ENDOTHELIAL DYSFUNCTION IN RHEUMATOID ARTHRITIS: THE ROLE OF INFLAMMATION AND CLASSICAL CARDIOVASCULAR DISEASE RISK FACTORS ON THE MICROVASCULATURE AND THE MACROVASCULATURE

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Abstract

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of the joints with predominant symptoms of pain, swelling and stiffness. Patients with RA are at increased risk of cardiovascular disease (CVD). The exact mechanism for this is unknown, but RA disease-related inflammation has been postulated to affect the vasculature and contribute to endothelial dysfunction. The studies presented in this thesis examine vascular function in patients with RA and explore associations with disease-related inflammation as well as CVD risk factors. A cross-sectional study was carried out with 99 RA patients and 32 healthy control participants who underwent assessments of microvascular endothelial function, macrovascular endothelial function and arterial stiffness (AIx). Microvascular and macrovascular endothelial function were similar in RA patients and healthy control participants, but AIx was higher in the RA patients, as was global CVD risk. RA disease-related inflammation was not associated with microvascular or macrovascular endothelial-dependent function, however, global CVD risk inversely correlated with microvascular endothelial-dependent function and macrovascular endothelial-independent function. A longitudinal study was conducted in 23 RA patients starting on anti-tumor necrosis factor-α (anti-TNF-α) treatment and all the above-mentioned assessments were repeated after 2 and 12 weeks of treatment. Treatment, which was successful in reducing disease activity at 2 and 12 weeks, resulted in an improvement in microvascular endothelial-dependent function at 2 weeks, but not at 12 weeks. There was no change in macrovascular endothelial-dependent function or arterial stiffness at any time point, nor in global CVD risk. Finally, a systematic review of the literature pertaining to endothelial function in RA was performed. This revealed that, on the whole, the evidence supporting a relationship between endothelial function and disease-related inflammation was not strong. The findings of these studies suggest that classical CVD risk may be a better predictor of endothelial function in RA than disease-related inflammation.

Acknowledgements

Contents

CHAPTER 6: THE ASSOCIATION BETWEEN INFLAMMATION, CARDIOVASCUALR DISEASE RISK FACTORS AND MICROVASCULAR AND MACROVASCULAR ENDOTHELIAL FUNCTION IN RHEUMATOID ARTHRITIS

CHAPTER 7: THE EFFECTS OF ANTI-TUMOR NECROSIS FACTOR-ALPHA ON MICROVASCULAR AND MACROVASCULAR ENDOTHELIAL FUNCTION IN RHEUMATOID ARTHRITIS

CHAPTER 8: GENERAL DISCUSSION

List of Figures

List of Tables

Chapter 6

Chapter 7

List of Abbreviations

ACE	Angiotensin Converting Enzyme		**EET**	Epoxyeicosatrienoic Acids
ACh	Acetylcholine		**eNOS**	Endothelial Nitric Oxide Synthase
ADP	Adenosine Diphosphate		**ER**	Endoplasmic Reticulum
ACR	American College of Rheumatology		**ESR**	Erythrocyte Sedimentation Rate
ANCOVA	Analysis of Co-variance		**ET**	Endothelin
ANOVA	Analysis of variance		**EULAR**	European League Against Rheumatism
AIx	Augmentation Index		**FBF**	Forearm Blood Flow
Anti-CCP	Anti-citrullinated Peptide Antibodies		**FMD**	Flow-mediated Dilatation
ATP	Adenosine Triphosphate		**FRS**	Framingham Risk Score
BK	Bradykinin		**GTN**	Glyceryl-trinitrate-mediated Dilatation
BMI	Body Mass Index		**GTP**	Guanosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate		**HAQ**	Health Assessment Questionnaire
cGMP	Cyclic Guanosine-3', 5-monophosphate		**HDL-C**	High-density Lipoprotein Cholesterol
CHD	Coronary Heart Disease		**HLA**	Human Leukocyte Antigen
cIMT	Carotid Intima-media Thickness		**HOMA**	Homeostasis Model Assessment
CVD	Cardiovascular Disease		**HR**	Heart Rate
CofV	Co-efficient of Variation		**ICAM-1**	Inter-cellular Adhesion Molecule-1
COX	Cyclooxygenase		**IL-1**	Interluekin-1
CRP	C-reactive Protein		**IL-6**	Interluekin-6
DAS28	Disease Activity Score 28		**IL-8**	Interluekin-8
DBP	Diastolic Blood Pressure		**IL-17**	Interluekin-17
DMARD	Disease Modifying Anti-Rheumatic Drug		**IMT**	Intima-media Thickness
ED	Endothelial Dysfunction		**IR**	Insulin Resistance
EDHF	Endothelium-Derived Hyperpolarizing Factor		**LDL-C**	Low-density Lipoprotein Cholesterol
EDTA	Ethylenediaminetetraacetic acid		**SD**	Standard Deviation

LNMMA	N^G monomethyl-L-arginine	**LDI**	Laser Doppler Imaging
MCP	Metacarpophalangeal	**SGc**	Soluble Guanylyl Cyclase
MCP-1	Monocyte-chemoattractant Protein-1	**SNP**	Sodium Nitroprusside
MIP	Macrophage Inflammatory Protein	**SOCa^{2+}**	Store-operated Calcium Channel
MLCK	Myosin Light Chain Kinase	**SP**	Substance P
MTP	Metatarsophlangeal	**TACE**	TNF-α Converting Enzyme
MTX	Methotrexate	**TC**	Total Cholesterol
NaCl	Sodium Chloride	**TG**	Triglycerides
NO	Nitric Oxide	**TNF-α**	Tumor Necrosis Factor-alpha
NSAID	Non Steroidal Anti-inflammatory Drugs	**TXA$_2$**	Thromboxane
NHS	National Health Service	**UK**	United Kingdom
NICE	National Institute of Clinical Excellence	**VAS**	Visual Analogue Scale
PC	Phosphorylchoine	**VIA**	Vascular Image Analysis
PGI$_2$	Prostacyclin	**WHO**	World Health Organisation
PIP	Proximal Interphalangeal	**VOP**	Venous Occlusion Plethysmography
PU	Perfusion Units	**VCAM**	Vascular Cell Adhesion Molecule
PWA	Pulse Wave Analysis	**VSMC**	Vascular Smooth Muscle Cells
PWV	Pulse Wave Velocity		
QUICKI	Quantitative Insulin Sensitivity Check Index		
RA	Rheumatoid Arthritis		
RCT	Randomised Control Trial		
RF	Rheumatoid Factor		
RH	Reactive Hyperaemia		
SBP	Systolic Blood Pressure		
SCORE	Systematic Coronary Risk Evaluation		

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2. **Sandoo A**, Toms T, Veldhuijzen van Zanten JJCS, Carroll D, and Kitas GD. 2009. Short-term effects of rituximab on flow-mediated dilatation may be mediated by intravenous glucocorticoids: Comment on the article by Gonzalez-Juanatey et al. *Arthritis and Rheumatism*. **61**. 854-855.

3. Stamatelopoulos KS, Kitas GD, Papamichael CM, Chryssohoou E, Kyrkou K, Georgiopoulos G, Protogerou A, Panoulas VF, **Sandoo A**, Tentolouris N, Mavrikakis M, Sfikakis PP. 2009. Atherosclerosis in rheumatoid arthritis versus diabetes: a comparative study. *Arteriosclerosis Thrombosis and Vascular Biology.* **29**(10). 1702-8

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5. Gasparyan AY, **Sandoo A,** Stavropolous-Kalinoglou, A, and Kitas GD. 2010. Mean platelet volume in patients with rheumatoid arthritis: the effect of anti-TNF therapy. *Rheumatology International*. Jan 12. Epub ahead of print.

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Chapter 1 (Part I): General Introduction

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of the joints with predominant symptoms of pain, swelling and stiffness. The pattern of disease activity often varies in patients, and can include periods of high disease activity (flares) interspersed with periods of low disease activity (remission). Poor control of disease activity can result in erosive bone damage which can then lead to joint destruction and physical disability [1]. The precise aetiology of RA is not known, however, recent concepts support the '*gene-environment interaction hypotheses'*, where a combination of environmental factors (such as smoking) and genetic factors (such as the shared epitope) can contribute to the onset of disease [2]. Patients with RA have a high mortality rate when compared to the general population, with cardiovascular disease contributing up to 50% of all deaths [3]. Currently, there is no cure for RA and so the goal of treatment is to suppress disease activity, reduce disease progression and adequately treat co-morbidities. The continuous development of new medications is enhancing the effective treatment of this condition.

Epidemiology

RA affects ~0.8% of the adult population of the United Kingdom (UK) and is more common in females than males [4]. The prevalence in some European countries appears to be lower than that in the UK (range 0.1 -0.5%) [5,6]. In other parts of the world the prevalence of RA also varies. For example, RA was found to be virtually absent in a rural population in Nigeria [7]. Interestingly, studies conducted in South Africa showed that the prevalence of RA was low amongst Bhantu-speaking people living in their traditional rural environments; however, the prevalence increased when this group of people migrated to the modern and industrialized town of Soweto [8,9]. This change in RA prevalence, along with the low incidence of RA in rural Nigeria, suggests that RA might be associated to lifestyle habits of industrialised areas. However, comparisons of the prevalence

between different countries must be interpreted with caution, as not all epidemiological studies use the same criteria to classify RA, and populations can differ on age and gender [4].

Aetiology

At present, the exact aetiopathogenesis of RA is not known, although current evidence suggests that the onset of RA may be triggered via an interaction between genetic, environmental and lifestyle factors. A discussion on these follows below.

a) Genetic Factors

There is evidence suggesting the cause of RA has a genetic basis. For example, population studies have shown that the prevalence of RA is greater in first degree relatives of patients with RA [10,11]. Furthermore, there is greater disease concordance in identical twins (15%) than in non-identical twins (4%) [12]. Alleles of the major histocompatibility complex class II (MHC II) have been identified as risk alleles for RA [13]. One of the most widely characterised genes is the Human Leukocyte Antigen (HLA) DRB1 gene [14]. The primary function of HLA is to encode viral peptides (produced when antigen-presenting cells engulf foreign pathogens) so that they can be identified by T-cells (which are released by the immune system to destroy virally-infected cells) [14]. RA susceptibility is dependent on multiple risk alleles of the HLA-DRB1 gene, and all share a conserved amino acid sequence, known as the rheumatoid epitope and are an integral part of the 'shared epitope hypothesis' [15]. RA risk alleles may also be located outside of the MHC, as a number of non-MHC alleles have recently been identified. Examples include PTPN22 [16], STAT4 [17], and TRAF1-C5 [18] amongst several others. However, alleles located on the MHC contribute to 30% of the genetic burden of RA as opposed to 3-5% of non-MHC alleles [19-21]. Interestingly, from the non-MHC risk alleles, PTNPN22 locus appears to confer the strongest risk for RA in European populations [16]. Another important finding is that both HLA-DRB1 and PTPN22 risk alleles strongly associate with more

severe forms of RA [22,23]. This has potentially important implications as it could allow categorisation of patients into different subsets of disease severity [24,25].

b) Hormonal Factors

The prevalence of RA varies between gender, with females 2-3 times more likely to develop RA than males [4]. Women who are nulliparous are at a much greater risk of RA [26], while pregnancy has a beneficial effect on RA [27]. Indeed, during pregnancy, there
is development of alloantibodies against the paternal HLA [28]. These alloantibodies are believed to inhibit the function of all HLA-DR alleles, and so reduce disease severity [29]. Collectively, these findings suggest that hormonal factors play an important role in the development of RA, possibly by interacting with genetic factors.

c) Infectious Agents

Although a number of different infectious agents for the pathogenesis of RA have been investigated, the most extensively investigated infectious agent in the RA population is the Epstein-Barr virus [30]. This virus shares the same HLA-DRB epitopes with type II collagen which is found in the cartilage of the joints. It is believed that exposure to the Epstein-Barr virus triggers a normal immunological response to the virus; however, due to similarities of the virus to type II collagen, an auto-immune response is triggered in the joints leading to increased inflammation in the synovium [31]. It is worthwhile to mention that rates of contracting Epstein-Barr virus are high in the general population, and one would therefore expect a similarly high prevalence of RA [32]. As this is not the case, the precise mechanisms of the cross-talk mentioned above need to be further elucidated.

d) Smoking

HLA-DR shared epitope (SE) genes play a major role in the development of RA [15]. Smoking appears to be a key environmental factor in the aetiology of RA [33,34] and has been demonstrated to interact with HLA-DR SE in RA patients with seropositive disease [35]. The precise mechanism for this interaction has not yet been elucidated, but protein citrullination appears to be a strong factor [36]. Protein citrullination is the post-transcriptional modification of arginine into citrulline which results in significant alterations in the structure and function of various proteins [37]. Antibodies to citrullinated proteins, such as anti-cyclic citrullinated peptide (anti-CCP) antibody, are biomarkers of protein citrullination and have been shown to precede the development of RA [38-40], as well as being possibly involved in the causation of RA [41]. Smoking has been shown to increase peptidylarginine deiminases (an enzyme responsible for catalysing arginine into citrulline) in alveolar tissue of healthy smokers, and not surprisingly, these individuals subsequently displayed evidence of protein citrullination in their bronchial alveolar lavage cells [42]. Another study conducted in RA patients revealed that HLA-DR SE risk alleles and smoking increased the risk of developing RA only in anti-CCP positive patients [36]. Importantly, smoking was associated with a higher risk of developing anti-CCP positive RA in patients with double copies of HLA-DR SE genes, with the risk being significantly lower in non-smokers with this pattern of SE gene expression [36]. Thus, exposure to cigarette smoke facilitates protein citrullination in the lungs, and the subsequent autoimmune response is more pronounced in individuals carrying the HLA-DR SE genes. Interestingly, exposure to silica (found in sand and quartz) has been shown to increase the risk of developing anti-CCP positive RA, and the effect of silica is stronger in patients exposed to smoking as well [43]. This suggests that exposure to environmental factors via inhalation may be an important pathway in the development of RA and supports the findings of the earlier work showing protein citrullination in alveolar tissue [36,42].

Pathogenesis

RA is an autoimmune disease in which alterations in immune function result in joint degradation and destruction. CD4+ T cells (also known as T helper cells) are regarded as major protagonists in initiating the inflammatory response in RA [44]. CD4+ T cells are not able to recognise antigens (a molecule that triggers an immune response) themselves and rely on antigen presentation by cells known as antigen presenting cells (APCs). Examples of APC's include macrophages, dendritic cells and B cells [44,45]. APC's form complexes with MHC II proteins which then internalise antigens via endocytosis and display fragments of the antigen on a groove located on their surface. Specialised T cell receptors located on CD4+ T cells which are specific to the endocytosed antigen recognise the antigens bound to MHC II proteins and the CD4+ T cell becomes activated [44].

CD4+ T cells can then stimulate the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and IL-17 [44]. The recruitment of inflammatory cells is mediated by chemotactic molecules such as IL-8, monocyte-chemoattractant protein-1 and macrophage inflammatory protein-α [46]. The next process is an increase in leucocyte-endothelial interactions [47]. Complement factors such as C3a and C5 attract leucocytes (e.g. CD4+ T cells, B cells and macrophages) to the endothelium, after which adhesion molecules such as E-selectin are involved in slow rolling of the leucocytes along the endothelial lining until firm adhesion occurs [48]. Intercellular adhesion molecules and vascular cell adhesion molecules then allow transmigration of leucocytes to the site of inflammation [48]. Pro-inflammatory cytokines play an active role in promoting leucocyte-endothelial interactions as they help to increase the expression of adhesion molecules [49]. These processes result in structural changes to the joint and are characterised by the formation of new blood vessels which increase delivery of more inflammatory cells to the synovium, hyperplasia of the synovial lining, and oedema [44]. Clinically, these processes result in pain and swelling of

the joint [47]. As inflammation continues there is further hyperplasia of the synovium which results in formation of thick, invasive tissue termed Pannus (see Figure 1) [44]. The formation of Pannus is a key event in the process of joint destruction as Pannus contains a high concentration of macrophages, T cells and B cells [47].
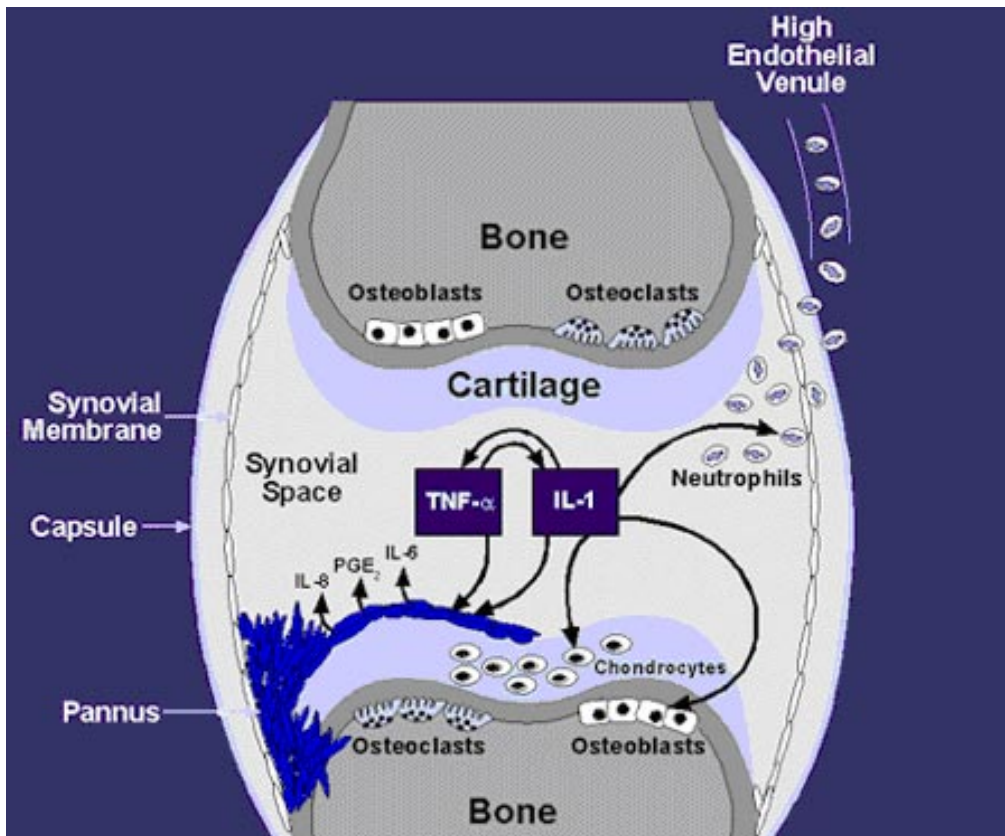
Figure 1. A joint affected by RA. Printed with permission from Alphaflex.com.

The macrophages that are present in the pannus release further pro-inflammatory cytokines which in turn stimulate mesenchymal cells like synovial fibroblasts, osteoclasts and chondrocytes to cause severe erosion to cartilage and bone tissue [50]. TNF- α, IL-1 and IL-6 are the main cytokines involved in stimulating these cells [51,52], and also stimulate matrix metalloproteinases which degrade the extracellular matrix leading to further bone erosion [48]. Another action of TNF- α and IL-1 involves blocking tissue-inhibitors of matrix metalloproteinases which perpetuates joint destruction [50].

The chronic inflammatory nature of RA occurs as a result of continued activation of CD4+ T cells by APC's, with B cells thought to be the most prominent APC in the synovium of RA patients [53]. Activated T cells also stimulate B cells to differentiate into plasma cells which then produce antibodies to specific antigens [1]. An example of one such antibody in RA is rheumatoid factor (RF), which is associated with severe articular disease [53]. In addition, RA patients who are seropositive for the RF antibody appear to be at a greater risk of mortality and morbidity form extra-articular manifestations such as cardiovascular disease [54]. Depletion of B cells using rituximab treatment has led to improvements in RA disease-severity [55], possibly due to reductions in T cell-mediated cytokine activity [45]. Thus, along with T cells, B cells also play an important role in regulating the immune response in RA, and may substantially contribute to chronic inflammation.

Clinical Features of Rheumatoid Arthritis

The symptoms of RA predominantly consist of pain, swelling and stiffness of the joints. The onset of disease typically occurs over several weeks or months, however, in approximately a third of people, the disease has a very rapid onset, and may occur over a few days or weeks [56]. The most commonly affected joints are the metacarpophalangeal (MCP), proximal interphalangeal (PIP) and metatarsophalangeal (MTP) joints as well as the wrists. In some patients the shoulders, elbows, knees and ankles may also be affected. An examination of the affected joints usually reveals the cardinal signs of inflammation (redness, tenderness, swelling and heat), and in early RA the MCP joints are typically affected first, although other joints can also be affected [56].

As the disease progresses, damage to the joints leads to physical deformities and eventual disability [57]. In patients with a disease duration greater than 10 years, 16% are severely disabled, while, only 17% of patients are free from any kind of disability [58]. Examples of irreversible deformities to the

joints include pes planus (loss of foot arches), hammer and claw toes, and deformities in the MCP and MTP joints due to joint subluxation (dislocation) [56].

Extra-articular manifestations are present in 40% of patients [59] and appear to be most common in patients with the highest disease activity [60]. Moreover, patients with extra-articular manifestations have increased morbidity and mortality [61]. Examples of such extra-articular manifestations include severe weight loss, osteoporosis and cardiovascular disease (CVD) [62-64]. Table 1 shows the extra-articular manifestations in RA. Due to its pertinence to the current project the discussion will focus only on CVD.

Table 1. The extra-articular manifestations of RA

Organ System	Extra-Articular Manifestation
Cardiovascular	Vasculitis, pericarditis, coronary artery disease, myocardial infarction
Pulmonary	Interstitial lung disease, pleural effusion, nodules,
Blood	Felty's syndrome, large granulocytic leukaemia, thrombocytosis, anaemia, splenomegaly, non-Hodgkin's lymphoma
Nervous	Neuropathy
Muscle	Rheumatoid cachexia
Bone	Osteopaenia and osteoporosis
Salivary Gland	Secondary Sjögren's syndrome with dry eyes and mouth
Skin	Cutaneous vasculitis, rheumatoid nodules
Occular	Scleritis, peripheral ulcerative keratitis, keratoconjunctivitis sicca, episcleritis

Turesson et al [59]

Diagnosis

The diagnosis of RA can be made using the patient's medical history, symptom patterns, as well as laboratory and radiographic features [56]. In 1987 the

American College of Rheumatology (ACR) established guidelines for classifying patients with RA (shown in Table 2). For patients to be diagnosed with RA, they must satisfy at least 4 out of 7 of the criteria, with criteria 1 - 4 being present for at least 6 weeks. These criteria have 89% specificity, and 94% sensitivity in distinguishing RA from other forms of arthritis [65].

Table 2. The 1987 revised criteria for the classification of RA by the ACR [65]

Criterion	Description
1. Morning Stiffness	Morning stiffness in and around the joints, lasting at least one hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least three joint areas (out of 14 possible; right or left PIP, MCP, wrist, elbow, ankle, MTP joints) simultaneously have had soft tissue swelling or fluid (not bony overgrowth) as observed by a physician
3. Arthritis of hand joints	At least one area swollen (as defined in criterion two) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement (as in criterion two) of the same joint areas on both sides of the body (bilateral involvement of PIP, MCP, or MTP joints without absolute symmetry is acceptable)
5. Rheumatoid nodules	Subcutaneous nodules over bony prominences or extensor surfaces, or in juxta-articular regions as observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal body decalcification localised in, or most marked adjacent to, the involved joints (osteoarthritis changes alone do not qualify)

Imaging

The use of newer imaging modalities such as ultrasonagraphy, magnetic resonance imaging (MRI) and computerised tomography (CT) are increasingly being used to diagnose RA much earlier than conventional radiology [66]. These imaging methods offer greater sensitivity in detecting synovitis, effusion, joint subluxations, joint space narrowing and bone oedema [66]. Furthermore, when compared with conventional methods, the increasing use of ultrasound has allowed for a greater success rate when performing joint aspirations, and greater accuracy when injecting steroids into the affected joints [67].

Assessment of Disease Activity

At present there is no cure for RA, but the disease can be effectively managed by monitoring the state of the patient's joints, and current levels of disease activity. The availability of validated assessment tools which are efficient and easy to perform allows the physician to adequately monitor the patient's disease activity. In the United Kingdom, the most frequently used assessment tool for disease activity is the disease activity score in 28 joints (DAS28) [68]. The DAS28 takes into account the total number of tender and swollen joints from 28 joints (fingers, wrists, elbows, shoulders, knees). In addition, it utilises a visual analogue scale (VAS) to indicate the patient's global health, as well as the erythrocyte sedimentation rate (ESR) [69]. The change in disease activity over subsequent visits to the physician or in response to treatment can be monitored by using the ACR and the European League against Rheumatism (EULAR) response criteria [70,71]. The ACR response criteria incorporate the percentage improvement in 7 variables (tenderness, swelling, physicians global disease activity, patients global disease activity, morning stiffness, pain and disability as well as the ESR score) and then categorises them into ACR20, ACR50 or ACR70 which correspond to a 20, 50 or 70% improvement in at least 5 of the 7 variables [71]. The EULAR response criteria incorporate changes in the DAS28 score from baseline values. The responses are then categorised as good, moderate or non-responders [70].

The Stanford Health Assessment Questionnaire (HAQ) can be used to assess the effects of disease activity or severity on the patient's functional ability. Patients rate their ability over the past week to carry out 20 activities within eight aspects of daily living (dressing/grooming, rising, eating, walking, hygiene, reach grip and errands/tasks) on a four point Likert scale ranging from 'without any difficulty' to 'unable to do'. For each aspect, patients also report whether they receive assistance from people or use specific devices (e.g. walking stick, stair-lifts, and bath seats). The scores are added together to give a single HAQ score, with a high HAQ score indicating reduced functional ability [72].

Pharmacological Approaches in Rheumatoid Arthritis

The management of RA involves the use of carefully selected pharmacological therapies which are based on the patient's disease severity, symptoms and response to previous medications. The aim of treatment is to reduce the inflammation in the joints and halt the destruction to the joints and bones, particularly if treatment is initiated early [73]. The most commonly used medications to treat RA are described in greater detail below.

a) Non Steroidal Anti-inflammatory Drugs

Traditional non steroidal anti-inflammatory drugs *(*tNSAIDs) were usually the first line of treatment administered by the physician [74]. There are a number of different tNSAIDs, with aspirin and ibuprofen being the better known types. The therapeutic target of tNSAIDs is to reduce inflammation and its associated symptoms, but they are unable to reduce RA disease progression [75]. The mode of action of tNSAIDs involves inhibition of the cyclooxygenase (COX) pathways that are involved in platelet aggregation (COX-1) and inflammation (COX -2) [76]. tNSAIDs can irritate the gastric mucosa of the stomach causing considerable gastrointestinal (GI) disturbances [77]. COX-1 derived prostaglandins can protect the GI tract [77], and their inhibition by NSAIDs results in GI disturbances. To counteract the GI disturbances, specific inhibitors of the COX-2 pathway were developed so that inflammation (and associated

pain) could still be reduced [78-81]. However, use of COX-2 inhibitors was reported to be linked with an increased risk of cardiac events [82]. The increased cardiac event risk was attributed to the *'prostanoid hypotheses'*, where inhibition of COX-2 (which is anti-thrombotic and promotes vasodilatation in the vasculature) would result in elevation of the COX-1 enzyme (which is pro-thrombotic and causes vasoconstriction) [83]. Crucially, the *'prostanoid hypotheses'* does not account for the fact that tNSAIDs are also associated with an increased risk of cardiac events, with the risk being equivalent to that of COX-2 inhibitors [84,85]. Furthermore, tNSAID inhibit COX-2 to the same extent as COX-2 specific inhibitors when administered at standard doses [86]. At present, the exact mechanism for the increased risk of cardiac events with both tNSAIDs and COX-2 inhibitors has not been elucidated. The deleterious effects off COX-2 inhibitors may be more pronounced in conditions which are likely to have high inflammatory levels in the vasculature such as RA and CVD, and consequently they are not recommended for long-term use in these patients [87].

b) Disease Modifying Anti-Rheumatic Drugs (DMARD)

As mentioned earlier, NSAIDs are effective in reducing inflammatory symptoms but with limited effects on reducing disease progression. In contrast, DMARD therapy greatly reduces, and in some cases completely suppresses disease progression [88]. The therapeutic benefit of DMARDs takes longer to occur, and so NSAIDs were prescribed first with DMARDs started later. However, it is now known that radiological damage to the joints occurs very early in RA [89], and delaying DMARD therapy can result in greater loss of functional ability [90]. Indeed, physicians are now prescribing DMARDs to treat early RA. One of the limitations of DMARDs is that although there are a number of different types, not all are effective in the patients. Thus, the dilemma for the physician is whether to start with combination DMARDs and then reduce one as the disease is adequately controlled or to start with one DMARD and progressively add other types until the disease is controlled [91,92]. The latter approach may have the benefit of limiting side effects of these medications but at the expense of early

control of disease activity, whilst the former approach has the opposite effect [93,94]. Current guidelines suggest that starting the patient on combination DMARD therapy is the way forward to halt disease progression early, but further research is still needed [91]. As stated previously there are a number of different DMARDs, with methotrexate, sulphasalazine and leflunomide being the most commonly prescribed, largely due to their superior benefit to side effect ratios over other DMARDs. The mechanism of action of DMARDs is not clear, but methotrexate has been shown to inhibit the production of IL-6, TNF-α and various adhesion molecules as well as inducing apoptosis of activated T-cells [95].

c) Biological Drugs

The recent emergence of biologic therapies has greatly advanced the treatment options available to the RA patient. Currently, the most common type of biologic drug being used to treat RA is anti-tumor necrosis factor-α (anti-TNF-α), which specifically inhibits the cytokine TNF-α [96]. TNF-α is mainly produced by CD4+ T-cells and macrophages in response to infections, however, overproduction can lead to deleterious effects [50,97]. It exerts potent pro-inflammatory affects by synthesising other pro-inflammatory cytokines such as IL-6, IL-1 and granulocyte colony-stimulating factor, as well as synthesizing adhesion molecules which attract leucocytes to the endothelium [98]. TNF-α is activated by TNF-α converting enzyme (TACE), and then binds to either p55 (also known as TNF-R1) or p75 (also known as TNF-R20) receptors to mediate its effects [98]. In the UK, three types of TNF-α inhibitors are most widely prescribed; adalumimab (fully human anti-TNF-α monoclonal antibody which inhibits activity of the TNF-α cytokine), etanercept (fusion protein which inhibits TNF-α receptors) and infliximab (chimeric anti-TNF-α antibody which also inhibits activity of the TNF-α cytokine) [96]. Recently, newer TNF-α inhibitors such as certolizumab pegol (recombinant humanised Fab' fragment which inhibits TNF-α activity) and golimumab (human monoclonal antibody which inhibits TNF-α activity) have been approved for treating RA. These agents have a rapid onset of action, reduce disease progression, and are able to work in combination with DMARD's such as

methotrexate [99-102]. Due to the large costs of this drug, prescription in the United Kingdom is governed by guidelines issued by the National Institute of Health and Clinical Excellence (NICE) which state that patients are only eligible for anti-TNF-α if they have severe disease and have had an inadequate response to at least two DMARDs (one of which must be methotrexate) [103]. Finally, anti-TNF-α must be administered with caution as there are concerns over its safety with reports of malignancies, heart failure and repeated infections in patients on these medications [104]. Recently, newer types of biologic drugs have also appeared on the market including rituximab (anti-CD20 agent), abatacept (selective co-stimulation of T-cells modulator), IL-1 receptor (anakinra), and IL-6 receptor (tocilizumab), and their effects are currently the subject of intense research [55,105-107].

d) Glucocorticoids

Glucocorticoids are another class of medication which are effective for the control of the inflammatory symptoms of RA, as well as for halting disease progression [108]. They can have systemic effects in the body when administered orally, intramuscularly and intravenously, or they may have local effects when administered directly into the joint using intra-articular injections [109]. However, glucocorticoids are not without side effects, some of which can be serious. Common side effects include osteoporosis, weight gain, muscle weakness, alopecia, cataracts and hypertension [110,111]. Interestingly, long-term glucocorticoid use does not associate with metabolic syndrome in RA patients, indicating that factors other than glucocorticoids, like disease activity, may elevate CVD risk in RA [112]. Due to their side-effects, glucocorticoids are typically prescribed on a short term basis to curtail a flare during active periods of the disease.

Summary

RA is a chronic inflammatory autoimmune disease which if not adequately controlled can lead to disability and a reduction in the quality of life. The

pathogenesis of RA involves a complex interplay of various immune cells which initiate inflammation and joint damage directly and indirectly. Treatment is targeted at reducing inflammatory symptoms and preventing radiological damage to the bones and cartilage with early treatment related to better prognosis. RA patients are also at greater risk of extra-articular manifestations, with CVD being a major determinant of morbidity and mortality in this group. The mechanisms which cause CVD need thorough investigation so that treatment can be targeted at effectively reducing CVD risk.

Cardiovascular Risk in Rheumatoid Arthritis

Data from recent meta-analyses have revealed that RA confers a 50-60% increased risk of mortality from CVD when compared to the general population [113-115]. Ischemic heart disease, cerebrovascular accidents and congestive heart failure appear to make the largest contribution to CVD mortality in RA [114,116,117]. Even patients with early RA (disease duration <2 years) appear to have increased cardiovascular morbidity [118,119], and there is evidence that atherosclerosis may occur before the diagnosis of RA is made [120]. Both classical CVD risk factors such as hypertension [121] and novel risk factors such as inflammation [122] are believed to accelerate atherosclerosis in RA [123,124] and contribute to CVD mortality. The following section discusses the impact of classical CVD risk factors and inflammation in RA patients.

Hypertension

In the general population the atherogenic potential of hypertension has been clearly demonstrated [125], and hypertension also appears to be a strong contributor to CVD in RA patients [126,127]. Hypertension is prevalent in 52-73% of RA patients [128], and in general, RA patients tend to exhibit higher blood pressures in comparison to healthy controls [129]. A multitude of inter-related factors may be contributing to the high prevalence including physical inactivity, which has been shown to strongly associate with the incidence of hypertension [130]. RA patients partake in significantly less physical activity than age and sex

matched individuals due to the pain, swelling and stiffness of the joints [131]. The sedentary lifestyle can then result in further complications such as obesity [132], which in itself is an independent predictor for hypertension in RA [128]. There is also strong evidence that inflammation may be a contributory factor for hypertension [133], with IL-6 and ICAM-1 being potential mediators of this link [134]. Medications used to treat RA may also confer a risk of developing hypertension as a systematic review of randomised control trials (RCTs) reported that patients on non-selective NSAIDs for at least 4 weeks showed a significant increase in systolic blood pressure (SBP) and diastolic blood pressure (DBP) when compared to baseline values [135]. Similarly, patients on selective COX-2 inhibitors have higher relative risk of developing hypertension when compared with patients receiving non-selective NSAID's albeit non-significantly [136]. Some DMARDs may also contribute to hypertension in RA, as leflunomide may increase SBP and DBP [137], possibly due to increased sympathetic drive [138]. Furthermore, ciclosporin use may also cause hypertension [139,140], as it can decrease vasodilatory molecules such as nitric oxide and prostacyclin, and increase vasoconstrictor molecules such as endothelin-1 [141]. In addition, ciclosporin increases sodium retention and vasoconstriction in the renal circulation leading to a reduction in glomerular filtration rate [141].

Dyslipidaemia

Dyslipidaemia is a strong predictor for CVD in the general population as well as in patients with RA [142]. Abnormal lipid patterns consist of high levels of low-density lipoprotein (LDL) and triglycerides (TG), and low levels of high-density lipoprotein (HDL) [143]. The prevalence of dyslipidaemia in RA varies between studies because of differences in population characteristics. For example, the prevalence of dyslipidaemia is 55% in patients with a variety of inflammatory arthritis [144], 68% in a sample of male RA patients [145], and 51% in a series of 400 patients from the Dudley Rheumatoid Arthritis Comorbidity Cohort (DRACCO) [146], when using United States National Cholesterol Education Program guidelines [147]. Adverse lipid profiles are evident in early [148] and

advanced RA [149], and it is thought that dyslipidaemia may precede the development of RA [150]. Similarly to hypertension, physical inactivity appears to have a major effect in altering the lipid profile, as patients undergoing exercise training programs often see large improvement in their lipid profile [151]. Exercise specifically increases levels of HDL cholesterol as well as decreasing TG levels and the total cholesterol:HDL ratio [152,153]. Inflammation too has an effect on altering the lipid profile of RA patients [123]. IL-6 and TNF-α increase free fatty acid release from adipose tissue and the liver which results in increased levels of TG [154]. In addition, the activity of lipoprotein lipase which is a catabolic enzyme responsible for the breakdown of triglycerides is downregulated by these cytokines (104). RA medications might also affect the lipid profile of RA patients. In one study patients were randomised to receive sulphasalazine or a combination of methotrexate, sulphasalazine and tapered doses of prednisolone (starting with 60 mg/day then tapered in 6 weekly steps to 7.5 mg/day and stopped completely after 28 weeks). The findings revealed that total cholesterol (TC) and HDL levels increased along with a reduction in the TC:HDL ratio in the group receiving the combination therapy [155]. Interestingly, improvements in the lipid profile were not observed after prednisolone was completely stopped (after 28 weeks) suggesting that glucocorticoid use may have more specific effects on lipids than all 3 treatments combined [155]. The favourable effect of glucocorticoids appears to be driven by increased HDL-C probably as a result of reduced disease-activity [149,155]. Despite the small effect of methotrexate and sulphaslazine on lipids mentioned in the above study, some specific DMARD's may exhibit beneficial effects on lipids profiles in RA, in particular hydroxychloroquine [156,157]. Hydroxychloroquine improves lipid profile via inhibition of very low density lipoprotein secretion from the liver [158] and inhibition of cholesterol synthesis [159]. On the other hand, DMARD's such a ciclosporin may have a negative impact on lipids. Studies in transplant patients (for which ciclosporine was originally indicated) reveal increased TG levels and reduced HDL levels after ciclosporin administration [160]. There is no available evidence on the effect of ciclosporin on lipids in RA and consequently further

research is needed. Newer biologic agents such as anti-TNF-α also appear to have a favourable impact on lipids in RA, as a systematic review of the literature revealed that, overall, TC and HDL-C were increased after treatment with anti-TNF-α agents [142]. However, anti-TNF-α has also been linked with increased TG levels [161-163], so studies showing favourable lipid profiles after treatment need to be interpreted with caution.

Insulin Resistance

Insulin resistance (IR) occurs as a consequence of abnormal production of insulin which leads to increased release of stored TG in fat cells, decreased uptake of glucose in skeletal muscle, and overproduction of glucose in the liver [123]. Collectively, these changes promote a high-glucose state which may then predispose the patient to type II diabetes and CVD [164]. The prevalence of IR in RA is not yet known [165]. However, impaired glucose levels have been found in RA patients with active disease [166] and high disease activity associates with IR [167]. This suggests that patients with the greatest disease activity are at a greater risk of developing insulin resistance. Indeed, inflammatory cytokines are involved in the process that leads to IR. For example, TNF-α causes a reduced uptake of glucose in the skeletal muscle [168], and blockade of TNF-α can result in large improvements in IR [169,170]. Controlling inflammation with DMARDs such as hydroxycholoroquine has been reported to reduce IR and fasting glucose levels through currently undetermined mechanisms [171]. However, long-term glucocorticoid use may contribute to the development of insulin resistance in RA [172], by decreasing skeletal muscle tissue [169]. Due to the limited amount of literature on IR in RA, there is a clear need for longitudinal studies which assess the extent of IR in RA and the contribution it makes to CVD risk.

Obesity

Obesity is a low-grade inflammatory condition characterised by a body mass index (BMI) greater than 30kg/m^2 [173] and is a major risk factor for CVD [174]. It is also implicated in the underlying causes of other CVD risk factors such as

hypertension, dyslipidaemia, and insulin resistance [175] and significantly contributes to atherosclerosis [176]. The chronic inflammation in RA can induce metabolic abnormalities [177], resulting in a loss of fat free mass [62], which coupled with a sedentary lifestyle, leads to accumulation of body fat while weight remains stable [178]. Adipocytes (fat cells underneath the skin) can secrete a variety of pro-inflammatory molecules known as adipokines [179]. Increased adiposity increases production of pro-inflammatory molecules, with low adiposity having an opposite effect [179]. In particular, adipocytes produce IL-6 [180,181], which in turn increases production of C-reactive protein (CRP) [182], and may therefore contribute to RA disease-related inflammation. Interestingly, obesity may be a risk factor for the development of RA [183,184], although this view has recently been challenged [185-187]. Obese RA patients with long disease duration (mean disease duration > 10 years) have higher disease activity and functional disability than normal weight patients, and this is perhaps due to the additional inflammatory load which is afforded by obesity [188]. Furthermore, obesity has been reported to contribute to the increased 10-year CVD risk probability [119], and associates with a number of other classical CVD risk factors [128,189]. Obesity may also affect the expression of pro-inflammatory genes that associate with CVD in RA [190]. Therefore, the impact of obesity on RA disease activity and CVD risk must be monitored carefully in these patients. Some medications used to treat RA, such as glucocorticoids, can contribute to the development of obesity [191]. In particular, glucocorticoids can redistribute fat so that there is greater central adiposity (relative to extremities) [192].

Inflammation

Atherosclerosis is an inflammatory condition, with high inflammatory level implicated for developing CVD [193]. Inflammatory markers such as IL-6, CRP and fibrinogen are associated with a high frequency of cardiovascular events [194-196]. In particular, CRP has received large attention due to its ability to independently predict cardiovascular events in the general population [197], which may in part, be due to its ability to directly contribute to the onset of CVD

[198]. In RA patients, inflammatory markers such as the CRP and ESR are elevated, and remain greater even in periods of low disease activity when compared to the general population [123]. In patients with inflammatory arthritis who were followed up for 10 years, CRP levels independently predicted CVD mortality [199]. The similarities between the inflammatory process of RA and atherosclerosis are remarkable. In both diseases, concentrations of IL-6 , CRP and TNF-α are elevated, and both have similar patterns of activation for T-cells and macrophages [122,200].

Inflammation in Atherosclerosis

The endothelium is the innermost layer of the blood vessels and consists of highly specialised cells responsible for vascular homeostasis and atheroprotection [201]. When the endothelium is exposed to injurious stimuli, the endothelial cells become dysfunctional – a process called endothelial dysfunction (ED) [202] (Figure 2). ED occurs in the initial stages of atherosclerosis and is characterised by increased vascular permeability. This allows transmigration of monocytes, lipids and T-cells into the vascular wall which is aided by adhesion molecules such as ICAM-1 and E-selectin. Differentiation of monocytes into macrophages helps retain LDL in the subendothelial space, which then forms into oxidised LDL. At this point, monocytes transform into macrophages and scavenge the oxidised LDL leading to formation of foam cells, which can be seen on the vascular wall as fatty streaks (See Figure 2) [203].

Endothelial Dysfunction
- Impaired vasodilatation
- Activation of pro-inflammatory cytokines
- Activation and adhesion of white blood cells
- Platelet aggregation

Structural Dysfunction
- Smooth muscle cell proliferation
- Formation of plaques
- Plaque rupture

Adventitia
Endothelium
Smooth Muscle
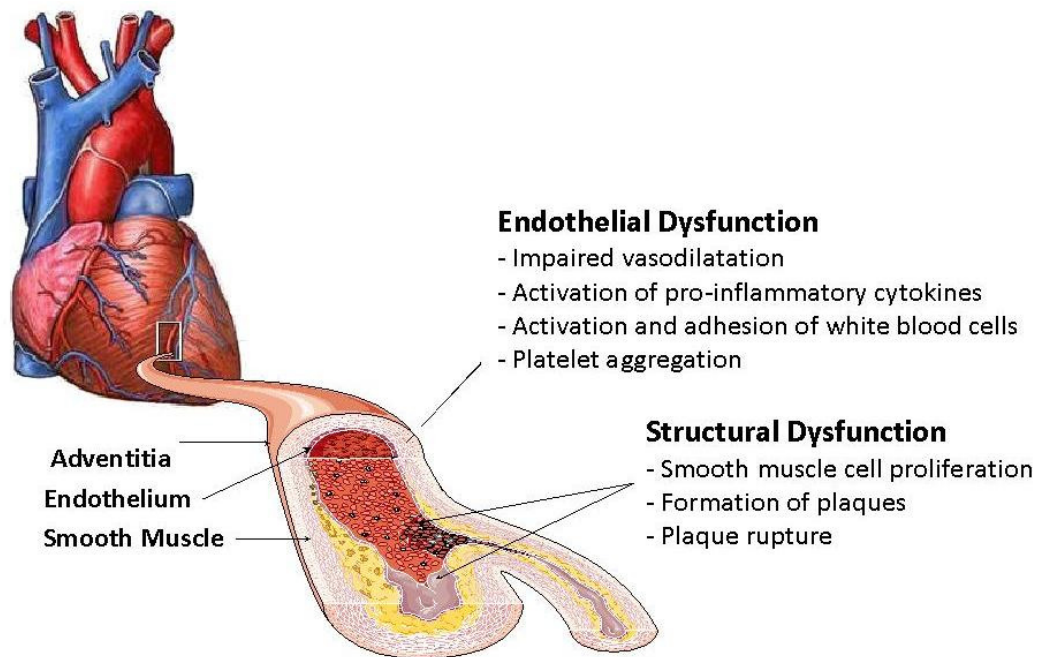
Figure 2. Progression of atherosclerosis in a coronary vessel.

Once foam cells are formed they produce pro-inflammatory cytokines and mediators such as IL-6, CRP and TNF-α. These inflammatory cells subsequently recruit further T-cells and macrophages which activate pro-inflammatory cytokines and contribute to continuously elevated inflammation in the vascular wall [204]. The next stage of atherosclerosis involves the migration of vascular smooth muscle cells (VSMC) towards the intima. The VSMC secrete large amounts of collagen which expands the extracellular matrix and forms a tough fibrous plaque containing a lipid rich core. This process occurs in the intermediate stage of plaque formation [203,205].

CRP released by foam cells causes destabilisation of the atheromatous plaque in the later stages of atherosclerosis by upregulation of matrix metalloproteinases resulting in the fibrous cap over the plaque to erode [206]. Erosion of the plaque results in increased platelet activity and leads to thrombus formation on the plaque [207]. This is largely mediated by the increased expression of tissue plasminogen activator inhibitor-1 which inhibits thrombolysis

[208]. As thrombosis increases, the plaque eventually progresses to an advanced stage and begins to intrude into the lumen causing stenosis and symptoms of myocardial ischemia [209]. Should the plaque rupture the thrombus could lead to complete occlusion of the vessels – a process which results in acute coronary syndromes [205].

Tumor Necrosis Factor-α

The concentration of TNF-α is markedly raised in RA [46] and TNF-α contributes significantly to the atherosclerotic process [210]. Specifically, TNF-α is responsible for the upregulation of vascular cell adhesion molecule-1 [211], which plays a well established role in atherosclerosis [212]. Further, it precedes the development of thickened intima by allowing formation of the fatty streak [213]. In RA patients, TNF-α enhances pro-thrombotic states such as dyslipidaemia [214], and recent evidence also reveals that TNF-α is involved in the more advanced processes of atherosclerosis, specific to formation of an advanced lesion, via the inhibition of endothelial progenitor cells which are involved in repairing endothelial injury [215]. In RA patients, endothelial progenitor cell numbers are decreased compared to the normal population, a phenomenon which reverses after TNF-α blockade [216]. Despite the evidence of TNF-α role in atherosclerosis, a number of large studies have failed to find a link between ant-TNF-α and decreased CVD risk. For example, analysis of the British Society of Rheumatology Biologics Register revealed that the occurrence of myocardial infarction did not differ between patients receiving anti-TNF-α and those that were anti-TNF-α naïve [217]. However, patients who showed a good clinical response after six months of anti-TNF-α treatment had a lower risk of myocardial infarction than patients who did not respond [217]. Another study revealed that biologic agents confer neither a higher or lower risk of developing a cardiac event when compared to patients receiving methotrexate only [218]. These findings suggest that it may be an overall reduction in disease-related inflammation that confers a cardioprotective effect rather than any specific effect of anti-TNF-α.

Summary

It is clear that CVD risk factors are highly prevalent in RA patients and result in a significant health burden. Interestingly, RA patients who are rheumatoid factor positive have twice the risk of mortality from CVD than the general population [54], suggesting that a complex interplay exists between inflammation and CVD in RA [219]. Of importance is the finding that once traditional CVD risk factors are statistically controlled, an elevated risk of CVD still appears to remain and can be attributed to high levels of inflammation [220]

Chapter 1 (Part II): Physiology of the Endothelium

Introduction

Once considered as a simple barrier between the blood and vessel wall, the endothelium is now regarded as a dynamic organ which lines the entire vascular system [201]. The endothelium controls vascular function by responding to various hormones, neurotransmitters and vasoactive factors which affect vasomotion, thrombosis, platelet aggregation and inflammation [201]. The balanced production of these vasoactive factors is atheroprotective, whereas a damaged endothelium causes disrupted production of these factors. The ensuing imbalance leads to endothelial dysfunction (ED), which is an early indicator of atherosclerosis [202]. Endothelial cells are located on the intima of all vessels (described in detail below), but display different structures and phenotypes depending on vessel type [221]. Endothelial cells in arteries and veins appear more continuous and thicker than those in capillaries which are fenestrated and thinner to allow for exchange of metabolites and gases [222]. In addition, endothelial cells can display heterogeneous responses to stimulation in different vascular beds, and even in different sections of the same vascular bed [223-225]. This suggests that ED may occur in selective vascular beds too [225].

Anatomy and Physiology of the Blood Vessels

The blood vessels provide the main link between the heart and the tissues. The vascular wall is made up of three layers; the intima (inner layer), the tunica medica (middle layer) and the tunica externa (outer layer) (see Figure 3). The intima consists of endothelial cells which regulate the function of the vessel by continuously responding to stimuli and releasing different vasoactive factors accordingly. The tunica media is a thick vascular layer consisting of smooth muscle cells, collagen and elastic tissue, which carry out functional tasks of the vessels such as vasodilatation, but also gives the vessel structural integrity. The tunica externa comprises of loose connective tissue that anchors the vessel to surrounding organs (Levick, 2003). The blood vessels are divided depending on

function, location and size into arteries, capillaries and veins. A brief description
of each is provided below.
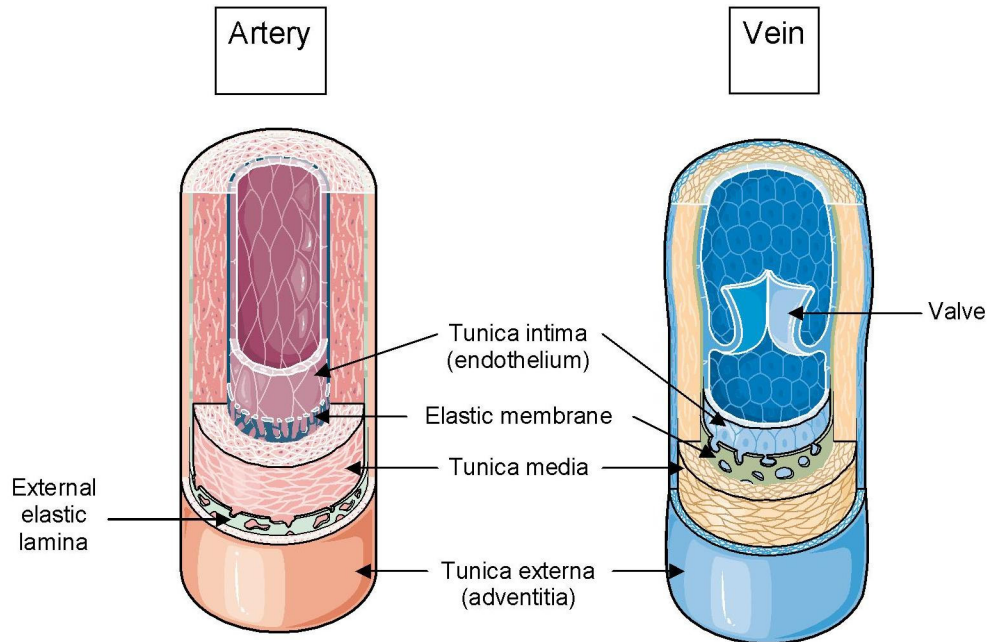
Figure 3. A cross-sectional view of an artery and vein

Arteries

The main function of the arteries is to supply the organs with blood. Given the
high pulse pressure in the arteries their walls are thicker than in other vessels.
Arteries can be divided into conducting arteries, conduit arteries and resistance
arteries based on their position in the arterial tree.

Conducting (elastic) arteries

These are the largest arteries in the body such as the aorta, pulmonary artery
and carotid artery which branch from the heart. Their walls contain a large
amount of elastic tissue which allows the vessel to expand and recoil to dampen
out the oscillatory changes in blood pressure as a result of intermittent ventricular
contractions. This ensures smooth bloodflow through the vessel which is
distributed towards specific organs [226].

Conduit (muscular) arteries

The conduit arteries are medium to small arteries which branch from conducting arteries to supply blood to a specific area of the body; examples are the brachial, radial and femoral arteries. Conduit arteries contain more smooth muscle than conducting arteries which enables them to regulate bloodflow by varying the diameter of the artery in response to different stimuli such as mechanical forces on the vascular wall (i.e. shear stress which is the dragging, frictional force exerted on the vessel wall by laminar bloodflow) or sympathetic nervous system activation [227].

Resistance arteries (arterioles)

The conduit arteries divide into resistance arteries, the arterioles. These are the smallest vessels of the arterial system and form part of the microcirculation. Resistance arteries are responsible for adequately perfusing the organ tissue with blood. Arterioles also help in slowing the blood flow to prevent damage to the adjacent capillaries. They consist mainly of smooth muscle cells which are highly innervated by sympathetic nerves, allowing the arterioles to regulate bloodflow to the tissue by dilating or constricting in response to sympathetic (de)activation [222]. Another stimulus that can cause dilation of arterioles is shear stress [228].

Capillaries

Capillaries are present in large numbers in nearly all tissues of the body, and like the arterioles are part of the microcirculation [222]. The passage of blood into the capillaries is controlled by pre-capillary sphincters which contain smooth muscle that allows them to contract or dilate (Figure 4). The opening of the sphincters depends on metabolic demand so not all capillaries contain blood continuously [229]. The main function of the capillaries is to enhance the diffusion of gases, metabolites and nutrients between the blood and the tissue. This is achieved by thin capillary walls which consist of a single layer of endothelial cells, thus, shortening the diffusion pathway between the blood and

tissue fluid. Efficiency of diffusion is further enhanced by the slow bloodflow which helps to increase the time available for diffusion [230].
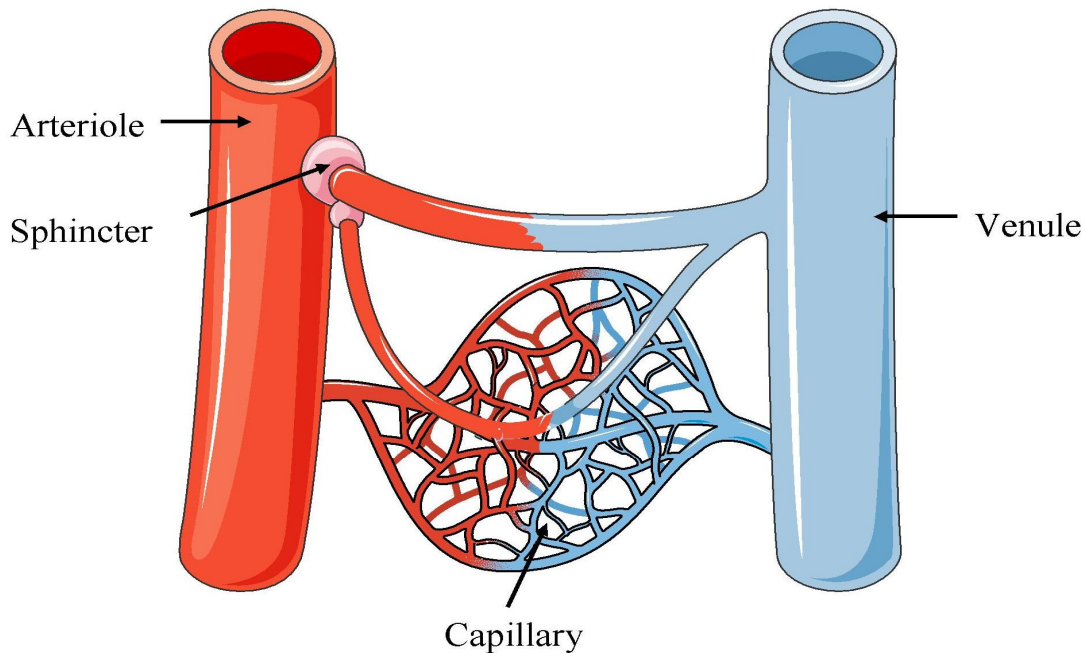
Figure 4. The human capillary network

Veins

The main function of the venous system is to return the blood to the heart. The capillaries flow into the venules, which are the microvessels of the venous system. Additional exchange of gases and metabolites occurs here as the venules have single layered walls, like the adjoining capillaries. The venules feed into the peripheral veins and then into the superior and inferior vena cavae, which are connected to the heart. In general, veins that are in close proximity to the heart have larger diameters. Given the lower blood pressure in the venous system compared to the arterial system, the vessel walls of veins are thinner and more compliant than arterial walls. This means that veins can accommodate large volumes of blood with only small increases in pressure. Mechanisms such as the skeletal muscle pump and respiratory pump as well as sympathetic nervous activation enable veins to return blood back to the heart. In addition,

veins contain valves to prevent backflow of blood while smooth muscle cells in the vascular wall allow veins to constrict and increase the blood pressure, both of which increase venous return (Figure 3) [229].

Regulation of Vascular Tone

The endothelium releases various vasoactive factors. These can be vasodilative such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium derived hyperpolarizing factor (EDHF) or vasoconstrictive such as thromboxane (TXA_2) and endothelin-1 (ET-1). These substances are discussed below.

Nitric Oxide

Nitric oxide (NO) is an endothelium-dependent vasodilator of the underlying smooth muscle and was first identified by Furchgott and Zawadzki [231]. NO has been shown to play an important role in the maintenance of basal vasodilator tone of the blood vessels [232]. NO is formed under the influence of the enzyme nitric oxide synthase (NOS), which converts the amino acid L-arginine to NO [233]. Three isoforms of NOS exist: neuronal isoform (nNOS) which produces NO to act as a neuronal messenger that regulates synaptic neurotransmitter release [234], macrophage or inducible isoform (iNOS) which is only expressed in cells that have been exposed to inflammatory mediators or other injurious stimuli that activate the macrophages [235], and endothelial NOS (eNOS) which produces nitric oxide in the vasculature [236]. The isoforms are classified by the cells they were originally found in, although, it is now known that expression of these isoforms also occurs in other cells, such as cardiac myocytes [237], skeletal muscle, blood platelets and the hippocampus [238]. Considering that the ability of a blood vessel to dilate is largely dependent upon the activity of eNOS, the present discussion will focus on this isoform.

Inactive eNOS is bound to the protein caveolin and is located in small invaginations in the cell membrane called caveolae [239]. When intracellular levels of Ca^{2+} increase, eNOS detaches from caveolin and is activated [239]. NO agonists can influence the detachment of eNOS from caveolin by releasing

Ca^{2+} from the endoplasmic reticulum (Figure 2) [240]. Examples of such NO agonists include bradykinin (BK), acetylcholine (ACh), adenosine tri-phosphate (ATP), adenosine di-phosphate (ADP), substance P and thrombin [241]. Once intracellular Ca^{2+} stores are depleted a signal (thus far unidentified) is sent to the membrane receptors to open Ca^{2+} channels allowing extracellular Ca^{2+} into the cell [242-244]. This process of Ca^{2+} regulation is known as store-operated Ca^{2+} entry or capacitative Ca^{2+} entry [245]. Ca^{2+} attaches to the protein calmodulin in the cytoplasm of the cell, after which it undergoes structural changes which allows it to bind to eNOS [246]. eNOS then converts $_L$-arginine into NO [233]. This pathway of NO production is represented in Figure 5 below. It is important to highlight that this mechanism of NO production is dependent on the levels of intracellular Ca^{2+} in the endoplasmic reticulum as well as Ca^{2+} which diffuses into the cell from extracellular stores. A reduction in Ca^{2+} causes the calcium-calmodulin complex to dissociate from eNOS, which in turn binds with caveolin and becomes inactivated [246].

The short term increase in NO is dependent on the intracellular Ca^{2+} but once this decreases additional mechanisms are activated to regulate NO production. One such mechanism is the phosphorylation of eNOS [247]. Phosphorylation of eNOS occurs via protein kinases [235], such as protein kinase A [240] and cyclic guanosine-3', 5-monophosphate (cGMP) protein kinase dependent II [247]. Shear stress initiates eNOS phosphorylation by the actions of protein kinase B (Akt) [248].

Shear stress results from increased bloodflow in the vessel and can increase NO production by eNOS phosphorylation but also through stimulating endothelial cell receptors [249] by allowing the transfer of blood-borne agonists to attach to endothelial cell receptors and increase intracellular Ca^{2+} [250]. In particular, shear stress activates specialised Ca^{2+}-activated K^+ channels on the endothelial cell surface, causing K^+ efflux and Ca^{2+} influx into the cell [251] (Figure 5). The contribution of Ca^{2+} and eNOS phosphorylation to NO production

is dependent on the duration of the shear stress. For example, intracellular Ca^{2+} release is dependent on shear stress of short durations [252], whereas shear stress of longer durations (>30 minutes) can deplete intracellular Ca^{2+} stores, and so NO production is dependent on eNOS phosphorylation [253].
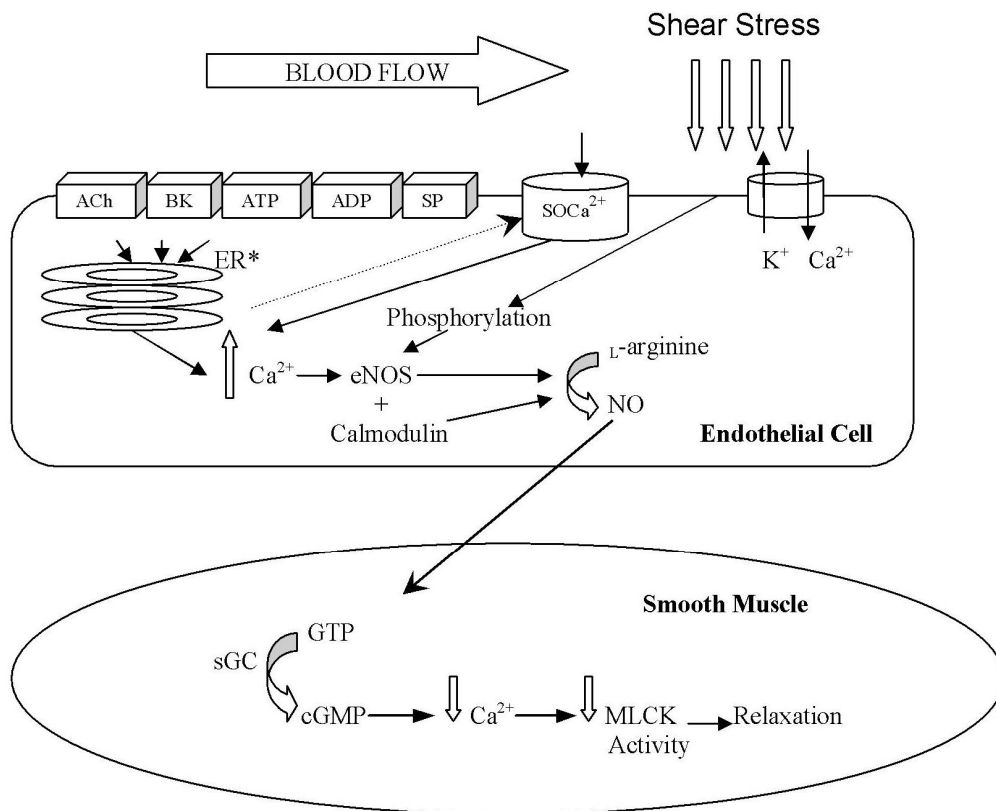
Figure 5. Endothelial nitric oxide production and it actions in the vascular smooth muscle cell. ACh= acetylcholine; BK= bradykinin; ATP= adenosine triphosphate; ADP= adenosine diphosphate; SP= substance P; $SOCa^{2+}$= store-operated Ca^{2+} channel; ER= endoplasmic reticulum; NO= nitric oxide; sGC= soluble guanylyl cyclase; cGMP= cyclic guanosine-3', 5-monophosphate; MLCK= myosin light chain kinase. *When Ca^{2+} stores of the endoplasmic reticulum are depleted a signal is sent to $SOCa^{2+}$ channel which allows extracellular Ca^{2+} into the endothelial cell.

Once synthesized, NO diffuses across the endothelial cell into the adjacent smooth muscle [254] (Figure 5), where it binds to the enzyme soluble guanylyl cyclase (sGC) [254]. The now activated enzyme increases the

conversion rate of guanosine triphosphate (GTP) to cGMP [255], which decreases smooth muscle tension [256]. Further, cGMP reduces Ca^{2+} release from the sarcoplasmic reticulum in the smooth muscle cell [257], and also helps to restore Ca^{2+} to the sarcoplasmic reticulum [258]. Both actions reduce the contraction of smooth muscle cells.

The mechanisms described above are continuously active and produce NO to maintain basal vasodilator tone [259]. By inhibiting NO activity using N^G monomethyl-L-arginine (L-NMMA), a dose dependent increase in blood pressure was found due to the vessels constricting [260,261]. The constriction was reversed when NO was administered [260,262], highlighting the importance of NO release in maintaining resting vasodilator tone. However, the vessel is also capable of dilating in the absence of NO. After removal of or damage to the endothelium, administration of glyceryl trinitrate (GTN) can still result in vasodilatation [232].

The mechanism by which GTN causes vasodilatation is not clear. Several researchers have suggested that GTN undergoes bioconversion to NO [232,263-265], but not all agree, as GTN has been found to cause vasodilatation without increasing NO [266-270]. Further, the breakdown products of GTN have been shown to activate sGC [271,272]. It is worth noting that other vasoactive agents such as calcium ionophore A23187 and isosorbide-dinitrate induce vasorelaxation without an increase in NO concentration [241]. Therefore, NO does not seem to be the only agent that can activate the sGC-cGMP pathway. Further research is needed to identify the precise mechanism of the agents, in particular, more research is needed *in vivo* due to the differences in response between intact or a denuded endothelium [201].

Aside from vasodilatation, NO is also involved in preventing platelet and leukocyte activation and adhesion to the vessel wall [273-276]. When the endothelium is damaged, the subsequent inflammation causes an increase in

leucocytes at the damaged site [277]. Inflammatory mediators such as TNF-α, interleukin-1 (IL-1) and chemokines stimulate the release of iNOS [278], which prevents leucocytes from adhering to the endothelium and reduces inflammatory mediators [279], as well as down-regulating and reducing the expression of adhesion molecules [276].

Prostacyclin and Thromboxane A$_2$

The synergistic actions of two prostanoids, prostacyclin (PGI$_2$) and thromboxane (TXA$_2$) also regulate vascular function [83]. Their production is catalysed by cyclooxygenase (COX) enzymes, of which there are two isoforms COX-1 and COX-2 [280]. COX-1 is expressed continuously in endothelial cells, whereas COX-2 is only expressed when the endothelium is damaged and exposed to inflammatory cytokines [281-283].

COX-2 converts arachidonic acid to prostaglandin H$_2$ (PGH$_2$), which is then synthesised into PGI$_2$ by prostacyclin synthase [284]. PGI$_2$ binds to the prostacyclin receptors (IP) [285], which are located on both platelets and vascular smooth muscle cells [286]. Activation of platelet IP receptors leads to inhibition of platelet aggregation [287-289]. PGI$_2$ binding to the smooth muscle cell IP receptor activates adenylate cyclase which induces the synthesis of cyclic adenosine monophosphate (cAMP) [290]. cAMP then activates protein kinase A [289], which allows relaxation of the smooth muscle in the same way as it does for NO [291,292]. It is worth noting that in the presence of NO, blocking PGI$_2$ production has no effect on vasodilatation [293,294]. However, when NO is blocked, the residual dilation is due to increased PGI$_2$ synthesis [295,296], suggesting that PGI$_2$ plays a compensatory role in dilation of the vessel when NO is reduced.

In contrast to PGI$_2$, TxA$_2$ causes platelet aggregation and vasoconstriction [297,298]. COX-1 converts arachidonic acid to PGH$_2$, after which TxA$_2$ is synthesised by thromboxane synthase [83,299]. TxA$_2$ mediates its effects by its

actions on thromboxane-prostanoid (TP) receptors which are located on platelets and their activation causes platelet aggregation [281,300]. The TP receptor is also found on smooth muscle cells, and activate phospholipase C [301]. This allows an increase in intracellular Ca^{2+} levels in the smooth muscle, leading to vasoconstriction [302].

The balance in the activity of PGI_2 and TxA_2 helps to maintain homeostasis in the healthy vessel. The importance of this balance becomes evident when using selective COX-2 inhibitors to reduce inflammation, which decreases the production of PGI_2 without affecting the production of TXA_2 [303]. However, administration of COX-2 inhibitors to patients with established CVD who are already receiving aspirin has been reported to improve endothelial function [304], although the authors of this study suggested that the improvement in endothelial function may have been due to the affects of aspirin on reducing platelet aggregation [304]. Further research is necessary to establish mechanisms by which COX-2 inhibitors may or may not increase CVD risk.

Endothelin-1

Endothelin (ET) is a vasoconstrictor which is expressed in the body in three isoforms, ET-1, ET-2, and ET-3 [305]. Endothelial cells only release ET-1 [306], thus the present discussion will focus only on this isoform. ET-1 is produced by converting Big ET-1 to ET-1 by endothelin converting enzyme [307]. Regulation of ET-1 production as well as its release is stimulated by inflammatory cells such as interleukins and TNF-α and decreased by NO and PGI_2 [305]. Shear stress causes a decrease in ET-1 expression, after initially promoting it (for review see Kedzierski & Yanagisawa [308]). ET-1 receptors have been identified both on smooth muscle cells (ET_A and ET_{-B2}) and endothelial cells (ET_{-B1}) [309,310]. The distribution of the different ET-1 receptors is dependent on the type of vascular bed, as veins show a reduced ET_A:ET_B receptor ratio compared with arteries [308]. When ET-1 binds to ET_A or ET_{-B2} receptors, smooth muscle Ca^{2+} channels open allowing extracellular Ca^{2+} into the cell. This causes

vasoconstriction in a similar way as TxA_2. Activation of ET-$_{B1}$ receptors on the endothelium causes vasodilatation by inducing the release of NO and PGI_2 [311-314]. In ED, ET-$_{B1}$ receptors on the endothelial cells are downregulated, while ET-$_{B2}$ receptors on smooth muscle cells are upregulated, thus enhancing vasoconstriction [315,316].

The effect of each receptor on the vasculature has been explored in patients with heart disease and in healthy participants. Selectively blocking ET$_A$ receptors in participants with ED reliably leads to vasodilatation [316-320]. However, blocking both ET$_A$ and ET$_B$ receptors in participants with ED results in greater vasodilatation than blocking ET$_A$ receptors only [316]. This finding suggests that the upregulation of smooth muscle ET$_B$ receptors has an additive effect on vasoconstriction in individuals with ED [315,316]. In healthy participants blocking ET$_B$ receptors leads to vasoconstriction [313,316,320,321], therefore, ET$_B$ receptors located on the endothelium predominantly regulate endothelial function in this group.

Apart from its vasoactive effects, ET-1 also causes inflammation and smooth muscle cell proliferation in the vessel. Binding of ET-1 to ET$_A$ receptors activates macrophages, increases neutrophil-vessel wall interactions, and elevates free radical concentrations, all of which lead to ED [322]. ET-1 causes smooth muscle cell proliferation by binding to ET receptors [323,324] or activating other growth factors such as platelet-derived growth factor [325]. This results in an increase in the intima-media thickness of the vessel wall [326,327], which can be reduced by blocking ET-1 receptors [328]. In addition, inhibition of ET$_A$ receptors in diseased vessels can reduce atherosclerosis, which again suggests that ET$_A$ receptors are active during ED [329].

Endothelium-derived Hyperpolarising Factor
Endothelium-derived hyperpolarising factor (EDHF) is a yet unidentified vasodilator substance which hyperpolarises the underlying smooth muscle by

making the membrane potential of the cell more negative [330-332]. EDHF is released when endothelial cells are activated by agonists such as BK and ACh [333]. NO and PGI_2 can also dilate the vessel by hyperpolarising the smooth muscle cells, albeit for a short period before the mechanisms discussed above take over [334]. However, when NO and PGI_2 are inhibited hyperpolarisation still occurs, suggesting the involvement of a third hyperpolarising factor [331,335,336]. A number of pathways have been implicated in causing the hyperpolarisation. Although the exact pathway is still unknown, attention so far has been paid to three factors in particular.

Activation of endothelial receptors and the subsequent increase in Ca^{2+} levels causes K^+ efflux from the cell [337-339]. The smooth muscle cell responds to changes in the extracellular K^+ levels and also releases K^+ out of the smooth muscle cell causing hyperpolarisation [340,341]. The change in the membrane potential of the smooth muscle cell reduces intracellular Ca^{2+} levels, resulting in relaxation [339].

Epoxyeicosatrienoic acids (EET) are products of arachidonic acid metabolism [342]. Although synthesised in the endothelial cell, they act by increasing K^+ efflux from the smooth muscle cells resulting in hyperpolarisation and relaxation [343,344]. However, in vessels where EET activity is inhibited, hyperpolarisation still occurs [345], suggesting that other mechanisms must be involved in hyperpolarising the smooth muscle cells.

Gap junctions are intercellular channels which can transfer signals from the endothelial cells to the smooth muscle cells [346,347]. In particular, gap junctions may transfer K^+ ions from the smooth muscle cells into the endothelial cell [348]. However, since most studies have only transferred artificial dye between the two cells it is difficult to establish exactly what is transferred under normal conditions [347,349,350].

Techniques to Assess Endothelial Function

Endothelial function is most commonly assessed in the peripheral circulation as direct assessment of endothelial function in the coronary arteries is highly invasive and associated with considerable risk for the participant. Several studies have reported close correlations between peripheral and coronary endothelial function [351-353]. In addition, assessments of endothelial function are good predictors of future cardiac events in individuals at risk of CVD and those with established CVD [354-358], and ED is common in individuals with CVD risk factors [359]. Most assessments of endothelial function involve the measurement of dilation in response to a stimulus, with impaired vasodilatation indicative of poor endothelial function. However, impaired vasodilatation can be the result of either the endothelium not sending the signals to the smooth muscle or of the smooth muscle cells not being able to respond to the signal and dilate. Therefore, in order to distinguish between ED and smooth muscle dysfunction, endothelium-dependent and endothelium-independent vasodilatation are typically assessed. Techniques that assess endothelial function in different vascular beds is shown in Figure 6 (presented on page 54) and described in more detail below.

Assessment of Microvascular Endothelial Function

Iontophoresis

The assessment of NO bioavailability in the microvasculature is conducted using iontophoresis [360]. Iontophoresis uses a small electrical current to pass negatively and positively charged vasoactive agents through the skin into the resistance vessels on the basis that like charges repel each other [361]. The amount of the agent that is delivered to the vessel depends on the density and duration of the current. The two most common agents used to test endothelial function are ACh and SNP [362]. The assessment is usually carried out in the forearm. Laser Doppler techniques are used to assess the perfusion in response to iontophoresis. Laser Doppler flowmetry (LDF) assesses perfusion of the vessel over a single point on the forearm [363]. Perfusion can also be assessed

by Laser Doppler imaging (LDI) which uses the same principles as LDF, but rather than scanning one point, a whole area of the forearm can be assessed [364] .

The ACh and SNP are administered in small chambers which are attached to the volar aspect of the forearm by watertight adhesive pads. The anodal chamber contains ACh, while SNP is present in the cathodal chamber. Both chambers are connected to an iontophoresis controller which delivers the current [363]. The vasoactive agents can be dissolved in fluid known as vehicles, e.g. deionised water or saline. However, these vehicles can also increase skin perfusion [362,365-367]. It has been suggested that use of a lower current density reduces the vasodilatory effects of the vehicles, but drug administration is also reduced [368]. However, a higher current density can be used with 0.5% sodium chloride (NaCl), as it does not elicit a vasodilatory response at this concentration [367]. External factors such as time of day, and menstrual cycle can affect microvascular bloodflow [369,370]. Therefore, it is advisable to follow established guidelines when administering this test [371].

Forearm Blood Flow and Venous Occlusion Plethysmography
Endothelial function of the forearm resistance vessels can be assessed using venous occlusion plethysmography (VOP) [372]. This assessment stops venous return from the forearm, while allowing arterial inflow; blood can enter the forearm but cannot escape resulting in a linear increase in forearm volume with time which is proportional to the incoming arterial blood flow [372]. The halt in venous return is achieved by inflating a blood pressure cuff placed around the forearm to below the diastolic blood pressure (typically 40mmHg) for 10 seconds, followed by 5 seconds of cuff deflation. The hand is excluded from the measurement by inflating a blood pressure cuff which is placed around the wrist to suprasystolic pressures. This reduces the variation in blood volume due to a high proportion of skin blood vessels susceptible to temperature variations. VOP can be assessed using automated equipment which can precisely control the

time for cuff inflation and deflation. The increase in forearm volume is assessed by mercury in rubber strain-gauge plethysmograph placed around the widest part of forearm. An increase in the length of the strain-gauge is detected by a change in electrical resistance and represents an increase in forearm blood flow (FBF). It is also important to assess FBF in the contra-lateral arm so that time-dependent changes in basal blood flow due to arterial pressure fluctuations can be accounted for [372]. The FBF response can also be assessed in response to Intra-brachial infusion of various vasoactive agonists (ACh, substance P, bradykinin) or antagonists (L-NMMA, indomethacin) [373].
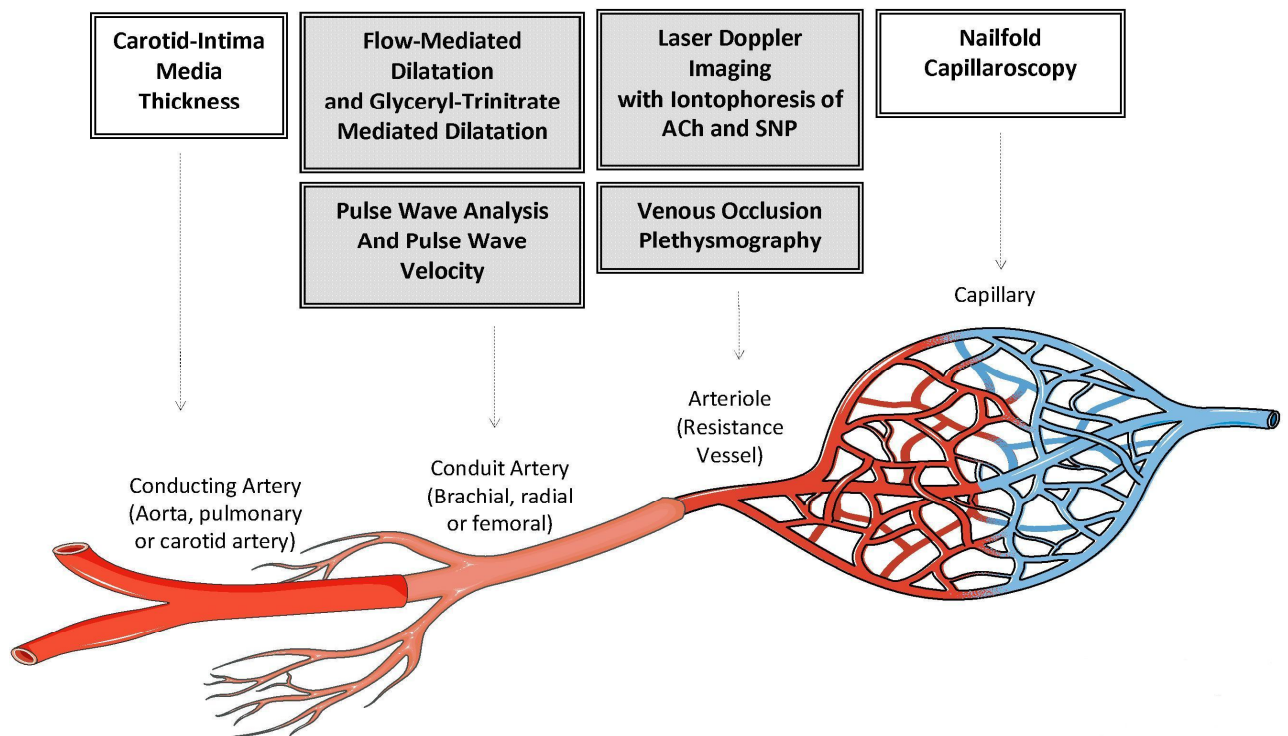
Figure 6. An overview of the assessments for endothelial function and vascular morphology performed in different vascular beds. ACh = Acetylcholine, SNP = Sodium nitroprusside. ☐ =Functional assessments ☐ = Morphological assessments

Nailfold capillaroscopy

Nailfold capillaroscopy is a technique to assess capillary morphology [374]. The technique involves the application of immersion oil to the nailfold epidermis of all

ten fingers. The nailfold is then placed under a microscope and abnormalities in the capillaries are characterised according to their size, number and morphological characteristics. Capillaroscopic abnormalities can be classified into three stages (early, active and late). The earliest change to capillary morphology is an enlargement in their size. A reduction in capillary number and structural impairments are seen in the active and later stages of microangiopathy [374,375].

Assessments of Macrovascular Endothelial Function

Flow-Mediated Dilatation

Flow-mediated dilatation (FMD) is a technique that increases blood flow through an artery to cause vasodilatation on the principal that the increased bloodflow produces shear forces on the endothelium and subsequently stimulates endothelial cells to release NO [248]. As indicated previously, reduced vasodilatation following an increase in shear forces is representative of impaired NO bioavailability [376]. Therefore, FMD is a good surrogate marker of NO bioavailability [377-379]. The FMD protocol involves a 2 minute baseline ultrasound scan of the brachial artery, after which a cuff placed around the wrist is inflated to 300 mmHg for 5 minutes. This causes tissue ischaemia and dilation of downstream resistance vessels via auto-regulatory mechanisms. When the cuff is released a sudden increase in bloodflow (reactive hyperaemia) through the brachial artery fills the dilated resistance vessels and in doing so exerts shear stress on the endothelial cells [380]. The resulting dilation, which peaks at 60-90 seconds after cuff release is dependent on NO activity [379]. FMD is expressed as the maximum percentage change in vessel diameter after cuff release relative to baseline vessel diameter [378], with a low percentage indicating poor endothelial function [373]. FMD is typically carried out in the brachial artery using high resolution ultrasound to assess the vessel diameter, but other arteries such as the radial and femoral artery have also been used to measure FMD [379,381]. Another method to quantify the dilation is strain-gauge plethysmography, with the

strain-gauge detecting the change in arm circumference following an increase in blood flow [382].

The protocol used for FMD is important as both occlusion duration and cuff placement have been shown to influence FMD. Five minutes of limb occlusion is adequate to evoke endothelium-dependent dilatation, with longer cuff durations showing a non-NO response [383]. Similarly, the placement of the cuff around the wrist is dependent on NO, whereas cuff placement on the upper arm is only partially mediated by NO [384]. Further, FMD responses can be affected by external factors such as sleep deprivation [385], hyperhomocysteinemia [386], caffeine [387], smoking [388], antioxidant therapy [389], menstrual cycle [390] and time of day [391]. Accordingly, it is important to control these factors [380].

Glyceryl Trinitrate

As described earlier, GTN produces dilation of the vessel by acting directly on the smooth muscle cells [232]. As such, the vasodilatory response to activated smooth muscle cells can be assessed by GTN administration. GTN is commonly administered as a vasodilator to cardiac patients presenting with angina as a tablet or oral spray, both of which are placed or sprayed directly under the tongue [392]. Typically, the assessment is carried out for 3-4 minutes, which is the time necessary for the vessels to reach peak dilatation [393].

Arterial Stiffness

Each time the heart contracts pressure waves are sent throughout the vasculature and the compliant arterial wall serves to dampen pressure oscillations that stem from the aortic root to aid smooth delivery of bloodflow to the tissues [226]. When the pressure waves reach branch points in the vasculature they are reflected back towards the heart. In a healthy individual the wave arrives during diastole to aid filling of the coronary vessels. However, in individuals with reduced arterial elasticity the pressure wave returns to the heart

much quicker and arrives during the systolic phase of the cardiac cycle. This serves to augment the afterload (the pressure the heart has to overcome to open the aortic semilunar valve) [332]. Some notable complications of arterial stiffness include insufficient myocardial perfusion leading to angina or a myocardial infarction, and left ventricular hypertrophy which may result in heart failure [394]. It is therefore not surprising that assessments of arterial stiffness are associated with a number of CVD risk factors such as ageing, smoking, hypertension and dyslipidemia [394]. Stiffening of the vascular wall can occur due to a reduction in NO production from endothelial cells, loss of smooth muscle tone [395], as well as degeneration of elastin fibres and increased collagen deposition in the vascular wall [396]. Consequently, arterial stiffness depends on functional and morphological changes in the vasculature [397].

A number of techniques can be used to assess arterial stiffness non-invasively from the peripheral circulation. The most widely used techniques at present are pulse wave analysis (PWA) and pulse wave velocity (PWV) due to their good reproducibility and ease of use [398]. These assessments have been reported to associate with coronary microvascular endothelial function [399]. PWA is the single measurement of radial artery pressure waveforms which are recorded using a transducer which flattens but not occludes the artery (applanation tonometery). The waveforms are calibrated against the standard brachial blood pressure which gives the maximum (systolic) and minimum (diastolic) points of the pressure curve. The pressure waveform is then mathematically transformed into a central aortic waveform which contains the first and second systolic peaks and displays the augmentation index (AIx). AIx is calculated as the difference between the second and first systolic peaks and is expressed as a percentage of the pulse pressure, with a high value indicating greater arterial stiffness [400]. To obtain PWV readings, arterial pressure waveforms are simultaneously derived from two arteries, usually the carotid and radial arteries, using an applanation tonometer. The distance between the two arteries is then measured and the wave transit time between these two points is

recorded to give a quantifiable PWV, with a greater PWV indicating quicker wave reflection back towards the heart and therefore greater arterial stiffness [401].

Carotid Intima-media thickness

Assessment of carotid-intima media thickness (cIMT) using B-mode ultrasound was first introduced in 1986 by Pignoli and colleagues [402]. The assessment detects thickening of the medial layer of the vascular wall and is a good predictor of cardiac events in patients with early atherosclerosis [403], and is also an important predictor for restenosis in patients who have undergone percutaneous coronary intervention [404]. In addition, increased cIMT has been reported to relate to a number of classical CVD risk factors such as ageing, hypertension, and dyslipidemia [405]. Changes in cIMT represents a sequence of events resulting from a decrease in NO bioavailability as well as an increase in ET-1 levels, which over time increase production of inflammatory cytokines, free radicals, adhesion molecules and thrombotic factors leading to smooth muscle proliferation [406-408]. Assessment of cIMT is typically performed in the common carotid artery, internal carotid artery and at carotid bifurcation points [397], and each site has a similar ability to predict future cardiovascular events [409].

Endothelial Dysfunction in Selected Clinical Populations

Endothelial Dysfunction and Cardiovascular Disease

Endothelial dysfunction is evident before the presentation of obstructive atherosclerotic lesions [193], and can even occur in children with a family history of cardiovascular disease [378]. The magnitude of ED increases in line with the accumulation of CVD risk factors [410]. Furthermore, endothelial function is a good prognostic marker of future cardiac events in patients with CVD [354-356]. Administration of L-arginine can increase NO bioavailability and improve endothelial function in patients with CVD risk factors [411]. In addition, medications that control CVD risk factors like anti-hypertensives or statins may

also have beneficial effects on endothelial function primarily through decreasing oxidative stress and lipid accumulation [359].

Endothelial Dysfunction and Hypertension

In hypertension, the delicate balance between vasodilators and vasoconstrictors produced by the endothelium is disrupted, with disturbance in the NO pathway leading to predominance of vasoconstrictors like ET-1, which contribute to high blood pressure [412]. Even though it is still unclear whether ED is the cause [413] or the consequence of elevated blood pressure [414], it appears to be an essential factor in hypertension [415]. Studies in humans have reported a significant impairment of the vasodilator response of small resistance vessels to ACh, but not to SNP, in hypertensive patients [416,417]. Additionally, impaired FMD identifies hypertensive patients at increased risk for non-fatal and fatal cardiovascular events [418], whereas the AIx is a predictor of cardiovascular mortality in subjects with essential hypertension [419]. Treatment with angiotensin-converting enzyme (ACE) inhibitors have been shown to improve endothelial function [420]. ACE inhibitors reduce oxidative stress and stimulate bradykinin to help increase NO bioavailability [421].

Endothelial Dysfunction and Diabetes

Individuals with type I and type II diabetes have evidence of both microvascular and macrovascular ED [362,422-424]. ED can even be evident in healthy individuals with a family history of diabetes [424], suggesting a genetic link. Patients with diabetes often have reduced NO bioavailability which results from increased oxidative stress [425], and oxidation of LDL due to hyperglycaemia [426]. Patients with type 1 diabetes have shown improved endothelial function when taking ACE inhibitors [427], through a reduction in oxidative stress, and an increase in NO bioavailability [421].

Endothelial Dysfunction and Rheumatoid Arthritis

The most common cause of mortality in rheumatoid arthritis (RA) patients is cardiovascular disease [121,428]. The amount of ED can be impacted by the severity of RA disease-related inflammation [429]. In general, RA patients have poorer endothelial function in both the microvasculature and the macrovasculature when compared to healthy individuals [430-435]. The effects of anti-inflammatory medications can improve endothelial function in different vascular beds [431,436-438], suggesting that inflammation may be impacting on the vasculature in RA [124,439]. See Chapter 2 for a systematic review on endothelial function in patients with RA.

Summary

The endothelium is important in maintaining vascular homeostasis and preventing the development of atherosclerosis. However, perturbation of its activity may lead to ED which, if left untreated, could progress to atherosclerotic lesion formation and subsequent cardiac events. Therefore, assessing endothelial function in patients at risk of cardiovascular disease is important to identify vascular abnormalities and may help monitor strategies and interventions that can improve endothelial function and lower CVD risk.

Chapter 2: Rheumatoid Arthritis and Endothelial Dysfunction: A Systematic Review of the Literature

Introduction

Rheumatoid Arthritis and Cardiovascular Disease

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of the joints with predominant symptoms of pain, swelling and stiffness [1]. Patients with RA also have a number of extra-articular manifestations, the most common of which is cardiovascular disease (CVD), accounting for up to 50% of all deaths [121,428]. RA patients have a worse outcome from acute CVD events than the general population [54,64]. The exact reasons for this remain undetermined. They may include a higher prevalence and severity of classical CVD risk factors such as hypertension [111,440], dyslipideamia [142,146], obesity [188] and physical inactivity [131] leading to metabolic abnormalities [441], but also a complex interplay between systemic inflammation, such CVD risk factors and vascular function in RA [219] [122,442].

The similarities between the inflammatory process in RA and in the blood vessels of atherosclerotic CVD are remarkable. In both conditions, concentrations of C-reactive protein (CRP), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) are elevated, and there are similar patterns of cellular activation consistent with chronic inflammation [122,200]. On the basis of this, and the observation that elevated inflammatory molecules such as CRP, IL-6 and TNF-α are associated with an increased risk for CVD events in the general population [194,195,443], it has been speculated that RA disease-related inflammation might be contributing to accelerated atherosclerosis [123,124]. Pro-inflammatory molecules may exert deleterious effects on the vascular endothelium which subsequently reduces the synthesis of NO and promotes endothelial dysfunction (ED) – an early indicator of atherosclerosis [444]. Pro-

inflammatory molecules may also have metabolic effects on adipose tissue, skeletal muscle, and the liver, that can contribute to the development of classical CVD risk factors such as dyslipidemia, insulin resistance, and obesity [168,188,445], which can also, in turn, contribute to ED [359]. Adequate control of disease-related inflammation can lead to improvements in CVD risk factors in RA [155,446]. Non-invasive assessments of vascular function and structure in patients with RA may provide an excellent means of disentangling these complex pathways and assess interventions that may reduce CVD risk in these patients.

The aims of the present work are to systematically review the current literature pertaining to vascular function and structure in RA with the aim of answering: (a) whether there is sufficient evidence that patients with RA have impaired vascular function and structure compared to normal controls; (b) whether there is sufficient evidence to delineate if such changes relate to systemic inflammation or classical CVD risk factors; and (c) whether any vascular changes in RA can be modified with therapy.

Methods

Following an RA-specific evidence-based tool for searching the literature [447], five databases [Medline, Cochrane Library, Cumulative Index to Nursing & Allied Health (CINAHL) research database, Google Scholar, and Excerpta Medica database (EMBASE)] were searched to identify publications from 1974 to January 1st 2010 in English pertaining to vascular endothelial function and RA in human participants. The Medical Subject Heading (MeSH) terms "rheumatoid arthritis" was employed in combination with specific terms related to assessments of the vasculature. The following terms along with the number of articles that came up with each search (displayed in brackets) were used: "endothelial function" (176), "laser doppler flowmetry" (12), "laser doppler imaging" (7), "forearm blood flow" (2), "venous occlusion plethysmography" (3), "flow mediated dilation" (5), "augmentation index" (6), "pulse wave analysis"(8),

"pulse wave velocity" (6), "carotid intima-media thickness" (63), and "atherosclerosis" (51). Full articles were retrieved for assessment if the information in the abstract fulfilled both of the following criteria: (i) involving RA patients and (ii) studying any of the above-mentioned factors relevant to cardiovascular endothelial function. Studies incorporating only participants with other types of inflammatory arthritis, degenerative arthritis or other inflammatory or connective tissue diseases were excluded. If the title and abstract did not provide sufficient information, the full-text manuscript was examined in order to evaluate if the article fitted the inclusion criteria. Conference proceedings, letters, reviews, editorials and comments were not included in this review. Initial searches identified 283 articles although many articles were duplicated when using the different keywords. From these articles 55 individual articles matched the inclusion criteria and were thus included in the analysis.

The reference lists of all of the identified articles were further examined in order to identify publications that were relevant to microvascular or macrovascular endothelial function, arterial stiffness or carotid intima-media thickness in RA; 22 additional articles met the inclusion criteria and were included into the analysis. These additional articles along with those found from the initial searches brought the total number of articles in the present review to 77. These included cross-sectional and longitudinal observational studies and randomised controlled trials (RCTs). The quality score of the cross-sectional and longitudinal studies was derived. This score was based on the criteria related to study design (e.g., choice of patient and control population, inclusion/exclusion criteria, power analyses), adherence to published protocol guidelines (e.g., laboratory conditions, participant preparation/condition, reproducibility) and statistical analysis (e.g., adjustment for group differences). A graded score was awarded depending on the adherence to these criteria, ranging from 2 points (mentioned in detail) to 0 points (not mentioned). Given the differences in aims between the studies, the total score varied between studies, therefore the scores were converted into percentages. The quality of the identified RCTs was

assessed using previously described procedures [447]. From the 77 publications, 48 were cross-sectional studies [126,219,430,433,435,448-490], 21 longitudinal studies without using randomisation [431,432,434,436,438,461,464,491-504], and eight were RCTs [496,505-511].

Results

Cross-Sectional Studies

Few data are available on microvascular function in RA patients (Table 1). The studies reveal subtle abnormalities in nailfold capillary microscopy [450], an attenuated response to endothelium-dependent and endothelium-independent microvascular stimuli assessed with venous occlusion plethymsography [448], and an increased hyperaemic vasodilatory response [451] in RA patients compared to healthy control participants. Microvascular endothelial function does not appear to be consistently associated with inflammatory markers, e.g., CRP was associated with endothelium-dependent function in some [448,449], but not all [451] studies. Given the scarcity of available studies and variety of methods applied, more research is needed to characterise microvascular endothelial function in RA patients.

All but one (74) of the interrogated studies of macrovascular endothelial function showed an attenuated endothelium-dependent macrovascular function in RA compared to control participants, whereas no differences in endothelium independent macrovascular function are reported [452,455] (Table 1). The decreased endothelium-dependent function, assessed with FMD, appears to be already evident within 1 year of RA diagnosis [454], but does not appear to be further influenced by disease duration [433,435]. Out of the studies that assessed the relationship between measures of disease activity (i.e., CRP, ESR, DAS28), four studies did not report an association between any of these factors and FMD [435,452,455,458]. Those that did find associations between levels of disease activity and FMD were characterised by inconsistencies that are difficult

to reconcile [433,451,454]. For example, FMD was associated with CRP but not ESR in the same group of patients [433,451]. Therefore, there is no strong evidence that FMD is influenced by disease-related factors.

In line with this, a comparison between RA and diabetes mellitus yielded no difference in FMD, even though CRP was significantly higher in RA [458]. In separate analyses, the same authors also reported that the presence of RA and the presence of diabetes were both independent predictors of poor FMD. However, in diabetes, this was shown to be due to classical CVD risk factors, which was not the case in RA [458]. Surprisingly few studies have examined the effects of classical CVD risk factors on macrovascular endothelial function in RA [433,455,456]. Associations were found for lipid levels in some [433,456], but not all studies [455]. Therefore, given the known associations between classical CVD risk factors and endothelial function, more research is necessary to explore these associations in RA. It is worth noting that endothelium-dependent macrovascular function in RA was lower than controls even when patients were matched for CVD risk or the comparison was statistically adjusted for CVD risk [455,457,458]. Taken together these studies suggest that there is ample evidence that endothelium-dependent macrovascular function is compromised in RA, but this does not appear to be consistently related to disease activity, and the influence of CVD risk factors is not clear.

Table 1. Overview of cross-sectional studies on microvascular endothelial function, macrovascular endothelial function, arterial stiffness and intima-media thickness in patients with rheumatoid arthritis

Authors	RA patients	Exclusion Criteria	Controls Participants	Vascular Assessment	Findings	Associations	Quality Index (%)
Microvascular Function							
Yki-Jarvinen et al 2003	20 RA	HD	33 healthy *	FBF with ACh, SNP	ESR, CRP ↑ in RA Vasodilatory response to ACh and SNP ↓ in RA	RA: low SNP with TNF-α and CRP (not IL-6 or ESR) High ACh with TNF-α (not CRP, ESR, IL-6) Basal flow with CRP, ESR, and TNF-α, not IL-6	67
Galarraga et al 2008	128 RA	HD, RF, CM		Laser Doppler imaging, ACh and SNP (peak value)	ACh ↓ in high CRP (> 10) SNP ↓ in high CRP (> 10) When patients split on basis of DAS28, no difference	Peak ACh with age and logCRP (multivariate)	61
Altomonte et al 1995	32 RA		32, age & sex matched	Nailfold capillary microscopy	Subtle abnormalities in RA	Not reported	22
Arosio et al 2007	65 RA	HD, RF	40 healthy*	LDF in response to hyperaemia	ESR, CRP ↑ in RA % increase ↑ in RA	No significant associations	96
Macrovascular Function							
Van Doornum et al 2003	25 RA	HD, RF	25 healthy, age & sex matched	FMD, GTN	FMD and GTN not different	No significant associations	96
Gonzalez-Juanatey et al 2003	55 RA	HR, RF	31 healthy, age & sex matched	FMD, GTN	FMD ↓ in RA, GTN no difference	No significant associations	73
Gerli et al 2004	87 RA	HD, RF, CM	33 (24 OA & 9 fibromyalgia), age & sex matched	FMD	FMD ↓ in RA	Not reported	50
Vaudo et al 2004	32 RA	HD, RF, CM	28 (8 fibromyalgia, 10 knee Osteoarthritis, 10 hand Osteoarthritis) age & sex matched	FMD	FMD ↓ in RA	FMD associated with CRP, CRP duration, average CRP, but not with ESR	75
Pingiotti et al 2007	50 RA	HR, RF, CM	26 healthy	FMD	FMD ↓ in RA	FMD associated with DAS28	32
Arosio et al 2007	65 RA	HD, RF	40 healthy*	FMD	FMD ↓ in RA	FMD associated with CRP	77

Kerekes et al 2008	52 RA	HD, RF, CM	40 healthy, age & sex matched	FMD, GTN	FMD ↓ in RA No difference in GTN	No significant associations	64
Rojas-Villarraga et al 2008	140 RA	HD		FMD	FMD<5%: 31%	Not reported	56
Soltesz et al 2009	14 RA, (50APS, 24SSc, 13PM)	HD, RF	36 healthy, age, sex & Framingham risk score matched	FMD	FMD ↓ in autoimmune disease	Not reported	36
Stamatelopoulos et al 2009	84 RA AND 48 RA	HD, RF, CM	84 healthy, age, sex & CV risk factor matched AND 48 healthy & 48 DM	FMD	FMD ↓ in RA No difference between remission and active group AND No difference in FMD between RA & DM	No significant associations AND Presence of RA independently associated with decreased FMD.	75
Arterial Stiffness							
Klocke et al 2003	14 RA	HD, RF, CM	14 healthy, sex, age, BP, & height matched	AIx	AIx ↑ in RA	No significant associations	70
Van Doornum et al 2003	25 RA	HD, RF	25 healthy, age & sex matched		SAC and LAC ↓ in RA	No significant associations	96
Wong et al 2003	53 RA (15 CAD, no CAD)	HD, CM	53 (15 CAD, 38 no CAD) age, sex, & CAD matched	PWA: SAE, LAE	SAE and LAE ↓ in RA No difference between CAD and no CAD	No significant associations	80
Roman et al 2005	80 RA	HD, CM	101 SLE, 105 healthy*	Arterial stiffness	Arterial stiffness ↑ in RA and SLE	RA and SLE patients only: arterial stiffness associated with CRP	55
Maki-Petaja et al 2006	77 RA	HD, RF, CM	142 healthy, age, sex, height & BMI matched	AIx PWV (aortic and brachial)	Aortic & brachial PWV↑ in RA No difference in AIx	Aortic PWV associated with CRP, but Not (cumulative) ESR or DAS28 No associations with brachial PWV or AIx	75
Arosio et al 2007	65 RA	HD, RF	40 healthy*	PWV	PWV ↑ in RA	No significant associations	77
Avalos et al 2007	57 RA, DD<6 years, 60 RA, DD>10 years		65 healthy, age, sex & race matched	PWV, AIx	PWV no difference AIx ↑ in late RA compared to early RA and controls	PWV & AIx not associated with CRP, ESR, DAS28 after adjustment for CVD risk	63

Wallberg-Jonsson et al 2008	30 RA		30 healthy, age & sex matched	AIx	No difference in AIx	AIx associated with retrospective inflammatory activity	44
Stamatelopoulos et al 2009	84 RA AND 48 RA	HD, RF, CM	84 healthy, age, sex & CV risk matched AND 48 healthy & 48 DM	PWV	PWV ↑ in RA AND No difference in PWV between RA and DM	No significant associations	75
Soltesz et al 2009	14 RA, (50APS, 24SSc, 13PM)	HD, RF	36 healthy, sex, age & Framingham risk score matched	AIx PWV	AIx and PWV ↑ autoimmune disease AIx ↑ in RA than SSc or PM	Not reported	36
Galarraga et al 2009	148 RA	HD, RF, CM		AIx	No difference in AIx between high (>10) and low CRP groups	AIx associated with CRP and DAS28	86
Crilly et al 2009	114 RA	HD		AIx	AIx higher in women than men	Increase in cumulative ESR positively associated with increase in AIx	100
Intima Medial Thickness							
Wallberg-Jonsson et al 2001	39 RA	RF	39 healthy, age & sex matched	carotid IMT femoral IMT	Mean cIMT ↑ in RA fIMT not different	No significant associations	60
Kumeda et al 2002	138 RA	HD, RF	94 healthy*	carotid IMT femoral IMT	cIMT ↑ in RA fIMT ↑ in RA	No significant associations	80
Park et al 2002	53 RA	HD, RF	53 healthy, age & post-menopausal matched	IMT	IMT ↑ in RA	No significant associations RA < 1 year, IMT ↓	90
Alkaabi et al 2003	40 RA	CM	40 healthy, age sex & postcode matched	IMT	IMT ↑ in RA	No significant associations	46
Gonzalez-Juanatey et al 2003	47 RA	HD, RF, CM	47 healthy, age & sex matched	IMT	IMT ↑ in RA	No significant associations	82
Del Rincon et al 2003	204 RA	RF, CM	102 healthy, age & sex matched	IMT	No difference in IMT	Significant associations with CRP and ESR in all participants	65
Wallberg-Jonsson et al 2004	39 RA		39 healthy, age & sex matched	carotid IMT femoral IMT		No significant associations Presence of RA associated with increase in cIMT	50
Gerli et al 2004	87 RA	HD, RF, CM	33 (24 OA & 9 fibromyalgia), age & sex matched	IMT	IMT ↑ in RA	Not reported	50

Study	RA	Controls	Other groups	Measure	IMT difference	Associations	Ref
Dessein et al 2005	74 RA	RF		IMT	N = 53 atherosclerosis N = 21 no atherosclerosis	No significant associations	81
Gerli et al 2005	101 RA	HD	75 with degenerative joint disease or other non-inflammatory rheumatic disorder, age & sex matched	IMT (8 measures)	Only mean Carotid Bifurcation-IMT ↑ in RA	No significant associations	73
Del Rincon et al 2005	631 RA (328 with plaque & 303 no plaque)			IMT	No difference in IMT	IMT associated with ESR	69
Gonzalez-Gay et al 2005	47 RA	HD, RF, CM		IMT	IMT greater in patients with highest quartile of CRP	IMT associated with CRP max CRP, and proportion CRP>10, average CRP, but not current CRP No association between IMT and ESR	88
Roman et al 2005	80 RA	HD, CM	101 SLE, 105 healthy*	IMT	No difference in IMT	No significant associations	55
Wada et al. 2005	50 RA	HD, RF	30 healthy*	IMT	IMT ↑ in RA	No significant associations CRP and ESR in healthy participants In all participants: RA status predictive	59
Dessein et al 2006	74 RA	RF		IMT	No difference in IMT between with NCEP-MetSyn and those without WHOMetsyn IMT higher with than without	Number of NCEP criteria not associated with IMT Number of WHO-MEtSyn associated with IMT	29
Grover et al 2006	57 RA	HD, RF, CM	45 healthy, age & sex matched	IMT	IMT ↑ in RA	No significant associations	64
Pahor et al 2006 Immunobiology	70 RA	HD, RF	40 healthy, age and sex matched	IMT	IMT ↑ in RA	Not reported	50
Pahor et al 2006 Rheumatol Int	70 RA	HD, RF	40 healthy, age & sex matched	IMT	IMT ↑ in RA	Significant association with CRP	50
Pamuk et al 2006	63 RA	HD, RF, CM	34 OA, age & sex matched	IMT	IMT ↑ in RA	No significant associations	70

Study	Sample	Factors	Comparison	Measure	IMT Result	Associations	Ref
Daza et al 2007	55 RA	RF	20 healthy*	IMT	IMT ↑ in RA	No significant associations	80
La Montagna et al 2007	45 RA	HD, RF	48 soft tissue disease patients*	IMT	IMT ↑ in RA	Associated with DAS28 IMT increases with 4 groups of DAS28	75
Surdacki et al 2007	30 RA	HD, RF, CM	20 healthy, age & sex matched	IMT	IMT ↑ in RA	No significant associations	89
Pingiotti et al 2007	50 RA	HR, RF, CM	26 healthy	IMT	IMT ↑ in RA	Associated with DAS28	32
Del Rincon et al 2007	631 RA			IMT	IMT ↑ in longest DD	No significant associations	57
Sherer et al 2007	100 RA	HD	69 with degenerative joint disease or other non-inflammatory rheumatic disorder	IMT (8 measures)	In RA: anti-HSP-65 positive RA lower mean and max CA-IMT		40
Sherer et al 2007	82 RA	HD	None	IMT (8 measures)		No significant associations	86
Coaccioli et al 2007	38 RA	HD, RF	30 healthy, age & sex matched, 42 DM, 37 obese	IMT	IMT ↑ in RA compared to healthy, not different from DM or obese	Not reported	35
Rojas-Villarraga et al 2008	140 RA	HD		IMT	High IMT: 54%	No significant associations	56
Ciftci et al 2008	30 RA	HD, RF, CM	32 healthy*	IMT	IMT ↑ in RA	No significant associations	80
Hannawi et al 2008	40 RA		40 healthy, sex, & CV risk matched	IMT	IMT ↑ in RA	Associated with CRP, DAS4	60
Kerekes et al 2008	52 RA	HD, RF, CM	40 healthy, age & sex matched	IMT	IMT ↑ in RA	No significant associations	64
Soltesz et al 2009	14 RA, (50APS, 24 SSc, 13PM)	HD, RF	36 healthy, age, sex & Framingham risk score matched	IMT	IMT ↑ in autoimmune disease	Not reported	36
Pereira et al 2009	71 RA	HD, RF, CM	53 healthy, age & sex matched	IMT	No difference in IMT	No significant associations	70

Stamatelopoulos et al 2009	84 RA AND 48 RA	HD, RF, CM	84 healthy, age, sex & CV risk matched AND 48 healthy & 48 DM	IMT	IMT ↑ in RA AND No difference in IMT between RA and DM	No significant associations	75
Schott et al 2009	93 RA		93 healthy, age, race & menopause matched	IMT	No difference in IMT	No significant associations	64

Note: RA: rheumatoid arthritis, FMD: flow-mediated dilatation, GTN: glycerine-trinitrate, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, DD, disease duration, HD: heart disease, which includes at least one of: hypertension, myocardial ischaemia, stroke, cerebrovascular disease, known/suspected CVD, atherosclerosis, peripheral vascular disease, cardiac arrhythmias, valvular heart disease. RF: risk factor for cardiovascular disease, which includes at least one of: family history of CVD, smoking, diabetes mellitus, hyperlipideamia, obesity. CM: comorbidity, which includes at least one of: renal disease, neoplasms, connective tissue disease, vasculitis, other inflammatory disease, recent surgery or illness, active infectious disease, hyperthyroidism malignancy. DM: diabetes mellitus.
*Populations not matched for age and sex, but age and sex do not differ between populations.

Overall, arterial stiffness was increased in RA compared to control participants (see table 1). Similar to the functional assessments described above, no consistent association between arterial stiffness and markers of disease activity is apparent. The number of studies that report an association between at least one disease activity measure and arterial stiffness is equal to the number of studies that do not find such an association. It is possible that it is not current but continuous inflammatory activity over the course of the disease that impacts negatively on the vasculature [512]. However, whereas longitudinal inflammatory burden, indexed by ESR, joint assessments, and physicians' global assessment since disease onset was predictive [463], cumulative assessment of ESR over the last 5 years was not [461]. This might imply that assessing disease activity only serologically, even when this is done longitudinally, is too simplistic to determine arterial stiffness. Radiographic damage using the Sharp score, an indirect marker of cumulative inflammatory activity on the joints, was found to be related to arterial stiffness [435]. Therefore, other RA-related assessments such as joint and physician's global assessment might provide a better reflection of the overall burden of RA. However, research is necessary to explore this in more detail.

The majority of the cross-sectional studies assessed IMT (Table 1), and show that overall IMT is increased in RA patients compared to healthy controls. This is confirmed by a recent systematic review and meta-analysis [513]. Like the functional vascular measures described above, IMT does not appear to be consistently associated with markers of disease activity (Table 1). Again, the majority of the studies reported current markers of inflammation, with a few exceptions [466,468,473]. Surprisingly, retrospective global disease activity was not associated with IMT [466], in contrast to the findings for arterial stiffness [463]. Longitudinal assessments of ESR and CRP revealed that CRP, but not ESR was related to IMT [473], despite a strong association between ESR and CRP. Therefore, more research may be necessary to determine why only CRP was related to IMT. Increased cIMT is already apparent in patients with a recent

diagnosis of RA [488]. However, whether IMT is further increased with disease duration is not clear from the available data. Even though several studies provide evidence for greater IMT with longer disease duration [219,467,468,487], others do not find such an association [126,469,476]. Caution should be exercised when interpreting these findings, as the impact of age on this association remains to be determined. IMT is known to increase with age in the general population [514], and is also the most consistent determinant in IMT in RA both in univariate and multivariate analyses [219,466,472,474,478,479,482,489,515]. Interestingly, in RA the age-induced increase in IMT was greater with longer disease duration [483]. However, the association between disease duration and IMT was no longer significant after correcting for age [490]. Therefore, more studies specifically looking at the changes over time are needed to clarify this.

Unfortunately, the comparison between RA and control participants has largely been done without correction for any factors that could impact IMT, such as CVD risk or its individual components. Various factors that are known to be associated with IMT in the general population have been explored in RA. For example, global cardiovascular risk, using the Framingham Risk Score, was associated with a higher IMT [456,456]. Even though not systematically, several individual CVD risk factors have been explored in relation to IMT. This showed that adverse lipid profile was related to IMT, not only in univariate [455,466,475,478] but also in multivariate analyses [475,479]. However, care should be taken when interpreting these results, as varying statistical analyses and multivariate models have been applied to assess the associations of individual risk factors in RA.

It is possible that CVD risk factors play a role in the association between IMT and inflammation in RA, given that ESR was associated with IMT only in the presence of CVD risk factors [219]. Direct comparison between the associations found in healthy participants and those found in RA patients might determine

whether inflammation affects IMT (and other vascular parameters) in RA patients in a different manner. This is likely given that the presence of RA has been reported to independently predict IMT [458,467,471,471,474].

Taken together, the cross-sectional studies reveal ample evidence for attenuated vascular function in patients with RA. Even though a large number of studies have been conducted in this area, the quality of these studies with regards to study design, adherence to published protocols, and appropriate statistical analyses, varies largely between studies (see Table 1). Few studies conducted power analyses for the comparison between groups, however, no data is available on appropriate power to examine factors associated with vascular function in RA. This has profound implications for the interpretation of the available data, and more research, specifically set out to explore the factors associated with vascular function in RA, is needed to understand the mechanism for vascular impairments in RA.

Given that RA disease-related inflammation is widely assumed to contribute to the elevated CVD risk through its impact on the vasculature [123,124], it is surprising to find that direct evidence for such an association is still lacking. In other populations, vascular function is associated with inflammation [516], although it is likely that the levels of inflammation that are generally seen in other populations are significantly lower than those in RA patients [458]. Accordingly, it remains possible that low to moderate grade inflammation characteristic of these other populations, such as diabetic or cardiovascular patients, is a good predictor of endothelial function, whereas high grade inflammation in RA is not predictive of vascular function or structure.

It is also possible that it is longstanding not current inflammation that impacts the vasculature in RA patients [512]. At present, the studies which have explored disease activity longitudinally have incorporated varying methods of quantifying accumulated disease activity, and also have varying results

[433,461,463,465-468,487]. Thus, even though RA has been shown to be predictive of greater arterial stiffness [458], as well as IMT [471,474], this does not seem to be due solely to current levels of disease-related inflammation. A comparison of RA and diabetes patients, for example, revealed similar vascular status despite higher levels of inflammation in the RA patients [458,486]. As vascular impairments cannot be fully explained by current levels of inflammation, other factors must be contributing. Unfortunately, to our knowledge, little attention has been paid to other potential influences. There is however, preliminary evidence of an interaction between inflammation and CVD risk factors affecting vascular function in RA [219]. A direct comparison between the association between vascular function and a range of potential determinants in different patient groups might help illuminate precisely which factors are particularly important in RA.

Longitudinal studies

The majority of studies that explored vascular parameters longitudinally examined the effects of a change in medication regimes (in particular anti-TNF-α treatment) (See Table 2). With one exception [434], microvascular endothelial function was shown to improve in response to successful treatment [432,436,492]. The effects of successful treatment on macrovascular endothelial function are similar [431,438,461,488,493-501]. Vascular function was no longer significantly different from control participants following treatment [432,436,461], even though markers of inflammation were still increased relative to controls [432]. Only two studies have reported the associations between change in disease activity (either assessed with DAS28, CRP, or ESR) and vascular function [488,499], with equivocal results. Changes in CRP and FMD were associated in one [488] but not the other study [499]. However, care should be taken when interpreting the presence or absence of reported associations given the small sample sizes in these longitudinal studies. In addition, only two studies reported a priori power calculations on the basis of changes in vascular parameters over time [496,498]. No power calculations, either a priori or post

hoc, were carried out for the associations between changes in disease activity and vascular function.

Few longitudinal studies are available on structural changes in the vasculature in RA (see Table 2). Most studies found that a reduction in disease activity as a result of anti-rheumatic treatment was not accompanied by an improvement in brachial arterial stiffness [461,492,496,517], even though an improvement was found in aortic arterial stiffness [461]. In contrast, one study reported that anti-TNF-α treatment, but not methotrexate, caused an improvement in arterial stiffness [464], and atorvastatin was reported to effect a decrease in arterial stiffness in the absence of changes in disease activity [518]. Even fewer longitudinal data are available for IMT; there are only four published studies. The results with regards to successful treatment are equivocal [499,501,504]. Nevertheless, change in IMT was found to be greater in RA patients compared to healthy control participants [503], as well as attenuated in patients on anti-TNF-α treatment compared to those on methotrexate [504]. None of the structural longitudinal studies provide evidence for a direct association between vascular parameters and measures of disease activity.

The influence of changes in classical CVD risk factors on changes in vascular function or structure has not received attention in the literature. Even though changes in lipid profiles have been explored in response to treatment, the results are equivocal. No reports are available on associations between changes in CVD risk factors and changes in vascular function or structure in RA. However, given the small sample sizes in the available studies, it remains possible that the studies are underpowered to analyse these associations. In sum, the longitudinal studies reveal that the vascular response to successful treatment is not clearly defined, and there is no consistent evidence for an association between changes in vascular parameters and changes in disease activity. However, given the small sample sizes of the studies and lack of appropriate power analyses, care should be taken when interpreting these data.

Table 2. Overview of longitudinal studies on microvascular endothelial function, macrovascular endothelial function, arterial stiffness and Intima-media thickness in patients with rheumatoid arthritis

Authors	RA patients	Exclusion Criteria	Intervention / Assessment Time Points	Vascular Assessment	Findings	Associations[1]	Quality Index (%)
Microvascular function							
Bergholm et al 2002	10 RA	HD	DMARDS / 0 & 6 months	FBF with ACh & SNP	CRP & ESR ↓ ACh ↑ SNP no change	Not reported	58
Hansel et al 2003	8 RA	HD, RF, CM	1) 2-4 days after IV MTX 2) etanercept / 2-4 days after MTX & 21 days after etanercept	FBF with ACh & GTN	No change	Not reported	50
Cardillo et al 2006	10 RA	HD, RF, CM	Intra-arterial saline infusion & Infliximab / Immediately following infusion	FBF with ACh & SNP	No change in CRP ACh ↑ SNP: no change	Not reported	59
Datta et al 2007	8 RA	HD, RF, CM	Anti-inflammatory treatment (various) / Pre & post acute treatment	LDI with iontophoresis (ACh & SNP)	CRP ↓ treatment ACh & SNP ↑ after treatment	CRP not associated with change in ACh	65
Komai et al 2007	15 RA	HD, CM	Infliximab (0, 2 & 6 weeks) / 0, 2 & 6 weeks.	FBF, FBF to GTN	DAS28, CRP, ESR ↓ at 2 & 6 weeks FBF ↑ at 2 & 6 weeks. No change in GTN	Not reported	25
Macrovascular function							
Hurlimann et al 2002	11 RA	HR, RF	Infliximab (0, 2 & 6 weeks) / 0 & 12 weeks	FMD GTN	DAS28, ESR ↓ at 12 weeks, CRP no change FMD ↑ at 12 weeks GTN no change	Not reported	46
Irace et al 2004	10 RA		Infliximab (0, 2 & 6 weeks) / 0, 2, and 6 weeks (before and after each infusion)	FMD, GTN	DAS28, ESR ↓ at 6 weeks, CRP no change FMD ↑ after each infusion FMD returned to baseline by next infusion.	Not reported	50
Gonzalez-Juanatey et al 2004	7 RA	HD, RF	At least 1 year anti-TNF treatment (infusion/8 weeks) / -2, +2, +7,+28 days	FMD, GTN	DAS28 ↑ at 7 days, CRP, ESR no change FMD ↑ at 2 and 7 days GTN no change	Not reported	67
Bilsborough et al 2006	9 RA		3 RA: Infliximab 6 RA: Etanercept / 0 & 36 weeks		DAS28 ↓ at 36 weeks FMD ↑ at 36 weeks GTN no change	Not reported	41

77

Study	N		Drug (timing)	Timepoints	Measures	Results	Association	Ref
Maki-Petaja et al 2006	9 RA	HD, RF, CM	Etanercept (2/week)	0, 4 & 12 weeks	FMD, GTN	DAS28, CRP, ESR ↓ at 4 & 12 weeks FMD↑ at 4 & 12 weeks GTN no change	Not reported	23
Gonzalez-Juanatey et al 2006	8 RA	HD, RF	Adalumimab (1/2 weeks)	0, +2 days, +2 weeks & +12 weeks	FMD, GTN	DAS28, CRP ↓ at 2 & 12 weeks. ESR ↓ at 12 weeks FMD ↑ +2day, +2week & +12 weeks GTN no change	No association between change in CRP & FMD	67
Ikonomidis et al 2008	23 RA	HD, CM	23 RA: Anakinra 19 RA: increase prednisone dose	0 & 30 days	FMD, GTN,	DAS28, CRP ↓ at 30 days, greater ↓ in anakinra than prednisone FMD ↑ at 30 days in anakinra GTN no change	Not reported	88
Gonzalez-Juanatey et al 2008	6 RA		Rituximab	0, 2 weeks & 6 months	FMD, GTN	DAS28, ESR, CRP ↓ 2 weeks & 6 months FMD ↑ at 2 weeks & 6 months GTN ↑ at 2 weeks	Not reported	50
Syngle et al 2008	24 RA	HD, RF, CM	Spironolactone	0 & 12 weeks	FMD, GTN	DAS28, CRP, ESR ↓ at 12 weeks FMD ↑ at 12 weeks GTN no change	Not reported	71
Sidiropoulos et al 2008	12 RA	HD	Anti-TNF	0, 3 months & every 2 months up-to 18months	FMD, GTN	DAS28 ↓ at 3 & 18 months CRP ↓ at 18 months ESR no change FMD no change at 3 months, ↑ at 18 months	18 months: no association between change in FMD and change in ESR, CRP, or DAS28	58
Bosello et al 2008	10 RA	HD, RF	Infliximab (0, 2, 6 & 14 weeks)	0, 2, 6 & 14 weeks (day before and after infusion)	FMD, GTN	DAS28 ↓ at 14 weeks ESR & CRP ↓ at 2 & 6 weeks FMD ↑ day after each infusion, but at baseline by next infusion GTN no change	Not reported	59
Kerekes et al 2009	5 RA	HD, RF, CM	Rituximab	0, 2, 6 &16 weeks	FMD, GTN	FMD ↑ in 4/5 patients at week 2 and week 6, & 5/5 at week 16	Change in FMD not associated with change in CRP	55
Hannawi et al 2009	31 RA		Combination DMARD	0 & 12 months	FMD, GTN	ESR & CRP ↓ at 12 months FMD and GTN↑ at 12months	Change in FMD associated with change in CRP	69

Arterial Stiffness

Study	N		Medication	Timepoints	Measure	Results	Associations	Ref
Maki-Petaja et al 2006	9 RA	HD, RF, CM	Etanercept (2/week)	0, 4 & 12 weeks	FMD, GTN	DAS28, CRP, ESR ↓ at 4 and 12 weeks Aortic PWV ↓ at 4 & 12 weeks Brachial PWV no change	Not reported	23
Komai et al 2007	15 RA	HD, CM	Infliximab (0, 2 & 6 weeks)	0, 2 & 6 weeks	FBF, FBF to GTN	DAS28, CRP, ESR ↓ at 2 & 6 weeks No change in PWV	Not reported	25
Galarraga et al 2009	47 RA	HD, RF, CM	21 Methotrexate 26 Etanercept	(0, 2 and 4 months)	AIx	Methotrexate: DAS28 ↓ at 2 & 4 months Etanercept: DAS28, logCRP, HAQ ↓ at 2 & 4 months AIx ↓ at 2 & 4 months in etanercept group only	Not reported	75
Intima media thickness								
Nagata-Sakurai et al 2003	62 RA	HD, RF	Stable medication	0, 18 - 36mths	IMT	Greater change in IMT in RA compared to healthy controls	Not reported	65
Del Porto et al 2007	30 RA	HD, RF, CM	14 RA: Infliximab 16 RA: etanercept	0 & 12 months	IMT	DAS44, CRP, ESR ↓ at 3 months & 12 months IMT ↓ at 12months	Not reported	77
Sidiropoulos et al 2008	12 RA	HD	Anti-TNF	0, 3 months & every 2 months up-to 18month	IMT	DAS28 ↓ at 3 & 18 months CRP ↓ at 18 months ESR no change IMT no change	Not reported	58
Kerekes et al 2009	5 RA	HD, RF, CM	Rituximab	0, 2, 6 & 16 weeks	IMT	IMT ↓ in 5/5 patients at week 2, 4/5 patients at week 6 & 3/5 patients at week 16	Change in IMT not associated with change in CRP	55

Note: RA: rheumatoid arthritis, FMD: flow-mediated dilatation, GTN: glycerine-trinitrate, IMT: intima-media thickness, AIx: augmentation index, PWV: pulse wave velocity, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, DAS28: Disease Activity Score, HD: heart disease, which includes at least one of: hypertension, myocardial ischaemia, stroke, cerebrovascular disease, known/suspected CVD, atherosclerosis, peripheral vascular disease, cardiac arrhythmias, valvular heart disease. RF: risk factor for cardiovascular disease, which includes at least one of: family history of CVD, smoking, diabetes mellitus, hyperlipideamia, obesity. CM: comorbidity, which includes at least one of: renal disease, neoplasms, connective tissue disease, vasculitis, other inflammatory disease, recent surgery or illness, active infectious disease, hyperthyroidism malignancy. DM: diabetes mellitus, PM: postmenopausal, OA: osteoarthritis [1]: associations with change in CRP, ESR and DAS28 only reported here.

Randomised Control Trials

There were only a small number of RCTs on vascular function or structure (see table 3). Briefly, IL-1ra antagonist was associated with an acute improvement in FMD [496], whereas 2 weeks of selective or non-selective COX inhibitors did not change FMD or AIx [506]; 56 weeks of anti-TNF-α decreased PWV, but not AIx or IMT [507]. Following 5 years of either prednisolone or no-prednisolone treatment, there was no difference in IMT or FMD between the treatment and no treatment arms of the trial [505]. However, due to the absence of baseline vascular assessment, this study does not provide information on changes in cIMT or FMD as a result of treatment. Four studies examined the effects of either statins or ACE inhibitors over a period of 2 to 8 weeks and demonstrated, that overall, these medications improved FMD and arterial stiffness [508-511], which is in line with studies in other populations [519,520]. These last studies also emphasise the potential importance of classical CVD risk factors in vascular function in RA, in particular the influence of lipid profiles. Statin treatment reliably results in an improvement in FMD [508,509,511], which can occur in the absence of a reduction in disease activity [509]. However, more detailed, and appropriately powered, studies are needed to explore the complex interplay between lipid profiles, disease activity and the vasculature in more detail. In sum, given the paucity of RCTs with limited sample size and lack of power calculations particularly in those studies testing anti-rheumatic medication, it is not possible to draw firm conclusions at this stage.

Table 3: Overview of randomised controlled trials on macrovascular endothelial function, arterial stiffness and intima-media thickness in patients with rheumatoid arthritis

Authors	RA patients	Exclusion Criteria	Intervention	Assessment Time Points	Vascular Assessment	Findings	Associations[1]	Jadad Score [447]
Anti rheumatic medication								
Hafstrom et al 2007	67 RA		34 prednisone for 2 years, then 13 continue, 21 stop prednisone treatment 33 no prednisone	Assessments at 5.3 years, no baseline values	IMT, FMD	IMT, FMD no difference between groups CRP, ESR no difference between groups	Not reported	1
Wong et al 2007	37 RA	HD, RF, CM	Placebo (12), Rofecoxib (12), Indomethacin (11) 2 weeks	0 & 2 weeks	FMD, GTN, AIx	No difference in disease activity change between groups No change in FMD, GTN, Aix in any group	Not reported	3
Ikonomidis et al 2008	23 RA	HD, CM	Anakinra or placebo 48hrs cross over	0 & 3 hours	FMD, GTN, PWV	CRP no change FMD ↑ anakinra GTN, PWV no change	Not reported	2
Wong et al 2009	26 RA	HD, RF	Infliximab (17) for 54 weeks or placebo (9) for 22 weeks	Baseline, 14 & 56 weeks	PWV, AIx. IMT,	DAS28 & ESR ↓ PWV ↓ 56 weeks IMT, AIx no change	Not reported	2
Statin and ACE inhibitors								
Tikiz et al 2005	43 RA	HD, RF	Placebo (15), Simvastatin (14), Quinapril (14) 8 weeks		FMD, GTN	Statin: CRP ↓ Statin: FMD ↑ No change in GTN	Not reported	2
Hermann et al 2005	20 RA	HD, RF, CM	Simvastatin & placebo 4 weeks crossover	0 & 4 weeks	FMD	No change in disease activity Statin: FMD ↑ GTN no change	Not reported	2
Flammer et al 2008	11 RA	HD, RF, CM	Ramipril & placebo 8 weeks crossover	0 & 8 weeks	FMD, GTN	No change in CRP FMD ↑ after ramipril GTN no change	Not reported	2
Maki-Petaja et al 2007	20 RA	HD, RF, CM	Ezetimibe & simvastatin 6 weeks crossover	0 & 6 weeks, and 0 & 6 weeks	FMD, GTN, AIx, PWV	DAS28, ESR & CRP ↓ PWV ↓ & FMD ↑ in both treatments AIx, GTN no change	Not reported	2

Note: RA: rheumatoid arthritis, FMD: flow mediated dilatation, GTN: glycerine-trinitrate, IMT: intima media thickness, AIx: augmentation index, PWV: pulse wave velocity, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, DAS28: Disease Activity Score, HD: heart disease, which includes at least one of: hypertension, myocardial ischaemia, stroke, cerebrovascular disease, known/suspected CVD, atherosclerosis, peripheral vascular disease, cardiac arrhythmias, valvular heart disease. RF: risk factor for cardiovascular disease, which includes at least one of: family history of CVD, smoking, diabetes mellitus, hyperlipideamia, obesity. CM: comorbidity, which includes at least one of: renal disease, neoplasms, connective tissue disease, vasculitis, other inflammatory disease, recent surgery or illness, active infectious disease, hyperthyroidism malignancy. [1]: associations with CRP, ESR and DAS28 only reported here.

Summary

The studies presented above provide clear evidence that vascular function and structure are impaired in patients with RA. RA has been increasingly reported to be associated with accelerated atherosclerosis [124,521], based on the assumption that high grade inflammation associated with RA induces accelerated atherosclerosis [123,124,522]. This assumption draws heavily on data from cross-sectional studies, which, irrespective of intrinsic issues regarding direction of causality, when considered, afford less than wholly compelling evidence that IMT is related to current inflammation. To our knowledge, there is only one study that assessed accelerated atherosclerosis. The increase in IMT was greater in RA patients compared to age and sex-matched healthy controls [503]. Unfortunately, with disease activity only assessed at baseline, a direct link between change in inflammation and IMT could not be explored. In addition, the study design did not allow for an assessment of the impact on IMT of variations in disease activity over the follow-up period.

Longitudinal studies are necessary to examine the concept of accelerated atherosclerosis. In order to determine how the fluctuations in disease activity influence vascular changes over time, measurements must be made over a protracted period. The vascular assessments described in this review are generally considered to be associated with an increased risk for cardiovascular death. There is evidence for this in the general population [523], but only one study with a small sample has reported that high levels of IMT are predictive of hard cardiac end-points in RA [524]. Therefore, in order to understand if and how vascular function is predictive for cardiovascular events, detailed longitudinal assessments are necessary. These assessments should include multiple vascular parameters as well as multiple potential determining factors. Once it is known what determines the impaired vascular function, interventions, either through medication and/or behavioural change, can be developed to improve vascular function as well as structure in RA.

Overview of Thesis

As explained earlier it is clear that rheumatoid arthritis (RA) is a condition associated with increased morbidity and mortality from cardiovascular disease (CVD). A significant part of this increased CVD risk is thought to result from chronically high disease-related inflammation which can perturb endothelial cell homeostasis leading to endothelial dysfunction (ED), accelerated atherosclerosis and subsequent cardiac events. The systematic review of the literature presented in Chapter 2 revealed that associations between disease-related inflammation and endothelial function may not be as strong as first thought. However, much of this research has focused on single vascular beds only with little attention being paid to assessing the effects of inflammation on endothelial function in different vascular beds (i.e. microvessels and macrovessels) in the same group of patients. In addition, factors which affect endothelial function in the general population, such as CVD risk factors, have not been simultaneously explored in the microcirculation and the macrocirculation in this group of patients. Thus, the present work includes a series of studies which examined differences in microvascular and macrovascular endothelial function between RA patients and healthy control participants (Chapter 5), explored associations between disease-related inflammation, classical CVD risk and endothelial function in RA (Chapter 6), and assessed the longitudinal effects of anti-inflammatory treatment on microvascular and macrovascular endothelial function in RA (Chapter 7).

Hypotheses

Chapter 5

The aim of the study presented in chapter 5 was to compare microvascular and macrovascular endothelial-dependent function between RA patients and healthy control participants. It was hypothesised that RA patients would have worse endothelial function in the microvasculature and the macrovasculature when compared to healthy control participants. A second aim of this study was to

examine the relationship between microvascular and macrovascular endothelial-dependent function in RA patients as only a few studies have previously examined such associations in RA, and *in vitro* research suggests that these two vascular beds are distinct from each other. It was hypothesised that these two vascular beds would be independent from each other in RA.

Chapter 6

The study presented in this chapter investigated predictors of endothelial function in RA. The specific aims were to examine associations between disease-related inflammation and microvascular and macrovascular endothelial-dependent function in RA. Similarly, associations between classical CVD risk factors and microvascular and macrovascular endothelial-dependent function in RA were also explored. Chronically high levels of systemic inflammation would be expected to impair endothelial function, thus, it was hypothesised that inflammation would be associated with microvascular and macrovascular endothelial function in RA. Classical CVD risk factors are strong predictors of endothelial function in the general population, and it was hypothesised that this association would remain for RA patients too.

Chapter 7

RA patients who were about to start anti-tumor necrosis factor-alpha (anti-TNF-α) therapy were followed up at 2 weeks and 12 weeks after treatment with simultaneous assessments of microvascular and macrovascular endothelial function. The aim of this study was to examine the longitudinal effects of lowering inflammation on microvascular and macrovascular endothelial function. It was hypothesised that a reduction in disease activity with treatment would improve microvascular and macrovascular endothelial function.

Chapter 3: General Methods

Participants

Participant Details for Studies Presented in Chapter 5 and 6

Ninety-nine rheumatoid arthritis (RA) patients were recruited from the rheumatology outpatient clinics of the Dudley Group of Hospitals NHS Trust, United Kingdom. All patients met the retrospective application of the 1987 revised RA criteria of the American College of Rheumatism [65]. Patients were excluded if they had previously confirmed acute coronary syndrome, established cardiovascular disease (CVD) or serious psychiatric disorder as indicated in their medical notes and/or on questioning during the initial consultation. Thirty-two healthy control participants were recruited from hospital staff and their friends. The exclusion criteria were the same as for the RA patients.

Participant Details for the study presented in Chapter 7

Twenty-nine RA patients who were due to start anti-tumor necrosis factor-alpha (anti-TNF-α) treatment were recruited from the Rheumatology Outpatient Clinics of the Dudley Group of Hospitals NHS Foundation Trust, United Kingdom. These patients were a subset of the ninety-nine RA patients that were recruited for the studies presented in chapters 6 and 7. All RA patients satisfied the National Institute of Clinical Excellence guidelines which state that patients are eligible for anti-TNF-α treatment if they have severe disease and have had an inadequate response to at least two DMARD's (one of which must be methotrexate). All patients met the retrospective application of the 1987 revised RA criteria of the American College of Rheumatism [65]. The exclusion criteria were previously confirmed acute coronary syndrome, established CVD or serious psychiatric disorder. From the 29 patients that were recruited, three patients withdrew from the study due to side-effects from the treatment, while three patients withdrew for personal reasons. Therefore, data were analysed for the 23 remaining patients.

Protocol

Participants reported to the vascular laboratory after a 12 hour overnight fast between 7:00 am and 11:00 am. They were asked to refrain from exercise 24 hours before the session, and from smoking 12 hours before the session. For ethical reasons, drug regimens were not interrupted. The laboratory was kept at a constant temperature of 22°c. All participants underwent a detailed clinical examination which included evaluation of their medical history and hospital records, and assessment of height, weight, body mass index (BMI) and body composition. In addition, demographic information was collected from all the participants by questionnaire. The disease activity score (DAS28) [68] and the Anglicised version of the Stanford Health Assessment Questionnaire (HAQ) [72] were also completed along with the patients global CVD risk scores. Participants were then asked to lie semi-recumbent on an armchair for the remainder of the session. Initially, patients were asked to lie quietly for 20 minutes, during which blood pressure measurements were taken. A blood sample was obtained immediately after this initial rest period. Participants then underwent assessments of arterial stiffness using pulse wave analysis and assessment of microvascular function using Laser Doppler imaging (LDI) with iontophoresis. This was followed by a further ten minutes rest. After this, macrovasculature endothelial-dependent function was assessed using flow-mediated dilatation (FMD) and, following an additional ten minutes of rest, assessment of glyceryltrinitrate-mediated dilatation (GTN).

Anthropometric and Body Composition

Height was measured to the nearest centimetre using a standard height measure (Seca 214 Road Rod, USA). Weight and body composition was assessed using a Tanita BC 418 MA Segmental Body Composition Analyser (Tanita Corporation, Tokyo, Japan). For this assessment the participants stood on pressure-contact footpads on a scale platform while a small unnoticeable electrical current (50 KHz, 800µA) was passed through the body. The principle of bio-impedance analysis is dependent upon the resistance to the electrical current from the

tissues of the body. Lean muscle tissue offers less resistance to the current due to high levels of water and electrolytes, whereas fat mass provides greater resistance to the electrical current. Details of the participants age, height, gender and body type (standard or athletic) were entered in to the analyser, which subsequently produced readings for percentage body fat (%) and body mass index (kg/m^2). The measurements of the Tanita body composition analyser are within +/- 4 percentage points from measurements obtained from a dual energy x-ray absorptiometry scanner.

Disease Activity Score

A modified disease activity score incorporating a 28 joint count (DAS28) was used to assess the patient's level of disease activity [68] (Appendix 1). The DAS28 is the most commonly used disease activity score in the United Kingdom at present [525], and has good validity [526]. The DAS28 takes into account the total number of tender and swollen joints from 28 joints (fingers, wrists, elbows, shoulders, knees). In addition, it utilises a visual analogue scale (VAS) with anchors of 0 (good health) and 100 (poor health) to indicate the patients global health on the morning of the test, as well as the erythrocyte sedimentation rate (ESR) [69]. All the variables are entered into the following equation to derive the DAS28 score:

$$DAS28 = 0.56x\sqrt{(tender\,28)} + 0.28x\sqrt{(swollen28)} + 0.70xin(ESR) + 0.014xVAS$$

With Tender28 is the number of tender joints and Swollen28 is the number of swollen joints [68].

Stanford Health Assessment Questionnaire

The Anglicised version of the 40 item Stanford Health Assessment Questionnaire (HAQ) [72] was used to assess functional disability in the RA patients (see Appendix 2). Patients rated their ability over the past week to carry out 20 activities within eight aspects of daily living (dressing/grooming, rising, eating,

walking, hygiene, reach, grip and errands/tasks) on a four point Likert scale ranging from 'without any difficulty' to 'unable to do'. For each aspect, patients reported whether they received assistance from others or used specific devices (e.g. walking stick, stair-lifts or bath seats). The highest scores from the eight areas were derived and increased by 2 if assistance was needed. These eight scores were then averaged to yield the HAQ score. A high HAQ score indicates greater functional disability. There is good agreement between self reported ratings of HAQ with those reported by spouses and observations by physiotherapists in patients with RA [527].

Global CVD Risk

To calculate global CVD risk, two separate CVD risk algorithms were used. The Framingham Risk Score (FRS) is a CVD risk calculator that incorporates a combination of CVD risk factors to estimate the likelihood of a fatal or non-fatal coronary heart disease (CHD) event (e.g. myocardial infarction) over the next 10-years [528]. In contrast, the Systematic Coronary Risk Evaluation (SCORE) is a CVD risk prediction chart specifically for European populations and unlike FRS, is not limited to just coronary events as it provides the 10 year risk score of any first fatal CVD event (e.g. stroke or ruptured abdominal aneurysm) [529]. Participants were also screened for the presence of the metabolic syndrome. The metabolic syndrome reflects an accumulation of several classical CVD risk factors, and confers a risk that is greater than the sum of its individual components [530]. All of these measures are described in detail below.

Framingham Risk Score

The risk factors used by the FRS are sex, age, total cholesterol level (TC), high density lipoprotein cholesterol (HDL-C) level, systolic blood pressure (SBP), diastolic blood pressure (DBP) as well as the presence of diabetes and smoking status [528]. All details were entered into an online FRS calculator (http://www.mdcalc.com/framingham-cardiac-risk-score) which used a scoring algorithm presented by Wilson and colleagues [528]. The level of CHD risk was

categorised into the following three categories 1) low risk (<10% CHD risk at 10 years), 2) intermediate risk (10-20% CHD risk in 10 years), 3) high risk (>20% CHD risk in 10 years) according to previously established criteria [531]. A high FRS has been shown to associate with coronary artery calcification in RA [532].

Systematic Coronary Risk Evaluation

The SCORE was calculated using validated risk tables. The variables incorporated into the table included age, sex, smoking, SBP, TC and HDL-C levels. Several tables containing the above variables have been designed, allowing SCORE to be calculated specifically for TC levels (TC SCORE) and TC:HDL ratios (TC:HDL SCORE) for both high and low risk populations [529]. In the present study, the high risk table was utilised due to the UK being classified as a high risk country for CVD. Participants can be classified at high risk of CVD if their 10 year risk of CVD mortality is ≥ 5% [529]. At present, there is no data on the validity of this utility in RA patients.

Metabolic Syndrome

Metabolic syndrome was classified according to World Health Organisation (WHO) criteria [533]. The mandatory criterion for diagnosis of metabolic syndrome is that the participants must have an impaired glucose tolerance test, or diabetes mellitus or insulin resistance. In addition, at least two out of five of the following criteria must also be present: obesity (BMI > 30 kg/m^2), hypertension (≥140/90 mmHg), low HDL cholesterol (men: < 0.9 mmol/l, women: < 1.0 mmol/l), high TG (≥ 1.7 mmol/l) and high albumin/creatinine ratio (≥ 30 mg/l).

Blood Pressure

Four blood pressure measurements of SBP and DBP were taken during the initial rest period at minutes 14, 16, 18 and 20 using an automatic blood pressure monitor (Datascope Accutor, USA). The four blood pressure measurements

were averaged to give a single resting value. Pulse rate was also obtained from the blood pressure monitor.

Endothelial Function

Pulse Wave Analysis

Non-invasive assessment of radial artery waveforms (pulse wave analysis) was recorded using an applanation tonometer (SphygmoCor Px Pulse Wave Analysis, ScanMed Medical Instruments, UK). After the recording of brachial blood pressure, the right radial artery was palpated to identify a suitable pulse. The applanation tonometer was positioned over the artery with enough pressure to flatten (but not occlude) the patient's radial artery. The applanation tonometer records the first and second systolic peaks and then displays the augmentation index (AIx). The AIx is calculated as the difference between the first and second systolic peak and is expressed as a percentage of the pulse pressure [400]. The pressure waveforms in the radial artery were recorded for an 11 second period. The software integrated in the analyser displayed an operator index which reflects the quality of the recorded waveform. If the operator index was low (< 65), another reading was taken. Three readings with an operator index > 65 were used for analysis. The average AIx of these three readings was calculated.

Laser Doppler Imaging with Iontophoresis

Endothelial function of the microvasculature was assessed non-invasively using Laser Doppler Imaging (Moor LDI 2 SIM, Moor Instruments Ltd, UK) by a single observer (AS). The participants remained in a semi-recumbent position in the armchair and their right arm was comfortably strapped to a firm pillow to prevent movement during the assessment. Two perspex chambers (internal diameter: 22mm) with an internal platinum electrode (ION 6, Moor Instruments Ltd, UK) were connected to an iontophoresis controller (MIC-Ie, Moor Instruments Ltd, UK). The chambers were attached to the volar aspect of the participant's right forearm using double sided adhesive pads. One chamber was connected to the

anodal connection of the iontophoresis controller and contained a 2.5ml dose of 1% acetylcholine (ACh, endothelial-dependent) (Sigma Chemical Co, USA). The second chamber was connected to the cathodal connection and contained a 2.5ml dose of 1% sodium-nitroprusside (SNP, endothelial-independent) (Sigma Chemical Co, USA). The vehicle for drug delivery was 0.5% sodium chloride. The chambers were covered by 32mm coverslips to prevent leakage of fluid. After a baseline scan, ten scans were recorded during iontophoresis of the vasoactive agents using a 30µA current, followed by two scans during recovery (i.e. when iontophoresis was stopped). The total duration of the assessment was 8 minutes 20 seconds. Measurements of perfusion were carried out offline by a single observer (AS) who was blinded to the identification of the participants.

The principles of LDI involve the reflection of a laser beam light from moving red blood cells (Doppler shift). The reflected laser light is then detected by photodetectors in the scanner head which converts the signal into arbitrary perfusion units (PU) and displays a colour coded image of vessel perfusion [371]. The distance of the laser beam from the site of iontophoresis was automatically measured by the Laser Doppler Imager. The LDI image analysis software was used to mark a region of interest within the outer diameter of the two chambers. The median perfusion units for each of the scans were used to identify the level of perfusion of each scan. Further analysis was conducted to identify the percentage increase in perfusion during iontophoresis relative to the baseline perfusion for ACh and SNP separately. For this, the following formula was used:

$$PercentageIncreasePerfusion = \left(\frac{PeakPerfusion - BaselinePerfusion}{BaselinePerfusion} \right) \times 100$$

Flow-Mediated Dilatation and Glyceryl-Trinitrate Medicated Dilatation
Assessment of macrovascular endothelial function was assessed by imaging the left brachial artery by an experienced ultrasonographer (AS) using Doppler

ultrasound equipped with a 5 MHz linear array transducer (Acuson Antares ultrasound system and SieClear, BW SieScape Imaging software, Siemens PLC). The assessment was conducted according to previously established guidelines [534]. Specifically, the brachial artery was scanned longitudinally 2-10 cm above the antecubital fossa. The depth and gain features were set to optimise the image and once a clear image had been obtained the ultrasound transducer was locked into place using a stereotactic clamp. An external computer was connected to the ultrasound machine so that all images could be recorded using vessel image analysis software (VIA) at 25 frames per second. To allow the software to automatically record vessel diameter, a predetermined region of interest was marked to detect and track the anterior and posterior walls [535]. The protocol for flow-mediation dilatation (FMD, endothelial-dependent) involved a 2 minute baseline scan of the artery, after which a cuff placed around the wrist was inflated to 300 mmHg for 5 minutes. The vessel was continuously scanned throughout the occlusion period. At 5 minutes the cuff was released to induce reactive hyperaemia and the subsequent dilatation of the vessel was imaged for a further 2 minutes. Then following ten minutes of rest, endothelial-independent responses were examined by asking the participant to place a 500 microgram GTN tablet (Alpharma, UK) under the tongue for five minutes while the vessel was imaged continuously. Analysis of the brachial artery diameters were performed offline by the same ultrasonographer who was blinded to the identification of the participant. The data were digitised at approximately 20 Hz, and were time stamped to the nearest second. These data were collapsed into one-second epochs and exported to a digital signal analysis package (Spike 2 v6, CED) where they were filtered with a 3 second moving average filter. The baseline diameter was established from the 120 seconds of data prior to the cuff-inflation. The baseline region was visually inspected and artefacts were excluded. The remaining baseline regions were averaged to produce the baseline diameter. For the FMD analysis, the post cuff-deflation region was automatically scanned for peak dilation and this peak was marked for visual inspection. If the peak had been misidentified, the operator had the opportunity

92

to select a more confined region within which the peak could then be identified. The peak value was then recorded as peak RH diameter. For the GTN data, an identical procedure was adopted to that used with FMD, except that the search for peak dilation was made in the region following the five minutes of drug administration. The equation used to calculate both FMD and GTN was as follows:

$$\%Increase = \left(\frac{PeakDiameter - BaselineDiameter}{BaselineDiameter} \right) \times 100$$

Blood Sample Analysis

Blood sampling

Blood was collected from the participant's antecubital vein using a 23G butterfly needle (Greiner Bio One GMBH, Austria). Two 4ml Z Serumsep Clot Activator Vacuette® tubes were collected for the assessment of C-reactive protein (CRP), TC, triglycerides (TG) and HDL-C. Blood for glucose analysis was collected in 2ml FE Sodium Flouride/K_3 ethylenediaminetetraacetic acid (EDTA) tubes. One K2EDTA Vacuette® tube was collected to measure erythrocyte sedimentation rate (ESR). A blood sample for measurement of insulin was collected in one 4ml Z Serumsep Clot Activator Vacuette® tube. One 9NC Coagulation Sodium Citrate 3.2% Vacuette® tubes was collected to measure fibrinogen. All samples were analysed immediately (see below for methods).

Laboratory Methods

A Vitros® 5.1 FS Chemistry system was used to measure TC, TG, HDL-C and glucose. The system incorporates the use of a slide which is specific to each biochemical test. The slide consists of multiple layers of analytical elements. A small amount of the participant's serum was placed on the slide and was then evenly spread so as to penetrate the underlying layers. The serum sample was

then entered into a reagent layer containing specialised dye. The dye attaches to the chemical to be measured from the serum. Reflectance spectrophotometry was used to measure the colour-complex which was formed when the chemical attaches to the dye. The amount of chemical bound dye is proportional to the concentration of the chemical which is being measured.

Measurement of CRP was also carried out by the Vitros$^®$ 5.1 FS Chemistry system using a heterogeneous sandwich immunoassay format. The format uses calcium as a capture agent, and a derivative of phosphorylchoine (PC) is covalently bound to polystyrene polymer beads. A signal was generated using monoclonal anti-CRP antibody labelled with horseradish peroxidase. The participant's serum was placed on the slide and was spread to penetrate the underlying layers of the slide. The CRP then binds to the PC-linked capture beads and the monoclonal anti-CRP antibody forming an insoluble sandwich complex. The slide was then washed using specialised fluid. The fluid also produces hydrogen peroxide needed for the enzyme-mediated oxidation of the lueco dye. The reflection density of the dye was measured and was proportional to the CRP concentration of the sample.

ESR was measured using the Starrsed Compact (Mechatronics BV, Netherlands). A total of 10ml of undiluted blood, anti-coagulated with EDTA is inserted in a vertical tube. The sedimentation (in millimetres) of the red blood cells at one hour gives the value of ESR.

Insulin was estimated from serum stored at -20°C. The Immunolite 2500 insulin was used on the Immulite 2500 analyser (Diagnostic Products Corporation, USA). A solid phase two-site chemi-luminescence immunometric assay was used to detect insulin. Insulin sensitivity was assessed by calculating the Homeostasis Model Assessment Insulin Resistance (HOMA IR) and Quantitative Insulin Sensitivity Check Index (QUICKI), as previously described [536,537].

Fibrinogen was measured by photo-optical clot detection based on the Clauss quantitative fibrinogen method. The measurement was performed in an automated coagulation analyser (ACL Futura Plus, Instrumentation Laboratory, UK). In the Clauss method, thrombin is added at 50-100 µ/mL to the plasma and the time it takes for clot formation (reaction time) is recorded. The rate of this clot formation is a function of fibrinogen concentration. Using a comparison with a standard curve, the reaction time is converted to give a value of fibrinogen expressed in mg/dL.

Rheumatoid Factor (RF) was determined using the manual particle agglutination method (MAST diagnostics, Merseyside, UK). The test involves an immunological reaction between RF in the serum and matching human IgG antibodies which are coated onto polysterene latex particles. Agglutination is observed when serum containing RF is mixed with the latex containing the human IgG antibodies. This process allows for the detection of serum RF and positive tests are quantified using an Enzyme-Linked Immuno-Sorbent Assay (ELISA). This involves the addition of diluted serum to wells which are coated in purified antigen allowing formation of antigen-antibody complexes. The wells are incubated at room temperature and unbound material is washed away. To immobilise the antibodies, horseradish peroxidase conjugated anti-IgG monoclonal antibody is added. The wells are then incubated again and washed as before. Following this, cycle tetra-methyl benzidine substrate is added to each well and change of the colour to dark blue confirms the presence of an antigen-antibody complex. Adding a stop solution turns the mixture yellow, the colour intensity measured by photospectrometry is proportional to the concentration of the antibodies in the original sample.

Data Handling and Statistical Analysis

All data entry and statistical analysis was performed using SPSS 15 (SPSS Inc, Chicago, Illinois). Data was checked for normal distribution using the

Kolmogorov-Smirnov test, and non-normally distributed variables were log transformed.

Chapter 4

Analysis of all physiological measurements was performed using a series of 2 Session (Session 1, Session 2) analyses of variance (ANOVA). For endothelial function, Pearson correlations were conducted to examine relationships between session 1 and session 2 endothelial function. In addition, coefficients of variation (CofV) were calculated for baseline and post-stimulus changes in perfusion (ACh and SNP) and vessel diameter (FMD% and GTN%) for session 1 and session 2 (temporal reliability). To calculate CofV the following equation was applied:

$$CofV = \left(\frac{SDofDifferenceBetweenTwoScores}{MeanofTwoScores} \right) \times 100$$

Chapter 5

Differences between patients and healthy controls were tested using univariate analysis of co-variance (ANCOVA) for continuous variables and Chi Squared test for discontinuous variables. The ANCOVA was preferred over the t-test as it allows factors that differ between groups to be co-varied in the analysis. As the healthy control participants were significantly younger than the RA patients all analysis presented in this chapter 5 were corrected for age using ANCOVA. Pearson's correlations were used to assess the relationships between microvascular and macrovascular endothelial-dependent function.

Chapter 6

To assess independent determinants of vascular function, linear regression (continuous variables) and logistic regression (discontinuous variables) were used. Inflammatory markers, global CVD risk and CVD risk factors were entered as independent variables with each measure of vascular function entered separately as the dependent variable. In addition, differences in general

characteristics and endothelial function between RA patients with low, moderate or high DAS28 were analysed using univariate ANOVA. For this analysis RA patients were categorised according to their DAS28 score into low disease activity (DAS28 <3.2), or high-disease activity (>5.1) according to previously established criteria [538]. RA patients were also split according to their endothelial function scores into 3 equal groups: low endothelial function, moderate endothelial function and good endothelial function. Univariate ANOVA was used to assess differences in general and disease-related characteristics between these three groups. Chi squared test was used to examine differences in medication use between the categories of endothelial function. Finally, the beta coefficients of all significant associations were compared to the corresponding beta coefficient in healthy controls. The beta coefficients were transformed into z scores for this analysis (described in greater detail in Chapter 6).

Chapter 7

Changes in each parameter of endothelial function, CVD risk and disease-related measurements were assessed using 3 X time (pre-treatment baseline, 2 weeks, and 12 weeks) repeated measures Analysis of Variance (ANOVA). Fisher LSD post-hoc tests were used for pair-wise comparisons where appropriate. Pearson correlations were used to examine whether changes in endothelial function related to changes in disease-related inflammation. The change in endothelial function and disease-related parameters at 2 and 12 weeks was calculated by subtracting the pre-treatment baseline values from the values obtained at 2 and 12 weeks.

Chapter 4: Examining the Reliability of Microvascular and Macrovascular Endothelial Function Measurement

Introduction

The endothelium is the innermost lining of the vasculature and is involved in the maintenance of vascular homeostasis via the regulation of a multitude of vasoactive processes. Disruption to these processes may predispose the vessel to atherosclerosis and increase the risk for cardiovascular disease (CVD) [539]. Peripheral endothelial function is a good indicator of early abnormalities in the vascular wall [202]. Further, measures of peripheral endothelial function have been shown to reflect coronary endothelial function [351-353], and as such are regarded as good predictors of cardiovascular disease [356,357,540,541]. This is perhaps hardly surprising given that atherosclerosis is now broadly appreciated to be a systemic disorder [193]. Peripheral vascular assessment typically quantifies the vasodilatory response of the vessel to a specific stimulus, with an attenuation of the dilatory response indicative of endothelial dysfunction [542]. Peripheral endothelial function can be measured in different vascular beds; Laser Doppler imaging (LDI) with iontophoresis of vasodilator agonists is used to assess microvascular endothelial function [371], whereas flow-mediated dilatation (FMD) in the brachial artery is used to assess macrovascular endothelial function [378].

The use of LDI with iontophoresis of vasodilator agonists has increased in recent years, largely due to its non-invasive nature and ease of use. Iontophoresis propels charged vasoactive agents such as Acetylcholine (ACh) and Sodium Nitroprusside (SNP), which are used to assess endothelium-dependent and endothelium-independent function respectively, into the skin using a weak electrical current [361]. Once through the skin, ACh binds to endothelial cell muscarinic receptors which release NO to cause vasodilatation. SNP directly activates smooth muscle cell receptors to allow for maximum

vasodilatation of the vessel [362]. Non-vasoactive substances such as sodium chloride (NaCl) or deionised water are used as vehicles for transporting the agents into the skin microvessels [367,543]. The LDI then simultaneously scans the iontophoresed areas to monitor changes in microvascular perfusion. Endothelial function is typically quantified as the percentage increase in perfusion in response to iontophoresis, relative to baseline perfusion. The advantage of LDI is that it can simultaneously scan multiple points in a given area and can therefore account for cellular movement artefacts and spatial differences of skin blood flow, both of which can affect the perfusion of the vessel [544,545]. Biological factors and behavioural factors have been reported to affect the reliability and repeatability of the technique. For example, circadian variation and smoking have been shown to influence microvascular function [369,546]. Controlling for such factors reduces the variability of the measurement and improves accuracy. It is important for laboratories to assure the stability of their assessments and to demonstrate that adequate standards of reproducibility for their specific protocols are met [371].

In the macrovessels, flow-mediated dilatation (FMD) and glyceryl-trinitrate mediated dilatation (GTN) are performed to assess endothelium-dependent and endothelium-independent function respectively [378]. FMD is typically carried out in the brachial artery. A cuff is used to occlude arterial blood flow for 5 minutes; release of this cuff causes a sudden increase in blood flow (reactive hyperaemia) through the brachial artery resulting in dilatation of the vessel. FMD is expressed as the percentage increase in post-cuff release vessel diameter relative to the baseline diameter. The baseline and post-cuff release diameter are quantified by ultrasound imaging of the vessel with subsequent assessments of the vessel diameter performed manually [378] or using automated edge detection software [535,547]. The use of GTN allows for assessment of the maximum vasodilator tone via GTN's action on smooth muscle cells [232]. Similar to FMD, the GTN response is quantified by the percentage increase in brachial artery diameter in response to GTN relative to baseline values.

FMD is a highly sensitive measure of endothelial function, as small changes in vascular diameter can elicit large FMD responses. For example, typical FMD values for healthy participants range from 5-10% [251], which corresponds to a 0.25-0.5mm change in arterial diameter for an artery with a diameter of 5mm. Given such small changes to the arterial diameter, careful attention must be paid to technical and biological factors that may influence the measurement. Recent technical advances include the use of automated wall tracking software which detect and calculate arterial diameters in real-time. This greatly reduces the variability found with manually measuring arterial diameters [535,547], which can be anywhere between 1.8-50% [548,549]. A study by Hijmering et al. 2001 [550] showed that by using automated wall-tracking software the reproducibility of baseline brachial artery diameter was excellent (coefficient of variation (CofV): 1.1%), although reproducibility of FMD was less impressive (CofV: 13.9%) The researchers suggested that the reproducibility of the baseline diameter was indicative of adequate control of technical factors, and that biological and behavioural factors may contribute to the larger variation observed with the FMD response. Indeed, FMD can be affected by a variety of biologic and behavioural factors such as sympathetic activation [551], sleep deprivation [385], caffeine consumption [387], smoking [388], antioxidant therapy [389] and time of day [391] (Table 1). Accordingly, it is important to control for these factors [380].

Table 1. Factors which can affect endothelial function

Authors	Factor	Vascular Bed
Elherik et al. (2002) [369]	Circadian variation	Microvasculature
Pellaton et al. (2002) [546]	Cigarette smoking	Microvasculature
Hijmering et al. (2002) [551]	Sympathetic activation	Macrovasculature
Takase et al. (2004) [385]	Sleep deprivation	Macrovasculature
Papamichael et al. (2005) [387]	Caffeine consumption	Macrovasculature
Lekakis et al. (1997) [388]	Cigarette smoking	Macrovasculature

Engler *et al.* (2003) [389]	Antioxidant therapy	Macrovasculature
Etsdua *et al.* (1999) [391]	Time of day	Macrovasculature

Although guidelines for both LDI and FMD have been established [371,380], normative data are yet to be published; this is mainly due to differences in methodology between studies [552] . At present, the guidelines stipulate that each individual vascular laboratory conduct their own test-retest reliability studies which assess stability of the methods over time (temporal reliability) and between the different assessors of the study (inter-assessor reliability). Thus, the aim of the present study was to assess the reliability of LDI and FMD assessments on two separate occasions as well as the reliability of the assessments between two assessors in a group of healthy participants who underwent measurement in conditions that carefully controlled for technical and biological factors.

Methods

Participants

Twelve healthy adults (age: 31.2 ± 6.2 years, body mass index: 24.1 ± 0.3 kg/m^2, 7 females) were recruited from the staff of the Rheumatology Department of the Dudley Group of Hospitals NHS Trust, United Kingdom. Participants had no known diseases and were not taking any vasoactive medication. They were asked to refrain from exercise for 24 hours and smoking for 12 hours before each session.

Study Protocol

All participants attended the vascular laboratory on two separate sessions after a 12 hour fast between 7:00am and 11:00am. The same observer conducted both sessions, with identical protocols. The laboratory was kept at 22°C. Upon arrival at the laboratory all procedures were explained. Following this, height and weight were measured. The participants were then asked to assume a semi-

recumbent position on a bed where they remained for the rest of the session. They initially rested for ten minutes, during which blood pressure measurements were initiated at minutes 4, 6, 8 and 10. At the end of the rest period a blood sample was taken (data not reported). Subsequently, endothelial function was assessed in the microvasculature using LDI with iontophoresis, after which there was another ten minutes rest. Subsequently, macrovascular endothelial function was measured using FMD and following a further 10 minutes rest; assessment of glyceryltrinitrate-mediated dilatation (GTN) was undertaken.

Anthropometric Assessment

Height was measured to the nearest 0.5cm using a standard height measure (Seca 214 Road Rod). Weight was assessed using a Tanita BC 418 MA Segmental Body Composition Analyser (Tanita Corporation, Tokyo, Japan). BMI was calculated as body weight divided by the square of the height (kg/m^2).

Blood Pressure

Systolic and diastolic blood pressure and pulse rate was recorded using a standard cuff placement over the brachial artery and a semi-automatic blood pressure monitor (Datascope Accutor, USA).

Assessment of Vascular Function

Microvascular endothelial function was assessed using iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) along with Laser Doppler Imaging. Flow-mediated dilatation (FMD) and glyceryl trinitrate-mediated dilatation (GTN) were used to assess macrovascular endothelial function. These assessments are described in greater detail in the General Methods section (see Chapter 3).

Statistical Analyses

The blood pressure and pulse rate data were averaged separately to yield a mean value. Subsequently, a series of 2 Session (Session 1, Session 2)

analyses of variance (ANOVA) was conducted on all physiological data. For the vascular assessments only, correlations were computed between session 1 and session 2. Coefficients of variation (CofV) were calculated for baseline and post-stimulus changes in perfusion (ACh and SNP) and vessel diameter (FMD% and GTN%) for session 1 and session 2 (temporal reliability). To calculate CofV the following equation was applied:

$$CofV = \left(\frac{SDofDifferenceBetweenTwoScores}{MeanofTwoScores} \right) \times 100$$

In addition, to assess the agreement between the vascular assessments, Bland and Altman plots were constructed [553].

Results

Participant Characteristics

Participant characteristics are displayed in Table 2. None of the variables varied over time ($p > .05$).

Table 2. Participant characteristics in each session

Participant Characteristics	Session 1	Session 2
Age (years)	31.2 ± 6.2	---------
Height (cm)	171 ± 12	---------
Weight (kg)	70.3 ± 8.9	70.2 ± 9.0
Body Mass Index (kg/m^2)	24.2 ± 3.0	24.3 ± 3.3
Systolic Blood Pressure (mmHg)	118 ± 9	118 ± 8
Diastolic Blood Pressure (mmHg)	71 ± 9	70 ± 9
Heart Rate (bpm)	67 ± 10	64 ± 8

Results are expressed as mean ± SD

Vascular Assessments

Microvascular Endothelial Function

Baseline perfusion for ACh and SNP is displayed Table 3. As can be seen, 2 Session ANOVAs revealed that there were no Session effects for baseline ACh and SNP perfusion. Correlational analyses showed strong positive associations between the two sessions in the baseline ACh and SNP perfusion for the assessor, r (10) = .97, p < .001, and r (10) = .99, p < .001 respectively,

Table 3 also shows the percentage increase in perfusion in response to ACh and SNP. For ACh% and SNP% no Session effects were evident. Correlational analyses yielded a strong positive association between the two sessions for percentage increase in response to ACh and SNP, r (10) = .99, p < .001, and r (10) = .99, p < .001 respectively.

Table 3: Baseline perfusion and percentage increase in perfusion in response to ACh and SNP

	Session 1	Session 2	Session Effect
Endothelial Function			F (1, 11) =
Baseline ACh Perfusion (PU)	40 ± 12	41 ± 9	0.19
Baseline SNP Perfusion (PU)	41 ± 19	39 ± 19	4.22
ACh (%)	584 ± 262	596 ± 266	2.89
SNP (%)	482 ± 240	490 ± 230	1.04

Results are expressed as mean ± SD

Macrovascular Endothelial Function

Baseline FMD and GTN diameter are reported in Table 4. ANOVA showed no Session effects for baseline FMD or GTN diameter. Correlational analyses showed a strong positive association between the two sessions in the baseline FMD and GTN diameters r (10) = .99, p < .001 and r (10) = .96, p < .001, respectively.

Percentage increase in the FMD and GTN sessions is displayed in Table 4. No Session effects were found for FMD or GTN percentage increases. Correlational analysis revealed strong associations between sessions in the FMD% and GTN% r (10) = .99, p < .001 and, r (10) = .95, p < .001, respectively.

Table 4: Baseline diameter and percentage increase in FMD and GTN diameters

	Session 1	Session 2	Session Effect
Endothelial Function			F (1,11) =
Baseline FMD Diameter (mm)	3.9 ± 0.7	3.8 ± 0.6	0.80
Baseline GTN Diameter (mm)	3.8 ± 0.6	3.7 ± 0.6	4.38
FMD (%)	6.1 ± 5.2	6.0 ± 4.8	0.03
GTN (%)	21.3 ± 5.8	21.0 ± 5.4	0.39

Results are expressed as mean ± SD

Co-efficient of Variation

The intra-assessor CofV for baseline perfusion of the ACh and SNP assessments were 4.3% and 3.9% respectively. For percentage change in perfusion in response to ACh and SNP, the intra-assessor CofV was 6.5% and 5.9% respectively.

For baseline diameters in the FMD and GTN assessments, the intra-assessor CofV was 2.5% and 5.0% respectively. For FMD% and GTN%, the intra-assessor CofV was 10.7 and 8.11% respectively.

Bland Altman Plots

For the microvascular assessments, Bland Altman Plots did not show any systematic bias in the response to ACh and SNP, and most values were within 2 standard deviations from the average (Figure 1a & b).

a)
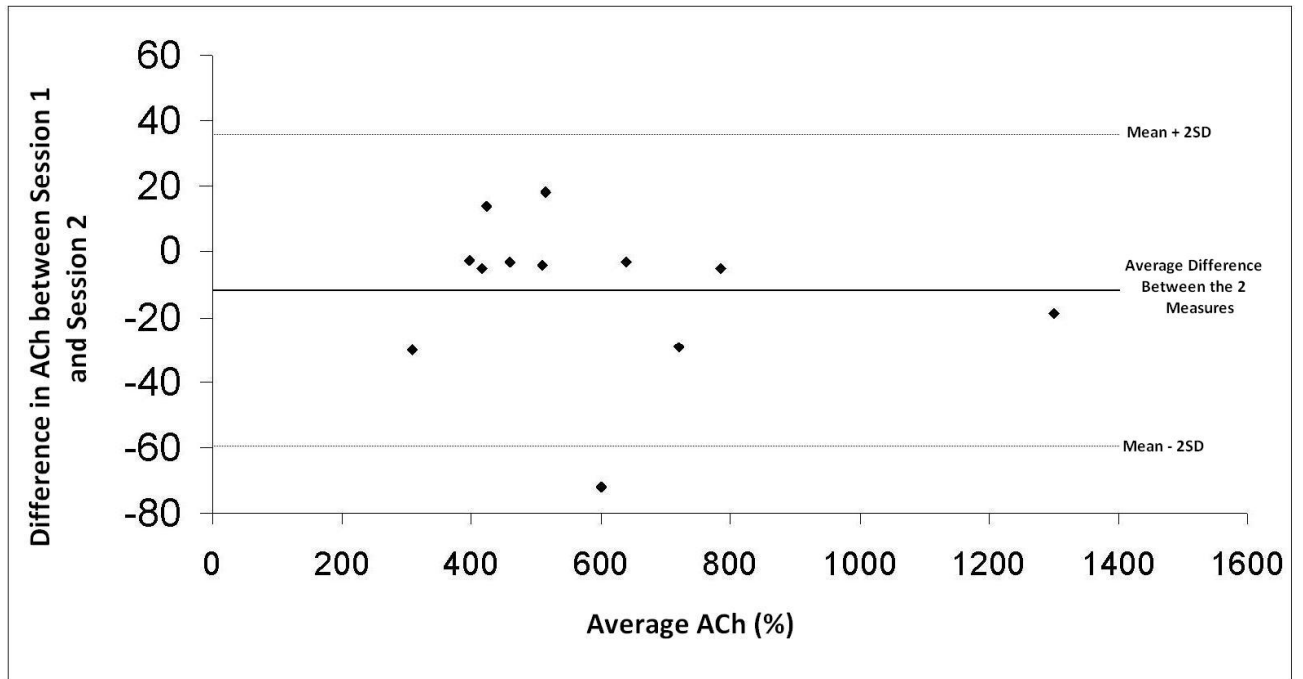
b)
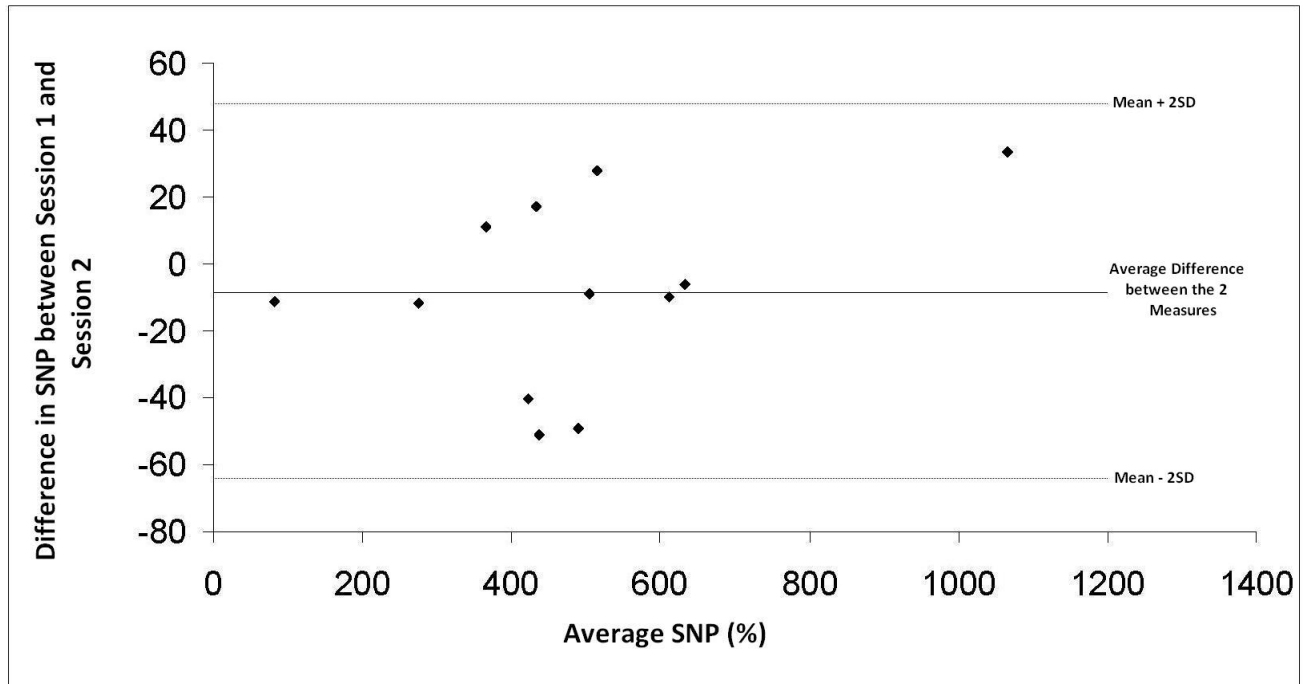
Figure 1. Bland Altman scores for ACh (a) and SNP (b) responses for session 1 and session 2.

There was no systematic bias in the macrovascular assessments, as all values were within 2 standard deviations from the average (Figure 2a & b).

a)
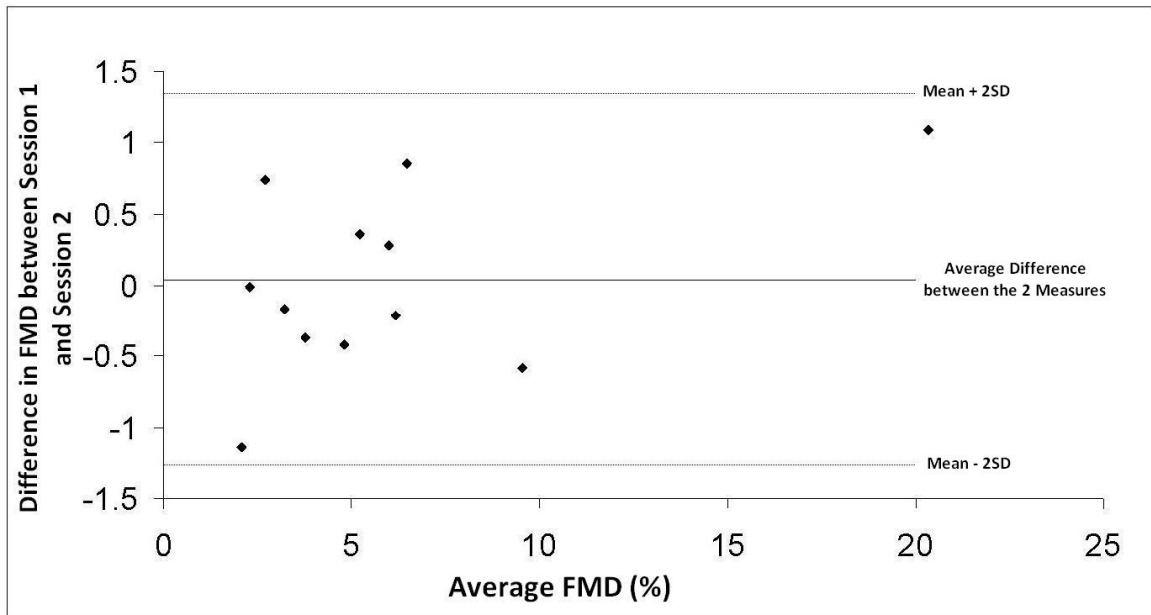
b)
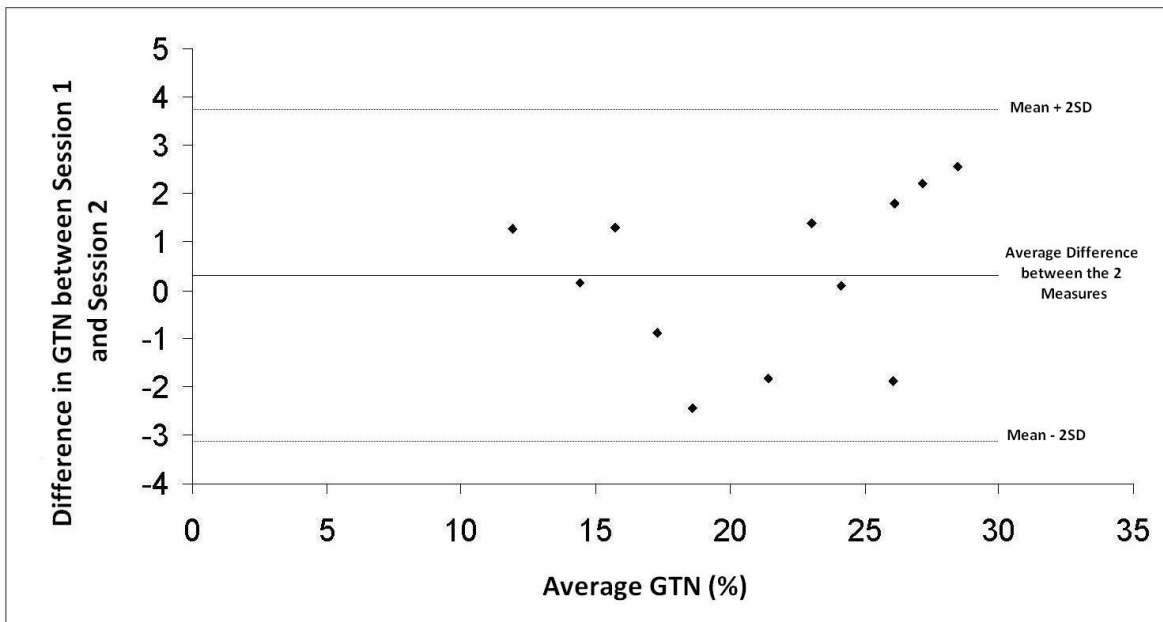
Figure 2. Bland Altman Scores for FMD (c) and GTN (d) responses for session 1 and session 2.

Discussion

Assessments of microvascular endothelial function using LDI with iontophoresis, and assessments of macrovascular endothelial function using FMD with Doppler Ultrasound demonstrated good intra-assessor reproducibility. The day to day variation in LDI seen in the present study was comparable to other studies that had adequately controlled for factors known to affect vascular function [552,554-556]. The findings of our study provide further support for the Standardisation Group of the European Society of Contact Dermatitis guidelines on assessment of microvascular blood flow using LDI [557], which stipulate that good intra-observer reliability can be achieved when biological and environmental factors are adequately controlled. Further, careful set up and management of equipment and use of validated protocols further reduces variability [367,371].

FMD is also susceptible to environmental and biological variations [380], and in contrast to LDI, is highly user-dependent. Reported CofV for FMD range from 1 – 84% [558], although not all studies calculate CofV in a uniform and consistent manner. For example, some studies report the CofV as the mean difference between measurements, which results in lower values [535,559]. However, even when the technique is performed by competent ultrasonographers with external factors controlled, there remains a high CofV. For example, the study by De Roos et al. [548] reported an intra-assessor CofV of 50.3%. Similarly, Tyldum et al. [560] found an intra-assessor CofV of 29.1%. In both of these studies, the analyses of the brachial artery diameters were carried out by manually identifying the vascular wall. Such analysis has been suggested to increase the variability seen with this technique, due to imaging artefacts such as false borders, noise from the ultrasound signal, and distorted vessels which all compromise the accuracy of the readings [547]. Recent developments in continuous automated edge-detection software have greatly improved the detection of the vascular wall boundaries [535,547]. In the present study, Vascular Image Analysis (VIA) software was used to measure the brachial artery diameter. The VIA software was developed using artificial neural networks

108

which '*learnt*' how to detect and differentiate between the vascular wall and non-wall tissues from a series of ultrasound scans of the carotid artery. The neural networks were able to correctly distinguish the vascular wall in 97% of the scans [561]. Additionally, the software takes continuous measurements to account for the cardiac cycle, allowing a more complete profile of the vessel diameter. In the present study, we maintained adequate control of external factors, and used the VIA software. This resulted in low CofV similar in magnitude to those observed in other studies that have used automated edge-detection software [550,562]. The actual contribution the VIA software makes to reproducibility does need further investigation in studies which incorporate repeated measurements of FMD, and compare the analysis of the diameters measured both manually and with the VIA software.

In conclusion, we found high reproducibility for measurements of endothelial function in both the microvasculature and macrovasculature. These techniques can therefore be usefully applied in clinical populations for diagnostic purposes and to predict risk for CVD [356,357,540,541].

Chapter 5: Assessing Microvascular and Macrovascular Endothelial Function in Patients with Rheumatoid Arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of the joints with predominant symptoms of pain, swelling and stiffness [1]. Patients with RA also have a number of extra-articular manifestations such as vasculitis and Felty's syndrome, which are associated with an increased risk of mortality [59]. One of the most common extra-articular manifestations is cardiovascular disease (CVD), accounting for up to 50% of all deaths in RA [121,428]. Interestingly, cardiovascular co-morbidity is evident in both long and short disease durations [119,563]. At present, the exact mechanism for CVD in RA is not known, although a number of factors have been postulated, including RA-related inflammation [439] and the presence of traditional risk factors such as hypertension, dyslipidemia and insulin resistance [149,167,440].

One of the earliest manifestations of CVD is endothelial dysfunction (ED), which occurs when the endothelial cells are exposed to injurious stimuli such as reactive oxygen species or lipid deposits [202,539]. Once exposed to these stimuli, levels of the atheroprotective molecule nitric oxide (NO) are reduced, leading to a reduction in endothelial cell function [539]. Endothelial function can be assessed in both the microvasculature and macrovasculature of the peripheral circulation [382], which are reflective of coronary endothelial function [351-353,379]. Peripheral assessments of endothelial function are thought to be good predictors of long-term cardiovascular events in individuals with atherosclerosis [354,356,404,564], peripheral vascular disease [540] and in healthy older participants [565]. ED has also been associated with a number of CVD risk factors such as hypertension, dyslipidemia, insulin resistance and obesity [359]. In RA patients, studies have shown poorer endothelial function when compared with controls of a similar age and sex in both the microvasculature and macrovasculature [431,436,455,459], and this has largely been attributed to high

systemic levels of inflammation [439] However, the majority of the studies have assessed endothelial function in a single vascular bed, with almost no studies investigating endothelial function in different vascular beds in the same participant.

Studies that have assessed associations between microvascular and macrovascular endothelial-dependent function in healthy individuals have reported mixed findings, with some reporting an association between microvascular and macrovascular endothelial-dependent function [566,567], but others reporting no association [568,569]. For example, improvement in microvascular endothelial-dependent function was unrelated to improvements in macrovascular endothelial-dependent function after an exercise intervention in healthy participants and participants with CVD [570]. Collectively, these findings do not provide conclusive evidence that the endothelial function of one vascular bed is associated with function in another. Further, there is evidence which suggests that endothelial cells in different segments of the vasculature respond differently to a given stimulus, and that the process of atherosclerosis is not the same in different vascular beds [571]. In diabetes, for example, it is known that vascular disease coexists in different sized vessels [572]. Initially, though, atherosclerotic changes occur primarily in the microvasculature [573], which may eventually contribute to the pathogenesis of macrovascular disease [574].

As mentioned above, the majority of studies that have examined endothelial function in RA patients have restricted their assessments to a single vascular bed, focusing mainly on the macrovasculature. To our knowledge, only one study has examined microvascular and macrovascular endothelial function at the same time in patients with RA [451]. Although endothelial function was reported to be impaired in both vascular beds, there was no association between the two vascular beds. Interestingly, 50% of RA patients with evidence of myocardial perfusion defects in response to a pharmacological challenge showed angiographically "pristine" coronary arteries, suggesting that their ischaemia was

due to microvascular dysfunction rather than overt epicardial coronary artery disease [575]. Therefore, the inter-relationship between microvascular and macrovascular endothelial function warrants further investigation

The first aim of the present study was to compare microvascular and macrovascular endothelial-dependent function between RA patients and healthy control participants. Due to the characteristically high levels of systemic inflammation in RA it was hypothesised that RA patients would have worse endothelial function in the microvasculature and the macrovasculature when compared to healthy control participants. The second aim was to examine relationships between microvascular and macrovascular endothelial-dependent function in RA, as only a few studies have previously examined such associations in RA, and *in vitro* research suggests that these two vascular beds are distinct from each other. It was hypothesised that these two vascular beds would not be associated with each other.

Methods

Participants

Ninety-nine patients with RA were recruited from the Rheumatology outpatient clinics of the Dudley Group of Hospitals NHS Foundation Trust, United Kingdom. Thirty-two healthy control participants were recruited from among the hospital staff. The participants are described in greater detail in the General Methods Chapter (Chapter 3). The study received local Research Ethics Committee approval and all participants gave their written informed consent according to the Declaration of Helsinki.

Study Protocol

Participants reported to a temperature controlled vascular laboratory (22°C) after a 12 hour overnight fast. All participants underwent a detailed clinical examination and demographic information was collected from all the participants

by questionnaire. The disease activity score (DAS28) [68] and the Anglicised version of the Stanford Health Assessment Questionnaire (HAQ) [72] were also calculated. Patients global CVD risk scores were measured using the Framingham Risk Score (FRS) and the Systematic Coronary Risk Evaluation for total cholesterol (TC SCORE) and total cholesterol high-density lipoprotein ratio (TC:HDL SCORE) [528,529]. Following this, the participants underwent assessments of arterial stiffness using pulse wave analysis and assessment of microvascular function using Laser Doppler imaging with iontophoresis, and assessment of macrovascular endothelial function using flow-mediated dilatation (FMD) and GTN-mediated dilatation (GTN). All the above assessments are described in greater detail in the General Methods chapter (Chapter 3).

Statistical analysis

Statistical analysis was performed using SPSS15 (SPSS Inc, Chicago, Illinois). Variables were tested for normality by the Kolmogorov-Smirnov test. Means and standard deviations (SD) were calculated for normally distributed continuous variables and proportions for categorical variables. Log transformation was performed for positively skewed variables as appropriate. Differences between patients and healthy controls were tested using univariate analysis of co-variance (ANCOVA) with age as the covariate for continuous variables and Chi Squared test for discontinuous variables. Pearson's correlations were used to assess the relationships between microvascular and macrovascular endothelial-dependent function.

 Results

Due to some loss of data, AIx was obtained for 11 healthy controls only. In addition, due to occasional problems with phlebotomy blood could not be obtained for every participant. Not all participants provided full demographic details in the questionnaire. The number of data points available for each parameter is displayed in all the tables that appear below.

Participant Characteristics and General Demographics

The general characteristics and demographic data are presented in Table 1

Univariate ANOVA showed that age, body fat (p = .005, η^2 = .062) and resting

SBP (p = .001, η^2 = .089) were greater in RA patients when compared to healthy

controls, however, when correcting for age, these differences were no longer

significant (Table 1). None of the healthy control participants were on any

medications. All RA patients and healthy control participants were British

Caucasians.

Table 1. The general characteristics of both groups

	RA Patients	N	Healthy Controls	N	*P* value*	Effect Size
Participant Characteristics						
Age (years)	56 ± 12	99	42 ± 12	32	.0001	η^2 = .014
Sex female N (%)	72 (73)	99	22 (69)	32	.66	η^2 = .038
Height (cm)	164 ± 9	99	168 ± 10	32	.18	η^2 = .015
Weight (kg)	80 ± 19	99	78 ± 17	32	.72	η^2 = .001
BMI (kg/m^2)	30 ± 6	99	28 ± 7	32	.34	η^2 = .008
Body Fat (%)	37 ± 9	99	31 ± 13	32	.07	η^2 = .028
Resting SBP (mmHg)	133 ± 16	96	121 ± 14	32	.08	η^2 = .025
Resting DBP (mmHg)	81 ± 10	96	77 ± 10	32	.46	η^2 = .004
Resting HR (bpm)	74 ± 13	96	70 ± 10	32	.08	η^2 = .025
General Demographics						
Employment N (%)						
Full-time	22 (24)	90	12 (63)	19	.000	------
Married or cohabiting N (%)	66 (68)	97	13 (72)	18	.73	------
Disease Characteristics						
RF Positive N (%)	70 (78)	90	------			
Disease duration (years)	11 ± 10	74	------			
DAS28	3.6 ± 1.3	93	------			
HAQ	1.7 ± .87	95	------			

RA Disease-Specific Medications			
Methotrexate	59 (60%)	99	------
Sulfasalazine	21 (21%)	99	------
Hydroxychloroquine	26 (26%)	99	------
Leflunomide	9 (9%)	99	------
Prednisolone	22 (22%)	99	------
NSAID	18 (18%)	99	------
COX II Inhibitors	11 (11%)	99	------
Analgesic	42 (42%)	99	------
Folic Acid	54 (54%)	99	------
Anti-TNF-α	11 (11%)	99	------
CVD Medications			
Anti-Hypertensive	25 (25%)	99	------
Statins/fibrate	12 (12%)	99	------
Beta-blocker	7 (7%)	99	------
Calcium-blocker	5 (5%)	99	------

Results are expressed as Number (percentage) or mean ± SD as appropriate. *Analysis corrected for age. BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate; RF = rheumatoid factor; DAS28 = disease activity score in 28 joints; HAQ = health assessment questionnaire; NSAID = non-steroidal anti-inflammatory drug; COX II = cyclooxygenase II; Anti-TNF = anti-tumor necrosis factor- alpha.

Serological Analysis

Univariate ANOVA revealed higher levels of TG (p = .001 η^2 = .082), insulin (p = .02 η^2 = .045), HOMA IR (p = .03 η^2 = .042), ESR (p = .000 η^2 = .152), CRP (p = .002 η^2 = .077), and fibrinogen (p = .000 η^2 = .205) in RA patients. QUICKI was found to be lower in RA patients (p = .02 η^2 = .046). When repeating the same analysis but controlling for age; insulin, HOMA and QUICKI were no longer different between the two groups (Table 2).

Table 2. Biochemical and haematological tests

	RA Patients	N	Healthy Controls	N	P value*	Effect Size
Biochemical Tests						
Total Cholesterol (mmol/l)	5.1 ± 1.0	93	5.0 ± 1.0	32	.65	η^2 = .002
HDL (mmol/l)	1.5 ± 0.3	93	1.4 ± 0.3	32	.95	η^2 = .000
Triglycerides (mmol/l)	1.5 ± 0.7	93	1.1 ± 0.6	32	.01	η^2 = .051
TC:HDL ratio	3.5 ± 8.5	93	3.6 ± 0.9	32	.84	η^2 = .000
Glucose (mmol/l)	4.6 (4.3 – 4.9)	91	4.6 (4.3 – 4.9)	32	.40	η^2 = .006
Insulin (pmol/l)	70.4 (40.6 – 105.5)	89	36.0 (27 – 82)	29	.23	η^2 = .012
HOMA IR	2.1 (1.1 – 3.2)	87	1.1 (0.7 – 2.9)	29	.28	η^2 = .010
QUICKI	0.35 ± 0.41	87	0.37 ± 0.05	29	.25	η^2 = .012
ESR (mmhr)	17.0 (8.8 – 28.3)	90	5.0 (2 – 9)	32	.001	η^2 = .088
CRP (mg/l)	5.0 (2.9 – 13.50)	93	2.9 (2.9 – 2.9)	32	.002	η^2 = .075
Fibrinogen (g/dl)	4.7 ± 1.2	89	3.5 ± 0.8	32	.000	η^2 = .159

Results are expressed as median (25th to 75th percentile values) or mean ± SD as appropriate.

*Analysis corrected for age. HDL = high density lipoprotein; TC = total cholesterol; HOMA IR = homeostasis model assessment insulin resistance; QUICKI = quantitative insulin sensitivity check index; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.

Presence of CVD risk factors

For the majority of the CVD risk factors, RA patients did not differ from healthy controls (see Table 3). However, Chi Squared test revealed a greater proportion of previous and current smokers, and a lower proportion of never smokers among the RA patients.

Table 3. CVD risk factors in RA patients and healthy controls

	RA Patients	N	Healthy Controls	N	*P* value	Effect Size
CVD Risk Factors						
Family history of CVD	45 (50)	90	6 (32)	19	.14	------
Diabetes	5 (6)	90	0	19	.29	------
Hypertension	32 (36)	90	3 (16)	19	.94	------
High Cholesterol	19 (21)	90	1 (5)	19	.11	------
Insulin Resistance	34 (39)	87	7 (24)	29	.20	------
Overweight	27 (30)	91	11 (37)	30	.40	------
Obese	52 (57)	91	13 (43)	30	.34	------
Smoking Status						------
Never smoked	38 (42)	91	17 (90)	19	0.001	
Previous smokers	36 (40)	91	2 (11)	19		
Current smokers	17 (19)	91	0	19		

Results are expressed as Number (%). Family history of CVD = self-report from demographic questionnaire; Diabetes = fasting glucose >7 mmol/l and/or oral hypoglycaemic medication or insulin use; hypertension = SBP >140mmHg, DBP >90mmHg or use of anti-hypertensive's; high cholesterol = fasting cholesterol >4.1 mmol/l or use of anti-hypercholesterolemics; insulin resistance = homeostasis model assessment ≥ 2.5 or quantitative insulin sensitivity check index ≤ 0.333; overweight: BMI ≥ 23-27.9; obese: BMI ≥ 28.

Global CVD Risk

The differences in the CVD risk scores between groups were analysed using univariate ANOVA and are shown in Table 4. RA patients were at a greater risk of CVD according to the FRS and TC SCORE criteria; for TC:HDL SCORE the difference did not quite meet the conventional criterion for statistical significance. The percentage of RA patients and healthy controls with metabolic syndrome was not different.

Table 4. CVD risk scores in RA patients and healthy controls

	RA Patients	N	Healthy Controls	N	P value	Effect Size
CVD Risk Score						
Framingham Risk Score	5 (3 – 10)	87	3 (2 -5)	19	.02	η^2 = .056
TC SCORE	1 (0 – 2)	64	1 (0 – 1)	18	.04	η^2 = .051
TC:HDL SCORE	1 (0 – 2)	64	0.5 (0 – 1)	18	.06	η^2 = .045
Metabolic Syndrome N (%)	13 (13)	89	3 (9)	32	.50	------

Results are expressed as median (25th to 75th percentile values) and Number (%). TC SCORE = total cholesterol systematic coronary risk evaluation; HDL = high density lipoprotein.

Vascular Assessments

Microvascular Endothelial function

Univariate ANOVA showed that baseline ACh perfusion was greater in RA patients even after controlling for age (Table 5). The peak ACh perfusion and the percentage increase in perfusion in response to ACh were lower in RA patients (p = .01, η^2 = .049 and p = .000, η^2 = .121 respectively), although these effects were no longer significant following adjustment for age. Baseline SNP perfusion, peak SNP perfusion and percentage increase in perfusion in response to SNP did not differ between groups.

Table 5. Microvascular endothelial function of RA patients and healthy controls

	RA Patients	N	Healthy Controls	N	P value*	Effect Size
Microvascular Function						
Baseline ACh Perfusion (PU)	37 (30 – 55)	94	31 (26 – 35)	30	.003	η^2 = .069
Peak ACh Perfusion (PU)	156 (93 – 210)	94	202 (140 – 257)	30	.92	η^2 = .000
Increase in Perfusion (ACh %)	236 (152 – 407)	94	456 (316 – 668)	30	.06	η^2 = .030
Baseline SNP Perfusion (PU)	36 (29 – 50)	94	33 (29 – 41)	30	.13	η^2 = .019
Peak SNP Perfusion (PU)	153 (99 – 205)	94	183 (112 – 235)	30	.35	η^2 = .007
Increase in Perfusion (SNP %)	261 (181 – 384)	94	386 (202 – 516)	30	.75	η^2 = .001

Results are expressed as median (25th to 75th percentile values). *Analysis corrected for age. ACh = acetylcholine; SNP = sodium nitroprusside

Macrovascular Endothelial Function

Baseline and peak FMD diameters were lower in RA patients and this difference survived adjustment for age (Table 6). There was no significant group difference in %FMD. Baseline GTN diameter, peak GTN diameter and %GTN did not differ between RA patients and healthy controls. For both groups, the baseline GTN diameter was similar to the baseline FMD diameter. AIx was found to be greater in RA patients than healthy controls, and this difference remained significant after controlling for age.

Table 6. Macrovascular endothelial function of RA patients and healthy controls

	RA Patients	N	Healthy Controls	N	*P* value*	Effect Size
Macrovascular Function						
Baseline FMD Diameter (mm)	3.3 (3.0 – 4.0)	96	3.6 (3.2 – 4.1)	32	.02	η^2 = .058
Peak FMD Diameter (mm)	3.6 (152 – 407)	96	4.0 (3.6 – 4.6)	32	.01	η^2 = .051
FMD (%)	9.5 (4.8 – 13.5)	96	11.4 (8.3 – 14.0)	32	.61	η^2 = .002
Baseline GTN Diameter (mm)	3.4 (3.0 – 3.8)	93	3.5 (3.2 – 4.2)	31	.06	η^2 = .028
Peak GTN Diameter (mm)	4.2 (3.7 – 4.7)	93	4.3 (4.0 – 5.1)	31	.08	η^2 = .025
GTN (%)	24.0 (16.3 – 30.4)	93	22.0 (19.4 – 29.0)	31	.30	η^2 = .009
AIx	33 (25 – 38)	84	27 (16 -35)	11	.04	η^2 = .044

Results are expressed as median (25th to 75th percentile values). *Analysis corrected for age. FMD = flow-mediated-dilatation; GTN = glyceryl-trinitrate-mediated dilatation; AIx = augmentation index.

Comparison of RA patients and Healthy Control Participants of Similar Ages

As RA patients were much older than the healthy control participants, all of the above analysis was repeated in RA patients and healthy control participants who were aged between 35 – 61 years (N = 57 RA patients, N = 21 healthy control participants). These age ranges were chosen as they allowed analysis of the maximum amount of participants in each group without there being a significant

difference in age between the two groups (RA patients age = 51 ± 7 years and healthy controls age = 49 ± 8 years, p = .29). This analysis revealed the same findings as the age-corrected analysis presented above.

Associations between Microvascular and Macrovascular Endothelial Function
For RA patients Pearson correlations showed that microvascular endothelial-dependent function (ACh) did not associate with macrovascular endothelial-dependent function (FMD). Similarly, microvascular endothelial-independent function (SNP) and macrovascular endothelial-independent function (GTN) were not associated with each other (see Table 7). In addition, no significant associations were found between microvascular peak ACh and SNP perfusion and macrovascular peak FMD and GTN diameters (data not shown).

Table 7. Correlations between the microvasculature and macrovasculature

		Macrovessels	
		Endothelial-dependent (FMD %)	Endothelial-independent (GTN %)
Microvessels	Endothelial-dependent (ACh %)	*r* (90) = .12, *p* = .27	----------
	Endothelial-independent (SNP %)	----------	*r* (89) = -.00, *p* = .94

ACh = acetylcholine; SNP = sodium nitroprusside; FMD = flow-mediated dilatation; GTN = glyceryl-trinitrate-mediated dilatation

Discussion
The present study found that microvascular and macrovascular endothelial function were similar in RA patients and healthy control participants after adjusting for age. However, AIx was higher in RA patients, as was global CVD risk. In addition, the assessments in the different vascular beds appeared to be independent of each other for both RA patients and healthy controls.

The finding that microvascular and macrovascular endothelial function is comparable between RA patients and healthy controls appears to contradict previous studies in RA patients which have shown impaired endothelial function

120

in the microvasculature [432,434,436,448,451,491] and in the macrovasculature [431,452,455,457,459,461,463,488,493,500]. However, these studies appear to differ to the present study in a variety of ways. First, although the general characteristics, such as age and gender, appear to be consistent between studies, RA disease-related inflammation in the present study was lower than that reported in many of the earlier studies [431,432,436,448,459,461,488,493,500]. Thus, high levels of disease-related inflammation at the time of the assessments may have contributed to the impairments in endothelial function reported in the other studies, especially as there is evidence that in healthy participants, acute inflammation can result in transient impairments in endothelial function [576]. Interestingly, the current findings were similar to those of Van Doornum et al 2003 [435] whose RA population had similar disease-related inflammation to the patients in the present study. It is noteworthy that the disease duration in the present study was comparable to other studies that reported impairments in endothelial function in RA relative to healthy participants [434,500]. Moreover, endothelial function was impaired in newly diagnosed patients and patients with low disease duration, but these patients also had high disease-related inflammation [432,451,491]. This might suggest that disease duration has a lesser influence on endothelial function than current disease-related inflammation.

In contrast to the present study, many of the previous studies had small samples [431,432,434,436,448,451,452,455,459,488,491,493,500]. It is now appreciated that small samples can increase the risk of type 1 as well as type 2 errors [577]. Post hoc power analyses were conducted using GPower3 [578] with significance set at .05 to determine the power to detect differences in microvascular and macrovascular endothelial function between RA patients and healthy control participants. On the basis of sample size, effect size, and the mean and standard deviation of the assessments of endothelial function in RA patients and healthy control participants, the obtained power to detect differences in microvascular endothelial-dependent function was .99 and for macrovascular

endothelial-dependent function .59. Thus, the obtained power for the comparison of microvascular endothelial function seems to be sufficient, whereas the power for the comparison in macrovascular function was low. It is important to highlight that the comparison of differences between measures were performed using analysis of co-variance with age as the covariate. Since it is not possible to co-vary when conducting power analyses, care should be taken when interpreting these power analyses.

The present study also differed from others in regards to the exclusion criteria that were applied. For example, participants with CVD risk factors were not excluded in the present study, but were in others [451,455,459,461]. Although CVD risk factors are known to impair endothelial function in the general population [202], their contribution to impairments in endothelial function in RA is not known. Moreover, in the present study, despite comparable endothelial function and CVD risk factors between participant groups, global CVD risk was greater in the RA patients. Thus, the extent of which CVD risk impairs endothelial function in RA requires further exploration. The influence of classical CVD risk on endothelial function is particularly interesting to explore in the context of systemic inflammation, given that inflammation has been reported to independently contribute to CVD [220].

In RA patients, a variety of assessments for endothelial function have been employed. For example, arterial stiffness can be assessed using AIx and pulse wave velocity, but these assessments are not always inter-correlated [461]. In the microcirculation, the majority of studies have assessed endothelial function using forearm blood flow with venous occlusion plethysmography (VOP) [432,434], and although to our knowledge no study has compared VOP with LDI in RA, methodological differences between assessments of endothelial function might contribute to the contrasting findings observed between studies. Importantly, from the studies which have reported poorer endothelial function, many did not report controlling for methodological and physiological factors that

can affect endothelial function [371,380]. For example, it is not always clear whether assessments were conducted at the same time of day for each participant or if the participants were fasted [436]. In addition, the reliability data for the vascular assessments are not consistently reported [431,432,434,491,493]. This has potential implications in the accuracy of the data and the interpretation of findings [371,380]. The assessments used in the current study have good reproducibility (see Chapter 3).

An interesting finding in the present study was that in the macrocirculation only AIx was impaired in RA patients. Unfortunately, AIx could only be obtained for 11 healthy participants. However, these 11 participants did not differ from the remaining healthy participants for known determinants of AIx such as HR, and SBP, although DBP was higher in those individuals where AIx had been obtained (p =.002) [400] . Additional analyses showed no difference in the SBP, DBP and HR between those RA patients and healthy participants where AIx was obtained. It is possible that the higher AIx in RA patients might reflect the increased global CVD risk that was evident in this group [579,580]. However, even in RA patients who are free from CVD and CVD risk factors, AIx has been reported to be higher than age and sex matched healthy controls [435,459,581]. Further, AIx has been shown to be positively associated with measures of cumulative inflammation independently of CVD risk factors [465]. If global CVD is a contributor to AIx, then impairments in the other assessments of endothelial function would also have been expected, as these too are affected by CVD [202]. Therefore, further examination of specific determinants of the separate measures of endothelial function in patients with RA is required.

AIx and FMD both examine endothelial function in the macrocirculation, but the presence of impairments in one but not the other highlights the inherent differences between these two techniques. FMD assesses vasodilatation in response to a stimulus (shear stress) and is fully dependent on NO [379], whereas AIx reflects resting vasomotor tone and compliance and is only partially

dependent on NO [582]. This suggests that along with NO, a number of other factors such as gene expression and smooth muscle integrity influence vascular compliance [401]. Indeed, no correlations between FMD and AIx in RA patients or healthy controls were found in the current study, which is in contrast to previous findings performed in non-RA patients with and without CVD [583]. In addition, at present it is not clear if impaired endothelial function results in arterial stiffness or vice-versa [226]. There is evidence that structural defects in the vessel as determined by carotid intima-media thickness are present in patients newly diagnosed with RA [488]. It is possible then that in RA patients free from overt CVD, impairments in vascular compliance might occur before ED of the brachial artery, but this requires further investigation.

The present study incorporated assessments of both microvascular and macrovascular endothelial function and showed that function in these two vascular beds was independent from one another. To our knowledge, only one other study has examined associations between small and large vessel endothelial function in RA patients, and reported, similarly to the current study, no associations [451]. Others studies in different populations have also found no correlation between microvascular and macrovascular beds [568-570]. In contrast, some studies have reported an association. However, in one study the participants were considerably younger than the present study (median age: 27 years) [566], and others tested a relatively small sample [567] or heterogeneous population [424].

The lack of association between microvascular and macrovascular endothelial function observed in the present study could be due to mechanistic differences between the assessments. It has been shown that SNP, FMD and GTN predominantly evoke maximum NO release as inhibition of NO completely abrogates the vasodilatory response to these stimuli [379,584]. However, NO inhibition only reduces 30-40% of the microvascular vasodilatory response induced by ACh [585], suggesting that other factors such as endothelium-derived

124

hyperpolarizing factor may also contribute to vasodilatation in the resistance vessels [334]. Further, the different stimuli applied by LDI and FMD involve distinct pathways to stimulate NO; LDI uses a pharmacological stimulus to activate NO, whereby, FMD uses a physiologic stimulus [586,587]. It is worth noting that correlations between peripheral and coronary endothelial function are stronger when the same stimulus is applied [352,588] than when different stimuli are applied [351]. Therefore, the distinct pathways of NO release evoked by differential stimulation of microvessels and macrovessels may in part, explain the lack of association between vascular beds observed in the present study. In addition, even when the same stimulus is applied to endothelial cells of different vascular beds, *in vitro* studies have reported that endothelial cells display heterogeneous responses, and this is even evident in different sections of the same vascular bed [223-225]. Moreover, in the context of RA, various disease-related parameters may exert differential effects in different sized vessels but this notion requires further investigation.

In summary, the present study found comparable endothelial function in the microvasculature and macrovasculature of RA patients and healthy controls, despite greater CVD risk in RA patients. In addition, microvascular and macrovascular endothelial function were independent of each other. Further research is needed to identify individual determinants of microvascular and macrovascular endothelial function in RA patients.

Chapter 6: The Association between Inflammation, Cardiovascular Risk Factors and Microvascular and Macrovascular Endothelial Function in Patients with Rheumatoid Arthritis

Introduction

Rheumatoid arthritis (RA) is an inflammatory disease of the joints which is also associated with an increased risk for cardiovascular disease (CVD) [121,428]. The inflammatory process of RA and CVD is remarkably similar [122,200], and on this basis, it has been speculated that RA disease-related inflammation might contribute to accelerated atherosclerosis [123,124]. Inflammation and classical CVD risk factors can both exert deleterious effects on the endothelium, leading to endothelial dysfunction (ED) in the general population [359,444]. However, studies that have examined associations between RA disease-related inflammation and microvascular or macrovascular endothelial function report equivocal findings; some find an association [430,448,449,463,498,500], whereas other do not [435,451,452,459]. To our knowledge only one study has examined associations between RA disease-related inflammation and endothelial-dependent function in different vascular beds; inflammation was associated with macrovascular but not with microvascular endothelial-dependent function [451]. There are no studies that have examined the correlation between classical CVD risk factors such as dyslipidemia and hypertension in the microcirculation and only a few studies have been conducted in the macrocirculation with inconsistent findings [433,455,456]. Therefore, the aims of the present study were a) to examine associations between disease-related inflammation and microvascular and macrovascular endothelial-dependent function in RA, b) to examine associations between classical CVD risk factors and microvascular and macrovascular endothelial-dependent function in RA, and c) to identify if any associations that were found were different in RA compared to healthy control participants.

Methods

Participants and Study Protocol

Ninety-nine patients with RA were recruited from the Rheumatology Outpatient Clinics of the Dudley Group of Hospitals NHS Foundation Trust, United Kingdom, and 32 healthy control participants were recruited from among hospital staff. The general characteristics of the participants have been described in greater detail in the previous chapter (Chapter 5) and the inclusion and exclusion criteria have been previously presented in the General Methods section (Chapter 3). The study protocol was identical to that of Chapter 5 and is therefore described in more detail in that chapter.

Statistical analysis

Statistical analysis was performed using SPSS15 (SPSS Inc, Chicago, Illinois). Variables were tested for normality using the Kolmogorov-Smirnov test. Means and standard deviations (SD) were calculated for normally distributed continuous variables and proportions for categorical variables. Log transformation was performed for positively skewed variables as appropriate. Differences in characteristics between RA patients and healthy control participants were assessed with univariate analysis of variance (ANOVA) with for continuous variables and Chi Squared test for discontinuous variables. To assess independent determinants of vascular function linear regression (continuous variables) and logistic regression (discontinuous variables) were used. Inflammatory markers, global CVD risk and CVD risk factors were entered as independent variables with each measure of vascular function entered separately as the dependent variable. These analyses were then repeated with RA as an independent variable.

Results

Participant Characteristics and Serological Analysis

The participants' general and disease-related characteristics along with serological and CVD risk factor analysis are presented in Chapter 5 (Tables 1 – 4).

Endothelial Function and RA Disease-Related Inflammation

Linear regression was performed with individual disease-specific parameters as independent variables and microvascular and macrovascular parameters entered separately as the dependent variables for RA. These analyses revealed that logCRP, logESR, fibrinogen, DAS28, HAQ and disease duration were not associated with microvascular or macrovascular endothelial-dependent and endothelial-independent function (see Table 1). No associations were found between arterial stiffness and individual disease-specific parameters.

Endothelial Function and DAS28 Cut-off Points

RA patients were categorised according to their DAS28 score into low disease activity (DAS28 <3.2, N= 36), or high-disease activity (>5.1, N = 12) according to established criteria [538]. Univariate ANOVA revealed that both groups were of a similar age, and from the vascular assessments, only microvascular endothelial-dependent function was significantly greater in patients with high disease activity (low disease activity: 280 ± 173, high-disease activity: 438 ± 336, p = .04, η^2 = .089).

Endothelial Function and Medications

The most commonly prescribed disease modifying anti-rheumatic drug (DMARD) was methotrexate (MTX). Univariate ANOVA showed that only macrovascular endothelial-dependent function was greater in patients receiving MTX than those who were not (10.2 ± 6.1% vs. 7.3 ± 5.1% respectively, p =.02). For all other RA medications and CVD medications (including NSAIDs) univariate ANOVA

revealed that microvascular and macrovascular endothelial function did not differ in patients receiving or not receiving these medications.

Endothelial Function and CVD Risk Factors

Linear regression was performed to examine associations between individual CVD risk factors (independent variables) and the separate measures of endothelial function (dependent variable) performed in the RA patients. For microvascular endothelial-dependent function, no associations were found with any of the serological (Table 2) or CVD risk factors (Table 3). Microvascular endothelial-independent function was positively associated with TC:HDL ratio, but not with any other CVD risk factor. In the macrocirculation, endothelial-independent function was negatively associated with SBP, insulin, HOMA, and positively associated with high cholesterol, hypertension and QUICKI. AIx was positively associated with QUICKI (Table 2). No other associations were found for macrovascular function. When the same analyses were conducted in healthy control participants, microvascular endothelial-independent function was associated with glucose (β= -.41, t (28) = 2.35, p = .03, R^2= .165), while macrovascular endothelial-independent function was associated with glucose (β= -.42, t (29) = 2.45, p = .02, R^2= .172), HOMA (β= -.44, t (24) = 2.52, p = .02, R^2= .196) and insulin resistance, (OR = 1.22 (1.00 – 1.47), p = .05).

Low, Moderate and High Endothelial Function

RA patients were split, according to their endothelial function scores in the microvasculature and the macrovasculature, into three equal groups: low endothelial function, moderate endothelial function and good endothelial function. Subsequent analysis with univariate ANOVA revealed that patients with lower microvascular endothelial-dependent and macrovascular endothelial-independent function were older than patients with moderate or high endothelial function (p = .007 and p = .004 respectively). For microvascular and macrovascular endothelial-dependent and endothelial-independent function as well as arterial

stiffness there was no difference in logESR, logCRP, DAS28 and disease duration between the different categories of endothelial function.

Chi Squared analysis was run to identify whether the proportion of medications the patients were receiving differed between patients with low, moderate or high endothelial function. This analysis revealed no differences in the proportion of patients receiving DMARDS, NSAIDs or CVD medications between the different categories of microvascular endothelial function. However, patients categorised into the high macrovascular endothelial-dependent function group had a greater proportion of methotrexate users (p = .05) than those in the moderate and low endothelial function groups. The proportion of patients on NSAIDs was greater in the low and high macrovascular endothelial-dependent function groups (p = .02). There were no differences in medication use for the macrovascular endothelial-independent categories or for the categories of arterial stiffness.

Table 1. Linear regression analysis for general and RA disease-related characteristics and endothelial function in RA

	Microvascular Function		Macrovascular Function		
	Endothelial-dependent	Endothelial-independent	Endothelial-dependent	Endothelial-independent	Arterial Stiffness
General Characteristics					
Age	β= -.37, R^2= .135***	β= -.20, R^2= .042*	β = -.24, R^2= .055*	β= -.36, R^2= .126***	β= .02, p = .87
BMI	β= .21, p = .05	β= .08, p = .41	β= -.12, p = .26	β= -.21, p = .05	β= -.11, p = .36
Resting SBP	β = -.11, p = .30	β= -.02, p = .88	β= -.11, p = .27	β= -.27, R^2= .066**	β= .04, p = .72
Resting DBP	β= .04, p = .67	β= .08, p = .44	β= -.12, p = .25	β= -.05, p = .61	β= .17, p = .14
Resting HR	β= .14, p = .19	β= .28, R^2= .078**	β = -.08, p = .40	β= .18, p = .08	β= .05, p = .65
RA Disease-related Characteristics					
Disease duration	β = -.12, p = .29	β= .21, p = .07	β= -.04 p = .76	β= -.12, p = .34	β= -.03, p = .84
LogCRP	β= -.61 p = .05	β= .10, p = .32	β= .15, p = .13	β= -.00, p = .97	β= .04, p = .73
LogESR	β= -.09, p = .41	β= .18, p = .10	β= -.01, p = .95	β= -.08, p = .46	β= .02, p = .88
Fibrinogen	β= .25, p = .12	β= .32 p = .30	β= .06, p = .58	β= -.06, p = .56	β= -.04, p = .74
DAS28	β= .15, p = .15	β= .02, p = .84	β= .06, p = .59	β= -.01, p = .93	β= .06, p = .59

General characteristics and RA disease-related characteristics were entered as independent variables, while microvascular and macrovascular endothelial function were entered as dependent variables in the regression analysis. SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; DAS28 = disease activity score in 28 joints. * p < .05, ** p < .01, *** p < .001. R^2 value is shown for all significant associations only.

Table 2. Linear regression between Serological factors and endothelial function in RA

	Microvascular Function		Macrovascular Function		
	Endothelial-dependent	Endothelial-independent	Endothelial-dependent	Endothelial-independent	Arterial Stiffness
Serological Factors					
Total Cholesterol	β = .06, p = .55	β = .20, p = .05	β = -.05, p = .63	β = .09, p = .41	β = .20, p = .07
HDL	β = -.13, p = .21	β =- .06, p = .56	β = -.00, p = .97	β = .15, p = .16	β = .09, p = .43
Triglycerides	β = -.12, p = .26	β = -.10, p = .34	β = .14, p = .19	β = -.04, p = .69	β = .12, p = .31
TC:HDL ratio	β = .19, p = .07	β = .24, R^2 =.058*	β = -.06, p = .55	β = -.09, p = .36	β = .09, p = .45
Glucose	β = -.10, p = .33	β =- .09, p = .40	β = -.07, p = .52	β = .02, p = .85	β = -.03, p = .81
Insulin	β = .13, p = .23	β = .04 p = .73	β = -.04, p = .71	β = -.25, R^2 =.065*	β = -.15, p = .20
HOMA IR	β = .09, p = .41	β =- .00, p = .95	β = .06, p = .61	β = -.23, R^2 =.052*	β = -.17, p = .15
QUICKI	β = -.07, p = .53	β = -.02, p = .88	β = .11, p = .30	β = .30, R^2 =.095**	β = .268, R^2 =.072*

Serological factors were entered as independent variables and endothelial function entered as dependent variables in the regression analysis.

HDL = high density lipoprotein; TC = total cholesterol; HOMA IR = homeostasis model assessment insulin resistance; QUICKI = quantitative insulin sensitivity check index. * p < .05, ** p < .01. R^2 value is shown for all significant associations only.

Table 3. Linear and binary regression between CVD risk factors and endothelial function in RA

	Microvascular Function		Macrovascular Function		
	Endothelial-dependent	Endothelial-independent	Endothelial-dependent	Endothelial-independent	Arterial Stiffness
CVD Risk Factors					
Family history of CVD	OR = 1.00 (0.99 -1.00)	OR = 1.00 (0.99 -1.00)	OR = 1.00 (0.93 -1.07)	OR = 0.99 (0.94 -1.04)	OR = 1.02 (0.97 -1.07)
Diabetes	OR = 1.00 (0.99 -1.00)	OR = 1.00 (0.99 -1.00)	OR = 0.98 (0.82 -1.17)	OR = 1.08 (0.94 -1.23)	OR = 1.08 (0.93 -1.25)
Hypertension	OR = 1.00 (1.00 -1.00)	OR = 1.00 (0.99 -1.00)	OR = 1.08 (0.99 -1.17)	OR = 1.07 (1.01-1.14)*	OR = 0.96 (0.92 -1.02)
High Cholesterol	OR = 1.00 (0.99 -1.00)	OR = 1.00 (0.99 -1.00)	OR = 1.04 (0.95 -1.14)	OR = 1.09 (1.02-1.17)**	OR = 1.04 (0.97 -1.11)
Insulin Resistance	OR = 1.00 (1.00 -1.00	OR = 1.00 (1.00 -1.00	OR = 1.01 (0.94 -1.10	OR = 1.05 (0.99 -1.11	OR = 1.03 (0.98 -1.09
Number of CVD Risk Factors	β = -.04, p = .69	β= -.06, p = .58	β= -.16, p = .11	β= -.29, R^2=.082**	β= -.03, p = .77
Global CVD Risk					
FRS	β = -.26, R^2=.068**	β = -.18, p = .09	β = -.12, p = .26	β = -.32, R^2=.101**	β = -.07, p = .51
TC SCORE	β = -.28, R^2=.077*	β = -.17, p = .18	β = -.23, p = .08	β = -.14, p = .30	β = -.11, p = .45
TC:HDL SCORE	β = -.26, R^2=.068*	β = -.17, p = .19	β = -.25, p = .05	β = -.17, p = .18	β = -.08, p = .55
Metabolic Syndrome	β = .10, p = .35	β = -.01 p = .89	β = .15, p = .16	OR: 1.17 (1.06 – 1.30)**	β = .18, p = .11

CVD risk factors and global CVD risk scores were entered as independent variables and endothelial function as dependent variables in the linear and binary regression analysis. Odd ratio (OR) with 95% confidence interval is presented for all binary regression analysis. CVD = cardiovascular disease; FRS = Framingham risk score; SCORE = systematic coronary risk evaluation; TC = total cholesterol; HDL = high density lipoprotein. * p < .05, ** p < .01. R^2 value is shown for all significant associations only.

Endothelial Function and Global CVD Risk

Linear regression analyses revealed that the FRS was negatively associated with microvascular endothelial-dependent function and macrovascular endothelial-independent function but not with any of the other vascular parameters (Table 5). TC SCORE and TC:HDL SCORE were only associated with microvascular endothelial-dependent function. In addition, there was a trend towards an association between macrovascular endothelial-dependent function and TC SCORE and TC:HDL SCORE. Logistic regression analysis revealed that macrovascular endothelial-independent function was associated with metabolic syndrome. Univariate ANOVA showed that patients with metabolic syndrome had a lower response to GTN than those with absence of metabolic syndrome (14.9 ± 6.5 vs. 23.7 ± 8.0%, p = .001). No associations were found between any of the above parameters when analysing data for healthy control participants only.

Individual Components of Global CVD Risk and Endothelial Function

Framingham Risk Score

Stepwise multivariate regression was performed to examine the relationship between the individual components of the global CVD risk scores (independent variables) and each parameter of endothelial function (dependent variables) that showed an overall association in the analysis presented above. The individual components that were entered for FRS were age, sex, TC, HDL-C, SBP, DBP, diabetes and smoking status. The entry probability was .05 and none of the variables were forced back into the model. This analysis showed that only age was significantly associated with microvascular endothelial-dependent function (Table 4). Examining the specific components of the FRS and macrovascular endothelial-independent function, revealed that sex, SBP and DBP were significantly associated.

Table 4. Relationship between individual components of the FRS and endothelial function

Multivariate Model	Microvascular Endothelial-dependent Function	Macrovascular Endothelial-independent Function*
Model 1		
Age	$\beta = -.36$, $t(82) = 3.45$, $p = .001$, $R^2 = .127$	
Sex		$\beta = .36$, $t(78) = 3.66$, $p = .000$
TC		
HDL-C		
SBP		$\beta = -.60$, $t(78) = 4.23$, $p = .000$
DBP		$\beta = .44$, $t(78) = 3.06$, $p = .003$
Diabetes		
Smoking		

The entry probability was .05 and none of the variables were forced back into the model. *The R^2 for this model was .272. TC = total cholesterol; HDL-C = high density lipoprotein-cholesterol; SBP systolic blood pressure; DBP = diastolic blood pressure.

Systematic Coronary Risk Evaluation

For the individual components of the TC SCORE, age, sex, smoking, SBP and TC were entered in a stepwise manner (see Table 5). For TC:HDL SCORE, the same variables were re-entered with TC being substituted for TC:HDL. In each analysis, microvascular endothelial-dependent function was entered as the dependent variable (as it was the only parameter that had shown an overall association with these risk scores in the previous analysis). As before, the entry probability for each variable was .05 and none of the variables were forced back into the model. This analysis revealed that only age was associated with TC SCORE and TC:HDL SCORE ($\beta = -.36$, $t(83) = 3.49$, $p = .001$, $R^2 = .117$ and $\beta = -.36$, $t(84) = 3.49$, $p = .001$, $R^2 = .117$, respectively).

Table 5. Relationship between individual components of the SCORE and endothelial function

Multivariate Model	Microvascular Endothelial-dependent Function
Model 1: TC SCORE	
Age	$\beta = -.36$, $t(83) = 3.49$, $p = .001$, $R^2 = .117$
Sex	
Smoking	
TC	
SBP	
Model 1: TC:HDL SCORE	
Age	$\beta = -.36$, $t(84) = 3.49$, $p = .001$, $R^2 = .117$
Sex	
Smoking	
TC:HDL	
SBP	

The entry probability was .05 and none of the variables were forced back into the model. TC = total cholesterol; HDL-C = high density lipoprotein; SBP systolic blood pressure.

Endothelial Function and Number of CVD Risk Factors

To examine whether endothelial function deteriorates as the number of CVD risk factors increase, the total number of CVD risk factors present for each participant were added together. The CVD risk factors that were included in the analysis were family history of CVD, smoking, diabetes, hypertension, hypercholesterolemia, insulin resistance, and obesity. Five per cent of patients had no CVD risk factors, 20% 1 CVD risk factor, 26% 2 CVD risk factors, 23% 3 CVD risk factors, 20% 4 CVD risk factors, 3% 5 CVD risk factors, 4% 6 CVD risk factors, and 0% 7 CVD risk factors. Linear regression revealed that for RA patients, macrovascular endothelial-independent function was inversely associated with the total number of CVD risk factors (Table 3). No associations were found when examining only the healthy control participants.

Comparison of Associations between RA Patients and Healthy Controls

Additional analyses were done to determine if the associations with endothelial function that are reported above vary between RA patients and healthy control participants. These analyses included the direct comparisons between beta-coefficients (β) in RA patients and healthy control participants. This comparison is done by transforming the beta-coefficients into z scores using the Fisher *z* score equation [589]. The following equation was used to transform the beta-coefficient into *z* scores:

$$z = (0.5)\log[(+\beta)/(1-\beta)]$$

Once the *z* score was obtained for the RA patients and the healthy controls, the statistical difference of the z scores between groups was computed using the following equation, which takes differences in group sizes into account:

$$z = (z_1 - z_2)/\sqrt{1/(N_1 - 3) + 1/(N_2 - 3)}$$

Where, z_1 is the z score for RA patients, z_2 is the z score for healthy controls, N_1 is number of RA patients and N_2 is number of healthy controls.

The analyses showed that the associations present in RA patients were not different to the associations found in healthy controls ($p > .40$).

Discussion

The findings of the present study revealed that RA disease-related inflammation was not associated with microvascular or macrovascular endothelial function. Similarly, individual CVD risk factors did not consistently relate to microvascular or macrovascular endothelial function. Global CVD risk, however, inversely correlated with microvascular endothelial-dependent function. Finally, the

magnitude of the associations that were present in RA patients were not different from the magnitude of the associations in healthy controls.

RA is characterised by increased systemic inflammation which has been suggested to impact on the vasculature and contribute to accelerated atherosclerosis [123,590]. However, the present findings suggest that inflammation does not relate to endothelial function in two different vascular beds. It is worth noting that there was a trend for an association between microvascular endothelial-dependent function and CRP. However, given the number of associations that were examined, this could be a chance finding. There are accepted ways for overcoming multiple comparisons such as the Bonferroni method which adjusts alpha for the number of comparisons undertaken. However, given that no significant associations were reported, the Bonferroni correction does not seem necessary. In the microvasculature, studies have reported conflicting associations between endothelial function and disease-related inflammation, as one study found an association with TNF-α but not with ESR or CRP [448], while in another endothelial function was associated with CRP only [449]. Further, some studies have not found any association [451]. Similarly, a number of studies in the macrovasculature found no associations between disease-related inflammation and macrovascular endothelial-dependent function [435,452,455,458], and studies that have reported associations present an inconsistent picture. For example, macrovascular endothelial function was reported to be associated with CRP but not ESR [433,461], while another study reported an association with DAS28 only [454]. Collectively, these findings suggest that the relationship between endothelial function and disease-related inflammation may not be as strong as has been previously suggested [123,590].

It is possible that it is not current but continuous long-term high levels of inflammation that are important for endothelial function in RA. It has been reported that CRP duration (average of 4 CRP measurements taken at separate times in a one year period x disease duration) is a better predictor of endothelial-

dependent function in RA patients than current CRP and ESR levels [433]. In addition, arterial stiffness has been shown to associate with retrospective ESR and CRP, but not with current levels of ESR and CRP [463,465], and retrospective ESR values show better associations with more advanced measures of (still subclinical) atherosclerosis such as cIMT [467,468,487]. On the other hand, aortic arterial stiffness has been associated with current CRP but not cumulative ESR and DAS28 [461], whereas another study reported that cIMT was not related to retrospective disease-related inflammation [466]. These contrasting findings are difficult to reconcile as the majority of the above studies used different methods to determine retrospective or cumulative inflammation. Moreover, the importance of RA disease duration must also be considered as patients with long disease duration might also have greater frequency of inflammatory fluctuations which could impact on endothelial function. It is worth mentioning that the present study and others have reported that endothelial function is independent from RA disease duration [433,435,461]. However, cIMT has been reported to be associated with disease duration [483,490], but this measure represents a later stage of atherosclerosis when the vasculature is more likely to be affected by continuous inflammatory insults over the course of the disease. It is possible that cyclical fluctuations of high and low disease activity which could acutely impact on the vasculature [512] could be more critical than disease duration on impacting endothelial function. Prospective studies that examine endothelial function and inflammatory levels over a longer period of time may provide greater insight on the interactions between inflammatory fluctuations and endothelial function in RA.

Disease-related inflammation is most commonly assessed using systemic inflammatory markers, but these markers may not accurately reflect local inflammatory changes in the vasculature. For example, Chia and colleagues [591] reported that infusion of TNF-α into the brachial artery induced a reduction in forearm blood flow in the infused arm, but not in the non-infused arm. Similarly, local inhibition of TNF-α in RA patients resulted in an immediate

improvement in endothelial function without affecting systemic inflammatory levels [491]. Thus, systemic markers of inflammation may be less sensitive to the local effects of inflammation on the vascular wall.

The majority of individual classical CVD risk factors did not associate with endothelial function in RA. In the general population the association between classical CVD risk factors and endothelial function has been well characterised [202,359]. To our knowledge, no study of RA patients has examined the impact of classical CVD risk factors on microvascular endothelial function, and only a few studies have examined the effects of classical CVD risk factors on macrovascular endothelial function [433,455,456]. Associations were present for lipid levels in some [433,456], but not all [455] studies. Classical CVD risk factors can improve after controlling RA disease-related inflammation, which suggests that inflammation can contribute to the development of classical CVD risk factors in patients with RA [155,446]. Further, ESR was only found to associate with cIMT in the presence of classical CVD risk factors [219]. These findings reveal that inflammation and classical CVD risk factors may interact to cause macrovascular atherosclerosis. In the present study, disease-related inflammation was not associated with any of the classical CVD risk factors (data not shown). As disease activity was low in the current study it is possible that such associations are unlikely in RA patients with low disease-related inflammation.

When classical CVD risk factors were incorporated into global CVD risk algorithms, associations were found with microvascular endothelial-dependent function, but not with macrovascular endothelial-dependent function. Further analysis revealed that age was the main contributor for these associations, and most likely accounted for the higher FRS and TC SCORE in RA patients relative to healthy controls. Multivariate regression analysis revealed no other individual CVD risk factor that contributed to the association between global CVD risk and microvascular endothelial-dependent function. This suggests that different CVD

risk factors may interact with each other to increase overall CVD risk rather than act independently in the vasculature. Indeed, previous studies have demonstrated that individual classical CVD risk factors are associated with each other in both RA [128] and the general population [592]. In the present study, an association between the number of classical CVD risk factors and microvascular and macrovascular endothelial-dependent function was not present, although a previous study in participants with CVD risk factors in the absence of CVD, reported that macrovascular endothelial-dependent function was reduced as the number of CVD risk factors increased [410]. In that study only four CVD risk factors were entered into the analyses and endothelial function did not differ between those participants without CVD risk factors and those with only one CVD risk factor [410]. This indicates that there may be a threshold for the number of CVD risk factors that are required before endothelial function deteriorates. Although seven CVD risk factors were entered in to the analyses in the current study, most patients had 1-4 CVD risk factors, with no patient having all 7 CVD risk factors. It is therefore possible that in RA 1-4 CVD risk factors may not be sufficient to affect endothelial function. Nevertheless, increased number of CVD risk factors may adversely affect the endothelium, but further research exploring the number of CVD risk factors that are required to impact different vascular beds in patients with RA is required.

Global CVD risk algorithms may under-represent risk of future cardiac events in clinical conditions such as diabetes and systemic lupus erythematosus [593,594]. A limitation of global CVD risk is that they only incorporate classical CVD risk factors which limit their use in RA, as novel CVD risk factors like inflammation can amplify CVD risk independently of classical CVD risk factors [220]. For example, two studies that compared RA patients with healthy controls matched for FRS reported lower macrovascular endothelial-dependent function and greater arterial stiffness and cIMT in the RA patients [455,457]. It has also been suggested that incorporating coronary artery calcification into the FRS algorithm would increase the accuracy of estimating future risk of CVD, as high

FRS score independently associates with coronary artery calcification in RA [532]. In the present study FRS was used along with the SCORE risk algorithm, and although calculated using classical CVD risk factors, the risk algorithms were based on different combinations of risk factors, and the use of more than one CVD risk algorithm provides better information on future CVD risk in RA [532].

The observation that microvascular but not macrovascular endothelial-dependent function was associated with FRS and SCORE risk algorithms highlights the importance of examining endothelial function in more than one vascular bed. Microvessels make up a much larger proportion of the vasculature than macrovessels [595], and may therefore have greater exposure to injurious stimuli [574]. Consequently, it is possible that even small increases in global CVD risk could have a greater effect on microvascular endothelial-dependent function. Microvascular abnormalities can occur before or occur alongside the development of CVD risk factors in healthy individuals and individuals with hypertension [596-598]. These findings are in line with the results from other clinical populations like diabetes, where microvascular dysfunction develops independently of macrovascular dysfunction [574], and may even contribute to the development of macrovascular disease [573]. Therefore, assessments which examine both vascular beds may provide more meaningful clinical information on vascular risk in RA. Further research is necessary to identify if microvasculature or macro-vasculature ED is predictive of clinical endpoints, both in RA and in other populations.

In the macrocirculation, endothelial-independent function was associated with the FRS, metabolic syndrome, parameters of insulin resistance, SBP, presence of high cholesterol and hypertension as well as the total number of CVD risk factors. Smooth muscle dysfunction occurs independently of ED in healthy individuals with CVD risk factors [599]. Further, macrovascular endothelial-independent function, but not macrovascular endothelial-dependent function was related to a reduction in SBP after 12 and 24 weeks of treatment in

patients with hypertension [600]. These findings indicate that CVD risk factors may differentially affect endothelial cell and smooth muscle function. In the current study, the endothelial-independent function was significantly lower in patients with hypertension, and there is some evidence that CVD risk factors like hypertension degrade cyclic guanosine monophosphate (cGMP) [601], a second messenger responsible for the relaxation of vascular smooth muscle cells [256]. In addition, *in vitro* studies have shown that soluble guanylyl cyclase, an enzyme responsible for activating cGMP, has reduced sensitivity to NO in hypertensive rats [602]. This means that even if adequate NO is released from the endothelial cells, abnormalities in smooth muscle cell signalling could still lead to a reduced vasodilatory response. The presence of insulin resistance is also thought to have direct effects on smooth muscle cells by promoting the actions of endothelin-1 which can lead to the proliferation of vascular smooth muscle cells [603]. This is supported by a study in RA patients which reported that components of the metabolic syndrome such as insulin resistance, were strongly associated with cIMT [475]. However, further studies are required to corroborate these findings in patients with RA, especially as functional and structural changes occur at distinct phases of atherosclerosis [604].

It is important to note that all associations found in the present study were not different between RA and healthy control participants. Thus, factors which affect endothelial function in the general population may have similar effects in RA. However, the presence of RA is an independent predictor of poor FMD [458], but this may not necessarily be due to inflammation, as endothelial function and cIMT are similar between RA and type II diabetes [458,486], despite inflammatory levels being greater in RA. Therefore, further research is needed to identify other RA specific factors such as physical inactivity [605], rheumatoid cachexia [606] and genes [452] that may also affect endothelial function in RA.

In conclusion, the present findings show that disease-related inflammation and individual classical CVD risk factors were not associated with microvascular

and macrovascular endothelial function in RA patients with low disease-related inflammation. Further longitudinal studies are needed to examine the effects of high disease-related inflammation and inflammatory fluctuations on microvascular and macrovascular endothelial function in patients with RA.

Chapter 7: The Effects of Anti-Tumor Necrosis Factor-alpha on Microvascular and Macrovascular Endothelial Function in Rheumatoid Arthritis

Introduction

Rheumatoid arthritis (RA) is characterised by an increased expression of pro-inflammatory cytokines which are involved in propagating joint damage [46]. Tumour necrosis alpha (TNF-a) is one such cytokine that plays a major role in the pathogenesis of RA due to its ability to regulate additional pro-inflammatory cytokines such as interleukin-1 (IL-1) and IL-6, as well as activating downstream inflammatory mediators like C-reactive protein (CRP) which then amplify the inflammatory response [98]. TNF-α also contributes to the inflammatory response in atherosclerosis [210], by direct deleterious effects on the endothelium [443]. For example, TNF-α can reduce nitric oxide (NO) bioavailability by down-regulating the expression of endothelial nitric oxide synthase (eNOS) [607]. TNF-α can also impair vascular regeneration by inhibiting the actions of endothelial progenitor cells (EPC) which are involved in repairing endothelial cell injury [215]. Indirect effects of TNF-α include alterations in the lipid profile which promote dyslipidemia [608,609], as well as impaired glucose metabolism leading to insulin resistance [610,611]. Inhibition of Tumor Necrosis Factor-α (anti-TNF- α) in RA patients with high disease-related inflammation can reduce disease activity and delay the progressive joint damage [612]. Preliminary evidence also suggests that anti-TNF-α therapy may cause a reduction of cardiovascular mortality in RA patients [613], via currently undetermined mechanisms.

Intra-arterial infusion of TNF-α can cause acute endothelial dysfunction (ED) [591], whereas local infusion of anti-TNF-α can acutely improve ED [491]. The effect of chronic anti-TNF-α treatment on microvascular and macrovascular endothelial function is not clear; some studies report long-term improvements [431,461,464,494,495,499], others report transient improvements

[438,492,493,500], or even no effect on endothelial function [434]. However, several studies included small sample sizes [438,461,493,499] and to our knowledge no studies have examined the longitudinal effects of anti-TNF-α on different sized vessels in the same group of patients with RA. The latter point is particularly important as it is possible that effective control of disease-related inflammation may exert differential effects in the microvasculature and macrovasculature.

Although RA disease-related inflammation has been postulated to adversely affect endothelial function [123,124,461], the available evidence does not consistently support this. Changes in disease-related inflammation are not related to changes in microvascular and macrovascular endothelial function in response to anti-inflammatory treatment [436,495,499,501]. Only one study actually found such a correlation [488]. Further, I reported that disease-related inflammation was not consistently associated with microvascular or macrovascular endothelial-dependent function in the previous chapter (Chapter 6). However, those findings need to be confirmed in a longitudinal study. Therefore, the aims of the present study were a) to examine the short-term effects of 2 and 12 weeks of treatment with anti-TNF-α on microvascular and macrovascular endothelial function, and b) to determine whether changes in microvascular and macrovascular endothelial function are associated with changes in disease-related inflammation in patients with RA.

Methods

Patients

Twenty-three RA patients who were due to start anti-TNF-α treatment were recruited from the Rheumatology Outpatient Clinics of the Dudley Group of Hospitals NHS Foundation Trust, United Kingdom. The patients are described in greater detail in Chapter 3. The study received local Research Ethics Committee

approval and all patients gave their written informed consent according to the Declaration of Helsinki.

Study Protocol

Patients reported to the vascular laboratory after a 12 hour overnight fast between 7:00 am and 11:00 am for three separate visits. The protocol was the same on each visit. Patients were asked to refrain from exercise 24 hours before the session, and from smoking 12 hours before the session. For ethical reasons, drug regimens were not interrupted prior to each assessment. The laboratory was kept at a constant temperature ($22 \pm 0.9\,^{o}C$). Patients were assessed prior to starting anti-TNF-α therapy (pre-treatment) and were re-assessed at 2 weeks and 12 weeks after initiation of treatment. For the 2 week assessment all patients had received a single dose of the medication. The 3-month assessments were conducted one week after the patients had received their last dose of anti-TNF-α. Fifteen patients were started on adalumimab, six on etanercept and 2 on Infliximab.

All patients underwent a detailed clinical examination which included evaluation of their medical history and hospital records, examination of height (Seca 214 Road Rod), weight, body mass index (BMI) and body composition (Tanita BC 418 MA Segmental Body Composition Analyser). Their disease activity score (DAS28) [68] and their scores on the Anglicised version of the Stanford Health Assessment Questionnaire (HAQ) [72] were also assessed on each occasion. In addition, demographic information was collected from all the patients by questionnaire. Following this, patients were asked to sit on a semi-recumbent armchair where they stayed for the remainder of the session. Initially, patients were asked to sit quietly for 20 minutes, during which blood pressure measurements were taken. A blood sample was obtained immediately after this initial rest period. The patients then underwent assessments of arterial stiffness using pulse wave analysis and assessment of microvascular function using Laser Doppler imaging with iontophoresis. This was followed by a further ten minutes

of rest. After this, macrovasculature endothelial function was assessed using flow-mediated dilatation (FMD) and, following an additional ten minutes of rest, assessment of GTN-mediated dilatation (GTN). These assessments are described in greater detail in the General Methods section (See Chapter 2).

Statistical analysis

Statistical analysis was performed using SPSS15 (SPSS Inc, Chicago, Illinois). Variables were tested for normality by the Kolmogorov-Smirnov test. Means and standard deviations (SD) were calculated for normally distributed continuous variables and proportions for categorical variables. Log transformation was performed for positively skewed variables as appropriate. Changes in each parameter of endothelial function, CVD risk and disease-related measurements were assessed using 3 X time (pre-treatment baseline, 2 weeks, 12 weeks) repeated measures Analysis of Variance (ANOVA). Where appropriate, Fisher LSD post-hoc tests were used for pair-wise comparisons. Endothelial function did not differ between the three different types of anti-TNF-α treatment at any time point, therefore all treatments were analysed together. The change in endothelial function and disease-related parameters at 2 and 12 weeks was calculated by subtracting the pre-treatment baseline values from the values obtained at 2 and 12 weeks. Pearson correlations were used to examine whether changes in endothelial function related to changes in disease-related inflammation.

Results

RA Disease-Specific Characteristics

The pre-treatment characteristics of the patients are presented in table 1. The disease duration ranged from 2 – 43 years. Fifteen (65.2%) of the patients were started on 40 mg of adalimumab, six (26.1%) on 50mg of etanercept and two (8.7%) on infliximab with a dosage of 3mg/kg. Sixteen patients (69.6%) were on methotrexate, 4 (17.4%) on hydroxychloroquine and 7 (30.4%) on

sulphasalazine. Six (26.1%) patients were on oral prednisolone up to a maximum dose of 7.5mg, 5 (21.7%) on oral anti-hypertensives, 6 (26.1%) on non-steroidal anti-inflammatory drugs (NSAIDs) and 1 (4.3%) on a cyclo-oxygenase II inhibitor (Coxib). There was no change in any of these medications or their doses during the follow-up period.

Table 1. The characteristics of the RA patients

	RA Patients
General Characteristics	
Age (years)	54 ± 15
Sex female N (%)	15 (65)
Height (cm)	165 ± 8
BMI (kg/m^2)	30 ± 6
Disease-related Characteristics	
RF Positive N (%)	20 (87)
Disease duration (years)	11 ± 11
CVD Risk Factors	
Family history of CVD	12 (40)
Diabetes	1 (3)
Hypertension	6 (20)
High Cholesterol	6 (20)
Insulin Resistance	10 (33)
Overweight	7 (23)
Obese	13 (43)
Smoking Status	
Never smoked	11 (48.8)
Previous smokers	7 (30)
Current smokers	5 (22)

Results are expressed as Number (%). Diabetes = fasting glucose >7 mmol/l and/or oral hypoglycaemic medication or insulin use; hypertension = SBP >140mmHg, DBP >90mmHg or use of anti-hypertensive's; high cholesterol = fasting cholesterol >4.1 mmol/l or use of anti-hypercholesterolemics; insulin resistance = homeostasis model assessment ≥ 2.5 or quantitative

insulin sensitivity check index ≤ 0.333; overweight: BMI ≥ 23-27.9; obese: BMI ≥ 28. BMI: body mass index, RF: rheumatoid factor.

Table 2 shows the RA disease-specific features at pre-treatment baseline, 2 weeks and 12 weeks after commencing treatment. The repeated measures (baseline, 2 weeks, 12 weeks) ANOVA revealed an overall effect for morning stiffness, CRP, ESR, fibrinogen, DAS28 and HAQ. Post hoc analyses revealed that morning stiffness, CRP, fibrinogen, DAS28 and HAQ were reduced after 2 weeks of treatment and remained so after 12 weeks of treatment. ESR improved 2 weeks after commencing treatment, but was similar to baseline levels at 12 weeks. None of the parameters differed between week 2 and week 12.

Table 2. Disease-related characteristics at baseline, 2 weeks and 12 weeks

	Baseline	2 Weeks	12 Weeks	Treatment Effect $F_{(2,22)}$ =
Morning Stiffness (mins)	116 ± 75	72 ± 81[a]	55 ± 83 [a]	4.33, p = .02
CRP (mg/l)	10 (4 – 14)	3 (2.9 – 6)[a]	5 (2.9 – 10)[a]	12.89, p = .000
ESR (mmhr)	16 (9 – 34)	10 (5 – 21)[a]	17 (5 – 27)	4.98, p = .01
Fibrinogen (g/L)	5.1 ± 1.0	4.2 ± .75 [a]	4.3 ± .91[a]	13.15, p = .000
DAS28	4.17± 0.96	2.74 ± 1.4[a]	2.64 ± 1.07 [a]	15.92, p = .000
HAQ	2.1 ± 0.5	1.3 ± 0.9 [a]	1.3 ± 0.9 [a]	17.18, p = .000

Results are expressed as median (25th to 75th percentile values) or mean ± standard deviation as appropriate. [a] = different from baseline. CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, DAS28: disease activity score, HAQ: health assessment questionnaire.

Serological and Cardiovascular Parameters

Serological and cardiovascular parameters are displayed in Table 3. ANOVA revealed an overall time effect for HDL cholesterol, SBP and DBP. Post hoc analysis showed that HDL cholesterol was higher at 2 weeks, but returned to baseline levels by 12 weeks. There was no change in glucose, insulin, HOMA IR and QUICKI at any of the follow up periods. SBP and DBP were lower at 2 and

12 weeks relative to baseline, but there was no difference in HR at any of the time points.

Table 3. Serological factors and CV parameters during treatment

	Baseline	2 Weeks	12 Weeks	Treatment Effect	Degrees of Freedom
Serological Analysis					
TC (mmol/l)	4.7 ± 0.9	5.0 ± 1.1	4.8 ± 0.9	F = 1.85, p =.46	2, 22
HDL (mmol/l)	1.4 ± 0.3	1.5 ± 0.3 [a]	1.4 ± 0.3[b]	F = 4.98, p =.01	2, 22
Triglycerides (mmol/l)	1.4 ± 0.6	1.4 ± 0.7	1.6 ± 0.2	F = 1.73, p =.20	2, 22
TC:HDL ratio	3.4 ± 0.8	3.3 ± 0.7	3.6 ± 0.1	F = 2.39, p =.10	2, 22
Glucose (mmol/l)	4.5 ± 0.5	4.4 ± 0.4	4.5 ± 0.5	F = 0.84, p =.44	2, 22
Insulin (pmol/l)	105 ± 116	97 ± 76	91 ± 64	F = 0.40, p =.60	2, 17
HOMA IR	3.2 ± 4.0	2.9 ± 2.3	3.0 ± 2.2	F = 0.30, p =.70	2, 14
QUICKI	0.35 ± 0.1	0.35 ± 0.1	0.34 ± 0.0	F = 0.33, p =.72	2, 14
CV Parameters					
Resting SBP (mmHg)	127 ± 15	122 ± 15 [a]	119 ± 15 [a]	F = 5.63, p =.007	2, 22
Resting DBP (mmHg)	80 ± 7	76 ± 7 [a]	75 ± 8 [a]	F = 6.99, p =.002	2, 22
Resting HR (bpm)	73 ± 13	72 ± 10	71 ± 12	F = 0.91, p =.55	2, 22

Results are expressed as mean ± standard deviation. [a] = different from baseline, [b] = different from 2 weeks. TC: total cholesterol, HDL: high density lipoprotein, HOMA IR: homeostasis model assessment insulin resistance, QUICKI: quantitative insulin-sensitivity check index, SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate.

Endothelial Function

Effect of Treatment

For microvascular function, resting microvascular perfusion did not differ between any of the time points (Table 4). ANOVA revealed an overall time effect for microvascular endothelial-dependent function. Post hoc analysis confirmed that the microvascular endothelial-dependent function was increased at 2 weeks, but not different from pre-treatment baseline at 12 weeks. In the macrocirculation,

the resting vessel diameters were the same across all the time points. Similarly, no differences were found in macrovascular endothelial-dependent and endothelial-independent function at any of the time points. Arterial stiffness remained similar to pre-treatment baseline at 2 weeks and at 12 weeks.

Table 4. Endothelial function during treatment

	Baseline	2 Weeks	12 Weeks	Treatment Effect	Degrees of Freedom
Microvascular Function					
Resting ACh Perfusion (PU)	41 ± 16	37 ± 11	38 ± 14	F =0.67, p =.51	2, 21
Increase in Perfusion ACh %	314 ± 214	423 ± 250[a]	348 ± 209[b]	F = 5.09, p =.01	2, 21
Resting SNP Perfusion (PU)	41 ± 17	39 ± 11	38 ± 9	F = 0.11, p =.90	2, 21
Increase in Perfusion SNP %	247 ± 126	284 ± 147	261 ± 152	F = 1.18, p =.32	2, 21
Macrovascular Function					
Resting FMD Diameter (mm)	3.5 ± 0.6	3.5 ± 0.6	3.5 ± 0.6	F = 0.10, p =.91	2, 19
FMD (%)	9.4 ± 6.8	12.0 ± 10.0	12.0 ± 8.1	F = 1.60, p =.21	2, 19
Resting GTN Diameter (mm)	3.6 ± 0.6	3.6 ± 0.6	3.6 ± 0.6	F = 0.04, p =.81	2, 19
GTN (%)	22 ± 7.4	23 ± 7.2	24 ± 7.2	F = 0.31, p =.74	2, 19
AIx	32 ± 9	31 ± 10	33 ± 9	F = 0.49, p =.62	2, 18

Results are expressed as mean ± standard deviation. [a]= different from baseline, [b] = different from 2 weeks. ACh: acetylcholine, SNP: sodium nitroprusside, FMD: flow-mediated dilatation, GTN: glyceryl tri-nitrate mediated dilatation, AIx: augmentation index.

Disease-related Inflammation and Endothelial Function

Pearson correlations were performed to examine the relationship between disease related parameters and endothelial function. This analysis revealed that absolute ESR and CRP were not related with any vascular parameter at any time point (p values > 07). Similarly, absolute DAS28 was not associated with any parameter of microvascular function (p values > 06); however, it was associated with baseline macrovascular endothelial-dependent function (r (22) = .45, p = .03). Changes (∆) in ESR, CRP and DAS28 relative to baseline were not

correlated with change in microvascular endothelial-dependent function at 2 weeks or at 12 weeks (p values > .10) (Table 5). Similarly, ΔESR and ΔDAS28 were not related with change in macrovascular endothelial-dependent function at 2 and 12 weeks. However, ΔCRP was associated change in macrovascular endothelial-dependent function at 2 weeks (r (22) = .52, p = .01).

Baseline ESR associated with the change in microvascular endothelial-dependent function at 2 weeks (r (22) = .48, p = .02) and baseline CRP was associated with change in microvascular endothelial-dependent function at 2 weeks (r (22) = .47, p = .02) and at 12 weeks (r (21) = .42, p = .05). Thus, those with high levels of disease-related inflammation at baseline showed the greatest improvement in microvascular function. No other significant correlations emerged from these analyses.

Table 5. Association between change in endothelial function and change in disease-related inflammation

	Change Score	Δ logESR 2 weeks	Δ logESR 12 weeks	Δ logCRP 2 weeks	Δ logCRP 12 weeks	Δ DAS28 2 weeks	Δ DAS28 12 weeks
		-0.25 ± 0.31	-0.11 ± 0.46	-0.67 ± 0.41	-0.49 ± 0.34	-1.50 ± 1.40	- 1.38 ± 1.28
Δ ACh (%) 2 weeks	110 ± 126	r (22) = - 0.16	r (21) = - 0.04	r (21) = - 0.26	r (22) = - 0.23	r (22) = - 0.16	r (22) = 0.11
Δ ACh (%) 12 weeks	29 ± 214	r (22) = - 0.22	r (20) = 0.03	r (21 = - 0.14	r (21) = 0.15	r (21) = 0.04	r (20) = 0.25
Δ SNP (%) 2 weeks	38 ± 83	r (22) = 0.06	r (21) = - 0.02	r (22) = - 0.29	r (21) = - 0.14	r (22) = 0.15	r (21) = 0.12
Δ SNP (%) 12 weeks	9 ± 120	r (21) = 0.04	r (20) = 0.10	r (21) = - 0.19	r (21) =- 0.22	r (21) = 0.14	r (21) = 0.18
Δ FMD (%) 2 weeks	2.6 ± 8.8	r (22) = 0.28	r (21) = 0.27	r (22) = 0.52*	r (22) = 0.26	r (22) = 0.23	r (21) = 0.24
Δ FMD (%) 12 weeks	2.6 ± 7.2	r (22) = 0.09	r (21) = 0.19	r (22) = 0.34	r (22) = 0.14	r (22) = 0.16	r (21) = 0.24
Δ GTN(%) 2 weeks	0.7 ± 6.7	r (22) = -0.13	r (21) = - 0.36	r (22) = 0.31	r (22) = - 0.17	r (22) = - 0.15	r (21) = - 0.02
Δ GTN (%) 12 weeks	1.2 ± 6.2	r (19) = 0.09	r (18) = - 0.13	r (19) = 0.29	r (19) = 0.24	r (19) = 0.25	r (19) = 0.06
Δ Alx 2 weeks	-1.1 ± 7.5	r (18) = 0.67**	r (17) = 0.30	r (18) = 0.27	r (18) = 0.04	r (18) = 0.24	r (17) = 0.11
Δ Alx 12 weeks	1.5 ± 8.1	r (19) = 0.44	r (18) = 0.18	r (19) = 0.16	r (19) = 0.09	r (19) = 0.06	r (18) = - 0.34

Change scores are expressed as mean ± standard deviation. * p = .01, **p = .002

Discussion

The present analyses revealed an improvement in microvascular endothelial-dependent function after 2 weeks of treatment with anti-TNF-α which returned to baseline after 12 weeks in RA patients who had newly started anti-TNF-α treatment. There was no change in macrovascular endothelial function during the 12 week follow-up period. In addition, microvascular and macrovascular endothelial function were not associated with disease-related inflammation at baseline, nor were the changes in microvascular and macrovascular endothelial function related to the changes in disease related inflammation. However, those with higher levels of ESR and CRP showed greater change in microvascular endothelial-dependent function.

The present result for microvascular endothelial-dependent function resonates with the findings of a previous study. Komai and colleagues [492] also found improvements after 2 weeks of treatment with anti-TNF-α when assessing microvascular endothelial-dependent function using forearm blood flow (FBF) response to ACh. In addition, although lower than that at 2 weeks, FBF was still increased after 6 weeks of treatment [492]. Therefore, it is possible that after an initial improvement, a gradual decrease in microvascular endothelial function occurs between 6 weeks and 12 weeks. Without a 6 week assessment in the current study this must remain speculation. In contrast, Hansel and colleagues [434] observed no change in microvascular endothelial-dependent function at 2 weeks in RA patients; however, these patients exhibited consistently lower disease-related inflammation than the present cohort. Given that elevated baseline inflammation was associated with greater change in endothelial function it is possible that change in endothelial function after treatment is unlikely in patients with low disease-related inflammation and this may explain the seemingly contrasting findings. Another study that examined microvascular endothelial function in patients with high baseline disease-related inflammation also found an improvement after anti-inflammatory treatment [436]. However, that pilot study had a small sample size and inconsistent follow-up period.

Macrovascular endothelial-dependent function did not change in response to anti-TNF-α treatment, which is in contrast to previous research [431,438,493-495,500]. Comparison of the patients included in all these studies revealed that baseline macrovascular endothelial-dependent function was better in the current study than in previous RA patient samples (9.4% vs. 2.8 – 7.0%, respectively). Studies which included a healthy control group showed lower baseline macrovascular endothelial-dependent function in the RA patients [431,493,500]. However in the present study, the baseline macrovascular endothelial-dependent function of the RA patients was comparable to healthy control participants described in the previous chapter (p = .33), therefore making an improvement in response to treatment unlikely. Furthermore, post hoc analyses were conducted using GPower3 [578] with significance set at .05 to determine the power to detect differences over time in microvascular and macrovascular endothelial function. On the basis of sample size, effect size, and correlations between the assessments at different time points, this analysis revealed that the obtained power was .99 to detect differences in microvascular and .98 to detect differences in macrovascular endothelial function. Thus, the obtained power in this study was sufficient to detect differences in endothelial function.

Arterial stiffness assessed with PWA remained unchanged throughout the study period which is a similar finding to a number of other studies that have examined PWA [461,517] and pulse wave velocity [492] in response to anti-TNF-α treatment in RA. In contrast to the present findings, Galarraga and colleagues [464] found a sustained improvement in PWA after 2 and 4 months of treatment with anti-TNF-α. However, their patients had greater disease-related inflammation at baseline than patients in the present study, and the subsequent improvement in disease-related inflammation might have contributed to the change in arterial stiffness. The present findings showed that arterial stiffness was reduced at 2 weeks, albeit non-significantly, and this change correlated with the change in ESR at 2 weeks. Therefore, it might be possible that anti-inflammatory treatment has an affect on arterial stiffness when baseline disease-related inflammation is high; an issue that warrants further investigation.

To our knowledge the current study was the first to examine the effect of anti-TNF-α on both microvascular and macrovascular endothelial function in the same patients. The present findings extend the previous chapters in that changes in microvascular and macrovascular endothelial function appear to be differentially regulated and may be selectively affected by disease-related inflammation in RA. Furthermore, there is increasing evidence that in early atherosclerosis, ED in the microvasculature may occur independently of ED in the macrovasculature [614,615]. It is well established that in type II diabetes the onset of microvascular disease occurs before macrovascular disease [572]. The reason for this has not yet been fully elucidated, but a number of *in vitro* studies have indicated that oxidative stress, endothelial-leucocyte interactions, platelet recruitment and pro-inflammatory cytokines like TNF-α can originate in small resistance vessels [616-619]. These molecules may subsequently spread to the macrovessels and initiate a pro-inflammatory state which can lead to lesion development [574]. These findings suggest that microvascular inflammation may occur before inflammatory changes in the macrovessels. Importantly, anti-TNF-α can ameliorate all the above factors in the vascular endothelium [443], and this translates into immediate reversal of microvascular ED when anti-TNF-α is infused into the brachial artery of RA patients with active disease [491]. Interestingly in the present study, higher baseline ESR and CRP were associated with greater change in microvascular endothelial-dependent function in response to anti-TNF-α treatment. Further, there was trend for lower microvascular endothelial-dependent function relative to previously assessed healthy controls (p = .07). This suggests that effective control of disease-related inflammation in those patients with the most active disease had more profound effects on microvascular endothelial function, where some underlying dysfunction may have been present.

The transient improvement in microvascular endothelial function mirrored the change in HDL-C. HDL-C associates with microvascular and macrovascular ED in healthy individuals and diabetics [620-622] as well as RA patients [466,471,477]. HDL-C prevents low density lipoprotein cholesterol (LDL-C) from oxidation and is involved in cellular efflux of accumulated LDL-C particles in the vasculature, which in turn prevents foam cell accumulation and subsequent ED [622]. In addition, HDL-C exerts very specific effects in

the vasculature including the reduction of TNF-α mediated superoxide release, stimulating production of EPC, and stimulation of NO in endothelial cells [623-626], all of which increase endothelial dependent vasodilatation of the vessel [623]. Given that both HDL-C levels as well as its anti-oxidant ability can increase with administration of anti-TNF-α in RA [142,627], it is possible that the improvement in microvascular endothelial function after 2 weeks of treatment with anti-TNF-α was mediated by increased HDL-C levels.

Treatment with anti-TNF-α effectively controlled CRP and DAS28 at all follow-up points in the current study, but disease-related inflammation was not associated with microvascular endothelial function and most parameters of macrovascular endothelial function. Surprisingly, DAS28 positively associated with macrovascular endothelial-dependent function at baseline. This association was unexpected, and implied that patients with high disease activity had better macrovascular endothelial function. It is possible that this association may have been a chance finding, given the number of associations that were performed. Interestingly, the change in CRP associated with the change in macrovascular endothelial-dependent function at 2 weeks, thus suggesting that lower CRP at 2 weeks may have contributed to the non-significant increase in macrovascular endothelial-dependent function observed in response to treatment.

Although the link between disease-related inflammation and endothelial function has been hypothesised [123,124], a number of cross-sectional studies have reported that disease-related inflammation is not associated with microvascular endothelial-dependent function [451] or macrovascular endothelial-dependent function [435,452,455,458]. Similarly, most longitudinal studies do not find correlations between change in disease-related inflammation and change in microvascular and macrovascular endothelial function in response to anti-inflammatory treatment [436,495,499,501], with only one study actually reporting an association [488]. Surprisingly, several studies did not report associations between changes disease-related inflammation and changes in microvascular or macrovascular endothelial function [431,434,438,491,493,494,500]. Collectively, these findings do not support a relationship between disease-related inflammation and

endothelial function, but further studies with larger sample sizes are required to confirm these findings.

The decrease in SBP and DBP after 2 and 12 weeks of treatment highlights the important role inflammation might play in regulating blood pressure in RA. In previous studies in RA patients there has been speculation regarding the role of systemic inflammation in relation to blood pressure. Patients on medium dose oral prednisolone (>=7.5mg) have been shown to have increased odds for hypertension even after adjustments for other risk factors [491]. However, it remains unclear whether this association reflects a deleterious side-effect of glucocorticoids or is due to increased systemic inflammation in patients who are on such treatment (channelling bias). The present findings suggest that systemic inflammation could be at least partially responsible for raised blood pressure in these patients. Indeed, the reduction in the blood pressure may have been a simple conditioning effect over time. However, data from the reliability study presented in Chapter 3 showed that SBP and DBP remained stable in 12 healthy control participants followed up on 4 occasions over a six week period. It is therefore likely that a reduction in TNF-α levels (reflected by the reduction in CRP and DAS28) contributed to the reductions in blood pressure, but further studies are needed to confirm these findings.

In conclusion, the present study revealed that treatment with anti-TNF-α resulted in a transient improvement in microvascular but not macrovascular endothelial function. Furthermore, the improvement could be mediated by a favourable effect of HDL-C on microvascular endothelial function. In addition, the longitudinal design of the present study revealed that systemic markers of inflammation did not associate with endothelial function in patients with RA.

Chapter 8: General Discussion

Summary

This thesis focussed on endothelial function in patients with rheumatoid arthritis (RA). Laser Doppler Imaging (LDI) with iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) was used for the assessment of microvascular endothelial-dependent and endothelial-independent function respectively. Macrovascular endothelial-dependent and endothelial independent function was measured using flow-mediated dilatation (FMD) and glyceryl-trinitrate-mediated dilatation (GTN) respectively. In addition, pulse wave analysis was used to characterise arterial stiffness. The work presented in Chapter 4 revealed that microvascular and macrovascular endothelial function were not significantly different between RA patients and healthy controls, arterial stiffness, however, was greater in RA. This piece of work was also one of the first in RA to examine endothelial function in various vascular beds in the same patient: both the cross-sectional studies presented in Chapter 4 and the longitudinal studies presented in Chapter 5 suggested that microvascular and macrovascular endothelial function were independent from each other.

One of the main hypotheses in this thesis was that disease-related systemic inflammation is a major determinant of microvascular and macrovascular endothelial function in RA. The results obtained here however, do not support this hypothesis. The relationship between endothelial function and disease-related inflammation has been largely assumed from data showing improvements in endothelial function after anti-inflammatory treatment [431,494,497], along with a small number of studies that have reported associations between inflammation and endothelial function [433,448,451,454], leading to the hypotheses that disease-related inflammation directly influences endothelial function [123,124]. The systematic review presented in Chapter 7 highlighted that the overwhelming majority of studies do not support the link between inflammation and endothelial function. This finding was also observed in our cross-sectional study presented in Chapter 5, which included a large sample of patients with different ages, disease severity and treatments. Furthermore, if disease-related inflammation does contribute to endothelial dysfunction (ED), then improvements in endothelial function after potent anti-inflammatory

treatment should be observed alongside lowered inflammatory markers, such as the ESR and CRP. However, the longitudinal studies presented in Chapter 6 showed that this was not the case, as improvement in microvascular endothelial-dependent function after anti-inflammatory treatment was not related to the change in disease-related inflammation. This is consistent with a number of other studies [436,495,499,501]. Interestingly, RA patients with very high disease-related inflammation at baseline had greater change in microvascular endothelial function after anti-TNF-α treatment (Chapter 6) suggesting that inflammation might still play a role in ED, but this is likely to be modest, or alternatively that there may be drug-specific effects that require further investigation. It is important to highlight that from the systematic literature review, it appears that the quality of studies that report no significant associations between disease-related inflammation and endothelial function is higher than studies which do find a significant association (See Table 1 of Chapter 7). Therefore, the findings of the experimental part of this thesis and the systematic review collectively suggest that factors other than inflammation need to be examined to decipher their impact on endothelial function in RA.

Microvascular endothelial-dependent function was not associated with individual CVD risk factors but was related with the Framingham Risk Score (FRS) and Systematic Coronary Risk Evaluation (SCORE). Interestingly, the improvement that was found in the microvasculature following two weeks of anti-TNF-α treatment occurred with a concomitant improvement in high-density lipoprotein cholesterol (HDL-C) (Chapter 6), as well as an improvement in systolic blood pressure (SBP) and diastolic blood pressure (DBP) at 2 and 12 weeks. This might suggest an interaction between inflammation and classical CVD risk factors that may then impact upon the microvasculature in RA. In contrast, macrovascular endothelial-dependent function was not linked either with markers of inflammation, or with classical CVD risk (either as individual CVD risk factors, or the FRS or SCORE risk algorithms), although classical CVD risk (FRS, the presence of the metabolic syndrome, along with individual CVD risk factors such as hypertension and hypercholesterolaemia) associated with macrovascular endothelial-independent function (Chapter 5). Collectively, these findings suggest differential regulation and impacts by inflammation and classical

CVD risk on the functional responses of the microvasculature and macrovasculature in patients with RA.

Implications

Factors that May Affect Endothelial Function in RA

As disease-related inflammation and endothelial function were not associated, other factors may be affecting the vasculature in RA. The present thesis suggests that classical CVD risk may play a predominant role. Classical CVD risk was associated with microvascular endothelial-dependent function and macrovascular endothelial-independent function in the analysis presented in Chapter 5. Thus, this hypothesis seems very plausible, especially as there is an increased prevalence in a number of classical CVD risk factors in RA [628]. In particular, the prevalence of dyslipidaemia [142] and hypertension [440] is elevated, and their control is worse in RA patients [128,146] compared to the general population [629]. Classical CVD risk factors can improve after controlling RA disease-related inflammation [155,446], which is indicative of a relationship between these two factors. The present work showed that the improvement in microvascular endothelial function after anti-inflammatory treatment was mirrored by an improvement in HDL-C (Chapter 6). The significance of this finding is put into context by the fact that the most evident lipid abnormality in RA is a reduction in HDL-C levels particularly during active disease [148,630,631], which may directly impair the vasodilatory capacity of the vessels [623-626,632]. Blood pressure was also lowered after anti-inflammatory treatment in the current work (Chapter 6), and several studies have suggested that inflammation may be an independent risk factor for hypertension in the general population [133,134,633]. Moreover, the improvement in microvascular endothelial function may contribute to the improvement in the blood pressure as microvessels play a major role in regulating total peripheral resistance – a key determinant of blood pressure. Importantly, inflammation associates with other measures of vascular function such as carotid intima-media thickness (cIMT) only in the presence of classical CVD risk factors [219]. Therefore, rather than inflammation exerting direct effects on the endothelium independently from other factors,

the magnitude of ED in RA may depend on subtle interactions between inflammation, classical CVD risk factors and endothelial function.

Factors other than classical CVD risk factors have also been postulated to affect endothelial function in RA. The aetiology of RA has a considerable genetic component [634], in particular, certain Human Leukocyte Antigen (HLA) alleles have consistently been shown to associate with RA, and these also appear to associate with worse macrovascular endothelial-dependent function in this group of patients [452]. The precise mechanism for this association is not clear, but the shared epitope associates with worse RA disease severity [635,636], and consequently, patients with greater disease severity may be predisposed to impairments in the vasculature which result in ED [637]. Interestingly, recent work from our group has shown a clear link between the shared epitope (and whether one has a "single dose" or a "double dose") with dyslipidaemia in patients with RA. We have also demonstrated the association of several polymorphisms, and their interplay with environmental factors, with hypertension or prevalent CVD in patients with RA [190,638-642]. Genetic polymorphisms may also impact directly on endothelial cell function. For example, polymorphisms in the nitric oxide synthase 3 gene (NOS 3), which is responsible for regulating endothelial nitric oxide synthase (eNOS) can result in reduced nitric oxide (NO) bioavailability [643]. Polymorphisms of the NOS 3 gene has been identified in individuals presenting with coronary vasoconstriction [644], however, little is known about NOS 3 gene expression in RA patients and further studies are warranted.

Limitations

A number of *in vitro* studies have revealed that endothelial cells display heterogeneous responses to stimulation in different vascular beds [224,225,571]. In the present work no association was found between microvascular and macrovascular endothelial-dependent function in RA which seemingly supports these *in vitro* observations. However, the techniques employed to examine microvascular and macrovascular endothelial-dependent function have a number of inherent differences. LDI and FMD involve distinct pathways to stimulate NO; LDI uses a pharmacological stimulus (ACh and SNP) to activate NO, whereas FMD uses a physiological stimulus (shear stress) [586,587]. Consequently, these

stimulus profiles may differentially activate NO. For example, FMD predominantly evokes maximum NO release as inhibition of NO completely abrogates the vasodilatory response to shear stress [379], but only 30-40% of the microvascular response to ACh is reduced by NO inhibition [585]. Thus, the independence of these vascular beds could be due to mechanistic differences in the techniques and it is therefore not possible to confirm if *in vivo* endothelial responses reflect *in vitro* observations [224,225,571]. Interestingly, correlations between peripheral and coronary endothelial function are stronger when the same stimulus is applied [352,588] than when different stimuli are applied [351], so use of a similar stimulus in the microvessels and the macrovessels may provide clearer information on the associations between these vascular beds. The techniques employed in the present study were selected because they are widely used in the literature.

Differences in methodology can make comparison of findings between studies difficult. For example, manual methods to detect and mark out the vessel diameter during FMD is common in a variety of studies [493,499,501,505], but this can be less accurate when compared with automated wall tracking software which detects and calculates arterial diameters in real-time and greatly reduces the variability found in the measurements [535,547]. In addition, different techniques have been employed to assess microvascular endothelial function (forearm blood flow (FBF) vs. LDI). FBF stimulates the vessels of the whole limb [372], whereas LDI only stimulates the tissues at the site of the iontophoresis chambers [371]. Therefore, it might be possible that recruitment of differing number of vessels elicit variable perfusion responses between the techniques. However, to our knowledge, FBF and LDI have not been directly compared, and further studies are necessary to identify if each technique provides a similar characterisation of endothelial function. Even studies that use only LDI to assess microvascular endothelial function have differences between each other. Unlike the current studies, others have incrementally increased the iontophoresis charge to deliver vasoactive agents in a stepwise manner [436,449]. However, the iontophoresis protocol used in the present work was adapted to suit the patients who may have discomfort in keeping inflamed joints still for long periods of time. Furthermore, initial pilot work showed that a plateau in the endothelial function

response could be achieved with the current protocol in healthy individuals (data not shown).

Global CVD risk was evaluated using the FRS [528] and SCORE [529], while the presence of the metabolic syndrome was also assessed [533]. To our knowledge these measurement scales have never been validated in inflammatory conditions such as RA. Some evidence in the general population suggests that the FRS algorithms may underestimate risk in the elderly (e.g. > 75) [645], while both the FRS and SCORE can underestimate CVD risk in almost a third of at risk females [645]. This may have important implications for RA where the majority of patients are elderly and female. Another limitation of these CVD risk algorithms is that they do not take into account novel CVD risk factors like disease-related inflammation which also contribute to the increased risk of CVD [220]. For example, some of the classical CVD risk factors incorporated in the algorithms such as blood pressure, lipids and insulin resistance can be affected by RA-disease related inflammation [111,155,446]. Therefore, algorithms which incorporate inflammation may provide better prognostic information on CVD risk in populations like RA. One such algorithm is the Reynolds risk score, which incorporates C-reactive protein (CRP) along with some of the classical CVD risk factors used for the FRS and SCORE [646]. Studies comparing conventional risk scores with Reynolds may give insight on whether Reynolds provides greater prognostic information on CVD risk than conventional risk utilities. It is worth mentioning that even Reynolds may have limitations as inflammatory markers such as CRP can fluctuate with the course of disease in RA patients, and it may therefore be difficult to determine 10 year CVD risk from a single CRP measurement. Alternatively, the recent recommendation by the European League Against Rheumatism (EULAR) cardiovascular group of multiplying the nationally recommended risk score by a factor of 1.5 [647] (to account for the additional risk afforded by RA and all the mechanisms this may encompass) could also be used in this context.

RA patients in the cross-sectional study were older than healthy control participants in the present work. Unfortunately, it proved virtually impossible to recruit healthy age-matched participants, as older people are likely to have clinical conditions that could affect

endothelial function. The longitudinal study did not have a no-treatment RA control group, for obvious ethical reasons. It is acknowledged that the inclusion of a patient group on stable medication could have strengthened the design of the study, as it would have allowed exploration of potential fluctuations in endothelial function. However, such a control group is likely to have lower baseline levels of disease-related inflammation making comparisons to patients with active inflammation difficult.

In the current work, participants were not asked to withhold their anti-rheumatic treatment or vasoactive medications prior to the vascular assessments as examining patients while they maintain their normal medication regime may provide a better reflection of the patient's arterial condition in an everyday setting. Furthermore, many of the anti-rheumatic medications have long half lives and would require a substantial period of abstinence to completely eradicate the drug and its effects from the system [1]. Methotrexate was the most commonly used anti-rheumatic medication and patients receiving this treatment had greater FMD (Chapter 5). No other analyses with anti-rheumatic medications were conducted due to small number of patients on each medication regime. It is therefore not possible to assess the effects of other anti-rheumatic medications on endothelial function. Additional analyses of the effects of vasoactive medication revealed that microvascular and macrovascular endothelial-dependent responses in RA patients receiving such medications (N = 46) did not differ when compared to patients who were not on these medications (p's >.36). Moreover, none of the healthy control participants were receiving any vasoactive medication, and endothelial-dependent responses in the microcirculation and macrocirculation were similar between RA patients not on vasoactive medications (N = 53) and healthy controls participants (p's >.06). Therefore, vasoactive medications appeared to have little impact on the current findings.

Future research

Current Inflammation versus Cumulative Inflammation
Although acute bouts of inflammation are important, the inflammatory burden over a period of time may be more crucial for the progression of atherosclerosis [648]. Further, the

metabolic effects from long-term inflammation can lead to the development of several classical CVD risk factors [168,188,445]. In RA, inflammation levels constantly fluctuate [1], and the endothelium is exposed to varying inflammatory loads. Patients with greater periods of elevated disease-related inflammation may develop more damage to the endothelium due to the higher cumulative inflammatory burden on the vasculature. Thus, characterising the inflammatory fluctuations over a period of time (cumulative inflammatory burden) may be a better predictor for ED in RA. Such an approach takes into account the inflammatory load during the course of the disease, with high inflammatory load likely to result in greater progression of atherosclerosis [648]. Chapter 4 revealed that arterial stiffness was greater in RA patients relative to healthy control participants but this was not related to current inflammatory levels. A number of studies have shown that cumulative inflammation shows better association with arterial stiffness (and vasodilatory function) than current inflammatory levels [433,463,465], and this is also evident for advanced measures of atherosclerosis like cIMT [467,468,487]. In line with this evidence it is tempting to speculate that continuous inflammatory insult may lead to higher arterial stiffness, but without recording cumulative inflammatory burden, this remains speculative. Further studies that explore relationships between cumulative inflammation and vascular assessments representing different stages of atherosclerosis are warranted.

Targeting Different Inflammatory Pathways

Treatment of RA involves several medications including disease-modifying anti-rheumatic agents (DMARDs) such as methotrexate. DMARDs are typically used early in the course of the disease and may exert beneficial effects both in terms of RA disease progression as well as CVD risk factors, endothelial function and eventual CVD outcome [118,142,441]. However, in patients who do not respond sufficiently well to DMARDs, biologic agents such as the anti-TNF-α, anti-IL6 receptor, anti-CD20 and selective co-stimulation modulators are often used. These agents exert their therapeutic effects by targeting different pathways of inflammation, and recent preliminary investigations have reported improvements in RA symptoms and endothelial function in response to some of these agents [496-498,501]. One advantage of examining the effects of various medications is that they might help to characterise the contribution of different inflammatory pathways on ED in RA. Such

findings could also be extended to patients with CVD, as RA and atherosclerosis share similar inflammatory pathogenic pathways [648].

CVD Risk Factors and Endothelial Function

At present, only a minority of studies have examined the impact of classical CVD risk factors on endothelial function and have mainly focused on lipids; these studies report an association between endothelial function and lipid levels [433,456]. However to our knowledge, there are no studies that have explored associations for other classical CVD risk factors and endothelial function in RA. Classical CVD risk factors substantially contribute to ED in the general population and in patients suspected of CVD [359], and as mentioned earlier, may be influencing the association between disease-related inflammation and ED in RA. Furthermore, interventions that specifically reduce CVD risk such as exercise have been hypothesised to have a beneficial effect on RA disease-related inflammation and endothelial function [649], yet no study has assessed this in RA [131]. Therefore, studies which look at the long-term effects of interventions such as exercise on disease-related inflammation and endothelial function might provide insight on the specific interactions present between inflammation, CVD risk and endothelial function.

Can Endothelial Function Predict Future Risk of Cardiac Events?

Assessments of endothelial function are regarded as good surrogate markers of nitric oxide (NO) bioavailability [379,585]. NO regulates a number of atherosclerotic processes including inhibition of platelet and leukocyte activation as well as their adhesion to the vessel wall [273-276]; this helps to maintain a favourable environment in the vessel [241]. Several studies in patients suspected of CVD and those with established CVD report that poor microvascular and macrovascular endothelial function at baseline are good predictors of atherosclerotic progression and future cardiac events [354-356]. In contrast, little is known about the prognostic value of endothelial function in RA, with only one study exploring the predictive value of cIMT for cardiac events [524]. After a follow up period of 5 years, during which 8 patients experienced a cardiac event, it was shown that cIMT of those with cardiac event was significantly higher at the start of the study compared to those without a cardiac event. [524]. However, even though these data are promising, they

should be interpreted with caution due to the small sample size of the study (47 patients in total), the significantly older age of the patients that had a cardiac event, and the lack of follow-up examination for cIMT [524]. A number of studies have reported impaired microvascular endothelial function in the coronary circulation of RA patients [575,650,651], which can be reversed with anti-inflammatory therapy [575,650], but these findings need to be supported with long-term studies that actually show a reduction in CVD morbidity and mortality as a result of an improvement in endothelial function.

Conclusion

The present work showed that in RA patients, classical CVD risk factors might have a greater impact than RA disease-related inflammation on the vasculature. Furthermore, the effects of CVD risk factors and disease-related inflammation may differentially impact microvascular and macrovascular endothelial function. Further studies are needed to confirm whether CVD risk factors affect vascular function, and if assessments of vascular function are predictive of long-term CV outcomes in RA.

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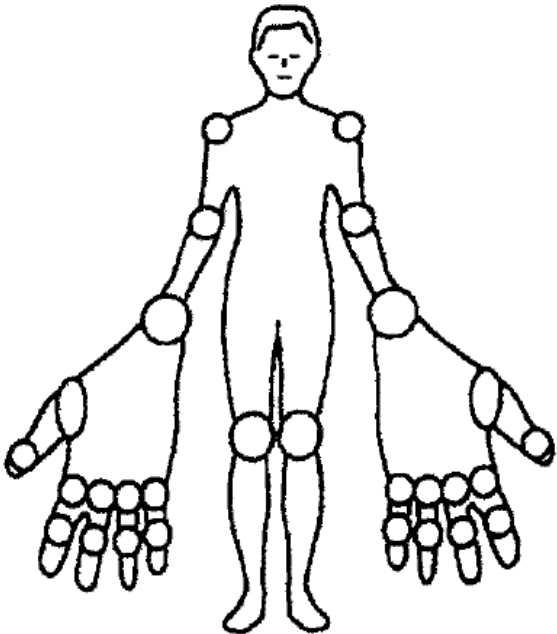
Appendix 1: Disease Activity Score
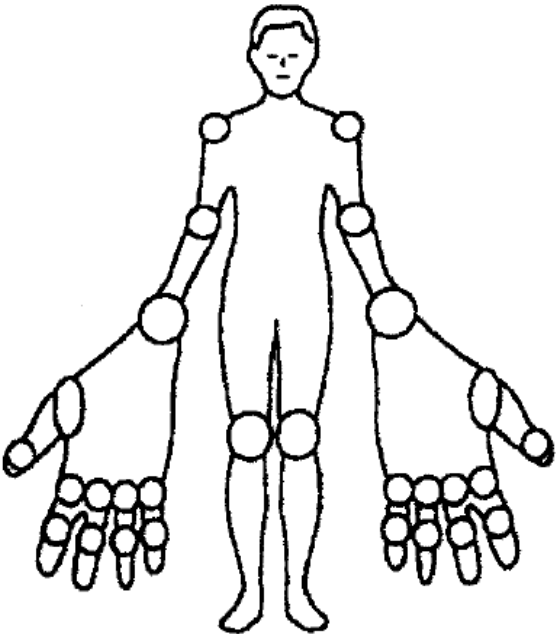
Please mark on the line above how your overall health has been in the last week

————————————————————————

Worst ever health   **Best ever health**

28 Joint Count

Swollen Joints	Tender joints

Total…………..   Total…………..

Appendix 2: Health Assessment Questionnaire

Name: _____   Date: _____

Please place an "x" in the box which best describes your abilities OVER THE PAST WEEK:

	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
DRESSING & GROOMING				
Are you able to:				
Dress yourself, including shoelaces and buttons?	☐	☐	☐	☐
Shampoo your hair?	☐	☐	☐	☐
ARISING				
Are you able to:				
Stand up from a straight chair?	☐	☐	☐	☐
Get in and out of bed?	☐	☐	☐	☐
EATING				
Are you able to:				
Cut your own meat?	☐	☐	☐	☐
Lift a full cup or glass to your mouth?	☐	☐	☐	☐
Open a new milk carton?	☐	☐	☐	☐
WALKING				
Are you able to:				
Walk outdoors on flat ground?	☐	☐	☐	☐
Climb up five steps?	☐	☐	☐	☐

Please check any AIDS OR DEVICES that you usually use for any of the above activities:

☐ Devices used for Dressing (button hook, zipper pull, etc.)   ☐ Built up or special utensils   ☐ Crutches

☐ Cane   ☐ Wheelchair

☐ Special or built up chair   ☐ Walker

Please check any categories for which you usually need HELP FROM ANOTHER PERSON:

☐ Dressing and grooming   ☐ Arising   ☐ Eating   ☐ Walking

Please place an "x" in the box which best describes your abilities OVER THE PAST WEEK:

	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO

HYGIENE

Are you able to:

Wash and dry your body?	☐	☐	☐	☐
Take a tub bath?	☐	☐	☐	☐
Get on and off the toilet?	☐	☐	☐	☐

REACH

Are you able to:

Reach and get down a 5 pound object (such as a bag of sugar) from above your head?	☐	☐	☐	☐
Bend down to pick up clothing from the floor?	☐	☐	☐	☐

GRIP

Are you able to:

Open car doors?	☐	☐	☐	☐
Open previously opened jars?	☐	☐	☐	☐
Turn faucets on and off?	☐	☐	☐	☐

ACTIVITIES

Are you able to:

Run errands and shop?	☐	☐	☐	☐
Get in and out of a car?	☐	☐	☐	☐
Do chores such as vacuuming or yard work?	☐	☐	☐	☐

Please check any AIDS OR DEVICES that you usually use for any of the above activities:

☐ Raised toilet seat   ☐ Bathtub bar   ☐ Long-handled appliances for reach

☐ Bathtub seat   ☐ Long-handled appliances in bathroom   ☐ Jar opener (for jars previously opened)

Please check any categories for which you usually need HELP FROM ANOTHER PERSON:

☐ Hygiene   ☐ Reach   ☐ Gripping and opening things   ☐ Errands and chores

Your ACTIVITIES: To what extent are you able to carry out your everyday physical activities such as walking, climbing stairs, carrying groceries, or moving a chair?

COMPLETELY	MOSTLY	MODERATELY	A LITTLE	NOT AT ALL
☐	☐	☐	☐	☐

Your PAIN: How much pain have you had IN THE PAST WEEK?
On a scale of 0 to 100 (where zero represents "no pain" and 100 represents "severe pain"), please record the number below.

Your HEALTH: Please rate how well you are doing on a scale of 0 to 100 (0 represents "very well" and 100 represents "very poor" health), please record the number below.