Investigation of atherosclerosis and the effects of anti-inflammatory therapy on plaque morphology in rheumatoid arthritis

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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School of Medicine
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Abstract

The University of Manchester
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PhD (Medicine)

Investigation of atherosclerosis and the effects of anti-inflammatory therapy on plaque morphology in rheumatoid arthritis

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Introduction
Rheumatoid arthritis (RA) is a systemic autoimmune condition, characterised by an inflammatory arthritis. It is associated with a 50% increased risk of cardiovascular (CV) mortality. Chronic inflammation is thought to lead to accelerated atherosclerosis in RA. There is some evidence to suggest that patients have a more inflammatory, unstable atherosclerotic plaque phenotype. The impact of advances in RA treatment, on cardiovascular co-morbidity remains unclear. The aims of the current study were to employ non-invasive imaging techniques to test the hypothesis that RA patients have more inflammatory, unstable atherosclerotic plaques compared to unaffected individuals and that treatment of active arthritis would lead to alterations in plaque composition and inflammation. Secondary aims were to evaluate the association of clinical phenotype and potential serological biomarkers of CV risk with plaque presence and phenotype.

Methods
A prospective pilot study of patients with active RA and age and sex matched controls was conducted. Subjects underwent clinical and serological evaluation, then carotid artery ultrasound was performed to screen for carotid plaque. Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) was performed on those with suitable plaque. A subgroup of patients had a carotid artery positron emission tomography (PET) scan. Patients were followed up with repeat clinical, serological and DCE-MRI assessments. The primary outcome evaluated was difference in plaque inflammation measured on DCE-MRI between patients and controls and in patients longitudinally. Secondary outcomes included differences in plaque composition on DCE-MRI, plaque inflammation on PET and the relationship of clinical, serological and imaging findings.

Results
130 patients and 52 controls were recruited and screened for carotid plaque. There was a higher prevalence of plaque on ultrasound in the patient group (53% vs 36%) and plaque was independently associated with high sensitivity C reactive protein (hsCRP). Carotid DCE-MRI data was analysed in 15 patients and 5 controls. There was no significant difference in plaque inflammation on DCE-MRI between the groups. However there was a significantly higher rate of plaque calcification in patients, despite similar plaque burden in both groups (73.3% vs 20%, p=0.038). All 15 patients exhibited features of high-risk plaque. Plaque inflammation was seen in all 13 patients in whom PET imaging was undertaken. No significant improvement in plaque inflammation was detected on DCE-MRI over time, which was in keeping with the lack of clinical improvement found in most cases.

Conclusions
Increased prevalence of atherosclerosis and differences in plaque phenotype were observed in this study and findings would support the hypothesis that patients have a more high-risk plaque phenotype. The high prevalence of calcified lesions in RA is a novel finding which warrants further investigation. The study was underpowered to detect significant changes in plaque inflammation, measured on DCE-MRI, between the groups and in patients over time. However, this study provides valuable data with which to plan a larger study to investigate the effects of anti-inflammatory therapy on atherosclerosis in RA in the future.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Acknowledgements

I would like to take this opportunity to express my thanks to Ian Bruce for first sparking my interest in academic rheumatology and for all the subsequent opportunities, support and guidance that he has provided over the last few years. I would also like to thank Yvonne Alexander and John Waterton for their advice and support during my PhD and also to James O’Connor, my advisor.

Completion of this PhD study would not have been possible without the help and collaboration of many people and organisations. I would particularly like to thank Penny Cristinacce, Heather Williams and Paul Hockings for all their help and support with the imaging aspects of the study. I would also like to thank Michael Jackson for his support with the flow cytometry methods and the team at the Specialist Assay Laboratory at CMFT, in particular Dr. Phillip Pemberton. I am grateful to the team at the Vascular Imaging Laboratory, University of Washington for the training and guidance that they provided throughout the study.

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I would like to thank the other clinical research fellows in the department, who have been a fantastic source of support and who made the last 3 years particularly enjoyable. Most importantly I would like to thank my partner Tim and my family for their unending support and encouragement, not just in the last 3 years but throughout my career.
Preface

I graduated from The University of St. Andrews in 2002 with a BSc. in Medical Science. I then completed my undergraduate medical training at The University of Manchester and graduated in 2005 with an MBChB (Honours). I undertook junior medical training in the North West of England, during which time I developed an interest in academic rheumatology. In 2009, I was successful in applying for an NIHR Academic Clinical Fellowship in Rheumatology in Manchester. During this post I began to develop my research interests and skills, under the supervision of Professor Ian Bruce. I also completed an MSc in Clinical Rheumatology, awarded by the University of Manchester in 2011. I was awarded a North West England Medical Research Council Clinical Pharmacology and Therapeutics Fellowship in 2011 to undertake this PhD.

I hope to continue to develop the research themes of this thesis. Through employing an integrated approach of imaging and laboratory techniques to investigate cardiovascular risk in rheumatoid arthritis, I hope to improve our ability to identify and treat patients at high risk of cardiovascular complications in the future.
Abbreviations

ACPA  Anti-citrullinated peptide antibody
ACR   American College of Rheumatology
AHA   American Heart Association
BMI   Body mass index
CAM   Cellular adhesion molecule
CMRI  Carotid MRI
CRP   C-reactive protein
CT    X-ray Computed tomography
CVD   Cardiovascular disease
DAS28 Disease activity score
DCE   Dynamic contrast enhancement
DMARD Disease modifying anti rheumatic drug
eGFR  Estimated glomerular filtration rate
EDTA  Ethylene diaminetetraacetic acid
EMP   Endothelial microparticles
ESR   Erythrocyte sedimentation rate
EULAR European League Against Rheumatism
FDG   Fludeoxyglucose
GSM   Grey scale measurement
HsCRP High sensitivity C-reactive protein
HR    Hazard ratio
HAQ   The Stanford Health Assessment Questionnaire
HD    High definition
HDL   High density lipoprotein
HR    Hazard ratio
ICAM  Intercellular adhesion molecule
ICC   Intra-correlation co-efficient
IF-8  Interferon gamma
IL-1  Interleukin 1
IL-6  Interleukin 6
IMT   Intima media thickness
IPH   Intraplaque haemorrhage
IRR   Incidence rate ratio
JSD   Jugular symphysis distance
ktrans Transfer contrast of contrast
LDL   Low density lipoprotein
LM    Loose matrix
LRNC  Lipid rich necrotic core
MAP   Mean arterial pressure
MI    Myocardial infarction
MP- RAGE Magnetization Prepared Rapid Acquisition Gradient Echo
MRI   Magnetic resonance imaging
NICE  National Institute of Health Research
OSEM  Ordered subset expectation maximisation
OR    Odds ratio
PD    Proton density
PET   Positron emission tomography
PPP   Platelet poor plasma
PWV  Pulse wave velocity
r    Correlation co-efficient
RA   Rheumatoid arthritis
RF   Rheumatoid factor
RMI  Wall remodelling index
SUV  Standardised uptake value
TBR  Target to background ratio
TC:HDL Total cholesterol to HDL ratio
TE   Echo time
TIA  Transient ischaemic attack
TOF  Time of flight
TNF  Tumour necrosis factor
TR   Repetition time
SMR  standardised mortality ratio
UA   Unstable angina
UHD  Ultra high definition
US   Ultrasound
UW   University of Washington
VCAM-1 Vascular cell adhesion molecule-1
Vp   Partial volume of blood
95%CI 95% confidence interval
1. Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterised by a symmetrical erosive inflammatory polyarthritis. Patients suffer with pain, stiffness and swelling of multiple joints and over time chronic inflammation can lead to joint damage and disability. Approximately 70% of patients will also have antibodies directed against rheumatoid factor (RF) or citrullinated peptides (ACPA) and although these antibodies do not drive the disease, they may be implicated in pathogenesis (1). Whist arthritis is the predominant feature of RA, it is a multi-system disease with a range of extra-articular manifestations including lung disease, subcutaneous nodules and vasculitis. Patients have a shortened life expectancy and considerable reduction in quality of life as a result of the disease.

1.1.1 Classification of RA

There are no diagnostic criteria for RA and in practice the diagnosis is made on the basis of clinical signs and symptoms, laboratory measures (including antibodies and acute phase reactants) and radiological findings. However, classification criteria were developed by the American College of Rheumatism (ACR) in 1957 in order to define the population for clinical and epidemiological research. The revised criteria published in 1987 showed good sensitivity and specificity in established disease and are outlined in table 1.1 (2). With an increasing need to classify patients early in the disease course, in order to evaluate treatment and outcomes in early disease, further revision was made in 2010 to improve classification in earlier disease (3). This updated classification criteria are described in Table 1.2. Validation studies have suggested that the new criteria are more sensitive and specific in early disease, however patients with negative antibody tests may not be classified using the new criteria, when they would have been classified using the 1987 criteria (4). Further validation studies are underway examining the ability of the new classification criteria to predict outcomes.
Table 1-1 The 1987 ACR Classification criteria for rheumatoid arthritis (2)

<table>
<thead>
<tr>
<th>At least 4 of the following criteria must be present for at least 6 weeks for a diagnosis of rheumatoid arthritis to be made</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Morning stiffness of joints more than 1 hour</td>
</tr>
<tr>
<td>➢ Soft tissue swelling over three or more joints observed by a physician</td>
</tr>
<tr>
<td>➢ Swelling of the proximal interphalangeal, metacarpophalangeal or wrist joints</td>
</tr>
<tr>
<td>➢ Symmetrical arthritis</td>
</tr>
<tr>
<td>➢ Subcutaneous nodules</td>
</tr>
<tr>
<td>➢ Positive test for rheumatoid factor</td>
</tr>
<tr>
<td>➢ Radiographic erosions or peri-articular osteopenia in hand or wrist joints</td>
</tr>
</tbody>
</table>
Table 1-2 The 2010 American College of Rheumatology/ European League Against Rheumatism classification criteria for rheumatoid arthritis. (3)

<table>
<thead>
<tr>
<th>Target population</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients who:</td>
<td></td>
</tr>
<tr>
<td>1) have at least 1 joint with definite clinical synovitis (swelling)</td>
<td></td>
</tr>
<tr>
<td>2) with the synovitis not better explained by another disease</td>
<td></td>
</tr>
</tbody>
</table>

**Classification criteria for RA**

Scored based algorithm: add score of categories A-D; a score of ≥6 is needed for classification of a patient as having definite RA

**A. Joint involvement**

- 1 large joint 0
- 2-10 large joints 1
- 1-3 small joints 2
- 4-10 small joints 3
- >10 small joints 5

**B. Serology (at least 1 result is needed for classification)**

- Negative RF and negative ACPA 0
- Low positive RF or low positive ACPA 2
- High positive RF or high positive ACPA 3

**C. Acute phase reactants (at least 1 result needed for classification)**

- Normal CRP and normal ESR 0
- Abnormal CRP or ESR 1

**D. Duration of symptoms**

- <6 weeks 0
- ≥6 weeks 1

RF= rheumatoid factor, ACPA= anti-citrullinated peptide antibody, CRP= c-reactive protein, ESR= erythrocyte sedimentation rate
1.1.2 Epidemiology of RA

The prevalence of RA using the 1987 criteria is estimated to be between 0.3 to 1.4% but there is a variation across the world (5). In the UK an inception cohort study found a prevalence of 0.8% in adults and an annual incidence of 0.36 in females and 0.15 in males per 100,000 patient years(6). Females are more likely to be affected and risk increases with age (6). There is a suggestion that the incidence of RA has decreased in recent years and also that the severity may also have lessened (7).

1.1.3 Aetiology of RA

The cause of RA still remains unclear but it is likely that environmental factors trigger the disease in genetically susceptible individuals.

1.1.3.1 Genetic risk factors

RA is a complex genetic condition with around 60% of the population susceptible due to genetic risk (8). Approximately 50% of the genetic risk is due to variations in the human leucocyte antigen (HLA) region (8). This area is referred to as the “shared epitope” and many studies have found variations in this region not only to be associated with development of RA but also disease severity, extra-articular disease and mortality (9;10) (11). Interestingly, the association appears strongest in patients who are seropositive (10).

With the advent of genome wide association studies a number of new risk loci have been identified. Okada et al. recently published results of a genome wide association study meta-analysis of just less 30,000 RA patients (12). In addition to confirming known risk loci, they also identified 42 risk loci bringing the total of known loci to over 100. While causative genes and functions are still under investigation, most loci appear to be associated with immune system regulation (12).
1.1.3.2 Environmental risk factors

Smoking

Smoking is associated with a two-fold increased risk of developing RA and is thought to contribute 18-25% of the attributable risk of developing RA (13). The risk is highest in seropositive patients (14). It has also been shown that there is a gene-environment interaction, with smokers who have 2 copies of the shared epitope having the highest risk of developing ACPA positive RA (10). Costenbader et al. demonstrated a linear relationship with number of years of smoking and risk of developing RA. There continued to be an increased risk even after cessation (Relative risk (RR) [95% confidence interval [95% CI]: 1.47 [1.23, 1.76]) (15)

Periodontitis

Periodontitis, which occurs as a result of bacterial infection of the gingiva, has been linked with risk of RA. Presence of periodontitis is more prevalent in RA patients than controls, in particular seropositive patients (16). Circulating levels of p. gingivalis have been associated with presence of anti-citrullinated peptide antibodies in subjects with no history of RA and presence of the bacteria is associated with the development of RA (16). While there is currently no temporal link to suggest causation, in vivo studies suggest that citrullination can occur in the presence of p. gingivalis and the potential role of periodontitis in the pathogenesis of RA remains an area of interest.

Obesity

A number of studies have found an increased risk of developing RA in patients who are obese(17) (18;19). Adipokines are hormones released from adipose cells, which not only exert effects on insulin regulation but also have immunological effects. Alteration in these hormones occurs in obesity and can stimulate pro-inflammatory cytokine production and
immune cell stimulation. Altered levels of circulating adipokines could potentially contribute towards the development of RA (20).

Other possible risk factors include alcohol intake, hormonal factors such as parity, diet and socio-economic status however the evidence for these factors remains weak (13).

1.1.3.3 Antibodies

One of the hallmark characteristics of RA is the presence of auto-antibodies. Presence of certain auto-antibodies is associated with adverse prognosis however their role in the pathogenesis and perpetuation of disease is yet to be proven.

Rheumatoid factor

Rheumatoid factor (RF) is a group of antibodies directed against the Fc portion of immunoglobulin G. They are present in approximately 60% of patients but are not specific to RA (21). RF production is thought to be stimulated by presence of immune complexes and can be induced during infections and also in other chronic autoimmune conditions such as Sjogren’s syndrome and autoimmune hepatitis (21).

Nielen et al. demonstrated that in many cases RF positivity actually preceded symptoms of arthritis and it has been shown that patients are more likely to become RF positive with increasing disease duration (22). Presence of RF, particularly in high titres is associated with worse prognosis and extra-articular disease (1). This could suggest a role in the disease process, however in vitro and animal studies have failed to identify a definitive pathogenic role for RF.

Anti-citrullinated peptide antibodies (ACPA)

Antibodies to citrullinated peptides (ACPA) are a more recent discovery and similar to RF are found in approximately 60% patients with early RA (23). Unlike RF their specificity for RA is around 90% (1). Citrullination occurs in dying cells and usually citrullinated proteins
are quickly phagocytosed, thus rarely detected by the immune system. In situations where large amounts of cell death or defective debris clearing occur, these proteins can be exposed to the immune system. It is thought that in genetically susceptible individuals (those with the shared epitope), antibodies to the citrullinated proteins can develop (24). The lung and the periodontum have both been shown to be sites of citrullination in the presence of stimuli such as smoking and *P. gingivalis* infection (25;26). Although antibody production is likely to be initiated at distant sites, B cells within the synovium also produce APCA (27). In vitro studies have also demonstrated cross reactivity with proteins in the joint suggesting a possible pathological role for ACPA (28).

Despite extensive research no comprehensive causation for RA has been found. There is an increasing recognition that RA is likely to be syndrome with different risk factors and immunological processes leading to the common clinical syndrome of RA.
1.1.4 Pathophysiology within the joint

The rheumatoid joint is characterised by synovial hypertrophy with prominent immune cell infiltration, endothelial activation and neovascularisation. The hypertrophied tissue forms an invasive “pannus” which degrades cartilage and over time causes bone erosion. If left untreated, this often leads to joint destruction.

1.1.4.1 Key cells found in the inflamed synovium

T cells

RA is traditionally thought of as a T helper 1 driven (TH1) disease and one of the hallmarks of RA is T cell auto-reactivity (29). Despite this no specific auto-antigens have been identified.

There is a predominance of memory T cells within the joint rather than naïve T cells and a subset of these T cells lack surface co-expression of CD28. These “CD28 null cells” may be activated in an antigen independent manner (30). They are also resistant to apoptosis and secrete high levels of interferon gamma and Tumour necrosis factor (TNF) into the joint, enhancing the inflammatory cascade (31). These cells are rarely found in healthy individuals and presence in the peripheral blood and synovium of RA patients has been associated with more severe disease (32).

Although the trigger for activation, differentiation and migration of TH1 cells are poorly understood they play a central role in controlling and perpetuating the inflammatory response within the joint, though pro-inflammatory cytokine production (33).

Other T cell subsets have also recently been implicated in pathogenesis. Th17 cells are a subset of T cells which are expanded in the RA synovium and are pro-inflammatory (34). They stimulate fibroblasts and chondrocytes via secretion of TNF and interleukin 17. Regulatory T cells, which promote immune tolerance are suppressed in RA and have less functional capacity, thus are ineffective in controlling the immune response within the joint (34).
Macrophages

Macrophages play a central role in synovial inflammation in RA (5;34;35). Macrophages line the synovium in health however their number is vastly increased in disease and there is a predominance of the M1 type macrophages. These have a pro-inflammatory phenotype, secreting cytokines such as TNF, interleukins 1 and 6 in addition to matrix metalloproteinases, which degrade cartilage. Macrophage numbers decrease following successful treatment of RA (33).

Other immune cells

Increased numbers of mast cells, plasma cells and neutrophils are found in the inflamed synovium and secrete pro-inflammatory cytokines, angiogenic and vasoactive substances which contribute to the inflammatory response and changes within the synovium (5;34).

Mesenchymal cells

Fibroblast like synoviocyte proliferation occurs in RA (5). These cells secrete pro-inflammatory cytokines but also cartilage degradation enzymes stimulating local destruction. Osteoclasts and chondrocytes are also both activated and secrete matrix degradation enzymes, which lead to bone and cartilage destruction.

Endothelial cells

During active inflammation endothelial cells become activated, expressing cellular adhesion molecules which attract and facilitate migration of immune cells (5). Increased permeability allows easier access of immune cells into the tissue.
1.1.4.2 \textit{Key signalling molecules}

The immune cells exert their effect through signalling with cytokines. Whilst there is a complex network of different cytokines with feedback mechanisms, there are a few cytokines, which appear as master regulators and have provided successful therapeutic targets in the treatment of RA.

\textbf{Tumour necrosis factor (TNF)}

TNF is one of the dominant cytokines involved in RA (34). It is secreted predominantly by macrophages but also by other cells of the innate and adaptive immune system (5). Experimental animal models have demonstrated the importance TNF plays in the development of inflammatory arthritis (36). It has pleotropic effects on many aspects of the inflammatory response in RA both within the joint and also in distant tissues (34). It is found in increased levels in the RA synovium (37). Table 1.3 summarises the effects of TNF in RA. Inhibition of TNF is a key therapeutic strategic in the treatment of RA and leads to improvement in joint and extra-articular disease in a significant proportion of patients (38;39).

\textbf{Interleukin-6 (IL-6)}

The importance of IL-6 has been a more recent discovery in comparison to TNF. It is secreted from a number of immune cells but also high levels are secreted by fibroblast like synoviocytes (40). IL-6 has a number of systemic effects, which are described in Table 1.3. The anti-IL-6 agent toculizimab is an effective treatment for RA emphasising its functional importance (41).

\textbf{Interleukin-1 (IL-1)}

The IL-1 family of cytokines are produced by most immune cells but in particular macrophages (5). Increased IL-1 is found in the RA synovium and animal models have
suggested a causative role in the development of inflammatory arthritis (42). Production is regulated by TNF and although stimulation is reciprocal, IL-1 it is thought to be downstream of TNF (43). The local and systemic effects of IL-1 are described in Table 1.3. IL-1 inhibition has been demonstrated to be clinically effective in some patients, but less so than the other cytokine targeted treatments in RA (39).
<table>
<thead>
<tr>
<th><strong>Table 1-3 Actions of key cytokines in RA (5;34;44;45)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumour necrosis factor (TNF)</strong></td>
</tr>
<tr>
<td><strong>Local effects</strong></td>
</tr>
<tr>
<td>Immune cells</td>
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<td></td>
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<tr>
<td>Synovial vasculature</td>
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<td></td>
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<tr>
<td>Mesenchymal cells</td>
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<td></td>
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<tr>
<td><strong>Systemic effects</strong></td>
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<tr>
<td><strong>Interleukin 6 (IL-6)</strong></td>
</tr>
<tr>
<td><strong>Local effects</strong></td>
</tr>
<tr>
<td>Differentiation and activation on B cells and T cells</td>
</tr>
<tr>
<td>Fibroblast like synoviocyte activation and proliferation</td>
</tr>
<tr>
<td>Endothelial cell activation</td>
</tr>
<tr>
<td>Cellular adhesion molecule up-regulation</td>
</tr>
<tr>
<td>Enhances osteoclast maturation</td>
</tr>
<tr>
<td><strong>Systemic effects</strong></td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td><strong>Interleukin 1 (IL-1)</strong></td>
</tr>
<tr>
<td><strong>Local effects</strong></td>
</tr>
<tr>
<td>Leucocyte activation</td>
</tr>
<tr>
<td>Endothelial cell activation</td>
</tr>
<tr>
<td>Fibroblast like synoviocyte proliferation</td>
</tr>
<tr>
<td>Stimulates pro-inflammatory respond</td>
</tr>
<tr>
<td>Particular role in stimulation of matrix metalloproteinase production</td>
</tr>
<tr>
<td>Suppression of proteoglycan production by chondrocytes.</td>
</tr>
<tr>
<td><strong>Systemic effects</strong></td>
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</table>
1.1.5 Treatment of RA

Disease modifying drug therapy is the cornerstone of treatment. However it has only been in the last 35 years that the use of methotrexate and other disease modifying anti-rheumatic agents (DMARDs) has been widely accepted. Prior to this, the mainstay of therapy has been non-steroidal anti-inflammatory agents and periodic use of glucocorticoids or gold salts. While these agents did improve joint symptoms they were associated with a significant burden of toxicity (46;47).

Two key advances in the management of RA have occurred in the last decade. The first is the recognition that early treatment using DMARDs, with the aim of remission leads to better long-term outcomes. A number of studies have demonstrated that with early initiation of disease modifying therapy (ideally within 3 months of symptoms onset) and quick escalation of therapy to target a low disease activity state, radiographic progression of disease is retarded (48-50). This approach has been recognised internationally and the National Institute of Clinical Excellence and Health published guidelines on the diagnosis and management of RA which reflect this (38). The current management strategy of RA is early referral and diagnosis with rapid control of inflammation using a combination of disease modifying agents, including methotrexate. Physiotherapy, occupational and psychological therapy are also important aspects of the modern management of RA.

The second key advance has been the development of biologic therapies. The first biological therapy licenced for the treatment of RA was infliximab, a monoclonal antibody directed against TNF. It is an intravenous agent and was proven to be clinically effective in treating patients who had failed to respond to methotrexate and also to slow radiographic progression (51). This drug provided an opportunity to treat a group of patients who until then, had suffered with chronic uncontrolled disease. Further anti-TNF drugs were licenced which were in sub-cutaneous form (39). Other monoclonal anti-bodies directed at key cytokines including IL-1 and IL-6 blockers were also developed, which are now in clinical use (39).

Rituximab is a monoclonal antibody, which targets B cells, which express CD20. It was initially licenced as a treatment for lymphoma, however a case report published in 1999 described the sustained improvement of RA, in a patient undergoing rituximab treatment for lymphoma (52). Further open labelled studies, then a randomised clinical trial confirmed the efficacy of rituximab in treating refractory RA (53;54). This was of particular
interest as the role of B cells in the pathogenesis or RA is poorly understood and as earlier discussed, RA is thought of as a predominantly T cell driven disease.

The advent of biologic therapy has revolutionised the care of refractory RA and clinical trials have also demonstrated efficacy in early disease. However, their expense and a theoretical latent increased risk of cancer, has meant that their use has been rationalised to those failing traditional DMARD therapy (55).

1.1.6 Clinical course and outcomes in RA

1.1.6.1 Disease activity

Rheumatoid arthritis is a chronic condition and patients follow a wide variety of trajectories. A longitudinal study of disease activity in individual early RA patients by Van Zeben et al. was published in 1994 (56). They found that only 50% of patients achieved sustained low disease activity state over a 6 year period. Eighteen percent of patients had fluctuating disease activity while seventeen percent had persistently high disease activity and the remainder either went from low to high or high to low disease activity. With the variation in clinical course and the treat to target approach currently used, a standardised measurement of disease activity is required.

To measure disease activity in clinical practice a composite score is used called the disease activity 28 score (DAS28). This score incorporates a tender and swollen joint count of 28 joints commonly affected in RA, acute phase reactants (C-reactive protein (CRP or Erythrocyte sedimentation rate (ESR)) and also a patient global visual analogue score of health. It can give an output of between 0 and 10 and the broad categories that these represent can be seen below in Table 1.4. This DAS28 has been validated for use both in clinical practice and clinical trial settings and treatment is targeted to aim for a low disease activity state (57;58). An improvement of the DAS28 of more that 1.2 is considered to be clinically significant (59).
Table 1-4 Disease activity states using DAS28 score (55)

<table>
<thead>
<tr>
<th>DAS28 score</th>
<th>Associated disease state</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2.6</td>
<td>Remission</td>
</tr>
<tr>
<td>2.6 - 3.2</td>
<td>Low disease activity</td>
</tr>
<tr>
<td>3.2-5.1</td>
<td>Moderate disease activity</td>
</tr>
<tr>
<td>&gt;5.1</td>
<td>High disease activity</td>
</tr>
</tbody>
</table>

There is evidence that new therapeutic strategies are having an impact on disease activity. The Norfolk Arthritis Register is an inception cohort study, which has captured and followed early arthritis patients since 1990. A study from this cohort demonstrated that the number of patients achieving remission within the first 3 year of diagnosis had significantly increased between 1990 and 2004 (60). Interestingly, there is a hypothesis that RA is becoming a milder disease with cohort studies demonstrating that at baseline patients present with a lower DAS28 score and in particular less swollen joints and lower ESR (61). It is likely that a combination of improvement in treatment strategies and also possibly a milder disease phenotype has led to an improvement in the disease activity that patients experience.

1.1.6.2 Extra-articular manifestations

Extra-articular disease occurs in approximately 40% of patients with RA (62). Manifestations are wide ranging both in terms of the organs involved but also in severity. Presence of extra-articular disease is associated with more severe articular disease and increased mortality (63). Common manifestations include subcutaneous nodules, inflammatory eye disease and sicca symptoms. Rarer manifestations include systemic vasculitis, amyloidosis and rapidly progressive interstitial lung disease. Recent studies have demonstrated a decline in incidence of vasculitis and amyloidosis when comparing RA patients treated in the last 15 years to those 30 years before (64-66). Conversely, rates of other manifestations, such as interstitial lung disease have in fact increased in more recent
years (67). This may be due to improvement in diagnostics but also increased use of drugs such as methotrexate, which may be associated with pulmonary toxicity.

### 1.1.6.3 Disease outcomes

**Joint damage**

Joint damage often occurs within the first 2 years of diagnosis and can progress rapidly in early disease, causing significant disability (68). There is clear evidence that effective early control of inflammation slows progression on joint damage. A study by Grigor et al. demonstrated that tight control of inflammation lead to significantly less joint damage at 18 months of follow up (49). Additionally, Finckh et al. studied 3 cohorts of early arthritis patients across 3 decades and found significantly less radiographic progression in the more recent cohort which appeared to be related to treatment effect (69).

**Disability**

Disability is most commonly measured using the Stanford Health Assessment Questionnaire (HAQ) which is a well validated disease specific measure of disability in RA (70). It has been shown to be sensitive to clinical changes but does not discriminate between disability due to active inflammation and damage (71). Interestingly most cohort studies examining progression of HAQ in different decades have failed to show a significant improvement in disability with the improvement in treatment strategies (72;73). It has been suggested that this may be due to changes in perception of illness and disability. As the treat to target strategy has only become widely practiced within the last 5 years it may be too early to detect the effect of this change.

**Mortality**

It has long been recognised that RA patients have increased mortality compared to the general population (74). A systematic review and meta-analysis by Dadoun et al.,
conducted in 2013, found a standardised mortality rate of 1.47 [95%CI: 1.19, 1.83] (75). Mortality trends over five decades were also examined. The study demonstrated that while incident mortality rates were reducing every decade, rates remained significantly higher than in the general population suggesting excess mortality persists despite improvements in treatment.

Cardiovascular disease is known to be the leading cause of death, with 50% of the excess mortality seen in RA, being attributable to cardiovascular disease (76). Avina-Zubieta et al. conducted a meta-analysis of cardiovascular mortality in RA and found a standardised mortality ratio of 1.59 (CI 95%: 1.46-1.73) for coronary heart disease and 1.52 (CI 95%: 1.40, 1.67) for stroke (76). Similar to all-cause mortality, standardised mortality rates for cardiovascular mortality have not fallen in the last 50 years emphasising the importance for further research into the causes, prediction and treatment of cardiovascular disease in RA (77).
1.2 Cardiovascular disease (CVD) in RA

1.2.1 Epidemiology

While pericardial disease is a recognised extra-articular manifestation of RA, the excess mortality is, in the most part due to atherosclerotic disease. A diagnosis of RA is associated with a 1.5 to 2 fold increased risk of myocardial infarction (MI), a similar burden of risk as is associated with type 2 diabetes (78).

Although risk of CVD increases with disease duration, excess risk is apparent in early arthritis and may in fact be increased prior to diagnosis (79). Maradit Kremers et al. demonstrated an increased incidence of MI in patients in the 2 years prior to fulfilling the 1987 ACR criteria for RA, suggesting the risk may predate the clinical onset of arthritis (80).

There also appears to be a different clinical phenotype of cardiovascular disease in RA compared with the general population. Patients are more likely to have atypical symptoms or silent ischaemia and have less warning symptoms prior to a major cardiac event compared with controls. There is also increased risk of sudden cardiac death and patients are more likely to have recurrent events and cardiac death following incident MI(80-83). Additionally there is not only an increased risk of stroke in RA (76) but also a higher case fatality in RA patients when compared to controls (84).

Some RA disease characteristics including antibody positivity, subcutaneous nodules and higher levels of inflammation are associated with increased risk of cardiovascular events (85-87). It is thought that chronic inflammation causes increased cardiovascular risk both through modulation of traditional risk factors and also possibly by directly affecting the vessel wall.

1.2.2 Genetic risk factors

Genetics studies have demonstrated that presence of some polymorphisms in the HLA-DRB1 gene, are associated with increased cardiovascular mortality within the RA population (10). Interestingly there is also evidence of an association with these alleles and cardiovascular risk in the general population too (88). The association in RA patients is most pronounced in smokers and those who were ACPA positive (10). It may be assumed that
the association is due to these polymorphisms also being associated with disease severity. However Mattey et al. found no evidence of interaction with disease severity markers, cardiovascular mortality and HLA-DRB1 polymorphisms, suggesting the relationship was independent of disease severity (89). Farragher et al. screened 19 alleles known to be associated with RA for an association with cardiovascular mortality (90). A variant of the CC21 allele, involved in leucocyte trafficking was found to be associated with increased cardiovascular mortality. Other studies have been less promising and there has yet been no convincing evidence to demonstrate an association with TNF and IL-6 receptor genes and cardiovascular mortality in RA (91).

1.2.3 The role of traditional risk factors

In RA there is an increased prevalence of some traditional cardiovascular risk factors while others are similar or indeed lower than in the general population.

Dyslipidaemia

Lipid profiles in RA differ from that of the general population. There is no increased prevalence of hyperlipidaemia at the time of diagnosis however patients tend to have an adverse lipid profile (92). A reduction in total cholesterol is seen in patients with active disease, however there is a preferential reduction in high density lipoprotein (HDL) compared with low density lipoprotein (LDL) leading to an adverse atherogenic index (93). On treatment of early arthritis an increase in parameters is often seen with an improvement in total cholesterol to HDL ratio (TC: HDL) (94).

In the general population LDL is a reliable marker of cardiovascular risk but this is not so in RA. Studies have shown that LDL is not associated with increased risk of cardiovascular events and that in fact there was a paradoxical increased risk in those with the lowest LDL measurements (although this did not achieve statistical significance) (95). Work by McMahon et al also demonstrated increased levels of “pro-inflammatory HDL” which promotes atheroma formation in RA patients (96).
Studies have shown that dyslipidaemia is under-diagnosed and under-treated by both primary and secondary care physicians in this population and under-treatment of dyslipidaemia may contribute to increased rate of events (92;97).

**Diabetes and insulin resistance**

There is an increased prevalence of diabetes in RA (OR 1.74, p=0.03) (98). It could be assumed that insulin resistance develops secondary to glucocorticoid use. However it has been shown that insulin resistance is associated with high levels of inflammation, disease activity and seropositivity in RA (99;100). As those with high disease activity often receive high cumulative glucocorticoid dose, it can be difficult to completely eliminate confounding factors. However, the higher incidence of insulin resistance in those with features of more severe disease, could partly explain the increased cardiovascular risk in that subgroup of RA patients. A study by Mirjafari et al. illustrated this point (100). Higher rates of insulin resistance were demonstrated in seropositive patients with early arthritis. The association with seropositivity, in an early disease cohort when there would be a low cumulative exposure to steroids, emphasises the relationship with insulin resistance and disease severity.

**Obesity**

Although patients with RA have increased waist circumference, therefore are deemed “centrally” obese, numerous studies have also shown that there is no significant difference in body mass index (BMI) when compared to the general population (101-103). Patients with high disease activity are in a catabolic state, where muscle is degraded more rapidly than fat. In this circumstance, patients often have normal or low BMIs but body fat content can be relatively high. A study by Giles et al. demonstrated that RA patients had higher distributions of subcutaneous and visceral fat compared to non- RA controls with the same BMI (104). It appears that BMI is a less reliable marker of obesity in RA than in the general population. Indeed studies have shown that low BMI can be associated with increased cardiovascular risk, possibly owing to the association of disease activity and catabolism (82).
**Metabolic syndrome**

Metabolic syndrome is a constellation of individual cardiovascular risk factors which, when present as a group, may confer a higher CVD risk that the additive effects of individual risk factors. The most recent and widely accepted definition was published by the International Diabetes Federation (IDF) in 2006 (105) and there recommended definition is outlined in Table 1.5. There is an increased prevalence of metabolic syndrome in the RA population (106). Chung et al. demonstrated that patients with both early and established RA has increased rates of the syndrome and that its presence was associated with evidence of subclinical atherosclerosis on x-ray computed tomography (CT) of the coronary arteries (99). RA patients had significantly higher rates of increased waist circumference, insulin resistance and lower levels of HDL. Presence of metabolic syndrome was associated with high levels of CRP.

Other studies have also demonstrated an association with metabolic syndrome in RA and raised levels of inflammatory markers. This suggests that inflammation may influence traditional cardiovascular risk factors (101;102).

Table 1-5 IDF definition of metabolic syndrome (105)

<table>
<thead>
<tr>
<th>Measured risk factors</th>
<th>Essential</th>
<th>Central obesity</th>
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<tbody>
<tr>
<td></td>
<td>Waist circumference &gt;102cm in males</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Waist circumference&gt;88cm in females</td>
<td></td>
</tr>
<tr>
<td>2 of more of:</td>
<td>Hypertension</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blood pressure &gt;130/85mmHg</td>
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</tr>
</tbody>
</table>

Dyslipidaemia

- Triglyceride levels> 1.7mmol/l
- Low HDL: males <1.03mmol/l and females <1.29mmol/l

Impaired fasting glucose

- Fasting glucose ≥5.6mmol/l
**Smoking**

There is an increased prevalence of smoking within the RA population (98). However, Gonzalez-Gay et al. compared the impact of traditional risk factors on cardiovascular events in a retrospective cohort study of RA patients (107). They found that although RA patients had a higher event rate, the hazard ratio (HR) for smokers versus non-smokers was significantly lower in the RA cohort compared with controls (HR 1.32 v 2.19, p=0.008). This suggests that smoking may have less of an impact on cardiovascular risk in RA patients than in the general population.

**Hypertension**

A systematic review by Panoulas et al. did find an increased prevalence of hypertension in RA compared with the general population. However, this finding was not uniform among the studies (108). In their review they found no evidence of any relationship with RA disease activity and hypertension although some medications used to treat RA, such as glucocorticoids and non-steroidal anti-inflammatory drugs are known to cause hypertension.

### 1.2.4 Cardiovascular risk prediction in RA

It is clear that some traditional risk factors may contribute to the increased risk of cardiovascular disease in RA but that their prevalence and impact may be altered by active joint disease and inflammation (107). Existing cardiovascular risk prediction models perform poorly in RA patients and the risk tends to be underestimated (109). This may be partly due to less of the proportion of risk being due to traditional risk factors but also measurements such as LDL are not as reliable markers of cardiovascular risk in RA. There is now a large body of evidence to suggest that when traditional risk factors are adjusted for, there is still a 50% increased risk of cardiovascular disease (110). It is now accepted that a diagnosis of RA is an independent predictor of cardiovascular mortality.

European guidelines suggest that risk estimates should be multiplied by a factor of 1.5 if two out of three are present: antibody positivity, nodules and disease duration greater
than 10 years (111). However there was recognition, in the guidelines that this was a crude adjustment and indeed did not account for the risk present in early disease. The guidelines also set out a research agenda aiming to address the issues (110).
1.3 The role of inflammation in atherosclerosis

Atherosclerosis, the underlying pathological mechanism for most ischaemic cardiovascular events, is now accepted to be a chronic inflammatory condition (112). Histological studies have shown that inflammation plays a key role in initiation, progression and stability of atherosclerotic lesions (113;114). There are many similarities in the key immune processes in both atherosclerotic lesions and inflamed RA synovium, as shown in figure 1.1. Additionally TNF, IL-6 and some matrix metalloproteinases which are increased in RA are also implicated in the progression of atherosclerosis (115). It could be that cytokines generated in the inflamed synovium have secondary effects on the vessel wall or alternatively, there may be a simultaneous process affecting both synovium and arteries in RA, as illustrated in figure 1.1.
Pathological features common to both synovitis and atherosclerosis (112;115).

A number of similarities are found between the pathological processes seen in both conditions, in particular endothelial activation, inflammatory cell infiltration, tissue neovascularisation and collagen degradation. It could be that these processes occur simultaneously in both the joint and vessel wall in RA or possibly that mediators produced in the synovium have distant secondary effects on the artery.
1.3.1 Stages of atherosclerosis

The stages of atherosclerosis, illustrated in figure 1.2., are discussed in more detail in subsequent sections.

**Figure 1-2 Development and rupture of atherosclerotic plaque.**

I. Normal arterial wall

II. Endothelial activation, LDL and immune cells enter the vessel

III. Modified LDL phagocytosed by macrophages forming foam cells.

IV. Neovascularisation and smooth muscle cell proliferation.

V. The plaque becomes more complex with calcium deposition, fibrous cap formation, intra-plaque haemorrhage and lipid core

VI. Increased immune cell infiltration and fibrous cap degradation eventually leads to rupture and thrombosis
**Endothelial dysfunction (Figure 1.2., stage II)**

In the earliest stage of atherosclerosis the innermost layer of the artery, the endothelium becomes dysfunctional. There is increased permeability and also increased expression of pro-inflammatory cytokines and cellular adhesion molecules such as vascular cellular adhesion molecule-1 (VCAM-1) (116). This attracts immune cells (predominantly monocytes) and facilitates their adhesion and transfer into the sub-endothelial layer. Small lipid molecules (such as LDL) are also able to cross the dysfunctional endothelium and be deposited in the vessel wall.

**Atherosclerotic plaque formation and progression (Figure 1.2., stage III)**

On entering the vessel wall, LDL molecules undergo modification (usually oxidisation). Monocytes differentiate into macrophages and take up the modified LDL to form foam cells. Macrophages stimulate further recruitment of immune cells including TH1 T cells by secreting TNF and IL-6 (117). TH1 cells promote foam cell formation via secretion of IL-1 and Interferon gamma (IF-γ). Smooth muscle cell proliferation is also stimulated and this, along with foam cell formation leads, to a localise thickening of the vessel wall which begins to form a plaque.

**Plaque progression, destabilisation and rupture (Figure 1.2., stage IV-VI)**

As the plaque grows it becomes more complicated in its composition. Angiogenesis occurs within the plaque but vessels are fragile and bleed causing intra-plaque haemorrhage. Additionally relative hypoxia causes foam cell apoptosis leading to deposition of a necrotic lipid core within the plaque. Both these processes stimulate the inflammatory response and further growth. The fibrous cap which covers the plaque becomes thinner, likely due to matrix metalloproteinase activity (118). If the fibrous cap erodes, the plaque can rupture and the thrombogenic content of the plaque is exposed to the circulation. A clot forms which can cause an occlusion in situ or embolize causing a clinical event. High levels of inflammation have been demonstrated on histology of ruptured plaques and imaging techniques have also demonstrated increased inflammation within vulnerable lesions.
Interestingly, the CD28null cells implicated in RA are also found in the shoulders of unstable plaque and presence of circulating CD28null cells is associated with a 9-fold increased risk of recurrent MI (119) (120). It has been proposed that these cells play a role in plaque destabilisation.

It was once thought that the key driver for clinical events was stenosis due to plaque growth. However, it is now known that plaque composition and inflammation are the key determinants of plaque stability and that rupture and clinical events can occur at non-stenosing lesions (121). With this in mind, the American Heart Association (AHA) published classification criteria for plaque grading which reflected the importance of compositional features, described in Table 1.6 (122). More recently a “high risk” plaque classification based on the structural and functional aspects of plaque, has been proposed (123) (Table 1.7).

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Grade</th>
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<tbody>
<tr>
<td>Intimal thickening</td>
<td>I</td>
</tr>
<tr>
<td>Intimal xanthoma</td>
<td>II</td>
</tr>
<tr>
<td>Diffuse intimal thickening with no calcification</td>
<td>III</td>
</tr>
<tr>
<td>Lipid core with fibrous cap +/- calcium</td>
<td>IV – V</td>
</tr>
<tr>
<td>Complex plaque with signs of surface defect/ haemorrhage or thrombus</td>
<td>VI</td>
</tr>
<tr>
<td>Calcified lesion</td>
<td>VII</td>
</tr>
<tr>
<td>Fibrotic plaque +/- calcium and no signs of surface disruption</td>
<td>VIII</td>
</tr>
</tbody>
</table>

**Table 1.6 AHA classification criteria of atherosclerotic plaque (122)**

**Table 1.7 Morphological features of high risk plaque (adapted from Virmani et al.) (123)**

- Plaque inflammation
- Spotty calcification
- Expansive outward wall remodelling
- Thin fibrous cap
- Large necrotic core
- Neoangiogenesis
- Intra-plaque haemorrhage
The histological evidence that inflammation plays a key role in atherosclerosis is supported by findings in population studies. High levels of inflammatory molecules including high-sensitivity CRP (hsCRP) and cellular adhesion molecules are independent predictors of future cardiovascular events in the general population (124) (125). There is increasing interest in the use of anti-inflammatory therapies for secondary prevention of myocardial infarction in the general population (126).
1.3.2 Atherosclerosis in RA

There is an increased prevalence of atherosclerosis in RA and studies have demonstrated that rates of progression are faster than in the general population (127) (128). A study screening for carotid plaque found that 46% of patients had plaque while only 19% of age-sex matched controls had plaque (129). This has been replicated in other studies (130).

In addition to having premature and accelerated atherosclerosis, there is some evidence that patients may have a more inflammatory plaque phenotype. A post mortem study by Aubrey et al. compared culprit lesions in patients and controls, who had died of acute MI (131). They demonstrated that patients had significantly more inflammation and less stenosis in the ruptured lesions, suggesting that plaques may be more rupture prone at an earlier stage of development. One other study, published in abstract form also demonstrated a similar pattern (132). There is very little other evidence regarding plaque phenotype in RA. One study compared atherosclerotic lesions on CT and found that patients had a higher prevalence of “high risk” lesions compared to controls, supporting histology findings (133). The clinical phenotype of more sudden major events and higher case fatality may also be in keeping with a more unstable, rupture prone phenotype but further work is required to characterise plaque phenotype in RA.

1.3.2.1 Potential mediators of accelerated atherosclerosis in RA

There is epidemiological and mechanistic evidence supporting the role that chronic inflammation plays in cardiovascular risk in RA. As in the general population, raised inflammatory markers independently predict cardiovascular events in RA. Studies have shown an association with baseline and cumulative CRP with subsequent cardiovascular events (134-136). In the general population, TNF levels independently predict subsequent myocardial infarction (137). Although increased levels of circulating TNF are found in RA, no clear association has been established with clinical events or subclinical atherosclerosis. One study by Mattey et al. demonstrated an association with soluble TNF receptors and subsequent cardiovascular mortality (89). However, other studies have failed to find an association with TNF levels and markers of subclinical disease (138) (139). There is also some evidence that IL-6 is associated with endothelial dysfunction (139). However, treatment with anti-TNF and anti-IL-6 agents, have both demonstrated a beneficial effect
on endothelial function (140;141). This suggests they may be implicated in accelerated cardiovascular disease.

A number of mediators of accelerated atherosclerosis in RA have been proposed however for the purpose of this review I will focus on those I propose to investigate in this thesis.

1.3.2.1.1 Cellular adhesion molecules

In the general population levels of circulating cellular adhesion molecules (CAMs) are predictive of future cardiovascular events (125). These molecules play are up-regulated in the inflamed synovium and play a key role in leucocyte trafficking within the joint(142). They have also been found to be increased in RA patients compared to controls (143). A cross sectional association with circulating CAMs and subclinical cardiovascular disease in RA patients has been demonstrated, however a link with clinical events had yet to proven (139). Increased circulating levels of these molecules may reflect a combination of endothelial activation in the synovium and the large arteries in RA.

1.3.2.1.2 Endothelial microparticles

Microparticles are small vesicles (diameter of 0.1-1µm) which are shed from activated or apoptotic cells. Initially thought to be inert particles, in vitro and animal studies have demonstrated that they have vasoactive properties and likely provide a method of cell to cell signalling (144). Microparticles can be derived from a number of cells including platelets, neutrophils and also the endothelium. Endothelial microparticles (EMPs) are shed from activated or damaged endothelium in response to stimuli such as CRP and pro-inflammatory cytokines(145). They have been shown to contain RNA and also cellular adhesion molecules, which can act locally and also may have downstream vascular effects (146).

In the general population, patients with acute coronary syndrome have raised levels of EMPs compared to those with stable ischaemic heart disease (147;148). Additionally one study found a 2.5 fold increase in EMP levels in patients with high risk coronary lesions on angiography compared with those who had low risk lesions (149). In a cohort of
haemodialysis patients, high EMP levels were associated with increased mortality at 50 month follow up (150). In subclinical disease EMP levels are associated with arterial stiffness and endothelial dysfunction (151). EMPs may play a role in both early subclinical disease and in plaque stability and clinical events.

In patients with RA, platelet derived microparticles have been found in the synovium and have been shown to amplify the inflammatory response in vitro (152;153). Little is known about EMPs in patients with rheumatoid arthritis however raised levels have been found in other auto-immune conditions such as SLE and have been associated with endothelial dysfunction (154).

1.3.2.1.3 CD28 null cells

As mentioned in earlier sections CD28 null cells have been implicated in both RA and unstable angina(119) (155). These cells differ in a number of ways from the more abundant CD4 positive CD28 positive T cells. They are pro-inflammatory, secrete high levels of interferon \(\theta\) (IF-\(\theta\)) and TNF (156). They can effectively kill endothelial cells in vitro and their ability to do so is enhanced in the presence of CRP (157). Additionally they are resistant to apoptosis, have a reduced signalling capacity from regulatory T cells and display ineffective B cell signalling, thus reducing antibody mediated responses (156) (158). *In vitro* TNF promotes down regulation of CD28 expression and the cells have been shown to promote synoviocyte proliferation (159).

In RA their presence has been associated with extra-articular disease, increased likelihood of requiring joint replacement and also in one study atherosclerosis on ultrasound (32;160). As previously discussed presence of these cells are associated with increased risk of recurrent MI and are preferentially associated with unstable plaque. It is thought that they secrete high levels of IF-\(\theta\), activate macrophages and produce matrix metalloproteinases (MMPs), which may destabilise the plaque thus increasing the risk of a clinical event occurring (157;161).

This unusual subset of T cells appears to play an important role in both diseases and their presence in RA may contribute to the development of atherosclerosis.
The mechanisms by which inflammation leads to increased risk of cardiovascular disease is still poorly understood. It may be that circulating cytokines directly damage the endothelium, initiating and accelerating atherosclerosis. However a number of cells and pathways are implicated in both diseases, suggesting that a common process mediated by inflammatory cells and proteins may affect both the arteries and the synovium simultaneously in RA.
1.3.3 The effects of treatment on cardiovascular risk in RA

The evidence so far suggests that there is still a widened mortality gap between RA patients and the general population despite advances in therapy. It may be that it takes many years before the recent changes in RA management have an impact on cardiovascular mortality.

There is evidence that the use of methotrexate has had an impact on cardiovascular event rates. A meta-analysis conducted by Micha et al. examined the association with methotrexate use and cardiovascular events (162). They included 10 studies, eight of which were in RA, one in inflammatory polyarthritis and one in psoriasis. They found a 21% reduction in cardiovascular events associated with methotrexate use (HR [95% CI]: 0.79 [0.73, 0.87]). There was a degree of heterogeneity among the studies; a significant proportion of studies were retrospective and also there was variability in disease duration in patients and adjustment for confounders such as underlying disease and glucocorticoid use. However the group used multiple analysis methods and found similar point estimates, supporting the hypothesis that methotrexate may be atheroprotective. A systematic review by Westlake et al., which focus on RA studies only, came to a similar conclusion (163).

The results of studies examining the effect of anti-TNF agents on cardiovascular events have been conflicting. A meta-analysis conducted by Barnabe et al. demonstrated an overall reduction in cardiovascular events in patients treated with anti-TNF agents when examining cohort studies (RR [95%CI]: 0.46[0.28, 0.77]) (164). However there was significant heterogeneity among study populations and many studies were deemed to be of low quality. Clinical trial data were also pooled but no significant difference was found within these data (RR [95% CI]: 0.85 [0.28, 2.59]). A systematic review conducted by Westlake et al. found no significant reduction in risk but concluded that due heterogeneity among studies, no formal meta-analysis could be conducted (165).

Data from the British Society for Rheumatology Biologics Register (BSRBR), a prospective case control study of RA patients initiating biologics in the UK was used to test the association of anti-TNF therapy and myocardial infarction (MI)(166). They found that although there was no significant difference in rates of MI between those treated with traditional DMARDs and anti-TNF therapy (Incident rate ratio (IRR) [CI 95%]: 1.44[0.56, 3.67]), there was a significant reduction in responders to TNF within the first 6 months (IRR [95% CI]: 0.36 [0.19, 0.69]). TNF is implicated in plaque instability and this finding could point to a plaque stabilisation effect of anti-TNF (167).
Studies examining the effects on subclinical cardiovascular disease have also shown mixed results. Tam et al. carried out a systematic review of the effects of anti-TNF therapy on progression of subclinical atherosclerosis in inflammatory arthritis (168). They included both observational and randomised controlled studies using intimal medial thickness measurements and arterial stiffness as endpoints. While findings were not uniform there appeared to be an improvement in subclinical markers of cardiovascular disease with anti-TNF. However the longest duration of follow up was 13 months and there were significant differences in study populations. An interesting study by Maki-Petaja et al. used $^{18}$F-fludeoxyglucose positron emission tomography (FDG-PET) to measure aortic inflammation in RA patients and controls (169). FDG-PET is a nuclear imaging technique, which can be used to quantify vascular inflammation. The study compared findings on PET between patients with active disease and controls and then patients before and after anti-TNF therapy. They demonstrated that RA patients had significantly increased levels of arterial inflammation compared with controls and that inflammation improved following treatment with anti-TNF. This study provided the first opportunity to visualise in vivo arterial inflammation in RA and demonstrated change with anti-TNF drugs.

Interestingly levels of aortic inflammation remained higher than in the control group even after anti-TNF therapy suggesting that low grade vascular inflammation may persist even in those deemed well treated. Another hypothesis to explain the continued increased cardiovascular mortality may be that this persistent low grade inflammation continues to drive atherosclerosis. In the general population a CRP of 2mg/dl or more is associated with increased cardiovascular risk (124). In a study of patients in remission, the mean CRP was 5mg/dl was found, suggesting they could remain at increased risk even when joint disease is satisfactorily treated (170). It could be hypothesised that further suppression is required to minimise cardiovascular risk. However, as the risk varies within the population and there is significant morbidity associated with anti-rheumatic therapies, a reliable method of identifying high risk patients and targeting treatment in this subpopulation is required.
1.4 Assessment of subclinical cardiovascular disease

While studies with clinical endpoints such as cardiovascular events are the gold standard, these types of studies often require large numbers of subjects and long follow up periods to answer the research question. They can also be costly and time consuming. With this in mind, there has been a growing interest in the development of biomarkers for cardiovascular risk. A biomarker is defined as “a physical sign or laboratory measurement that occurs in association with a pathological process and that has putative diagnostic and/or prognostic utility” (171).

Additionally, the study of clinical events gives limited information about the underlying pathological mechanism and natural history of disease. Current clinical techniques for arterial assessment include angiography and carotid ultrasound, which give accurate information about stenosis but limited information on plaque composition or inflammation. As a result, patients with high risk, non-stenosing lesions may not be identified and targeted for treatment appropriately.

Spurred on by these unmet needs, there have been great advances in arterial imaging. A wide variety of invasive and non-invasive imaging techniques have now been employed to evaluate atherosclerosis and cardiovascular risk.

1.4.1 Carotid artery imaging

The carotid artery is a medium size vessel, close to the skin surface. Its size and location make it an ideal vessel to assess non-invasively. The area where the carotid artery bifurcates, known as the carotid bulb is a common site of atherosclerotic plaque occurrence, thought in part to be due to its turbulent flow. The carotid artery is also a clinically relevant site to study atherosclerosis, as carotid plaque commonly results in embolic stroke and it is also a reliable biomarker of overall cardiovascular risk (172). The Joint American Cardiology guidelines now advise inclusion of carotid ultrasound to assess cardiovascular risk in moderate risk populations (173).

Non-invasive coronary imaging is more difficult as vessel calibre is small, the arteries are deep and respiratory and cardiac motion often cause artefact. Invasive imaging with angiography and intra-luminal ultrasound provides accurate information about luminal
stenosis and some detail on plaque composition, however its use is limited by the intrusive nature of the procedure. X-ray computed tomography (CT) can be used to quantify coronary artery calcium which has been shown to be predictive of future coronary events but significant doses of radiation are required and little detail on plaque composition or inflammation is available (174). Non-invasive coronary imaging is evolving, however currently the carotid artery provides the most reliable site to investigate atherosclerosis and a surrogate marker of subclinical cardiovascular disease.

1.4.2 Carotid artery ultrasound

Ultrasonography (US) is a well-established modality with which to assess the carotid artery. It is used in both clinical practice and research settings to assess cardiovascular risk (173;175). Reliable information can be gathered about plaque presence, lumen size and wall thickness and these measures correlate well with future clinical outcomes in population-based studies (172). With the use of high resolution probes, information can be gathered about high risk features such as cap irregularities, presence of lipid core or haemorrhage, however views can be limited due to calcium deposition within the plaque (176). A semi-quantitative score of plaque echogenicity, known as grey scale measurement has also been shown to identify high risk lesions although there is a significant degree of inter-operator variability (177). Recent advances have been made with the use of microbubbles as contrast, which can demonstrate differences in symptomatic and asymptomatic plaques (178). Studies using this technique demonstrated good correlation with microvasculature and contrast enhancement on matching histology, although again studies were limited by calcium deposition (179).

Ultrasound provides a cheap non-invasive method of assessing presence and size of plaque and gives some information about plaque morphology. However there are some concerns about reproducibility due to the high level of operator dependence (180). Also, calcium deposits are a common feature of complex high-risk plaque and the limitation of visualising plaque with calcium can be problematic.

While presence of plaque on US is predictive of future events, a recent meta-analysis showed that regression on US following drug therapy is not associated with reduction in future cardiovascular events (181). Therefore, other imaging methods are required to
characterise plaque composition in detail, detect vulnerability and change more accurately. Newer modalities including magnetic resonance imaging (MRI) and PET have been developed which provide a method for more detailed evaluation.
1.4.3 Carotid MRI

Carotid MRI (CMRI) is an imaging technique, developed in the last decade, which allows detailed visualisation of carotid plaque. Using multiple sequences of black blood, bright blood and time of flight angiography (TOF) imaging (examples given in figure 1.3-1.5); plaque dimensions, composition and more recently, functional aspects including inflammation and sheer stresses can be evaluated. An in depth review of the physics underpinning this imaging modality is beyond the scope of this thesis and the main focus is to review the potential utility of CMRI for investigation of atherosclerosis and as a biomarker of cardiovascular risk.

![Figure 1-3. Coronal MRI time of flight angiography of the carotid arteries](image)
The blood appears bright allowing the outline of the vessel to be seen including the common carotid artery, the bifurcation (where plaques most commonly occur) and the internal and external carotid arteries.
Figure 1-4 “Black blood” sagittal image where the blood signal is suppressed so the lumen appears black and the vessel walls can be seen (marked in blue). A plaque can be seen at the bifurcation (outlined in red) with calcification within (marked in yellow).
Figure 1-5 Axial images of the carotid bifurcation, corresponding to the sagittal image seen in figure 1.4. The top left image is a “black blood” sequence where the blood is suppressed and the vessel wall and plaque components can be quantified. The top right image illustrates normal vessel wall (in blue), a plaque area (outlined in red) and calcification within the plaque (outlined in yellow). The bottom left image is the corresponding axial “bright blood” sequence taken at the same slice position with vessel wall, plaque and calcification also highlighted in the bottom right image.
Initially the technique was developed using ex-vivo carotid endarterectomy specimens from symptomatic patients. It was then applied to in-vivo subjects in cross sectional, longitudinal and interventional studies. Techniques have advanced greatly with the use of 3 Tesla MRI, refined imaging protocols, surface coils and semi-automated software. This has allowed sub-millimetre resolution with good signal to noise ratio and accurate quantification of plaque constituents (182).

Studies using CMRI have shown high sensitivity and specificity with good levels of intra- and inter-observer variability (183-185). Today, detailed non-invasive assessment of atherosclerotic plaque can be achieved using CMRI to inform our understanding of plaque progression, predictors of stroke and effects of intervention on atherosclerotic plaque.

1.4.3.1 Structural CMRI

1.4.3.1.1 Validation studies

The availability of specimens from patients undergoing carotid endarterectomy has allowed thorough validation of CMRI against histology. Not only plaque dimensions but also quantification of specific plaque components has been well studied (3).

Plaque burden

While plaque size and luminal stenosis is not necessarily the key trigger for an acute clinical episode, it is still a strong predictor of future events (186). A number of studies have validated the quantification of plaque “burden” i.e. plaque dimensions and volume. A strong correlation with CMRI and histology findings (correlation co-efficients (r) 0.90 -0.92) was demonstrated (187;188). Plaque dimensions are usually measured on black blood sequences and measurements can be expressed as absolutes or expressed as percentages of lumen or total vessel. “Normalised wall index” is an expression of measured wall area divided by the sum of the wall and luminal area in that slice. This gives an estimate of plaque burden, which takes into account how much of the vessel wall is imaged and is known to independently predict cardiovascular events (189).
Most histological studies have been in symptomatic patients with large advanced plaques however, Saam et al. examined plaque phenotype and burden in asymptomatic subjects and compared them to ultrasound findings. They were able to reliably characterise plaque dimensions and components in smaller lesions (190).

**Plaque components**

As discussed earlier, the presence of certain plaque characteristics is highly suggestive of rupture prone plaque and a number of studies have characterised these components using CMRI.

**Calcification**

Calcification is recognised as a feature of advanced atherosclerotic plaque however calcification can begin early in the atherosclerotic plaque process. They are two proposed models for plaque calcification (191). The first is the passive model where a tissue micro-environment provides favourable conditions for calcium precipitation (192). Conditions that favour calcium precipitation include tissue necrosis and cholesterol deposition, both of which are a feature of complex atherosclerotic plaque. This model of calcification is most relevant in patients with metabolic disorders such as chronic kidney disease and is more strongly associated with diffuse vessel calcification than atherosclerotic calcification (191).

The second, more favoured model is the “active model” of atherosclerotic calcification (191). *In vitro* studies have demonstrated the ability of arterial cells to differentiate into osteoblast like cells, which are involved in bone formation (193). Key cells and signalling molecules involved in bone formation are found in human atherosclerotic plaques and notably higher levels are found in areas of plaque with calcification (194-196). Inflammatory cytokines including TNF are known to up-regulate the signalling pathways for mineralisation in bone and also thought to up-regulate calcification within the plaque (197). Thus chronic inflammation may also drive plaque calcification, in addition to other processes such as thinning of the fibrous cap within plaque.

While calcium nodules are a feature of advanced complex plaque, if found in the presence of other high risk features, such as LRNC, are predictive of plaque rupture (123). On MRI, calcium nodules are visible as hypo-intense lesions in black blood imaging sequences. A
number of studies have shown that calcification on CMRI was strongly correlated with histology findings on endarterectomy specimens \( r = 0.74, p<0.001 \) (184). Calcification of carotid plaque on MRI has been shown to predict subsequent stroke and is also associated with more severe coronary artery disease (181) (198). X-ray computerised tomography (CT) can be used to measure calcium content in both the coronary and carotid arteries (199) and coronary calcification is predictive of future cardiovascular events. Carotid MRI has the advantage of quantifying calcium in the context of other plaque features thus providing a more accurate grading of plaque severity.

**Lipid Rich Necrotic Core (LRNC)**

In complex plaques, a lipid rich necrotic core (LRNC) consisting of lipid deposits, other debris from apoptotic foam cells and loose fibrous matrix is found underneath the fibrous cap. The LRNC can be visualised using CMRI on a number of black blood imaging sequences. Measurements of LRNC on CMRI correlates well with LRNC on matching histology specimens \( \text{kappa [95%CI]}=0.98 [0.93, 100] \) (200). The use of a gadolinium-based contrast agent such as gadopentetate or gadoterate improves the delineation of the LRNC against other structures, particularly the fibrous cap, making it appear more hypo-intense on post contrast T1 weighted images. Cai et al. found a strong correlation between findings on contrast enhanced MRI and histology \( r=0.87, p<0.05 \) (201). The presence of LRNC of greater than 23% of the total plaque volume is thought to signify an increased risk of rupture(123), therefore accurate quantification of this component has important clinical implications.

**Intraplaque Haemorrhage (IPH)**

Intraplaque haemorrhage (IPH) is known to occur in advanced plaques. As a plaque grows, angiogenic factors stimulate neovascularisation within the plaque. These vessels are fragile and often rupture causing local haemorrhage. Early in the development of CMRI, differentiation of IPH and luminal thrombus was challenging. However, a number of studies have since shown that IPH can be reliably identified using CMRI, as a hyper-intense on T1 weighted, time of flight and magnetisation prepared rapid acquisition gradient echo (MP-RAGE) sequences. Chu et al. carried out CMRI on 27 pre-endarterectomy patients and found a sensitivity and specificity of 96% and 74% respectively for IPH when images were
compared with histology (202). Furthermore Kampshulte et al. demonstrated that IPH could be distinguished from thrombus with 96% accuracy (203;203). Histological studies have shown that IPH is associated with high risk plaques and if IPH occurs at the shoulder of plaque it may lead to weakening of the plaque surface, causing destabilisation (204;205).

*Fibrous cap*

On CMRI the fibrous cap is visualised best on bright blood and time of flight sequences. Hatsukami et al. performed CMRI on 22 patients due for carotid endarterectomy and demonstrated that fibrous cap integrity and thickness could be identified using contrast MRI with 89% accuracy (206). Subsequent studies have also shown good correlation with CMRI measurements of fibrous cap disruption and areas with histological specimen from carotid endarterectomy patients (207;208). At a thickness of less than 65 micrometres there is a high risk of rupture, which leads to exposure of the thrombogenic content of plaque to the circulation resulting in thrombosis (123). Thin or ruptured plaque has been shown to be strongly associated with symptomatic lesions in both MRI and histological studies (209).

1.4.3.1.2 Classification of carotid plaque

The evaluation of individual plaque components and volume has been well validated but Cai et al. demonstrated that CMRI could be used to reliably classify plaque using the American Heart Association classification system (201;210). They performed CMRI on 60 patients prior to carotid endarterectomy and compared histological classification. A weighted kappa level of agreement of 0.79 was found between MRI and histological grading. Sensitivity and specificity for early lesions were 67% and 100%; type VI lesions, 82% and 91% and type VIII lesions of 56% and 100%, respectively.

When looking specifically at identification of the culprit lesion in symptomatic patients, CMRI has also been shown to accurately characterise these lesions. Moody et al. demonstrated that sub-acute thrombus could be identified on CMRI in 16 patients with acute stroke (211) Using contrast enhancement Kampshulte et al. demonstrated that thrombus and fibrous cap rupture could be reliably identified. Thrombus was visualised
with 96% accuracy and was seen as a hyper-intense lesion on T1 weighted and TOF images (203).

The evidence so far suggests that CMRI and histological findings correlate closely and thus CMRI allows accurate non-invasive assessment of plaque phenotype when histology is not necessarily available. This provides an excellent opportunity to study plaque in both early and advanced stages.

1.4.3.1.3 Population studies

The properties of CMRI have made it useful in population studies. The technique has been employed in existing large cohort studies such as the Atherosclerosis Risk in the Community (ARIC) study and the Multi-ethnic Study of Atherosclerosis (MESA) (212;213). These and other studies have investigated associations and predictors of plaque progression and clinical events.

**Symptomatic versus asymptomatic plaque**

King-Im et al. compared CMRI findings in 20 symptomatic and 20 asymptomatic subjects who had carotid artery stenosis of greater than 50% (214). Despite having equivalent plaque burden, symptomatic patients had a significantly higher incidence of plaque rupture (36.5% vs 8.7%, p=0.004), thrombus (46.5% vs 14%, p<0.001), large LRNC (63% vs 28%, p=0.002) and complicated type VI plaque (61.5% vs 28.1%; p = 0.001) compared with asymptomatic individuals. Findings of thrombus or thin fibrous cap were associated with a 5.25 and 7.94 fold increased chance of having symptoms (OR [95%CI: 5.25 [2.08, 13.24] and 7.94[2.93, 25.51] respectively). This study emphasises the fact that plaque composition is a key determinant for clinical events irrespective of plaque burden. CMRI could provide a reliable method of identifying culprit lesions to target intervention.

**Plaque composition at different grades of stenosis**

Saam et al. performed CMRI on 175 asymptomatic patients with varying degrees of luminal stenosis (190). They found that 21% of plaques with <50% stenosis were classified as AHA type VI complex lesions and that 8.1% of plaques which caused <15% of stenosis were also
of this type. These high risk lesions may have been missed using current clinical methods of assessment thus CMRI provides additional clinically significant information.

**Association of traditional and novel risk factors**

The ARIC study is a large U.S prospective multicentre study, which has been established for over 20 years (212). A sub-study investigated carotid plaque burden and composition using CMRI in over 1700 participants. At the time of scanning, a number of traditional and novel markers of cardiovascular risk were measured. Traditional risk factors had also been measured at baseline, a mean of 18 years earlier.

When examining longitudinal associations they found that a number of traditional risk factors were independent predictors of plaque burden at the 18 year follow up (see table 1.8). LDL, total cholesterol and age at baseline also predicted presence of LRNC. However the significance disappeared on adjustment for wall thickness.

<table>
<thead>
<tr>
<th>Table 1-8 Predictors of increased wall thickness (all p&lt;0.05) in the ARIC study (212)</th>
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<tbody>
<tr>
<td>• Greater age</td>
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<tr>
<td>• Increased total and low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>• Male sex</td>
</tr>
<tr>
<td>• White race</td>
</tr>
<tr>
<td>• Diabetes</td>
</tr>
<tr>
<td>• Hypertension</td>
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<tr>
<td>• Smoking</td>
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When comparing presence of traditional risk factors at the time of CMRI, they found that levels of LDL were associated with plaque burden while pro-atherogenic ratios of lipids were associated with presence of LRNC. BMI and blood glucose were inversely associated with fibrous cap thickness.

These findings suggest that although traditional risk factors may predict plaque burden, they are of less value in predicting plaque composition. One feature of the study was that diabetes and BMI increased over time, while cholesterol levels were reduced. It may be that changes in risk factors during the 18 year interim period, influenced plaque progression, which was not captured in the study. This could account for the discrepancy.
between longitudinal and cross sectional findings related to plaque composition. Alternatively it may be that more traditional risk factors influence plaque composition in a more transient way, however further work is required to address this question.

When examining novel risk markers, they also found a significant association of genetic polymorphisms coding for platelet activation and matrix metalloproteinase production (215) (216) with plaque burden and composition. Circulating markers of platelet and monocyte activation and chemokine levels were also associated plaque composition on CMRI (217) (218).

Other cross sectional studies have also demonstrated that atherogenic lipid profiles are associated with increased plaque burden and that LDL is specifically associated with LRNC size (213). The studies so far have improved our understanding of the role of traditional and some novel risk factors in plaque progression however a number of unanswered questions remain.

Comparing populations

CMRI offers the opportunity to compare plaque phenotype in different populations. It has been shown in CMRI studies that type 2 diabetic patients have 2.59 increased chance of having high risk plaque compared with non-diabetic patients, with the same grade of stenosis (OR [95% CI]: 2.59 [1.15-5.81]) (219). This may mean stratifying risk on the degree of stenosis alone, is even less effective in the diabetic patients than it is in the general population.

Significant differences in plaque phenotype have also been demonstrated between the sexes and also different ethnicities (220) (221). This could help account for the differences in event rates and response to treatments between different populations.

Predictors of clinical events

CMRI has been utilised in longitudinal studies to identify plaque features, which are predictive of future clinical events and also outcomes following treatment. Gupta et al. recently performed a meta-analysis of the predictive value of presence of IPH, LRNC and thinning/rupture of the fibrous cap (222). They included 9 eligible studies and found that all
3 features predicted subsequent ipsilateral cerebrovascular events (hazard ratios are summarised in Table 1.9).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Hazard ratio (95%CI)</th>
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<tbody>
<tr>
<td>IPH</td>
<td>4.59 (2.91, 7.24)</td>
</tr>
<tr>
<td>LRNC</td>
<td>3.00 (1.51, 5.95)</td>
</tr>
<tr>
<td>Thin/ ruptured fibrous cap</td>
<td>5.93 (2.65, 13.20)</td>
</tr>
</tbody>
</table>

Table 1-9 Association of plaque components and subsequent events Adapted from a meta-analysis by Gupta et al. (222)

Subsequent to this meta-analysis, data from the MESA cohort at 5 year follow up after MRI have been published. This demonstrated a significant association of presence of LRNC and the wall remodelling index (RMI) with cardiovascular events at 5 years (189). They also demonstrated that the incorporation of CMRI findings into a Framingham based cardiovascular risk model, significantly improved performance of the model.

**Triggers for plaque progression**

CMRI studies examining plaque progression have shown that plaques with IPH at baseline had more accelerated increase in volume, decrease in luminal diameter and increase in size of LRNC (205). This suggests IPH could be a trigger for plaque progression and destabilisation. Underhill et al. demonstrated that LRNC at baseline was associated with a 2.6 fold increase in fibrous cap disruption, a key trigger of clinical events (OR[95%CI] per 5% increase LRNC size:2.6[ 1.5-4.6]) (223). Through identifying these key drivers of progression and destabilisation, new therapeutic strategies may be developed.

**Predicting complications of intervention**

Carotid artery stent insertion and carotid endarterectomy are treatment options for symptomatic carotid artery stenosis. Both can be complicated by intracerebral events. Two studies using pre-procedural CMRI have shown that patients with features of unstable plaque are at higher risk of this complication. CMRI therefore could be used in a clinical setting to stratify risk and direct therapy appropriately (224;225).
1.4.3.1.4  Interventional studies

CMRI has been employed in a number of clinical trials to assess the effects of therapy on both plaque burden and composition. A pilot study carried out by Saam et al. found that 14 participants were required to find a 5% change in plaque thickness, a 10% change in volume and 20% change in LRNC at 3 months in order to achieve 80% power (226).

Contrary to trials with clinical endpoints, CMRI studies can be carried out in small numbers over shorter time periods. This makes it ideal for use in proof of concept studies in addition to giving detailed information on direct effects of therapy on subclinical disease.

CMRI has also been used to assess the effects of statin therapy on plaque. Lee et al. demonstrated a mean reduction of 31% in plaque wall volume after 3 months of statin therapy (227). Corti et al. also found a significant reduction in plaque area and thickness after 12 months of treatment with statins with a maximal reduction in plaque area of 19% at 24 months (228).

Others have found change in individual components of plaque. Zhao et al. published a study of a placebo controlled trial of lipid lowering therapy (229). They found a significant reduction in indices of plaque burden, size of LRNC and an increase in fibrous tissue following therapy, suggesting plaque stabilisation. These changes were not significantly associated with changes in lipid levels, suggesting modes of action independent of lipid lowering properties. Underhill et al. also demonstrated a significant reduction in volume of LRNC following statin therapy (230).

CMRI and the risk of coronary artery disease

While most studies have used CMRI to examine stroke risk, there have also been some studies in patients with coronary artery disease. Zhao et al. demonstrated that increased carotid wall volume and calcium content was significantly associated with coronary calcium scores on CT, suggesting that MRI could be used to investigate and monitor atherosclerosis in CAD patients (231). A longitudinal study by Noguchi et al. demonstrated that presence of high intensity signal on T1 weighted imaging, thought to represent IPH, was a significant predictor of future coronary events (232). These findings compliment the large body of
ultrasound studies, which have demonstrated the predictive value of carotid plaque for all cause cardiovascular events (175).

1.4.3.2 Measuring Inflammation and neovascularisation on dynamic contrast enhanced MRI

As previously discussed, inflammation and neovascularisation are thought to play a role in plaque progression and are key features of vulnerable lesions. Kerwin et al. proposed the use of dynamic contrast enhanced MRI (DCE MRI) to quantify neovascularisation and inflammation within the plaque (233). DCE MRI is a well-established technique used in oncology. Sequential images of an area of interest are taken as contrast is injected. Using mathematical modelling of contrast kinetics within the target tissue, microvascularisation can be quantified (234). In solid cancers, DCE imaging can be used to guide choice of therapy and assess response (235).

1.4.3.2.1 Image acquisition and analysis

Unlike tumours, carotid plaques are small discrete structures so carotid DCE is technically challenging. Kerwin et al. incorporated a DCE sequence within the existing structural MRI protocol where high resolution T1 weighted images were acquired in quick succession before, during and after gadolinium based contrast injection. In this first study, the slice with the largest plaque area, which had good differentiation between lumen and plaque, was chosen for analysis (233). A region of interest was drawn around the plaque area and the signal intensity at each time point was measured. A two compartmental Patlak model was then used to model contrast kinetics. This model uses the tissue and blood concentration of contrast, taking the blood concentration from the centre of the artery lumen (or near-by jugular vein in cases where there was severe stenosis). Certain assumptions are required for a Patlak model (listed below in Table 1.10) and it is only appropriate for use if low contrast concentrations are employed over short time periods, as with carotid DCE. Although one of the assumptions for this model is that the contrast decays exponentially, in carotid DCE, the change in signal intensity within the artery over time is measured. Thus, the measured signal decay can be used within the model rather than assuming exponential decay. Using this model, the fraction of blood volume found
within the plaque (Vp) can be estimated. This was proposed to represent density of microvessels within the plaque. The transfer constant of contrast from blood to the extracellular space (Ktrans), is also estimated using this model. Ktrans was thought to be influenced by both microvessel density and vessel permeability, a proxy for inflammation.

<table>
<thead>
<tr>
<th>Table 1-10 Assumptions for Patlak model of contrast kinetics (adapted from Kerwin et al.) (233)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Concentration of contrast is proportional to signal intensity</td>
</tr>
<tr>
<td>• Contrast agent concentration within the plaque consisted of a blood component and also an extravascular extracellular space component</td>
</tr>
<tr>
<td>• Plasma concentration decays exponentially (although this assumption is not required for carotid DCE, as signal intensity is measured within the artery lumen over time)</td>
</tr>
<tr>
<td>• Reflux of contrast form plaque back to plasma is negligible</td>
</tr>
</tbody>
</table>

1.4.3.2.2 Validation studies

In the first human study, DCE-MRI was performed on 20 symptomatic patients prior to carotid endarterectomy. A strong correlation was found between Vp and microvascular density (r=0.80, p<0.001) (233). A subsequent study matching histology and DCE-MRI findings confirmed the correlation with Vp and microvessel density but also of Ktrans and macrophage density (r =0.75, p<0.05) (236).

Gaens. et al. recently assessed validity and reproducibility of DCE MRI in a prospective study. There were slight differences in the imaging protocol, compared with the studies performed by Kerwin et al., however they validated findings against histology and compared 3 different models for analysis (237). They demonstrated that the Patlak model was the most reproducible model compared with other models (intraclass correlation coefficient (ICC) for Ktrans and VP was 0.79 and 0.48 respectively) and confirmed that negligible contrast reflux was seen within the first 7 minutes after injection. Due to an outlier in the Vp measurement ICC was less than expected and on removal a significant improvement in ICC was seen (although data was not shown in the paper).
1.4.3.2.3 Population studies

While there are less data published on DCE-MRI in population studies, higher Ktrans values were found in symptomatic lesions compared with asymptomatic lesions and values correlated with CRP and smoking status (238).

Sun et al. demonstrated an association of Ktrans measured in the adventitial of plaque with presence of intra-plaque haemorrhage, providing further evidence of an association with neovascularisation and haemorrhage. Prospective studies examining risk prediction are still lacking (239).

The technique has been used to evaluate the effects of drugs on plaque inflammation and neovascularisation. Dong et al. examined DCE imaging of hypercholesterolaemic patients on lipid lowering therapy and found a significant reduction in Ktrans at 1 year follow up. This finding was independent of changes in lipid levels (240).

1.4.3.3 Future developments

The capabilities and applications of CMRI are still advancing. In more recent times, CMRI has been used to study the mechanics of flow and sheer stress in the carotid artery and their effects on plaque. Associations between plaque composition, stability and sheer stress have been found, suggesting that flow dynamics may influence plaque development and rupture (241).

There has also been the development of more specific inflammation tracers for using MRI. Tracers containing superparamagnetic nanoparticles allow visualisation of phagocytic macrophage activity on MRI (242). The lack of commercial availability of tracers and long complex imaging protocols has hampered use in a wider research setting.

CMRI has so far shed light on the natural history of plaque development at a number of stages. It has also has begun to help stratify risk of clinical events and inform on effects of drug therapy on plaque. The accuracy with which components and, in particular, inflammation can be quantified, could improve our understanding in the RA population. It is now accepted that inflammation is a key driver of cardiovascular disease and studies investigating the use of anti-inflammatory drugs are ongoing. DCE imaging could provide an
excellent method of directly quantifying the anti-inflammatory effects of these drugs on vascular inflammation.

There are some limitations with CMRI. Patients are required to be still for around 30-40 minutes and motion artefact sometimes can be an issue. This is minimised with neck supports, none the less some scans can be of insufficient quality. Additionally specialist skills are required in analysis of scans even with the aid of semi-quantitative software. Despite this, CMRI provides an excellent tool with which to investigate atherosclerosis and in the future could help stratify risk of stroke and aid drug development.

1.4.3.4 Summary

Up to the time of study set up in 2011, there was clear evidence to suggest that CMRI could be employed to evaluate differences in plaque burden and morphology in small groups of patients (n=14 per group) and that DCE measurements in large plaques correlated with inflammation and neovascularisation on histology. There was evidence to suggest that findings on CMRI could be used to detect changes following therapy and could be to predict not only cerebral events but also events in the coronary circulation. These findings suggest its potential utility as a biomarker of overall cardiovascular risk.
1.4.4 Positron emission tomography (PET)

PET is a nuclear imaging technique, which can be used to quantify metabolic processes. When combined with CT or MRI, metabolic activity can be mapped to a precise anatomical location. Radioactively labelled Fludeoxyglucose (\(^{18}\)FDG) is a commonly used tracer in PET imaging. FDG is a glucose analogue, which is transported into cells via trans-membrane glucose transporters. It is assumed to be taken up on a 1:1 ratio with glucose. Once inside the cell it undergoes the first phosphorylation step of glucose metabolism after which means it is unable to leave most cells (with the exception of liver and skeletal muscle). However due to the structural differences between FDG and glucose, no further metabolic steps can occur and the FDG is trapped irreversibly within the cell. Cells which are metabolically active, consume more glucose therefore preferential uptake of FDG is seen in metabolically active tissues. The radioactive label has a short half-life and while in the target tissue it begins to decay. This causes positrons to be emitted, which collide with nearby electrons causing photons to be emitted at 180 degree angles. The photons can be detected when the patient is lying in a circular PET scanner and overtime an image can be built up of the FDG accumulation within a tissue. There is poor spatial resolution within a PET image, therefore registration with CT and MRI allows more precision of anatomical location of signal. While FDG-PET is not specific to a particular process it is very sensitive to inflammation. This property sparked interest in its’ potential to quantify atherosclerotic plaque inflammation, in particular as activated macrophages have higher metabolic activity compared with other cells and are abundant in unstable plaque. The use of FDG-PET to quantify plaque inflammation has become of increasing interest in this area of research.

1.4.4.1 Measuring atherosclerotic plaque inflammation using FDG-PET-CT

There are challenges associated with imaging such small lesions using a technique with poor spatial resolution and there are varied published methods of image acquisition processing and analysis.
1.4.4.1.1 Image acquisition

Patients are injected with the FDG tracer and then are generally advised to rest quietly prior to the PET scan. Factors which can influence reliability of the scan include time from injection to imaging acquisition, movement during the rest period and fasting status of the patient (243). Minimising movement and speech, reduces uptake in the muscles surrounding the carotid artery, which may otherwise mask signal from the artery. Additionally, fasting prior to the scan is recommended, as this minimises the concentration of glucose in the blood which competes with the FDG to be taken up into cells (243).

Studies have reported varying times from injection to image acquisition from 60 minutes to 180 minutes (244;245). Recommendations published by Rudd et al. in 2013 suggested at least 90 minutes between injection and imaging, in order to obtain optimal measurements of plaque inflammation (244). A more recent study in 2014 examined the effect with varying blood glucose levels and injection to imaging time on images(243). They found that blood glucose at the time of injection negatively correlated with FDG uptake, and injection to scan time positively correlated with FDG uptake. Their recommendations were that for optimal quantification blood glucose pre-injection should be below 7mmol/L and that FDG should circulate for 150 minutes.

CT scans, and more recently MRI, can be performed on a combined scanner which makes image co-registration with FDG uptake optimal. However, in order to identify plaque reliably on CT, high resolution and contrast use, are required which carries additional radiation dosage. Combined PET-MRI scanners are not yet widely available and often PET and MRI images are acquired on different scanners. This can cause problems for image co-registration but use of supports to reproduce neck and head position help minimise this problem.

1.4.4.1.2 Imaging analysis

There is currently no one standardised way to analyse carotid FDG-PET. Three different techniques have been described in the literature. A region of interest is drawn around the plaque on multiple slices and then FDG signal intensity can be measured within that region. The most commonly used method of quantifying carotid FDG uptake is using the standardised uptake value (SUV), which is the measured FDG uptake (MBq/ml) in the
plaque divided by the injected activity per kilogram body weight. This method is the most widely used in oncology and is often used in the quantification of carotid plaque FDG uptake (246). A mean value of all slices in the region of interest can be taken. However, more commonly the maximum uptake value (SUV\text{max}) is measured. The second method is to express the FDG uptake in the region of interest as a ratio of the signal from a vein such as the superior vena cava, which is called target to background ratio (TBR). The third method requires multiple blood sampling so that the rate at which FDG is transported into cells and is metabolised can be calculated using pharmacokinetic modelling. This technique expresses the FDG uptake as a transfer constant K_i (247). While this is considered the gold standard for measuring FDG uptake it is rarely used in practice due to the requirement for multiple sampling.

A reproducibility study performed by Izquierdo-Garcia et al. compared the three methods of measuring FDG uptake within plaque using the K_i models as the gold standard and also performed scans at two time points to demonstrate inter-scan reproducibility (247). They found that SUV was the most reproducible parameter (ICC: 0.90 versus ICC: 0.54 for TBR). However TBR correlated better with K_i (r=0.58 vs r=0.46 for SUV). Other reproducibility studies have demonstrated a high level of inter-scan reproducibility and inter and intra-observer agreement (ICC [95%CI]: 0.86[0.69-0.95], 0.94[0.85-0.98] and 0.88[0.73-0.95] respectively) in short term studies (248).

1.4.4.1.3 Histological validation studies

A number of pre-clinical studies in rabbit models of atherosclerosis have demonstrated a significant correlation of FDG-uptake and macrophage content of plaque and less consistently with degree of vessel thickening. Rudd et al. carried out the first human study employing FDG-PET to examine plaque inflammation (249). They performed FDG-PET on 8 patients with symptomatic carotid artery stenosis and compared findings to the contralateral artery. Increased FDG uptake was seen in all symptomatic lesions compared with only some asymptomatic lesions. Then they went on to compare with histological findings on endarterectomy specimens from the same patients and showed dense macrophage infiltration, which correlated with FDG uptake on PET. The final part of their experiment included performance of autoradiography of three of the histology specimens, which had been incubated with \(^3\)H-DG. This demonstrated tracer uptake between the lipid
core and fibrous plaque of the plaques. Although this was not pinpointed to a specific cell type it could signify the inflammation associated with fibrous cap thinning and the process of plaque destabilisation seen in vulnerable lesions. Subsequent studies have confirmed the association of FDG uptake with macrophage content and also an association with lipid content (250;251). Hypothetically neovascularisation and increased vessel permeability may allow increased delivery and diffusion of FDG into plaques.

1.4.4.1.4 Population studies

A number of studies have retrospectively analysed FDG uptake in the aorta, carotid and iliac arteries of patients who had undergone whole body PET for suspected malignancy. These studies demonstrated an association with traditional risk factors (such as hypercholesterolaemia), circulating MMP-3 levels (which may be implicated in fibrous cap erosion), CRP and a history of cardiovascular disease (252) (253). One study by Rominger et al. examined the rate and association of subsequent cardiovascular events in a cohort of patients, with no prior cardiovascular disease, who had had a whole body FDG-PET-CT (254). 932 patients were included in the study and were followed for a median of 29 months. There was an event rate of 1.6% and arterial FDG uptake was independently associated with risk of a cardiovascular event. The study suggested a TBR of more than 1.6 was associated with a significant increased risk of a cardiovascular event. Limitations of these studies include their retrospective nature, that fact they were carried out in a specific population (cancer patients who may have been on therapy) and that image acquisition was not specifically optimised for vessel imaging. Prospective studies examining the utility of FDG-PET for predicting cardiovascular events are forming part of the High Risk Plaque Initiative, a multi-national biomarker study aiming to develop better stratification tools for atherosclerotic plaque (255).

Tahara et al examined the prevalence of inflamed carotid plaques identified on ultrasound screening and the association with cardiovascular risk factors and CRP (246). Patients on statins and those with inflammatory disease, cancer or acute coronary syndrome were excluded. Inflammatory lesions were defined as greater than or equal to 1 times the mean plus standard deviation of the SUV in study patients, which was 1.60. FDG-PET was performed in 41 participants with plaque confirmed on ultrasound and 12 of these patients had evidence of an inflammatory lesion (29.3%). Patients with inflamed plaques had higher
BMI, waist circumference and a higher frequency of anti-hypertensive use, but there was no difference in CRP or lipid parameters between the groups.

### 1.4.4.1.5 Interventional studies

Carotid FDG-PET has been employed to evaluate the effects of existing and novel treatments on carotid plaque inflammation. Mizoguchi et al. demonstrated significant reduction in FDG uptake associated with pioglitazone use at 4 month follow up when compared with patients taking another oral hypoglycaemic (256). Effects were independent of glucose reduction. Further studies also demonstrated a significant reduction in plaque inflammation in the aorta and carotid arteries following 3 months of treatment with simvastatin (250;257). PET has provided a valuable endpoint in small proof of concept studies of new agents including Dalcetrapib and also on a new MAP kinase inhibition molecule (258;259). As with CMRI, change can be detected in smaller numbers over shorter time periods using FDG-PET-CT although further prospective studies are required to validate imaging findings with cardiovascular risk.

### 1.4.5 Comparison of DCE-MRI and FDG-PET findings in the carotid artery

A number of studies have compared these two inflammation imaging methodologies and surprisingly only weak correlations are found. Truijman et al. carried out FDG-PET and DCE-MRI in TIA patients and found a correlation co-efficient of 0.30 between Ktrans and TBR (p=0.04) (260). Wang et al. compared both techniques in symptomatic and asymptomatic patients with plaque. There was no significant correlation across the study but a significant correlation was found in the symptomatic group(r=0.59, P=0.006) (245).

While both techniques image the broad process of inflammation, neither directly visualise inflammation. DCE-MRI relies on the assumption that the vessels in inflamed plaques are more permeable thus allowing more contrast into the extra cellular space leading to higher Ktrans values. However Ktrans is also dependent on microvessel density. FDG-PET measures metabolic activity, as a proxy for inflammation and it may be that neovascularisation and increased metabolic activity may occur at different times within the
plaque. Dual assessment with both techniques may provide a more comprehensive assessment of the functional status of plaque.

1.4.6 Carotid artery imaging in RA

There has been a substantial body of work investigating atherosclerosis using carotid artery ultrasound in RA patients. Plaque presence has been shown to predict future cardiovascular events not only in the general population but also in RA (261). Higher prevalence of carotid plaque has been found on ultrasound in patients compared to controls and higher rates of progression have been observed (262;263). Ultrasound studies have demonstrated an association with inflammation and presence and progression of carotid plaque in RA (262;263). Additionally, Van Sijl et al., demonstrated a higher prevalence of an outward wall remodelling pattern in RA patients compared to controls on carotid ultrasound (264). Outward remodelling is known to be a feature of high risk plaque (123).

Only one study has used high resolution ultrasound to assess carotid plaque morphology in RA. Semb et al. compared grey scale measurements (GSM) of plaque on ultrasound in RA patients and controls (265). No significant difference in GSM was found between the groups. However, patients with active disease did have lower GSM than those in remission (p=0.03), suggesting patients with active disease may have more unstable plaque. On review of the MRI and PET literature there are no published studies describing the use of these techniques to investigate carotid atherosclerosis in RA.
1.5 Other measures of subclinical cardiovascular disease

1.5.1 Arterial stiffness

Arterial stiffness refers to the ability of the artery to expand and recoil in response to blood flow. Increased stiffness is strongly associated with aging but is also a predictor of cardiovascular events independent of age, sex and blood pressure (266;267). Over time, thickening of the intima-medial layer occurs with smooth muscle cell hyperplasia, fibrosis and loss of elastin. This leads to reduced distensibility and elastic recoil within the arterial tree, which is thought to promote atheroma formation. Although these structural changes influence stiffness, the vascular endothelium is also a key regulator and endothelial dysfunction can cause a reversible increase in vascular stiffness.

Increased arterial stiffness has been shown to correlate with cardiovascular mortality, left ventricular dysfunction and recurrence of ischaemic coronary event (267;268). In the general population, improvement in arterial stiffness has been demonstrated following treatment with anti-hypertensive agents (269).

HsCRP is an independent predictor of arterial stiffness (270) suggesting inflammation may influence vascular tone. In RA, patients have been shown to have higher arterial stiffness compared to age and sex matched controls (271;272). Some, but not all of these studies, have found associations with arterial stiffness and disease activity and severity (272). Conflicting results have also been found when examining changes with RA-related therapy (273;274). The heterogeneity of RA populations, study design and methodologies used in these studies could explain some of the variability in results.

1.5.1.1 Measuring arterial stiffness

Pulse wave velocity (PWV) is considered the “gold standard” direct method which measures the velocity of a pulse of blood flow between two points in the aorta (275). Reduced distensibility leads to an increased velocity along the vessel. This can be measured non-invasively using 3 different techniques. The first is using a piezoelectric method where pressure sensitive transducers are applied to the skin above the carotid and femoral arteries and a measurement is taken between these two sites. The distance between the
two sites is then divided by the transit time between the carotid and femoral waves to give PWV (276). The second method is using planimetry tonometry. A sensor is applied to the skin over the radial artery which evaluates the arterial waveform in the context of peripheral blood pressure. By applying a transfer function equation to the radial waveform, the aortic PWV can be estimated (277).

The third method is using an arteriograph machine, which evaluates the oscillometric pressure curves of the brachial artery using plethysmography. A pressure cuff is applied over the brachial artery and the distance between the jugulum and the symphysis pubis is measured (JSD). Initially the blood pressure in the brachial artery is measured using the pressure cuff and then the cuff automatically inflates to 35mmHg above the measured systolic blood pressure. Fluctuations in the brachial artery pressure are sensed by the cuff and pulse waves are recorded and transmitted to a computer. The PWV is calculated by evaluating the timings of wave reflections in the pulse with the additional information of the JSD. Comparison of the three techniques has shown high levels of agreement on PWV measurement (276). However, the first 2 techniques require experienced operators and this is thought to have hampered their use in clinical practice (276). The arteriograph is a small, automated machine, which can be attached to a laptop and measurements have been shown to correlate strongly with centrally measured PWV (r=0.95, p<0.001) (278).

1.5.2 Endothelial function

Endothelial dysfunction is known to be one of the earliest signs of cardiovascular disease and can be detected before any structural defect can be seen.

Endothelial function is dynamic and where it can be an early sign of cardiovascular disease it can also be used to reflect early improvement in cardiovascular health following therapy. In the general population, improvement in endothelial dysfunction has been demonstrated with statin therapy even before cholesterol lowering has occurred (279;280). In patients with RA, endothelial dysfunction can be detected early in disease and some studies have demonstrated improvement following successful suppression of disease activity (127;281).
1.5.2.1 Measuring endothelial function

There are a number of methods employed to assess endothelial function including flow mediated dilatation (FMD) which measures the degree of reactive brachial artery vasodilatation in response to sheer stress using ultrasound. Other methods include measurement of blood biomarkers, which reflect endothelial activation and damage.

Circulating levels of cellular adhesion molecules and endothelial microparticles are both reflective of endothelial function, as discussed earlier. Serial measurements of these molecules can be performed to detect changes in endothelial function over time and have been used in interventional studies to assess effects of therapy in the general population (282).

1.6 Summary of the literature and relevance

Inflammation is thought to contribute to premature atherosclerosis in RA. Plaque composition and inflammation play a critical role in acute cardiovascular events in the general population. RA patients have less warning symptoms, higher risk of sudden cardiac death and this may be due to having more unstable inflammatory atherosclerotic plaques. It is also unclear how current RA treatments affect atherosclerosis progression and plaque stability.

Imaging techniques such as CMRI and FDG-PET could provide methods of characterising atherosclerosis in RA and assessing changes within the arteries following treatment. Other subclinical measures of cardiovascular disease such as endothelial dysfunction and arterial stiffness may also be used to provide insight into the effects of anti-inflammatory therapy on the vasculature.

Non-invasive imaging is an accepted method to study atherosclerosis in more detail. Population studies have shown that newer modalities such as CMRI and FDG-PET may provide new insight into angiogenesis and composition of plaque in disease. They also allow plaque inflammation and instability to be considered.

RA is known to be associated with increased cardiovascular risk and studies have demonstrated increased prevalence of arterial stiffness and presence of carotid plaque on ultrasound. Only a few studies have begun to employ more detailed imaging and to our
knowledge, neither CMRI nor FDG-PET has been used to investigate carotid plaque morphology and inflammation in RA. Several key issues need to be assessed when considering the use of these imaging methods in RA. Firstly, the technical feasibility in a disease with background inflammation, joint stiffness and neck discomfort; secondly, the percentage of lesions assessable by these techniques in an RA population and thirdly whether differences in plaque inflammation and morphology can be detected between RA patients and unaffected subjects using these methods. All of these issues are vital to increase our understanding of inflammation and angiogenesis of plaque in RA and also to determine how anti-inflammatory therapy and suppression of inflammation may influence plaque characteristics in RA patients.
1.7 Hypothesis and aims

1.7.1 Hypothesis

Rheumatoid arthritis is associated with increased prevalence of atherosclerosis and may be associated with a more inflammatory, unstable plaque phenotype. This thesis aims to investigate differences in plaque phenotype between RA and the general population and explore relationships with disease activity and plaque inflammation.

The key hypothesis tested in this thesis is:

RA patients have more unstable, inflammatory carotid plaque compared with the general population and plaque composition and inflammation can be altered by treating active joint disease.

1.7.2 Aims

The aims of this thesis are:

1) To establish whether a distinct inflammatory phenotype can be detected in carotid plaques of RA patients using CMRI and FDG-PET biomarkers.

2) Determine whether differences can be detected in plaque morphology and inflammation after treatment of active joint disease using CMRI.

3) To identify clinical characteristics and serological biomarkers that correlate with plaque presence, inflammation and morphology.

4) To test the feasibility of using MRI and FDG-PET-CT to evaluate carotid plaque in the RA population.
As a result of this study, the aim is to understand more about the atherosclerotic plaque in RA, identify clinical, serological and imaging biomarkers for higher CVD risk and assess the effects of therapy on plaque characteristics.
2 Materials and methods

2.1 Introduction

This chapter will describe the methods used to test the hypothesis and aims described in chapter 1. This will include an overview of the study, details of study design and imaging and laboratory methods used in the thesis.

2.2 Study Setting

This study was based at the Arthritis Research UK Centre for Epidemiology and the study sponsor was The University of Manchester. The study was based in secondary care but with recruitment of control participants from primary care.

2.3 Study funding and support

This study was funded from 4 grants. The main funding was for a three year North West England Medical Research Council Clinical Pharmacology and Therapeutics Research Fellowship. Two small grants were awarded from the Astra Zeneca University of Manchester Strategic Alliance Fund. Further funding, to support the study, was provided by the Manchester NIHR Musculoskeletal Biomedical Research Unit (BRU). The study was adopted onto the NIHR Clinical Study Portfolio, which led to support with recruitment of patients and controls.
2.4 Overview of study design

This was a prospective case control pilot with longitudinal follow up. RA patients with active arthritis and age and sex matched controls were recruited to the study. While subjects were recruited from multiple centres in the North West of England, the research visits were undertaken at a single centre, the University of Manchester. Clinical assessments were performed at the National Institute for Health Research/ Wellcome Trust Manchester Clinical Research Facility (MCRF), imaging and laboratory tests were performed within the University of Manchester and Central Manchester Hospitals Foundation Trust.
2.5 Ethical Approval

Ethical approval was obtained from the National Research Ethics Committee (Lancaster Committee, reference number 12/NW/0117, appendix 1). Approval was also obtained from the Administrations of Radioactive Substances Advisory Committee (appendix 2). Local approval was obtained for the Trusts and Primary Care Groups listed in Table 2.1. Written informed consent was obtained from each participant in accordance with Good Clinical Practice in Research Guidelines (appendix 3).

Table 2-1. Centres where local ethical approval was obtained

- Central Manchester University Hospitals NHS Foundation Trust (CMFT)
- University Hospital of South Manchester NHS Foundation Trust (UHSM)
- Salford Royal NHS Foundation Trust (SRFT)
- The Pennine Acute Hospitals NHS Trust
- East Cheshire NHS Trust
- Pennine MSK Musculoskeletal Partnership
- NHS Manchester
- NHS Oldham
- NHS Salford
2.6 Inclusion and exclusion criteria

Patients with a physician reported diagnosis of RA were invited to participate in the study. At the first visit, confirmation that patients met the 1987 classification criteria was undertaken (described in Table 1.1). The ACR criteria were chosen at the time of study design as they are well validated against outcomes and are particularly specific for established disease. Other inclusion and exclusion criteria can be seen in Tables 2.2 and 2.3.

<table>
<thead>
<tr>
<th>Table 2-2 Inclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Age 40-65 years old</td>
</tr>
<tr>
<td><strong>For RA patients only:</strong></td>
</tr>
<tr>
<td>• Diagnosis of RA &gt; 1 year</td>
</tr>
<tr>
<td>• DAS28 score &gt; 3.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2-3 Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Statin therapy within the last 2 months</td>
</tr>
<tr>
<td>• Chronic kidney disease with an eGFR &lt; 60mL/min/1.73m²</td>
</tr>
<tr>
<td>• History of vasculitis</td>
</tr>
<tr>
<td>• Previous allergy to contrast</td>
</tr>
<tr>
<td>• Unstable cervical spine</td>
</tr>
<tr>
<td>• History of carotid endarterectomy</td>
</tr>
<tr>
<td>• Cervical spine surgery with metal insertion</td>
</tr>
<tr>
<td>• Metallic implants which are contraindicated in MRI scanning e.g. cardiac pacemaker</td>
</tr>
<tr>
<td>• BMI &gt; 38kg/m²</td>
</tr>
<tr>
<td>• Inability to give consent</td>
</tr>
<tr>
<td>• Pregnancy or breast feeding</td>
</tr>
</tbody>
</table>
2.7 Participant recruitment

2.7.1 Patient recruitment

Patients were recruited from six rheumatology centres within the North West of England (listed in Table 2.1). Patients were identified and approached by member of the local team and if agreeable were provided with an information leaflet. They were then contacted at least 24 hours after the initial approach to discuss the study further and arrange a study visit if agreeable. Posters advertising the study were also placed within outpatient departments with contact details. If a patient contacted the study team, a leaflet was sent out and a follow up phone call was carried out to arrange a study visit if agreeable.

2.7.2 Control recruitment

Control participants were recruited by 4 different methods. The first was the “best friend system”, whereby participating patients could invite a friend to act as a control participant. The second method was via mailshot co-ordinated through the NIHR Primary Care Research Network. Database searches for suitable control participants, was conducted at three GP practices. Letters of approach, which included an information leaflet and a reply slip, were sent out to eligible patients, by their own medical practitioner. Once a reply slip was received, the participant was contacted to discuss the study further and arrange a study visit. Other methods of recruitment included local advertisement within The University of Manchester and participating GP practices.
2.8 Participant assessments

2.8.1 Visit one

Clinical assessments

Participants attended in the morning, having fasted overnight. Following written informed consent, a medical history was taken. In particular screening for cardiovascular risk factors and in RA patients a full rheumatological history was taken (detailed in Table 2.4).

<table>
<thead>
<tr>
<th>All participant history</th>
<th>RA history</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Smoking status</td>
<td>• Disease duration</td>
</tr>
<tr>
<td>• Significant family history of CVD</td>
<td>• Current and previous DMARD use</td>
</tr>
<tr>
<td>• History of diabetes</td>
<td>• Glucocorticoid use within the last 12 months</td>
</tr>
<tr>
<td>• History of hypertension</td>
<td>• Extra-articular manifestations including nodules</td>
</tr>
<tr>
<td>• History of hypercholesterolaemia</td>
<td></td>
</tr>
<tr>
<td>• Full drug history</td>
<td></td>
</tr>
<tr>
<td>• History of other medical conditions</td>
<td></td>
</tr>
</tbody>
</table>

A 28 tender and swollen joint count and assessment for rheumatoid nodules and other extra-articular clinical manifestations, was also performed in RA patients.

Anthropomorphic measurements and bloods were taken as described in table 2.5. Blood was also drawn to test novel risk markers included in table 2.5 and details on analysis methods for these tests will be discussed in subsequent sections.

Patients were also then asked to complete The Stanford Health Assessment Questionnaire (HAQ) and visual analogue score of global health. From the clinical and serological measurements a DAS28 score was calculated in RA patients.
Table 2-5 Variables measured

<table>
<thead>
<tr>
<th>Clinical</th>
<th>Serological</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Blood pressure</td>
<td>• Urea and electrolytes (including creatinine)</td>
</tr>
<tr>
<td>• Pulse</td>
<td>• ESR</td>
</tr>
<tr>
<td>• Weight</td>
<td>• High sensitivity CRP (hsCRP)</td>
</tr>
<tr>
<td>• Height</td>
<td>• Fasting glucose</td>
</tr>
<tr>
<td>• Waist circumference</td>
<td>• Fasting lipid profile</td>
</tr>
<tr>
<td>• Hip circumference</td>
<td>• Pregnancy test (if applicable)</td>
</tr>
</tbody>
</table>

**Novel markers**
- CD4+CD28null cells
- Endothelial microparticles (EMPs)
- Cellular adhesion molecules: I-CAM, VCAM-1, e-selectin, p-selectin
- IL-6
- TNF

**For RA patients**
- RF and ACPA

**Vascular assessments**

**Aortic Pulse wave velocity**

Aortic pulse wave velocity (PWV) was measured using an automated Tensiomed Arteriograph machine (Tensiomed, Hungary). While still fasted, patients were positioned supine on a bed and asked to rest quietly for at least 5 minutes. A blood pressure cuff was then applied round the non-dominant arm above the ante-cubital fossa. This was attached to the arteriograph machine, which is in turn attached to a laptop (set up seen in figure 2.1). A measurement was taken from the symphysis pubis to the sternal notch. This data was then entered into the arteriography programme on the laptop and an automated measurement was then performed which included automated analysis of arterial wave forms at different stages of cuff deflation (as previously described in the introduction). An automated read out of PWV was generated on the laptop. This method of measuring PWV has previously been validated at our centre in a cohort of patients with autoimmune condition including RA (283).
Figure 2-1 The set up for measurement of arterial stiffness using the Tensiomed arteriograph (Tensionmed, Hungary). With the subject supine, a cuff is applied to the upper arm. The cuff is attached to the automated arteriograph machine, which inflates and deflates the cuff in order to quantify aortic PWV. The waveforms appear on the laptop screen with a calculated PWV value.
Carotid artery ultrasound

An ultrasound of both carotid arteries was performed on a Philips iU22 machine using a 9-3MHz probe with specific protocol settings for carotid artery ultrasound (a detailed imaging protocol is described in subsequent imaging methods section). If suitable plaque was identified, subjects were invited to have an MRI scan.

2.8.2 Visit 2

2.8.2.1 CMRI scan

Participants with suitable plaque attended the University of Manchester Translational Imaging Unit at Salford Royal Hospitals NHS Foundation Trust within two weeks of the first visit. A CMRI with contrast injection was performed on a 3 Tesla Philips Achieva MRI scanner (Philips Healthcare, Netherlands). Details of image acquisition and processing are described in section 3.9.2. This would constitute the end of the study for control participants.

2.8.3 Visit 3-FDG-PET sub study

A carotid artery FDG-PET-CT scan was performed in a subgroup of patients (not in controls) within 2 weeks of the MRI scan. Additional exclusion criteria were applied at this stage, listed in table 2.6. Patients undergoing MRI scanning, who did not meet any of the additional exclusion criteria, were invited to take part in the sub study.

If agreeable, patients attended the Department of Nuclear Medicine, CMFT where a PET-CT scan was performed on a Siemens Biograph mCT time of flight PET/CT scanner (Siemens, Germany). Details of the imaging acquisition and analysis are described in subsequent sections on imaging methods.
2.8.4 Visit 3/4 (follow up assessment)

Following the MRI scan, patients were back under the care of their own clinician and as they had active disease, it was expected that their RA therapy would be escalated or changed with the aim of controlling disease. Members of the research team made contact with the patients and the clinical team within 3 to 6 months of the initial scan. In order to evaluate the effect of suppressing inflammation on plaque inflammation and morphology, repeat assessments was planned for when a significant improvement in clinical disease activity had been noted (an improvement in DAS28>1.2). If, on contacting the subjects, disease activity had not significantly improved, the follow up visit was delayed until a significant improvement had been noted.

Clinical assessments

Participants attended having fasted overnight and consent to continue with the study was reconfirmed. A history was retaken in particular noting any changes in medications, new diagnoses or symptoms. Clinical examination of joints was performed with the 28 swollen and tender joint counts. Patients then completed the visual analogue score of perceived global health. Blood was drawn for the tests listed in table 2.7. A DAS28 score was calculated.
Table 2.7 Serological tests on follow up visit

- HsCRP
- ESR
- Cellular adhesion molecules
- Endothelial micro particles
- Fasting lipid profile

**MRI assessment**

Following breakfast the patients then underwent repeat CMRI with identical sequences to the first MRI. Slices were positioned as close to the ones in the original scan as possible. At this point participation in the study was complete.
2.9 Imaging methods

The following section describes in detail the methods used for imaging in the study including acquisition, processing and analysis.

2.9.1 Ultrasound

2.9.1.1 Imaging protocol

Participants were positioned flat on a bed with their head rotated laterally and chin tilted caudally to optimise the carotid bulb view. Firstly the carotid bifurcation was visualised in cross section. The probe was passed above and below the carotid bulb at least 2 cm in each direction. This was performed at different angles around the circumference of the carotid artery as, if only one probe position is used, plaque on the lateral wall can be missed (personal communication T. Hatsukami). Images were captured with and without doppler measurements to assess for presence of plaque and stenosis.

A longitudinal assessment of the artery was then undertaken 2cm above and below the carotid bifurcation and circumferentially around the vessel. Plaque was defined as the presence of 2 out of the following parameters: Intima medial thickness (IMT) >1.5mm, wall protrusion or increased wall echogenicity (284). If plaque was present a minimum of 3 sets of measurements of plaque dimensions were taken. All images were anonymised and stored. Example images of normal artery and artery with plaque can be seen in figure 2.2 to 2.4.

Although accepted plaque definition includes a minimum IMT of 1.5mm, an IMT of at least 2mm is required for reliable DCE-MRI assessment of plaque (240). Therefore, only patients with plaque greater than 2 mm thickness went forward to have an MRI scan. Ultrasound scans were performed by three operators. Details on training and validation are described in appendix 4.
Figure 2-2 Ultrasound of a normal carotid artery. In this longitudinal view, the vessel walls are seen in grey and the lumen appears black as demonstrated by the arrows. The common carotid artery (CCA) bifurcates (marked *) into the internal and external carotid artery (marker ICA and ECA respectively). The intimal medial layer can be seen as a thin layer along the inner edge of the vessel wall (marked by the yellow arrow).
Figure 2-3 A carotid artery ultrasound with evidence of plaque in the common carotid artery. The yellow arrow points to the plaque, which appears as a raised lesion protruding into the lumen and is of mixed echogenicity. Normal vessel wall can be seen on the superficial vessel wall (red arrow). The dimensions of the plaque are measured using calipers and thickness and length measurements can be seen in the bottom left corner of the image.
Figure 2-4 Doppler ultrasound image of the carotid artery. This the same view of the carotid artery as is seen in figure 2.3 but with a doppler measurement applied. Doppler measures blood flow by quantifying the intensity changes in signal within a specified area. The Doppler scale can be seen in the top right corner of the image. The higher the signal intensity change, the higher the Doppler signal. The lumen can be seen to be filled with colour, which signifies the blood flow within the lumen. A reduction in the luminal diameter and can be seen in the area where there is plaque (B) compared to an area of the vessel without plaque (A).
2.9.2 CMRI

2.9.2.1 MRI set up and protocol optimisation

The study was conducted in collaboration with The Vascular Imaging Laboratory, University of Washington (UW) who pioneered the CMRI technique. The candidate visited the UW research group to present the study and learn about imaging acquisition and principles of analysis prior to completion of the study protocol. A second visit was undertaken once the study had started, to discuss progress and to complete formal training on quality assurance of MRI images and plaque identification. Details of protocol optimisation and the candidates training in quality assurance are described in appendix 4.

Equipment specifications

An 8 channel surface coil was purchased, which was specifically designed for carotid artery imaging (seen in figure 2.5). Supporting software and an imaging protocol, provided by the UW research group, was uploaded onto the Philips Achieva 3 Tesla scanner (Philips Healthcare, Netherlands) at the Translational Imaging Unit, University of Manchester. A protocol optimisation study with 5 healthy volunteers was undertaken prior to the start of the case-control study.

Imaging acquisition

The imaging protocol validated by the UW team was implemented without any changes to ensure accurate imaging acquisition. After venous cannulation, participants were positioned on the scanner bed with their head in a fixed position head rest and the surface coils positioned on either size of the trachea (see figure 2.5). The head position was aligned with the long axis of the scanner. At this point a measurement from tip of the mandible to sternal notch was measured if the patient was proceeding to have a PET-CT scan.
Once in the scanner, an initial survey and a 2 dimensional time of flight (2D-TOF) sequence were taken in order to plan subsequent sequences. An index artery was chosen on the basis of ultrasound findings. The bifurcation in the index artery was identified on the TOF sequence and subsequent sequences were centred round this slice (see figure 2.6). Details of each imaging sequence can be seen in table 2.8.

The DCE MRI sequence was performed following structural imaging sequences. Only 4 slices are acquired for DCE, due to the need for good temporal resolution. Therefore a brief review of the structural images was performed prior to DCE. This was to confirm plaque presence and align DCE slices to include both the bifurcation and the plaque in the index artery. Gadopentate (Bayer Healthcare Pharmaceuticals), a gadolinium based contrast agent was injected after the first 2 frames of the DCE sequence, at a concentration of 0.05mmol/kg and at a rate of 1cc/second. A further 18 frames were acquired during and after contrast administration. A post contrast enhanced T1 weighted sequence was taken immediately after the DCE sequence. Total scan time was approximately 50 minutes.

Figure 2-5 Custom made surface coils for carotid MRI. The subject lies on a fixed position head rest, which helps to reduce motion during the scan and provide optimal neck position for imaging the carotid arteries. The pads with in-build surface coils are placed on either side of the trachea overlying the carotid arteries.
The 2D time of flight (TOF) angiography is used to plan slice position on subsequent sequences.

Image I. demonstrates an example of a 2D TOF sequence with the yellow arrow pointing to the carotid bifurcation of the index artery.

Slices are then positioned centering around the bifurcation.

Image II. shows the slice positioning for an axial sequences with the red lines centered around the bifurcation.

Figure 2-6 Identification of the bifurcation on 2D-TOF and schematic of slice positioning
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Example image</th>
<th>Details</th>
</tr>
</thead>
</table>
| Survey          |               | TR: 6.30ms  
TE: 2.38ms  
Flip angle: 15°  
Slice thickness: 4mm  
Number of slices: 33  
Matrix: 256 x 256  
Voxel dimensions: 0.9375 x 0.9375 x 4mm |
| 2D-TOF          |               | TR: 25.15ms  
TE: 3.93ms  
Flip angle: 40°  
Slice thickness: 2mm  
Number of slices: 36  
Matrix: 256 x 256  
Voxel dimensions: 0.625 x 0.625 x 2mm |
| Sagittal Oblique|               | Oblique sagittal slices through each vessel wall  
Multi-slice double inversion recovery (MDIR) for blood suppression  
TR: 2000ms  
TE: 7.50ms  
Flip angle 90°  
Slice thickness: 2mm  
Number of slices: 6  
Matrix: 256 x 256  
Voxel dimensions: 0.3125 x 0.3125 x 2mm |
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Example image</th>
<th>Details</th>
</tr>
</thead>
</table>
| Proton density weighted turbo spin echo (PD) | ![Image](image1.png) | TSE motion sensitized driven equilibrium for blood suppression  
TR: 4800ms  
TE: 10ms  
Flip angle: 90°  
Slice thickness: 2mm  
Number of slices: 16  
Matrix: 268 x 268  
Voxel dimension: 0.2857 x 0.2857 x 2mm |
| T2-weighted turbo spin echo | ![Image](image2.png) | MDIR for blood suppression  
TR: 4800ms  
TE: 50ms  
Flip angle: 90°  
Slice thickness: 2mm  
Number of slices: 16  
Matrix: 268 x 268  
Voxel dimensions: 0.2857 x 0.2857 x 2mm |
| 3D-TOF | ![Image](image3.png) |  
TR: 20ms  
TE: 4.88ms  
Flip angle: 20°  
Slice thickness: 2mm  
Number of slices: 48  
Matrix: 268 x 268  
Voxel dimensions: 0.2857 x 0.2857 x 2mm |
Table 2.8. Details of imaging sequences acquired (Cont.)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Example image</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetisation prepared rapid acquisition gradient echo (MP-RAGE)</td>
<td></td>
<td>TR: 8.68ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TE: 5.24ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flip angle: 15°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slice thickness: 2mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of slices: 48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Matrix: 268 x 268</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Voxel dimensions: 0.2857 x 0.2857 x 2mm</td>
</tr>
<tr>
<td>T1- 3D Turbo field echo</td>
<td></td>
<td>TR: 8.40ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TE: 3.90ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flip angle: 8°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For co-registration with PET-CT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slice thickness: 2mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of slices: 156</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Matrix: 224 x 224</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Voxel dimensions: 1 x 1 x 2mm</td>
</tr>
<tr>
<td>T1 Weighted turbo spin echo</td>
<td></td>
<td>Quadruple inversion recovery for flow suppression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TR: 800ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TE: 10ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flip angle: 90°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slice thickness: 2mm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of slices: 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Matrix: 268 x 268</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Voxel dimensions: 0.2857 x 0.2857 x 2mm</td>
</tr>
</tbody>
</table>
Table 2.8. Details of imaging sequences acquired (Cont.)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Example image</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamic contrast enhanced T1 weighted fast field echo</td>
<td></td>
<td>TR: 126ms&lt;br&gt;TE: 4.61ms&lt;br&gt;Flip angle: 50°&lt;br&gt;Slice thickness: 3mm&lt;br&gt;Matrix: 260 x 260&lt;br&gt;Voxel dimensions: 0.3125 x 0.3125 x 3mm&lt;br&gt;Number of slices: 4&lt;br&gt;Number of frames: 15&lt;br&gt;Contrast injection on third frame&lt;br&gt;Time between frames: 17.5sec</td>
</tr>
<tr>
<td>Contrast enhanced T1 -weighted turbo spin echo</td>
<td></td>
<td>Quadruple inversion recovery&lt;br&gt;TR: 800ms&lt;br&gt;TE: 10ms&lt;br&gt;Flip angle: 90°&lt;br&gt;Slice thickness: 2mm&lt;br&gt;Number of slices: 16&lt;br&gt;Matrix: 268 x 268&lt;br&gt;Voxel dimensions: 0.2857 x 0.2857 x 2mm</td>
</tr>
</tbody>
</table>
Initial image review and quality assurance

Following data acquisition, scans were stored in an anonymised format. A review was undertaken by the candidate with the aim of identifying any clinically relevant findings on the MRI (stenosis>70%, plaque ulceration or thrombus formation) and also to evaluate imaging quality. The data was then transferred over a secure server to the Vascular Imaging Laboratory, University of Washington. Images were then reviewed by a second reader for quality assurance purposes.

Quality assurance

A standardised quality assurance procedure was carried out and a form was completed for each scan (appendix 5). Each sequence was reviewed and graded from 1 (poor quality) to 4 (excellent quality). In general a grade of 2 or above was acceptable. Confirmation was then recorded that plaque was present and that there was adequate coverage of plaque in the DCE sequences. A final recommendation was given whether the scan should be repeated or not.

2.9.2.2 Imaging analysis

MRI scans were analysed by 2 validated readers at the Vascular Imaging Laboratory, UW using semi-automated software (CASCADE, Seattle, WA). Readers were blinded to the clinical details and were not aware whether they were patients or control subjects. Scans were analysed in 3 batches and where data at 2 time points was available for subjects, these datasets were analysed together. This was to reduce inter and intra-reader variability in individual subjects and to ensure measurements were taken from the same plaque if more than one was present within the artery.
2.9.2.2.1 Plaque burden and morphology analysis

CASCADE is a semi-automated analysis tool which has been validated against manual analysis and histological components on endarterectomy specimens (Cascade, Seattle, WA) (285). The steps taken to analyse plaque morphology are set out below:

*Step 1: Identification of bifurcation*

The slice with the index artery bifurcation is found in all contrast weightings. The bifurcation is used as the landmark to co-register all images together so that sections of the vessel wall can be reviewed in multiple contrast weightings at once.

*Step 2: Identification of inner and outer wall boundaries*

A semi-automated boundary detection tool is applied to the T1 weighted sequence, with manual correction by the reader to ensure correct marking of the boundaries of vessel wall (an example can is seen in figure 2.7). This was then applied to the other sequences and corrected manually as required to ensure that the vessel wall boundaries are accurately outlined in all sequences.
Figure 2-7 An example of boundary definition on an axial T1 weighted sequences. Contours are drawn around the inner and outer boundaries of the vessel wall (inner boundary can be seen in red, outer boundary in blue). The plaque can be seen as a thickening in the wall (highlighted by the arrow).

Step 3: Identification of individual plaque components

A morphology enhanced probabilistic plaque segmentation (MEPPS) algorithm is then applied to the plaque (285). This model was developed using regions outlined on histology specimens and matching signal intensities within each component. A model was developed based on the probability of each pixel being from a specific tissue component on MRI. Components including necrotic core, intra-plaque haemorrhage, calcium and loose matrix are segmented. Manual refinement of regions is then applied to ensure good segmentation.
Step 4: Quantification of plaque dimensions and components

Following segmentation of wall and individual components measurements can be taken of plaque and component areas in each slice.

Measurements taken in each slice included:

- Lumen area
- Wall area
- Maximum wall thickness
- Mean wall thickness
- Minimum wall thickness
- Calcium area
- Loose matrix area (LM)
- Lipid rich necrotic core area (LRNC)
- Intra-plaque haemorrhage area (IPH)

2.9.2.2.2 DCE MRI analysis

CASCADE software was also used for DCE MRI analysis and the steps for analysis are described below. The UW group published data on inter-rated reproducibility using this technique (ICC=was 0.95) (238).

Step 1: Motion correction

A region of interest is drawn around the carotid artery and smoothing algorithms are applied to correct for motion artefact. An example can be seen in figure 2.8.
Figure 2-8. Motion correction of images

Image A. demonstrates the first image taken in the DCE series. The key structures are highlighted including the internal carotid artery (ICA) wall and lumen.

A.

Image B. demonstrates a time series of DCE images acquired before, during and after contrast injection. The green horizontal line has been placed at the superior wall of the ICA in the first image. Over time the superior wall of the ICA can be seen to gradually drift below the green line, suggesting motion artefact. The same time series can be seen after motion correction in image C. The artery wall appears to be aligned in all images and the small white dot seen in the lumen is also in the same position in each sequence improving the accuracy of measurement of change in signal intensity over time.

B. Before motion correction

C. After motion correction
Step 2: Generation of an arterial input function

Using change in signal intensity in the lumen in each frame, an arterial input function (AIF) is generated as seen in figure 2.9.

**Figure 2-9 Generation of an arterial input function.** Signal intensity is measured in each image at the same point in the lumen (marked by the white dot in image A). The signal intensity is then plotted over time to generate an arterial input function (AIF), which can be seen in image B. Each white dot in the curve represents a single time-point measured signal intensity and the large dots represent the time frames of bolus arrival and peak signal intensity.
Step 3: Estimation of VP and Ktrans

Using the previously described Patlak model, the VP and Ktrans for each pixel can be estimated based on the arterial input function and the changes in signal intensity in each pixel over time. An example of the measured signal intensity and model fit can be seen in figure 2.10. A colour coded vasa- vasorum (V-V) image is then generated with the VP measurements appearing in red and Ktrans measurements in green (seen in figure 2.10).

![Pixel Curve](image)

**Figure 2-10 Estimation of DCE parameters**

An example of the signal intensity measurement in one pixel over time (red curve) with the model fit overlaid (yellow curve) can be seen in top image. This pixel curve is generated from the small box seen in Image B (highlighted by the white circle).

A V-V parameter map is generated from all the pixel measurements in one slice (image B). The red codes for Vp and green for Ktrans signal intensity. As a high proportion of plasma is seen in the lumen of the artery and the vein, very high Vp measurements are found here, so the lumen appear as an intense red. Ktrans measurements within the plaque and in the adventitia can be seen in green (as highlighted in image B).
Step 4: Mapping Vp and Ktrans to plaque

Finally contours of the plaque are outlined on the T1 weighted sequence and co-registered with the DCE images (as seen in figure 2.11). An average of the Ktrans and Vp measurements in each pixel within the plaque boundaries is calculated for each slice. Then a mean Ktrans and VP value for the whole plaque is calculated by taking a weighted mean of the value in each slice (the mean is weighted for the number of pixels measurements in each slice. Thus, it is more representative of the volume of the whole plaque rather than giving slices with large and small plaque areas equal weighting).

Figure 2-11 Measurement of DCE parameters. The inner and outer boundaries of the vessel wall measured on the T1 weighted sequence are applied to the V-V map (as seen in image B) and from here measurements of ktrans and Vp within plaque are taken.
2.9.2.2.3 Further calculations by the candidate

Following receipt of the results from the UW, the candidate carried out further calculations for each dataset based on the results. These are summarised in Table 2.9.

<table>
<thead>
<tr>
<th>Table 2-9. Further calculations made by the candidate</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Plaque volume (plaque area x slice thickness for each slice where wall thickness&gt;1.5mm)</td>
</tr>
<tr>
<td>• Calcium volume (calcium area x slice thickness for each slice where wall thickness&gt;1.5mm)</td>
</tr>
<tr>
<td>• LRNC volume (LRNC area x slice thickness for each slice where wall thickness&gt;1.5mm)</td>
</tr>
<tr>
<td>• The percentage of plaque volume which was LRNC (100[LRNC volume/ plaque volume])</td>
</tr>
<tr>
<td>• The percentage of plaque volume which was calcium (100[calcium volume/ plaque volume])</td>
</tr>
<tr>
<td>• Wall remodelling index (wall area/[wall area + lumen area] where wall thickness&gt;1.5mm)</td>
</tr>
</tbody>
</table>

2.9.3 FDG-PET-CT imaging methods

2.9.3.1 Protocol development and optimisation

FDG-PET-CT was performed at the Department of Nuclear Medicine, CMFT under the supervision of Dr Mary Prescott, Consultant in Nuclear Medicine and Dr Heather Williams, Medical Physicist. The imaging protocol was developed based on previously published papers on FDG-PET-CT and in collaboration with colleagues at University College London Hospitals who have previous experience of carotid FDG-PET-CT. A customised head rest was developed to mimic the head position during MRI, to allow co-registration of PET and MRI images (as seen figure 2.12). The protocol was not optimised on healthy volunteers.
due to the radiation dose however checks were made using dummy models, to ensure optimal positioning.

2.9.3.2 Equipment specification

The Siemens Biograph mCT time of flight PET/CT scanner was used in the study (Siemens, Germany). Key features of this scanner includes acquisition of time of flight (TOF) information during imaging, which has improved the spatial resolution to 4.1mm, image contrast and signal to noise ratio compared with scanners without the TOF function (286).

The administered dose was halved compared to routine oncology imaging in order to reduce the dose to patient volunteers and the acquisition time extended to compensate for the lower administered dose and the longer rest period (2 hours versus 1 hour) compared to standard 18F-FDG PET for oncology or neurology indications. PET parameters were selected to optimise image contrast and resolution to aid visualisation of low 18F-FDG uptake in small carotid plaques, based on experience with brain 18F-FDG PET-CT and phantom studies. The CT parameters were selected to provide a low-quality image sufficient for attenuation correction purposes and to use in registering the MR to CT data, whilst limiting the radiation dose to the patient. The positioning strategy was evaluated using volunteers positioned on both scanners.
Figure 2-12 Head rest for PET scan. A customised head rest was made to replicate the head position in the MRI head rest. A loose strap was also placed to discourage movement during the scan. Measurements from the sternal notch to the chin were taken before both MRI and PET scans to ensure the degree of neck flexion was similar. This would allow co-registration of MRI and PET images.
2.9.3.3 Imaging acquisition

Patients attended the department having fasted 6 hours prior to arrival. Height and weight was measured then blood glucose was checked using a glucometer. Providing the fasting glucose was less than 10mmol/L, a cannula was then inserted and 200MBQ of FDG was injected. The patients then rested in a quiet room, wearing a soft cervical collar for 2 hours.

Following the rest, subjects were asked to lie on the PET scanner bed with the headrest and with the head aligned with the long axis of the scanner. Chin to sternal notch distance was measured and if it was different from the measurement taken during the MRI scan then the patient would be repositioned to ensure the measurements matched. The imaging acquisition protocol described in table 2.10 was then performed over a 20 minute period. The data was acquired in “list mode” which is where every pair of photos captured are logged as they come in with a time stamp and reconstruction is performed off line after image acquisition.

<table>
<thead>
<tr>
<th>Table 2-10 PET Image acquisition protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ CT topogram from top of ears to sternal notch</td>
</tr>
<tr>
<td>➢ CT for semi-localisation over 2 PET bed positions, ensuring the carotid bifurcations were within the axial extent.</td>
</tr>
<tr>
<td>➢ 2-bed PET acquisition, 10 minutes per bed.</td>
</tr>
</tbody>
</table>
2.9.3.4 Imaging processing and co-registration

Reconstruction and co-registration were performed by Dr. Heather Williams, Senior Medical Physicist at CMFT. Most reconstructions and co-registrations were performed on the Siemens clinical work station however some reconstructions were performed on the research workstation but using the same code with support from Dr Matt Kelly of the Siemens Healthcare software development group in Oxford. 3 reconstructions were performed: ordered subset expectation maximisation (OESM) reconstruction, high definition (HD) reconstruction and ultra-high definition reconstruction (UHD).

OESM is the standard reconstruction used in most centres. This uses an iterative process whereby data generated from an estimated image is compared to the recorded PET data and then the data from the estimated image is improved based on the comparison. This process is repeated several times to bring the estimated data close to the recorded data. Instead of comparing the data set as a whole, smaller subsets of data all undergo iteration, which improves the rate at which the estimated and actual data coverage are compared. Following this a Gaussian filter is applied to smooth out random fluctuations in signal intensity, which appear as image noise and undermine the accurate interpretation of the underlying structures within the image. For this study 3 iterations were used in 24 subsets and a 256 matrix and 5mm post filter was applied, which is similar to that used in routine $^{18}$F-FDG PET-CT oncology imaging and is expected to be similar to reconstruction algorithms used to produce published data.

HD reconstruction takes account of the varied spatial resolution of the field of view. On the scanner used, the spatial resolution varies from 4mm in the centre to 6mm at the edges of the field. HD reconstruction employs the same iterative process as OESM but further modelling is performed based on this variation within the field. This leads to sharper image quality. In the current study the same number of iterations and subsets were used but a finer matrix (400) and a 1mm post filter was used. These settings are similar to those used to image small changes in uptake the cortical folds of the brain (H. Williams personal communication).

UHD makes use of the time of flight information available on this particular scanner. A pair of photons hit the detectors on opposite sides of the ring within the PET scanner these are registered within a few nanoseconds. The PET detector applies a timing window wide enough for related photons to be recorded as coincident, but short enough to reject
gamma photons, which are not related to each other. On the Siemens Biograph mCT, this timing window is 4ns in duration. However, the application of this timing window means that PET scanners can only confirm that the photons were emitted at some point along the line connecting the two detector elements (see figure 2.13). However, the current generation of detectors used within PET scanners can respond much more quickly than this; for example, the timing resolution of the Siemens Biograph mCT at CMFT is 530ps. These detectors are capable of qualifying the difference in arrival time for the two gamma photons within the 4ns timing window and hence localise the photon emission as originating from within an 8 cm sub-section of the line connecting the detector. This means that the $^{18}$F-FDG uptake can be localised more accurately and image contrast improved. This is particularly important, as the carotid plaque is a small structure with a low signal compared with other tissues such as tumours or cortex. The UHD incorporates this information as well as the HD changes previously described. Using this technique, 4 iterations in 24 subsets were performed on a 400 matrix with 1mm post filter, which were recommended on the basis of previous phantom studies (H. Williams, personal communication).
Figure 2-13 Schematic of difference between OESM and UHD reconstruction

A. A positron emitted from the decaying FDG trapped within the target tissue quickly collides with an electron (\(\text{\(e^+\)}\)) and emits a pair of photons at 180° angles. These photons are detected by gamma cameras, which are positioned in a spiral around the patient (seen as green circles in figure A). Depending on how quickly the camera detects the gamma ray the more accurately the position of the original collision occurred along the axis between the cameras.

B. Using OESM reconstruction (blue line) the collision can have occurred anywhere along the axis. Using the UHD reconstruction point of collision can be narrowed down to within 8 cm (red line).
2.9.3.5 Imaging analysis

The images were reviewed within 7 days by a nuclear medicine consultant to screen for any clinically relevant abnormalities. The scans were then analysed as a batch. The candidate, who was blinded to the PET images, identified plaque within the index artery on the T1-weighted MRI image. Locations were noted for each slice, which has evidence of plaque.

The medical physicist then used these to draw the regions of interest on the co-registered PET-MRI dataset. A region of interest was drawn around the whole vessel in each slice where plaque had been identified (as seen in figure 2.14 and 2.15). As the spatial resolution of the scan was 4.1mm and some plaques were only 2mm thick, an assumption was made that the highest signal was coming from the plaque rather than the lumen or other structures within 4.1mm proximity of the region of interest boundary. In each slice a SUV\text{max} measurement was taken within the region of interest then the highest SUV\text{max} of the slices was taken as the SUV\text{max} of the plaque.
Figure 2-14 An example of a region of interest from where SUV measurements were taken. This figure shows an axial PET-MRI slice of the left common carotid artery. The lumen of the artery appears as a high intensity MRI signal and there is a plaque in the posterolateral border of the lumen. A region of interest has been drawn around the vessel including the area of the plaque (seen as a red boundary).
**Figure 2-15 Sagittal image with a defined region of interest for SUV measurement.**
This is the corresponding sagittal image to figure 2.14. A small portion of the bifurcation can be seen as a high intensity signal highlighted by the top arrow. The red boundary is the region of interest across the selected slices where plaque has been identified on MRI.
Further exploratory measurements were to be taken including $SUV_{\text{max}}$ of the nearby jugular vein, from which to calculate a target to background ratio (TBR). Finally a $SUV_{\text{max}}$ measurement was taken in a non-plaque segment of the artery to assess whether there was preferential inflammation within the plaque or whether plaque inflammation was merely a reflection of generalised vascular inflammation in RA (an example can be seen in figures 2.16 and 2.17).

The medical physicist was blinded to disease activity and DCE results, although they were aware of a limited medical history to ensure the patient was suitable for PET-CT imaging and able to comply with the imaging procedure.

![Figure 2-16 A PET-MRI image with a region of interest around non-atheromatous wall.](image)

This is an axial PET-MRI image from the same subject as in figure 2.16. This slice included a portion of the common carotid artery where no plaque has been noted. A region of interest has been drawn around the vessel including the wall (red boundary), from which SUV measurements are then taken.
Figure 2-17 Sagittal PET-MRI image with defined regions of interest for SUV measurement. This sagittal PET-MRI image highlights the regions of interest for both atheromatous wall (red boundary highlighted by the top arrow) and the non-atheromatous wall (the boundary highlighted by the middle arrow) in the same subject as figures 2.15-2.17.
2.10 Laboratory methods

2.10.1 Blood tests performed at Central Manchester Pathology Laboratories

Standard processing of clinical blood tests (listed below) was undertaken using standard protocols at CMFT pathology laboratory.

- Urea and electrolytes
- ESR
- Fasting lipid profile
- Fasting glucose
- RF
- ACPA

The research bloods described below were analysed by Dr Philip Pemberton at the Clinical Research Department at CMFT using standard ELISA techniques. Detailed protocols for each technique are found in appendix 6. Assay characteristics can be seen in table 2.11.

Table 2-11. ELISA assay characteristics for research tests performed at CMFT

<table>
<thead>
<tr>
<th>Test</th>
<th>Dynamic range</th>
<th>Minimum detection limit</th>
<th>Intra-assay CoV (%)</th>
<th>Inter-assay COV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs-CRP</td>
<td>15mg/l</td>
<td>0.1 mg/l</td>
<td>4.7</td>
<td>6.2</td>
</tr>
<tr>
<td>IL-6</td>
<td>600pg/ml</td>
<td>0.5pg/ml</td>
<td>-</td>
<td>17.18</td>
</tr>
<tr>
<td>TNF</td>
<td>1ng/ml</td>
<td>2pg/ml</td>
<td>5.9</td>
<td>13.1</td>
</tr>
<tr>
<td>E-selectin</td>
<td>6ng/ml</td>
<td>0.1ng/ml</td>
<td>6.2</td>
<td>8.4</td>
</tr>
<tr>
<td>P-selectin</td>
<td>5ng/ml</td>
<td>30pg/ml</td>
<td>4.0</td>
<td>9.0</td>
</tr>
<tr>
<td>I-CAM</td>
<td>2ng/ml</td>
<td>0.1ng/ml</td>
<td>4.5</td>
<td>9.3</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>1ng/ml</td>
<td>35pg/ml</td>
<td>3.0</td>
<td>14.8</td>
</tr>
</tbody>
</table>
2.10.2 Quantification of CD28null cells

4ml samples of blood was collected in an EDTA vacutainer tube and immediately placed on ice. Samples were not stored but analysed on the morning of collection.

The protocol was researched and optimised prior to the start of the clinical study. The optimisation and final protocol can be seen in appendix 7. In brief, two samples of 100µL of whole blood were prepared for flow cytometry. Both samples were incubated with an Fc receptor blocking reagent which reduces non-specific binding of conjugated antibodies. After 15 minutes Vioblue-conjugated anti-human CD45 antibodies were added to both samples. To one sample, Phycoerythrin (PE)-conjugated anti-human CD28 antibodies and allophycocyanin (APC)-conjugated anti-human CD4 anti-bodies were added. PE and APC isotype control antibodies were added to the second sample which acted as a benchmark for background interference. After 15 minutes incubation, red cell lysis solution was added and the samples were incubated for a further 15 minutes at room temperature.

Flow cytometry was then performed on each sample by Dr. Michael Jackson, at the Faculty of Life Sciences Core Facility, on a Cyan APD Flow cytometer (DAKO Systems). First, all CD45 positive events were selected and then forward and side scatter was used to identify the lymphocyte population. Within the lymphocyte population PE (CD28) was plotted against APC (CD4) and flow cytometry was stopped when approximately 50,000 CD45 positive lymphocyte events had been counted. Figure 2.18 demonstrates the gating for the experiment.

Quantification of the percentage of APC positive events that were PE negative was calculated using summit software offline. This was done in batches, blinded to patient/control status.
Figure 2-18 Flow cytometry analysis of CD28null T cells.

Step 1: The viobule fluorescence (CD45 marker) was plotted on the X axis against side scatter (SS) which represents cellular granularity on the Y axis. All events which demonstrated viobule fluorescence (i.e. CD45 positive) were selected.

Selection of all CD45 positive (viobule fluorescent) events.
Step 2: The forward scatter (FS) which represents cell size and side scatter (SS) are plotted for all the selected events. The lymphocyte region within this plot is then selected for further analysis.
Step 3: APC (CD4) fluorescence is the plotted against PE (CD28) fluorescence in the events selected in step 2. Quadrants are then defined to identify the proportion on APC(CD4) positive PE (CD28) negative events. The absolute and percentage counts of CD4+CD28null T cells can be seen in “R5” in image C. In this example there are very few CD4+CD28null T cells.
2.10.3 Quantification of Endothelial Microparticles (EMPs)

The protocol for collection, storage and analysis of EMPs was developed and validated within the research group. The standardised protocol was employed by the candidate in the current study (co-efficient of variance 9.8%, B. Parker, personal communication).

Sample collection and storage

Blood for EMP was drawn before any other sample to minimise EMP release due to cell damage as a result of venepuncture. 4.5ml of venous blood was collected in a citrated vacutainer and immediately placed on ice then transported to the Core Technology Facility at University of Manchester to be prepared for storage.

The sample underwent two-step centrifugation. First the sample was spun at 1700g for 10 minutes at 4°C, after which the plasma layer was harvested and transferred to a fresh test tube. This was then spun at 20,000g for 10 minutes at 4°C. The platelet poor plasma (PPP) was then removed and put into 200μl aliquots and stored at -80 degrees centigrade.

2.10.3.1 Measurement of EMP levels

EMPs were measured using flow cytometry. Once thawed, PPP samples were incubated with fluorescently labelled antibodies to specific surface markers. The antibodies used are listed in table 2.12. The full protocol can be seen in appendix 8.
Table 2-12. Conjugated antibodies used in EMP analysis

<table>
<thead>
<tr>
<th>Conjugated Antibody</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efluoro450-conjugated anti-human Annexin V</td>
<td>Annexin V binds to externalised phosphatidylserine, which is found on all types of microparticles, thus acts as a micro-particle marker.</td>
</tr>
<tr>
<td>Phycoerythrin(PE)-conjugated anti-human CD31</td>
<td>CD31 is an endothelial cell marker and is used to identify microparticles of endothelial cell origin.</td>
</tr>
<tr>
<td>Allophycocyanin(APC)-conjugated anti-human CD42B</td>
<td>CD42B is a platelet surface marker and is used to identify platelet micro-particles and exclude them from analysis.</td>
</tr>
</tbody>
</table>

50µl of fluorescent counting beads were also added to the sample, in order to quantify the number of EMP events. Following incubation with the antibodies for 10 minutes, flow cytometry was performed on a Cyan APD Flow cytometer (DAKO Systems). Initially all events were viewed on forward and side scatter (see Figure 2.19). The beads were identified and background interference was identified and excluded. Gating was then applied to exclude any APC positive events (Platelets derived events). Finally all PE (CD31) and eFluor450 (annexin V) events were selected and counted. Analysis was stopped when the fluorescent bead count reached 1000.
All events are included in a forward scatter (FS, representing size) and side scatter (SS, representing granularity) plot. The counting beads are identified and counted (R1 at the bottom of the image). Background interference is identified and excluded (R3) in the above image.
Following removal of all APC (platelet) events, PE (CD31, endothelial marker) fluorescence was plotted against Annexin V (microparticle marker). Events positive for both markers represent EMP events and were quantified (R6 in image B).
EMS numbers per ml were then calculated offline by first calculating the volume of plasma analysed using the formula below:

\[
v = \frac{z}{x} \times \frac{y}{20}
\]

Where \(v\) = volume of plasma assayed, \(z\) = total sample volume, \(x\) = total number of beads added, \(y\) = number of beads counted and 20 is the dilution factor.

Then to generate the number of EMPs the number of dual positive events was divided by the plasma volume and then multiplied by 1000 to generate the number of EMPs per 1000μL of plasma. Samples were analysed sequentially in batches, irrespective of patient/control status.

### 2.11 Data management

All data was recorded on a clinical report form (CRF) for each patient. No personal identifiable details were written on the CRF to ensure patient confidentiality and all CRFs were kept in a locked filing cabinet within a locked office in The University of Manchester. Data was transcribed on to a password-protected database, which was designed specifically for the study. Only members of the research team had access to the CRFs and database.

MRI images were fully anonymised before transfer over a secure server to the University of Washington. A confidentiality agreement formed part of the contract with the University of Washington so no data could be used for any other purposes other than to analyse and report back on this study.

PET-CT images were uploaded onto the CMFT password secured radiology system so that images were available to clinicians involved in patient care.
2.12 Statistical methodology

2.12.1 Primary outcome

The primary outcome measure in this study was plaque inflammation as measured by Ktrans on CMRI. The primary outcomes assessed were the difference in Ktrans between patients and controls and the change in Ktrans over time in RA patients.

2.12.2 Secondary outcomes

Secondary outcomes specified a priori were:

- Differences in plaque burden and morphology on CMRI between patients and controls and patient at different time points

- Correlation of clinical and serological markers with plaque presence on ultrasound and plaque inflammation on MRI

- Correlation of the change in hsCRP, DAS28, LDL with change in Ktrans and VP on CMRI in patients

- Correlation of SUV\textsuperscript{max} and TBR carotid plaque with DAS28, HS-CRP and LDL

- Correlation of PET and CMRI imaging biomarkers

- Evaluate the association of potential biomarkers of cardiovascular risk (EMPs, CAMs and CD28 null cells) with presence and phenotype of plaque in patients

- Generate data with which to plan a larger interventional study using CMRI in the RA population. This would include prevalence of plaque suitable within the RA population, numbers needed to screen in order to gather enough MRI data sets, range of Ktrans values and feasibility and tolerability of CMR and PET imaging in RA patients.
2.12.3 Sample size estimation

At the time of study set up, little data was available for the reproducibility of Ktrans, on which to base a power calculation. There was however, published data on sample size calculations for plaque morphology. As previously mentioned 14 participants per group were required to detect a significant change in wall thickness and lipid core at 3 months. As this was a pilot study no formal power calculation was performed but based on the published data on plaque morphology and the expert opinion of the team at the University of Washington and Bio Imaging Institute, University of Manchester the aim was to collect 30 MRI datasets in patients at 2 time points (i.e. 60 scans) and 14 MRI datasets in controls at 1 time point.

A local study of plaque prevalence in established RA and controls, demonstrated 53% of patients and 29% of controls had carotid plaque on ultrasound(287). On this basis a sample size of 80 patients and 60 control participants was planned, to identify sufficient subjects to invite for MRI and PET scans.

2.12.4 Statistical analysis

The funding provided by the Astra Zeneca-University of Manchester Strategic Alliance included funding for collection and analysis of 12 MRI and PET datasets in patients. An interim analysis was planned once this data was collected, to provide a report to the funding body and also to assess if any changes to the study design were required in order to meet the primary outcome of the study. Following this analysis the remainder of imaging data was analysed at the close of the study.

All statistical analysis was performed using STATA® 11 software. Descriptive analysis was first undertaken. Histograms were used to assess the distribution of continuous data. Where normal distribution was found, means and standard deviations were reported however those with non-normal distributions, medians and interquartile ranges were reported. In normally distributed data parametric tests were used to compare groups and in non-normally distributed variables non-parametric tests were used.

Spearman’s correlation co-efficient testing was used to evaluate correlations of imaging, serological and clinical variables. When evaluating the associations of clinical and
serological variables with presence of plaque on ultrasound logistic regression was used. Univariate analysis was initially performed then any variables with a significance level of $p<0.1$ or any variable with a known association with the outcome were entered into a multivariable logistic regression model. No corrections were made for multiple testing.
2.13 Contribution of the candidate

I jointly conceived and developed the study with my supervisors and collaborators (listed in table 2.13). I acted as Principle Investigator for the study and applied for all regulatory approvals (with the exception of the radiation licence, which was applied for by Dr. Mary Prescott). I was responsible for recruitment and study co-ordination. I performed most clinical assessments, although some were performed by an advanced nurse practitioner who had been specifically trained in rheumatological assessment, including DAS28 scores for the study (Mrs Sujamole Subin). I developed the study specific ultrasound protocol and following training and validation with 2 colleagues, performed the majority of the carotid ultrasounds (greater than 100). I also trained in CMRI image acquisition and was responsible for planning and supervising CMRI scans to ensure optimal imaging quality and adequate coverage of plaque for DCE analysis was achieved during the scans. I trained and validated in quality assurance assessment of MRI scans and performed initial review of MRI scans to exclude any clinically relevant findings. I acted as one of the two readers for quality assurance from May 2012 (after the second training visit at UW). I prepared and analysed all EMPs and CD28null cell samples. I identified the regions of interest on MRI for PET measurements and was involved planning the methodology for analysis of PET data. I was responsible for data preparation, statistical analysis and interpretation of results.

Table 2-13 List of collaborators on the study

| • Dr. Penny Cristinacce, Research Associate, Bio-imaging Institute, University of Manchester |
| • Dr. Paul Hockings, Principle Scientist, Personalised Healthcare and Biomarkers, Astra Zeneca |
| • Dr. Heather Williams, Senior Medical Physicist, Department of Nuclear Medicine, CMFT |
| • Dr. Mary Prescott, Nuclear Medicine Consultant, Department of Nuclear Medicine, CMFT |
| • Dr. Jaqueline James, Nuclear Medicine Consultant, Department of Nuclear Medicine, CMFT |
| • Dr. Philip Pemberton, Department of Clinical Pathology, CMFT |
| • Professors Chun Yuan and Thomas Hastukami and the Vascular Imaging Laboratory Research Group, University of Washington |
| • Mr Michael Jackson, Flow Cytometry Technician, Faculty of Life Sciences, University Of Manchester |
3 Carotid atherosclerosis and arterial stiffness in RA

3.1 Introduction

Increased cardiovascular risk in RA is thought to be due to a combination of increased prevalence of traditional risk factors and RA specific factors, in particular chronic inflammation. There is epidemiological evidence to suggest that inflammation plays a significant role in the increased cardiovascular mortality seen in RA. Increased prevalence of atherosclerosis had previously been demonstrated in RA and evidence from histological and imaging studies is beginning to point towards a more inflammatory rupture prone plaque phenotype in RA. With recent advances in imaging techniques such as DCE-MRI and PET, it is now possible to non-invasively evaluate carotid plaque inflammation and morphology.

The aims of the current study were to employ these techniques to investigate atherosclerotic plaque phenotype in RA and evaluate the effect of treating active arthritis on plaque inflammation and composition. Secondary aims included assessing the feasibility of using these two techniques in a cohort of patients with active joint disease and to evaluate clinical and serological measures associated with the presence and phenotype of carotid plaque.

3.2 Aims

The aims of the current chapter were to:

- To describe cohort characteristics at baseline of patients and control subjects
- To compare prevalence of carotid plaque on ultrasound in patients and age and sex matched controls
- To evaluate factors associated with carotid atherosclerosis in RA patients
- To compare aortic PWV in patients and controls
- To evaluate the factors associated with aortic PWV in patients
3.3 Methods

In order to evaluate the main aims of the study, a prospective pilot study of RA patients with active established disease and age and sex matched controls was conducted. Detailed methods are described in Chapter 2 but in brief: clinical and serological evaluation of traditional cardiovascular risk factors and RA related factors were undertaken. Aortic PWV was measured using an arteriograph and carotid artery ultrasound was performed to screen for carotid plaque. Subjects with plaque greater than 2mm thick (the minimum required for DCE-MRI analysis of plaque) went on to have a DCE-MRI of the carotid arteries. FDG-PET-CT scans were also performed in a subgroup of RA patients. Repeat clinical, serological and MRI assessments were performed at a follow up visit in patients. The aim was to collect MRI datasets in 30 patients and 14 controls at in order to compare plaque inflammation and composition in patients and controls and patients over time. It was estimated that 80 patients and 60 controls would be required to complete MRI data collection.

After 6 months of recruitment, an interim analysis was performed to assess progress in the study. At that stage, 57 RA patients had been recruited and based on the rate of MRI data collection (which will be discussed in more detail in a subsequent chapter), it became apparent that a larger sample size would be required than initially estimated. Therefore the recruitment targets were increased to include 160 patient and 120 control subjects.

3.4 Results

3.4.1 Overview of recruitment and participant flow through the study

Figure 3.1 summarises the recruitment and flow of participants through the study. 130 patients and 52 controls were recruited to the study. A more detailed description of patient flow, cohort characteristics and findings at each time point will be described in subsequent sections. The area in blue highlights the participants included in the analysis described in the current chapter.
Figure 3-1 Participant flow though the study
3.4.2 Cohort characteristics at baseline

A total of 130 patients and 52 controls were recruited to the study. There was no significant difference in age between the groups (median [IQR] age 55.5[48.8, 61.9] years and 56.4[48.0, 60.4] years, in patients and controls respectively, p=0.97). 99(76.2%) patients were female, while 41 (78.84%) controls were female (p=0.761).

3.4.2.1 Traditional cardiovascular risk factors

Table 3.1 summarises participant reported traditional cardiovascular risk factors in patients and controls. Two patients (1.54%) had a history of clinical cardiovascular disease, one had a history of stable angina and another had a history of transient ischaemic attack. One (1.9%) control participant had a history of stroke. Patients were more likely to have been treated for hypertension in the past or at the time of assessment compared to controls (20% vs 7.69%, p=0.049) and there was a trend toward higher rates of smoking in the patient group. Otherwise there was no significant difference in participant reported prevalent traditional risk factors.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases (n=130)</th>
<th>Controls (n=52)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.5 (48.8, 61.9)</td>
<td>56.4 (48.0, 60.4)</td>
<td>0.973</td>
</tr>
<tr>
<td>Gender (female)*</td>
<td>99 (76.2)</td>
<td>41 (78.9)</td>
<td>0.761</td>
</tr>
<tr>
<td>Current smoker*</td>
<td>18 (13.9)</td>
<td>3 (5.77)</td>
<td>0.104</td>
</tr>
<tr>
<td>Ex-smoker*</td>
<td>47 (36.2)</td>
<td>18 (34.6)</td>
<td>0.432</td>
</tr>
<tr>
<td>Never smoked*</td>
<td>65 (50.0)</td>
<td>28 (53.8)</td>
<td>0.151</td>
</tr>
<tr>
<td>Family history of CVD*</td>
<td>43 (33.1)</td>
<td>17 (32.7)</td>
<td>0.974</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>26 (20.00)</td>
<td>4 (7.69)</td>
<td>0.049</td>
</tr>
<tr>
<td>Dyslipidaemia*</td>
<td>9 (6.92)</td>
<td>5 (9.62)</td>
<td>0.515</td>
</tr>
<tr>
<td>Diabetes*</td>
<td>2 (1.54)</td>
<td>1 (1.92)</td>
<td>0.822</td>
</tr>
<tr>
<td>Clinical CVD*</td>
<td>2 (1.54)</td>
<td>1 (1.92)</td>
<td>0.841</td>
</tr>
</tbody>
</table>

Blood pressure and fasting bloods for serum glucose and lipids were measured in all participants and the results are summarised in table 3.2. As has been reported by others, total and LDL cholesterol levels were lower in RA patients (shown in table 3.2). There was also a trend towards lower HDL levels in patients, although this did not reach statistical
significance. We also noted a higher systolic blood pressure in the RA cohort consistent with the higher prevalence of known hypertension (136[124, 148] mmHg vs 127[117, 140] mmHg p=0.006) but there was no significant difference in diastolic pressure.

RA patients had significantly higher levels of hsCRP and IL-6 compared to controls but there was no significant difference in TNF levels between the groups (seen in table 3.2). A comparison was made between controls, patients on anti-TNF therapy and patients not on anti-TNF therapy. A dot plot of TNF levels in the three groups can be seen in figure 3.2. There was no difference in levels between patients on and off anti-TNF therapy and controls. Levels of TNF, IL-6 and hsCRP were undetectable in a significant number of subjects (see figure 3.3). Due to the limits of detection of these assays, hsCRP, IL-6 and TNF levels were converted to quartiles for subsequent analyses.

Table 3-2 Measurement of traditional risk factors and circulating cytokines, median (IQR)

<table>
<thead>
<tr>
<th>Test</th>
<th>Cases (n=130)</th>
<th>Control (n=52)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.1 (4.6, 5.8)</td>
<td>5.5 (4.9, 6.3)</td>
<td>0.019</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.97 (2.52, 3.60)</td>
<td>3.28 (2.65, 3.82)</td>
<td>0.049</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.60 (1.26, 1.96)</td>
<td>1.72 (1.42, 2.04)</td>
<td>0.078</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.9 (0.7, 1.4)</td>
<td>1.0 (0.8, 1.4)</td>
<td>0.591</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>3.22 (2.68, 4.13)</td>
<td>3.27 (2.54, 3.87)</td>
<td>0.633</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.9 (4.6, 5.3)</td>
<td>5.2 (4.7, 5.7)</td>
<td>0.021</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>136 (124, 148)</td>
<td>127 (117, 140)</td>
<td>0.006</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>79 (73, 85)</td>
<td>81 (76, 89)</td>
<td>0.134</td>
</tr>
<tr>
<td>hsCRP levels (mg/l)</td>
<td>2.89 (1.01, 6.18)</td>
<td>0.79 (0.35, 2.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNF levels (pg/ml)</td>
<td>1 (1, 45.3)</td>
<td>1 (1, 1)</td>
<td>0.125</td>
</tr>
<tr>
<td>IL-6 levels (pg/ml)</td>
<td>1.99 (0.25, 4.69)</td>
<td>0.52 (0.25, 1.46)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 3-2 Levels of circulating TNF in patients on and off anti-TNF and controls with bars representing median.

Figure 3-3 The percentage of patients and controls with detectable circulating cytokine levels.
3.4.2.2 Disease characteristics of RA cohort

All RA patients met the ACR 1987 classification criteria for RA at baseline assessment. RA-related disease characteristics are summarised in table 3.3. Patients had established disease, with a median (IQR) disease duration of 10.2 (5.3, 20.9) years at the time of assessment. 107 (82.3%) patients were positive for ACPA and 97 (74.6%) were positive for RF antibodies. In total 114 (87.7%) were classed as seropositive with at least one antibody present on testing at baseline. 26 (20.0%) patients had subcutaneous nodules and in total 35 (26.92%) had extra-articular disease defined as a history or findings on clinical examination of: nodules, rheumatoid lung disease, vasculitis, RA related skin ulcers, RA related eye disease, amyloidosis or Felty’s syndrome (see figure 3.4).

At time of assessment, median DAS28 score was 4.62 (3.76, 5.47) and the distribution of patients with low, moderate and high disease activity states can be seen in figure 3.5. Six patients were in DAS28 remission, 14 patients had low disease activity, 41 had moderate disease activity and 59 had high disease activity. The median HAQ score was 1.36 (0.50, 2.13) and 97 (74.6%) had a HAQ score greater than 1. As HAQ scores are generated by taking a mean of ordinal values, HAQ was divided into quartiles for analysis. The distribution of HAQ scores and median values within each bracket can be seen in figure 3.6.
Figure 3-5 Distribution of disease activity according to DAS28 score (where remission is defined as a DAS28<2.6; low disease activity between 2.6 and 3.2; moderate disease activity between 3.2 and 5.1 and high disease activity as DAS28>5.1).

Figure 3-6 Quartiles of HAQ score (where the median (range) for brackets 1,2,3,4 was 0.25(0, 0.5), 1.0625 (0.625, 2.125), 1.8125(1.5, 2.125), 2.857(2.25, 5) respectively).
### Table 3-3 Disease characteristics in patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (IQR)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset (years)</td>
<td>44.6 (34.0, 50.4)</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>10.2 (5.3, 20.9)</td>
</tr>
<tr>
<td>ACPA positive*</td>
<td>107 (82.3)</td>
</tr>
<tr>
<td>RF positive*</td>
<td>97 (74.6)</td>
</tr>
<tr>
<td>Seropositive (RF or ACPA positive)*</td>
<td>114 (87.7)</td>
</tr>
<tr>
<td>HAQ score</td>
<td>1.36 (0.50, 2.13)</td>
</tr>
<tr>
<td>Nodules*</td>
<td>26 (20.0)</td>
</tr>
<tr>
<td>Extra-articular manifestations*</td>
<td>35 (26.9)</td>
</tr>
<tr>
<td>Swollen joint count</td>
<td>6 (3, 10)</td>
</tr>
<tr>
<td>Tender joint count</td>
<td>8 (4, 13)</td>
</tr>
<tr>
<td>Visual analogue score (0-100)</td>
<td>45 (22, 60)</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>14 (7, 27)</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.62 (3.76, 5.47)</td>
</tr>
<tr>
<td><strong>Current therapy</strong></td>
<td></td>
</tr>
<tr>
<td>Any DMARD (including biologic therapy)*</td>
<td>115 (88.5)</td>
</tr>
<tr>
<td>Combination DMARD therapy*</td>
<td>36 (27.7)</td>
</tr>
<tr>
<td>Methotrexate*</td>
<td>80 (61.5)</td>
</tr>
<tr>
<td>Sulfasalazine*</td>
<td>22 (17.1)</td>
</tr>
<tr>
<td>Hydroxychloroquine*</td>
<td>31 (24.0)</td>
</tr>
<tr>
<td>Leflunomide*</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Azathioprine*</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Biologic therapy*</td>
<td>38 (29.2)</td>
</tr>
<tr>
<td>Anti-TNF therapy*</td>
<td>32 (24.8)</td>
</tr>
<tr>
<td>Rituximab*</td>
<td>3 (2.33)</td>
</tr>
<tr>
<td>Abatacept*</td>
<td>1 (0.78)</td>
</tr>
<tr>
<td>Tocilizumab*</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Oral glucocorticoids*</td>
<td>15 (11.5)</td>
</tr>
<tr>
<td>Ever had oral glucocorticoids*</td>
<td>69 (53.1)</td>
</tr>
</tbody>
</table>

*Frequency (%)  

115 (88.5%) patients were taking disease modifying therapy (including biologics) at baseline. 35 (27.9%) were taking combination therapy with traditional DMARDs. 38 (29.2%) patients were on biologic therapy, most of who were taking anti-TNF drugs (n=32). 15 (11.5%) were taking oral glucocorticoids at baseline and 69 (53.1%) patients had been treated with oral glucocorticoids at some point during their disease course.
3.4.3 Prevalence and associations of carotid plaque

The prevalence of carotid plaque on ultrasound was significantly higher in patients than in controls (69 [53.08%] vs 19 [36.54%], p=0.043). Non-parametric tests (Mann Whitney U and chi squared tests) were used to assess clinical and serological features associated with plaque in patients and controls. Stepwise multivariable logistic regression was then performed to evaluate factors associated with carotid plaque in patients. Variables known to be associated with plaque were included in the model as were any novel factors, which had a significance level of p<0.1.

3.4.3.1 Associations with carotid plaque

Table 3.4 summarises factors associated with plaque in patients and controls. In patients, age, smoking, hsCRP and IL-6, DAS28 score, ESR and HAQ score were all significantly associated with presence of carotid plaque. In controls plaque was significantly associated with male gender.
Table 3-4 Association of clinical and serological features with presence of carotid plaque. Median (IQR) or frequency (%) where *. Quartiles where†.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with plaque</th>
<th>Patients without plaque</th>
<th>p-value</th>
<th>Controls with plaque</th>
<th>Controls without plaque</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=69</td>
<td>N=61</td>
<td></td>
<td>N=19</td>
<td>N=33</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>58.5 (51.2, 62.7)</td>
<td>52.4 (46.5, 56.7)</td>
<td>&lt;0.001</td>
<td>57.7 (48.9, 60.1)</td>
<td>56.2 (46.7, 60.3)</td>
<td>0.55</td>
</tr>
<tr>
<td>Male*</td>
<td>16 (23.2)</td>
<td>15 (24.6)</td>
<td>&lt;0.852</td>
<td>8 (42.1)</td>
<td>1 (12.0)</td>
<td>0.036</td>
</tr>
<tr>
<td>Smoking*</td>
<td>13 (18.8)</td>
<td>5 (8.19)</td>
<td>0.015</td>
<td>2 (10.5)</td>
<td>1 (3.0)</td>
<td>0.25</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>15 (21.7)</td>
<td>11 (18.0)</td>
<td>0.598</td>
<td>2 (10.5)</td>
<td>2 (6.1)</td>
<td>0.374</td>
</tr>
<tr>
<td>Diabetes*</td>
<td>0</td>
<td>2 (3.28)</td>
<td>0.122</td>
<td>1 (5.2)</td>
<td>0</td>
<td>0.124</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>140 (129, 152)</td>
<td>135 (123, 144)</td>
<td>0.032</td>
<td>125 (116, 115)</td>
<td>127 (118, 132)</td>
<td>0.69</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81.5 (76, 89)</td>
<td>81 (76, 87)</td>
<td>0.496</td>
<td>79 (73, 91)</td>
<td>78 (74, 82)</td>
<td>0.439</td>
</tr>
<tr>
<td>TC: HDL</td>
<td>3.38 (2.70, 4.36)</td>
<td>3.12 (2.62, 2.92)</td>
<td>0.33</td>
<td>3.37 (2.96, 2.77)</td>
<td>2.91 (2.41, 3.80)</td>
<td>0.086</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>3.02 (2.48, 3.66)</td>
<td>2.93 (2.54, 3.37)</td>
<td>0.637</td>
<td>3.27 (2.95, 3.97)</td>
<td>3.28 (2.44, 3.59)</td>
<td>0.315</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.54 (1.21, 1.91)</td>
<td>1.67 (1.3, 1.97)</td>
<td>0.191</td>
<td>1.63 (1.43, 2.01)</td>
<td>1.87 (1.42, 2.09)</td>
<td>0.229</td>
</tr>
<tr>
<td>hsCRP (mg/l) †</td>
<td>-</td>
<td>-</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
<td>0.981</td>
</tr>
<tr>
<td>IL-6 (pg/ml) †</td>
<td>-</td>
<td>-</td>
<td>0.001</td>
<td>-</td>
<td>-</td>
<td>0.542</td>
</tr>
<tr>
<td>TNF (pg/ml) †</td>
<td>-</td>
<td>-</td>
<td>0.200</td>
<td>-</td>
<td>-</td>
<td>0.340</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>10.51 (5.90, 21.9)</td>
<td>9.09 (4.55, 19.4)</td>
<td>0.280</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.83 (4.3, 5.66)</td>
<td>4.48 (3.21, 5.15)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HAQ score†</td>
<td>-</td>
<td>-</td>
<td>0.006</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>20 (8, 33)</td>
<td>8 (5, 20)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RF positive</td>
<td>52 (75.4)</td>
<td>43 (68.3)</td>
<td>0.435</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ACPA positive</td>
<td>57 (82.6)</td>
<td>47 (74.6)</td>
<td>0.325</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Seropositive*</td>
<td>61 (88.4)</td>
<td>53 (86.9)</td>
<td>0.765</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Extra-articular disease*</td>
<td>18 (26.1)</td>
<td>17 (28.3)</td>
<td>0.77</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Current oral glucocorticoid therapy</td>
<td>8 (11.6)</td>
<td>7 (11.7)</td>
<td>0.989</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ever treated with glucocorticoids</td>
<td>135 (52.2)</td>
<td>34 (56.7)</td>
<td>0.617</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.4.3.2 *Independent associations of plaque in patients*

Table 3.5 summarises the results of logistic regression of factors associated with plaque in patients. A stepwise logistic regression model was used to evaluate independent associations of plaque in patients. Age, gender, systolic blood pressure, smoking, TC:HDL, DAS28, HAQ, hsCRP and IL-6 were included in the model. Smoking and hsCRP were independently associated with carotid plaque.

### 3-5 Logistic regression to evaluate associations between plaque and clinical and serological variables in patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted OR (CI 95%)</th>
<th>OR (CI95%) with age adjustment</th>
<th>OR (95%CI) in fully adjusted model†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>1.09 (1.03, 1.15)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gender (Male)</td>
<td>0.92 (0.41, 2.07)</td>
<td>0.89 (0.36, 2.18)</td>
<td>-</td>
</tr>
<tr>
<td>Smoking</td>
<td>3.80 (1.22, 11.87)</td>
<td>6.36 (1.76, 23.09)</td>
<td>6.29 (1.39, 589.7)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>1.02 (1.00, 1.04)</td>
<td>1.01 (1.00, 1.04)</td>
<td>-</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>1.11 (0.85, 1.44)</td>
<td>1.15 (0.86, 1.55)</td>
<td>-</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>1.02 (0.99, 1.06)</td>
<td>1.02 (0.97, 1.05)</td>
<td>-</td>
</tr>
<tr>
<td>DAS28</td>
<td>1.66 (1.18, 2.31)</td>
<td>1.53 (1.08, 2.18)</td>
<td>-</td>
</tr>
<tr>
<td>HAQ</td>
<td>1.63 (1.11, 2.41)</td>
<td>1.55 (1.02, 2.33)</td>
<td>-</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>1.05 (1.02, 1.08)</td>
<td>1.04 (1.01, 1.08)</td>
<td>-</td>
</tr>
<tr>
<td>Extra-articular disease</td>
<td>0.92 (0.45, 1.90)</td>
<td>0.81 (0.37, 1.75)</td>
<td>-</td>
</tr>
<tr>
<td>Seropositivity</td>
<td>1.17 (0.41, 3.34)</td>
<td>1.17 (0.55, 5.30)</td>
<td>-</td>
</tr>
<tr>
<td>Current glucocorticoid use</td>
<td>1.03 (0.35, 3.06)</td>
<td>0.96 (0.29, 3.14)</td>
<td>-</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>1.52 (1.10, 2.12)</td>
<td>1.41 (1.00, 1.99)</td>
<td>2.4 (1.23, 4.70)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.45 (1.06, 1.98)</td>
<td>1.40 (1.01, 1.96)</td>
<td>-</td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>1.12 (0.87, 1.47)</td>
<td>0.99 (0.74, 1.32)</td>
<td>-</td>
</tr>
</tbody>
</table>

†Stepwise logistic regression performed with significance value set at p<0.1. Variables included in model: age, gender, smoking, systolic blood pressure, TC:HDL, DAS28, HAQ, hsCRP, IL-6.
3.4.3.3 Factors associated with plaque in controls

Table 3.6 summarises the associations with of plaque in controls. On univariate analysis male gender was significantly associated with carotid plaque and this remained significant on age adjustment.

Table 3-6 Logistic regression to evaluate associations between plaque and clinical and serological variables in controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted OR (95% CI)</th>
<th>Age Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>1.02 (0.94, 1.01)</td>
<td>-</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>4.22 (1.04, 17.16)</td>
<td>6.09 (1.29, 28.70)</td>
</tr>
<tr>
<td>Smoking</td>
<td>4.0 (0.33, 49.10)</td>
<td>3.8 (0.31, 48.40)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>1.01 (0.98, 1.05)</td>
<td>1.02 (0.98, 1.06)</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>1.03 (0.98, 1.10)</td>
<td>1.04 (0.98, 1.10)</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>1.51 (0.84, 2.71)</td>
<td>1.53 (0.78, 2.98)</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>0.39 (0.01, 1.69)</td>
<td>0.261 (0.47, 1.42)</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>1.17 (0.87, 1.59)</td>
<td>1.19 (0.86, 1.66)</td>
</tr>
</tbody>
</table>
3.4.4 Aortic pulse wave velocity (PWV)

PWV was measured in a subset of 98 patients and 44 control subjects. Baseline characteristics of this subgroup can be seen in Table 3.7.

Table 3-7 Baseline characteristics of the subgroup with PWV data. Median(IQR) or frequency (%)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.5 (49.4, 61.0)</td>
<td>56.7 (47.1, 60.5)</td>
<td>0.829</td>
</tr>
<tr>
<td>Gender (male)*</td>
<td>24 (24.9)</td>
<td>8 (18.2)</td>
<td>0.405</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>21 (21.4)</td>
<td>2 (4.7)</td>
<td>0.013</td>
</tr>
<tr>
<td>Current smoker*</td>
<td>12 (12.2)</td>
<td>2 (4.65)</td>
<td>0.119</td>
</tr>
<tr>
<td>Ex-smoker*</td>
<td>37 (37.7)</td>
<td>13 (30.2)</td>
<td>0.163</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>99.5 (92.7, 107.7)</td>
<td>94.3 (88.7, 102.0)</td>
<td>0.014</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>3.12 (2.63, 4.07)</td>
<td>3.01 (2.44, 3.89)</td>
<td>0.67</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>2.48 (1.01, 5.75)</td>
<td>0.73 (0.34, 1.71)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.99 (0.25, 4.69)</td>
<td>0.52 (0.25, 1.46)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>1 (1, 45.3)</td>
<td>1 (1)</td>
<td>0.123</td>
</tr>
<tr>
<td>ESR (mm/Hr)</td>
<td>13 (7, 27)</td>
<td>5 (2, 12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.58 (3.98, 5.51)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HAQ</td>
<td>1.19 (0.5, 2.06)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>10.5 (5.14, 21.86)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Extra-articular disease*</td>
<td>26 (26.3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Seropositivity*</td>
<td>86 (87.8)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Arteriography was only performed on those who had abstained from smoking for 8 hours prior to the study visit and the tests were carried out while subjects were fasted, as per the manufacturers’ recommendations. PWV measurements in patients and controls can be seen in figure 3.7. There was no significant difference in PWV between patients and controls (mean [SD]: 9.64 [2.91] m/s vs 9.16 [2.46] m/s in patients and controls respectively, p=0.34).
Evaluation of associations of PWV with clinical and serological markers was performed in patients and controls separately. The association with current smoking could not be evaluated, as all smokers had smoked on the morning of assessment. The association with previous smoking was examined. Findings are summarised in Table 3.8. In patients, PWV was significantly associated with age, mean arterial pressure (MAP) but also DAS28 score, RF and disease duration.
### Table 3.8 Associations of clinical and serological characteristics with PWV in patients and controls.

*Spearman r when variables are continuous. †Quartiles

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman r*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.27</td>
<td>0.09</td>
<td>0.49</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>-</td>
<td>0.18</td>
<td>0.07</td>
</tr>
<tr>
<td>Hypertension</td>
<td>-</td>
<td>0.71</td>
<td>0.23</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>-</td>
<td>0.21</td>
<td>-</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>0.39</td>
<td>0.03</td>
<td>0.13</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>-0.04</td>
<td>0.65</td>
<td>0.26</td>
</tr>
<tr>
<td>hsCRP †</td>
<td>-</td>
<td>0.65</td>
<td>0.46</td>
</tr>
<tr>
<td>IL-6 †</td>
<td>-</td>
<td>0.22</td>
<td>0.12</td>
</tr>
<tr>
<td>TNF †</td>
<td>-</td>
<td>0.82</td>
<td>0.06</td>
</tr>
<tr>
<td>DAS28 score</td>
<td>0.24</td>
<td>0.03</td>
<td>-</td>
</tr>
<tr>
<td>ESR (mmHg)</td>
<td>0.59</td>
<td>0.77</td>
<td>-</td>
</tr>
<tr>
<td>HAQ †</td>
<td>-</td>
<td>0.46</td>
<td>-</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>0.29</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>Extra-articular disease</td>
<td>-</td>
<td>0.31</td>
<td>-</td>
</tr>
<tr>
<td>RF positive</td>
<td>-</td>
<td>0.04</td>
<td>-</td>
</tr>
<tr>
<td>ACPA positive</td>
<td>-</td>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>Current glucocorticoid use</td>
<td>-</td>
<td>0.87</td>
<td>-</td>
</tr>
<tr>
<td>SUVmax carotid plaque</td>
<td>0.45</td>
<td>0.17</td>
<td>-</td>
</tr>
</tbody>
</table>

### 3.4.4.1 Factors independently associated with PWV in patients

Linear regression models were used to evaluate factors independently associated with PWV in patients. PWV was normally distributed so no transformation was required.

Univariate analysis was performed then adjustment was made for age and blood pressure, as PWV is known to be strongly associated with these variables. Finally, a multivariable model was performed to test for independent associations with PWV based on known important predictors and those that had a p value <0.1 on univariate analysis. Results are displayed in table 3.9. Current smokers could not be included in the analysis as no current smokers had abstained from smoking prior to tests, thus relationship could not be reliably evaluated. Previous smoking was included as a variable.

In patients, PWV was significantly associated with age and mean arterial pressure (MAP). DAS28 score was also a significant predictor of PWV and remained significant after
adjustment for age and blood pressure. Disease duration was also associated on univariate analysis but became insignificant following age and blood pressure adjustment.

Age, MAP, disease duration, DAS28, and seropositivity were included in the stepwise regression model. Age and DAS28 were independent predictors of PWV.

Table 3-9 Factors independently associated with PWV on linear regression

<table>
<thead>
<tr>
<th>RA related variable</th>
<th>β co-efficient (CI 95%)</th>
<th>Age and MAP adjusted β co-efficient (95% CI)</th>
<th>Fully adjusted β co-efficient (95% CI)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.098 (0.023, 0.173)</td>
<td>-</td>
<td>0.10 (0.014, 0.191)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>0.522 (-0.735, 1.781)</td>
<td>0.547 (-0.668, 1.762)</td>
<td>-</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>0.070 (0.028, 0.105)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>-0.249 (-0.734, 0.235)</td>
<td>-0.200 (-0.671, 0.271)</td>
<td>-</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>0.049 (-1.005, 0.103)</td>
<td>0.031 (-0.025, 0.087)</td>
<td>-</td>
</tr>
<tr>
<td>DAS28</td>
<td>0.729 (0.225, 1.232)</td>
<td>0.676 (0.18, 1.168)</td>
<td>0.70 (0.190, 1.210)</td>
</tr>
<tr>
<td>HAQ</td>
<td>0.548 (-0.957, 1.192)</td>
<td>0.539 (-0.98, 1.177)</td>
<td>-</td>
</tr>
<tr>
<td>ESR (mm/Hr)</td>
<td>0.019 (-0.013, 0.053)</td>
<td>0.014 (-0.019, 0.047)</td>
<td>-</td>
</tr>
<tr>
<td>Extra-articular disease</td>
<td>0.005 (-1.213, 1.224)</td>
<td>-0.033 (-1.21, 1.147)</td>
<td>-</td>
</tr>
<tr>
<td>RF positive</td>
<td>1.06 (-0.397, 2.4491)</td>
<td>1.19 (-0.227, 2.615)</td>
<td>-</td>
</tr>
<tr>
<td>Current glucocorticoid use</td>
<td>-0.649 (-1.983, 1.854)</td>
<td>-0.163 (-3.729, 7.792)</td>
<td>-</td>
</tr>
<tr>
<td>hsCRP</td>
<td>0.070 (-0.380, 0.512)</td>
<td>0.136 (-0.317, 0.587)</td>
<td>-</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.499 (-0.699, 1.699)</td>
<td>0.516 (-0.71, 1.745)</td>
<td>-</td>
</tr>
<tr>
<td>TNF</td>
<td>-0.315 (-1.394, 0.814)</td>
<td>-0.315 (-1.294, 0.814)</td>
<td>-</td>
</tr>
</tbody>
</table>

†Fully adjusted stepwise linear regression, variables included in the model: age, MAP, DAS28, seropositivity
3.5 Discussion

Key findings in the chapter include:

• There was a higher prevalence of carotid plaque in patients compared with age and sex matched controls (53.08% patients vs. 36.5% of controls, p=0.043).

• In patients, the presence of carotid plaque was independently associated with levels of hs-CRP and smoking (OR [95%CI]: 2.4[1.23, 4.70] and 6.29[1.39, 589.7] respectively).

• No significant difference in pulse wave velocity (PWV) was found between patients and controls however in patients PWV was independently associated with age and RA disease activity as measured by DAS-28 (β co-efficient [95% CI]: 0.10[0.014, 0.191) and 7.0 [0.19, 1.21] respectively).

Patient characteristics

76.15% of patients were female which is similar to population estimates of gender distribution however there was a lower age at onset compared to the publish literature.

The median age at onset in the current study was of 44.6 years compared to the mean age of onset of 54 years, described in the Norfolk Arthritis Register, an inception cohort study of inflammatory polyarthritis patients (288). There was a higher prevalence of ACPA and RF positivity than is generally found in RA (74.62% vs 60% and 87.6% vs 60%) (21;23). Antibody positivity is known to be associated with more severe disease, thus it may follow that disease activity is harder to control in seropositive patients (1). By recruiting patients with established but active disease, a bias towards seropositive patients will have occurred. Additionally, RF positivity also increases with disease duration, so this may have contributed to the higher than expected rate (22).

The prevalence of extra-articular disease was lower than is generally described in the literature (26.9% compared with 40%) (62). It may have been expected that a higher rate of extra-articular manifestations would be found in this cohort, who had a median disease duration of more than 10 years and a high rate of seropositivity (which is associated with
extra-articular disease). However, extra-articular manifestations were either patient reported, or found on examination at baseline assessment. No review of medical records was undertaken, so we may have underestimated the prevalence. Additionally, unless a formal diagnosis of secondary Sjogren’s Syndrome was stated, patient reported history of sicca symptoms was not included as an extra-articular manifestation. This was because sicca symptoms can often be caused by common factors such as medication or contact lens use rather than as a manifestation of RA. This could also have led to an underestimation of extra-articular involvement.

**Disease activity**

20 (15.4%) of patients were deemed to have low disease activity at baseline despite being referred to the study team with active disease. In many cases this was due to participants receiving intramuscular glucocorticoids at the time of referral, which had taken effect by the time of the study visit. In clinical practice there is often a pressing need to treat inflammation due to pain and discomfort, thus glucocorticoids are often administered at the time of consultation. This can lead to difficulties in carrying out research assessments while disease activity remains high, as time lines are often short. However, it would have been unethical to delay treatment for the purposes of the study. Patients with low disease activity did not progress to MRI scanning, however they did contribute to the analysis of serological and ultrasound findings.

**Treatment**

Despite the majority of patients having active disease, more than two thirds were on either monotherapy (61.6%) or no disease modifying therapy (11.5%). In some cases the use of monotherapy or glucocorticoids alone was due to previous complications such as infections or abnormal liver function tests. However, a number of patients had only ever tried one DMARD agent. There is clear evidence that use of combination DMARD therapy is more effective in reducing disease activity and radiographic progression in early disease. Although it is assumed that this would also apply in established disease, the research in established disease is lacking. Patients were recruited from 6 centres, with approximately 20 consultants in charge of care and the use of monotherapy could not be attributed to
single centre practice. An audit of the diagnosis and management of patients with RA was carried out in the North West of England in 2011. Similarly, this found that approximately one third of patients were initiated on combination DMARD therapy at diagnosis (289). This could suggest that despite the evidence, use of combination therapy in RA has yet to be widely taken up into clinical practice. 29% of patients were on biologic therapy. Although prevalence rates are not available for the use of biologic therapy for RA within the UK, this rate seems quite high compared to that generally found in clinical practice. There are a number of possible reasons for this including selection bias, as patients on biologic drugs attend clinic more regularly and DAS28 scores are required to justify continuation of biologic use.

**Traditional cardiovascular risk factors in patients and controls**

Only 2 (1.5%) patients and 1(1.9%) control had a history of clinical cardiovascular disease. The prevalence of clinical disease was likely to be lower than is found in the whole population as the use of statin therapy was an exclusion criterion. Current guidelines for secondary prevention of both stroke and acute coronary syndrome recommend initiation of statin therapy (290;291). Additionally screening for cardiovascular risk factors in both RA patients and the general population (over 40 years old) is recommended in national guidelines (292). It is likely that a significant proportion of those at highest risk were screened and started on statin therapy thus were not included in the study. Therefore, it could be hypothesised that participants in the current study have a lower cardiovascular risk than would be found in the whole population.

There were significant differences in prevalent cardiovascular risk factors between patients and controls. Treated hypertension and systolic blood pressure were a significantly higher within the RA cohort. This would align with the systematic review performed by Panoulas et al., which found that an increased prevalence of hypertension within the RA population (108).

There was a higher frequency of smokers in the RA cohort, although this did not reach statistical significance. Higher rates of smoking have been found in other case controls studies of established RA and smoking is known to be a risk factor for the development of RA(98) (293).
The rate of prevalent diabetes was similar between the two populations however 3 patients had evidence of raised fasting glucose at baseline consistent with diabetes. It is likely that there was a selection bias against diabetics in the current study as the guidelines for management of diabetes includes the use of statins, thus many diabetics would not have been eligible to take part.

As would be expected with the exclusion criteria, there was a low prevalence of participants known to have dyslipidaemia at inclusion. However there were significant differences in lipid levels between the 2 groups. Patients had significantly lower levels of total cholesterol and LDL and there was a trend towards lower levels of HDL although this was not statistically significant. These lower lipid levels are in keeping with the existing literature which suggests that active RA patients have lower levels of both LDL and HDL (93). However in other studies it has generally been found that there is a preferential lowering if HDL and in the current study the significant difference was in LDL levels.

**Levels of cytokines in patients and controls**

Levels of hsCRP, IL-6 and TNF were measured in 130 patients and 40 controls using a standardised ELISA method. As would be expected, significantly higher levels of hsCRP and IL-6 were found in patients compared with controls. This confirms the findings of other studies examining levels of circulating IL-6 in patients with active RA with age and sex matched controls (139;294).

Surprisingly, there was no significant difference in levels of TNF between patients and controls. It could have been presumed to be due to a significant number of patients being on anti-TNF agents (n=32). However there was no difference in levels between those on and off anti-TNF drugs and controls. This suggests that the lack of difference was not due to anti-TNF therapy use. As TNF is a dominant cytokine involved in RA pathogenesis, it would be expected that increased levels would be found in patients with active disease, but there have been conflicting results in other studies. Klimek et al. demonstrated increased circulating levels in a study of 38 patients with early RA compared to age and sex matched controls (294). However, a study by Vazquez-Del Mercado et al. detected TNF in the synovial fluid but not in peripheral blood of 14 RA patients using an ELISA method (295).

Importantly, this study also performed retro-transcriptase polymerase chain reaction for
mRNA expression of cytokines including TNF and found significantly higher levels of TNF in patients than in controls using this method. In the current study around 50% of patients had undetectable TNF levels using ELISA. It is possible that due to the limitations of the ELISA assay a full comparison of TNF levels could not be made.

Prevalence and factors associated with carotid plaque

An increased prevalence of carotid plaque was detected in patients compared with controls, with more than 50% of patients having carotid plaque. Presence of carotid plaque measured on ultrasound is known to be a validated biomarker for cardiovascular risk in the general population (175). Inclusion of carotid plaque has been shown to significantly improve cardiovascular risk prediction models in the general population (175). Evans et al. also demonstrated that presence of carotid plaque was a significant predictor of incident acute coronary syndrome at follow up in an RA population (296). The findings in the current study would support the existing literature that suggests RA patients have higher cardiovascular risk than age and sex matched controls.

Numerically these rates are similar to those found in a previous study of plaque prevalence in RA, published by our group (129). It could be expected that the rate of plaque would be lower in this study, as patients on statins were excluded thus biasing the cohort towards those with lower cardiovascular risk. However, Toms et al. demonstrated a low screening rate for dyslipidaemia in the RA population in the UK (92). Therefore this may not be a substantial underestimation of true plaque burden. Secondly it could be that, although the rate of plaque presence is similar, the burden of plaque is less than would be found had patients on statins been included. No formal grading of plaque severity was performed in this study. However, the fact more than half of plaques (37/69) found in patients were less than 2.5mm thick, suggests that most of these patients have early atherosclerosis.

The association of traditional risk factors with carotid plaque

Smoking was the only traditional risk factor associated with carotid plaque. Smokers were 6.29 times more likely to have carotid plaque than non-smokers. Smoking is well known to be associated with carotid atherosclerosis and cardiovascular disease in the general population. In RA, smoking may also mediate additional effects on atherosclerosis through
reducing drug response, thus increasing inflammation (297). This emphasises the importance of smoking cessation advice in RA patients, not only to help with joint disease but also to reduce cardiovascular risk.

On univariate analysis, age and systolic blood pressure were the only other traditional risk factors associated with presence of carotid plaque in the RA cohort. A study by Wallberg-Jonsson examined the associations of carotid plaque in 39 RA patients and also found significant associations between smoking, age and carotid plaque in RA patients (298). Contrary to the current study, an association was also found with total and LDL cholesterol levels. In this study the lack of association with any lipid parameters could be due to the exclusion of those on statin therapy, which may have led to exclusion of a large portion of patients with adverse lipid profiles. A study by Gonzalez-Juatney et al. also examined traditional CVD and RA related factors associated with presence of plaque in a case control study. They found no association between carotid plaque and LDL or HDL levels in patients. However, they had excluded those with prevalent cardiovascular risk factors or clinical cardiovascular disease (299).

The association of clinical and serological biomarkers of inflammation with carotid plaque

HsCRP was independently associated with carotid plaque in patients. Each increase in quartile of hsCRP was associated with a 2.4 fold increased risk of having carotid plaque. As previously discussed, CRP has been shown to be a predictor of clinical cardiovascular events in both the general population and in RA. Del Rincon et al. also studied the association of CRP with carotid plaque and on full adjustment for traditional risk factors found over an 8 fold increased risk of carotid plaque in those in the highest quartile of CRP(OR [95%CI]: 8.31 [1.98, 34.94]) (300). In Addition, Giles et al. demonstrated that higher CRP levels were associated with a faster rate of plaque progression in RA patients(301). It has to be noted, however that CRP measurement, at a single time point is likely be less important than cumulative CRP measurement over time, as this would better represent the overall burden of inflammation. This was beyond the scope of this study but examining the relationship with cumulative burden of inflammation and cardiovascular risk is an interesting area of further study in future work.
On univariate analysis ESR was significantly associated with plaque, even after age adjustment. Del Rincon et al. also found that ESR was independently associated with carotid plaque in RA (300). ESR was not included in the multivariable model, as it was used to calculate DAS28 and was closely correlated with hsCRP, however findings on age adjusted analysis support the hypothesis that inflammation plays a key role in cardiovascular risk in RA.

A significant association was also found between IL-6 levels but not TNF levels and carotid plaque in the current study. IL-6 levels have been shown to predict cardiovascular mortality in the general population, independent of traditional risk factors (Relative risk [95%CI]: 1.25 [1.19–1.32]) (137). Additionally genetic studies have suggested a causative role in coronary artery disease (302). In RA there is less evidence for an association with IL-6 levels and cardiovascular risk. Dessein et al. studied 74 RA patients and found an association with IL-6 levels and markers of endothelial dysfunction (139). A prior study by our group also failed to find an association with IL-6 levels and carotid plaque, although only 48 patients were included in the study (129). Additionally Kerekes et al. studied 52 patients with established RA and did not find a significant association between carotid intimal thickening and IL-6 on ultrasound (138). However both these studies were in patients with stable disease and had significantly smaller sample sizes than in the current study. IL-6 is known to strongly influence CRP production in the liver and our findings of both biomarkers being associated with plaque would be consistent with this, adding to the validity of results (303).

A recent meta-analysis by Kaptoge et al. found that soluble TNF levels were predictive of subsequent non-fatal MI in the general population, independent of traditional risk factors (relative risk [95%CI]: 1.17[1.09, 1.25] (137). In RA, levels of soluble TNF receptors (thought to be a more stable measure than TNF) were found to be an independent predictor of cardiovascular mortality in a cohort of RA patients (hazard ratio (HR) [95%CI]:1.22 [1.05, 1.49] (89). The direct association of circulating TNF and atherosclerosis in RA has not been established. The previously described study by Kerekes et al. failed to find an association with carotid intimal thickening and TNF levels and Dessein et al. found TNF levels to be associated with only 1 out of 3 measures of endothelial dysfunction (138;139). The lack of association with TNF levels and measures of subclinical atherosclerosis may be due to poor sensitivity of the assay or, as described by Mattey et al., the short-term variability in soluble TNF levels within the blood. Perhaps further evaluation of the association of TNF receptor
levels with carotid plaque may shed more light on the relationship of TNF and atherosclerosis in RA.

**Association of RA disease characteristics with carotid plaque**

A significant association of disease activity (as measured by DAS28) with plaque was found. Few studies have examined the association of DAS28 with carotid atherosclerosis and have mainly focused on the individual components, in particular CRP and ESR. A study by Innala et al. evaluated the association of baseline DAS28 and risk of clinical cardiovascular event at 5 year follow up (304). They found significant association between baseline DAS28 and also cumulative DAS28 and clinical events at follow up (HR [95%CI]: 1.063 [1.021, 1.106] and 1.025 [1.010, 1.040]). It is unlikely that disease activity, at a single time point has a significant causative effect on plaque formation but rather, a cumulative effect of disease activity over time. The DAS28 score is a composite score including tender joint counts and visual analogue score of global health. Joint tenderness can be due to damage and secondary osteoarthritis, not just acute inflammation. Additionally the global health score can also be affected by joint damage and disability contributing to poor health. For this reason the DAS28 in patients with established disease is likely to partially reflect burden of inflammation as well as acute inflammatory activity. This may have explained part of the association of DAS28 with carotid plaque. After adjustment for hsCRP the association was not significant. This suggests that while DAS28 is associated with increased cardiovascular risk, serological markers of inflammation, such as hsCRP, are likely to be better predictors of future cardiovascular events in RA.

We also found that HAQ was significantly associated with plaque even in an age-adjusted analysis. A study from the Norfolk Arthritis Register found that HAQ score 1 year after diagnosis, was an independent predictor of cardiovascular mortality at 10 year follow up (HR [95%CI] was 1.49[1.12, 1.97]) (305). The association of HAQ with carotid plaque has not been assessed in published studies to date, but Kumeda et al. did find a significant correlation between HAQ score and carotid artery intimal medial thickness in patients with established RA (regression co-efficient: 0.305, p<0.05) (306). While HAQ can be influenced by current disease activity, it is recognised as a marker of overall disease burden and severity and could reflect the cumulative burden of inflammation over time.
In previous studies disease duration, seropositivity and extra-articular manifestations have been associated with cardiovascular disease (307). These findings were not replicated in our study, although the association with disease duration approached significance. It is possible that the high prevalence of seropositivity in this cohort made it hard to detect an association.

These results confirm the important role that inflammation plays in increased cardiovascular risk in RA. The lack of association with other traditional risk should be interpreted with caution in this study, as the exclusion of statins has likely led to lower rates of dyslipidaemia and diabetes than in the general RA population. However, taken in the context of other published studies, these results highlight the relatively smaller role that traditional risk factors play in cardiovascular risk in RA and also the importance of RA as a risk factor for cardiovascular disease.

In summary, our results demonstrated an increased prevalence of carotid plaque in RA patients compared to controls. This confirms the increased burden of atherosclerosis in RA, as has been previously reported. In this cohort with active established disease, we found that the key factors associated with plaque were smoking and hsCRP. This suggests that a combination of traditional risk factors and inflammation drive plaque formation.

Whether plaque in RA is also more “unstable” or inflamed in nature requires more detailed analysis of plaque characteristics using more novel imaging methods, which will be the focus of the next chapter.

**Aortic PWV**

PWV is a well validated marker of arterial stiffness and is known to predict future cardiovascular events in the general population (266;267). In the current study we found no significant difference in PWV between patients and controls. In the published literature a number of studies have demonstrated differences in arterial stiffness between RA patients and age and sex matched controls. Maki-Petaja *et al.* compared PWV in 77 patients with active arthritis of a similar age to the current study (mean [SD] age 54[19]) (272). A statistically significant increase in PWV was found in patients (median [IQR] PWV: 8.35[7.14, 10.24] ms$^{-3}$ vs 7.52[6.56, 9.18] ms$^{-3}$, p=0.005). In the current study, there was no difference in age and gender distributions between the groups, to explain a lack of
difference found. One explanation may be that there were more smokers in the RA group who therefore had to be excluded from this portion of the study. Smoking is known to be associated with increased arterial stiffness (308). By excluding a higher proportion of smokers from the patient group, this may have led to a disproportionate loss of subjects with higher arterial stiffness in the patient group.

In patients, PWV was independently associated with disease activity and age. This again highlights the strong influence of inflammation on subclinical cardiovascular disease in RA. Other studies have demonstrated an association between arterial stiffness and disease activity, which would be consistent with findings in the current study (272;309). Seropositivity was significantly associated with PWV on age-adjusted analysis but did not remain in the multivariable model. This could suggest that part of the effect of seropositivity on cardiovascular disease is attributable to the increased disease activity associated with antibody positivity.

Summary

PWV and carotid plaque measure different aspects of subclinical cardiovascular disease but both were independently associated with measures of inflammation in RA patients. It is clear from findings in this chapter that a combination of classical risk factors and inflammation contribute to cardiovascular risk in RA. We have demonstrated an increased burden of atherosclerosis in RA. However, a more detailed assessment of plaque phenotype and its associations with the risk factors discussed here, will be the subject of the next chapter.
4 Evaluation of carotid plaque inflammation and morphology in RA using non-invasive imaging techniques

4.1 Introduction

As demonstrated in Chapter 3, there is an increased prevalence of atherosclerosis in patients with RA, which is independently associated with serological markers of inflammation (310). Atherosclerosis is known to be a chronic inflammatory condition, with both arms of the immune system playing a key role in initiation, progression and destabilisation of atherosclerotic lesions (114). Clinical events occur as a result of plaque rupture. It was once thought that degree of stenosis was the key determinant of plaque rupture. However, histology and imaging studies have demonstrated that plaque composition and inflammation are, in fact the key triggers for plaque rupture and clinical events (123). In RA, there is some evidence to suggest that patients have a more inflammatory rupture prone plaque phenotype. Aubrey et al. demonstrated higher levels of inflammatory infiltrates in coronary lesions of RA patients who had died of myocardial infarctions compared to controls. Additionally, a study comparing coronary lesions in RA patients and controls, using CT, found a higher proportion of high risk plaques in RA patients. The clinical phenotype of cardiovascular events in RA would also be consistent with a more unstable plaque phenotype. Patients have less warning symptoms prior to a major event and have worse outcomes following myocardial infarction and stroke (81)(84).

CMRI is a well validated tool with which to quantify carotid plaque volume and evaluate compositional features. Findings on CMRI have been closely correlated with histological specimens and are associated with stroke and coronary disease risk (183;212;221). Measurement of Ktrans on DCE-MRI is strongly correlated with microvessel density and macrophage content on carotid endarterectomy specimens and provides a non-invasive method of measuring plaque inflammation and neovascularisation(226).

FDG-PET-CT is a nuclear imaging technique, which can also be employed to evaluate carotid plaque inflammation. FDG uptake within the carotid artery correlates with inflammation on histological specimens and has been shown to be associated with traditional cardiovascular risk factors and subsequent cardiovascular events (231; 244).
Evaluation of atherosclerosis in the carotid artery is of relevance in the RA population. Firstly, findings in the carotid artery are predictive of events in other vascular territories and also carotid atherosclerosis can cause stroke, the incidence of which is increased in the RA population.

The primary aim of this study was to employ CMRI and PET to test the hypothesis that RA patients have more unstable inflammatory plaque phenotype compared to controls and that treatment of active joint disease will have an effect on plaque inflammation and composition.

4.2 Aims

The specific aims of the current chapter were to:

- Employ CMRI and PET imaging to test the hypothesis that RA patients have more inflammatory unstable carotid plaque compared to controls and that plaque inflammation and composition can be alter by treating active joint disease

- Evaluate the feasibility of using these techniques in RA patients with active disease

- To evaluate the associations between plaque inflammation and RA disease characteristics and serological measurements

- Evaluate the correlation between plaque inflammation as measured on PET and CMRI

4.3 Methods

A prospective pilot study of patients with active disease and age and sex matched controls was conducted. After undergoing clinical and serological evaluation of traditional and novel cardiovascular risk factors, carotid artery ultrasound was performed with the aim of identifying carotid plaque. The standard definition of carotid plaque includes a minimum thickness of 1.5mm however DCE-MRI measurements can only be accurately be performed
in plaques which are more than 2mm thick. For this reason, only subjects with “suitable” plaque, i.e. plaque more than 2mm proceeded to have an MRI scan.

In those with suitable plaque, CMRI was performed on a 3 Tesla Philips Achieva Scanner (Philips, Netherlands) using a carotid artery surface coil. Details of the imaging acquisition and analysis are described in Chapter 2. Initial quality assurance of scans was undertaken within 1 week of scan to assess imaging quality, check for any clinically relevant findings and to verify that the data was suitable for DCE analysis. Scans were then analysed in batches at the Vascular Imaging Laboratory, University of Washington. The primary outcome for the study was the difference in the Ktrans of plaque, measured on CMRI, between patients and controls and in patients over time. Secondary outcomes included measurement of plaque volume and morphological characteristics.

An FDG-PET-CT of the carotid arteries was performed in consecutive patients who had no history of cancer, poorly controlled diabetes or recent infection. Scans were performed within 2 weeks of MRI and images were co-registered with T1 weighted MRI images. Plaque was identified on MRI and FDG uptake was measured in this area on PET. Analyses were performed by a senior medical physicist, who was blinded to the DCE-MRI findings.

Repeat clinical, serological and MRI assessments were performed in patients when disease activity was deemed to have improved.

It was estimated that a sample size of 80 patients and 60 controls would be required to collect MRI datasets in 30 patients and 14 controls. An interim analysis was performed 6 months after recruitment began in order to evaluate progress. At this point, 57 patients had been recruited to the study. Figure 4.1 summarises the progression of patients in the study. On ultrasound screening, 30 patients (52.6%) had carotid plaque. However only 12(21%) had plaque, which was more than 2mm thick, thus were eligible for MRI. 10 patients completed an MRI scan and FDG-PET-CT was also performed in 5 patients. Imaging quality was deemed sufficient in all 10 MRI cases but only 6 of 10 had plaque suitable for DCE analysis.
As a result of the interim analysis, 3 significant changes were made to the study design, in consultation with our collaborators at the University of Washington. Firstly, an increase in threshold to proceed to MRI was increased to plaque thickness > 2.5mm in order to minimise measurement error on ultrasound and reduce the chances of discrepancy of ultrasound and MRI findings. Sample size was also increased to 120 controls and 160 patients and the upper age limit for inclusion was increased to 70 years old.
4.4 Results

4.4.1 CMRI findings at baseline

130 patients and 52 controls were recruited to the study and baseline characteristics of these subjects were described in detail in the last chapter. Age and sex was closely matched between the 2 groups. An overview of progression of subjects through the study is summarised in figure 4.2.

Figure 4-2 Overview of the participant recruitment and numbers through the study
Plaque was found in 69 (53.1%) patients on ultrasound. 34 of the 69 had plaque which was suitable for MRI. Two participants were excluded prior to MRI (one due to low disease activity, one due to raised BMI). One further patient was unable to attend the MRI appointment and five failed to complete the MRI scan (one due to joint pain, one due to difficulties with coughing and three due to claustrophobia). On quality assessment, datasets in the 26 completed scans had satisfactory imaging quality. Plaque deemed suitable for DCE analysis was found in 15 out of the 26 completed MRI scan (57.7%). In the other 11 cases plaque was deemed too thin for DCE measurements to be reliably undertaken.

Plaque was found in 19 (36.5%) control subjects on ultrasound screening however 11 of these were suitable for MRI. One subject did not complete the scan due to claustrophobia. 4 of the 10 completed scans (40%) were suitable for DCE analysis. In a 5th case, plaque was of suitable size but due to an error in slice positioning DCE analysis was possible. In this case plaque burden and composition were analysed.

### 4.4.1.1 Cohort characteristics

The clinical and serological characteristics of the participants who had MRI datasets suitable for analysis are summarised in table 4.1. There were no significant differences in age and sex. There were no significant differences in classical risk factors between the groups although there was a trend towards higher systolic blood pressure in the patient group.

**Table 4-1 Differences in traditional risk factors between patients and controls, median(IQR) or frequency (%) where**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n=15)</th>
<th>Controls (n=5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.6 (56.2,64.9)</td>
<td>58.9 (52.5, 66.6)</td>
<td>0.631</td>
</tr>
<tr>
<td>Female*</td>
<td>11 (73.3)</td>
<td>3 (60.0)</td>
<td>0.372</td>
</tr>
<tr>
<td>Current smoker*</td>
<td>2 (13.3)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>2.96 (2.13, 2.28)</td>
<td>3.87 (3.28, 4.33)</td>
<td>0.081</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.55 (1.17, 2.01)</td>
<td>1.33 (1.25, 1.72)</td>
<td>0.860</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>3.33 (2.48, 4.04)</td>
<td>4.36 (3.77, 4.87)</td>
<td>0.176</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>147 (129, 161)</td>
<td>127 (117, 140)</td>
<td>0.079</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>87 (79, 89)</td>
<td>78 (71, 89)</td>
<td>0.342</td>
</tr>
<tr>
<td>Pulse wave velocity (ms⁻²)</td>
<td>9.7 (8.0, 11.9)</td>
<td>9.2 (7.7, 10.2)</td>
<td>0.364</td>
</tr>
<tr>
<td>Known hypertension*</td>
<td>1 (6.67)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Diabetic*</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Clinical CVD*</td>
<td>1 (6.67)</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
In keeping with our selection criteria for MRI analysis (larger plaque size), the subgroup of patients, in whom MRI analysis was performed, were significantly older than the rest of the RA cohort (median age 59.62[56.24, 64.49] years vs 54.18[48.00, 60.9] years, p=0.004) They also had higher ESR levels (30[17, 46] mm/hr vs 13[7, 27] mm/hr, p=0.002). There was no significant difference in other classical cardiovascular risk factors or disease characteristics. Clinical characteristics and therapy are summarised in table 4.2.

Table 4-2 Disease characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (IQR) or frequency (%) where *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease duration (years)</td>
<td>6.66 (2.89, 14.9)</td>
</tr>
<tr>
<td>DAS28 score</td>
<td>4.78 (4.19, 5.97)</td>
</tr>
<tr>
<td>HAQ score</td>
<td>1.31 (0.50, 2.31)</td>
</tr>
<tr>
<td>RF positive*</td>
<td>12 (80.0)</td>
</tr>
<tr>
<td>ACPA positive*</td>
<td>14 (93.3)</td>
</tr>
<tr>
<td>Seropositive*</td>
<td>14 (93.3)</td>
</tr>
<tr>
<td>Extra-articular disease*</td>
<td>7 (46.7)</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>30 (17, 46)</td>
</tr>
<tr>
<td>HsCRP (mg/ml)</td>
<td>4.29</td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>1 (1, 192.9)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>3.01 (0.25, 4.69)</td>
</tr>
<tr>
<td>Current therapy</td>
<td></td>
</tr>
<tr>
<td>Combination DMARD therapy*</td>
<td>5 (33.3)</td>
</tr>
<tr>
<td>Anti-TNF therapy*</td>
<td>2 (13.3)</td>
</tr>
<tr>
<td>Tocilizumab*</td>
<td>1 (6.7)</td>
</tr>
<tr>
<td>Current oral glucocorticoid therapy*</td>
<td>2 (13.3)</td>
</tr>
</tbody>
</table>
4.4.1.2 Plaque characteristics

There was evidence of contrast enhancement in all cases (an example of this can be seen in figure 4.3). There was no significant difference in Ktrans or Vp measurements between cases and controls. Plots of DCE parameters in patients and controls can be seen in figure 4.4. and 4.5 respectively.

Figure 4-3 Dynamic contrast enhanced MRI images of plaque in the left carotid artery. Gradual increase in plaque enhancement over time can be seen in images i. to iii. Image iv. represents the signal intensity seen within the plaque over time with the measured intensity represented by the red line and the model fit as the yellow line. Image v. demonstrates the calculated vasa-vasorum image with Ktrans of the plaque in green and VP in red (here seen in the lumen of the artery and vein).
Figure 4-4 Dot plot of Ktrans measurements within plaque in patients and controls (bar represent median Ktrans in each group).
Table 4-3 Plaque characteristics on MRI in patients and controls. Median(IQR) or frequency (%) where *

<table>
<thead>
<tr>
<th>Plaque characteristics</th>
<th>Patients (n=15)</th>
<th>Controls (n=5)^</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque volume (mm³)</td>
<td>351.6 (217.1, 453.1)</td>
<td>309.9(149.9, 706.1)</td>
<td>0.813</td>
</tr>
<tr>
<td>Ktrans plaque (min⁻¹)</td>
<td>0.045 (0.030, 0.785)</td>
<td>0.082(0.057, 0.104)</td>
<td>0.194</td>
</tr>
<tr>
<td>VP plaque (0-1)</td>
<td>0.070(0.050, 0.097)</td>
<td>0.048(0.043, 0.274)</td>
<td>0.484</td>
</tr>
<tr>
<td>RMI</td>
<td>0.510(0.480,0.600)</td>
<td>0.600 (0.485, 0.645)</td>
<td>0.483</td>
</tr>
<tr>
<td>Calcium present*</td>
<td>11 (73.3)</td>
<td>1 (20.0)</td>
<td>0.038</td>
</tr>
<tr>
<td>Calcium % volume of plaque</td>
<td>1.5 (0, 7.3)</td>
<td>0 (0,0)</td>
<td>0.079</td>
</tr>
<tr>
<td>LRNC present*</td>
<td>13 (86.7)</td>
<td>4 (80.0)</td>
<td>0.560</td>
</tr>
<tr>
<td>LRNC % volume of plaque</td>
<td>10.7 (4.6, 14.4)</td>
<td>20 (16.0, 24.9)</td>
<td>0.149</td>
</tr>
<tr>
<td>Loose matrix present*</td>
<td>6 (40.0)</td>
<td>3(60.0)</td>
<td>0.212</td>
</tr>
<tr>
<td>Thin/disrupted fibrous plaque†</td>
<td>12 (85.7)</td>
<td>4 (80)</td>
<td>0.761</td>
</tr>
</tbody>
</table>

^n=4 for DCE measurements in controls; †Measurement not performed in 1 patient

There was no significant difference in plaque volume or RMI between the groups, suggesting equivalent size plaques in both groups. On evaluation of plaque morphology, there was a significantly higher prevalence of plaque calcification in patients compared with controls. There was a high prevalence of LRNC and also thin or disrupted fibrous cap in both groups. All 15 patients had a least one feature of high risk plaque (defined as...
calcification, thin fibrous cap or LRNC). In controls, 4/5 had at least one feature of high risk plaque.

Figure 4-6 Prevalence of specific plaque characteristics in patients and controls on CMRI

4.4.1.3 Correlation of imaging parameters with clinical and serological markers

Correlation of Ktrans of plaque with LDL, hsCRP, TNF and IL-6 were made across the groups at baseline using spearman rank test but no correction for multiple testing was made. Correlation of DAS28 with Ktrans was also evaluated in patients. Results for the whole cohort are displayed in figures 4.7 to 4.12. No significant correlation between Ktrans and any of the described serological markers or DAS28 was found. There was a trend towards a weak correlation between LDL and lipid core volume ($r=0.382$, $p=0.096$). The associations
of hsCRP and LDL with Ktrans were also assessed in the patient group and no significant association was found ($r=-0.01$, $p=0.979$ and $0.266$, $p=0.337$ respectively).

Figure 4-7 LDL levels plotted against Ktrans of plaque
Figure 4-8 IL6 levels plotted against Ktrans measurement of plaque

\[ r = 0.13, \ p = 0.59 \]

Figure 4-9 TNF levels plotted against Ktrans measurement of plaque

\[ r = 0.19, \ p = 0.44 \]
Figure 4-10 HsCRP levels plotted against Ktrans measurement of plaque

Figure 4-11 DAS28 score plotted against Krans of plaque in patients
The relationship between calcification and Ktrans values was interrogated. Ktrans tended to be higher in those without calcification when evaluated across the groups (as shown in figure 4.13) although this was not statistically significant (p=0.339). In patients, there was an inverse correlation between volume of calcium within the plaque and Ktrans measurement, which showed a trend towards significance (r=-0.45, p=0.093).
Figure 4-13 Ktrans measurement in plaques with and without calcification (p=0.339)

Figure 4-14 Ktrans measurement of plaque plotted against volume of calcium within the plaque

$r=-0.45, p=0.093$
4.4.2 Change in CMRI findings in patients over time

Of the 15 patients who were included in the analysis at baseline, 10 had follow up data suitable for DCE analysis. 4 patients were unable to attend a follow up visit (2 due to work commitments, 1 due to coughing difficulties as a result of bronchiectasis during the first scan, 1 due to prolonged hospital admission for pneumonia). The last participant had a follow up MRI but there was inadequate coverage during DCE sequences and a repeat scan was not possible due to time constraints.

4.4.2.1 Cohort characteristics

The median (IQR) follow up was 7(6, 13) months. Of the 10 patients included in follow up analysis, 2 had started a new disease modifying therapy (tocilizumab=1, rituximab=1) and 1 patient had started oral glucocorticoids. A total of 3 patients were on oral glucocorticoids at time of follow up and 2 patients had been given an intramuscular glucocorticoid injection between visits. One patient had been on no DMARDs at baseline and at follow up, the only treatment given had been an intramuscular glucocorticoid injection. At the time of follow up, 3 patients had started statin therapy.

Changes in individual DAS28 scores can be seen in figure 4.15. 3 patients had a clinically significant improvement in disease activity (DAS28 decreased by >1.2) and 2 patients had a significant deterioration (DAS28 increased by >1.2). The remaining 5 patients had no significant change in disease activity. Median values of the relevant clinical, serological and imaging measurements are described in table 4.4.
Table 4-4 Differences in clinical, serological and imaging variables at follow up. Median (IQR)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>Follow up</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS28</td>
<td>4.83 (4.43, 6.33)</td>
<td>4.46 (4.02, 5.54)</td>
<td>0.123</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>5.75 (4.79, 6.89)</td>
<td>4.29 (1.19, 5.37)</td>
<td>0.043</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>2.96 (2.13, 3.28)</td>
<td>2.85 (1.96, 3.26)</td>
<td>0.174</td>
</tr>
<tr>
<td>Plaque volume (mm$^3$)</td>
<td>361.4 (217.1, 459.5)</td>
<td>308.8 (212.9, 465.1)</td>
<td>0.413</td>
</tr>
<tr>
<td>RMI</td>
<td>0.51 (0.48, 0.61)</td>
<td>0.51 (0.47, 0.59)</td>
<td>0.875</td>
</tr>
<tr>
<td>LRNC volume (mm$^3$)</td>
<td>40.1 (14.86, 52.92)</td>
<td>24.0 (0, 43.42)</td>
<td>0.039</td>
</tr>
<tr>
<td>Ktrans (min$^{-1}$)</td>
<td>0.062 (0.030, 0.081)</td>
<td>0.056 (0.041, 0.075)</td>
<td>0.625</td>
</tr>
<tr>
<td>Vp (0-1)</td>
<td>0.0693 (0.0506, 0.0964)</td>
<td>0.0257 (0.0195, 0.0661)</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Wilcoxon matched pairs test was used to assess for significant changes between the 2 time points.

Figure 4-15 Change in DAS28 scores in patients between baseline and follow up assessment
4.4.2.2 Change in MRI measurements

Changes in plaque volume, Ktrans, Vp and LRNC volume can be seen in figure 4.17 and median values are summarised in table 4.4. Wilcoxon matched pairs test was used to assess for significant changes in parameters between the two time points. There was a significant change in LRNC volume between baseline and follow up (p=0.039) and Vp (p=0.013). In one case there was a vast reduction in plaque volume (409.3mm³ to 213mm³ as shown in figure 4.18), which was accompanied with a reduction in LRNC. On removal of this case there was only a trend towards reduction in LRNC volume (p=0.078). There was also a statistically significant change noted in Vp of plaque between time points (p=0.049).
Figure 4-17 Change in LRNC volume in plaque from baseline to follow up

Figure 4-18 Change in Vp measurements in plaque from baseline to follow up
Figure 4-19 Change in Ktrans measurements in plaque from baseline to follow up

Figure 4-20 Change in plaque volume measurements from baseline to follow up
We evaluated the relationship of change in $K_{\text{trans}}$ with change in DAS28, hsCRP and LDL. The correlation of change in LDL with change in LRNC volume was also tested using spearman rank correlation. Findings are summarised in figures 4.21 to 4.24. No significant relationship was found between any of these factors and MRI measurements of interest. There was no correlation with change in $V_p$ and hsCRP, DAS28 or LDL on spearman rank test ($r=-0.53$, $p=0.117$; $r=-0.19$, $p=0.602$; -0.006, $p=0.987$ respectively).

![Graph](image)

*Figure 4-21 Change in $K_{\text{trans}}$ of plaque plotted against change in hsCRP levels*
Figure 4-22 Change in ktrans of plaque plotted against change in DAS28 score

Figure 4-23 Change ktrans plotted against change in LDL cholesterol levels
Figure 4-24 Change in LRNC volume of plaque plotted against change in LDL cholesterol levels

$r = -0.25, p = 0.48$
4.4.3 Findings on carotid artery FDG-PET-MRI

FDG-PET-CT was performed in 13 patients. MRI data were available in all cases, however in 4 cases plaque were deemed too small for DCE analysis. These cases were still included in analysis, as previously published studies have used ultrasound measurements of 1.5mm to define plaque for PET and in the 4 cases it was noted that plaque was present on ultrasound, but just not large enough to satisfy the minimum size requirement for DCE. Co-registration was achieved in all subjects. In a few cases there was minor mis-registration between PET and MRI and an example of this can be seen in figure 4.30. In these circumstances, differences in position of other landmarks on MRI and PET were used as a guide to identify the region on interest on PET, which corresponded to the region of interest, which had been pre-specified on MRI. Cases were also reviewed by a second senior medical physicist to ensure the measurements were taken from the correct region.

Ordered subset expectation maximisation (OSEM), high definition (HD) and ultra-high definition (UHD) reconstructions were performed on all datasets. Unfortunately HD reconstruction in one case and OSEM reconstruction in another case was lost due to a technical failure of the PET workstation early in the study. UHD was available in all cases. Maximum SUVs were measured in the pre-defined region of interest in the region of the plaque, an area of the vessel wall where no plaque had been noted and where possible a measurement in the region of the jugular vein was also taken. An example of a co-registered PET-MRI in UHD can be seen in figure 4.25.
Figure 4-25 An example of a PET-CT-MRI UHD reconstruction. This is a sagittal view where the carotid artery can be seen running almost vertically in the centre of the image highlighted by the 3 pronged arrow. An area of increased FDG uptake (highlighted by the arrow on the far left of the image) can be seen in the region of the plaque, as a coloured area corresponding to high intensity on the colour scale (bottom right of the image). Other key structures are also highlighted which are used as landmarks including the brain and spinous processes.
4.4.3.1 Measurement of $SUV^{\text{max}}$ in carotid plaque

Values for $SUV^{\text{max}}$ of plaques for each patient in different reconstructions can be seen in figure 4.26. Median (IQR) $SUV^{\text{max}}$ in plaque on OSEM, HD and UHD reconstruction were 1.88 (1.75, 2.28), 2.24 (2.04, 2.75) and 2.18 (2.00, 2.65) respectively. There was no significant difference between the reconstruction methods ($p=0.106$). A Bland Altman plot comparing OESM and UHD measurements can be seen in figure 4.27. All but one subject lies within the 95% limits of agreement and the bias is -0.079 suggesting close agreement between the two methods.

Figure 4-26 The $SUV^{\text{max}}$ values of patients on OSEM, HD and UHD reconstructions.
Further analysis was performed using UHD generated values, as this was the most advanced reconstruction method. The association of $SUV_{\text{max}}$ with age, gender, plaque volume, hsCRP and DAS28 score was evaluated. There was a significant correlation with $SUV_{\text{max}}$ and hsCRP levels ($r=0.58$, $p=0.04$) although this was borderline in view of the multiple comparisons made in a small dataset. There was no significant association with age, gender, DAS28 score or plaque volume ($r=0.13$, $p=0.697$; $p=0.634$; $r=0.37$, $p=0.201$; $r=0.20$, $p=0.609$ respectively).
r = 0.58, p = 0.04

Figure 4-28 SUV$^{\text{max}}$ in plaque plotted against hsCRP levels

In the 9 cases where DCE analysis had been undertaken, no association with Ktrans and SUV$^{\text{max}}$ was found ($r = -0.14$, $p = 0.74$).
4.4.3.2 Target to background ratio (TBR)

TBR was calculated by dividing the $SUV_{\text{max}}$ in the plaque by the $SUV_{\text{max}}$ as measured in the centre of the jugular vein. In one case, the jugular vein was ill defined on MRI so no reading was available. The median (IQR) $SUV_{\text{max}}$ in the jugular vein was 1.85 (1.67, 2.34). TBR values in the 12 cases are illustrated in figure 4.29. The median (IQR) TBR was 1.03 (0.95, 1.35).

In a number of cases high $SUV_{\text{max}}$ was measured in the jugular vein, due to avid uptake in an adjacent lymph node, which led to “bleeding” of the signal into the region of the jugular vein (an example is seen in figure 4.30). Additionally, in other cases the jugular vein lay directly beside the artery wall where the plaque was situated and due to spatial resolution it was difficult to separate the signals (see figure 4.31).

![Figure 4-29 TBR measurements in each individual case](image)
Figure 4-30 An example of an FDG-PET-MRI, where minor mis-registration has occurred. FDG signal is seen posterolaterally to the spine on MRI. The FDG signal from the plaque is also slightly displaced. There is a lymph node with high FDG signal adjacent to the jugular vein. The spatial resolution on PET means that some signal from both the plaque and more significantly the lymph node contribute to the SUV<sub>max</sub> measured in the jugular vein.

Figure 4-31 An axial MRI image through the carotid bifurcation. The carotid plaque in this case abuts the jugular vein. Due to the low special resolution of PET, FDG signal originating from the plaque could contribute to the signal measured in the jugular vein.
4.4.3.3 SUV$^{\text{max}}$ in non-atheromatous artery wall

SUV$^{\text{max}}$ was measured in a non-atherosclerotic region of the carotid artery to assess if uptake in the plaque reflected generalised vessel wall inflammation or was localised to the plaque. The median (IQR) SUV$^{\text{max}}$ in the non-atheromatous region was 2.23 (1.62, 2.56). There was no significant difference between median measurements of SUV$^{\text{max}}$ in the 2 regions (p=0.357). However, there was only a trend towards a correlation between vessel wall and plaque measurements in individual patient ($r=0.42$, $p=0.078$). The median (IQR) ratio of SUV$^{\text{max}}$ in plaque and non-atheromatous artery was 1.05 (0.94, 1.46) and in 5 out of 13 cases uptake more than 30% higher was seen in the plaque compared with the vessel wall (figure 4.32). There was no significant correlation between SUV$^{\text{max}}$ in non-atheromatous wall and CRP ($r=0.35$, $p=0.238$).

![Figure 4-32](image_url)

**Figure 4-32** The ratio of plaque inflammation to non-atheromatous wall inflammation in each case (with dotted red line highlighting the ratio of 1.3)
4.5 Discussion

The key findings in this chapter include:

- A significantly higher prevalence of plaque calcification on CMRI was seen in patients compared with controls, despite plaque burden being equivalent.

- No significant difference in plaque inflammation was found between patients and controls on CMRI

- A significant reduction in LRNC volume was found on CMRI in patients at follow up.

- Plaque inflammation was demonstrated in all 13 patients who underwent FDG-PET-MRI.

MRI data collection and sample size estimation

In order to test the primary hypothesis that patients with RA have a more inflammatory, unstable plaque phenotype compared with controls, collection of 30 MRI datasets in patients and 14 in controls was planned. As this was a pilot study, no formal power calculation was performed. At the time of study set up there were no published data available on which to base sample size estimation on Ktrans measurement, however there was data to suggest that a minimum of 14 subjects per group were required to detect a significant change in plaque morphology (226).

Despite the changes following interim analysis, 15 data sets in patients and 4 in controls were collected which were suitable for DCE analysis. In total, 69 patients had plaque on ultrasound of which 32 were eligible for MRI. The majority of those with plaque were excluded due to the plaque not meeting the minimum required thickness for DCE analysis (n=35). Two further patients were excluded due to high BMI or low disease activity. Six patients did not complete the scan. Therefore whilst 26 datasets were of satisfactory imaging quality only 15 had plaque suitable for DCE analysis. Generally patients tolerated the imaging acquisition well with rates of early termination of scans similar between the groups.

A comparison of patient flow, before and after the changes made after interim analysis can be seen in table 4.5. Despite the changes made, the proportion of completed datasets suitable for DCE analysis did not change significantly (60% and 56.3%).
Table 4-5. Differences in rates of plaque prevalence and data collection, before and after the changes instituted after interim analysis. Frequency and % of total recruited shown.

<table>
<thead>
<tr>
<th>Milestone</th>
<th>Prior to interim analysis</th>
<th>Following interim analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients recruited</td>
<td>57 (100%)</td>
<td>73 (100%)</td>
</tr>
<tr>
<td>Any plaque found on ultrasound</td>
<td>30 (52.6%)</td>
<td>39 (53.4%)</td>
</tr>
<tr>
<td>Plaque suitable for MRI</td>
<td>12 (21.1%)</td>
<td>20 (27.4%)</td>
</tr>
<tr>
<td>Completed MRI</td>
<td>10 (17.5%)</td>
<td>16 (21.9%)</td>
</tr>
<tr>
<td>MRI data suitable for analysis</td>
<td>6 (10.5%)</td>
<td>9 (12.3%)</td>
</tr>
</tbody>
</table>

At the time we designed the study, there was very little in the published literature regarding the dropout rate, at the key steps from screening to DCE analysis. The majority of studies using DCE-MRI, up to that point, had been in symptomatic plaque in patients prior to endarterectomy i.e. in large plaques. 3 published studies subsequently described the use of carotid artery DCE in patients who had not been undergoing subsequent intervention for carotid disease.

The study most like the current protocol was conducted at the Vascular Imaging Laboratory, University of Washington (UW, our collaborators in this study). It was a single centre interventional study. Patients with plaque resulting in >15% carotid artery stenosis on ultrasound, were included in the study (240). In this group 25.2% of plaques were more than 2mm thick on MRI, thus suitable for DCE analysis. Although this study did not have a specific inclusion of plaque thickness>2mm it would be expected that many plaques causing 15% stenosis would be greater than 2mm thick. This is in agreement with the current study, that there is a significant discrepancy in plaque thickness estimates on ultrasound and MRI.

A second study, also by the UW group, described the findings of a multi-centre reproducibility study (245). In this study a measurement of plaque thickness on MRI was used as criteria to proceed to DCE MRI. Using this MRI based threshold, 85.4% of scans were then suitable for DCE analysis at 2 time points. However, it is not clear whether the screening threshold for this study was 1mm or 2mm thickness as there is a discrepancy between the methods and results sections of the paper. 2mm is generally accepted as a more suitable cut point for such studies.
A third study from a different centre, studied patients with clinical cardiovascular disease, approximately 86% of whom had symptomatic carotid atherosclerosis (311). Patients with carotid artery stenosis greater than 30% on ultrasound were included. 95.6% of datasets were suitable for DCE analysis. Taken together, data from the current study and the recently published studies, suggest that either use of MRI to screen for plaque suitability or the inclusion of only larger plaque, as measured on ultrasound, is likely to yield a higher percentage of datasets suitable for DCE analysis. Also, studies of asymptomatic plaque tend to yield a lower conversion rate to suitable scans, in part due to the size of such plaques.

Chen et al. also tested reproducibility of carotid DCE using the UW imaging protocol employed in the current study (312). Scans were performed in 51 patients twice, two weeks apart. Inter-scan variability was assessed and the co-efficient of variation (CoV) was calculated for both Ktrans and Vp. On analysis of 35 paired datasets, a CoV was found of 25% and 62% for Ktrans and Vp respectively. The high variability in Vp measurement was significantly associated with plaque size (spearman r=-0.45, p=0.007) and when analysis was restricted to plaques with an area greater than 25mm², the CoV reduced to 28%. This suggests that Vp measurement is unreliable in small plaques. The authors concluded that a sample size of 50 MRI datasets, are required, per arm, in order to detect a significant difference in Ktrans measurement. This estimate does not take in to account imaging acquisition error, motion artefact or plaques not being of suitable size for DCE analysis.

Data on DCE analysis in other conditions such as in solid tumours have found a CoV in the region of 15%(234). It is possible that the increased variability of carotid DCE measurements may be related to size of target tissue. This would be consistent with the significant improvement of CoV of Vp measurement in larger lesions. Another possibility is the choice of kinetic model. Three models were compared by Gaens et al., in a reproducibility study of carotid DCE (311). The Patlak model was found to be the most reliable model (mean relative fit uncertainty [SE] was 10 [%±1] % for Ktrans compared with 20[%±1] % and 33[%±3]% for to alternative models). There were differences in the number of time points imaged and injection rate between this and the current study, so results may not be directly comparable. Additionally both this and the histological validation work by Kerwin et al. were performed in large lesions (236). It is possible that a different model or possibly even more simple analyses, such as, quantification of contrast enhancement within the plaque, may yield more reliable results in small lesions.
On the basis of findings in the current study 12 patients are required to be screened to acquire 1 suitable MRI dataset. Thus in order to adequately power the study, 600 subjects would have to have been recruited to each arm. This may be a conservative estimate in the context of studying RA patients. Vascular inflammation as measured by FDG-PET-CT was markedly higher in patients compared with controls (169). Although this is a different method of measuring vascular inflammation, it could be hypothesised that there may be a larger difference in Ktrans measurements between patients and controls, than would be apparent when comparing groups with non-inflammatory disease. Additionally the interventional study by Dong et al. was able to detect a significant difference in Ktrans measurement in 28 participants in a single centre study, suggesting smaller numbers may be needed in a single centre setting (240). Despite this it is very likely that the current study was considerably underpowered to test the primary hypothesis.

Discrepancy between ultrasound and MRI findings

There was a significant and unexpected discordance between findings of plaque on MRI and ultrasound in the current study. This could point towards a significant false positive rate of identification of plaque on ultrasound or false negative rate on MRI. On review of the literature, little is known about the rates of false positives and negatives in asymptomatic subjects using either modality. As previously mentioned in carotid artery ultrasound is a widely accepted tool with which to assess carotid artery wall thickness and is known to predict clinical cardiovascular events in population studies (172). However ultrasound can be highly operator dependent and over-estimation of plaque thickness could potentially have occurred in this study, leading to a false positive results.

Riley et al. examined intra and inter-operator reproducibility of carotid ultrasound in a cohort of over 800 asymptomatic subjects (313). The mean (SD) difference in wall thickness measurements between the same sonographer at 2 time points was -0.01 +/- 0.13 mm and between 2 sonographers was 0.00 +/- 0.15 mm. This would point towards a relatively small magnitude of measurement error. There was a high level of agreement between the 3 sonographers in the current study (95%). However, the senior sonographer trained the other two operators, which could have led to systematic error in measurement. In an attempt to address this, the first 10 cases where plaque had been identified, were reviewed by the head of the Vascular Physiology Laboratory at CMFT. In all cases, there was
an agreement with the original findings of plaque. However this was only a review of static images.

On discussion with the collaborating team at The Vascular Imaging Laboratory, University of Washington, another potential source of error on ultrasound was highlighted. Commonly a small collateral branch can occur at the carotid artery bifurcation. Although not widely described in the literature, on ultrasound this branch can give the impression of a plaque or lead to an overestimation of plaque size if its origin is in close proximity. An example of this occurring in one of the subjects in this study can be seen in figure 4.33.

Figure 4-33 A case where a lesion thought to represent plaque was in fact deemed to be a collateral branch originating in the bifurcation on review of MRI images.

A. Image A. shows a longitudinal view of the carotid artery on ultrasound. Localised thickening can be see in the deep vessel wall at the bifurcation (highlighted by red bar) which was identified as plaque on the basis that it was more than 1.5mm thick, increased wall echogenicity and protrusion into the lumen.
Image B. demonstrates the corresponding sagittal view of the artery on MRI. The portion of the vessel wall that corresponds to the thickening seen on ultrasound, is highlighted by the arrow on the far left of the image. There is no significant thickening or plaque on this image. No collateral vessel is apparent on this view.
Image C. is an axial slice through at the level where the plaque was identified on ultrasound. Again this confirms absence of plaque along the wall but a small collateral artery can be seen branching off the bifurcation (highlighted by the top two red arrows). It is thought that this vessel accounts for the appearances on ultrasound.

There is also the possibility of false negatives on carotid MRI, although the rates would be expected to be lower than the US false positive rate as MRI is less operator-dependent. On review of images with the collaborators, it was noted that in some cases significant flow artefact was present in the lumen. Plaque most commonly occurs in the bifurcation where flow artefact is also most commonly found. In some cases the inner border of the vessel wall could not be reliably defined, as flow artefact was present adjacent to the wall. This was deemed as “no plaque” although there may have been plaque, which could not be defined. An example of this can be seen in figure 4.34.
Figure 4-34. A T1 weighted axial image through the carotid bifurcation. Significant flow artefact can be seen in the lumen adjacent to the area of the wall where plaque had been identified on ultrasound.

Although the discrepancy between the two imaging modalities is not widely acknowledged in the literature, similar rates of discordance have been noted in other studies (personal communication, Dr. Dongxiang Xu, University of Washington). Studies are on going at the Vascular Imaging Laboratory, University of Washington to further investigate the rates of agreement of plaque measurement on MRI and ultrasound.

MRI results

In those who underwent MRI scanning, there were no statistically significant differences in traditional risk factors between the two groups, although there was a trend towards lower LDL cholesterol levels and higher systolic blood pressure in patients.

On comparison of patients in which DCE was undertaken and the rest of the RA cohort, the only differences were older age and ESR levels. Both of these factors have been associated with plaque development in RA, therefore this is not an unexpected observation (310).
**Plaque burden**

We found no significant difference in plaque volume or RMI between the two groups. Although it should be noted that plaque thickness was a “suitability” criterion for MRI scanning to occur. Therefore the volume in this population was to some extent pre-selected. This would suggest a similar burden of plaque between the two groups.

**Calcification**

Despite similarities in plaque burden and selection criteria, patients were significantly more likely to have calcified plaque than controls (73.3% vs 20%). Although these are small numbers in the current study, particularly in the control group, the prevalence of calcification in the control group is similar to those found in the published literature. Dong et al. studied a cohort of patients with a history of coronary artery disease, but who had no symptomatic cerebrovascular disease, using CMRI. In a cohort of 28 patients, 8 (29%) patients had calcification (240). This suggests increased prevalence of carotid plaque calcification is a real observation in RA.

Carotid plaque with calcification is classed as an advanced type IV-VII lesion (122) and is associated with clinical risk of stroke and more severe coronary heart disease (180) (198). Increased prevalence of vascular calcification in RA has been noted in previous studies. Chung et al. found an increased prevalence and severity of coronary calcification in RA patients compared with controls on CT, which was associated with high levels of ESR (314). One study examined the prevalence of carotid artery calcification in RA, as measured on CT. RA patients were significantly more likely to have carotid calcification compared with controls (OR = 5.7, CI 95% 1.7–18.7, p = 0.004) (315). This study also confirmed an association with calcification and CRP levels. It is clear from the literature that increased vascular calcification is found in RA but until now this has been thought to reflect increased overall atherosclerotic burden. In the current study, despite a similar burden of plaque in both groups, patients had a significantly higher prevalence of calcification. This would suggest that calcification is not merely a reflection of atherosclerotic burden but points towards a different, more calcified plaque phenotype in RA.
Chronic inflammation and in particular TNF is thought to drive the calcification of atherosclerotic lesions (191). Additionally calcification has been associated with increased levels of CRP, TNF and IL6 in the RA population (199) (316). It could be hypothesised that the increased circulating levels of pro-inflammatory cytokines in RA drive a more calcified plaque phenotype and further work is warranted to investigate this.

Other features of high-risk plaque

Although in the current study there were no significant differences in fibrous cap thickness and LRNC between the groups there was a high prevalence of these two high-risk features compared to the published literature. U-King-Im et al. evaluated plaque phenotype in 57 lesions in a group of 18 subjects with >50% carotid artery stenosis on ultrasound (214). On CMRI 28/57 (49.1%) lesions had thin or ruptured fibrous cap, significantly lower than either group in the current study. Underhill et al. found prevalence of LRNC to be 28% in a cohort of 108 asymptomatic subjects with >50% stenosis (223). There was a higher than expected prevalence in the control group of these features, likely due to the small sample size. When compared with the published literature, it does appear that RA patients have a higher frequency of high-risk plaque features. In fact, when applying Virmani’s proposed classification, all patients had at least one feature of high risk plaque (123).

Few other studies have examined carotid plaque phenotype in RA patients. Semb et al. compared grey scale measurement (GSM) of plaque on ultrasound, a semi-quantitative measurement, which is known to be associated with more rupture prone plaque on histology (265;317). While there is an association found between GSM and cardiovascular events, there is significant variability in the technique (177). In the study by Semb et al., no difference in GSM was found between patients and controls. Interestingly, there were significantly lower GSM measurements in patients with active disease, which could suggest a more high risk phenotype in those with uncontrolled disease.

Coronary artery CT can be used to evaluate plaque phenotype in a semi-quantitative fashion. Lesions can be classified as non-calcified, calcified or mixed (a mixture of calcification and other material such as lipid core). The highest risk lesions on CT are non-calcified or mixed plaques. One study used this technique to evaluate the prevalence of different types of lesions in RA patients. Karpouzas et al. demonstrated a higher frequency of mixed and non-calcified coronary lesions in RA patients compared to controls (133). This
would also support the hypothesis that RA patients have a more unstable high-risk plaque phenotype. In the current study, calcification was accompanied by loose matrix or LRNC, in all but one patient, suggesting a mixed plaque phenotype in most cases. Although findings in the carotid and coronary arteries are not directly comparable, particularly when different imaging modalities are used, our findings are in keeping with Karpouzas et al. and support the hypothesis that patients with RA have a higher risk plaque phenotype.

**DCE MRI findings**

There was no significant difference in Ktrans or Vp measurement between cases and controls. Whilst no difference was found, the study was, in retrospect, underpowered to detect a difference. In addition, the high prevalence of calcification within the RA group will have influenced assessment. Previous studies have shown a trend towards an inverse association between calcification and Ktrans measurement (236;238). Calcium nodules are avascular, thus there would be no measurable Ktrans in calcified regions of plaque. In the presence of significant calcification, it is likely that any Ktrans signal may be reduced. In our cohort, Ktrans values tended to be higher in non-calcified lesions. Interestingly, whilst not significant, the r value (-0.45) would suggest that the correlation between calcium volume and Ktrans measurement was a real correlation. The high rate of calcification in patients compared with controls could therefore explain the similarity in Ktrans values between the groups. Therefore, techniques to exclude calcification from the region of interest may provide a more accurate measurement of ktrans, if technically achievable.

Hypothetically Ktrans can be influenced by factors such as flow rate and perfusion pressure. No formal measurement of these factors was taken at the time of scanning. However, pulse and blood pressure was assessed at baseline and no subjects had significant tachycardia or bradycardias at assessment and there were no significant differences in blood pressure or PWV measurements between groups undergoing MRI. Additionally, no subjects had a history of cardiac failure or other conditions which may lead to lower perfusion pressure, therefore it is unlikely that circulatory differences between the 2 groups, had a significant impact on differences in Ktrans measurement.
Correlation of MRI with clinical and serological biomarkers

Our ability to test multiple associations between imaging and serological biomarkers was limited due to sample size. Previous studies have demonstrated a correlation between hsCRP and Ktrans and also LDL levels with LRNC (238;240). These associations were tested in addition to the association with Ktrans and IL-6 and TNF across the groups, at baseline. In patients, the correlation of Ktrans and DAS28 score was also tested. Ktrans was not significantly associated with these serological or clinical measures of inflammation. There was a trend towards an association between LDL levels and LRNC. While Kerwin et al. did find an association with CRP and Ktrans, this was in a population of symptomatic patients with large carotid plaques (238). Dong et al. found no significant correlation of CRP with Ktrans in the study of 28 asymptomatic patients without inflammatory polyarthritis (240). It is possible that both this and the current study were underpowered to detect the association, or alternatively that the association is most apparent in unstable rupture prone lesions, as was the case in study by Kerwin et al.. The trend towards an association between LRNC and LDL levels is in keeping with previous studies (230).

Longitudinal analysis of MRI in patients over time

One of the main aims of the study was to evaluate the effects of anti-inflammatory therapy on plaque inflammation and morphology. This was an observational study thus, control over intervention and escalation of treatment was not within the remit of the study team. Current guidelines for the management of RA recommend escalation in treatment with the aim of achieving a DAS28 score of less than 3.2, then maintaining patients in a low disease activity state (55). Control of disease activity is known to have a significant impact on progressive joint damage and is could be hypothesised that it may reduce RA related co-morbidity (49). It was expected that following the baseline visit, patients would have further changes to their management which would lead to a reduction of disease activity. However, only 3 patients had a significant improvement in DAS28 at the follow up visit. 5 patients had no significant change in disease activity and 2 patients had significantly higher disease activity at follow up.

Only 2 patients started a new DMARD and one started oral glucocorticoids in the interim. Otherwise there had been no escalation in treatment, other than intramuscular glucocorticoids in 2 cases. This suggests that the current guidelines are not being widely
applied in routine clinical practice. Alternatively these longstanding patients may by the nature of them having active disease at this stage, represent a harder to treat subgroup. The minimal change in disease activity and hsCRP over time therefore made evaluation of the effects of disease suppression difficult to assess.

It was expected that disease activity would have significantly improved by 3 to 6 months after baseline however on contacting patients, in most instances follow up was delayed as no significant improvement had been noted. There was a concern that if follow up was left too long, time would become as significant confounder in the analysis. It was decided during the study to include some participants who had not significantly improved to assess the stability of Ktrans measurements in the absence of significant change in disease activity. The longest follow up was 13 months and in this case the patient had preferred to hold off follow up until her symptoms had improved, otherwise all participants were followed up within 12 months. The varying follow up (4-13 months) between patients may also still have led to some confounding, despite attempts to limit the duration of follow up.

Statins are known to have an effect on plaque inflammation and plaque morphology and 3 out of the 10 patients followed up had started a statin (230). At baseline, letters were sent to the patient with details of their traditional risk factors and advice was given to contact their general practitioner (GP) for further management if required. Additionally results were also sent to the patient’s GP. A number of patients were dyslipidaemic at baseline however in some cases, after consultation with their GP, patients had opted to try dietary modification in the first instance and had declined statin therapy. A number of concerns were voiced by patients about starting statins including poly-pharmacy and the possible worsening of musculoskeletal pain related to the drug.

Despite the problems outlined above there was a significant reduction in LRNC at follow up. The change in LRNC was in part driven by findings in one participant. At follow up the plaque in this participant had regressed from a total volume of $409.3 \text{mm}^3$ to $213 \text{mm}^3$ with an associated reduction in LRNC. There had been no change in medication (including no initiation of statin therapy), disease activity or lipid levels during the follow up period. The patient had continued to smoke but had lost 14 pounds in weight while following a low carbohydrate diet. When this case was excluded, a trend towards reduction of LRNC remained ($p=0.07$). On review of the literature there is evidence that lesions can occasionally regress, however this is mainly reported in the context of drug therapy (318).
The images were discussed with the team at UW to ensure that the same lesion was being analysed at both time points and confirmation of this was given.

There was also a statistically significant change in Vp measurements between baseline and follow up. However, changes were not correlated with clinical or serological inflammation or LDL and in view of the high CoV for Vp measurement in the recent studies (64%), the true significance of this difference is uncertain.

Summary

In summary, the assessment of carotid plaque by MRI in RA patients is feasible however only around 50% of plaques deemed suitable on ultrasound can be fully assessed by MRI and work by collaborators is on going to better understand this discrepancy. RA plaques are more likely to be calcified and this also confounds the ability to measure Ktrans accurately. Finally our observational design did not allow us to see large a decrement in disease activity in this cohort and therefore we cannot comment on how anti-inflammatory therapy and a major reduction in CRP may influence DCE parameters. In spite of this the LRNC results did show a significant reduction over time and this may in part be due to modulation of CV risk factors in the cohort.

We can conclude that plaque morphology in RA seems to have the characteristics of high risk lesion and that a larger study with a prospective intervention is needed to better understand the relationship of inflammation and disease related factors with plaque characteristics in RA.
FDG-PET-CT findings

FDG-PET-MRI was used to evaluate plaque inflammation in 13 patients with active RA. FDG uptake is known to correlate strongly with macrophage content of carotid plaque and has been used to evaluate plaque inflammation in observational and interventional studies (249;251). This was the first time FDG-PET-MRI of carotid plaque had been attempted by our group or in patients with RA, to our knowledge. Scans were well tolerated by patients and in all cases adequate imaging quality was achieved. PET-CT and MRI scans were performed on different scanners, which posed significant potential challenges for co-registration of images. A head support was designed for PET-CT, which, as far as possible, reproduced the head position on MRI. Additionally time was taken to ensure mandible to sternal notch distance was the same and that there was no cervical rotation during both scans. In all cases co-registration was achieved although in some subjects minor differences in landmarks could be detected. Using the discrepancies between landmarks such as the spinal cord, alterations in the region of interest on PET were made, to take account of misregistration, with guidance from a second senior medical physicist. This could have led to a degree of inaccuracy in the measurement of FDG uptake, however not acknowledging and acting upon misregistration would also have led to inaccurate measurement. This problem is not confined to the current study as combined PET-MRI is not widely available and previous studies using MRI have also had to co-register datasets from different scanners (247;319).

There was a trend towards higher SUV$^{\text{max}}$ using the HD and UHD reconstruction. HD reconstruction takes account of the variability in spatial resolution within the field of view and leads to sharper images particularly in smaller structures. UHD adds to this by localising within 8cm the point where photon emission has occurred allowing better localisation of signal, which is of particular value in tissues where there is a low signal. Use of UHD tends to give higher values than standard OSEM reconstruction. Although there was a trend towards higher values using UHD reconstruction this did not reach statistical significance, which make a fairer comparison with the published literature possible.
**Plaque inflammation as measured by SUV\textsuperscript{max}**

SUV\textsuperscript{max}, the most commonly used method of measuring FDG uptake, is calculated by dividing the measured tissue concentration of FDG by the injected concentration per kilogram body weight. This method is used widely in oncology (320). It has been shown to be the most reproducible measure of carotid plaque inflammation on PET and correlates strongly with macrophage content of plaque \((r=0.70, p<0.001)\) (247;321). In this study, carotid plaque inflammation was seen in all cases with SUV\textsuperscript{max} ranging from 1.69 to 6.11. Although there was no control cohort for comparison, Tahara et al. examined the prevalence of plaque inflammation in an asymptomatic population using FDG-PET-CT (246). In this study, 100 patients with no history of clinical cardiovascular disease, diabetes, inflammatory disease, cancer or statin use were screened for carotid plaque using ultrasound. Exclusion criteria were similar to the current study. Those with plaque went on to have an FDG-PET-CT. Plaque inflammation was defined as an SUV\textsuperscript{max} > 1 x standard deviation above the mean (1.39+0.21) of the cohort, a value of 1.60. 41 participants were found to have plaque, 12 of whom had evidence of plaque inflammation on FDG-PET-CT. This suggests that the prevalence of inflammatory plaque in patients without inflammatory or clinical cardiovascular disease is around 30%. On the basis of this publication, all subjects in the current study would be classed as having inflammatory plaque. This strongly supports the hypothesis that patients with active arthritis have an inflammatory atherosclerotic plaque phenotype.

There was one outlier in our cohort, who had particularly high SUV\textsuperscript{max}. This raises suspicion that the signal could partly be due to an adjacent structure such as lymph node. However, this subject had the highest disease activity and the largest plaque of all 13 cases and was the only subject on no DMARD therapy. All these factors could potentially contribute a higher SUV\textsuperscript{max} value.

The correlation of plaque inflammation with CRP has been found in other studies. This finding does support the role inflammation plays in accelerated atherosclerosis in RA although should be interpreted with caution in view of the lack of correction for multiple testing (322).
Target to background ratio (TBR)

TBR is another commonly used method to quantify arterial inflammation. This is a ratio of the uptake in plaque versus background blood FDG signal, commonly measured in the superior vena cava (323). TBR has also been shown to significantly correlate with macrophage content on histology ($r=0.85$, $p<0.001$) and TBR>1.6 in the aorta is predictive of a subsequent cardiovascular event (254;321). In the current study, TBR measurements were calculated using the jugular vein, as the superior vena cava was not included in the imaging acquisition protocol. This produced a range of results (TBR range 0.62-1.91) as high uptake was seen in the region of the jugular vein in many cases. High FDG uptake in lymph nodes has previously been noted in RA patients. Kubota et al. carried out whole body FDG-PET-CT in patients with RA and found that 15/18 participants had significant uptake in the axillary lymph nodes (324). A second study by Vijayant et al. also found significant FDG uptake in the lymph nodes of 10/17 RA patients on whole body PET (325). Anatomically, the anterior cervical lymph node chain lies along the lateral aspect of the jugular vein. Due to the limited spatial resolution of PET it would be impossible to extract a true background blood signal from the jugular vein in the presence of nearby inflamed lymph node. Additionally in some cases the carotid plaque was located on the posterolateral border of the artery, abutting the vein and could also have contributed to the jugular vein signal. These results suggest that calculation of TBR based on blood signal in the jugular vein, may not be a reliable method of quantifying carotid artery FDG uptake in RA patients.

Difference in FDG uptake in plaque versus non-atheromatous vessel wall

Generalised vessel wall inflammation, even in the absence of plaque has been shown to be associated with cardiovascular risk factors and subsequent cardiovascular events (254). Previously, Maki- Petaja et al. demonstrated that patients with active RA, had evidence of increased FDG uptake in the aortic wall compared with controls (169). It could be hypothesised that in the current study, FDG uptake in carotid plaque was a reflection of generalised vascular inflammation, as seen in the Maki-Petaja study, rather than a localised inflammation within the plaque. In order to explore this, SUV\textsuperscript{max} measurements were taken in a non-atheromatous region of the same carotid artery. FDG uptake was demonstrated in non-atheromatous wall in most cases (range 1.46 to 3.04) confirming the finding of generalised arterial inflammation in RA patients. While median (IQR) SUV\textsuperscript{max} values in atheromatous and non-atheromatous vessel wall were similar, there was only a moderate
correlation between measurements in individual patients ($r=0.41$, $p=0.078$). In 5 cases the SUV$^{\text{max}}$ was more than 30% higher in the plaque than in the vessel wall. This could suggest that carotid plaque inflammation does not merely reflect generalised vessel inflammation, but in some cases represents a focused active inflammatory process within the plaque.

**Correlation of PET and MRI findings**

In the current study no association was found between Ktrans and SUV$^{\text{max}}$ of plaque ($r=-0.14$, $p=0.74$). Previous studies have demonstrated a weak or, in some cases, no association between Ktrans and FDG uptake. Calgano et al. compared DCE MRI and PET measurements in 40 subjects with coronary artery disease(326). There were significant differences in imaging acquisition and analysis protocols in both modalities, when compared with the current study. However, an inverse correlation between Ktrans and SUV$^{\text{max}}$ ($r=-0.22$, $p<0.05$) within the same lesion was demonstrated. The authors hypothesised that inflammation (as measured by FDG-PET) occurs in response to tissue hypoxia at an earlier stage of atherosclerosis than neovascularisation (measured on DCE MRI) and that using combining both imaging modalities would provide complementary information about plaque vulnerability. The small size in the current study ($n=9$) means that although, no definitive conclusions can be drawn, findings are consistent with the published literature, which suggest that there is, at best a weak correlation, between Ktrans and FDG uptake in carotid plaque.

**Limitations and methodological considerations**

There were some limitations is in the analysis of FDG-PET in this study. Firstly the spatial resolution of PET is limited to 4.1mm and some lesions were only 2mm thick. Signal measured in the region of plaque could therefore have potentially included signal from other structures. As far as possible signal was localised to plaque but contribution of metabolic activity from nearby structures could not be completely excluded. Additionally, some patients who underwent FDG-PET were subsequently deemed not to have large enough plaque for MRI (i.e less than 2mm thick). These patients were still included in the study, as standard definition of plaque includes lesions greater than 1.5mm thick on ultrasound and the lower limit for a “negative MRI” was 2mm. Previously published studies have used ultrasound to identify carotid plaque before going on to use FDG-PET (246).
Additionally, FDG-PET has been used to evaluate carotid vessel wall inflammation in nonatheromatous wall in other published work (169). Thus the decision was taken not to exclude patients, who had met ultrasound but not MRI criteria for plaque.

**Summary**

In summary we have shown that FDG-PET is a feasible and acceptable technique to use in RA patients. Although there were some technical issue with co-registration and the proximity of lymph nodes to the jugular vein, we demonstrated increased FDG uptake within the plaque in all cases. There was also uptake demonstrated in non-atheromatous artery wall suggesting generalised vascular inflammation occurs in RA. However in some cases there appeared to be preferential uptake within the plaque suggesting an active inflammatory process within the plaque rather than uptake being a marker of generalised wall inflammation.
5 Evaluation of potential non-imaging biomarkers of cardiovascular risk in RA

5.1 Introduction

Findings in the study have so far highlighted the increased burden of atherosclerosis and the importance of inflammation in atherogenesis in RA. Inflammation is thought to lead to accelerated atherosclerosis both through the modulation of traditional risk factors and by directly affecting the vessel wall. The mechanisms by which inflammation mediates its effects on the artery remain unclear. As discussed in Chapter 1, certain cells and proteins have been implicated in the both RA and atherosclerosis in the general population and these will be the focus of investigation in the current chapter.

Cellular adhesion molecules (CAMs) are expressed on the surface of activated endothelium and facilitate leucocyte chemotaxis and adhesion the vessel wall. CAMs are shed from the vessel wall and can be measured in the circulation. In RA increased CAMs are found in inflamed synovium and have been associated with subclinical cardiovascular disease (262). In the general population CAMs levels are predictive of subsequent cardiovascular events and act as a marker of endothelial dysfunction.

CD28null cells are an abnormal subset of CD4 T cells which secrete high levels of IF-\(\gamma\), have cytolytic properties and are resistant to apoptosis (327). Expanded numbers are found in some patients with RA and their presence is associated with more severe disease. Expanded populations of these cells have also been demonstrated in patients with acute coronary syndrome and are an independent predictor of recurrent events (328). These cells have been associated with increased IMT on ultrasound in RA patients and may mediate deleterious effects on plaque (32).

EMPs are small vesicles, which are shed from activated or damaged endothelium and are associated with more severe coronary disease in the general population. They have also been found in patients with active SLE and are associated with other measurements of endothelial dysfunction. To our knowledge EMPs levels have never been studied in RA however they may provide insight into endothelial dysfunction.
5.2 Aims

The specific aims of this chapter are to:

- Compare CAMs, CD28null cells and EMPs in RA patients and age and sex matched controls
- Evaluate the association of these serological measures with clinical and serological measures of cardiovascular risk, RA disease activity and subclinical cardiovascular disease

5.3 Methods

Blood was drawn for measurement of CAMs, CD28null T cells and EMPs at baseline in patients and controls. Details on sample preparation and analysis techniques are described in detail in Chapter 2. In brief, CAM5 levels were measured in serum, using a standard ELISA assay, by Dr P. Pemberton at CMFT. The proportion of CD4 T cells which lacked CD28 co-expression were measured in whole blood samples using flow cytometry. EMP levels were also measured in platelet poor plasma using flow cytometry.

Levels of each serological measure were compared in patients and controls and then association with clinical, serological and imaging variables evaluated using non-parametric statistics and regression models.

5.4 Results

5.4.1 Cellular adhesion molecules

Cellular adhesion molecules (CAMs) were measured in 130 patients and 40 controls at baseline. Patients had significantly higher levels of Inter-cellular adhesion molecule (I-CAM) and E-selectin compared with controls. There were no significant differences in levels of vascular cellular adhesion molecule -1 (VCAM-1), nor in P-selectin between patients and
controls (p=0.749 and 0.140 respectively). Figures 5.1 to 5.4 demonstrate the differing levels of each CAM between the groups.

Figure 5-1 Levels of I-CAM in patients and controls.
Figure 5-2 Levels of VCAM-1 in patients and controls

![Graph showing VCAM-1 levels in patients and controls with a p-value of 0.748.]

Figure 5-3 Levels of E-selectin in patients compared to controls

![Graph showing E-selectin levels in patients and controls with a p-value of <0.001.]

Figure 5-4 Levels of P-selectin in patients compared to controls

P-selectin levels (ng/ml)

Patients

Controls

p = 0.140
**Association of CAMs with subclinical cardiovascular disease**

The association of CAM levels with carotid plaque and PWV was evaluated in patients and findings are summarised in figures 5.5 to 5.12.

E-selectin and I-CAM levels were associated with plaque presence and there was a trend towards an association between VCAM-1 and plaque presence. VCAM-1 was significantly associated with PWV and there was a trend towards an association between PWV and I-CAM and but not with e-selectin. There was no association between p-selectin and either marker of subclinical cardiovascular disease.

**Figure 5-5 I-CAM levels in patients with and without carotid plaque**

![I-CAM levels in patients with and without carotid plaque](image)

$p = 0.032$
Figure 5-6 VCAM-1 levels in patients with and without carotid plaque

\[ p = 0.083 \]

Figure 5-7 E-selectin levels in patients with and without plaque

\[ p = 0.043 \]
Figure 5-8 P-selectin levels in patients with and without plaque

Figure 5-9 Correlation between ICAM levels and PWV
Figure 5-10 Correlation between VCAM-1 levels and PWV

$r = 0.36, p < 0.001$

Figure 5-11 Correlation between E-selectin levels and PWV

$r = 0.13, p = 0.18$
The association of CAMs levels with Ktrans of plaque on MRI and SUV$_{\text{max}}$ of plaque on FDG-PET was also evaluated in the patients with imaging results (n=15 and n=13, respectively). There was a strong correlation between Ktrans and e-selectin ($r=0.70$, $p=0.004$) and a moderate correlation with p-selectin which showed a trend towards significance ($r=0.45$, $p=0.09$). There was also a trend towards a moderate correlation between SUV$_{\text{max}}$ and VCAM-1 ($r=0.51$, $p=0.07$). However no correction was made for multiple comparisons. Dot plots can be seen in figure 5.13 to 5.15.
Figure 5-13 Correlation of Ktrans with E-selectin levels

![Graph showing correlation between Ktrans (min⁻¹) and E-selectin levels (ng/ml)]

$r = 0.70$, $p = 0.004$

Figure 5-14 Correlation of P-selectin levels with ktrans measurements

![Graph showing correlation between Ktrans (min⁻¹) and P-selectin levels (ng/ml)]

$r = 0.45$, $p = 0.089$
Figure 5.15 Correlation between SUV$^{\text{max}}$ of plaque and VCAM-1. 

\[ r = 0.51, p = 0.072 \]
Factors associated with CAM levels in patients

Stepwise linear regression was used to evaluate if any factors were independently associated with levels of CAMs. All 4 CAMs were non-normally distributed so were log transformed to satisfy the assumption of normality required for linear regression. A significance level was set at p<0.1 for the multivariable models in all cases.

I-CAM levels

Table 5.1 summarised the results of the regression of log transformed I-CAM. Smoking, DAS28 and IL-6 were independently associated with ICAM levels. On univariate analysis smoking, systolic blood pressure, HAQ, RF positivity, hsCRP were significantly associated with levels of I-CAM.

Table 5-1 Factors associated with log transformed I-CAM on linear regression

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Unadjusted β coefficient [95% CI]</th>
<th>Age adjusted β coefficient [95% CI]</th>
<th>Fully adjusted β coefficient [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.006 (-0.002, 0.015)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (male)</td>
<td>0.386 (-0.121, 0.198)</td>
<td>0.068 (-0.098, 0.235)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>0.251 (0.070, 0.434)</td>
<td>0.276 (0.082, 0.468)</td>
<td>0.205 (0.024, 0.151)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>0.004 (0.001, 0.008)</td>
<td>0.005 (0.001, 0.009)</td>
<td></td>
</tr>
<tr>
<td>TC:HDL</td>
<td>0.041 (-0.009, 0.091)</td>
<td>0.041 (-0.009, 0.091)</td>
<td></td>
</tr>
<tr>
<td>DAS28</td>
<td>0.106 (0.056, 0.158)</td>
<td>0.105 (0.051, 0.158)</td>
<td>0.074 (0.009, 0.138)</td>
</tr>
<tr>
<td>HAQ</td>
<td>0.078 (0.007, 0.148)</td>
<td>0.076 (0.002, 0.149)</td>
<td></td>
</tr>
<tr>
<td>RF positivity</td>
<td>0.211 (0.059, 0.363)</td>
<td>0.234 (0.076, 0.391)</td>
<td></td>
</tr>
<tr>
<td>Extra-articular disease</td>
<td>0.020 (-0.132, 0.172)</td>
<td>-0.010 (-0.165, 0.145)</td>
<td></td>
</tr>
<tr>
<td>IL-6†</td>
<td>0.110 (0.054, 0.166)</td>
<td>0.110 (0.059, 0.174)</td>
<td>0.087 (0.023, 0.151)</td>
</tr>
<tr>
<td>hsCRP†</td>
<td>0.123 (0.064, 1.181)</td>
<td>0.115 (0.054, 0.176)</td>
<td></td>
</tr>
</tbody>
</table>

*Subclinical cardiovascular disease*

| Carotid plaque                 | 0.169 (0.356, 0.302)             | 0.151 (0.006, 0.297)               |                                      |
| PWV (ms⁻¹)                     | 0.024 (-0.002, 0.049)            | 0.021 (-0.005, 0.048)              |                                      |

†Fully adjusted model included: age, systolic BP, smoking, DAS28, RF positivity, IL-6, hsCRP, carotid plaque, significance value set at 0.1

E-Selectin levels

Findings are summarised in table 5.2. E-selectin was independently associated with DAS28, RF positivity and IL-6. On univariate analysis there was also an association with HAQ, hsCRP and carotid plaque and all remained on age adjustment.
**Table 5-2 Factors associated with log transformed E-selectin levels on linear regression in patients**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Unadjusted β co-efficient [95% CI]</th>
<th>Age adjusted β co-efficient [95% CI]</th>
<th>Fully adjusted† β co-efficient [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.002 (-0.026, 0.030)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>-0.132 (-0.629, 0.367)</td>
<td>-0.117 (-0.631, 0.397)</td>
<td>-</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.496 (-0.164, 1.156)</td>
<td>0.632 (-0.040, 1.304)</td>
<td>-</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>-0.003 (-0.016, 0.010)</td>
<td>0.002 (-0.011, 0.015)</td>
<td>-</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>0.064 (-0.938, 0.222)</td>
<td>0.059 (-0.100, 0.212)</td>
<td>-</td>
</tr>
<tr>
<td>DAS28</td>
<td>0.356 (0.195, 0.515)</td>
<td>0.388 (0.226, 0.549)</td>
<td>0.247 (0.041, 0.453)</td>
</tr>
<tr>
<td>HAQ†</td>
<td>0.228 (0.024, 0.432)</td>
<td>0.263 (0.063, 0.463)</td>
<td>-</td>
</tr>
<tr>
<td>RF positivity</td>
<td>0.757 (0.286, 1.227)</td>
<td>0.800 (0.319, 1.281)</td>
<td>0.783 (0.201, 1.365)</td>
</tr>
<tr>
<td>Extra-articular disease</td>
<td>0.019 (-0.287, 0.674)</td>
<td>0.116 (-0.365, 0.597)</td>
<td>-</td>
</tr>
<tr>
<td>IL-6†</td>
<td>0.282 (0.104, 0.461)</td>
<td>0.295 (0.115, 0.476)</td>
<td>0.261 (0.048, 0.474)</td>
</tr>
<tr>
<td>hsCRP†</td>
<td>0.253 (0.065, 0.442)</td>
<td>0.523 (0.061, 0.444)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Subclinical cardiovascular disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid plaque</td>
<td>0.411 (-0.008, 0.831)</td>
<td>0.524 (0.079, 0.968)</td>
<td>-</td>
</tr>
<tr>
<td>PWV (ms⁻¹)</td>
<td>0.034 (-0.054, 0.122)</td>
<td>0.052 (-0.036, 0.141)</td>
<td>-</td>
</tr>
</tbody>
</table>

†Stepwise linear regression model, significance value set at 0.1. Variables included in the model: age, systolic blood pressure, DAS28, HAQ score, RF positivity, IL-6, hsCRP, carotid plaque

VCAM-1 levels

VCAM-1 levels were independently associated with IL-6 and PWV. Systolic blood pressure, DAS28, CRP and carotid plaque were all significantly associated with VCAM-1 on univariate analysis.

**Table 5-3 Factors associated with log transformed VCAM-1 on linear regression in patients.**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Unadjusted β co-efficient [95% CI]</th>
<th>Age adjusted β co-efficient [95% CI]</th>
<th>Fully adjusted† β co-efficient [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.006 (6x10⁻⁵, 0.012)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>0.070 (-0.03, 0.175)</td>
<td>0.029 (-0.077, 0.136)</td>
<td>-</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.025 (-0.105, 0.155)</td>
<td>0.012 (-0.121, 0.145)</td>
<td>-</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>0.003 (4x10⁻⁵, 0.005)</td>
<td>0.003 (8x10⁻⁵, 0.005)</td>
<td>-</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>-0.008 (-0.041, 0.026)</td>
<td>-0.006 (-0.039, 0.026)</td>
<td>-</td>
</tr>
<tr>
<td>DAS28</td>
<td>0.039 (0.003, 0.077)</td>
<td>0.040 (0.002, 0.077)</td>
<td>-</td>
</tr>
<tr>
<td>HAQ†</td>
<td>0.030 (-0.018, 0.079)</td>
<td>0.031 (-0.017, 0.078)</td>
<td>-</td>
</tr>
<tr>
<td>RF positivity</td>
<td>-0.005 (-0.111, 0.102)</td>
<td>0.024 (-0.084, 0.131)</td>
<td>-</td>
</tr>
<tr>
<td>Extra-articular disease</td>
<td>0.078 (-0.023, 0.179)</td>
<td>0.076 (-0.023, 0.175)</td>
<td>-</td>
</tr>
<tr>
<td>IL-6†</td>
<td>0.004 (1x10⁻³, 0.008)</td>
<td>0.004 (1x10⁻³, 0.009)</td>
<td>0.045 (0.004, 0.087)</td>
</tr>
<tr>
<td>hsCRP†</td>
<td>0.009 (0.003, 0.015)</td>
<td>0.009 (0.004, 0.014)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Subclinical cardiovascular disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid plaque</td>
<td>0.093 (0.004, 0.181)</td>
<td>0.070 (-0.022, 0.164)</td>
<td>-</td>
</tr>
<tr>
<td>PWV (ms⁻¹)</td>
<td>0.031 (0.014, 0.047)</td>
<td>0.030 (0.013, 0.047)</td>
<td>0.031 (0.014, 0.048)</td>
</tr>
</tbody>
</table>

†Stepwise linear regression model, significance value set at 0.1. Variables included in the model: age, systolic blood pressure, DAS28, IL-6, hsCRP, PWV

228
P-selectin was independently associated with hsCRP. On univariate analysis there was an association with hsCRP and DAS28.

Table 5-4. Factors associated with log transformed P-selectin on linear regression

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Unadjusted β coefficient [95% CI]</th>
<th>Age adjusted β coefficient [95% CI]</th>
<th>Fully adjustedβ coefficient [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.006 (-0.001, 0.014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (male)</td>
<td>0.046 (-0.097, 0.189)</td>
<td>0.047 (-0.098, 0.192)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>0.020 (-0.157, 0.196)</td>
<td>0.007 (-0.173, 0.187)</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>7x10^-4 (-0.003, 0.004)</td>
<td>7x10^-4 (-0.003, 0.004)</td>
<td></td>
</tr>
<tr>
<td>TC:HDLD</td>
<td>0.013 (-0.031, 0.058)</td>
<td>0.018 (-0.025, 0.061)</td>
<td></td>
</tr>
<tr>
<td>DAS28</td>
<td>0.069 (0.018, 0.120)</td>
<td>0.055 (0.002, 0.107)</td>
<td></td>
</tr>
<tr>
<td>HAQ†</td>
<td>-0.011 (-0.074, 0.050)</td>
<td>-0.007 (-0.071, 0.056)</td>
<td></td>
</tr>
<tr>
<td>RF positivity</td>
<td>-0.52 (-0.192, 0.087)</td>
<td>-0.012 (-0.153, 0.129)</td>
<td></td>
</tr>
<tr>
<td>Extra articular disease</td>
<td>-0.016 (-0.164, 0.121)</td>
<td>-0.036 (-0.172, 0.099)</td>
<td></td>
</tr>
<tr>
<td>IL-6†</td>
<td>0.057 (0.004, 0.109)</td>
<td>0.052 (5x10^-4, 0.105)</td>
<td></td>
</tr>
<tr>
<td>hsCRP†</td>
<td>0.063 (0.008, 0.117)</td>
<td>0.061 (0.007, 0.115)</td>
<td>0.089 (-0.030, 0.147)</td>
</tr>
<tr>
<td>Carotid plaque</td>
<td>7x10^-4 (-0.121, 0.123)</td>
<td>-0.040 (-0.169, 0.087)</td>
<td></td>
</tr>
<tr>
<td>PWV (ms^-1)</td>
<td>-0.007 (-0.029, 0.0154)</td>
<td>-0.007 (-0.031, 0.016)</td>
<td></td>
</tr>
</tbody>
</table>

†Stepwise linear regression model, significance value set at 0.1. Variables included in the model: age, systolic blood pressure, DAS28, IL-6, hsCRP
5.4.2 CD28null cell populations

CD28null cells were measured using flow cytometry in 91 patients and 25 controls. Baseline characteristics of those included in this subgroup analysis are summarised in table 5.5.

Table 5-5 Baseline characteristics of subjects in whom CD28null cell frequency was measured. Median (IQR) or frequency (%) where *

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>56.2 (49.4, 62.6)</td>
<td>56.7 (47.1, 60.3)</td>
<td>0.605</td>
</tr>
<tr>
<td>Gender (male)*</td>
<td>23 (25.3)</td>
<td>5 (21.7)</td>
<td>0.725</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>14 (15.4)</td>
<td>0</td>
<td>0.045</td>
</tr>
<tr>
<td>Smoking*</td>
<td>9 (9.9)</td>
<td>2 (8.0)</td>
<td>0.588</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>136 (124, 148)</td>
<td>126 (116, 134)</td>
<td>0.022</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81 (76, 89)</td>
<td>78 (73.4, 82)</td>
<td>0.012</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>3.14 (2.67, 4.08)</td>
<td>2.89 (2.34, 3.68)</td>
<td>0.239</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>15 (2, 10)</td>
<td>15 (8, 27)</td>
<td>0.003</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>2.96 (0.99, 6.19)</td>
<td>0.79 (0.30, 1.13)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.84 (0.25, 4.33)</td>
<td>0.25 (0.25, 1.56)</td>
<td>0.015</td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>1 (1, 5.1)</td>
<td>1 (1, 1)</td>
<td>0.062</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.59 (3.59, 5.49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAQ</td>
<td>1.187 (0.375, 2.125)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF positive</td>
<td>78 (85.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACPA positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extra-articular disease*</td>
<td>23 (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid plaque*</td>
<td>52 (57.1)</td>
<td>10 (40.0)</td>
<td>0.128</td>
</tr>
<tr>
<td>PWV (ms⁻³)</td>
<td>9.50 (7.50, 11.40)</td>
<td>8.40 (7.40, 11.50)</td>
<td>0.808</td>
</tr>
<tr>
<td>ktrans plaque (min⁻¹)</td>
<td>0.045 (0.030, 0.785)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUVmax plaque</td>
<td>2.18 (1.99, 2.65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUVmax vessel wall</td>
<td>2.23 (1.62, 2.56)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The median (IQR) percentage of CD4 cells which lacked CD28 expression was 0.9 (0.38, 3.38) % and 0.66 (0.36, 5.2) % in patients and controls respectively (p=0.822). Although there was no significant difference, there was a higher range in patients compared with controls (as shown on figure 5.16). The presence of an expanded CD28null cell population was defined as >2.5% based on previous literature (327). Expanded populations were found in 28 (30.8%) of patients and 10 (40%) of control participants (p=0.38). CD28null cells levels were divided into quartiles for further analysis. Levels within each quartile can be seen in figure 5.17. The median (max, min) was 0.23 (0.045, 0.362), 0.487 (0.381, 0.841), 1.69(0.902, 4.443) 7.829(4.737, 24.24) % in quartiles 1,2,3,4 respectively.
Figure 5-16 Levels of CD28null cells in patients and controls. Median (range) was 0.9 (0.045, 24.24) % in patients and 0.66 (0.149, 13.12)% in controls (p=0.822)

Figure 5-17 Quartiles of CD28null cells. Median (max, min) was 0.23 (0.045, 0.362)% , 0.487 (0.381, 0.841)% , 1.69(0.902, 4.443)% , 7.829(4.737, 24.24)% in quartiles 1,2,3,4 respectively.
Factors associated with CD28null cell frequency in patients

Ordinal logistic regression was used to evaluate independent associations with CD28null cell frequency. Univariate analysis was initially performed, followed by age adjusted regression. Finally, variables known to be associated with CD28null cells or found to be significant on univariate analysis were entered into a stepwise ordinal logistic regression model with significance value set < 0.1. The results are detailed in table 5.6. Gender and hypertension remained in the model but hypertension was the only significant variable, which was independent association with quartiles of CD28null cells.

Table 5-6 Ordinal logistic regression to test associations of CD28null cell frequency in patients

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Unadjusted OR [95%CI]</th>
<th>Age adjusted OR [95%CI]</th>
<th>Fully adjusted OR [95%CI]†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.018 (-2.938, 2.453)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gender (male)</td>
<td>-0.885 (-1.738, 0.032)</td>
<td>0.028 (-0.021, 0.078)</td>
<td>-0.124 (-2.598, 0.114)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.42 (0.315, 2.528)</td>
<td>1.345 (0.227, 2.464)</td>
<td>1.603 (0.058, 2.148)</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>-1.321 (-2/58, -0.062)</td>
<td>-0.024 (-0.368, 0.319)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>0.430 (-0.782, 1.643)</td>
<td>0.433 (-0.823, 1.689)</td>
<td></td>
</tr>
<tr>
<td>HAQ score</td>
<td>0.287 (-1.591, -0.021)</td>
<td>0.290 (-0.130, 0.711)</td>
<td></td>
</tr>
<tr>
<td>DAS28 score</td>
<td>0.088 (-0.199, 0.376)</td>
<td>0.066 (-0.224, 0.357)</td>
<td></td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>0.125 (-0.193, 0.444)</td>
<td>0.0599 (-0.267, 0.387)</td>
<td></td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>0.0981 (-0.896, 0.385)</td>
<td>0.098 (-0.198, 0.396)</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.302 (-0.017, 0.621)</td>
<td>0.279 (-0.0451, 0.603)</td>
<td></td>
</tr>
<tr>
<td>RF positive</td>
<td>0.935 (0.073, 1.798)</td>
<td>0.800 (-0.083, 1.684)</td>
<td></td>
</tr>
<tr>
<td>Extra-articular disease</td>
<td>-0.038 (-0.876, 0.799)</td>
<td>-0.184 (-1.047, 0.679)</td>
<td></td>
</tr>
</tbody>
</table>

†Full adjusted model using stepwise ordinal logistic regression. Variables entered into model: age, gender, smoking, hypertension, TC:HDL, IL-6, RF positivity. Significance value set at <0.1.

The association with measurements of subclinical cardiovascular disease was also evaluated using non-parametric statistics. There was a significant association between SUV<sup>max</sup> and quartiles of CD28null cells (p=0.045) but there was no association with plaque presence, Ktrans measurement or PWV (p=0.447, 0.672 and 0.274 respectively).
5.4.3 Endothelial microparticles

Endothelial microparticles (EMPs) were measured in 118 patients and 40 controls. Baseline characteristics are summarised in table 5.7. Findings were representative of the whole cohort.

Table 5-7 Cohort characteristics of subjects with EMP measurements. Median (IQR) or frequency (%) where *

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>55.5 (49.3, 61.9)</td>
<td>56.4 (46.7, 60.3)</td>
<td>0.819</td>
</tr>
<tr>
<td>Gender (male)*</td>
<td>29 (24.6)</td>
<td>8 (20.0)</td>
<td>0.555</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>4 (10.0)</td>
<td>20 (16.95)</td>
<td>0.290</td>
</tr>
<tr>
<td>Smoking*</td>
<td>16 (13.6)</td>
<td>2 (5)</td>
<td>0.092</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>136 (124, 148)</td>
<td>127 (117, 134)</td>
<td>0.003</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81 (76, 89)</td>
<td>78 (74, 85)</td>
<td>0.079</td>
</tr>
<tr>
<td>TC:HDL</td>
<td>3.27(2.68, 4.23)</td>
<td>2.96 (2.45, 3.52)</td>
<td>0.069</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>14.5 (7, 27)</td>
<td>5 (2, 10.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>2.54 (0.99, 6.18)</td>
<td>0.815 (0.28, 1.74)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>2.15 (0.34, 4.69)</td>
<td>0.57 (0.25, 1.73)</td>
<td>0.007</td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>1 (1, 45.3)</td>
<td>1 (1, 112.8)</td>
<td>0.305</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.6 (3.76, 5.47)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HAQ</td>
<td>1.38 (0.5, 2.13)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Seropositivity*</td>
<td>103 (87.29)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Extra-articular disease*</td>
<td>32 (27.12)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Markers of subclinical cardiovascular disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid plaque*</td>
<td>66 (55.9)</td>
<td>12 (30.0)</td>
<td>0.005</td>
</tr>
<tr>
<td>PWV (ms⁻²)</td>
<td>9.7 (7.5, 11.8)</td>
<td>8.45 (7.5, 10.9)</td>
<td>0.411</td>
</tr>
<tr>
<td>Non-invasive imaging markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ktrans plaque (min⁻¹)</td>
<td>0.045 (0.030, 0.785)</td>
<td>0.082 (0.057, 0.104)</td>
<td>0.194</td>
</tr>
<tr>
<td>SUV^{max} plaque</td>
<td>2.18 (1.99, 2.65)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUV^{max} vessel wall</td>
<td>2.23 (1.62, 2.56)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

There was a trend towards higher levels in patients compared to controls however this did not reach statistical significance (27246.06[9180.63, 79223.28] events/ml vs 12468.81[5134.66, 47352.94] events/ml, p=0.064).
The association of EMPs with traditional risk factors, RA related factors and measured of subclinical cardiovascular disease was evaluated. Non-parametric tests were used and results are summarised in table 5.8. There was no association between EMP levels and any of the factors evaluated.
Table 5-8 Associations of clinical and serological characteristics with EMP levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients Spearman r*</th>
<th>p</th>
<th>Controls Spearman r*</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>-0.006</td>
<td>0.542</td>
<td>-0.247</td>
<td>0.115</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>-</td>
<td>0.687</td>
<td>-</td>
<td>0.478</td>
</tr>
<tr>
<td>Hypertension</td>
<td>-0.134</td>
<td>0.177</td>
<td>0.069</td>
<td>0.684</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>0.111</td>
<td>0.244</td>
<td>-0.229</td>
<td>0.451</td>
</tr>
<tr>
<td>TC:HDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HsCRP</td>
<td>-</td>
<td>0.658</td>
<td>-</td>
<td>0.833</td>
</tr>
<tr>
<td>IL-6</td>
<td>-0.618</td>
<td>0.191</td>
<td>-0.229</td>
<td>0.451</td>
</tr>
<tr>
<td>TNF</td>
<td>-0.624</td>
<td>0.451</td>
<td>-</td>
<td>0.843</td>
</tr>
<tr>
<td>DAS28</td>
<td>-0.058</td>
<td>0.582</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HAQ</td>
<td>-</td>
<td>0.965</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RF positive</td>
<td>-0.774</td>
<td>0.244</td>
<td>-</td>
<td>0.451</td>
</tr>
<tr>
<td>ACPA positive</td>
<td>0.310</td>
<td>0.015</td>
<td>-</td>
<td>0.929</td>
</tr>
<tr>
<td>Extra-articular disease</td>
<td>-0.585</td>
<td>0.819</td>
<td>-0.015</td>
<td>0.349</td>
</tr>
<tr>
<td>Subclinical cardiovascular disease measures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PWV (ms⁻¹)</td>
<td>0.023</td>
<td>0.761</td>
<td>-0.09</td>
<td>-</td>
</tr>
<tr>
<td>Carotid plaque</td>
<td></td>
<td>0.136</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUVmax carotid plaque</td>
<td>-0.10</td>
<td>0.770</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUVmax vessel wall</td>
<td>-0.09</td>
<td>0.761</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ktrans plaque</td>
<td>-0.01</td>
<td>0.959</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Spearman rank correlation used or where variables were categorical Mann Whitney U test or Kruskall Wallis tests were used.
5.5 Discussion

Key findings in this chapter included:

- Higher levels of some CAMs were found in patients compared to controls and were associated with subclinical markers of cardiovascular disease and clinical and serological measures of inflammation.

- No difference in levels of circulating CD28null cell was found between patients and controls. However, CD28 null cells levels were associated with hypertension in patients.

- There was a trend towards higher EMP levels in patients compared with controls.

CAMs

CAMs act as markers of endothelial activation and play an important role in both synovitis and atherosclerosis. The function of the selectins is to facilitate leucocyte rolling along the endothelium while the role of VCAM-1 and E-CAM is to facilitate adhesion of leucocytes to the endothelium. Increased CAM levels are associated with endothelial activation in the capillaries of inflamed synovium and the medium and large vessels (125;142). The soluble forms are shed from activated endothelium and act as a marker of cell surface expression. CAMs are known to have other intra-cellular signalling properties however it is not clear whether the soluble form of these molecules are biologically active within the circulation (329). In the general population CAMs are known to independently predict cardiovascular events(125).

In the current study, significantly increased levels of I-CAM and E-selectin were found in patients compared to controls. Levels of I-CAM were independently associated with disease activity, smoking and IL-6 levels, whilst e-selectin levels were independently associated with DAS28, IL-6 and RF positivity. Although VCAM-1 and p-selectin were not significantly higher in patients, VCAM-1 was independently associated with IL-6 and p-selectin was independently associated with hsCRP. These findings demonstrate an important link between CAM levels and the inflammatory pathways involved in RA.
We also examined the association of CAM levels with subclinical markers of cardiovascular disease. We found that I-CAM and e-selectin levels were significantly higher in patients with carotid plaque. These findings are consistent with other studies, which have demonstrated an association with CAMs and sub-clinical atherosclerosis in RA (262). We also found a significant association between VCAM-1 and aortic PWV, which remained independent even after full adjustment. These findings suggest that endothelial dysfunction and inflammation are associated with atherogenesis in RA. The relationship between CAMs and plaque inflammation, as measured on MRI and PET was explored in a subgroup of patients. A strong correlation between Ktrans of plaque and e-selectin was found in addition to a moderate (but non-significant) correlation with p-selectin. Also, there was also a trend towards a moderate correlation between SUVmax in plaque on PET and VCAM-1. Although the sample size was small and no corrections were made for multiple testing, these findings suggest that CAMs may be implicated in plaque inflammation and these associations have not been reported in the published literature before.

To what degree CAM levels are due to macrovascular endothelial activation rather than synovial inflammation is unclear. In addition, it is not known whether CAMs generated in the synovium can have distant effects on the macrovasculature. However the associations with both clinical and serological measures of inflammation and also plaque presence and inflammation found in this study, suggest that these molecules may play an important role in atherogenesis in RA.

**CD28null cells**

The CD28null cell population is known to be expanded in a subgroup of patients with RA and is associated with more severe features of disease such as extra-articular disease and joint replacement (160). One study has also shown an association with carotid IMT on ultrasound in RA patients (32). In patients with acute coronary syndrome, they are an independent predictor of recurrent coronary events and have been implicated in plaque destabilisation (330). In the current study it was hypothesised that this cell population would be expanded in RA patients compared to controls and the aim was to explore the association of these cells with clinical phenotype and carotid plaque in RA patients. The frequency of these cells did not differ between the groups although the range was higher in RA patients.
CD28 null cells were defined according to previous literature which suggested a cut off 2.5% of all CD4 positive T cells (327). In the current study, the CD28null cell population was expanded in 30.8% of patients which is consistent with findings in other studies (155). However, unexpectedly there was also an expansion in a significant proportion of controls (40%). Samples were collected consecutively and on many occasions, patients and controls samples were analysed at the same time. The offline quantification was done on consecutive samples with no information on the case/control status. It is therefore unlikely that a systematic error in collection and quantification affected the control group disproportionately. An increased frequency of these cells in known to occur in end stage renal disease and coronary artery disease however subjects with significant renal impairment were excluded from the study and there were similar rate of cardiovascular disease in both groups (n=1 in controls and n=2 in patients). The samples were only available in 25 patients and higher than expected prevalence may partly be due to the small sample size.

In patients, CD28null cells were not associated with the presence of plaque however were associated with plaque inflammation as measured on FDG-PET. CD28null cells are known to be increased in patients with unstable angina compared to those with stable ischaemic heart disease and have been shown to be preferentially found in unstable atherosclerotic plaques compared to stable lesions (328). It is hypothesised that CD28null cells are a marker of plaque instability and may lead to degradation of the fibrous cap mediated through increased IF-γ production (327). The findings in the current study could suggest that CD28null cells are associated specifically with an unstable inflammatory plaque phenotype in RA rather than all plaque although findings should be interpreted with caution as no correction was made for multiple testing. However the number of patients with FDG-PET-MRI results was small (n=13). CD28null cells were also shown to be associated with age, hypertension and RF positivity. Due to the small number of patients with FDG-PET results, adjustments for these factors could not be made.

Ordinal logistic regression was performed to assess for independent associations with CD28null cell frequency. Although RF positivity was associated on univariate analysis, on stepwise ordinal regression only hypertension and gender remained in the model, with hypertension alone remaining significant. The previously described paper by Gerli et al. which examined the associations of CD28 null cells, found no association with either hypertension or gender in a cohort of RA patients (32). Few other studies have evaluated
the associations of CD28null cells with traditional risk factors in RA and this warrants further investigation.
EMPs

EMPs are known to be associated with unstable cardiac disease in the general population and have also been found to be increased in other auto-immune conditions (148;148;154). There are no published data on levels of EMPs in RA. In the current study there was a trend towards higher levels of EMPs in patients, which did not reach statistical significance (p=0.064). Moreover, there was no association with markers of inflammation or with subclinical markers of cardiovascular disease. Parker et al. found significantly higher levels of EMPs in patients with active SLE compared to controls (157548 [59906, 272643]/ml vs 41024 [30179, 98082] ml, p=0.003) (154). There was also a significant correlation with endothelial dysfunction, measured as flow mediated dilatation and also with improvement in disease activity over time (p=0.006 and p=0.02 respectively). From this and other studies, EMPs may play an important role in cardiovascular risk in auto-immune disease but findings in this study do not support their role as a biomarker of cardiovascular risk in RA.

Summary

In summary we have found serological markers, which were significantly associated with both clinical and serological measures of inflammation in addition to subclinical cardiovascular disease. The associations of CAM levels and CD28null cells with plaque inflammation, as measured on PET and MRI also suggest that they may be implicated in plaque inflammation and stability. The lack of longitudinal comparison of serological measurements in this study makes it impossible to fully assess the potential of these measurements as biomarkers of cardiovascular risk in RA. Additionally caution should be noted, as there was no correction for multiple testing. However, the findings have supported their importance as mediators of accelerated atherosclerosis in RA and further investigation is warranted.
6 Discussion

The primary aim of this PhD project was to test the hypothesis that patients with RA have a more unstable, inflammatory carotid plaque phenotype compared to the general population and that plaque inflammation can be altered by treating active joint disease.

The secondary aims of the study were:

- To test the feasibility of employing CMRI and FDG-PET imaging in a cohort of RA patients with active arthritis
- To identify clinical characteristics and serological biomarkers that correlated with plaque presence, inflammation and morphology.

6.1 Testing of the primary hypothesis

In order to test the primary hypothesis a pilot study of RA patients and age and sex matched controls was conducted with the aim of comparing plaque inflammation and morphology on MRI. The primary outcome measure for the study was the Ktrans value measured in carotid plaque. Ktrans is a calculation of the transfer constant of contrast in the plaque over time. It is a composite marker of both microvascular density and vascular permeability and has been shown to correlate well with these 2 parameters on plaque histology (238). However during the course of the study further information on the reliability of Ktrans measurement was published (245). It became apparent that a significantly larger sample size was required to adequately test the primary hypothesis, than was possible to include in the current study. Although there was no difference in the primary outcome measure between patients and controls, significant information was obtained about plaque morphology on MRI and plaque inflammation using FDG-PET.

On MRI, there was evidence of significantly higher prevalence of plaque calcification compared to controls with equivalent sized lesions. Calcification is a feature of advanced complex plaque and presence in the carotid artery is associated with increased risk of subsequent stroke and also more severe coronary artery disease (189). Although numbers in the control group were small, the prevalence of calcification was similar to other
published studies in the general population (240). This finding would support the hypothesis that RA patients have a more advanced/unstable plaque phenotype compared with controls. In support of this, all patients had at least one feature of high risk plaque, as defined by Virmani, et.al. (123). Additionally compared to the literature, there was a higher prevalence of LRNC and thin fibrous cap, pointing towards a more unstable phenotype.

PET was performed in patients only at baseline. Significant plaque inflammation was demonstrated in all 13 subjects on PET. Although no control group was available for PET imaging, the frequency of plaque inflammation in asymptomatic subjects is estimated at 30% using FDG-PET, in the published literature. The fact that all 13 patients had significant plaque inflammation would support the primary hypothesis that patients have a more inflammatory plaque phenotype and could indicate that patients have simultaneous joint and plaque inflammation. However, the relationship with plaque and joint inflammation would need to be further investigated in a larger longitudinal study in order to prove this hypothesis.

Although this was a small pilot study, the findings are consistent with the clinical phenotype of cardiovascular disease. In particular, the clinical picture of less warning symptoms and more sudden severe cardiovascular events in RA.

It was also hypothesised that treatment of active joint disease would lead to alteration in plaque inflammation as measured on MRI. Unfortunately, in the current study testing of this hypothesis was hampered both by the lack of clinical improvement in the majority of cases. It was to be expected that in some cases active joint disease may be resistant to treatment but the fact that a significant improvements was only see in 3 out of 10 cases was surprising. This made it impossible to evaluate the relationship between clinical disease activity and plaque inflammation. Additionally only 3 participants had a significant alteration in therapy which meant that the evaluation of the effects of anti-inflammatory therapy on plaque inflammation (independent of clinical response) could not be evaluated. Although observational studies provide a more clinically relevant setting in which to evaluate this question, this study suggests that a more controlled study such as an interventional trial may be a better method of testing the hypothesis.
6.2 Secondary aims

6.2.1 Feasibility of CMRI and FDG-PET-CT in patients with RA

One of the secondary aims of this study was to test the feasibility of CMRI and FDG-PET imaging to evaluate atherosclerosis in RA. Neither technique has been employed to characterise carotid plaque in an RA population. Although the majority of cardiovascular events that occur in RA are in the coronary circulation, there is also a significantly increased risk of stroke (76). The size and location of the carotid artery allows more detailed evaluation of plaque composition and inflammation, than is currently possible in the coronary arteries, using non-invasive imaging methods. Additionally, the presence of carotid atherosclerosis and plaque phenotype are predictors of cardiovascular events in both the cerebral and coronary territories (186;198). Therefore the ability to evaluate carotid plaque phenotype in vivo would serve to further our understanding of the pathogenesis of atherosclerosis in RA.

The first aspect was the suitability of using these scans within the specific population studied. Both the MRI and FDG-PET-CT scans required the patients to be still for prolonged periods which may not have been possible to due to active joint disease and in particular cervical spine involvement. Other aspects, which may have been a barrier, were patient concerns regarding claustrophobia and also the radiation dosage associated with FDG-PET. Although the revised targets for recruitment were not reached, there were no significant difficulties in recruiting patients to the study. Time and clinical capacity for screening were the rate limiting factors for recruitment during the PhD. On the whole, patients were happy to proceed with MRI and PET scanning, when plaque was found and in most cases scans were completed successfully. This suggests that patients found CMRI and FDG-PET-CT acceptable techniques to employ in clinical studies of RA.

Good imaging quality was achieved using both scanning techniques and registration of images was possible in all cases. However, the discrepancy between ultrasound and MRI findings led to significant problems in the use of MRI. With the benefit of the results in the current study and the recently published literature, it appears that the utility of DCE is limited to larger lesions rather than those included in the current study (245;311). DCE-MRI provides valuable and reliable information about plaque neovascularisation and inflammation in patients with symptomatic carotid lesions but the relationship with
measurements in smaller plaques is less well validated (238). Additionally the high prevalence of calcium found in the RA group may also potentially confound Ktrans measurement in this particular disease cohort. It is likely that further validation work and optimisation of the technique may improve its validity for use in smaller lesions. New techniques are emerging where DCE MRI can be performed on vessel wall less than 2mm thick using blood suppression (331). This “black blood” DCE technique has not been widely validated but may provide a method of evaluating smaller lesions more reliably using DCE-MRI.

The use of MRI to measure plaque burden and compositional features including calcification and lipid core is well validated (184). This technique provided valuable information on differences in plaque morphology in this small study and could be very useful in larger, longer term studies of plaque morphology in RA. There was evidence of changes in plaque volume and LRNC, even in the small group of 10 patients whom were followed up. Although there was no obvious association found between cardiovascular risk factors or disease related factors and these changes, it highlights the opportunity for studying evolution of lesions in small numbers over short time periods. This could provide the opportunity to study the natural history of atherosclerotic lesions in RA, in a depth of detail, not possible with other non-invasive techniques.

On the basis of the current study, FDG-PET-MRI also appears to be a promising technique with which to quantify plaque inflammation in RA. Although the numbers were small, good quality images and measurements of plaque inflammation were obtained in all subjects. Of note the proximity of the cervical lymph nodes to the jugular vein posed a particular problem for measurement of TBR in RA patients. TBR had been used increasingly when examining plaque inflammation in both the carotid artery and aorta (243) and the relationship of TBR with incident cardiovascular events is more validated than the relationship with SUVmax (254). In previous studies the vena cava has been used as the background reference point. In the current study, a small field of view was chosen in order to minimise radiation dosage and scanning time thus the vena cava was not available for measurement. The jugular vein may provide a valid reference for background uptake in other populations. However, findings in the current study suggest that in RA patients, metabolic activity in the lymph nodes can lead to falsely high background uptake within the
jugular vein. In future studies in RA the decision to include a larger field of view on PET would have be need taken in order to reliably calculate a TBR in this population.

6.2.2 Clinical and serological biomarkers of increased cardiovascular risk

Another secondary aim was to investigate clinical and serological factors associated with carotid plaque presence, inflammation and morphology. In the study of 130 patients and 52 age and sex matched controls we found significant differences in traditional cardiovascular risk factors at baseline between the two groups. As had been found in previous studies, hypertension was more prevalent in the RA group and higher systolic blood pressure was also noted in patients. Additionally significant differences were found in lipid levels between patients and controls. Patients had lower levels of total cholesterol and LDL and a trend towards lower levels of HDL compared to controls. However these differences should be interpreted with caution, in the context of the exclusion of those on statin therapy. Although screening is recommended in both groups, RA patients are more likely to have been screened due to high frequency of clinical encounters. Additionally if the recommended multiplication factor was used, patients were more likely to start statins than controls with equivalent lipid levels. Additionally active inflammation is known to lead to a reduction in the levels of total and LDL cholesterol, which was also noted in this study.

Patients were significantly more likely to have carotid plaque compared with age and sex matched controls, consistent with previously published studies. While age, blood pressure and smoking were all associated with plaque presence, no association was found with lipid levels. On multivariable analysis smoking was the key traditional risk factor independently associated with carotid plaque. The emphasises the important but lesser role that traditional risk factors play in cardiovascular disease in RA compared with the general population. These findings are in keeping with the study by Gonzalez Gay et al. which demonstrated that the relative contribution of traditional risk factors to cardiovascular events was less in RA than controls (111). None the less, the strong influence of smoking on cardiovascular risk in RA should be emphasised when cardiovascular risk is being discussed with patients. Smoking cessation is likely not only to improve cardiovascular risk but may also have additional positive effects on treatment response in RA (297).

Significant associations of disease related factors with presence of carotid plaque were demonstrated in this study. Clinical disease activity (DAS28), disability (HAQ) and levels of
IL-6, hsCRP and ESR were all associated with carotid plaque. All these are direct markers of, or heavily influenced by inflammation. These findings highlight the importance of inflammation as a driver for atherosclerosis in RA. HsCRP was an independently associated with carotid plaque with a 2.4 fold increase of every increase in quartile of hsCRP. HsCRP is an independent predictor of cardiovascular events in the general population and it appears in this study to be strongly associated with subclinical cardiovascular disease in RA (124). Interestingly, hsCRP was also associated with plaque inflammation on FDG-PET-MRI. This further supports the link between active RA and atherosclerotic plaque inflammation. The DAS28 score was also strongly and independently associated with arterial stiffness, another measure of subclinical cardiovascular disease. The only other independent association was age. This again demonstrates the important link between active joint disease and vascular dysfunction in RA.

Findings in this study and the published literature highlight the prominent role inflammation plays in cardiovascular risk in RA. However, there are no current guidelines addressing the direct link between inflammation and cardiovascular risk in RA. Although European guidelines advise a multiplication factor in the presence of certain disease characteristics, there is no specific advice regarding the targeting of inflammation to lower cardiovascular risk. There is evidence that treatments, such as, methotrexate are associated with cardiovascular risk reduction but questions remain about the amount of inflammation suppression required to achieve adequate risk reduction and whether any agents such as TNF blockers have any specific benefits over and above suppression of inflammation. In the current study, we were unable to evaluate the effects of suppressing inflammation on plaque inflammation. However the fact that several clinical and serological markers of active disease were linked with carotid plaque presence and phenotype and arterial stiffness emphasises the importance of addressing this question.

The role of potential novel biomarkers of cardiovascular risk in RA was also evaluated. Populations of CD28null cells have been found to be expanded in RA patients, compared with controls in other studies. In the general population, the presence of these cells has been associated with recurrent cardiovascular events. In the current study, these cells were not associated with the presence of carotid plaque but were significantly associated with plaque inflammation. This would complement histology studies, which suggested that CD28null cells were preferentially expanded in unstable plaque. These cells have been also been implicated in plaque destabilisation. In the context of the published literature, our
findings suggests that these cells could act as a marker of high risk plaque in RA but further studies would be required to confirm this.

The role of EMPs as a potential biomarker of cardiovascular risk was also explored. To our knowledge, this is the first study examining EMP levels in RA. In other inflammatory diseases, they have been associated with markers of endothelial dysfunction (154). Although there was a trend towards higher levels of EMPs in patients compared with controls, there was no association with subclinical cardiovascular disease or with clinical and serological disease activity markers. Although this is a small study, the findings do not support a role for EMPs in cardiovascular risk prediction in RA.

Finally soluble CAMs, are known to predict cardiovascular events in the general population. Increased levels of CAMs were found in RA patients compared with controls. Although CAM levels were associated with carotid plaque in the patient group, this was not independent of disease activity. However VCAM-1 was independently associated with aortic PWV and in the subgroup of patients with MRI and PET data, significant correlations were found between CAMs and imaging measures of plaque inflammation. This supports their role in atherogenesis in RA. CAMs are up-regulated in synovial capillary endothelium of RA patients, in addition to the endothelium of large and medium vessels. The findings in the current study suggest that the influence of joint disease on CAMs levels may limit the predictive value of CAM levels in cardiovascular risk estimation in RA but further investigation of their role atherosclerosis in RA is warranted.
6.3 Limitations of the study

There are several limitations of this study, which should be considered when interpreting the results. The first is that the exclusion criteria limit the generalizability of the results to the whole RA population. The exclusion of those on statin therapy and also those with significant renal impairment are likely to have led to a cohort with lower cardiovascular risk than is generally found in the RA population. Although the same exclusion criteria were applied to controls, it is possible that the influence of traditional risk factors such as diabetes, on presence and phenotype of plaque in RA was under-estimated. Statins are known to influence plaque inflammation and morphology on MRI and FDG-PET. Had participants on statins been included, it would have made it more difficult to characterise plaque phenotype and inflammation in RA. Despite this being a cohort biased towards lower cardiovascular risk, more than 50% of patients still had evidence of carotid plaque on ultrasound.

Another limitation study was the heterogeneity of RA therapies used by patients in the study. DMARD therapies are known to have varying effects on atherosclerosis and patients were on a broad range of regimes; from no DMARD therapy to combination therapy with biologic drugs. No conclusions on the effect of medication used, could be drawn due to the heterogeneity. In addition, steroid use is known to be associated with increased cardiovascular mortality. Cumulative steroid use was patient-reported in the study and not verified with medical records. The variability and lack of accurate data on steroid use made it difficult to account for this in analysis.

There were also limitations in the control group, in particular the low prevalence of traditional risk factors and carotid plaque. The exclusion of those on statin therapy led to a very low risk control group and may not be representative of risk factors in the general population. The lack of PET data in the control group was also a significant limitation of the study.

A further limitation was the sample size. With the information in the recently published literature, it is clear that the study was underpowered to detect a difference in the primary outcome measure (Ktrans) (311;312). This was a pilot study and information became available only after study set up. Recruitment was also lower in the control group. This was
partly due to the fact that patients needed to be recruited earlier in the study to allow for time for follow up. However this also had a significant impact when comparing both imaging and serological biomarkers. Additionally, no corrections were made for multiple comparisons, which may be particularly important when interpreting the correlation of clinical and serological measurements with imaging biomarkers in the study. Some interesting, biologically plausible and potentially important associations were found however no firm conclusions can be drawn on the basis of the results. Further work would be required to further investigate findings from this study.

As previously mentioned FDG-PET-CT was not performed in the control group, as it was felt that the radiation dose may not be unjustified, particularly in the setting of a pilot study. Findings on FDG-PET have therefore been compared to the published literature. This causes many difficulties, as there is no standard imaging acquisition or analysis protocol for carotid artery PET. While the findings do suggest a high prevalence of plaque inflammation in RA, further studies with a control group are required. Adjusting for plaque calcification on DCE-MRI may also need to be factored in to future studies.

Finally, a major limitation in the study was the lack of clinical improvement in disease activity in patients undergoing follow up. While this is an interesting finding and highlights the difficulties of measuring change in observational studies, it meant that part of the primary hypothesis and the association of serological biomarkers over time could not be evaluated. This was compounded but the fact that Ktrans was the only measurement expected to change within the follow up period and it was unlikely that a sample size of 10 was large enough to detect a change, even if there had been improvement in disease activity.
7 Final conclusions and future work

Despite the limitations described above, the current study confirms that RA patients have a higher prevalence of subclinical cardiovascular disease than age and sex matched controls and also provides preliminary evidence to suggest that patients have a more advanced and inflammatory unstable plaque phenotype. The importance of disease related factors and inflammation in cardiovascular risk in RA has also been highlighted and although the effect of anti-inflammatory therapy was not evaluated, the case was made for further investigation of this in the future.

As part of this study, two novel imaging modalities have been established at the University of Manchester and proof of concept was demonstrated for their use to investigate atherosclerosis in RA. CMRI provided valuable information on plaque morphology and could be employed in larger prospective studies. It is clear DCE-MRI is most valid in large carotid plaque and that a larger sample size would be required to detect significant differences on carotid DCE MRI, using the current analysis protocol. However, it may be that optimisation of kinetic modelling or a more simple analysis may improve its applicability to smaller lesions or reduce the requirement for such a large sample size. The MRI data from the current study can provide a dataset in which to investigate this further. A pump -priming grant has been awarded to the candidate, to develop a method of measuring vessel wall inflammation on MRI, based on the change in signal intensity after contrast injection. This would allow evaluation of vessel wall inflammation irrespective of the degree of vessel wall thickening. Secondly, further work is planned to improve the DCE analysis methodology, in conjunction with MRI physicists within the University of Manchester, who have significant experience in DCE MRI in the context of other tissues.

FDG-PET-MRI of the carotid arteries appears to be an effective method with which to measure plaque inflammation in RA and was well tolerated by patients. Consideration to the cumulative radiation dosage would have to be given when planning longitudinal studies. However using the current protocol, radiation dose was significantly lower than is used when performing a CT of the thorax. Funding for a new combined PET-MRI scanner has recently been secured by The University of Manchester. This provides an excellent opportunity to develop combined imaging methods to comprehensively evaluate atherosclerotic plaque morphology and function in one imaging session. Plans to take the
imaging of atherosclerosis using combined PET/MRI forward are in the early stages of development but will form part of the candidate’s forthcoming National Institute of Health Research Clinical Lectureship.

Currently it is known that patients are 50% more likely to die from cardiovascular disease compared to the general population and that despite new treatment strategies, this mortality gap persists. Current methods for estimating cardiovascular risk in RA perform poorly and there is no reliable method to identify high risk patients early in the disease course. Additionally, an effective strategy to specifically target cardiovascular risk in RA remains elusive. The benefit of drugs targeting traditional risk factors in the RA population is unknown and targeting of inflammation to reduce cardiovascular risk is not established.

This study has provided a proof of concept for using MRI and PET imaging in the investigation of cardiovascular risk in RA. An integrated approach of using these techniques in conjunction with detailed clinical phenotyping and serological biomarkers may provide an effective method for improving stratification and treatment of cardiovascular risk in RA.
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8 Appendices

Appendix 1 - NRES Approval letter

NHS
Health Research Authority
NRES Committee North West - Lancaster
Burton House
3rd Floor
4 Minshull Street
Manchester
M1 3JZ

16 February 2012

Dr Sarah Skeoch
ARUK Epidemiology Unit
Stretford Building, The University of Manchester
Oxford Road, Manchester
M13 9PT

Dear Dr Skeoch

Study title: Investigation of atherosclerosis and the effects of anti-inflammatory therapy on plaque morphology in patients with rheumatoid arthritis

REC reference: 12/NW/0117

The Research Ethics Committee reviewed the above application at the meeting held on 09 February 2012. Thank you for attending to discuss the study.

Ethical opinion

The Chair welcomed you to the REC and thanked you for attending to discuss the study. The Committee told you they had found this a very interesting, good, and hopefully worthwhile study.

The Committee asked for clarification of the recruitment process and you said they will not be new patients – you are looking for patients who have been established as having the disease for a year and whose symptoms are not very severe. They will be regular attendees at the clinic for whom their rheumatoid treatment could be improved. They will be given a Participant Information Sheet and reply slip and you will telephone them after a couple of weeks. You will have their telephone number from the records.

You confirmed that if lipids are raised the participants will go on to statins and can still be entered into the study, as they if they have carotid plaques. You expect about a third will need statins but you are excluding those who are already being treated with statins so there will be enough participants to detect a significant difference from RA treatment alone.

The Committee asked whether you have a statistician on the team and you confirmed that you have. The statistician has reviewed the sample size, although this study is a pilot.

The Committee asked for some minor changes to the Participant Information Sheet as below. The Committee was pleased to note that a research user group has been involved in the design of the Participant Information Sheet.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

A Research Ethics Committee established by the Health Research Authority.
Appendix 2- ARSAC Approval

ARSAC Support Unit

Dr Mary Cordelia PRESCOTT
Nuclear Medicine Centre
Central Manchester University Hospitals
Oxford Road
Manchester
M 5 9WJ

Dear Dr PRESCOTT

Administration of Radioactive Medicinal Products to Persons

The Medicines (Administration of Radioactive Substances) Regulations 1978, Amended By
The Medicines (Administration Of Radioactive Substances) Regulations 1995
Certificate for the Administration of Radioactive Medicinal Products

I am writing on behalf of the Secretary of State to enclose certificate number RPC 60/2000/28723, which authorises you to administer the radioactive medicinal products listed in the Schedule to the certificate for the purposes there specified. The normal place of administration of the radioactive medicinal products and the usual level of administered activity for the purpose of the certificate are understood to be those set out in your certificate application which forms the basis of this authorisation.

Every clinical research investigation involving the use of radioactive medicinal products should be checked and approved by a local research ethics committee. In all instances ultimate approval for the projects as a whole will lie with the ethics committee which should ensure that the applicant holds the necessary authorisation and takes note of any comments made by the ARSAC.

This certificate is valid until the date shown on the certificate or until the research project is completed if before this date. If you wish to seek variation or extension, you should do so by making an application in good time to the above address. A list of current research projects is also enclosed.

Further information is available in the “Notes for Guidance on the Clinical Administration of Radiopharmaceuticals and the Use of Sealed Radioactive Sources” copies of which are available from this office and the website www.ARSAC.org.uk.

If you should have any queries, then please do not hesitate to contact me at the above address, quoting the above RPC number on all correspondence.

Yours sincerely

ARSAC Support Unit

www.ARSAC.org.uk
Appendix 3- Consent form

A study of inflammation and atherosclerotic plaque morphology in Rheumatoid Arthritis using Carotid Magnetic Resonance Imaging

PATIENT CONSENT

Name of researcher: Dr Sarah Skeoch, Tel: 0161 275 1614

1. I have read and understand the information sheet on this project dated 16/04/2013 and have been given a copy to keep. I have been able to ask questions about the project and I understand why the research is being done and any risks involved.

2. I agree to give a sample of blood for use in this research. I understand how the sample will be collected, that giving a sample for this is voluntary and that I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected.

3. I understand that sections of my medical notes and data collected during the study may be looked at by responsible individuals from The University of Manchester or Salford Royal Foundation Trust or from regulatory authorities where it is relevant to my taking part in research. I give my permission for these individuals to have access to my records.

4. I understand that my GP will be informed of my participation in this study.

5. I understand that I will be informed if any of the results of the medical tests done as part of the research are important for my health, and with my permission, my GP and/or consultant will also be informed.

6. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test.

7. I know how to contact the research team if I need to, and how to get information about the results of the research.

8. I understand that my blood sample is being gifted to medical research.

9. I agree to take part in this study.

_________________________  ____________________________  ____________________________
Name of patient  Date  Signature

_________________________  ____________________________  ____________________________
Name of person taking consent  Date  Signature
(if different from researcher)

_________________________  ____________________________  ____________________________
Researcher  Date  Signature

Plaque Morphology in RA, Consent  Version 3.0, 16/04/2013
Page 1 of 1
Appendix 4- Optimisation of imaging techniques

Ultrasound training and validation

3 operators performed scans during the study. GB was a vascular physiologist, experienced in carotid ultrasound. SS, the candidate and Mrs Sujamole Subin (SSu), the advanced practitioner were trained by GB to perform carotid artery ultrasound over a 4 month period. After a period of observation and training, 23 cases (46 arteries) were scanned by all 3 operators. Overall there was 95.6% agreement between the 3 operators. In one case SS recorded plaque that was not identified by GB or SSu and in a second case SSu failed to identify plaque, which has been recorded by GB and SSu. GB left the trust 6 months into recruitment, so the remainder of scans were performed by SS and SSu.

MRI imaging optimisation

5 carotid MRI scans were performed in healthy volunteers prior to the start of the patient study. One scan included contrast injection. All scans were reviewed by previously validated readers at the Vascular Imaging Laboratory, University of Washington (UW). All five scans were deemed of sufficient quality. At this point all parties were satisfied with the imaging acquisition set up and the decision to proceed to the patient study was made.

MRI quality assurance training

The candidate undertook a period of training in quality assurance at the Vascular Imaging Laboratory, UW. Following tutorials, the candidate evaluated 65 CMRI datasets which had previously been graded by an experienced reader. The aim of each assessment was to check:

- Images were of sufficient quality for analysis
- Plaque was present and coverage was adequate
- Identify an index artery for analysis
The candidate’s evaluation was then compared to the existing QA check. There was a 98.5% agreement on imaging quality and presence and coverage of plaque.
Appendix 5- Imaging quality check

MRI Image Quality Check

MRI Study Details:
Subject ID:
Date of MRI:
MRI exam #:

Image Quality Review:

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1. Is image quality sufficient?
2. Is coverage sufficient?
3. Should scan be repeated?
4. Index side:
Comments:

Name of Reviewer
Signature of Reviewer

Date of Review
Email or Phone

298
Appendix 6 - ELISAs performed at CMFT by Dr. P. Pemberton

IL-6, TNF, IL-6, TNFα, E-selectin, ICAM, P-selectin & VCAM assays

Materials

1. DuoSet ELISA development kits for IL-6, TNFα, E-selectin, ICAM, P-selectin & VCAM (R&D Systems, Abingdon, UK).

Analysis is based on the sandwich ELISA principle:

Each kit contains a coating antibody, a biotin-labelled detection antibody, streptavidin-peroxidase and a recombinant standard.

2. Phosphate-buffered saline, 10x concentrate (Sigma-Aldrich, Poole, Dorset)

3. Immulon 4HBX Microtitre plates (Thermo Scientific, Rochester, NY, USA)

4. Reagent Diluent, 1% BSA in PBS (x1)

5. Wash buffer, 0.05% Tween-20 in PBS (x1)

6. Substrate solution, Tetramethylbenzidine (Sigma-Aldrich, Poole, Dorset)

7. Stop solution, 1M sulphuric acid

Method

1. Capture antibody in PBS (x1) is added to a microtitre plate and incubated overnight at RT to create a solid phase.

2. After washing (x3), the plate is blocked by incubation for 1hr with Reagent Diluent

3. After washing (x3), standards and serum (x1) are then incubated for 2hr with the solid phase antibody (that captures the antigen).

4. After washing (x3), biotin-labelled detection antibody is added & incubated for 1hr.

5. After washing (x3), streptavidin-peroxidase is added & incubated for 30min.

6. After washing (x3), substrate TMB solution is added and colour develops in proportion to the amount of bound HRP.

7. Colour development is stopped by addition stop solution.

8. Colour intensity is then measured at $\lambda = 450$nm on a plate reader (Dynex Technologies, Worthing, UK).
9. A standard curve is generated using Fig P software (Hamilton, ON, Canada) and concentrations of cytokine in serum calculated.

**High-sensitivity C-reactive protein in house assay**

**Materials**

1. Rabbit anti-human CRP antibodies (unlabelled and horse-radish peroxidase- labelled), calibrators and controls were obtained from Abcam (Cambridge, UK).

2. Phosphate-buffered saline, 10x concentrate (Sigma-Aldrich, Poole, Dorset)

3. Immulon 4HBX Microtitre plates (Thermo Scientific, Rochester, NY, USA)

4. Reagent Diluent, 1% (w/v) BSA in PBS (x1)

5. Wash buffer, 0.1% Tween-20 in PBS (x1)

6. Sample diluent, 1% (w/v) BSA / 0.1% (v/v) Tween-20 in PBS (x1).

7. Substrate solution, o-phenylenediamine tablets (Sigma-Aldrich, Poole, Dorset)

8. Stop solution, 1.5M sulphuric acid

**Method**

1. Capture antibody in PBS (x1) is added to a microtitre plate and incubated overnight at RT to create a solid phase.

2. The plate is blocked by incubation for 1hr with Reagent Diluent

3. After washing (x3), standards, controls and serum (x1000 in Reagent diluent) are incubated for 2hr with the solid phase antibody (that captures the antigen).

4. After washing (x3), horseradish peroxidase labelled detection antibody is added & incubated for 1hr.

5. After washing (x3), substrate OPD solution is added and colour develops in proportion to the amount of bound HRP.

6. Colour development is stopped by addition stop solution.

7. Colour intensity is then measured at $\lambda = 490$nm on a plate reader (Dynex Technologies, Worthing, UK).
8. A standard curve is generated using Fig P software (Hamilton, ON, Canada) and concentrations of hs-CRP in serum calculated

**Assay Characteristics**

**hsCRP**

Source: in house assay

Assay Dynamic Range: up to 15mg/L

Analytical sensitivity: minimum detection limit calculated from the mean plus two standard deviations of 8 replicate analyses of reagent blank was 0.1mg/L.

Intra-assay variation: n = 14, CV = 4.7%.

Inter-assay variation: n = 19, CV = 6.2%.

**IL-6**

Source: R&D Systems DuoSet development kit DY206.

Assay Dynamic Range: up to 600pg/ml

Analytical sensitivity: minimum detection limit calculated from the mean plus two standard deviations of 9 replicate analyses of reagent blank was 0.5pg/ml.

Inter-assay variation: n = 28, CV = 17.18%.

Inter-assay variation: not determined

**TNF**


Assay Dynamic Range: up to 1ng/ml

Analytical sensitivity: minimum detection limit calculated from the mean plus two standard deviations of 11 replicate analyses of reagent blank was found 2pg/ml.

Intra-assay variation: n = 8, CV = 5.9%.

Inter-assay variation: n = 8, CV = 13.1%.

**E-selectin**

Source: R&D Systems DuoSet development kit DY724.

Assay Dynamic Range: up to 6ng/ml
Analytical sensitivity: minimum detection limit calculated from the mean plus two standard deviations of 16 replicate analyses of reagent blank and was 0.1ng/ml.

Intra-assay variation: n = 9, CV = 6.2%.

Inter-assay variation: n = 13, CV = 8.4%.

**I-CAM**

Source: R&D Systems DuoSet development kit DY720.

Assay Dynamic Range: up to 2ng/ml

Analytical sensitivity: minimum detection limit calculated from the mean plus two standard deviations of 12 replicate analyses of reagent blank and was 0.1ng/ml.

Intra-assay variation: n = 16, CV = 4.5%.

Inter-assay variation: n = 23, CV = 9.3%.

**P-selectin**

Source: R&D Systems DuoSet development kit DY137.

Assay Dynamic Range: up to 5ng/ml

Analytical sensitivity: minimum detection limit calculated from the mean plus two standard deviations of 16 replicate analyses of reagent blank and was 30pg/ml.

Intra-assay variation: n = 16, CV = 4.0%.

Inter-assay variation: n = 23, CV = 9.3%.

**VCAM-1**

Source: R&D Systems DuoSet development kit DY809.

Assay Dynamic Range: up to 1ng/ml

Analytical sensitivity: minimum detection limit calculated from the mean plus two standard deviations of 8 replicate analyses of reagent blank and was 35pg/ml.

Intra-assay variation: n = 16, CV = 3.0%.

Inter-assay variation: n = 32, CV = 14.8%.
Appendix 7 - CD28null cell protocol

Reagents

- $F_c$ blocking reagent (Milteyni®)
- Vioblue-conjugated anti-human CD45 antibodies (BD Biosciences®)
- PE- mouse anti-Î²G2a, Î’ isotype control antibody (Biolegend®)
- APC mouse anti-Î²G2a, Î’ isotype control (Biolegend®)
- PE-anti-human CD28 antibody (BD biosciences®)
- BD FACS™ lysing solution (BD Biosciences®)
- De-ionised water

Sample preparation

1) 4ml blood collected in an EDTA vacutainer tube which is then placed on ice and transferred to the laboratory.

2) Vortex sample well then add 100µl to a tube labelled “A” and also 100µl to one labelled “B” where “A” is the isotype control and “B” is for the CD4/CD28 binding antibodies.

3) Add 10µl of $F_c$ blocking reagent (Milteyni®) to sample A and sample B, vortex and incubate in the dark at room temperature for 15 minutes.

4) After 15 minutes, add 5µl of Vioblue-conjugated anti-human CD45 antibodies (BD Biosciences®) to both samples, vortex and incubate for a further 15 minutes at room temperature in the darkness.

5) Add 5µl of PE- mouse anti-Î²G2a, Î’ isotype control antibody (Biolegend®) and 5µl of APC mouse anti-Î²G2a, Î’ isotype control (Biolegend®) to sample A, vortex and incubate for 15 minutes in darkness, room temp.

6) Add 5µl of PE-anti-human CD28 antibody (BD biosciences®) and 5µl APC- anti-human CD4 antibody (BD biosciences®) to sample B, vortex and incubate for 15 minutes in darkness.

7) Dilute 100µl BD FACS™ lysing solution in 900µl de-ionised water and vortex well.

8) After samples have incubated for 15 minutes, add 500µl of 1:10 concentration lysing solution, vortex and incubate for another 15 minutes in darkness at room temperature.

9) Transfer samples to the flow cytometry facility for analysis.
Appendix 8 - EMP protocol

Reagents

- Efluoro450-conjugated anti-human Annexin V apoptosis detector kit (eBioscience®)
- PE-conjugated anti-human CD31 (BD biosciences®)
- APC-conjugated anti-human CD42B (BD biosciences®)
- Fluorospheres flow counting beads (Beckman Coulter®)

Sample preparation and storage

1) 4.5ml of blood drawn into a citrated vacutainer bottle, placed on ice and transported to the laboratory.
2) Perform centrifugation of the sample: 1700g at 4°Celsius for 10 minutes
3) Harvest the plasma layer and add to 2 tubes
4) Perform centrifugation on the samples: 20,000g at 4°Celsius for 10 minutes
5) Harvest the platelet poor plasma (PPP), leaving the platelet pellet in the tube and store in 200-400µl aliquots at -80 °Celsius

Labelling of samples

1) Pipette 50µl of thawed PPP to a test-tube
2) Dilute 10µl of buffer concentrate from the apoptosis kit to 900µl of de-ionised water
3) Add 900µl of the buffer solution to the PPP sample
4) Add 5µl of FITC- conjugated annexin V to the PPP sample
5) Add 5µl of PE- conjugated CD31
6) Add 5µl of APC-conjugated anti-CD42b
7) Incubate for 10 minutes in the dark
8) Add 50µl of fluorescent counting beads
9) Transfer to the flow cytometry facility for analysis