ADIPOKINES AS BIOMARKERS OF SUBCLINICAL CARDIOVASCULAR DISEASE IN RHEUMATOID ARTHRITIS

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, South Africa,

2021

Declaration

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

I hereby certify that the studies contained in this thesis have been approved by the Committee for Research in Human Subjects, University of the Witwatersrand, Johannesburg. The ethics approval number is M170986, which forms part of a larger study M120562 renewed as M170592.

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory condition that confers an increased risk for the development of cardiovascular disease, independent of conventional cardiovascular risk factors. Systemic high-grade inflammation is a key determinant in the development of cardiovascular disease. The mechanisms whereby RA contributes to subclinical cardiovascular disease is under investigation. Adipokines are intricately linked to the regulation of various inflammatory processes and may be a mechanistic link to subclinical cardiovascular disease. Several adipokines are altered in RA compared to the general population. Whether adipokines are reliable biomarkers of subclincial cardiovascular disease in RA warrant investigation. This thesis thus focused on exploring the contribution of the adipokines nesfatin, visfatin and vaspin to the development of subclinical cardiovascular disease, including atherosclerosis, arterial stiffness and cardiac function, in patients with RA.

Atherosclerosis contributes largely to the increased cardiovascular disease mortality in RA. Nesfatin and visfatin concentrations are altered in RA compared to the general population. Nesfatin is an anti-inflammatory molecule and is associated with reduced risk of atherosclerosis in the general population. In contrast, visfatin concentrations are increased in chronic inflammatory conditions and are directly associated with atherosclerosis. The first study examined the potential impact of nesfatin and visfatin on metabolic risk factors, endothelial activation, atherosclerosis and plaque vulnerability mediators in 232 patients with established RA. Adipokine concentrations, endothelial activation marker concentrations including E-selectin, vascular adhesion molecule-1, intracellular adhesion molecule-1, monocyte chemoattractant protein-1, angiopoietin-2 and asymmetric dimethylarginine and plaque stability mediators, including matrix metalloproteinases (MMP) 2, 3 and 9 were assessed by ELISA. Common carotid intima-media thickness (cIMT) and carotid artery plaque were assessed by ultrasound. Independent associations of nesfatin and visfatin concentrations with metabolic risk factors, endothelial activation, carotid atherosclerosis and altered plaque stability were determined in multivariate regression analysis. Rheumatoid factor (RF) positivity was associated with nesfatin (β (SE) = 0.65 (0.14), p<0.0001) and visfatin levels (β (SE) = 0.16 (0.07), p = 0.03). Visfatin concentrations were related to diastolic blood pressure (β (SE) = 4.54 (1.70), p = 0.01) and diabetes (β (SE) = 0.09 (0.05), p = 0.04). Nesfatin levels were associated with cIMT (β (SE) = -0.02 (0.01), p = 0.007). Nesfatin (β (SE) = 0.12 (0.03), p = 0.001) and visfatin concentrations (β (SE) = 0.23 (0.07), p = 0.001) were related to those of matrix metalloproteinase-2 (MMP-2), a plaque stability mediator. The nesfatin-MMP-2 and visfatin-MMP-2 relations were stronger in RF negative compared to RF positive patients. This study showed that nesfatin was associated with reduced atherosclerosis and increased plaque stability mediator levels in RA. Visfatin was related to adverse cardio-metabolic risk in RA. Increased MMP-2 expression

in relation to visfatin may represent a compensatory mechanism aimed at reducing cardiovascular risk in RA.

Vaspin concentrations are also altered in RA. Vaspin concentrations have been associated with atherosclerotic changes and have been identified as an independent prognostic marker of adverse cardiac events. The second study examined the potential impact of vaspin on metabolic risk factors, endothelial activation, carotid atherosclerosis, and plaque vulnerability mediators in 170 RA patients. Relationships between vaspin and endothelial activation, plaque vulnerability mediators and atherosclerosis were identified in multivariate regression analysis. Vaspin concentrations were associated with RF positivity (β (SE) = 0.03 (0.10), p = 0.002). Vaspin was not associated with any cardiovascular disease markers, endothelial activation, or atherosclerosis in both univariate and multivariate analysis. The relationship between MMP-2 and vaspin concentrations were, however, influenced by the presence of major cardiovascular risk factors (β (SE) = -0.08 (0.03), p = 0.02). In subgroup analysis, vaspin levels were positively associated with those of MMP-2 in patients with no major cardiovascular risk factors (β (SE) = 0.42 (0.16), p = 0.01), but not in patients where one or more risk factors were present (β (SE) = 0.06 (0.06), p = 0.35). The presence of major cardiovascular risk factors also impacted on the vaspin-angiopoetin-2 relationship, where vaspin levels were positively associated with those of angiopoetin-2 in patients with no major cardiovascular risk factors (β (SE) = 1.13 (0.05), p = 0.02). This study showed that vaspin concentrations were associated with reduced risk of plaque rupture, but only in those with low cardiovascular disease risk.

Impaired arterial function mediates cardiovascular events in non-RA persons. RA patients experience increased arterial stiffness. Several adipokines are associated with arterial stiffness in the general population. The third study investigated the relationships between nesfatin, visfatin and vaspin and measures of arterial function in 173 patients with RA. Arterial function measures of arterial stiffness, pressure pulsatility and wave reflection were assessed using applanation tonometry. In multivariate analysis, there were no significant associations between nesfatin or vaspin concentrations and any markers of arterial function. Visfatin concentrations were inversely associated with central augmentation pressure (β (SE) = -0.18 (0.06), p = 0.004), augmentation index (β (SE) = -0.15 (0.07), p = 0.02), reflection magnitude (β (SE) = -0.14 (-0.06), p = 0.04), central systolic blood pressure (β (SE) = -0.13 (0.06), p = 0.02) and central pulse pressure (β (SE) = -0.14 (0.06), p = 0.03). In stratified analysis, these associations remained significant only in older person and those with greater disease severity. The study showed that increased visfatin concentrations were associated with reduced wave reflection markers in RA patients who are older than 50 years of age and in patients with increased disease severity. Although this finding may seem paradoxical, recent evidence has shown decreased wave

reflection in older adults and those exposed to inflammation, in the presence of increased arterial stiffness. The reduced wave reflection increases the pulsatile energy that is absorbed in the periphery. Therefore, reduced wave reflection, especially in older adults is associated with increased risk of target organ damage. The results are in keeping with previous studies that suggest visfatin may be associated with adverse vascular remodelling.

RA patients experience an increased risk of developing left ventricular (LV) diastolic dysfunction and heart failure with a preserved ejection fraction. The role of adipokines as biomarkers for the development of heart failure is not well described. The final study investigated the association between nesfatin, visfatin and vaspin and diastolic function markers in 170 RA patients. Cardiac function was assessed by echocardiography. In multivariate regression analysis, visfatin concentrations were independently associated with E/e', an index of LV filling pressure (β (SE) = 0.21 (0.08), p = 0.008), and left atrial volume index (LAVI) (β (SE) = 0.17 (0.07), p = 0.02). In stratified analysis the association between visfatin and increased filling pressures remained significant in younger patients and those with lower disease severity. In stratified analysis, nesfatin concentrations were associated with reduced risk of LV concentric hypertrophy (relative wall thickness and LV mass index) in younger patients and in those with a shorter disease duration. Vaspin concentrations were associated with reduced LV relaxation (reduced lateral wall mitral annular velocity) in older patients. This study showed disparate associations between visfatin, nesfatin and vaspin concentrations and markers of adverse cardiac structure and function, and that these associations are impacted by age and disease severity.

In conclusion, the findings reported in this thesis contribute to the understanding of the roles of nesfatin, visfatin and vaspin as possible mediators of subclinical cardiovascular disease in RA patients. The findings suggest that nesfatin is associated with reduced atherosclerosis and increased plaque stability mediator levels and a decreased risk of adverse LV remodelling in RA. Vaspin concentrations are associated with reduced risk of plaque rupture and vessel stability, but only in those in the early stages of disease and with low cardiovascular disease risk. Visfatin relates to adverse cardio-metabolic risk in RA and is a likely biomarker for adverse vascular remodelling, plaque stability and increased LV filling pressures. Taken together, these adipokines may improve risk stratification for cardiovascular disease in RA patients. Publications and presentations arising from this thesis.

Publications and presentations arising from this thesis

*** Robinson, C**., Tsang, L., Solomon, A., Woodiwiss, A. J., Gunter, S., Mer, M., Hsu, H. C., Gomes, M., Norton, G. R., Millen, A., & Dessein, P. H. (2018). Nesfatin-1 and visfatin expression is associated with reduced atherosclerotic disease risk in patients with rheumatoid arthritis. *Peptides*, *102*, 31–37.

Oral presentation

Robinson, C., Gunter, S., Norton G.R., Woodiwiss, A.J., Tsang, L., Dessein, P.H., Millen A.M.E. Nesfatin-1 and Visfatin expression I associated with reduced atherosclerotic disease risk in patients with rheumatoid arthritis. 45th Annual Conference of the Physiological Society of Southern Africa, Pretoria, South Africa, August 2017.

Poster Presentation

Robinson, C., Gunter, S., Norton G.R., Woodiwiss, A.J., Tsang, L., Dessein, P.H., Millen A.M.E. Nesfatin-1 and Visfatin expression are associated with reduced atherosclerotic disease risk in patients with rheumatoid arthritis. Annual European Congress of Rheumatology 2017, Madrid, Spain, June 2017.

** This paper was published in a well-respected peer-reviewed journal and already widely cited at the time of submitting this thesis (citations: 17). Our intent is to submit the additional chapters for publication.*

Statement of contribution to the research

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

The cross-sectional study was designed in conjunction with Prof Patrick Dessein and Prof Aletta Millen. As part of a team of researchers including Professors Millen and Dr Gunter, we performed all echocardiographic and applanation tonometry assessments and Professors Dessein and Solomon performed the clinical assessments. I performed all data analysis, interpreted the data, and wrote the manuscripts for this thesis with the help of my supervisors Professors Millen and Mer and with the input of Prof Dessein. All the co-authors on the individual papers contributed to data interpretation, critically revising the manuscripts, and provided expert opinions.

Acknowledgments

I wish to extend my immense gratitude to Prof Aletta Millen. Her guidance, attention to detail and clinical knowledge resulted in successful completion of this thesis. Thank you for never letting me lose hope!

Similarly, I wish to thank Prof Mervyn Mer without whom this project would not have been completed. His guidance, advice and professional opinion was of immense value and will continue to govern my clinical career. Prof, your humility remains my inspiration!

To my parents, family and friends – your patience and endless motivation made this possible.

This thesis is dedicated to Jaden van der Merwe –may you always have to courage to challenge convention and turn dreams into reality. I remain your biggest fan.

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Abbreviations

A	trans-mitral blood flow velocity in the late (atrial) period of left ventricular diastolic filling
a’	peak velocity during late (atrial) diastole
ACE	angiotensin converting enzyme
ACPA	anti-citrullinated peptide antibody
ACRP30/ADPOQ	adiponectin
ADMA	asymmetric dimethylarginine
AKt	protein kinase B
AIx	aortic augmentation index
AMPK	adenosine monophosphate-activated protein kinase
Ang-2	angiopoeitin-2
Anti-CCP	anti-citrullinated protein antibody
AP	augmented pressure
ARB	angiotensin receptor blockers
BMI	body mass index
BP	blood pressure
Ca^{2+}	calcium
CAD	coronary artery disease
cAMP	cyclic adenosine 3’,5’-monophosphate
CDAI	Clinical Disease Activity Index
cGMP	cyclic guanosine monophosphate
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration

CI	confidence interval
cm	centimetre
cm/s	centimetre per second
CO_2	carbon dioxide
cPP	central pulse pressure
CRP	C-reactive protein
cSP	central systolic pressure
CVD	cardiovascular disease
DAS28	Disease Activity Score in 28 joints
DBP	diastolic blood pressure
DD	diastolic dysfunction
DMARD	Disease-modifying antirheumatic drugs
E	trans-mitral blood flow velocity in the early period of left ventricular diastolic filling
e’	peak velocity during early diastole at the mitral annulus
e’/a’	ratio of early to late mitral annulus relaxation velocity
E/A	ratio of early to late diastolic filling velocity
E/e’	index of LV filling pressures
ECG	electrocardiogram
EF	ejection fraction
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate

EULAR	European League Against Rheumatism
Ft	time to the peak of the forward wave
GFR	glomerular filtration rate
HAQ-DI	Stanford Health Assessment Questionnaire disability index
HDL	high density lipoprotein
HFpEF	heart failure with preserved ejection fraction
HFrEF	heart failure with reduced ejection fraction
HLA	human leukocyte antigen
HOMA-IR	Homoeostatic Model Assessment of insulin resistance
hs-CRP	high-sensitivity C-reactive protein
ICAM-1	intercellular adhesion molecule 1
IL 6	interleukin 6
IL-1β	interleukin 1beta
IMT	intima-media thickness
IQR	interquartile range
IVST	interventricular septum thickness
Kg	kilogram
kg/m^2	kilogram per meter squared
LA	left atrial
LAVI	left atrial volume index
LDL	low-density lipoprotein
LV	left ventricular
LVEDD	left ventricular end diastolic diameter

LVESD	left ventricular end systolic diameter
LVESV	left ventricular end systolic volume
LVM	left ventricular mass
LVMI	left ventricular mass indexed to body surface area
MCP-1	monocyte chemoattractant protein-1
MESA	Multi-Ethnic Study of Atherosclerosis
ml	millilitre
ml/m^2	millilitre per $meter^2$
mm Hg	millimetres of mercury
mmol/l	millimole per litre
MMP	matrix metalloproteinase
NAD	nicotinamide adenine dinucleotide
NAMPt	nicotinamide phosphoribosultransferase
NF-kB	nuclear factor kappa B
NMN	nicotinamide mononucleotide
Nmnat	nicotinamide-nucleotide adenylyltransferase
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drug
NUCB2	nucleobindin-2
OLETF	Otsuka Long Evnas Toushima fatty
OR	Odds ratio
Pb	backward wave pressure
PBEF	pre-B cell enhancing factor

Pf	forward wave pressure
PKA	protein kinase A
PP	pulse pressure
PPamp	pulse pressure amplification
PPc	central aortic pulse pressure
pPP	peripheral pulse pressure
PRPP	phophoribosyl pyrophosphate
PTPN-22	protein tyrosine phosphatase, non-receptor type-22
PWT	posterior wall thickness
PWV	pulse wave velocity
r	correlation coefficient
RA	rheumatoid arthritis
RBP-4	retinol binding protein-4
RF	rheumatoid factor
RM	reflection magnitude
RWT	relative wall thickness
SD	standard deviation
SE	standard error
SERCA	sarco/endoplasmic reticulum Ca^{2+} -ATPase
sFRP-5	secreted frizzled-related protein-5
Std	standardised
SVF	stromal vascular fraction
TDI	Tissue Doppler Imaging

T_{INF}	time of inflection point
TNF-α	tumour necrosis factor alpha
US	ultrasound
VCAM-1	vascular cell adhesion molecule 1
α	alpha
β	beta

Preface

Cardiovascular disease (CVD) contributes significantly to global all-cause mortality rate. According to the World Health Organisation, CVD results in an estimated 17.9 million deaths annually. In low- and middle-income countries, an epidemiological transition has been proposed as one of the reasons for increased CVD burden. In developing countries, the increased prevalence of CVD burdens the already resource-limited health care systems. The need for accurate and cost-effective risk stratification is imperative in the prevention and management of CVD.

Although traditional cardiovascular risk factors contribute substantially to the incidence of CVD, they fail to account for all cardiovascular events. One possible non-traditional risk factor that has recently received considerable attention as a risk factor for CVD is chronic systemic inflammation. In this regard, systemic inflammation is associated with cardiovascular events independent of traditional CVD risk factors. Furthermore, disorders associated with chronic high-grade inflammation markedly exacerbate the risk for cardiovascular events.

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic inflammation. RA patients have double the risk for cardiovascular events compared to the general population. In patients with RA, traditional risk factors such as diabetes, hypertension, dyslipidaemia, and obesity, fail to fully account for the increased risk of cardiovascular events. Although the presence of chronic inflammation is often offered as explanation, the exact mechanisms whereby systemic inflammation enhances CVD risk in RA are currently under investigation. Numerous biomarkers of cardiovascular disease and inflammation have been investigated in recent literature to improve risk stratification in RA patients.

In this regard, the involvement of adipokines have recently enjoyed attention to explore the pathogenesis of CVD. The current focus on adipose tissue as a bioactive endocrine organ with systemic involvement, was fueled by the discovery of the cytokine-like hormone, leptin in 1994. The subsequent discovery of adiponectin played a key role in understanding the pathophysiology of obesity-linked disease cascades. Numerous adipokines have since been associated with cardiovascular events and subclinical cardiovascular disease in the general populations. In this regard leptin, visfatin and resistin have been associated with an increased CVD risk, while others such as omentin, apelin and adiponectin display cardioprotective properties. In addition, many adipokines display pro-inflammatory properties. Hence it is not surprising that adipokines have received attention as a possible mediator of CVD in high-grade inflammatory conditions. Indeed, adipokine concentrations are often altered in RA. Nevertheless, the contribution of adipokines to RA disease progression and the development of CVD is poorly understood.

Taken together, patients with RA are at increased risk for cardiovascular disease. Although traditional risk factors contribute to the increased prevalence of cardiovascular diseases, inflammation in RA plays a key role in the development of cardiovascular disease. To improve cardiovascular disease risk stratification in RA, several biomarkers, including adipokines have received considerable attention. However, the unique interaction between inflammation, traditional risk factors and altered fat metabolism in RA may impact the production and mechanisms of action of adipokines in this population. Therefore, the influence of adipokines in cardiovascular disease risk and the complex interplay between traditional risk factors, inflammation, fat metabolism and cardiovascular disease in RA, identifying useful biomarkers to enhance cardiovascular disease risk stratification in RA warrants investigation. It is within this conceptual framework that the present thesis was prompted. This thesis addresses some outstanding issues regarding the role of adipokines in the increased risk of CVD in patients with RA. In an attempt to do so, three topical adipokines were evaluated namely nesfatin, visfatin and vaspin with specific focus on subclinical cardiovascular disease. Briefly I evaluated the association of these adipokines with demographic variables and patient characteristics of a cohort of patients with confirmed RA and varying levels of disease activity. Furthermore, the associations between nesfatin, visfatin and vaspin and markers of subclinical cardiovascular disease including endothelial dysfunction, atherosclerosis, arterial stiffness, and LV diastolic dysfunction, were explored.

The present thesis comprises a series of semi-independent chapters each with an introduction, methods, results and discussion. To provide a broad overview of current literature and to highlight the reasons for conducting this research, a literature review is provided in chapter 1. The impact of nesfatin and visfatin on metabolic risk factors, endothelial activation, carotid atherosclerosis, and plaque vulnerability mediators are reported in chapter 2. Chapter 3 is dedicated to the impact of vaspin on metabolic risk factors, endothelial activation, carotid atherosclerosis, and plaque vulnerability mediators. In chapter 4 details the associations between all three adipokines with markers of arterial stiffness while chapter 5 evaluates the association between these adipokines and cardiac geometry and function. A summary of the findings of each chapter in the context of the literature is provided in Chapter 6. Thereafter a list of references and the appendices are provided.

Statitistical analysis for each study is detailed in respective chapters outlined above. Briefly, statistical analyses were performed using IBM SPSS statistics (version 25.0, IBM, USA) and SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Data are expressed as mean (standard deviation) for normally distributed variables or median (interquartile range) for non-normally distributed variables. Dichotomous variables were expressed as percentages. A mixed regression model was employed to determine the various relationships of age, race, and gender with that of the adipokine concentrations.

Subsequently, associations of adipokine concentrations with Patient characteristics were assessed in demographic adjusted models. Associations between adipokine concentrations and the relevant subclinical CVD markers evaluated in each chapter, were assessed in both univariate and multivariate regression models. As several traditional risk markers and RA characteristics may impact the relationship between adipokines and cardiovascular risk, relevant interactions terms were assessed and stratified analyses in respective subgroups were performed. Statistical significance was set as $p < 0.05$.

Chapter 1: Literature Review

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a prototypic, chronic inflammatory condition that affects 0.5 to 1% of the population (Silman & Pearson, 2002). RA is characterised by destruction of cartilage and bone of synovial joints, due to synovial inflammation and hyperplasia (McInnes & Schett, 2011). In addition, abnormal activity of synovial cells or synoviocytes in the joints of RA patients causes disparate inflammatory cascades and stimulates an overproduction of inflammatory cytokines which leads to systemic inflammation. One such cascade comprises the overproduction and overexpression of tumour necrosis factor alpha (TNF-α) (Lloyd *et al.*, 2010; Feldman *et al.*, 1996), eliciting a response from additional cytokines such as interleukin-6 (IL-6). Chronic systemic inflammation in RA contribute to extra-articular manifestations that lead to organ dysfunction, progressive disability, and increased mortality (Smolen *et al*., 2016). Although the exact aetiology is unknown, RA comprises complex interactions between genetic- and environmental factors (Deane *et al*., 2017; Traylor *et al.*, 2017; McInnes & Schett, 2011).

In this regard, twin studies yielding concordance rates of 15-30% in monozygotic twins and 5% in dizygotic twins (Silman & Pearson, 2002), suggest that genetic factors contribute to RA prevalence (McInnes & Schett, 2011). The human leukocyte antigen (HLA)-DRB1 locus confers disease susceptibility in patients who test positive for rheumatoid factor and/or anti-citrullinated protein antibodies (ACPA) (Kurowska *et al*., 2017; McInnes & Schett, 2011; Gregersen *et al*., 1987) Gene interactions between (HLA)-DRB1 and PTPN22 have been described thereby adding to the complexity of the exact aetiology in RA patients (Kallberg *et al.*, 2007). Despite the genetic predisposition, environmental factors contribute to the prevalence of RA. With regards to gene-environment interactions, smoking increases the risk for developing RA in patients presenting with (HLA)-DR4 alleles (Kallberg *et al.*, 2007; Symmons *et al*., 1997). Smoking also confers increased risk for ACPA-positive patients in the presence of (HLA)-DRB1 alleles (McInnes & Schett, 2011; Klareskog *et al.,* 2006). Infectious agents triggering the initiation and formation of immune complexes have also been associated with RA (Li *et al*., 2013; Wilder & Crofford, 1991). Infectious agents implicated in the pathogenesis of RA include Epstein-Barr virus, cytomegalovirus, parvo- and rubella virus (Alamanos & Drosos, 2005). In addition, the majority of RA patients are women. Studies suggest the susceptibility to RA in women are higher when using oral contraceptives and reduced with parity (Alamanos & Drosos, 2005; Silman & Pearson 2002). Nevertheless, the factors predisposing individuals to RA are still under investigation.

The current diagnostic criteria for RA as defined by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) incorporates both RF and ACPA in serology criteria to be met in addition to the presence of joint involvement, acute-phase reactants and patient reported

disease activity (Aletaha *et al.*, 2010). Previously, the presence of rheumatoid factor (RF) was considered the hallmark autoantibody to confirm RA. Subsequently, studies have reported low disease specificity for RF in individuals with RA (Song & Kang, 2010). More recently, ACPA has been identified as a disease specific marker detected in 70-90% of RA patients (Song & Kang, 2010; Suzuki *et al.*, 2003; Schellekens *et al.*, 2000). Current practice recognises two major subtypes of RA, classified according to the presence or absence of ACPA (Guo *et al.*, 2018). The presence of ACPA, detected by commercially available enzyme-linked immunosorbent assay (ELISA), that assesses cyclic citrullinated peptide antibodies (anti-CCP), confers increased risk for disease severity and in contrast with RF, can be detected before clinical manifestation of the disease (Kurowska *et al*., 2017; Rantapää-Dahlqvist *et al.*, 2003).

Currently there is no cure for RA and timeous diagnosis is encouraged to minimize adverse outcomes and to prevent disability. The mainstay of treatment is the use of disease modifying anti-rheumatic drugs (DMARDs) with constant surveillance to detect the development of extra-articular manifestations as a result of high-grade circulating inflammation. The cornerstone of DMARD therapy is aimed at reducing circulating inflammation and preventing disease progression (Smolen *et al*., 2016). Although DMARDs have proven effective at reducing the effects of circulating inflammation and slowing disease progression, its effectiveness in the prevention and management of extra-articular manifestations, especially cardiovascular disease, is controversial (Davis *et al*., 2011).

1.2 Cardiovascular disease in rheumatoid arthritis

It is well established that compared to the general population, RA patients have an increased risk of cardiovascular mortality and cardiovascular events (Avina-Zubeita *et al.*, 2008). It is estimated that individuals with RA have a two-fold greater risk for developing cardiovascular disease, a risk profile similar to that of patients with longstanding diabetes mellitus (Crowson *et al.*, 2013; Van Halm *et al.*, 2009; Solomon *et al.*, 2006; Maradit-Kremers *et al.*, 2005). Cardiac involvement contributes to as much as 50% of mortality in RA patients (Sarzi-Puttini *et al*., 2010). Cardiac complications described in RA include various clinical presentations such as pericarditis, arrhythmias, valvular disease, coronary heart disease and heart failure (Turiel *et al*., 2010). Furthermore, RA patients also have an increased risk for subclinical cardiovascular disease including endothelial dysfunction, arteriosclerosis, atherosclerosis and left ventricular dysfunction (Avina-Zubeita *et al.*, 2008).

The mechanisms underlying the heightened CVD risk in RA has received considerable attention in recent years. In this regard, certain traditional CVD risk factors are more prevalent in RA, yet conventional risk stratification underestimates the risk of CVD in RA (Dessein & Semb, 2013). In this regard, the EULAR task force suggests that current CVD risk stratification models, such as the

Framingham risk score, should be adjusted to account for the increased risk in RA patients (Agca *et al.*, 2017). Although traditional CVD risk factors contribute to the increased CVD risk in RA, even in the absence of conventional cardiovascular risk factors CVD risk is heightened in RA (del Rincon, 2001; Kramer & Giles, 2011). It has been established that systemic high-grade inflammation is a key contributing factor to the heightened CVD risk in RA (Gonzalez *et al.*, 2008; Gonzalez-Gay *et al*., 2007; Sattar & McInnes, 2005). Moreover, inflammation has also been linked to sub-clinical cardiovascular diseases. The section below will highlight the contribution of systemic inflammation to subclincial cardiovascular disease, specifically artherosclerosis, arterial stiffness and left ventricular dysfunction in RA patients.

1.3 Subclinical cardiovascular disease in rheumatoid arthritis

1.3.1 Atherosclerosis in rheumatoid arthritis

The presence of atherosclerosis is one of the principal predictors of coronary artery disease. The MESA study showed that the addition of atherosclerosis presence to risk stratification scores significantly improved the prediction of coronary artery disease when compared to when using conventional risk factors alone (McClelland *et al*., 2015). RA patients have an increased prevalence of atherosclerosis and coronary artery events compared to the general population (Ruscitti *et al*., 2019). Compared to age and sex-matched control subjects, RA patients have more unstable carotid plaques that are more prone to rupture (Urman *et al*., 2018). Both traditional and RA disease-related risk factors have been associated with atherosclerosis in RA patients (Arida *et al.*, 2018; Crowson *et al.*, 2013; Steiner & Urowitz, 2009; Gonzalez-Gay *et* al., 2005). In non-RA individuals, the development of atherosclerosis is often the result of complex interactions between various metabolic and cardiovascular risk factors such as obesity, diabetes, dyslipidaemia, hypertension, and smoking. In the general population, an atherogenic lipid profile, which is characterised by increased total cholesterol and low-density lipoprotein (LDL) concentrations and a decreased high-density lipoprotein (HDL) concentration, are well-known causes of atherosclerosis (Kobiyama & Ley, 2018). Increased concentrations of LDL ultimately cause inflammation in medium and large arteries and is considered the underlying causes of the formation of atherosclerotic plaque (Kobiyama & Ley, 2018). In RA patients, lipid profiles are altered compared to the general population. RA patients have decreased total cholesterol and LDL concentrations and a disproportionate suppression of high-density lipoprotein (HDL). Despite the lower LDL concentrations, RA patients have an increase atherosclerotic risk that is largely attributed to the disproportionate ratio between total cholesterol and HDL cholesterol (atherogenic index) (Steiner & Urowitz, 2009). These altered lipid concentrations in RA have largely been attributed to high-grade systemic inflammation (Erum *et al*., 2017; Crowson *et al.*, 2013). Furthermore, it has been reported that systemic inflammation also alters protein structures, resulting in altered function of the

lipoproteins (Toms *et al*., 2010). The altered function of lipoproteins decreases the anti-atherogenic effects of HDL, thus contributing to atherosclerosis and plaque formation (Charles-Schoeman *et al.,* 2015).

Besides altering lipoprotein structure and function, inflammation also has a direct adverse effect on the endothelium. Indeed, inflammation-induced endothelial activation is one of the earliest precursors of atherosclerosis in the general population (Gimbrone & Garcia-Cardena, 2016; Sitia *et al*., 2010). Because of the abundant evidence outlining the involvement of inflammatory and immunoregulatory mediators in atherogenesis, the atherosclerotic process is classified and described as a chronic inflammatory disease (Ridker *et al.,* 2007). Considering the role of inflammation in the development of endothelial dysfunction and its progression to atherosclerosis in the general population, it is not surprising that endothelial activation is considered one of the earliest markers of cardiovascular disease in RA (Dessein *et al*., 2005). Endothelial activation cause adhesion of leukocytes to the endothelial cells which increases its permeability and therefore significantly contributes to the formation and progression of atherosclerotic plaques (Gimbrone & Garcia-Cardena, 2016). Indeed, markers of endothelial activation and dysfunction are strongly associated with atherosclerotic CVD in RA (Khan *et al.*, 2010; Dessein *et al.*, 2005). Besides the role of inflammation in the formation of plaque, it is suggested that inflammation also contributes to the erosion of plaque. The accelerated progression of atherosclerosis contributes to cardiac events that arise from the rupture of plaque, resulting in luminal occlusion and thrombosis (Mahmoudi *et al.*, 2017; Rothwell *et al*., 2003). Cytokines such as interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) mediates the destabilisation of atherosclerotic plaques (Pasceri & Yeh, 1999). In this regard, inflammatory cytokines impact matrix metalloproteinases (MMPs) which play an important role in plaque stability (Vacek *et al.*, 2015). Taken together, RA patients are at increased risk of atherosclerotic cardiovascular disease. Systemic inflammation has been shown to impact the atherogenic index, endothelial dysfunction and mediators of plaque stability and vulnerability. In addition to the link with endothelial dysfunction and atherosclerosis, inflammation has also been linked to other subclinical cardiovascular diseases. In this regard another possible mechanism for the increased cardiovascular burden in patients with RA, is altered arterial function.

1.3.2 Arterial stiffness in rheumatoid arthritis

In the general population, ageing and risk factor exposure are associated with smooth muscle cell hyperplasia, destruction of elastic tissue and increased collagen deposition in the interstitium of large vessels, which all contribute to stiffening of large arteries (Ghebre *et al.,* 2016; Barodka *et al.*, 2011). Arterial functional changes such as increased arterial stiffness, increased pressure pulsatility and

increased wave reflection independently predict cardiovascular events and cardiovascular mortality (Vlachopuloos *et al*., 2010; Wang *et al*., 2012; Townsend *et al.,* 2015). The contribution of altered arterial function to increased risk of cardiovascular events is manifested through several mechanisms (Mitchell *et al.*, 2004). The section below will briefly describe the various measures of arterial functional changes and their relation to cardiovascular events and mortality.

Increased arterial stiffness increases the magnitude and propagation speed of the aortic pressure waveform that travels through the arterial tree (Mitchell, 2009). Pulse wave velocity (PWV) is a well-established measure of arterial stiffness and measures the rate at with aortic pressures are transmitted (Lee & Joo, 2019; Vlachopoulos *et al.*, 2010). Carotid-femoral PWV is the current gold standard for non-invasive measurement of arterial stiffness and is considered a direct measure of large artery stiffness (Townsend *et al*., 2015; Laurent *et al*., 2006; Pannier *et al*., 2002). A landmark study evaluating associations between various measures of arterial stiffness and cardiovascular events, showed that carotid femoral PWV is the best indicator of cardiovascular risk (Mitchell *et al.*, 2010). Increased carotid femoral PWV is associated with increased risk for adverse cardiovascular events and cardiovascular mortality (Townsend *et al*., 2015; Mitchell *et al.*, 2010a,b; Vlachopoulos *et al.,* 2010; Boutouyrie *et al.*, 2002; Laurent *et al.,* 2001; Meaume *et al.*, 2001). Increased arterial stiffness increases impedance and therefore with each cardiac cycle, the heart needs to generate greater force to pump against the increased resistance (Mitchell, 2009; Nichols & Edwards, 2001). As a result, the forward wave pressure magnitude and the central aortic systolic pressure increases. In addition, increases in arterial stiffness reduces coronary perfusion (Vlachopoulos *et al*., 2011). In this regard, as the aortic pressure waveform travels towards the periphery, changes in the properties and diameter of the vessel results in the forward traveling wave, generated by ventricular contraction, to be reflected back to the heart. Hence the aortic pressure waveform is comprised of both the forward traveling wave and the reflected (backward traveling) wave (Barodka *et al.*, 2011; Vlachopoulos *et al*., 2011; Mitchell *et al.*, 2010a). Increased arterial stiffness increases the speed of wave reflection which causes the reflected wave to arrive earlier in the cardiac cycle thereby augmenting central systolic and reducing diastolic pressure (Vlachopoulos *et al*., 2011). In this regard, the pressure gradient generated by the early return of the reflected wave adds to the cardiac workload because of the increased central systolic pressure, which is compounded by the reduced diastolic pressure that decreases coronary flow (Chirinos *et al.*, 2012). Increases in both central systolic pressure and increased central aortic pulse pressure have been independently associated with cardiovascular outcomes (Said *et al.*, 2018; Townsend *et al.*, 2015; Vlachopoulos *et al*., 2010; Pini *et al.*, 2008).

In healthy individuals, brachial pulse pressure is estimated to be approximately 10 mmHg higher than central aortic pulse pressure because of the change in the elastic properties as one moves distally along

the arterial tree (Barodka *et al.*, 2011; Vlachopoulos *et al*., 2011; Mitchell *et al.*, 2010a,b). This difference in central to peripheral pulse pressure is known as pulse pressure amplification (PPamp). With increased stiffness and the resultant increase in central pulse pressure, the pulse pressure amplification between central large arteries and peripheral arteries are reduced. In this regard, reduced pulse pressure amplification has also been associated with increased cardiac workload and adverse cardiovascular events (Benetos *et al.,* 2010; 2012).

Increased arterial stiffness and increased wave reflection may also contribute to cardiovascular events as a result of increased pressure pulsatility that can lead to target organ damage (Vasan *et al*., 2019; Mitchell *et al.*, 2011; Hashimoto & Ito, 2009). In healthy individuals, large compliance vessels maintain a constant flow, thereby buffering against pulsatile forces and protecting against end organs against damage (Cecelja & Chowienczyk, 2012; Steppan *et al.*, 2011). With increased wave reflection, detrimental pulsatile forces in peripheral arteries are increased. Consequently, a portion of the pulsatile energy is returned to the aorta and secondly the pulsatile energy in the periphery may damage the microcirculation (Mitchell *et al*., 2011; Vlachopoulos *et al*., 2011). Aortic augmentation index (AIx) has been considered a marker of wave reflection and is determined by the augmented central pressure in late systole divided by central pulse pressure (Vlachopoulos *et al.*, 2011). Although augmentation index has been associated with cardiovascular events (Vlachopoulos *et al*., 2010; Chirinos *et al*., 2005; Ueda *et al.,* 2004), others have raised concerns that the determinants of AIx incorporates a large portion of the forward wave and hence it underestimates the reflected wave (Booysen *et al*. 2015; Baksi *et al.*, 2009). Therefore, the use of wave separation analysis to determine the forward and backward wave forms are recommended (Chirinos *et al*., 2012; Wang *et al.,* 2010). With regards to the backward wave, both the amplitude and the timing of the backward wave have been associated with cardiovascular outcomes and target organ changes (Heusinkveld *et al*., 2019; Booysen *et al.*, 2015; Chirinos *et al*., 2012; Weber *et al.*, 2012), independent of central systolic pressure and arterial stiffness (Weber *et al*., 2012; Wang *et al*., 2010).

Although arterial stiffness is associated with ageing and risk factor exposure, chronic inflammation also impacts large vessel remodelling. Inflammation increases collagen deposition and destruction of elastic tissue and accelerates smooth muscle cell proliferation (Park & Lakatta, 2012). Indeed, RA patients have increased arterial stiffness, central aortic pressure, and wave reflection compared to the general population (Ambrosino *et al.*, 2015a,b; Klocke *et al*., 2003). Although traditional risk factors are associated with altered arterial function in RA, circulating inflammatory markers and disease duration are independent contributors to increased arterial stiffness and wave reflection (Gunter *et al.,* 2017; Vázquez-Del Mercado *et al.,* 2017; Fan *et al.*, 2014; Provan *et al*., 2011). In RA, arterial stiffness is independently associated with cardiovascular events (Ikdahl *et al*., 2016). Furthermore, our

group has recently shown that wave reflection impacts the development of atherosclerosis (Gunter *et al*., 2018) and LV diastolic dysfunction (Mokotedi *et al.,* 2019) in RA.

1.3.3 Left ventricular diastolic dysfunction in rheumatoid arthritis

Although atherosclerosis and arteriosclerosis contribute to ischemic heart disease and the increased CVD mortality in RA, heart failure accounts for approximately 13% of the cardiovascular mortality in RA (Nicola *et al*., 2005). Patients with RA have twice the risk of developing heart failure compared to the general population (Avina-Zubeita *et al*., 2008; Nicola *et al*., 2005).

Heart failure can be classified into two broad phenotypes, namely heart failure with a reduced ejection fraction (HFrEF) and heart failure with a preserved ejection fraction (HFpEF) (Harper *et al*., 2018). Heart failure with a reduced ejection fraction is characterised by impaired pump capacity and hence systolic dysfunction. HFrEF was previously considered the most prevalent form of heart failure (Hajouli & Ludhwani, 2020; Harper *et al*., 2018). More recently, increasing incidence of cases of HFpEF have been reported (Shah *et al.*, 2020; Oktay & Shah, 2015). In these patients, ventricular filling is inadequate as a result of impaired myocardial relaxation during diastole (Obokata *et al*., 2020). Indeed, left ventricular (LV) diastolic dysfunction is one of the pre-clinical conditions most frequently believed to progress to HFpEF (Wan *et al*., 2014; Aziz *et al.,* 2013).

The pathogenesis of LV diastolic dysfunction and HFpEF is poorly understood. An aging population and the increased burden of disease of lifestyle contribute significantly to the development of LV diastolic dysfunction and HFpEF (Harper *et al.*, 2018; Oktay & Shah, 2015). In this regard, a recent paradigm suggested that chronic systemic inflammation is the underlying mechanism that link several comorbid conditions including obesity, diabetes mellitus and hypertension to the development of LV diastolic dysfunction (Paulus & Tschöpe, 2013). Indeed, inflammation is strongly linked to HFpEF (Paulus & Tschöpe, 2013). In conditions of high-grade inflammation, patients more frequently have HFpEF than patients with heart failure in the general population (Liang *et al*., 2010; Davis *et al*., 2008). Studies evaluating phenotypes of heart failure in RA populations, suggest a higher prevalence of LV diastolic dysfunction in these patients. A recent study suggests that 55% of patient with RA present with LV diastolic dysfunction compared to 9% with systolic dysfunction (Renjith *et al.,* 2017). Considering the proposed paradigm that inflammation may be the mechanistic link in the pathogenesis of LV diastolic dysfunction, the increased prevalence is LV diastolic dysfunction seen RA may be as a result of inflammation (Van Linthout & Tschöpe, 2017; Paulus & Tschöpe, 2013). Davis *et al.* (2011), showed that cardiac dysfunction in RA is not as a result of traditional CVD risk factors, but rather an intricate interplay between various factors such as mediators of inflammation and disease modifying antirheumatic drugs (DMARDs).

Inflammation is believed to contribute to several mechanisms involved in the development of LV diastolic dysfunction (Glezeva *et al.,* 2014). In this regard, there are three broad phenotypes that of LV diastolic dysfunction (Lewis *et al*., 2017). First, increased collagen volume and cross-linking contribute to extracellular matrix remodelling and cardiac fibrosis (Lewis *et al*., 2017; Zile *et al*., 2015). Myocardial fibrosis decreases the compliance and increases the stiffness of the ventricle (Zile *et al*., 2015). Inflammation increases the production and cross-linking of collagen, thereby increasing LV stiffness (Westermann *et al*., 2011). Second, during diastole adequate relaxation is dependent on the reuptake of calcium into the sarcoplasmic reticulum via the sarco-endoplasmic reticulum Ca^{2+} transport ATPase (SERCA) pump (Biesiadecki *et al*., 2014). Impairments in the reuptake of calcium prevents the dissociation of actin and myosin cross-bridges, which prevents active relaxation (Shah *et al.,* 2016; Lewis *et al*., 2017). Inflammation impairs the reuptake of calcium via SERCA during diastole (Wu *et al.*, 2011). Third, during diastole, the cytoskeletal protein titin, functions like a spring that allows the recoil of the cardiomyocytes (Lewis *et al*., 2017; Shah *et al*., 2016; Zile *et al*., 2015). The phosphorylation status of titin impacts the ability of the protein to recoil (Zile *et al*., 2015). Microvascular inflammation has been implicated in the hypophosphorylation of titin, which impairs its ability to recoil during diastole and therefore cause a stiff ventricle (Hutchinson *et al.*, 2015; Paulus & Tschöpe, 2013). Taken together, the associations between increased proinflammatory markers and the mechanisms underlying LV diastolic dysfunction and the high prevalence of HFpEF in RA patients suggest that systemic inflammation play an important role in LV diastolic function abnormalities. Hence it is not surprising that controlling systemic inflammation has been suggested as a potential therapeutic target for the management of LV diastolic dysfunction and prevention of HFpEF (Shah *et al*., 2016; Glezeva *et al*., 2014).

In summary, RA patients have increased cardiovascular mortality compared to the general population. Several sub-clinical cardiovascular diseases are also more prevalent in RA than in the general population. Conventional risk factors fail to account for the increased prevalence of subclinical cardiovascular diseases in RA. Hence alternative CVD risk profiling strategies need to be evaluated, specific for RA populations.

1.4 Adipokines as biomarkers of cardiovascular disease

The role of adipose tissue in systemic metabolism and as a mediator of inflammatory changes is no longer disputed (Berg & Scherer, 2005; Ouchi *et al.*, 2003). Indeed, the importance of white adipose tissue as an endocrine organ that secretes bioactive molecules, including hormones and proteins has been confirmed (Di Raimo *et al.*, 2015). White adipose tissue is predominantly present subcutaneously and in the viscera. White adipose tissue is comprised of adipocytes, pre-adipocytes, fibroblasts, vascular cells and immune cells, collectively referred to as stromal-vascular fraction (SVF) of adipose

tissue (Di Raimo *et al.*, 2015). Protein secretion by adipocytes as well as SVF of adipose tissue, contributes to mechanisms of vascular insult and atheromatous changes (Ouchi *et al.*, 2011; Berg & Scherer, 2005). These soluble, bioactive proteins that exhibit both pro- and anti-inflammatory effects, are referred to as adipocytokines (Scrivo e*t al.*, 2013). Trayhurn & Wood (2004) recommend the use of the abbreviated term adipokine as a more accurate depiction of protein functionality and origin. The term adipocytokine suggest that the bioactive proteins secreted by adipose tissue are cytokines or cytokine-like, which holds true in the case of IL-6 for instance, but not for all adipokines (Trayhurn & Wood, 2004).

In obesity the immunological activity of adipose tissue is altered (Ouchi e*t al.*, 2011). In the presence of obesity, hypertrophic adipocytes and stromal cells in the adipose tissue mediate systemic inflammatory changes that in turn affect a myriad of pathogenic pathways. In addition to changes in cellular composition, the expression of adipokines varies depending on the site of adipose deposit (Samaras *et al.*, 2010; Fried e*t al.*, 1998). Circulating adipokine levels are thus representative of both the extent of adiposity as well as its biological activity. Nevertheless, adipokines can also be synthesized by synoviocytes, osteoblasts, osteoclasts, chondrocytes and inflammatory cells in joints (Carrión *et al*., 2019).

Numerous adipokines have been identified and investigated in the past three decades. Complement factor D or the serine protease adipsin, was the first adipokine identified (Cook *et al.*, 1987). This was followed by the identification of TNF as a pro-inflammatory molecule that is secreted by adipose tissue and implicated in various inflammatory cascades in the presence of obesity (Ouchi e*t al.*, 2011; Hotamisligil e*t al.*, 1993). The current focus on adipose tissue as a bioactive endocrine organ was fueled by the discovery of the cytokine-like hormone, leptin (Trayhurn & Wood, 2004; Zhang *et al.*, 1994). The subsequent discovery of adiponectin (ACRP30/ADIPOQ) played a key role in understanding the pathophysiology of obesity-linked disease cascades (Hu e*t al.*, 1996; Maeda e*t al*, 1996). In addition to TNF-α, IL-6 and leptin (Farooqi *et al.*, 2002; Santos-Alvares *et al.*, 1999; Lord *et al.*, 1998), some of the most pertinent pro-inflammatory adipokines that have been identified include resistin (Jamaluddin *et al.*, 2012; Bokarewa *et al.*, 2005; Verma *et al.*, 2003), retinol binding protein 4 (RBP-4) (Kotnik e*t al.*, 2011; Balagopal *et al.*, 2007), and chemerin (Dessein e*t al.*, 2014d; Rourke e*t al.*, 2013). Anti-inflammatory adipokines include adiponectin, omentin, apelin and secreted frizzled-related protein 5 (SFRP-5) (Ouchi e*t al.*, 2010; Farkhondeh *et al.,* 2020). There is a plethora of evidence that these and other adipokines play a vital role in several biological and physiological functions, including satiety regulation, inflammatory and immune function, glucose and lipid metabolism, and blood pressure control (Recinella *et al*., 2020). Furthermore, dysregulation of these and other adipokines are believed

to be important mediators of systemic inflammation, hypertension, insulin resistance and hence cardio-metabolic risk (Recinella *et al*., 2020; Berg & Scherer, 2005).

The production of adipokines are altered in certain conditions of high-grade inflammation, including RA (Gomez *et al*., 2011). Altered adipokines concentrations have been implicated in the pathophysiology, disease activity and disease severity of RA (Recinella *et al*., 2020; Szumilas *et al.,* 2020; Chaparro-Sanabria *et al*., 2019; Gomez *et al.*, 2011). Although some adipokines are considered pro-inflammatory or anti-inflammatory, many of these adipokines have shown to exert dual roles (Szumilas *et al.,* 2020). As a result, the exact physiological mechanisms of these adipokines are not fully understood. Nevertheless, the expression of several adipokines in RA patients are associated with circulating inflammatory markers and markers of disease activity and severity (Recinella *et al*., 2020). Furthermore, our group and other have shown that altered adipokine concentrations are associated with cardiovascular disease risk factors and cardiovascular outcomes. In this regard several adipokines including chemerin, apelin, RBP-4, resistin, omentin, leptin and adiponectin have been associated with endothelial activation, atherosclerosis and/or risk of plaque rupture (Robinson *et al*., 2017; Gunter *et al*., 2017; Dessein *et* al., 2013; Kang *et* al., 2013; Dessein *et al*., 2014a; 2014b; 2014c; 2014d). Nevertheless, several confounding factors including population specific characteristics, genetics, ethnicity, gender, and other cardio-metabolic risk factors impact the relationships between adipokines and cardiovascular disease risk markers and markers of endothelial function and atherosclerosis (Gunter *et al.,* 2017; Robinson *et al*., 2017; Dessein *et al*., 2014a; 2014c; Dessein *et* al., 2013). This suggests that the pathophysiological context needs to be considered when adipokines are employed to enhance cardiovascular risk stratification in RA. Moreover, the evidence that adipokine concentrations are associated with other subclinical cardiovascular disease markers are scarce (Gunter *et al*., 2018; Turkyilmaz *et al.*, 2013). Hence, the association between adipokines and arterial function and LV diastolic function requires elucidation.

Although several of the abovementioned adipokines have been implicated in RA disease pathogenesis and CVD risk, several adipokines have gained attention in recent literature. In this regard, the adipokines nesfatin, visfatin and vaspin have been identified as novel biomarkers of vascular and cardiac function in the general population. Moreover, their expression in RA seem to be altered compared to the general population. The involvement of these adipokines in CVD risk in RA is currently not clear. *Therefore, in the present thesis I aimed to determine the contribution of these adipokines as possible CVD risk stratification markers in patients with RA.* The section below will briefly highlight what is currently known about the adipokines nesfatin, visfatin and vaspin.

1.4.1 Nesfatin

First described by Oh-I *et al* (2006), nesfatin is an anorexigenic peptide secreted by the hypothalamic nuclei. Evidence suggest that nesfatin is involved in energy homeostasis and appetite suppression, independent of the leptin pathway (Yosten & Samson 2012; Goebel-Stengel *et al.*, 2011; Shimizu *et al.*, 2009). In addition to the regulation of feeding behaviour, nesfatin is an important mediator of body fluid regulation (Yosten & Samson, 2010; Yosten & Samson, 2009) with central nervous system secretion involving the hypothalamus and brainstem (Oh-I *et al.*, 2006). Expression of Nucleobindin2 (NUCB2), a precursor peptide for nesfatin-1, has also been reported in gastric mucosa, adipose tissue and human pancreatic islets (Prinz *et al.*, 2016; Ishida *et al.*, 2012; Riva *et al.*, 2011 Stengel, 2009). Although this adipokine has recently been implicated as an important mediator of cardiometabolic pathways, the mechanisms involved are contradictory. In this regard, nesfatin concentrations are reported to be decreased in patients with acute myocardial infarctions (MI) when compared to the plasma nesfatin concentrations of patients with stable angina pectoris and coronary artery disease (Dai *et al*., 2013). Nesfatin-1 levels are also inversely associated with coronary artery stenosis and plaque morphology features (Kuyumcu, 2018). In diabetic patients with peripheral arterial disease, nesfatin concentrations were inversely correlated to disease progression and severity, suggesting that nesfatin may be protective against atherosclerotic pathogenesis (Ding, 2015). Furthermore, animal studies suggested that nesfatin is involved in the pathogenesis of vascular remodelling (Mori *et al.,* 2018; Zhang *et al*., 2018). However, results are contradictory (Mori *et al.,* 2018; Zhang *et al*., 2018). Therefore, the exact involvement of nesfatin in the development of CVD remains uncertain and requires further investigation.

In patients with osteoarthritis, there is controversy whether nesfatin is pro- or anti-inflammatory (Recinella *et al.,* 2020; Carrión *et al*., 2019). Furthermore, in osteoarthritis it has been proposed that nesfatin may reduce cardiovascular risk (Carrión *et al.,* 2019). In RA, one study has shown that nesfatin concentrations are associated with increased disease severity and inflammatory profiles (Kvlividze *et al.*, 2019), but this has not been confirmed by others (Naghashian *et al.,* 2019). Furthermore, the role of nesfatin-1 in cardiovascular disease risk among RA patients is currently uncertain.

1.4.2 Visfatin

Visfatin was initially identified as pre-B-cell colony enhancing factor (PBEF) and is alternatively referred to as NAMPt, for the functional homology with nicotinamide phosphoribosyltransferase (Al-Suhami & Shehad, 2013; Samal *et al*., 1994). Expression of visfatin is not limited to subcutaneous and visceral adipocytes. In addition, visfatin is expressed in activated inflammatory cells, cardiomyocytes as well as in perivascular and epicardial adipose tissue (Romacho *et al*., 2013). Indeed, several studies have

shown associations between visfatin and inflammatory and oxidative stress markers in various population groups (Hognogi & Simiti, 2016; Mattu & Randeva, 2013; Erten *et al.*, 2008). However, the evidence that visfatin is associated with cardiovascular disease risk markers are contradictory. In patients with metabolic syndrome, visfatin has been implicated in cardio-metabolic manifestations (Takebayashi *et al.,* 2007; Chen *et al*., 2006). Increased concentrations of extracellular visfatin has been correlated with vascular smooth muscle cell proliferation (Wang *et al.,* 2009) and with mediating plaque instability, and hence increasing the risk for ischaemic heart disease (Liu *et al*., 2009). Some suggest there is an association between visfatin and increased carotid artery stiffness (La Favor *et al.,* 2011), while others reported no association between visfatin and markers of arterial function, including pulse wave velocity (Kato *et al.,* 2009). In addition, visfatin has been associated with cardiac remodelling, possibly via the activation of the renin-angiotensin system (RAS) (Malavazos *et al.,* 2008; Huang *et al.*, 2011). Furthermore, it has been shown that cardiomyocytes can secrete Visfatin/Nampt when stressed and that exogenous Nampt contributes to cardiac to hypertrophy and fibrosis (Pillai *et al.,* 2013). In contrast, in patients with end-stage renal dysfunction, visfatin was not associated with left ventricular mass, however, it was associated with impaired left ventricular diastolic function (Erten *et al.*, 2008). In the elderly, visfatin was associated with chronic heart failure (Wang *et al.,* 2014). The contrition to remodelling was mediated through calcineurin/ nuclear factor of activated T-cell (NFAT) signalling pathway (Pillai *et al.,* 2013).

In RA, circulating visfatin concentrations are increased compared to controls and are associated with RA disease activity (Lee & Bae, 2018; Otero *et al*., 2006). However, one study showed that visfatin concentrations are not associated with inflammation or cardiovascular disease risk factors in treated RA patients (Gonzalez-Gay *et al.*, 2010). Nevertheless, the role of visfatin in subclinical cardiovascular disease in RA is currently uncertain and requires elucidation (Franco-Trepat *et al.*, 2019).

1.4.3 Vaspin

Vaspin, visceral adipose tissue-derived serpin or sometimes referred to as serpinA12, has been recognised as a serine protease inhibitor (serpine), with a 40% homology with alpha-1 antitrypsin (Hida *et al.*, 2005). The potential antiprotease properties of this serpine has previously been demonstrated in a type 2 diabetes mellitus animal model in Otsuka Long-Evans Tokushima fatty (OLETF)-rats, where vaspin levels were positively correlated with the presence of type 2 diabetes, abdominal obesity, insulin resistance and dyslipidaemia (Li *et al*., 2011; Hida *et al*., 2005). Vaspin levels were increased when obesity and insulin levels peaked but decreased with worsening of diabetes and body weight reduction (Hida *et al*., 2005). In the same study, insulin therapy resulted in increased vaspin levels, suggesting that vaspin is protective in states of metabolic disease. Human expression of vaspin is reported in both visceral and subcutaneous adipose tissue with the liver as primary site of production

(Escote *et al.*, 2017). Other organs expression vaspin include the pancreas, skin, skeletal muscle and hypothalamus (Nicholson *et al*., 2019; Escote *et al.*, 2017). Klöting *et al*. (2006) reported that vaspin is related to obesity and insulin resistance, and that it is not expressed in lean individuals with a BMI<25 kg/m^2. Similarly, others have shown expression of vaspin is increased in subcutaneous adipose tissue of older, obese patients compared to aged-matched controls (Nicholson *et al*., 2019). A meta-analysis reported a significant association between vaspin concentrations and obesity (Feng *et al*., 2014). The increased vaspin levels in patients with metabolic disease alludes to the potential of this adipokine to serve as a biomarker for cardiovascular disease (Zhou *et al*., 2019; Dimova & Tankova, 2015).

With regard to the effects of vaspin on the cardiovascular system the results are controversial. It has been suggested that vaspin may protect against atherosclerotic disease progression through upregulations of phophoinositide-3-kinase/Akt signalling (Qi *et al.*, 2017; Jung *et al.*, 2011). Vaspin has been described for its anti-apoptotic and anti-atherosclerotic effects, as it limits apoptosis of endothelial cells caused by free fatty acids (Sawicka *et al*., 2016). Furthermore, vaspin inhibits endothelial activation molecules, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin (Jung *et al.,* 2014). Low vaspin levels have been associated with coronary artery disease severity and acute coronary syndrome (Zhang *et al*., 2013; Kobat *et al.*, 2012; Kadoglou *et al.*, 2011; Aust *et al.*, 2009). In contrast, others have suggested increased vaspin concentrations are associated with the presence of coronary artery disease in patients with type 2 diabetes mellitus (Hao *et al*., 2016). Moreover, despite reported associations between vaspin levels carotid-IMT and plaque (Esaki *et al.*, 2014; Karbek *et al.*, 2014; Li *et al.*, 2012) evidence on whether serum vaspin levels are increased or decreased in acute settings is contradictory (Karbek *et al*., 2014; Choi *et al*., 2011). The association of vaspin with arterial stiffness and LV diastolic dysfunction is not well documented. In this regard, only one study, in postmenopausal women reported an association between vaspin concentrations and pulse wave velocity (Tanna *et al*., 2017).

In RA, some have reported that serum vaspin concentration are higher than in healthy controls and associated with inflammatory markers (Ozgen *et al*., 2010). In contrast others have shown no association between serum vaspin and inflammatory markers (Senolt *et al*., 2010). Taken together, it is unclear whether vaspin is protective or detrimental for the development of CVD. Furthermore, despite the associations between vaspin and atherosclerosis in other population groups, this has not been reported in RA (Ozgen *et al*., 2010). Further investigations to determine the role of vaspin in subclinical cardiovascular disease in RA are warranted. *Therefore, in this study I aimed to determine the association between adipokine concentrations and cardiovascular disease risk factors, rheumatoid characteristics, and markers of subclinical cardiovascular disease, including atherosclerosis, arterial function, and LV diastolic function.*

1.5 Problem statement

Patients with RA are at increased risk for cardiovascular mortality. Several sub-clinical cardiovascular diseases, including atherosclerosis, arterial stiffness and LV diastolic dysfunction contribute to the development of cardiovascular disease and ultimately cardiovascular mortality in RA. Although traditional risk factors contribute to the increased prevalence of these sub-clinical cardiovascular diseases, inflammation plays a key role in the pathogenesis of these conditions. Hence, currently recommended cardiovascular risk stratification tools that are largely based on traditional cardiovascular disease risk factors, have major limitations when used to predict CVD risk in RA patients. Therefore, identification of useful biomarkers to enhance CVD risk stratification in this context is required.

The role of adipokines in the pathogenesis and risk stratification of CVD has received considerable attention. Several adipokines have been associated with cardiovascular events and subclinical cardiovascular disease in the general population and several clinical populations. However, the production and mechanisms of action of adipokines may be of particular importance in RA, as the disease is characterized by high levels of inflammation, altered fat metabolism and fat distribution and a high prevalence and earlier onset of cardiovascular disease. Indeed, adipokine concentrations and expressions are different in RA compared to the general population. Given the influence of adipokines in cardiovascular disease risk and the complex interplay between inflammation, fat metabolism and cardiovascular disease in RA, identifying useful biomarkers to enhance cardiovascular disease risk stratification in RA warrants investigation. Moreover, several confounding factors have shown to impact the relationship between adipokines and CVD risk. Therefore, an investigation into the factors that impact the relationship between adipokines and CVD, specifically in this context, could further strengthen risk stratification in RA.

1.6 Aims

The aims of this thesis were to determine the associations between the adipokines nesfatin, visfatin and vaspin in relation to:

- traditional cardiovascular disease risk factors and RA specific characteristics.
- markers of endothelial activation, atherosclerosis, and plaque stability mediators, independent of traditional CVD risk factors and RA characteristics.
- markers of arterial function including arterial stiffness (pulse wave velocity), wave reflection (reflected wave pressure, reflection magnitude and augmentation index), pressure pulsatility

(aortic systolic and pulse pressure, pulse pressure amplification and peripheral pulse pressure) and forward wave pressure, independent of traditional CVD risk factors and RA characteristics.

- markers of left ventricular geometry and systolic and diastolic function, independent of traditional CVD risk factors and RA characteristics.

Chapter 2: Nesfatin-1 and visfatin expression is associated with reduced atherosclerotic disease risk in patients with rheumatoid arthritis

The data in this chapter have been published as:

Robinson, C., Tsang, L., Solomon, A., Woodiwiss, A. J., Gunter, S., Mer, M., Hsu, H. C., Gomes, M., Norton, G. R., Millen, A., & Dessein, P. H. (2018). Nesfatin-1 and visfatin expression is associated with reduced atherosclerotic disease risk in patients with rheumatoid arthritis. *Peptides*, *102*, 31–37.

2.1 Abstract

Nesfatin is an anti-inflammatory molecule that reduces atherosclerotic cardiovascular risk. Visfatin has pro-inflammatory properties and is pro-atherogenic. The study examined the potential impact of nesfatin and visfatin on subclinical cardiovascular disease in 232 (113 black and 119 white) consecutive rheumatoid arthritis (RA) patients from 2 centres. Independent relationships of nesfatin and visfatin concentrations with metabolic risk factors, endothelial activation, carotid atherosclerosis and altered plaque stability were determined in multivariable regression models. Visfatin concentrations were related to diastolic blood pressure (β=4.536, p=0.01) and diabetes prevalence (β=0.092, p=0.04). Nesfatin levels were associated with carotid intima-media thickness (β=-0.017, p=0.008). Nesfatin (β=0.116, p=0.001) and visfatin concentrations (β=0.234, p=0.001) were related to those of matrix metalloproteinase-2 (MMP-2), a plaque stability mediator. Nesfatin and visfatin concentrations were interrelated (Spearman's rho=0.516). Rheumatoid factor (RF) positivity was associated with nesfatin (β=0.650, p<0.0001) and visfatin levels (β=0.157, p<0.03). The nesfatin-MMP-2 and visfatin-MMP-2 relations were both stronger in RF negative compared to RF positive patients (interaction p=0.01 and p=0.04, respectively). Nesfatin is associated with reduced atherosclerosis and increased plaque stability mediator levels in RA. Visfatin is related to adverse cardio-metabolic risk in RA. Increased MMP-2 expression in relation to visfatin may represent a compensatory mechanism aimed at reducing cardiovascular risk in RA.

2.2 Introduction

Rheumatoid arthritis (RA) is a prototypic high-grade inflammatory disease resulting in joint destruction. Patients with RA experience adverse metabolic risk factor profiles with increased endothelial activation (Dessein *et al*., 2005) and atherosclerosis (Ambrosino *et al.*, 2015a) and disease activity related arterial plaque vulnerability to rupture (Semb *et al*., 2007) that translate into a ~50% increase in cardiovascular mortality (Avina-Zubeita *et al.,* 2008). Whereas systemic inflammation is associated with increased cardiovascular risk in RA, the underlying mechanisms remain incompletely understood (Castaneda *et al.,* 2016). In this regard, evidence in support of an involvement of several adipokines in joint inflammation and damage as well as the enhanced cardiovascular risk in RA was reported (Szekanecz *et al.*, 2015).

Nesfatin-1 (nesfatin) was first described by Oh-I and colleagues in 2006 as an anorexigenic molecule that is secreted by the hypothalamus (Oh-I et al., 2006). Nesfatin is a nucleobindin 2 derived peptide that is involved in energy homeostasis and appetite suppression independent of the leptin pathway (Yosten & Samson, 2009). Nesfatin is also secreted by subcutaneous adipose tissue, the gastric mucosa and pancreatic endocrine cells and testes (Ayada *et al*., 2015). Recent studies revealed that nesfatin is an anti-inflammatory molecule that may reduce cardiovascular risk (Ding *et al.,* 2015; Dai *et al.,* 2013). Nesfatin increases free fatty acid utilization (Dong *et al*., 2013) and improves glucose and lipid metabolism (Ayada *et al.*, 2015). Nesfatin concentrations are inversely associated with atherosclerosis extent (Ding *et al.,* 2015; Dai *et al.,* 2013). Nesfatin levels are reduced in patients with acute myocardial infarction but not in those with stable angina (Dai *et al.,* 2013). Low nesfatin concentrations may therefore contribute to plaque vulnerability. In type 2 diabetic patients, nesfatin levels were lower in those with compared to those without peripheral artery disease (Ding *et al.,* 2015). Scotece and colleagues recently reported that nesfatin is expressed in human and murine chondrocytes (Scotece *et al*., 2014). Interestingly, as applies to the effects of the anti-inflammatory adipokine adiponectin in RA (Ehling *et al.*, 2006), nesfatin had pro-inflammatory properties in the Scotece study (Scotece *et al*., 2014). However, to our knowledge, the role of nesfatin in RA and its associated enhanced cardiovascular risk has not been reported.

Visfatin was described as an insulin mimetic molecule by Fukuhara and colleagues in 2005 (Fukuhara *et al.*, 2005a). The respective manuscript was subsequently withdrawn as the glucose lowering effects could not be reproduced (Fukuhara *et al.*, 2005b). Visfatin was identical to the previously reported pre-B cell colony-enhancing factor and to have the same enzymatic activity as nicotinamide phosphoribosyltransferase (NAMPT) (Romacho *et al.*, 2013). Visfatin is expressed in not only visceral adipocytes but also in subcutaneous, perivascular and epicardial fat and activated immune cells

(Romacho *et al*., 2013). Visfatin is a pro-inflammatory molecule (Stofkova, 2010; Moschen *et al.*, 2007) that can enhance cardiovascular risk. In recent studies, visfatin was shown to reduce β cell function (Lopez-Bermejo *et al.*, 2006) and increase insulin resistance and glucose and lipid concentrations (Kieswich *et al.*, 2016; Gouranton *et al*., 2014). Visfatin enhances endothelial activation (Kim *et al.*, 2008), impairs endothelium-dependent relaxation in rat and human mesenteric micro vessels through NAMPT activity (Vallejo *et al*., 2011), is associated with atherosclerosis (El-Shishtawy *et al.*, 2016; Kadoglou *et al.*, 2010) and contributes to arterial plaque vulnerability (Kadoglou *et al.*, 2012; Dahl *et al.*, 2007). A recent meta-analysis confirmed that large circulating visfatin concentrations are associated with excess adiposity, type 2 diabetes, insulin resistance, metabolic syndrome and cardiovascular diseases (Chang *et al.*, 2011).

Visfatin concentrations are increased in RA (Otero *et al.*, 2006). Visfatin contributes to the pathophysiology of RA (Brentano *et al.*, 2007). Visfatin levels were not associated with metabolic risk factors in patients with severe RA undergoing anti-TNF-α-therapy (Gonzalez-Gay *et al.*, 2010). By contrast, in a recent investigation among untreated early RA patients, visfatin concentrations were related to insulin resistance and increased total and LDL cholesterol and triglyceride levels (El-Hini *et al.*, 2013). Visfatin was not associated with coronary artery calcification scores in RA (Rho *et al.*, 2010). In a study that included 29 RA patients, visfatin was not related to carotid intima-media thickness (IMT) (Ozgen *et al.*, 2011). Also, no relationship was found between 2 NAMPT polymorphisms and disease susceptibility and cardiovascular risk in RA (Garcia-Bermudez *et al.*, 2011). The role of visfatin in cardiovascular risk among RA patients requires further investigation.

Herein, the study examined the potential impact of nesfatin and visfatin on metabolic risk factors, endothelial activation, carotid atherosclerosis, and plaque vulnerability mediators in a relatively large bi-ethnic group of patients with established treated RA.

2.3 Methods

2.3.1 Patients

This study was approved by the Human Research Ethics Committee (Medical) (approval number: M06-07-33) and performed according to the principles outlined in the Helsinki declaration. Charlotte Maxeke Johannesburg Academic Hospital and Milpark Hospital in Johannesburg, South Africa served as recruiting centres for 232 (113 black, 119 white) consecutive patients. All participants met the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA (Aletaha *et al*., 2010). Informed, written consent was obtained from each patient.

2.3.2 Patient characteristics and conventional metabolic risk factors

Previously described methods were employed to record patient characteristics as well as conventional metabolic risk factors (Dessein *et al.*, 2014b). These included demographic characteristics, lifestyle factors, anthropometric measures, blood pressure variables, lipid levels, glucose metabolism parameters, kidney function, RA characteristics and medication use. In brief, anthropometric measures were recorded according to standard protocols. Body mass index (BMI), waist circumference and waist to hip ratio were employed as indicators of generalized and abdominal adiposity, and fat distribution, respectively. Disease activity was assessed by the Clinical Disease Activity Index (CDAI) and Disease Activity Score in 28 joints (DAS28). Disease severity markers included the number of deformed joints and rheumatoid factor (RF) status. Glomerular filtration rate (GFR) was estimated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. Cardiovascular drug and synthetic disease modifying agent use was recorded.

Metabolic risk factors included systolic and diastolic blood pressure, high-density lipoprotein, low-density lipoprotein, triglyceride concentrations, lipid ratios and glucose levels. Patients with an average systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure of ≥ 90 mmHg and/or current antihypertensive medication use were diagnosed with hypertension. Dyslipidaemia was considered present when the cholesterol/HDL ratio was $\underline{\geq}$ 4. Diabetes was defined as a fasting plasma glucose concentration ≥ 7 mmol/l or/and the use of glucose lowering agents.

2.3.3 Endothelial activation and plaque vulnerability mediators

Solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine HS, R&D Systems, Inc., Minneapolis, MN, USA) were used to measure endothelial activation and plaque vulnerability markers. Endothelial activation molecules comprised soluble E-selectin, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and monocyte chemoattractant protein 1 (MCP-1), angiopoietin 2 and asymmetric dimethylarginine (ADMA). Their lower detection limits were 0.009 ng/l, 0.6 ng/l, 0.096 ng/l, 5.0 pg/ml, 1.2 pg/ml, and 0.05 µmol/l respectively; their inter-assay and intra-assay coefficients of variation were 7.9 and 5.8, 7.0 and 3.1, 5.5 and 4.6, 5.7 and 5.8, 8.9 and 5.9, and 7.8 and 8.9%, respectively.

Altered plaque vulnerability mediator molecules comprised matrix metalloproteinases (MMP)-2, -3 and -9. Their lower detection limits were 3.5 ng/ml, 0.3 pg/ml and 10 pg/ml, respectively; the inter-assay and intraassay coefficients of variation were 10.0 and 12.0%, respectively, for each of the 3 measurements.

2.3.4 Atherosclerosis

Carotid artery ultrasound (US) measurements were made in accordance with the Mannheim consensus on carotid ultrasound (Touboul *et al.*, 2007). High-resolution B-mode ultrasound (Image Point, Hewlett Packard and SonoCalc IMT, Sonosite Inc.) was performed using linear array 7.5 MHz probes. Carotid intima-media thickness (IMT) was measured by obtaining images over at least 1 cm of the distal common carotid arteries and 1 cm proximal to the carotid bifurcation. Arterial plaque was identified in the extra-cranial carotid tree (common and internal and external carotid arteries as well as the bulb) and defined as a focal structure that encroaches into the arterial lumen of at least 0.5mm or 50% of the surrounding intima-media thickness value, or demonstrates a thickness of > 1.5 mm as measured from the media-adventitia to the intima-lumen interface. Ultrasound was performed by two experienced operators that were blinded to the clinical profiles of the patients. The intra-observer coefficients of variation were 5.8% and 4.1% (for the operator in private and public sector, respectively); the inter-observer coefficient of variation was 8.0% for measurements made by two operators in 23 randomly selected patients.

2.3.5 Nesfatin-1 and visfatin

Nesfatin-1 and visfatin concentrations were determined by a solid-phase sandwich ELISA (QuantikineHS, R&D Systems, Inc., Minneapolis, MN, USA). The lower detection limit was 31.3 pg/ml for nesfatin-1 with inter-assay and intra-assay coefficients of variation <10 % and <8%, respectively. The lower detection limit for visfatin was 0.778 ng/ml with inter-assay and intra-assay coefficients of variation <15 % and <10%, respectively.

2.3.6 Data management and analysis

Continuous variables were expressed as mean (SD), or median (interquartile range (IQR)) when non-normally distributed. Non-normally distributed characteristics were also logarithmically transformed prior to their inclusion in multivariable statistical analysis. Categorical variables were expressed as proportions or percentages.

The relationships of age at disease onset, age at time of the study, gender and population origin with nesfatin and visfatin concentrations were determined in a mixed regression model. Associations of other patient characteristics with nesfatin and visfatin levels were assessed in demographic characteristic adjusted models.

The independent relationships of nesfatin-1 and visfatin concentrations with metabolic risk factors, atherosclerosis and MMP levels were evaluated in multivariate linear or logistic regression models with adjustment for potential confounders and/or mediators as identified in the previous analysis.

Amongst patients with RA, those with severe disease are particularly at high risk for CVD. In this regard, the adipokine effects on cardiovascular risk depend on pathophysiological context which includes not only disease characteristics but also adiposity status and population origin in RA patients. We therefore determined the impact of the respective patient characteristics on nesfatin and visfatin concentration-subclinical CVD relations by adding appropriate interaction terms and their individual components to the models. Further, the interaction of visfatin-nesfatin concentrations with subclinical CVD, was assessed. Statistical computations were made using IBM SPSS statistics (version 23.0, IBM, USA). Statistical significance was set as $p < 0.05$.

2.4 Results

2.4.1 Patient characteristics

Descriptive statistics of the recorded patient characteristics are given in Table 2.1. The mean (±SD) age at time of the study was 57.1 (10.8) years with a mean disease duration of 13.6 (9.3) years. The median (interquartile range) CDAI was 7 (2-14). All patients received synthetic disease modifying agents. Of the participants, 59.3% were hypertensive and 18.2% had dyslipidaemia; 40.3% had carotid artery plaque.

2.4.2 Associations of patient characteristics with adipokine concentrations

Table 2.2a and 2.2b give the associations of patient characteristics with nesfatin-1 and visfatin concentrations, respectively, at $p \leq 0.15$. This was done to identify potential confounding variables in subsequent analyses. In demographic feature adjusted models, black population origin (β (SE) = 0.313 (0.124), p = 0.01), rheumatoid factor positivity (β (SE) = 0.650 (0.140), $p < 0.0001$), prednisone use (β (SE) = 0.925 (0.388), p = 0.02) and insulin therapy (β (SE) = -0.019 (0.009), p = 0.04) were significantly associated with nesfatin concentrations. The median (IQR) nesfatin concentrations were 4.9 (0.1-12.5) ng/ml in black and 7.6 (1.3-18.3) ng/ml in white Africans with RA, and 1.1 (0.1-7.2) ng/ml in rheumatoid factor negative and 8.3 (1.3-18.2) ng/ml in rheumatoid factor positive patients. None of the traditional cardiovascular risk factors were associated with nesfatin. Age at time of the study (β (SE) = 3.792 (1.511), p = 0.01) and rheumatoid factor positivity (β (SE) = 0.157 (0.071), p=0.03) were associated with visfatin levels. The median (IQR) visfatin concentrations were 39.0 (23.8-63.9) ng/ml in rheumatoid factor negative and 65.7 (38.1-94.7) ng/ml in rheumatoid factor positive patients with RA.

Table 2.1. Recorded characteristics in 232 patients with RA

Demographic characteristics		**DAS28**	**2.9 (1.7)**
Age at disease onset, years	43.5 (13.0)	Clinical Disease Activity Index	7 (2-14)
Age at study time, years	57.1 (10.8)	Erythrocyte sedimentation rate, mm/hr	12 (5-27)
Female gender, %	83.5	C-reactive protein, g/l	5.3 (2.2-12.5)
Black, %	48.3	Interleukin 6, pg/ml	3.5 (2.2-5.9
White, %	51.7	Leukocytes, n/nl	5.8 (4.8-7.6)
Lifestyle factors		Deformed joints, number	9 (9)
Exercise, %	36.8	Extra-articular manifestations, %	7.6
Alcohol use, %	20.9	**Disease modifying agents**	
Current smoking, %	6.8	Methotrexate, %	84.3
Anthropometry		Chloroquine, %	66.1
Body mass index, kg/m^2	27.5 (6.0)	Leflunomide, %	29.7
Waist circumference, cm	91 (13)	Sulphasalazine, %	19.1
Waist to hip ratio	0.86 (0.80-0.91)	Azathioprine, %	14.8
Metabolic risk factors		Tetracycline, %	11.9
Hypertension, %	59.3	Cyclophosphamide. %	3.4
Systolic BP, mm Hg	134 (21)	Penicillamine, %	3.0
Diastolic BP, mm Hg	82 (13)	**TNF- α blockers, %**	3.8
Total cholesterol, mmol/l	4.8 (1.0)	**Prednisone, %**	2.5
HDL cholesterol, mmol/l	1.5 (1.3-1.9)	**Non-steroidal anti-inflammatory agents, %**	17.7
LDL cholesterol, mmol/l	2.7 (0.8)	**Endothelial activation markers**	
Triglycerides, mmol/l	1.0 (0.-1.4)	E-selectin, ng/ml	39.2 (18.6)
Total C – HDL C ratio	3.1 (2.5-3.6)	ICAM-1, ng/ml	291 (208-352)
Total C – HDL C ratio ≥ 4, %	18.2	VCAM-1, ng/ml	891 (669-1040)
Non-HDL cholesterol, mmol/l	3.2 (0.9)	MCP 1, pg/ml	544 (274-680)
Diabetes, %	12.3	Angiopoietin 2, pg/ml	2.86 (2.05-3.34)
Glucose, mmol/l	4.7 (4.4-5.2)	Asymmetric dimethylarginine, µmol/l	0.70 (0.50-0.80)
CKD-EPI (ml/min/1.73m^2)	95 (20)	**Plaque vulnerability markers**	
Framingham Risk Score	5.3 (6.7)	MMP-2, ng/ml	1409 (604-2369)
Cardiovascular agent use		MMP-3, pg/ml	10.8 (4.2-23.5)
Antihypertensives, %	30.1	MMP-9, ng/ml	4608(3413-6842)
Statins, %	28.4	**Carotid atherosclerosis**	
Ezetimibe, %	0.8	Intima-media thickness, mm	0.702 (0.119)
Insulin, %	1.7	Plaque, %	40.3
RA characteristics		**Adipokines**	
Disease duration, years	13.6 (9.3)	Nesfatin, ng/ml	6.6 (0.1-15.0)
HAQ-DI	0.38 (0.00-0.88)	Visfatin, ng/ml	60.2 (32.5-86.8)
Rheumatoid factor positive, %	75.7		

Continuous variables expressed as mean (SD) or median (interquartile range) as appropriate. RA: rheumatoid arthritis; BMI: body mass index; BP: blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein; C: cholesterol; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; HAQ-DI: Stanford Health Assessment Questionnaire Disability Index; DAS28: Disease Activity Score in 28 joints; TNF-α: tumour necrosis factor-alpha; ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; MCP: monocyte chemoattractant molecule; MMP: matrix metalloproteinase.

Table 2.2a. Associations of patient characteristics with **nesfatin*** concentrations at p ≤ 0.15

Characteristics	**β (SE)**	**P**
Demographic characteristics		
Age at time of the study	-0.579 (0.751)	0.44
Age at disease onset	-0.002 (0.005)	0.82
Female sex	0.249 (0.169)	0.14
Black	0.313 (0.124)	**0.01**
Rheumatoid arthritis features		
Rheumatoid factor positive	0.650 (0.140)	**<0.0001**
Erythrocyte sedimentation rate*	0.246 (0.148)	**0.10**
Leukocytes*	0.050 (0.027)	**0.06**
Treatment		
Prednisone	0.925 (0.388)	**0.02**
Insulin	-0.019 (0.009)	**0.04**

Associations were assessed in demographic characteristic adjusted models. β: regression coefficient; SE: standard error. Significant associations are shown in bold. * Logarithmically transformed.

Table 2.2b. Associations of patient characteristics with **visfatin*** concentrations at p ≤ 0.15

Characteristics	**β (SE)**	**P**
Demographic characteristics		
Age at time of the study	3.792 (1.511)	**0.01**
Age at disease onset	2.678 (1.816)	0.14
Female sex	-0.004 (0.083)	0.97
Black	-0.037 (0.061)	0.55
Body mass index	-0.009 (0.005)	0.11
Rheumatoid arthritis features		
Rheumatoid factor positive	0.157 (0.071)	**0.03**
Deformed joints*	0.103 (0.060)	0.08
Treatment		
Cyclophosphamide	-0.304 (0.168)	0.07
Insulin	-0.409 (0.234)	0.08
Metabolic risk factors		
Diastolic blood pressure	4.536 (1.697)	**0.01**
Diabetes	0.092 (0.045)	**0.04**

Relationship of visfatin with metabolic risk factors assessed in models adjusted for: age, sex, race, body mass index, rheumatoid factor positivity, deformed joints, cyclophosphamide, and insulin treatment. β: regression coefficient; SE; standard error. Significant associations shown in bold. * Logarithmically transformed.

2.4.3 Associations of nesfatin and visfatin concentrations with metabolic risk factors

Nesfatin concentrations were not related to metabolic risk factors. As also shown in Table 2.2b, in potential confounder adjusted models, visfatin levels were associated with diastolic blood pressure (ß (SE) = 4.536 (1.697) p = 0.01) and diabetes (β (SE) = 0.092 (0.045), p = 0.04). The median (IQR) nesfatin concentrations were 59.6 (32.6-85.7) ng/ml in patients without and 76.5 (29.5-103.2) ng/ml in those with diabetes.

2.4.4 Independent associations of nesfatin and visfatin concentrations with endothelial activation, plaque vulnerability mediators and atherosclerosis.

As given in Table 2.3, neither nesfatin nor visfatin concentrations were associated with endothelial activation markers in univariate and multivariable adjusted analysis. In univariate analysis, nesfatin levels were directly associated with MMP-2 (β (SE) = -0.499 (0.134), p < 0.0001) and inversely with MMP-3 (β (SE) = -0.255 (0.105), p = 0.02) and carotid IMT (β (SE) = -0.022 (0.008), p = 0.007). In potential confounder adjusted models, the relationships of nesfatin concentrations with those of MMP-2 (β (SE) = 0.116 (0.033), p = 0.001) and carotid IMT (β (SE) = -0.017 (0.008), p = 0.04) remained significant. Upon additional adjustment for CDAI or waist circumference, nesfatin levels remained related to those of MMP-2 (β (SE) = 0.117 (0.034), p = 0.001 and β (SE) = 0.014 (0.001), p = 0.05, respectively). Visfatin concentrations were directly associated with those of MMP-2 in both univariate (β (SE) = 0.204 (0.063), p = 0.002) and multivariable analysis (β (SE) = 0.234 (0.067), p = 0.001). Upon additional adjustment for CDAI or waist circumference, visfatin levels remained related to those of MMP-2 (β (SE) = 0.236 (0.067), p = 0.001 and β (SE) = 0.226 (0.067), p = 0.001, respectively).

Visfatin concentrations were directly associated with those of MMP-2 in both univariate (β (SE) = 0.204 (0.063), p = 0.002) and multi-variable analysis (β (SE) = 0.234 (0.067), p = 0.001). Upon additional adjustment for CDAI or waist circumference, Visfatin levels remained related to those of MMP-2 (β (SE) = 0.236 (0.067), p = 0.001 and β (SE) = 0.226 (0.067), p = 0.001, respectively).

Table 2.3. Relationships of nesfatin and visfatin concentrations with those of endothelial activation markers and matrix metalloproteinases and atherosclerosis

	Nesfatin*				**Visfatin***			
	Univariate		**Multivariate**		**Univariate**		**Multivariate**	
Characteristics	**β (SE)**	**P**	**β (SE)**	**P**	**β (SE)**	**P**	**β (SE)**	**p**
Endothelial activation								
E-selectin	0.004 (0.003)	0.22	0.298 (1.422)	0.84	-0.002 (0.002)	0.32	-2.928 (2.895)	0.31
ICAM-1*	-0.141 (0.374)	0.71	0.000 (0.013)	0.98	0.181 (0.188)	0.34	0.004 (0.026)	0.89
VCAM-1*	0.213 (0.347)	0.63	-0.009 (0.011)	0.68	0.069 (0.221)	0.76	-0.015 (0.023)	0.51
MCP-1*	0.031 (0.235)	0.89	-0.008 (0.021)	0.69	0.136 (0.118)	0.25	0.014 (0.043)	0.75
Angiopoietin 2*	0.378 (0.310)	0.22	0.143 (0.319)	0.66	0.199 (0.152)	0.19	0.133 (0.156)	0.40
ADMA*	0.148 (0.397)	0.71	0.012 (0.012)	0.33	0.242 (0.194)	0.22	0.038 (0.024)	0.11
Plaque vulnerability								
MMP-2*	**0.497 (0.134)**	**<0.0001**	**0.116 (0.033)**	**0.001**	**0.204 (0.063)**	**0.002**	**0.234 (0.067)**	**0.001**
MMP-3*	**-0.255 (0.105)**	**0.02**	-0.070 (0.039)	0.08	-0.082 (0.083)	0.32	0.219 (0.165)	0.19
MMP-9*	0.357 (0.270)	0.19	0.014 (0.018)	0.56	-0.016 (0.034)	0.65	-0.073 (0.069)	0.83
Atherosclerosis								
C-IMT	**-0.022 (0.008)**	**0.007**	**-0.017 (0.008)**	**0.04**	-0.008 (0.017)	0.62	-0.025 (0.016)	0.11
	OR (95% CI)		**OR (95% CI)**		**OR (95% CI)**		**OR (95% CI)**	
Plaque	0.789 (0.599-1.040)	0.09	0.808 (0.579-1.127)	0.21	1.263 (0.706-2.257)	0.43	0.981 (0.468-2.058)	0.96

Associations of nesfatin concentrations with endothelial activation, plaque vulnerability and atherosclerosis were assessed in age, gender, black, rheumatoid factor positive, erythrocyte sedimentation rate, leukocyte count and prednisone and insulin treatment adjusted models. Relationships of visfatin levels with endothelial activation, plaque vulnerability and atherosclerosis were determined in age, gender, black, BMI, rheumatoid factor positive, deformed joint count, cyclophosphamide and insulin treatment, diabetes and diastolic blood pressure adjusted models β: regression coefficients; SE: standard error; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular adhesion molecule 1; MCP-1: monocyte chemoattractant protein 1; ADMA: asymmetric dimethylarginine; MMP: matrix metalloproteinase; C-IMT: carotid intima media thickness. Significant relationships are shown in bold. *Logarithmically transformed.

2.4.5 Impact of patient characteristics on the associations of nesfatin and visfatin concentration with those of plaque stability mediators and carotid IMT

Population origin and anthropometric measures did not impact on the associations of nesfatin and visfatin concentrations with those of plaque stability mediators and carotid IMT. Among RA characteristics, rheumatoid factor positivity influenced nesfatin-MMP-2 (interaction p = 0.01) and visfatin-MMP2 (interaction p = 0.04) relationships. However, as shown in Table 2.4, nesfatin and visfatin concentrations were each independently associated with those of MMP-2 in rheumatoid factor negative (β (SE) = 0.155 (0.074), p = 0.04 and β (SE) = 0.615 (0.197), p = 0.003, respectively) as well as rheumatoid factor positive patients (β (SE) = 0.098 (0.04), p = 0.01) and (β (SE) = 0.163 (0.073), p = 0.02, respectively).

The concentrations of nesfatin and visfatin were strongly correlated (Spearman's rho = 0.516, p < 0.0001). However, the interaction term nesfatin x visfatin was not independently associated with endothelial activation, plaque stability mediator concentrations and atherosclerosis.

Table 2.4. Independent relationships of nesfatin and visfatin concentrations with those of matrix metalloproteinase-2 among rheumatoid factor negative and positive patients

		Matrix metalloproteinase 2*			
		Nesfatin*		**Visfatin***	
Group	**n**	**β (SE)**	**P**	**β (SE)**	**P**
Rheumatoid factor negative	176	0.155 (0.074)	0.04	0.615 (0.197)	0.003
Rheumatoid factor positive	56	0.098 (0.039)	0.01	0.163 (0.073)	0.02

Associations of nesfatin concentrations with those of matrix metalloproteinase-2 were assessed in age, gender, black, rheumatoid factor positive, erythrocyte sedimentation rate, leukocyte count and prednisone and insulin treatment adjusted models. Relationships of visfatin levels with those of matrix metalloproteinase-2 were determined in age, gender, race, BMI, rheumatoid factor positive, deformed joint count, cyclophosphamide and insulin treatment, diabetes and diastolic blood pressure adjusted models. ß: regression coefficients; SE: standard error. *Logarithmically transformed.

2.5 Discussion

In the present study, we report for the first time that in patients with established treated RA, (1) visfatin concentrations are independently associated with increased diastolic blood pressure and diabetes, (2) nesfatin concentrations are related to reduced carotid IMT and nesfatin and visfatin levels are directly associated with those of MMP-2. Further, nesfatin and visfatin levels were strongly interrelated (Spearman's rho = 0.516, $p < 0.0001$). MMP concentrations are increased in obesity and patients with active RA (Traurig *et al*., 2006). The nesfatin-MMP-2 and visfatin-MMP-2 associations were independent of adiposity and RA activity in the present study.

We recently reported that apelin, which is another anti-inflammatory adipokine, is associated with increased MMP-2 and reduced MMP-9 concentrations in RA (Gunter *et al*., 2017). MMP-2 increases smooth muscle migration and proliferation that results in enhanced fibrous cap formation and thereby reduces plaque vulnerability to rupture (Newby, 2015; Nagase *et al.*, 2006; Watanabe & Ikeda, 2004). Congruently, MMP-2 concentrations are larger in stable compared to unstable arterial plaques. By contrast, MMP-3 and MMP-9 are implicated in increased plaque vulnerability (Newby, 2015; Nagase *et al.*, 2006; Watanabe & Ikeda, 2004). In this regard, omentin that is another anti-inflammatory adipokine, was associated with reduced MMP-3 levels in RA (Robinson *et al.*, 2017). Taken together, our previous studies and current results indicate that anti-inflammatory adipokines may exert a plaque stabilizing effect in RA, which is in line with previous reports in non-RA subjects.

Nesfatin was also inversely associated with carotid IMT in the present study. This further supports a potential protective effect of nesfatin against cardiovascular disease in patients with RA. Carotid IMT and plaque constitute different atherosclerotic phenotypes (Spence *et al.,* 2004). Arterial intima-media thickening represents mostly tunica media vascular smooth muscle hypertrophy in response to blood pressure and is associated with stroke risk factors and events. In contrast, arterial plaque indicates the presence of severe intimal atherosclerosis that is more strongly related to coronary artery disease and its risk factors. Whether nesfatin protects particularly against cerebrovascular disease in RA merits further investigation.

In contrast to nesfatin concentrations, those of visfatin were associated with metabolic risk factors including diastolic blood pressure and diabetes. An association between visfatin polymorphisms and diastolic blood pressure was reported in both schoolchildren and obese children (Korner *et al.*, 2007). The relationship between visfatin concentrations and diabetes is well documented in non-RA subjects (Chang *et al*., 2011).

Given that visfatin is strongly implicated in increased plaque instability (Kadoglou *et al*., 2012; Dahl *et al.,* 2007) our finding of a direct relationship between concentrations of visfatin and those of MMP-2 was unexpected. Interestingly in this regard, whereas nesfatin and visfatin reportedly had overall contrasting effects on inflammation and cardiovascular disease risk in non-RA studies, we found that the concentrations of these 2 molecules were also directly correlated in RA. Further, rheumatoid factor positivity was associated with increased levels of both nesfatin and visfatin, and the nesfatin-MMP-2 as well as visfatin-MMP-2 relationships were stronger in rheumatoid factor negative compared to rheumatoid factor positive patients. Taken together, previously reported findings on the cardiovascular effects together with our current findings suggest that increased MMP-2 concentrations in relation to those of visfatin may represent a compensatory mechanism aimed at reducing visfatin mediated cardiovascular risk in RA. An association between rheumatoid factor and visfatin was previously reported in RA (Matsui *et al.*, 2008).

A comprehensive panel of subclinical cardiovascular disease risk markers was assessed and adjusted consistently for potential confounders in our analysis. A major limitation of this investigation is that no healthy control group was included. It therefore remains to be determined whether the potential effects of nesfatin and visfatin on atherosclerotic cardiovascular disease risk as identified in our study, are RA specific. Although models were consistently adjusted for sex in multivariable analysis, the fact that only 38 of the participating RA patients were men, may still have impacted the results. The study was cross-sectionally designed and thereby calls for future longitudinal investigations. Circulating concentrations of biomarkers do not necessarily represent their tissue levels.

In conclusion, nesfatin is associated with reduced atherosclerosis and increased plaque stability mediator levels in RA. Visfatin is associated with adverse metabolic risk factors in RA. Increased MMP-2 production in RA may partly occur to compensate for visfatin mediated enhanced cardiovascular risk.

Chapter 3: Associations between vaspin concentrations, vascular remodelling and plaque vulnerability is impacted by cardiovascular disease risk factors in rheumatoid arthritis

3.1 Abstract

Vaspin protects against metabolic risk and atherogenesis. Whether vaspin is associated with metabolic risk factors and cardiovascular disease risk in rheumatoid arthritis requires elucidation. This study aimed to determine the potential impact of vaspin on metabolic risk factors, endothelial activation, carotid atherosclerosis, and plaque vulnerability mediators in 170 RA patients. Vaspin concentrations, endothelial activation marker concentrations including E-selectin, vascular adhesion molecule-1, intracellular adhesion molecule-1, monocyte chemoattractant protein-1, angiopoietin-2 and asymmetric dimethylarginine (ADMA), and plaques stability mediators such as matrix metalloproteinases (MMP) 2, 3 and 9 were assessed using ELISA. Common carotid intima-media thickness (cIMT) and carotid artery plaque were assessed by ultrasound. Relationships between vaspin and endothelial activation, plaques stability mediators and atherosclerosis were identified in multivariable mixed linear regression models. Vaspin concentrations were associated with RF positivity (β (SE) = 0.03 (0.10), p = 0.002). Vaspin was not associated with any cardiovascular disease markers, metabolic risk markers, endothelial activation, or atherosclerosis in both univariate and multivariate analysis in all patients. The relationship between MMP-2 and vaspin concentrations were, however, influenced by the presence of major cardiovascular risk factors (β (SE) = -0.08 (0.03), p = 0.02). In sensitivity analysis, vaspin levels were positively associated with those of MMP-2 in patients with no major cardiovascular risk factors (β (SE) = 0.42 (0.16), p = 0.01), but not in patients where one or more risk factors were present (β (SE) = 0.06 (0.06), p = 0.35). The presence of major cardiovascular risk factors also impacted on the vaspin-angiopoetin-2 relationship, where vaspin levels were positively associated with those of angiopoetin-2 in patients with no major cardiovascular risk factors (β (SE) = 1.13 (0.05), p = 0.02). In conclusion, vaspin concentrations were associated with reduced risk of plaque rupture and vessel stability, but only in those in the early stages of disease and with low cardiovascular disease risk.

3.2 Introduction

Vaspin (visceral adipose tissue-derived serpin) belongs to the family of serine protease inhibitors and display 40% homology with alpha-1 antitrypsin (Hida *et al.*, 2005). Vaspin is expressed in both visceral and subcutaneous adipose tissue and is found in the liver, pancreas, skeletal muscle, skin and hypothalamus (Nicholson *et al*., 2019; Escote *et al.*, 2017). Increasing evidence emphasize the role of vaspin in obesity and related metabolic disorders (Recinella *et al*., 2020). The potential antiprotease properties of this serpine has previously been demonstrated in animal models of obesity and type 2 diabetes mellitus where vaspin levels were positively correlated with metabolic abnormalities (Li *et al*., 2011; Hida *et al*., 2005). Vaspin levels were increased when obesity and insulin concentrations peaked but decreased with worsening of diabetes and body weight reduction (Hida *et al*., 2005). In humans, vaspin concentrations are also associated with obesity and insulin resistance (Feng *et al*., 2014).

Nevertheless, administration of vaspin to animal models of metabolic dysfunction have shown improved glucose tolerance, insulin sensitivity and reduced food intake and reduced expression of other pro-inflammatory molecules and adipokines (Hida *et al*., 2005; Recinella *et al*., 2020). Moreover, insulin therapy resulted in increased vaspin levels, suggesting that vaspin is protective in states of metabolic disease (Hida *et al*., 2005). Considering the association of vaspin with the presence of metabolic disease, the contribution of this adipokine to the development of cardiovascular disease warrants investigation (Dimova & Tankova, 2015; Feng *et al*., 2014).

The outcomes of studies investigating the role of vaspin in CVD, remain inconsistent. Recent evidence suggests that vaspin may play a role in the development of atherosclerosis (Choi *et al.*, 2011). In this regard, vaspin have been associated with the presence of plaque and carotid-IMT (Esaki *et al*., 2014; Karbek *et al*., 2014; Li *et al.*, 2012). In contrast, others have shown serum vaspin concentrations are reduced in patients with coronary artery disease (Kobat *et al*., 2012; Kadoglou *et al*., 2011). It is thought that vaspin might be increased in diseased states to protect against adverse remodelling through the inhibition of endothelial activation molecules. In this regard, vaspin was highly expressed in vascular smooth muscle cells and macrophages in coronary plaque (Sato *et al*., 2018). Evidence suggests that vaspin contributes indirectly to the inhibition of endothelial activation markers by inhibiting NF-kB following AMPK activation (Jung *et al*., 2014). The same study demonstrated that activation of NF-kB, which is induced by TNF- α, can be reduced by administration of vaspin (Jung *et al.*, 2014). Although some have suggestion that vaspin could serve as prognostic marker in patients with acute coronary syndrome (Zhou *et al.*, 2019), other proposed that vaspin cannot be used as a biomarker for diagnosis

of coronary artery disease (Stančík *et al*., 2017). These contrasting finding highlight the need to elucidate the role of vaspin as a biomarker for CVD.

In patients with RA, increased endothelial activation and atherosclerosis result in significantly higher cardiovascular morbidity and mortality rates when compared to a non-RA population (Dessein *et al*., 2015). It is estimated that patients with RA have a 2-fold increased risk for the development of CVD which cannot be fully explained by traditional risk factors (Avina-Zubeita *et al.,* 2008). Indeed, systemic inflammation contribute to the increased CVD risk in RA (Crowson *et al*., 2018). Several adipokines have been implicated in the pathogenesis of RA and have been associated with CVD risk in RA (Recinella *et al*., 2020; Carrión *et al*., 2019). In RA, reports on the contribution of serum vaspin levels to RA are contradictory. Increased vaspin levels have been reported in RA patients (Senolt *et al*., 2010; Ozgen *et al*., 2010). Although some have reported associations between vaspin and RA disease progression (Maijer *et al*., 2015), others have shown no association between vaspin and CRP levels or white cell count (Senolt *et al*., 2010). Moreover, reducing inflammation with anti-inflammatory treatments in RA resulted in increased vaspin levels (Klaasen *et al*., 2012). Whether vaspin is associated with atherosclerotic CVD in RA is currently not known. Therefore, the role of vaspin in CVD risk in RA patients requires further investigation. This study aimed to examine the potential role of vaspin on metabolic risk factors, endothelial activation, carotid atherosclerosis, and plaque vulnerability mediators in a group of RA patients.

3.3 Methods

3.3.1 Patients

This study was conducted according to the principles outlined in the Helsinki declaration. Approval for the study was granted by the Human Research Ethics Committee of the Witwatersrand under the reference number M120562. A total of 170 patients were recruited for the study. All participants provided written informed consent. Study sites included both Charlotte Maxeke Johannesburg Academic Hospital and Milpark Hospital in Johannesburg, South Africa. All participants had established rheumatoid arthritis and met the criteria defined by the European League Against Rheumatism classification criteria for RA (Aletaha *et al*., 2010).

3.3.2 Patient characteristics, conventional metabolic risk factors and RA disease characteristics

Demographic and patient characteristics of patients were recorded, and the presence of metabolic risk factors were evaluated. Age at disease onset and the age at the time of study were documented. For

lifestyle variables, regular exercise, alcohol consumption and smoking were recorded. Body weight and height were obtained using a digital scale and stadiometer, respectively. Body mass index was calculated according to standard approaches. Waist and hip circumference were measured with a tape measure. Brachial blood pressure measurements comprised the average of 3 readings obtained by using an automated ambulatory blood pressure monitor. Standard laboratory blood tests of renal and liver function, haematological parameters, lipids, and glucose were performed. Dyslipidaemia was diagnosed when the atherogenic index, i.e., the cholesterol–HDL cholesterol ratio, was >4, or the use of lipid-lowering agents. Diabetes was considered as the use of glucose-lowering agents or a fasting plasma glucose ≥7 mmol/l.

For rheumatoid disease characteristics, disease activity was estimated by the Disease Activity Score in 28 joints (DAS28) (Prevoo *et al.,* 1995) as well as the Clinical Disease Activity Index (CDAI) (Gulfe *et al.*, 2009). C-reactive protein concentrations were determined by immunoturbidimetric methods. Disease severity was assessed by a rheumatologist by means of deformity joint counts. Rheumatoid factor status and anti-citrullinated protein antibody (ACPA) status were recorded. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula was used to determine estimated glomerular filtration rate (eGFR) as a measure of renal function. The Framingham risk score was calculated to assess the 10-year risk of CVD which included coronary artery disease, stroke, peripheral arterial disease, or heart failure (D'Agostino, 2008). The use of this score in risk stratification of patients with RA, has previously been validated by Dessein *et al.* (2015).

3.3.3 Endothelial activation and plaque vulnerability mediators

Solid-phase sandwich enzyme-linked immunosorbent assays (ELISA) (Quantikine HS, R&D Systems, Inc., Minneapolis, MN, USA) were used to measure endothelial activation and plaque vulnerability markers. Endothelial activation markers included soluble E-selectin, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and monocyte chemoattractant protein 1 (MCP-1). The lower detection limits were 0.009, 0.6, 0.096 and 5.0 pg/ml, respectively. The interassay and intraassay coefficients of variation were 7.9 and 5.8, 7.0 and 3.1, 5.5 and 4.6, and 5.7 and 5.8 %, respectively. In addition to the conventional endothelial activation molecules, asymmetric dimethylarginine (ADMA) and angiopoietin 2 were evaluated as markers of endothelial function and vascular remodeling, respectively. The lower detection limits of ADMA and angiopoietin 2 were 0.05 µmol/l and 1.2 pg/ml, respectively. The interassay and intraassay coefficients of variation were 7.8 and 8.8, and 8.9 and 5.9 %, respectively. A Z-score comprising of all the mentioned endothelial activation and remodeling markers was also calculated. Altered plaque vulnerability mediator molecules comprised matrix metalloproteinases 2, -3

and -9. Their lower detection limits were 3.5 ng/ml, 0.3 and 10 pg/ml, respectively. The interassay and intraassay coefficients of variation were 10.0 and 12.0 %, respectively, for each of the three measurements.

3.3.4 Atherosclerosis

Carotid artery ultrasound (US) measurements were made in accordance with the Mannheim consensus (Touboul *et al*., 2012) using a high-resolution B-mode ultrasound with a linear array 7.5 MHz probe (Sonocalc IMT, Sonosite Inc, Bothell, Washington). Carotid IMT was measured by obtaining images of at least one cm length of the distal common carotid arteries. This was measured at the optimal angle of incidence where both branches of the internal and external carotid artery were clearly visible. Both left and right common carotid arteries were assessed and the c-IMT measurement comprised the mean of left and right IMT measurements. Plaque in the carotid tree was defined as focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding intima-media thickness value or demonstrates a thickness of > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface. Ultrasound was performed by two experienced operators that were blinded to the clinical profiles of the patients. The intra-observer coefficients of variation were 5.8% and 4.1% (for the operator in private and public sector, respectively); the inter-observer coefficient of variation was 8.0% for measurements made by two operators (23 randomly selected patients).

3.3.5 Vaspin

Vaspin concentrations were determined by a solid-phase sandwich ELISA (QuantikineHS, R&D Systems, Inc., Minneapolis, MN, USA). The lower detection limit was 4.66 pg/ml for vaspin with inter-assay and intra-assay coefficients of variation 6.5 % and 5.0%, respectively.

3.3.6 Data analysis

Statistical analysis was performed using IBM SPSS statistics (version 23.0, IBM, USA). Continuous variables are reported as mean (±SD) for normally distributed variables or median (IQR) for variables not normally distributed. Discrete data are reported as proportions. Associations between vaspin and age at the time of study, age at time of disease onset, gender and race were assessed in mixed regression model. Associations between vaspin and all additional demographic variables and patient characteristics were assessed in models adjusted for age, race and gender to determine possible confounders for further analysis. The association of vaspin concentrations with risk factors for metabolic disease as well as markers of endothelial function, atherosclerosis and MMP levels, were determined using linear

regression models. To assess the impact of possible confounding factors as determined in prior analysis, associations between vaspin and endothelial function, atherosclerosis and MMPs were determine in multivariate linear or logistic regression models.

As RA patients with increased number of cardiovascular disease risk factors and greater disease severity are at a particularly high risk for CVD, stratified analysis was performed to assess the impact of disease severity and established risk factors for the development of CVD on the relationship between vaspin and markers of endothelial activation and atherosclerosis. To perform stratified analysis, appropriate interaction terms and their individual components were added to the regression models. In the case that the interaction term was significantly associated with outcome variables, stratified analysis was performed. Statistical significance was set at $p < 0.05$.

3.4 Results

3.4.1 Patient characteristics

Recorded characteristics of the study population are detailed in Table 3.1. The mean (SD) age at the time of the study was 58.3 (10.5) years with a mean disease duration of 14.9 (9.6) years. Majority of the participants were women (81.8%) with a mean BMI of 27.0 (5.7) kg/m^2. Of the total population, 57.6% were hypertensive, 11.2% had diabetes mellitus and 16.2 % had a total cholesterol-HDL ratio > 4. The mean DAS28 score was 2.9 (1.7) and the population had a mean (SD) disease duration of 15 (19.6) years. 76.3 % of patients tested positive for rheumatoid factor and all patients received synthetic disease modifying agents. The median (IQR) c-IMT was 0.69 (0.63-0.76), while 43% of the population had atherosclerotic plaque.

3.4.2 Associations between vaspin and patient characteristics

Associations between vaspin and other demographic and rheumatoid as well as cardiovascular risk factors are shown in Table 3.2. Vaspin concentrations were not associated with age, sex or gender (all $p>0.05$). Vaspin concentrations were not associated with any metabolic or cardiovascular disease risk markers (all $p > 0.05$). Among the RA disease characteristics, RF positivity was associated with vaspin concentrations (β (SE) 0.03 (0.10), $p=0.002$) in age, sex and race adjusted analysis.

Table 3.1. Recorded characteristics in 170 patients with RA

Demographic characteristics		**DAS28**	**2.5 (1.4)**
Age at disease onset, years	43.3 (12.8)	Clinical Disease Activity Index	7 (2-14)
Age at study time, years	58.3 (10.5)	ESR, mm/hr	9 (4-24)
Female gender, %	81.8	C-reactive protein, g/l	5.1 (2.2-13.0)
Black, %	42.4	Interleukin 6, pg/ml	3.6 (2.0-5.8)
White, %	57.6	Leukocytes, n/nl	5.9 (4.8-7.7)
Lifestyle factors		Deformed joints, n	9 (10)
Exercise, %	40.5	Extra-articular manifestations, %	9.4
Alcohol use, %	24.3	**Disease modifying agents**	
Current smoking, %	6.5	Methotrexate, %	82.9
Anthropometry		Chloroquine, %	62.4
Body mass index, kg/m^2	27 (5.7)	Leflunomide, %	30.6
Waist circumference, cm	89 (13)	Sulphasalazine, %	17.6
Waist to hip ratio	0.85 (0.80-0.91)	Azathioprine, %	12.9
Metabolic risk factors		Tetracycline, %	12.4
Hypertension, %	57.6	Cyclophosphamide, %	2.9
Systolic BP, mm Hg	132 (20)	Penicillamine, %	1.2
Diastolic BP, mm Hg	82 (12)	**TNF- α blockers, %**	4.7
Total cholesterol, mmol/l	4.8 (1.0)	**Prednisone, %**	1.8
HDL cholesterol, mmol/l	1.5 (1.3-1.9)	**NSAIDs, %**	18.8
LDL cholesterol, mmol/l	2.6 (0.8)	**Endothelial activation markers**	
Triglycerides, mmol/l	1.0 (0.8-1.4)	E-selectin, ng/ml	35 (24-50.0)
Total C – HDL C ratio $\geq$ 4, %	16.2	ICAM-1, ng/ml	282 (214-353)
Diabetes, %	11.2	VCAM-1, ng/ml	858 (666-1038)
Glucose, mmol/l	5.2 (2.3)	MCP 1, pg/ml	405 (263-651)
eGFR, ml/min/1.73m^2	99 (28)	Angiopoietin 2, pg/ml	2.59 (2.08-3.35)
Framingham Risk Score	5.3 (6.8)	ADMA, µmol/l	0.65 (0.50-0.80)
Cardiovascular agent use		**Plaque vulnerability markers**	
Antihypertensives, %	48.2	MMP-2, ng/ml	1513 (809-2511)
Statins, %	28.2	MMP-3, pg/ml	10.5 (3.1-27.9)
Ezetimibe, %	1.2	MMP-9, ng/ml	4570 (3371-6745)
Insulin, %	1.8	**Carotid atherosclerosis**	
RA characteristics		Intima-media thickness, mm	0.69 (0.63-0.76)
Disease duration, years	14.9 (9.6)	Plaque, %	42.9
HAQ-DI	0.38 (0.00-0.93)	**Adipokines**	
RF positive, %	76.3	Vaspin, ng/ml	1.9 (0.9-4.2)

Continuous variables expressed as mean (SD), median (interquartile range) or proportions as appropriate. RA: rheumatoid arthritis; BMI: body mass index; BP: blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein; C: cholesterol; eGFR: estimated glomerular filtration rate; HAQ-DI: Stanford Health Assessment Questionnaire Disability Index; RF: rheumatoid factor; DAS28: Disease Activity Score in 28 joints; ESR: erythrocyte sedimentation rate; TNF-α: tumour necrosis factor-alpha; NSAIDs: non-steroidal anti-inflammatory drugs; ICAM: intercellular adhesion molecule; VCAM: vascular adhesion molecule; MCP: monocyte chemoattractant molecule; ADMA: Asymmetric dimethylarginine MMP: matrix metalloproteinase.

Table 3.2. Associations between vaspin and demographic characteristics, rheumatoid characteristics, and traditional cardiovascular disease risk factors

	β (SE)	**P**
Demographic characteristics		
Age at time of the study	0.78 (0.04)	0.58
Age at disease onset	0.56 (0.03)	0.75
Female sex	-0.20 (0.36)	0.58
Race	0.06 (0.07)	0.34
Cardio-metabolic risk factors		
Smoking	0.24 (0.18)	0.20
Body mass index	-0.01 (0.01)	0.32
Diabetes	-0.01 (0.17)	0.97
Hypertension	-0.03 (0.10)	0.73
Dyslipidaemia	0.07 (0.10)	0.50
Estimated glomerular filtration rate	0.001 (0.003)	0.86
Rheumatoid characteristics		
Disease duration	0.001 (0.005)	0.86
Clinical disease activity index	0.001 (0.004)	0.76
Disease activity score in 28 joints	0.10 (0.06)	0.08
Rheumatoid factor positivity	**0.33 (0.10)**	**0.002**

Relationship of vaspin with cardio-metabolic and RA disease characteristics were assessed in a model adjusted for: age, sex, race. β: regression coefficient; SE: standard error. Significant associations shown in bold. * Logarithmically transformed.

3.4.3 Independent associations of vaspin concentrations with markers of endothelial function, atherosclerosis and plaque vulnerability mediators

Vaspin concentrations were not associated with any marker of endothelial activation, including VCAM-1, ICAM-1, or E-selectin in univariate or multivariate analysis (all p>0.05) (Table 3.3). Vaspin concentrations were not associated with any other marker of endothelial function or vascular remodelling in univariate or multivariate analysis. Vaspin concentrations were not associated with atherosclerosis or plaque vulnerability mediators in univariate or multivariate analysis (all p>0.05) (Table 3.3).

3.4.4 Impact of cardiovascular disease risk factors and disease duration on the associations between vaspin and markers of vascular remodelling and plaque vulnerability mediators

The relationship between vaspin concentrations and MMP-2 and angiopoietin-2 were influenced by the presence of major cardiovascular risk factors (p=0.02). In subgroup analysis shown in Table 3.4 vaspin levels were positively associated with those of MMP-2 in patients with no major cardiovascular risk factors (ß (SE) 0.42 (0.16), p=0.01) but not in patients where one or more risk factors were present (ß (SE) 0.06 (0.06), p=0.35). Vaspin levels were positively associated with those of angiopoietin-2 in patients with no major cardiovascular risk factors (ß (SE) 1.13 (0.05), p=0.02), but not in those with more than one risk factor (p=0.55). The relationship between MMP-9 and vaspin was influenced by disease duration. Vaspin concentrations were inversely associated with MMP-9 in patients with disease duration of less than 15 years (ß (SE) -0.11 (0.05), p=0.03), but not in those with a disease duration greater than 15 years (p=0.69) (Table 3.4).

Table 3.3. Relationships of vaspin* concentrations with those of endothelial activation markers, plaque vulnerability markers and atherosclerosis

	Univariate		Multivariate	
Characteristics	**β (SE)**	**P**	**β (SE)**	**P**
Endothelial activation				
E-selectin	0.004 (0.003)	0.96	0.29 (1.42)	0.93
ICAM-1*	-0.14 (0.38)	0.59	0.001 (0.01)	0.65
VCAM-1*	0.21 (0.35)	0.69	-0.01 (0.01)	0.25
MCP-1*	0.03 (0.24)	0.73	-0.01 (0.02)	0.48
Angiopoietin 2*	0.38 (0.31)	0.41	0.14 (0.32)	0.70
ADMA*	0.15 (0.40)	0.84	0.01 (0.01)	0.50
Z-score		0.78		0.73
Plaque vulnerability				
MMP-2*	0.50 (0.13)	0.11	0.12 (0.03)	0.08
MMP-3*	-0.26 (0.11)	0.79	-0.07 (0.04)	0.82
MMP-9*	0.36 (0.27)	0.30	0.01 (0.02)	0.20
Atherosclerosis				
C-IMT	-0.02 (0.01)	0.91	-0.02 (0.01)	0.59
	OR (95% CI)		**OR (95% CI)**	
Plaque	0.79 (0.30-1.04)	0.99	0.81 (0.58-1.13)	0.80

Multivariate regression analysis vaspin concentrations versus endothelial activation, plaque vulnerability and atherosclerosis included the following confounders: age, gender, race, and rheumatoid factor positive. β: regression coefficient; SE: standard error; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular adhesion molecule 1; MCP-1: monocyte chemoattractant molecule; ADMA: asymmetric dimethyl arginine, MMP: matrix metalloproteinase, C-IMT: carotid intima media thickness; OR: odds ratio; CI, confidence interval. *Logarithmically transformed.

Table 3.4. Independent relationships of vaspin concentrations with those of matrix metalloproteinase-2 and -9 and angiopoietin-2 in stratified analysis

Group	n	β (SE)	P
Matrix metalloproteinase-2			
No major risk factors	52	**0.42 (0.16)**	**0.01**
More than 1 risk factor	118	0.06 (0.06)	0.35
Matrix metalloproteinase-9			
Disease duration < 15 years	88	**-0.11 (0.05)**	**0.03**
Disease duration ≥ 15 years	82	0.02 (0.05)	0.69
Angiopoietin-2			
No major risk factors	52	**1.13 (0.05)**	**0.02**
More than 1 risk factor	118	0.02 (0.03)	0.55

Associations of vaspin concentrations with matrix metalloproteinase-2; matrix metalloproteinase-9 and angiopoietin-2 were assessed in age, gender, race and rheumatoid factor positive adjusted models. ß: regression coefficients; SE: standard error. Significant associations are shown in bold. *Logarithmically transformed.

3.5 Discussion

The present study demonstrates an association between vaspin concentrations, and the presence of established RA as defined by RF positivity. In contrast to non-RA population groups, vaspin was not associated with traditional markers of metabolic or cardiovascular diseases. In the total population vaspin concentrations were not associated with any marker of endothelial function, plaque vulnerability mediators or atherosclerosis. However, the association between atherosclerotic CVD risk and vaspin were impacted by the presence of CVD risk markers and by disease duration. In this regard, vaspin concentrations were directly associated with MMP-2 concentrations and with angiopoietin-2 concentrations in patients who had no major risk factors for the development of CVD. An inverse association was noted with MMP-9 concentrations in patients with a shorter disease duration.

Although vaspin has previously been strongly linked to obesity and metabolic dysfunction in animal models and the general population (Recinella *et al*., 2020; Feng *et al*., 2014), in this RA cohort, the results were not replicated. Klöting *et al.* (2006), previously suggested that the expression of vaspin mRNA is modulated in a fat deposit site specific manner. Furthermore, Nicholson *et al* (2018) showed that vaspin was expressed more in subcutaneous adipose tissue in obesity. It is well known that in RA, the high-grade inflammatory environment alters fat metabolism and fat distribution. In this regard, RA patients often show a decreased subcutaneous and increased visceral adiposity, known as rheumatoid cachexia, which are strongly linked to increased CVD risk (Summers *et al*., 2010; Giles *et al*., 2010). Furthermore, some suggest that inflammatory markers and disease severity are associated with altered vaspin concentrations in RA (Senolt *et al*., 2010; Ozgen *et al*., 2010). Therefore, in the particular RA cohort, the presence of high-grade inflammation or possibly the adipose tissue distribution may limit vaspin as a biomarker for metabolic disease risk in this context.

The impact of the context of RA on the use of vaspin as a biomarker, is further highlighted by the associations between vaspin and atherosclerotic CVD risk markers in the current study. In this regard, in all patients no association was shown between vaspin concentrations and any marker of endothelial activation, endothelial function, atherosclerosis or plaque vulnerability mediators. However, in interaction analysis, both the presence of traditional CVD risk factors and RA disease duration impacted the relationship between vaspin concentrations and markers of vascular remodelling and plaque vulnerability mediators.

In the current study a significant association was shown between vaspin concentrations and MMP-2 concentrations, but only in patients with low risk for CVD. Furthermore, the results showed that higher

vaspin concentrations were associated with a decreased concentration of MMP-9, in patients with a disease duration less than the median (15 years). MMP-2 is an important mediator of plaque stability through fibrous cap formation, thereby decreasing vulnerability to rupture (Newby, 2015; Nagase *et al.*, 2006; Watanabe & Ikeda, 2004). MMP-2 also enhances platelet aggregation in response to vascular injury and might play a role in angiogenesis (Sebastiano *et al*., 2017; Jiao *et al*., 2012). In contrast to the protective role of MMP-2, MMP-9 contributes to cardiovascular disease progression through the degradation of extra-cellular matrix resulting in inflammation and fibrosis (Yabluchanskiy *et al.*, 2013) and increasing plaque vulnerability (Newby, 2015; Nagase *et al.*, 2006; Watanabe & Ikeda, 2004). This suggests that in patients at lower CVD risk and those with a shorter disease duration, vaspin may reduced atherosclerotic CVD risk by mediating increased plaque stability. This is in keeping with previous findings that suggest the favourable role of vaspin in CVD progression. In this regard, others have suggested that vaspin may protect the blood vessel by preventing free-fatty acid induced apoptosis in endothelial cells (Jung *et al*., 2011), decreasing the generation of reactive oxygen species (Liu *et al*., 2014) and suppression of the migration and proliferation of vascular smooth muscle cells and increasing collagen production (Sato *et al*., 2018).

The present study also reports an association between vaspin and angiopoietin-2 in patients who had no cardiovascular disease risk factors. Angiopoietin-2 is expressed in smooth muscle cells and endothelial cells and acts predominantly as an antagonist for angiopoietin-1. Angiopoietin-1 is expressed mainly by mesenchymal cells and is involved in normal angiogenesis and the stabilization of mature vessels (Nicolini *et al*., 219; Thurston & Daly, 2012; Suri *et al*., 1998; Davis *et al.*, 1996). The activity of angiopoietin-2 is dependent on the inflammatory status and is secreted in response to endothelium activation to aid in the sensitization of the endothelium to cytokines (Fiedler & Augustin, 2006). Traditionally angiopoietin-2 is described as a mediator of adverse inflammatory changes, increased endothelial activation, adverse vascular remodelling, progression of atherosclerosis and destabilizing the vessel (Nicolini *et al*., 2019; Trollope & Golledge, 2011). Nevertheless, the role of aniopoietin-2 in CVD is dose-dependent (Tressel *et al*., 2008). In this regard, at physiological concentrations or a transient upregulation of angiopoietin-2, stimulates revascularization, while high concentrations or prolonged exposure to angiopoietin-2 exacerbate vessel destabilization (Nicolini *et al*., 2019; Fiedler & Augustin, 2006). Moreover, Daly *et al.* (2006) suggests that angiopoietin- 2 secretion is not in response to increased angiopoietin-1 (as antagonist) but rather in response to decreased angiopoietin-1, activity, thus serving as a protective mechanism that ensures endothelial cell survival in response to pathological changes. Angiopoietin-2 is also suggested to be protective against adipose-related inflammatory changes (An *et al.,* 2017). These dose-dependent effects of

angiopoietin-2 may explain the relationship between vaspin and angiopeitin-2 in only the group with no cardiovascular disease risk factors. A possible mechanism for the vaspin/Ang-2 relationship may involve the P13K/Akt/eNos pathway as both these biomarkers have been implicated as mediators of this pathway (Ju *et al*., 2014). The lack of association between vaspin concentrations and endothelial activation markers in the current study supports the possibility that the vaspin angiopoietin-2 relations may be cardioprotective.

This study has some limitations. It is important to consider that adipokine and endothelial marker expression are very often varied at different stages of disease progression and might be both protective and detrimental depending on physiological status. Considering the evidence that both MMP2 and angiopoietin-2 may be protective and/or detrimental in the progression of atherosclerotic CVD, the possibility that vaspin may be associated with adverse CVD risk cannot be excluded. Further, serum and tissue levels of vaspin may not be related. Although a comprehensive panel of atherosclerotic disease risk markers was assessed, the results did not include a healthy control group, and hence it is not certain whether the results are RA specific. The study was not statistically powered to perform stratified analysis is men and women to determine the role of sex on these results. Considering that our population were predominantly women, the result may not be applicable in men. The cross-sectional design of the study prohibits the making inferences about cause and effect.

Taken together, our results showed an association between vaspin and MMP-2 as well as vaspin and angiopoietin-2, in patients with established RA but no cardiovascular risk factors. These findings suggest that vaspin plays a role in vessel and plaque stability in response to early inflammatory changes that has not yet resulted in increased cardiovascular risk. In patients with known cardiovascular risk factors, these compensatory effects are lost. This hypothesis is supported by previous associations reported between MMP2 and angiopoietin-2 in coronary heart disease (Wu *et al*., 2016). Furthermore, in patients with short disease duration, vaspin was inversely associated with MMP-9, further supporting that vaspin exerts protective properties which are no longer noted once the disease progresses. In conclusion, vaspin concentrations may protect against vessel instability by preventing plaque rupture, but only in patients with low cardiovascular disease risk and a short disease duration. This suggests vaspin may be a biomarker that enhance cardiovascular disease risk stratification in RA patients, especially in those in the early stages of disease progression.

Chapter 4: Visfatin, but not nesfatin or vaspin, is associated with reduced wave reflection in rheumatoid arthritis

4.1 Abstract

Rheumatoid arthritis patients experience impaired arterial function and increased arterial stiffness. Impaired arterial function mediates cardiovascular events and are associated with atherosclerosis and target organ damage in RA. Nesfatin, visfatin and vaspin are associated with metabolic risk factors and atherosclerosis in RA. Whether these three adipokines are associated with indices of arterial stiffness in patients with rheumatoid arthritis, needs elucidation. In 173 patients with RA, arterial function measures including arterial stiffness, pressure pulsatility and wave reflection were assessed using applanation tonometry. The association between nesfatin, visfatin and vaspin concentrations and arterial function measures were determined in multivariable regression analysis. The impact of RA disease severity and cardiovascular disease risk profiles on the relationship between the adipokines and arterial function markers were determined in stratified analysis. There were no significant associations between nesfatin or vaspin concentrations and any markers of arterial function. Visfatin concentrations were inversely associated with central augmentation pressure (β (SE) = -0.18 (0.06), p = 0.004), augmentation index (β (SE) = -0.15 (0.07), p = 0.02), reflection magnitude (β (SE) = -0.14 (-0.06), p = 0.04), central systolic blood pressure (β (SE) = -0.13 (0.06), p = 0.02) and central pulse pressure (β (SE) = -0.14 (0.06), p = 0.03). In stratified analysis, these associations remained significant only in older person and those with greater disease severity. In conclusion, these results show that increased visfatin concentrations were associated with reduced wave reflection markers in RA patients who are older than 50 years of age and in patients with increased disease severity. Although this finding may seem paradoxical, recent evidence has shown decreased wave reflection in older adults and those exposed to inflammation, in the presence of increased arterial stiffness. The reduced wave reflection increases the pulsatile energy that is absorbed in the periphery. Therefore, reduced wave reflection, especially in older adults indicates an increased risk of target organ damage. Hence our results support previous studies suggesting that visfatin may be associated with adverse vascular remodelling and increased risk of target organ damage in older patients.

4.2 Introduction

Arterial functional changes including increased arterial stiffness, increased pressure pulsatility and increased wave reflection independently predict cardiovascular events (Townsend *et al*., 2015; Vlachopulous *et al.*, 2010; Wang *et al.*, 2010). The contribution of altered arterial function to increased risk of cardiovascular events is manifested through several mechanisms (Mitchell *et al.*, 2004). First, increased arterial stiffness increases the propagation speed of the aortic pressure waveform, which increases the speed and magnitude of wave reflection that results in an increased systolic and a decreased diastolic blood pressure. The increased pulse pressure results in an increased cardiac workload which is compounded by a decreased cardiac perfusion pressure (Mitchell *et al*., 2009) Second, increased pressure pulsatility and increased wave reflection can lead to target organ damage (Vasan *et al*., 2019; Mitchell *et al.*, 2011; Hashimoto & Ito, 2009).

In patients with RA, the increased burden of cardiovascular disease is well established (Cavalli & Favalli, 2018). High grade inflammation plays a significant role in the increased CVD burden (Crowson *et al*., 2018). Several mechanisms whereby inflammation increases CVD in RA have been investigated. One of the possible mechanisms whereby systemic inflammation compound the CVD burden in RA is impaired arterial function and increased arterial stiffness. Indeed, RA patients, have increased arterial stiffness (Ambrosino *et al.*, 2015b). Although traditional risk factors are associated with altered arterial function in RA, circulating inflammatory markers and disease duration are independent contributors to increased arterial stiffness and wave reflection (Gunter *et al.*, 2017; Vázquez-Del Mercado *et al.,* 2017; Fan *et al.*, 2014; Provan *et al*., 2011). Furthermore, increased arterial stiffness and wave reflection contribute to the development of atherosclerosis (Gunter *et al*., 2018; Ikdahl *et al*., 2016) and LV diastolic dysfunction (Mokotedi *et al.,* 2019) in RA.

Adipokines are well-recognised markers of inflammatory status (Graßmann *et al*., 2017; Manusco, 2016). In RA, regulation of several adipokines and their association with sub-clinical cardiovascular disease are altered compared to the general population (Recinella *et al*., 2020; Carrión *et al*., 2019; Fatel *et al*., 2018; Abella *et al*., 2014; Neumann *et al*., 2011). A large portion of adipokines have been implicated in endothelial dysregulation and the development of atherosclerosis in RA (Ruscitti *et al.,* 2018). In this regard, regulation of nesfatin (Kvlividze *et al*., 2019), visfatin (Lee & Bae, 2018; Mirfeizi *et al*., 2014; Bao *et al.,* 2009) and vaspin (Maijer *et al.,* 2015) are altered in RA. However, the involvement of the aforementioned adipokines in inflammatory and disease profiles are contradictory (Recinella *et al*., 2020). As presented in chapters 2 and 3, nesfatin, visfatin and vaspin were all associated with altered plaque vulnerability (MMP2) and/or vascular remodelling in RA. Considering

the link between MMP-2 expression and the development of arterial stiffness (Lyle & Raaz, 2017; Peeters *et al.,* 2017; Yasmin *et al*., 2005) and the association between arterial function and atherosclerosis (Gunter *et al*., 2017), it raises the questions whether these adipokines are associated with altered arterial function.

Despite the association between several adipokines and markers of arterial function (Bielecka-Dabrowa *et al.,* 2020), the contribution of nesfatin, visfatin and vaspin to arterial stiffness is not well-known. One recent animal study suggested that nesfatin is involved in the pathogenesis of arterial stiffness (Mori *et al.,* 2018). These authors proposed that nesfatin exerts vasoprotective effects, possibly through the enhancement of nitric oxide (NO) production in vascular endothelial cells (Mori *et al.,* 2018). The association between visfatin and arterial stiffness is contradictory. Some suggest there is an association between visfatin and increased carotid artery stiffness (La Favor *et al.,* 2011), while others reported no association between visfatin and markers of arterial function, including pulse wave velocity (Kato *et al.,* 2009). With regards to vaspin, only one study, in a non-RA population, reported an association with pulse wave velocity in postmenopausal women (Tanna *et al*., 2017). In patients with RA, currently no studies have investigated the association between markers of arterial function with nesfatin, visfatin, and vaspin. *Thus, the aim of this study was to evaluate the associations of nesfatin, visfatin and vaspin with markers of arterial function in patients with RA.*

4.3 Methods

4.3.1 Patients

One hundred and seventy-three patients, with established RA as defined by the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for the diagnosis of RA (Aletaha *et al.,* 2010), were recruited from the Milpark Hospital in Johannesburg, South Africa. This study was conducted according to principles outlined in the Helsinki declaration and was approved by the Human Research Ethics Committee at the University of the Witwatersrand (approval number: M170592). Participation was voluntary and participants provided written informed consent.

4.3.2 Patient characteristics, conventional metabolic risk factors and RA disease characteristics

Patient characteristics and the presence of conventional metabolic risk factors were assessed and documented by a specialist physician. Briefly, anthropometric measures were recorded according to standard protocols. Body mass index (BMI), waist circumference and waist-to-hip ratio were employed as indicators of generalised and abdominal adiposity. Disease activity was assessed by the Clinical Disease Activity Index (CDAI) and Disease Activity Score in 28 joints (DAS28). The number of deformed

joints, extra-articular manifestations, rheumatoid factor (RF) status, anti-citrullinated protein antibody (ACPA) status, C-reactive protein concentrations and erythrocyte sedimentation rate (ESR) were determined. Glomerular filtration rate (GFR) was estimated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. Cardiovascular drug and synthetic disease modifying agent use were recorded. Cardio-metabolic risk factors recorded included systolic and diastolic blood pressure, high-density lipoprotein, low-density lipoprotein, triglyceride concentrations, lipid ratios and glucose and insulin levels. Patients with an average systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure of ≥ 90 mmHg or current antihypertensive medication use were diagnosed with hypertension. Dyslipidaemia was classified when the cholesterol/HDL ratio was $\underline{\geq}$ 4. The Homeostasis Model Assessment of insulin resistance (HOMA-IR) was calculated, and diabetes was defined as a fasting plasma glucose concentration ≥ 7 mmol/l or the use of glucose lowering agents.

4.3.3 Arterial function

As markers of aortic function, central aortic pressure, central augmentation pressure, augmentation index (AIx), forward (Pf)- and backward wave (Pb) pressure and pulse wave velocity (PWV) were determined as previously described (Gunter *et al*., 2017). Briefly, after participants rested for 15 minutes in the supine position, consecutive waveforms were recorded by applanation tonometry for an 8-second period at the radial, femoral, and carotid pulses. A high-fidelity SPC-301 micromanometer (Millar Instrument, Inc., Houston, Texas), interfaced with a computer utilising SphygmoCor software, version 9.0 (AtCor Medical Pty. Ltd., West Ryde, New South Wales, Australia), were employed. Recordings were discarded when the systolic or diastolic variability of consecutive waveforms exceeded 5%, or when the amplitude of the waveform signal was less than 80 mV. The waveform was calibrated by auscultatory measurement of the brachial BP obtained immediately before the recordings. From the radial pressure waveform, the central aortic pressure waveform was derived using a validated, generalised transfer function inbuilt in the SphygmoCor software. Central (aortic) pulse pressure (cPP) was calculated as aortic systolic (cSP) minus central diastolic BP. Pulse pressure amplification (PPamp) was calculated as the ratio of brachial to central pulse pressure. The magnitude of the forward and reflected wave components of the aortic pressure waveform was determined by wave separation analysis using a modified triangular waveform. Reflection magnitude (RM) was calculated as reflected wave amplitude (Pb)/forward wave amplitude (Pf). Aortic PWV was determined by measuring sequential recordings of the arterial pressure waveform at the carotid and the femoral arteries. The pulse wave velocity was calculated by dividing the distance the wave travels by the transit time.

4.3.4 Adipokines

Adipokine concentrations were determined by a solid-phase sandwich ELISA (RayBiotech Inc., Georgia, USA) according to manufacturer instructions. The lower detection limit was 31.25 pg/ml for nesfatin-1 with inter-assay and intra-assay coefficients of variation <10 % and <8%, respectively. The lower detection limit for visfatin was 0.778 ng/ml with inter-assay and intra-assay coefficients of variation <15 % and <10%, respectively. The lower detection limit was 4.66 pg/ml for vaspin with inter-assay and intra-assay coefficients of variation 6.5 % and 5.0%, respectively.

4.3.5 Data analysis

Database management and statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Continuous variables were expressed as mean (SD) when normally distributed, or median (IQR) when non-normally distributed. To improve normality, non-normally distributed variables were logarithmically transformed prior to their inclusion in regression analysis. Categorical variables were expressed as proportions or percentages. The relationships between age at disease onset, age at time of the study, gender, and race and adipokine concentrations were determined in mixed model regression analysis. Associations of other patient characteristics with adipokine concentrations were assessed in demographic characteristic adjusted models. Characteristics that were associated with nesfatin, visfatin or vaspin were included as potential confounders in subsequent regression analysis. The independent relationships of nesfatin, visfatin and vaspin concentrations with markers of arterial function were evaluated in univariate and multivariate linear regression models with adjustment for potential confounders and/or mediators as identified in the previous analysis. As age, sex, race, height, weight, heart rate and mean arterial pressure are established confounders in the context of arterial function, these variables were additionally included as confounders in multivariate regression analysis (Townsend *et al*., 2015; Booysen *et al.,* 2015; Gunter *et al.,* 2017).

The risk of cardiovascular disease is particularly high amongst patients with more severe disease and those with an adverse traditional cardiovascular disease profile. Therefore, the impact of RA disease severity and cardiovascular disease risk profiles on the relationship between the adipokines and arterial function markers was determined through interaction analysis. Stratified analysis was performed when appropriate (interaction $p < 0.05$). Results were considered statistically significant when $p<0.05$.

4.4 Results

4.4.1 Patient characteristics

Recorded characteristics of the study population are shown in Table 4.1. Majority (82%) of participants were women and the mean (SD) age at the time of the study was 58.4 (12.3) years with a median (IQR) disease duration of 14.8 (8.9-22.5) years. Of the participants, 70.4 % were ACPA positive and 74.0% were rheumatoid factor positive. Disease manifestations were well-controlled, and the median (IQR) CRP concentrations were 3.3 (1.3-8.0) mg/l and the mean (SD) disease activity score in 28 joints (DAS28) was 2.9 (1.7). The mean (SD) BMI of the participants was 26.9 (5.2) kg/m^2. Of the patients, 10.4% were smokers, 42.2% were hypertensive, 50.3% had dyslipidaemia and 5.2% had diabetes mellitus. The median (IQR) pulse wave velocity was 7.3 (6.2-9.4) m/s. The median (IQR) nesfatin, visfatin and vaspin concentrations were 21.6 (7.8-41.9) ng/ml, 26.1 (12.3-47.0) ng/ml and 308.4 (184.7-413.7) pg/ml respectively.

4.4.2 Associations of patient characteristics with adipokine concentrations

Nesfatin

Nesfatin concentrations were significantly associated with CRP concentrations (partial r (95% CI) 0.16 (0.01-0.31), p=0.04), methotrexate use and abatacept use (partial r (95% CI) 0.18 (0.03-0.32), p=0.02 and 0.19 (0.04-0.34), p=0.01 respectively) (Table 4.2a). Nesfatin concentrations were not associated with any cardiovascular disease or metabolic risk markers

Visfatin

Visfatin concentrations were associated with the presence of extra-articular manifestations (partial r (95% CI) 0.19 (0.04-0.33), p=0.01) and with azathioprine use (partial r (95% CI) -0.15 (-0.29-0.002), p=0.046) (Table 4.2b).

Vaspin

Vaspin concentrations were associated with systolic blood pressure (partial r (95% CI) 0.16 (0.01-0.31), p=0.03), the use of oral glucose lowering agents and HOMA-IR (partial r (95% CI) 0.22 (0.07-0.36), p=0.005) and 0.28 (0.13-0.41), p=0.0003 respectively) (Table 4.2c). Vaspin concentrations were also associated with prednisone use and the presence of diabetes (partial r (95% CI) prednisone use: 0.17 (0.03-0.31), p=0.02); diabetes: 0.20 (0.05-0.34), p=0.01 respectively).

Table 4.1. Recorded characteristics in 173 patients with RA

Demographic characteristics			
Age at study time, years	58.4 (12.3)	CDAI	5.3 (1.0-12.8)
Age at disease onset, years	42.1 (14.0)	DAS 28	2.9 (1.7)
Female gender, n (%)	141 (82)	ESR, mm/h	12 (4-25)
Lifestyle factors		CRP, mg/l	3.3 (1.3-8.0)
Exercise, %	31.8	Interleukin 6, pg/ml	7.8 (3.5-14.2)
Alcohol use, %	32.3	Leukocytes, n/nl	5.5 (4.5-6.9)
Current smoking, %	10.4	Deformed joints, n	0 (0-10)
Anthropometry		Extra-articular manifestations, %	18.5
Body mass index, kg/m^2	26.9 (5.2)	Stanford HAQ	0.4 (0-0.9)
Waist circumference, cm	91.9 (13.2)	**Synthetic disease modifying agents**	
Waist-to-hip ratio	0.88 (0.09)	Methotrexate, %	75.1
Metabolic risk factors		Chloroquine, %	49.1
Hypertension, %	42.2	Leflunomide, %	40.5
Systolic BP, mm Hg	128 (15)	Sulphasalazine, %	15.6
Diastolic BP, mm Hg	81 (8)	Tetracycline, %	15.6
Heart rate, bpm	71.8 (11.9)	Azathioprine, %	6.9
Total cholesterol, mmol/l	4.5 (1.0)	Current DMARDS, n	2.0 (1.1)
HDL cholesterol, mmol/l	1.63 (0.47)	**Biological disease modifying agents**	
LDL cholesterol, mmol/l	2.39 (0.85)	TNF-α inhibitors, %	8.7
Triglycerides, mmol/l	0.95 (0.71-1.30)	Abatacept, %	2.9
Cholesterol-HDL ratio	2.70 (2.28-3.23)	**NSAID, %**	34.1
Cholesterol-HDL ratio >4, %	9.2	**Prednisone use, %**	2.3
Dyslipidaemia, %	50.3	**Arterial function measures**	
Diabetes, %	5.2	PWV, m/s	7.3 (6.2-9.4)
Glucose, mmol/l	4.9 (4.5-5.1)	Augmentation index, %	31.0 (11.3)
HOMA-IR	1.4 (1.0-2.0)	Central systolic BP, mm Hg	126 (16)
GFR, ml/min/1.73m^2	93.1 (23.4)	Central pulse pressure, mm Hg	42 (14)
Framingham score	1.13 (0.57-2.37)	Brachial pulse pressure, mm Hg	46 (14)
Cardiovascular agents		Pulse pressure amplification	1.15 (0.27)
Antihypertensives, %	41.6	Forward wave pressure, mm Hg	28 (8)
Statins, %	43.4	Backward wave pressure, mm Hg	21 (8)
Ezetimibe, %	10.4	Reflection magnitude	0.75 (0.23)
Oral glucose lowering agents, %	1.7	Time to peak forward wave, msec	138.4 (39.3)
Insulin, %	1.7	Time to wave reflection, msec	254.6 (19.0)
RA characteristics		**Adipokines**	
RA duration, years	14.8 (8.9-22.5)	Nesfatin, ng/ml	21.6 (7.8-41.9)
Rheumatoid factor positive, %	74.0	Visfatin, ng/ml	26.1 (12.3-47.0)
ACPA positive, %	70.4	Vaspin, pg/ml	308.4 (184.7-413.7)

Continuous variables expressed as mean (SD), median (interquartile range) or proportions as appropriate. RA: rheumatoid arthritis; BP: blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein; HOMA-IR: homeostasis model for insulin resistance; GFR: glomerular filtration rate; ACPA: anti-citrullinated antibody positive; CDAI: clinical disease activity index; DAS28: Disease Activity Score in 28 joints; ESR: erythrocyte sedimentation rate; HAQ: Health Assessment Questionnaire; TNF-α: tumour necrosis factor-alpha; NSAID: non-steroidal anti-inflammatory drugs; PWV: pulse wave velocity.

Table 4.2a. Associations of patient characteristics with **nesfatin*** concentrations (n = 160)

	Partial r (95% CI)	**P**
Demographic characteristics		
Age at time of the study	-0.02 (-0.17-0.14)	0.81
Age at disease onset	-0.04 (-0.19-0.12)	0.65
Female sex	0.09 (-0.07-0.24)	0.28
Black	0.07 (-0.09-0.22)	0.39
Inflammatory markers		
C-reactive protein*	**0.16 (0.01-0.31)**	**0.04**
Disease-modifying anti-rheumatic drugs		
Methotrexate	**0.18 (0.03-0.32)**	**0.02**
Abatacept	**0.19 (0.04-0.34)**	**0.01**

Associations were assessed in demographic characteristic adjusted models. CI: confidence interval. Significant associations shown in bold. *Logarithmically transformed.

Table 4.2b. Associations of patient characteristics with **visfatin*** concentrations (n = 173)

	Partial r (95% CI)	**P**
Demographic characteristics		
Age at time of the study	0.10 (-0.05-0.25)	0.18
Age at disease onset	0.10 (-0.05-0.25)	0.18
Female sex	0.05 (-0.10-0.20)	0.51
Black	-0.02 (-0.17-0.13)	0.76
RA disease markers		
Extra-articular manifestation	**0.19 (0.04-0.33)**	**0.01**
Disease-modifying anti-rheumatic drugs		
Azathioprine	**-0.15 (-0.29- -0.002)**	**0.046**

Associations were assessed in demographic characteristic adjusted models. CI: confidence interval. Significant associations shown in bold. *Logarithmically transformed.

Table 4.2c. Associations of patient characteristics with **vaspin*** concentrations (n=173)

	Partial r (95% CI)	**P**
Demographic characteristics		
Age at time of the study	-0.06 (-0.20-0.09)	0.47
Age at disease onset	-0.06 (-0.21-0.09)	0.45
Female sex	0.04 (-0.11-0.19)	0.62
Black	0.03 (-0.12-0.18)	0.67
Cardio-metabolic risk factors		
Systolic blood pressure	**0.16 (0.01-0.31)**	**0.03**
Diabetes	**0.20 (0.05-0.34)**	**0.01**
HOMA-IR*	**0.28 (0.13-0.41)**	**0.0003**
Treatment		
Oral glucose lowering agents	**0.22 (0.07-0.36)**	**0.005**
Prednisone	**0.17 (0.03-0.31)**	**0.02**

Associations were assessed in demographic characteristic adjusted models. CI: confidence interval. Significant associations shown in bold. *Logarithmically transformed.

4.4.3 Associations of markers of arterial function with nesfatin, visfatin and vaspin

Table 4.3 details the univariate relationship between markers of arterial stiffness and the adipokine concentrations. No associations were noted between either nesfatin or vaspin concentrations and any of the marker of arterial function. Visfatin concentrations were inversely associated with central augmentation pressure (Std β (SE) -0.15 (0.08), p=0.05), augmentation index (Std β (SE) -0.18 (0.08), p=0.02), reflection magnitude (Std β (SE) -0.16 (0.07), p=0.03) and time to wave reflection (Std β (SE) -0.19 (0.07), p=0.01).

In multivariate analysis (Table 4.4), there were no significant associations between nesfatin or vaspin concentrations and any markers of arterial function. In multivariate adjusted analysis, the inverse associations between visfatin and central augmentation pressure (Std β (SE) -0.18 (0.06), p=0.0038), augmentation index (Std β (SE) -0.15 (0.07), p=0.02) and reflection magnitude (Std β (SE) -0.14 (-0.06), p=0.04) remained significant. The association between visfatin concentrations and time to wave reflection was no longer significant (p=0.08). In addition, visfatin was inversely associated with central systolic pressure (Std β (SE) -0.13 (0.06), p=0.02) and central pulse pressure (Std β (SE) -0.14 (0.06), p=0.03).

4.4.4 Independent relationships of visfatin concentrations with arterial function measures in stratified analysis

In stratified analysis (Table 5), visfatin was associated with central systolic pressure (Std β (SE) -0.16 (0.07), p=0.02), central pulse pressure (Std β (SE) -0.16 (0.08), p=0.05), central augmentation pressure (Std β (SE) -0.21 (0.08), p=0.01) and augmentation index (Std β (SE) -0.17 (0.08), p=0.04), reflected wave pressure (Std β (SE) -0.16 (0.07), p=0.03) in patients who were 50 years or older, but not in patients younger than 50 years (all p>0.05). In rheumatoid factor positive patients, visfatin was associated with central pulse pressure (Std β (SE) -0.16 (0.08), p=0.03), central augmentation pressure (Std β (SE) -0.20 (0.07), p=0.01), augmentation index (Std β (SE) -0.16 (0.08), p=0.14), reflected wave pressure (β (SE) -0.17 (0.07), p=0.02) and reflection magnitude (Std β (SE) -0.20 (0.08), p=0.001). In patients where rheumatoid factor was negative, visfatin was associated with central systolic pressure (Std β (SE) -0.20 (0.09), p=0.05). In patients with a CRP concentration higher than the median, visfatin was associated with central augmentation pressure (Std β (SE) -0.18 (0.08), p=0.03) and reflected wave pressure (Std β (SE) -0.16 (0.08), p=0.05), but not in patients with CRP concentrations less than the median (all p>0.05).

Table 4.3. Univariate associations between markers of arterial function and nesfatin, visfatin and vaspin concentrations

	Nesfatin* (n=160)		Visfatin* (n=173)		Vaspin* (n=173)	
	Std β (SE)	**P**	**Std β (SE)**	**P**	**Std β (SE)**	**P**
Pulse wave velocity*	-0.02 (0.08)	0.81	-0.03 (0.08)	0.75	-0.08 (0.08)	0.30
Central systolic blood pressure	0.03 (0.08)	0.69	-0.06 (0.08)	0.41	0.04 (0.08)	0.60
Central pulse pressure	-0.04 (0.08)	0.63	-0.07 (0.08)	0.38	0.03 (0.07)	0.70
Brachial pulse pressure	-0.01 (0.08)	0.86	-0.01 (0.08)	0.93	0.08 (0.08)	0.30
Central augmentation pressure	0.05 (0.08)	0.50	**-0.15 (0.08)**	**0.05**	0.05 (0.08)	0.50
Augmentation index	0.07 (0.08)	0.36	**-0.18 (0.08)**	**0.02**	0.04 (0.08)	0.63
Forward wave pressure	-0.04 (0.08)	0.62	0.02 (0.08)	0.81	0.07 (0.08)	0.37
Backward wave pressure	-0.03 (0.08)	0.69	-0.09 (0.08)	0.24	0.02 (0.08)	0.78
Pulse pressure amplification	0.06 (0.08)	0.50	0.11 (0.08)	0.14	0.04 (0.08)	0.57
Reflection magnitude	-0.01 (0.08)	0.91	**-0.16 (0.08)**	**0.03**	-0.02 (0.08)	0.83
Time to peak forward wave	-0.08 (0.08)	0.33	-0.12 (0.08)	0.12	0.03 (0.08)	0.66
Time to wave reflection	-0.02 (0.08)	0.80	**-0.19 (0.07)**	**0.01**	-0.04 (0.08)	0.59

Std β: standardised beta coefficient; SE: standard error. Significant associations shown in bold. *Logarithmically transformed

Table 4.4. Multivariate adjusted associations between markers of arterial stiffness and nesfatin, visfatin and vaspin concentrations

	Nesfatin* (n=160)		Visfatin* (n=173)		Vaspin* (n=173)	
	Std β (SE)	**P**	**Std β (SE)**	**P**	**Std β (SE)**	**P**
Pulse wave velocity*	-0.03 (0.07)	0.66	-0.10 (0.07)	0.14	-0.07 (0.08)	0.37
Central systolic blood pressure	0.02 (0.06)	0.71	**-0.13 (0.05)**	**0.02**	-0.03 (0.06)	0.62
Central pulse pressure	-0.03 (0.07)	0.63	**-0.14 (0.06)**	**0.03**	0.00 (0.07)	0.98
Brachial pulse pressure	-0.03 (0.07)	0.64	-0.08 (0.07)	0.25	0.01 (0.07)	0.94
Central augmentation pressure	0.06 (0.07)	0.35	**-0.18 (0.06)**	**0.004**	0.01 (0.07)	0.84
Augmentation index	0.08 (0.07)	0.28	**-0.15 (0.07)**	**0.02**	0.02 (0.07)	0.81
Forward wave pressure	-0.04 (0.08)	0.59	-0.08 (0.07)	0.29	0.07 (0.08)	0.41
Backward wave pressure	-0.03 (0.06)	0.69	**-0.14 (0.06)**	**0.01**	-0.00 (0.06)	0.96
Pulse pressure amplification	0.00 (0.08)	0.97	0.10 (0.07)	0.17	0.00 (0.08)	0.99
Reflection magnitude	-0.00 (0.07)	0.98	**-0.14 (0.06)**	**0.04**	-0.04 (0.07)	0.56
Time to peak forward wave	-0.05 (0.08)	0.55	-0.11 (0.08)	0.13	-0.07 (0.08)	0.45
Time to wave reflection	-0.04 (0.07)	0.56	-0.12 (0.07)	0.08	0.02 (0.07)	0.82

Associations of nesfatin concentrations with arterial function markers were assessed in age, sex, race, mean arterial pressure, heart rate, height, weight, C-reactive protein, methotrexate use and abatacept use adjusted models. Associations of visfatin concentrations with arterial function markers were assessed in age, sex, race, mean arterial pressure, heart rate, height, weight, extra-articular manifestations, and azathioprine use adjusted models. Associations of vaspin concentrations with arterial function markers were assessed in age, sex, race, mean arterial pressure, heart rate, height, weight; systolic blood pressure, oral glucose agent use, HOMA-IR, prednisone use, diabetes adjusted models. Std β: standardised beta coefficients; SE: standard error. Significant relationships are shown in bold. *Logarithmically transformed.

Table 4.5. Independent associations between visfatin* concentrations and markers of pressure pulsatility and wave reflection in stratified analysis

		cSP		cPP		cAP		AIx		Pb		RM	
	n	**Std β (SE)**	**P**	**Std β (SE)**	**P**	**Std β (SE)**	**P**	**Std β (SE)**	**P**	**Std β (SE)**	**P**	**Std β (SE)**	**P**
Age													
<50 years	47	-0.03 (0.08)	0.72	-0.05 (0.09)	0.53	-0.04 (0.08)	0.59	-0.03 (0.11)	0.76	-0.07 (0.08)	0.43	-0.1 (0.12)	0.37
≥50 years	126	**-0.16 (0.07)**	**0.02**	**-0.16 (0.08)**	**0.05**	**-0.21 (0.08)**	**0.01**	**-0.17 (0.08)**	**0.04**	**-0.16 (0.07)**	**0.03**	-0.13 (0.07)	0.09
Rheumatoid factor													
RF negative	45	**-0.2 (0.09)**	**0.05**	-0.11 (0.12)	0.35	-0.25 (0.13)	0.06	-0.27 (0.15)	0.07	-0.13 (0.11)	0.26	-0.04 (0.14)	0.77
RF positive	128	-0.12 (0.07)	0.09	**-0.16 (0.08)**	**0.03**	**-0.20 (0.07)**	**0.01**	**-0.16 (0.08)**	**0.04**	**-0.17 (0.07)**	**0.02**	**-0.2 (0.08)**	**0.001**
CRP													
CRP < 3.3units	85	-0.13 (0.08)	0.11	0.11 (0.09)	0.23	-0.15 (0.08)	0.09	-0.15 (0.09)	0.11	-0.09 (0.09)	0.28	-0.03 (0.08)	0.69
CRP ≥3.3 units	88	-0.14 (0.08)	0.07	-0.14 (0.08)	0.09	**-0.18 (0.08)**	**0.03**	-0.13 (0.09)	0.14	**-0.16 (0.08)**	**0.05**	-0.17 (0.09)	0.08

Associations of visfatin concentrations with arterial function markers were assessed in age, sex, race, mean arterial pressure, heart rate, height, weight, extra-articular manifestations and azathioprine use adjusted models. Std β: standardised beta coefficient; SE: standard error; cSP: central systolic pressure; cPP: central pulse pressure; cAP: central augmentation pressure; AIx: augmentation index; Pb: backward wave pressure; RM: reflection magnitude. Significant associations shown in bold. ***** Logarithmically transformed.

4.5 Discussion

The present study reports for the first time that in RA patients, visfatin was inversely associated with markers of wave reflection and pressure pulsatility, independent of confounders. The association between visfatin and wave reflection and pressure pulsatility markers were influenced by age and disease profiles. No associations between visfatin and arterial function markers were seen in younger patients (<50 years of age) or those with more favourable disease profiles. However, the inverse association between visfatin and arterial function markers remained significant in older patients (> 50 years) and those with an adverse disease profile. Nesfatin and vaspin concentrations were not associated with any markers of arterial stiffness, pressure pulsatility or wave reflection.

In this cohort of RA patients, none of the adipokines were associated with patient characteristics, including age, age at disease onset, sex or race. However, nesfatin concentrations were associated with CRP concentrations and with methotrexate and abatacept use. Visfatin concentrations were higher in patients with adverse disease profiles (increased extra-articular manifestations) and were reduced in those with used azathioprine. These results are in keeping with previous reports that showed inflammatory marker and disease severity status may impact nesfatin (Kvlividze *et al.*, 2019) and visfatin concentrations (Lee & Bae, 2018; Otero *et al*., 2006) in RA patients. Similar to previous reports, we showed that vaspin concentrations were associated with cardio-metabolic risk markers and metabolic disturbances (Recinella *et al*., 2020; Feng *et al*., 2014). These results are in contrast to our previous reports, that did not show any associations between vaspin concentrations and metabolic risk markers (section 4.3.2). These discrepant results may be explained by the demographic characteristics and the disease profiles of the patients in the two cohorts. The patients in the current study had better disease control and they were more homogenous in terms of socio-economic status, race and metabolic disease risk markers compared to the previous study (Chapter 3). Hence these differences in the cohorts may have impacted our findings.

The current study showed no association between visfatin concentrations and pulse wave velocity. This is in keeping with previous studies that reported no association between visfatin and pulse wave velocity in the general population (Kato *et al*., 2009). Despite the lack of association between visfatin and arterial stiffness, an inverse association was shown between visfatin concentrations and markers of wave reflection, including central augmentation pressure, backward wave pressure, augmentation index and reflection magnitude. This association between increased visfatin concentrations and reduced wave reflection may seem controversial. In this regard, several studies using wave separation analysis reported associations between wave reflection and target organ changes (Heusinkveld *et al*.,

2019; Bello *et al.,* 2017; Booysen *et al*., 2015; Chirinos *et al*., 2012; Weber *et al*., 2012; Wang *et al.,* 2010). With ageing, because of the stiffer central aorta, it has been suggested that both the forward wave and backward wave amplitude increases in older adults. A logical assumption is that the increased speed of the aortic pulse waveform would results in an earlier return of the reflected wave reflection or a distal shift in reflection points. Where this has traditionally been the accepted view, questions arise about the validity of this assumption (Kondiboyina *et al*., 2020).

Various studies have shown that with age, large artery stiffness results in increased PWV, yet the changes in the time of inflection point (T_{INF}) are negligible (Phan *et al*., 2020; Izzo, 2014; Mitchell *et al.*, 2010; Namasivayam *et al*., 2009). In addition, very little change in medium sized PWV have been reported with ageing (O'Rourke & Nichols, 2005). Besides the changes in transit time, recent evidence suggests that the reflected wave magnitude may be reduced, rather than increased in older adults (Kondiboyina *et al*., 2020; Motau *et al*., 2020). Indeed, already in 1985, Mitchell proposed a paradigm that suggests a reduced, rather than an increased wave reflection is associated with target organ damage. In this regard, pulse waves generated from the heart moves in a distal direction where reflected waves are generated at sites of impedance mismatch (Mitchell, 1985). The return of the pulsatile energy to the heart prevents the pulsatile energy transmission to the microcirculation of low impendence organs such as the brain and the kidneys (Mitchell, 1985). In young and healthy individuals, because of the compliant large arteries, the stiffness gradient between the central and peripheral arteries creates an impedance mismatch, which generate reflected waves and hence limits the transmission of pulsatile pressure to end organs (Mitchell, 1985). In older individuals, although increased arterial stiffness is believed to increase central pulse pressure via an increase in wave reflection, it has been shown that increased arterial stiffness may in fact reduce wave reflection (Kondiboyina *et al*., 2020; Mitchell *et al*., 2011). Mitchell (1985) proposed that in older adults, the decrease in the stiffness gradient between the central and peripheral arteries reduces the impedance mismatch and hence reduces wave reflection. However, despite a lower central pulse pressure, lower wave reflection increased the pulsatile energy being absorbed in the peripheral vasculature which were associated with an increased risk of end organ damage (Mitchel *et al*., 2011). Indeed, recent evidence supported the theory that a reduced wave reflection increases the hydraulic energy transmission in the vessels, and hence increase the risk of target organ damage (Kondiboyina *et al*., 2020). Others have also shown that increased backward wave pressure after the age of 50 years do not contribute to target organ changes (Motau *et al*., 2020; Kolkenbeck-Ruh *et al*., 2019; De Buyzere *et al*., 2018), and that in fact backward wave magnitude decrease after 50 years (Motau *et al*., 2020). Moreover, we and others have recently shown that is it the timing rather than the magnitude of the

backward wave that contributes to adverse cardiac remodelling (Mokotedi *et al*., 2019; Chirinos *et al*., 2012). However, increased backward waves do contribute to end organ damage, especially LV remodelling, in the earlier years as it increases LV afterload (Motau *et al*., 2020; Kolkenbeck-Ruh *et al*., 2019). Indeed, in stratified analysis the results showed that the associations between visfatin concentrations and reduced wave reflection were significant in older adults (>50 years), but not in younger patients. Moreover, the relatively high average age of the population may have contributed to the association between visfatin and wave reflection in the total cohort.

This study further showed that the association between visfatin concentrations and wave reflection markers were impacted by disease profiles. In this regard, in patients that were RF positive and those that had CRP concentrations greater than the median, the association between visfatin and wave reflection remained significant, but not in those with a more favourable disease profile. This association may be explained by the influence of inflammation on wave reflection. Similar to the effects of age, it has previously been suggested that acute inflammation may also decrease wave due to peripheral vasodilation (Vlachopoulos *et al*., 2005). This was recently confirmed by Schroeder *et al.* (2019) who evaluated the effect of acute inflammation on wave reflection in healthy individuals. The authors concluded that acute inflammation results in reduced wave reflection posing an increased risk to microvascular damage of the cerebral vessels, likely as a result of increased transmitted pulsatility (Schroeder *et al*., 2019). Obesity and diabetes, characterised by low grade inflammation, have also been associated with reduced wave reflection (Maple-Brown *et al*., 2005). Taken together, our results suggest that higher visfatin concentrations mediate reductions in wave reflection that may indicate an increased risk for end organ damage, especially in older adults and those with adverse disease profiles.

Indeed, visfatin has been implicated in adverse vascular remodelling. Increased concentrations of extracellular visfatin has been associated with vascular smooth muscle cell proliferation via nicotinamine mononucleotide (NMN)-mediated ERK1/2 and p38 signalling (Wang *et al.,* 2009). Briefly, visfatin/nampt displays enzymatic activity as the rate-limiting step in the synthesis of NAD, where NMN is synthesized from nicotinamide (Vitamin B3) and 5-phosphoribosyl-pyrophosphate (PRPP). NMN is then converted to NAD by nicotinamide/nicotinic acid mononucleotide adenylyltransferase (Nmnat) (Imai, 2009). Indeed, vascular smooth muscle cell proliferation is the hallmark of adverse vascular remodelling (Lacolley *et al.*, 2018; Romacho *et al*., 2013) Besides the direct contribution to vascular remodelling, visfatin may contribute indirectly to vascular remodelling. Visfatin has been associated with inflammatory markers and oxidative stress markers, which contribute to adverse vascular remodelling (Mattu & Randeva, 2013; Lui et *al.*, 2009).

Despite previous involvement of nesfatin and vaspin in plaque vulnerability mediations, no associations between either nesfatin or vaspin concentrations and indices of arterial stiffness or wave reflection are reported. To my knowledge, currently there is no evidence that either of these adipokines are involved in arterial functional changes in RA patients and warrant further investigation.

This study has some strengths and limitations. A comprehensive evaluation of arterial functional variables was performed with consideration of a wide range of potential confounders in the statistical analysis. However, the was cross-sectional, hence drawing inferences on the direction of causality are limited. Furthermore, the lack of a healthy control groups limits the generalisability of the results to other RA populations. Assessing a comprehensive set of arterial function measures, multiple associations were evaluated. However, the results remained consistent when continuous and dichotomized outcomes were used in differently adjusted multivariable models. As all hypertensive patients received antihypertensive agents, the findings may not apply in RA patients with untreated or/and uncontrolled hypertension. The triangulation method of aortic wave separation was used to determine aortic forward and backward wave pressure. Although this method has previously been validated, future studies should determine the association between adipokines and the forward and backward waves using simultaneous recording of actual aortic flow and pressure in RA. Although carotid femoral PWV is considered the gold standard, non-invasive measure of arterial stiffness, body surface estimate may underestimate the PWV.

In conclusion, the study reports that visfatin was inversely associated with wave reflection, in older patients with adverse disease profiles. The findings suggest that visfatin may be involved in vascular remodelling which contribute to decreased wave reflection and possibly an increased risk for target organ changes, especially in older patients with more severe disease. The exact mechanism whereby visfatin contribute to adverse vascular pathology needs further investigation. Furthermore, whether nesfatin, visfatin and/or vaspin are associate with target organ damage in RA require elucidation.

Chapter 5: Nesfatin, visfatin and vaspin are differentially associated with cardiac geometry and left ventricular diastolic dysfunction in patients with rheumatoid arthritis

5.1 Abstract

RA patients experience an increased risk of developing heart failure with a preserved ejection fraction (HFpEF) compared to the general population. Left ventricular (LV) diastolic dysfunction is a pre-clinical disorder that frequently progresses to HFpEF. Although systemic inflammation plays a mechanistic role in the development of LV diastolic dysfunction and is implicated in altered adipokine physiology, the role of adipokines as biomarkers for the development of LV diastolic dysfunction is not well described. This study aimed to determine the association between nesfatin, visfatin and vaspin and LV diastolic function markers in RA. In 170 RA patients, markers of cardiac geometry and LV diastolic function were assessed using echocardiography. Left ventricular diastolic function parameters included the ratio of early-to-late transmitral inflow velocity (E/A), mitral annular tissue lengthening in early (e') and late diastole (a') and the ratio of E/e'. In multivariate regression analysis, visfatin concentrations were independently associated with E/e', an index of LV filling pressure (β (SE) = 0.21 (0.08), p = 0.008), and left atrial volume index (LAVI) (β (SE) = 0.17 (0.07), p = 0.02). In stratified analysis the association between visfatin and increased filling pressures remained significant in younger patients and those with lower disease severity. In stratified analysis, nesfatin concentrations were associated with reduced risk of LV concentric hypertrophy (relative wall thickness and LV mass index) in younger patients and in those with a shorter disease duration. Vaspin concentrations were associated with reduced LV relaxation (reduced lateral wall mitral annular velocity) in older patients. In conclusion, the results showed disparate associations between visfatin, nesfatin and vaspin concentrations and markers of adverse cardiac structure and function, and that these associations are impacted by age and disease severity.

5.2 Introduction

Patients with established RA, have a two-fold risk for developing cardiovascular disease that often results in heart failure (Crowson *et al.*, 2013; Van Halm *et al.*, 2009; Maradit-Kremers *et al.*, 2005; Solomon *et al.*, 2006). Studies evaluating phenotypes of heart failure in RA populations, suggest an increased prevalence of LV diastolic dysfunction in these patients (Schau *et al*., 2015; Liang *et al.,* 2010). A recent study suggests that 55% of patients with RA present with LV diastolic dysfunction compared to 9% with systolic dysfunction (Renjith *et al.,* 2017). Similarly, RA patients have a higher prevalence of LV diastolic dysfunction compared to non-RA individuals (Schau *et al.*, 2015; Aslam *et al.*, 2013; Liang *et al*., 2010). In this regard, patients with LV diastolic dysfunction are often asymptomatic and timeous identification is crucial in the prevention of progression of LV diastolic dysfunction to HFpEF (Targońska-Stępniak *et al.*, 2019; Wan *et al*., 2014).

Chronic inflammation is associated with the development of HFpEF (Glezeva *et al.,* 2014). In patients with comorbid diseases such as obesity, hypertension and diabetes, it is believed that systemic inflammation may be the underlying mechanisms that explains the development of LV diastolic dysfunction (Van Linthout & Tschöpe, 2017; Paulus & Tschöpe, 2013). Several inflammatory markers such CRP and cytokines including TNF-α, IL-1β and IL- 6 are implicated in the mechanisms underlying LV diastolic dysfunction and HFpEF (Mocan *et al*., 2019). The high prevalence of LV diastolic dysfunction cannot be fully explained by traditional risk factors (Crowson *et al*., 2018; Crowson *et al*., 2005). Several RA diseases characteristics, inflammatory markers and DMARDs have been associated with LV diastolic dysfunction in RA (Davis *et al.*, 2011; Liang *et al*., 2010).

Various biomarkers have been evaluated for their potential to improve risk stratification and early detection of LV diastolic dysfunction. In this regard, MMPs represent changes in elastin and collagen ratios that results in clinical progression from hypertension and/or related CVD risk to symptomatic HFpEF (Spinale *et al.,* 2013; Zile *et al.,* 2013). Similarly, galectin-3 has been associated with severity of HFpEF (deFilippi & Felker, 2010). B-type natriuretic peptide (BNP) and N-terminal pro-BNP (NT-proBNP) are well-validated biomarkers of cardiac dysfunction with elevated concentrations reported in patients with HFpEF (Van Veldhuisen *et al*., 2013). The role of adipokines as biomarkers for the development of heart failure is not well described (Horbal *et al*., 2020; Francisco *et al*., 2016; Straburzyńska-Migaj *et al.*, 2012). Nevertheless, the complex interplay between adipokines, systemic inflammation atherosclerosis in RA has received considerable attention (Recinella *et al*., 2020; Carrión *et al*., 2019). Furthermore, we have recently shown in RA patients that other adipokines are associated with arterial functional changes (Gunter *et al*., 2018) and that arterial function is linked to the development of LV

diastolic dysfunction (Mokotedi *et al*., 2019). Moreover, in the present thesis, results have shown that nesfatin, visfatin and vaspin concentrations are linked to plaque vulnerability, and the visfatin is linked to wave reflection that may ultimately contribute to target organ damage. Therefore, whether the adipokines may play a role in the development of LV diastolic dysfunction in RA warrant investigation. Therefore, the aim of this study was to determine the associations between nesfatin, visfatin and vaspin and markers of cardiac geometry and function in RA patients.

5.3 Methods

5.3.1 Patients

This present study was conducted according to principles outlined in the Helsinki declaration and was approved by the Human Research Ethics Committee at the University of the Witwatersrand (approval number: M170592). Patients were recruited from Milpark Hospital in Johannesburg, South Africa. Participation was voluntary and patients had established RA as defined by the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for the diagnosis of RA (Aletaha *et al.,* 2010). All participants provided written informed consent. Of the total study population, 170 patients who had high quality echocardiography images were included in this study.

5.3.2 Patient characteristics, conventional metabolic risk factors and RA disease characteristics

Patient characteristics, conventional cardio-metabolic risk factors and RA disease characteristics were recorded. Description of the demographic parameters, cardiovascular disease risk factors and RA characteristics assessed are provided in chapter 4, section 4.3.2 of the present thesis.

5.3.3 Echocardiography

Echocardiography was performed using a Sonosite M-Turbo ultrasound (SonoSite® Inc., Bothell, WA, USA) according to the American Society of Echocardiography convention (Lang *et al*., 2015). With the patient in the partial left decubitus position, LV dimensions were determined using two-dimensional directed M-mode echocardiography in the parasternal long axis view. The LV end systolic diameter (LVESD) and LV end diastolic diameter (LVEDD) and the interventricular septal wall thickness (IVST) and posterior wall thickness (PWT) in systole and diastole were measured. The LV dimensions were measured only when appropriate visualisation of both the right and the left septal surfaces occurred and where the endocardial surfaces of both the septal and posterior wall were clearly visible. Left ventricular end diastolic (LVEDV) and systolic (LVESV) volumes were assessed using the Teichholz method (Teichholz *et al*., 1976). LV ejection fraction (EF) was calculated as [(LVEDD- LVESD) / LVEDV]

x 100. Left ventricular mass was determined using a standard formula (Lang *et al*., 2015) and indexed to body surface area. LV relative wall thickness (RWT), a marker of concentric remodelling, was calculated as (PWT in diastole x 2) / LVEDD) (Ganau *et al.*, 1992). Left ventricular hypertrophy was identified when left ventricular mass index (LVMI) was >95 g/m^2 for women and >115 g/m^2 for men (Lang *et al.*, 2015). Concentric hypertrophy was characterised when RWT >0.42. Left ventricular diastolic function was assessed using pulsed Doppler, and tissue Doppler imaging (Nagueh *et al.*, 2016). Transmitral flow patterns were recorded at the mitral valve leaflet tips using pulsed Doppler in the apical four chamber view. From the mitral valve, the inflow velocity during early (E) and late (atrial-A) diastole were measured. The ratio of early to late diastolic filling velocity (E/A) was calculated as a marker of LV relaxation. Patients were considered to have impaired relaxation if the E/A ratio was < 0.8 (Nagueh *et al*., 2016).

The velocity of myocardial tissue lengthening was measured using Tissue Doppler Imaging (TDI). The cursor was placed at the septal and lateral corners of the mitral annulus in the apical four chamber view and the peak velocities during early (e') diastole were recorded. Because mitral E is dependent on ventricular relaxation as well as left atrial driving forces (pressures), while e' is dependent on relaxation alone, the E/e' ratio was calculated as an index of left ventricular filling pressures. An E/e' ratio > 12 was considered as an increased filling pressure (Mitter *et al*., 2017). In addition, the left atrial area was determined using planimetry at end-systole in the apical four chamber or two chamber views. Left atrial volume was calculated by the area-length method and was indexed to body surface area (LAVI), as an index of filling pressure.

Echocardiography was performed by two experienced operators that were blinded to the clinical data and cardiovascular disease risk factor profiles of the patients. All echocardiographic images were reviewed by a single experienced operator. Intra- and inter-observer studies were conducted on 20 individuals. For the inter-observer variability, the Pearson's correlation coefficients for LV end-diastolic diameter, septal wall thickness, posterior wall thickness, E, A and e' were 0.71, 0.84 and 0.81, 0.95, 0.92 and 0.92 respectively ($p<0.0001$ for all) and the coefficient of variation for LV end-diastolic diameter, septal wall thickness, posterior wall thickness, E, A and e' were 2.7%, 2.9%, 2.6%, 1.9%, 3.9% and 4.2% respectively. There were no significant differences between the measures on unpaired t-tests ($p>0.2$ for all). For the intra-observer variability, the Pearson's correlations for repeat measures ranged between 0.74 and 0.98 for the one and 0.82 and 0.94 for the other observer for all the aforementioned measures (all $p<0.001$). The respective coefficients of variation ranged between 1.4%

and 4.7% for the one and 1.2% and 5.4% for the other observer for all the aforementioned measures. There were no differences in the repeat measures on paired t-tests for either observer ($p>0.2$ for all)

5.3.4 Adipokines

Adipokine concentrations were determined by a solid-phase sandwich ELISA (RayBiotech Inc., Georgia, USA) according to manufacturer instructions. A description of the adipokine measures is provided in chapter 4, section 4.3.4 of the present thesis.

5.3.5 Data analysis

Database management and statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Continuous variables were expressed as mean (SD), or median (IQR) when normally or non-normally distributed, respectively. Categorical variables were expressed as proportions. Variables the were not normally distributed were log-transformed prior to inclusions in regression analysis. The relationships between demographic, cardiometabolic and RA disease characteristics and adipokine concentrations were determined as described in chapter 4, section 4.3.5 of the present thesis. The independent relationships of nesfatin, visfatin and vaspin concentrations with markers of LV remodelling and LV diastolic function were evaluated in univariate and multivariate linear and logistic regression models. Characteristics that were associated with nesfatin, visfatin or vaspin were included as potential confounders in multivariate regression analysis. We recently showed that several traditional risk factors impact LV diastolic function in RA (Mokotedi *et al*., 2017). Therefore, age, sex, race, systolic blood pressure, diastolic blood pressure, heart rate and waist-to-hip ratio were additionally included as confounders in multivariate regression analysis. As cardiac remodelling is strongly associated with the development of LV diastolic dysfunction, left ventricular mass index was included in additional regression models with LV diastolic function variables.

As the risk of cardiovascular disease is particularly high amongst patients who are older and those with more severe RA disease profiles, the study determined the impact of RA disease severity and cardiovascular disease risk profiles on the relationship between the adipokines and arterial function markers in interaction analysis. When interaction terms were associated with outcome variables independent of the individual factors, stratified analysis was performed. Results were considered statistically significant when $p<0.05$.

5.4 Results

5.4.1 Patient characteristics

Recorded characteristics of the study population is detailed in Table 5.1. Of the participants, 82.3% were women and the mean (SD) age at the time of the study was 58.2 (12.6) years. Of the participants, 40.8% were hypertensive, of which all received anti-hypertensive medication. Disease severity in the population was low and all patients received synthetic disease modifying agents. Of the study sample, 22.9% showed impaired LV relaxation (E/A<0.85), 41.8% had increased filling pressures (E/e' >12), 19.4% had left ventricular hypertrophy (increased LVMI) and 54.1% had concentric LV remodelling (RWT >0.42).

5.4.2 Associations of patient characteristics with adipokine concentrations

Associations between adipokine concentrations and demographic, cardio-metabolic and RA disease characteristics were reported in Chapter 4, section 4.4.2 in Tables 4.2a, 4.2b and 4.2c. Based on this analysis, in associations with nesfatin, the following variables were included as confounders in multivariate regression models: CRP concentrations, methotrexate and abatacept use. For associations with visfatin, extra-articular manifestations and azathioprine use were included as confounders in multivariate regression analysis models. For associations with vaspin, systolic blood pressure, the use of oral glucose lowering agents, HOMA-IR, prednisone use, and diabetes were included as confounders in multivariate regression analysis models.

Table 5.1. Recorded characteristics in 170 patients with RA

Demographic characteristics			
Age at study time, years	58.2 (12.6)	Rheumatoid factor positive, %	73.6
Age at disease onset, years	41.9 (13.9)	ACPA positive, %	69.8
Female gender, n (%)	140 (82.3)	CDAI	5.3 (1.0-12.8)
Lifestyle factors		DAS 28	2.9 (1.7)
Exercise, %	32.7	ESR, mm/h	12 (4-25)
Alcohol use, %	32.1	CRP, mg/l	3.3 (1.3-8.0)
Current smoking, %	10.3	Interleukin 6, pg/ml	7.8 (3.5-14.2)
Anthropometry		Leukocytes, n/nl	5.5 (4.5-6.9)
Body mass index, kg/m2	26.1 (5.3)	Deformed joints, n	0 (0-10)
Waist circumference, cm	91.3 (13.3)	Extra-articular manifestations, %	18.4
Waist-to-hip ratio	0.88 (0.08)	Stanford HAQ	0.4 (0-0.9)
Metabolic risk factors		**Synthetic disease modifying agents**	
Hypertension	40.8	Methotrexate, %	75.9
Systolic BP, mm Hg	128 (15)	Chloroquine, %	48.9
Diastolic BP, mm Hg	81 (9)	Leflunomide, %	40.2
Heart rate, bpm	72 (12)	Sulphasalazine, %	16.1
Total cholesterol, mmol/l	4.5 (1.0)	Tetracycline, %	16.7
HDL cholesterol, mmol/l	1.64 (0.47)	Azathioprine, %	6.9
LDL cholesterol, mmol/l	2.40 (0.86)	Current DMARDS, n	2.0 (1.1)
Triglycerides, mmol/l	0.95 (0.70-1.29)	**Biological disease modifying agents**	
Cholesterol-HDL ratio	2.70 (2.28-3.24)	TNF-α inhibitors, %	8.1
Chol-HDL ratio >4, %	9.2	Abatacept, %	2.3
Dyslipidaemia, %	50	NSAID, %	33.3
Diabetes, %	5.2	Prednisone use, %	2.3
Glucose, mmol/l	4.8 (4.5-5.1)	**Echocardiography**	
HOMA-IR	1.4 (0.98-1.90)	E/A	1.01 (0.82-1.32)
GFR, ml/min/1.73m2	91.7 (23.2)	E/e'	11.08 (8.01-13.37)
Framingham score	1.09 (0.56-2.37)	Lateral e' (cm/s)	9.6 (7.68-12.2)
Cardiovascular agents		Septal e' (cm/s)	7.94 (6.38-9.64)
Antihypertensives, %	40.8	LAVI	20.06 (16.01-24.39)
Statins, %	43.1	RWT	0.43 (0.38-0.50)
Ezetimibe, %	10.3	LVMI	78.76 (66.14-93.24)
Oral glucose lowering agents, %	1.7	**Adipokines**	
Insulin, %	1.7	Nesfatin, ng/ml	21.6 (8.46-41.5)
RA characteristics		Visfatin, ng/ml	26.0 (12.3-47.0)
RA duration, years	14.8 (8.9-22.5)	Vaspin, pg/ml	308.4 (185.3-413.7)

Continuous variables expressed as mean (SD), median (interquartile range) or proportions as appropriate. RA: rheumatoid arthritis; BP: blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein; GFR: glomerular filtration rate; ACPA: anti-citrullinated protein antibody; CDAI: clinical disease activity index; DAS28: Disease Activity Score in 28 joints; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; HAQ: Health Assessment Questionnaire; TNF-α: tumour necrosis factor-alpha; NSAID: non-steroidal anti-inflammatory drugs E/A: ratio of early to late diastolic filling velocity; E/e': index of left ventricular filling pressure; e': peak mitral annular velocity during early diastole; LAVI: left atrial volume index; RWT: relative wall thickness; LVMI: left ventricular mass indexed to body surface area.

5.4.3 Associations between nesfatin, visfatin and vaspin and indices of LV remodelling and LV diastolic function

Table 5.2 details the associations between nesfatin, visfatin and vaspin and markers of LV remodelling and LV diastolic function. In univariate analysis, no associations were noted between nesfatin or vaspin and markers of LV remodelling or LV diastolic function. In multivariate analysis, no associations were noted for both nesfatin and vaspin and any of the markers of LV remodelling or LV diastolic function.

In univariate analysis, visfatin concentrations were associated with increased E/e' (Std β (SE) 0.18 (0.08), p=0.02), increased LAVI (Std β (SE) 0.21 (0.08), p=0.005) and reduced lateral wall e' (Std β (SE) -0.16 (0.08), p=0.04). In multivariate analysis, the association between visfatin and E/e' (Std β (SE) 0.21 (0.08), p=0.008). and LAVI (Std β (SE) 0.17 (0.07), p=0.02) remained significant, while the association with lateral wall e' was no longer significant (p=0.16). When further including relative wall thickness as a potential confounder in the multivariate regression model, these results were materially unaltered.

5.4.4 Independent relationships between adipokine concentrations and LV remodelling and LV diastolic function in stratified analysis

In stratified analysis, nesfatin concentrations were associated with a reduced LVMI in patients that were younger than 50 years (Std β (SE) -0.36 (0.15), p=0.03) and in those that had a CDAI less than the median (Std β (SE) -0.21 (0.11), p=0.05). Nesfatin was also associated with a lower RWT in patients who had a disease duration less than 15 years (Std β (SE) -0.31 (0.14), p=0.03). Higher visfatin concentrations were associated with increased filling pressures in patients who were younger than 50 years (Std β (SE) 0.41 (0.14), p=0.008). Higher visfatin concentrations were consistently associated with increased filling pressures (E/e') and increased LAVI in patients who had shorter disease duration and DAS28 and CDAI scores less than the median (all $p<0.05$). Higher vaspin concentrations were associated with a higher lateral e' in those that are older than 50 years (p=0.03) and who had a DAS28 score less than the median (p=0.05).

5.4.5 Independent contribution of visfatin concentrations to increased filling pressures

Figure 5.1 shows that a one SD increase in visfatin was associated with an increased filling pressure in all patients (OR (95%CI) 1.45 (1.02-1.98), p=0.04) and in stratified analysis in women only (OR (95%CI) 1.45 (1.02-2.06), p=0.04). When including LVMI as an additional confounder in regression models, the results were materially unaltered (p=0.03 and p=0.02, respectively).

Table 5.2. Associations between adipokine concentrations and LV diastolic function variables in univariate and multivariate regression analysis

	Nesfatin* (n=159)		Visfatin* (n=170)		Vaspin* (n=170)	
	Std β (SE)	**P**	**Std β (SE)**	**P**	**Std β (SE)**	**P**
Univariate analysis						
E/A*	0.01 (0.01)	0.30	0.01 (0.08)	0.95	0.03 (0.08)	0.71
E/e'*	-0.01 (0.08)	0.90	**0.18 (0.08)**	**0.02**	-0.07 (0.08)	0.40
Lat e'*	0.12 (0.08)	0.15	**-0.16 (0.08)**	**0.04**	0.10 (0.08)	0.21
Sep e'*	0.08 (0.08)	0.32	-0.11 (0.08)	0.16	-0.08 (0.08)	0.27
LAVI	0.07 (0.08)	0.40	**0.21 (0.08)**	**0.01**	0.05 (0.08)	0.54
RWT	-0.03 (0.08)	0.72	0.00 (0.08)	1.00	-0.07 (0.08)	0.39
LVMI*	-0.07 (0.08)	0.35	0.13 (0.08)	0.10	0.00 (0.08)	0.97
Multivariate – Model 1						
E/A*	0.04 (0.07)	0.60	0.08 (0.07)	0.27	0.06 (0.08)	0.46
E/e'*	-0.04 (0.09)	0.62	**0.21 (0.08)**	**0.01**	-0.04 (0.09)	0.66
Lat e'*	0.06 (0.07)	0.41	-0.09 (0.07)	0.16	0.11 (0.07)	0.13
Sep e'*	0.05 (0.08)	0.48	-0.09 (0.07)	0.18	-0.06 (0.08)	0.40
LAVI	0.11 (0.08)	0.19	**0.17 (0.07)**	**0.02**	0.06 (0.09)	0.48
RWT	-0.10 (0.09)	0.26	-0.01 (0.08)	0.92	-0.08 (0.09)	0.37
LVMI*	-0.03 (0.07)	0.69	0.08 (0.07)	0.25	0.00 (0.08)	0.98
Multivariate – Model 2						
E/A*	0.05 (0.08)	0.56	0.10 (0.07)	0.18	0.06 (0.08)	0.44
E/e'*	0.05 (0.09)	0.54	**0.23 (0.08)**	**0.003**	-0.04 (0.09)	0.67
Lat e'*	0.07 (0.07)	0.34	-0.09 (0.07)	0.19	0.11 (0.07)	0.14
Sep e'*	0.06 (0.08)	0.47	-0.08 (0.07)	0.23	-0.06 (0.08)	0.42
LAVI	0.13 (0.08)	0.13	**0.20 (0.07)**	**0.01**	0.07 (0.09)	0.40
RWT	-0.10 (0.09)	0.23	0.01 (0.08)	0.94	-0.08 (0.09)	0.37

For model 1, associations for nesfatin were assessed in age, sex, race, systolic blood pressure, diastolic blood pressure, heart rate, waist-to-hip ratio, CRP, methotrexate use and abatacept use adjusted models. Associations for visfatin were assessed in age, sex, race, systolic blood pressure, diastolic blood pressure, heart rate, waist-to-hip ratio, extra-articular manifestations and azathioprine use adjusted models. Associations for vaspin were assessed in age, sex, race, systolic blood pressure, diastolic blood pressure, heart rate, waist-to-hip ratio, oral glucose agent use, HOMA-IR, prednisone use, diabetes adjusted models. For model 2, LVMI was included as an additional confounder. Std β: standardised beta coefficients; SE: standard error. E/A: ratio of early to late diastolic filling velocity; E/e': index of left ventricular filling pressure; e': peak velocity during early diastole; LAVI: left atrial volume index; RWT: relative wall thickness; LVMI: left ventricular mass indexed to body surface area. * logarithmically transformed.

Table 5.3. Independent associations of adipokine concentrations with measures of LV diastolic function in stratified analysis

	n	Std β (SE)	P	Std β (SE)	P
Nesfatin* vs		**RWT**		**LVMI**	
Age					
<50 years	47	-0.11 (0.19)	0.59	**-0.36 (0.15)**	**0.03**
≥50 years	109	-0.10 (0.11)	0.35	-0.03 (0.09)	0.75
Disease duration					
<15 years	80	**-0.31 (0.14)**	**0.03**	-0.21 (0.14)	0.13
>15 years	76	-0.05 (0.11)	0.65	0.03 (0.08)	0.69
DAS28					
<2.9	86	-0.17 (0.09)	0.08	-0.05 (0.08)	0.52
>2.9	70	-0.06 (0.17)	0.7	0.11 (0.14)	0.42
CDAI					
<5.3	76	-0.09 (0.12)	0.49	**-0.21 (0.11)**	**0.05**
>5.3	78	-0.15 (0.13)	0.24	0.07 (0.10)	0.48
Visfatin* vs		**E/e'***		**LAVI**	
Age					
<50 years	47	**0.41 (0.14)**	**0.008**	0.21 (0.13)	0.13
≥50 years	126	0.15 (0.10)	0.13	0.16 (0.09)	0.07
Disease duration					
<15 years	84	**0.27 (0.12)**	**0.03**	**0.23 (0.11)**	**0.04**
>15 years	83	0.17 (0.11)	0.13	0.17 (0.10)	0.1
DAS28					
<2.9	91	**0.29 (0.11)**	**0.01**	**0.31 (0.11)**	**0.004**
>2.9	76	0.22 (0.12)	0.06	0.03 (0.11)	0.81
CDAI					
<5.3	81	**0.55 (0.12)**	**0.0001**	**0.35 (0.13)**	**0.01**
>5.3	86	0.15 (0.09)	0.13	0.13 (0.09)	0.18
Vaspin* vs		**Lateral e'***			
Age					
<50 years	44	-0.02 (0.16)	0.87		
≥50 years	116	**0.20 (0.09)**	**0.03**		
Disease duration					
<15 years	79	0.11 (0.10)	0.28		
>15 years	81	0.10 (0.11)	0.33		
DAS28					
<2.9	90	**0.21 (0.10)**	**0.05**		
>2.9	70	-0.11 (0.10)	0.28		
CDAI					
<5.3	76	0.16 (0.11)	0.15		
>5.3	81	0.03 (0.11)	0.72		

* logarithmically transformed; DAS28: disease activity score in 28 joints; CDAI: clinical disease activity index.

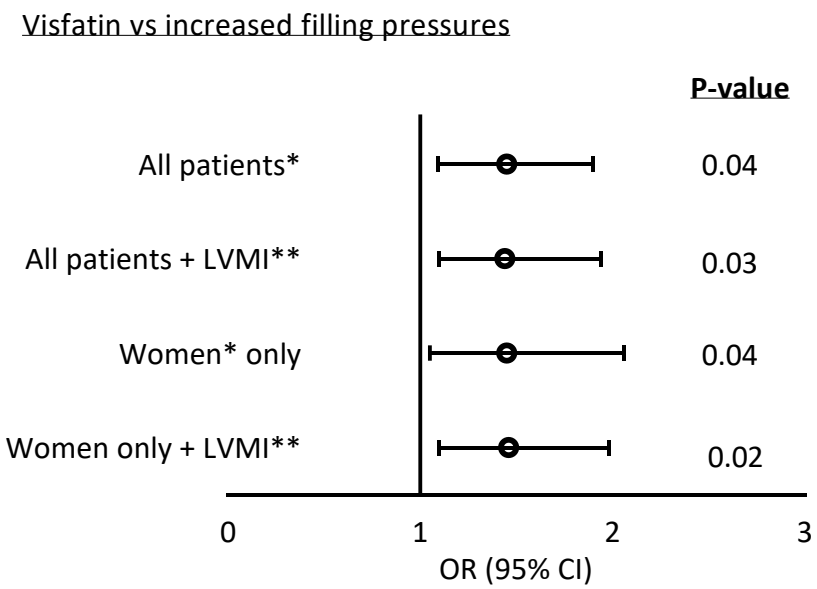

Figure 5.1. Association of 1SD increase in visfatin concentrations with raised filling pressures (E/e'>12) in all patients and in women only. *Confounders included age, sex, race, systolic blood pressure, diastolic blood pressure, heart rate, waist-to-hip ratio, extra-articular manifestations and azathioprine use; **Confounders as for * + left ventricular mass index (LVMI). OR: odds ratio; CI: confidence interval; LVMI: left ventricular mass index.

5.5 Discussion

The main findings of the current study are that higher visfatin concentrations were associated with increased filling pressures as evidenced by E/e' and left atrial volume index (LAVI). In stratified analysis, the association between visfatin and increased filling pressures persisted in patients younger than 50 years and patients with low disease activity and shorter disease duration. Similarly, increased visfatin concentrations were associated with increased filling pressure in women only and remained significant when including left ventricular mass index (LVMI) as a potential confounder. In the total population nesfatin and vaspin concentrations were not associated with markers of LV remodelling or LV diastolic function. In subgroup analysis, however, nesfatin concentrations were inversely associated with relative wall thickness (RWT) in patients with short disease duration and with LVMI in younger patients and those with lower disease activity. In subgroup analysis, vaspin concentrations were associated with increased lateral e' in older patients and patients with lower disease activity.

The current study improves our understanding regarding the potential for adipokines nesfatin, visfatin and vaspin as possible risk stratification biomarkers for cardiac dysfunction. Our results showed that visfatin is associated with markers of increased LV filling pressures and increased left atrial hypertrophy. Furthermore, the study showed that a one SD increased in visfatin was significantly associated with an increased risk of increased filling pressures (E/e' >12). As women are at increased risk for the development of LV diastolic dysfunction (Duca *et al.,* 2018; Ferreira *et al.*, 2015), in sensitivity analysis in women only the results showed that increased visfatin remained significantly associated with increased filling pressures. In this regard, increased filling pressure is a hallmark feature of LV diastolic dysfunction and is independently linked to the development of HFpEF and adverse cardiovascular outcomes (Aurigemma *et al.*, 2001). Furthermore, increased LAVI is considered in the diagnosis of LV diastolic dysfunction (Nagueh *et al*., 2016). Increased LV filling pressures has a direct impact on left atrial remodelling, and hence LAVI is also considered a prognostic marker for LV diastolic dysfunction (Nagueh *et al*., 2016). Increases in LV filling pressure is often a consequence of adverse LV remodelling, greater LV stiffness and impaired LV relaxation (Lyle & Brozovich, 2018; Selby *et al*., 2011; Kasner *et al.,* 2007; 2011). However, when including LVMI as a possible confounder in the multivariate regression models, visfatin remained independently associated with filling pressures. Nevertheless, in the current study visfatin concentrations were not directly related to markers of concentric remodelling (RWT or LVMI) or impaired relaxation (e' or E/A). This suggests that visfatin may not directly impact LV remodelling or LV relaxation. This is in keeping with previous results demonstrating that RA disease is an independent predictor of LV diastolic dysfunction, regardless of cardiac remodelling (Mokotedi *et al*., 2017).

Previous studies have suggested that visfatin may be involved in adverse LV remodelling (Erten *et al*., 2008; Malavazos *et al.,* 2008). However, these results are not consistent (Erten *et al.*, 2008). Furthermore, it has been suggested that the association between visfatin and left ventricular hypertrophy may be mediated by other inflammatory cytokines such as IL-6 and TNF-alpha, rather than a direct effect (Erten *et al.*, 2008). Lower visfatin concentrations were reported in patients with systolic heart failure when compared to healthy controls (Straburzyńska-Migaj *et al*., 2012). Others have also shown that visfatin concentrations are 1.7-fold higher in patients with HFpEF compared to patients with HFrEF, suggesting that visfatin may be associated with LV diastolic dysfunction (Toczylowski *et al*., 2019). Wang *et al*., (2014) also reported an association between visfatin and the functional class of heart failure.

With regards to the possible mechanisms whereby visfatin contribute to impaired cardiac function, it has been shown that cardiomyocytes can secrete Visfatin/Nampt when stressed and that exogenous Nampt contributes to cardiac remodelling inducing both hypertrophy and fibrosis (Pillai *et al.,* 2013). The contribution to remodelling was mediated through calcineurin/ nuclear factor of activated T-cell (NFAT) signalling pathway (Pillai *et al.,* 2013). In addition, visfatin has also been linked to the protein kinase A (PKA) pathway. In this regard, a recent study suggests that intracellular cyclic adenosine 3',5'-monophosphate (cAMP) regulates the expression of visfatin/nampt (Mitani *et al*., 2020). Increased concentrations of cAMP activate PKA, resulting in increased contractility and compliance as it allows for Ca^{2+} influx (MacLeod, 2016). However, PKA is also responsible for timeous relaxation of the ventricle (Lyle & Brozovich, 2018). Overexpression of PKA is associated with both hypertrophy and fibrosis that contribute to LV diastolic dysfunction (Frey & Olson, 2003; Lyle & Brozovich, 2018). However, whether visfatin is involved directly in the other mechanism of LV diastolic dysfunction, including calcium reuptake and titin phosphorylation (Lewis *et al*., 2017), is currently not known.

Interestingly, the study showed that the association between visfatin and increased filling pressures remained significant in younger patients with more favourable disease profiles. This is in contrast to the result in Chapter 4, where the associated between visfatin and the risk of target organ damage were shown in only in older patients with more adverse disease profiles. This suggests that visfatin concentrations and its impact on vessel remodelling and LV functional impairments may be impacted by age and disease status. Nevertheless, our findings suggest that visfatin may contribute to ventricular stiffening and/or impaired ventricular relaxation in the absence of hypertrophy. The exact mechanisms whereby visfatin contribute to both vascular and cardiac dysfunction and the impact of age and stages of disease on these mechanisms need further investigation.

Although nesfatin was not associated in all patients, in stratified analysis, nesfatin was associated with reduced relative wall thickness in patient with a disease duration less than 15 years, and with a lower LVMI in patients younger than 50 years and in those with a clinical disease activity index less than the median. These results suggest that in younger patients and those with more favourable disease profiles, nesfatin is protective again adverse LV concentric remodelling and LV hypertrophy. Although no study has evaluated the effect of nesfatin on LV diastolic dysfunction in humans, some evidence suggests that nesfatin receptors may be expressed in cardiomyocytes (Schalla & Stengel, 2018). Indeed, some animal studies using isoproterenol-induced myocardial infarction models in isolated rat heart preparations, showed that nesfatin may be cardioprotective by reducing infarct size (Tasatargil *et al*., 2017; Angelone *et al*., 2013). Furthermore, nesfatin also showed to reduce proinflammatory cytokines in rat hearts (Tasatargil *et al*., 2017). Similarly, these findings are in keeping with the results presented in chapter 2 of the present thesis, showing that nesfatin may be protective of adverse vessel remodelling, especially in those with more favourable disease profiles. Nevertheless, the mechanisms whereby nesfatin protect against adverse cardiac remodelling and left ventricular hypertrophy require further investigation.

Vaspin was not associated to any cardiac structural and functional markers in the total sample. In stratified analysis, increased vaspin concentrations were associated with increased lateral wall e' in older patients and in those with a DAS28 score less than the median. This suggest that higher vaspin concentrations were protective against a decrease in e', a marker of impaired relaxation. Previous results also suggest that vaspin may protect against adverse cardiac remodelling, possibly through its anti-inflammatory properties in patients with coronary artery disease (Zhou *et al.,* 2019). Furthermore, a recent study in a diabetic rat model, also showed protective effects of vaspin against myocardial injury by reducing inflammatory pathways (Li *et al*., 2019). The association noted in older patients, might be explained by the fact that vaspin concentrations increases with age (Xu *et al*., 2017). Furthermore, these results are in keeping with the result reported in chapter 3, that showed vaspin was protective against adverse vessel remodelling. Nevertheless, the associations between vaspin and relaxation were weak and requires confirmation in larger longitudinal studies.

This study has strengths and further limitations. The study investigated a relatively large group of RA patients. Subequent analysis accounted for a comprehensive range of carefully identified potential confounding characteristics. Our study design was cross-sectional and age- and sex matched healthy controls were not investigated, which precludes the identification of cause-effect relationships. Future longitudinal and interventional studies should include healthy matched controls to determine whether these findings are unique to RA. LV mass was determined using linear echocardiographic. Although

this method is validated, it employs the Devereux formula, which assumes a prolate ellipsoid shape of the LV. Future studies should determine LV mass using real-time 3D echocardiography, which relies on the direct measurement of the LV without geometric assumptions regarding LV shape and wall thickness.

In conclusion, the study reports for the first time that visfatin concentrations are associated with increased filling pressures, a hallmark characteristic of LV diastolic dysfunction. This association was influenced by age, disease activity and disease duration, but could not be explained by sex or changes in LV geometry. Although no associations were noted between markers of LV diastolic dysfunction and both nesfatin and vaspin, these adipokines exhibited cardio-protective properties in smaller subgroups.

Chapter 6: **Summary and conclusion**

6.1 Summary

Rheumatoid arthritis is a chronic inflammatory disease characterised by joint involvement with extra-articular manifestations that lead to organ dysfunction, as a result of disparate inflammatory cascades. RA confers a 2-fold increased risk for the development of CVD, which is not fully explained by conventional cardiovascular risk factors (Crowson *et al*., 2018; Avina-Zubeita *et al*., 2008). Cardiac involvement contributes to as much as 50% of mortality in patients with RA (Avina-Zubeita *et al*., 2008). Although poorly understood, systemic inflammation in RA is implicated in the mechanisms underlying the increased for CVD in RA (Crowson *et al*., 2018).

The need for improved CVD risk stratification in RA patients is well documented (Dessein & Semb, 2013). Adipokines have enjoyed considerable attention as possible biomarkers of CVD that may improve risk stratification in RA. Therefore, in the current thesis, I conducted a series of studies designed to advance our understanding of the roles of nesfatin, visfatin and vaspin as possible mediators of increased cardiovascular disease in patients with RA. Firstly, we established the relationship of these adipokines with demographic, traditional CVD risk factors and RA characteristics. Thereafter, this thesis evaluated the impact of nesfatin, visfatin and vaspin on three different markers of subclinical cardiovascular disease, namely atherosclerosis, arteriosclerosis, and LV diastolic dysfunction. A summary of the key findings of each chapter is provided in Table 6.1

6.1.1 Associations of adipokines with atherosclerotic CVD in RA

Patients with RA experience adverse metabolic risk factor profiles with increased endothelial activation and atherosclerosis (Ambrosino *et al.*, 2015a; Dessein *et al*., 2005). Indeed, atherosclerosis contributes to a substantial portion of RA related CV events (Avina-Zubeita *et al*., 2012). Often arterial plaque vulnerability related to disease activity result in plaque rupture that translate into a ~50% increase in cardiovascular morbidity and mortality (Avina-Zubeita *et al.,* 2008; Semb *et al*., 2007). Whereas systemic inflammation is associated with increased cardiovascular risk in RA, the underlying mechanisms remain incompletely understood. In this regard, evidence in support of an involvement of several adipokines in joint inflammation and damage as well as the enhanced cardiovascular risk in RA has been reported. We recently investigated the association of various matrix metalloproteinases with that of adipokines in patients with RA (Gunter *et al*., 2018; Robinson *et al*, 2017; Dessein *et al*., 2014d; 2014c; 2014b; 2014a). MMP's are zinc-dependant metallo-endopeptidases that are involved in the degradation of the extracellular matrix. In inflammatory conditions, such as RA, increased expression of MMP's contribute to both the degradation of cartilage and systemic manifestation (Araki & Mumura, 2017). Function and expression of MMP's are dependent on subtypes with five major

Table 6.1. Summary of key findings

CHAPTER	ADIPOKINES	KEY FINDINGS
Chapter 2: Nesfatin-1 and visfatin expression is associated with reduced atherosclerotic disease risk in patients with rheumatoid arthritis	Nesfatin Visfatin	The present findings suggest that nesfatin is protective against the development of CVD in patients with RA. This is evidenced by the associations between nesfatin and reduced cIMT and between nesfatin and MMP-2, a mediator of plaque stability. Visfatin is associated with increased levels of MMP-2, which might suggest a compensatory protective mechanism in patients with RA.
Chapter 3: Associations between vaspin concentrations, vascular remodelling and plaque vulnerability is impacted by cardiovascular disease risk factors in rheumatoid arthritis	Vaspin	Vaspin concentrations were directly associated with MMP-2 concentrations in patients who had no major traditional CVD risk factors. An inverse association was noted with MMP-9 concentrations in patients with a shorter disease duration. This suggests that vaspin concentrations may be associated with a reduced risk of plaque rupture, but only in those in the early stages of disease and with low CVD risk. Vaspin was also associated with angiopoietin-2 in patients who had no traditional CVD risk factors, which further supports the possibility that the vaspin may be cardioprotective.
Chapter 4: Visfatin, but not nesfatin or vaspin, is associated with reduced wave reflection in rheumatoid arthritis	Nesfatin Visfatin Vaspin	Visfatin was not associated with increased arterial stiffness (PWV), however it was inversely associated with markers of wave reflection, including augmentation pressure, backward wave pressure, augmentation index and reflection magnitude. The association between visfatin and wave reflection markers were influenced by age and disease profiles. Results showed that visfatin may contribute to adverse vessel remodelling and hence the risk for target organ damage in RA. There were no associations between either nesfatin or vaspin concentrations and indices of arterial stiffness or wave reflection.
Chapter 5: Nesfatin, visfatin and vaspin are differentially associated with cardiac geometry and left ventricular diastolic dysfunction in patients with rheumatoid arthritis	Nesfatin Visfatin Vaspin	Higher visfatin concentrations were associated with increased filling pressures as evidenced by the association between visfatin and E/e' and left atrial volume index. Visfatin concentrations were not related to markers of concentric remodelling (RWT or LVMI) or impaired relaxation (e' or E/A). This suggests that visfatin is directly associated with increased filling pressures, a cornerstone of LV diastolic dysfunction, but that is may not directly impact LV remodelling or LV relaxation. Nesfatin was associated with a lower risk of adverse myocardial remodelling in those that were younger, had a shorter disease duration and lower disease activity. Lower vaspin concentrations associated with improved LV relaxation in older patients and those with lower disease activity.

groups identified namely collagenases (MMP-1,-8,-13), gelitanases (MMP-2,-9), stomelysins (MMP-3,-10,-11), matrilysins (MMP-7,-26) and membrane sybtypes (Araki & Mimura, 2017; Itoh, 2015). We recently reported that apelin, which is another anti-inflammatory adipokine, is associated with increased MMP-2 and reduced MMP-9 concentrations in RA (Gunter *et al*., 2017). MMP-2 increases smooth muscle migration and proliferation that results in enhanced fibrous cap formation and thereby reduces plaque vulnerability to rupture (Newby, 2015; Nagase *et al.*, 2006; Watanabe & Ikeda, 2004). Congruently, MMP-2 concentrations are larger in stable compared to unstable arterial plaques. By contrast, MMP-3 and MMP-9 are implicated in increased plaque vulnerability (Newby, 2015; Nagase *et al.*, 2006; Watanabe & Ikeda, 2004). In this regard omentin, another anti-inflammatory adipokine, was associated with reduced MMP-3 levels in RA (Robinson *et al.*, 2017). Taken together, our previous studies indicate that anti-inflammatory adipokines may exert a plaque stabilizing effect in RA, which is in line with previous reports in non-RA subjects.

The first study showed that in patients with established treated RA, nesfatin concentrations were related to reduced carotid IMT and increased MMP-2 levels. The association between nesfatin and MMP-2 were independent of adiposity and RA activity These results support a potential protective effect of nesfatin against cardiovascular disease in patients with RA, by reducing cIMT and increased plaque stability. In contrast, visfatin concentrations were independently associated with increased diastolic blood pressure and diabetes, and increased MMP-2. The visfatin-MMP-2 association was independent of adiposity and RA activity. Given that visfatin is strongly implicated in increased plaque instability (Kadoglou *et al*., 2012; Dahl *et al.,* 2007) our finding of a direct relationship between concentrations of visfatin and those of MMP-2 was unexpected. Interestingly in this regard, whereas nesfatin and visfatin reportedly had overall contrasting effects on inflammation and cardiovascular disease risk in non-RA studies, we found that the concentrations of these 2 molecules were also directly correlated in RA. Further, rheumatoid factor positivity was associated with increased levels of both nesfatin and visfatin, and the nesfatin-MMP-2 as well as visfatin-MMP-2 relationships were stronger in rheumatoid factor negative compared to rheumatoid factor positive patients. Taken together, previously reported findings on the cardiovascular effects together with our current findings suggest that increased MMP-2 concentrations in relation to those of visfatin may represent a compensatory mechanism aimed at reducing visfatin mediated cardiovascular risk in RA.

In addition to the nesfatin and visfatin, the second study examined the potential impact of vaspin on metabolic risk factors, endothelial activation, carotid atherosclerosis and plaque vulnerability mediators in a group of RA patients. Increasing evidence emphasize the role of vaspin in obesity and related metabolic disorders (Recinella *et al*., 2020; Feng *et al*., 2014). Despite its association with

metabolic disorders, the role of vaspin in CVD is inconsistent. Some suggest vaspin may play a role in the development atherosclerosis (Choi *et al.*, 2011) and that it could serve as prognostic marker in patients with acute coronary syndrome (Zhou *et al.*, 2019). Other proposed that vaspin cannot be used as a biomarker for diagnosis of coronary artery disease (Stančík *et al*., 2017). In RA, reports on the contribution of serum vaspin levels to RA are contradictory. Whether vaspin is altered in RA and whether it contributes to disease pathogenesis is not certain (Maijer *et al*., 2015; Klaasen *et al*., 2012; Senolt *et al*., 2010; Ozgen *et al*., 2010).

The second study, in contrast to previous reports, showed that in RA patients vaspin was not associated with metabolic disease risk markers. However, vaspin concentrations were associated with the presence of established RA as defined by RF positivity. Vaspin concentrations were directly associated with MMP-2 concentrations in patients who had no major traditional CVD risk factors. An inverse association was noted with MMP-9 concentrations in patients with a short disease duration. This suggests that vaspin concentrations were associated with reduced risk of plaque rupture, but only in those in the early stages of disease and with low CVD risk. Our study also reports an association between vaspin and angiopoietin-2 in patients who had no traditional CVD risk factors. Although angiopoietin-2 is traditionally described as a mediator of adverse inflammatory changes, increased endothelial activation, adverse vascular remodelling, progression of atherosclerosis and destabilizing the vessel (Nicolini *et al*., 2019; Trollope & Golledge, 2011), the activity of angiopoietin-2 is dependent on the inflammatory status (Fiedler & Augustin, 2006), and its role in CVD is dose-dependent (Tressel *et al*., 2008). In this regard, at physiological concentrations or a transient upregulation of angiopoietin-2, stimulates revascularization, while high concentrations or prolonged exposure to angiopoietin-2 exacerbate vessel destabilization (Nicolini *et al*., 2019; Fiedler & Augustin, 2006). Hence, these dose-dependent effects of angiopoietin-2 may explain the relationship between vaspin and angiopeitin-2 in only the group with no cardiovascular disease risk factors. The protective role of vaspin might be lost in patients with existing cardiovascular risk factors. Furthermore, the lack of association between vaspin concentrations and endothelial activation markers in the current study supports the possibility that the vaspin angiopoietin-2 relations may be cardioprotective.

Taken together, our results suggest that in a cohort of RA patients that comprised of patients from both private and public healthcare, nesfatin and vaspin may be protective against atherosclerotic CVD by increased plaque stability. Similar to previous evidence showing that visfatin is associated with adverse vessel remodelling, these results demonstrated that increased visfatin concentrations may enhance atherosclerotic CVD risk by increasing cIMT. However, in patients with adverse CVD risk,

visfatin may upregulate MMP2 concentrations as a compensatory mechanism aimed at reducing visfatin mediated cardiovascular risk in RA.

6.1.2 Associations of adipokines with arterial stiffness, altered wave reflection and pressure pulsatility in RA

Another mechanism that may contribute to the increased CVD risk in RA patients, is increased arterial stiffness. Arterial stiffness predicts cardiovascular events in the general population and RA populations, independent of traditional cardiovascular risk factors (Mitchell *et al*., 2010; Klocke *et al.*, 2003). Pulse wave velocity, a marker of arterial stiffness and AIx, a measure of wave reflection comprises the two arterial function measures that are mostly investigated in studies on CVD risk stratification (Ambrosino *et al.*, 2015b; Vlachopoulos *et al.*, 2010). In addition, the measurements of wave reflection and pressure pulsatility were included as these factors were also shown to independently contribute to CVD risk in non-RA persons (Weber *et al.*, 2012; Wang *et al.*, 2010; Mitchell *et al.*, 2004).

RA patients have increased arterial stiffness, central aortic pressure, and wave reflection compared to the general population (Ambrosino *et al*., 2015; Klocke *et al*., 2003). Although traditional risk factors are associated with altered arterial function in RA, circulating inflammatory markers and disease duration are independent contributors to increased arterial stiffness and wave reflection (Gunter *et al*., 2017; Vázquez-Del Mercado *et al*., 2017; *Fan et al*., 2014; Provan *et al*., 2011). Despite the association between several adipokines and markers of arterial function the contribution of nesfatin, visfatin and vaspin to arterial stiffness is not well-known.

The third study, in a cohort of RA patients form private healthcare, reports for the first time that visfatin was not associated with increased arterial stiffness (PWV), however it was inversely associated with markers of wave reflection, including augmentation pressure, backward wave pressure, augmentation index and reflection magnitude. The association between visfatin and wave reflection markers were influenced by age and disease profiles. No associations between visfatin and wave reflection markers were seen in younger patients (<50 years of age) or those with more favourable disease profiles. However, the inverse association between visfatin and wave reflection markers remained significant in older patients (> 50 years) and those with an adverse disease profile. This association between increased visfatin concentrations and reduced wave reflection may seem controversial. However, recent evidence supports a paradigm suggested by Mitchell (1985) that a reduced, rather than an increased wave reflection is associated with higher risk for target organ damage (Kondiboyina *et al*., 2020; Phan *et al*., 2020). Briefly, the reduced wave reflection limits the reflection of the pulsatile energy back to the heart, hence the hydraulic energy needs to be absorbed

by peripheral microvasculature, and hence can cause target organ damage (Kondiboyina *et al*., 2020; Mitchell *et al*., 2011). Furthermore, increased age and inflammatory status are associated with adverse vessel remodelling and have both been linked to reduced wave reflection (Motau *et al.*, 2020; Schroeder *et al.*, 2019; Maple-Brown *et al.*, 2005). This impact of age and inflammation on the contribution of the reflected wave was confirmed by the study. Results showed that visfatin may contribute to adverse vessel remodelling and hence the risk for target organ damage in RA.

Despite previous involvement of nesfatin and vaspin in plaque vulnerability mediations, no associations between either nesfatin or vaspin concentrations and indices of arterial stiffness or wave reflection, are reported. Nevertheless, in this cohort of patient, vaspin concentrations were strongly linked to adverse metabolic profiles. These results are in keeping with current literature (Recinella *et al*., 2020), but in contrast to the results reported in the cohort of both private and public healthcare in chapter 3. The differences in demographic characteristics, disease profiles, metabolic risk factors, adiposity and socio-economic status between the two cohorts may have impacted these results.

6.1.3 Associations of adipokines with left ventricular diastolic dysfunction in RA

The findings in Chapter 4 raise the question whether visfatin is indeed associated with target organ damage. In this regard, RA patients have a two-fold risk for developing HFpEF compared to the general population (Nicola *et al*., 2005). Studies evaluating phenotypes of heart failure in RA populations, suggest an increased prevalence of LV diastolic dysfunction in these patients (Liang *et al*., 2010). Evidence suggests that both low- and high-grade inflammation contribute to the development of heart failure in non-RA patients (Paulus & Tschöpe, 2013). In RA, although traditional risk factors contribute to the development of LV diastolic dysfunction, RA disease characteristics, inflammation and altered arterial function play an important role in the pathogenesis of LV diastolic dysfunction (Mokotedi *et al*., 2019; Mokotedi *et al.,* 2017, Davis *et al.*, 2011; Liang *et al*., 2010). The contribution of adipokines to LV diastolic dysfunction is not well studied.

The result from the fourth study showed that higher visfatin concentrations were associated with increased filling pressures as evidenced by the association between visfatin and E/e' and left atrial volume index. In stratified analysis, the association between visfatin and increased filling pressures persisted in patients younger than 50 years and patients with low disease activity and shorter disease duration. As women are at increased risk of developing LV diastolic dysfunction, stratified analysis in women only showed that increased visfatin concentrations were associated with increased filling pressures. Taken together, these results suggest that visfatin is directly associated with increased

filling pressures, a cornerstone of LV diastolic dysfunction. Increases in LV filling pressure is often a consequence of adverse LV remodelling, greater LV stiffness and impaired LV relaxation (Lyle & Brozovich, 2018; Selby *et al*., 2011; Kasner *et al.,* 2007; 2011). Indeed, several mechanisms contribute to the development of LV diastolic dysfunction. In this regard, LV concentric remodelling and increased LV fibrosis, impaired relaxation because of impaired calcium reuptake and cardiomyocyte stiffness as a result of altered titin phosphorylation all contribute to the phenotypes of LV diastolic dysfunction (Lewis *et al*., 2017; Zile *et al*., 2015). Nevertheless, when including LVMI, as a marker of LV remodelling and fibrosis in the analysis, visfatin remained independently associated with increased LV filling pressures. Moreover, in the current study visfatin concentrations were not directly related to markers of concentric remodelling (RWT or LVMI) or impaired relaxation (e' or E/A). This suggests that visfatin may not directly impact LV remodelling or LV relaxation. This is in keeping with previous results demonstrating that RA disease is an independent predictor of LV diastolic dysfunction, regardless of cardiac remodelling (Mokotedi *et al*., 2017).

No associations were noted between markers of LV diastolic dysfunction and both nesfatin and vaspin in the total sample. In subgroup analysis, however, nesfatin was inversely associated with concentric remodelling (RWT) in patients with short disease duration and with LVMI in younger patients as well as in patients with low disease activity. Although no study has evaluated with effect of nesfatin on LV diastolic dysfunction, our current findings are in keeping with previous animal studies suggesting that nesfatin is cardioprotective (Schalla & Stengel, 2018; Tasatargil *et al.*, 2017). Also, in subgroup analysis, vaspin was directly associated with lateral e' in older patients and patients with low disease activity. These results suggest that vaspin concentrations were protective against impaired LV relaxation. Again, this is in keeping with previous studies suggesting that vaspin protects against adverse cardiac remodelling (Zhou *et al.,* 2019), especially as vaspin increases in older adults (Xu *et al*., 2017).

Taken together, the results from this cohort confirms our earlier findings that both nesfatin and vaspin may be protective against CVD. In this regard nesfatin may impact LV concentric remodelling, while vaspin may impact LV relaxation. Visfatin contributes to the increased CVD risk by adverse vessel remodelling that result in reduced wave reflection. Visfatin is also associated with increased LV filling pressures, independent of LV fibrosis and remodelling. Importantly, the various adipokines were associated with disparate markers of LV remodelling and LV diastolic function. Overall, the results of these studies support previous suggestions that management of not only traditional CVD factors but also inflammation and RA disease characteristics is vital in reducing the overall risk of CVD, as these factors impacted the relationships between adipokines and various subclinical CVD risk markers. Finally, the consideration and use of adipokines may improve CVD risk stratification in patients with

RA. A summary of the relationship between the adipokines and subclinical cardiovascular markers in RA are provided in Figure 6.1.

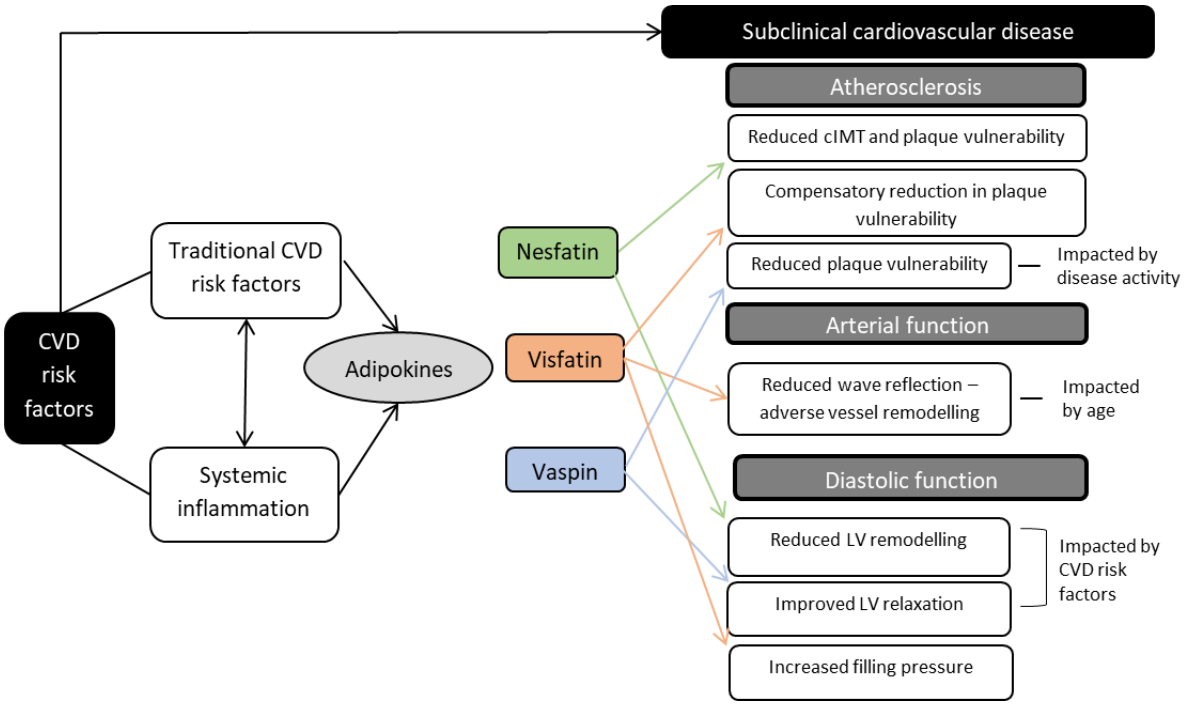

Figure 6.1. Graphical representation of relationship between adipokines and subclinical cardiovascular disease in patients with RA

6.2 Limitations

This study has several strengths and limitations. The specific limitations were discussed in each of the chapters of the thesis, however, there are some limitations that warrant emphasis. First, age- and sex-matched controls were not included in the cohorts of RA patients, which precludes drawing inferences regarding cause and effect. Since the present study did not include a control group, it remains uncertain as to whether our findings are RA specific. Future longitudinal investigations are needed to substantiate these findings. This thesis evaluated the effects of these adipokines on subclinical cardiovascular disease markers in two different cohorts of RA patients. Although several findings were confirmed in the two cohorts, differences in socio-economic status, race, disease profiles and other demographic factors may have impacted our results. It is important to consider that expression of the adipokines nesfatin, visfatin and vaspin are dependent on multifactorial regulation and influenced by fat area and distribution, renal function, iron metabolism, inflammatory- and hormone profile amongst others. In addition, expression of these adipokines could be regulated by other endothelial markers which were not explored. Although three relatively new adipokines were assessed, inclusion of more

well-known adipokines in the results may have improved the interpretation of these results. Also, circulating concentrations of biomarkers is not necessarily representative of tissue levels.

With regards to arterial function measurement, although the use of the in-built generalised transfer function to determine the central waveform has been validated, the accuracy of central blood pressure obtained using transfer functions may be suboptimal. In addition, the use of the 'triangulation method' for wave separation analysis has been validated. However, the assumptions intrinsic to the use of this method are not ideal as backward and forward wave pressures are overestimated compared to Doppler measures. Simultaneous recording of actual aortic flow and pressure should be addressed in future studies.

The additional evaluation of endothelial dysfunction as measured by flow mediated dilatation could have provided further evidence in support of our findings in chapter 2 and 3 in the present thesis. Lastly, although the echocardiography measures employed in this study are validated and according to current principals, other more sensitive techniques aimed at identifying early pre-clinical LV systolic and diastolic dysfunction such as Speckle Tracking echocardiography may have provided further insight to the role of adipokines on cardiac function.

6.3 Conclusion

In conclusion, the findings reported in this thesis, contribute to our understanding of the possible roles of nesfatin, visfatin and vaspin as possible mediators of increased cardiovascular disease in patients with RA. The findings suggest that nesfatin is associated with reduced atherosclerosis and increased plaque stability mediator levels in RA. However, these associations are impacted by traditional risk factors and RA disease characteristics. Similarly, vaspin concentrations are associated with reduced risk of plaque rupture and vessel stability, but only in those in the early stages of disease and with low cardiovascular disease risk. Both nesfatin and vaspin were not associated with markers of arterial stiffness or wave reflection. However, nesfatin was associated with a reduced risk of adverse LV remodelling and hypertrophy in younger patients with more favourable disease profiles, while vaspin was related to improved LV relaxation markers. In contrast, visfatin relates to adverse cardio-metabolic risk in RA suggesting a role in adverse vascular remodelling and plaque stability. Visfatin is a likely biomarker for adverse vascular remodelling, plaque stability and increased LV filling pressures. Taken together, these findings suggest that adipokines may improve risk stratification for cardiovascular disease in RA patients and merits further study. As traditional risk factors and RA disease profiles impacted the relationships between adipokines and subclinical CVD risk markers, the

findings in this thesis further support recommendations that both traditional cardiovascular risk management and adequate RA control should be targeted in the prevention of CVD.

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Appendices

Appendix A: Human ethics clearance certificates

R14/49 Mrs Chanel Robinson et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170986

NAME: (Principal Investigator)	Mrs Chanel Robinson et al
DEPARTMENT:	Physiology Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Adipokines as Biomarkers of Subclinical Cardiovascular Disease in Rheumatoid Arthritis
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under Primary Study M170592
SUPERVISOR:	Prof Aletta Millen and Prof Patrick Dessein
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	23/10/2017

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

DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

R14/49 Prof Patrick Dessein et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170592

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

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

DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: Confirmation of contribution to work by primary supervisor

To whom it may concern

I hereby confirm that Chanel Robinson has substantially contributed to the conception of each of the studies, data collection, interpretation of the results and writing of the manuscripts that are included in this PhD thesis

Yours sincerely

Associate Prof Aletta Millen

Appendix C: Publication of "Nesfatin-1 and visfatin expression is associated with reduced atherosclerotic disease risk in patients with rheumatoid arthritis"