BIOMARKERS OF ENDOTHELIAL DYSFUNCTION IN TREATED AND UNTREATED RATS WITH COLLAGEN INDUCED RHEUMATOID ARTHRITIS

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DECLARATION

I, Mikayra Singh, 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 Screening Committee, University of the Witwatersrand, Johannesburg. The ethics approval number is 2017/03/21/C.

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Signed on the 16th day of October 2020

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ABSTRACT

Background. High-grade chronic inflammation increases the risk for cardiovascular disease (CVD). Endothelial dysfunction is one of the earliest markers of inflammation-induced CVD. Treatment with anti-inflammatory drugs has shown improvement in endothelial function. However, the mechanisms whereby inflammation and anti-inflammatory drugs impact endothelial function are not well understood. Alterations in microRNA (miRNA), short non-coding RNAs that post-transcriptionally regulate gene expression, have been linked to various disease states. Evidence supports miRNAs as novel biomarkers and predictors of endothelial dysfunction and CVD. However, the effect of inflammation on miRNA expression and the mechanisms by which biologic anti-inflammatory treatments alter miRNA gene regulation to affect downstream signalling and ultimately endothelial function and CVD risk requires investigation.

Methods. Three-month-old, male and female Sprague-Dawley rats were randomly divided into four groups. One group served as the control group (n=21) and received a saline subcutaneous injection. The other three groups were exposed to an arthritis inducing protocol where rats received a subcutaneous injection of bovine type-II collagen emulsified in incomplete Freund's adjuvant at the base of the tail. Upon signs of inflammation, one group received no treatment (collagen induced arthritis (CIA) group, n= 27), one group received a tumour necrosis factor alpha (TNF-α) receptor blocker (anti-TNF-α group, n=13) every three days for six weeks and one group received an interleukin 6 (IL-6) receptor blocker (anti-IL-6 group, n=15) once a week for 6 weeks. Body weight, blood pressure and arthritis scores in the hind paws were measured throughout the study. At termination, vascular function was measured using applanation tonometry and vascular responses to vasodilators and vasoconstrictors in mesenteric and renal arteries (vascular reactivity) were measured using a wire myograph. Blood was obtained and serum concentrations of TNF-α, IL-6 and C-reactive protein (CRP) were measured by ELISA. Total RNA and total miRNA were extracted from cardiac tissue and serum, respectively, and reverse transcribed. Real time PCR was used to determine the relative expression of miR146a-5p, miR155-5p, vascular adhesion molecule-1 (VCAM-1) and pentraxin-3 (PTX-3). Group differences in body weight, blood pressure and arthritis

scores were measured by repeated measures analysis of variance (ANOVA). Group differences in inflammatory cytokine concentrations were measured by a two-way ANOVA with a Tukey post-hoc test. Group differences in relative miRNA expression were measured by a Kruskal-Wallis test. Associations between relative miRNA expression and inflammatory markers, vascular function markers and cardiac function markers were determined by Pearson's correlations.

Results. Body weight and blood pressure did not change over the course of the study and did not differ between the groups (all $p>0.05$). Circulating inflammatory marker concentrations and arthritis scores were higher in all rats exposed to the collagen induced inflammatory protocol compared the control group (all $p<0.05$). The expression of VCAM-1 and PTX-3 were higher in the CIA and anti-IL-6 groups (both $p<0.05$), but not the anti-TNF-α group ($p>0.05$) compared to the control group. Relative miR146a-5p expression was higher in the CIA group compared to the anti-TNF-α group ($p=0.01$). Relative miR155-5p expression was higher in the CIA group compared to the control group ($p=0.05$). There were no differences in the relative expression of miR146a-5p or miR155-5p between the control and anti-TNF-α groups ($p>0.05$). Higher circulating CRP concentrations were associated with the relative expression of miR146a-5p ($r=0.31$, $p=0.04$) and miR155-5p ($r=0.62$, $p=0.009$), but the association was only seen in the absence of anti-inflammatory treatments. Relative miR146a-5p expression was associated with the sensitivity of mesenteric arteries to vasodilators in control and inflammation groups ($r=0.39$, $p=0.04$), but not when the anti-inflammatory treatments groups were included in the analysis ($r=-0.09$, $p=0.52$). Relative miR155-5p expression was associated with markers of arterial stiffness, renal artery sensitivity to vasodilators and maximal relaxation responses in the control and inflammation groups (all $p<0.05$), but not when treatment groups were included. Both miR146a-5p ($r=0.35$, $p=0.008$) and miR155-5p ($r=0.31$, $p=0.02$) were associated with relative mRNA expression of PTX-3, independent of anti-inflammatory treatment.

Conclusion. Exposure to high-grade inflammation and TNF-α blocker treatment impact the expression of miR146a-5p and miR155-5p. Although miR155-5p was associated with markers of vascular reactivity and large artery function, these relationships are affected

by anti-inflammatory treatment. The association between miR146a-5p and miR155-5p and endothelial function in a high-grade inflammatory model is not affected by anti-inflammatory treatment. Taken together, our results suggest that exposure to high-grade inflammation alters upstream gene regulation of endothelial function, which may be affected by TNF-α blocker treatment.

DEDICATION

I dedicate this dissertation to my parents, Krishen and Pratima Singh for showing me unwavering love and support throughout my life, and to my brother, Mikhail Singh for providing me with strength and encouragement like no other.

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Everything I achieve, I owe to God.

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Lastly, I'd like to thank my grandmother Neila Seeban, for her continuous thoughts and prayers and my best friend Matthew Pather and his family, for motivating me to achieve everything I wished for.

STATEMENT OF CONTRIBUTION TO DATA COLLECTION AND ANALYSIS

I declare that I designed this study in conjunction with my supervisors Professor AME Millen and Dr S Gunter. I was part of a team of researchers (including Prof Millen, Prof Michel, Dr Mokotedi, Dr Gunter, Ms Manilall) and postgraduate students responsible for collecting data for a larger study, of which this thesis forms a part. I was responsible for optimising the technique and collecting data for the main outcome variable in this thesis, namely microRNA expression. I performed the data analysis for this thesis with the help of Dr S Gunter and Prof AME Millen. I wrote this thesis which was reviewed by Prof Millen and Dr Gunter.

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ABBREVIATIONS

Ach	Acetylcholine
ADMA	Asymmetric dimethylarginine
AIx	Augmentation index
BP	Blood pressure
CIA	Collagen induced arthritis
CI	Confidence interval
CNTF	Ciliary neurotrophic factor
CRP	C-reactive protein
CT-1	Cardiotrophin-1
CV	Cardiovascular
CVD	Cardiovascular disease
DMARDs	Disease modifying anti-rheumatic drugs
ECM	Extracellular matrix
EDCFs	Endothelium-derived contracting factors
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
E-selectin	Endothelial leukocyte adhesion molecule
FMD	Flow mediated dilatation
HFpEF	Heart failure with a preserved ejection fraction
HUVECs	Human umbilical vein endothelial cells

ICAM-1	Intercellular adhesion molecule 1
IFNγ	Interferon gamma
IL-11	Interleukin 11
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
iNOS	Inducible nitric oxide
IRAK-1	Interleukin-1 receptor associated kinase
IVST	Interventricular septal thickness
Jarid 2	Jumonji AT rich interactive domain
LDL	Low density lipoprotein
LIF	Leukemia inhibitory factor
LV	Left ventricular
MEF2A	Myocyte enhancer factor 2A
miRNA	MicroRNA
MTX	Methotrexate
NNT-1/BSF-3	Novel neurotrophin-1/B cell stimulatory factor -3
NO	Nitric oxide
OSM	Oncostatin M
Phe	Phenylephrine
pre-miRNA	Precursor miRNA

pri-miRNA	Primary miRNA
PTX-3	Pentraxin-3
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
RT	Reverse transcriptase
RT-PCR	Real time quantitative PCR
SHIP 1	Src homology 2 domain-containing inositol-5-phosphatase 1
sIL-6R	Soluble Interleukin-6 receptor
SOCS1	Suppressor of cytokine signalling 1
T2DM	Type 2 Diabetes Mellitus
TIMP-2	Tissue inhibitor of metalloproteinases 2
TLR	Toll like receptor
TNFR	TNF-α receptors
TNF-α	Tumour necrosis factor-α
TRAF6	TNF associated receptor 6
Treg	Regulatory T cells
VCAM-1	Vascular adhesion molecule 1

CHAPTER 1
INTRODUCTION

Despite a significant effort aimed at preventing and treating cardiovascular disease (CVD), it remains one of the primary causes of death globally and has remained so for the past 15 years (WHO, 2018). Cardiovascular disease accounts for more deaths annually compared to any other non-communicable diseases, including cancer and respiratory diseases (Al-Mawali, 2015). Although traditional risk factors, such as hypertension and obesity contribute substantially to CVD, they fail to fully account for all CVD events (deGoma *et al*., 2012). Non-traditional risk factors have been suggested to contribute to CVD risk (deGoma *et al*., 2012).

Inflammation is considered a risk factor for CVD and is strongly associated with cardiovascular (CV) events, including stroke, myocardial infarction and heart failure (Libby, 2006; Liang *et al*., 2010; Willerson & Ridker, 2004). Indeed, patients with chronic inflammatory diseases, including rheumatoid arthritis (RA), have increased CV morbidity and mortality (England *et al*., 2018). RA is an autoimmune, rheumatic disease that is characterised by chronic inflammation that originates in the synovial joints and progresses to a systemic disease. Patients with RA have a higher prevalence of subclinical CVD, including atherosclerosis, arterial stiffness and cardiac dysfunction (Aviña-Zubieta *et al*., 2008; Aslam *et al*., 2013; Ambrosino *et al*., 2015). Although chronic systemic inflammation is implicated in the increased CVD risk in RA, the direct mechanisms by which inflammation heightens CVD risk is currently under investigation.

One possible mechanism whereby inflammation contributes to CVD risk is through changes in endothelial function. Endothelial dysfunction is considered one of the earliest signs of subclinical CVD (Chadderdon *et al*., 2014). During chronic inflammation, there is an abundance of free radicals produced and secreted by the endothelial cells, that cause oxidative stress and alters nitric oxide signalling (Chadderdon *et al*., 2014). A local inflammatory response is also elicited, during which leukocytes migrate through the vasculature and increases vascular smooth muscle cell proliferation. Over time, these processes cause endothelial dysfunction, which substantially increases the risk of CVD (Castellon & Bogdanova, 2016). Endothelial dysfunction commonly progresses to arterial dysfunction and/or atherosclerosis (Deanfield *et al*., 2007). Myocardial microvascular inflammatory endothelial activation has also recently been implicated in adverse

cardiomyocyte function. More specifically, microvascular endothelial activation directly and negatively affects cardiomyocytes through altered paracrine signalling, ultimately promoting the development of heart failure with a preserved ejection fraction (Franssen *et al*., 2016; Paulus & Tschöpe, 2013). Consequently, emphasis has been placed on identifying novel biomarkers for efficient detection and therapeutic strategies that target endothelial dysfunction.

MicroRNAs (miRNA) are regulators of gene expression and play a major role in cell signalling through gene silencing pathways. Therefore, aberrantly expressed miRNAs have been implicated in several diseases, including chronic inflammatory conditions. Specifically, studies have shown that miR146a-5p and miR155-5p are upregulated in RA, and their expression is strongly induced through cytokine signalling pathways (Jung *et al*., 2019). Inappropriate expression of miR146a-5p and miR155-5p may present a novel mechanism whereby vascular integrity is lost, leading to endothelial dysfunction (Ardekani & Naeini, 2010). In this regard, little is known about the upstream gene regulation that may control vascular integrity. Considering its relationship with inflammation and vascular integrity, miRNAs may be considered potential novel biomarkers of endothelial dysfunction.

Conventionally, RA is treated with synthetic disease modifying anti-rheumatic drugs (DMARDs), which reduces RA disease activity by suppressing the immune system. However, recently there has been a shift to biologic DMARDs, which has shown greater disease control and remission (Ma & Xu, 2013; Engler & Skosana, 2016; Benjamin & Lappin, 2019). Despite positive reports on the effects of biologic DMARDs on inflammation and RA disease progression, the effects on CVD risk have been controversial (Baniaamam *et al*., 2018). The controversial results are largely as a result of confounding factors and study design limitations in human RA studies. Further research in pre-clinical studies is required to determine the mechanism by which biologic DMARDs affect CVD risk.

It is well known that chronic inflammation impairs vascular integrity directly at the site of the vessels. It has also been suggested that inflammation may cause inappropriate expression of miRNAs that are responsible for the regulation of endothelial function. Indeed, evidence supports the role of miRNAs as a novel biomarker and predictor of endothelial dysfunction (Araldi & Suárez, 2016). However, the role of biologic DMARD therapies in the modulation

of miRNA expression is not well studied. Therefore, there is a need to determine whether high-grade inflammation impacts upstream gene regulation of endothelial function and whether biologic DMARD therapy alters the regulation of miRNAs.

After this introductory chapter, the thesis is structured into four chapters. Chapter 2 provides a concise review of the current literature, wherein the main reasons for conducting this study will be emphisized. Chapter 3 will outline the study design and methods used. In chapter 4 the results from the present study will be presented and in chapter 5 the main findings and results will be discussed in the context of the literature.

CHAPTER 2
LITERATURE REVIEW

2.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases worldwide (Cooper & Stroehla, 2003). The signature characteristic of RA is synovitis that results in the destruction of cartilage and bone (McInnes & Schett, 2011). In addition to synovial inflammation and hyperplasia, RA is characterised by autoantibody production including anti-citrullinated protein antibody and rheumatoid factor. Following an autoimmune response, naïve T-cells differentiate into T-helper (Th) 1 cells and/or Th17 cells that, in turn, drive inflammation via interleukin (IL) 17 release and by cognate interactions with adjacent macrophages. Macrophage-derived cytokines including tumour necrosis factor-α (TNF-α), IL-1, IL-6, IL-15, and IL-18 drive many of the pro-inflammatory pathways involved in RA (McInnes & Schett, 2007), which are produced by all cells involved in the regulation of the immune response. Of these inflammatory cytokines, TNF-α is most strongly associated with the pathogenesis of RA. TNF-α forms part of the acute phase reaction, that also stimulates the release of other inflammatory cytokines such as granulocyte colony stimulating factor (GM-CSF), IL-1, IL-6 and IL-8 (Visanthi *et al.,* 2007). TNF-α is also responsible for the upregulation of the endothelial expression of various soluble adhesion molecules including vascular adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule (E-selectin) (Szekanecz & Koch, 2000). These signalling pathways lead to the characteristic chronic, high-grade inflammatory state, in RA patients (Kraan *et al*., 2007). Increased concentration of circulating inflammatory cytokines result in extra-articular complications that affects several organ systems (Sattar *et al*., 2003). In the presence of extra-articular manifestations and comorbidities, this autoimmune disease is characterised by substantial disability as well as an increase in patient mortality (Alamanos & Drosos, 2004). One of the organ systems that bears significant damage from the increased circulating inflammation in RA is the cardiovascular system. Indeed RA is associated with a significantly increased risk of CVD (Urman *et al*., 2018).

2.2 Cardiovascular disease in rheumatoid arthritis

Compared to the general population, the risk of CVD is doubled in RA patients (Crowson *et al*., 2013), and is comparable to the heightened CVD risk in patients with type 2 diabetes

mellitus (T2DM) (Peters *et al*., 2009). Moreover, subclinical CVD, including atherosclerosis and vascular dysfunction, is significantly more prevalent in RA compared to the general population (Ambrosino *et al*., 2015; Skeoch & Bruce, 2015; Roman *et al*., 2006). The prevalence of atherosclerosis is increased three-fold in patients with RA compared to the general population (Roman *et al.,* 2006). Although conventional CVD risk factors such as hypertension, T2DM and obesity certainly contribute to the increased prevalence of CVD in RA, these factors do not fully account for all CV events (Jagpal & Navarro-Millán, 2018). Systemic inflammation has been implicated in the increased CVD risk in RA (DeMizio & Geraldino-Pardilla, 2020). The mechanisms whereby systemic inflammation in RA patients contribute to CVD risk are currently under investigation (Soubrier *et al*., 2014).

2.3 Endothelial dysfunction as a risk marker for cardiovascular disease

The vascular endothelium is a single, differentiated layer of simple squamous epithelium, which lines the vasculature and the heart (Stan, 2010). The endothelium is known as a vital mediator of vascular tone and homeostasis as it synthesises and secretes vasodilators and vasoconstrictors. The endothelial-derived vasodilatory factors including nitric oxide (NO), prostacyclin, bradykinin and endothelium-derived hyperpolarising factor (EDHF) and endothelium-derived contracting factors (EDCFs), comprising endothelin 1, angiotensin II, and vasoconstrictor prostanoids (Crilly *et al*., 2009; Fitch *et al*., 2001). Importantly, healthy endothelial function requires a precise balance between vasoconstriction and vasodilation, which ensures optimal perfusion pressure to organs (Deanfield *et al*., 2007).

In chronic disease states and illness, the endothelium is exposed to circulating molecules which affects its function. Acute phase proteins, such as CRP, and inflammatory cytokines have been implicated in the altered endothelium function (Carnevale *et al*., 2014; Clapp *et al*., 2004; Steyers & Miller, 2014). Cytokines induce and upregulate the endothelial expression of various soluble adhesion molecules including VCAM-1, ICAM-1 and E-selectin (Szekanecz & Koch, 2000). The increased expression of these adhesion molecules is known as endothelial activation. Figure 2.1 depicts the process of endothelial action. Once these adhesion molecules are expressed on the endothelium, circulating monocytes and macrophages use these adhesion molecules to attach. Once attached, monocytes

enter the arterial wall intima via diapedesis, where they secrete additional proinflammatory cytokines (Liao, 2013; Dessein *et al*., 2013; Schoenfeld *et al*., 2015). The interaction between chemokines and growth factors also act on neighbouring smooth muscle cells to induce proliferation. This leads to altered endothelial function, and is referred to as endothelial dysfunction. With endothelial dysfunction, the endothelial cells fail to secrete the necessary mediators required to maintain vascular homeostasis (Hadi *et al*., 2005). The consequence of endothelial dysfunction is the reduced ability of the vessels to vasodilate, therefore perfusion pressure and oxygen delivery to organs are reduced (Deanfield *et al*., 2007).

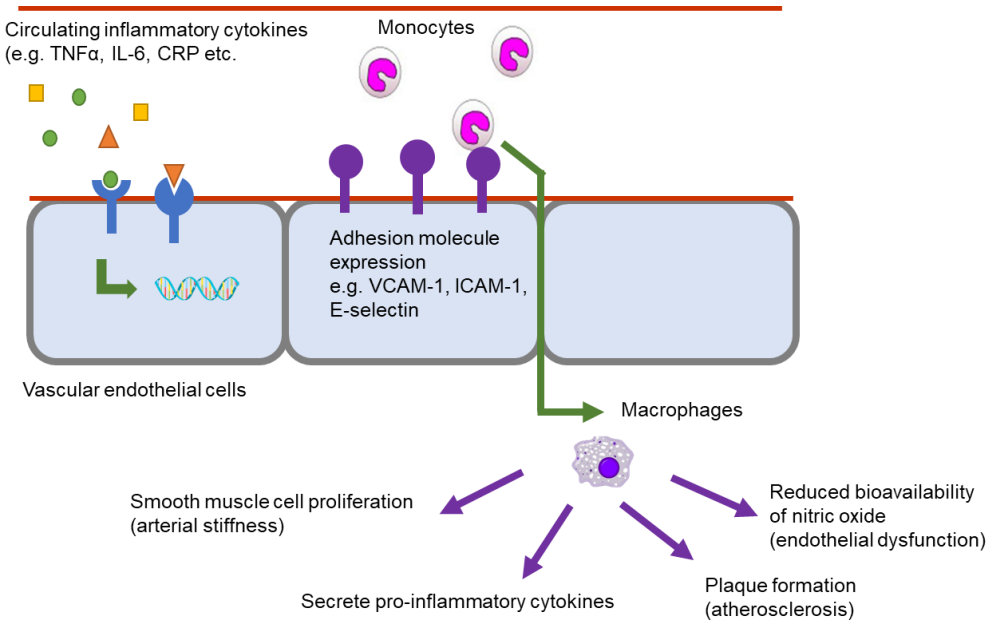

Figure 2.1. A summary of the process of endothelial activation. Inflammtory cytokines induce the upregulation of adhesion molecule VCAM-1, ICAM-1 and E-selectin in a process known as endothelial activation. The adhesion of blood cells to these molecules initiates a cascade of events which ultimately lead to arterial stiffess, atherosclerosis and endothelial dysfunction.

Endothelial dysfunction is an independent prognostic marker for the risk of future CV events (Bonetti *et al*., 2003; Lerman & Zeiher, 2005). Indeed endothelial dysfunction has been linked to atherosclerosis, arterial stiffness, myocardial infarction, stroke and heart failure (Camici *et al*., 2015; Shirwany & Zou, 2010; Anderson, 2006). Exposure to all major

traditional risk factors, including ageing, smoking, T2DM, hypertension and hypercholesterolemia has shown to increase endothelial activation, and/or the development of endothelial dysfunction (Liao, 2013; Brunner *et al*., 2005). However, inflammation has been shown to be a key driver of endothelial dysfunction, independent of traditional risk factors. In this regard, patients with RA and other inflammatory conditions have consistently shown a higher prevalence of endothelial dysfunction. Multiple studies have recognised inflammation-induced endothelial dysfunction as an important independent early predictor of atherosclerotic CVD as well as heart failure (Mudau *et al*., 2012; Dessein *et al*., 2005; Davignon & Ganz, 2004).

2.4 Endothelial dysfunction as a marker of CVD in inflammation

Chronic systemic inflammation is one of the key drivers of endothelial dysfunction. In this regard, inflammation alters NO signalling and impairs vasodilatory responses (Kobayasi *et al*., 2010; Coene *et al*., 1985). Early experiments in animals that used vascular reactivity as a marker of endothelial function, demonstrated marked impairments in relaxation responses to the administration of acetylcholine, an α_2 receptor mediated vasodilator in animals exposed to inflammation (Coene *et al*., 1985). Moreover, augmented responses to vasoconstrictive substances such as phenylephrine, are commonly seen in inflammation-induced endothelial dysfunction (Hamilton & Watts, 2013; Tabit *et al*., 2010). Indeed, endothelial dysfunction is one of the characteristic features of chronic inflammatory disorders such as RA (Aviña-Zubieta *et al*., 2008; Dessein *et al*., 2005). However, what is the evidence that endothelial dysfunction is associated with sub-clinical CVD in RA?

2.4.1 Endothelial function and atherosclerosis

It is now well recognised that inflammation-induced endothelial activation is one of the earliest precursors of atherosclerosis (Sitia *et al*., 2010; Gimbrone & Garcia-Cardena, 2016). The abundance of evidence outlining the role of inflammatory and immunoregulatory mediators in atherogenesis has led to the formulation of the Inflammatory Hypothesis of Atherothrombosis (Hansson, 2005; Libby, 2003; Ross, 1999), which describes the atherosclerotic process as a chronic inflammatory disease (Ridker *et al*., 2007). The role of

inflammation in the atherosclerotic process is summarised in Figure 2.2 Therefore, endothelial activation and dysfunction are considered the turning point towards a pro-atherogenic phenotype. Endothelial activation significantly contributes to the formation and progression of atherosclerotic plaques as a result of increased permeability and leukocyte adhesion (Gungor *et al*., 2017; Gimbrone & Garcia-Cardena, 2016). Indeed, in RA several markers of endothelial function including flow mediated dilation, ICAM, VCAM, e-selectin and endothelial leukocyte adhesion molecule concentrations have been associated with various markers of atherosclerotic CVD including cIMT and plaque (Verma et al., 2017; Khan et al., 2010; Versari et al., 2009; Dessein et al., 2005).

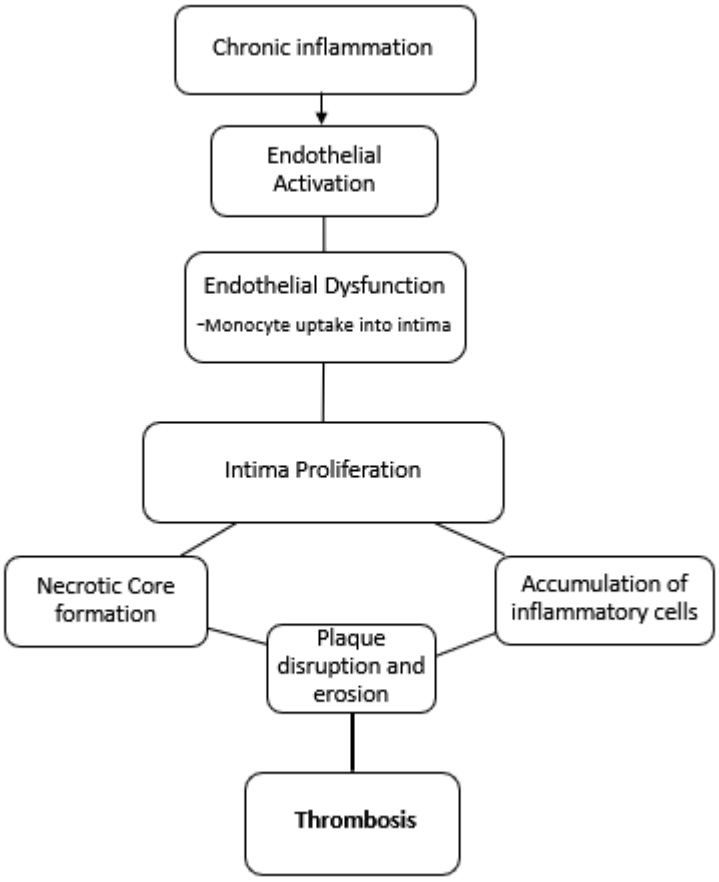

Figure 2.2. A summary of the Inflammatory Hypothesis of Atherothrombosis. Adapted from Ambrose *et al*., 2019.

2.4.2 Endothelial function and arteriosclerosis

Besides atherosclerosis, endothelial dysfunction has also been linked to the development of arteriosclerosis, which is defined the thickening and hardening of the walls of the arteries, that is typically associated with old age. Chronic inflammation causes a decrease in the production of elastin and an increase in collagen production in the extracellular matrix (ECM), which may lead to structural stiffening of the arterial vasculature (arteriosclerosis) (Park & Lakatta, 2012). Elastin and collagen comprise a large proportion of the ECM of the vessel wall, and their turnover is regulated by enzymes known as matrix metalloproteases (Galis & Khatri, 2002). Inflammatory cytokines cause a decrease in the production of elastin by inducing the release of matrix metalloproteases and by decreasing activity of the endogenous tissue inhibitor of metalloproteinases 2 (TIMP-2) (Park & Lakatta, 2012). Moreover, inflammation induces arterial stiffness through the inflammatory cytokine-activation of advanced glycation end products which form cross-links between collagen and bring about stiffer arteries Additionally, advanced glycation end products promote oxidative stress and decrease NO release, thereby altering endothelial function and further contributing to arterial stiffness (Mozos *et al*., 2017). Therefore, inflammation-induced endothelial dysfunction contributes to increased vascular stiffness and impaired arterial distensibility (Ramsey *et al*., 1995). (Mozos *et al*., 2017). Figure 2.3 provides a summary of the effect of inflammation on advanced glycation end products.

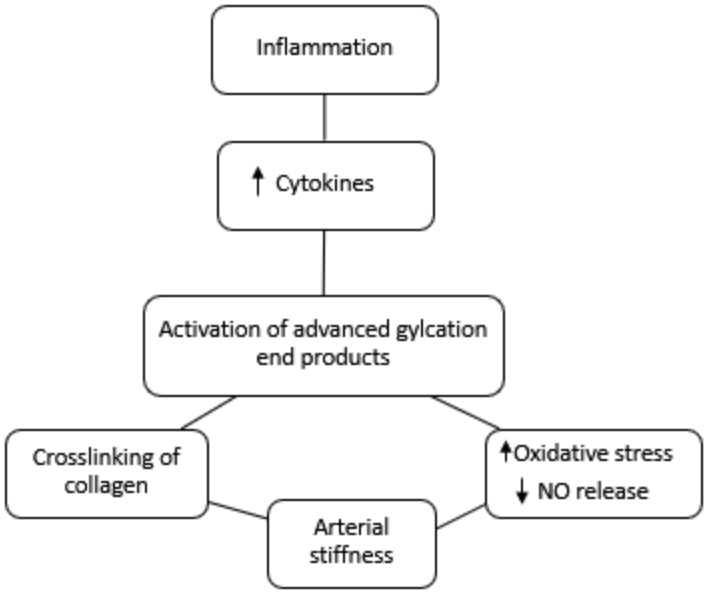

Figure 2.3. A summary of the effect of inflammation on advanced glycation end products. Inflammation induces the activation of advanced glycation end products which, through crosslinking of collagen and a decrease in NO release, causes arterial stiffness.

Arterial stiffness is an indication of structural and functional changes of the vessel wall and is associated with an increased risk of CV events (Adel *et al*., 2016; Zieman *et al*., 2005). Pulse wave velocity, a non-invasive measure of arterial stiffness, is a predictor of CV events independent of traditional risk factors (Vlachopoulos *et al*., 2010). When arteries are stiff, the pulse wave travels down the arterial tree at a faster velocity. At the reflection sites, when the pressure wave is reflected back to the heart, with stiffer arteries the reflected wave returns to the heart earlier, during systole (Chirinos *et al*., 2012; Nichols & Edwards, 2001). This earlier return of the reflected wave elevates systolic blood pressure which increases the workload of the heart and reduces diastolic blood pressure, which reduces myocardial perfusion (Chirinos *et al*., 2012). Altered wave reflection, as frequently measured by augmentation index (Alx), is associated with greater CVD risk (Janner *et al*., 2012). Indeed, arterial stiffness and wave reflection is increased in RA (Ambrosino *et al*., 2015) and are independently associated with inflammatory cytokines (Gunter *et al*., 2017; Klocke *et al*., 2003).

Taken together, inflammation is strongly implicated in the development of endothelial dysfunction. Inflammation-induced endothelial dysfunction contributes to the increased CVD risk in RA. Indeed, inflammation-induced endothelial dysfunction has been associated with atherosclerosis and arteriosclerosis in RA. Hence it is clear that the development of endothelial dysfunction in RA may initiate a cascade of events that can contribute to CV events. In order to develop useful therapeutic targets for the treatment of inflammation-induced CVD, it is important to understand the mechanisms whereby inflammation may cause endothelial dysfunction.

2.5 Mechanisms of inflammation-induced endothelial dysfunction

Several mechanisms whereby inflammation may induce endothelial dysfunction have been investigated. Inflammation causes an increase in circulating pro-inflammatory cytokine concentrations, such as IL-6, TNF-α and interleukin-1 beta (IL-1β) (Kany *et al*., 2019).These pro-inflammatory cytokines have pleotropic effects on various tissues whereby they induce further activation of immune cells resulting in an vicious cycle of cytokine release and cumulative inflammatory responses (Kany *et al*., 2019). The resulting condition is an overproduction of reactive oxygen species (ROS). ROS is a by product of aerobic respiration produced intracellularly in the mitochondrial respiratory chain and under normal physiological conditions is maintained within a normal range, however, when there are exogeneous stimuli such as TNF-α present, this results in an overproduction of ROS known as oxidative stress (Bae *et al*., 2011; Ray *et al*., 2012). Oxidative stress causes the oxidation of low density lipoprotein (LDL) (Cervantes Gracia *et al*., 2017). Endocytosis of the oxidised LDL occurs within macrophages and accumulates over time in the intima of the vessel and promotes plaque formation and atherosclerosis (Hansson, 2006).

Although endothelial activation is involved in the process of atherosclerosis as described above, endothelial activation occurs prior to the development of endothelial dysfunction. Figure 2.4 provides a brief summary of the process whereby endothelial dysfunction ultimately leads to endothelial dysfunction. In this regard, continued activation of the endothelium induces endothelial cells to undergo functional change through ROS production and disruption in NO signalling (Aletaha *et al*., 2010). NO is the predominant endothelium-derived vasodilator in the systemic arterial system. Under normal

physiological conditions, after its secretion from the endothelium, NO diffuses into the vascular smooth muscle where it acts as a potent vasodilator which causes vascular smooth muscle relaxation (Fitch *et al*., 2001; Sandoo *et al*., 2011). Besides its vasodilatory effects, under normal physiological conditions, NO scavenges ROS in the vessels, and in doing so removes ROS. Therefore, NO is an important mediator of vascular health. However, endothelium activation by proinflammatory cytokines causes endothelial dysfunction through the production of ROS which decreases NO bioavailability and through the uncoupling of the endothelial nitric oxide synthase (eNOS) within the endothelium (Aletaha *et al*., 2010). Endothelial NOS is the enzyme that produces NO by converting L - arginine to L-citrulline, using co-factors (Yokoyama & Hirata, 2007). An increase in ROS results in dysfunction of these co-factors, causing eNOS to produce superoxide instead of NO in a process known as uncoupling (Yokoyama & Hirata, 2007; Varadharaj *et al*., 2015).

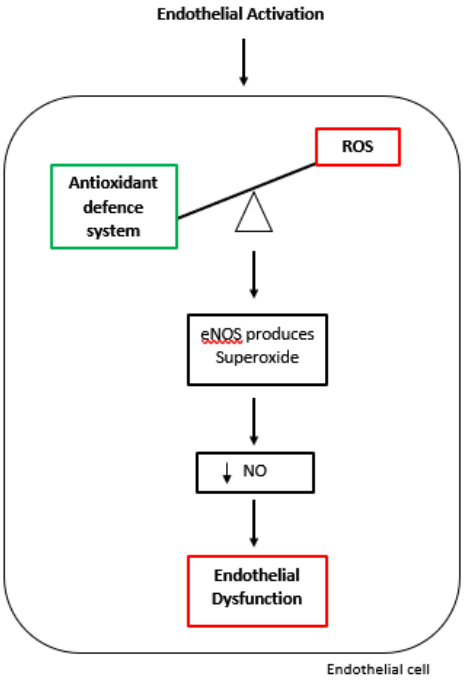

Figure 2.4. An overview of the process whereby inflammation induces oxidative stress in the vasculature. Endothelial activation causes the production of ROS which induces endothelial dysfunction through decreased NO production and increased superoxide production. Adapted from Incalza *et al.,* 2018.

Low levels of ROS are maintained by various antioxidant enzymes and are necessary for vascular function under normal conditions (Wink *et al*., 2001; Xia *et al*., 1998). However, oxidative stress occurs when the excessive production of ROS surpasses the controlling capacity of cellular antioxidants, which results in endothelial cell injury (Xia *et al*., 1998). Uncoupling of eNOS causes it to produce superoxide instead of NO which aggravates oxidative stress levels within the endothelium (Wink *et al*., 2001; Xia *et al*., 1998). The amplified production of superoxide results in the formation of peroxynitrite, an inhibitor of cyclic guanylyl cyclase, which subsequently inhibits the antioxidant enzyme superoxide dismutase and further progresses oxidative stress (Lubos *et al*., 2008). Both the increase in ROS production and reduction in NO bioavailability disrupt vasomotor tone. Therefore this imbalance results in an impairment of endothelium-dependent vasodilation, which is the defining characteristic of endothelial dysfunction (Bonetti *et al*., 2003).

Inflammatory cytokines not only uncouple eNOS, but also upregulate inducible nitric oxide (iNOS). In this regard, iNOS is capable of increasing and maintaining NO at pathologically high concentrations, resulting in excessive ROS production and significant vascular damage (Clancy *et al*., 1998). In contrast to the protective effect of NO at low concentrations, increased concentrations of NO leads to excessive vascular permeability, impaired cellular signalling and subsequent destruction of the vascular wall (Clancy *et al*., 1998).

Given its involvement and its predictive significance for CV events, there has been considerable interest in the detection and monitoring of endothelial dysfunction in the clinical setting (Davignon & Ganz, 2004). As endothelial dysfunction is largely reversible, therapeutic strategies are now aimed at early detection of endothelial dysfunction, prior to the onset of arterial dysfunction, hypertension, atherosclerosis or heart failure with a preserved ejection fraction. Consequently, the identification of clinical biomarkers for the detection of endothelial dysfunction is of clinical relevance.

2.6 Biomarkers of inflammation-induced endothelial dysfunction

While measures, such as catheterization, intracoronary infusion of acetylcholine and quantitative coronary angiography (Flammer *et al*., 2012), are considered the gold standard

for the assessment of endothelial function and vascular reactivity, these methods are invasive and not frequently used due to its high cost (Si *et al*., 2018). Less invasive approaches to measure endothelial function in humans, such as flow mediated dilatation (FMD), have been used more frequently in clinical studies. FMD is an ultrasound-based technique (Raitakari & Celermajer, 2000) that is widely used for investigating endothelial function. However there are several limitations and challenges with this method, including it being operator dependent, difficult to perform and the inconsistent interpretation of the results (Si *et al*., 2018; Šejda *et al*., 2005; Raitakari & Celermajer, 2000). Furthermore, while functional assessment can identify endothelial damage, it does not provide information on possible underlying mechanisms of endothelial dysfunction (Burger & Touyz, 2012). Hence, several studies have criticised the use of FMD as a valid marker of endothelial dysfunction (Pereira *et al*., 2018; Harris *et al*., 2010; Woodman *et al*., 2001).

Biomarkers have become a more reliable and feasible method for measuring endothelial function and for elucidating pathophysiological processes in several disease states (Mayeux, 2004). Several biomarkers of endothelial function have been identified in the literature (Neves *et al*., 2016; Hunt *et al*., 2015;). Although a thorough discussion of each of these biomarkers is beyond the scope of this thesis, the function and relevance of some of the most pertinent biomarkers will be highlighted here.

Following endothelial activation, the concentration of glycoproteins and adhesion molecules such as VCAM-1, ICAM-1 and E-selectin are significantly increased as explained previously in section 2.4 (Krieglstein & Granger, 2001). The measurement of ICAM-1, VCAM-1 and E-selectin is therefore considered a validated measure and biomarker of endothelial activation in clinical studies (Paulus *et al*., 2011). Although these markers signify endothelial activation, they do not necessarily reflect endothelial dysfunction. In this regard, elevated levels of asymmetric dimethylarginine (ADMA) is considered a marker of endothelial function (Matrozova *et al*., 2016; Sibal *et al*., 2010). ADMA is a naturally occurring NOS inhibitor that reduces NO availability and impairs endothelial function (Matrozova *et al*., 2016; Sibal *et al*., 2010).

Pentraxin-3 (PTX-3) has been identified as a novel and possibly more accurate biomarker of endothelial function (Yasunaga *et al*., 2014). PTX-3 is an acute phase protein that activates the classical complement pathway and represents primary local activation of innate immunity and inflammation (Fornai *et al*., 2016). In contrast to the acute phase protein C-reactive protein (CRP), which is synthesised in the liver after stimulation by IL-6, PTX-3 is produced directly by endothelial cells in the inflamed vasculature upon endothelial activation (Yasunaga *et al*., 2014; Breland *et al*., 2010; Hollan *et al*., 2010; Norata *et al*., 2010; Jaillon *et al*., 2007). PTX-3 has been described as a possible link between inflammation and CVD, as it possesses multifaceted properties extending beyond immunity, to include functions related to vascular and CV regulation (Fornai *et al*., 2016; Dubin *et al*., 2012). In this regard, PTX-3 concentrations are correlated with the presence and severity of endothelial dysfunction, as well as the severity of coronary atherosclerosis in various clinical populations (Nerkiz *et al*., 2015; Witasp *et al*., 2013; Hamad *et al*., 2012; Suliman *et al*., 2008). PTX-3 is involved in both of the above mentioned molecular mechanisms leading to vascular damage, and is significantly upregulated during inflammation (Fornai *et al*., 2016; Parlak *et al*., 2012; Norata *et al*., 2010) . PTX-3 impairs endothelial function through a P-selectin/matrix metalloproteinase (MMP) 1 pathway, and disrupts NO signalling (Carrizzo *et al*., 2015). PTX-3 is also thought to exert detrimental effects on the atherosclerotic process since it promotes plaque formation, amplifies vascular inflammation and interferes with plaque stability (Fornai *et al*., 2016;; Deban *et al*., 2010; Bassi *et al*., 2009).

In addition to the usefulness in the clinical detection of early stages of endothelial dysfunction, the aforementioned biomarkers have also provided essential mechanistic information into the pathways whereby inflammation induces endothelial dysfunction. Although several of these endothelial function biomarkers are expressed locally, it has been suggested that upstream gene regulation also impacts vascular function. In this regard, miRNAs have been suggested as promising diagnostic tools, however research supporting these suggestions is limited.

2.7 MicroRNAs as regulators of endothelial function

MicroRNAs are short non-coding RNAs which function to post-transcriptionally regulate gene expression. MicroRNAs supress gene expression by inhibiting translation of messenger RNA (mRNA) or inducing mRNA degradation (Urbich *et al*., 2008). The regulation of miRNAs has been shown to be connected to complex gene regulatory networks (Shukla *et al*., 2011). The majority of human miRNAs are encoded within the introns and long non-coding transcripts of the protein coding exons. However, miRNA codons can be present in both exons and introns depending on the regulatory process, known as alternative splicing (O'Brien *et al*., 2018; Shukla *et al*., 2011). The biogenesis of miRNAs is proposed to occur following the transcription of miRNA genes by RNA polymerase II to form primary miRNA (pri-miRNA). Thereafter they are processed post-transcriptionally, which results in precursor miRNA (pre-miRNA) (O'Brien *et al*., 2018). This post transcriptional process occurs within the nucleus and is facilitated by Drosha, a class 2 RNase III catalytic enzyme (Wahid *et al*., 2010), which cleaves pri-miRNA into hairpin structures (Han *et al*., 2004). The pre-miRNA is then transported to the cytoplasm, where it is acted on by Dicer, a protein from the RNase III family (Wahid *et al*., 2010) that further cleaves the hairpin structure and produces an approximately 22 nucleotide miRNA duplex (Koscianska *et al*., 2011). The miRNA duplex undergoes a process that determines which of the two strands become the active strand. Some of the features that play a role in the determination of the active strand include thermodynamics, i.e. which strand has the weakest 5′ binding; and the presence of excess purines (Meijer *et al*., 2014). Based on these features, the active strand becomes separated from the subsequently degraded passenger strand and forms the mature miRNA. The mature miRNA is then coupled with a binding molecule, known as argonaute protein, to form a RNA-induced silencing complex (RISC) (Siomi & Siomi, 2010). Figure 2.5. shows a summary of the process of synthesizing microRNAs and how they silence mRNA post-transcriptionally.

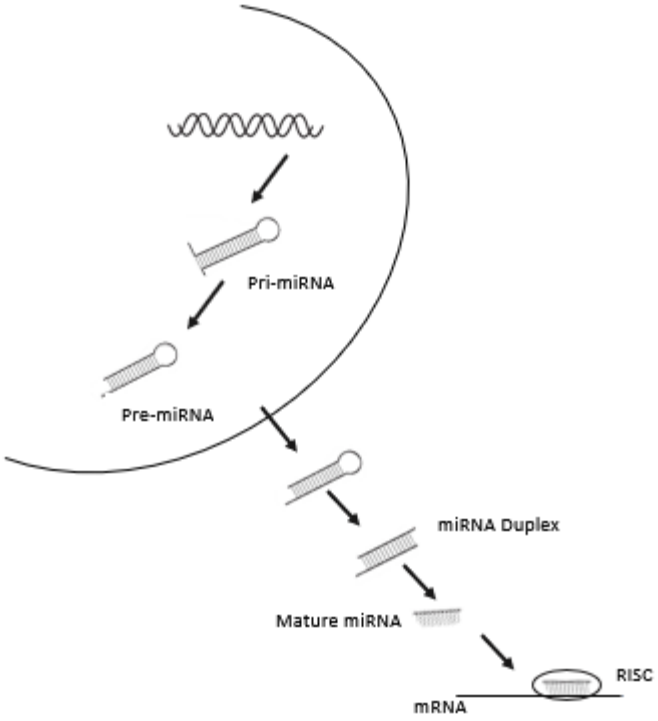

Figure 2.5. A summary of the process of synthesizing microRNAs and how they silence mRNA post-transcriptionally. Biosynthesis of miRNA begins with the transcription of the transcription of miRNA genes to form primary miRNA (pri-miRNA), which is processed post-transcriptionally, forming precursor miRNA (pre-miRNA). The pre-miRNA is then transported to the cytoplasm, where it is cleaved to produce a miRNA duplex of which one of the two strands become the active strand. The active strand forms the mature miRNA. The mature miRNA is then coupled with binding molecules to form an RNA-induced silencing complex (RISC). Adapted from Davis *et al*., 2015.

In the human genome alone, more than 2600 miRNA genes have been identified and potentially over 46000 miRNA-mRNA duplex regions (Platnikova *et al*., 2019; Hammond, 2015). These miRNAs are believed to regulate up to one third of the genome. Therefore, miRNAs are one of the largest classes of gene-regulatory molecules (Siomi & Siomi, 2010). Recent evidence points to several mechanisms whereby miRNAs regulate gene expression, including gene silencing via miRISC, miRNA-mediated translational activation and miRNA-mediated transcriptional and post-transcriptional gene regulation within the nucleus (O'Brien *et al.,* 2018). The biogenesis of miRNAs is tightly regulated, with differentiation and maturation being stringently controlled. Importantly, miRNAs often

interact with the transcription factors that regulate their expression, generating double negative feedback loops (Platnikova *et al*., 2019; Siomi & Siomi, 2010; Li *et al*., 2009).

Several studies have found that miRNAs play crucial roles in various cell signalling cascades and pathways. Thus aberrantly expressed, dysfunctional or dysregulated miRNA may lead to a multitude of diseases (Chen & Zhang, 2013). Some of the diseases in which the regulation of miRNAs have been implicated include myocardial infarction, autoimmune diseases and cancer (Ardekani & Naeini, 2010).

Although several molecules and growth factors regulate endothelial function, little is known about the complex upstream regulation of gene expression and translation regulating vascular integrity (Urbich *et al*., 2008). As the dysregulation of specific miRNAs have been implicated in vascular integrity and inflammation, these could potentially serve as biomarkers of endothelial dysfunction (Ait-Aissa *et al.,* 2020; Fernández-Hernando & Suárez, 2018; Khalyfa *et al*., 2016) Hence miRNAs may contribute to the risk stratification of CVD in patients with RA (Castro-Villegas *et al*., 2015). Although several miRNAs are implicated in the regulation of endothelial function or inflammation, an extensive discussion of these miRNAs are beyond the scope of this thesis. Nevertheless, two miRNAs that have been upregulated in RA and have been associated with inflammation-induced endothelial dysfunction, miR155-5p and miR146a-5p, will be discussed here.

Both miR155-5p and miR146a-5p are upregulated in RA (Duroux-Richard *et al*., 2011; Murata *et al*., 2010; Pauley *et al*., 2008). These miRNAs have been implicated in the regulation of NO signalling, expression of adhesion molecules and/or inflammatory pathways (Ayeldeen *et al*., 2018; Moran-Moguel *et al*., 2018; de Lucia *et al*., 2017; Heggermont *et al*., 2017; Zhang *et al*., 2017; Wang *et al*., 2012; Filková *et al*., 2012). The section below will briefly highlight the involvement of the aforementioned miRNAs in inflammation and endothelial function.

MiR155-5p is strongly implicated in the regulation of innate immune signalling pathways, and has been extensively studied in RA patients (Moran-Moguel *et al*., 2018). Increased TNF-α concentrations have been associated with aberrant overexpression of miR155-5p, which further enhances circulating TNF-α and lL-6 production via positive feedback (Filková

et al., 2012). TNF-α has been reported to impair NO/cGMP mediated vascular relaxation, through NF-κB-dependent biogenesis of miR155-5p (Choi *et al*., 2018). However, the association between miR155-5p and vascular adhesion molecules, arterial stiffness, vascular reactivity and cardiac geometry and function has not been fully explored in RA patients.

MiR146a-5p has gained importance for its role as a modulator of cell function and differentiation in both the adaptive and innate immune responses. MiR146a-5p is therefore a vital part of many cell pathways and maintains the integrity of cell functions. Lu *et al* (2010) found that miR146a-5p was extensively expressed in and was essential for the function of regulatory T (Treg) cells. Treg cells are specialised T-cells which function to maintain tolerance to self-antigen by suppressing the immune response (Kondelkova *et al*., 2010). Inadequate expression of miR146a-5p leads to a larger quantity of Treg cells with impaired function, resulting in diminished immunological tolerance due to substantial activation of lymphocytes and massive tissue infiltration (Rusca & Monticelli, 2011; Lu *et al*., 2010). Indeed, impaired Treg function has previously been reported in RA (Boissier *et al.,* 2009).

Studies have found that serum levels of miR146a-5p was significantly increased in systemic lupus erythematosus, lupus nephritis (Labib *et al*., 2019) and in both the RA and T2DM patients (Ayeldeen *et al*., 2018). Furthermore, miR146a-5p is significantly related to inflammation, vascular ageing, ventricular dysfunction and heart failure (Heggermont *et al*., 2017; de Lucia *et al*., 2017). Additionally, one study found that miR146a-5p indirectly regulates the expression of VCAM-1 and ICAM-1, thus bringing about an effect on the function of the endothelium (Lo *et al*., 2017). However, research investigating the regulation of miR146a-5p, and its effect on endothelial function within populations with high-grade inflammation is limited and further investigation is warranted. Furthermore, although these miRNAs are implicated in inflammation-induced endothelial dysfunction, little is known on the effect of anti-inflammatory treatments on miRNA expression in RA patients.

2.8 Anti-inflammatory treatment effects on endothelial dysfunction

Traditionally RA has been treated with synthetic disease-modifying anti-rheumatic drugs (DMARDs), of which the most common is methotrexate (MTX) (Smolen *et al*., 2007).

However, synthetic DMARDs are not always effective in achieving and maintaining disease regression (Curtis & Singh, 2011). Currently, more than half of RA patients do not achieve sustained drug-induced disease remission (Nagy & van Vollenhoven, 2015). Biologic DMARDs are more effective at maintaining optimal disease control (Curtis & Singh, 2011). In this regard, biologic treatments reduce the aberrant effect of inflammation by either competing for receptor binding or by high affinity binding of antibodies to cytokines, thereby interfering with cytokine function or production (Benjamin and Lappin, 2019; Hamilton and Clair, 2000).

A number of biologic DMARDs are currently available. One of the first marketed and most frequently used is TNF-α inhibitors. More recently, IL-6 and IL-1β blockers have been developed. Although biologic DMARDs have shown to improve disease activity and progression, their effects on CVD risk in RA are controversial. In this regard, treatment with TNF-α antagonists and IL-6 inhibitors have demonstrated improvements in endothelial function in RA (Murdaca *et al*., 2013; Protogerou *et al*., 2011). TNF-α antagonists have been shown to reverse vascular endothelial dysfunction by improving endothelium dependent vasodilation (Steyers and Miller, 2014) and reducing aortic stiffness (Mäki-Petäjä *et al*., 2006). Indeed, TNF-α antagonists have also been found to decrease the levels of biomarkers of endothelial activation (Klimiuk *et al*., 2009). IL-6 antagonists similarly prevent endothelial dysfunction by increasing NO production, decreasing aortic stiffness and decreasing inflammation (Kaur *et al.,* 2014; Protogerou *et al*., 2011). Despite these positive reports on endothelial function, their effects on CVD in RA is controversial (Baniaamam *et al*., 2018; Rao *et al*., 2015; Barnabe *et al*., 2011).

Taken together, chronic inflammation has numerous pathological effects, both locally at the site of vascular activation, and during gene regulation occurring upstream. There is mounting evidence to support miRNAs as novel biomarkers and predictors of endothelial dysfunction. However, whether inflammation affects the relationship between these specific miRNAs and alterations to endothelial function requires investigation. Moreover, the mechanisms by which anti-inflammatory treatments alter miRNA signalling to affect downstream sequences and ultimately endothelial function and CVD risk are uncertain. It is currently not known whether biologic therapies are effective in restoring miRNA levels.

Further investigation is required to determine the direct effects of biologic DMARDs on biomarkers of endothelial function and miRNAs, without the influence of confounding factors commonly noted in human RA studies.

2.9 The collagen induced arthritis model

Human studies investigating the effects of TNF-α and IL-6 antagonists on cardiovascular health have thus far been hindered by several confounding factors. These include the concomitant use of Methotrexate with biological treatments, additional use of cardiovascular drugs and clinical heterogeneity of study populations due to differences in RA disease characteristics. Therefore, the use of animal models of RA can provide greater insight into the sole effect of biological agents on endothelial function, without the influence of confounding or mediating factors (Totoson *et al.*, 2016). Although several animal models have been proposed, the structural and immunological changes of the collagen-induced arthritis (CIA) rat model best resemble that of RA (Bolon *et al*., 2011) and is therefore the most widely used model (Steyers & Miller, 2014).

2.10 Problem statement

In chronic inflammatory diseases, such as RA, patients have an increased risk of developing CVD. While some of the increased CVD risk is attributed to traditional risk factors, it does not fully explain the excessive CV morbidity and mortality in RA patients. Chronic inflammation in RA is considered to be a major contributor to the increased CVD risk. The mechanisms whereby inflammation increases CVD are currently being investigated.

A known prognostic marker of CVD is endothelial dysfunction, which has been shown to be linked to inflammation. While acute inflammation causes the activation of the endothelium, chronic inflammation causes its dysregulation and dysfunction. Additionally, chronic inflammation upregulates specific miRNAs which are known to be associated with endothelial dysfunction. Therefore, miRNAs could be a potential biomarker of inflammation-induced endothelial dysfunction. However, there is currently insufficient evidence to support this claim and hence further investigation is warranted.

Furthermore, while biologic anti-inflammatory drugs currently used to treat RA reduce the severity of inflammation, the effect of these drugs on the expression of miRNA and by extension its impact on endothelial function is unknown. Thus, the potential role of miRNAs in endothelial dysfunction warrants investigation to 1) elucidate the effect of inflammation on miRNA expression in high-grade inflammation and 2) the effect of biologic treatments on miRNA expression and its possible downstream effects on endothelial function.

2.11 Aim

This study aims to determine the effect of biologic anti-rheumatic drug treatment on novel and conventional biomarkers of endothelial function in a CIA rat model.

2.12 Study Objectives

The specific objectives are to determine

- the effect of inflammation on the expression of miRNAs (miR155-5p and miR146a-5p)
- the effect of biologic DMARD treatment on the expression of miRNAs (miR155-5p and miR146a-5p)
- the association between circulating inflammatory markers, endothelial function markers and miRNA expression in untreated and treated rats exposed to high-grade inflammation

CHAPTER 3
METHODS

3.1 Animals

Three-month-old, male and female Sprague-Dawley rats were obtained from the Central Animal Services (CAS) of the University of the Witwatersrand as part of a larger study. Seventy-one rats (male n=30; female n=41) from which high-quality RNA could be extracted were included in this study. Rats were kept in temperature-controlled rooms on a 12-hour light- dark cycle and were housed individually. Male and female rats were housed in separate rooms. All measurements and procedures were approved by the Animal Research Ethics Committee of the University of the Witwatersrand (approval number 2017/03/21C, Appendix A).

3.2 Study design

All rats were habituated for two weeks, during which they were weighed, and blood pressure and arthritis scores were measured weekly. Water and standard rat chow were provided at all times during the habituation period and the study. Following the habituation period, rats were randomly divided into four groups. The first group was the control group (n=20, male=7, female=13) and received subcutaneous saline injections (0.2 ml) at the base of the tail. The other three groups, namely the inflammation (n=27, male=12, female=15), the anti-TNF-α group (n=13, male=6, female=7) and the anti-IL-6 groups (n=11, male=5, female=6) were exposed to an arthritis inducing protocol. Arthritis was induced by subcutaneous injections of Bovine Type II collagen emulsified in incomplete Freund's adjuvant (Chondrex) at the base of the tail. To guarantee a high incidence of arthritis, 7 and 21 days after the first immunization, rats received a booster injection. In the groups that were exposed to the arthritis protocol, upon the first signs of inflammation (approximately three weeks after primary immunization) all intervention groups received 1-4 mg/kg pain relief medication (Tramazec). Two of the experimental groups were further treated with biologic anti-rheumatic drugs. The anti-TNF-α group received a TNF-α inhibitor (Etanercept; 10 mg/kg) every third day for six weeks. The anti-IL-6 group received an IL-6 receptor blocker (Tocilizumab; 8mg/kg) once a week for six weeks. Following the treatment period, rats were anaesthetised to obtain arterial stiffness measurements. At termination, blood samples were obtained, centrifuged and serum samples were stored in -80˚Celcius

freezers. Heart tissue was collected, and renal and mesenteric arteries were isolated for vascular reactivity measures.

3.3 Arthritis score

Signs of inflammation were scored in the hind paws of the rats in order to determine the severity of arthritis. Signs and severity of inflammation were scored on a scale of zero to four as previously described (Mokotedi *et al*., 2019). A cumulative score from the scale for two hind paws (with a maximum of eight) were used to represent disease severity. The scoring is described as follows: 0) no swelling or focal redness in the paw, 1) slight swelling or focal redness in the paw, 2) low to moderate oedema in the paw, 3) pronounced oedema in the paw and restriction of paw use, 4) excessive oedema with paw rigidity and deformity.

3.4 Blood pressure

A non-invasive, tail cuff blood pressure amplifier (Biopac Systems, Santa Barbara, CA, USA) was used to measure blood pressure. Prior to the measurement the tail was placed on a heating pad for ten minutes. Rats were placed in restrainers with adjustable restriction panels, where the average of three blood pressure was measured at midday, once a week.

3.5 Arterial stiffness

Prior to termination, rats were anaesthetised by intramuscular injections of ketamine (100 mg.kg-1) and xylazine (5 mg.kg-1). The neck and inner right thigh of the rats were shaved, and rats were placed on a heating pad in the supine position. Arterial stiffness was measured by applanation tonometry (PulsePen device, DiaTecne, Milan, Italy), during which one probe was placed on the common carotid artery pulse and one probe on the femoral artery pulse, in order to record pulse waves simultaneously. The two probes were fixed in the correct position on the arteries by means of mechanical arms, equipped with micro-regulators. Thereafter, pressure signals were transmitted to a computer by means of an optical fibre that ensures electromagnetic isolation for the rat undergoing the test. Following acquisition of the tonometric pulse waves, the distance from the carotid to the

femoral probe sites were measured. Pulse wave velocity was calculated as the distance between the two recording sites divided by the time delay between the two arterial waveforms at each site, and was thus reported as meters per second. Using inbuilt software, the central aortic waveform was derived using a validated transfer function. From the central aortic wave form, the inbuilt software derived the central augmentation pressure (AP), the augmentation index (AIx) and the forward (Pf) and backward wave (Pb) pressures (Westerhof et al., 2006). The augmentation index was calculated as (second systolic peak/first systolic peak) x 100.

3.6 Vascular reactivity

While under anaesthesia, rats were terminated by thoracotomy and the hearts were 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 renal and mesenteric arteries were carefully isolated, and 2 mm rings of the arteries were cleaned of connective tissue and fat. The cleaned artery rings were suspended in a wire myograph (model 610M, Danish Myo Technology, Aahrus, Denmark). The wire myograph system is capable of accurately measuring the reactivity of small isolated blood vessels of 100 µm to 2.5 mm in diameter. The blood vessels were mounted as a ring and threaded over two parallel stainless-steel wires. The wires were secured to two supports or "jaws" with one support attached to a precision micrometre (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), to produce a maximal contraction response, which served as the reference contraction. The artery preparations 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 KCI (80mM)-induced increases in tension. The relaxation responses, induced by acetylcholine, an endothelium-dependent vasodilator (ACh, 0.1 nM to 10 mM), were assessed in half-log increments during phenylephrine (Phe, 10 μM)-induced contractions. Vascular relaxation was expressed as a percentage of the baseline tension during contractions induced by Phe (10 μM). Vasodilators and vasoconstrictor solutions were obtained from Sigma (St. Louis, MO, USA).

3.7 Circulating inflammatory markers

Blood samples were used to measure serum IL-6, TNF-α and CRP concentrations, using solid-phase sandwich enzyme linked immunosorbent assay (ELISA) (Elabscience Biotechnology Co. Ltd, Wuhan, China). The coefficients of variation were <10% for all kits. The lower detection limit for IL-6, TNF-α and CRP was 62.50 pg/ml, 78.13 pg/ml and 0.31 ng/ml, respectively.

3.8 Expression of miRNAs

3.8.1 RNA extraction and cDNA synthesis

Total RNA was extracted from blood serum using the MagMAX™ mirVana™ Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA), using manufacturer's instructions. Serum samples were subjected to protein digestion using Proteinase K, after which the samples were lysed. RNA binding was then performed using binding beads and a magnetic stand (Thermo Fischer Scientific, Waltham, USA) to separate it from the supernatant. This was followed by two wash steps to ensure all debris had been removed and the RNA was pure. Lastly the RNA was then eluted to recover the purified RNA into the elution plate.

cDNA templates were prepared using TaqMan™ Advanced miRNA cDNA synthesis kits (Thermo Fisher Scientific, Waltham, MA). cDNA synthesis consisted of a Poly (A) tailing reaction, where after an adaptor ligation reaction was performed. The reaction plate was then incubated in a thermal cycler. After which, a reverse transcription step occurred using reverse transcriptase (RT). Thereafter the miR-Amplification reaction was performed to a final volume of 30 μL. Production of cDNA (>1000ng/μl) was confirmed using a NanoDrop

OneC spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The undiluted miR-Amp reaction contents were then stored at -20°C pending the real-time quantitative PCR reaction.

3.8.2 Real time quantitative PCR (RT-PCR)

Comparative gene expression RT-PCR was performed using cDNA (~1µg), pre-designed VIC labelled probe mixes for the reference miRNA (miR191-5p, 0.25 µL, Taqman assay ID: rno480971_mir), the miRNA of interest (0.5 µL Taqman Advanced miRNA Assay), Taqman Fast Advanced Master Mix (5 µL) and 3.25 µL of RNase-free water, to make up a total volume of 10 µL per reaction well. Cycling conditions consisted of enzyme activation 95°C for 20 seconds, followed by denaturing at 95°C for 1 second and annealing and extending at 60°C for 20 seconds for a total of 40 cycles.

Previous studies have conventionally made use of SnU6 or RNU6 as endogenous controls. However, several studies have reported the expression of these genes to be unstable and therefore not appropriate for use as reference genes (Xiang *et al*., 2014; Schwarzenbach *et al*., 2015). SnU6 and RNU6 do not reflect the biochemical character of miRNA molecules in terms of their transcription, processing and tissue specific expression patterns and may respond differently to the extraction, reverse transcription and PCR amplification steps (Serafin *et al*., 2014; Benz *et al*., 2013; Gee *et al*., 2011). Several studies have advocated miR191-5p to be stably expressed, making it a more suitable reference gene (Bignotti *et al*., 2016; Li *et al*., 2015; Zheng *et al*., 2013; Hu *et al*., 2012; Shen *et al*., 2011; Peltier & Latham, 2008). MiR191-5p was found to be the most suitable reference gene for serum miRNA after comparing it to one other potential endogenous reference gene (miR26a-5p) using RefFinder algorithm (Panina *et al*., 2018). MiR191-5p was found to be most consistently expressed in serum, with a distinguishably low cycle threshold value standard deviation. The literature also reports that it has a low co-efficient of variation (Bargaje *et al*., 2010; Thermo Fisher Scientific Inc., 2016). Comparative miRNA expression was assessed for miR146a-5p (Taqman assay ID: rno481451) and miR155-5p (Taqman Assay ID: rno480953_mir) in duplex reactions with miR191-5p as the endogenous control, in duplicate plates. Analysis was performed using the Applied Biosystems real-time PCR Analysis Modules Software.

3.9 Expression of endothelial dysfunction markers

Total RNA was extracted from homogenized rat heart LV samples using an Illustra minispin RNA extraction kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. Reverse transcription was performed to produce cDNA from the isolated RNA (2 µl) using SuperScript™ IV VILO™ cDNA synthesis master mix (18 µl, Thermo Fisher Scientific, Life Technologies, Carlsbad, USA), incubated at 25°C for 10 minutes followed by incubation at 50°C for 10 minutes and inactivated at 85°C for 5 minutes in a thermal cycler (T100, BioRad, California, USA). Production of cDNA (>50ng/µl) was confirmed using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Comparative gene expression RT-PCR was performed using cDNA (~2 µg), pre-designed Taqman probe mixes for the reference gene HPRT1 (0.25 µl, Taqman assay ID: Rn01527838_g1, Thermo Fisher Scientific, Life Technologies, Carlsbad, USA), the gene of interest (0.5 µl, Taqman gene expression assay) and Taqman advanced PCR master mix (5 µl) in a final volume of 10 µl.

Cycling conditions consisted of an initial hold at 50°C for 2 minutes, a hot start hold at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute using a StepOne Plus Real-Time PCR System (Thermo Fisher Scientific, Life Technologies, Austin, USA). Hypoxanthine guanine phosphoribosyl-transferase 1 (HPRT1, Taqman assay ID: Rn01527838_g1) was found to be the most suitable reference gene for rat LV tissue. Comparative gene expression of VCAM-1 (0.5 µl, Taqman assay ID: Rn00563627_m1, Thermo Fisher Scientific, Life Technologies, Carlsbad, USA) and PTX-3 (0.5 µl, Taqman assay ID: Rn01769850_m1, Thermo Fisher Scientific, Life Technologies, Carlsbad, USA), was assessed in duplex reactions with HPRT1 (0.25 µl) in duplicated plates.

3.10 Analysis of gene expression data using the ΔΔCt method

The ΔCt values were calculated by subtracting the Ct value for the reference gene, (miR191-5p or HPRT1), from the Ct value of the target gene for each sample. The ΔΔCt values were then calculated by subtracting the ΔCt value for each sample from the mean of the ΔCt values determined for samples from the control animals that were not inoculated

with collagen, within the same PCR plate. The fold-change in relative expression was then calculated as $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

3.11 Statistical Analysis

Statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., USA). Continuous data was expressed as means ± SD for normally distributed variables or median and interquartile range (IQR) for non-normally distributed variables. Changes in blood pressure and body mass over the study and between groups were determined by repeated measures analysis of variance (ANOVA) followed by a Tukey *post hoc* test. To compared arthritis scores between groups and to baseline, a Friedman was performed. For normally distributed data, two-way ANOVAs followed by Tukey *post hoc* tests were performed to assess differences between groups at termination for all measured markers. For data not normally distributed, a Kruskal-Wallis test followed by a Dunn's *post hoc* test was used to determine differences in biomarkers between groups. As there were no differences in the circulating inflammatory markers or the miRNA expressions between males and females, results were pooled. Associations between circulating endothelial function markers and miRNA expressions were determined by Pearson's correlation coefficients in control and inflammation groups and in all groups in separate models. MicroRNA data were log transformed, and PTX-3 data were square rooted, to improve normality prior to inclusion in linear regression analyses. As biologic DMARD treatment was believed to impact on the association between circulating cytokine concentrations and miRNA expression, these associations were first reported in the control and inflammation groups only, and thereafter in all groups. All graphs and figures were prepared using Graphpad Prism software, version 8 (Graphpad, USA). Differences were considered statistically significant if $P \leq 0.05$.

CHAPTER 4
RESULTS

4.1 Body weight and blood pressure

There were no differences in body mass between the groups at baseline ($p>0.05$). Body mass was not different at termination compared to baseline in any of the groups (all $p>0.05$, Figure 4.1A). The average (±SEM) body mass of the anti-TNF-α group (379.5±32.3g) at termination was lower than the body mass of the control group (505.9±25.4g, $p=0.05$) at termination. There were no other differences in body mass between the groups at termination (all $p>0.05$).

Systolic blood pressure at termination was similar to systolic blood pressure at baseline in all groups (all $p>0.05$, Figure 4.1B). Diastolic blood pressure did not differ at termination compared to baseline in any of the groups (all $p>0.05$, Figure 4.1B). There were no differences in systolic or diastolic blood pressure at baseline or at termination between the groups (all $p>0.05$).

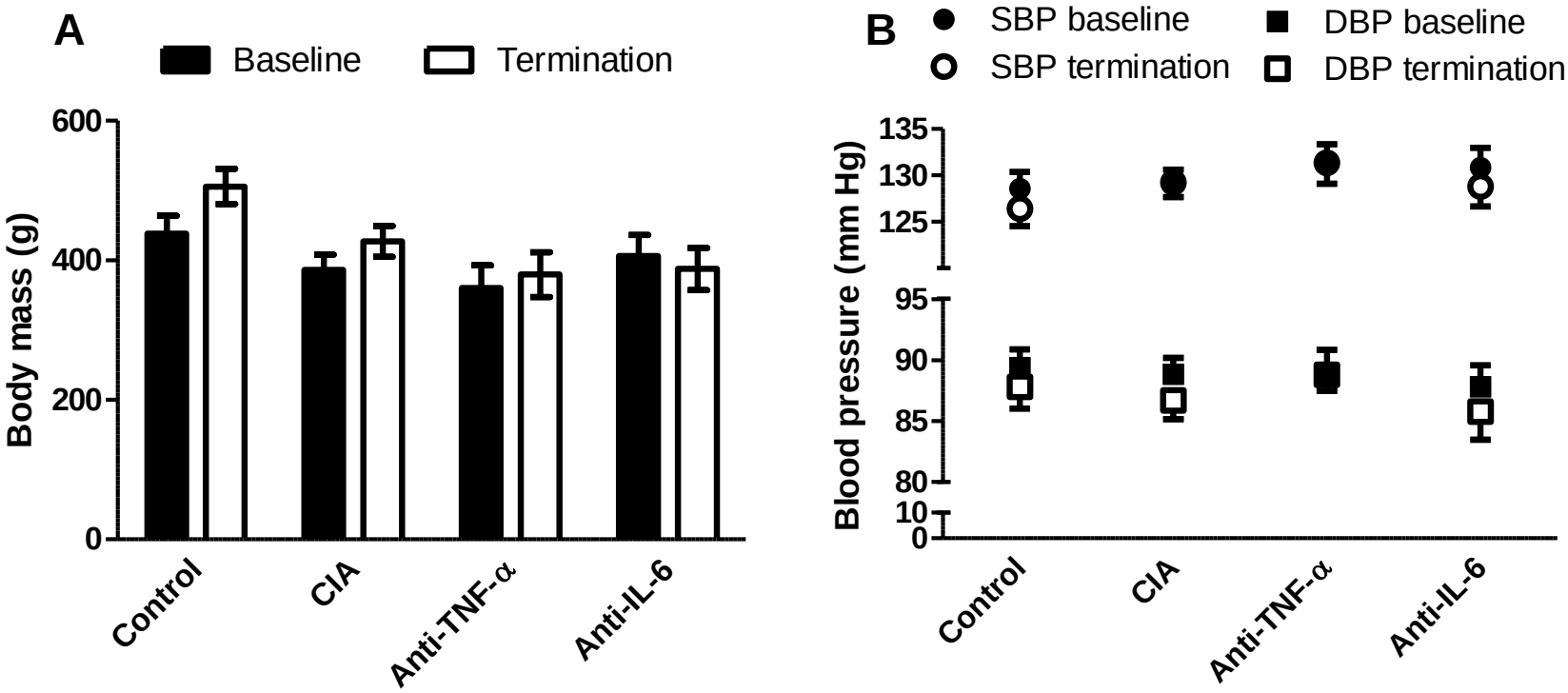

Figure 4.1. Body weight (A) and blood pressure (B) at baseline and at termination in control, CIA, anti-TNF-α and anti-IL-6 groups. CIA, collagen induced arthritis; SBP, systolic blood pressure; DBP, diastolic blood pressure.esults are presented as means ± SEM. Group differences were determined by repeated measures ANOVA followed by a Tukey post-hoc test.

4.2 Arthritis scores, circulating inflammatory marker concentrations and endothelial function marker expressions

Arthirits scores changes significantly over time in the groups exposed to the arthritis protocol (χ^2 = 41.1, DF = 3, p<0.0001). Arthritis scores in the hind paws of the control group was zero at baseline and did not change over the course of the study compared to baseline (Table 4.1). The median arthritis scores in the CIA, anti-TNF-α and anti-IL-6 groups were significantly higher at termination compared to baseline (all p<0.0001) and compared to the control group (all p<0.0001) (Table 4.1).

The circulating CRP concentrations were higher in all groups exposed to inflammation compared to the control group (F = 26.5, DF = 3, p < 0.0001). The average (±SEM) CRP concentrations in the CIA (0.57±0.04 ng/ml, p<0.0001), anti-TNF-α (0.41±0.05 ng/ml, p=0.001) and anti-IL-6 groups (0.65±0.05 ng/ml, p<0.0001) compared to the control group (0.13±0.04 ng/ml) (4.2B). The CRP concentration in the anti-TNF-α group was lower compared ot he anti-IL-6 group (p = 0.009) and there was a trend towards a lower mean value compared to the CIA group (p = 0.08). The average (±SEM) circulating TNF-α concentrations were higher in the CIA (181.8±8.4 pg/ml, p<0.0001), anti-TNF-α (143.5±11.9 pg/ml, p=0.02) and anti-IL-6 groups (162.8±11.5 pg/ml, p=0.0003) compared to the control group (97.2±9.8 pg/ml) (F = 14.8, DF = 3, p < 0.0001; Table 4.1). There was a trend towards a lowerd circulating TNF-α concentrations in the anti-TNF-α group compared to the CIA group (p=0.051). The average (±SEM) serum IL-6 concentrations were higher in the CIA (31.6±1.9 pg/ml, p<0.0001), anti-TNF-α (26.8±2.7 pg/ml, p<0.0001) and anti-IL-6 groups (30.8±2.6 pg/ml, p<0.0001) compared to the control group (16.9±2.2 pg/ml) (F = 9.8, DF = 3, p < 0.0001; Table 4.1). There were no other differences in the circulating inflammatory marker concentrations between the CIA, anti-TNF-α and anti-IL-6 groups (all p>0.05).

The relative mRNA expression of VCAM-1 were different between the groups (F = 5.73, DF = 3; p = 0.001). The mean (±SEM) relative mRNA expression of VCAM1 was significantly higher in the CIA group (1.48 ± 0.48; p = 0.008) and the anti-IL-6 group (1.61 ± 0.66; p = 0.01), but not the anti-TNF-α group (1.18± 0.09; p = 0.97), compared to the

control group (1.15 ± 0.55). There was a trend towards a lower VCAM-1 expression in the anti-TNF-α group compared to the CIA ($p = 0.08$) and anti-IL-6 ($p = 0.07$) groups.

The relative mRNA expression of PTX-3 were significantly different between the groups ($\chi^2 = 15.2$, DF = 3, $p = 0.002$). The median (IQR) relative mRNA expression of PTX-3 was significantly higher in the CIA (3.03 (1.72 – 4.53); $p = 0.04$) and anti-IL-6 (2.87 (0.84-3.99); $p = 0.01$) groups, but not the anti-TNF-α group (1.69 (1.36-1.80); $p = 0.54$) compared to controls (0.85 (0.36-1.22)). The PTX-3 expression was higher in the CIA group compared to the anti-TNF-α group ($p = 0.02$).

Table 4.1. Arthritis scores, circulating inflammatory marker concentrations and expression of endothelial function markers in control inflammation, anti-TNF-α and anti-IL-6 groups

	Control Mean ± SEM/ Median (IQR)	CIA Mean (±SEM) / Median (IQR)	Anti-TNF-α Mean (±SEM) / Median (IQR)	Anti-IL-6 Mean (±SEM) / Median (IQR)
n	20	27	13	11
<u>Paw arthritis score</u>				
Baseline	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)
Termination	0 (0 – 0)	3 (2 – 6)*	3 (2 – 5)*	3 (2 – 8)*
<u>Cirulcating inflammatory marker concentrations</u>				
CRP (ng /ml)	0.13 ± 0.04	0.57 ± 0.04*	0.41 ± 0.05*	0.65 ± 0.05*†
TNF-α (pg/ml)	97.2 ±9.8	181.8 ± 8.4*	143.5 ± 11.9*	162.8 ± 11.5*
IL-6 (pg/ml)	16.9 ± 2.2	31.6 ± 1.9*	26.8 ± 2.7*	30.8 ± 2.6*
<u>Endothelial function marker relative mRNA expression</u>				
VCAM-1	1.15 ± 0.55	1.48 ± 0.48*	1.18± 0.09	1.61 ± 0.66*
Pentraxin 3	0.85 (0.36 - 1.22)	3.03 (1.72 – 4.53)* †	1.69 (1.36 - 1.80)	2.87 (0.84 - 3.99)*

CIA, Collagen induced arthritis *p<0.05 versus control; †p<0.01 versus anti-TNF-α. Group differences for arthiritis scores were determined by Friedman test for repeated measures. Group differences at termination were determine by an ANOVA followed by Tukey *post* hoc test for normally distributed variables and by Kruskal-Wallis test followed by a Dunn's *post hoc* test for non-normally distributed variables.

4.3 Relative miRNA expression

The relative expression of miR146a-5p were different between the gropus (χ^2 =11.2, DF = 3, p = 0.01). The relative expression of miR146a-5p were significantly higher in the CIA group (median (IQR): 1.5(1.0-2.7)) compared to the anti-TNF-α group (median (IQR): 0.5(0.4-0.8); p=0.05). Although there was a trend towards a higher expression of miR146a-5p in the CIA group (median (IQR): 1.5(1.0-2.7)) compared to the control group (median (IQR): 1.3(0.8-1.5); p=0.09), this difference did not reach statistical significance. There were no differences in the relative expression of miR146a-5p between the control and anti-TNF-α groups (p=0.35). The relative expression of miR155-5p were different between the gropus (χ^2 =7.1, DF = 3, p = 0.048). The relative expression of miR155-5p was significantly higher in the CIA group (median (IQR): 4.5(1.9-9.0)) compared to the control group (median (IQR): 1.9(0.3-4.2); p=0.05). There was a trend towards a lower expression of miR155-5p in the anti-TNF-α (median (IQR): 2.2(0.4-2.8); p=0.1) group compared to the CIA group. There were no differences between the relative expression of miR155-5p between the control and anti-TNF-α groups (p=0.98).

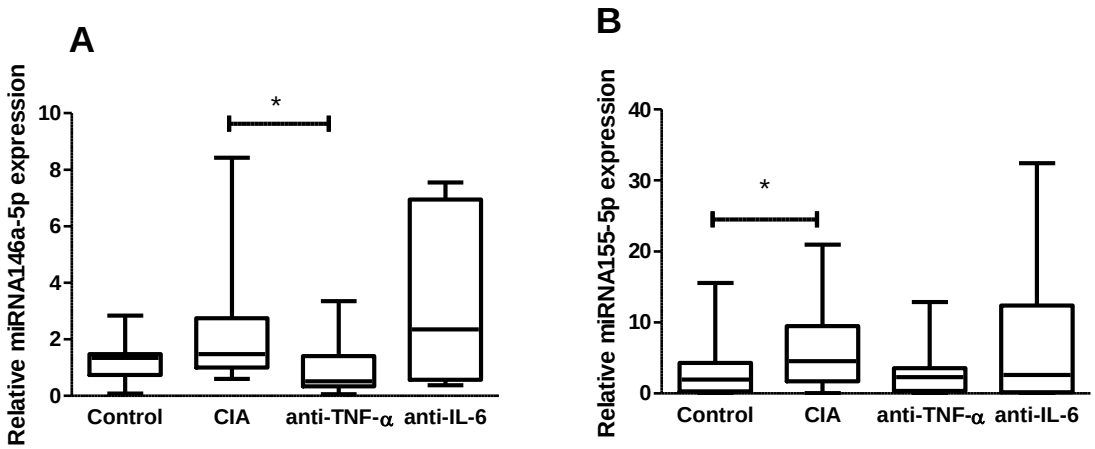

Figure 4.2. Relative expression of miR146a-5p (A) and miR155-5p (B) in the control CIA, anti-TNF-α and anti-IL-6 groups. Data are presented as median (IQR). *p<0.05. Group differences were determine by a Kruskal-Wallis test followed by a Dunn's *post hoc* test.

4.4 Association between circulating inflammatory markers and relative miRNA expression

There was a significant association between the relative expression of miR146a-5p and circulating CRP (r=0.31, p=0.04) and circulating TNF-α (r=0.33, p=0.03) concentrations in the control and inflammation groups only (Figure 4.3, top left panel). When including the drug treatment groups in the model, the association between miR146a-5p and circulating CRP (r=0.32, p=0.007) and circulating TNF-α (r=0.30, p=0.02) concentrations remained significant (Figure 4.3, bottom left panel). The relative expression of miR155-5p was associated with circulating CRP concentrations (r=0.39, p=0.009) in the control and inflammation groups (top right panel), but it was no longer significant when including all groups in the model (r=0.21, p=0.08) (Figure 4.3, bottom right panel). Circulating IL-6 and TNF-α concentrations were not associated with relative expression of miR155-5p in the control and inflammation groups or in all groups (all p>0.05) (Figure 4.4, right panel).

4.5 Association between miRNA expression and vascular function in high-grade inflammation

There was a significant association between miR146a-5p and miR155-5p in the control and inflammation group (r=0.35, p=0.02) and in all groups (r=0.44, p=0.0003; Figure 4.5). There was no association between the relative expression of miR146a-5p and blood pressure or any marker of large artery function (all p>0.05) (Table 4.2). A higher relative expression of miR146a-5p was associated with increased sensitivity of the mesenteric arteries to acetylcholine (r=0.39, p=0.04) in the control and inflammation groups (Table 4.2). However, when all groups were included in the analysis, this association was no longer significant (r=-0.09, p=0.52). In the control and inflammation groups alone, there was a trend towards a significant association between relative expression of miR146a-5p and relative mRNA expression of PTX-3 (r=-0.30, p=0.09). When all groups were included in the model, higher relative expression of miR146a-5p was associated with higher relative mRNA expression of PTX-3 (r=0.35, p=0.008) and there was a trend towards a significant relationship with VCAM-1 (r=0.26, p=0.05).

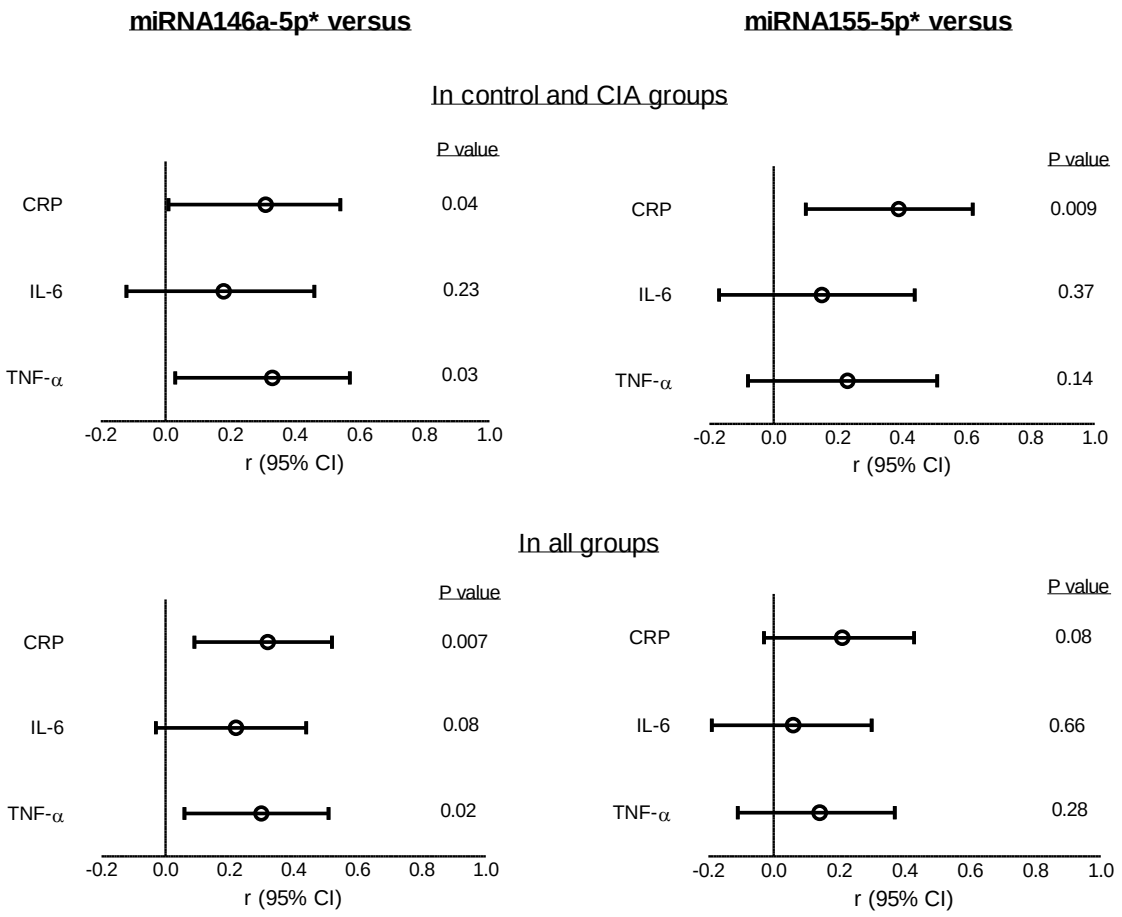

Figure 4.3. Associations between circulating inflammatory markers and miR146a-5p (left panel) and miR155-5p (right panel) in control and inflammation groups (n=44) (top) and in all groups (n=67) (bottom). Associations were determine using Pearson's correlations. Open circles represent correlation coefficients and error bars represent 95% confidence intervals (CI).*logarithmically transformed variables.

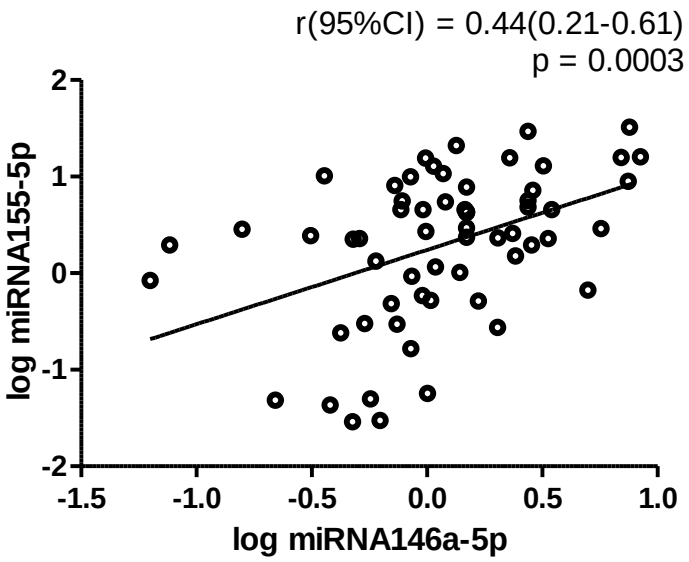

Figure 4.4. Association between miR146a-5p and miR155-5p (n=67). Association was determined by Pearson's correlation.

The relative expression of miR155-5p was not associated with systolic (r=0.17, p=0.29) or diastolic (r=0.26, p=0.09) blood pressure. When only the control and inflammation groups were included in the analysis, higher relative expression of miR155-5p was associated with higher augmentation pressure (r=0.48, p=0.01) and augmentation index (r=0.47, p=0.01), but not when all groups were included in the model (both p=0.24). Higher relative expression of miR155-5p was associated with increased sensitivity of the renal arteries to acetylcholine (r=0.58, p=0.005), and with a reduced maximal acetylcholine induced relaxation response (r=-0.53, p=0.01). In all groups the association between the relative expression of miR155-5p and renal artery sensitivity (r=0.25, p=0.13) and maximal relaxation (r=-0.28, p=0.09) responses did not differ. The relative expression of miR155-5p was significantly associated with the relative mRNA expression of PTX-3 in the control and inflammation groups (r=0.46, p=0.007) and remained significant when all groups were included in the model (r=0.31, p=0.01). There were no relations between the relative expression of miR155-5p and the relative VCAM-1 mRNA expression in either the control and inflammation groups alone (r=0.19, p=0.26) or in all groups (r=0.20, p=0.12).

Table 4.2. Associations between miR146a-5p and miR155-5p and markers of vascular function in the control and inflammation groups alone (n=44) and in all groups (n=67)

	miR146a-5p*				miR155-5p*			
	Control & inflammation		All groups		Control & inflammation		All groups	
	r(95%CI)	p	r(95%CI)	P	r(95%CI)	p	r(95%CI)	p
Systolic BP	0.21(-0.10-0.47)	0.18	0.002(-0.24-0.24)	0.98	0.17(-0.15-0.45)	0.29	-0.05(-0.29-0.19)	0.68
Diastolic BP	-0.08(-0.37-0.22)	0.60	0.003(-0.24-0.24)	0.98	0.26(-0.05-0.53)	0.09	0.20(-0.05-0.42)	0.11
Pulse wave velocity	0.16(-0.23-0.50)	0.42	0.06(-0.23-0.34)	0.70	0.31(-0.08-0.62)	0.11	0.23(-0.06-0.48)	0.12
Augmentation pressure	0.09(-0.29-0.45)	0.63	0.12(-0.17-0.39)	0.42	**0.48(0.11-0.73)**	**0.01**	0.17(-0.12-0.44)	0.24
Augmentation index	0.02(-0.36-0.39)	0.93	0.08(-0.20-0.36)	0.58	**0.47(0.10-0.73)**	**0.01**	0.17(-0.12-0.44)	0.24
Forward wave pressure	0.22(-0.17-0.55)	0.25	0.09(-0.20-0.36)	0.56	-0.18(-0.53-0.23)	0.38	-0.16(-0.43-0.13)	0.27
Backward wave pressure	-0.06(-0.42-0.32)	0.76	0.10(-0.19-0.37)	0.49	-0.31(-0.62-0.09)	0.11	-0.12(-0.39-0.17)	0.39
Mesenteric Ach sensitivity*	**0.39(0.01-0.68)**	**0.04**	-0.09(-0.37-0.19)	0.52	0.24(-0.22-0.61)	0.29	0.10(-0.19-0.38)	0.49
Mesenteric Ach relaxation	0.01(-0.38-0.39)	0.95	-0.09(-0.37-0.19)	0.51	0.38(-0.05-0.70)	0.08	0.01(-0.28-0.29)	0.96
Renal Ach sensitivity*	0.18(-0.28-0.58)	0.44	-0.02(-0.36-0.32)	0.89	**0.58(0.19-0.81)**	**0.005**	0.25(-0.09-0.54)	0.13
Renal Ach relaxation	-0.28(-0.64-0.19)	0.23	-0.01(-0.35-0.33)	0.96	**-0.53(-0.79--0.12)**	**0.01**	-0.28(-0.56-0.06)	0.09
Mesenteric Phe sensitivity*	0.03(-0.33-0.38)	0.87	0.02(-0.27-0.31)	0.87	0.17(-0.22-0.51)	0.38	0.09(-0.21-0.37)	0.56
Mesenteric Phe contractility	0.10(-0.25-0.43)	0.57	0.05(-0.23-0.33)	0.71	0.21(-0.17-0.53)	0.27	0.11(-0.18-0.38)	0.46
Renal Phe sensitivity*	0.01(-0.34-0.35)	0.98	-0.005(-0.29-0.28)	0.98	-0.25(-0.56-0.13)	0.19	-0.20(-0.47-0.09)	0.17
Renal Phe contractility	-0.05(-0.39-0.30)	0.78	-0.10(-0.38-0.19)	0.50	0.14(-0.24-0.48)	0.47	0.02(-0.28-0.31)	0.92
VCAM-1 expression	0.22(-0.13-0.52)	0.21	**0.26(0.004-0.48)**	**0.05**	0.19(-0.15-0.49)	0.26	0.20(-0.06-0.44)	0.12
PTX-3 expression[#]	0.38(0.03-0.64)	0.03	**0.36(0.10-0.57)**	**0.007**	**0.46(0.13-0.69)**	**0.007**	**0.31(0.07-0.54)**	**0.01**

Associations were determined by Pearson's correlations. *logarithmically transformed variables; [#]square rooted variable.

CI, confidence interval; BP, blood pressure; Ach, acetylcholine; Phe, phenylephrine, VCAM-1, vascular adhesion molecule 1. PTX-3 pentraxin 3.

CHAPTER 5
DISCUSSION

Chronic inflammation in auto-immune diseases, such as RA, impact endothelial function, which contribute to an increased risk of CVD (Chadderdon *et al*., 2014; Willerson & Ridker., 2004). Several studies have been conducted to elucidate the mechanisms whereby inflammation causes endothelial and vascular dysfunction. In this regard, various biomarkers of endothelial dysfunction have been identified. While some biomarkers are well characterised and used clinically, there has been a recent interest in the gene component of the inflammatory response, and in particular the regulation and expression of miRNAs that affect the endothelial lining. In the present study, we investigated whether biologic disease modifying drugs (TNF-α antagonists and IL-6 antagonists) affected the gene regulation (miRNA expression) of inflammation-induced endothelial dysfunction. We also determined whether the expression of miRNAs were associated with inflammation-induced vascular and cardiac dysfunction.

The main findings of the current study were that exposure to high-grade inflammation was associated with an increased expression of miR146a-5p and miR155-5p, and that treatment with biologic DMARDs impacted the inflammation-induced expression of these miRNAs. This was particularly apparent in the anti-TNF-α treatment group, as both miR146a-5p and miR155-5p expressions were similar to the control group and miR155-5p expression was significantly lower in the anti-TNF-α treatment group compared to the CIA group. Both miR146a-5p and miR155-5p were associated with endothelial function markers, as measured by PTX-3, independent of DMARD treatment. Increased expression of miR155-5p was associated with markers of endothelial, vascular and cardiac function; however, treatment with biologic DMARDs impacted this association.

5.1 High-grade inflammation alters miRNA expression

A high-grade inflammatory state, as measured by increased arthritis scores, was induced in all rats exposed to the experimental procedure, consistent with previous studies using the CIA model (Mokotedi *et al*., 2019). The concentrations of circulating cytokines (CRP, IL-6 and TNF-α) were elevated in the inflammation group, in agreement with previous clinical RA studies (McInnes *et al*., 2016; Dessein *et al*., 2005). Besides the upregulation of traditional markers of inflammation in this CIA model, this study advances our understanding of the genes that are upregulated in high-grade inflammation.

In a typical immune response to a pathogen, as many as 100 genes are upregulated (Barry *et al*., 2017). These genes are carefully regulated to achieve pathogen clearance while at the same time, preventing the consequences of unregulated expression, such as cancer (O'Connell *et al*., 2007). The present study showed that miR155-5p was significantly upregulated in response to exposure to high-grade systemic inflammation. Higher circulating CRP concentrations were associated with increased expression of miR155-5p. Our results are in agreement with previous clinical studies where greater expressions of miR155-5p were observed in RA (Alivernini *et al*., 2018; Kurowska- Long *et al*., 2013; Stolarska *et al*., 2011) and lupus erythematosus patients (Chen *et al*., 2017) compared to the general population. It has been estimated that miR155-5p can target up to 140 genes and modulatory proteins, and therefore it is considered to be a multifunctional miRNA (Xiaoyan *et al*., 2017). MiR155-5p expression is induced in monocytes, macrophages and dendritic cells upon toll like receptor (TLR) or interferon gamma (IFNγ) stimulation and drives their inflammatory response by repressing the production of proteins that are inhibitors of innate cell activation (Alivernini *et al*., 2018). These inhibitors of innate cell activation include 1) Src homology 2 domain-containing inositol-5-phosphatase 1 (SHIP 1), which are inhibitors of the cell survival and growth TLR/Pi3/Akt kinase pathway, 2) suppressor of cytokine signalling 1 (SOCS 1), a type of cytokine receptor and STAT pathway inhibitor and 3) Bcl6, an inhibitor of NF-κβ (Alivernini *et al*., 2018). Based on the suppression of the inhibitors of innate cell activation, miR155-5p is considered a key modulator of the immune response, and excessive expression could contribute to the induction and maintenance of chronic inflammation, autoimmunity and fibrosis (Alivernini *et al*., 2018). MiR155-5p therefore enhances the intensity of the inflammatory response, and may induce negative effects in inflammatory conditions such as RA.

In the current study, although not significant, there was a trend towards a higher relative expression of miR146a-5p in the inflammation group compared to the control group. The expression of miR146a-5p was associated with increased circulating CRP and TNF-α concentrations, with similar trends observed for circulating IL-6 concentrations. Previously it has been suggested that miR146a-5p is a biomarker of systemic inflammation (Cunha *et al*., 2016). Similar to our results, others have reported increased

expression of miR146a-5p in RA (Li *et al*., 2014; Pauley *et al*., 2008) and other inflammatory conditions (Wang *et al*., 2012). Proinflammatory cytokines induce the transcription of miR146a-5p via TLR binding (Fabbri *et al*., 2013). Increased miR146a-5p expression targets multiple receptor adaptors, including TNF associated receptor 6 (TRAF6) and interleukin-1 receptor associated kinase (IRAK-1), to suppress excessive NF-κβ signalling (Taganov *et al*., 2006). Although miR146a-5p is upregulated in states of chronic inflammation, some have suggested that it acts as a negative regulator of inflammation, whereby it inhibits NF-κβ activity, and thereby attenuates inflammatory cytokine production (Feng *et al*., 2017; Gao *et al*., 2015). This suggests that miR146a-5p may be involved in a novel mechanism of negative feedback regulation of TLR and cytokine receptor signalling.

In this regard, it has been proposed that the presence of miR146a-5p indicates the presence of systemic inflammation, but that gene regulation is aimed at repression of the unrestrained inflammatory response. However, studies conducted on miR146a-5p associated signalling have reported controversial results. Although some studies have confirmed miR146a-5p signalling to function via this negative feedback loop (Roos *et al*., 2016; Ramkaran *et al*., 2014), other studies have not been able to demonstrate similar miR164a-5p negative feedback signalling (Alipour *et al*., 2013; Zilahi *et al*., 2012). Additionally, defective NF-κβ-miR146a-5p negative feedback loops have been reported in low grade inflammatory conditions such as T2DM, where despite increased miR146a-5p expression, TRAF6 concentrations were decreased and NF-κβ signalling remained increased (Yousefzadeh *et al*., 2015). One study also found age related dysregulation in miR146a-5p negative feedback regulation (Jiang *et al*., 2012). Our results and these previous findings suggest that miR146a-5p signalling has not been fully characterised and that further research is required.

5.2 The effect of biologic DMARD therapy on miRNA expression

The relative expression of miR146a-5p in the anti-TNF-α group was significantly lower compared to the inflammation group, with similar trends observed for miR155-5p expression. Although research investigating the effect of biologic DMARDs on miRNA expression is limited, one previous study reported increased miR146a-5p expression

following TNF-α blocker treatment (Castro-Villegas *et al*., 2015). These contrasting findings could possibly be explained by several methodological differences between the studies. The study by Castro-Villegas and colleagues (2015) included patients that previously did not respond to synthetic DMARD therapy, and some of the included patients were on combination therapies. In the current study, rats were exposed to the inflammation for only 10 weeks and to the drugs for six weeks. It is possible that reciprocal regulation between the inflammatory cytokines and miR146a-5p is dependent on RA disease duration and severity. The greater disease activity and longer duration of RA in the previous compared to the current study may indicate that severe dysregulation of miRNAs might have been present prior to their study. Furthermore, using biologic and synthetic DAMRDs in combination may have confounding effects on miRNA expression.

Moreover, the previous study reported the expression of individual miRNAs relative to their baseline (fold change), which further limits the comparability between the studies (Castro-Villegas *et al*., 2015). We did not measure miRNA expression at baseline or at the onset of inflammation prior to treatment, and therefore the miRNA expression observed in our study may have been measured at a different time point within the immune response relative to resolution of the inflammation.

While Castro-Villegas (2015) argued that the upregulation of miR146a-5p resulted in the significant suppression of the target genes (inflammatory cytokines), it must be considered that these factors reciprocally regulate each other and thus the opposite may also be true. While inflammatory stimuli increase the expression of these miRNAs, TNF-α blocker treatment is aimed specifically at inhibiting the binding of circulating TNF-α to its receptor, and therefore blocks the activation of the inflammatory signalling cascade induced by TNF-α (Reddy *et al*., 2016). In this regard, presently, two types of TNF-α receptors (TNFR) have been identified. First, the membrane bound TNFR is located on the membrane of cells and upon binding of TNF-α, it responds in a pro-inflammatory manner by releasing other cytokines and inflammatory mediators. Second, the soluble TNFR binds circulating TNF-α, which effectively deactivates TNF-α and blunts the immune response. Enbrel, the anti-TNF-α treatment used in the current study, is a soluble TNF-α receptor. Enbrel is a dimeric fusion protein, consisting of an extracellular ligand

portion (Klimiuk *et al*., 2009) of the TNF-α receptor, which is combined with a portion of IgG (Frenzel *et al*., 2016). Enbrel binds competitively to circulating TNF-α, thereby mimicking the effect of the naturally occurring soluble TNFR and inhibits binding of circulating TNF-α to the membrane bound TNFR (Reddy *et al*., 2016). Therefore, Enbrel competitively inhibits cellular signalling elicited by TNF-α, and effectively modulates the inflammatory response. Importantly, an etanerceptt/TNF- α complex is formed that not only inactivates TNF- α, but also prolongs its half life (Goldenberg, 1999; Moreland *et al.,* 1999). Receptor inhibition is therefore commonly accompanied by increased circulating concentrations of the cognate ligand (Mann *et al.,* 2008). Therefore, TNF- α inhibition could have decreased the expression of miR146a-5p, despite no decreases in circulating TNF- α concentrations (as measured by ELISA). Indeed, in the current study we showed a reduced expression of miR146a-5p in the anti-TNF-α group despite a high concentration of circulating TNF-α.

Although studies investigating the effect of TNF-α blocker therapies on miRNA expression are currently scarce, several studies have shown improvements in endothelial and vascular function in response to TNF-α blocker therapy. Previous studies showed that TNF-α blocker therapy reduced the concentration of circulating ICAM-1 and E-selectin (Klimiuk *et al*., 2009; Sidiropoulos *et al*., 2009; Komai *et al*., 2007). Others showed a transient improvement in endothelial function, as measured by flow mediated dilatation and brachial ultrasonography (Gonzalez-Juanatey *et al*., 2004; Irace *et al*., 2004). Two studies, conducted in arthritic populations, also demonstrated decreases in ADMA concentrations following TNF-α blocker therapy (Spinelli *et al*., 2014; Angel *et al*., 2012). Improvements in large artery stiffness after TNF-α blocker treatment were also previously reported in the literature (Dulai *et al*., 2012; Tam *et al*., 2012; Galarraga *et al*., 2009). In these studies, pulse wave velocity and augmentation index were used to measure arterial stiffness. Therefore, in RA, TNF-α blocker therapy may impart positive effects on the endothelium and vasculature. Hence in the current study, the reduction in miR146a-5p expression, a well-known gene regulator of inflammation-induced endothelial dysfunction, in response to TNF-α blocker therapy, supports the beneficial effects of these drugs on the vasculature in high-grade inflammatory conditions.

In the current study, IL-6 blocker therapy, in contrast, did not ameliorate the expression of either miR146a-5p or miR155-5p. In this regard, some animals seemed to respond well to IL-6 blockers while others were adversely affected, as seen by the large spread in the miRNA expression data for the anti-IL-6 group. Indeed, not all patients with RA respond equally well to Tocilizumab treatment (Koehm *et al*., 2020). Nevertheless, to our knowledge there are currently no studies that have investigated the effect of IL-6 blocker therapy on miRNA expression. However, some studies have reported effects of IL-6 blocker therapy on vascular function in RA. In this regard, there seem to be controversy regarding the effects of IL-6 blockers on markers of vascular and endothelial function (Ursini *et al*., 2017). Hence the same may be true for the effect of IL-6 blocker treatment on miRNA expression.

The lack of consistent findings in response to IL-6 blocker treatment may be as a result of either the efficacy of the drug treatment, or the drug mechanism of action. In this regard, one previous study in rat adjuvant induced arthritis that showed slowed disease progression after treatment, injected rats with Tocilizumab intra-planar three times a week (Abdel-Maged *et al*., 2018). Although the dosage of IL-6 blocker therapy in the current study was given according to previously reported literature, it may be that the frequency of the injections was not high enough given the increased metabolic rates in rats compared to humans.

With regards to the drug mechanism of action, Tocilizumab is a monoclonal antibody targeted against IL-6 and is effective in ameliorating excessive inflammation associated with IL-6 overproduction (Liu *et al*., 2016). The human body contains a membrane bound IL-6 receptor (IL-6R) and a soluble IL-6 receptor (sIL-6R). Soluble IL-6R are produced either through alternative mRNA splicing (Muller-newen *et al*., 1996), or through proteolytic cleavage of membrane bound IL-6R (shedding) (Müllberg *et al*., 1995). In contrast to that of the TNFR, binding of IL-6 to its soluble receptor also elicits a pro-inflammatory response (Peters *et al*., 1996), and in fact, notably widens the spectrum of cells responsive to IL-6 (Tamura *et al*., 2018). Tocilizumab functions by binding competitively to the IL-6 binding site of both the membrane bound IL-6 receptor and the soluble IL-6 receptor (Mihara *et al*., 2012). It has been reported that sIL-6 is significantly

upregulated following Tocilizumab therapy, suggesting that IL-6 signalling does change following Tocilizumab treatment (Levi *et al*., 2013). It is widely accepted that sIL-6R signalling exerts pro-inflammatory effects whereas membrane bound IL-6R signalling exerts anti-inflammatory effects and is required for regeneration (Rose-John, 2012). The specific effects of Tocilizumab on these signalling pathways require further investigation.

It must also be mentioned that IL-6 signalling is made more complex by the presence of various alternative signalling pathways through the other members of the IL-6 cytokine family. Besides IL-6, this cytokine family consists of interleukin 11 (IL-11), leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and novel neurotrophin-1/B cell stimulatory factor -3 (NNT-1/BSF-3) (Senaldi *et al*., 1999; Pennica *et al*., 1995; Paul *et al*., 1990; Stöckli *et al*., 1989; Malik *et al*., 1989; Gearing *et al*., 1987). A characteristic feature of the IL-6 related cytokines is that members within this family exert similar and overlapping effects on given tissues and that one cytokine can promote a wide variety of biological functions on various tissues and cells (Kishimoto *et al*., 1995). Therefore, despite blocking IL-6, alternative pro-inflammatory signalling may still be possible through upregulation of the other IL-6 family members. In this regard, studies investigating the effect of IL-6 blockers on endothelial and vascular function have shown inconsistent results. While some smaller studies reported improvements in endothelial or vascular function (Bacchiega *et al*., 2017; Kume *et al*., 2011; Protogerou *et al*., 2011;), one large (n=132) placebo controlled study showed no improvements in vascular function (McInnes *et al*., 2015). Further to this, a large population-based study reported that up to 22% of patients did not respond to Tocilizumab (Levi *et al*., 2013). This underscores the possibility that various alternative signalling pathways may be probable. Taken together, these DMARDs are expected to significantly impact the association between inflammation and miRNA expression, based on their mechanisms of action. Indeed, our results showed that miR155-5p was correlated to increased circulating CRP concentrations in control and inflammation groups only, but when the drug treated groups were included in the analysis, the association was no longer significant.

However, based on the above mechanisms of DMARD action, it must also be mentioned that, following administration of the biologic DMARDs, the concentration of circulating cytokines may no longer be an accurate measure of inflammation. Although these cytokines might be present in circulation, and will be detected by ELISAs, they are likely to no longer be biologically active, as they cannot bind to their receptors to elicit the intended response. Although some studies in RA have reported reduced circulating cytokine concentrations following biologic DMARD treatments, others reported no change in circulating inflammatory markers (Levi *et al*., 2013;Edwards *et al*., 2012; Moutsopoulos *et al*., 2008; Mercado *et al*., 1999) . Therefore, the association between these circulating cytokines and the expression of miRNAs may be impacted by biologic DMARDs. Nevertheless, studies elucidating the effect of biologic DMARDs on miRNA expression and the mechanisms underlying these effects are warranted.

5.3 MiRNAs as a risk marker for endothelial and arterial dysfunction in high-grade inflammation

5.3.1 MiR146a-5p as a marker of vascular function

Upregulation of miR146-5p in RA is thought to occur as a compensatory mechanism to repress inflammation-induced endothelial activation and dysfunction (Cheng *et al*., 2013; Yin *et al*., 2016). Through the inhibition of intermediary adaptor molecules, miR146a-5p suppresses NFκβ signalling and reduces expression of adhesion molecules (Yin *et al*., 2016; Cheng *et al*., 2013). In this regard, the current study showed that miR146a-5p was associated with mesenteric artery sensitivity to vasodilators (acetylcholine) in the control and inflammatory groups. Previous studies conducted by our group showed inflammation to significantly reduce the vasodilation response of mesenteric arteries to acetylcholine (Mokotedi *et al*., 2019). Other animal studies, using high-grade inflammatory models, have reported similar reductions in endothelial function (Dooley *et al*., 2015; He *et al*., 2013). Importantly, these changes in endothelial function occurred despite unchanged blood pressure (Mokotedi *et al*., 2019).

In the current study, despite associations with endothelial function markers, miR146a-5p was not associated with blood pressure or arterial function markers, including

augmentation pressure and pulse wave velocity. Although there are limited studies investigating the association between miR146a-5p and blood pressure or arterial function, one previous study reported increased miR146a-5p expression in inflamed plaques of peripheral arterial disease patients (Aquila *et al*., 2017). Other studies reported miR146a-5p promoted vascular smooth muscle cell proliferation (Xue *et al*., 2019; Cao *et al*., 2015). Nevertheless, a clear association between miR146a-5p and arterial function has not previously been reported. Hence, miR146a-5p appears to be more closely related to endothelial function, than vascular function. This is in line with various clinical studies where inflammation-induced endothelial dysfunction precedes arterial dysfunction (Soltész *et al*., 2009; Doshi *et al*., 2001). Although inflammation has been associated with arterial function changes in human studies (Ambrosino *et al*., 2015), these associations are more commonly seen following longer RA disease durations (Wållberg-Jonsson *et al*., 2008). Hence the short duration of the inflammatory protocol in the current study may have precluded associations between miR146a-5p and arterial function. Alternatively, it is possible that miR146a-5p may not be a sufficient biomarker of arterial dysfunction in high-grade inflammation.

With regards to the impact of biologic DMARDs, despite associations between miR146a-5p and endothelial dependent relaxation in the control and inflammation groups, upon inclusion of biologic DMARD treatment groups, these associations were no longer significant. Interestingly, when including DMARD treatment groups in the correlations, miR146a-5p was related to vascular adhesion molecules, as measured by VCAM-1 and endothelial integrity, as measured by PTX-3. In this regard, it is well known that miR146a-5p, VCAM-1 and PTX-3 are increased in RA (Li *et al*., 2014; Pauley *et al*., 2008; Luchetti *et al*., 2000; Littler *et al*., 1997). In a previous rat study using CIA, circulating VCAM-1 concentrations were significantly higher in the group inoculated with collagen compared to controls (Mokotedi *et al*., 2019). Biologic DMARD therapy has been shown to reduce inflammation and disease activity (Curtis & Singh, 2011). This reduces inflammation-induced miR146a-5p expression, whereby miR146a-5p is significantly downregulated following TNF-α blocker treatment. DMARD therapy also reduces the expression of circulating vascular adhesion molecules (Klimiuk *et al*., 2009; Sidiropoulos *et al*., 2009; Komai *et al*., 2007). Therefore, the association between miR146a-5p and vascular

function markers that are frequently affected in high-grade inflammation is in line with previous evidence.

Nevertheless, these findings provide further evidence to support our previous suggestion that biologic DMARDs impact miR146a-5p expression and its relationship with inflammation-induced endothelial dysfunction. Further research is required to understand the role of miR146-5p in the regulation of endothelial dysfunction in high-grade inflammatory conditions such as RA, and to elucidate the molecular mechanisms whereby DMARDs affect the regulatory function of miR146a-5p.

5.3.2 *MiR155-5p as a marker of vascular function*

In the current study, miR155-5p was directly related to markers of endothelial dysfunction. In this regard we have shown an association between increased expression of miR155-5p and an increased sensitivity of the renal arteries to vasodilators but a decreased maximal vasodilatory response. Acetylcholine is an endothelium dependent vasodilator and therefore our results suggest that miR155-5p expression is associated with impaired endothelium dependent relaxation.

Interestingly, high concentrations of NO as seen in inflammatory conditions, upregulates the expression of miR155-5p (Yuhas *et al*., 2014). Additionally, miR155-5p reportedly promoted NO production in LPS-stimulated mouse microglial N9 cells (Cardoso *et al*., 2012). These findings suggest the presence of a positive feedback loop between high concentrations of NO and miR155-5p that accelerates inflammatory reactions and impairs vascular function. It must be noted that basal concentrations of NO inhibits miR155-5p, resulting in a negative feedback mechanism (Sun *et al*., 2012). These findings are consistent with the suggestion that basal concentrations of NO are protective, whereas high concentrations of iNOS impairs vascular integrity, increases ROS and damages the endothelium (Yuhas *et al*., 2014; Clancy *et al*., 1998).

The association between miR155-5p and vascular reactivity is further supported by the association between miR155-5p and increased PTX-3 expression in the current study. In this regard the mechanism of PTX-3 induction is similar to miR155-5p, where it is produced directly by endothelial cells, smooth muscle cells, macrophages, neutrophils

and dendritic cells, in response to TLR agonists TNF-α, IL-1β, and other inflammatory mediators (Garlanda *et al*., 2005). MiR155-5p would therefore be expected to parallel PTX-3 activation. Our results confirm previous findings that the expression of miR155-5p and PTX-3 are closely linked (Sun et al., 2015). Further investigations should elucidate the mechanisms whereby these biomarkers are associated.

Despite these positive associations with PTX-3, the literature reports contrasting findings regarding miR155-5p expression and endothelial function. MiR155-5p expression has been associated with various cellular pathways and mechanisms involved in vascular endothelium activation and dysfunction, mainly by promoting monocyte recruitment to the vascular wall (Syed *et al*., 2015; Nazari-Jahantigh *et al*., 2012). One study that used a miR155-5p antagonist showed improvements in endothelial function markers and reduced local production of inflammatory cytokines (Kurowska-Stolarska *et al*., 2011). In contrast, other studies have suggested that miR155-5p represses expression of endothelial markers and impairs monocyte recruitment to the vascular wall. In Angiotensin II stimulated human umbilical vein endothelial cells (HUVECs) increased miR155-5p expression reversed the upregulation of Ets-1 genes, namely VCAM-1, and MCP-1 (Zhu *et al*., 2011; Zhan *et al*., 2005). An important consideration is whether these *in vitro* results can be extrapolated to human or animal studies, where the genes of interest may be under the control of various regulators. Indeed, the regulation of miRNAs are complex, and the cellular pathways relating to endothelial function are under the control of several epigenetic modulators (Lee & Chiu, 2019; Khullar *et al*., 2017).

Furthermore, although overexpression of miR155-5p resulted in a reduction in ICAM-1 and VCAM-1 expressions in two non-inflammatory cell culture studies, these results were blunted following cytokine stimulation (Cerutti *et al*., 2016; Wu *et al*., 2014). In fact, VCAM-1 concentrations did not change in response to upregulated miR155-5p expression, following TNF-α stimulation in one of these studies (Wu *et al*., 2014). This suggests that miR155-5p may participate in either a positive or negative feedback manner, as explained for NO signalling. It appears that miR155-5p has a pleiotropic role in controlling NF-κβ signalling and may contribute to endothelial dysfunction under high-grade inflammatory conditions (Cheng *et al*., 2014).

Given the effect of inflammation on miR155-5p expression as explained above, the role of biologic DMARDs on these associations should be considered. In RA, TNF-α blocker therapy has consistently shown improvement in endothelial function (Ursini *et al*., 2017). The results from our study show that TNF-α blocker therapy prevented inflammation-induced upregulation of miRNA 155-5p. and miR155-5p remained associated with endothelial function, as measured by PTX-3, independent of DMARD treatment. TNF-α blocker therapy also prevented inflammation-induced expression of VCAM and significantly reduced PTX-3 expression. This indicates that improvements in endothelial function were likely present following treatment and that miR155-5p may be a candidate biomarker of endothelial function in these treatment populations.

Considering the strong link between endothelial function and the development of large artery dysfunction, we wanted to determine whether miR155-5p is directly related to indicators of large artery function. We found significant associations between miR155-5p and augmentation index and augmentation pressure. However, we found no association between miR155-5p and pulse wave velocity. Similar to our findings, previous studies using pharmacological inhibition or gene deletion of miR155-5p found improved vascular functional recovery following stroke, via improvements in microvascular integrity and reductions in the expressions of inflammatory mediators (Pena-Philippides *et al*., 2016; Caballero-Garrido *et al*., 2015; Wen *et al*., 2015). Although pulse wave velocity is considered the gold standard, non-invasive measure of large artery function (Vlachopoulos *et al*., 2010), a recent study showed that RA disease activity and inflammation is not independently associated with pulse wave velocity (Gunter *et al*., 2017). In contrast, the same study showed that RA disease activity and cumulative inflammatory load is independently associated with wave reflection (Gunter *et al*., 2017). Augmentation index and augmentation pressure are frequently used indicators of wave reflection (Kelly *et al*., 1989).

Although biologic DMARDs did not affect the relationship between miR155-5p and endothelial function, the associations between miR155-5p and arterial function were altered in the treatment groups. Upon the inclusion of biologic DMARD groups, miR155-5p expression was no longer associated with measures of large artery function. This

suggests that the association between miR155-5p and large artery function may not be a direct relationship, but rather indirect via endothelial function. Further research to determine the mechanism whereby these DMARDs alter miR155-5p expression and regulation of large artery function is warranted. Moreover, studies elucidating the mechanisms whereby biologic DMARDs alter gene regulation of endothelial and large artery function are required.

5.4 Limitations

This study has further limitations. Although the CIA model has been deemed the most closely linked to RA in humans and is widely used (Steyers & Miller, 2014; Bolon *et al*., 2011), there may be several differences in mechanisms that we are unable to account for. Hence miRNA signalling in high-grade inflammation may be different in rats. We made use of miR191-5p as our endogenous control. Recent studies have recommended the use of miRNAs as reference genes, instead of SnU6 or RNU6. We acknowledge that there is ongoing discussion in the literature regarding the most appropriate endogenous control in miRNA studies. Nevertheless, our study design utilised a validated duplex reaction approach (Martens-Uzunova *et al*., 2019; Smoczynska *et al*., 2019).

We determined the expression of two miRNAs relative to endothelial function; however, the interpretations of results may have been strengthened if the concentrations of downstream adaptor proteins and signalling mechanisms (eg. TRAF-6, TRIF, MyDD88, SOS-1 and SOCS1) were also measured. The study design may have also impacted the interpretation of our result. Future studies should aim to characterise the inflammatory model by measuring the concentrations of inflammatory markers and miRNAs over time, and thus determining the expression of miRNAs relative to the immune response and the onset of endothelial dysfunction.

In the present study, we determined the relative left ventricular mRNA expressions of VCAM and PTX-3. Future studies should consider measuring circulating measures of these endothelial function markers, so as to provide a more direct comparison with circulating miRNA concentrations.

The current study made use of a humanised TNF-α dimer and a humanised anti-IL-6 monoclonal antibody. It is possible that these treatments did not bind effectively to the rat cytokines and the receptors. Future studies should consider using a rat specific TNF-α inhibitor and a rat specific IL-6 receptor blocker. In this study we included both male and female rats. Sample sizes did not allow for sensitivity analysis. However, miRNA expressions, endothelial function, arterial function and cardiac function did not differ significantly between males and females and therefore results were pooled for the sexes.

5.5 Conclusions

The results of the current study provided new insights into the effect of inflammation on miR146a-5p and miR155-5p expression. Our results suggest the possibility that these miRNAs are extensively involved in the regulation of the inflammatory response, and that these miRNAs were upregulated in response to cytokines.

We further investigated whether the expression of miRNAs were related to endothelial function. MiR146a-5p was directly associated with endothelial dysfunction, and based on its negative regulatory role in inflammation, it is suggested that this upregulation occurred in order to ameliorate the inflammatory induced endothelial dysfunction. MiR155-5p was extensively involved in the detrimental remodelling of the vascular system. MiR155-5p was also strongly related to endothelial dysfunction.

Finally, we investigated the effect of biologic DMARDs on miRNA regulation relative to endothelial dysfunction. Our results paradoxically show that miR146-5p is directly related to endothelial function when considering all groups. Further research is required to determine the mechanisms whereby these drugs elicit their responses. MiR155-5p was strongly related to endothelial dysfunction, independent of DMARD therapy, and therefore our results suggest that miR155-5p may be a valid biomarker for endothelial dysfunction in high-grade inflammatory conditions. Furthermore, it appears that TNF-α blocker therapy may confer positive effects on miRNA regulation as well as endothelial dysfunction. Further investigations to elucidate the effects of IL-6 blocker therapy on miRNA expression are required.

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APPENDIX

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

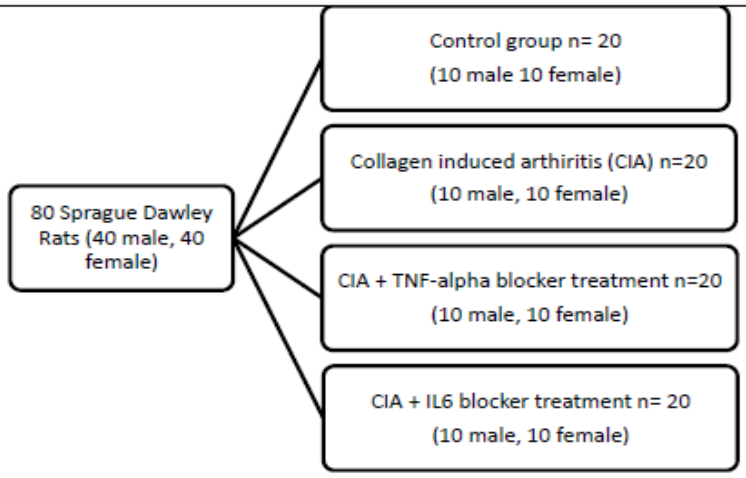
i. Specific modification / extension requested:

- 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

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 6 May 2019

Certificate reference: 2017/03/21C
Category: O
Applicant: Lebogang Mokotedi
Department: Physiology
Tel: 011 717 2262; **Email**: Lebogang.Mokotedi@wits.ac.za

RE: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that **Mikayra Singh (869659)**, Department of **Physiology** (WITS), does not require full Animal Ethics Research Committee clearance to undertake the work titled **"Biomarkers of endothelial dysfunction in treated and untreated rats with collagen induced rheumatoid arthritis".**

Reason for waiver
Data from the project entitled "Cardiovascular morphology and function in a rat model of rheumatoid arthritis" has been collected from 2017 to 2018 on 125 rats. From these experiments, specimen samples were collected for future analysis. Mikayra Singh will be using these specimen samples (blood samples) as part of her MSC degree.

Details of the study
The aim of the study is to determine the effect of biologic anti-rheumatic treatment drugs on both novel and conventional biomarkers of endothelial function in Sprague Dawley rats exposed to collagen induced arthritis. Blood samples collected from the previous approved ethics application will be used for this study. Blood samples will be used to measure circulating endothelial function markers (ICAM, VCAM, E-selectin and pentraxin-3) and the expression of miRNAs (miR-155, miR-126-3p, miR-499 and miR-146a) involved in the regulation of vascular function between the treatments and control groups.

Please contact me should you require further information.

Yours sincerely

Geoffrey Candy
Chair : Animal Ethics Research Committee, University of the Witwatersrand

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