The effects of TNF-alpha blocker and IL-6 blocker therapy on vascular function in a collagen induced arthritis rat model

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DECLARATION

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

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

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ABSTRACT

Endothelial dysfunction and arterial stiffness are associated with increased cardiovascular morbidity and mortality in rheumatoid arthritis (RA). Chronic systemic inflammation appears to play a vital role in the development of endothelial dysfunction and arterial stiffness in patients with RA. Although biologic disease modifying antirheumatic drugs (DMARDS), including tumour necrosis factor alpha (TNF-α) blockers and interleukin-6 (IL-6) blockers have shown reduced inflammatory status and disease progression in RA patients, their effect on cardiovascular disease risk is inconsistent. The role of biologic DMARDS on endothelial function and arterial stiffness in the collagen induced arthritis (CIA) rat model, has not been investigated.

Male (n=47) and female (n=31) Sprague-Dawley rats were randomly divided into the control (n=22), inflammation (n=26), tumor necrosis factor-alpha blocker (TNF-α blocker; n=15) and interleukin-6 blocker (IL-6 blocker; n=15) groups. To induce arthritis, rats in the inflammation, TNF-α blocker and IL-6 blocker groups received a 0.2 ml subcutaneous injection of bovine type-II collagen emulsified in incomplete Freund's adjuvant at the base of the tail. Following the onset of inflammation, the TNF-α blocker group received 10mg/kg intraperitoneal injections of Etanercept (TNF-α receptor blocker) every three days for six weeks and the IL-6 blocker group received 8mg/kg intraperitoneal injections of Tocilizumab (IL-6 receptor blocker) once a week for 6 weeks. The control group received a 0.2 ml injection of saline at the base of the tail. Body weight, food intake, blood pressure, arthritis scores and paw thickness measurements at the tarsometatarsal and ankle joints were assessed every two weeks. After six weeks of treatment, arterial stiffness and vascular reactivity in mesenteric and renal arteries were assessed using the pulse pen and wire-myograph, respectively. Serum concentrations of TNF-α, IL-6 and C-reactive protein (CRP) were measured by ELISA. Group differences in all arthritis scores were measured by repeated measures analysis of variance (ANOVA). Group

differences in inflammatory and vascular function markers were assessed using a two-way ANOVA with a Tukey post-hoc test. Associations between inflammatory markers and vascular reactivity changes were determined by Pearson's correlations.

There were no significant differences in body weight, food intake and blood pressure between the groups at baseline and at termination (all $p>0.05$). Arthritis scores and paw thickness measurements were significantly increased in the inflammation, TNF-α blocker and IL-6 blocker groups compared to the control group at termination (all $p<0.01$). There were no differences in arthritis scores or paw thickness measurements between the inflammation, TNF-α blocker and IL-6 blocker groups ($p<0.05$). Circulating TNF-α concentrations were significantly higher in the inflammation, TNF-α blocker and IL-6 blocker groups compared to the control group ($p<0.0001$, $p=0.02$, $p=0.001$ respectively). There were no differences in the contractile responses in mesenteric or renal arteries between any of the groups (all $p>0.05$). Acetylcholine-induced relaxation in mesenteric ($p<0.0001$) and renal arteries ($p<0.0001$) was significantly impaired in the inflammation compared to control group. In the IL-6 blocker group, ACh-induced relaxation in both mesenteric ($p<0.0001$) and renal arteries ($p<0.0001$) were significantly greater compared to the inflammation group. There were no differences in ACh-induced relaxation between the inflammation and TNF-α blocker groups in either renal or mesenteric arteries (all $p>0.05$). Markers of arterial stiffness were not different between the groups (all $p>0.05$). Greater serum concentrations of TNF-α ($r=-0.36$, $p=0.01$), IL-6 ($r=-0.30$, $p=0.02$) and CRP ($r=-0.34$, $p=0.01$) were associated with impaired KCl-induced contractions (lower E_{max}) in mesenteric arteries. Greater serum concentrations of TNF-α ($r=-0.68$, $p<0.0001$), IL-6 ($r=-0.54$, $p=0.001$) and CRP ($r=-0.68$, $p<0.0001$) were associated with impaired ACh-induced relaxation (lower E_{max}) in renal arteries.

In conclusion, chronic high-grade inflammation impairs vascular relaxation, but not contraction, in resistance and large arteries. Despite unchanged circulating inflammatory

marker concentrations, IL-6 blocker treatment reduced the inflammation-induced impairments in endothelial function in resistance and large arteries. However, the mechanisms underlying the improved vascular function requires further investigation.

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TABLE OF CONTENTS

ABBREVIATIONS

ACh	Acetylcholine
AGEs	Advanced glycation end products
AIA	Adjuvant induced arthritis
AIx	Augmentation index
BP	Blood pressure
BW	Body weight
$CaCl_2$	Calcium chloride
CAS	Central animal services
cGMP	Cyclic guanosine monophosphate
CIA	Collagen induced arthritis
COX-2	Cyclooxygenase-2
CRP	C-Reactive protein
CV	Cardiovascular
CVD	Cardiovascular disease
DAS28	Disease activity score in 28 joints
DBP	Diastolic blood pressure
DMARDs	Disease modifying anti-rheumatic drugs
EC_{50}	Half maximal response

ECM	Extracellular matrix
ED	Endothelial dysfunction
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
E_{max}	Maximum response
eNOS	Endothelium derived nitric oxide
E-selectin	Endothelial leukocyte adhesion molecule
ESR	Erythrocyte sedimentation rate
FMD	Flow mediated dilation
ICAM-1	Intercellular adhesion molecule 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
Jak-STAT	Janus kinase/signal transducer and activator of transcription
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
$MgSO_4$	Magnesium sulphate
MMPs	Matrix metallopeptidases
mN	Millinewton
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate

NO	Nitric oxide
Phe	Phenylephrine
PWV	Pulse wave velocity
RA	Rheumatoid arthritis
ROS	Reactive Oxygen Species
SBP	Systolic blood pressure
SEM	Standard error of the mean
SNP	Sodium nitroprusside
TIMP-2	Tissue inhibitor of metalloproteinases 2
TNF-α	Tumour necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1

LIST OF FIGURES

Chapter 3

Chapter 4

LIST OF TABLES

Chapter 2

Chapter 4

CHAPTER 1

INTRODUCTION

Rheumatoid arthritis (RA) is a common, chronic systemic inflammatory disorder characterised by progressive joint destruction (Kaufmann *et al.,* 2013). Although RA is a synovial-joint inflammatory disease, RA leads to chronic systemic inflammation, which results in several extra-articular manifestations (Shetty *et al.,* 2014; Ridgley *et al.,* 2018). Extra-articular manifestations in RA are associated with severe disease activity and premature mortality (Cojocaru *et al.,* 2010). In this regard, cardiovascular disease (CVD) has been identified as one of the largest contributing factors to the increased mortality in RA (Cuchacovich and Espinoza, 2009). Although traditional CVD risk factors comprising obesity, hypertension, dyslipidaemia and diabetes mellitus are known to enhance the risk of mortality in RA, these risk factors do not fully account for all cardiovascular events (del Rincon *et al.,* 2001; van Halm *et al.,* 2006). In this regard, chronic systemic inflammation contributes to the increased cardiovascular events and risk of cardiovascular mortality in patients with RA (Bacon *et al.,* 2002). However, the mechanisms involved in inflammatory induced CVD risk is currently under investigation.

Atherosclerosis is known to contribute to the increased CVD mortality in RA patients (Aviña-Zubieta *et al.,* 2008; Georgiadis *et al.,* 2006). Risk factor exposure (del Rincón *et al.,* 2001) and chronic systemic inflammation (Sattar, McCarey, Capell, and McInnes, 2003) are associated with the development of arteriosclerosis and atherosclerosis in RA (Marti *et al.,* 2012; Sitia *et al.,* 2010). Circulating inflammatory cytokines, including tumour necrosis factor -alpha (TNF-α) and interleukin 6 (IL-6), are likely to contribute the connection between RA and atherosclerosis (Cuchacovich and Espinoza, 2009). In this regard, although the source of inflammation in RA is the synovial tissue, inflammatory mediator and pro-inflammatory cytokines enter the systemic circulation, where it affects various organ systems. In particular, exposure of the vascular endothelium to these inflammatory cytokines, damages vascular endothelial cells (Sattar *et al.,* 2003). Damage to the endothelium, which results in endothelial dysfunction, is a mechanism believed to underlie both atherosclerosis and arteriosclerosis

(Fitch *et al.*, 2001). The endothelium ensures the regulation and the balance between vasodilation and vasoconstriction of vessels and hence controls vascular homeostasis. When this balance is disrupted, such as in states of chronic inflammation, endothelial dysfunction occurs and results in damage to arterial walls (Davignon, 2013). In addition to its effects on endothelial function, chronic inflammation may impair vascular function resulting in increased arterial stiffness (Klocke *et al.*, 2003). Endothelial dysfunction and increased arterial stiffness have been acknowledged as independent predictors of CVD. Furthermore, both endothelial dysfunction and increased arterial stiffness are more frequent in patients with RA when compared to healthy individuals (Irace *et al.*, 2004). Although some evidence supports a role of high-grade inflammation in the pathogenesis of vascular dysfunction in RA patients, the exact inflammatory mechanisms mediating this role are uncertain and are currently under investigation.

Traditionally, RA has been managed with synthetic disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate (MTX) (Gaffo *et al*., 2006; Sizova, 2008). However, failure to fully control disease activity using synthetic DMARDs has led to the development of novel biologic agents. Over the past decade, treatment strategies for RA have markedly improved due to the use of biologic DMARDs, which have demonstrated considerable benefits over synthetic DMARDs (Strand and Singh, 2007). Although the use of biologic DMARDs have been proven effective in controlling joint damage and systemic inflammatory effects (Taylor, 2003), there are some contradictory findings on the effects of biologic DMARDs on vascular function. In addition to the conflicting results of biologic DMARDs on vascular function, the mechanisms whereby biologic DMARDs affect vascular function is not well-defined.

In the present thesis, chapter 2 begins with a literature review that summarises the current knowledge in the literature on the effects of inflammation on CVD specifically the risk for endothelial dysfunction and arterial stiffness in RA. Chapter 2 also highlights the current

effects of anti-inflammatory treatment of vascular function in RA and will provide the reasons for conducting the various experiments in this thesis. Chapter 3 outlines the methodological procedures employed in the study and Chapter 4 presents the results obtained in the current study. Chapter 5 will discuss the findings in the context of the current literature and will highlight the conclusion and limitations of the current study.

CHAPTER 2

LITERATURE REVIEW

2.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder (Aletaha *et al*., 2010), affecting about 1% of the global population (Olivier *et al*., 2018). In developed countries, the prevalence on RA ranges from 0.5 to 1%, whereas in developing countries such as South Africa, the prevalence of RA is less than 0.5%. The lower prevalence of RA in developing countries is most likely the result of under reported cases, as the systems in place within healthcare sectors are rudimentary (Olivier *et al*., 2018). Interestingly, most studies in developing nations show an increased prevalence in modern and industrialised areas in comparison with rural areas (Chopra and Abdel-Nasser, 2008; Solomon *et al*., 1975). This supports suggestions that RA might be associated with lifestyle habits of urbanised areas. In the South African population, the ratio of women to men with RA is 3:1, which is in accordance with the gender distribution of RA globally (Olivier *et al*., 2018).

RA is characterised by progressive joint swelling, tenderness and destruction, particularly in the small joints of the hands and feet (Kaufmann *et al*., 2013). Joint damage in RA is caused by influx of inflammatory mediators, known as cytokines in synovial tissue of joints, and is usually only visible externally in more advanced stages of disease progression (Aletaha *et al*., 2010). Chronic synovial inflammation in RA patients results in joint deformities, through the excessive release of cytokines and chemokines which result in severe pain, functional limitation and deformity (del Rincón *et al*., 2001). Although the precise aetiology of RA is not fully understood, it is a complex disease that is believed to be caused by an interplay between genetic and environmental factors, which lead to the early development of perturbations in both the innate and adaptive immune system (Asquith *et al*., 2009). In addition to the synovial-based inflammation, RA is a disease characterised by systemic inflammation, which results in a number of extra-articular manifestations (Cojocaru *et al*., 2010). The presence of extra-articular manifestations in RA is associated with severe disease course and premature mortality

(Cojocaru *et al.*, 2010). Cardiovascular disease (CVD) is considered the major extra-articular manifestation in RA and may account, at least in part, for the increased premature mortality in this population (Cuchacovich and Espinoza, 2009).

2.2 Cardiovascular disease risk in RA

RA patients have a 1.28 to 3-fold increase risk of mortality compared to the general population (Avina-Zubeita *et al.*, 2008). The risk of CVD in RA patients is comparable to that of diabetes mellitus (Peteres *et al.*, 2009; Stamatelopoulos *et al.*, 2009) and persists even after accounting for traditional cardiovascular risk factors (del Rincón *et al.*, 2001). The reasons for the increased CVD in RA are not fully understood. In this regard, traditional risk factors including smoking, hypertension, obesity and insulin resistance are strongly linked to CVD in RA patients (Panoulas *et al*., 2010; Baghdadi *et al*., 2015; La Montagna *et al*., 2007). Furthermore, there is a complex interplay between risk factor development, systemic inflammation and CVD (Bacon *et al*., 2002). Although these traditional CVD risk factors do relate to the increased CVD rates, it cannot fully explain the high incidence of CV events in this population (del Rincón *et al.*, 2001; van Halm *et al*., 2006). Clinical and serological RA features such as high inflammatory markers and joint swelling are associated with increased CVD mortality in patients with RA (Solomon *et al.*, 2004; Gabriel, 2008). After controlling for traditional risk factors, RA is considered a major risk factor for premature ischaemic heart disease (Aviña-Zubieta *et al.*, 2008). These observations underpin complex interrelations between traditional and disease-related risk factors, all of which contribute to heightened CVD risk in patients with RA. The following section will highlight the role of RA disease-related risk factors as possible contributors to the increased CVD risk in RA.

2.3 High-grade inflammation in increased CVD risk in RA

Systemic inflammatory markers such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) have been associated with a high incidence of cardiovascular events, independent of traditional risk factors in RA patients (Maradit-Kremers *et al.*, 2005). Increased CRP concentrations have been associated with preclinical markers of CVD including arterial stiffness (Ghada *et al.*, 2018) and endothelial dysfunction (Dessein *et al*., 2005) in patients with RA. Such observations suggest that RA disease-related inflammation might contribute to the accelerated atherosclerosis. Although the exact mechanism by which chronic high-grade inflammation causes atherosclerosis in RA is poorly understood, growing evidence supports the notion that increased pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) disrupt endothelial homeostasis leading to impaired vascular function which is an early step in atherogenesis (Sandoo *et al*., 2011). In this regard, one possible mechanism that may account for the increased CVD with high-grade inflammation could be through impaired vascular function (Sandoo *et al.*, 2011).

2.3 Impaired vascular function in RA

Atherosclerosis contributes to a large portion of CV events and mortality in RA (del Ricon *et al*., 2007; Bonetti *et al*., 2003). Endothelial dysfunction is currently considered the fundamental pathophysiological abnormality that explains the development of atherosclerosis, which can sequentially lead to changes in tunica intima of the vascular wall and increase arterial stiffness (Palombo and Kozakova, 2016; Della Corte *et al.*, 2016). In addition, increased arterial stiffness results in higher central systolic pressures leading to increased vascular wall stress, which could cause breakdown of the vascular wall elastin and endothelial dysfunction. Thus, the interrelationship between endothelial dysfunction and arterial stiffness are associated with the development and maintenance of atherosclerotic conditions (Bonetti *et al*., 2003). Arterial stiffness and endothelial dysfunction are associated with virtually all known risk factors for

CVD (Masaki *et al.*, 2016) and independently predict the risk of future cardiovascular events in the general population (Bonetti *et al.*, 2003).

In RA patients, arterial stiffness in increased and endothelial dysfunction is more prevalent than in the general population (Irace *et al.*, 2004; Metsios *et al*., 2009; Roman *et al.*, 2005). The more severe endothelial dysfunction and increased arterial stiffness in RA have been attributed to chronic high-grade systemic inflammation (Roman *et al*., 2006). Indeed, several studies have reported that circulating inflammatory markers and disease severity in RA patients are associated with arterial stiffness and endothelial dysfunction (Wallberg-Jonsson *et al*., 2008; Galarraga *et al.*, 2009; Crilly *et al.*, 2009; Roman *et al.*, 2005; Vaudo *et al*., 2004). Since vascular dysfunction plays a key role in the development of atherosclerosis in RA patients, the role of inflammation in the development of vascular abnormalities warrants investigation. Before describing the role of inflammation on endothelial dysfunction and arterial stiffness in RA, the sections below briefly highlight the pathophysiology of endothelial dysfunction and arterial stiffness.

2.4 Understanding the pathophysiology of endothelial dysfunction and arterial stiffness

2.4.1 Endothelial dysfunction

Endothelial dysfunction plays a key role in the development and progression of atherosclerosis (Sandoo *et al.*, 2011). Endothelial dysfunction is a pathologic state characterised by damage to the endothelium which results from an imbalance between vasodilatory and vasoconstrictive responses of the vascular endothelium (Sandoo *et al*., 2011). The endothelium, a monolayer of cells lining the circulatory system, plays an important role in the maintenance of vascular homeostasis by releasing endothelial-derived vasodilatory factors including nitric oxide (NO), prostacyclin, bradykinin and endothelium-derived hyperpolarising factor (EDHF) (Fitch *et al*.,

2001). NO is the predominant endothelium-derived vasodilator in the systemic arterial system and is synthesised from L-arginine by the enzyme nitric oxide synthase (eNOS) (Fitch *et al*., 2001). Physiological and pharmacological stimuli such as shear stress, acetylcholine and bradykinin activate eNOS via calcium (Ca^{2+}) /calmodulin- dependent mechanisms (Crilly *et al*., 2009). Once NO is released from the endothelial cells, it diffuses into the vascular smooth muscle cells and acts as a potent endothelium-derived vasodilator by stimulating the enzyme guanylate cyclase (Crilly *et al*., 2009). The conversion of cyclic guanosine triphosphate into cyclic guanosine monophosphate (cGMP) leads to the activation of cGMP-dependent protein kinase which decreases intracellular Ca^{2+} and ultimately leads to vascular smooth muscle relaxation (Sandoo *et al*., 2011).

In addition to its vasodilatory effects, NO plays a key role in maintaining the vascular wall in a quiescent state by inhibiting inflammation, thrombosis and cellular proliferation (Crilly *et al*., 2009). The endothelium also modulates vasomotion by the generation of endothelium-derived contracting factors (EDCFs), including angiotensin II, endothelin 1 and vasoconstrictor prostanoids (Crilly *et al*., 2009). These vasoconstrictor agents predominantly act in a local capacity but may exert some effects systemically, and could possibly play a role in the regulation of arterial structure and remodelling (Crilly *et al*., 2009).

Several studies have showed impaired endothelium-dependent function in RA patients, assessed by reduced flow-mediated dilatation (FMD) (Gonzalez-Juanatey *et al*., 2013; Gerli *et al*., 2004; Vaudo *et al*., 2004; Pingiotti *et al*., 2007; Arosio *et al*., 2007) or by impaired ACh-induced vasodilation (Mäki-Petäjä *et al*., 2008; Galarraga *et al*., 2008). Moreover, impaired endothelium-dependent vasodilation has been linked to increased circulating inflammatory cytokines in RA (Arisio *et al*., 2007; Galarraga *et al*., 2008; Mäki-Petäjä *et al*., 2008; Pingiotti *et al* 2007).

2.4.2. Arterial stiffness

Arterial stiffness is an early sign of structural and functional changes of the vessel wall and is associated with an increased risk of CV events (Zieman *et al.*, 2005; Adel *et al.*, 2016). Arterial stiffness depends on the functional and morphological changes occurring within the walls of major conduit arteries, which leads to a decreased buffer capacity of the arteries (Roman *et al.*, 2005; Nichols and O'Rourke, 1998; Shirwany and Zou, 2010). With each contraction, pressure waves are sent throughout the vasculature and the compliant arterial wall acts as a pressure buffer, dampening the oscillatory pulse of blood ejected by the left ventricle (Chirinos *et al*., 2012). In healthy individuals, left ventricular ejection generates a forward travelling pulse wave with a relatively slow velocity at 5-7m/s (Safar *et al*., 2003). Due to changes in impedance and resistance to pulsatile blood flow as the forward wave travels through different compositions of the arterial wall, a reflected wave is generated which returns to the heart during diastole (Nichols and Edwards, 2001; Mitchell *et al*., 2010). Arterial stiffness occurs when there is reduced arterial elasticity, which results in the forward pressure wave traveling at a faster velocity resulting in the reflected wave returning much sooner to the heart during the systolic phase of the cardiac cycle (Chirinos *et al*., 2012; Nichols and Edwards, 2001). Earlier return of the reflected wave results in increased systolic blood pressure which increases the workload of the heart and reduced diastolic blood pressure (Chirinos *et al*., 2012). This results in insufficient myocardial perfusion, which can lead CV complications including myocardial infarction and heart failure (Chirinos *et al.*, 2012).

A balance between the production and degradation of collagen and elastin regulates arterial rigidity. The degeneration of elastin fibres and increased collagen deposition in the vascular wall determines the increase in wall stiffness (Zieman *et al*., 2005). The decreased elastin production is mediated through the activation of various matrix metalloproteinases (MMPs).

Although structural changes (decreased elastin and increased collagen) in the vascular wall are a major determinant of arterial stiffness, the endothelium also plays an important role in arterial stiffness (Wilkinson *et al.*, 2002; Wilkinson *et al.*, 2002; Kinlay *et al.*, 2001). Reduction in NO production from endothelial cells, which results in reduced vascular tone, also causes stiffening of the vascular wall (Wilkinson *et al.*, 2002; Wilkinson *et al.*, 2002; Kinlay *et al.*, 2001).

Although a number of techniques can be used to assess arterial stiffness non-invasively, carotid-femoral pulse wave velocity (PWV) is the most widely used non-invasive technique to assess arterial stiffness due to its good producibility and ease of use (Laurent *et al.*, 2006). In addition to aortic stiffness measurements, aortic wave reflection is measured using augmentation index (AIx). AIx is calculated from the pressure waveform of an artery and is expressed as the percentage of central pulse pressure produced by the rise in systolic pressure due to the overlap of the forward and reflected pressure waves (Jain *et al.*, 2014). However, recent studies have reported that AIx is not an accurate index of wave reflection (Wang *et al.*, 2010; Chirinos *et al.*, 2012) as it underestimates the contribution of the reflected wave (Booysen *et al.*, 2015). Instead, wave reflection measured using wave separation analysis (which separates the forward and reflected waves) is a better measure of wave reflection intensity than AIx (Wang *et al.*, 2010; Chirinos *et al.*, 2012).

Several studies have shown an association between markers of chronic high-grade systemic inflammation with markers of arterial stiffness in RA, including PWV (Mäki-Petäjä *et al.*, 2006; Roman *et al.*, 2005), AIx (Wallberg-Jonsson *et al.*, 2008; Galarraga *et al.*, 2009; Crilly *et al.*, 2009) and reflected wave pressure using separation analysis (Gunter *et al.*, 2017). Given the evidence that inflammatory markers are associated with endothelial dysfunction and arterial stiffness in cross-sectional studies (Wallberg-Jonsson *et al.*, 2008; Galarraga *et al.*, 2009; Crilly *et al.*, 2009; Roman *et al.*, 2005; Vaudo *et al.*, 2004; Mäki-Petäjä *et al.*, 2006; Gunter *et al.*, 2017), the question arises, what are the mechanisms explaining the associations between RA

or associated inflammatory changes and measures of vascular function? The section below briefly highlights the mechanisms whereby inflammation may mediate endothelial dysfunction and arterial stiffness.

2.5 Mechanisms of inflammation-induced endothelial dysfunction and arterial stiffness

Endothelial function relies on the tight regulation of nitric oxide and antioxidant signalling pathways (Gutterman *et al*., 2005). A number of studies support the notion that chronic high-grade inflammation results in a dysregulation of these pathways through the upregulation of proinflammatory signalling pathways that act in a positive feedback manner to amplify one another and reduce nitric oxide bioavailability (Lao, 2013). Chronic high-grade inflammation results in increased circulating levels of inflammatory cytokines such as IL-6, TNF-α, and interleukin 1 beta (IL-1β) which are expressed by macrophages, monocytes and neutrophils (Totoson *et al*., 2016a). These cytokines up-regulate the expression of endothelial cell expression of various soluble adhesion molecules such as vascular adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule (E-selectin) (Totoson *et al*., 2016; Lao, 2013; Matsuzawa and Lerman, 2014). Endothelium activation by proinflammatory cytokines leads to endothelial dysfunction by decreasing NO bioavailability through the production of reactive oxygen species (ROS) and through eNOS uncoupling within the endothelium (Aletaha *et al.*, 2010; Lao, 2013; Matsuzawa and Lerman, 2014). Under normal physiological conditions, low levels of ROS are maintained and are necessary for vascular function by various antioxidant enzymes such as superoxide dismutases and peroxiredoxins (Xia *et al*., 1998; Wink *et al*., 2001). Oxidative stress occurs when the production of ROS exceeds the capacity of cellular antioxidants which results in endothelial injury (Xia *et al*., 1998). In addition, inflammatory cytokines trigger endothelial dysfunction by uncoupling eNOS, which results in a decreased NO bioavailability (Haruna *et*

al., 2006). eNOS uncoupling results in the alteration of eNOS from a primarily NO-synthesising enzyme, to a superoxide-producing enzyme, which exacerbates endothelial oxidative stress levels (Xia *et al*., 1998; Wink *et al*., 2001). The enhanced generation of superoxide results in the formation of peroxynitrite, which inhibits cyclic guanylyl cyclase and further enhances oxidative stress by inhibiting superoxide dismutases (Lubos *et al*., 2008). The combined increased vascular ROS production and decreased vascular NO bioavailability impairs vasomotor tone. This imbalance results in an impairment of endothelium-dependent vasodilation, which is the defining characteristic of endothelial dysfunction (Bonetti *et al*., 2003).

Besides the impaired endothelial function, evidence also points to inflammation-induced arterial stiffness. High-grade inflammation may cause arterial stiffness by numerous pathophysiological mechanisms. Firstly, chronic inflammation is associated with endothelial dysfunction (Hingorani *et al*., 2000), which may lead to arterial stiffness through the reduced NO bioavailability and increased ROS production. Chronic inflammation may lead to structural stiffening of large vessels by decreasing elastin production and increasing collagen production in the extracellular matrix (Park and Lakatta, 2012). Inflammatory cytokines promote the release of a number of inducible MMPs accompanied by a decreased activity of its tissue inhibitor of metalloproteinases 2 (TIMP-2), which results in decrease elastin production (Park and Lakatta, 2012). In addition to the activation of MMPs, inflammatory cytokines activate advanced glycation end products (AGEs) which cross-link with either collagen or elastin proteins resulting in stiffer arteries (Mozos *et al*., 2017). Additionally, AGEs affect endothelial function by promoting oxidative stress and decreasing NO release (Mozos *et al*., 2017). The resulting combined effects of increased MMP production together with the reduced production of NO contribute to arterial stiffening.

2.6 Impaired vascular function in animal models of RA

Endothelial dysfunction has been extensively studied in the adjuvant-induced arthritis model (Bilsborough *et al*., 2006; Bosello *et al*., 2008; Cardillo *et al*., 2006; Gonzalez-Juanatey *et al*., 2006; Hurlimann *et al*., 2002; Ikonomidis *et al*., 2008; Irace *et al*., 2004; Komai *et al*., 2007; Maki-Petaja *et al*., 2006; Rongen *et al*., 2018; Ruiz- Limón *et al*., 2017; Sidiropoulos *et al*., 2008). However, there is currently limited evidence to support the role of inflammation on vascular function in the collagen-induced arthritis (CIA) model. The CIA model is an experimental model that shares both immunological and pathological features most similar to human RA compared to other experiment models (Bendele, 2001; Brand *et al*., 2007). The CIA model was first discovered by Trentham *et al* in 1977 and is induced by the subcutaneous injection of type II collagen emulsified in either complete (inactivated mycobacteria suspended in mineral oil) or incomplete (mineral oil) Freund's adjuvant, at the base of the tail. Clinical signs of arthritis usually develop three to five weeks after primary immunisation (Bendele, 2001; Brand *et al*., 2007). As in human RA, CIA is characterised by pronounced synovial hyperplasia, infiltration of inflammatory cells and bone and cartilage erosion (Holmdahl *et al*., 2000). In addition, other pathologic features that characterises both CIA and human RA is the production of collagen type II-specific antibodies (Holmdahl *et al*., 2000) and rheumatoid factor (Bendele, 2001). The most noticeable difference between the CIA model and human RA is that CIA is more susceptible in males than in females (Bendele, 2001), unlike human RA which is three times more frequent in women than in men (Olivier *et al*., 2018).

We have recently shown a consistent association between circulating inflammatory markers and markers of endothelial dysfunction in small resistance (mesenteric) in the CIA model (Mokotedi *et al*., 2019). Hence, it is possible that endothelial dysfunction can be explained by high circulating inflammatory markers independent of traditional risk factors. However, the impact of inflammation on endothelial function differs between different vascular beds

(Sandoo *et al*., 2011), and endothelial function appears to be affected earlier in small than in large arteries (Bordy *et al.*, 2018; Totoson *et al*., 2016b). This highlights the importance of assessing endothelial function in different vascular beds. Importantly, the effect of inflammation on arterial stiffness has not been investigated in the CIA model. Whether inflammation-induced endothelial dysfunction translates into arterial stiffness in the CIA rat model is unknown. *Hence in the present thesis, I aimed to determine the direct effects of circulating inflammatory markers on endothelial function in small resistance mesenteric and large conduit renal arteries. In addition, I aimed to determine the direct effects of inflammation on arterial function in the CIA rat model.*

2.7 Anti-inflammatory treatment effects on vascular function

Traditionally, disease activity and severity in RA has been managed with synthetic disease modifying anti-rheumatic drugs (DMARDs), including methotrexate, hydroxychloroquine and sulfasalazine (Gaffo *et al*., 2006; Sizova, 2008; Smolen *et al*., 2007). However, due to the inability to fully control disease activity using synthetic DMARDs (Curtis and Singh, 2011), recent advances in the understanding of the pathogenesis of RA has led to the development of new therapeutic agents. In the last 10 years, disease activity and severity has markedly improved with the use of biologic DMARDs (Ramiro *et al.*, 2017; Singh *et al.*, 2016). Currently, a number of biologic DMARDs are available, including TNF-α antagonists (Etanercept (Enbrel), Infliximab and Adalimumab), IL-6 receptor blockers (Tocilizumab (Actemra) and Sarilumab) and IL-1 receptor blockers (Anakinra) (Hamilton and Clair, 2000; Wasserman, 2011). These biologic DMARDs target specific cytokines in biologic pathways regulating the inflammatory response in RA (Hamilton and Clair, 2000; Wasserman, 2011). Biologic DMARDs bind specifically to selected cytokines, to prevent binding to the membrane receptor, thus interfering with the biological function of the cytokine (Guo *et al.*, 2018). Although the use of biologic DMARDs impede disease progression and improve systemic

inflammation more effectively than synthetic DMARDs (Strand and Singh, 2007), their effects on vascular function are controversial. The section below highlights what is currently known about biologic DMARD effects on RA disease management and specifically vascular function.

2.7.1 Biologic DMARD effects on endothelial function

TNF-α antagonists have been very successful in the treatment of disease activity and severity in RA (McInnes and Schett, 2017). As a result, majority of investigations of biologic DMARD effects on endothelial function were focussed on TNF-α antagonist therapy. Table 2.1 summarises the effect of biological DMARD therapy on endothelial function in clinical studies. Majority of these studies have reported that TNF-α antagonists including Etanercept (Maki-Petaja *et al*., 2006; Bilsborough *et al.*, 2006), Infliximab (Cardillo *et al.*, 2006; Komai *et al.*, 2007; Hurlimann *et al.*, 2002; Bilsborough *et al.*, 2006; Sidiropoulos *et al.*, 2009; Bosello *et al.*, 2008) and Adalimumab (Sidiropoulos *et al.*, 2009) improve endothelial function as measured by FMD. Despite these encouraging findings, the improved endothelial function associated with long term TNF-α antagonist therapy seems to be transient and that endothelial function returns to pre-infusion states one month after infliximab administration (Gonzalez-Juanatey *et al*., 2004; Irace *et al.*, 2004; Bosello *et al.*, 2008). In contrast others reported no changes in endothelial function despite effective reduction of disease activity after TNF-α antagonist therapy (Cardillo *et al.*, 2006; Rongen *et al.*, 2018).

Il-6 receptor blocker treatment studies are still fairly new but their importance in treating CVDs in RA patients is being established as it is involved in many immunological processes in RA (Genovese *et al*., 2008). Only a few studies have investigated the effects of IL-6 receptor blocker therapy on endothelial function in patients with RA (Ikonomidis *et al.*, 2019; Protogerou *et al.*, 2011; Ruiz- Limón *et al.*, 2017). These studies showed that endothelial function was improved after 3- 6 months of tocilizumab therapy (Ruiz- Limón *et al*., 2017).

Given the evidence that IL-6 plays a pivotal role in the pathogenesis of RA and is associated with accelerated vascular risk (Sattar *et al*., 2003), more studies are needed to investigate the therapeutic role of IL-6 receptor blocker therapy on endothelial function. Although the studies on the effects of IL-1β inhibitors have been limited, two studies from the same group have shown that the use of an IL-1β inhibitor (Anakinra) improved impaired endothelial function as well as coronary aortic function (Inkonomidis *et al*., 2008). However, further studies investigating the effects of IL-β inhibitors on endothelial function is warranted.

2.7.2 Biologic DMARD effects on arterial stiffness in RA patients

A number of clinical studies have examined the effects of biologic DMARD therapy on arterial stiffness in RA (Bordy *et al.*, 2018; Totoson *et al*., 2016b; Maki-Petaja *et al*.,2006; Pieringer *et al*., 2010; Komai *et al*., 2007; Ikonomidis *et al.*, 2008; Van Doornum *et al*., 2005; Galarraga *et al*., 2009; Angel *et al.*, 2010). Table 2.2 summarises the effects of biologic DMARD therapy on arterial stiffness in RA. Some studies have reported that TNF-α antagonists improved arterial stiffness including PWV (Angel *et al*., 2010; Maki-Petaja *et al.*, 2006) and AIx (Galarraga *et al*., 2010), while others have reported that TNF-α antagonists and IL-6 blockers have no effect on arterial stiffness despite improved disease activity (Komai *et al*., 2007; Ikonomidis *et al.*, 2008; Van Doornum *et al.*, 2005; Wong *et al.*, 2007). Similarly, a recent meta-analysis reported that TNF-α antagonists induced no changes in arterial stiffness measurements despite improvements in the inflammatory status of RA patients (Mathieu *et al*., 2013). Although there have been no studies on the effects of IL-6 blockers on arterial stiffness, one study by Inkonomidis *et al*. (2008) found that the used of the IL-1β inhibitors (Anakinra) showed little to no improvement in arterial stiffness. Nevertheless, more evidence on the effects of biologic DMARDS, other than TNF-α receptor blockers, are needed.

In summary, evidence from clinical studies reveal that the effects of biologic DMARD therapy on vascular function is not clearly defined. This could be as a result of the patient's clinical response to treatment as it has been reported that patients who respond better to treatment showed a tendency of improved vascular function compared to non-responders (Galarraga *et al.*, 2009). These findings suggest that the reduction in disease-related inflammation could improve vascular function. However, most of these clinical studies have some limitations, including the very small sample sizes, heterogeneous study designs, no adjustments for confounding variables and no prospective follow-ups. In addition, the major complication in understanding the effects of biologic DMARDs in clinical studies is that these drugs are usually used in combination with synthetic DMARDs and cardiovascular drugs (Pieringer *et al.*, 2010). Thus, the independent contribution of biologic DMARDs to vascular function remains uncertain. In addition, the cross-sectional nature of the above-mentioned studies precludes investigating the mechanisms whereby biologic DMARDs affect vascular function and requires further investigation. In this regard, the use of animal models of chronic inflammation remains the prime setting to assess the direct effects of biologic DMARDs on the vascular function with limited confounding factors.

To date, only one study in the adjuvant-induced arthritis model has investigated the effects of TNF-α antagonists on endothelial function (Totoson *et al.*, 2016b). This study showed that treatment with TNF-α antagonists improved the inflammatory status and reduced the inflammation-induced impairments in endothelial function in the adjuvant-induced arthritis model (Totoson *et al.*, 2016b). There are currently no studies that have evaluated the effects of biologic DMARDs on vascular function in the CIA rat model. Whether the use of TNF-α antagonists and IL-6 blockers improve disease-related inflammation and vascular function in the CIA rat model is unknown. *Hence, in the present thesis, I aimed to determine the effects of*

Table 2.1. Summary of clinical studies showing the effects of biologic DMARDS on endothelial function in RA patients

Authors	n	Treatment	Treatment administered	Findings
Bilsborough et al., 2006	9	Infliximab Etanercept	0 and 36 weeks	DAS28 was decreased. FMD was increased.
Bosello et al., 2008	10	Infliximab	0, 2, 6 and 14 weeks (day before and after infusion)	DAS28 was decreased, ESR and CRP concentrations decreased. FMD increased day after each infusion but returned to baseline by next infusion.
Cardillo et al., 2006	10	Saline & Infliximab	Immediately following infusion	No changes were observed in CRP concentrations. ACh-induced relaxation was improved. No changes were observed in SNP-induced relaxation.
Gonzalez-Juanatey et al., 2006	8	Adalimumab	0, 2 days, 2 weeks and 12 weeks	DAS28, CRP and ESR concentrations were decreased. FMD increased.
Hurlimann et al., 2002	11	Infliximab	0 and 12 weeks	DAS28 and ESR concentrations were decreased. No change in CRP. FMD increased.
Ikonomidis et al., 2008	23	Anakinra	0 and 30 days	DAS28 and ESR concentrations were decreased. FMD increased 30 days after treatment.
Ikonomidis et al., 2008	23	Anakinra	0 and 3 hours	No changes in CRP concentrations. FMD was increased after 3 hours.
Ikonomidis et al., 2019	40	Tocilizumab	3 months	CRP concentrations were decreased. FMD improved after 3 months.
Irace et al., 2004	10	Infliximab	0, 2, and 6 weeks (before and after each infusion)	DAS28 and ESR concentrations were decreased, no change in CRP. FMD increased after each infusion, returned to baseline by next infusion.
Komai et al., 2007	15	Infliximab	0, 2 & 6 weeks.	DAS28, CRP and ESR concentrations were decreased. FBF increased
Maki-Petaja et al., 2006	9	Etanercept	0, 4 & 12 weeks	DAS28, CRP and ESR concentrations were decreased. FMD increased.
Protogerou et al., 2011	16	Tocilizumab	3 & 6 months	CRP concentrations were decreased. FMD improved after 3 months.
Rongen et al., 2018	6	Etanercept	6 months	No difference from baseline

Ruiz- Limón et al., 2017	20	Tocilizumab	Every week for 6 months	DAS28, E-selectin and VCAM-1 were decreased. Increased endothelial function (baseline). FMD improved after 6 months.
Sidiropoulos et al., 2008	12	Adalimumab Infliximab	0, 3 months and every 2 months up to 18 months	DAS28 increased, CRP concentrations decreased. FMD increased.

CRP: C-reactive protein, DAS28: Disease Activity Score 28 joint count, ESR: erythrocyte sedimentation rate, FMD: flow-mediated dilatation, VCAM-1: vascular cell adhesion molecule 1, E-selectin: endothelial leukocyte adhesion molecule

Table 2.2 Summary of clinical studies showing the effects of biologic DMARDs on arterial stiffness in RA patients

Authors	**n**	**Treatment**	**Treatment administered**	**Findings**
Angel et al., 2010	35	Adalimumab Etanercept Infliximab	0 and 3 months	PWV and circulating ESR and CRP concentrations were decreased. No changes in AIx.
Galarraga et al., 2009	26	Etanercept	0, 2 and 4 months	DAS28, CRP concentrations and AIx were decreased.
Ikonomidis et al 2008	23	Anakinra	0 and 30 days	DAS28 and CRP concentrations were decreased. No changes in PWV
Komai et al., 2007	15	Infliximab	0, 2 and 6 weeks	DAS28, CRP, ESR concentrations were decreased. No changes in PWV
Maki-Petaja et al., 2006	9	Etanercept	0, 4 and 12 weeks	DAS28, CRP, ESR concentrations and aortic PWV were decreased No change in PWA and brachial PWV
Pieringer et al., 2010	17	Infliximab	0 and 7 weeks	ESR, CRP & DAS28 concentrations were decreased. AIx was increased.
Van Doornum et al., 2005	14	Etanercept Adalimumab Infliximab	0 and 6 weeks	ESR, CRP & DAS28 concentrations were decreased No changes in AIx
Wong et al., 2009	17	Infliximab	0 and 56 weeks	DAS28 & ESR were decreased. No changes in PWV and AIx.

AIx: augmentation index, CRP: C-reactive protein, DAS28: Disease Activity Score 28 joint count, ESR: erythrocyte sedimentation rate, PWA: pulse wave analysis, PWV: pulse wave velocity, E-selectin: endothelial leukocyte adhesion molecule

the TNF-α blocker Etanercept as well as the IL-6 blocker Tocilizumab on inflammation-induced vascular dysfunction in the CIA rat model.

2.8 Problem statement

Patients with RA have an increased risk of developing atherosclerosis. Endothelial dysfunction and arterial stiffness play a key role in the development of atherosclerosis and subclinical CVD in RA and are consequently important targets for reducing cardiovascular risk in patients with RA. However, whether exposure to high-grade inflammation directly impairs endothelial function and large artery stiffening needs further elucidation. In addition, biologic DMARDs are showing promising disease outcomes, and have been reported to reduce cardiovascular risk in patients with RA. However, clinical studies on the effects of biologic DMARDs on endothelial dysfunction and arterial stiffness in RA have provided conflicting results. Whether their effects depend on the beneficial impact on the inflammatory status or vascular function needs to be confirmed. In this context, a better understanding of the role of inflammation and the role of biologic DMARD therapy on the vasculature may identify possible therapeutic targets for the treatment of endothelial dysfunction and arterial stiffness and the prevention and management of atherosclerosis in patients with RA.

2.9 Aim

The aim of this study was to determine the effect of biologic DMARDs on endothelial function and arterial stiffness in Sprague Dawley rats exposed to collagen-induced arthritis (CIA).

The specific objectives of the current study are to determine

1. the effect of inflammation on endothelial function in mesenteric and renal arteries of Sprague Dawley rats exposed to CIA using the wire-myograph system.

2. the effect of inflammation on arterial stiffness in Sprague Dawley rats exposed to CIA using the PulsePen device.

3. whether treatment with TNF-α blockers or IL-6 blockers alters inflammation-induced changes in vascular function in Sprague Dawley rats exposed to CIA.

4. the association of circulating inflammatory markers with markers of vascular function in Sprague Dawley rats exposed to CIA, and those treated with TNF-α blockers (Etanercept) or IL-6 blockers (Tocilizumab).

CHAPTER 3

METHODS

3.1 Animals

One-hundred, three-month old Sprague-Dawley rats (female, n=40; male, n=60) were obtained from the Central Animal Service (CAS) at the University of the Witwatersrand. Rats were housed individually in a temperature-controlled room with a 12-hour light-dark cycle. During the intervention three rats died (females, n=3) and nineteen (females, n=11 and males, n=8) rats did not show any signs of inflammation after exposure to the arthritis protocol and were therefore excluded from the study. Seventy-eight rats (female, n=31 and male, n=47) were included in the final study. All procedures were approved by the animal ethics screening committee of the university of the Witwatersrand (AESC number: 2017/03/21C).

3.2 Experimental design

An outline of the experimental design is presented in Figure 3.1. Rats were habituated for two weeks where they had unrestricted access to drinking water and standard rat chow (Nutritionhub, Stellenbosch, South Africa). During the habituation period, blood pressure (BP) was measured twice a week, body weight (BW), arthritis scores and paw thickness were measured once a week. Following the habituation period, rats were randomly divided into the control (n=22, 9 females and 13 males), inflammation (n=26, 8 females and 18 males), tumour necrosis factor-alpha blocker (TNF-α blocker; n=15, 7 females and 8 males) and interleukin-6 blocker (IL-6 blocker; n=15, 7 females and 8 males) groups. To induce arthritis, rats in the inflammation, TNF-α blocker and IL-6 blocker groups received a 0.2 ml subcutaneous injection of bovine type-II collagen emulsified in Freund's adjuvant at the base of the tail. Rats in the control group received a 0.2 ml subcutaneous injection of saline at the base of the tail. Following the onset of inflammation (~day 28), the TNF-α blocker group received intraperitoneal injections of Etanercept (Enbrel) (TNF-α receptor blocker) every third day and the IL-6 blocker group received intraperitoneal injections of Tocilizumab (Actemra) (IL-6 receptor blocker) once a week for 6 weeks. Body weight, food intake, blood pressure, arthritis

scores and paw thickness measurements at the tarsometatarsal and ankle joints were assessed every two weeks. After six weeks of treatment rats were anaesthetised by intramuscular injections of ketamine (100 $mg.kg^{-1}$) and xylazine (5 $mg.kg^{-1}$) to obtain non-invasive arterial stiffness measurements after which rats were terminated by thoracotomy. Vascular reactivity was assessed in mesenteric and renal arteries. A blood sample was collected to measure serum concentrations of TNF-α, IL-6 and C-reactive protein (CRP).

3.3 Induction of collagen-induced arthritis

After the two-week habituation period, systemic high-grade inflammation was induced by injecting an arthritis-inducing emulsion into the rats as previously described (Jasemian *et al.*, 2011). The arthritis-inducing emulsion was prepared by dissolving bovine type-II collagen (Chondrex cat. #20021, Redmond, WA, USA) in 0.05M acetic acid by gently stirring overnight at 4°C. Equal amounts of dissolved bovine type-II collagen (2 mg/ml) and incomplete Freund's adjuvant (Chondrex cat. #7002, Redmond, WA, USA) were mixed using an electric homogeniser until a white emulsion formed. The emulsion was prepared immediately prior to immunisation. Rats in the Inflammation, TNF-α blocker and IL-6 blocker groups were immunised with 0.2 ml (200 µg) of the emulsion by a subcutaneous injection at the base of the tail. Rats in the Inflammation, TNF-α blocker and IL-6 blocker groups received 0.1 ml (100 µg) booster injections on day 7 and day 21 following the primary immunisation to ensure a high incidence and severity of arthritis. Control rats received a subcutaneous injection of 0.2 ml physiological saline solution at the base of the tail. All procedures were performed under general anaesthesia (isoflurane).

3.4 Drug intervention

Upon the first sign of inflammation (~ day 28), as identified by swelling, oedema or joint rigidity in the hind paws, rats in the TNF-α blocker group received intraperitoneal injections of

TNF-α receptor blocker (Etanercept) (Pfizer, New York, United States) at a dosage of 10 mg/kg every three days for 6 weeks. Rats in the IL-6 blocker group received intraperitoneal injections of 8 mg/kg IL-6 receptor blocker (Tocilizumab) (Roche, Basel, Switzerland) once a week for 6 weeks (Totoson *et al*., 2016b). These concentrations were previously reported in the literature (Totoson *et al*., 2016b; Kobayashi *et al*., 2016; Hançerli *et al*., 2017) and are in line with treatment guidelines in clinical RA (Baniaamam *et al*., 2018). Rats in the Inflammation, TNF-α blocker and IL-6 blocker groups also received a daily injection of Tramazac between 1 and 4 mg/kg (Zydus Healthcare, Johannesburg, South Africa) upon the first signs of swelling in the paws, to alleviate the pain.

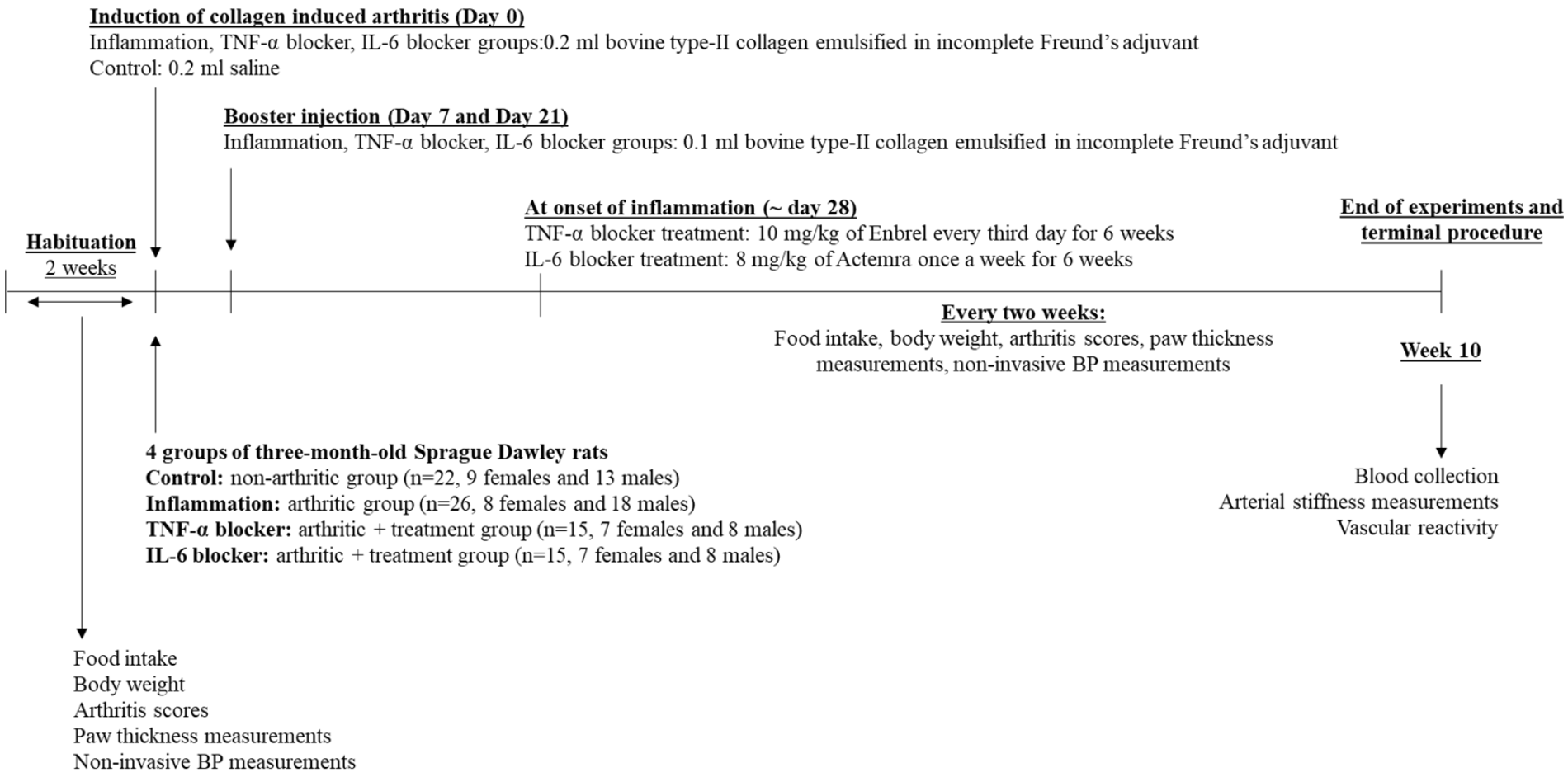

Figure 3.1 Experimental design

3.5 Arthritis scores

To evaluate the severity of arthritis, the hind paws of the rats were scored as previously described (Kim *et al*., 2010). Swelling was scored on a scale of zero to four, where a score of zero was assigned if the hind paw presented no swelling or focal redness. If the hind paw had slight swelling or focal redness a score of one was given. If the hind paw had low-to-moderate oedema a score of two was given. If the hind paw had pronounced oedema with limited use of the joint a score of three was given. If the paw had excessive oedema with deformity and joint rigidity the paw was given an arthritis score of four. The cumulative score for both hind paws for each rat (with a possible maximum of eight) was used to represent the overall disease severity and progression. As arthritis scores only provide a subjective measure of the severity of arthritis, the hind paw thickness was measured at the tarsometatarsal and ankle joints using a digital calliper (Mastercraft, South Africa) every two weeks.

3.6 Blood pressure

Blood pressure was measured every two weeks, as well as taken the day prior to termination and under anaesthesia in order to calibrate the Pulse Pen (PulsePen device, DiaTecne, Milan, Italy). Using the tail cuff technique which employs non-evasive blood pressure (NIBP) Amplifiers (Biopac Systems, Santa Barbara, CA, USA). Blood pressure was measured three times and the average of the last two measures were used. To obtain blood pressure, rats were placed in restrainers and tails were heated for ten minutes. Measurements were taken at midday to avoid diurnal variation. Rats were habituated to the restrainers for two weeks before the first measurement to enable them to adapt to the procedure.

3.7 Arterial stiffness

Prior to termination, rats were anaesthetised by intramuscular injections of ketamine (100 $mg.kg^{-1}$) and xylazine (5 $mg.kg^{-1}$) to measure aortic stiffness by applanation tonometry

(PulsePen device, DiaTecne, Milan, Italy). The neck and inner right thigh of the rats were shaved and placed on a heating pad in the supine position. The instrument was calibrated using the tail-cuff blood pressure obtained immediately prior to the measuring of the pulse wave. The carotid and femoral pulse waves were recorded simultaneously by placing one probe on the common carotid artery and one probe on the femoral artery. The two probes were positioned and fixed on the arteries by means of mechanical arms, equipped with micro-regulators. Once the probes were in place, pressure signals were transmitted to a computer by means of an optical fibre that ensures the electromagnetic isolation for the rat undergoing the test. After the tonometric pulse wave acquisition, the sites where probes were placed were marked on the skin and the distance from the carotid to the femoral were measured. Pulse wave velocity (PWV) was calculated as the distance between the two recording sites divided by the time delay between the two arterial waveforms at each site. Other measurements obtained from the PulsePen device include the forward (Pf) and backward waves (Pb) and augmentation pressure (AP) and augmentation index (AIx), all which were used to determine arterial stiffness.

3.8 Vascular reactivity

After the arterial stiffness measurements, the anaesthetised rats were terminated by thoracotomy and the heart was removed from the thoracic cavity. The kidneys and the mesentery were removed and placed in a cold, modified Krebs-Ringer bicarbonate (control) solution (mmol/l: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11.1 glucose). The arteries from the kidney and mesentery were isolated and rings (2 mm long) were cleaned of fat and connective tissue and suspended in a wire myograph (model 610M, Danish Myo Technology, Aahrus, Denmark). The wire myograph system is designed to measure the reactivity of small isolated blood vessels of 100 μm to 2.5 mm in diameter. Four blood vessels were mounted as rings for each rat (2 mesenteric and 2 renal arteries) and threaded over two parallel stainless-steel wires.

The wires were secured to two supports or "jaws" with one support attached to a precision micrometer (allowing manual control of vessel circumference and stretch). The other support was attached to a force transducer for measurements of force/tension development. 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). The preparation was kept in a heated chamber filled with an oxygenated, physiological salt solution at 37°C. 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. Two rings (1 mesenteric artery and 1 renal artery) were exposed to increasing concentration of KCl (1 to 120 mM) and 2 rings (1 mesenteric artery and 1 renal artery) were exposed to increasing concentrations of 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. Following the vasoconstriction responses, the heated chambers were rinsed 3 times with the Krebs-Ringer bicarbonate solution and Phe (10^{-5}M) was added to preconstrict the vessels. Once a stabilised constriction was obtained, two rings (1 mesenteric artery and 1 renal artery) were exposed to increasing concentrations of acetylcholine (an endothelium-dependent vasodilator (ACh, 0.1 nM to 10 mM)), and 2 rings (1 mesenteric artery and 1 renal artery) were exposed to increasing concentrations of sodium nitroprusside (endothelium-independent vasodilator (SNP, 1 nM to 100 mM)). Vascular relaxation was expressed as a percentage of the baseline tension during contractions induced by Phe (10 μM). KCl, Phe, SNP and ACh were purchased from Sigma (St. Louis, MO, USA).

3.9 Enzyme-linked immunosorbent assay

After thoracotomy (as described in section 3.8 above), blood was collected into serum clot activator tubes and allowed to clot for 30 minutes on ice. Blood was then centrifuged and stored at -80°C for further analysis. Serum concentration of TNF-α, IL-6 and CRP were determined using enzyme-linked immunosorbent assay kits (Elabscience Biotechnology Co. Ltd, Wuhan, China). The lower detection limit for TNF-α, IL-6, and CRP were 78.13 pg/ml, 62.50 pg/ml and 0.31 ng/ml respectively, all with coefficients of variation of <10%.

3.10 Statistical analysis

Statistical analysis was performed using SAS software, version 9.4 (SAS Institute Inc., USA). Data are expressed as means ± standard error of the mean (SEM). Differences in food intake, body weight, arthritis scores, paw thickness, blood pressure and the vascular reactivity dose-response curves between the groups were evaluated using repeated-measures analysis of variance (ANOVA) followed by Tukey post-hoc tests. The sensitivity (EC_{50}) and the maximal (E_{max}) responses to vasodilators and vasoconstrictors were determined from regression analysis logistic sigmoid function curves (GraphPad Software Inc, San Diego, CA). The EC_{50} was log transformed to improve normality. A two-way ANOVA followed by a Tukey post hoc test was used to determine differences in inflammatory markers, arterial stiffness measurements and the $logEC_{50}$ and E_{max} responses between the groups. Associations between inflammatory markers and the sensitivity ($logEC_{50}$) and maximal (E_{max}) to ACh-induced relaxation were determined using Pearson's correlations. Differences were considered statistically significant if $p < 0.05$. As there were no sex differences in any of the blood pressure or vascular measurements; the male and female data were grouped to increase statistical power.

CHAPTER 4

RESULTS

4.1 Body weight and food intake changes

Table 4.1 shows the body weight and food intake at baseline and at termination. There were no significant differences in body weight and food intake within or between the groups at baseline or at termination ($p>0.05$).

Table 4.1 The body weight and food intake at baseline and termination between the different groups in the CIA rat model.

	Control	**Inflammation**	**TNF-α blocker**	**IL-6 blocker**
Baseline				
Body weight (g)	426.8 ± 25.3	430.7 ± 23.3	398.8 ± 30.7	406.9 ± 30.7
Food intake (g)	182.3 ± 14.5	205.7 ± 13.3	160.7 ± 17.5	144.2 ± 17.5
Termination				
Body weight (g)	486.7 ± 25.3	454.4 ± 23.3	411.9 ± 30.7	387.6 ± 30.7
Food intake (g)	182.3 ± 14.5	151.6 ± 11.8	160.7 ± 17.5	144.2 ± 17.5

Data presented as mean ± SEM.

4.2 Arthritis scores and paw thickness

Figure 4.1 shows the degree of inflammation (A), the arthritis scores (B), and paw thickness at the tarsometatarsal joint (C) and ankle joint (D) in the four groups of rats. Inflammation developed in approximately 28 days after the first immunisation in hind paws of the rats. The inflammation, TNF-α blocker and IL-6 blocker groups showed clear signs of joint swelling and oedema compared to control rats (Figure 4.1 A, pictures taken at termination).

The arthritis scores were significantly increase in the inflammation group at weeks six (3.39 ± 0.50), eight (3.5 ± 0.49) and 10 (3.06 ± 0.47) compared to baseline (0 ± 0) (all $p<0.0001$). The arthritis scores were significantly increased in the TNF-α blocker group at weeks six (4.33 ± 0.55), eight (4.26 ± 0.53) and 10 (3.70 ± 0.52) compared to baseline (0 ± 0) (all $p<0.0001$). The arthritis scores were significantly increased in the IL-6 blocker group at weeks six (3.93 ± 0.52), eight (4.14 ± 0.51) and 10 (3.07 ± 0.44) compared to baseline (0 ± 0) (all $p<0.0001$; Figure 4.1 B).

The arthritis scores were significantly increased at weeks four, six, eight and 10 in the inflammation group (2.17 ± 0.43, 3.39 ± 0.50, 3.5 ± 0.49, 3.06 ± 0.47 respectively) compared to the control group (0 ± 0.43; $p=0.004$, 0 ± 0.50; $p<0.0001$, 0 ± 0.49; $p<0.0001$, 0 ± 0.47; $p=0.0001$ respectively). The arthritis scores were significantly increased at weeks six, eight and 10 in the TNF-α blocker (4.33 ± 0.55, 4.26 ± 0.53, 3.70 ± 0.52 respectively) and IL-6 blocker (3.93 ± 0.52, 4.14 ± 0.51, 3.07 ± 0.44 respectively) groups compared to the control group (all $p<0.0001$). There were no differences in the arthritis scores between the inflammation, TNF-α blocker and IL-6 blocker groups at any time point (all $p>0.05$; Figure 4.1 B).

The paw thickness at the tarsometatarsal joints in the inflammatory group was significantly increased at week 10 (6.01 ± 0.23mm) compared to baseline (4.71 ± 0.14mm) ($p<0.0001$; Figure 4.1 C). The paw thickness at the tarsometatarsal joints in the inflammation, TNF-α

blocker and IL-6 blocker groups were increased at week 10 when compared to baseline although not significantly ($p>0.05$). The paw thickness at the tarsometatarsal joints were significantly increased at week 10 in the inflammation group (6.01 ± 0.23mm) compared to the control group (4.98 ± 0.28mm; $p=0.03$). There were no differences in the paw thickness at the tarsometatarsal joints between the inflammation, TNF-α blocker and IL-6 blocker groups throughout the different weeks ($p>0.05$; Figure 4.1 C).

The paw thickness at the ankle joints in the inflammatory group was significantly increased at week 10 compared to baseline ($p=0.0005$; Figure 4.1 D). Although paw thickness at the ankle joints were higher at week 10 compared to baseline in the TNF-α blocker and IL-6 blocker groups, these differences did not reach statistical significance ($p>0.05$). The paw thickness at the ankle joints were significantly increased at week 10 in the inflammation group (9.59 ± 0.23mm) compared to the control group (8.41 ± 0.28mm; $p=0.01$). There were no differences in the paw thickness at the ankle joints between the inflammation, TNF-α blocker and IL-6 blocker groups throughout the study ($p>0.05$; Figure 4.1D).

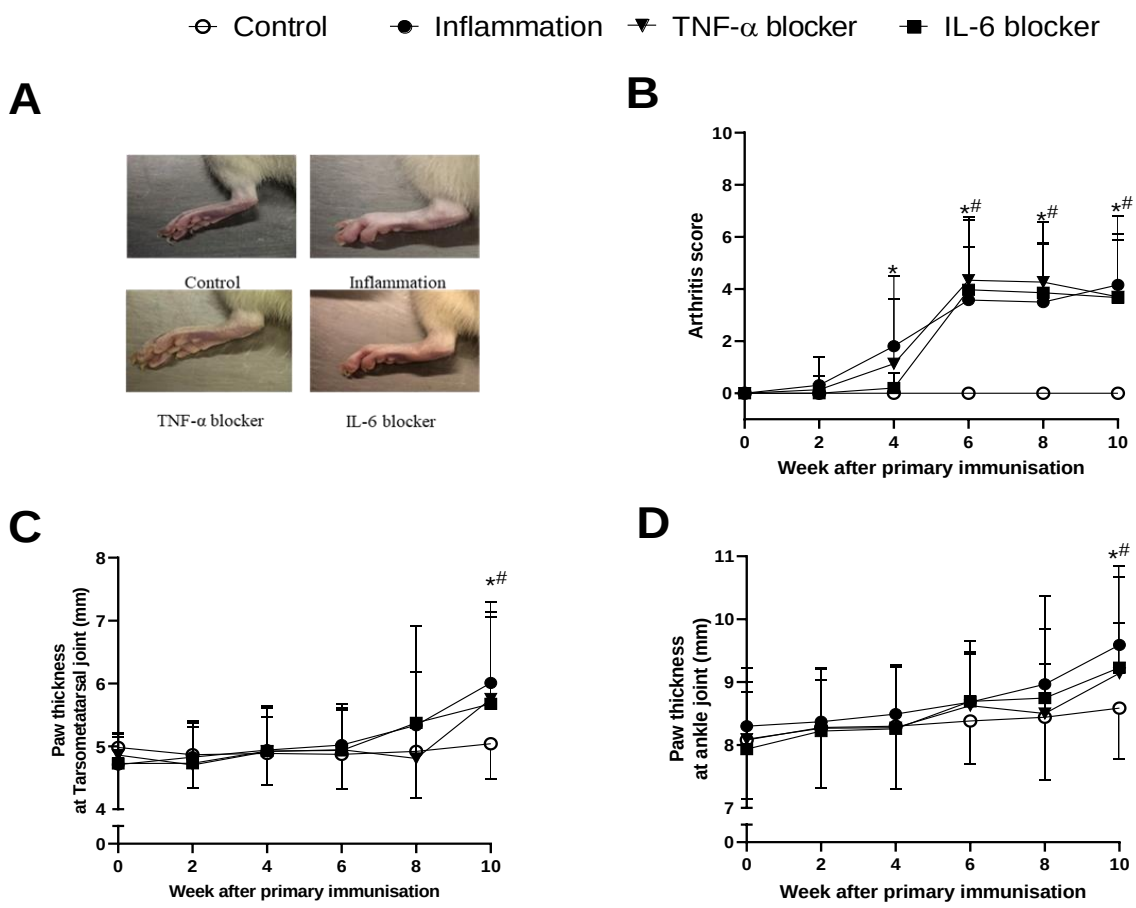

Figure 4.1 The effects of TNF-α blocker and IL-6 blocker on the degree of inflammation in hind limbs **(A)**, arthritis scores **(B)** and paw thickness at tarsometatarsal **(C)** and ankle joints **(D)**. Data expressed as means ± SEM. # $p<0.05$ compared to baseline, * $p<0.05$ compared to control group.

4.3 Inflammatory markers

The serum concentrations of TNF-α were significantly higher in the inflammation (187.48 ± 9.44pg/ml), TNF-α blocker (146.17 ± 11.17pg/ml), and IL-6 blocker (162.78 ± 11.56pg/ml) groups compared to the control (100.26 ± 9.92pg/ml) group ($p<0.0001$, $p=0.02$, $p=0.001$ respectively; Figure 4.2 A). The serum concentrations of TNF-α were significantly higher in the inflammation group (187.48 ± 9.44pg/ml) compared to the TNF-α blocker group (146.17 ± 11.17pg/ml; $p=0.03$; Figure 4.2 A). There were no significant differences between the inflammation and IL-6 blocker groups ($p=0.35$; Figure 4.2 A).

The serum concentrations of IL-6 were significantly higher in the inflammation (32.17 ± 2.11pg/ml) and IL-6 blocker (30.77 ± 2.71pg/ml) groups compared to the control (19.23 ± 2.26pg/ml) group ($p=0.001$, $p=0.01$ respectively; Figure 4.2 B). Serum IL-6 concentrations were not significantly different between the TNF-α blocker (27.91 ± 2.61pg/ml) group and the control group (19.23 ± 2.26pg/ml; $p=0.07$ Figure 4.2 B). There were no significant differences between the inflammation and IL-6 blocker groups ($p=0.98$; Figure 4.2 B).

The serum concentrations of CRP were significantly higher in the inflammation (0.62 ± 0.04ng/ml), TNF-α blocker (0.44 ± 0.06ng/ml), and IL-6 blocker (0.65 ± 0.06ng/ml) groups compared to the control (0.15 ± 0.05ng/ml) group ($p<0.0001$, $p=0.001$, $p<0.0001$ respectively; Figure 4.2 C). The serum concentrations of CRP were significantly higher in the IL-6 blocker group (0.65 ± 0.06ng/ml) compared to the TNF-α blocker group (0.44 ± 0.06ng/ml; $p=0.04$; Figure 4.2 C). There was a trend towards a lower CRP serum concentration in the TNF-α blocker group (0.44 ± 0.06ng/ml) compared to the inflammation group (0.62 ± 0.04ng/ml, $p=0.06$; Figure 4.2 C). There were no significant differences in CRP concentrations between the inflammation and IL-6 blocker groups ($p=0.96$; Figure 4.2 C).

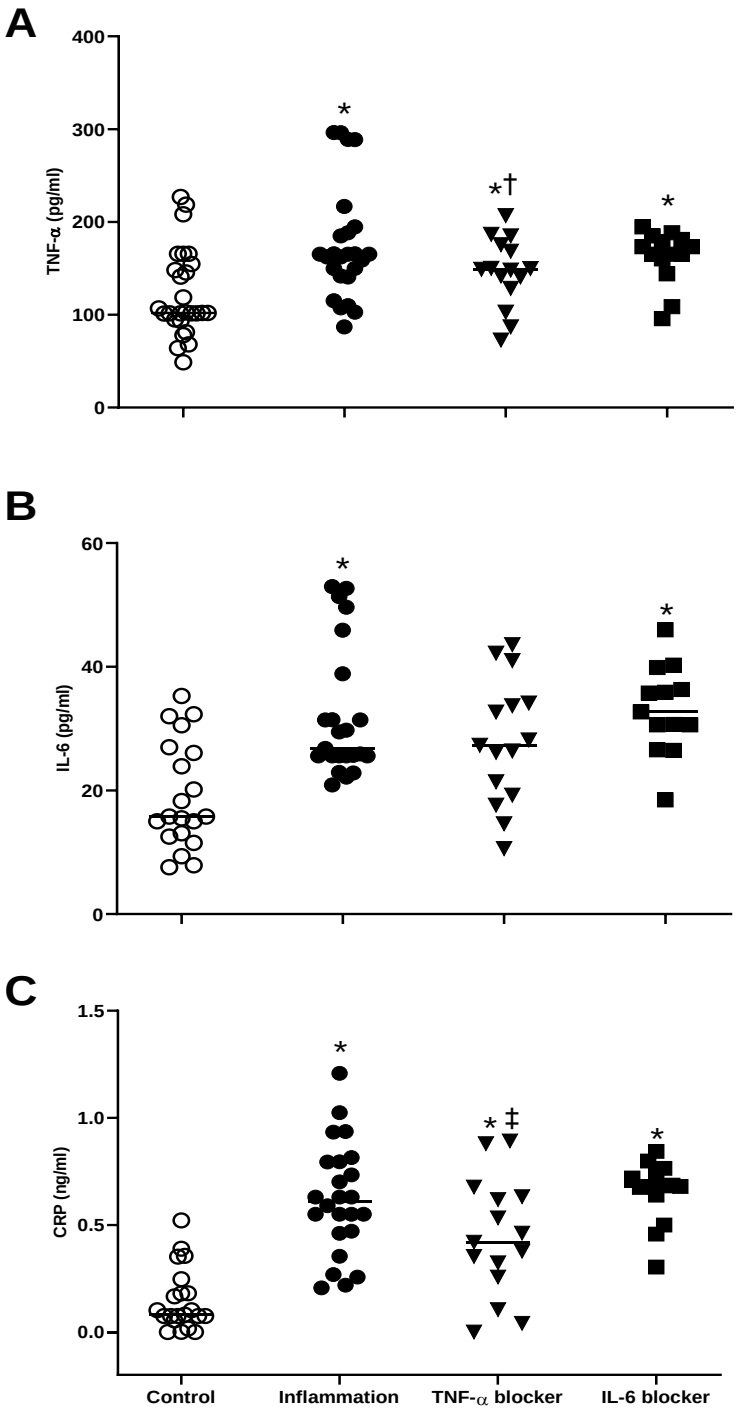

Figure 4.2 The effects of TNF-α blocker and IL-6 blocker on circulating serum TNF-α **(A)**, IL-6 **(B)** and CRP **(C)** concentrations. * $p<0.05$ compared to the control group. † $p<0.05$ compared to the inflammation group. ‡ $p<0.05$ compared to the IL-6 blocker group.

4.4 TNF-α blocker and IL-6 blocker treatment effect on inflammation induced vascular reactivity changes

4.4.1 Contractile responses

Figure 4.3 shows the contractile dose response curves of potassium chloride (KCl) and phenylephrine (Phe) in mesenteric and renal arteries of the four groups. There were no significant differences in the KCl-induced and Phe-induced contractile responses in either the mesenteric or renal arteries ($p>0.05$; Figure 4.3 A-D). The sensitivity ($LogEC_{50}$) and maximum contractile responses (E_{max}) values derived from these curves are presented in Table 4.2. The sensitivity ($LogEC_{50}$) and maximum contractile responses (E_{max}) to KCl and Phe in mesenteric and renal arteries were similar between the groups ($p>0.05$; Table 4.2).

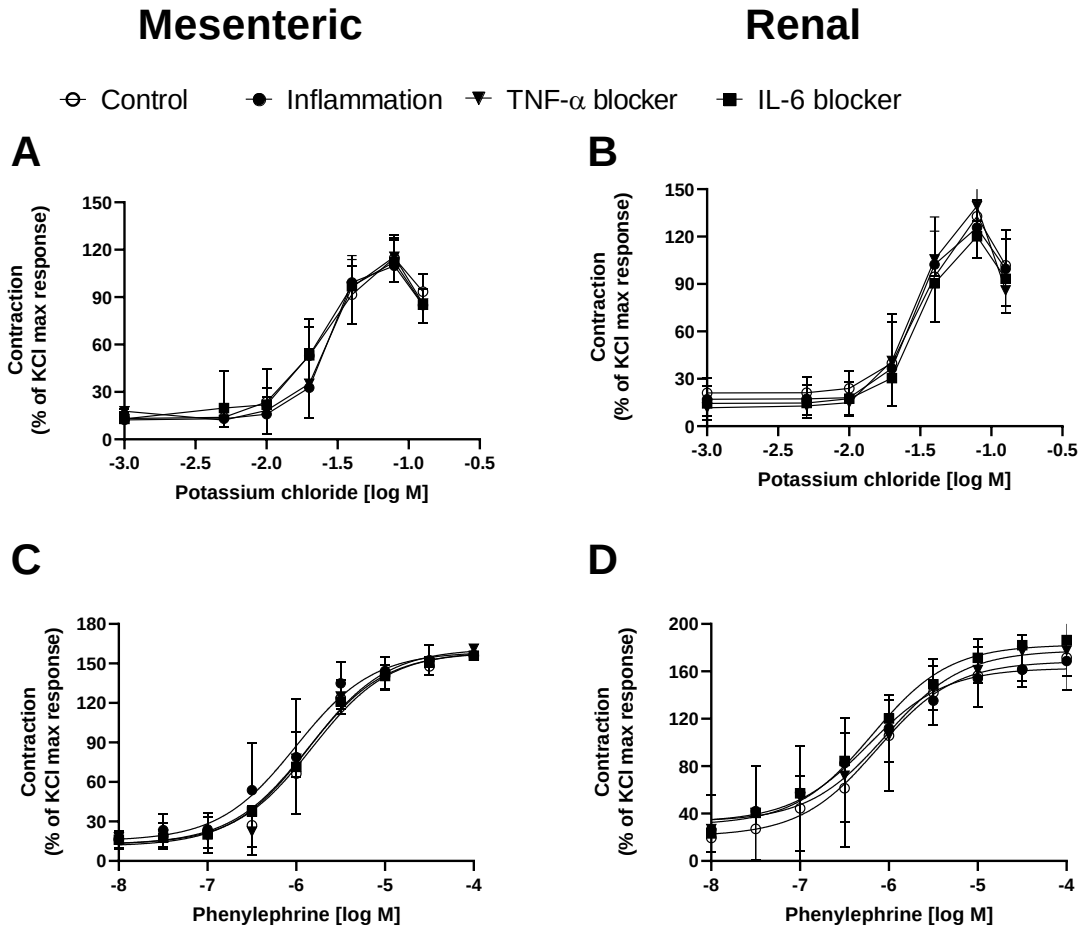

Figure 4.3 The effects of TNF-α blocker and IL-6 blocker on the contractile dose response curves of potassium chloride (KCl) (**A** and **B**) and phenylephrine (Phe) (**C** and **D**) in mesenteric (left panel) and renal (right panel) arteries.

Table 4.2 The effects of TNF-α blocker and IL-6 blocker on the sensitivity ($LogEC_{50}$) and maximum responses (E_{max}) to potassium chloride (KCl) and phenylephrine (Phe) in mesenteric and renal arteries.

		Mesenteric arteries				Renal arteries			
		Control	**Inflammation**	**TNF-α blocker**	**IL-6 blocker**	**Control**	**Inflammation**	**TNF-α blocker**	**IL-6 blocker**
KCl	$LogEC_{50}$ (M)	-1.54 ± 0.05	-1.51 ± 0.04	-1.46 ± 0.05	-1.63 ± 0.05	-1.35 ± 0.05	-1.37 ± 0.04	-1.54 ± 0.06	-1.38 ± 0.05
	E_{max} (mN)	146.51 ± 5.42	135.58 ± 4.95	139.51 ± 5.25	127.27 ± 5.83	164.96 ± 5.55	162.69 ± 5.19	149.09 ± 6.94	155.26 ± 5.36
Phe	$LogEC_{50}$ (M)	-5.88 ± 0.09	-6.07 ± 0.07	-5.92 ± 0.09	-5.92 ± 0.09	-6.17 ± 0.14	-6.18 ± 0.11	-5.98 ± 0.15	-6.44 ± 0.14
	E_{max} (mN)	158.95 ± 1.94	160.83 ± 1.65	158.47 ± 2.02	158.49 ± 2.21	172.10 ± 8.30	166.30 ± 6.49	174.53 ± 9.18	175.19 ± 8.71

Data represented as mean ± SEM

4.4.2 Relaxation responses

4.4.2 1 Mesenteric arteries

Figure 4.3 shows the relaxation dose response curves of acetylcholine (ACh) and sodium nitroprusside (SNP) in mesenteric and renal arteries. In mesenteric arteries, the ACh-induced relaxation responses at $10^{-8.5}$ to 10^{-6} M were significantly impaired in the inflammation and the TNF-α blocker groups compared to the control group (all $p<0.0001$; Figure 4.3 A). The ACh-induced relaxation responses at $10^{-8.5}$ to 10^{-6} M were significantly higher in the IL-6 blocker group compared to the inflammation and the TNF-α blocker groups (all $p<0.0001$; Figure 4.3 A). There were no differences in the relaxation responses between the control and IL-6 groups at any of the ACh concentrations.

There were no significant differences in the relaxation responses for SNP-induced relaxation between the groups in mesenteric arteries (all $p>0.05$; Figure 4C).

The sensitivity ($LogEC_{50}$) and maximum relaxation responses (E_{max}) values derived from these curves are presented in Table 4.3. The $LogEC_{50}$ of ACh-induced relaxation was significantly impaired in the inflammation (-6.17 ± 0.03M; $p<0.0001$) and the TNF-α blocker (-6.22 ± 0.02 M; $p<0.0001$) groups compared to the control group (-7.37 ± 0.03 M; Table 4.3). There were no differences in $LogEC_{50}$ of ACh-induced relaxation between the control (-7.37 ± 0.03 M) and IL-6 blocker groups (-7.34 ± 0.03 M; $p>0.05$; Table 4.3).The $LogEC_{50}$ of ACh-induced relaxation was significantly impaired in the inflammation group (-6.17 ± 0.03 M) and the TNF-α blocker (-6.22 ± 0.02 M; $p<0.0001$) groups compared to the IL-6 blocker group (-7.34 ± 0.03 M; $p<0.0001$; Table 4.3). There were no differences in the E_{max} of ACh-induced relaxation in the mesenteric arteries between the groups (all $p>0.05$; Table 4.3). There were no significant differences in $LogEC_{50}$ and E_{max} for SNP-induced relaxation between the groups in mesenteric arteries (all $p>0.05$; Table 4.3).

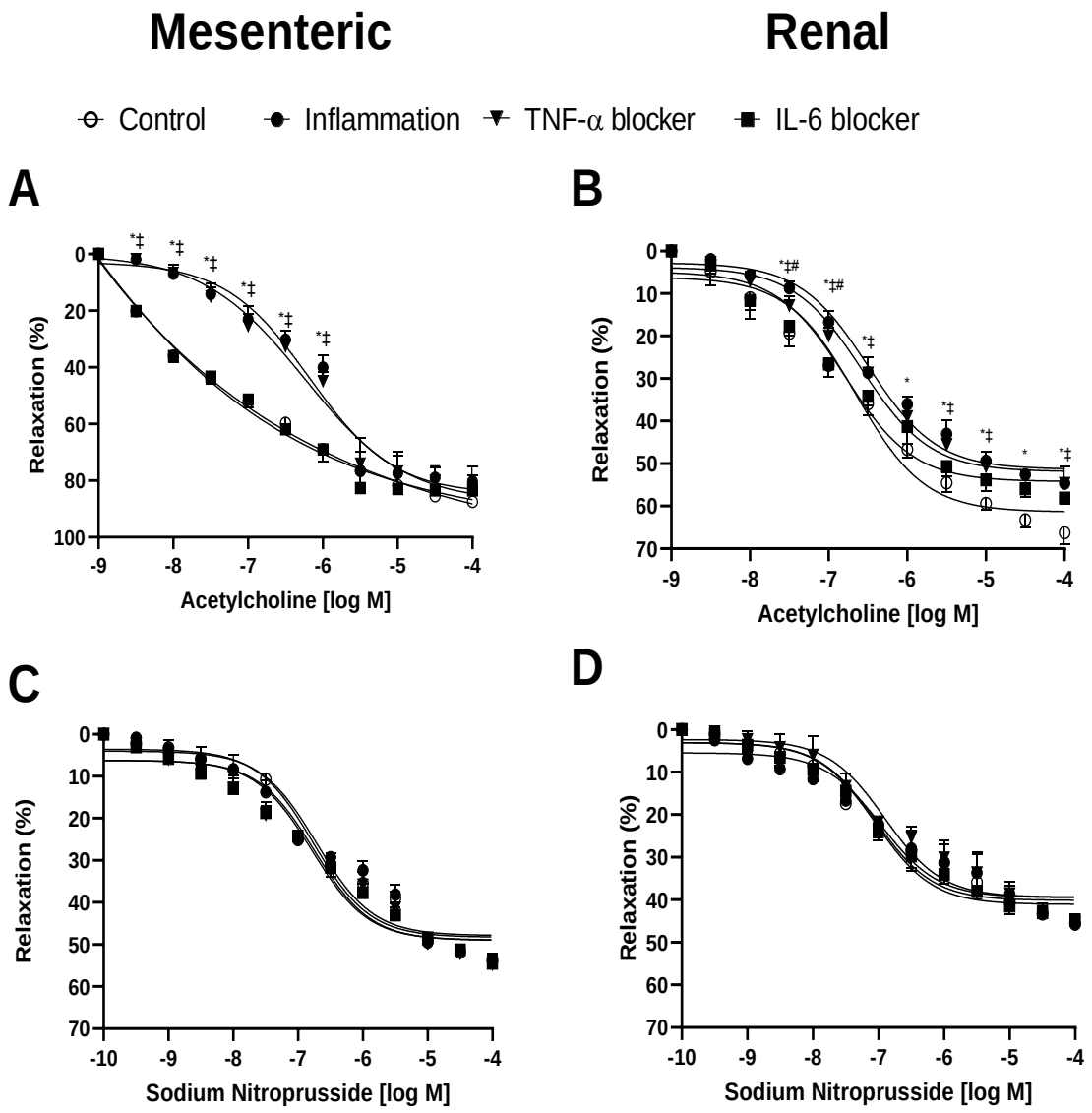

Figure 4.4 The effects of TNF-α blocker and IL-6 blocker on the relaxation dose response curves of acetylcholine (ACh) **(A and B)** and sodium nitroprusside (SNP) **(C and D)** in mesenteric (left panel) and renal (right panel) arteries. * $p<0.05$ Control group compared to the Inflammation and the TNF-α blocker groups. ‡ $p<0.05$ IL-6 blocker group compared to the Inflammation and the TNF-α blocker groups. # $p<0.05$ TNF-α blocker group compared the Inflammation group.

Table 4.3 The effects of TNF-α blocker and IL-6 blocker on the sensitivity (EC_{50}) and maximum responses (E_{max}) to acetylcholine (ACh) and sodium nitroprusside (SNP) in mesenteric and renal arteries.

		Mesenteric arteries				Renal arteries			
		Control	**Inflammation**	**TNF-α blocker**	**IL-6 blocker**	**Control**	**Inflammation**	**TNF-α blocker**	**IL-6 blocker**
ACh	$LogEC_{50}$ (M)	-7.37 ± 0.03	-6.17 ± 0.03*	-6.22 ± 0.02*	-7.34 ± 0.03†#	-6.65 ± 0.02	-6.51 ± 0.03*	-6.58 ± 0.02	-6.78 ± 0.03*†#
	E_{max} (mN)	78.97 ± 1.21	83.55 ± 1.58	82.14 ± 1.12	79.54 ± 1.24	61.36 ± 0.41	51.31 ± 0.47*	51.90 ± 0.42*	54.35 ± 0.51*†#
SNP	$LogEC_{50}$ (M)	-6.74 ± 0.03	-6.72 ± 0.02	-6.74 ± 0.03	-6.74 ± 0.03	-7.02 ± 0.05	-6.99 ± 0.05	-6.87 ± 0.06	-7.05 ± 0.06
	E_{max} (mN)	49.62 ± 0.17	49.57 ± 0.15	49.81 ± 0.18	49.81 ± 0.18	41.44 ± 0.53	41.84 ± 0.58	40.54 ± 0.63	40.89 ± 0.67

Data expressed as means ± SEM. *p<0.05 compared to the Control group. †p<0.05 compared to the Inflammation group. # p<0.05 compared to the TNF-α blocker group.

4.4.2.2 Renal arteries

In the renal arteries, ACh-induced relaxation responses at $10^{-7.5}$ to 10^{-4} M were significantly impaired in the inflammation and the TNF-α blocker groups compared to the control group (all $p<0.0001$; Figure 4.2 B). The ACh-induced relaxation responses at $10^{-7.5}$ to $10^{-6.6}$ M were significantly improved in the IL-6 blocker group compared to the inflammation and the TNF-α blocker groups (all $p<0.01$; Figure 4.3 B). The ACh-induced relaxation responses at $10^{-7.5}$ to 10^{-7} M were significantly higher in the TNF-α blocker group compared to the inflammation group ($p=0.001$ and $p=0.03$ respectively; Figure 4.3 B). The ACh-induced relaxation responses at $10^{-5.5}$, 10^{-5} and 10^{-4} M were significantly higher in the IL-6 blocker group compared to the inflammation and the TNF-α blocker groups (all $p<0.05$; Figure 4.3 B). There were no differences in the relaxation responses between the control and IL-6 groups at any of the ACh concentrations.

There were no significant differences in the relaxation responses for SNP-induced relaxation between the groups in renal arteries (all $p>0.05$; Figure 4D).

The sensitivity ($LogEC_{50}$) and maximum relaxation response (E_{max}) values derived from these curves are presented in Table 4.3. The $LogEC_{50}$ of ACh-induced relaxation was significantly impaired in the inflammation (-6.51 ± 0.03M; $p=0.001$) and the TNF-α blocker (-6.58 ± 0.03 M; $p=0.02$) groups compared to the control group (-6.65 ± 0.02 M; Table 4.3). There were no differences in $LogEC_{50}$ of ACh-induced relaxation between the control (-6.65 ± 0.02 M) and IL-6 blocker groups (-6.78 ± 0.03 M; $p>0.05$; Table 4.3).The $LogEC_{50}$ of ACh-induced relaxation was significantly impaired in the inflammation group (-6.65 ± 0.02 M) and the TNF-α blocker (-6.58 ± 0.02 M; $p<0.0001$) groups compared to the IL-6 blocker group (-6.78 ± 0.03 M; $p<0.0001$; Table 4.3). The E_{max} of ACh-induced relaxation was significantly impaired in the inflammation (51.31 ± 0.47 mN; $p<0.0001$), TNF-α blocker (51.90 ± 0.42 mN; $p<0.0001$)

and the IL-6 blocker (54.35 ± 0.51 mN; $p<0.0001$) groups compared to the control group (61.36 ± 0.41 mN; Table 4.3). There were no significant differences in $LogEC_{50}$ and E_{max} for SNP-induced relaxation between the groups in renal arteries (all $p>0.05$; Table 4.3).

4.5 Pulse wave analysis

Table 4.4 shows the effects of inflammation, and TNF-α blocker and IL-6 blocker treatment on arterial stiffness measures. There were no significant differences observed between the groups in any of the arterial stiffness measures (all $p>0.05$).

Table 4.4 The effects of TNF-α blocker and IL-6 blocker on arterial stiffness measures.

	Control	Inflammation	TNF-α Blocker	IL-6 Blocker
Systolic blood pressure	128 ± 2	129 ± 2	132 ± 2	129 ± 2
Diastolic blood pressure	88 ± 2	89 ± 1	88 ± 2	90 ± 2
Pulse pressure	40 ± 2	40 ± 2	44 ± 2	39 ± 2
Pulse wave velocity	4.71 ± 0.21	4.73 ± 0.22	5.14 ± 0.22	4.66 ± 0.22
Augmentation index	4.18 ± 1.15	6.18 ± 1.21	4.01 ± 1.21	3.72 ± 1.21
Mean arterial pressure	94 ± 15	125 ± 16	95 ± 16	95 ± 16
Augmentation pressure	1.57 ± 0.25	1.72 ± 0.26	1.58 ± 0.26	1.60 ± 0.26
Pressure of forward wave	29.31 ± 0.76	26.01 ± 0.69	27.83 ± 0.89	27.76 ± 0.89
Pressure of reflected wave	8.54 ± 0.73	8.11 ± 0.76	8.35 ± 0.76	7.25 ± 0.76

Data expressed as mean ± SEM.

4.6 Associations between circulating inflammatory markers and vascular reactivity

Figure 4.4 shows the associations between circulating inflammatory markers and the $LogEC_{50}$ and E_{max} of KCl and Phe-induced contraction in mesenteric (left panel) and renal (right panel) arteries. There were no associations between inflammatory markers and the $LogEC_{50}$ (Figure 4.4 A and B) of KCl-induced contraction in both mesenteric and renal arteries ($p>0.05$). Greater serum concentrations of TNF-α ($r=-0.36$, $p=0.01$), IL-6 ($r=-0.30$, $p=0.02$) and CRP ($r=-0.34$, $p=0.01$) were associated with impaired KCl-induced contractions (lower E_{max}) in mesenteric arteries (Figure 4.4 C). There were no associations between inflammatory markers and the E_{max} (Figure 4.4 D) of KCl-induced contraction in renal arteries ($p>0.05$). There were no associations between inflammatory markers and the $LogEC_{50}$ (Figure 4.4 E and F) and E_{max} (Figure 4.4 G and H) of Phe-induced contraction in both mesenteric and renal arteries (all $p>0.05$).

Figure 4.5 shows the associations between circulating inflammatory markers and the $LogEC_{50}$ and E_{max} of ACh and SNP-induced relaxation in mesenteric (left panel) and renal (right panel) arteries. There were no associations between inflammatory marker concentrations and the $LogEC_{50}$ (Figure 4.5 A and B) of ACh-induced relaxation in mesenteric and renal arteries or the E_{max} of ACh-induced relaxation in mesenteric arteries (all $p>0.05$; Figure 4.5 C). Greater serum concentrations of TNF-α ($r=-0.68$, $p<0.0001$), IL-6 ($r=-0.54$, $p=0.001$) and CRP ($r=-0.68$, $p<0.0001$) were associated with impaired ACh-induced relaxation (lower E_{max}) in renal arteries (Figure 4.5 D). There were no associations between inflammatory markers and the EC_{50} (Figure 4.5 E and F) and E_{max} (Figure 4.5 G and H) of SNP-induced relaxation in both mesenteric and renal arteries ($p>0.05$).

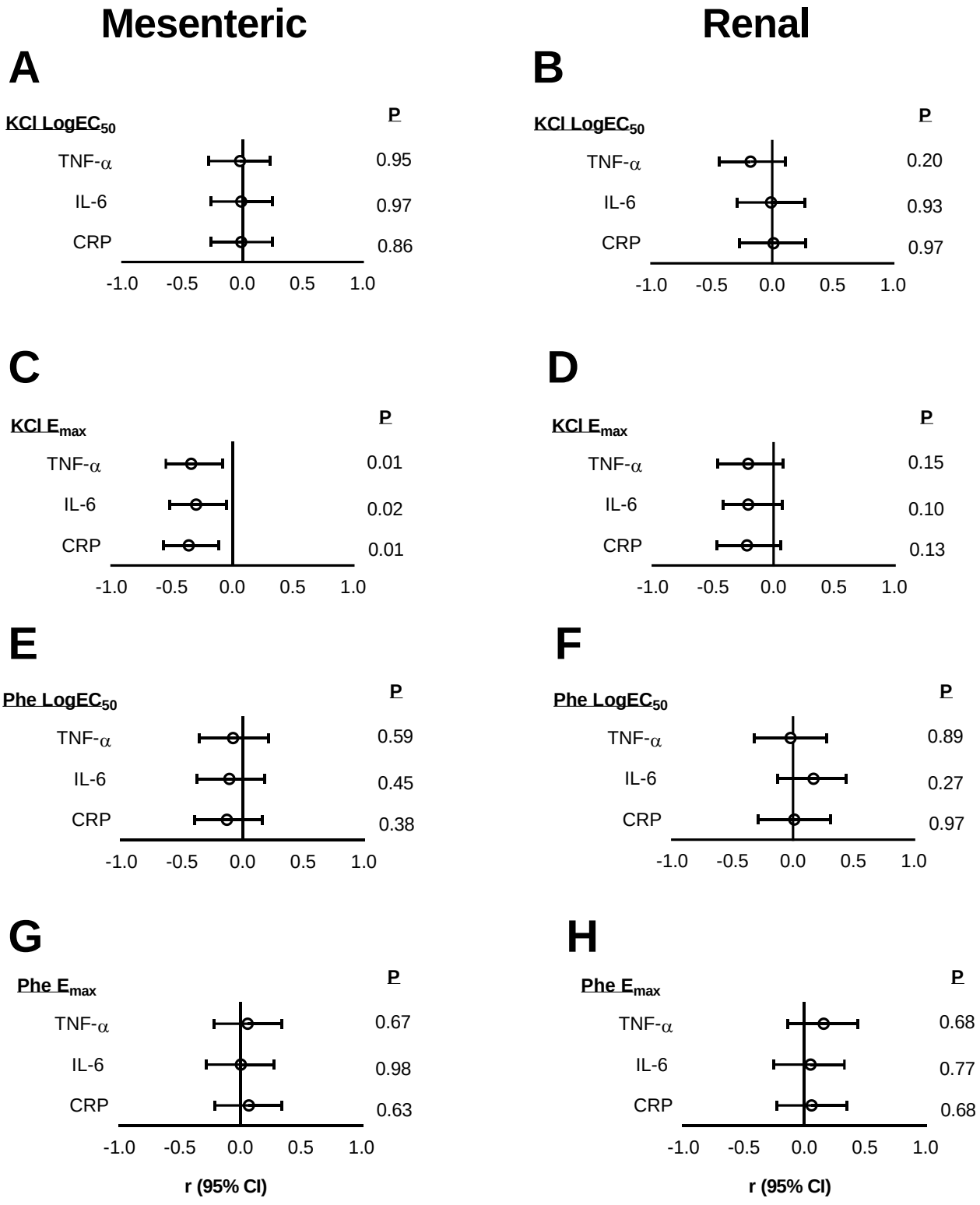

Figure 4.4 The associations between inflammatory markers and the $logEC_{50}$ **(A, B, E and F)** and E_{max} **(C, D, G and H)** of KCl and Phe-induced contraction in mesenteric (left panel) and renal (right panel) arteries. Open circles represent the correlation coefficient (r).

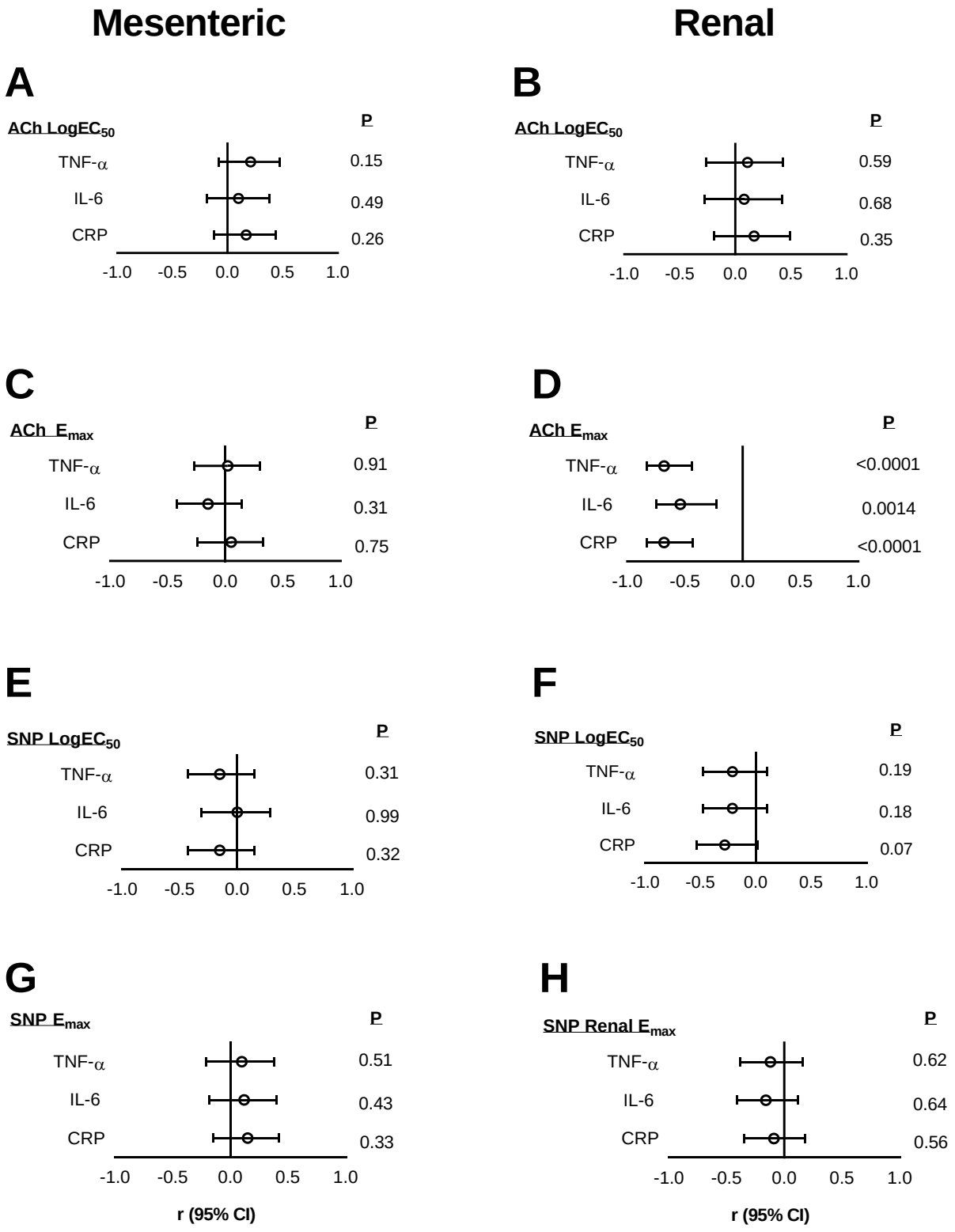

Figure 4.5 The associations between inflammatory markers and the $logEC_{50}$ **(A, B, E and F)** and E_{max} **(C, D, G and H)** of ACh and SNP-induced relaxation in mesenteric (left panel) and renal (right panel) arteries. Open circles represent the correlation coefficient (r).

CHAPTER 5

DISCUSSION

The present study examined the effects of high-grade systemic inflammation on endothelial function as well as arterial stiffness and determined whether treatment with TNF-α blockers and IL-6 blockers altered inflammation-induced changes in vascular function in Sprague Dawley rats exposed to CIA. The main findings of the current study are that exposure to high-grade inflammation impaired ACh-induced relaxation in both mesenteric and renal arteries. However, the SNP-induced relaxation responses were similar between the groups in both mesenteric and renal arteries. These findings suggest that the overall impairment is likely to be endothelial-dependent rather than endothelial-independent. Although the endothelial-dependent relaxation responses were altered, contractile responses were not altered by high-grade systemic inflammation in both mesenteric and renal arteries. Despite the lack of statistical significance between the groups, high concentrations of circulating inflammatory markers were associated with impaired KCl-induced contraction and impaired ACh-induced relaxation in renal arteries. Neither high-grade inflammation nor treatment with anti—inflammatory drugs affected arterial stiffness. Although treatment with IL-6 blockers failed to decrease the circulating inflammatory cytokine concentrations, it improved inflammation-induced endothelial dysfunction in both mesenteric and renal arteries. These findings suggest that endothelial function is adversely affected by exposure to high-grade inflammation and IL-6 blocker therapy may ameliorate inflammation-induced endothelial dysfunction.

5.1 Effects of high-grade inflammation on endothelial function

In the present study, ACh-induced relaxation was impaired in both the mesenteric and renal arteries in rats immunised with CIA compared to control rats. In mesenteric arteries, the sensitivity to vasodilators (Ach) were reduced after exposure to inflammation. Whereas in renal arteries, both the sensitivity and maximal relaxation responses to vasodilation (Ach) were impaired in the group exposed to inflammation. These results are consistent with the impaired endothelial function reported in small resistance as well as large conduit arteries in RA patients

(Dessein *et al*., 2005; Galarraga *et al*., 2008; Vaudo *et al*., 2004) and in animal models of RA (He *et al*., 2013; Reynolds *et al*., 2012; Totoson *et al*., 2015, 2016b). In the present study, endothelial dysfunction in renal arteries was associated with high circulating inflammatory markers, which is consistent with previous clinical RA studies (Galarraga *et al*., 2008; Vaudo *et al*., 2004). In addition to these clinical RA studies, associations between circulating inflammatory markers and impaired ACh-induced relaxation have previously been reported in animal models of RA (Mokotedi *et al*. 2019; Totoson *et al*., 2016b). Traditional cardiovascular risk factors such as hypertension are strongly related to the development of CVD in patients with RA. However recent studies suggest that endothelial dysfunction in RA occurs independent of traditional risk factors (Erre *et al*., 2018; Sandoo *et al*., 2012; Vaudo *et al*., 2004). Similar to other animal studies (Mokotedi *et al*., 2019; Totoson *et al*., 2015), our results confirm that endothelial dysfunction cannot be explained by blood pressure changes as there were no differences in blood pressure between the groups.

Indeed, a number of studies have showed that inflammation affects endothelial function directly, as inflammatory cytokines such as TNF-α and IL-6 have been shown to cause endothelial activation which causes a decrease in NO bioavailability and results in impaired endothelial function (Aletaha *et al*., 2010; Lao, 2013; Matsuzawa and Lerman, 2014). Studies have determined that CRP is an important marker in the development of RA as CRP levels are used to determine disease activity as well as long term prognosis (Dessein *et al*., 2004). This is vital as one of the major effects in the upregulation of IL-6 is the production of CRP, and rising CRP concentrations promote the secretion of IL-6 in a positive feedback loop (Dessin *et al*., 2004; Del Giudice and Gangestad, 2018). We have previously shown that exposure to high-grade inflammation upregulates endothelial activation markers associated with endothelial dysfunction in rats exposed to CIA (Mokotedi *et al*., 2019). In addition to endothelial activation, chronic exposure to inflammation results in the uncoupling of eNOS which

increases the production of ROS and exacerbates endothelial oxidative stress (Xia *et al.*, 1998; Wink *et al.*, 2001). The resulting imbalance leads to impaired endothelium-dependent relaxation which is a defining characteristic of endothelial dysfunction (Bonetti *et al.*, 2003). Taken together, our results show that exposure to high-grade inflammation results in endothelial dysfunction in both small and large arteries. Future studies are required to elucidate the mechanisms of how high-grade inflammation alters endothelial function in both micro- and macrovessels.

In the present study, the impaired endothelial function in mesenteric and renal arteries was not accompanied by concomitant contractile dysfunction. Although exposure to high-grade inflammation did not impair contractile responses, high circulating inflammatory markers were associated with impaired KCl maximal contractile responses. Indeed, acute exposure to high-grade inflammation has been reported to result in vascular contractile dysfunction in rats exposed to CIA for three weeks (Reynolds *et al.*, 2012). Hence high-grade inflammation could possibly affect contractile responses in the early stages of the disease which would probably explain the associations observed in the current study. Future studies should investigate the time-course effect of high-grade inflammation on contractile responses.

5.2 Effect of high-grade inflammation on arterial stiffness

In the present study, exposure to high-grade inflammation and anti-inflammatory treatment had no effect on large artery function measures. In contrast with the present study, several clinical studies have reported that PWV, central systolic blood pressure and AIx are increased in patients with RA (Irace *et al.*, 2004; Metsios *et al.*, 2009; Roman *et al.*, 2005). In addition, several studies have shown associations between high circulating inflammatory markers with markers of arterial stiffness including PWV (Mäki-Petäjä *et al.*, 2006; Roman *et al.*, 2005), AIx (Wallberg-Jonsson *et al.*, 2008; Galarraga *et al.*, 2009; Crilly *et al.*, 2009) and reflected

wave using separation analysis (Gunter *et al*., 2017) in patients with RA. A number of studies have postulated that exposure to high-grade inflammation causes increased collagen deposition and degeneration of elastin fibres in the vascular wall, which results in vascular remodelling and increased arterial stiffness in patients with RA (Hattori *et al.,* 2003). While there is ample evidence in clinical studies, to the best of our knowledge, the effect of high-grade inflammation on arterial stiffness has not been investigated in animal models of RA. Although exposure to high-grade inflammation impaired endothelial function in the present study, this did not translate into increased arterial stiffness. As disease duration is a strong predictor of arterial stiffness in patients with RA (Vázquez-Del Mercado *et al.,* 2017), the results of the present study imply that exposure to high-grade inflammation for nine weeks may have been too short to cause changes in arterial stiffness measurements. Alternatively, despite applanation tonometry being the gold-standard for measuring large artery function non-invasively in humans, this technique may not be sensitive enough in animals to detect small differences in large artery function. Lastly, applanation tonometry in the current study was measured under anaesthesia, which is known to affect various body systems, including the vasculature. Hence it may be possible that the effects of the anaesthesia may have masked inflammation induced changes in large artery function. In this regard, anaesthesia is known to have vasodilatory effects in vitro. Ludvigsen et al. (2014) reported that the endothelial response was influenced by anaesthesia, despite no statistically significance decrease in blood pressure. Future studies should determine whether high grade inflammation for a longer period, or large artery function measured under difference circumstance and using a more invasive approach shows different results in large artery function.

5.3 Effect of TNF-alpha inhibitor therapy on the inflammatory profile and endothelial function

In the present study, treatment with Etanercept improved the circulating TNF-α concentrations. In addition, although circulating IL-6 concentrations were significantly higher in the rats exposed to CIA compared to the control rats, no differences where observed in the circulating IL-6 concentrations between the rats in the control group and the rats receiving the TNF-α blocker therapy. These results imply that TNF-α blocker therapy suppressed the inflammatory responses induced by CIA. Our results are consistent with clinical studies which have showed that the administration of TNF-α blockers improve the inflammatory profiles of RA patients (Bilsborough *et al.,* 2006; Bosello *et al.,* 2008; Hurlimann *et al.,* 2002; Irace *et al.,* 2004; Komai *et al.,* 2007; Maki-Petaja *et al.,* 2006). In addition to clinical studies, animal studies have also showed that TNF-α blocker therapy improves the inflammatory profiles of rats exposed to high-grade inflammation (Chakravarthy *et al.,* 2014, Totoson *et al.,* 2016a). Although our studies and prior animal studies showed improvement in endothelial function, unlike the aforementioned studies, we did not show improvements in circulating inflammatory markers. The discrepancy between our study and previous animal studies may be as a result of methodological differences between the studies. In this regard, the aforementioned studies used different strains of rat (Lewis rats) compared to our study and the model of inducing inflammation were difference between the studies. Another discrepancy between our study and the others was the duration of drug administration, in our study rats were on treatment for a period of six weeks whereas the other studies administered the treatment for a period of three to four weeks (Chakravarthy *et al.,* 2014, Totoson *et al.,* 2016a). Furthermore, the discrepancies may also be explained by the drug actions as described below.

TNF-α is a proinflammatory cytokine with pleiotropic biological activities. TNF-α binds to either a membrane-bound TNF receptor or a soluble TNF receptor (Narayanan *et al.,* 2011).

Binding of TNF-α to membrane-bound TNF receptors promote the activation of numerous pathways which lead to the production of numerous inflammatory cytokines (Narayanan *et al.*, 2011). However, binding of TNF-α to soluble TNF receptors results in the deactivation of TNF-α thus damping the immune response (Narayanan *et al.*, 2011). Etanercept, a fully soluble human dimeric fusion protein produced by recombinant DNA, is composed of two soluble TNF receptor extracellular domains linked to the Fc portion of human immunoglobulin G1 (IgG1) (Spencher-Green, 2000). Etanercept mimics the actions of the naturally occurring soluble TNF receptor and functions as a TNF-α inhibitor by competitively binding to TNF-α, preventing the interaction of TNF-α with membrane bound receptors and hence inhibiting the induced proinflammatory activity (Madhusudan *et al.*, 2005; Mohler *et al.*, 1993). Etanercept has a more profound biologic effect than a naturally occurring soluble TNF receptor as it has a prolonged half-life in the bloodstream and is therefore effective in preventing the activation of the inflammatory cascade (Madhusudan *et al.*, 2005). Drugs get metabolised in rats up to ten times faster than in humans, despite this increased metabolism the efficacy of the drug is not affected (Sawada *et al.*, 1985). The study conducted by Totoson et al. (2016a) only administered the drug for a total of three weeks, giving injections every third day whereas in our study the rats were on treatment for a period of six weeks. Some clinical studies have stated that although TNF-α blockers are effective in decreasing symptoms of inflammation as well as endothelial dysfunction they have a transient effect, and endothelial function returns to its impaired state after some time. Hence the different time frames between the studies may have affected the inflammatory profiles in the rats. Taking together, it is not surprising that TNF-α blocker therapy has been considered as a potential treatment strategy for improving inflammatory profiles of RA patients.

Although treatment with TNF-α blockers improve systemic inflammation and cardiovascular risk in RA patients, their effects on endothelial function are controversial. Some studies have

reported that TNF-α blocker therapy improved endothelial function by reducing the expression of endothelial adhesion molecules in the synovial tissue of RA patients (Maki-Petaja *et al.*, 2006; Tak *et al.*, 1996) and by decreasing the soluble forms of intracellular adhesion molecule-1 (ICAM-1) and E-selectin in RA patients (Klimuik *et al.*, 2005; Komai *et al.*, 2007; Sidiropoulos *et al.*, 2009). Others have reported transient improvements in endothelial function (Gonzalez-Juanatey *et al.*, 2004; Irace *et al.*, 2004; Bosello *et al.*, 2008), while others have reported no changes in endothelial function (Cardillo *et al.*, 2006; Rongen *et al.*, 2018). In addition, a recent meta-analysis concluded that TNF-α blocker therapy produced no changes in endothelial dysfunction in RA patients (Mathieu *et al*., 2013). The discrepant results among these clinical studies could be as a result of methodological design between these studies. Since clinical studies have several confounding factors and have led to inconsistent results, the use of animal models of RA is a benchmark choice to study the impact of TNF-α blocker therapy on vascular function.

Only one previous study using an adjuvant-induced arthritis model has investigated the effects of TNF-α blocker therapy on endothelial function (Totoson *et al.*, 2016b). Totoson *et al.*, (2016b) found that treatment with TNF-α blockers for three weeks improved endothelial function by decreasing the production of ROS and enhancing NOS activity (Totoson *et al.*, 2016b). In contrast, the present study showed that treatment with TNF-α blockers for six weeks failed to improve endothelial function in rats exposed to CIA. These discrepancies may be explained by the different protocol used to induce arthritis in the aforementioned study. Nevertheless, our results support reports in human studies that have shown either transient or no effects of TNF-α blocker therapy on endothelial function. Although the TNF-α blocker therapy marginally improved the circulating TNF-α blocker concentrations in our study, the CRP and IL-6 concentrations were not lower compared to the inflammation group. This suggests that impaired vascular function in high grade inflammation may not be mediated by

TNF-α. Furthermore as some clinical studies have reported that vascular benefits of TNF-α blocker therapy may be transient (Gonzalez-Juanatey *et al*., 2004; Irace *et al.*, 2004; Bosello *et al.*, 2008), future studies need to investigate the time-course effect and the underlying mechanisms responsible for the TNF-α blocker effects on endothelial function.

5.4 Effect of IL-6 blocker therapy on the inflammatory profile and on endothelial function

In the present study, treatment with Tocilizumab did not improve circulating inflammatory cytokine concentrations, as the cytokine concentrations in rats receiving IL-6 blocker therapy were similar to the rats exposed to CIA. In contrast to our results, clinical studies have showed that the administration of IL-6 blockers improve the inflammatory profiles of RA patients (Ikonomidis *et al.*, 2019; Protogerou *et al.*, 2011; Ruiz- Limón *et al*., 2017).

Tocilizumab is a recombinant humanised anti-IL-6 receptor monoclonal antibody which competitively binds to the IL-6 binding site of monocytes and B cells (Mihara *et al.*, 2011; Nishimoto and Kishimoto 2008). IL-6 is a pleiotropic cytokine that predominantly regulates the immune and inflammatory response (Nishimoto and Kishimoto, 2008; Wollert and Drexler, 2001). IL-6 transmits its signal through binding to membrane-bound IL-6 receptors or soluble IL-6 receptors (Wollert and Drexler, 2001). The membrane-bound IL-6 receptor consists of the IL-6α receptor chain which binds IL-6 directly and a signal-transducing glycoprotein 130 (gp130) subunit (Wollert and Drexler, 2001). The IL-6α receptor chain on the membrane bound IL-6 receptor is solely expressed on the surface of hepatocytes and leukocytes, which results in anti-inflammatory processes (Wollert and Drexler, 2001). In the context of inflammation, IL-6 acts on cells through the formation of a complex with the soluble IL-6 receptor and gp130 which triggers downstream trans-signalling via Janus-activated kinase/ signal transducers and activators of transcription (Jak-STAT) pathways on different target cells (including

endothelial, smooth and neural cells) resulting in the IL-6 proinflammatory effect (Nishimoto and Kishimoto 2008). In this regard, Tocilizumab prevents the binding of IL-6 to both the membrane bound IL-6 receptor as well as the soluble IL-6 receptor (Nishimoto and Kishimoto 2008). Tocilizumab suppresses the proinflammatory effects of IL-6 by competitively binding to the soluble IL-6 receptor which disrupts the Jak-STAT pathways, hence damping the inflammatory role of IL-6 (Nishimoto and Kishimoto 2008). Although the pro-inflammatory effect of IL-6 are reduced, the mechanism of action of the Tocilizumab suggests that it may not necessarily reduce the circulating IL-6 concentrations, in fact it may even increase the circulating concentrations through negative feedback (Luna *et al*., 2014).

Although a number of studies have reported that IL-6 blocker therapy suppresses the inflammatory profile of RA patients, the effect of Tocilizumab on endothelial dysfunction has been minimally explored in RA patients. To the best of our knowledge only three studies have reported on the protective effects of Tocilizumab on endothelial function in RA patients (Ikonomidis *et al.*, 2019; Protogerou *et al.*, 2011; Ruiz- Limón *et al.*, 2017). Endothelial function assessed by FMD was improved in 16 RA patients treated with Tocilizumab for 3 and 6 months (Protogerou *et al.*, 2011). Furthermore, inhibition of IL-6 activity by Tocilizumab improved endothelial function as assessed by coronary flow reserve and FMD of the brachial artery in 40 RA patients (Ikonomidis *et al.*, 2019). Ruiz- Limón *et al.*, (2017) found that there was a marked improvement in endothelial function after a period of 6 months of Tocilizumab therapy. It was suggested that the improved endothelial function as a result of Tocilizumab was via a decreased in the activation of the endothelium as measured by a reduced VCAM-1 and E-Selectin (Ruiz- Limón *et al*., 2017). This suggestion was supported by *in vitro* studies that showed treatment with Tocilizumab to the co-culture of RA monocytes with endothelial cells plus IL-6 reduced the inflammatory profile and improved endothelial function by promoting a down regulation of VCAM-1 and ICAM-1 (Ruiz- Limón *et al*., 2017). Additionally,

Tocilizumab improved endothelial function by the inhibition of nuclear factor-κB which subsequently reduces eNOS production (Ruiz- Limón *et al.*, 2017). In agreement with these clinical studies, we have showed for the first time that IL-6 blocker therapy significantly improves endothelial function in both mesenteric and renal arteries in rats exposed to CIA. In mesenteric arteries IL-6 blocker treatment improved the sensitivity (EC_{50}) of the vessel to vasodilator, whereas in renal arteries both the sensitivity and maximal response to vasodilator (Ach) were improved compared to the inflammation group. Furthermore, IL-6 blockers improved the vasodilator response in the endothelial dependent pathway, as it was only significant in response to stimulus by acetylcholine. Sodium-nitroprusside induced vasodilation was not different between the groups. Sodium-nitroprusside is potent endothelial independent vasodilator, which suggest that the inflammatory induced vascular dysfunction is likely to be caused by endothelial damage and that IL-6 blocker may reduce the effects of circulating inflammatory markers on the endothelial cells, despite high circulating cytokine concentrations. Our data suggest that the mechanisms governing endothelial function and circulating inflammatory cytokines are distinct and remain to be elucidated to better understand the cardiovascular benefits of IL-6 blocker therapy in RA. Nevertheless, our results suggest that IL-6 may be a possible therapeutic target to prevent adverse vascular remodelling in disorders characterised by high-grade inflammation. Future studies need to investigate the cellular mechanisms responsible for the improvement of endothelial function upon IL-6 blockade.

5.5 Limitations

The present study has further limitations. We used of a non-invasive approach (Pulse-Pen) rather than catheter-based systems to assess aortic stiffness. Although the PulsePen device is a validated tool to estimate arterial stiffness in humans (Salvi *et al.*, 2019) and the manufacturers

indicate its applicability in animal studies, validation studies using the applanation tonometry in animal studies are scarce and requires further investigation.

Future studies should assess aortic stiffness using invasive catheter-based methods. Although we have demonstrated associations between inflammatory markers and impaired endothelial function, the direct casual effect of inflammation on impaired endothelial function can only be implied. Hence, future studies should investigate whether inflammation directly causes the overproduction of cyclo-oxygenase-2 (COX-2) activity and upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which could account for the endothelial dysfunction. The present study investigated the role of biologic DMARDs on vascular function using a humanised soluble TNF-α inhibitor and a humanised anti-IL-6 monoclonal antibody. As the humanised treatments may have not bound effectively to the rat cytokines and receptors, future studies need to used rat specific TNF-α inhibitor and IL-6 receptor blocker therapy. The effects of biologic DMARDS on circulating inflammatory markers and vascular function were only assessed at a single time point, 10 weeks after the primary immunisation injection. Future studies should investigate the time-course effect of inflammation and biologic DMARDs on vascular function.

5.6 Conclusion

In conclusion, the evidence presented in the present thesis adds to our understanding of the role of high-grade inflammation and biologic DMARDs on vascular function. In this regard, the present study showed that exposure to high-grade systemic inflammation *in vivo* results in endothelial dysfunction in both mesenteric and renal arteries which is not translated into arterial stiffness in the CIA rat model. Circulating inflammatory markers were associated with impaired endothelial function in CIA. The use of TNF-α inhibitor therapy improved the inflammatory profiles of rats exposed to high-grade inflammation but failed to improve

vascular function in CIA. Our results do not undermine the beneficial effects of TNF-α inhibitor therapy on cardiovascular risk in RA but suggest that mechanisms other than the reduction in endothelial dysfunction are likely to be involved. The use of IL-6 blocker therapy improved inflammation-induced endothelial dysfunction by ameliorating ACh-induced relaxation in both mesenteric and renal arteries. In this regard, the inhibition of IL-6 appears to have favourable effects on vascular function and is thus a potential therapeutic target for the management of endothelial dysfunction in RA. Future studies should elucidate the molecular mechanisms whereby IL-6 blocker therapy improves vascular function.

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APPENDIX A

Animal ethics clearance certificate

AESC 2012 M&E

Please note that only type written applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Lebogang Mokotedi

b. Department: School of Physiology

c. Experiment to be modified / extended

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

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

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

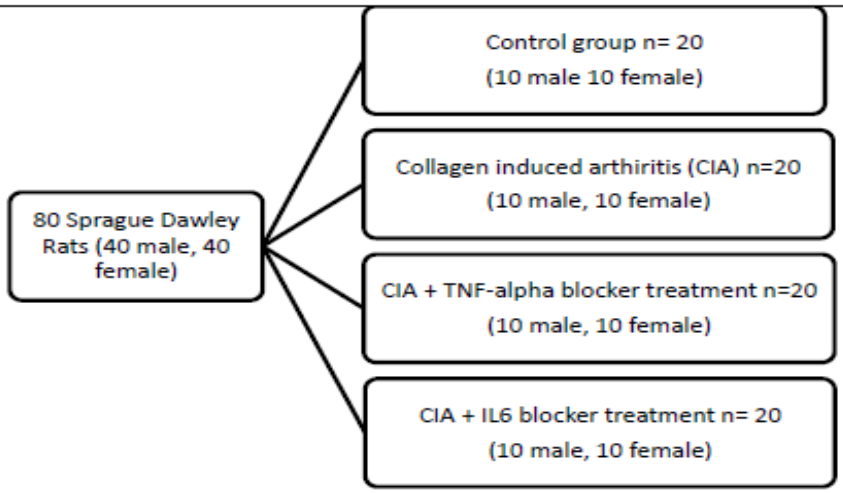
i. Specific modification / extension requested:

- Addition of co-workers

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

- Additional rats required

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



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

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

The addition of co-workers:

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

Additional rats required:

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

Addition of drug treatment regimen to reduce inflammation:

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

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

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

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

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Date:07/05/2018

Signature:

RECOMMENDATIONS

Date: 22/5/2018

Signature:

Chairman, AESC