analyses are highly reliable in the assessment of LV function (Kleijn *et al.*, 2012). Thirdly, the effects of CIA on cardiac function were assessed in male rats. Future studies should determine whether inflammation impairs cardiac function in female rats. Lastly, we measured circulating CRP levels using a conventional CRP assay kit which is not sensitive enough to detect lower CRP levels, especially for the control group. Future studies should determine circulating CRP levels using both conventional and hs-CRP assay kits.

In conclusion, in this study, we showed that exposure to high-grade inflammation *in vitro* causes impaired LV diastolic function and greater subclinical myocardial deformation despite a normal blood pressure and preserved ejection fraction. These findings add to the evidence that

systemic inflammation can mediate myocardial remodelling that leads to diastolic dysfunction and myocardial deformation. Although evidence suggests that inflammation directly affects the cardiomyocyte, a new paradigm recently suggested that systemic inflammation induces impaired microvascular function which alters myocardial function. Investigating the role of inflammation on endothelial function in a pre-clinical model of RA not confounded by traditional risk factors may cast further light on the mechanisms responsible for adverse cardiovascular outcomes in RA.

Chapter 4: Vascular Cell Adhesion Molecule-1 Mediates Inflammation Induced Endothelial Dysfunction in Collagen-Induced Arthritis

The data in this chapter have been submitted for publication in the Arthritis & Rheumatism Journal:

Mokotedi L, Millen AME, Mogane C, Gomes M, Woodiwiss AJ, Norton GR, Dessein PH, Michel FS. Vascular Cell Adhesion Molecule-1 Mediates Inflammation Induced Endothelial Dysfunction in Collagen-Induced Arthritis. *Arthritis Rheum* (under review).

4.1. Abstract

Background. Evidence supporting the role of inflammation on endothelial dysfunction in the context of rheumatoid arthritis (RA) is limited. The aim of the present study was to determine the role of high-grade inflammation on endothelial function and its association with biomarkers of endothelial dysfunction in collagen-induced arthritis.

Methods. Sprague-Dawley rats were randomly divided into a control (n=12) or collagen-induced arthritis (CIA; n=21) group. To induce arthirtis, bovine type-II collagen emulsified in incomplete Freund's adjuvant was injected at the base of the tail. Nine weeks after the primary immunisation, vascular reactivity in mesenteric and saphenous arteries was assessed using wire myography. Serum concentrations of inflammatory markers (tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β), C-reactive protein (CRP)) and biomarkers of endothelial dysfunction (vascular cell adhesion molecule-1 (VCAM-1) and asymmetric dimethylarginine (ADMA)) were measured by ELISA.

Results. Acetylcholine-induced relaxation in mesenteric arteries was significantly impaired in the CIA group compared to controls ($p=0.01$), while the responses to potassium chloride, phenylephrine and sodium nitroprusside were similar between the groups ($P>0.05$). No differences in vascular reactivity in saphenous arteries were observed between the groups ($P>0.05$). Compared to controls, TNF-α, IL-6, IL-1β, CRP (all $p<0.00001$) and VCAM-1 ($p=0.02$) were elevated in the CIA group, however ADMA was similar between the groups ($p=0.83$). TNF-α ($r=0.38$; $p=0.03$), IL-6 ($r=0.37$; $p=0.03$), IL-1β ($r=0.41$; $p=0.02$) and CRP ($r=0.36$, $p=0.04$) were significantly associated with VCAM-1. VCAM-1 was inversely associated with the maximal relaxation (E_{max}) to acetylcholine in mesenteric arteries ($r=-0.49$; $p=0.01$).

Conclusion. Exposure to high-grade inflammation impairs endothelium-dependent relaxation in mesenteric arteries of arthritic rats. Inflammation-induced increases in VCAM-1 concentrations may be an early predictor of impaired endothelium-dependent relaxation in microvessels.

4.2. Introduction

Rheumatoid arthritis (RA) is associated with a greater risk of cardiovascular mortality (Aviña-Zubieta *et al.*, 2008). The increased incidence of cardiovascular events in RA (Avina-Zubieta *et al.*, 2012) is predominantly as a result of accelerated atherosclerosis (Riise *et al.*, 2001) which cannot be fully explained by traditional cardiovascular risk factors (del Rincón *et al.*, 2001). High-grade inflammation reportedly plays a key role in the onset and progression of atherosclerosis in RA (Libby, 2008).

Endothelial dysfunction, as measured by flow-mediated dilation, is a common feature in RA (Di Minno *et al.*, 2015) and is associated with markers of atherosclerosis (González-Juanatey *et al.*, 2011). However various methodological concerns with regard to flow-mediated dilation (Bots *et al.*, 2005; Yeboah *et al.*, 2012), has necessitated the use of surrogate circulating biomarkers of endothelial dysfunction. Indeed circulating biomarkers of endothelial dysfunction including soluble intercellular adhesion molecules or asymmetric dimethylarginine (ADMA) relates to endothelial dysfunction in RA (Wållberg-Jonsson *et al.*, 2008; Şentürk *et al.*, 2016). Biomarkers of endothelial dysfunction have shown strong relations with atherosclerosis in RA (Wållberg-Jonsson *et al.*, 2002; Dessein *et al.*, 2005). Besides their role as biomarkers of endothelial dysfunction, they lend insight to the possible mechanisms responsible for the endothelial dysfunction. In this regard, expression of the cell surface protein vascular adhesion molecule 1 (VCAM-1) has been implicated in the recruitment of lymphocytes which mediate vascular remodelling (Liao, 2013). ADMA, an endogenous inhibitor of nitric oxide (NO) production causes a reduced nitric oxide synthase (eNOS) which also leads to endothelial dysfunction (Sibal *et al.*, 2010).

In RA, chronically elevated pro-inflammatory cytokine concentrations are believed to cause functional and structural changes in both endothelial and smooth muscle cells, resulting in endothelial dysfunction (Steyers and Francis, 2014). Indeed inflammatory markers have been associated with endothelial dysfunction in RA patients (Klimiuk *et al.*, 2002; Yki-Järvinen *et al.*, 2003; Vaudo *et al.*, 2004; Dessein *et al.*, 2005; Arosio *et al.*, 2007; Galarraga *et al.*, 2008). Treatment with anti-inflammatory agents improve endothelial function in RA patients (Kotani *et al.*, 2016; Ruiz-Limón *et al.*, 2017). However, as a result of confounding factors and the cross-sectional nature of the majority of the aforementioned studies, the independent contribution of inflammation to impaired endothelial function is uncertain. In this regard, several animal models of RA have been developed in an attempt to understand the mechanisms of inflammation-induced vascular dysfunction in high grade inflammation.

Inflammation-induced endothelial dysfunction has been reported in adjuvant-induced arthritis (Totoson *et al.*, 2015, 2016). However, the collagen-induced arthritis (CIA) model is

thought to provide a more accurate model of human RA compared to other experiment models (Bendele, 2001; Brand *et al.*, 2007; Hegen *et al.*, 2008). However, to date, only a few studies have investigated endothelial function in CIA (Reynolds *et al.*, 2012; He *et al.*, 2013; Dooley *et al.*, 2015; Palma Zochio Tozzato *et al.*, 2016). In this regard, endothelial dysfunction has been reported in mice aorta (He *et al.*, 2013) and coronary or digital arteries of sheep (Dooley *et al.*, 2015), while others have shown unaltered endothelial function in mice aorta (Reynolds *et al.*, 2012; Palma Zochio Tozzato *et al.*, 2016). These conflicting results emphasises the need to elucidate the role of inflammation on endothelial function in CIA.

The use of biomarkers of endothelial function and hence the mechanisms underlying endothelial dysfunction in pre-clinical studies are limited. Therefore, the present study aimed to investigate the impact of high-grade inflammation on endothelial function and to determine the relationship between systemic inflammatory markers, biomarkers of endothelial dysfunction and vascular reactivity in a model of CIA.

4.3. Materials and Methods

4.3.1. Animals and experimental design

All experimental procedures were approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC number: 2017/03/21C) and conducted in compliance with the Guide for the Care and Use of Laboratory Animals. A description of the number of animals used and the experimental design is provided in chapter 3, page 46 of the present thesis.

4.3.2. Arthritis induction and assessment

A description of the induction and evaluation of arthritis is provided in chapter 3, page 46 of the present study.

4.3.3. Non-invasive blood pressure measurements

A description of the assessment of blood pressure is provided in chapter 3, page 47 of the present thesis.

4.3.4. Blood glucose and triglyceride concentrations

Three days before termination, rats were fasted overnight and fasting blood glucose and triglyceride concentrations were determined from a drop of blood obtained from the tail vein using a pinprick. Glucose concentrations were determined using a calibrated glucometer (Ascensia EliteTM Blood glucose meter, Bayer Corporation, Mishawaka, USA) and triglycerides were determined using a calibrated Accutrend triglyceride analyzer (Roche, Mannheim, Germany).

4.3.5. Vascular reactivity

Nine weeks after the first immunisation, rats were sacrificed with an overdose of ketamine (200mg.kg-1) and xylazine (10mg.kg-1) followed by thoracotomy. The intestines and the saphenous arteries were removed and placed in a cold modified Krebs-Ringer bicarbonate (control) solution with the following composition (mmol/l): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11.1 glucose. Second branches of mesenteric arteries and the saphenous branch of the femoral arteries were cleaned of fat and connective tissue and the arteries were cut into 2 mm rings and suspended in a wire myograph (model 610M; Danish Myo Technology, Aahrus, Denmark). The rings were suspended between 2 stainless steel wires with a diameter of 40 mm in an organ chamber filled with control solution which was continuously bubbled with 95% O_2 and 5% CO_2 and kept at a constant temperature of 37°C. One of the wires was connected to a movable holder supporting a tension transducer (Grass Instrument Co, Quincy, MA) to collect the isometric force measurements using a data acquisition system (PowerLab 4SP; ADInstruments). The rings were placed under an optimal resting tension as previously described (Christon *et al.*, 2005). After a 60-minute stabilisation period, the arteries were exposed to potassium chloride (KCl, 80 mM), which produced a maximal contraction response and served as the reference contraction. Preparations were exposed to increasing concentration of potassium chloride (1 to 120 mM) or phenylephrine (Phe, 1nM to 0.1 mM (in a half-log increment)). Vascular contraction was expressed as the percentage of KCl (80 mM)-induced increases in tension. The relaxation responses induced by acetylcholine, an endothelium-dependent vasodilator (ACh, 0.1 nM to 10 mM), or sodium nitroprusside, an endothelium-independent vasodilator (SNP, 1 nM to 100 mM) were assessed in half-log increments during phenylephrine (10 μM)-induced contractions. Vascular relaxation was expressed as a percentage of the baseline tension during contractions induced by phenylephrine (10 μM). Potassium chloride, phenylephrine, sodium nitroprusside and acetylcholine were purchased from Sigma (St. Louis, MO, USA).

4.3.6. Enzyme-linked immunosorbent assay

A description of the assessment of inflammatory markers is provided in chapter 3, page 49 of the present thesis. In addition to inflammatory cytokines, serum levels of vascular cell adhesion molecule-1 (VCAM-1) and asymmetric dimethylarginine (ADMA) (biomarkers of endothelial dysfunction) were measured using ELISA kits according to the instructions of the manufacturer. Each sample was tested in duplicate. The lower detection limit for VCAM-1 and ADMA were 12.50 pg/ml and 1.5ng/ml respectively, all with coefficients of variation of <10%.

4.3.7. Statistical analysis

Statistical analysis was performed using the SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Data are expressed as means ± SEM. Unpaired, two-tailed t-tests were performed to determine differences in body weight, arthritis scores, paw thickness, blood pressure, vascular reactivity measures and inflammatory markers between the control and CIA groups. The sensitivity (EC_{50}) and the maximal (E_{max}) responses were determined from regression analysis logistic sigmoid function curves (GraphPad Software Inc, San Diego, CA). Associations between inflammatory markers and biomarkers of endothelial dysfunction and the sensitivity (EC_{50}) and maximal (E_{max}) ACh-induced relaxation were determined by Pearson's correlation coefficients. $P< 0.05$ was considered statistically significant.

4.4. Results

4.4.1. Characterisation of the experimental model

The general characteristics of the experimental model including the disease severity scores and blood pressure are described in chapter 3, page 50 of the present study. Briefly, the arthritis score ($P<0.00001$), hind paw thickness at the tarsometatarsal joint ($P<0.0001$) and the ankle joint ($p=0.0007$) were significantly increased in the CIA group compared to controls nine weeks following the first immunisation (Figure 4.1). There were no differences in systolic (control: 133.2 ± 0.9 mm Hg; CIA: 136.5 ± 2.9 mm Hg) or diastolic (control: 92.4 ± 2 mm Hg; CIA: 94.2 ± 1.2 mm Hg) blood pressure between the control and CIA groups.

4.4.2. Fasting blood glucose and triglyceride concentrations

No differences were observed in blood glucose (control: 4.03 ± 0.59 mmol/L; CIA: 4.07 ± 0.61 mmol/L; $p=0.91$) or blood triglyceride concentrations (control: 1.45 ± 0.29 mmol/L; CIA: 1.46 ± 0.07 mmol/L; $p=0.97$) between the control and CIA groups.

4.4.3. Vascular reactivity differences in control and CIA groups

The maximal contractile response to KCl was similar between the experimental groups in mesenteric arteries (control: 14.1 ± 1.0 mN; CIA: 15.3 ± 0.7 mN; $p=0.36$) and saphenous arteries (control: 14.8 ± 1.2 mN; CIA: 14.9 ± 0.9 mN; $p=0.87$). The vascular contractile responses to KCl and Phe in mesenteric and saphenous arteries are shown in Figure 4.2. The sensitivity (EC_{50}) or maximal (E_{max}) contraction to KCl and Phe were similar between the control and CIA groups in both the mesenteric and saphenous arteries (Figure 4.2; $P>0.05$).

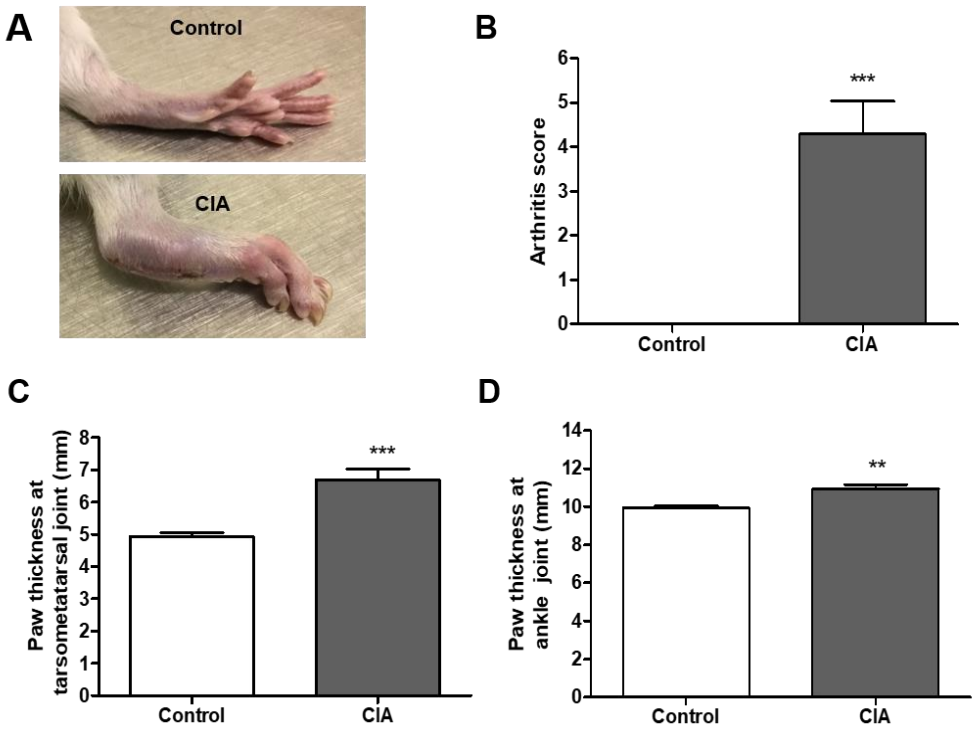


Figure 4.1 Changes in **(A)** hind limbs, **(B)** arthritis scores, **(C)** paw thickness at the metatarsal joint and **(D)** paw thickness at the ankle joint in control (n=12) and CIA (n=21) rats nine weeks after the primary immunisation. Data expressed as means ± SEM. **P < 0.01 compared to control group. ***P < 0.0001 compared to control group.

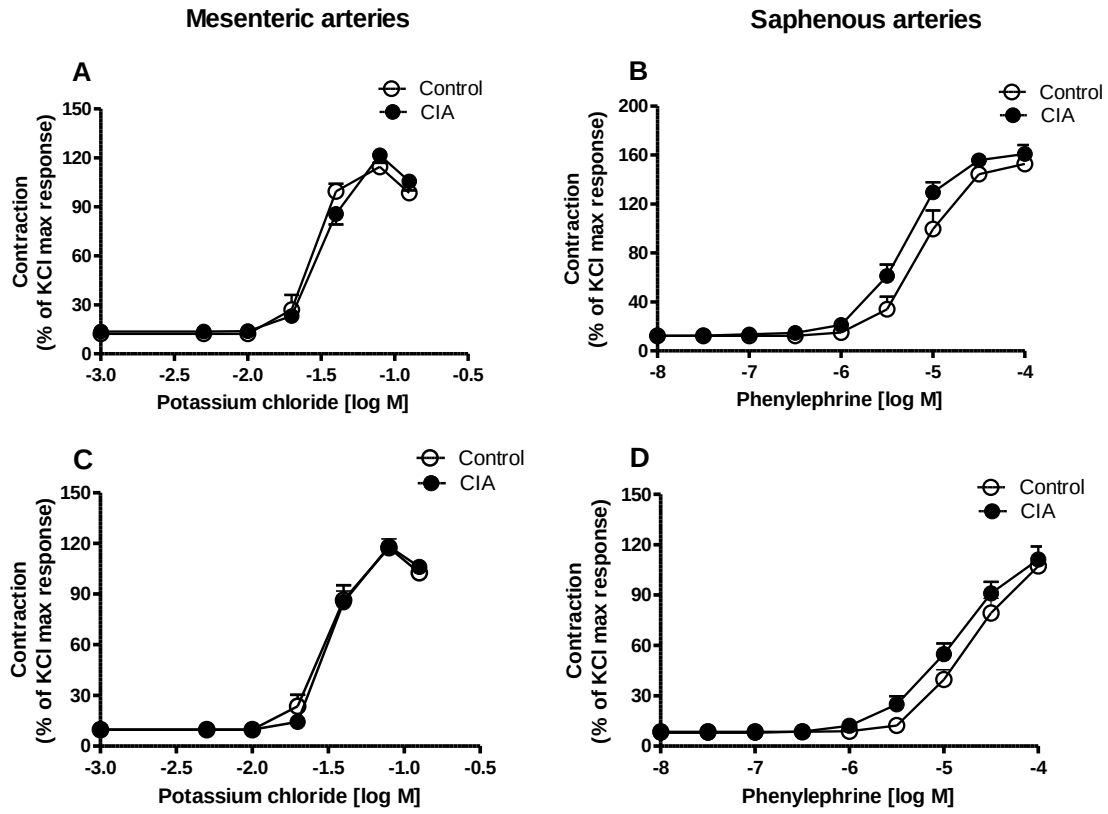


Figure 4.2 Cumulative dose-response curves to **(A** and **B)** potassium chloride and **(C** and **D)** phenylephrine in the second branch of mesenteric arteries (left panel) and saphenous arteries (right panel) of control (n=11) and CIA (n=19) rats. Contractile responses are expressed as a percentage of the KCl maximal response. Open circles represent the control group and closed circles represent the collagen-induced arthritis (CIA) group.

Phe (10 μM)-induced contraction were similar in the mesenteric and saphenous arteries before addition of ACh (mesenteric arteries: control: 24.1 ± 1.6 mN; CIA: 22.9 ± 1.5 mN; p=0.62 and saphenous arteries: control: 18.9 ± 1.9 mN; CIA: 17.8 ± 1.2 mN; p=0.61) or SNP (mesenteric arteries: control: 20.8 ± 1.9 mN; CIA: 20.4 ± 1.3 mN; p=0.89 and saphenous arteries: control: 17.2 ± 1.6 mN; CIA: 17.9 ± 1.6 mN; p=0.74).

Figure 4.3 shows the concentration-response curves to ACh or SNP exposure in mesenteric and saphenous arteries of control and CIA groups. The sensitivity (EC_{50}) to ACh was significantly reduced in mesenteric arteries of CIA rats compared to controls (control: 6.55 ± 0.21 μM, CIA: 32.4 ± 0.94 μM; p=0.05) (Figure 4.3A). The maximal response (E_{max}) to ACh in mesenteric arteries was significantly lower in CIA rats compared to controls (control: 83.30 ± 3.49 %, CIA: 60.91 ± 6.29 %; p=0.01). The EC_{50} and E_{max} to ACh in saphenous arteries were similar between the two experimental groups (P>0.05; Figure 4.3B). The EC_{50} and the E_{max} to SNP in mesenteric (Figure 4.3C) and saphenous (Figure 4.3D) arteries were similar between the control and CIA groups (P>0.05).

4.4.4. Systemic inflammatory markers and biomarkers of endothelial dysfunction

The serum concentrations of TNF-α (control: 277.9 ± 25.4 pg/ml; CIA: 645.9 ± 28.8 pg/ml), IL-6 (control: 100.4 ± 21.1 pg/ml; CIA: 377.8 ± 20.7 pg/ml), IL-β1 (control: 104.5 ± 15.9 pg/ml; CIA: 245.5 ± 11.5 pg/ml) and CRP (control: 0.2 ± 0.1 ng/ml; CIA; 1.0 ± 0.1 ng/ml) were significantly greater in the CIA group compared to controls (all p<0.0001). The serum concentrations of VCAM-1 were significantly greater in CIA rats compared to controls (control: 67.7 ± 11.3 pg/ml; CIA: 111.0 ± 12.4 pg/ml; p=0.02). No differences were observed in serum concentrations of ADMA between the groups (control: 10.8 ± 2.4; CIA: 11.5 ± 2.1; p=0.83).

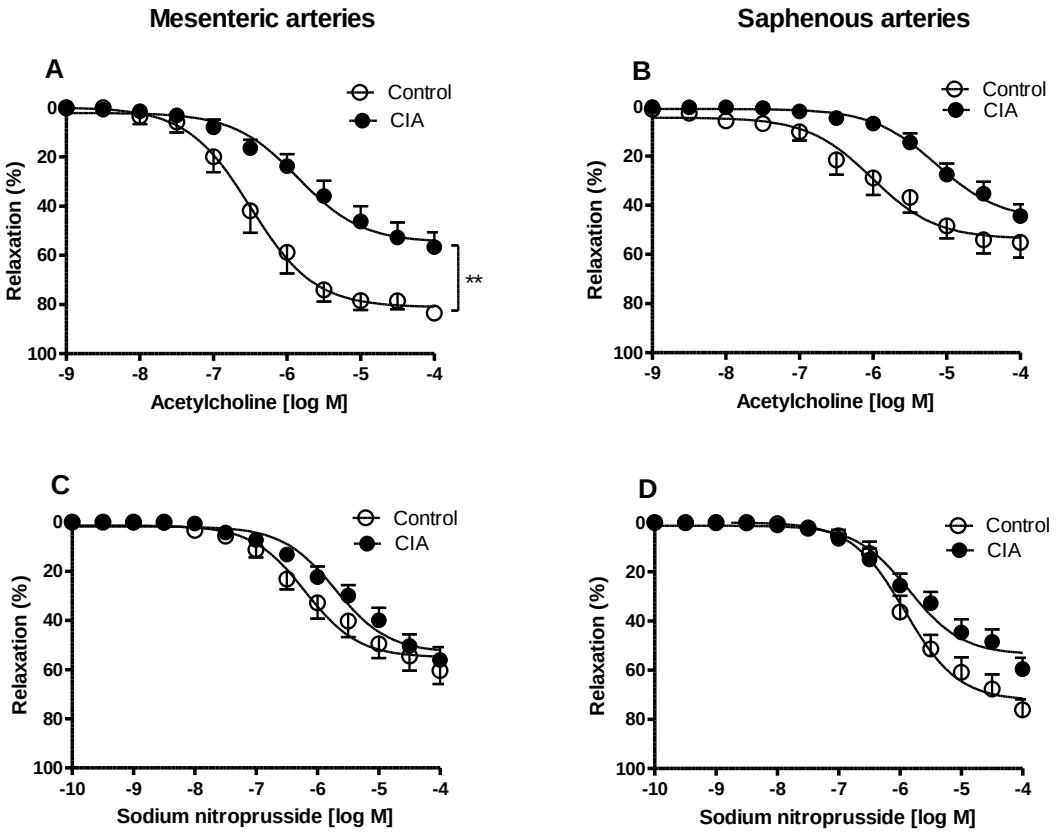


Figure 4.3 Cumulative dose-response curves to **(A** and **B)** acetylcholine and **(C** and **D)** and sodium nitroprusside in the second branch of mesenteric arteries (left panel) and saphenous arteries (right panel) of control (n=11) and CIA (n=19) rats. Contractile responses are expressed as a percentage of the KCl maximal response. Open circles represent the control group and closed circles represent the collagen-induced arthritis (CIA) group.

4.4.5. Systemic inflammatory markers associated with impaired endothelium-dependent relaxation in mesenteric but not saphenous arteries

Figure 4.4 shows the relationship between systemic inflammatory markers and E_{max} of ACh-induced relaxation in mesenteric arteries. Greater serum concentrations of TNF-α (r=-0.49; p=0.01; Figure 4.4A), IL-6 (r=-0.51; p=0.004; Figure 4.4B), IL-1β (r=-0.55; p=0.001; Figure 4.4C) and CRP (r=-0.48, p=0.01; Figure 4.4D) were associated with lower E_{max} ACh-induced relaxation in mesenteric arteries. No significant associations were found between TNF-α (r=0.31; p=0.06), IL-6 (r=0.31; p=0.07), IL-1β (r=0.32; p=0.10) and CRP (r=0.32, p=0.12) and the EC_{50} of ACh-induced relaxation in mesenteric arteries. No associations were found between inflammatory makers and the E_{max} (TNF: r= 0.32; p=0.11, IL-6: r=0.25; p=0.21, IL-1B: r=0.18; p=0.35, CRP: r=0.19; p=0.34) or EC_{50} (TNF: r=0.38; p=0.04, IL-6: r=0.31; p=0.10, IL-1B: r=0.20; p=0.29, CRP: r=0.29; p=0.14) of ACh-induced relaxation in saphenous arteries.

4.4.6. Systemic inflammatory markers associated with biomarkers of endothelial dysfunction

Figure 4.5 shows the associations between systemic inflammatory markers and biomarkers of endothelial dysfunction. Greater serum concentrations of TNF-α (r=0.39; p=0.03), IL-6 (r=0.37; p=0.04), IL-1β (r=0.41; p=0.02) and CRP (r=0.36, p=0.04) were associated with greater serum concentrations of VCAM-1. Serum concentrations of TNF-α (r=-0.06; p=0.76), IL-6 (r=-0.06; p=0.76), IL-1β (r=-0.09; p=0.61) and CRP (r=-0.11, p=0.56) were not correlated with serum concentrations of ADMA.

4.4.7. VCAM-1 associated with impaired endothelium-dependent relaxation in mesenteric but not saphenous arteries

Figure 4.6 shows the associations between serum concentrations of VCAM-1 or ADMA and the E_{max} of ACh-induced relaxation in mesenteric arteries. Greater concentrations of VCAM-1 were associated with lower E_{max} of ACh-induced relaxation in mesenteric arteries (r=-0.49; p=0.01, Figure 4.6A). Concentrations of ADMA were not associated with the E_{max} ACh-induced relaxation in mesenteric arteries (r=-0.09; p=0.65; Figure 4.6B). No associations were found between VCAM-1 (r=0.34; p=0.08) or ADMA (r=-0.02; p=0.92) concentrations and the EC_{50} of ACh-induced relaxation in mesenteric arteries. No associations were found between biomarkers of endothelial dysfunction and the E_{max} (VCAM-1: r=-0.06; p=0.76, ADMA: r=0.28; p=0.13) or EC_{50} (VCAM-1: r=-0.07; p=0.70, ADMA: r=0.35; p=0.07) of ACh-induced relaxation in saphenous arteries.

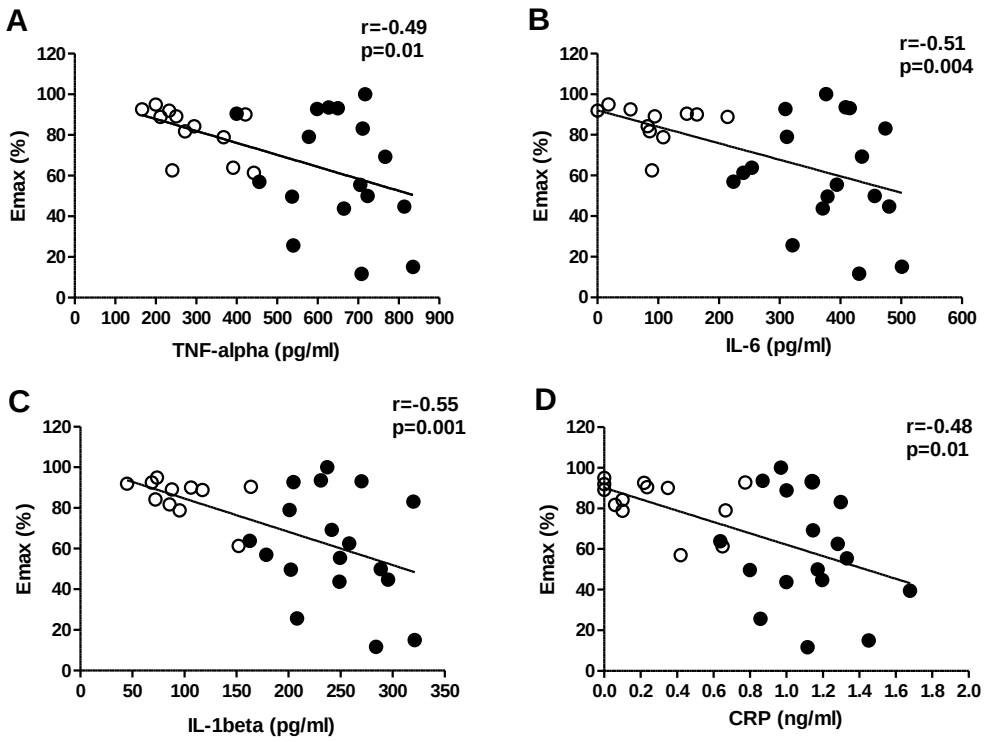


Figure 4.4 Correlation analysis between **(A)** tumor necrosis factor-alpha (TNF-alpha), **(B)** interleukin-6 (IL-6), **(C)** interleukin 1-beta (IL-1beta) and **(D)** C-reactive protein (CRP) with the maximal relaxation (E_{max}) to acetylcholine in the mesenteric arteries of control and CIA groups. Open circles represent the control group and closed circles represent the CIA group.

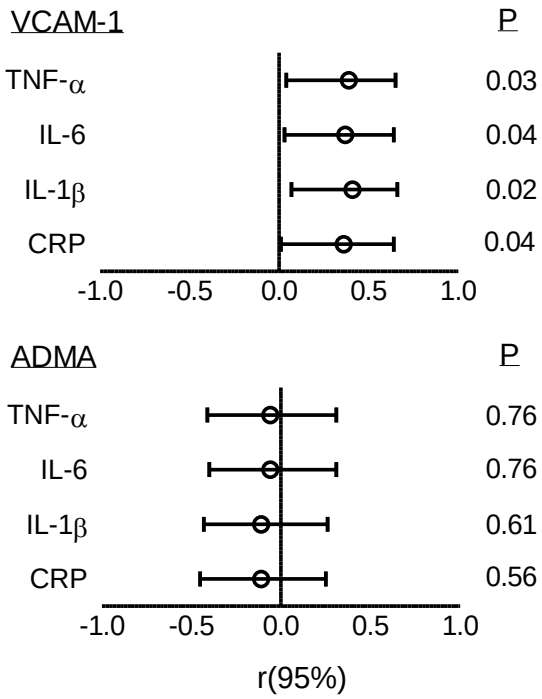


Figure 4.5 Correlation analysis between tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), interleukin 1-beta (IL-1beta) and C-reactive protein (CRP) with vascular adhesion molecule-1 (VCAM-1) and asymmetric dimethylarginine (ADMA) in the control and CIA groups. Open circles represent the correlation coefficient (r) and horizontal lines represent the 95% confidence intervals (CI).

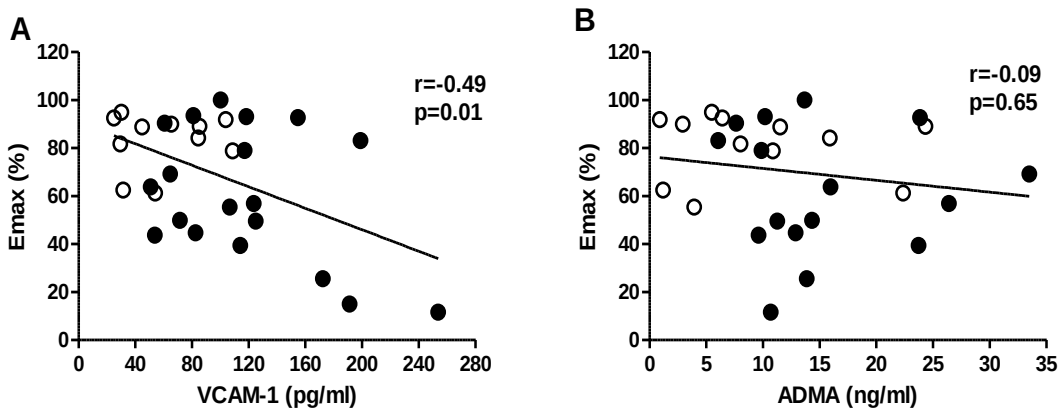


Figure 4.6 Correlation analysis between **(A)** vascular adhesion molecule-1 (VCAM-1) and **(B)** asymmetric dimethylarginine (ADMA) with the maximal relaxation (E_{max}) to acetylcholine in the mesenteric arteries of the control and CIA groups. Open circles represent the control group (n=11) and closed circles represent the CIA group (n=19).

4.5. Discussion

The present study demonstrated associations between systemic inflammatory markers, circulating VCAM-1 concentrations and impaired endothelium-dependent relaxation in a model of CIA. First, exposure to inflammation impaired ACh-induced relaxation but not SNP-induced relaxation of mesenteric arteries, suggesting the endothelial nature of the dysfunction induced by inflammation. The impaired endothelium-dependent relaxation was not accompanied by concomitant contractile dysfunction in mesenteric arteries. Secondly, high-grade inflammation resulted in increased circulating VCAM-1 concentrations, which were associated with impaired endothelium-dependent relaxation in mesenteric arteries. In contrast, ADMA concentrations were not affected by inflammation and did not relate to impaired relaxation in mesenteric arteries. These results enhance our understanding of the nature of inflammation induced impaired vascular function.

In the present study, endothelial dysfunction was associated with inflammatory markers. These results are in accordance with previous cross-sectional studies in RA that reported impaired endothelium-dependent relaxation in patients with adverse inflammatory profiles (Vaudo *et al.*, 2004; Arosio *et al.*, 2007; Galarraga *et al.*, 2008). Associations between circulating inflammatory markers and impaired ACh-induced relaxation has previously been shown in an animal study using adjuvant-induced arthritis (Totoson *et al.*, 2016). Although systemic inflammation-endothelial dysfunction relations in cross-sectional RA-studies have been adjusted for potential confounders (Vaudo *et al.*, 2004; Mäki-Petäjä *et al.*, 2008; Galarraga *et al.*, 2008), traditional risk factor profiles are different in RA compared to the general population (Zegkos *et al.*, 2016). Traditional risk factors contribute to the heightened cardiovascular mortality in RA (Baghdadi *et al.*, 2015). Recent findings indicate that traditional risk factors such as obesity, dyslipidemia and hypertension, are independently associated with endothelial dysfunction (Vaudo *et al.*, 2004; Rojas-Villarraga *et al.*, 2008; Sandoo *et al.*, 2012; Erre *et al.*, 2018). Consistent with previously reported findings in animal models of RA, our results confirm that endothelial dysfunction is not explained by metabolic (He *et al.*, 2013) or blood pressure-dependent mechanisms (Totoson *et al.*, 2015). Indeed, evidence suggests that inflammatory cytokines may modify the phenotype of endothelial cells, thereby leading to endothelial dysfunction (Sattar *et al.*, 2003; Zhang, 2008; Sprague & Khalil, 2009).

The present study showed no change in ADMA concentrations. Systemic inflammatory markers and impaired endothelium-dependent relaxation of mesenteric arteries were not associated with circulating ADMA concentrations. In contrast to clinical studies in RA (Şentürk et al., 2016; Spasovski et al., 2013) increased circulating levels of ADMA, are frequently reported and is believed to reflect endothelial dysfunction (Şentürk *et al.*, 2016). ADMA acts as an eNOS inhibitor by competitive inhibition of cellular L-arginine uptake by

endothelial cells or by increasing superoxide oxide production which further decreases NO bioavailability leading to endothelial dysfunction (Veresh *et al.*, 2008; Antoniades *et al.*, 2009; Sibal *et al.*, 2010). In the adjuvant-induced arthritis model, one study reported similar ADMA levels in rats exposed to inflammation compared to rats receiving anti-inflammatory drugs (Verhoeven et al., 2017). However, the absence of a control group in the aforementioned study limits the interpretation of these results. Similar to our results, in a previous study showed no changes in ADMA concentrations 8 weeks post immunisation compared to the control group in the CIA model (Chen *et al.*, 2013). However, no associations were reported between ADMA, inflammatory markers and endothelium dysfunction (Chen *et al.*, 2013). Our results suggest that the inflammation-induced endothelial dysfunction is not a result of eNOS inhibition by ADMA. Hence ADMA may not be an early biomarker of small resistance artery endothelial dysfunction in CIA rats.

Similar to described findings in RA patients (Klimiuk *et al.*, 2002; Dessein *et al.*, 2005; Wållberg-Jonsson *et al.*, 2008), the present study showed increased circulating VCAM-1 concentrations in the CIA group which were associated with systemic inflammatory markers and endothelial dysfunction. Previously, in the adjuvant-induced arthritis model, impaired endothelium-dependent relaxation of mesenteric arteries have been reported in the presence of increased circulating intercellular adhesion molecule 1 (ICAM-1) concentrations but not VCAM-1 concentrations (Totoson *et al.*, 2015). However, no association between ICAM-1 or VCAM-1 and endothelial dysfunction was demonstrated in the aforementioned study (Totoson *et al.*, 2015). These discrepancies may be explained by the different protocols used to induce arthritis in the latter and current studies. To the best of our knowledge, the current study is the first to demonstrate associations between VCAM-1 and impaired endothelium-dependent relaxation in an animal model of RA.

VCAM-1, a marker of endothelial activation, is expressed by endothelial cells and leukocytes in response to endothelial cell injury, often as a result of increased circulating inflammatory cytokines (Zhang, 2008; Liao, 2013). Endothelial activation increases the production of reactive oxygen species inside the endothelium which inactivate and reduce intracellular concentrations of NO (Gryglewski *et al.*, 1986; Yoshizumi *et al.*, 1993).

In the present study, endothelial dysfunction was impaired in mesenteric but not in saphenous arteries. Similarly, endothelial dysfunction has been reported in small resistance arteries at early stages of arthritis (Totoson *et al.*, 2015). However, ACh-induced relaxation was not impaired in rodent aorta in the early (Reynolds *et al.*, 2012; Palma Zochio Tozzato *et al.*, 2016), but rather during the later stages of arthritis (He *et al.*, 2013; Totoson *et al.*, 2016). Taken together, we and others have shown that inflammation affects endothelial function in small resistance arteries in the early stages of arthritis. It is most likely that larger arteries are affected in longer duration, more severe arthritis. Indeed, evidence suggests that

microvascular endothelial dysfunction contributes to cardiovascular disease development and precedes macrovascular endothelial dysfunction (Bordy *et al.*, 2018).

Nevertheless, ACh-induced relaxation in larger arteries, such as the saphenous branch of the femoral artery, is largely NO-dependent, whereas in smaller resistance arteries, such as the mesenteric arteries, endothelium-derived hyperpolarizing factor (EDHF) plays a greater role (Zygmunt *et al.*, 1995; Wigg *et al.*, 2001). The association between VCAM-1 and endothelial dysfunction in only mesenteric arteries, suggests that the role of VCAM-1 as a mediator of inflammation-induced resistance artery endothelial dysfunction extends beyond altered NO activity. Further studies are required to elucidate the mechanisms of how VCAM-1 alters endothelial function in microvessels.

In conclusion, the present study showed that exposure to high-grade inflammation *in vitro* impairs endothelium-dependent relaxation of small resistance arteries. Endothelial activation (VCAM-1) but not ADMA was associated with systemic inflammation and resistance artery endothelial dysfunction in CIA. These findings add to the evidence that systemic inflammation impairs endothelial function which may increase the risk for CVD in RA. Although it has been suggested that inflammation-induced microvascular endothelial dysfunction is involved in the development of diastolic dysfunction, evidence suggests that long-term exposure to inflammation also alters large artery function. In the general population arterial stiffness and altered aortic hemodynamics contribute to diastolic dysfunction. Whether large artery function affects diastolic function in RA requires elucidation.

Chapter 5: Early wave reflection and arterial stiffness are associated with diastolic dysfunction in rheumatoid arthritis

The data in this chapter have been submitted for publication in the International Journal of Cardiology:

Mokotedi L, Gunter S, Robinson C, Michel FS, Solomon A, Norton GR, Woodiwiss AJ, Tsang L, Dessein PH, Millen AME. Early wave reflection and arterial stiffness are associated with diastolic dysfunction in rheumatoid arthritis. *Int J Cardiol* (under review).

5.1. Abstract

Background. Rheumatoid arthritis (RA) impacts arterial and diastolic function. Arterial wave reflection relates to atherosclerosis in RA. This study examined whether arterial properties can determine diastolic function in RA.

Methods. Arterial function and left ventricular function were assessed by applanation tonometry using SphygmoCor software and echocardiography, respectively, in 173 RA patients. Arterial function measures included carotid-femoral pulse wave velocity (PWV), central systolic and pulse pressure, pulse pressure amplification, and the magnitude and timing of the forward and reflected waves. Diastolic function parameters comprised the ratio of early-to-late transmitral blood flow velocity (E/A) and ratio of E to the mean of the lateral and septal wall myocardial tissue lengthening (e'). Relationships were identified in multivariate regression models.

Results. The timing of the reflected wave (partial r=0.17, p=0.03) was associated with E/A; PWV (partial r=0.19, p=0.02) was related to E/e'. The timing of the reflected wave (OR=0.53 (95% Cl=0.33-0.85)), forward wave magnitude (OR=1.71 (95% CI=1.15-2.53)) and pulse pressure amplification (OR=0.58 (95% CI=0.39-0.89)) were associated with impaired relaxation (E/A <0.8); PWV (OR=1.64 (95% CI=1.13-2.38)) was related to increased left ventricular filling pressure (E/e' >12). The timing of the reflected wave (OR=0.66 (95% CI=0.47-0.92)) and PWV (OR=1.86 (95% CI=1.20-2.90)) were related to diastolic dysfunction according to previous criteria; timing of the forward wave (OR=1.71 (95% CI=1.11-2.66)) was associated with diastolic dysfunction according to recent criteria.

Conclusion. Early wave reflection and arterial stiffness are associated with left ventricular impaired relaxation and increased filling pressure, respectively, in RA.

5.2. Introduction

Rheumatoid arthritis (RA) is associated with an increased cardiovascular disease mortality risk (Aviña-Zubieta *et al.*, 2008) of which 13% is attributable to heart failure (Nicola *et al.*, 2006). Patients with RA are particularly prone to heart failure with a preserved ejection fraction (HFpEF) (Davis *et al.*, 2008; Aslam *et al.*, 2013). To date, no therapeutic intervention was shown to benefit HFpEF (Borlaug & Paulus, 2011; Pitt *et al.*, 2014). Left ventricular (LV) diastolic dysfunction is considered the pre-clinical measure that is most frequently associated with the pathophysiology of (Burke *et al.*, 2014) and progression to (Wan *et al.*, 2014) HFpEF. The pathophysiologic mechanisms underlying the development of LV diastolic dysfunction and ultimately HFpEF await further elucidation.

Altered large artery function plays an important role in overt heart failure (Mitchell *et al.*, 2001; Kawaguchi *et al.*, 2003; Desai *et al.*, 2009; Kang *et al.*, 2010; Kitzman *et al.*, 2013; Tsao *et al.*, 2015). As LV remodelling starts prior to the onset of heart failure symptoms, early identification of arterial changes that can lead to subclinical LV dysfunction is pertinent in cardiovascular event prevention. Accordingly, two recent studies revealed an association between comprehensively assessed arterial function measures and LV diastolic dysfunction in non-RA subjects (Kaess et al., 2016; Peterson et al., 2016).

Altered aortic hemodynamics contributes to the development of cardiac dysfunction through changes in the steady state (mean arterial pressure) and/or pulsatile components of blood pressure (Wang *et al.*, 2010; Vlachopoulos *et al.*, 2010*b*, 2010*a*; Chirinos *et al.*, 2012*a*; Ben-Shlomo *et al.*, 2014; Cooper *et al.*, 2015). Whereas steady-state pressure influences cardiac remodelling, changes in aortic function were shown to mediate pulsatile hemodynamics that further increases the load on the heart. Indeed, pulsatile hemodynamics contribute to target organ damage and cardiovascular events independent of steady state pressure (Wang *et al.*, 2010; Vlachopoulos *et al.*, 2010*b*; Weber *et al.*, 2012; Chirinos *et al.*, 2012*a*; Zamani *et al.*, 2015; Cooper *et al.*, 2015; Sibiya *et al.*, 2015). Arterial pulsatility estimation is often limited to the pulse pressure measurement. However, the central pulse pressure waveform is determined by both forward and backward wave components (Avolio *et al.*, 2009). Aortic stiffening increases the characteristic impedance, which participates in enhancing forward wave pressure (Cooper *et al.*, 2015). Aortic stiffening also increases the magnitude and speed of the reflected wave (Avolio *et al.*, 2009), which thereby arrives at the heart during systole. This results in an increased LV afterload and decreased coronary perfusion (Chirinos *et al.*, 2012*b*). Importantly, backward waves rereflect off the closed ventricular chamber during systole and aortic valve during diastole and thereby become forward traveling waves (Phan *et al.*, 2016). These rectified reflections contribute largely to the overall forward wave, particularly in the setting of arterial stiffening (Phan *et al.*, 2016). Large artery stiffening and wave reflection further account for reduced pulse pressure

amplification that occurs with vascular aging and exposure to cardiovascular risk factors (Avolio *et al.*, 2009).

Besides affecting cardiac function by enlarging pulse pressure, aortic stiffening can impact the heart through impaired mechanical ventricular-arterial coupling (Bell *et al.*, 2015, 2017). Also, whether increased wave reflection impacts LV function beyond that of arterial stiffening has treatment implications (Gunter et al., 2018).

Systemic inflammation comprises an increasingly implicated mechanism in arterial function (Jain *et al.*, 2014) diastolic dysfunction and HFpEF (Paulus & Tschöpe, 2013). In this regard, RA patients experience enhanced arterial stiffness (Ambrosino *et al.*, 2015) and an increased prevalence of diastolic dysfunction that is related to disease duration (Montecucco *et al.*, 1999), extra-articular manifestations (Gonzalez-Juanatey *et al.*, 2004) and circulating interleukin-6 concentrations (Liang *et al.*, 2010). We recently reported that disease characteristics and/or traditional cardiovascular risk factors are associated with both altered aortic (Gunter *et al.*, 2017) and diastolic function (Mokotedi *et al.*, 2017) in RA. Aortic wave reflection predicts the presence of very high-risk subclinical atherosclerosis to a clinically useful extent (Gunter et al., 2018) in RA. To our knowledge, whether specific aortic functional changes are associated with cardiac function and, if so, which factors mediate these relationships in patients with RA, has not been reported.

The aim of the present study was to determine large artery functional characteristics that are associated with alterations in LV function, independent of traditional cardiovascular risk factors and disease features in patients with RA.

5.3. Methods

5.3.1. Patients

One hundred and eighty-six patients that were seen at the Milpark hospital and met the American College of Rheumatology /European League Against Rheumatism criteria for RA (Aletaha *et al.*, 2010), were invited and agreed to participate in the study. Among these, 173 patients that did not have heart failure and in whom high-quality echocardiography and arterial function measures could be obtained, were included in the study. This investigation obtained approval from the University of the Witwatersrand Human (Medical) Research Ethics Committee (approval number: M06-07-33; protocol number: M120562 renewed as M170592) in Johannesburg, South Africa, and was conducted in line with the principles of the Helsinki declaration. All participants provided written, informed consent.

5.3.2. Demographic features, cardiovascular risk factors and RA characteristics

A description of the demographic parameters, cardiovascular disease risk factors and RA characteristics assessed is provided in chapter 2, page 20 of the present thesis.

5.3.3. Arterial function

Arterial waveforms were assessed by applanation tonometry at the radial, carotid and femoral artery pulses, each for 10 seconds using a high-fidelity SPC-301 micromanometer (Millar Instrument, Inc., Houston, Texas) interfaced with a computer employing SphygmoCor, version 9.0 software (AtCor Medical Pty. Ltd., West Ryde, New South Wales, Australia) as previously described (Norton *et al.*, 2012) (Figure 5.1). When the systolic or diastolic variability on consecutive waveforms were >5% or the amplitude of the pulse wave signal was <80mV, recordings were discarded. Brachial blood pressure measurements were taken immediately before the recordings were used to calibrate the pulse wave. An in-built validated transfer function in the SphygmoCor software was used to obtain the central (aortic) waveform. Central pulse pressure (cPP) was calculated as the difference between central systolic and diastolic blood pressure. Augmentation index (Alx) was calculated as (second systolic peak/ first systolic peak) x 100 and the pulse pressure amplification as the radial pulse pressure/aortic pulse pressure. Aortic pulse wave velocity (PWV) was measured from sequential waveform measurements at carotid and femoral sites using applanation tonometry and SphygmoCor software (Figure 5.1). The time delay in the pulse waves between the carotid and femoral sites was determined using an electrocardiograph derived R wave as a fiducial point. The distance which the pulse wave travels was determined as the difference between the distance from the femoral sampling site to the suprasternal notch, and the distance from the carotid sampling site to the suprasternal notch. Aortic carotid-femoral PWV was calculated as distance (metres) divided by transit time (seconds). Using SphygmoCor software, the aortic waveform was separated into the aortic forward (Pf) and backward (Pb) waves assuming a triangular flow wave (Westerhof *et al.*, 2006) (Figure 5.2). The time to wave reflection (Pb time) and time to the peak of the forward wave (Pf time) were determined from the separated forward and backward waves. The reflected magnitude (RM) was determined as Pb/Pf x 100. All measurements were made by a single experienced operator who was unaware of the cardiovascular risk factor profiles of the patients.

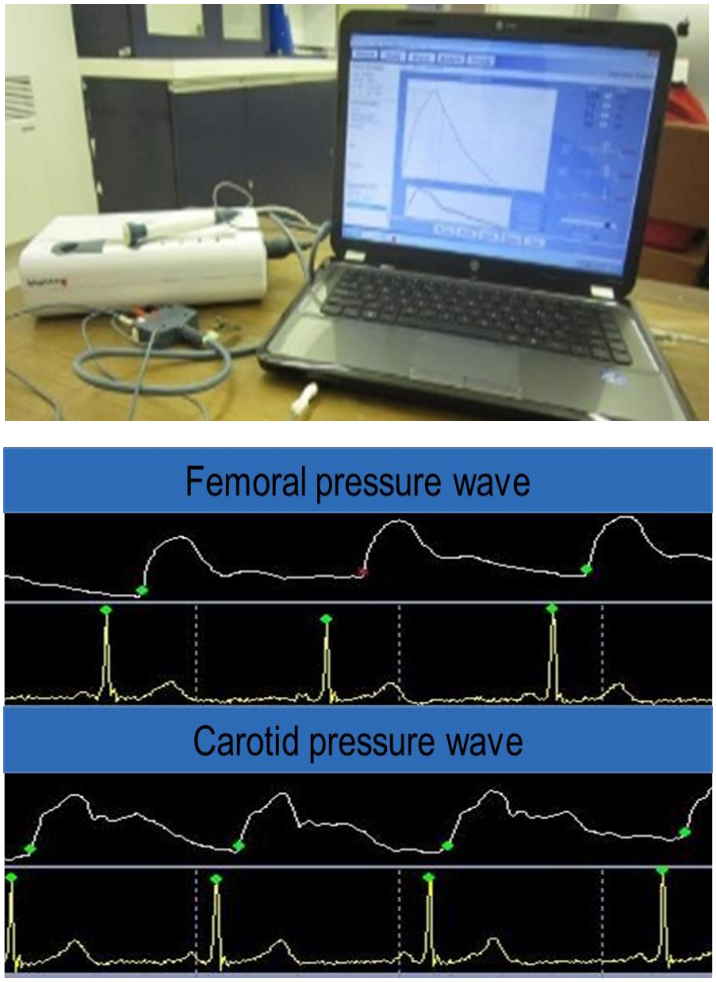


Figure 5.1 Photograph of the SphygmoCor device (upper panel) coupled to an applanation tonometer, which was attached to a computer, and used to determine aortic haemodynamics. The lower panel shows examples of the femoral and carotid pressure waves together with the electrocardiographic trace showing R waves which were used as fiducial points to determine the time delay in the waves between the carotid and femoral sites (green dots on both ECG and pressure waveform)

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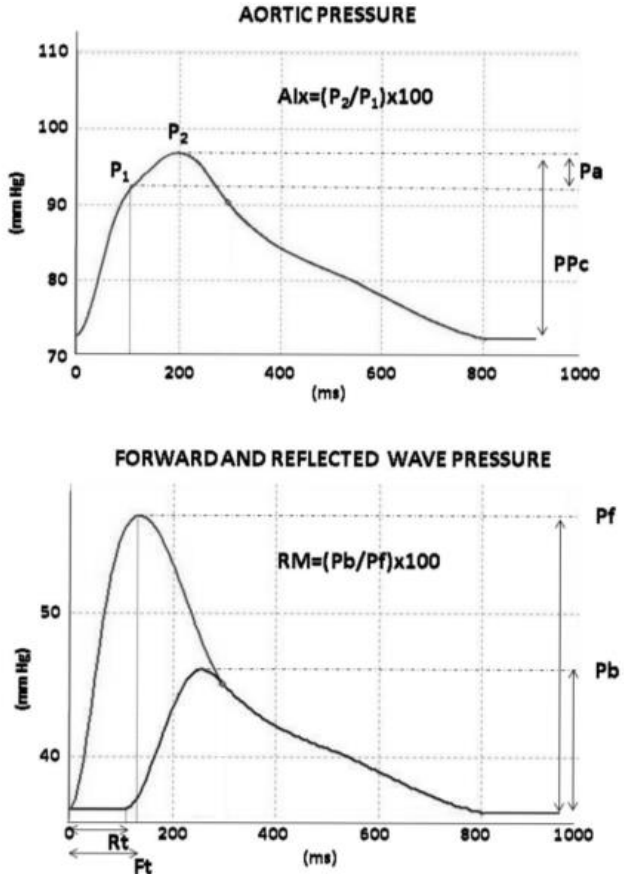

Figure 5.2 Example of the aortic pressure waveform obtained to determine aortic functional parameters. The aortic pressure wave was separated into the forward and backward waves using the triangulation method. Pa, augmented pressure; PPc, central aortic pulse pressure; AIx, augmentation index; Pf, forward wave pressure; Pb, backward (reflected) wave pressure; RM, reflection magnitude; Ft, time to peak forward wave; Rt, reflected wave time; P1, first systolic peak; P2, second systolic peak.

5.3.4. Echocardiography

Echocardiography was performed according to the American Society of Echocardiography convention, with the patient in the partial left decubitus position using a Sonosite M-Turbo ultrasound (SonoSite® Inc., Bothell, WA, USA) (Lang *et al.*, 2015). A description of the echocardiographic approach to the assessment of cardiac geometry, LV diastolic and systolic function is provided in chapter 2 page 21 of the present thesis.

5.3.5. Statistical analysis

Statistical analysis was performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC). Continuous variables were expressed as mean (SD) for normally distributed data or median (interquartile range (IQR)) for non-normally distributed data. Categorical characteristics were reported as percentages. Non-normally distributed variables were logarithmically transformed in order to improve skewness and kurtosis. Multiple linear and backward logistic regression analysis were performed to determine the independent relations between aortic and diastolic function and diastolic dysfunction, respectively. Based on reported evidence, age, sex, race, height, weight, mean arterial pressure and heart rate were included in the known confounder adjusted multivariate models (Wang *et al.*, 2010; Ben-Shlomo *et al.*, 2014; Cooper *et al.*, 2015; Gunter *et al.*, 2017). In the fully adjusted multivariate models, additional potential confounders included those factors that were associated with aortic function or diastolic function measures in RA (Gunter *et al.*, 2017; Mokotedi *et al.*, 2017). As LV mass and concentric hypertrophy are strongly associated with diastolic function, the relationships of LV mass index and relative wall thickness with diastolic function parameters were determined. In univariate analysis, LV mass index was more strongly and consistently associated with diastolic function parameters than relative wall thickness and, hence, was forced in additional regression models with diastolic dysfunction as dependent variable. As it was recently suggested that inflammation is strongly implicated in diastolic dysfunction (Paulus & Tschöpe, 2013), CRP and IL-6concentrations as well as the DAS28 were forced additionally into separate models. Deformed joint count as a marker of cumulative inflammation was also forced into separate models. Sensitivity analysis was performed in women as 80% of participants were female. Significance was set at $p \leq 0.05$.

5.4. Results

5.4.1. Patient characteristics

Table 5.1 gives the recorded characteristics. Mean (SD) age was 58.4 (12.4) years with a median (IQR) disease duration of 14.6 (8.9-22.0) years. All of the 39.9% of patients with hypertension were receiving anti-hypertensive treatment; 49.7% and 5.2% of the participants had dyslipidaemia and diabetes mellitus, respectively. The majority of patients (74.6%) tested rheumatoid factor (74.6%) or/and anti-citrullinated peptide antibody (70.4%)

positive. Disease activity was well controlled with a mean DAS28 of 2.8 (1.7) and median (IQR) Clinical Disease Activity Index of 6 (1-13); 89.6% of patients were receiving conventional synthetic disease-modifying agents and 11.0% used biologic disease-modifying agents.

Median (IQR) pulse wave velocity was 7.3 (6.2-9.4) m/sec and mean (SD) central systolic and pulse pressure were 126 (15) mm Hg and 43 (14) mm Hg, respectively; 18.8% of the patients had LV hypertrophy, 55.9% had concentric remodelling and 21.2% had a reduced ejection fraction. Of the participants, 22.9% had a reduced E/A (<0.8), 42.1% had an increased E/e' (>12), 57.8% had a decreased lateral e' (<10cm/sec) and 2.9% had an increased left atrial volume index.

5.4.2. Associations of arterial function measures with left ventricular (LV) geometry

Pulse pressure amplification was related to relative wall thickness in a fully adjusted multivariable model (partial r=-0.17, p=0.04). There were no other associations between arterial function markers and LV relative wall thickness of LV mass index (data not shown). In multivariable backward logistic regression analysis, no associations between arterial function parameters and LV hypertrophy were found (data not shown).

5.4.3. Associations of arterial function measures with diastolic function

Table 5.2 gives the independent relationships of arterial function measures with those of diastolic function. The time to wave reflection was associated with E/A in the known confounder (partial r=0.19, p=0.03) and fully adjusted model (partial r=0.17, p=0.03). PWV was related to E/e' in the known confounder (partial r=0.20, p=0.01) and fully adjusted model (partial r=0.19' p=0.02); central systolic pressure was associated with E/e' in the known confounder (partial r=0.16, p=0.04) but not fully adjusted model. Arterial function was not related to left atrial volume index (data not shown). Figure 5.3 depicts the known confounder adjusted means and SDs of arterial function variables stratified by tertiles of diastolic function. The time to wave reflection increased across tertiles of E/A. Pulse wave velocity increased across tertiles of E/e'.

Table 5.1 Recorded characteristics in 173 patients with RA

Demographic characteristics		EA manifestations, %	23
Age at study time, years	58.4 (12.4)	Stanford HAQ-DI	0.4 (0.0-0.9)
Age at disease onset, years	42.2 (14.1)	Conventional synthetic DMARD	
Female sex, %	80.92	Methotrexate, %	75.7
Lifestyle factors		Chloroquine, %	48.0
Exercise, %	31.8	Leflunomide, %	40.5
Alcohol use, %	32.4	Sulphasalazine, %	15.0
Current smoking, %	10.4	Tetracycline, %	15.6
Anthropometry		Azathioprine, %	6.9
Body mass index, kg/m^2	26.7 (5.3)	Synthetic DMARD, n	2.0 (1.1)
Waist circumference, cm	92 (13)	Synthetic DMARD, %	89.6
Waist-hip ratio	0.88 (0.09)	Biological DMARD	
Cardiometabolic risk markers		TNF-α inhibitors, %	8.7
Systolic BP, mm Hg	128 (15)	Abatacept, %	2.3
Diastolic BP, mm Hg	81 (8)	Biologic DMARD, %	11.0
Hypertension, %	39.9	NSAID, %	33.0
Total cholesterol, mmol/l	4.5 (1.0)	Prednisone use, %	2.3
HDL cholesterol, mmol/l	1.64 (0.47)	GFR, ml/min/1.73m^2	91 (23)
LDL cholesterol, mmol/l	2.4 (0.9)	Arterial function	
Triglycerides, mmol/l	1.0 (0.7-1.3)	PWV, m/sec	7.3 (6.2-9.4)
Cholesterol-HDL ratio	2.7 (2.3-3.2)	AIx, %	31.4 (11.1)
Dyslipidemia, %	49.7	Central systolic BP, mm Hg	126 (15)
Glucose, mmol/l	4.8 (4.5-5.1)	Central pulse pressure, mm Hg	43 (14)
Diabetes, %	5.2	Brachial pulse pressure, mm Hg	47 (13)
HOMA-IR	1.4 (1.0-1.9)	Pulse pressure amplification	1.14 (0.27)
Cardiovascular agents		Forward wave pressure, mm Hg	28 (9)
Antihypertensives, %	39.9	Backward wave pressure, mm Hg	21 (8)
Statins,%	42.8	Reflection magnitude	0.75 (0.23)
Ezetimibe,%	9.4	Time to peak forward wave, msec	138 (39)
Oral glucose lowering agents,%	1.7	Time to wave reflection, msec	255 (19)
Insulin,%	1.7	Echocardiography	
RA characteristics		LV mass index, g/m^2	78.8 (66.2-92.7)
RA duration, years	14.6 (8.9-22.0)	LV relative wall thickness	0.44 (0.08)
Rheumatoid factor positive, %	74.6	Stroke volume, ml	55.3 (24.2)
ACPA positive, %	70.4	Ejection fraction, %	58.6 (13.4)
CDAI	6 (1-13)	Lateral e', cm/s	9.6 (7.7-12.1)
DAS 28	2.8 (1.7)	Septal e', cm/s	7.8 (6.4-9.6)
ESR, mm/h	12 (4-25)	E/A	1.0 (0.8-1.3)
CRP, mg/l	3.3 (1.3-8.0)	E/e'	11.2 (7.9-13.5)
Interleukin 6, pg/ml	7.5 (3.5-14.2)	Left atrial volume index, ml/m^2	20.5 (6.0)
Leukocytes, n/nl	5.5 (4.6-7.0)	DD by previous criteria, %	59.4
Deformed joints, n	0 (0 - 11)	DD by new criteria, %	22.9

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

Table 5.2 Association of arterial stiffness properties with diastolic function markers

	E/A [a]		E/e' [a]	
	partial r	P	partial r	P
Known confounder adjusted model [b]				
PWV [a]	-0.07	0.41	**0.20**	**0.01**
AIx	0.008	0.92	-0.02	0.80
cSP	0.07	0.36	**0.16**	**0.04**
cPP	0.07	0.35	0.12	0.11
pPP	0.05	0.55	0.14	0.08
PPamp	-0.02	0.83	0.02	0.79
Pf	0.04	0.64	0.15	0.06
Pb	0.05	0.54	0.10	0.19
RM	0.002	0.98	-0.05	0.49
Pf time	0.12	0.14	0.04	0.6
Pb time	**0.19**	**0.01**	-0.07	0.35
Fully adjusted model [c]				
PWV [a]	-0.10	0.22	**0.19**	**0.02**
AIx	-0.05	0.56	-0.06	0.51
cSP	0.07	0.39	0.09	0.22
cPP	0.04	0.66	0.04	0.58
pPP	0.02	0.81	0.03	0.73
PPamp	0.04	0.64	0.03	0.74
Pf	0.03	0.73	0.08	0.29
Pb	-0.003	0.97	0.06	0.43
RM	-0.02	0.76	-0.04	0.63
Pf time	0.09	0.26	0.04	0.62
Pb time	**0.17**	**0.03**	-0.06	0.46

[a] Characteristics that were logarithmically transformed

[b] Known confounder adjusted model: age, sex, race, height, weight, heart rate, mean arterial pressure

[c] Fully adjusted model included:

E/A - age at disease onset, sex, race, height, mean arterial pressure, heart rate, disease duration, waist-hip ratio, erythrocyte sedimentation rate, glomerular filtration rate

E/e' - age, sex, race, height, diastolic blood pressure, heart rate

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

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

cSP - total cholesterol-HDL cholesterol ratio, statin therapy, chloroquine therapy

cPP - total cholesterol-HDL cholesterol ratio, statin therapy, chloroquine therapy

pPP - total cholesterol-HDL cholesterol ratio, C-reactive protein concentrations, statin therapy, tetracycline therapy, chloroquine therapy

PPamp - waist-hip ratio, total cholesterol concentrations, glucose concentrations, leflunomide therapy, TNF-α inhibitor therapy

Pf - total cholesterol-HDL cholesterol ratio, statin therapy

Pb - number of DMARD

RM - HDL cholesterol concentrations, TNF-α inhibitor therapy, leflunomide therapy

Pb time - waist-hip ratio, erythrocyte sedimentation rate, white cell count, ACPA status

PWV, pulse wave velocity; AIx, augmentation index; cSP, central systolic pressure; cPP, central pulse pressure; pPP, peripheral pulse pressure; PPamp, pulse pressure amplification; Pf forward wave pressure; Pb, backward wave pressure; RM, reflection magnitude; Pf time, time to the peak of the Pf; Pb time, time to wave reflection; HDL, high density lipoprotein; TNF-α, tumour necrosis factor alpha; DMARD, disease modifying anti-rheumatic drugs; ACPA, anti-citrullinated peptide antibody.

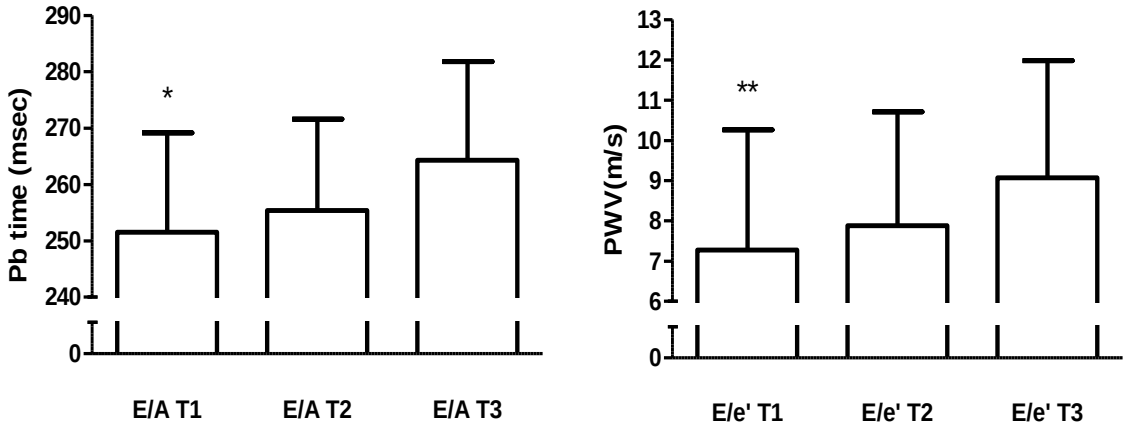

Figure 5.3 Adjusted means of arterial function properties across tertiles of diastolic function. Adjusted for known confounders: age, sex, race, height, weight, heart rate, mean arterial pressure; *p=0.04 versus tertile 3; **p=0.004 versus tertile 3; Pb time, time to wave reflection; PWV, pulse wave velocity; T, tertile

5.4.4. Independent relations of arterial function measures with impaired LV relaxation and increased filling pressure

Table 5.3 gives the associations of arterial function measures (1 SD increase) with impaired LV impaired relaxation and increased filling pressure. The time to wave reflection was associated with a decreased E/A in the known confounder adjusted (OR=0.48 (95% Cl=0.31-0.74)) and fully adjusted backward logistic regression models (OR=0.53 (95%CI=0.33-0.85)). Upon forcing PWV or central pulse pressure additionally into this model, the time to wave reflection remained associated with a reduced E/A (OR (95%CI)) with PWV in the model =0.57 (0.35-0.94); OR (95% CI) with central pulse pressure in the model =0.53 (0.33-0.85)). Pulse pressure amplification (OR=0.58 (95% CI=0.39-0.89)) and time to the peak of the forward wave (OR=1.71 (95% CI=1.15-2.53)) were related to a reduced and increased E/A, respectively, in the fully adjusted model. In the known confounder adjusted model, PWV (OR=1.69 95% CI=1.14-2.49)), central systolic pressure (OR=1.57 (95% CI=1.04-2.37)) and peripheral pulse pressure (OR=1.41 (95% Cl=1.00-1.99)) were related to an increased E/e'. PWV remained associated with an increased E/e' (OR=1.64 (95% CI=1.13-2.83)) in the fully adjusted model. When central pulse pressure was additionally forced into this model, PWV remained associated with an increased E/e' (OR=1.65 (95% CI=1.13-2.37)).

As also shown in Table 5.3, when we performed sensitivity analysis in patients with isolated diastolic dysfunction (ejection fraction ≥50%), the results were not materially altered. When LV mass index or CRP and interleukin-6 concentrations and DAS28 or deformed joint count was additionally forced into the models shown in Table 5.3, the results were also materially unaltered (data not shown).

In sensitivity analysis among women (Table 5.4), PWV (OR=2.03 95% CI=1.20-3.44)) and the time to wave reflection (OR=038 (95% CI=0.20-0.71)) were associated with an increased and reduced E/A, respectively. PWV was associated with an increased E/e' (OR=1.58 (95% CI=1.04-2.40)).

Table 5.3 Association of arterial function properties (1 SD increase) with impaired relaxation and increased filling pressure

	Decreased E/A (<0.8)		Increased E/e' (>12)	
	OR (95% CI)	**P**	**OR (95% CI)**	**P**
Known confounder adjusted model [a]				
PWV			1.69 (1.14-2.49)	0.008
cSP			1.57 (1.04-2.37)	0.03
pPP			1.41 (1.002-1.99)	0.048
Pb time	0.48 (0.31-0.74)	0.001		
Fully adjusted model [b]				
PWV			1.64 (1.13-2.38)	0.009
PPamp	0.58 (0.39-0.89)	0.01		
Pf	1.71 (1.15-2.53)	0.008		
Pb time	0.53 (0.33-0.85)	0.009		
Fully adjusted model [b] **in those with isolated diastolic dysfunction**				
PWV			1.59 (1.02-2.48)	0.04
PPamp	0.43 (0.25-0.74)	0.002		
Pf	2.16 (1.31-3.56)	0.003		
Pb time	0.41 (0.23-0.71)	0.002		

[a] Known confounder adjusted model: age, sex, race, height, weight, heart rate, mean arterial pressure

[b] Fully adjusted model: age at disease onset, sex, race, heart rate, disease duration, waist-hip ratio, diastolic blood pressure, azathriopine therapy, triglyceride concentrations.

PWV, pulse wave velocity; cSP, central systolic pressure; cPP, central pulse pressure; pPP, peripheral pulse pressure; PPamp, pulse pressure amplification; Pf forward wave pressure; Pb time, time to wave reflection.

Table 5.4 Associations of arterial function properties (1 SD increase) with impaired relaxation, increased filling pressure and diastolic dysfunction in women (n=142)

	Decreased E/A (<0.8)		Increased E/e' (>12)		DD new criteria		DD previous criteria	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
PWV	1.80 (1.06-3.04)	0.03	1.58 (1.04-2.40)	0.01			2.03 (1.20-3.44)	0.009
cSP							1.60 (1.06-2.40)	0.02
PPamp								
Pb time	0.38 (0.20-0.71)	0.002					0.66 (0.46-0.95)	0.03

Adjustors: age at disease onset, sex, race, heart rate, disease duration, waist-hip ratio, diastolic blood pressure, azathriopine therapy and triglyceride concentrations.

PWV, pulse wave velocity; cSP, central systolic pressure; PPamp, pulse pressure amplification; Pb time, time to wave reflection.

5.4.5. Independent relations of arterial function measures with LV diastolic dysfunction according to previous and current criteria sets

Figure 5.4 shows arterial function measures (1 SD increase) that predicted the presence of diastolic dysfunction according to previous (Redfield *et al.*, 2003) and recent (Nagueh *et al.*, 2016) classification criteria. Upon applying recently reported criteria (Redfield *et al.*, 2003), forward wave time was associated with the presence of diastolic dysfunction in the known confounder (OR (95% CI)=1.43 (1.00-2.04)) and fully adjusted model (OR (95%CI)=1.58 (1.05-2.36)). Upon applying previously reported criteria (Nagueh *et al.*, 2016), in the known confounder adjusted model, PWV (OR (95% CI)=1.97 (1.25-3.12)) and central systolic pressure (OR (95% CI)=2.03 (1.27-3.24)) were directly associated with diastolic dysfunction whereas the time to wave reflection was inversely (OR (95% CI)=0.64 (0.45-0.90)) related to diastolic dysfunction. In the fully adjusted model, PWV (OR (95%CI) =1.86 (1.20-2.90)) and the time to wave reflection (OR (95%CI) =0.66 (0.47-0.92)) remained associated with diastolic dysfunction. As also given in Figure 5.4, among patients with isolated diastolic dysfunction (ejection fraction ≥50%), the relationships of PWV, the time to wave reflection and time to the peak of the forward wave with diastolic dysfunction persisted. In addition, central systolic pressure predicted the presence of diastolic dysfunction according to both recent and previous recommended criteria. Upon forcing LV mass index or markers of current or cumulative inflammation additionally into the models, the results were materially unaltered (data not shown).

As also shown in Table 5.4, in sensitivity analysis among women, arterial function measures did not predict the presence of diastolic dysfunction according to recent criteria (Nagueh *et al.*, 2016). PWV (OR (95%CI) =2.03 (1.20-3.44)), central systolic pressure (OR (95%CI) =1.60 (1.06-2.40)) and the time to wave reflection (OR (95%CI) =0.66 (0.46-0.95)) were associated with diastolic dysfunction according to previous criteria (Redfield *et al.*, 2003).

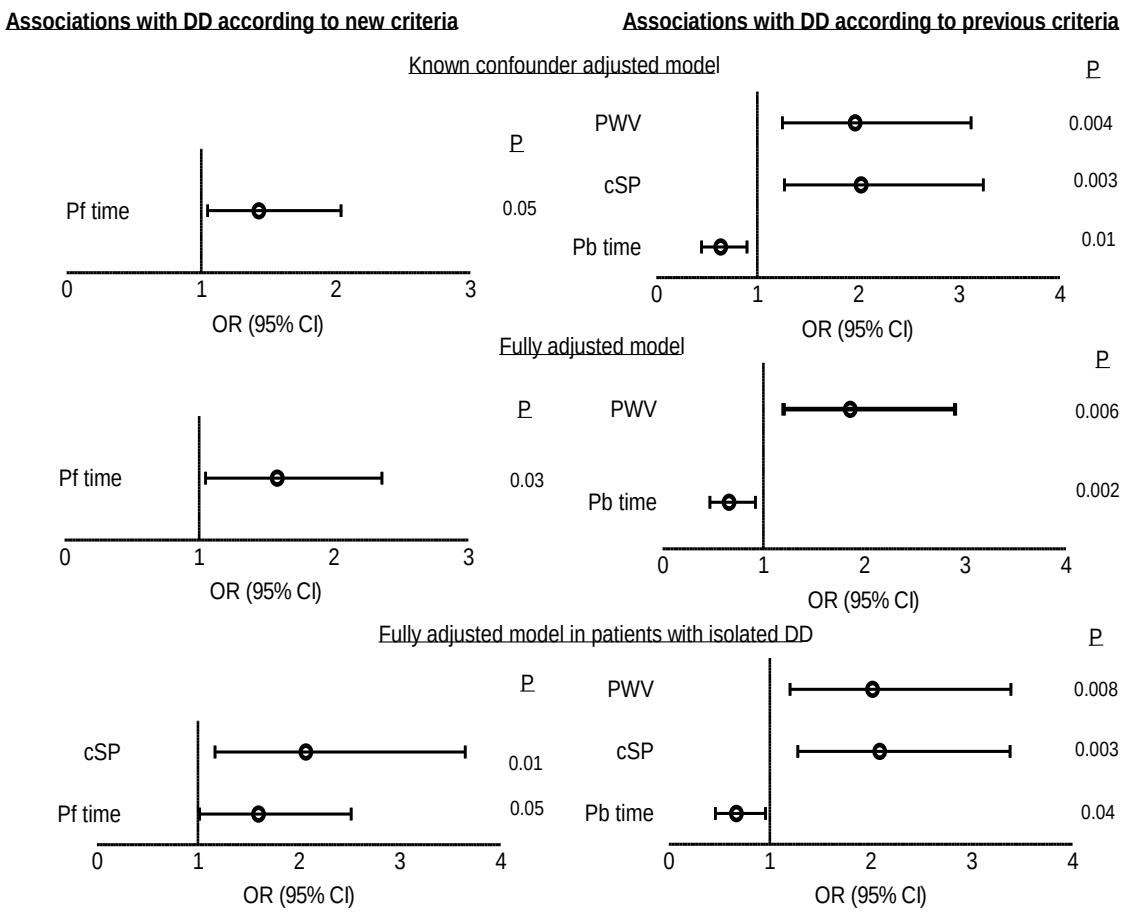

Figure 5.4 Associations of arterial function properties (1 SD increase) with diastolic dysfunction according to new and previous criteria sets. Known confounder adjusted model: age, sex, race, height, weight, heart rate, mean arterial pressure. Fully adjusted model: age at disease onset, sex, race, heart rate, disease duration, waist-hip ratio, diastolic blood pressure, azathriopine therapy, triglyceride concentrations. DD, diastolic dysfunction; Pf time, time to the peak of the forward wave; PWV, pulse wave velocity; cSP, central systolic pressure; Pb time, time to wave reflection

5.5. Discussion

This study evaluated the potential impact of arterial stiffness, wave reflection and pressure pulsatility on echocardiographically determined LV function in patients with RA. We report for the first time that an early return of the reflected wave is independently associated with impaired LV relaxation and arterial stiffness is related to increased LV filling pressure in RA. These results were reproduced when the analysis was restricted to patients with a normal systolic function or isolated diastolic dysfunction as well as women with RA. Reflected wave timing and arterial stiffness predicted the presence of diastolic dysfunction according to the criteria proposed by Redfield and colleagues (Redfield *et al.*, 2003) but not those by Nagueh et al (Nagueh *et al.*, 2016).

Arterial stiffening and early return of the reflected wave increase LV load and reduce coronary perfusion. We found that the associations of arterial stiffness and reflected wave timing with impaired diastolic function were not explained by central pulse pressure in the present study. This indicates that patients with RA may experience impaired mechanical coupling of the left ventricle to the proximal aorta whereby large artery stiffness and wave reflection load the heart directly. In support of impaired mechanical ventricular-arterial coupling as an underlying mechanism of diastolic function, strategies aimed at reducing cardiac afterload are reportedly unsuccessful in patients with HFpEF (Borlaug & Paulus, 2011; Pitt *et al.*, 2014). Impaired myocardial-arterial coupling is more frequent in non-RA women than men (Chirinos *et al.*, 2012*b*). This may explain the increased susceptibility to heart failure among women. Eighty-one percent of RA patients in the present study were women.

The relationships of arterial stiffness and reflected wave timing with diastolic function also persisted after adjusting for current and cumulative inflammation markers in the present investigation. We and others have previously reported evidence towards a role of inflammation in arterial function and/or diastolic function in patients with RA (Montecucco *et al.*, 1999; Gonzalez-Juanatey *et al.*, 2004; Liang *et al.*, 2010; Ambrosino *et al.*, 2015; Gunter *et al.*, 2017). These current results suggest that arterial functional changes can impact diastolic function irrespective of the inflammatory burden in RA. However, as disease activity was overall well controlled in this cohort, our findings may not apply in patients with moderate or markedly active RA.

The associations of arterial stiffness and reflected wave timing with diastolic function were equally consistent in our analyses. Reflected wave timing was related to impaired LV relaxation and diastolic dysfunction independent of arterial stiffness. Current hypertension

guidelines recommend consideration of arterial stiffness in refining cardiovascular risk stratification (Williams *et al.*, 2018). This is because arterial stiffness predicts cardiovascular disease beyond brachial blood pressure in hypertensive persons (Vlachopoulos *et al.*, 2010*a*; Ben-Shlomo *et al.*, 2014). Recent studies, particularly those in which separation analysis was applied and as was done in the current investigation, have identified wave reflection as an additional and equally important independent contributor to cardiovascular outcomes (Wang *et al.*, 2010; Weber *et al.*, 2012; Kaess *et al.*, 2016). Congruently, we recently documented that reflected wave pressure and reflection magnitude were strongly associated with subclinical atherosclerosis in hypertensive as well as normotensive RA patients (Gunter et al., 2018). Arterial stiffening was not related to atherosclerosis in RA (Gunter et al., 2018). Strikingly, the present study revealed that reflection wave timing rather than the absolute pressure was associated with LV relaxation in RA. In keeping with this finding, a previous investigation by Chirinos and colleagues disclosed that the loading sequence is more important than the actual pressure in determining myocardial wall stress among Asklepios Study participants (Chirinos *et al.*, 2012*b*).

Compared to early wave reflection and arterial stiffness, pressure pulsatility measures were less consistently related to diastolic function in patients with RA. Besides reflection wave timing, pulse pressure amplification and forward wave pressure but not arterial stiffness were independently associated with impaired LV relaxation (E/A <0.8). This reinforces the importance of wave reflection in impaired diastolic function among patients with RA.

In all RA patients, central systolic blood pressure was not associated with diastolic dysfunction. By contrast, in a sensitivity analysis among patients without concurrent systolic dysfunction, central systolic blood pressure predicted the presence of diastolic dysfunction according to both the reported criteria by Nagueh and colleagues (Nagueh *et al.*, 2016) and Redfield et al (Redfield *et al.*, 2003). In this regard, pressure pulsatility ultimately arises from the interaction between LV contractility (stroke volume) and arterial properties (Tsimploulis *et al.*, 2018). The current results, therefore, suggest that in RA patients with diastolic dysfunction and concurrent impaired systolic function, central systolic blood pressure does not impact diastolic function due to reduced LV ejection. As only 21.2% of our patients had a reduced ejection fraction, the finding on the central systolic blood-diastolic dysfunction relation should be investigated further in future larger studies. This issue has clinical implications since low systolic blood pressure is reportedly associated with increased mortality in heart failure patients with and without preserved ejection fraction (Dulai *et al.*, 2012).

Whereas early wave reflection and arterial stiffness were consistently associated with core features of diastolic dysfunction in RA, these arterial properties predicted the presence of diastolic dysfunction as defined according to the criteria by Redfield and colleagues (Redfield *et al.*, 2003) but not Nagueh et al (Nagueh *et al.*, 2016). Forward wave timing was not related to LV relaxation or filling pressure but was associated with diastolic dysfunction as defined by Nagueh and colleagues (Nagueh *et al.*, 2016). Diastolic dysfunction is a complex condition. Both a recent population study (Rasmussen-Torvik *et al.*, 2017) and an RA investigation (Mokotedi *et al.*, 2017) documented marked disparities in the estimated prevalence of diastolic dysfunction and its associated risk factors upon application of the different reported classification criteria sets. Previously reported findings together with our present results indicate that the identification of persons at risk of developing HFpEF by applying currently available definitions requires further study.

The findings of this study have treatment implications in patients with RA. We previously reported that markers of current and cumulative inflammation, as well as traditional cardiovascular risk factors, are associated with wave reflection in RA (Gunter *et al.*, 2017). Taken together with our present findings, treatment of the respective risk factors may improve wave reflection and thereby LV relaxation and diastolic dysfunction in RA. Tumor necrosis factor-α treatment is associated with reduced arterial stiffness in RA (Dulai *et al.*, 2012; Gunter *et al.*, 2017). Therefore, TNF-α inhibitors and possibly other disease-modifying agents may reduce arterial stiffness mediated LV filling pressure in RA. Further, considering that 1) increased wave reflection precedes arterial stiffening (Peterson et al., 2016); 2) wave reflection is reduced by angiotensin converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB) and calcium channel blockers (CCB) independent of blood pressure control, unaltered by diuretics and increased by beta-blockers (BB) (Dudenbostel & Glasser, 2012) and 3) the association of wave reflection with diastolic dysfunction was independent of blood pressure in the current study, ACEI, ARB, CCB and diuretics may comprise first-line agents for hypertension in RA patients. This contrasts with the current European League Against Rheumatism recommendations on cardiovascular disease risk management in RA (Agca *et al.*, 2017). The effects of disease activity control and different antihypertensive agents on diastolic function in patients with RA should be evaluated in future longitudinal and mechanistic studies.

The present study has strengths and further limitations. We investigated a relatively large group of RA patients and accounted for a comprehensive range of carefully identified potential confounding characteristics. We evaluated different functional arterial properties that were each previously documented to predict cardiovascular events in non-RA persons (Avolio *et al.*, 2009; Wang *et al.*, 2010; Vlachopoulos *et al.*, 2010*b*, 2010*a*; Weber *et al.*, 2012; Chirinos *et al.*, 2012*a*;

Ben-Shlomo *et al.*, 2014; Cooper *et al.*, 2015). Our study design was cross-sectional and thereby precludes the identification of cause-effect relationships. As all hypertensive patients were receiving antihypertensive agents and blood pressure control was adequate in the present cohort, the findings on arterial stiffening may not apply in RA patients with untreated or/and uncontrolled hypertension. Diastolic function and arterial function are dependent on loading conditions and heart rate. Heart rate and blood pressure were accounted for in all diastolic function and arterial function analyses in this study. Finally, I used the 'triangulation method' of aortic wave separation to determine aortic forward and backward wave pressure. Although this method was validated, it may not be optimal (Kips *et al.*, 2009). Future studies should determine the impact of the forward and backward waves on LV function using simultaneous recording of actual aortic flow and pressure in RA.

In conclusion, this study demonstrates that aortic early wave reflection and arterial stiffness are associated with LV impaired relaxation and increased filling pressure, respectively, in patients with RA. The relationships of arterial function with diastolic dysfunction were independent of steady state and pulsatile pressure. This suggests that impaired direct ventricular-arterial coupling mediates diastolic dysfunction in RA. Different classification criteria sets markedly impact the associations of arterial function measures with estimated diastolic dysfunction in RA. As there are currently no effective treatment strategies for HFpEF, our results indicate that aortic stiffness and wave reflection are potential therapeutic targets for the management of diastolic dysfunction in RA.

Chapter 6: Summary and conclusion

6.1. Summary

In order to institute effective treatment strategies for HFpEF, it is important to better understand the pathophysiological mechanisms underlying the development of diastolic dysfunction. Recently, chronic systemic inflammation has been proposed as the underlying cause for HFpEF independent of the aetiology. Therefore, in the current thesis, I conducted a series of studies designed to advance our understanding of the role of chronic high-grade inflammation on cardiac function.

6.1.1. Traditional risk factors associated with diastolic dysfunction in RA

Diastolic dysfunction is more prevalent in persons with RA compared to the general population (Nicola *et al.*, 2005; Aslam *et al.*, 2013) and is central to the development of HFpEF (Wan *et al.*, 2014). In the general population, aging and other traditional cardiovascular risk factors are strongly associated with the development of diastolic dysfunction (Masoudi *et al.*, 2003; Bhatia *et al.*, 2006; Owan *et al.*, 2006; Lee *et al.*, 2009; Borlaug & Paulus, 2011). It is well known that exposure to chronic inflammation alters the traditional CVD risk profile in persons with RA (Chung *et al.*, 2008; Libby, 2008; Liang *et al.*, 2010; Innala *et al.*, 2011; Choy *et al.*, 2014). Although in RA, a number of studies have shown associations between disease duration, disease severity and diastolic function (Montecucco *et al.*, 1999; Di Franco *et al.*, 2000; Arslan *et al.*, 2006; Rexhepaj *et al.*, 2006; Canturk *et al.*, 2006; Liang *et al.*, 2010; Davis *et al.*, 2011; Tomáš *et al.*, 2013), there is limited knowledge on the traditional risk factors that contribute to the development of diastolic dysfunction (Montecucco *et al.*, 1999). Furthermore, a large portion of these studies reported only on E/A as a marker of diastolic dysfunction in RA (Montecucco *et al.*, 1999; Di Franco *et al.*, 2000; Rexhepaj *et al.*, 2006; Canturk *et al.*, 2006; Udayakumar *et al.*, 2007; Tomáš *et al.*, 2013). Although it has been shown that a reduced E/A is associated with adverse cardiovascular outcomes (Aurigemma *et al.,* 2001), diastolic function variables as measured by TDI are more sensitive and more strongly associated with diastolic dysfunction (Kasner *et al.*, 2007). In 2003 the American Society of Echocardiography recommended using a combination of pulse Doppler and TDI indices in the classification of diastolic dysfunction (Redfield *et al.*, 2003). However, the recently published criteria for the classification of diastolic dysfunction does not consider the E/A ratio in persons with normal ejection fraction (Nagueh *et al.*, 2016) and has not been applied in RA. In this regard applying different classification criteria for diastolic dysfunction alters the prevalence and associated risk factors (Rasmussen-Torvik *et al.*, 2017).

In the present thesis, I identified traditional cardiovascular risk factors that were consistently associated with measures of diastolic function/dysfunction, independent of

established confounders. I demonstrated that waist-hip ratio is associated with impaired LV relaxation (E/A) whereas a low diastolic blood pressure is associated with increased LV filling pressure (E/e'), independent of LV mass. Further, I showed for the first time in RA that using different classification criteria sets alters the estimated prevalence and risk factors associated with diastolic dysfunction. Upon applying previous criteria (Redfield *et al.*, 2003), I found that the prevalence of diastolic dysfunction was markedly higher than when applying the current criteria (Nagueh *et al.*, 2016). A low diastolic blood pressure was independently associated with diastolic dysfunction using previous criteria whereas the waist-hip ratio was associated with diastolic dysfunction using the current criteria. Although the relationship between central obesity and diastolic dysfunction has been extensively described in the general population (Ammar *et al.*, 2008; Libhaber *et al.*, 2009; Russo *et al.*, 2011; Aljaroudi *et al.*, 2012; Millen *et al.*, 2014), in RA this association is of clinical importance as it has been shown that central adiposity is linked to chronic inflammation (Giles *et al.*, 2010) and is associated with atherosclerosis (Solomon *et al.*, 2012). Considering that low diastolic blood pressure contributed to increased filling pressure (E/e') and diastolic dysfunction, the present study suggests that low diastolic blood pressure predisposed RA patients to diastolic dysfunction as a result of the decreased coronary perfusion which ultimately leads to stiffening of the ventricle. These results support the recent recommendation for persons with RA, that not only disease activity and severity is controlled for CVD management, but also risk factors related to lifestyle (Agca *et al.*, 2017). A further important clinical implication of these findings is that the criteria used to identify persons with diastolic dysfunction need careful consideration. Future studies are required to determine the classification criteria sets that are most applicable to persons with RA.

6.1.2. Inflammation caused diastolic and systolic dysfunction in a pre-clinical model of chronic inflammation

Both traditional risk factors and inflammation are implicated in the development of diastolic dysfunction in RA. Yet it is difficult to discern the independent contribution of inflammation on diastolic dysfunction as a result of the interdependence of inflammation and traditional risk factors in RA. Only one study has reported associations between inflammation and diastolic dysfunction, independent of traditional risk factors (Liang *et al.*, 2010). However, the cross-sectional nature of the study precludes drawing conclusions on cause and effect relationships. Nevertheless, treatment with anti-inflammatory drugs improves myocardial functional abnormalities (Abbate *et al.*, 2010, 2013; Baniaamam *et al.*, 2018) which supports the role of inflammation on diastolic dysfunction. Although diastolic abnormalities are more common in RA, a number of studies recently showed a combination of diastolic and pre-clinical systolic dysfunction despite preserved ejection fraction (Sitia *et al.*, 2012; Fine *et al.*, 2014; Hamdy *et al.*,

2017; Midtbø *et al.*, 2017; Lo Gullo *et al.*, 2018). RA disease characteristics are associated with accelerated myocardial systolic dysfunction in RA (Løgstrup *et al.*, 2014; Fine *et al.*, 2014; Baktir *et al.*, 2015).

In an effort to understand the direct role of chronic systemic inflammation on cardiac function, in the second study, I set out to determine the role of high-grade inflammation on diastolic and systolic function using pulsed Doppler, TDI and STE in a pre-clinical model of collagen-induced arthritis in rats (an animal model most closely related to RA). I showed for the first time that the collagen-induced arthritis model induced concentric myocardial remodelling which resulted in impaired LV relaxation and increased ventricular stiffness. These diastolic abnormalities were present independent of hypertension and were strongly associated with circulating inflammatory markers. In addition, LV filling pressure was increased in the collagen-induced arthritis model however it was not associated with inflammatory markers. This suggests that increased LV filling pressures may not be directly linked to inflammation but may rather be increased through impaired active relaxation and myocardial stiffness induced by inflammation. Although traditional risk factors are associated with impaired diastolic function as indicated in chapter 2, chapter 3 confirms that systemic inflammation independently impairs diastolic function. As low disease activity reduces the cardiovascular risk burden in RA (Agca *et al.*, 2017), adequate control of inflammation and disease activity is essential for the prevention of diastolic dysfunction and hence cardiovascular risk in RA. Furthermore, despite preserved LV chamber function and contractility, indices of pre-clinical systolic function including myocardial deformation and motion makers were impaired in the collagen-induced arthritis model. Myocardial deformation but not contractility was associated with circulating inflammatory markers. The present findings underscore the importance of early recognition of pre-clinical myocardial dysfunction using STE and timely management of inflammation and disease activity to help prevent the progression of asymptomatic subclinical LV dysfunction in RA.

6.1.3. Inflammation caused endothelial dysfunction in a pre-clinical model of chronic inflammation

Although evidence suggests that inflammation has a direct role on myocardial remodelling, a new paradigm for myocardial remodelling has recently been proposed. In this regard, it is suggested that inflammation impairs microvascular endothelial function which results in the production of reactive oxygen species leading to altered intracellular pathways in the myocardium which impairs cardiac function (Paulus & Tschöpe, 2013). A limited number of studies have shown a direct role of inflammation on endothelial function of macrovascular arteries in animal models of RA (Totoson *et al.*, 2015, 2016). However microvascular endothelial

dysfunction precedes impairments of larger arteries (Totoson *et al.*, 2015; Bordy *et al.*, 2018). Soluble adhesion molecules such as VCAM-1 and the eNOS inhibitor ADMA have been associated with endothelial dysfunction in a number of pathologic conditions (Juonala *et al.*, 2007; Antoniades *et al.*, 2011; Vasilev *et al.*, 2013) and in RA (Klimiuk *et al.*, 2002; Wållberg-Jonsson *et al.*, 2008; Di Minno *et al.*, 2015; Şentürk *et al.*, 2016). Whether inflammation-induced endothelial dysfunction is mediated by these biomarkers has important treatment implications for the prevention of CVD in RA.

Hence in the third study, I evaluated the role of inflammation on endothelial function and whether VCAM-1 or ADMA mediate the inflammation-induced endothelial dysfunction in a preclinical model of RA. I found that inflammation impaired endothelium-dependent relaxation but not endothelium-independent relaxation in small resistance mesenteric arteries. This underscores the endothelial nature of the dysfunction induced by inflammation. However endothelial function was unaltered in larger saphenous arteries, confirming previous suggestions that the microvasculature is affected prior to larger elastic arteries. Circulating inflammatory markers were elevated in the collagen-induced arthritis rats and were strongly related to the impaired endothelium-dependent relaxation in mesenteric arteries. The changes in endothelial function were not a result of differences in blood pressure or metabolic profiles between the groups. These findings confirm that systemic inflammation independently impairs endothelial function and highlights the importance of evaluating small artery endothelial function as an early diagnostic indicator of cardiovascular risk in RA patients. Similar to our finding in Chapter 3, these results reinforce the importance of adequate control of inflammation and disease activity to prevent endothelial dysfunction and progression to cardiovascular disease in RA (Kotani *et al.*, 2016; Ruiz-Limón *et al.*, 2017).

Circulating VCAM-1 but not ADMA concentrations were elevated in the arthritic rats. The increased VCAM-1 concentrations were strongly associated with both inflammatory markers and endothelium-dependent relaxation in mesenteric arteries. The present findings indicate that endothelial activation may be involved in the inflammation-induced endothelial dysfunction. Endothelial activation promotes the production of reactive oxygen species which reduce intracellular NO concentrations (Gryglewski *et al.*, 1986; Yoshizumi *et al.*, 1993). As EDHF plays a greater role in small arteries, another important finding of the present study is that the role of VCAM-1 as a mediator of inflammation-induced small resistance artery endothelial dysfunction extends beyond altered NO activity. As microvascular endothelial dysfunctions impairs cardiac function (Paulus & Tschöpe, 2013), the impaired small resistance artery function in this study, may have indirectly contributed to impaired cardiac function as reported in chapter 3 of the present study.

6.1.4. Aortic stiffness and wave reflection associated with diastolic dysfunction in RA

Our findings in chapter 4 raise the questions whether inflammation-induced endothelial and vascular dysfunction predicts the development of diastolic dysfunction. In this regard, endothelial dysfunction is central to the development of atherosclerosis in RA (González-Juanatey *et al.*, 2011). Endothelial dysfunction also impairs arterial function and is related to arterial stiffness (Wållberg-Jonsson *et al.*, 2008; Sandoo *et al.*, 2011). Indeed persons with RA experience increased arterial stiffness (Ambrosino *et al.*, 2015). Impaired arterial function increases the workload of the heart and reduces coronary perfusion (Franklin, 2007; Chirinos *et al.*, 2012*b*). Aortic functional abnormalities have been related to diastolic dysfunction in the general population (Peterson et al 2016; Kaess et al 2016; Ikonomides et al 2008; Borlaug et al 2007; Canepa et al 2014). In the final investigation of this thesis, I assessed in RA patients whether large artery function, including PWV and wave reflection indices, are associated with diastolic dysfunction and whether systemic inflammation influences this process.

I found that an earlier return of the reflected wave was associated with impaired relaxation, whereas increased aortic stiffness was associated with increased filling pressures of the LV. Although traditional cardiovascular risk factors impact diastolic function as was reported in chapter 2, the arterial function-diastolic function relations identified in chapter 5 were independent of traditional cardiovascular risk factors. The relationship between arterial function and diastolic function was also independent of central haemodynamics and cumulative inflammatory markers. These results suggest that aortic stiffening independently impacts the heart through impaired mechanical ventricular-arterial coupling. Importantly markers of arterial function were differentially associated with diastolic dysfunction, depending on the classification criteria sets used. Aortic stiffness and an earlier return of the reflected wave were associated with diastolic dysfunction when employing previously published criteria (Redfield et al 2003), but not the current criteria (Nagueh *et al.*, 2016). These results substantiate our conclusions in chapter 2 of the present thesis; that the optimal criteria for identifying diastolic dysfunction in RA need careful consideration. Another important clinical implication of the present findings is that with adequate management of arterial function, diastolic dysfunction may be prevented. Recently our group showed associations with wave reflection and atherosclerosis (Gunter et al 2018). Thus adequate management of arterial function may reduce the risk of diastolic dysfunction in RA.

In summary, in the context of the reportedly high prevalence of diastolic dysfunction in RA, our current results indicate that traditional risk factors and inflammation play an important role in the development of diastolic dysfunction, either via direct myocardial remodelling or

indirectly via altered endothelial function and arterial function. Adequate inflammation control and CVD risk management may be pivotal in preserving diastolic function via direct effects on the myocardium or indirectly via the preservation of endothelial and arterial function. Taken together, the main findings of the present thesis are that: (1) central obesity and low diastolic blood pressure are independently associated with diastolic function in RA; (2) high-grade inflammation impairs myocardial function via alterations in myocardial remodelling independent of hemodynamic load; (3) inflammation impairs endothelial function of small resistance arteries via endothelial activation; (4) arterial stiffness and increased wave reflection speed is related to impaired diastolic function independent of hemodynamic load and traditional cardiovascular disease risk factors in RA.

6.2. Limitations

The specific limitations of each study of the present thesis have been addressed in detail in each data chapter however, there are some limitations that are worthy of emphasis. First, majority of the participants in the cross-sectional human data were white as patients were recruited from a local private hospital. Although the prevalence of RA is similar among black and white urbanized South Africans (Solomon et al 2005), there are major differences in the disease activity, severity and treatment characteristics in RA patients from the private versus the public healthcare sector. RA patients from public healthcare sectors have a higher inflammatory burden and a higher risk of CVD than RA patients from private healthcare sectors (Dessein *et al.,* 2009; Solomon *et al.,* 2005). Therefore, generalizing the results in chapter 2 and chapter 5 of this thesis to the general South African population from the public healthcare sector would be inappropriate as the results are more reflective of a particular niche within the South African health care system. Future studies should investigate the role of traditional risk factors, inflammation and the vasculature on diastolic dysfunction in the public health care sector. Second, age- and sex-matched controls were not included in the cross-sectional human data which precludes drawing inferences regarding cause and effect. However, a control group was included in the animal data which supported the results obtained from the human data. Third, I did not report on the changes in mitral inflow with the Valsalva maneuver as the measurement has not been standardised (Nagueh *et al.*, 2016) and cannot be accurately performed by all patients. Also, I did not report on the tricuspid regurgitation velocity and hence may have overlooked some participants with LV diastolic dysfunction. Tricuspid regurgitation velocity should be included in future studies. Fourth, STE is capable of identifying LV dysfunction earlier than conventional methods. The additional evaluation of pre-clinical systolic function using STE in chapter 2 and chapter 5 could have provided further insight to the role of inflammation on cardiac function, in light of the findings in chapter 3 of the present thesis. In addition, although

the use of the generalised arterial transfer function for the derivation of central waveform has been validated (Chen *et al.*, 1997), the accuracy of central blood pressure obtained using transfer functions has largely been criticised as the calibration underestimates central blood pressure (O'Rourke & Avolio, 2003; Hope *et al.*, 2004). Hence aortic pressures may have been underestimated using this approach. In addition, the use of the 'triangulation method' for wave separation analysis has been validated. However, the assumptions intrinsic to the use of this method are not ideal as backward and forward wave pressures are overestimated compared to Doppler measures (Kips *et al.*, 2009). Simultaneous recording of actual aortic flow and pressure should be addressed in future studies. Lastly, additional evaluation of endothelial function using the flow-mediated dilation method in chapter 5 of the present thesis could have provided further evidence in support of the findings of the impaired endothelial function in chapter 4.

6.3. Conclusion

In conclusion, the evidence presented in the present thesis adds to our understanding of the pathophysiological mechanisms that may play an important role in the development of LV diastolic dysfunction in patients with RA. In this regard, I showed that traditional cardiovascular risk factors including central obesity and a low diastolic blood pressure are associated with markers of impaired diastolic function in RA. Furthermore, I showed that inflammation directly causes diastolic dysfunction in a pre-clinical model of RA. These results further support the reported recommendations to not only manage traditional cardiovascular risk factors but also inflammation in order to reduce the overall cardiovascular risk in RA. In addition, I also showed that inflammation causes pre-clinical systolic dysfunction using STE despite preserved ejection fraction. Therefore, STE may aid in the identification and early detection of pre-clinical systolic dysfunction which will improve the prognosis of these patients. Moreover, I demonstrated that inflammation impairs endothelial function and that large artery stiffening is associated with impaired diastolic function abnormalities. This supports the recently suggested mechanism that diastolic dysfunction may be mediated via inflammation-induced alterations in vascular function. Hence, evaluation of the overall vascular status may be a valuable surrogate that could aid novel therapeutic approaches for the management of diastolic dysfunction in RA.

Chapter 7: References

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Zile MR, Gottdiener JS, Hetzel SJ, McMurray JJ, Komajda M, McKelvie R, Baicu CF, Massie BM & Carson PE (2011). Prevalence and significance of alterations in cardiac structure and function in patients with heart failure and a preserved ejection fraction. *Circulation*; DOI: 10.1161/Circulation.110.011031.

Zygmunt PM, Ryman T & Hogestatt ED (1995). Regional differences in endothelium-dependent relaxation in the rat: contribution of nitric oxide and nitric oxide-independent mechanisms. *Acta Physiol Scand* **155,** 257–266.

Chapter 8: Appendices

8.1. Appendix A: Human ethical clearance certificates

R14/49 Prof Patrick Dessein et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170592

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

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

DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

R14/49 Ms Lebogang Mokotedi et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170987

NAME: (Principal Investigator)	Ms Lebogang Mokotedi et al
DEPARTMENT:	Physiology Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	The Impact of Cardiovascular Disease Risk Factors, Inflammation and the Vasculature on Diastolic Function in Rheumatoid Arthritis
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under Primary Study M170592
SUPERVISOR:	Prof Aletta Millen and Dr Frederic Michel
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	23/10/2017

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review September and will therefore be due in the month of September each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 26/10/2017

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

8.2. Appendix B: Animal ethical 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/Apr/17

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

Works 2000/lain0015/AESCCert.wps

8.3. Appendix C: Modification of animal ethics clearance

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND
ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Lebogang Mokotedi

b. Department: Physiology

c. Experiment to be modified / extended

	AESC NO		
Original AESC number	**2017**	**03**	**21C**
Other M&Es :			-

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:	-	
g.	Total number of animals allocated to the experiment to date:	30	
h.	Number of animals used to date:	30	

i. Specific modification / extension requested:
- To limit food intake in some of the rats in the control group to match food intake in the experimental group
- To request 5 additional adult male SD rats to include in the control and experimental groups
- To measure body weight twice a week
- To include an additional co-worker. Mr Conrad Katlego Mogane (student number: 363984) His contact details are as follow: Cell: 0834393713 Email: 1991con@gmail.com

j. Motivation for modification / extension:

From the pilot study we have observed that the rats receiving bovine collagen emulsified in incomplete Freund's adjuvant (n=6) eat less and lose weight compared to the control rats (n=2). We would therefore like to restrict 10% of the food intake (Stofkova et al 2010 and Chowdhury; Rayford 2001) in half of the rats in the control group so that it is equal to the food intake in the experimental group to rule out any nutrient effect on diastolic function. We also request five additional rats (two for the control and three for the experimental groups) so that we have an n=7 and not n=6. An n=7 is more appropriate for statistical significance for echocardiography. In addition, due to the weight loss we propose to measure body weight twice a week to closely monitor the rats for the end point for humane interventions. Mr Conrad Katlego Mogane will be assisting with the blood pressure measurements and terminal procedures.

1. Stofkova A, Zelezna B, Romzova M, Ulicna O, Kiss A, Skurlova M, Jurcovicova J. Effect of feeding status on adjuvant arthritis severity, cachexia, and insulin sensitivity in male Lewis rats. Mediators Inflamm 2010; doi: 10.1155/2010/398026.
2. Chowdhury P and Rayford P. Effect of food restriction on plasma cholecystokinin levels and exocrine pancreatic function in rats. Ann Clin Lab Sci 2001; 31(4):376-382.

Date: 7th September 2017 Signature:

RECOMMENDATIONS: APPROVED. I. Restriction of feed intake. II. Five additional rats. III. Increased frequency of weighing for welfare monitoring. Iv. Inclusion of C Mogane as a co-worker.

CONDITIONS: The rats should be closely monitored for welfare purposes and termination end points strictly adhered to.

Date: 12 September 2017 Signature:

Chairman, AESC

8.4. Appendix D: Student's contribution to article (s) and agreement of co-author(s)

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, **Lebogang Palesa Mokotedi**, student number **793464**, declare that this Thesis/Dissertation/Research Report is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/Dissertation/Research Report .

Signature of Student  **Date 12-12-2018**

Name of Primary Supervisor: Associate Prof Aletta Millen

Signature of Primary Supervisor  **Date 12-12-2018**

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/Dissertation/Research Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

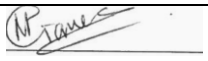
Article 1: Title: The Impact of Different Classification Criteria Sets on the Estimated Prevalence and Associated Risk Factors of Diastolic Dysfunction in Rheumatoid Arthritis

Journal name, year, volume and page numbers: International Journal of Rheumatology: 2017, 1-13.

Authors	Name	Signature	Date
1st author	Lebogang Mokotedi		12-12-2018
2nd author	Sulé Gunter		12-12-2018
3rd author	Chanel Robinson		12-12-2018
4th author	Gavin Norton		12-12-2018
5th author	Angela Woodiwiss		12-12-2018
6th author	Linda Tsang		12-12-2018
7th author	Patrick Dessein		12-12-2018
8th author	Aletta Millen		12-12-2018

Article 2: Title: Inflammation induced changes in left ventricular diastolic and systolic function in collagen-induced arthritis.

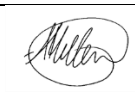
Submitted to Cardiovascular Research (under review)

Authors	Name	Signature	Date
1st author	Lebogang Mokotedi		12-12-2018
2nd author	Frederic Michel		12-12-2018
3rd author	Conrad Mogane		12-12-2018
4th author	Monica Gomes		12-12-2018
5th author	Angela Woodiwiss		12-12-2018
6th author	Gavin Norton		12-12-2018
7th author	Patrick Dessein		12-12-2018
8th author	Aletta Millen		12-12-2018

Article 3: Title: Vascular Cell Adhesion Molecule-1 Mediates Inflammation Induced Endothelial Dysfunction in Collagen-Induced Arthritis

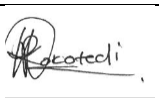
Submitted to Arthritis & Rheumatism (under review)

Authors	Name	Signature	Date
1st author	Lebogang Mokotedi		12-12-2018

2nd author	Aletta Millen		12-12-2018
3rd author	Conrad Mogane		12-12-2018
4th author	Angela Woodiwiss		12-12-2018
5th author	Gavin Norton		12-12-2018
6th author	Patrick Dessein		12-12-2018
7th author	Frederic Michel		12-12-2018

Article 4: Title: Early wave reflection and arterial stiffness are associated with diastolic dysfunction in rheumatoid arthritis.

Submitted to International Journal of Cardiology (under review)

Authors	**Name**	**Signature**	**Date**
1st author	Lebogang Mokotedi		12-12-2018
2nd author	Sulé Gunter		12-12-2018
3rd author	Chanel Robinson		12-12-2018
4th author	Frederic Michel		12-12-2018
5th author	Ahmed Solomon		12-12-2018
6th author	Gavin Norton		12-12-2018

7th author	Angela Woodiwiss		12-12-2018
8th author	Linda Tsang		12-12-2018
9th author	Patrick Dessein		12-12-2018
10th author	Aletta Millen		12-12-2018

Comments by primary supervisor:

The publications emanating from the human data on RA patients presented in this thesis forms part of a larger on-going study in persons with rheumatoid arthritis where the risk factors for cardiovascular disease are investigated. Lebogang contributed to a major portion of the data collection as part of a team of researchers (SG, CR, AM, LT and PD). She analyzed the data for both these papers (paper 1 and paper 4) and interpreted the data. She wrote the manuscripts to which the other authors contributed expert opinions and provided editorial input.

The publications emanating from the animal data on collagen induced arthritis presented in this thesis was solely attributed to the PhD work of Lebogang. The conceptualization of the study was done in conjunction with her supervisors. Lebogang was responsible for all the animal handling, establishing of the collagen induced arthritis model and termination of the animals. Data collection was done in conjunction with her supervisors and Mr Conrad Mogane. Lebogang performed all the data analysis and wrote both manuscripts (paper 2 and paper 3). The remainder of the authors assisted with interpretation of data and provided editorial input and expert opinion.