The Role of sIL-6R Mediated Trans-signaling in Cardiovascular Morbidity in Rheumatoid Arthritis

A thesis submitted in candidature for the degree of Doctor of Philosophy

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January 2018

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Abbreviations

ACR- American College of Rheumatology

anti-CCP - Anti-cyclic citrullinated peptide

BP- Blood pressure

BSR – British Society for Rheumatology

CAD- Coronary artery disease

CIA- Collagen induced arthritis

CIMT- Carotid intima-media thickness

CKD- Chronic kidney disease

COX - Cyclooxygenase

CRP- C-reactive protein

CV – Cardiovascular

CVD – Cardiovascular disease

DMARDS - Disease modifying anti-rheumatic Drugs

ED- Endothelial dysfunction

ESR- Erythrocyte sedimentation rate

EULAR - European League Against Rheumatism

FMD-Fow-mediated vasodilatation

HDL- High density lipoprotein

HLA- Human leucocyte antigen

Hs-CRP- High sensitivity CRP

IL- Interleukin

LDL- Low density lipoprotein

MI- Myocardial infarction

MTX – Methotrexate

NICE- National Institute for Health and Care Excellence

NSAIDS – Non-steroidal anti-inflammatory drugs

RA- Rheumatoid arthritis

RRS- Reynolds risk score

SCCPs- sIL-6R-regulated CVD Candidate Proteins

SCORE - Systematic Coronary Risk Evaluation Score

sgp130- Soluble gp130

TNF-α- Tumour necrosis factor – α

VCAM-1 – Vascular Cell Adhesion Molecule -1

Presentations and posters arising

Davies R, Williams JO, Sime K, Hughes E, Jordan LA, Rawlings C, Lang D, Rose-John S, Jones SA, Williams AS, Choy EH. Therapeutic Blockade of Interleukin-6 Trans-Signalling Restores Vascular Function in Murine Collagen Induced Arthritis [abstract]. Arthritis Rheumatol. 2016; 68 (suppl 10). ***Poster presentation and poster tour at American College of Rheumatology 2016***

Davies R, Daniela Iacono , Lauren A. Jordan , Jessica O Williams , Charlotte Rawlings , Derek Lang , Anwen S. Williams and Ernest H. Choy. Serum Vascular Cell Adhesion Molecule 1 Levels are Associated with Vascular Dysfunction and Increased Cardiovascular Risk in an Animal Model and Patients with Rheumatoid Arthritis. Rheumatology (2016). 55 (suppl 1): i54. doi: 10.1093/ rheumatology/kew102.004. ***Oral presentation at Rheumatology 2016.***

Davies R, Jin H, Sime K, Williams J, Thompson C, Jones G, Allen-Redpath K, Jones S, Hughes T, Rose-John S, Williams A, Choy E. IL-6 trans-signaling causes accelerated atherosclerosis in disease prone animals Annals of the Rheumatic Diseases 2017;76:208-209. ***Poster presentation and poster Tour at EULAR 2017***

Davies R, Thompson C, Jin H, et al. Patients with early rheumatoid arthritis have increased cardiovascular risk at the time of diagnosis. Annals of the Rheumatic Diseases 2017;76:1173.

Acknowledgments

Firstly, I would like to express my sincere gratitude to my supervisors; Professor Ernest Choy for his unwavering support, enthusiasm and vast knowledge and Professor Anwen Williams for her extensive experience, immense knowledge and support. Guidance from the offset from you both has been invaluable.

The work carried out in this thesis would not have been possible without the help, support and guidance of numerous people in the Tenovus building, as well as the financial support from Arthritis Research UK. I owe a great deal to all the patients that have taken the time to be part of these studies: thank you.

To lab colleagues and, more importantly, friends: Charlie, Jess, Katie, Gareth, Lauren, Rav, Anne, Saydul, Selinda, Sun. Your help with science has been invaluable and your chats and cake more so. Many thanks for excellent advice/guidance from Stefan Rose-John, Simon Jones and Gareth Jones. Tim Hughes, Julian Halcox, Libby Ellins, Keith: thank you for all the cardiovascular advice and help.

To Mum/Dad, thank you for your unwavering support, love, and instillation of belief. Loz, Ella, Georgia, Onion, Diff ladies, the Rowlands' and all family friends near and far, thanks for everything and I can't wait to be able to play again! Lastly, Matt, for your support, patience and being the light at the end of the tunnel. Thank you.

DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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Abstract

Background

Cardiovascular mortality in patients with RA is 50% higher than the general population. Although well established that the incidence of CVD is increased, the precise cause is unclear. There is increasing recognition that systemic inflammation is a major driver of increased CV risk. IL-6 is implicated in CVD in the general population but its role in CVD in RA is undefined. Of the two modes of IL-6 signaling, evidence demonstrates that trans-signaling is pro-inflammatory whereas classical signaling has regenerative or anti-inflammatory effects. The aim of this thesis is to examine the role of IL-6 trans-signaling in CVD in RA by experimental and translational studies.

Methods

Myography was used to determine the effect of IL-6 trans-signaling blockade, using sgp130Fc, on aortic constriction in mice with CIA. Serum CCL2 and VCAM-1 were measured. The effect of IL-6 trans-signaling, using Hyper-IL-6, on atherosclerotic plaque size and fibrous cap thickness was assessed in ApoE$^{-/-}$ mice. Arterial and plaque VCAM-1 expression was assessed. The relationship between sIL-6R-regulated CVD Candidate Proteins (SCCPs) and CVD in established RA was investigated in a cross-sectional study. An observational longitudinal study investigated whether SCCPs were associated with presence and progression of subclinical atherosclerosis in early RA, using carotid ultrasound to measure CIMT.

Results

Sgp130Fc reduced arthritis severity and restored vascular dysfunction in CIA. This was associated with reduced serum CCL2 and VCAM-1. In ApoE$^{-/-}$ mice, Hyper-IL-6 increased plaque size and VCAM-1 expression in the brachiocephalic artery. In established RA, VCAM-1 correlated with disease activity and CV risk. In early RA, baseline RA disease activity was associated with CIMT change at 6 months. Patients that were 'rapid progressors', in terms of CIMT change at 12 months, had higher baseline VCAM-1, HbA1c, total cholesterol:HDL ratio and LDL cholesterol.

Discussion

IL-6 trans-signaling appears to play a pivotal role in vascular dysfunction and atherosclerosis in mouse models. In early RA, proteins regulated by IL-6 trans-signaling are associated with progression of subclinical atherosclerosis. Inflammation from RA onset in CVD susceptible individuals may accelerate atherosclerosis. Findings suggest that IL-6 trans-signaling blockade may be beneficial to RA patients, and perhaps for atherosclerosis in the general population.

1 Chapter 1- General Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory chronic polyarthritis that leads to joint destruction, deformity and loss of function. It is the most common inflammatory arthritis globally, affecting approximately 1% of the UK population (Symmons et al, 2002). It is a systemic disorder but is predominantly characterised by symmetrical persistent synovial inflammation in diarthrodial joints (Tak and Bresnihan, 2000). Within the joint the inflamed synovial lining proliferates and forms pannus which erodes into cartilage and bone, leading to joint damage (Bartok and Firestein, 2010).

Its consequences include pain, disability and increased mortality. Costs of RA in the UK, including indirect costs and work related disability, have been estimated at £3.8-£4.75 billion per year (Pugner et al, 2000). Although its course is heterogeneous and variable, within two years of diagnosis patients usually experience moderate disability and after 10 years 30% are severely disabled (National Collaborating Centre for Chronic Conditions 2009). Extra-articular manifestations can include vasculitis, scleritis, pulmonary fibrosis, rheumatoid nodules, amyloidosis, serositis and lymphadenopathy (Cojocaru et al, 2010). Systemic manifestations include anaemia, fatigue, depression, osteoporosis and cardiovascular disease (CVD) (Choy, 2012), the latter being responsible for the increased mortality in RA (Meune et al, 2009).

1.2 Aetiology of RA

The aetiology of RA is unknown, although some environmental and genetic factors have been associated with its development. These include specific human leucocyte antigen (HLA) alleles, smoking and viral infection. HLA-DR4 correlates strongly with RA (Fugger and Svejgaard, 2000). However, concordance rates in monozygotic twins are only around 15% (Silman et al, 1993), suggesting that environmental factors play a large role. Smoking also appears to increase the risk of developing RA and to have an adverse effect on disease progression (Masdottir et al, 2000). Numerous infectious agents have been suggested as potential causes or triggers for RA, including Epstein–Barr virus (EBV), mycoplasma organisms (Schaeverbeke et al, 1997) and peridodontopathic bacteria (Routsias et al, 2011). Bacterial and viral products, such as EBV, have been found in the joints of RA patients at synovial biopsy (Takei et al, 1997). However, it has also been postulated that this may be due to persistence of infection due to altered T cell responses, rather than pathogens playing a causative role (Toussirot et al, 2000). It is well known that certain pathogens can lead to a reactive arthritis, but this usually remits spontaneously (Carter and Hudson, 2009). Hormonal factors are thought to play a role in the aetiology of RA; RA is 2-4 times more frequent in females than males (Kvien et al, 2006). RA also tends to improve during pregnancy and recur postpartum (Hazes et al, 2011). RA is associated with the production of autoantibodies, notably rheumatoid factor and anti-citrullinated proteins (Song and Kang, 2010).

1.3 The Normal Joint

In order to understand the processes involved in RA, it is important to appreciate normal joint structure and environment and how these change in disease. There are three types of human joints: fibrous, synovial and cartilaginous. Joints affected in RA are predominately synovial. These are the most common joints in humans and include the metacarpophalangeal joints, hips, shoulders and knees. Synovial joints consist of the articular surfaces of two bones which are covered by hyaline cartilage. The cartilage functions in weight bearing, shock absorption and to reduce friction during movement. A fibrous capsule surrounds the joint and is comprised of two layers; an outer fibrous membrane and the inner synovial membrane. The joint capsule is filled with synovial fluid which provides lubrication and allows oxygen and nutrients to pass to the cartilage (Knedla et al, 2007). The synovial membrane is composed predominately of synovial fibroblasts and macrophages (Knedla et al, 2007). The synovium is infiltrated by capillaries and venules and also has lymphatic drainage. It is the central area of pathology in a number of inflammatory joint diseases, including RA (Smith, 2011). In the normal joint, bone turnover is carefully balanced. Osteoclasts resorb bone by attaching themselves to the periosteum and releasing proteolytic enzymes and acid, causing degradation of the bone and its membrane (Clarke, 2008). Osteoblasts synthesise bone; secreting bone extracellular matrix proteins which become mineralised to form bone (Clarke, 2008).

1.4 Pathogenesis of RA

The pathogenesis of RA is complex and not fully elucidated. However, several mechanisms underlying the development of the disease have been recognised. It is proposed that in genetically predisposed individuals the repeated activation of both the innate and adaptive immune system leads to the breakdown of self-tolerance, leading to autoantigen presentation and antigen-specific T and B cell activation (Picerno et al, 2015). Several cell types, most notably dendritic cells, express pattern-recognition molecules, such as Toll-like receptors, which bind to various self and foreign structures and become activated. These then act on cells of the adaptive immune system. This culminates in synovial inflammation and hyperplasia, with leucocytes, predominately T-lymphocytes and macrophages, infiltrating the synovial compartment via activated endothelial cells expressing various adhesion molecules (Smolen et al, 2007). There is also angiogenesis and increased synovial fluid production, containing polymorphonuclear cells with some T cells and macrophages. The inflammatory exudate overlying synovial cells on the inside of the joint capsule, pannus, contains osteoclasts and this destroys bone, leading to bone erosion. Bone repair by osteoblasts usually does not occur in active RA (Smolen and Steiner, 2003). The growth of the synovial membrane is accompanied by neovascularisation. Cartilage is also degraded by enzymes secreted by chondrocytes, neutrophils and synoviocytes (Choy, 2012).

Activated T and B cells produce cytokines and chemokines. Cytokines play key roles in the pathogenesis of RA, notably TNF-α and IL-6, both of which are used as therapeutic targets for treatment in RA. Other cytokines such as IL-1 and IL-17, although implicated in RA pathogenesis, have not been validated as successful therapeutic targets in RA.. Chemokines, such as CXCL8 and CXCL9, also play important roles in RA, including cell-cell interactions, migration and chemoattraction (Koch, 2005). These pathological changes are summarised in Figure 1.

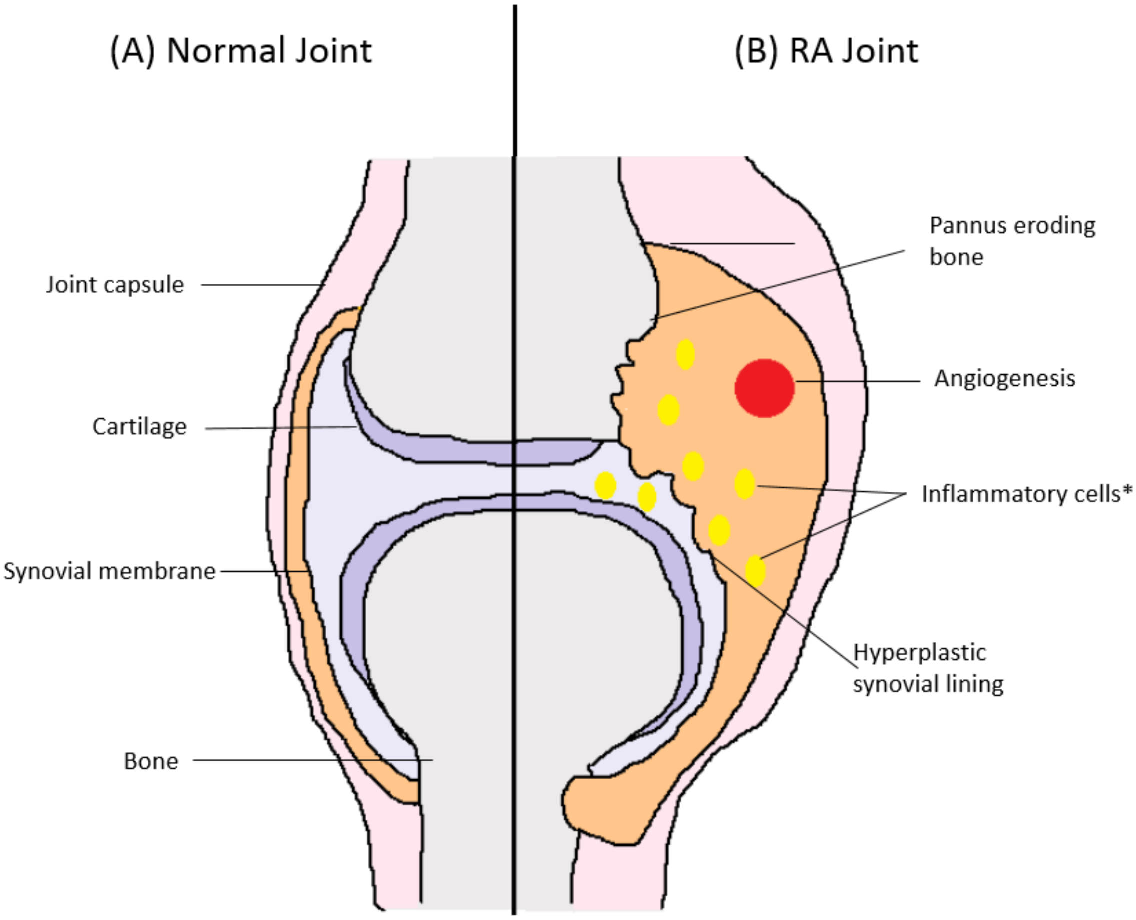

Figure 1. Overview of a (A) Normal joint and (B) Joint affected by RA. In the RA joint inflammatory cells (macrophages, T and B cells, dendritic cells, plasma cells and mast cells) invade the synovium and surrounding areas. The synovial lining becomes hyperplastic, neovascularised and pannus erodes bone.

1.5 Cardiovascular risk in RA

Mortality is increased in RA mainly due to CVD. Indeed, cardiovascular mortality in patients with RA is up to 50% higher than the general population (Meune et al, 2009; Avina-Zubieta et al, 2008; Peters et al, 2010; Solon et al, 2003; de Groot et al, 2010;

Gullick and Scott, 2011; Meune et al, 2010; Gabriel, 2008) and is of the magnitude seen in diabetes mellitus (Luqmani et al, 2006). Although it is well established that incidence of CVD is increased in RA, the precise cause is unclear. While traditional cardiovascular risk factors, such as smoking, diabetes and hypertension contribute to this increased mortality in RA patients, they do not fully explain the increase in risk (Dessein et al, 2005; del Rincon, 2001). The increased cardiovascular risk in RA is related to the severity and duration of inflammation (Kelt, 2009). Certain inflammatory cytokines, such as interleukin-6 (IL-6), have been implicated in the pathogenesis of both RA (Srirangan and Choy, 2010) and CVD (Hartman and Frishman, 2014).

Cardiovascular events occur on average ten years earlier in RA patients than the general population (Bacon et al, 2002). Cardiovascular manifestations in RA include arrthymias, myocarditis, pericarditis, cardiac amyloidosis, coronary vasculitis, congestive heart failure, and atherosclerosis (Voskuyl, 2006). The focus of this thesis is atherosclerosis, which is the cause of the majority of cardiovascular deaths in RA. As well as having an increased incidence of CV events, RA patients also experience significantly lower survival rates after a myocardial infarction or stroke compared to those without RA (Solomon et al, 2006). Despite this, standardised admission rates for cardiovascular disease are not raised; suggesting either that CVD in RA goes unrecognised before the fatal event or has a higher case fatality than in the general population (Goodson et al 2005). In addition to an increase in actual cardiovascular events, RA patients have a higher prevalence of preclinical atherosclerosis than the general population, such as asymptomatic carotid artery atherosclerotic plaques (Roman et al, 2006).

The term coronary microvascular dysfunction (CMD) has been introduced to describe abnormalities in the regulation of myocardial blood flow (MBF) which are not explained by disease of the epicardial coronary arteries. A study using PET to measure MBF found that patients with RA and SLE had reduced coronary flow reserve in the absence of significant coronary artery disease (Recio-Mayoral et al 2009). There are also differences in the presentation and outcome of heart failure in those with and without RA. Despite RA patients having more subtle presentation of heart failure, and having more preserved myocardial function, mortality from heart failure in RA patients is significantly higher (Davies et al 2008).

The Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients with Rheumatoid Arthritis (TRACE-RA) study was designed to assess whether atorvastatin 40mg daily was superior to placebo for the primary prevention of CVD events in patients with RA (Kitas et al, 2015). However, the study was terminated early, after more than 3,000 patients of the 4,000 target population had been recruited, due to a low incidence of the primary cardiovascular end point. This highlights the fact that using cardiovascular events as outcome for exploratory studies is not appropriate since sample size is prohibitively large. The following study therefore uses carotid ultrasound as a surrogate for determining subclinical atherosclerosis in RA.

1.6 Hypotheses for increased CV risk in RA

There are a number of theories put forward to explain the increased risk of CVD in RA patients. Firstly, RA patients have an increased prevalence of traditional cardiovascular risk factors. These include smoking and hypertension (Panoulas et al, 2008). Secondly, due to the nature of the disease, some RA patients are less active than the general population, and inactivity in the general population has been linked to cardiovascular disease (Bijnen et al, 1994). Thirdly, some drugs used in the management of RA have been implicated, outlined in section 3 below. Lastly, there is increasing evidence that systemic inflammation plays an important role in the increased risk in RA patients. These theories are discussed in more detail below.

1. Traditional CV risk factors are more prevalent in RA patients. Certain traditional cardiovascular risk factors, such as smoking (Wolfe, 2000; McEntegart et al, 2001; Solomon et al, 2003), diabetes mellitus and reduced HDL cholesterol levels appear more prevalent in RA patients (Boyer et al, 2010). There is conflicting evidence in the literature regarding hypertension in RA patients;

some studies do not show a difference between RA patients and healthy subjects (Roman et al, 2006; Maradit-Kremers et al, 2005; Solomon et al, 2003), while others have shown an increased prevalence in RA patients (Han et al, 2006). An important concept in RA patients is the 'lipid paradox'. In the general population the risk of atherosclerosis development increases progressively with increasing LDL cholesterol levels and declines with increasing levels of HDL cholesterol, however, in RA, the presence of a proinflammatory state leads to a decrease of total cholesterol, HDL cholesterol and LDL cholesterol in patients (Robertson et al 2013). In contrast, reduction in inflammation coincides with increases in serum lipid values (Myasoedova et al 2011). These changes are complex and of importance are relative changes in the pro and anti-inflammatory lipoprotein components. Chronic inflammation leads to oxidative changes that alter HDL structure and reduce apolipoprotein-A-I in patients with active RA (Charles-Schoeman et al 2009). Several studies have shown an increased prevalence of diabetes mellitus in RA (Han et al, 2006; Dessein et al, 2002) but others have shown a similar prevalence to controls (Solomon et al, 2003). Several studies have found a higher prevalence of hyperlipidaemia in RA (Han et al, 2006). A recent large, retrospective cohort study of UK patients found that there were no differences in the frequency of testing and treatment of CV risk factors between RA patients and non-RA patients (Alemao et al, 2016). Thus, higher CV risk seen in RA patients is unlikely to be due to differences in traditional CV risk factor management.

2. **Inactivity of RA patients.** Patients with RA have high levels of inactivity (Sokka et al, 2008) for various reasons, includuing physical pain, poor joint movement, fear of pain or joint damage, fatigue and mood disturbance. It is known that in the general population lack of exercise increases the risk of CV disease development and exercise reduces this risk (Gielen et al, 2009). However, this does not fully explain the increase in CV risk, which increases early in the disease course of RA, where inactivity may not be present, or may have only

been present for a short time period. Also, as treatments for RA improve, disease should become less disabling, such that levels of inactivity may reduce.

3. Drugs used in RA management. Some drugs used in RA management have associated cardiovascular risk, such as NSAIDs and corticosteroids. Others seem to have a protective role.

    a. NSAIDS are commonly used for symptomatic treatment of RA, both before and after definitive diagnosis, to treat symptoms such as pain and swelling. Most NSAIDs act as nonselective inhibitors of the enzyme cyclooxygenase (COX), inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes. COX catalyses the formation of prostaglandins and thromboxane from arachidonic acid. Selective cyclo-oxygenase-2 (COX-2) inhibitors are selective inhibitors of the COX-2 isozyme (FitzGerald and Patrono, 2001). A meta-analysis reports that selective COX-2 inhibitors are associated with a moderate increase in the risk of vascular events, as are high dose regimens of ibuprofen and diclofenac, but high dose naproxen is not (Kearney et al, 2006).

    b. Glucocorticoids are often used as a means of rapidly reducing inflammation in RA. Adverse effects of long-term glucocorticoid use, especially at high doses, are well recognised in the general population. These include effects on body weight and fat distribution (Macfarlane et al, 2008), blood pressure (Whitworth et al, 2000) and insulin resistance (Andrews and Walker, 1999). There are conflicting reports of their effects on lipid profile (Choi et al, 2005). In RA the effects of glucocorticoids on cardiovascular risk are not fully elucidated, in part because glucocorticoids reduce systemic inflammation which is thought to play a major role in CV risk in RA. In an inception cohort study of low-dose prednisolone use during the first 2 years of RA disease, the incidence of ischaemic coronary artery events was similar in those taking prednisolone and those not (Ajeganova et al, 2014). However, those taking prednisolone had an increased long-term risk of ischaemic cerebrovascular event and there was a trend towards reduced survival in the prednisolone group.

c. Several disease modifying anti-rheumatic drugs (DMARDS) are used to treat RA. The most commonly used are methotrexate (MTX), sulphasalazine (SSZ) and hydroxychloroquine (HCQ.) A case control study found that MTX and, to a lesser extent, SSZ were associated with significantly lower CVD risk compared to RA patients who had never used SSZ, HCQ or MTX (van Halm et al, 2006) While another study found a protective effect of MTX on cardiovascular risk (Choi et al, 2002), others have not (Singh and Cameron, 2012). Interestingly, there is an ongoing trial assessing MTX efficacy as secondary prevention in non-RA patients with established CVD (Everett et al, 2013). Hydroxychloroquine has been found to have a favourable effect on lipid profiles (Morris et al, 2011) and insulin sensitivity (Mercer et al, 2012). Leflunomide has been associated with hypertension (Rozman et al, 2002).

d. Biological therapies

   i. TNF-α blocking agents. Most studies have found that TNF-α blocking agents are associated with a reduced cardiovascular risk in RA (Solomon et al, 2013; Jacobsson et al, 2005; Greenberg et al, 2011; Naranjo et al, 2008). Other studies have shown no difference in risk with anti TNF (Solomon et al, 2006; Dixon et al, 2007) and some have shown that there may be an increased cardiovascular risk (Suissa et al, 2006). TNF-α blocking agents are contraindicated in RA patients with heart failure on the basis that clinical trials which used these drugs in non-RA heart failure produced disappointing results in patients with moderate to severe heart failure (Cacciapaglia et al, 2011). A more recent study found that patients with RA receiving a TNF inhibitor had a decreased risk of MI compared with patients with RA receiving non-biologcial DMARDs over the medium term (Low et al 2017). A mouse model of arthritis, the Tg197 arthritis model, develops TNF- driven and mesenchymalsynovial fibroblast (SF)-dependent polyarthritis (Ntari et al 2018). These mice also develop heart valve disease alongside chronic polyarthritis. Both pathologies were dependant on TNF in this model.

ii. Anti-IL-6R antibody (Tocilizumab). In a study of patients receiving Tocilizumab there was an association between the baseline total cholesterol:HDL ratio and an increased risk of major adverse cardiovascular events (Rao et al, 2015). The risk of cardiovascular events while receiving treatment, however, was associated with control of disease activity but not lipid changes. Studies have consistently shown that tocilizumab is associated with increased lipid levels in the context of decreased inflammatory marker levels (Nishimoto et al, 2010; Emery et al, 2008). Although lipid changes alongside reduction in inflammation are seen with all biological and non-biological DMARDs, theses are most well described and are more apparent with Tocilizumab. The ENTRACTE study compared rates of major cardiovascular outcomes in RA patients treated with Tocilizumab or Etanercept (Giles et al 2016). By week 4, total cholesterol, LDL, HDL, and triglycerides increased significantly in the Tocilizumab arm compared with the Etanercept arm, but there was no significant difference in major cardiovascular events over a mean follow up of 3.5 years. Data from phase 3 studies and extension studies have shown rates of MI were lower with tocilizumab versus placebo

(Schiff et al, 2011) and analysis of the long-term safety (n = 4171; median treatment duration 3.9 years) found a stable rate of cardiovascular events over time with tocilizumab exposure (Genovese et al, 2013; Schiff et al, 2011). Also, tocilizumab does not appear to increase CIMT in RA (Kume et al, 2011).

iii. Rituximab, a monoclonal anti-CD20 antibody which causes B cell depletion, does not appear to increase risk of cardiovascular events at 6 months and in longer term follow up (van Vollenhoven et al, 2010) and appears to have beneficial effects on lipid profile (Raterman et al, 2013). Ait Oufella et al (2010) showed that mature B cell depletion using a CD20-specific monoclonal antibody caused a significant reduction of atherosclerosis in mouse models of atherosclerosis.

4. Systemic inflammation in RA. The excess cardiovascular burden in RA persists after adjustment for traditional CV risk factors (del Rincon et al, 2001; Maradit-Kremers, 2005). There is accumulating data implicating systemic inflammation as the major driver of this increased CV risk (Gabriel, 2008; del Rincon et al, 2001; Arts et al, 2014). Inflammation contributes to the onset and pathogenesis of CVD in the general population. Epidemiological studies have shown that circulating CRP (Ridker and Cook, 2004) and IL-6 (Ridker et al, 2000) are associated with higher risk of CVD in the general population, independent of known CVD risk factors. It should be noted that evidence indicates that the CRP does not cause CVD, whereas IL-6 appears to play a causative role (Welsh et al 2017). High IL-6 levels are associated with increased mortality in patients with acute coronary syndromes (Biasucci et al, 1999). Levels of CRP and IL-6 are much higher in RA patients than the general population, indeed there is a significant association between inflammation measured by erythrocyte

sedimentation rate (ESR) or CRP, and risk of CVD in RA (Chung et al, 2008; Book et al, 2005; Crilly et al, 2009; Graf et al, 2009; del Rincon et al, 2005; Gonzalez-Gay et al, 2005). There is also evidence that reducing inflammation in RA lowers CV risk (van Halm et al, 2006; Dixon et al, 2007; Choy and Sattar, 2005).

RA is associated with anti-citrullinated proteins (anti-CCP) antibodies. Within atherosclerotic plaque citrullinated proteins are prevalent in non-RA patients (Sokolove et al 2013). Certain anti-CCP antibodies were associated with atherosclerotic burden. These observations suggest that citrullinated epitopes within atherosclerotic plaque may be targeted by RA-associated anti-CCP antibodies which may form immune complexes, locally increasing plaque inflammation and progression. This may in part explain the accelerated atherosclerosis observed in patients with RA. Citrullination has also been found to be higher in the myocardium of RA patients compared to non-RA patients (Giles et al 2012).

Advances in basic science have established the fundamental role of inflammation in all stages of atherosclerosis, from plaque formation, to instability and eventual rupture (Gonzalez Gay et al, 2005; Hansson and Hermansson, 2011; Libby et al, 2011). The pathophysiology of atherosclerosis is discussed in section 1.13.1.

1.7 Assessing Cardiovascular Risk in RA

National and International guidelines, including those of the British Society for Rheumatology (Luqmani et al, 2009), National Institute for Health and Clinical Excellence (2009) and European League Against Rheumatism (EULAR) (Peters et al, 2010) recommend annual assessment of cardiovascular risk in RA patients. EULAR recommendations for cardiovascular management were based on a systematic literature review. Traditional cardiovascular risk factor assessment equations, such as the Framingham risk score (FRS) and Systematic Coronary Risk Evaluation Score

(SCORE) (Conroy et al, 2003) models, underestimate cardiovascular risk in RA. Therefore, EULAR recommends multiplying such traditional cardiovascular risk scores by 1.5 for RA patients who meet two of three criteria consisting of (1) disease duration >10 years, (2) positive rheumatoid factor or anti-cyclic citrullinated peptide (anti-CCP) serology, and (3) presence of severe extra-articular manifestation, to account for the unexplained increased cardiovascular risk in RA (Peters et al, 2010). However, recent studies have shown that even modified cardiovascular risk scores, such as recommended by EULAR still underestimate overall cardiovascular risk (Arts et al, 2014; Rosales-Alexander et al, 2014). Thus, there is a need for a better means of risk stratification. EULAR guidelines were updated in 2016 and recommend optimal control of RA disease activity, CV risk assessment at least once every 5 years, consideration of carotid ultrasound as screening for asymptomatic atherosclerotic plaques as part of the CVD risk evaluation (Agca et al 2016).

1.8 Cardiovascular risk algorithms

See Table 1 for comparison of different cardiovascular risk algorithms.

a) SCORE

The SCORE model is based on gender, age, smoking, systolic blood pressure and total cholesterol (Conroy et al, 2003). There are different charts for patients from a high or low CV risk region of Europe. It is derived from a large dataset of prospective European studies and predicts fatal atherosclerotic CVD events over a ten year period.

b) The Framingham risk score

The Framingham Risk Score (FRS) was first developed based on data obtained from the Framingham Heart Study in the USA and was first published in 1998 (Wilson et al, 1998) and the current version in 2002 (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2002). It performs well in North America (Eichler et al, 2007) but less so elsewhere (Brindle et al, 2003). The updated version includes age, gender, systolic BP, smoking status, total cholesterol and HDL cholesterol and whether the patient is on treatment for hypertension. The endpoint definition is angina pectoris and cardiovascular death.

c) The QRISK2 score

The QRISK2 is the only validated CV risk score to include RA as an independent CV risk factor. It includes age, systolic blood pressure, smoking status, total cholesterol:HDL ratio, body mass index, ethnicity, geographical measures of deprivation, family history of early CVD, chronic kidney disease, RA, atrial fibrillation, diabetes mellitus, and antihypertensive treatment. In the UK current National Institute for Health and Care Excellence (NICE) guidelines recommend using this to assess CV risk (NICE, 2014). The NICE guidelines state that if a patient has a greater than 10% risk of CVD event over the next ten years when calculated using the QRISK2 then primary prevention with lipid lowering therapy (such as statins) should be initiated (NICE, 2014). The algorithm is updated annually to reflect changes in populations, data quality and national guidelines. A prospective open cohort study found that the QRISK2 was better calibrated to the UK population than the FRS and has better discrimination (Hippisley-Cox et al, 2008).

d) The Reynolds risk score (RRS)

This score includes age, total cholesterol, HDL cholesterol, systolic blood pressure, diabetes mellitus assessed by haemoglobin A1c (for women only), current smoking, parental history of MI before age 60 years and serum high sensitivity CRP (hs-CRP) (Ridker et al, 2007). Endpoints assessed are cardiovascular death, nonfatal MI, non-fatal stroke and coronary revascularization.

e) The American College of Cardiology/American Heart Association (ACC/AHA) 2013 guideline.

This score includes age, gender, ethnicity, total cholesterol and HDL cholesterol, smoking status, diabetes, treatment for hypertension, systolic and diastolic blood pressure. Endpoints assessed are fatal and non-fatal MI and stroke.

Risk algorithm	**SCORE**	**Framingham**	**Reynolds**	**QRISK2**	**ACC/AHA**
Age	✓	✓	✓	✓	✓
Gender	✓	✓	✓	✓	✓
Systolic blood pressure	✓	✓	✓	✓	✓
Diastolic blood pressure					✓
Smoking	✓	✓	✓	✓	✓
Total Cholesterol	✓	✓	✓		✓
HDL cholesterol		✓	✓		✓
Cholesterol:HDL ratio				✓	
Rheumatoid arthritis				✓	
Deprivation score				✓	
On treatment for hypertension		✓		✓	✓
hsCRP			✓		
Diabetes		*			✓
Family history of early CVD			✓	✓	
CKD				✓	
AF				✓	
Ethnicity				✓	✓
Endpoint definition	Fatal atherosclerotic CVD events	Angina pectoris, CV death	MI, stroke, coronary revascularization, CV death	MI AP, CAD, stroke, and TIA	Fatal and non-fatal MI and stroke

Table 1. Summary of various cardiovascular risk algorithms. hsCRP (high sensitivity CRP), CKD (Chronic Kidney disease), AF(Atrial Fibrillation). *removed from updated version.

Which risk score performs best in RA?

The QRISK2 score is the only validated score to include RA as an independent risk factor for CVD. However, it does not factor in time since RA diagnosis as suggested by EULAR and may, therefore, over predict risk in patients recently diagnosed with RA. However, recent data suggests that patients with RA may have similar chances to develop CV events early and late in their disease course (Kerola et al, 2012a; Kerola et al, 2012b).

One study used data collected prospectively from the Nijmegen early rheumatoid arthritis inception cohort in the Netherlands to assess the discriminatory ability of different cardiovascular risk algorithms in early RA (Arts et al, 2014). Algorithms assessed were SCORE, FRS, RRS and QRISK2. Discriminatory ability for CV risk prediction was estimated by the area under the receiver operating characteristic curve. Calibration was assessed by comparing the observed versus expected number of events. SCORE, FRS and RRS primarily underestimated CV risk at middle and low observed risk levels and mostly overestimated CV risk at higher observed risk levels. The QRISK2 primarily overestimated observed CV risk. Of note, depending on the model used, up to 32% of observed CV events occurred in RA patients who were classified as low risk for CV disease.

It could be thought that the RRS may be good for assessing risk in patients with RA as it incorporates the inflammatory marker hs-CRP in addition to traditional risk factors. However, many patients with RA have high levels of CRP during the course of their disease (generally much higher than the general population) and thus it is unclear if adding hs-CRP to a CV risk algorithm improves the predictive performance in RA.

In order to improve identification of patients at increased CV risk, different approaches have been suggested. The cut-off point for primary prevention could be lowered. However, this could also lead to overtreatment, as most patients in low risk groups do not develop cardiovascular events (Arts et al, 2014). Alternatively, the CV risk score could be adjusted by a certain correction factor for patients with RA, such as suggested by the EULAR recommendations (Peters et al, 2010). This particular multiplicator was chosen as a meta-analysis showed a mean standardised mortality ratio (SMR) of 1.2 for inception cohorts (2 years' disease duration) compared with a mean SMR of 1.9 for established disease (Ward, 2001). The EULAR guideline authors chose 1.5 as a multiplicator as available comparative studies did not adequately adjust for important confounders (including social class and physical activity), and few adjusted for all established CV risk factors using continuous data. Thus, it is possible that the excess CV risk in RA, over and above traditional risk factors, has been overestimated. Hence, a multiplication factor of 1.5 (rather than 2.0), was chosen on the basis of the evidence from observational SMR reporting studies as well as expert opinion (Peters et al, 2010).

Drawback of current risk algorithms

One problem with risk assessments are that they may be falsely reassuring for some patients who are defined as having low risk when they may actually have multiple marginal abnormalities. An important component of multivariate risk models for the estimation of CVD risk is that many of the risk factors (e.g. age, hypertension, serum LDL-cholesterol) are recognised as producing a graded increase in risk. In addition, the relative effects of traditional risk factors differ according to the particular vascular disease outcome being evaluated (Wilson, 2008). Some risk algorithms do not include patient important CVD outcomes such as stroke, heart failure or development of symptomatic peripheral artery disease. Therefore, there is a real need to better understand the aetiology of CVD in RA as current risk algorithms may lead to both over- and under-estimation of risk in different patients. Moreover, if a biomarker could be identified which could identify RA patients at high risk of CVD, they could be selected for more invasive investigation or management of risk.

1.9 Vascular function

In order to understand vascular dysfunction it is important to understand the normal vascular structure and function.

1.10 Normal structure of arteries

To withstand regular pumping of the heart and thus high pressures, arteries have thick muscular walls. The artery wall consists of three layers, summarised in Figure 2.

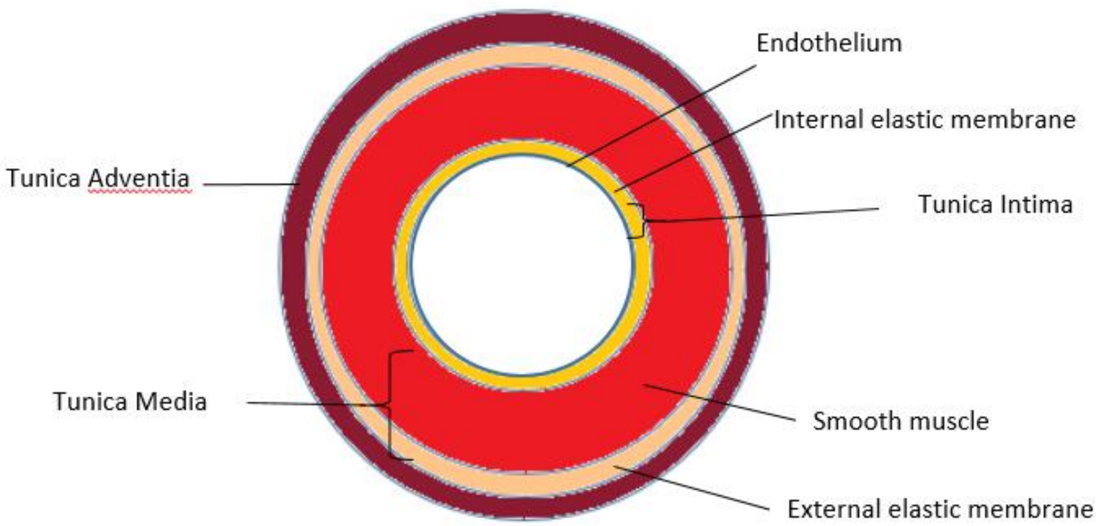

Figure 2. A diagram of the normal arterial wall in cross section.

- Tunica Adventitia (or tunica externa)

  This is the outer layer rich in collagenous ECM molecules. It contains fibroblasts, blood vessels, and perivascular nerves. It also has a role in immune surveillance and inflammatory cell trafficking and contains the vasa vasorum, which maintains the medial layer and provides access for macrophage and leukocyte migration into the intima (Majesky et al, 2011).

- Tunica Media

This consists of vascular smooth muscle cells (VSMCs) arranged concentrically with bands of elastin fibres between the layers. VSMCs enable blood vessels to constrict to allow vessel resistance (Rensen et al, 2007). When these cells contract they decrease the diameter of the blood vessel (i.e. vasoconstriction). When the VSMCs relax the blood vessel increases in diameter (i.e. vasodilation).

- Tunica intima

The innermost layer consists of a single layer of endothelial cells supported by an underlying internal elastic lamina. The endothelial cells are in direct contact with blood flow and form an interface between the circulating blood and the cells of the vessels (Rajendran et al, 2013). Intact endothelium mechanically separates platelets and their pro-coagulant products from intravascular, subendothelial and tissue coagulation factors, and also inhibits pro-coagulant proteins (Rajendran et al, 2013). It controls the passage of materials and white blood cells into and out of the circulation, reduces turbulence of blood flow and participates in fibrinolysis by the production of tissue plasminogen activator. The endothelium responds to numerous circulating factors, altering vascular tone and architecture. Several pathological conditions including hypercholesterolemia, diabetes mellitus and chronic inflammation disrupt the homeostatic mechanisms of the endothelial layer. This results in increased adhesiveness of the endothelium to leucocytes and in altered permeability of the endothelium. This also leads to heightened reactivity of the adjacent vascular smooth muscle. Endothelial dysfunction is discussed in more detail in section 1.12.1.

1.11 Vascular smooth muscle constriction

Circulating endothelium-derived factors, hormones, neurotransmitters and shear stress all contribute to vascular smooth muscle tone, and thus lumen diameter (Wynne et al 2008). The final product is the control of blood pressure and organ blood flow. All VSMCs, regardless of the stimulus, produce contraction through cross-bridge cycling between actin and myosin filaments (Wynne et al 2008). In smooth muscle, this process is initiated by calcium-mediated change in the thick (myosin) filaments (Hilgers and Webb 2005). An increase in free intracellular calcium results from either increased flux of calcium into the cell through calcium channels or by release of calcium from internal stores, for example sarcoplasmic reticulum. The free calcium binds to calmodulin. Calcium-calmodulin activates myosin light-chain kinase, which phosphorylates myosin light chains (MLC) in the presence of ATP (Hilgers and Webb 2005). MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and thus, smooth muscle contraction.

1.12 Non-invasive assessment of vascular function and CVD in RA

It is known that patients with RA have increased incidence of cardiovascular disease. There are various methods (invasive and non-invasive) used to measure both vascular function and structural abnormalities in RA and the general population. Coronary angiography is the gold standard test for identifying the presence and extent of atherosclerotic coronary artery disease. This invasive procedure is time consuming, costly and confers risk to the patient, such as bleeding, arrhythmia, infection, pain, blood vessel perforation and myocardial infarction. Non-invasive techniques confer less risk. Each has its benefits and drawbacks, discussed in sections 1.12.1 to 1.12.6.

1.12.1 Endothelial dysfunction

Endothelial dysfunction (ED) is one of the key early events in atherogenesis; it precedes structural atherosclerotic changes (Raitakari and Celermajer, 2000). However, it is also present in the late stages of atherosclerosis when it can lead to constriction or thrombosis of vessels (Raitakari and Celermajer, 2000). ED can be assessed invasively, by infusion of substances such as acetylcholine or substance P, which enhance the release of endothelial nitric oxide (Tousoulis et al, 2005). It can also be assessed noninvasively by measurement of endothelial-dependent flow-mediated vasodilatation (ED-FMD) of peripheral arteries, measured by ultrasonography (Corretti et al, 2002). Here, arterial diameter is measured in response to an increase in shear stress (induced by inflation and then deflation of a sphygmomanometer cuff around the limb, distal to the scanned part of the artery), which causes endothelium-dependent dilatation. In the general population ED-FMD is a predictor of cardiovascular events (Bonetti et al, 2003), and is associated with risk factors of CVD. Endothelial dysfunction is a reversible disorder; interventions such as cholesterol lowering and smoking cessation can improve it (Bonetti et al, 2003).

ED-FMD is routinely expressed as the percentage change in arterial diameter (FMD%) from a resting baseline. Several studies have shown impaired FMD% in RA patients compared to controls (Fan et al, 2012; Chatterjee et al, 2012; Temiz et al, 2015). In one study median FMD% was significantly lower in RA patients compared to controls and according to the standard cut off value (FMD of 4.5%), 17 RA patients (48.57%) and 4 controls (11.43%) had abnormal FMD% (Chatterjee et al, 2012).

1.12.2 Arterial distensibility

Arterial distensibility is a measure of vascular elastic behaviour, it measures arterial ability to contract and expand with cardiac relaxation and pulsation. Decreased arterial distensibility alters arterial flow dynamics and disturbs coronary perfusion. In animal models, a there is an alteration in arterial distensibility in the early stages of atherosclerosis of the aorta, before other vessel wall changes such as increased vessel wall thickness and plaque formation (Hironaka et al, 1997). In humans, a decrease in distensibility (or increased artery wall stiffness) is related to risk factors for clinical vascular disease (Dart et al, 1991) and can predict those at increased risk of future coronary artery disease and stroke in apparently healthy individuals (Mattace-Raso et al, 2006). Of note, in the latter study, aortic pulse wave velocity was an independent predictor of coronary heart disease and stroke but carotid distensibility was not. However, this study used the brachial pulse pressure rather than the carotid pulse pressure, which may have led to an underestimation of the distensibility. In the atherosclerotic process, functional changes in the vascular wall may occur before anatomical changes such as intima media thickening or plaque formation. Previous studies have shown reduced carotid distensibility in women with RA compared to female controls and these changes correlated with disease severity (Turesson et al, 2005). There was no detected difference in men with RA compared to male controls.

1.12.3 Echocardiography

Transthoracic echocardiography (TTE) uses ultrasound to assess cardiac function and structure. It is safe, low cost and can also provide information on ischaemia when combined with pharmacological stress or exercise (Erhayiem et al, 2014). TTE is fairly easily available and well tolerated (Armstrong et al, 2012). However, adjacent structures can interfere with picture quality, obesity or acoustic shadowing from the lungs can be problematic, and reproducibility is limited by reporting variability

(Armstrong et al, 2012). Studies using TTE have found that patients with RA have a higher incidence of valvular heart disease, pericarditis and aortic plaques than controls (Guedes et al, 2001), and also higher incidence of abnormal left ventricular remodelling than controls (Myasoedova et al, 2013).

1.12.4 Cardiac MRI

Cardiac magnetic resonance (CMR) imaging can assess both anatomy and function of the heart, as well as tissue characterisation. It can assess left ventricular mass, aortic distensibility, myocardial strain and myocardial ischemia (Erhayiem et al, 2014). There is no radiation exposure, it is a reproducible method and provides better accuracy than TTE for several measurements (Armstrong et al, 2012). Unlike echocardiography, cardiac MRI can produce images without interference from adjacent bone or air. However, examination time is significantly longer than other non-invasive methods and requires more patient cooperation. Claustrophobic patients may have difficulty tolerating the procedure and installation and operation of the MRI equipment is expensive. Due to the magnets used in MRI, it is contraindicated in patients with ferromagnetic objects in situ, such as cardiac pacemakers and intracranial metal. In patients with RA mean left ventricular mass, left ventricular ejection fraction and cardiac output were both lower than in controls (Giles et al, 2010). Other studies have shown myocardial abnormalities in RA patients without known cardiac disease and abnormal CMR findings were associated with a higher RA disease activity (Kobayashi et al, 2010). There are differences seen in the RA population compared to the general population in terms of LV mass; although there are conflicting results in the literature. High LV mass in the general population is a poor prognostic indicator, but in RA patients, studies have shown reduced LV mass compared to pubished controls (Bissell et al 2014).

1.12.5 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/computed tomography (CT)

Positron emission tomography (PET)/ computed tomography (CT) imaging of atherosclerosis using the metabolic marker fluorodeoxyglucose (FDG) allows quantification of arterial inflammation in vessels and plaques (Tawakol et al, 2013). It was shown to improve prediction of future CVD events in the general population (Figueroa et al, 2013). However, resources are expensive and require advanced technical skill to perform and interpret, and not all centres will have the required equipment. RA patients were found to have significantly higher arterial FDG uptake compared with matched controls, after adjusting for atherosclerosis risk factors and statin use (Emami et al, 2014). In this study, arterial FDG uptake correlated with synovial activity. As well as arterial inflammation, a recent study has shown increased FDG uptake in the myocardium of RA patients compared to controls (Amigues et al, 2016).

1.12.6 Carotid Ultrasound Scan (USS)

High-resolution B-mode ultrasound is a non-invasive method, used to assess the atherosclerotic process in various arteries. Atherosclerosis does not take place uniformly in the arterial tree, preferentially developing at arterial branches and opposite to flow dividers, as these sites are exposed to low or disturbed blood flow and low shear stress (Warboys et al, 2011). Regions exposed to unidirectional high flow are spared. These findings may be due to the fact that in areas of low blood flow, atherogenic cells or material have better access to the endothelium. Also, in areas of low shear stress, adhesion molecules are induced on endothelial cells (O'Keeffe et al, 2009). Major sites for the atherosclerotic process are the coronary arteries, carotid and cerebral arteries, areas of the aorta and the large arteries to the lower extremities. The carotid arteries are easily accessible and atherosclerosis here reflects disease in other arteries such as the coronary arteries. Autopsy studies have shown positive correlations between carotid and coronary plaque burden (Mitchell and Schwartz, 1962). However, the presence of carotid plaque alone has not been sufficient to identify patients with inflammatory joints diseases at risk for CAD, and a combination of ultrasonographic measurements has been suggested for risk stratification in these patients (Svanteson et al 2017). Skeoch et al (2017) used Dynamic Contrast Enhanced MRI (DCE-MRI) and FDG-PET in RA patients. A higher prevalence of plaque calcification was noted in RA compared to controls, despite similar plaque size. There was also plaque inflammation detected in 12/13 RA patients scanned.

US allows assessment of plaque and measurement of intima-media thickness (IMT). IMT is the combined width of the intima and media layers of an artery, measured from the border between the vessel lumen and the intima and the border between the media and adventitia (Urbina et al, 2006). Far-wall carotid IMT (CIMT) accurately represents the intima-media thickness compared with direct histological examination, whereas near wall CIMT measurements may underestimate the intima–media thickness (Wong et al, 1993). Prospective longitudinal studies have shown that detection of plaque or CIMT thickening on USS are associated with an increased risk of myocardial infarction (Salonen and Salonen, 1991; van der Meer et al, 2004) and stroke (Gupta et al, 2015) in the general population. In RA, CIMT predicts development of CV events (Gonzalez-Juanatey et al, 2009). In this study RA patients without traditional CV risk factors who had CIMT values >0.90 mm had an increased risk of CV events over a 5-year follow-up period. Another study found that RA patients with carotid plaque had a higher risk of acute coronary syndromes (ACS); those with unilateral plaques had a 2.5 times increased risk and those with bilateral plaques had a 4.3 times increased risk (Evans et al, 2011). Also, mean CIMT has been shown to be significantly higher in RA patients (s (0.50 $\pm$ 0.16 mm) compared to controls (0.44 $\pm$ 0.09 mm) (Chatterjee et al, 2012).

In view of its merits of low cost, reproducibility, non-invasiveness, good correlation with coronary atherosclerosis and good predictive value in terms of CV risk, this thesis used carotid USS and measurement of CIMT and plaque as a surrogate marker for atherosclerosis, as well as measures of arterial distensibility.

1.13 Inflammation and CVD atherosclerosis in the general population

Although great progress has been made in the prevention and treatment of cardiovascular disease, it is still the leading cause of death in the Western world (Braunwald, 1997). Advances in basic science have established the fundamental role of inflammation in all stages of atherosclerosis, from plaque formation to instability and eventual rupture (Gonzalez-Gay et al, 2005; Hansson and Hermansson, 2011; Libby et al, 2011). Both the innate and adaptive immune responses are involved in atherosclerosis, regulated by various cytokines. To appreciate the role of inflammation in the atherosclerosis process it is important to understand the pathogenesis of atherosclerosis.

1.13.1 Pathogenesis of atherosclerosis

Lesions of atherosclerosis occur mainly in medium and large muscular and elastic arteries (Ross, 1999). As mentioned in section 1.12.6, atherosclerosis does not take place uniformly in the arterial tree, developing at arterial branches and opposite flow dividers, where there is often disturbed blood flow. Although thought of as a disease of aging, early lesions can develop in childhood. 'Fatty streaks', the earliest type of lesion, are seen in infants and young children (Napoli et al, 1997). These are in fact inflammatory lesions, consisting of T lymphocytes and monocyte derived macrophages (Stary et al, 1994).

Atherosclerotic lesions start to develop under a dysfunctional and leaky endothelium (Falk, 2006), augmented by factors such as high plasma LDL concentration (Ramji and Davies, 2015). As the process advances, endothelial cells may disappear, leaving denuded areas. Certain molecules and lipoprotein particles extravasate through this leaky endothelium into the subendothelial space (Falk, 2006). Here, lipoproteins become oxidized and therefore atherogenic. Once activated by various stimuli, the endothelium becomes activated and the expression of adhesion molecules such as

VCAM-1, ICAM-1, E selectin and P selectin are upregulated (Falk, 2006). Consequently, leucocytes, namely monocytes and T cells, are recruited to the endothelium, attracted to it by chemokines, such as CCL2 (Deshmane et al, 2009). These cells then migrate through the endothelium into the intima. Once here, monocytes differentiate into macrophages and these then ingest modified LDL to become foam cells (Libby et al, 2002). Sustained accumulation of modified LDL plus disturbed lipid homeostasis causes apoptosis and necrosis of foam cells (Ramji and Davies, 2015). This results in a necrotic core of the plaque and amplification of the inflammatory response (Moss and Ramji, 2016).

T and B lymphocytes are also found in the intima during lesion development. These lymphocytes, as well as vascular wall cells, secrete growth factors and cytokines that promote the migration and proliferation of smooth muscle cells (SMCs). SMCs also undergo characteristic phenotypical changes, can up-regulate adhesion molecules (Ikeda et al, 1993) and secrete cytokines (Ikeda et al, 1991). SMCs also express enzymes that can degrade the collagen and elastin in response to inflammatory stimulation (Libby et al, 2002). This allows SMCs to invade through the elastic laminae and collagenous matrix of the plaque (Libby et al, 2002). SMCs also secrete extracellular matrix (ECM) proteins that stabilise plaque by forming a fibrous cap over it.

1.13.2 Plaque rupture

As the plaque evolves, inflammatory mediators induce the expression of collagenases by foam cells within the intimal lesion and also inhibit collagen synthesis (Libby et al, 2002). This leads to thinning of the plaque fibrous cap, rendering it weak and susceptible to rupture (Bentzon et al, 2014). When a plaque ruptures, a gap in the fibrous cap exposes the thrombogenic core of the plaque to blood (Bentzon et al, 2014). This then leads to platelet aggregation, coagulation and thrombus, which causes most acute complications of atherosclerosis (Libby et al, 2002).

1.14 Biomarkers of inflammation in risk prediction in the general population

As suggested above, inflammation is involved in all stages of atherosclerosis. Several studies have shown relationships between various biomarkers of inflammation and cardiovascular risk, in apparently healthy individuals as well as in patients with cardiovascular disease. There are several biomarkers that have been studied. Epidemiological studies have shown that circulating CRP (Ridker and Cook, 2004), IL-6 (Ridker et al, 2000) and fibrinogen (Danesh et al, 2005) are associated with higher risk of CVD in the general population, independent of known CVD risk factors. In RA, levels of these pro-inflammatory molecules and cytokines are increased.

1.14.1 C-reactive protein (CRP)

For several reasons, CRP has been studied as a biomarker of CVD for clinical application. In healthy individuals without inflammatory diseases or infections, levels of hsCRP remain stable over long periods of time (Danesh et al, 2004). Most laboratories in developed countries used for routine clinical care also measure CRP and there is no diurnal variation (Meier-Ewert et al, 2001). CRP can be found within the atherosclerotic lesion, in the vascular intima, where it co-localizes with foam cells (Torzewski et al 1998). Most clinical studies report that CRP is an independent predictor of cardiovascular events (Paffen and DeMaat, 2006), even after adjusting for other cardiovascular risk factors such as age, smoking, obesity, hypercholesterolemia and hypertension. However, others have reported that although CRP predicts risk of MI, the increase in risk could be largely explained by the presence of other risk factors (Doggen et al, 2000). However, in RA, levels of CRP are generally much higher than the general population and are more subject to fluctuations (Aotsuka et al, 2005).

1.14.2 Interleukin-6 (IL-6)

Various studies have examined the association of IL-6 and cardiovascular risk and disease. Elevated IL-6 levels in healthy men were associated with increased risk for future MI independently of hs-CRP (Ridker et al, 2000). Levels of IL-6 are higher in those with acute coronary syndromes compared to those with stable angina (Ozdemir et al, 2008). High IL-6 levels are associated with increased mortality in patients with acute coronary syndromes (Biasucci et al, 1999). Among women with prevalent CVD, those with high IL-6 levels had >4-fold risk of death compared to women with low IL-6 levels (Volpato et al, 2001). There is an increasing understanding of the complexity of IL-6 signalling and several studies have studied the IL-6 receptor (IL-6R).

1.15 Reduction of inflammation can reduce risk of CVD in the general population

Large studies in the general population have highlighted that inflammation is a key driver of atherosclerosis. The JUPITER study (Ridker et al 2008), found that in a trial of over 15,000 apparently healthy individuals without hyperlipidaemia but with elevated high sensitivity CRP, statin reduced the number of major cardiovascular events. The CANTOS study found that Canakinumab, a monoclonal antibody targeting interleukin-1β, led to a significantly lower rate of recurrent CV events then placebo, independent of lipid lowering (Ridker et al 2017).

Studies have shown that statin therapy lowers CRP levels independently of lipid levels, supporting the notion that statins have anti-inflammatory effects (Zakynthinos et al, 2008). One study has shown that statins limit both protein and RNA levels of IL-6-induced CRP in human hepatocytes (Arnaud et al, 2005). In vivo animal and human studies show that statins can improve endothelial function. This improvement may be partly due to reduced LDL levels, however several studies have found that endothelial function is restored before significant changes in serum cholesterol levels are seen (Anderson et al, 1995). Statins also inhibit the expression of VCAM-1 and ICAM-1, therefore reducing immune-cell recruitment within the vessel wall and reducing inflammation (Jain and Ridker, 2005). Statins also induce eNOS accumulation within endothelial cells, therefore reducing the effects of reduced NO availability in the inflammatory setting (Jain and Ridker, 2005).

1.16 Animal models of RA and atherosclerosis

Animal models for both RA and atherosclerosis are well established. They provide crucial tools to investigate the pathophysiology of these diseases and to test therapeutic strategies in vitro prior to use in humans. Assessments of disease and treatment outcomes can be made in well-controlled environments, without having to allow for individual variability between human patients. It is also possible to obtain tissue from animal studies that would be difficult or impossible to obtain from living humans. Animal models of RA and atherosclerosis provide the unique opportunities to unravel the pathophysiological features of vascular dysfunction and cardiovascular disease in these settings.

1.16.1 Animal models of arthritis

Animal models have been used extensively in studies of rheumatoid arthritis. The aetiology of RA is multifactorial, with both genetic and environmental components. Presentation and progression of RA in humans is very variable. In animal models, conditions can be tightly controlled and thus results can be compared and data can be reproduced. Animal models of RA have significantly advanced our understanding of the pathogenesis of RA and contributed to several major advances in its treatment, most notably the development of anti-TNF agents.

In terms of studying the pathogenesis of cardiovascular disease in RA, it is not feasible to sample human vascular tissue. Therefore using animal models of RA allows us to gain a greater understanding of the functional and histological abnormalities within vascular tissue that may contribute to cardiovascular disease in RA. Studies have shown that even 10 years before disease onset in RA, patients have serum abnormalities such as presence of rheumatoid factors (Nielen et al, 2004) and antibodies against citrinullated self-proteins (Mandl et al, 2005). Also, patients who go on to develop RA have dyslipidaemia with a more atherogenic lipid profile than matched blood donors at least 10 years before onset of symptoms (van Halm et al, 2007). Patients with RA have rapid progression of CVD soon after RA onset (Innala et al, 2011). Animal models allow us to study the early, and even pre-disease RA phase.

Several animal models of inflammatory arthritis exist, each with their own advantages and limitations. Models can be induced, such as antigen induced arthritis or collagen-induced arthritis (CIA), or animals can be genetically manipulated to spontaneously develop arthritis, such as the TNF-alpha-transgenic mouse, K/BxN mouse, and the Skg mouse (Asquith et al, 2009). Table 2 summarises several rodent models of arthritis and their key features. In this thesis the CIA model was used for several reasons; its systemic nature, ease of induction, reproducibility of results, and previous experience of this model in the department. Moreover, there are previous reports of vascular dysfunction in the literature in this model.

Model	Animal Strain	Examples of arthritogens	Arthritogenic effector mechanism	Mechanism of action	Pattern of joint involvement	Pathological features	Vascular function
Adjuvant – induced arthritis	Male Wistar or Lewis rat	Heat-killed.*M. tuberculosis* H37Ra or *M. smegmatis*	-	T cell and neutrophil mediated	Polyarticular	Marked bone resorption, and periosteal bone proliferation	Reduced acetylcholine (ACh)-induced endothelium-dependent vasorelaxation (Prati et al, 2011)
Antigen Induced arthritis	Mouse/rat	Methylated bovine.serum albumin (BSA)	-	T cell mediated	Monoarticular	Cartilage and bone destruction	Unknown (no systemic manifestations)
Collagen Induced arthritis	Male DBA/1 mice	Type II collagen (CII)	-	T and B cell mediated, complement-dependent	Polyarticular	Cartilage and bone destruction, synovitis, and periosteal proliferation	Reduced ACh-induced endothelium-dependent vasorelaxation (He et al, 2013) Reduced aortic constriction (Reynolds et al, 2012)
Streptococcal cell wall induced arthritis	Rat	Group A/B/C streptococci	-	T-cell and monocyte-mediated	Polyarticular	Cartilage and bone destruction	Unknown
K/BxN	Mouse	-	Crossreactive autoantibodies against glucose-6-phosphate isomerase	Complement activation and mast cell degranulation	Polyarticular	Cartilage and bone destruction	Mild aortic valve inflammation (Binstadt et al, 2009) Vascular function unknown
SKG	Mouse	-	A defective thymic selection of T cells due to a mutation in the SH2 domain of ZAP 70	T cell mediated	Polyarticular	Cartilage and bone destruction	Unknown

Table 2. Summary of animal models of RA and effects on vascular function

1.16.1.1 Collagen Induced Arthritis

CIA has proven to be an important model of human RA, helping to define the role of many of its cellular and molecular mediators (Myers et al, 1997), and is the most widely studied model of RA. Murine CIA (mCIA) has been very important in the testing and development of numerous agents, such as tumour necrosis factor inhibitors. Arthritis is induced by immunisation with an emulsion of type II collagen (CII) (usually heterologous, chicken or bovine) and complete Freund's adjuvant; subsequently auto-antibodies to collagen II are produced.

CIA shares several pathological features with RA. Both are polyarticular, systemic diseases. The target tissue in RA is cartilage, and collagen II is a key protein here. In terms of histological findings, several shared pathological features between RA and mCIA include synovial hyperplasia, mononuclear cell infiltration, and cartilage degradation (Brand et al, 2007). In RA it is well established that inherited susceptibility is associated with genes encoding the human MHC class II molecules (HLA-DR4 and HLA-DR1 alleles) (Todd et al, 1988). Similarly, susceptibility to CIA is linked to expression of specific MHC class II genes (Brand et al, 2007).

Differences between mCIA and RA

There are differences between RA and mCIA. In mCIA rheumatoid factor is not present. There is no female preponderance as there is in RA, and CIA is generally monophasic, although some relapsing models have been described (Boissier et al, 1987). In mCIA animals develop antibodies to collagen, while this is not the case in a large proportion of patients with RA (Bevaart et al, 2010). Of note, neutrophils are abundantly present in the synovial tissue of mice with CIA (Suzuki et al, 1997), which is in contrast to RA synovium, where there are relatively few neutrophils, and more T cells, plasma cells, macrophages, and B cells (Tak et al, 1997). Also, in RA there are marked variations in the synovial cell infiltrate in biopsy specimens from different patients, even in early disease (Tak et al, 1997), reflecting the heterogeneity of RA.

Mechanisms of CIA development

The pathogenesis of CIA involves both T-cell and B-cell-specific responses to collagen II, a major protein constituent of joint cartilage. B cells are of critical importance as they produce antibodies to collagen II. Indeed, B cell-deficient mice are resistant to CIA (Svensson et al, 1998). The role of T cells in CIA are twofold; they provide help to B cells for the production of anti-CII antibodies (Corthay et al, 1999) and also play a role in joint inflammation through activation of other cells, e.g. synovial macrophages.

Anti-collagen II antibodies bind to collagen II in the joint, resulting in complex formation and complement activation, C3 deposition and cleavage of C5 (Wang et al, 1995). These events trigger a local inflammatory response during which T cells, monocytes and granulocytes are attracted to the joint, resulting in further immune activation and production of chemokines (Luross and Williams, 2001). At the time of the clinical onset of arthritis, IL-1, IL-10, TNFα, and IL-6 can be detected at the site of inflammation in the joint. More recently described cytokines such as IL-17, IL-21, IL-23, IL-32, and IL-33 have all been reported to aggravate CIA (Moudgil et al, 2011).

The symptoms of CIA begin around day 21 post immunisation. Within a few days after arthritis onset the affected joint shows an inflammatory reaction. The synovium, which is normally hypocellular, becomes infiltrated with macrophages, neutrophils and B and T cells. The histopathology is similar to that observed in RA, with formation of an erosive pannus tissue which invades cartilage and bone. Cartilage is damaged by matrix-degrading enzymes and osteoclasts induce bone erosion. This can lead to remodelling and ultimately destruction of the joint. The severity of arthritis and disease course varies depending on mouse strain, type and dose of collagen immunised. These factors are discussed in more detail in section 3.1.1. Most mouse strains are more resistant to development of arthritis after immunization with homologous (mouse) CII. However, some studies have shown that immunisation with homologous CII can produce a more chronic form of CIA, with periods of remission and exacerbations (Boissier et al, 1987; Holmdahl et al, 1985).

CVD and CIA

The risk of CVD associated with RA appears to precede the ACR criteria–based diagnosis of RA (Maradit-Kremers et al, 2005). Therefore the CIA model offers an excellent opportunity to identify early changes within the vasculature that lead to CVD.

Reynolds et al found a relationship between arthritic disease in DBA/1 mice and contractile dysfunction in rings of isolated aorta (Reynolds et al, 2012). However, contractile dysfunction was not accompanied by overt endothelial dysfunction as endothelium-dependent relaxation responses to acetylcholine were similar to those seen in tissues from non-immunized control mice. Also, relaxation responses to exogenous nitric oxide (NO) were unaffected. These findings suggest that there is sufficient endothelium-derived NO bioavailability during mCIA, and that vascular smooth muscle responds both to endogenous and exogenous NO to produce vasodilation. This is unexpected given the fact that endothelial dysfunction is found early in RA. However, the mouse model of CIA occurs over days rather than weeks or months, and it may possible be that if experiments were left to run for longer, endothelial dysfunction may have occurred.

Other studies have shown that CIA is associated with increased aortic expression of VCAM-1 (Denys et al, 2016). VCAM-1 mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium and has been shown to play a crucial role in early atherosclerosis (Cybulsky et al, 2001). Interestingly in this study by Denys et al, at 15 weeks after the first CIA immunisation, atherosclerotic plaque formation in the aorta was detected with CIA mice fed a high fat diet, and non-immunised mice fed a high fat diet, but not in CIA alone. Previous work in our department by Williams et al showed that CIA drives an increase in systemic inflammation, represented by increased macrophages in the aorta and perivascular adipose tissue (PVAT) of mice with CIA (Williams et al, 2016). This is relevant to RA patients, as they are known to have more inflammation in both their aorta (Greenburg et al, 2012) and in atherosclerotic plaques (Aubry et al, 2007) compared with non-RA controls. Also, we know that systemic inflammation in RA is associated with increased cardiovascular risk. Other work from our department has shown that vascular calcification is not responsible for the vascular dysfunction seen in CIA. This work also showed that aortic collagen and elastin become dysregulated during CIA and show a fibrosis like phenotype (unpublished yet).

1.16.2 Animal models of atherosclerosis

Mouse models of atherosclerosis have proved useful to study the development and progression of atherosclerotic lesions. Knockout and transgenic mouse models of atherosclerosis have been valuable in understanding the molecular and cellular mechanisms involved and in evaluating the effectiveness of treatment (Zadelaar et al, 2007). The predominant lipoprotein in wild type mice is HDL, making them relatively resistant to atherosclerosis (Getz et al, 2012). Therefore mice have been genetically modified to produce changes in lipid metabolism, making them prone to atherosclerosis. The extent to which these models serve as accurate models of human diseases is debateable. Unlike humans, mice rarely develop atherosclerosis in the coronary arteries but more so in the aortic root (Getz and Reardon, 2012). Also, atherosclerotic lesions tend to be less complex than human lesions (Getz and Reardon, 2012). Despite these drawbacks, mouse models of atherosclerosis provide valuable insights into lesion pathogenesis and the effect of various drugs on the disease.

There are several animal models of atherosclerosis. Large animal models include rabbits, pigs and nonhuman primates. Due to the relative ease of genetic manipulation and the relatively quick time frame for atherosclerosis development, mouse models are currently the most extensively used (Getz and Reardon, 2012). The most widely used knock out mouse models are the LDL receptor deficient mouse (LDLR$^{-/-}$) and the ApoE$^{-/-}$ mouse. The LDLR$^{-/-}$ mouse has delayed clearance of VLDL and LDL from plasma. They have a moderate increase of plasma cholesterol level and develop atherosclerosis slowly on normal chow diet (Zaragoza et al, 2011). Unlike the ApoE$^{-/-}$ mice, no systematic pathological analysis of lesion development in the LDLR$^{-/-}$ mice has been reported (Getz and Reardon, 2012), and they also do not develop a fibrous cap (Breslow, 1996). ApoE$^{-/-}$ mice have been created by homologous recombination in embryonic stem cells (Nakashima et al, 1994). These mice develop a range of atherosclerotic lesions from fatty streaks to fibrous plaques, distributed throughout the arterial tree. Atherosclerotic lesions show several features of the inflammatory-fibroproliferative response characteristic of atherosclerosis in humans (Nakashima et al, 1994). $ApoE^{-/-}$ mice do have some disadvantages; plasma cholesterol in this model is largely carried on lipoprotein remnants rather than the LDL, which is generally the most frequent carrier of cholesterol in human atherosclerosis (Getz and Reardon, 2012). Due to the complex lesions that develop rapidly and their comparability with human lesions, the $ApoE^{-/-}$ mouse was used in this thesis.

Relevance to RA patients

Although there are obvious differences in the $ApoE^{-/-}$ model and RA patients, RA patients are known to have dyslipidaemia more than ten years before the onset of arthritis, with higher total cholesterol, triglyceride and Apo B levels and lower HDLc levels than matched controls (van Halm et al, 2006). Interestingly, CRP had only a marginal influence on the differences in lipid levels between patients and controls in this study. Therefore, utilising the $ApoE^{-/-}$ model and enhancing systemic inflammation (for example by using tools to enhance IL-6 trans-signaling), presents a way to study the effect of dyslipidaemia and inflammation, processes that occur in early or even pre-RA. In addition, RA patients have increased inflammation in plaques and increased proportion of unstable plaques compared to non-RA patients at autopsy (Aubry et al, 2007). Using this mouse model also allows examination of other defined outcomes such as plaque size, expression of SCCPs in plaque and fibrous cap thickness.

1.17 IL-6 in RA and CVD

1.17.1 IL-6

IL-6 is a pleiotropic cytokine, a 26-kDa glycopeptide. It is produced by various cell types such as B cells, T cells, fibroblasts, osteoblasts, monocytes, and endothelial cells (Srirangan and Choy, 2010). It is involved in a wide range of biological activities including immune regulation, haematopoiesis and inflammation (Hunter and Jones, 2015). IL-6 has hormone like characteristics that affect many systems and processes such as lipid metabolism (Glund and Krook, 2008), neuropsychological behaviour (Rohleder et al, 2012) and the neuroendocrine and vascular systems (Hou et al, 2008). IL-6 deficiency can lead to compromised innate and adaptive immunity in mice (Kopf et al, 1994) and in humans (Puel et al, 2008).

Serum levels of IL-6 in a normal physiological state are low (2.6-6.5 pg/mL (Kim et al, 2011) but can increase rapidly in inflammatory states, such as infection. In severe inflammatory states, such as septic shock, levels can reach the ng/ml range (Calandra et al, 1991). In RA, serum levels range from around 1.5–234.0 pg/ml (Robak et al, 1998). In RA, IL-6 is seen at high levels in synovial fluid and IL-6 in the joint has been shown to induce pannus formation, osteoclast activation and mediate chronic synovitis (Srirangan and Choy, 2010). IL-6 can activate cells via two signaling pathways, IL-6 classical signaling and IL-6 trans-signaling, discussed in more detail below and outlined in Figure 3.

1.17.2 IL-6 signaling

In classical signaling, IL-6 binds to membrane bound IL-6R (mIL-6R). The complex of IL-6/mIL-6R then associates with another protein, gp130, which then dimerizes and instigates intracellular signalling (Jones et al, 2001). IL-6R is only present on certain cells, these are hepatocytes, and some leukocyte subpopulations, including monocytes, neutrophils, and some T cells and B cells (Rose-John, 2012). Thus, classical signaling only affects certain cells. In addition to signal transduction through mIL-6R, there is also ‘trans-signaling’. Here, soluble IL-6R (sIL-6R), which is generated by either alternative splicing or ectodomain shedding (Dayer and Choy, 2010), triggers gp130 signaling by first binding to IL-6 (Rose-John, 2012). As gp130 is ubiquitously expressed, trans-signaling enables IL-6/sIL-6R to activate cells that lack the IL-6R. SIL-6R is released by monoctyes and activated T cells (Briso et al, 2008). Importantly, unlike other soluble cytokine receptors such as soluble TNF-αR, sIL-6R does not act antagonistically, thus limiting the IL-6 cytokine activity, but rather acts as an agonist.

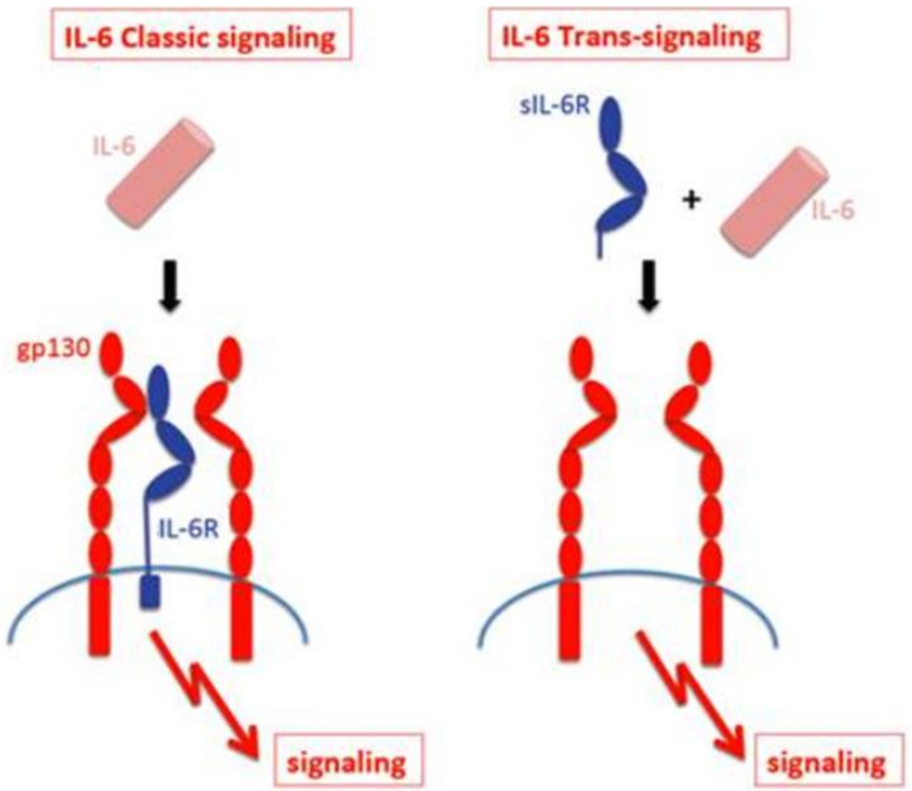

Figure 3. Two modes of IL-6 signaling. IL-6 classic-signaling requires membrane bound IL-6R and is therefore restricted to cells that express this: hepatocytes, some epithelial cells and some leukocytes. IL-6 trans-signaling requires sIL-6R and can affect any cell of the body since gp130 is ubiquitously expressed. (Adapted from Rose-John, 2012)

A soluble from of gp130, (sgp130) is found in the circulation. This is formed from translation from alternatively spliced mRNA (Rose-John, 2012). Sgp130 blocks trans-signaling by binding to the complex of IL-6/sIL-6R so that this cannot bind to gp130 (Figure 4). IL-6 alone does not interact with sgp130, therefore signaling via membrane-bound IL-6R is not inhibited by sgp130 (Jostock et al, 2001). Serum levels of sgp130 are around 400 ng/mL (Narazaki et al, 1993). sIL-6R levels are around 75ng/ml. As mentioned previously, serum IL-6 levels in a normal physiological state are 2.6-6.5 pg/mL. Thus, sgp130 is the most abundant protein, followed by sIL-6R and then IL-6 at much lower levels. This implies that under physiological conditions, IL-6 will be bound to sIL-6R, which is in turn bound to sgp130, and is, therefore neutralised. In extreme conditions such as sepsis, levels of IL-6 will increase profoundly and levels of sIL-6R increase. These will exceed levels of sgp130 which change little during inflammation (Rose-John, 2012), and IL-6 can therefore act systemically, by classical signaling if levels exceed sIL-6R, or by trans-signaling if sIL-6R levels are higher. What is not clear however, are specific levels of these cytokines and receptors at the sites of inflammation.

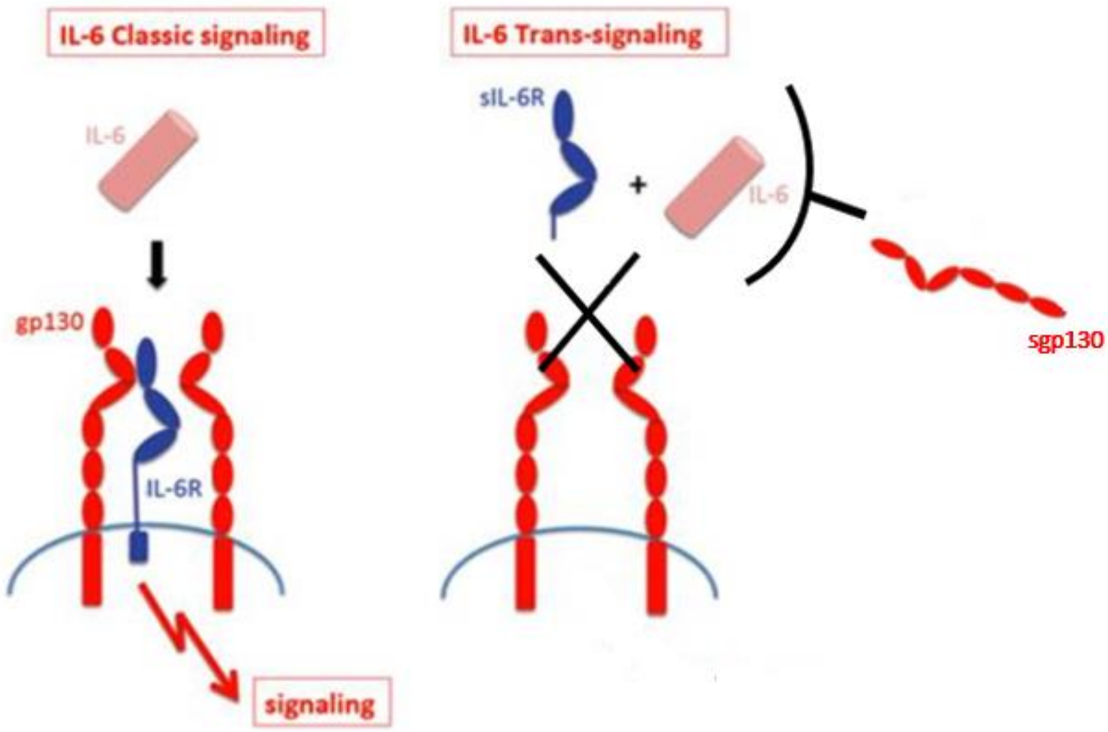

Figure 4. Blockade of IL-6 trans-signaling by sgp130. Sgp130 only binds to the complex of IL-6/sIL-6R and therefore only blocks IL-6 trans-signaling. Excess IL-6 will continue to bind to mIL-6R and classical trans-signaling will continue. Adapted from Rose-John, 2012.

JAK/STAT pathway

Once the complex of IL-6/IL-6R (either IL-6/mIL-6R or IL-6/sIL-6R) has bound to gp130, gp130 dimerises, leading to the activation of Janus Kinases and the activation of transcription factors of the STAT (signal transducers and activators of transcription) family. STATs are latent transcription factors that reside in the cytoplasm until activated. Gp130-associated kinases Jak1, Jak2, and Tyk2 become activated upon stimulation, and the cytoplasmic tail of gp130 is phosphorylated. This mediates the recruitment of STAT1, STAT3 and STAT5 proteins (Hunter and Jones, 2015). Subsequently, STAT3 also becomes phosphorylated, forms dimers and translocates to the nucleus, where it regulates transcription of target genes (Heinrich et al, 1998). These genes include those that encode acute-phase proteins. STAT3 also upregulates the transcription of genes encoding the SOCS3 proteins, which inhibit STAT3 activation (Nishimoto and Kishimoto, 2006).

1.17.3 Pro and anti-inflammatory activities of IL-6

There is increasing evidence that trans-signaling is pro-inflammatory whereas classical signaling has important regenerative or anti-inflammatory effects, such as regeneration of damaged intestinal epithelium and wound healing, discussed in more detail below. Two research tools have been developed to differentiate the activities of classical and trans-signaling. Hyper-IL-6 is a complex of IL-6/sIL-6R bound by a flexible peptide linker (Fischer et al, 1997). This specifically induces trans-signaling. Sgp130Fc is an engineered fusion protein which consists of the extracellular portion of gp130 linked to the Fc domain of a human IgG1 antibody. Sgp130Fc blocks IL-6 trans-signaling without affecting IL-6 classic signaling.

Evidence that trans-signaling is pro-inflammatory

In a mouse sepsis model using caecal puncture and ligation (CLP) classical versus trans-signaling was examined (Barkhausen et al, 2011). This model has a 60% mortality untreated. Mice given neutralising IL-6 antibody did not have a significant increase in survival but 100% of mice given sgp130Fc survived. CLP led to an upregulation of the acute phase protein serum amyloid A (SSA). This SAA induction was inhibited by global IL-6 blockade but was unaffected by treatment of the mice with sgp130Fc. Also, sgp130Fc inhibited epithelial apoptosis whereas anti IL-6 antibody did not.

In another experiment, mice were infected with Mycobacterium tuberculosis (Mtb) and administered either sgp130Fc or anti-TNF (Sodenkamp et al, 2012). With anti-TNF antibody treatment there was a 10-100 fold increase in mycobacterial colony forming units in the lung, liver and spleen. In contrast, administration of sgp130Fc did not interfere with protective immune responses after infection with Mtb and there was no increase of bacterial burden. Sgp130Fc-overexpressing transgenic (sgp130Fc^{tg}) mice were also infected with Mtb. These mice were capable of controlling mycobacterial growth. This is of importance in RA as other biological agents, especially anti-TNF agents are associated with an increased rate of reactivation of tuberculosis (Stallmach et al, 2010).

Table 3 summarises the effect of sgp130Fc on animal models of human diseases

Human disease	**Mouse model**	**Effect of sgp130fc**
Rheumatoid arthritis	Antigen induced arthritis (Nowell et al, 2003) Collagen induced arthritis (Nowell et al, 2009)	Reduced disease severity Reduced disease severity
Sepsis	Caecal puncture and ligation (Barkhausen et al, 2011)	Increased survival
Tuberculosis	Pulmonary infection with mycobacterium tuberculosis (Sodenkamp et al, 2012)	No interference with protective immune responses
Acute inflammation	Air pouch model (Rabe et al, 2008)	Reduction in infiltrating inflammatory cells
Atherosclerosis	$LDLr^{-/-}$ mice (Schuett et al, 2012)	Reduction in atherosclerosis
Ovarian cancer	Human ovarian tumour xenograft model in NOD/SCID mice (Lo et al, 2010)	Reduced ascites formation and enhanced tumour sensitivity to paclitaxel

Table 3 Effect of sgp130Fc on animal models of human diseases

Evidence that IL-6 has anti-inflammatory or regenerative effects

$IL\text{-}6^{-/-}$ mice exhibited less tumours but more inflammation than wild type mice in an inflammatory colon cancer model (Grivennikov et al, 2009). The $IL\text{-}6^{-/-}$ mice had impaired regeneration of the irritated intestinal epithelium. Another mouse model found that $IL\text{-}6^{-/-}$ mice failed to control bacterial numbers 2-3 weeks after infection with Citrobacter rodentium and exhibited increased mortality (Dann et al, 2008). From these studies it was concluded that the regenerative activities of IL-6 are needed for wound healing in the intestine.

$IL\text{-}6^{-/-}$ mice have impaired wound healing with reduced expression of IL-1, chemokines, adhesion molecules, transforming growth factor-β1, and vascular endothelial growth factor at the wound sites compared to wild type mice (Lin et al, 2003). In $IL\text{-}6^{-/-}$ mice there was reduced leukocyte infiltration, re-epithelialization, angiogenesis, and collagen accumulation. Wild type mice were also given neutralizing anti-IL-6 monoclonal antibody which significantly delayed wound closure. These findings suggest that IL-6 plays an important role in wound healing.

1.17.4 IL-6 and sIL-6R in RA

Serum IL-6 is higher in patients with RA than controls, correlates with CRP and duration of morning stiffness (Madhok et al, 1993). In RA, IL-6 is found at high levels in synovial fluid and IL-6 in the joint has been shown to induce pannus formation, osteoclast activation and mediate chronic synovitis (Srirangan and Choy, 2010). Several cell types within the joint can produce IL-6, but the main source is from fibroblast-like synoviocytes (Bartok and Firestein, 2010). Serum sIL-6R levels in RA patients have been found to be significantly higher than those of control subjects (Kohno et al, 1998). In this study, sIL-6R was detectable in synovial fluid, but at a lower level than in the serum, in contrast to IL-6 which was much higher in the synovial fluid than serum. One important source of sIL-6R is circulating neutrophils. Previous work by Jones et al (1999) found CRP causes a 3-fold increase in sIL-6R release by neutrophils. CRP levels are substantially elevated in patients with active RA. CRP-stimulated release of sIL-6R by circulating neutrophils could lead to an increase in circulating sIL-6R levels.

Tocilizumab, a humanised monoclonal antibody against the IL-6R is a licensed treatment for RA. This is an effective treatment; reducing disease severity, inflammatory markers and slowing progression in terms of radiologic damage (Smolen et al, 2011). Tocilizumab blocks both classical and trans-signaling (Rose-John, 2012). Although a very effective treatment for RA, several side effects have been reported. These include abnormalities in liver function tests (LFTs), increases in serum cholesterol levels, increase in infection rates, and a risk of gastrointestinal perforation if the patient has pre-existing diverticulitis (Navarro-Millán et al, 2012). These potential side effects are thought to be due to the blockade of classical signaling rather than trans-signaling.

1.17.5 IL-6 and sIL-6R in CVD

Inflammation contributes to the onset and pathogenesis of CVD in the general population. Epidemiological studies have shown that circulating CRP (Ridker and Cook, 2004) and IL-6 (Ridker et al, 2000) are associated with higher risk of CVD in the general population, independent of known CVD risk factors. High IL-6 levels are associated with increased mortality in patients with acute coronary syndromes (Biasucci et al, 1999). Both IL-6 and sIL-6R levels are increased in patients presenting with acute myocardial infarction compared to controls and those with stable coronary artery disease (CAD) (Anderson et al, 2013), but sgp130 showed no significant change between AMI, CAD, and control patients.

A non-synonymous allelic variant in the IL-6R gene (rs2228145):358Ala increases serum sIL-6R by 35% but reduces surface IL-6R expression (Ferreira et al, 2013). Two large-scale genetic and biomarker studies found a causal link between IL-6R gene polymorphism and development of CVD in the normal population (IL6R Genetics Consortium Emerging Risk Factors Collaboration, 2012; Interleukin-6 Receptor Mendelian Randomisation Analysis (IL6R MR) Consortium, 2012). However, in RA, IL-6R polymorphism is not associated with CVD (López-Mejías et al, 2011). This is not unexpected since sIL-6R level is 2-3 fold higher in RA patients than the general population (Usón et al, 1997), hence the impact of RA on circulating sIL-6R level is greater than IL-6R genotype.

Although IL-6R is associated with atherosclerosis, human endothelial cells express gp130 but not IL-6R. sIL-6R mediated trans-signaling is known to activate endothelial cells to express vascular adhesion molecules and bind neutrophils (Modur et al, 1997), inducing vascular inflammation. sIL-6R correlates with markers of endothelial function and inversely correlates with pulse wave propagation time (Weiss et al, 2013). A study by Klouche et al (1999) describes an autocrine stimulation loop of IL-6/sIL-6R. Human vascular SMC constitutively express only scant amounts of IL-6R and so do not respond to stimulation with IL-6. The study showed that SMC also do not constitutively express significant levels of gp130, so that they would not be appreciably sensitive to trans-signaling by the IL-6/sIL-6R complex. However, it was found that treatment of SMC with IL-6/sIL-6R induced up-regulation of gp130 mRNA and surface protein expression. This was accompanied by secretion of IL-6 by the SMC. Alongside this, there was induction and secretion of CCL2, up-regulation of ICAM-1, and marked cell proliferation. Thus, presence of the IL-6/sIL-6R seems to drives an amplification loop that causes a proinflammatory state of SMCs.

In animal models of atherosclerosis, there is evidence that IL-6 plays an important role, although specific pathways are not completely understood. Administration of exogenous IL-6 to $ApoE^{-/-}$ mice enhances atherosclerotic lesion formation, suggesting a pivotal role for IL-6 in plaque progression (Huber et al, 1999). In contrast, Schieffer et al showed that $ApoE^{-/-}$-$IL\text{-}6^{-/-}$ double knockout mice had higher serum cholesterol and increased atherosclerotic lesion formation compared to $ApoE^{-/-}$ mice (Schieffer et al, 2004). These experiments show that IL-6 plays a pivotal role in the atherosclerosis process but do not examine the role of classical versus trans-signaling. Another study found that administration of sgp130Fc reduced atherosclerosis in $LDLR^{-/-}$ mice (Schuett et al, 2012).

1.17.6 IL-6 effects of lipids

IL-6 has various and complex effects on lipid metabolism. IL-6 increases VLDLR expression in several tissues and this decreases triglyceride levels (Hashizume et al, 2009). In healthy volunteers administration of IL-6 caused an increase in total cholesterol (Lyngso et al, 2002). IL-6 was infused intravenously for 2.5 hours giving rise to circulating concentrations of approximately $35ngl^{-1}$. During infusion of IL-6 the heart rate, oxygen uptake and energy expenditure increased significantly and the respiratory quotient decreased significantly. The calculated lipid oxidation rate increased significantly while the carbohydrate oxidation rate decreased significantly. In the control study all parameters remained constant. IL-6 infusion gave rise to increased net glycerol release in subcutaneous adipose tissue while the net release of fatty acids did not change significantly. Conversely, administration of IL-6 to middle-aged and old rhesus monkeys (Ettinger et al, 1995), and cancer patients (Veldhuis et al, 1995) resulted in a decrease of total cholesterol levels.

In patients with myocardial infarction circulating IL-6 levels are correlated negatively with total cholesterol levels (Brugadas et al, 1996). The same is true of patients after major surgery (Akgun et al, 1998) and those on haemodialysis (Bologa et al, 1998). Interestingly, low serum cholesterol levels are among the most consistent predictors of mortality in patients with end-stage renal disease undergoing haemodialysis, thought to be due to cytokine-mediated acute-phase reaction to acute or chronic inflammation and in the latter study, IL-6 level was the strongest predictor of mortality.

1.18 SIL6R-regulated CVD Candidate Proteins (SCCPs)

As indicated in section 1.17.2, human endothelial and vascular smooth muscle cells do not express the IL-6R but can respond to the complex of IL-6/sIL-6R. Increasing data suggests that trans-signaling, which is regulated by sIL-6R, plays important roles both in RA and CVD. Previous work, using the human primary cell based BioMAP® system has examined biological pathways driven by sIL-6R (Tan et al, 2013). The BioMAP® system uses human primary cell based cultures and puts them under various stimulation conditions. Biomarker responses are measured and stored in a database. They can be used to predict outcomes, for example of a drug or cytokine. The results are quantitative and reproducible. The effects of sIL-6R on cells implicated in the pathogenesis of atherosclerosis have been examined. These cells examined were endothelial, peripheral blood mononuclear and coronary artery smooth muscle cells. sIL-6R was found to regulate the release of several proteins, including VCAM-1, Interleukin-8 (CXCL-8), Monocyte Colony Stimulating Factor (M-CSF), CXCL9, CCL2, Thrombomodulin, Tissue Factor and Matrix Metalloproteinase-1 (MMP-1). I have collectively called these proteins sIL-6R-regulated CVD Candidate Proteins (SCCPs). While there are various other proteins that are regulated by sIL-6R, I have selected those that have been implicated in cardiovascular disease. Many of these SCCPs have also been linked to rheumatoid arthritis. Table 4 summarises what is known about their role in CVD and RA. This thesis uses this data to investigate the association of these proteins in animal models of RA and atherosclerosis, and also in patients with RA, investigating their potential predictive role of CVD in RA.

Protein regulated by sIL-6R	**Levels in RA**	**Levels in CVD**
Matrix Metalloproteinase-1	Increased, correlates with disease activity (Green et al, 2003)	Elevated MMP-1 predicts presence of advanced CVD in normal population (Hwang et al, 2009)
CCL2	Increased in serum and synovial fluid (Koch et al 1992) Correlates with CIMT (Södergren et al, 2010)	CCL2 gene inactivation decreases progression of atherosclerotic lesions (Linic et al, 2013) Increased expression in atherosclerotic lesions (Egashira, 2003)
M-CSF	Increased in RA patients (Kawaji et al, 1995) Correlates with disease activity (Rioja et al, 2008)	Found in atherosclerotic plaques (Ait-Oufella et al, 2011) Risk factor adverse outcomes in angina & elevated in acute coronary syndromes (Saitoh et al, 2000) Strong predictors of cardiac events in those with angina (Rallidis et al 2004)
CXCL9	Increased in synovium (Iwamoto et al, 2008), unknown in serum	Increased expression in plaques (Mach et al, 1999)
VCAM-1	Correlates with disease activity (Navarro-Hernández et al, 2009)	Rapidly induced by proatherosclerotic conditions, including early lesions (O'Brien et al, 1993)
CXCL-8	Correlates with disease activity (Slavić et al, 2005)	Correlates with carotid intimal thickness in RA patients (Benucci et al, 2013)
Thrombomodulin	Conflicting data (Ichikawa et al 1993, Abdul-Monieum et al, 2001; Hanyu et al, 1999)	Decreased (Wei et al, 2011) Expression reduced in atherosclerotic lesions (Lasik et al, 2001)
Tissue factor	Unknown (involved in pannus formation (Chen et al, 2013))	Increased in plaques in unstable angina or myocardial infarction compared to stable angina (Annex et al, 1995). Serum levels higher in unstable than stable angina (Soejima et al, 1999)

Table 4. SCCPs in RA and CVD

1.19 Project summary

Patients with RA are at increased risk of cardiovascular disease compared to the general population. Atherosclerosis and RA are both diseases in which inflammation plays a pivotal role in pathogenesis and progression. IL-6 has been implicated in pathogenesis of both RA and CVD, in particular there is increasing evidence that IL-6 trans-signalling is pro-inflammatory. Although several studies have examined relationships between IL-6 and cardiovascular disease and/or rheumatoid arthritis, none have examined the role of IL-6 trans-signaling in CVD in RA. In this thesis animal models of RA were used to examine the role of IL-6 trans-signalling in vascular dysfunction in RA.

Patients with RA have increased markers of inflammation within atherosclerotic plaques compared to the general population. To examine the direct effect of IL-6 trans-signalling on atherosclerosis, an animal model was used, the ApoE$^{-/-}$ mouse. Here, for the first time, the effect of trans-signalling versus classical signalling was examined on plaque size.

Cardiovascular risk scores, even with modification as recommended by EULAR, underestimate CVD risk in RA patients. Previous work using the BioMAP® system has identified several proteins that are regulated by sIL-6R. Most of these SCCPs have been implicated in both CVD and RA. As CVD is increased in early RA, I have investigated the association of SCCPs with progression of subclinical atherosclerosis in this patient group. Potential candidate proteins may serve as predictors of progression of subclinical atherosclerosis or even as novel therapeutic targets for reducing CVD in RA.

1.20 Hypothesis and Aims

The overall aim of the thesis was to examine the role of IL-6 trans-signalling in cardiovascular disease in rheumatoid arthritis using both human and animal studies.

I hypothesise that sIL-6R-mediated trans-signaling is a major driver of CVD in RA. This hypothesis was addressed through the following aims:

1.

A. To investigate whether IL-6R KO mice are protected against vascular dysfunction in collagen-induced arthritis (CIA).
B. To investigate whether blocking IL-6 trans-signalling using sgp130Fc restores vascular function in CIA

2. To determine whether IL-6 trans-signaling accelerates atherosclerosis in Apo-$E^{-}/^{-}$ mice.

3. To determine whether SCCPs are associated with progression of CVD using carotid intimal thickness (CIMT) as a surrogate marker for subclinical atherosclerosis.

2 Chapter 2 - Materials and general methods

2.1 Chemicals and buffers:

Citrate buffer (0.2M): 8.4 g citric acid, was dissolved in 200 ml dH_20 then pH adjusted to 3.95 by the addition of a potassium hydroxide solution.

Tetramethylbenzadine (TMB): 240 mg of TMB was dissolved at a concentration of (0.1 M) in 5 ml of dimethyl sulphoxide (DMSO) and 5 ml of ethanol, and stored at 4°C.

ELISA Developing Buffer: was freshly prepared on each occasion by the addition of 10 µl Hydrogen Peroxide and 100 µl TMB to 10 ml of Citrate Buffer per 96 well plate.

Miller's elastin (Fisher Scientific catalogue number LAMB-1080-D): 1:1 dilution with distilled water.

Oxalic acid (Fisher Scientific, catalogue number 75688): 1% oxalic acid (1 g in 100 mL distilled water).

Potassium permanganate (Fisher Scientific catalogue number P/6520/53): 2 g of potassium permanganate was dissolved in 400 mL of distilled water.

Van Gieson stain solution: (Fisher Scientific 88011)

Ethylenediaminetetraacetic acid (EDTA) decalcification solution: 70g of (EDTA) in 900ml of PBS (pH 7)

S-nitrosothiol (SNO): SNO was made by adding 500ul of n-acetlycyteine to 500ul nitrite. This was kept on ice in the dark. A spectrometer was used to calculate to the molar concentration. Serial dilutions were performed to get concentrations of 1nM to 10mM.

Krebs Buffer

Reagent	mM
NaCl	109.17
KCl	2.68
KH_2PO_4	1.18
$MgSO_4.7H_2O$	1.22
$NaHCO_3$	25.00
Glucose	10.99
$CaCl_2.H_2O$	1.71

Table 5. Composition of Krebs buffer

High potassium Krebs buffer

Reagent	mM
NaCl	39.36
KCl	59.99
KH_2PO_4	1..18
$MgSO_4.7H_2O$	1.22
$NaHCO_3$	25.00
Glucose	10.99
$CaCl_2.H_2O$	1.71

Table 6. Composition of high potassium Krebs buffer

2.2 General methods

Specific methods are detailed in methods sections in each chapter

2.2.1 ELISA

Levels of SCCPS were measured using duoset ELISA development kits from R&D systems in accordance with manufacturer's instructions. Standards and samples were assayed in duplicate. Concentrations used in each ELISA are detailed in the relevant methods section in each chapter.

Protocol for ELISA on murine serum

Step	Process	Time
1	96-well ELISA plate coated with 50µl of capture antibody	Overnight
2	Excess liquid was removed from the wells by inverting and blotting against paper towels. Plate washed three times with 150µl per well of 0.1% (v/v) Tween in PBS	-
3	300µl blocking buffer to each well	1 hour minimum
4	Repeat step 2	-
5	Add 50µl of standard and samples. Cover and incubate for 2 hours at room temperature	2 hours
6	Repeat step 2	-
7	50µl detection antibody per well. Cover and incubate for 2 hours at room temperature	2 hours
8	Repeat step 2	-
9	50µl of streptavidin-HRP, cover and place in the dark	20 minutes
10	50µl of substrate solution to each well, cover and place in dark	Up to 10 minutes-observe colour
11	50µl stop solution to each well	
12	Immediately determine optical density of each well at 450nm.	

Table 7. Protocol for ELISA on murine serum

Protocol for ELISA on human serum

Step	Process	Time
1	96-well ELISA plate coated with 100µl of capture antibody	Overnight
2	Excess liquid was removed from the wells by inverting and blotting against paper towels. Plate washed three times with 150µl per well of 0.1% (v/v) Tween in PBS	-
3	300µl blocking buffer to each well	1 hour minimum
4	Repeat step 2	-
5	Add 100µl of standard and samples. Cover and incubate at room temperature	2 hours
6	Repeat step 2	-
7	100µl detection antibody per well. Cover and incubate at room temperature	2 hours
8	Repeat step 2	-
9	100µl of streptavidin-HRP, cover and place in the dark	20 minutes
10	100µl of substrate solution to each well, cover and place in dark	Up to 10 minutes-observe colour
11	50ul stop solution to each well	
12	Immediately determine optical density of each well at 450nm.	

Table 8. Protocol for ELISA on human serum

3 Chapter 3 - Effect of IL-6R mediated trans-signalling on Vascular Function in CIA

3.1 Introduction

As discussed in the general introduction, CIA offers an excellent model to examine the systemic effects of RA.

3.1.1 CIA in DBA-1 versus C57BL/6 mouse strains

For this thesis both the DBA-1 and the C57BL/6 mouse strain were used. It was shown previously that susceptibility to CIA is linked to the $H\text{-}2^q$ haplotype (DBA-1 mice) and that $H\text{-}2^b$ (C57BL/6) mice were less sensitive to CIA development (Stevenson et al, 2006). Several studies have since shown that it is possible to induce CIA in C57BL/6 but there is both reduced incidence and reduced severity of arthritis, compared with DBA/1 mice (Bevaart et al, 2010). Generally, in DBA-1 mice arthritis starts at around day 25-28 and arthritis incidence reaches 80-100% (Brand et al, 2007). In C57BL/6 mice arthritis can start at around day 30, but incidence is variable. Several groups have induced CIA in C57BL/6 mice (Campbell et al, 2000; Campbell et al, 1998; Kai et al, 2006), although there have been difficulties with reproducibility in this strain (Pan et al, 2004). Table 9 summarises the literature on mCIA in C57BL/6, in terms of reagents used to induce arthritis, time course and arthritis incidence. Some groups have also used lipopolysaccharide (LPS) to boost arthritis incidence and severity in C57BL/6 mice.

The main advantage of using the C57BL/6 strain is that most transgenic and knockout strains of mice are on his background. Indeed, the in-house colony of IL-6R knockout mice was on this background. However, CIA in DBA-1 mice is more reproducible and onset of arthritis is quicker than in C57BL/6. An ideal scenario would be to back-cross the genetically modified strain onto the DBA/1 background but this is time consuming and costly. C57BL/6 mice with CIA can develop a chronic form of CIA which resembles human RA in terms of disease course, histological findings, and response to drugs commonly used in RA (Inglis et al, 2007). In DBA-1 mice the T-cell proliferative response to type II collagen in immunised mice peaks before the onset of arthritis and is in decline by the time of disease onset; in C57BL/6 however the T cell response is strong and sustained (Inglis et al, 2007). Hence, the C57BL/6 strain may be more useful to assess T-cell activity and to evaluate T-cell-targeted therapies (Inglis et al, 2007).

For this chapter, we used both C57BL/6 and DBA-1 mice; IL-6 and IL-6KO mice are only available on the C57BL/6 background and DBA-1 mice were used as CIA in this model is better characterised than in C57Bl/6, and arthritis in C56BL/6 mice takes approximately 60 days to appear.

3.1.2 Vascular function in CIA

Investigating the effect of RA on vascular function is challenging to conduct on human vascular tissue. The CIA model offers a useful tool to assess early changes within the vasculature that lead to CVD. Previous work by Reynolds et al (2012) found a relationship between arthritic disease in DBA/1 mice and contractile dysfunction in rings of isolated aorta. Here, we were able to assess the effect of absence of IL-6 and IL-6R, and also the effect of selective blockade of IL-6 trans-signalling, on contractile dysfunction in CIA.

Paper	Age CIA induced, sex & source of mice	Reagents used and route given	Dose TB per mouse	Type collagen	Dose collagen per mouse	Incidence of arthritis	Time stopped	Used LPS?
Inglis et al, 2007	10-12wks male Harlan	ID Bovine, chicken, or mouse T2 collagen in CFA with *M. tuberculosis.*	150ug on day 0	Type II bovine, mouse or Chicken	200ug on day 0	Only chicken collagen induced arthritis - incidence 61.7%	2wks after onset (early arthritis) or 6-8wks after onset (late arthritis)	No
Campbell et al, 2000	>8wks, male & female, in house (B6, B6/N11, B6:129c, B6:C3HeB/FeJd)	ID T2 Chicken collagen and CFA with *M. tuberculosis*.	250ug on day 0 and 21	Type II Chicken	100ug on day 0 and 21	incidence of 60–70% by day 60	100 days	No
Asquith et al, 2010	12 wks, male, Harlan (Indianapolis)	T2 chicken collagen/ CFA ID day 0. T2 chicken collagen/PBS day 21 IP.	150ug on day 0	Type II chicken	200ug of collagen on d0 and 200ug in PBS on day 21	75% of mice receiving a normal diet and 54% of mice on high-fat diet developed diseaseby day 42.	Day 42	Yes (20-40ug on D26 if no arthritis
Bäcklund et al, 2012	7-12 wks male, in house	ID chicken or rat in CFA with Mycobacterium butyricum, or in IFA with M. tuberculosis.	166.5ug (M. butyricum or M. tuberculosis)	chicken or rat Type II	100 or 200ug on day 0. Some mice were boosted 5 weeks later with 50ug of CII in IFA	all three CII preparations induced arthritis in with a comparable phenotype (28% incidence)	50 days	No
Chu et al, 2003	8-10wks female, Jackson	ID Bovine CII and CFA with M tuberculosis.	125ug on day 0	Bovine type II	100ug bovine cII on day 0	3/30 (10%)	65	No
Pan et al, 2004	8-10 weeks male, Jackson (Bar Harbor, pathogen free	ID Bovine CII and CFA containing M. Tuberculosis.	200ug on day 0	Bovine type II	100ug on day 0 and then 100ug in IFA on day 21	5/35 Bl6 mice had arthritis by day 35 (14%)	Day 35 all stopped	No
Mould et al, 2008	Female 8-12 weeks. C57bl6/J	ID Chicken T2 collagen and CFA with M. tuberculosis.	125ug on day 0 only	Chicken type II	100ug . Then on day 8 OR day 21, 100ug collagen with IFA only (100ul).	First arthritis evident on day 25. 89% incidence in control c57bl6 mice .	Day 69	No
Geboes et al 2009	Female, Ludwig institute cancer research. Age unclear	Followed Campbell et al protocol	250ug on day 0 and 21	Chicken type II	100ug on day 0 and 21 mouse	60% incidence (5/9 wild type c57bl6), mean day of onset 27	Day 37	No

Table 9 Summary of CIA induced in C57BL/6 mice in the literature

3.1.3 Tools to investigate the role of IL-6 signalling on vascular function in CIA

Using IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice allows us to investigate the complete absence of IL-6 and IL-6R on vascular function in CIA. As functions mediated by IL-6R require binding to its receptor, it may be expected that IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice would have the same phenotype. Although they do share similarities, there have also been differences observed phenotypically. For example, whereas IL-6$^{-/-}$ mice have impaired wound healing, IL-6R$^{-/-}$ mice heal almost as well as WT mice (McFarland-Mancini et al, 2010). Here in Cardiff there are in-house colonies of IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice. It has been shown that IL-6$^{-/-}$ mice have delayed CIA onset and reduced CIA severity (Saiai et al, 1999), others have shown that IL-6$^{-/-}$ mice are completely protected from CIA (Alonzi et al, 1998). Vascular function in CIA in these mice has not been previously assessed. In this chapter, for the first time, we assess the induction of CIA in IL-6R$^{-/-}$ mice and compare with IL-6$^{-/-}$ mice. We also assess, for the first time, the effect of CIA induction in these mice on vascular function. The advantage of using knock-out mice is the knowledge that there is a complete absence of IL-6 or IL-6R. However, in these mice, both IL-6 signalling pathways are redundant and thus we cannot gather information about the role of classical versus trans-signalling in vascular function in CIA in this setting. Also, using knock-out mice has reduced clinical relevance as these mice have a total lack of cytokine activity, compared to a reduction in cytokine activity; the latter seen generally with anti-cytokine therapies. Also, the complete absence of IL-6 or IL-6R from conception may result in other cytokines being overexpressed. As mentioned in section 1.17.3, using sgp130Fc allows us to investigate the effect of selective blockade of IL-6 trans-signalling. However, using antibody blockade has several possible disadvantages in terms of practicalities: how well the antibody works depends on multiple factors including binding affinity of the antibody, dosing, tissue penetrance and technical ability of the investigator e.g. performing intraperitoneal injections.

3.2 Hypothesis and Aims

As can be seen from Table 9, there is variable penetrance and incidence of CIA in C57BL/6 mice. Therefore it was necessary to characterise CIA in these mice in our facility before inducing CIA in knockout strains and assessing vascular function. The vascular constriction response was also determined in these animals to assess the impact of CIA on vascular function. IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice were also used alongside sgp130Fc treatment to characterise the role of IL-6 and IL-6 trans-signaling in both CIA progression and associated vascular dysfunction. This chapter details methodology, results and discussion under the following hypothesis and objectives:

Hypothesis: IL-6R mediated trans-signalling is a major driver of vascular dysfunction in CIA.

In order to address this hypothesis the following five aims were identified:

1. To establish a protocol for the induction of CIA in C57BL/6 WT mice.
    a. Further, to characterise CIA in C57BL/6 mice in terms of incidence, time course, and severity of arthritis.

2. To assess vascular constriction and relaxation responses in aortic tissue from age and sex matched C57BL/6 mice with CIA and without CIA.

3. To assess the effect of complete absence of IL-6 signalling on CIA induction, using IL-6R$^{-/-}$ and IL-6$^{-/-}$ mice, and compare arthritis time course, severity and penetrance with CIA induction in wild-type age-and sex matched C57BL/6 mice.

4. To assess the effect of complete absence of IL-6 signalling on vascular constriction and relaxation responses in aortic tissue, using IL-6R$^{-/-}$ and IL-6$^{-/-}$

mice immunised with CIA compared with wild type immunised/arthritic C57BL/6 mice and non-immunised controls.

5. To assess the effect of blocking IL-6 trans-signalling using sgp130Fc on vascular function in DBA-1 mice with CIA.

3.3 Materials and methods

3.3.1 Sgp130Fc

Spgp130Fc was obtained from two sources, both of which had been used previously in the department. The first was from a gift from Professor Rose Jose in Kiel University. The second was from R&D systems (Q6PDI9).

3.3.2 Etanercept

The TNF receptor fusion protein, etanercept, was used as a positive control in experiments. This is an established treatment for rheumatoid arthritis and its efficacy has been proven in both mouse and human studies. It is a combination protein consisting of the extra-cellular portion of two of the 75 kd-TNF receptors (TNF-R2) for TNF combined with a human Fc portion of human lgG1 (Horiuchi et al, 2010). Etanercept (Enbrel®, Amgen) was sourced from the clinical rheumatology department in the University Hospital of Wales.

3.3.3 Mice

All work with mice was performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and under the authority of the Home Office Personal (I56CC73C0) and Project (30/2928) Licences. Wild type C57BL/6 mice (specific strain C57Bl/6JOlaHsd) were sourced from Harlan, UK. DBA-1 mice were sourced from Charles River, UK. Mice had free access to normal chow diet and water and had a light dark cycle of 12 hours. The temperature range in the animal house was 19-23°C and humidity 55 $\pm$ 10%. Mice were purchased at 7 weeks of age and allowed a 1 week settling in period before arthritis was induced at 8 weeks, unless otherwise stated. Mice were euthanised under schedule 1 of the United Kingdom Animals (Scientific Procedures) Act 1986 by inhalation of carbon dioxide and death confirmed by palpation.

3.3.4 Knockout mice

Specific knock out strains used (IL-6$^{-/-}$ and IL-6R$^{-/-}$) were sourced from in-house colonies. Due to breeding difficulties within these colonies, and in keeping with the ethical principles of replacement, reduction and refinement, both female and male mice were used to utilise available mice. Age and sex matched wild type C57BL/6 mice were used as controls. Knock out mice were 18 weeks old at day 0. Control wild type C57BL/6 mice (specific strain C57Bl/6JOlaHsd) were sourced from Harlan, UK at 17 weeks and were 18 weeks old at day 0.

Numbers, gender and strain immunised, along with age and sex-matched control are shown in Table 10.

Strain	Gender	Age at day 0	Number immunised with CIA	Number not immunised with CIA
C57BL/6 WT	Male	18 weeks	2	2
IL-6R^{-}/$^{-}$	Male	18 weeks	2	0
C57BL6 WT	Female	18 weeks	5	5
IL6^{-}/$^{-}$	Female	18 weeks	3	0
IL-6R^{-}/$^{-}$	Female	18 weeks	3	0

Table 10. Characteristics of knockout mice and matched wild-type mice undergoing CIA protocol

3.3.5 Establishing a protocol for induction of CIA in C57BL/6 mice

Two strengths of type II collagen (CII)/complete Freund's adjuvant (CFA) were used in this chapter. See Table 11 for a summary of the concentration/doses used in each protocol.

3.3.5.1 Preparation of CII/CFA (low dose)

Type II collagen (CII) from chicken sternal cartilage (Sigma Aldrich Ltd) (5mg) was dissolved overnight in 2.5ml of 10mM acetic acid and kept on a cold plate overnight at 4°C with continuous stirring. CFA was prepared by grinding 100mg heat-killed *Mycobacterium tuberculosis* H37Ra to a fine powder using a pestle and mortar, in a fume hood. To this, 20ml Freund's incomplete adjuvant was added, providing a final concentration of 5mg/ml. This was stored at -20°C.

3.3.5.2 Preparation of CII/CFA emulsion

At the time of immunisation an equal volume of CII solution and CFA were mixed and passed through a glass syringe 20 times to ensure thorough mixing. To check that an emulsion had formed a drop was placed on in water; if it floated it was of the correct consistency.

	Low dose CII		**High dose CII**	
	Dose per mouse on day 0 and 21	**Concentration per injection per mouse**	**Dose per mouse on day 0 and 21**	**Concentration per injection per mouse**
Type II collagen	100ug	1mg/ml	250ug	2.5mg/ml
Mycobacterium tuberculosis	250ug	2.5mg/ml	500ug	5mg/ml

Table 11. Doses of collagen and mycobacterium tuberculosis in low and high dose CII/CFA preparations.

3.3.6 Injection of mice with emulsion

Mice were anesthetised with isoflurane (4L/min) and oxygen (2-4L/min) and immunised with the collagen and CFA emulsion. Mice were injected on day 0 via the intradermal route. 50 µl was injected twice at adjacent sites, on the right lateral side of the tail base. From day 0 to day 21 mice were inspected at least 3 times per week to check general health status and injection sites. Two booster injections of 50 µL were given on day 21, on the left lateral side of the tail base. From day 20, as an analgesic, Temgesic (400 mcg/L) was crushed and suspended in the drinking water of the mice and changed daily.

3.3.7 Assessment of arthritis severity

Animals were observed daily from day 21 for the development of arthritis except where specified.

Clinical gradation of arthritis severity

Arthritis severity was scored in each paw using a scale from 0 to 5, see Table 12. The sum of the four paw scores was calculated for each mouse, this was the total paw score. Paw swelling was also measured in hind paws daily for each mouse using a spring calliper gauge. Mice were also weighed and inspected daily.

Severity limits

If any mouse reached any one of the following at any point in the experiment it would have been removed from the experiment and killed. These humane end points were defined by the procedure project licence:

- A paw score of 5 in one paw or a combined score above 14
- A greater than 20% weight loss in two consecutive weigh-ins

Scoring arthritis severity

Individual paw score	Clinical features
0	No evidence of erythema or swelling
1	Erythema and mild swelling confined to the tarsals or ankle joint
2	Erythema and mild swelling extending from the ankle to the tarsals
3	Erythema and moderate swelling extending from the ankle to metatarsal joints
4	Erythema and severe swelling encompasses the ankle, foot and digits
5	Deformed paw/ankylosis

Table 12. Individual Paw Scoring System in mCIA

3.3.8 Isolating blood serum

Once palpation had confirmed death of the mouse a cardiac puncture was performed to remove blood. Using a 25 gauge needle, approximately 1ml of whole blood was taken. This was placed into vacutainers coated with clot activator and stored on ice. Samples were then centrifuged at 1600g for 10 minutes at 4°C. The serum was then aliquoted in 50ul aliquots and stored at -80°C.

3.3.9 Dissection technique

The dorsal skin, peritoneum, ribs and lungs were removed to expose the heart. The abdominal aorta was observed and snipped distally. The left ventricle of the heart was perfused slowly with 1ml of Krebs solution to gently flush the aorta. The thoracic aorta was exposed and carefully dissected so that any force on it was kept to a minimum. This was placed in fresh Krebs buffer for use in myography. Under a dissecting microscope, fat was removed from half of the aorta and two 2mm rings of thoracic aorta were cut for use in myography. The rest of the aorta was placed in 70% (v/v) methanol for 1 week for use in histology.

Hind paws were removed, and then fixed in neutral buffered formal saline for 1 week prior to processing for immunohistological evaluation.

3.3.10 Assessment of Vascular Function- Myography

Isometric tension studies using a myograph were used to assess vascular function ex vivo in isolated aortae. The thoracic aorta was cut into rings of 2 mm length, using a ruler for scale. Each ring was mounted in separate wells of a Mulvany myograph (Danish Myo-Technology, Aarhus, Denmark) and bathed in Krebs buffer for 20 minutes. Each ring was held between two prongs, one connected to a force transducer to allow ring tension measurement and the other connected to a micrometer to allow manual increase in tension. Each well was continually gassed with 95% oxygen/5% carbon dioxide. The temperature was maintained at 37°C by the steel block that the myography well was placed on. Myograph output was recorded and analysed using Myodaq and Myodata (Aarhus, Denmark) software respectively.

After mounting, the rings were subjected to equilibration for 20 minutes and it was ensured there was no tension on the rings. Each ring was then zeroed (the tension set at 0 mN). Then, over a period of 10 minutes, the tension on each ring was increased by 0.5mN per minute to reach a baseline tension of 5mN. This baseline tension had been used previously in DBA-1 mice by Reynolds et al (2012). Tissues were then rested for 20 minutes and the tension adjusted so that it was maintained at 5 mN. Tissues were then exposed to 60 mM K^+ for 5 minutes to condition the vessels to contraction. Baths were then emptied and refilled with fresh Krebs buffer 8 times to wash them, and then left to re-equilibrate for a further 20 minutes in 5 ml fresh Krebs. If the baseline tension had altered during this time then the tension was manually adjusted to reach 5 mN. Tissues were then sequentially exposed to increasing concentrations of 5-hydroxytryptamine (5-HT) (10^{-9} to 10^{-5}M) in half log increments.

For experiments also examining relaxation responses tissues were then washed 8 times, left to re-equilibrate for 20 minutes and then re-constricted to using 5-HT to reach 80% of their maximal constriction established previously. This was usually achieved with a final 5-HT well concentration of 10^{-7}. Once the vessel constriction had reached a plateau at 80% of their maximal constriction, increasing concentrations were added of Acetylcholine (ACh) (10^{-9} to 10^{-5}M), in half log increments. ACh causes relaxation of the vessel via endothelium-derived nitric oxide generation. Tissues were then washed and reconstricted to 80% maximal with 5-HT. Increasing concentrations of S-nitrosothiol (SNO) (see Section 2.1 in general methods) were then added (10^{-9} to 10^{-5}M), in half log increments. SNO causes relaxation of the vessel via exogenously donated nitric oxide.

3.3.11 Myography analysis

The myodata programme was used to record constriction and relaxation responses. 5-HT-induced contraction responses were calculated as the increase in tension above the resting tension. Relaxation responses to ACh and SNO were measured and expressed as a percentage of the 5-HT-induced tone.

3.3.12 Histological assessment of arthritis severity

Sample preparation

Hind paws were cut so that the knee and ankle were separated at the mid tibia. Excess fat and connective tissue was removed and then joints were placed into histocassettes.

Decalcification

In order to section the joints for histology they were decalcified with EDTA for 2 weeks at 4°C. The EDTA solution was changed every 2-3 days. In order to ensure bones were decalcified, radiographs were taken of the joints using a Kodak FX-Pro after 2 weeks.

Shandon tissue processor cycles

Joints were dehydrated using a Thermoshandon Tissue Processing Path Centre machine. The paws were immersed in increasing concentrations of ethanol; 70% (v/v) for 30 minutes, 90% (v/v) for 90 minutes, 100% ethanol for 60 minutes (repeated 3 times). Paws were then immersed in xylene for three incubation of an hour each. Joints were then immersed in four changes of wax and then removed from the tissue processor.

Tissue embedding

After removal from the tissue processor joints were set in paraffin wax using the Thermoshandon Histocentre.

Sectioning of embedded joints

Serial 7µm thick sections of wax-embedded joints were cut using a microtome and mounted on Superfrost Plus glass slides. Slides were then placed for 3 hours in the oven at 56°C to ensure adherence of the tissue to the slide and also remove paraffin from tissue sections.

3.3.13 Haematoxylin and Eosin Staining

This staining method allows grading of severity of arthritis histologically. Haematoxylin stains nuclei purple and eosin stains cytoplasm and connective tissue pink. This allows distinction between different parts of the joint and identification of changes in the normal structure.

After sectioning and baking of the slides were treated as follows:

Process	Rationale	Time
3 X xylene washes	Slide rehydration and wax clearing	3 X 5 minutes
2 X 100% IMS washes	Slide rehydration	2 X 3 minutes
90% IMS wash	Slide rehydration	3 minutes
Distilled water	Equilibrate	5 minutes
Haematoxylin	Nuclear stain	1 minute
Running tap water	Removal of excess haematoxylin	Until water runs clear
Distilled water	Equilibrate	1 minute
Eosin	Eosinophilic structure stain	30 seconds
Running tap water	Removal of excess eosin	Until water runs clear
Distilled water	Equilibrate	1 minute
90% ethanol	Slide dehydration and wax clearing	3 minutes
2 X 100% ethanol	Slide dehydration and wax clearing	2 X 3 minutes
3 X xylene	Slide dehydration and wax clearing	3 X 5 minutes
Mount with DPX and coverslip	Preservation of staining	

Table 13. Haematoxylin and Eosin Staining protocol

3.3.14 Grading of arthritis severity histologically

Sections were scored for synovial hyperplasia, cellular infiltration, cellular exudate, and cartilage and bone degradation, with 0 representing a normal joint, outlined in Table 14. The maximal score for each joint was 14.

Component	**Subcomponent**	**Grade**
Subsynovial inflammation	• Normal • Focal inflammatory infiltrates, adiposity hardly affected (10% inflammatory cells, 90% adipose tissue) • Focal inflammatory infiltrate equals adiposity (50% inflammatory cells, 50% adipose tissue) • Random inflammatory infiltrates dominating cellular histology (70% inflammatory cells, 30% adipose tissue) • Substantial inflammatory infiltrate with severe loss of adiposity (90% inflammatory cells, 10% adipose tissue) • Ablation of adiposity due to inflammatory infiltrate (100% inflammatory cells, 0% adipose tissue)	0 1 2 3 4 5
Synovial exudate	• Normal • Evidence of inflammatory cells in space • Moderate numbers of inflammatory cells in space, possibly with evince of fibrin deposits. • Substantial number of inflammatory cells with large fibrin deposits	0 1 2 3
Synovial hyperplasia and pannus formation	• Normal (1-3 layers thick) • Over three–layer thick synovial lining, showing evidence of thickening and/or invasion of joint space • Over three–layer thick synovial lining 'creeping' over cartilage surfaces and/or finger-like processes into joint pace • Over three layer thick synovial lining showing substantial covering of cartilage surfaces with evident cartilage loss	0 1 2 3
Cartilage/bone erosion	• Normal • Detectable loss of cartilage • Detectable erosion of underlying bone by pannus activity • Pannus has destroyed a significant part of the bone	0 1 2 3

Table 14. Histological grading system for arthritis severity in mCIA

3.3.15 Immunohistochemistry

The following protocol was used for immunohistochemistry to determine expression of pSTAT3 (Table 15). Antibody concentrations are outlined in Table 16. Identification of pSTAT-3 (phosphorylated STAT3) was carried out. pSTAT3 was measured to allow identification of tissues activated by IL-6; in response to IL-6 (either classical signaling or trans-signaling), STAT3 is phosphorylated.

Process	Rationale	Time
Xylene	Rehydration and wax clearing	2 X 5 minutes
100 % ethanol	Slide rehydration	2 x 5 minutes
90% ethanol	Slide rehydration	5 minutes
70% ethanol	Slide rehydration	5 minutes
Distilled water	Equilibrate	5 minutes
Citrate buffer in water bath at 95°C	Antigen retrieval	60 minutes
Allow to cool on bench		30 minutes
Distilled water	Wash	2 x 5 minutes
3% H_2O_2	Peroxidase block	10 minutes
Distilled water	Wash	5 minutes
Distilled water	Wash	5 minutes
10% normal goat serum in TBS/T	Decrease non-specific binding of antibody	60 minutes
Avidin block	Decrease non-specific binding of antibody	10 minutes
TBS-T	Wash	5 minutes
Biotin block	Decrease non-specific binding of antibody	10 minutes
TBS/ T	Wash	5 minutes
Primary antibody or isotype control antibody	Identification of target protein and control IgG staining	Overnight
TBS/T	Remove excess antibody	3 x 5 minutes
Detection antibody	Identification of bound primary antibody	1 hour
TBS/T	Remove excess antibody	3 x 5 minutes
Streptavidin-HRP	Identify bound Antibody in sections	20 minutes
TBS/T	Wash	3 x 5 minutes
DAB substrate	Allow identification of positive staining	1-20 minutes
Distilled water	Rinse	1 minute
Harris Haematoxylin	Nuclear stain	1-15 seconds
Running tap water	Remove excess stain	Until water runs clear
70% ethanol	Dehydration of slides and clearing	5 minutes
90 % ethanol	Dehydration of slides and clearing	5 minutes
2 x 100 % ethanol	Dehydration of slides and clearing	2 x 5 minutes
2 x Xylene	Dehydration of slides and clearing	2 x 5 minutes
Mount with DPX and coverslip	Preservation of staining	

Table 15. Immunohistochemistry protocol for pSTAT3

Reagent	pSTAT3
Primary antibody	Rabbit anti mouse pSTAT3 (cell signalling technologies) 40ug/ml
Primary antibody concentration	1:50
Isotype	Rabbit IgG 1.622mg/ml
Isotype concentration	1:50
Secondary antibody	Biotinylated Goat Anti-Rabbit IgG Antibody (vector laboratories)

Table 16. pSTAT3 immunohistochemistry reagents and concentrations for ELISA

3.3.16 Assessing the effect of blockade of IL-6 trans-signaling on CIA

3.3.16.1 Establishing a concentration, dosing schedule, source and route of administration of sgp130Fc to inhibit CIA

For all CIA experiments in DBA-1 mice, low dose CII/CFA emulsion was used and mice were immunised on day 0 and day 21, in the same method as in C57BL/6 mice.

Figures 5, 6 and 7 are schematic representations of the experimental approach and Table 17 summarises the experimental approach.

3.3.16.2 Dose and dosing schedule of sgp130Fc

Sgp130Fc has been previously used in the department to inhibit CIA. Previous studies by Nowell et al 2009 were used to calculate the dose of sgp130Fc. The half-life of sgp130fc is 72 hours (Sommer et al, 2014). In experiment 1 Sgp130Fc at 2.5mg/kg was given from day 21, on alternate days, for 7 doses (see Figure 5 for schematic representation). An age and sex-matched group were immunised with CIA and administered sterile PBS 100ul IP on alternate days from day 21 for 7 doses. Mice were sacrificed on day 34 and myography was performed. As these myography experiments were time consuming and required great accuracy, 3 mice were killed per day. Therefore to ensure mice were all sacrificed on day 34, day 0 and day 21 were staggered so that 2 mice were immunised with low-dose type II collagen and CFA on each day 0 and 21.

As previous work in DBA-1 mice in the department found that arthritis severity can increase rapidly from day 21, another dosing schedule of sgp130Fc was used, with increased frequency of administration. Here (Experiment 2, Figure 6), sgp130Fc 2.5mg/kg was administered daily from day 21 to day 27. An age and sex-matched group were immunised with CIA and administered sterile PBS 100ul IP daily from day 21 to day 27. All mice were sacrificed on day 28. As myography was performed on all mice, to ensure accuracy whilst performing experiments, 3 mice were killed per day. Therefore day 0 and day 21 were staggered so that 2 mice were immunised with low dose type II collagen and CFA on each day 0 and 21.

3.3.16.3 Route of sgp130Fc administration

Previous work in the department has administered sgp130Fc intraperitoneally. Sgp130Fc is a large protein, its molecular weight is 186kDa. Previously studies have also administered sgp130Fc intravenously with good effects in monkeys with CIA (unpublished). Therefore, in Experiments 1 and 2, sgp130Fc was administered intraperitoneally. Following this, as IP sgp130Fc did not work as was previously found, in experiment 3, spg130Fc was administered intravenously.

In experiment 3 (Figure 7), sgp130Fc was administered at 2.5mg/kg on day 21 and again on day 28. In this experiment, etanercept served as a positive control and mice were immunised with CIA and administered 2.5mg/kg etanercept on day 21 and day 28. Mice were sacrificed on day 30. So that myography could be performed accurately, the experiment was run in two batches and the immunisation days for each batch were staggered.

3.3.16.4 Control mice

For all experiments, a group of mice served as age and sex-matched non –immunised controls.

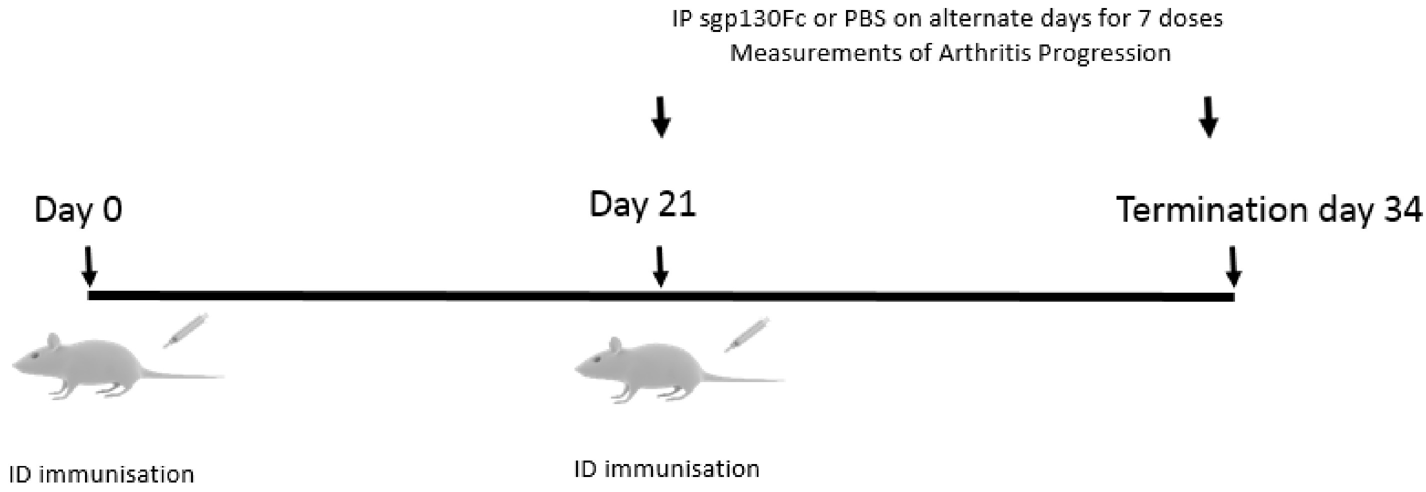

Figure 5. Representation of experimental procedure for treatment of mice with sgp130Fc IP, Experiment 1

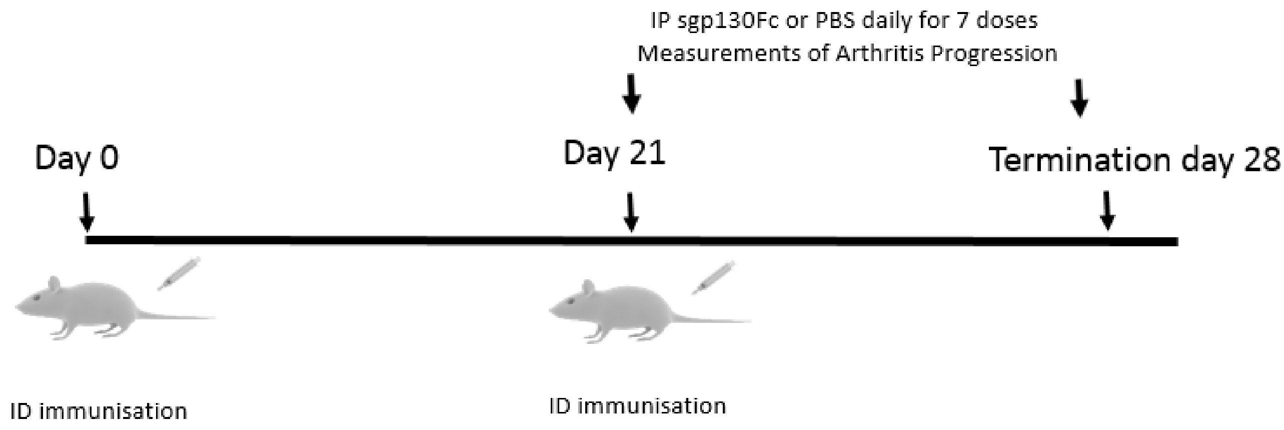

Figure 6. Representation of experimental procedure for treatment of mice with sgp130Fc IP, Experiment 2.

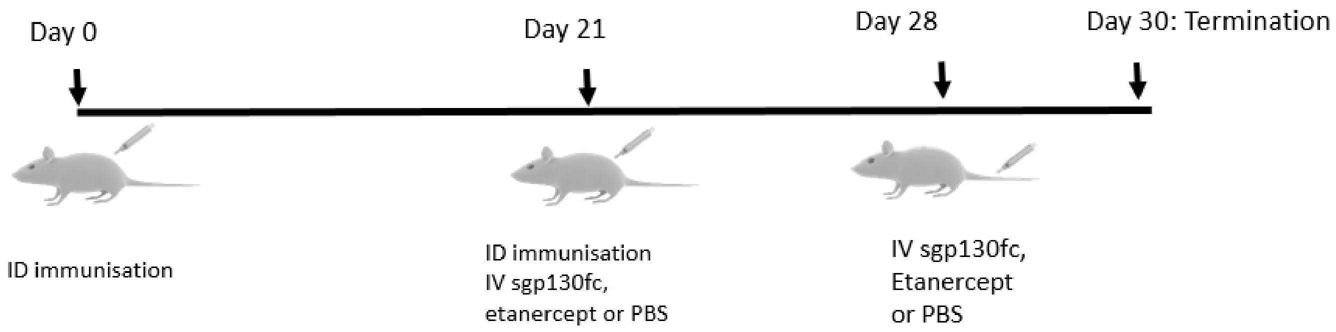

Figure 7. Representation of experimental procedure for treatment of mice with either IV sgp130Fc, PBS or etanercept, experiment 3.

	Experiment 1	**Experiment 2**	**Experiment3**
Concentration of sgp130Fc	2.5mg/kg	2.5mg/kg	2.5mg/kg
Source of sgp130Fc	Kiel University	R&D systems	Kiel University
Day of first sgp130Fc administration	21	21	21
Frequency of administration	Alternate days	Daily	Weekly
Route of sgp130Fc administration	IP	IP	IV

Number of doses of sgp130Fc	7	7	2
Day experiment ended	34	28	30
Number mice immunised with CIA and administered sgp130Fc	4	4	10
Number mice immunised with CIA and administered PBS	4	4	10
Number of non-immunised controls	4	4	10

Table 17. Summary of experimental approach of blocking IL-6 trans-signaling using sgp130Fc in CIA in DBA-1 mice.

3.3.17 ELISA measurements of CCL2, VCAM-1 and sgp130

It has been shown in human studies that CCL2 and VCAM-1 are associated with both rheumatoid arthritis and cardiovascular disease. Both are SCCPs and have been found, using the Biomap system, to be regulated by IL-6 trans-signaling. To determine whether sgp130Fc was detectable in the circulation of mice to whom it was administered, human sgp130 was measured in mouse serum.

Approximately 1ml of whole blood was obtained from mice after sacrifice, from which 200 µl of serum was aliquoted after centrifuging at 4°C at 10,000 rpm for 10 minutes. Serum CCL2, VCAM-1 and sgp130 were measured using duoset ELISA development kits from R&D systems in accordance with manufacturer's instructions, at concentrations in Table 18. See Table 7 in general methods for ELISA protolol for murine serum.

The optical density was then immediately measured at 450nm. A standard curve was plotted, and protein concentrations were calculated from this.

Protein measured	**Capture antibody working concentration**	**Detection antibody working concentration**	**High standard concentration**
Mouse CCL2	200 ng/ml	50 ng/ml	250 pg/ml
Mouse VCAM-1	1 µg/ml	100 ng/ml	8000 pg/ml
Human Sgp130	4 µg/ml	400 ng/ml	10000 pg/ml

Table 18. Antibody and standard concentrations used in ELISA in CIA experiments

3.3.18 Statistics

Statistics used were dependant on the experiments performed. Where two groups were compared the paired means student's t-test was used. Where multiple groups are compared, a one way ANOVA and post hoc Bonferroni test were performed. All results were expressed as the mean $\pm$ SEM. Differences that were considered significant were $p < 0.05$.

Sample size estimation

This was based on a previous study in CIA in C57BL/6 mice showing 75% of animals developed an inflammatory arthritis (Asquith et al, 2010). A reduced incidence of arthritis in 30% of animals is considered biologically relevant. A group size of 12 animals is necessary to achieve 90% statistical power p<0.05 in a primary ANOVA screen followed by post-hoc Bonferroni corrected T-test. However, taking into consideration the principles of reduction in animal studies, less animals were used in the first instance.

3.4 Results

All results including text, tables and error bars in figures are mean $\pm$ SEM.

Establishing a protocol for CIA induction in C57BL/6 mice

3.4.1 No clinical or subclinical arthritis in C57BL/6 mice using low dose collagen and CFA

There was no clinical sign of arthritis 66 days after the initiation of the CIA protocol using low dose CII/CFA regimen. Non-immunised control mice had a trend towards greater weight gain than immunised mice from day 0 to 66 (7.1 $\pm$ 0.65 g versus 5.7 $\pm$ 0.5 g) but this was not significant (Figure 8). Although there was no arthritis clinically, four hind paws were taken from four immunised mice and four control mice to check for subclinical inflammation. There was no difference in histology between immunised mice and controls, all paws were scored as 0. Figure 9 shows examples of knee and ankle joint from C57BL/6 non-immunised control mice and immunised, non-arthritic mice.

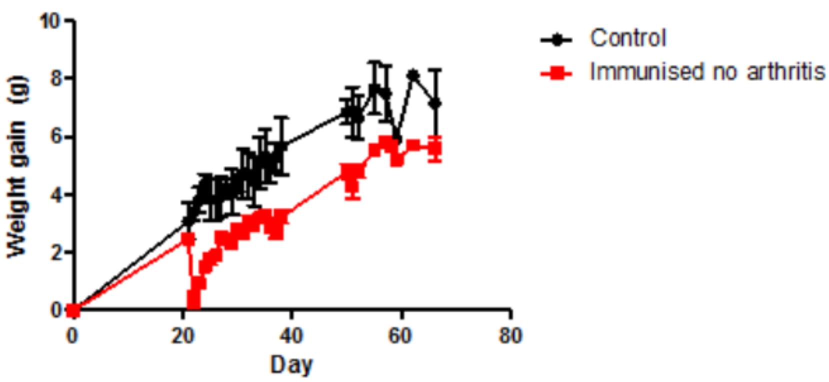

Figure 8. Mean weight gain for control mice versus C57BL/6 mice immunised with CIA. No significant difference between mean weight gain in C57BL/6 non-immunised control mice (7.1 $\pm$ 0.65 g), n=4 and immunised, non-arthritic mice (5.7 $\pm$ 0.5 g), n=8.

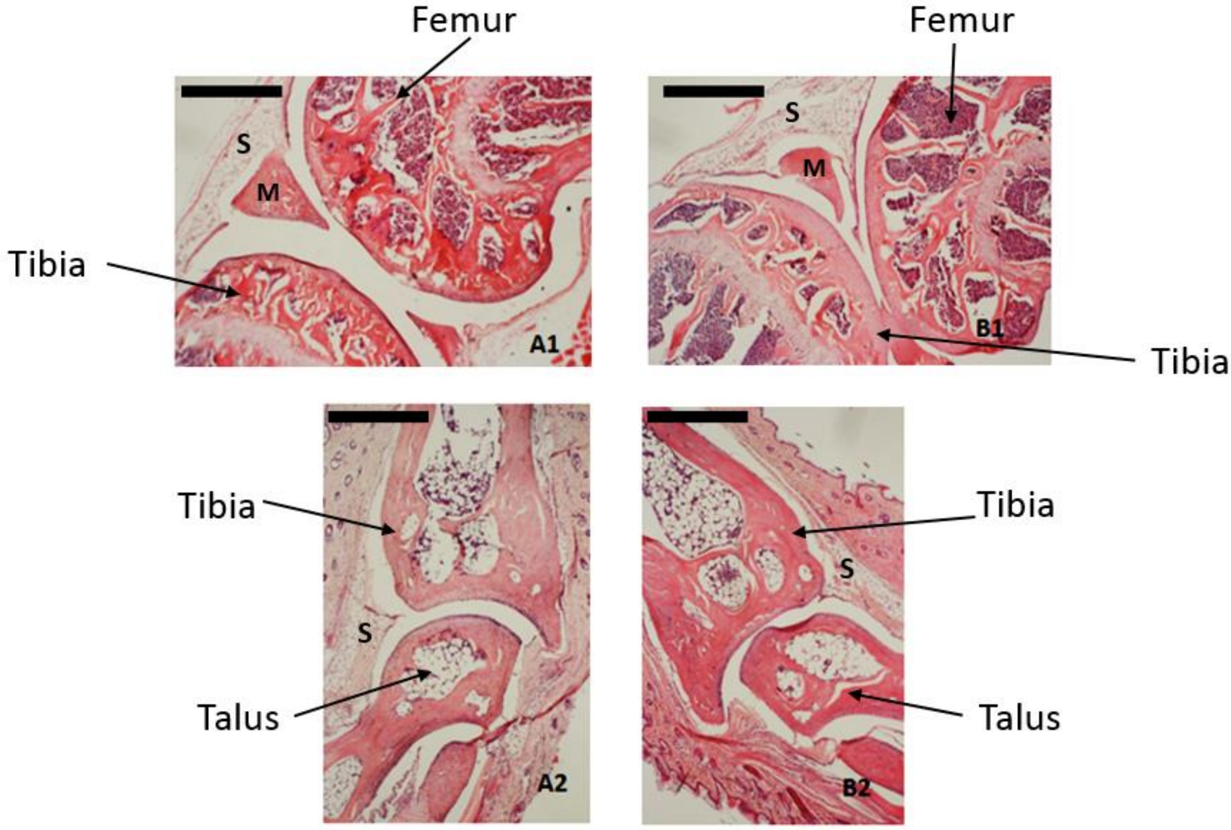

Figure 9. H&E staining of knee and ankle from C57BL/6 non-immunised control mice and immunised, non-arthritic mice. (A1) non-immunised control mouse knee. (B1) immunised, non-arthritic mouse knee. (A2) non-immunised control mouse ankle. (B20 immunised, non-arthritic mouse ankle. All given score of 0, representing a normal joint. Scale bar represents 0.5 mm. M = meniscus, S = synovium

3.4.2 No significant difference in constriction or relaxation responses in immunised C57BL/6 non-arthritic mice and control mice

In C57BL/6 mice immunised with low dose collagen and CFA there was no significant difference in maximal developed tension to 5-HT in control (9.8 ± 1.1 mN), and immunised, non-arthritic mice (7.11 ± 1.1) (Figure 10A). Similarly, there was no statistically significant difference in percentage relaxation to ACh in control (123 ±

22%) and immunised non-arthritic mice (95 + 8%) (Figure 10B). There was also no statistically significant difference in percentage relaxation to SNO in control (100 + 1.7%) and immunised non-arthritic mice (97 + 1.4 %) (Figure 10C).

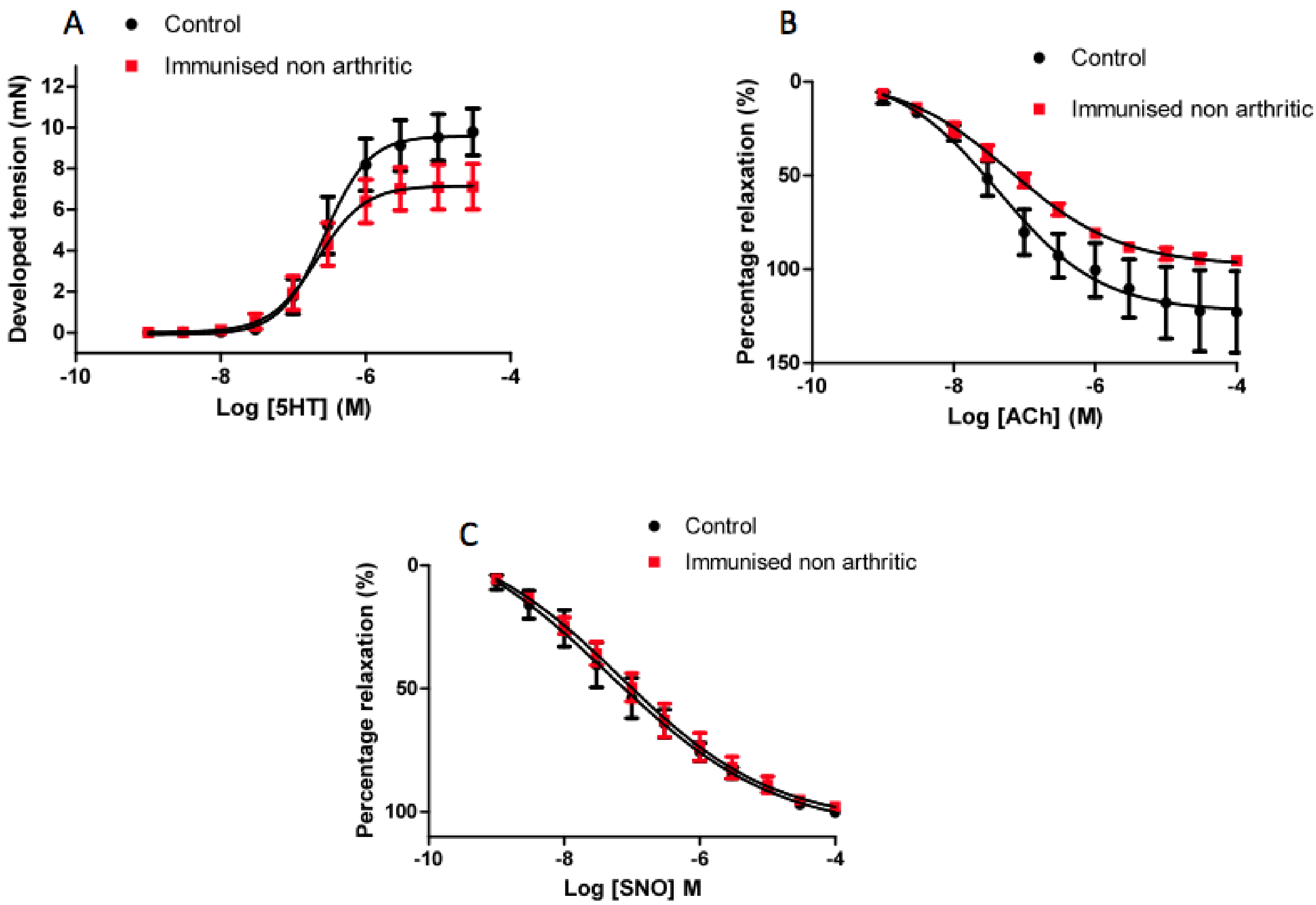

Figure 10. Vascular responses of aorta of C57BL/6 mice. (A) Constriction responses to 5-HT in C57BL/6 control mice compared to immunised, non-arthritic mice. No significant difference in maximal developed tension to 5-HT in vessels from control (9.8 + 1.1 mN), n = 4 and immunised, non-arthritic mice (7.11 + 1.1 mN), n = 8. (B) Relaxation responses to ACh in C57BL/6 control mice compared to immunised, non-arthritic mice. Vessels were constricted to 70-80% of their previously induced maximal contraction using 5-HT and then relaxed using ACh. No significant difference in percentage relaxation to ACh in control (123 + 22%) and immunised non-arthritic mice (95 + 8%). (C) Relaxation responses to SNO in C57BL/6 control mice compared to immunised, non-arthritic mice. Vessels were constricted to 70-80% of their previously induced maximal contraction using 5-HT and then relaxed using SNO. No significant difference in percentage relaxation to SNO in control (100 + 1.7%) and immunised non-arthritic mice (97 + 1.4 %)

3.4.3 Arthritis induction in C57BL/6 mice using high dose collagen and CFA

In total 10 of 16 C57BL/6 mice developed an arthritis (62.5%). This experiment was run in two batches so that myography could be performed at the same time points, as only a certain number of myography experiments could be run on the same day. In the first experiment 7 of 8 mice developed arthritis (87.5%), and in the second experiment 3 out of 8 mice developed arthritis (38%) (Figure 11). One mouse developed arthritis (maximal paw score 1) which then spontaneously resolved, thus at the time of sacrifice, 9 out of 16 mice had an arthritis. Arthritis onset in those mice that developed arthritis was between day 26 and 43. Mice with CIA had significantly lower weight gain (2.0 $\pm$ 0.7g) than control mice (4.9 $\pm$ 2.5g), $p = 0.015$ (Figure 12A). There was a significant negative correlation between total paw score and weight gain at the time of sacrifice for C57BL/6 mice, $R = -0.86$, $p < 0.0001$ (Figure 12B).

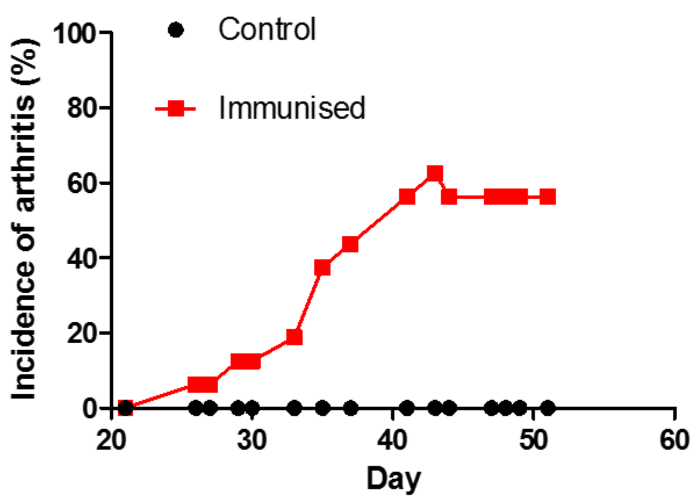

Figure 11. Incidence of arthritis in C57BL/6 mice immunised with high dose collagen and CFA. 10 out of 16 mice (62.5%) developed an arthritis. Results are presented from two experiments combined

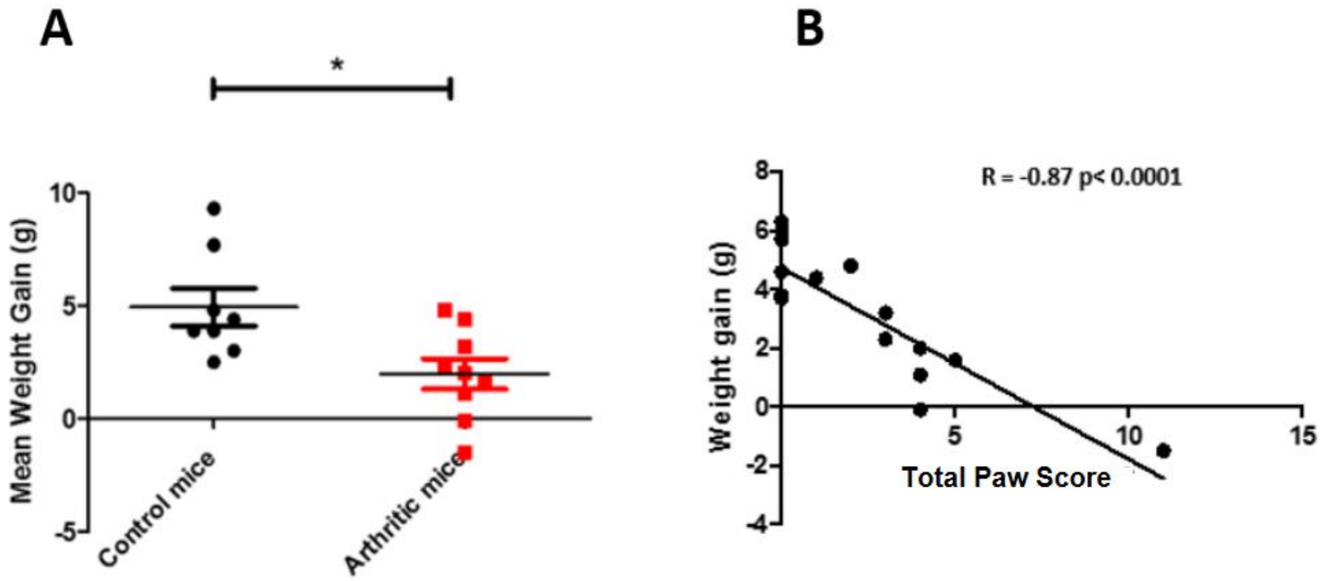

Figure 12. (A) Mean weight gain significantly higher in C57BL/6 non-immunised control mice (4.9 $\pm$ 2.5 g) compared to arthritic mice (2.0 $\pm$ 0.7g), *p* < 0.05 (B) Significant negative correlation between total paw score and weight gain at the time of sacrifice for C57BL/6 mice immunised with CIA using high dose collagen and CFA protocol. (R -0.87, *p* <0.0001).

3.4.4 Induction of arthritis confirmed histologically in C57BL/6 mice immunised with high dose collagen and CFA

Five knees from control mice and five knees from immunised mice scored as having arthritis clinically were stained with H&E to check for histological signs of arthritis (Figure13). This confirmed findings in keeping with CIA, with subsynovial inflammation, synovial exudate, synovial hyperplasia, pannus formation and cartilage and bone erosions.

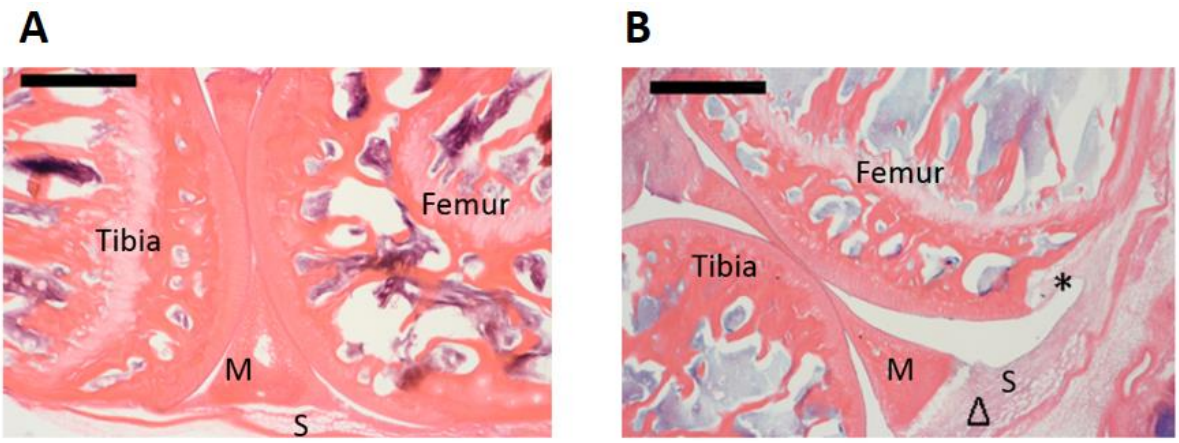

Figure 13. Arthritis confirmed histologically by H&E staining in C57BL/6 mice immunised with high dose collagen and CFA. A) Control mouse knee, score 0. B) Arthritic mouse knee, score 4. * represents erosion of underlying bone by pannus activity, Δ focal inflammatory infiltrates in synovium. Scale bar represents 0.5mm. M = meniscus, S = synovium.

3.4.5 Arthritis significantly reduces the vascular constriction response in aortic tissue but does not alter relaxation responses

Mice with clinical arthritis had significantly lower mean maximal aortic contraction (7.8 ± 3.1 mN), compared to non-immunised control mice (11.4 ± 2.7 mN), $p < 0.05$ (Figure 14). There was no significant difference between maximal relaxation in control (93 ± 6.9 %), arthritic (77 ± 13 %) and immunised non-arthritic mice (91 ± 18 %). There was no significant difference in maximal relaxation to SNO between control (99.5 ± 7.0 %), arthritic (98.0 ± 4.8 %) and immunised non-arthritic mice (101.7 ± 5.5 %). Of the nine mice that had a clinical arthritis at the time of termination, there was no significant correlation between maximal developed tension and total paw score (Figure 15).

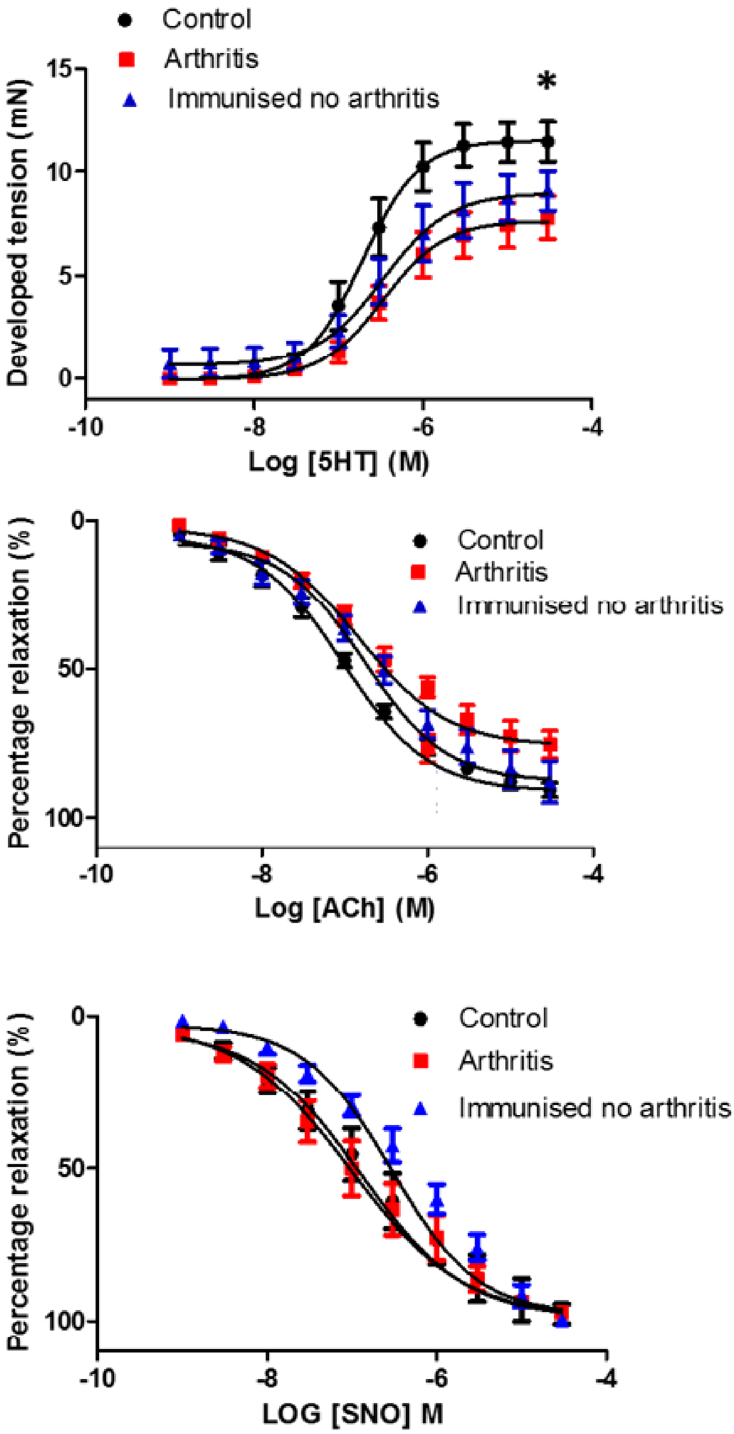

Figure 14. Vascular responses in the aorta of control, immunised and arthritic C57BL/6 mice to 5-HT, ACh and SNO. (A) Mean maximal contraction for control (11.4 ± 2.7 mN), n =8 was significantly higher than arthritic mice, n= 9 (7.8 ± 3.1 mN) *p* < 0.05, but not immunised non-arthritic mice (9.1 ± 2.5 mN), n=7. (B) No significant difference between maximal relaxation in control (93 ± 6.9 %), arthritic (77 ± 13 %) and immunised non arthritic mice (91 ± 18 %). (C) No significant difference in maximal relaxation to SNO between control (99.5 ± 7.0 %), arthritic (98.0 ± 4.8 %) and immunised non arthritic mice (101.7 ± 5.5 %).

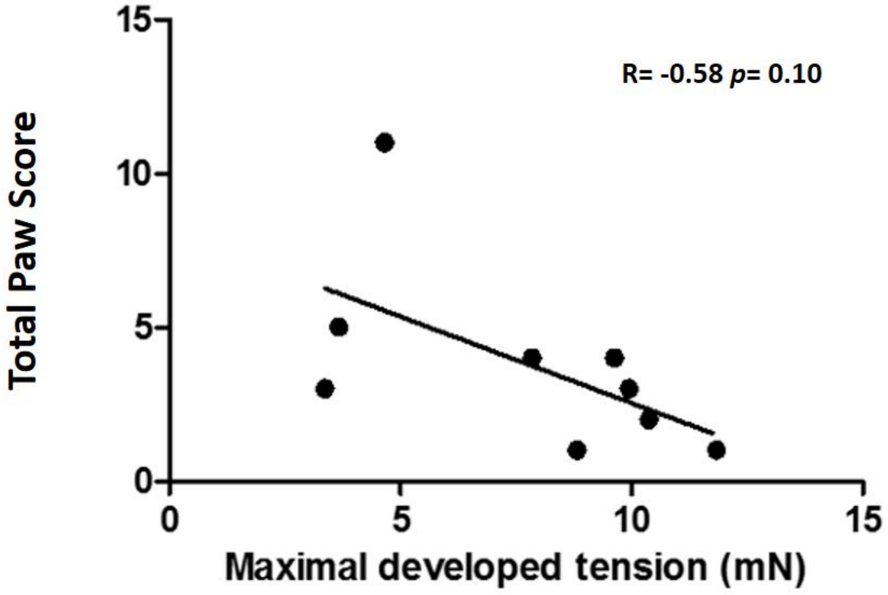

Figure 15. No significant correlation between maximal developed tension and total paw score in C57BL/6 arthritic mice, n= 9.

3.4.6 Complete absence of IL-6 signalling using IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice confers resistance to CIA

No IL-6$^{-/-}$ or IL-6R$^{-/-}$ mice developed arthritis when immunised with CIA using high dose CII/CFA (Figure 16). One of five (20%) immunised WT females developed arthritis on day 23, developed a moderate arthritis by day 33 but then regressed to mild at day 49. The plan was to allow the arthritis to progress to severe but in view of an improvement clinically the mouse was sacrificed on day 49. One of two (50%) immunised WT male mice developed CIA.

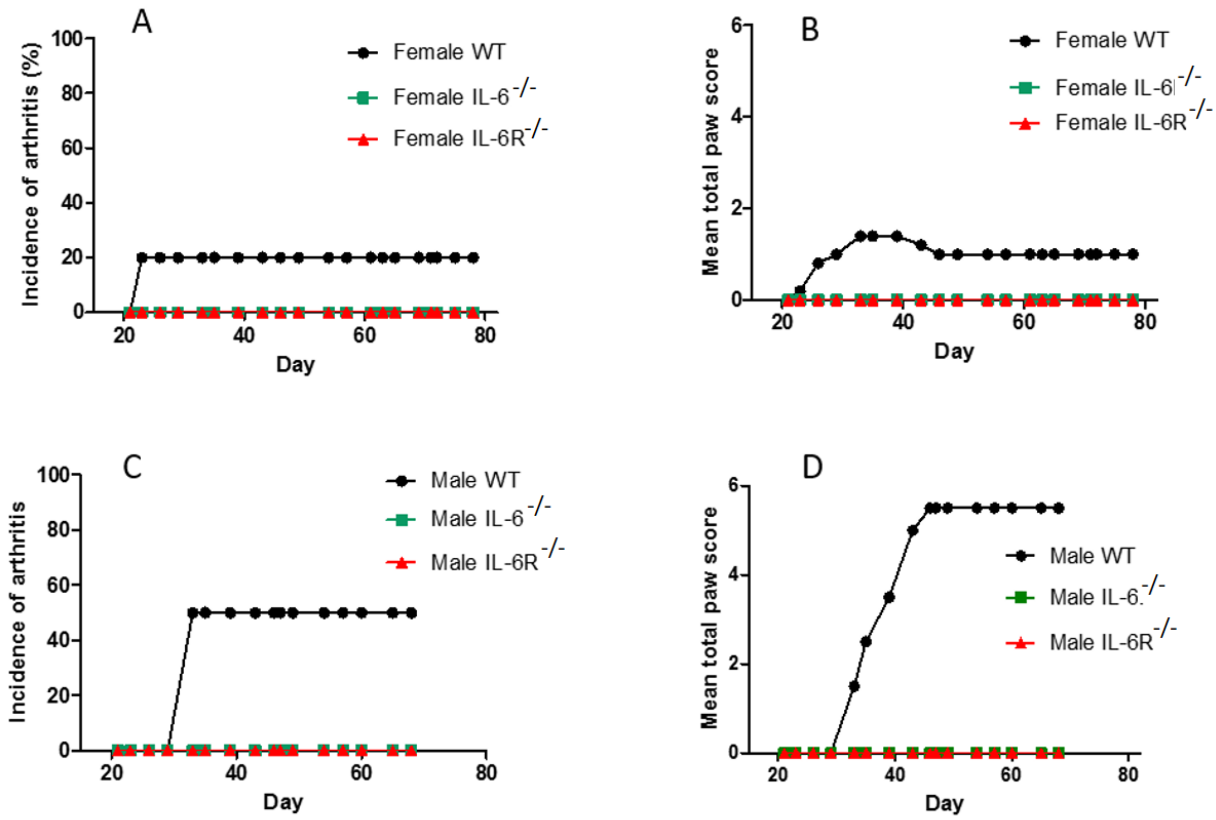

Figure 16. Incidence of arthritis for C57BL/6 WT and IL-6 KO and IL-6R KO mice in (A) female and (C) male mice. No IL-6$^{-/-}$ or IL-6R$^{-/-}$ mice developed arthritis. Mean total paw score for (B) females, n = 3 per group in IL-6$^{-/-}$ and IL-6R$^{-/-}$ and n = 5 WT and (D) males, n = 2 per group.

3.4.7 Female immunised IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice have reduced aortic constriction compared to wild type mice.

Mean maximal developed aortic tension for female control wild-type mice (13.5 $\pm$ 0.6 mN) and wild type immunised-non arthritic mice (12.3 $\pm$ 0.6 6 mN) was significantly higher than IL-6$^{-/-}$ (8.5 $\pm$ 0.5 mN) and IL-6R$^{-/-}$ (6.4 $\pm$ 0.7 mN) mice, $p < 0.01$ (Figure 17). Maximal developed tension for the single wild type arthritic mouse was 12.2 mN. There was no significant difference in percentage relaxation to ACh between the groups (Figure 18). The mean percentage relaxation to ACh for control mice was (89.3 $\pm$ 2.3 %), immunised non arthritic mice (75.7 $\pm$ 9.1 %), IL-6$^{-/-}$ mice (90.8 $\pm$ 6.1 %), IL-6R$^{-/-}$ mice (85.4 $\pm$ 1.3 %) and for the wild type arthritic mouse was 83.2 %. There was also no significant difference in percentage relaxation to SNO between the groups

(Figure 19). Mean percentage relaxation to SNO for control mice (101.9 ± 1.1 %), immunised non-arthritic mice (103.0 ± 1.5%), IL-6$^{-/-}$ mice (97.0 ± 7.0 %), IL-6R$^{-/-}$ mice (101.0 ± 2.8 %) and the wild type arthritic mouse 106.1%.

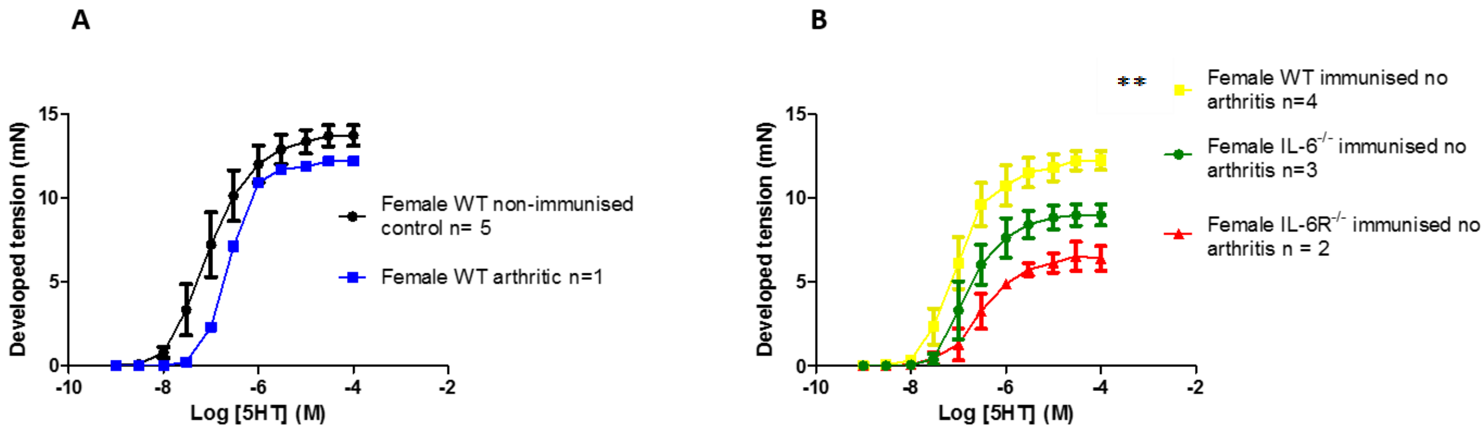

Figure 17. (A) Vasoconstriction concentration–response curves to 5-HT in aortic rings from female non-immunised control and female wild type arthritic mice. Maximal developed tension for the single wild type arthritic mouse was 12.2 mN and for control mice (13.5 ± 0.6 mN). (B) Vasoconstriction concentration–response curves to 5-HT in aortic rings from female immunised non arthritic wild type IL-6$^{-/-}$and IL-6R$^{-/-}$ mice. Mean maximal developed tension for female wild type immunised-non arthritic mice (12.3 + 0.6 mN) was significantly higher than IL-6$^{-/-}$ (8.5 + 0.5 mN) and IL-6R$^{-/-}$ (6.4 + 0.7 mN) mice **p < 0.01.

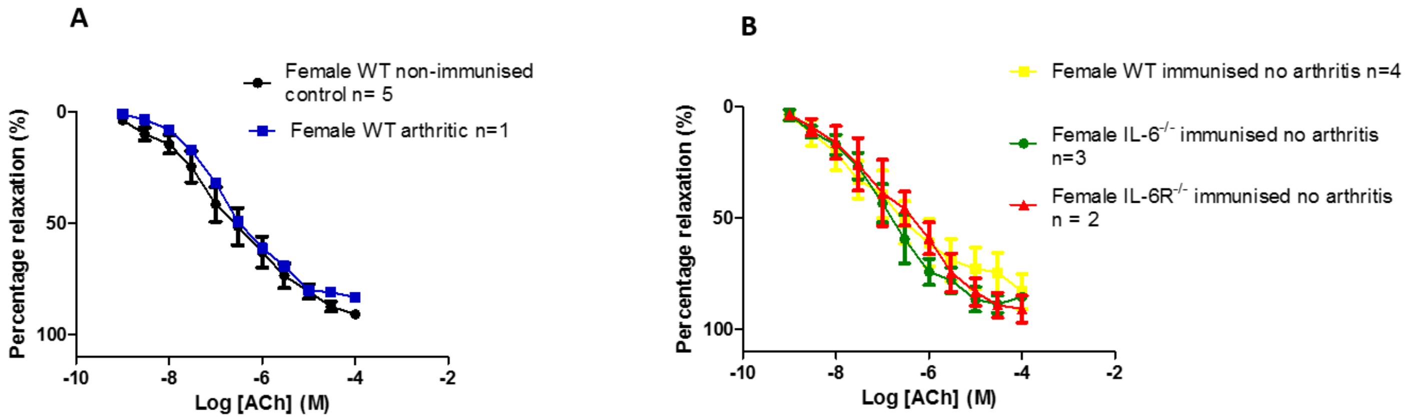

Figure 18. (A) Relaxation–response curves to ACh following pre-constriction to 70–80% of their appropriate Rmax response to 5-HT in aortic rings from female non-immunised control and wild type arthritic mice. No significant difference in mean percentage relaxation to ACh for control mice (89.3 $\pm$ 2.3 %) and the wild type arthritic mouse (83.2 %). (B) Relaxation–response curves to ACh following pre-constriction to 70–80% of their appropriate Rmax response to 5-HT in aortic rings from female immunised, non-arthritic wild type, IL-6$^{-/-}$and IL-6R$^{-/-}$ mice. No significant difference in mean percentage relaxation to ACh for immunised WT non arthritic mice (75.7 $\pm$ 9.1 %) immunised IL-6$^{-/-}$ mice (90.8 $\pm$ 6.1 %) and immunised IL-6R$^{-/-}$ mice (85.4 $\pm$ 1.3 %)

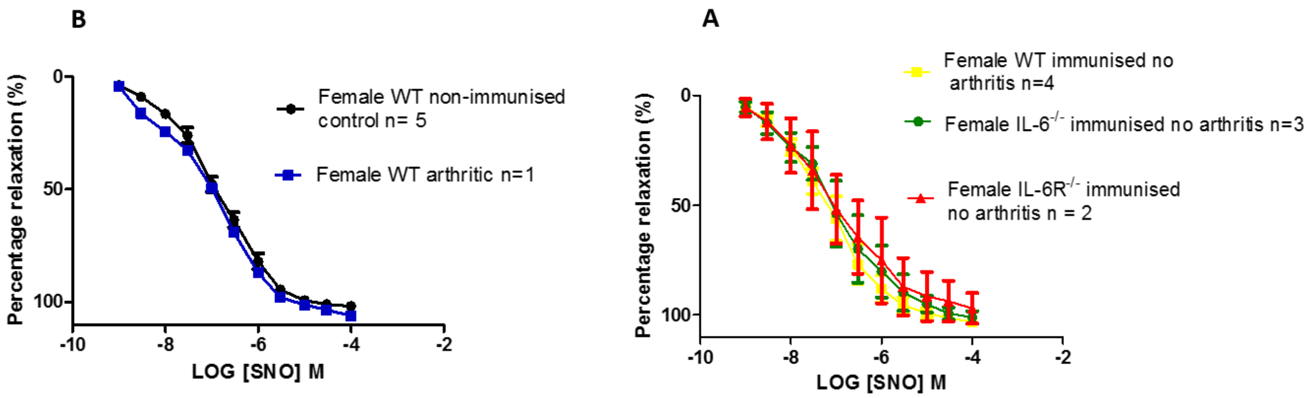

Figure 19. (A) Relaxation–response curves to SNO following pre-constriction to 70–80% of their appropriate Rmax response to 5-HT in aortic rings from female wild type non-immunised control, and arthritic mice. No significant difference in percentage relaxation between control mice (101.9 $\pm$ 1.1 %) and the wild type arthritic mouse (106.1%). (B) Relaxation–response curves to SNO following pre-constriction to 70–80% of their appropriate Rmax response to 5-HT in aortic rings from female immunised, non-arthritic wild type, IL-6$^{-/-}$and IL-6R$^{-/-}$ mice. No significant difference in percentage relaxation between percentage relaxation to SNO for WT immunised non-arthritic mice (103.0 $\pm$ 1.5%), immunised IL-6$^{-/-}$ mice (97.0 $\pm$ 7.0 %) and immunised IL-6R$^{-/-}$ mice (101.0 $\pm$ 2.8 %)

3.4.8 Trend for reduced aortic constriction in male IL-6R$^{-/-}$ mice compared to non-immunised control wild type

Mean maximal constriction to 5-HT in the male control WT mouse was 12.4 mN and male WT arthritic mouse 3.1 mN (Figure 20A). Although numbers in each group were only 1 or 2, there appears to be a trend for higher mean maximal constriction to 5-HT in the immunised non-arthritic mouse (11.0 mN) compared to the IL-6R$^{-/-}$ mice (3.0 $\pm$ 2.1 mN) (Figure 20B). There was no significant difference in percentage relaxation to ACh between the groups; percentage relaxation to ACh in the control mouse was 92.7%, immunised non arthritic mouse 77.3%, arthritic mouse 82.4% and mean for IL-6R$^{-/-}$ mice was 75.9 $\pm$ 1.9 % (Figure 21). There was also no difference in percentage relaxation to SNO; percentage relaxation to SNO in the control mouse was 115.4%, immunised non arthritic mouse 110.4%, arthritic mouse 104.7% and mean for IL-6R$^{-/-}$ mice was 105.2 $\pm$ 0.2 % (Figure 22). The male mouse experiment did not have an IL-6$^{-/-}$ mouse included as there were none available.

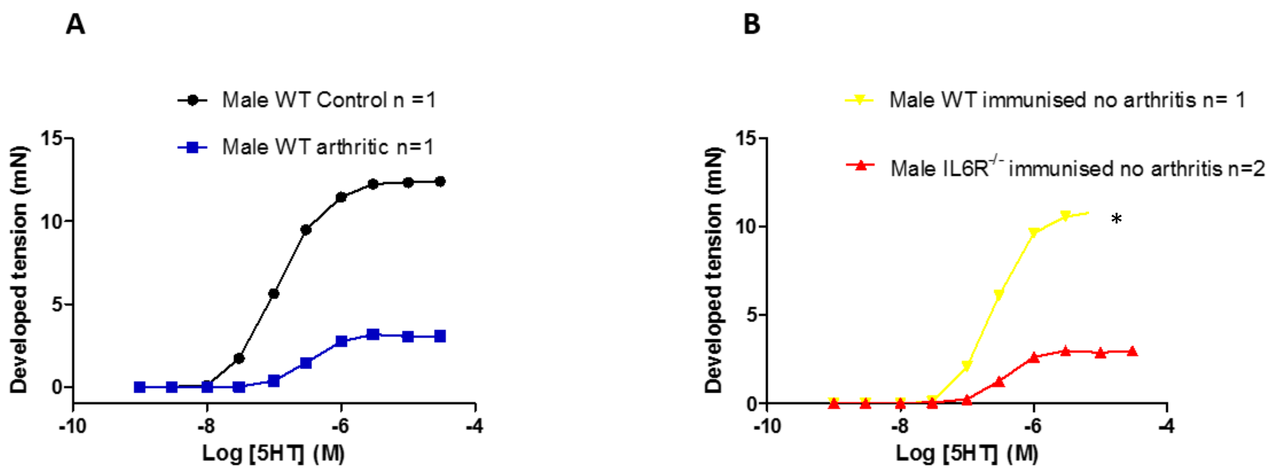

Figure 20. (A) Vasoconstriction concentration–response curves to 5-HT in aortic rings from male wild type non-inmmunised control, and wild type arthritic mice. Mean maximal developed tension in the control mouse (12.4 mN) and wild type arthritic mouse (3.1 mN). (B) Vasoconstriction concentration–response curves to 5-HT in aortic rings from male immunised non-arthritic wild type and IL-6R$^{-/-}$ mice. Mean maximal developed tension to 5HT was significantly higher in the wild type immunised non arthritic mouse (11.0 mN) compared to the IL-6R$^{-/-}$ mice (3.0 $\pm$ 2.1 mN) * p < 0.05.

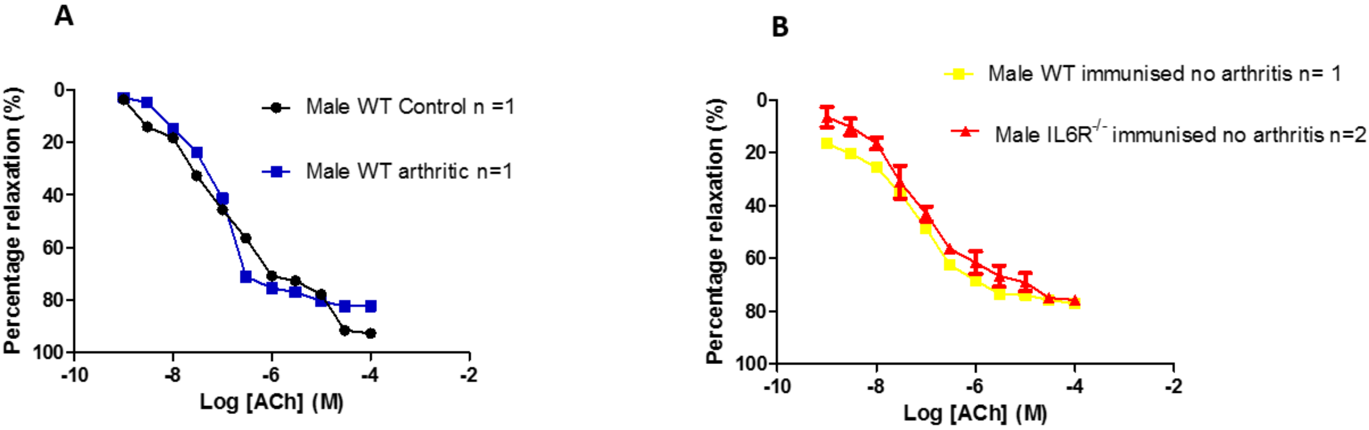

Figure 21. Relaxation–response curves to ACh following pre-constriction to 70–80% of their appropriate Rmax response to 5-HT in aortic rings from (A) Male non-immunised control wild type mice and male wild type arthrititc mice and (B) Male immunised non arthritic wild type mince and IL-6R$^{-/-}$ mice. No significant difference in percentage relaxation between the groups. Mean percentage relaxation to ACh in WT control mouse 92.7 %, WT arthritic mouse 82.4 %, immunised non-arthritic mouse 77.3 %, and IL-6R$^{-/-}$ immunised mouse (75.9 $\pm$ 1.9 %)

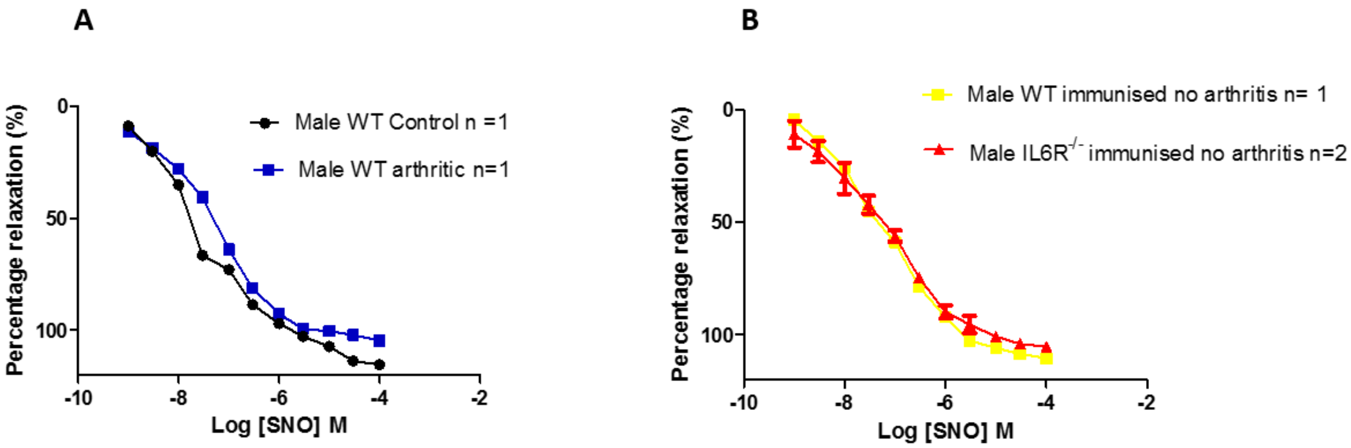

Figure 22. Relaxation–response curves to SNO following pre-constriction to 70–80% of their appropriate Rmax response to 5-HT) in aortic rings from (A) Male non-immunised control wild type mice and male wild type arthritic mice and (B) Male immunised non arthritic wild type mince and IL-6R$^{-/-}$ mice. No significant difference in percentage relaxation between the groups. Percentage relaxation to SNO in the WT control mouse was 115.4 %, WT arthritic mouse 104.7 %, WT immunised non arthritic mouse 110.4%, and mean for IL-6R$^{-/-}$ mice was 105.2 $\pm$ 0.2 %.

3.4.9 The effect of blockade of IL-6 trans-signaling on CIA using sgp130Fc

3.4.9.1 No effect of sgp130Fc on arthritis incidence or severity in DBA-1 mice when administered intraperitoneally

In experiment 1 and 2, where mice were administered sgp130Fc intraperitoneally, there was no effect on arthritis onset, incidence or severity (Figure 23). Of those mice immunised with CIA and administered PBS, in experiment 1 arthritis incidence was 100% and in experiment 2 was 75%. There was no effect whether sgp130Fc was administered on alternate days (Experiment 1) or daily (Experiment 2); there was no significant difference in mean total paw score on day 34 for mice administered IP sgp130Fc in Experiment 1 (8.5 ± 1.7) and those administered PBS (8.3 ± 1.5) (Figure 24). Mean total paw score on day 28 for IP sgp130Fc in Experiment 2 was 3.8 ± 1.5, PBS (2.0 ± 1.4), not significant.

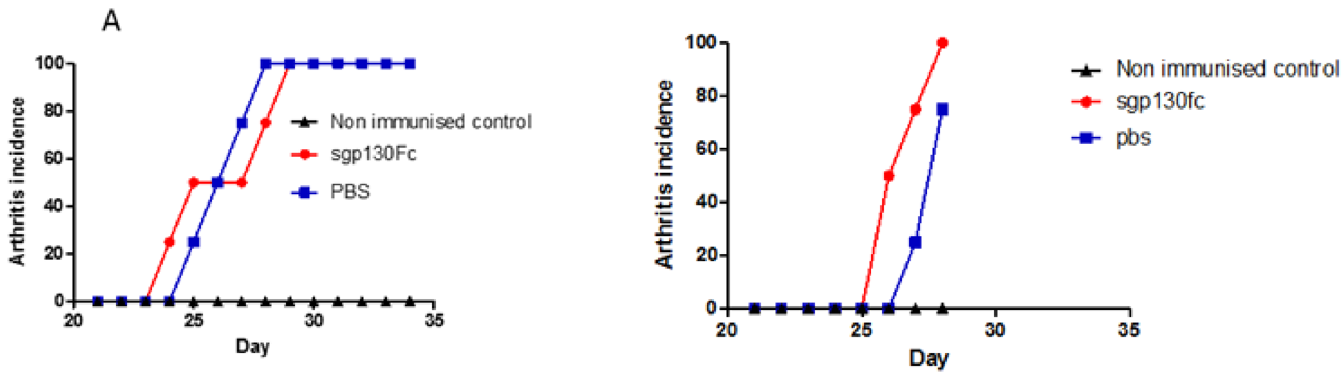

Figure 23. Arthritis Incidence in DBA-1 mice treated with IP sgp130Fc. (A) Experiment 1: 100% arthritis incidence in DBA-1 mice immunised with CIA and administered either PBS or sgp130Fc on alternate days from day 21, n =4 in each group. (B) Experiment 2: 100 % incidence in DBA-1 mice immunised with CIA and administered sgp130Fc daily from day 21 and 75% arthritis incidence in those administered PBS, n = 4 in each group.

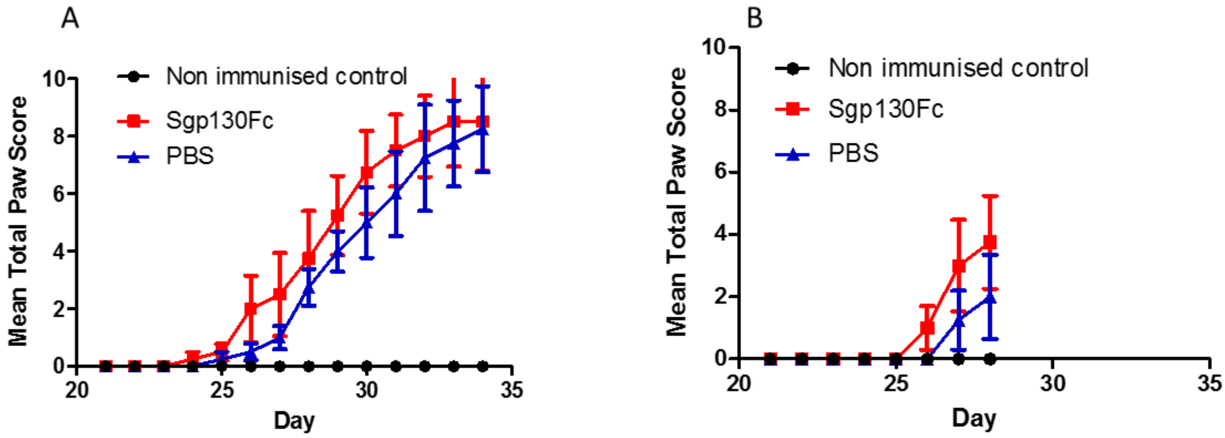

Figure 24. Mean total paw score over time for mice immunised with CIA and administered sgp130Fc IP. (A) Experiment 1: IP sgp130Fc or PBS IP from day 21 on alternate days for 7 doses, no significant difference, n = 4 in each group. (B) Experiment 2: IP sgp130Fc or PBS from day 21 daily for 7 doses, no significant difference, n = 4 in each group

3.4.9.2 No effect on aortic constriction responses in mice with CIA administered IP sgp130Fc

There was no statistically significant difference in maximal developed tension in mice with CIA administered sgp130Fc compared to PBS. In Experiment 1 there was no difference in maximal developed tension in those administered sgp130Fc (mean maximal developed tension 7.88 $\pm$ 1.0 mN) compared to those administered PBS (6.55 $\pm$ 1.0 mN), but mean maximal developed tension was statistically significantly higher in non-immunised control mice (11.3 $\pm$ 0.7 mN), $p < 0.05$ (Figure 25A). There was no difference in maximal developed tension in those treated with sgp130Fc in Experiment 1 (mean maximal developed tension 6.0 $\pm$ 0.9 mN), compared to those administered PBS (5.2 $\pm$ 1.0 mN), or non-immunised control mice (7.89 $\pm$ 0.7 mN) (Figure 25B).

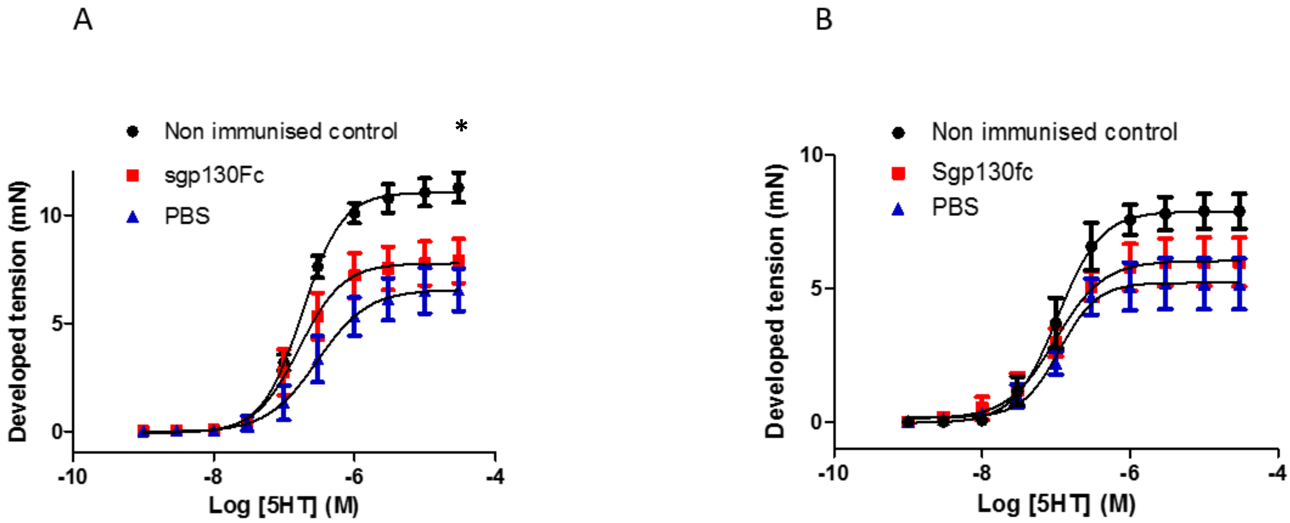

Figure 25. Vasoconstriction concentration–response curves to 5-HT in aortic rings from DBA-1 mice immunised with high dose collagen and CFA and administered IP sgp130Fc or PBS. (A) Sgp130Fc sourced from Kiel, Germany. No difference in mean maximal developed tension in those administered sgp130Fc (7.88 + 1.0 mN) compared to those administered PBS (6.55 + 1.0 mN), but mean maximal developed tension was significantly higher in non-immunised control mice (11.3 + 0.7 mN), ****p* **< 0.05. n = 4 in each group (B) sgp130Fc sourced from R&D systems. No significant difference in maximal developed tension in mice administered agp130Fc or PBS. n = 4 in each group.**

3.4.9.3 Detection of sgp130Fc in serum in DBA-1 mice administered IP or IV sgp130Fc

ELISA was performed to measure human sgp130 in mouse serum from those mice administered both sgp130Fc from Kiel and from R&D systems. In all mouse serum samples where sgp130Fc was administered intraperitoneally, all absorbance values for sgp130 were below the lower limit of detection (0.23 OD), indicating absence in serum. In the same ELISA, human samples were also tested, and all samples tested contained sgp130. In mice administered intravenous sgp130Fc, human sgp130 was detectable in the serum (mean concentration 205 + 36 pg/ml, taken 48 hours after administration), but not in mice administered PBS or etanercept), n = 4 per group (Figure 26).

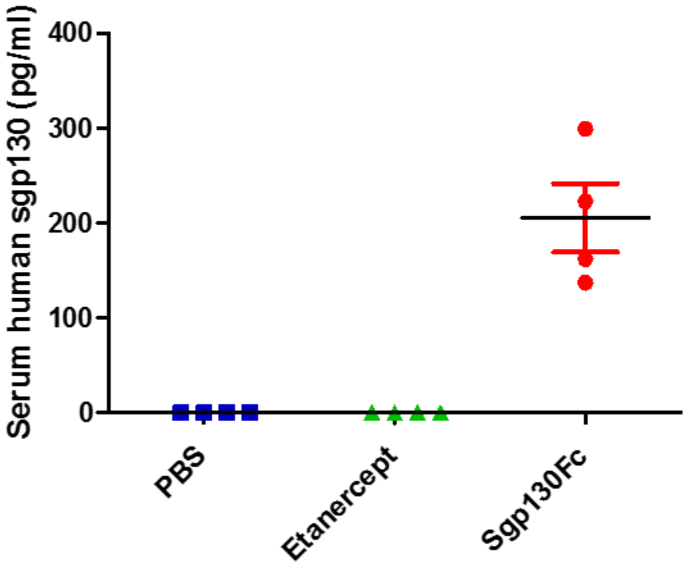

Figure 26. Measurement of human sgp130 in serum in mice administered human sgp130Fc IV. Human sgp130 was detected in serum from mice administered sgp130Fc (mean concentration 205 ± 36 pg/ml), but not in etanercept or PBS administered groups (n=4 in each group). Blood was collected 48 hours after the second intravenous dose of sgp130Fc.

3.4.9.4 Intravenous sgp130Fc reduced arthritis incidence and severity in immunised DBA-1 mice

Intravenous sgp130Fc reduced arthritis incidence in DBA-1 mice immunised with low dose collagen and CFA compared to those administered PBS. Arthritis incidence on day 29 was 90% for PBS-administered mice, 50% for sgp130fc-administered mice and 50% for etanercept-administered mice (Figure 27A). Mean total paw score for PBS–administered mice (4.3 ± 3.4) was significantly higher than sgp130Fc–administered mice (1.5 ± 1.8), and etanercept-administered mice (1.2 ± 1.7), $p < 0.05$ (Figure 27B)

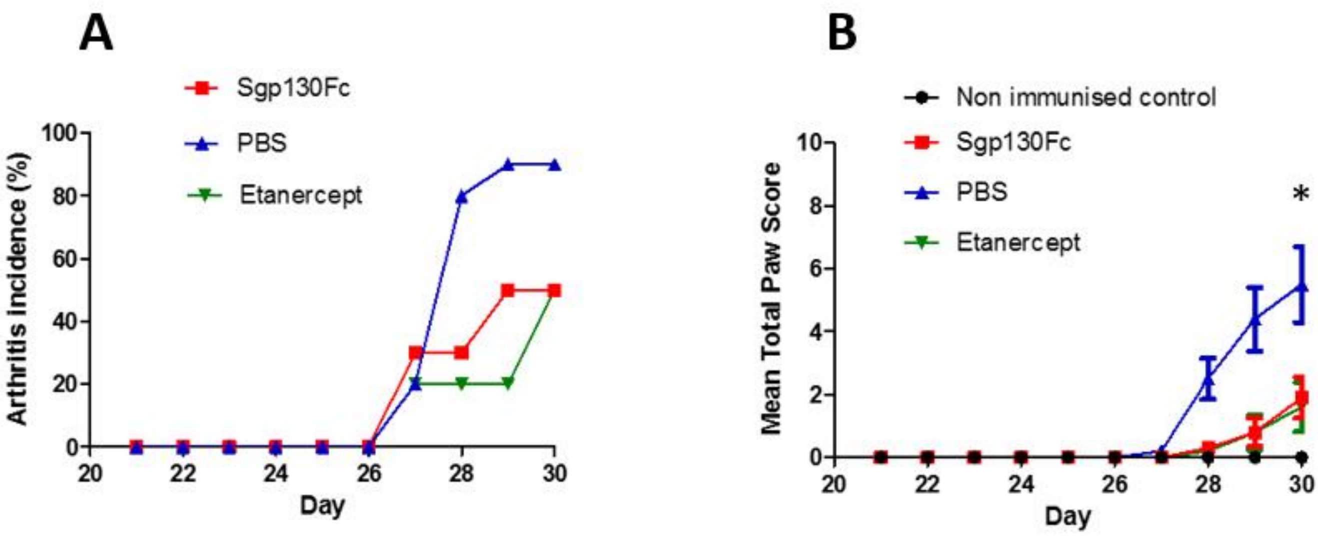

Figure 27. (A) Arthritis incidence and (B) Paw score over time for mice immunised with CIA and administered intravenous sgp130Fc, etanercept or PBS. Significant reduction in mean total paw score at day 30 in mice administered sgp130Fc (1.5 + 1.8), or etanercept (1.2 + 1.7), compared to those administered PBS (4.3 + 3.4), *p <0.05. n = 10 in each group

3.4.9.5 Intravenous sgp130Fc restored vascular function in CIA

There was a significant reduction in maximal developed tension in mice with CIA administered PBS (5.8 + 2.4 mN) compared to non-immunised control mice (8.5 + 1.8 mN), p < 0.05. There was no significant difference in mean maximal developed tension between sgp130Fc (8.1 + 2.0 mN), etanercept treated mice (7.7 + 1.9 mN) and non-immunised controls (Figure 28).

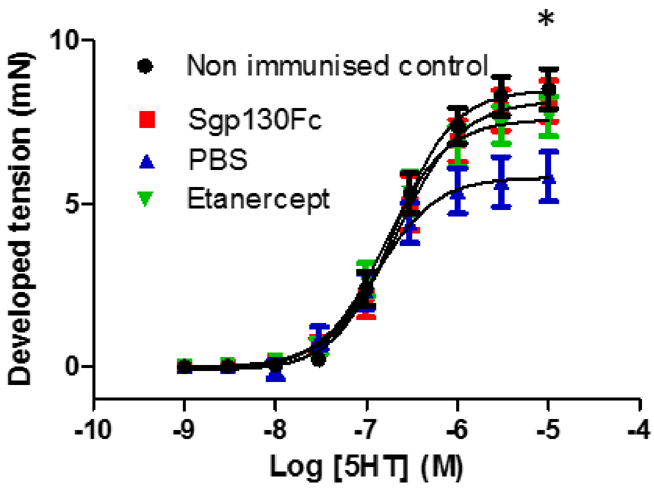

Figure 28. Effect of IV sgp130Fc on aortic constriction in DBA-1 mice immunised with CIA and administered either sgp130FC, PBS or etanercept, compared with non-immunised controls. Graphs show vasoconstriction concentration–response curves to 5-HT (following pre-constriction to 80% of their appropriate Rmax response to 5-HT) in aortic rings N = 10 in each group. Significant reduction in maximal developed tension in mice with CIA administered PBS (5.8 + 2.4 mN) compared to non-immunised control mice (8.5 + 1.8 mN), *p* < 0.05. No significant difference in mean maximal developed tension between sgp130Fc (8.1 + 2.0 mN), etanercept treated mice (7.7 + 1.9 mN) and non-immunised controls.

3.4.9.6 IV sgp130Fc reduced serum CCL2 and VCAM-1 levels in immunised mice compared to those administered PBS

Serum CCL2 was significantly higher in mice with CIA treated with PBS (117.3 + 14.5 pg/ml) compared to those treated with IV sgp130Fc (41.87 + 12.1 pg/ml), $p < 0.001$ and non-immunised controls (16.9 + 4.8 pg/ml) $p < 0.001$ (Figure29A). CCL2 was significantly higher in CIA mice administered etanercept (116.7 + 36.7 pg/ml) compared to those treated with IV sgp130fc ($p < 0.01$) and non-immunised controls ($p < 0.001$).

Serum VCAM-1 was significantly higher in PBS-administered mice (3144 + 366 ng/ml) compared to controls (1861 + 160 ng/ml), mice administered etanercept (2077 + 173 ng/ml) and mice administered IV sgp130Fc (1979 + 136 ng/ml), $p < 0.01$ (Figure29B). There was no significant difference in serum VCAM-1 between etanercept or sgp130fc treated groups compared to non-immunised controls. In immunised DBA-1 mice there was a significant positive correlation between paw score and both serum VCAM-1 and serum CCL2 level (Figure30).

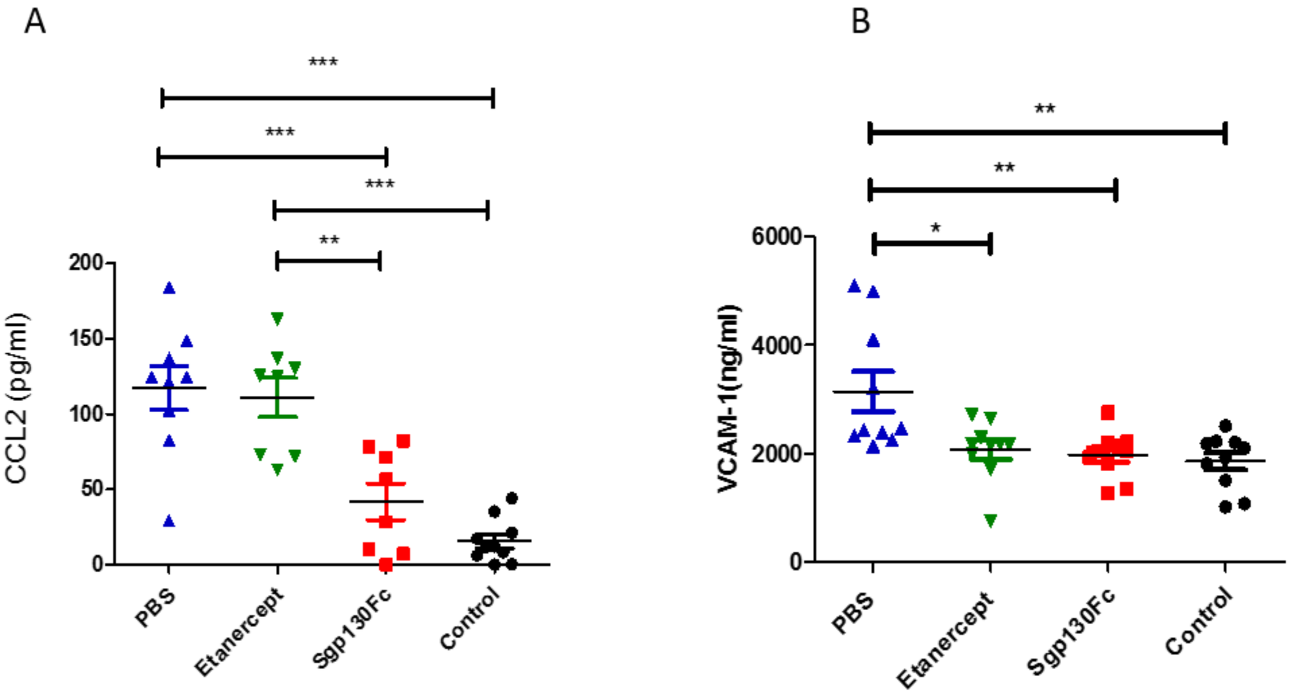

Figure 29. Serum levels of (A) CCL2 and (B) VCAM-1 in DBA-1 mice. (A) Significantly higher CCL2 in mice with CIA administered PBS (117.3 + 14.5 pg/ml) compared to sgp130Fc (41.87 + 12.1 pg/ml), and non-immunised controls (16.9 + 4.8 pg/ml). Significantly higher CCL2 in mice immunised with CIA and administered etanercept (116.7 + 36.7 pg/ml) compared to sgp130Fc and controls, n = 9 in each group. (B) Serum VCAM-1 was significantly higher in PBS treated mice (3144 + 366 ng/ml) compared to controls (1861 + 160 ng/ml), mice administered etanercept (2077 + 173) and mice administered sgp130Fc (1979 + 136). There was no significant difference in serum VCAM-1 between etanercept or sgp130fc treated groups compared to controls, n = 10 in each group. *p <0.05, ** p <0.01, *** p < 0.001

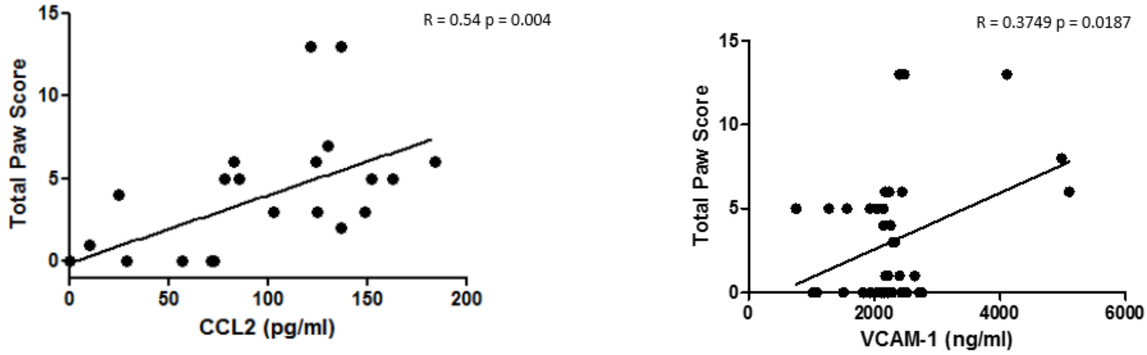

Figure 30. Correlations between serum CCL2 or VCAM-1 and total paw score in DBA-1 mice immunised with low dose collagen and CFA. Significant positive correlation between serum CCL2 and total paw score, R = 0.54, *p* = 0.004. Significant positive correlation between serum VCAM-1 and total paw score, R = 0.3749, *p* = 0.00187

3.4.9.7 PSTAT3 not detected in the aorta of arthritic mice

PSTAT3 was present in the synovium of arthritic joints of mice with CIA (Figure 31). pSTAT3 was not present in the aorta of control mice or in the aorta of mice with CIA (Figure 32).

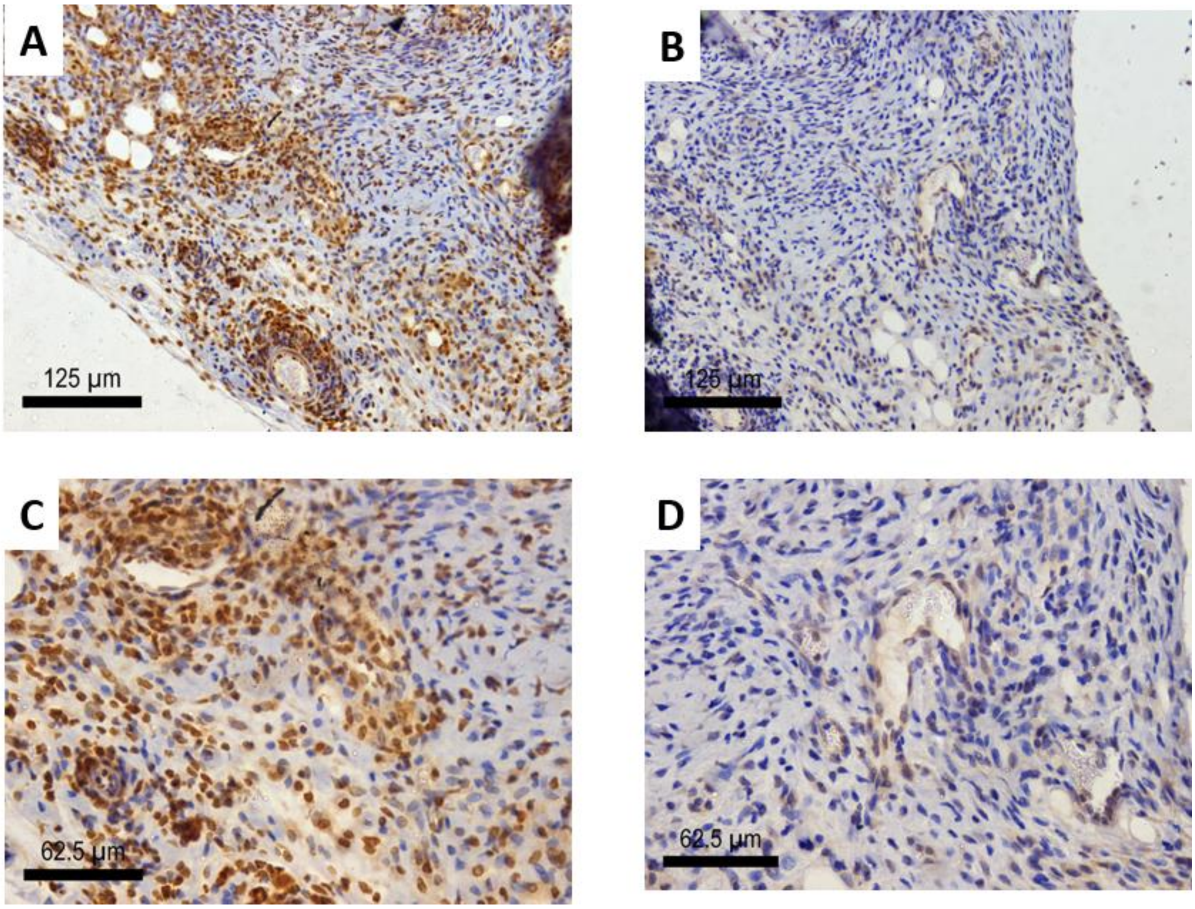

Figure 31. pSTAT3 staining in synovium from arthritic joints in DBA-1 mice with CIA. (A) Positive staining for pSTAT3 in synovium at X 20 magnification (B) Isotype control at X 20 magnification. (C) Positive staining for pSTAT3 in synovium at X 40 magnification (D) Isotype control at X 40 magnification

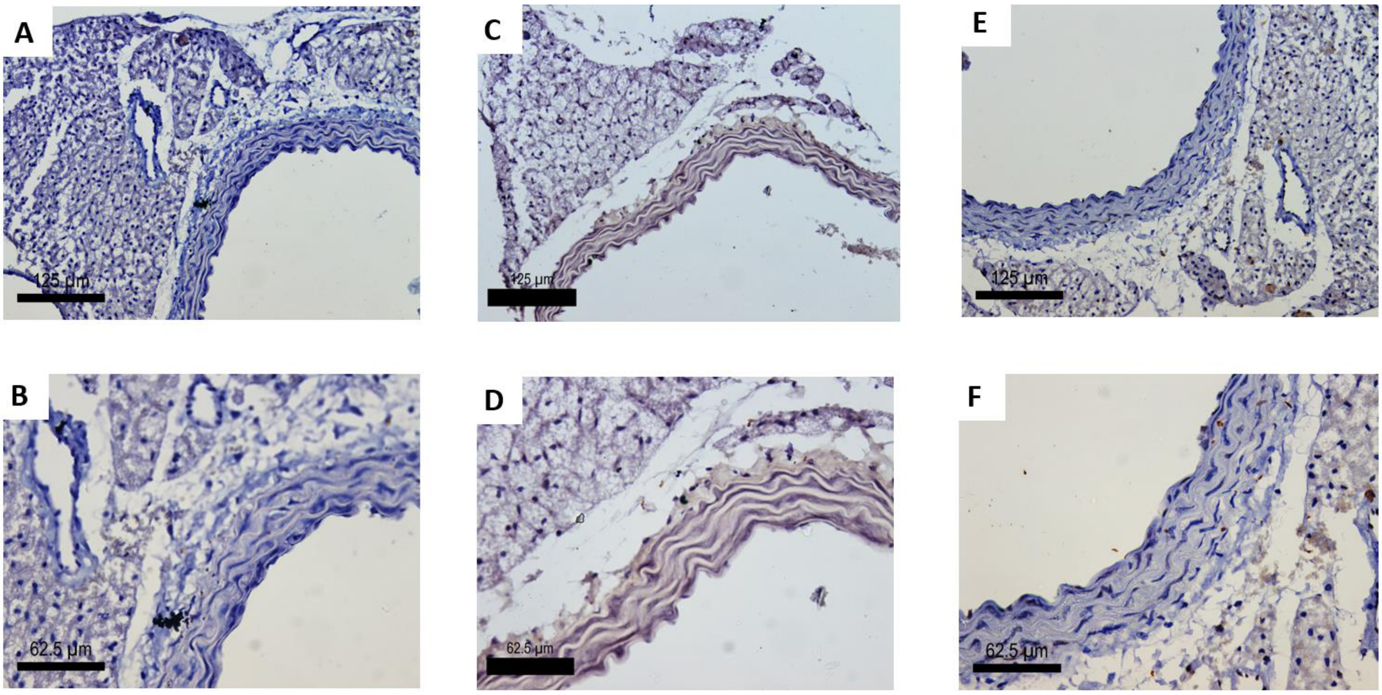

Figure 32. No pSTAT3 staining in the aorta of control DBA-1 mice and arthritic DBA-1 mice. (A) Negative staining for pSTAT3 in arthritic mouse aorta, X 20 magnification. (B) Isotype control aorta from arthritic mouse, X 40 magnification. (C) Negative staining for pSTAT3 in control mouse aorta, X 20 magnification (D) Negative staining for pSTAT3 in control mouse aorta, X 40 magnification (E) Isotype control aorta from arthrtitic mouse, X 20 magnification (F) Isotype control aorta from arthrtitic mouse, X 40 magnification.

3.5 Discussion

3.5.1 Arthritis induction in wild type C57BL/6 and DBA-1 mice

When using the same doses of collagen and CFA that are used to induce CIA in DBA-1 mice (low dose collagen and TB) no C57BL/6 mice developed arthritis. When using high dose collagen and CFA, CIA was successfully induced in C57BL/6 mice but with a variable severity and time course. This is in keeping with the literature, in which there are differing reports of arthritis incidence and severity of CIA in C57BL/6 mice. It is known that this reduced susceptibility to CIA is linked to the H-2^b haplotype in C57BL/6 mice, and that the H-2^q haplotype in DBA-1 mice confers greater susceptibility to CIA, with greater clinical scores and more paw swelling than C57BL/6 mice (Bevaart et al, 2010). Of note, in the two experiments using high dose collagen and CFA, the first incidence of arthritis was 87.5% and the second 38%. These mice were sourced from the same company, were the same age, were housed in the same environment and the same methods were used to induce arthritis. Possible factors in this variability in incidence could involve the quality of collagen preparation, quality of immunisation injections and slight changes in environment, such as noise level. It has previously been observed that fighting amongst male mice reduces the incidence of arthritis (Inglis et al, 2007). Although no fighting was reported and no wounds were observed, we cannot rule out the possibility of fighting amongst mice. In DBA-1 mice, arthritis was induced successfully with low dose collagen and CFA, with an incidence of 100% in the first experiment and 75% in the second. This is in line with the literature on arthritis incidence in DBA-1 mice (Brand et al, 2007).

3.5.2 Effect of CIA on weight gain

Mice with CIA had significantly lower weight gain than control mice; this is in line with previous literature (Yongfeng et al, 2016; Filippin et al, 2013). There was a significant negative correlation between weight gain and arthritis severity for immunised mice. From day 21 onwards food pellets were left in the bottom of the cage, as well as in the food hopper of the cage lid, to ensure that arthritic mice could reach these easily and were not disadvantaged by having to reach up to get food on their hind paws. Therefore ability of the mice to reach food should not have impacted on their weight gain. The cause of reduced weight gain in arthritic mice may be anorexia or systemic inflammatory response causing inflammatory cachexia. Studies have found that daily food intake did not differ between controls and CIA animals (Filippin et al, 2013). Another study found that in adjuvant arthritis induced in rats, pair fed animals (mice with arthritis and controls fed the same amount), those with adjuvant arthritis lost 20% of their body weight at the end of experimentation, and pair-fed animals lost only 5% body weight, indicating that anorexia alone does not explain inflammatory cachexia (Roubenoff et al, 1997). In these mice, weight loss correlated with TNF-α production by spleen mononuclear cells.

What is unknown in this chapter is the components of the mass weighed, and whether the reduction in weight gain is due to less fat or less muscle gain. Previous studies in CIA have shown that there is a correlation between arthritis score and locomotion; mice with more severe arthritis scores had reduced locomotion (Hartog et al, 2009). Those with higher levels of locomotion had higher muscle weight. Studies in patients with RA have shown that energy and protein intake were similar to control patients but body cell mass (the mass of all the metabolically active tissue of the body) was lower in RA patients (Roubenoff et al, 1994).

3.5.3 Effect of CIA on vascular responses in WT C57BL/6 mice

In line with previous work in DBA-1 mice (Reynolds et al, 2012), arthritic C57BL/6 mice had impaired vasoconstriction to 5-HT compared to non-immunised control mice. Contractile dysfunction was not accompanied by overt endothelial dysfunction; endothelium-dependent relaxation responses to Acetylcholine were similar between groups. Moreover, relaxation responses to exogenously donated nitric oxide (using SNO) were unaffected. This again is in line with previous work by Reynolds et al (2012). There was no significant relationship between maximal developed tension and arthritis severity at the time of sacrifice, although there was a trend towards higher maximal developed tension with lower total paw score. However, the number of mice that developed an arthritis was relatively small (nine) and so this may have become significant with a greater group numbers. Previous work by Reynolds et al found there was a significant reduction in maximal developed tension in mild and moderate graded mice compared to the severe grade. See Reynolds et al (2012) for information about arthritis severity grading. Due to the variable day of onset of arthritis and variable progression of arthritis severity for each mouse, at the time of sacrifice mice had variable total paw scores (although all were in the mild category except for one). Mice also had had arthritis for a variable number of days at the time of sacrifice. In view of this, constriction responses may have differed between arthritic mice.

The reason for the observed reduction in aortic constriction in CIA has been examined previously in our department. Aortic and plasma levels of MMP-9 were increased with increasing contractile dysfunction (Williams et al, 2016). MMP-9 is type IV collagenase enzyme, and thus can degrade the extracellular matrix in the aorta. A previous study found that inhibition of cytokine-induced NO expression in rat aortic smooth muscle cells was associated with a selective, dose-dependent increase in MMP-9 expression and synthesis (Upchurch et al 2001). Other work in our department has found that aortic collagen and elastin become dysregulated during CIA and show a fibrosis like phenotype (unpublished yet).

3.5.4 CIA and vascular responses in IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice

No IL-6$^{-/-}$ mice developed arthritis, in line with previous work in IL-6$^{-/-}$ mice (Alonzi et al, 1998). Here, for the first time, we show that IL-6R$^{-/-}$ mice are resistant to CIA. This is in line with the evidence that the action of IL-6 is necessary for the development of CIA.

Although small numbers, there appears to be impaired vasoconstriction in immunised IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice. This is interesting, as there is much evidence that

IL-6 is implicated in the pathogenesis of CVD and correlates with CV risk in humans (Hartman and Frishman, 2014). Previous work found no significant difference in phenylephrine induced constriction between WT and IL-6$^{-/-}$ mice (Coles et al, 2007), but constriction responses have not been assessed in IL-6R$^{-/-}$ mice previously. Another previous study has found that IL-6$^{-/-}$ mice were protected against angiotensin II induced endothelial dysfunction and hypertrophy in carotid arteries (Schrader et al, 2007). Unfortunately, breeding of the in-house IL-6$^{-/-}$ and IL-6R$^{-/-}$ colonies was poor and no further mice were available to increase numbers to assess constriction responses. It would be of interest to assess constriction responses in non-immunised IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice and compare with immunised IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice; perhaps baseline constriction responses would be unaffected, but the addition of an inflammatory insult, such as immunisation with CFA, could promote the vascular dysfunction seen in this experiment. We know from several previous studies that IL-6 plays homeostatic roles in lipid metabolism and atherosclerosis (Schieffer et al, 2004). What has not been fully elucidated is the role of IL-6 in homeostatic vascular function. It is known that IL-6 is needed for epithelial cell repair, for example in the gastrointestinal tract. Perhaps IL-6 is also needed for vascular endothelial cell repair and to protect against early insult to the vasculature. IL-6 is known to play a role in lipid metabolism and ApoE$^{-/-}$-IL-6$^{-/-}$ double knockout mice have greater atherosclerosis than ApoE$^{-/-}$ mice. If complete absence of IL-6 promotes atherosclerosis then perhaps IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice have early atherosclerotic changes such as fatty streaks which may be contributing to reduced aortic constriction. One way to assess this would be to histologically examine the aortas of both immunised and non-immunised IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice for early atherosclerosis.

As mentioned, although IL-6$^{-/-}$ and IL-6R$^{-/-}$ mice share similarities, some differences have been seen phenotypically. Whereas IL-6$^{-/-}$ mice have impaired wound healing, IL-6R$^{-/-}$ mice heal almost as well as WT mice (McFarland-Mancini et al, 2010). In this study, 100% of IL-6$^{-/-}$ mice developed ulcers at the wound boundary, compared to just 18% of IL-6R$^{-/-}$ mice and 8% of WT mice. It is unclear whether these ulcers were provoked by persistent irritation from an open wound, phenotypic defects in wound contraction or psychological/stress-induced from defects in IL-6. Surprisingly, mice deficient in both IL-6 and IL-6R, or IL-$6^{-/-}$ mice treated with an IL-6R blocking antibody, showed better wound healing than IL-$6^{-/-}$ mice. This suggests that absence of IL-6R is beneficial to wound healing. The reasons for this is unclear but there are other members of the IL-6 family; ciliary neurotrophic factor (CNTF) (Schuster et al, 2003) and IL-27 (Crabe et al, 2009), which can bind to IL-6R in vitro but with lower affinity than IL-6. The relevance of these findings remains unclear in vivo.

A disadvantage of using IL-$6^{-/-}$ or IL-$6R^{-/-}$ mice in this model is that these mice are resistant to arthritis. Therefore, we cannot be sure if any change in vascular response is just due to the absence of arthritis and its associated systemic inflammation. IL-$6^{-/-}$ mice do produce antibodies to collagen II in response to CIA, but levels peak later and are reduced compared to IL-$6^{+/+}$ mice (Sasai et al, 1999). In addition, IL-$6^{-/-}$ mice have a complete lack of IL-6 and so any effect on the vasculature could be due to absence of either IL-6 classical or trans-signaling, or both. Similarly with IL-$6R^{-/-}$ mice there is neither classical IL-6 signaling, nor IL-6 trans-signaling, as there is no IL-6 or sIL-6R available. Therefore, it was decided that to further understand the role of trans-signaling on vascular function in mCIA, sgp130Fc would be used to specifically block IL-6 trans-signaling, whilst allowing classical signaling to continue.

3.5.5 Effect of sgp130fc on arthritis severity and vascular function

When administered intraperitoneally, there was no effect of sgp130Fc on arthritis incidence or severity compared to PBS controls. This is in contrast to previous work (Nowell et al, 2006). Sgp130Fc consists of the extracellular portion of gp130 linked to the Fc domain of a human IgG1 antibody. Its molecular weight is 186kDa. Since these previous experiments were performed, the production process of sgp130Fc has changed. Human sgp130 was undetectable, when checked by ELISA, in mice administered given sgp130Fc IP, suggesting it did not reach the vasculature. One reason for this may be that the change in sgp130Fc production process may have changed its structure, perhaps conferring a greater propensity to aggregate and making it unable to cross the peritoneal membrane. There was also no difference in maximal aortic developed tension in those given IP sgp130Fc, probably as this did not affect arthritis severity. When administered intravenously, human sgp130 was detectable in the serum. Therefore the reason for IP sgp130Fc having no effect is likely to be due to its route of administration. Other possible reasons for sgp130Fc being ineffective when administered IP are poor administration skill, although this seems less likely as previous experiments with the same investigator using IP injections have been successful.

When administered intravenously, both sgp130Fc and etanercept reduced arthritis severity in CIA compared with mice administered PBS. Paw scores were more than double in the PBS group compared to sgp130Fc and etanercept groups. Sgp130Fc and etanercept each reduced arthritis incidence by 55%. This is similar to the literature; Wooley et al (1993) found a 61% reduction in CIA incidence in anti-TNF treated mice on day 55. Carty et al found a 50% reduction in incidence of mCIA when mice were administered sgp130Fc compared to PBS (from thesis but not published). Rose et al (2013) developed a mouse model called K/BxAg7 which develop an erosive inflammatory arthritis, followed by atherosclerosis after 12 weeks. These mice have dyslipidaemia and increased IL-6 levels. The study found that etanercept reduced arthritis and atherosclerotic burden in these mice.

For the first time this chapter demonstrates that both sgp130Fc and etanercept prevented the reduction in constriction responses seen in PBS treated mice. As both of these drugs also improved the arthritis severity, it is difficult to know whether this effect on the vasculature is related to reduced arthritis severity, or whether the drugs are having a direct effect on the blood vessels. Interestingly, although the arthritis incidence in sgp130Fc and etanercept groups was 50%, constriction responses in these mice were not significantly different from non-arthritic control mice, perhaps suggesting a greater effect of both drugs on the vasculature than on the joints. Within individual groups: sgp130Fc, etanercept and PBS there was no significant correlation between paw score and maximal developed aortic contraction. There was a trend within each group for a negative correlation, and this was most pronounced in the PBS group. Therefore, we cannot say that there is a direct relationship between improvement in arthritis severity and improvement in vascular function with sgp130Fc and etanercept.

Serum CCL2 was significantly higher in mice with CIA administered PBS than those administered sgp130Fc. CCL2 recruits monocytes, memory T cells and dendritic cells to sites of inflammation and is known to be increased in serum and synovial fluid of RA patients (Koch et al, 1992). CCL2 levels also correlate with CIMT in RA patients (Södergren et al, 2010). CCL2 gene inactivation significantly decreases progression of atherosclerotic lesions (Linic et al, 2013) and CCL2 expression is increased in atherosclerotic lesions and injured arteries (Egashira et al, 2003). CCL2 is produced by many cell types, including endothelial, fibroblasts, epithelial, smooth muscle, mesangial, astrocytic, and microglial cells. However, monocytes and macrophages are the major source of CCL2 (Deshmane et al, 2009). Macrophage numbers in the aorta have been shown to be increased in CIA (Williams et al, 2016). The reduction in CCL2 in mice administered sgp130Fc seen in this work is in line with the literature; work by Nowell et al (2003) has shown that IL-6 trans-signaling (via Hyper-IL-6) increased arthritis severity and controlled intrasynovial mononuclear leukocyte recruitment through CCL2. In the same study, treatment of IL-6$^{+/+}$ mice with sgp130Fc significantly reduced the intensity of CCL2 staining to levels comparable with that seen in IL-6$^{-/-}$ mice. Treatment with etanercept did not reduce CCL2 levels compared to PBS. Previous studies have found that TNF-α enhances the expression of CCL2 in astroctyes (Barna et al, 1994) and human proximal tubular epithelial cells (Ho et al, 2008) but to my knowledge, there are no studies reporting the effect of TNF-α on expression of CCL-2 on the vascular endothelium. As both sgp130Fc and etanercept restored vascular function but etanercept did not reduce serum CCL2, we cannot conclude that the improvement in vascular responses with these drugs is solely due to reduced CCL2.

Serum VCAM-1 levels were significantly higher in mice with CIA administered PBS than those administered IV sgp130Fc, etanercept or non-immunised control mice. Previous work using the Biomap® system has shown that IL-6 trans-signaling regulated the release of VCAM-1 from endothelial cells, PBMCs and coronary artery smooth muscle cells. Thus, the reduction seen in serum VCAM-1 levels in those administered sgp130Fc compared to PBS would fit with this. Mice administered etanercept also had lower VCAM-1 than those administered PBS. TNF-α is known to increase the expression of VCAM-1 in human endothelial cells (Kim et al, 2001; Haraldsen et al, 1996) and a study in patients with RA found that serum VCAM-1 levels were significantly lower after treatment with infliximab, an anti-TNF antibody (Klimiuk et al, 2004). Previous work in CIA has shown that qRT-PCR VCAM-1 expression in the aorta was higher in CIA versus non immunised mice (Denys et al, 2013). Interestingly, the expression of IL-6 in the aorta was similar in all groups. VCAM-1 induces immune-cell recruitment within the vessel wall and therefore inflammation. Thus, the improved vascular constriction response seen with both sgp130Fc and etanercept could be due to the reduction in VCAM-1 and therefore reduction in recruitment of inflammatory cells into the aorta.

pSTAT3 was not detected in the aorta of either control or arthritic mice in this study, but was present in the arthritic joints. This positive staining in the joint is in keeping with previous work in AIA which has reported rapid activation of STAT-3 following arthritis induction, with pSTAT3 staining in arthritic joints (Nowell et al, 2009). In this study, activation of STAT-3 was significantly impaired in IL-6$^{-/-}$ mice. A previous study in human aortic tissue has shown that pSTAT3 staining only occurred in aneurysmal tissue, and not in non-aneurysmal tissue (Liao et al, 2010). Studies in ApoE$^{-/-}$ mice have shown positive staining for pSTAT3 in the aorta (Madrigal-Matute et al, 2010). As blockade of trans-signalling restored vascular function in CIA, it could be proposed that in the aorta of arthritic mice, IL-6 trans-signaling pathways should be at play, and thus pSTAT3 should be detectable in the aorta of arthritic mice. However, this maybe time dependant and perhaps if mice were culled earlier, this may have been detectable. Another reason for the lack of pSTAT3 staining in the aortic tissue could be tissue dependant; the pSTAT3 stain has been used previously in the laboratory with good results in joints, but has not been used previously in aortic tissue.

3.6 Conclusion

This work confirms the variable penetrance and severity of CIA in C57BL/6 mice. The work in this chapter also shows validation of previous findings of vascular dysfunction in CIA, in that arthritic wild type mice have reduced aortic constriction to 5-HT. In line with previous work, IL-6$^{-/-}$ were resistant to CIA. For the first time, we show that IL-6R$^{-/-}$ are also resistant to CIA. Interesting trends for reduced vascular constriction in immunised IL-6$^{-/-}$ and IL-6R$^{-/-}$ have been presented. We have shown that selective blockade of IL-6 trans-signalling using intravenous sgp130Fc reduces arthritis severity and restores the vascular dysfunction associated with CIA. We have also shown that blockade of TNF-α also restores vascular dysfunction in CIA. Blockade of IL-6 trans-signaling was associated with a reduction in serum CCL2 and VCAM-1. This work demonstrates the importance of IL-6 trans-signaling in vascular dysfunction in an animal model of CIA. However, the relevance of this in human RA remains to be established. Also, the role of IL-6 trans-signaling in the 'end points' of vascular dysfunction, namely atherosclerosis, remains to be elucidated. Therefore the following chapters will explore the role of IL-6 trans-signaling in atherosclerosis in an animal model and the association of IL-6 trans-signaling-regulated proteins with progression of atherosclerosis in patients with RA.

4 Chapter 4 – The Effect of IL-6R mediated trans-signalling on Atherosclerosis in the ApoE$^{-/-}$ Mouse

4.1 Introduction

Cardiovascular risk is increased in RA and strongly associated with inflammation. This increase in cardiovascular risk and cardiovascular events is present soon after RA diagnosis. This suggests that inflammation in RA could accelerate pre-existing subclinical atherosclerosis. By using an animal model of atherosclerosis we can examine the effect of inflammation, specifically the effect of IL-6 trans-signaling, on atherosclerosis development in susceptible animals. As mentioned in section 1.16.2 of the general introduction, there are several animal models of atherosclerosis. However, the ApoE$^{-/-}$ mouse model detailed in this chapter was chosen because of its lesion comparability with humans and the timescale of lesion development in this model (Asquith et al, 2010).

4.1.1 The ApoE$^{-/-}$ mouse

Apolipoprotein E (ApoE) is a class of apolipoprotein found in chylomicrons and intermediate density lipoproteins and functions primarily as a lipid transporter. It plays a protective role in atherosclerosis. ApoE has three major isoforms; ApoE2, ApoE3 and ApoE4. It functions as a ligand for receptors that clear very low–density lipoprotein (VLDL) remnants and chylomicrons. ApoE is predominantly synthesized in the liver but is also found in the brain, spleen, lung, kidney, ovary, testis, peripheral nerves and muscle (Zhang et al, 2011). It is also synthesized by macrophages and monocytes in vessels, and has local effects on inflammatory reactions in atherosclerotic vessels (Curtiss, 2000), such as inhibiting SMC proliferation and migration induced by oxidised LDL and platelet-derived growth factor (Ishigami et al, 2000). ApoE exerts its biological functions by binding to the low-density lipoprotein receptor (LDLR) family, effectively mediating the uptake of lipoproteins by cells

(Mahley, 1988). Therefore, $ApoE^{-/-}$ mice have delayed clearance of lipoprotein particles and even on a normal chow diet have increased cholesterol and triglyceride levels compared to their normal litter-matched controls (Jawieri et al, 2004).

The initial stages of atherosclerosis in the $ApoE^{-/-}$ mouse develop after 5-6 weeks on a normal chow diet. This involves the attachment of monocytes to the endothelial wall, then subsequent passage into the endothelial space. After 10 weeks, fatty streaks appear and at around 20 weeks fibrous plaques develop. Lesions in $ApoE^{-/-}$ mice on Western-type diet occur earlier and contain more lipid than those on chow diet (Nakashima et al, 1994). On a western diet fatty streaks occur at around 8 weeks and by 15 weeks early fibrous plaques are seen (Nakashima et al, 1994).

4.1.2 The effect of IL-6 on atherosclerosis on the $ApoE^{-/-}$ mouse

In humans, long term IL-6 levels are associated with CVD risk as strongly as some key established CV risk factors (Danesh et al 2008). There are conflicting results of the effect of IL-6 on atherosclerosis in $ApoE^{-/-}$ mice. A study by Huber et al showed that IL-6 increased atherosclerotic lesion size in both C57BL/6 and $ApoE^{-/-}$ mice 1.9- to 5.1-fold compared to saline-treated animals (Huber et al, 1999). Mice were administered recombinant IL-6 (5000u) weekly whilst fed either high fat or normal chow diet for 6 to 21 weeks. In contrast to this, $ApoE^{-/-}$-IL-$6^{-/-}$ double knockout mice had higher serum cholesterol and increased atherosclerotic lesion formation compared to $ApoE^{-/-}$ mice (Schieffer et al, 2004). Therefore these studies have shown an increase in atherosclerosis when IL-6 is administered above physiological levels, but also in the complete absence of IL-6. However, the studies do not specifically examine the role of IL-6 trans-signaling versus classical IL-6 signalling in atherosclerosis.

One study has examined the role of IL-6 trans-signaling in atherosclerosis development in $LDLR^{-/-}$ mice (Schuett et al, 2012). Here, blockade of IL-6 trans-signaling, by administration of sgp130Fc, reduced atherosclerosis in $LDLR^{-/-}$ mice without affecting serum lipid levels. This study addressed the effect of blocking IL-6 trans-signaling on atherosclerosis in a non-inflammatory setting. These mice do not have inflammatory disease so their IL-6 and IL-6R levels will be similar to normal individuals and patients with ischaemic heart disease. However, in RA patients levels of IL-6 and IL-6R are 10 fold higher. This chapter uses Hyper-IL-6 to reflect these increased IL-6/IL-6R levels and examines their effect on atherosclerosis in the ApoE$^{-/-}$ mouse model.

4.1.3 Assessment of atherosclerosis in mice

Assessment of atherosclerosis has mostly been made by quantifying the area of plaques, usually in the aorta, aortic root or brachiocephalic artery. Two main methods have been used, the en face method and cross sectional analysis. The en face method involves spitting the aorta down its entire length and pinning it to expose the luminal endothelium. The aorta can then be stained for lipids, e.g. with Oil red O. In cross sectional analysis, the brachiocephalic artery or aortic root are most often used. This allows serial quantification of cross sections, and also staining for other uses, such as immunohistochemistry. Imaging software can be used for both methods to accurately measure plaque size.

As well as plaque size, other measures are necessary to assess plaque stability. Plaque stability is a key factor in atherosclerosis assessment as this is directly linked to risk of a rupture, which in turn can lead to occlusive thrombosis and death. Markers of plaque stability include thickness of fibrous cap, and presence and extent of intraplaque hemorrhage, inflammation and necrotic core (van der Wal and Becker, 1999) (Figure 33). There is increasing data that the brachiocephalic artery is a useful site to examine plaque size and stability in mice (Jackson et al, 2007; Teupser et al, 2003). The brachiocephalic artery is a short vessel which arises from the arch of the aorta (Figure 34). It branches into the right subclavian and right common carotid arteries. In this chapter, we also examine the effect of Hyper-IL-6 and IL-6 on fibrous cap thickness in the brachiocephalic artery in the ApoE$^{-/-}$ mouse.

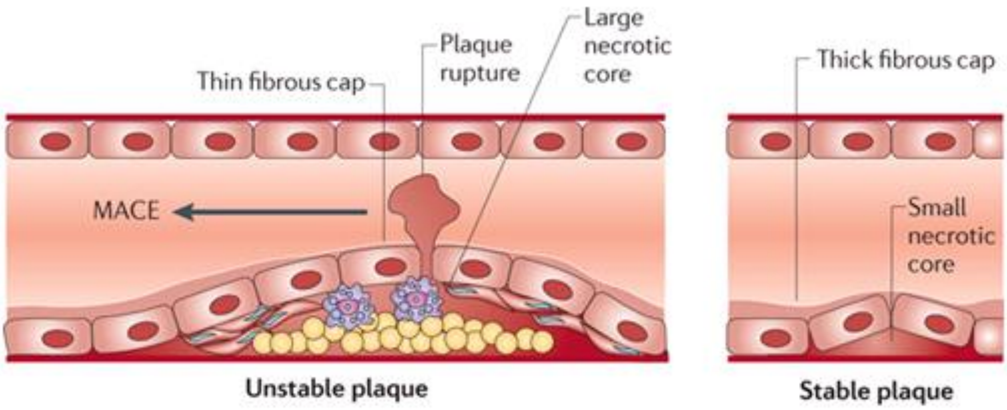

Figure 33. A schematic diagram of an artery showing a ruptured unstable plaque versus a stable plaque. Unstable plaques are characterised by increased numbers of inflammatory cells and mediators, large necrotic cores and thin fibrous caps. When the fibrous cap is eroded, prothrombotic material leaks out, resulting in thrombus formation. In contrast, stable plaques have less inflamamtion, smaller necrotic core and thicker fibrous cap. (MACE = major adverse cardiac events) Taken from Charo et al, 2011.

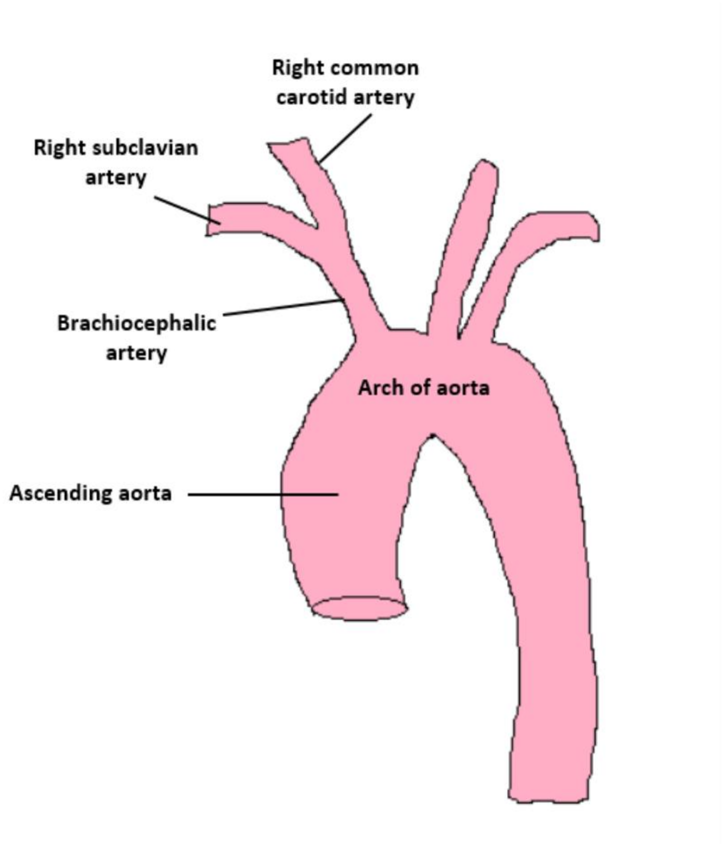

Figure 34. A schematic diagram of the brachiocephalic artery. This branches off the arch of the aorta and then divides into the right subclavian artery and the right common carotid artery.

4.2 Hypothesis and aims

The aim of this chapter was to determine the effect of IL-6 trans-signaling on atherosclerosis development in the ApoE$^{-/-}$ mouse. Using this model allows examination of other defined outcomes such as plaque size, expression of SCCPs in plaque and fibrous cap thickness.

This chapter details methodology, results and discussion under the following hypothesis and aims:

Hypothesis: IL-6 trans-signaling accelerates atherosclerosis in ApoE$^{-/-}$ mice.

In order to address this hypothesis the following four aims were identified:

1. To determine the effect of IL-6 trans-signaling, using Hyper-IL-6, on aortic plaque size and to compare any effect with that caused by IL-6 and PBS using ApoE$^{-/-}$ mice
2. To determine the effect of IL-6 trans-signaling, using Hyper-IL-6, on brachiocephalic plaque size and to compare any effect with that caused by IL-6 and PBS using ApoE$^{-/-}$ mice
3. To analyse the effect of IL-6 trans-signaling on fibrous cap thickness by treating ApoE$^{-/-}$ mice with Hyper-IL-6 and comparing against results obtained by IL-6 and PBS administration.
4. To measure levels of VCAM-1 in serum and the brachiocephalic artery from ApoE$^{-/-}$ mice treated with Hyper-IL-6, IL-6 and PBS.

4.3 Specific methods

4.3.1 Mice

All ApoE$^{-/-}$ mice were purchased from Charles River, UK at 7 weeks of age. A previous study demonstrated no significant difference in lesion size formation between

C57BL/6 mice housed under two conditions that inferred a range of infective states (Mallat et al, 1999). There are many variables that could potentially affect atherosclerosis development other than pathogen status, for example sterilisation status of food, water source, and bedding composition. As RA patients are exposed to multiple environmental agents, including infectious agents, it was felt that conventional housing was more likely to mirror this environment than pathogen-free housing.

At 8 weeks of age mice were fed high-fat diet containing 21% (wt/wt) pork lard and supplemented with 0.15% (wt/wt) cholesterol for 8 weeks. Experiments were performed in accordance with the home office approved project licence (PL 302822). Mice had free access to water and had a light dark cycle of 12 hours. The temperature range in the animal house was 19-23°C and humidity 55 $\pm$ 10%.

To ensure atherosclerosis development in our facility over this time period, under the above conditions, 6 mice were initially sourced and fed as above. This test batch was then sacrificed and atherosclerosis was quantified. Once it was shown that these conditions produced atherosclerosis the following experimental approach was taken. Mice were divided into three groups and drugs were administered intraperitoneally as follows:

Group	N number	Drug	Dose per mouse	Volume per administration	Frequency of administration	Duration
1A	6	Hyper-IL-6	0.5µg	100µl	Twice weekly	8 weeks
1B	6	IL-6	0.16µg	100µl	Twice weekly	8 weeks
1C	6	PBS	N/A	100µl	Twice weekly	8 weeks

Table 19. Drugs and doses administered to ApoE$^{-/-}$ mice in Experiment 1. See section 4.3.2 for rationale for drug doses

In order to check the reproducibility of the initial result a second experiment was undertaken (Experiment 2). An additional experimental group was introduced in this experiment; 1 µg Hyper-IL-6 was tested in order to assess whether the effect upon IL-6 trans-signalling was concentration dependent.

Group	N number	Drug	Dose per mouse	Volume per administration	Frequency of administration	Duration
2A	6	Hyper-IL-6	0.5µg	100µl	Twice weekly	8 weeks
2B	6	IL-6	0.16µg	100µl	Twice weekly	8 weeks
2C	6	PBS	N/A	100µl	Twice weekly	8 weeks
2D	6	Hyper-IL-6	1µg	100µl	Twice weekly	8 weeks

Table 20. Drugs and doses administered to ApoE$^{-/-}$ mice in Experiment 2.

4.3.2 Rationale for drug doses

No previous studies in the literature could be found which had administered Hyper-IL-6 to mice over a period of 8 weeks; most studies administered Hyper-IL-6 only as one dose (Nechemia-Arbely et al, 2008; Rakemann et al, 1999). Therefore an approximate equivalent dose was chosen to that of levels of circulating IL-6/sIL-6R complex in human inflammatory arthritis. In a study by De Benedetti et al (1994), levels of circulating IL-6/sIL-6R complex in systemic JIA were 6.8-8.9 ng/ml. From my own work in patients with RA, serum levels of IL-6/sIL-6R complex were between 0-16.5 ng/ml (n= 83). Peters et al (1998) administered 0.4 µg IP of Hyper-IL-6 to mice and sacrificed them 4 hours after administration. Serum levels of Hyper-IL-6 were approximately 10 ng/ml, thus for this experiment 0.5 µg of Hyper-IL-6 was thought to be an appropriate dose. An equivalent molar concentration of IL-6 was used; the molecular weight of IL-6 is 20kDa and Hyper-IL-6 60kDa therefore 0.16 µg of IL-6 was chosen.

Hyper-IL-6 was a gift from Professor Rose John in Kiel University and IL-6 was sourced from R&D systems (206-IL).

4.3.3 Monitoring of mice

Mice were weighed twice weekly and monitored at least 3 times per week for general health status. A side effect that can occur with high fat feeding in ApoE$^{-/-}$ mice is skin irritation, manifest as scratching and scaly skin which can lead to ulceration. This tends to occur after several months of high fat feeding. If mice in this experiment had developed ulcers from these effects then they would have been euthanised using a humane method.

4.3.4 Collection of Experimental Samples

Mice were culled using a schedule 1 approach and blood collected by cardiac puncture for serum analysis. The chest cavity was opened and the heart continuously perfused with PBS. The right atrium was snipped to allow blood to flow out. The chest and neck were dissected to allow observation of the brachiocephalic and carotid arteries. The brachiocephalic artery was carefully dissected and removed with a piece of the aortic arch. This was immediately embedded in OCT and snap frozen in liquid nitrogen (Figure 35). The heart was removed, cut and placed in OCT and immediately frozen.

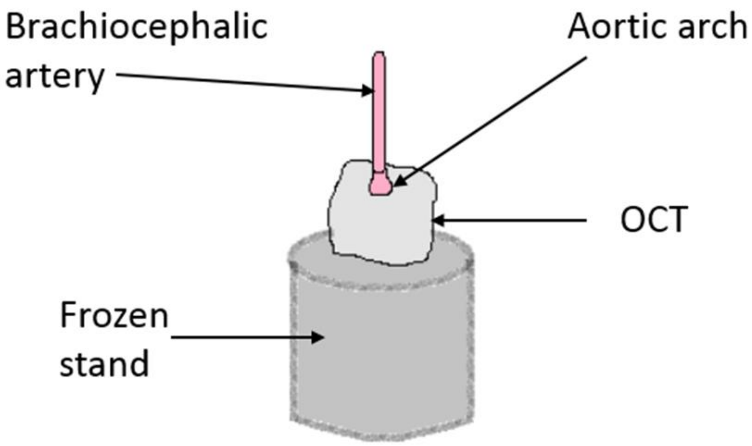

Figure 35. Diagram of mounting procedure of the brachiocephalic artery. After dissection the brachiocephalic artery, along with a piece of the aortic arch was suspending into OCT which is mounted on a frozen stand. This was then immersed in liquid nitrogen to snap freeze it.

4.3.5 Sectioning of cardiac tissue and brachiocephalic artery

The OCT-embedded heart was mounted in a cryostat set at -22°C. Serial aortic root cross-sections of 7µm thickness were taken at the level of all three leaflets of the aortic valve and mounted on glass slides (Superfrost Pink). Slides were then stored at -80°C.

The OCT-embedded brachiocephalic artery was mounted in a cryostat set at -22°C. The section was orientated so that the aortic arch was cut first. Sections were cut through the aorta until the brachiocephalic artery was reached. Serial section of 7um thickness were cut and mounted on glass slides (Superfrost pink).

4.3.6 Staining tissue with Oil-red O for lipid content

Oil red O stock was prepared by dissolving Oil red O (2mg/ml) (Sigma UK) in Isopropyl Alcohol (Fischer scientific). Then an equal concentration of Oil red O stock was mixed with 30% Isopropyl Alcohol and filtered through a 0.22 micron filter. This was allowed to stand for 15 minutes before use.

Slides were air dried for 20 minutes. Then the protocol was performed as below.

Procedure	**Duration**	**Purpose**
Distilled water	Dip in	Remove residue
30% Isopropyl Alcohol	5 minutes	Rinse
Working Oil Red O	20 minutes	Stains lipids
30% Isopropyl Alcohol	Dip in	Differentiate
Distilled water	Dip in	Rinse
Harris Haematoxylin	1 minute	Stains nuclei
Distilled water	Until Haematoxylin stops leaching out	Remove excess stain
Mounted in glycerol and coverslip		Keep moist and preserve stain

Table 21. Protocol for Oil Red O staining of frozen sections

4.3.7 Determination of plaque size

Lesion size was determined by computer-assisted morphometry on stained sections. Morphometry was performed using Image ProPlus™ software version 4.0 (Media Cybernetics).

4.3.8 Immunohistochemistry

Immunohistochemistry of the brachiocephalic lesions was performed to examine staining for VCAM-1. VCAM- was stained for as in human RA patients this correlated with disease activity and CV risk and in CIA models CIA is associated with increased aortic expression of VCAM-1 (Denys et al, 2016). Slides were taken out of the freezer and left at room temperature before the following protocol was performed.

Process	Rationale	Time
Acetone at -20°	To fix slide	5 minutes
PBS	Wash	5 minutes
10% H202	Block endogenous peroxide	15 minutes
PBS	Wash	5 minutes
10% normal goat serum in TBS/T	Decrease non-specific binding of antibody	15 minutes
Remove serum		
Avidin block	Decrease non-specific binding of antibody	10 minutes
TBS/T	Wash	5 minutes
Biotin block	Decrease non-specific binding of antibody	10 minutes
Remove biotin		60 minutes
TBS/T	Wash	5 minutes
Primary antibody or isotype control antibody	Identification of target protein and control IgG staining	Overnight (at 4°C)
Allow to stand at room temperature		1 hour
PBS	Remove excess antibody	3 X 5 minutes
Detection antibody : biotinylated anti-rabbit IgG, affinity purified from goat, Vector labs #BA-10000) at 1:200 dilution in TBS/T	Identification of bound primary antibody	60 minutes
TBS/T	Remove excess antibody	3 x 5 minutes
ABC reagent from kit 30 mins	Identify bound Antibody in sections	30 minutes
TBS/T	Wash	3 x 5 minutes
DAB substrate	Allow identification of positive staining	1- 20 minutes
Distilled water	Rinse	1 minute
Harris Haematoxylin	Nuclear stain	10 seconds
Distilled water	Remove excess stain	Until water runs clear
Cover with glycerol and coverslip	Prevent drying	

Table 22. Protocol for immunohistochemistry for staining for VCAM-1 on frozen brachiocephalic sections.

4.3.9 Analysis of VCAM-1 staining

Firstly the brachiocephalic artery and plaque was examined to determine the location of the most intense VCAM-1 staining. In order to do this, specific areas of the same artery were examined, for example the arterial wall underlying plaque, the plaque itself and the arterial wall opposite the plaque. Photoshop was used to determine the total number of pixels in the selected area of the picture (Figure 36A). The number of brown pixels, representative of positive staining, was then calculated.

To compare the percentage staining of VCAM-1 in brachiocephalic arteries and plaque in the different experimental groups the entire brachiocephalic arterial wall and plaque of individual slides were examined (Figure 36B). The number of brown pixels, representative of positive staining, was then calculated. The positive staining was calculated as a percentage of the total region and was corrected for isotype control values.

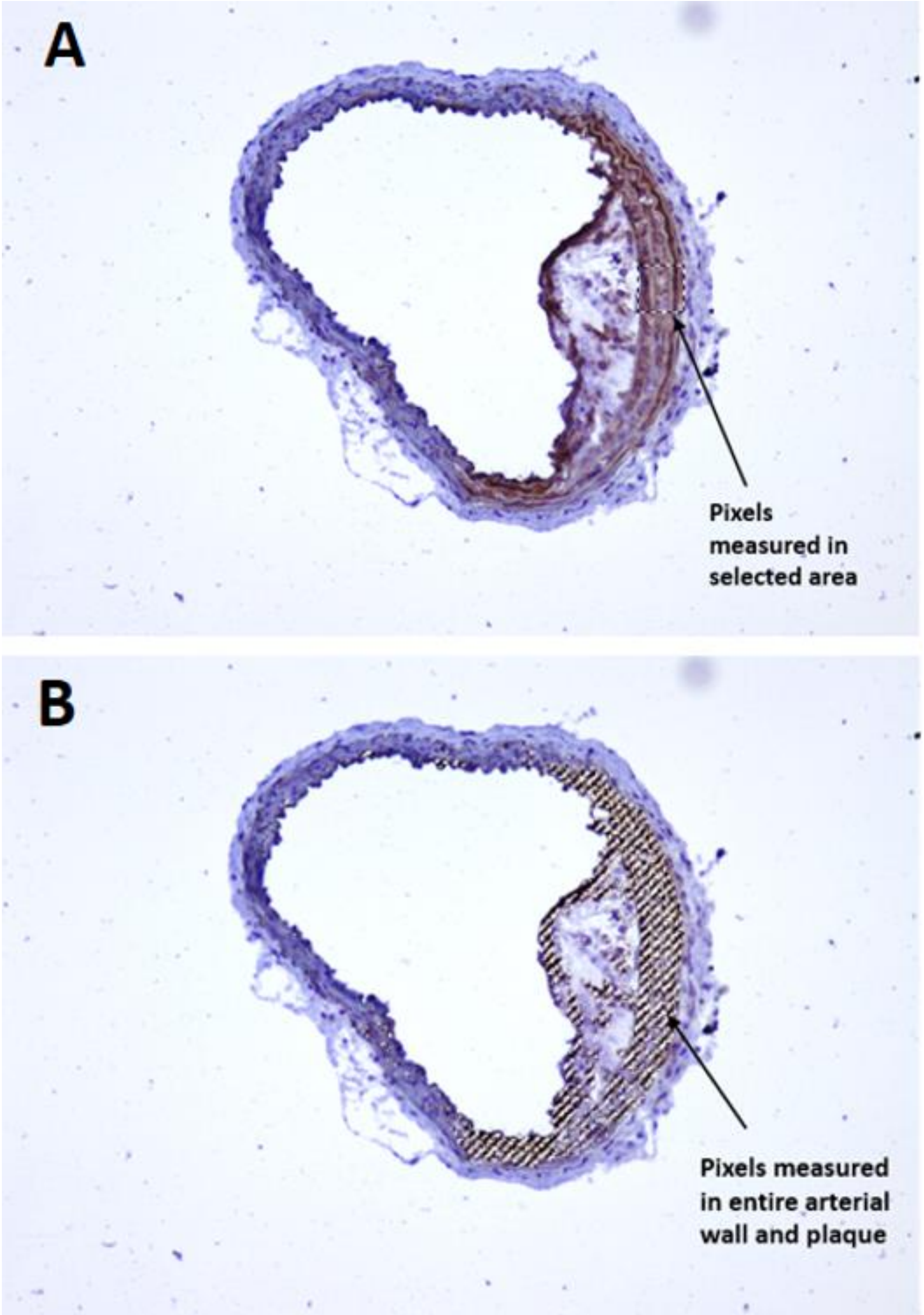

Figure 36. Examples of brachiocephalic artery sections stained for VCAM-1 and approach to measurement of staining. (A) VCAM-1 staining measured in specific areas of brachiocephalic artery or plaque. (B) VCAM-1 staining measured in the brachiocephalic artery and plaque

4.3.10 Elastin Van Geison (EVG) stain

EVG was used to stain elastin and collagen. This allows identification on the fibrous cap. Slides were taken out of the freezer and the following protocol was performed.

Procedure	**Duration**	**Purpose**
Air dry sections	30 minutes	
70% ethanol	15 minutes	Hydration
Distilled water	1 minute	Rinse
Potassium permanganate	10 minutes	Oxidising agent
Distilled water	Until stain runs clear	Rinse
1 % Oxalic acid	10 minutes	Decolourise
Distilled water	5 minutes	Rinse
Miller's elastin	Overnight on Rocker	Stains elastic fibres blue/black
70% ethanol	Dunk 7 times	
Distilled water	1 minute X 2	Rinse
Van Gieson Solution	10 minutes	Stains collagen red
Blot slides dry		Get rid of most of stain
Oven bake at 60°C	30 minutes	Increases adherence to slide
70% ethanol	3 minutes	Dehydration
90% ethanol	3 minutes	Dehydration
100% ethanol	3 minutes	Dehydration
100 % Xylene	5 minutes	Dehydration
Mount with DPX and coverslip		To preserve stain

Table 23. Protocol for Elastin Van Gieson Stain.

4.3.11 Fibrous cap thickness analysis

Image Pro-Analyser was used to measure the fibrous cap thickness, after staining with EVG. Five separate measurements were taken for each individual fibrous cap and the mean value was calculated for the best estimate. Fibrous cap thickness at the widest and thinnest part of the plaque were also recorded.

4.3.12 Serum

Using a 25 gauge needle, approximately 1ml of whole blood was taken by cardiac puncture. This was placed into vacutainers coated with clot activator and stored on ice. Samples were then centrifuged at 1600g for 10 minutes at 4°C. The serum was then aliquoted in 50ul aliquots and stored at -80°C. Serum CCL2 and VCAM-1 were measured using duoset ELISA development kits from R&D systems in accordance with manufacturer's instructions, at concentrations in Table 24. See Table 7 in general methods section for ELISA protolol for murine serum. Serum was sent for analysis of total cholesterol, HDL, LDL, triglycerides and free fatty acids at the Medical Research Council Harwell laboratory, UK.

Protein measured	**Capture antibody working concentration**	**Detection antibody working concentration**	**High standard concentration**
Mouse CCL2	200 ng/ml	50 ng/ml	250 pg/ml
Mouse VCAM-1	1 µg/ml	100 ng/ml	8000 pg/ml

Table 24. Concentrations of CCL2 and VCAM-1 antibodies and standards for ELISA.

4.3.13 Statistical Analysis

Analysis of statistical difference between the groups was carried out using a one way ANOVA with a Bonferroni post hoc test. $p < 0.05$ was considered significant.

4.3.14 Sample Size Estimation

Previous studies have shown that $ApoE^{-/-}$ increases atherosclerotic plaque by 0.4 $\pm$ 0.05 mm^2 (Asquith et al, 2010). Six animals per group will have 90% statistical power to demonstrate a significant difference with $p < 0.05$.

4.4 Results

4.4.1 Lipid rich plaques present in ApoE$^{-/-}$ mice after 8 weeks of high fat feeding

After 8 weeks of high fat feeding, 6 of 6 mice had plaques in the aortic root (Figure 37) and brachiocephalic artery (Figure 38). In the brachiocephalic artery the mean plaque area was 0.014 $\pm$ 0.0047 mm^2 and mean plaque percentage was 7.72 $\pm$ 1.51%. In the aortic root mean plaque size was 0.22 $\pm$ 0.007 mm^{2}and mean plaque percentage was 19.8 $\pm$ 1.46%.

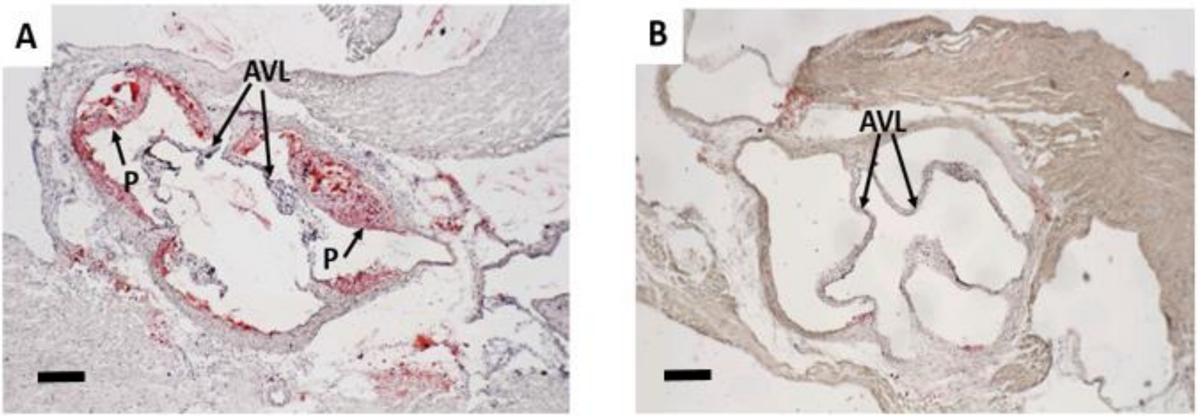

Figure 37. Aortic root section from ApoE$^{-/-}$ mouse and wild type C57BL/6 mouse stained with Oil red O (A) Aortic root from ApoE$^{-/-}$ mouse fed high fat diet from 8 weeks and sacrificed at 16 weeks, lipid rich plaques seen. (B) Wild type C57BL/6 mouse at 16 weeks at the same level of the aortic root, no plaque seen. Both at X 4 magnification. P (Plaque), AVL (Aortic valve leaflets). Scale bars = 200 µm

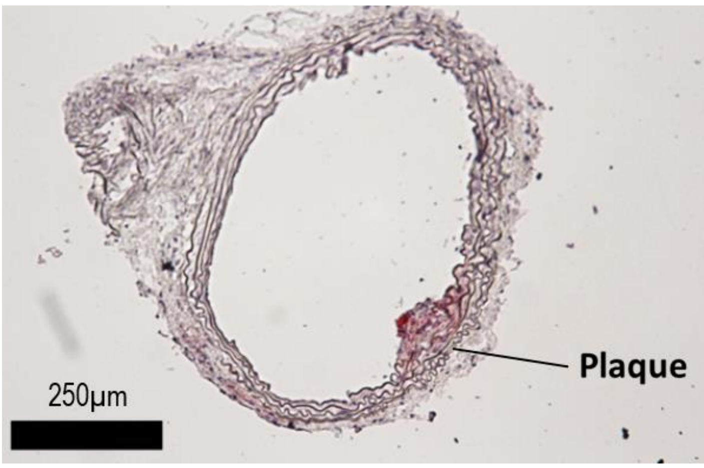

Figure 38. Brachiocephalic artery from ApoE$^{-/-}$ mouse stained with Oil red O. Lipid in plaque can be seen stained red. At X 10 magnification.

4.4.2 Skin irritation in mice administered Hyper-IL-6

The following results are grouped for experiments 1 and 2. Three of 12 mice (25%) mice in the Hyper-IL-6 0.5 µg group and 1 of 6 (16.7%) of mice in the Hyper-IL-6 1 µg group developed signs of skin irritation that required the application of clay treatment. No other mice in other groups developed signs of skin irritation.

4.4.3 Lower weight gain in mice administered Hyper-IL-6 1 µg

Mice administered Hyper-IL-6 1 µg (mean weight gain 3.5 $\pm$ 1.0 g) had significantly lower weight gain than those administered PBS (6.1 $\pm$ 2.2 g), IL-6 (6.5 $\pm$ 1.6 g) or Hyper-IL-6 0.5 µg (7.4 $\pm$ 1.8 g) (Figure 39).

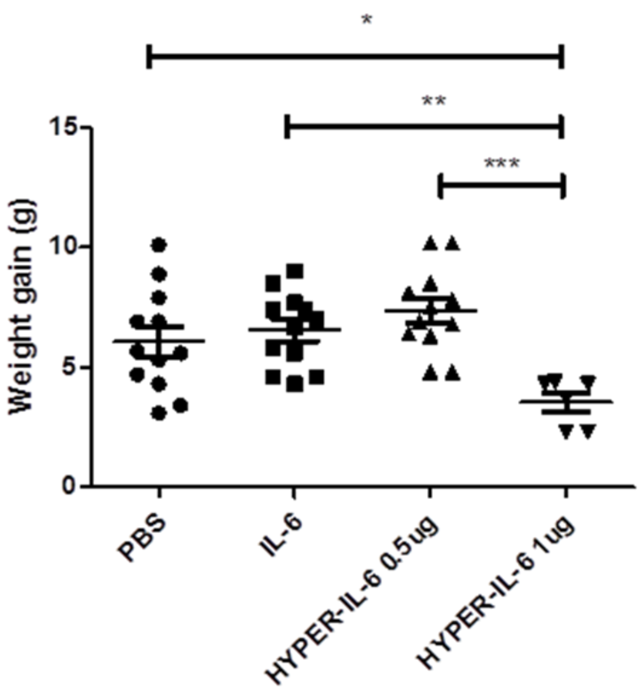

Figure 39. Weight gain in 16 week old ApoE$^{-/-}$ mice fed a high fat diet from 8 weeks and administered either PBS, IL-6, Hyper-IL-6 0.5µg or Hyper-IL-6 1µg twice weekly for 8 weeks. Weight gain was significantly lower in mice administered Hyper-IL-6 1µg than those administered PBS, IL-6 or Hyper-IL-6 0.5 µg. *p < 0.05, **p < 0.01, ***p < 0.0001

4.4.4 No significant difference in aortic root plaque size between mice administered PBS, IL-6 or Hyper-IL-6

There was no difference in aortic root plaque area or aortic plaque percentage between the groups. Mean plaque area for PBS (0.225 $\pm$ 0.01 mm^2), IL-6 (0.217 $\pm$ 0.01 mm^2), Hyper-IL-6 0.5 µg (0.228 $\pm$ 0.011 mm^2), Hyper-IL-6 1 µg (0.218 $\pm$ 0.001 mm^2) (Figure 40A). Similarly, there was no significant difference in percentage plaque in the aortic root; mean percentage plaque for PBS (21.9 $\pm$ 1.9 %), IL-6 (22.9 $\pm$ 1.1 %), Hyper-IL-6 0.5 µg (25.0 $\pm$ 1.5 %) , Hyper-IL-6 1 µg (24.1 $\pm$ 0.1 %) (Figure 40B).

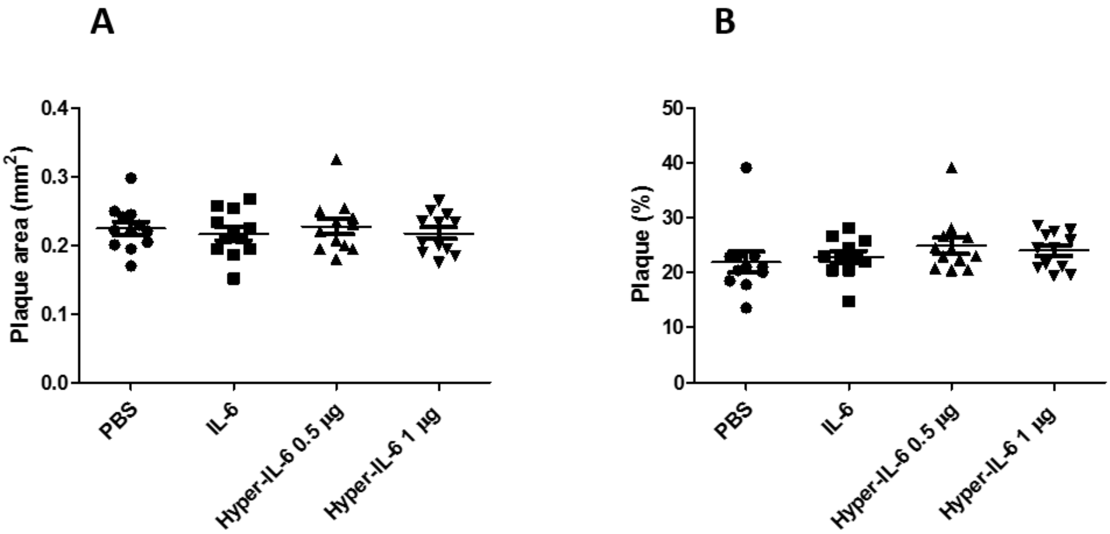

Figure 40. No difference in aortic plaque area (A) or aortic plaque percentage (B) in ApoE$^{-/-}$ mice aged 16 weeks fed a high fat diet and administered either PBS, IL-6, Hyper IL-6 0.5µg or Hyper-IL-6 1µg twice weekly for 8 weeks.

4.4.5 Significantly larger plaque and plaque percentage in the brachiocephalic arteries of mice administered Hyper-IL-6 compared to IL-6 and PBS

Mice treated with Hyper-IL-6 1µg had significantly larger brachiocephalic plaque size (0.73 $\pm$ 0.04 mm^2) than those administered PBS (0.018 $\pm$ 0.01 mm^2), $p < 0.001$, and IL-6 (0.033 $\pm$ 0.017 mm^2), $p = 0.015$ (Figure41A). Mice administered Hyper-IL-6 1µg had significantly higher percentage plaque in the brachiocephalic artery (45.3 + 18.1 %) compared to those administered PBS (10.38 + 6.7 %), *p* < 0.001 or IL-6 (20.1 + 10.2 %), *p* = 0.002 (Figure 41B). Mice administered Hyper-IL-6 0.5µg had significantly higher percentage plaque (27.7 + 16.2 %) than PBS administered mice, *p*= 0.015. Examples of brachiocephalic cross sections from each group are shown in Figure 42. There was a significant negative correlation between weight gain and brachiocephalic plaque size and brachiocephalic plaque percentage area (Figure 43).

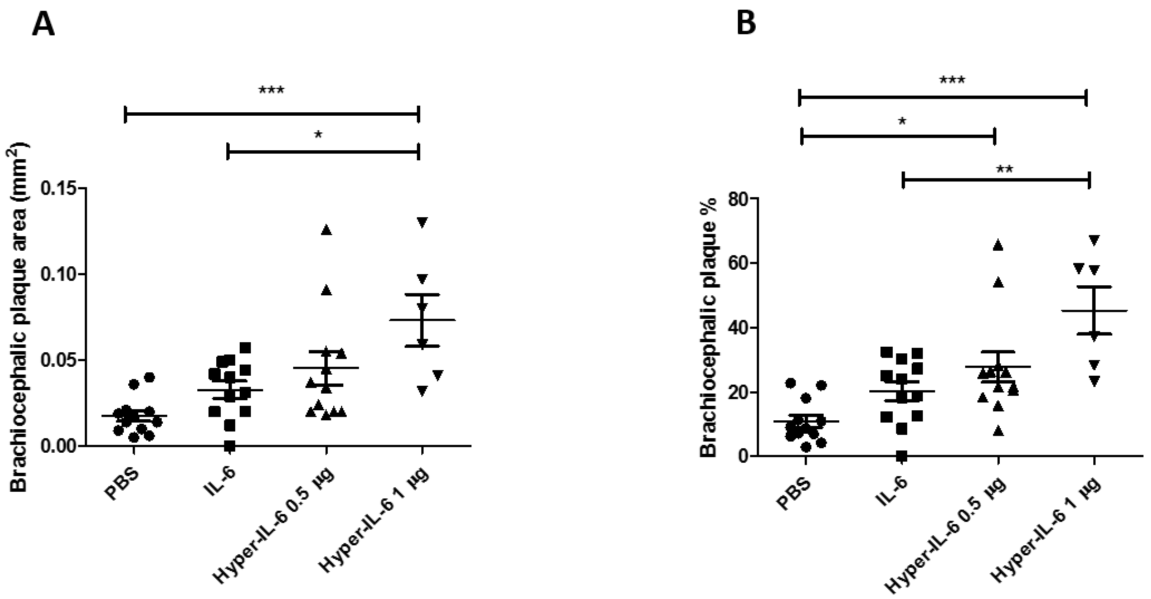

Figure 41. Brachiocephalic plaque size and plaque percentage in 16 week old ApoE-/- mice fed high fat diet from age 8 weeks and administered either PBS, IL-6 or Hyper-IL6. (A) Significantly higher brachiocephalic plaque area in mice administered Hyper-IL- µg (mean plaque area 0.73 + 0.04 mm^2) compared to those administered PBS (0.018 + 0.01 mm^2), *p* < 0.001 or IL-6 (0.033 + 0.017 mm^2), *p* = 0.015. (B) Significantly higher percentage plaque area in mice administered Hyper-IL-6 1 µg (45.3 + 18.1 %) compared to those administered PBS (10.38 + 6.7 %), *p* < 0.001or IL-6 (20.1 + 10.2 %), *p* = 0.002. Significantly higher percentage plaque in mice administered Hyper-IL-6 0.5 µg (27.7 + 16.2 %) compared to mice administered PBS *p*= 0.015.

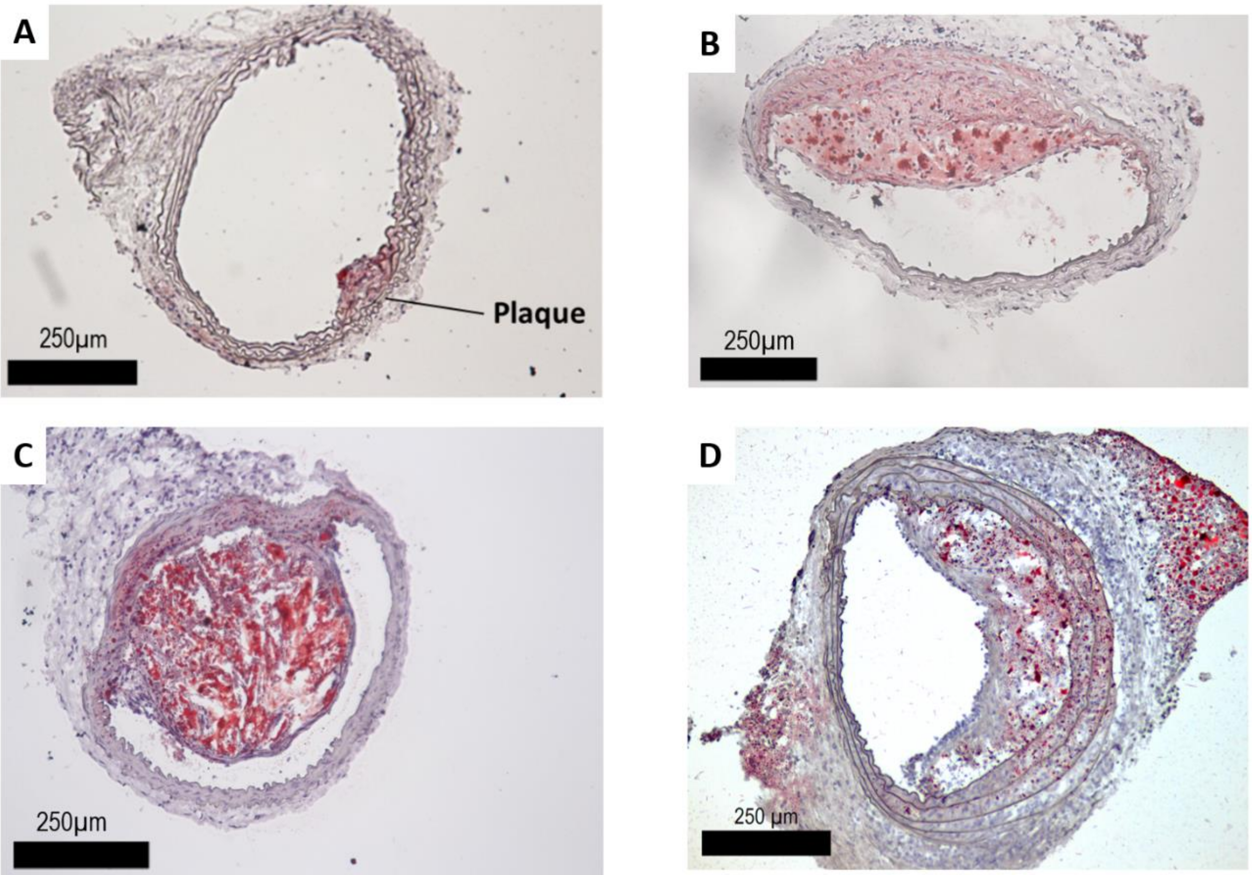

Figure 42. Examples of cross sectional images from brachiocephalic arteries from ApoE$^{-/-}$ mice aged 16 weeks fed a high fat diet from age 8 weeks and administered the following drugs twice weekly for 8 weeks from age 8 weeks (A) Mice administered PBS (B) Mice administered IL-6 (C) Mice administered Hyper-IL-6 0.5 μg (D) Mice administered Hyper-IL-6 1 μg.

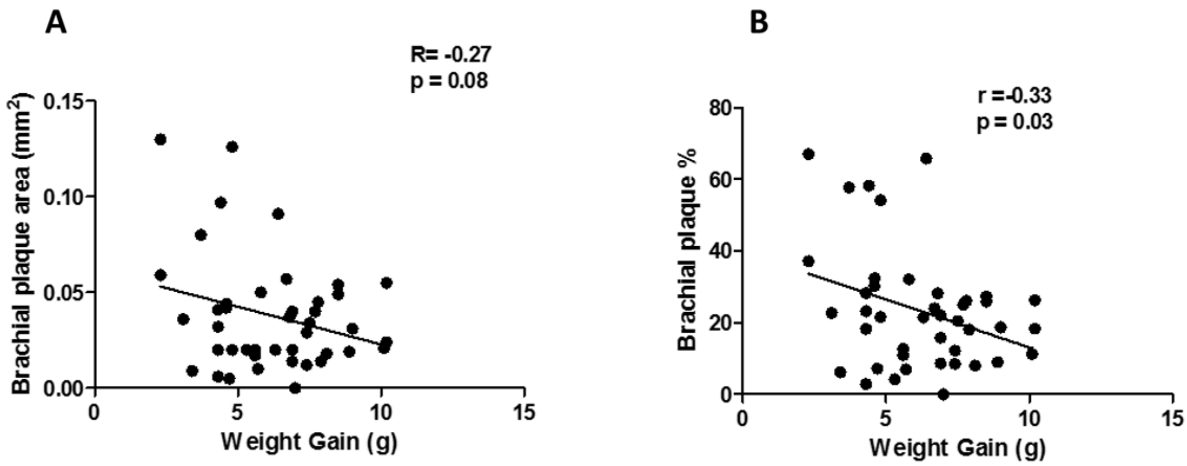

Figure 43. A) No significant correlation between weight gain and brachiocephalic plaque area in ApoE$^{-/-}$ mice aged 16 weeks fed high fat diet for 8 weeks R = -0.27, *p* = 0.08. (B) Significant negative correlation between weight gain and brachial plaque percentage in ApoE$^{-/-}$ mice aged 16 weeks fed high fat diet for 8 weeks, r = -0.33, *p*= 0.03.

4.4.6 VCAM-1 staining in the brachiocephalic artery

Firstly, brachiocephalic arteries of the same mouse were examined for VCAM-1 staining. VCAM-1 staining was mostly localised to the artery wall underlying plaque. Some VCAM-1 staining was observed within plaque (especially the plaque cap) and in the arterial wall opposite the plaque (Figure 44). This was quantified in 8 mice, 2 from each group. Mean percentage staining for VCAM-1 was significantly higher in the artery wall underlying plaque (33.7 ± 11.0 %), then in the plaque itself (13.65 ± 7.0 %) and lowest in the arterial wall opposite the plaque (2.0 ± 2.5 %) , $p < 0.001$ (Figure 45).

Percentage VCAM-1 staining in the brachiocephalic artery from all mice in all groups was then compared. For this analysis the entire arterial wall and entire plaque in cross section was examined. Percentage VCAM-1 staining in the brachiocephalic sections (including vessel wall and plaque) was significantly higher in mice administered Hyper IL-6 0.5 µg and Hyper-IL-6 1 µg (mean percentage staining 11.7 ± 4.07 % and 12.8 ± 4.24 %) compared to mice administered PBS (mean 5.7 ± 1.1 %)

but not mice administered IL-6 (mean 10.2 ± 2.5 %) (Figure 46). There was a significant positive correlation between percentage VCAM-1 staining in the brachiocephalic artery and both brachiocephalic plaque area (R = 0.44, *p* = 0.03) and brachiocephalic plaque percentage (R = 0.47, *p* = 0.02) (Figure 48).

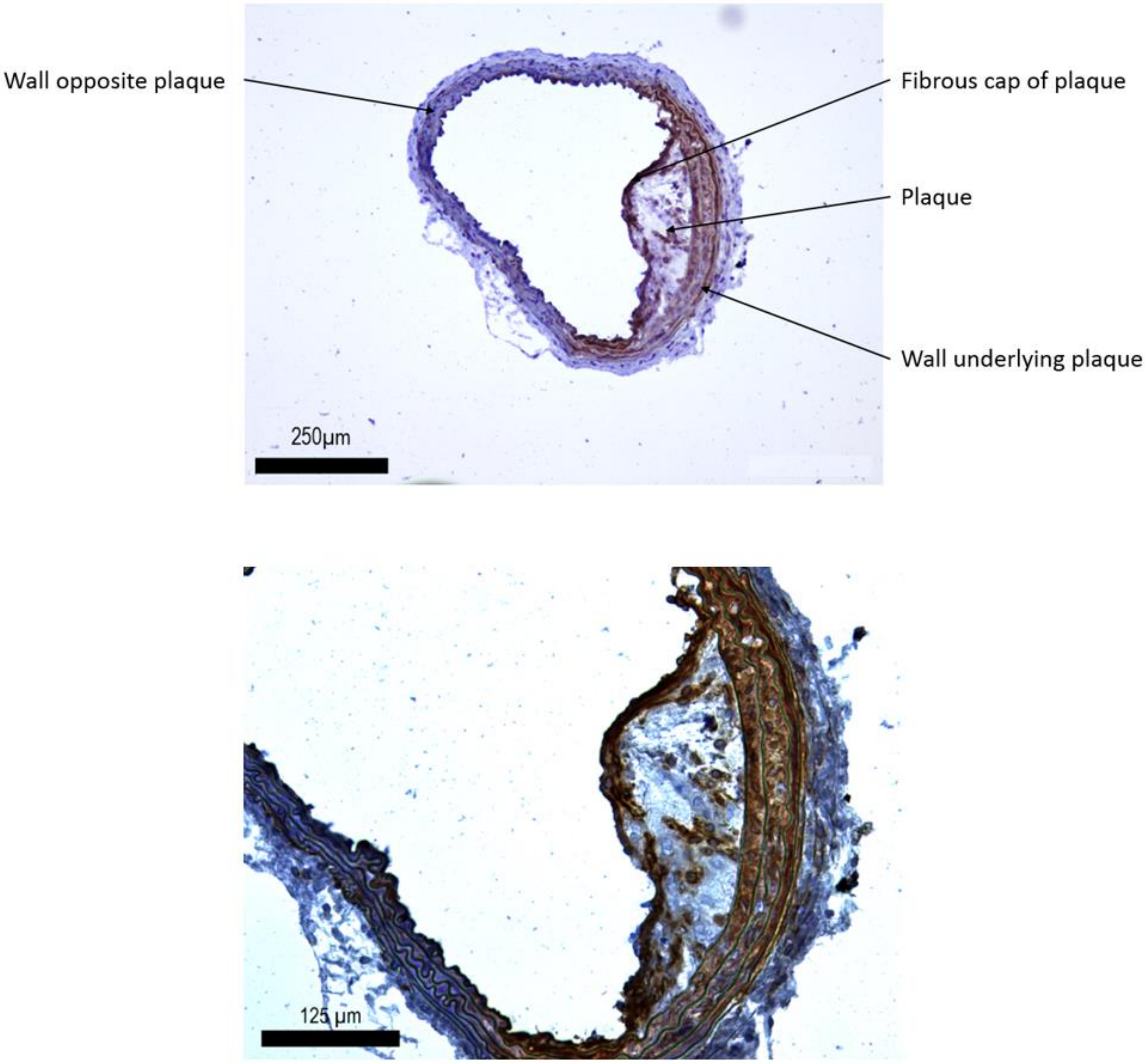

Figure 44. Brachiocephalic artery stained for VCAM-1 at (A) X 4 magnification. VCAM-1 staining (brown) can be seen mostly in the arterial wall underlying the plaque, in the fibrous cap and some in the plaque. Little VCAM-1 staining in the arterial wall opposite plaque. (B) X 20 magnification. VCAM-1 staining (brown) can be seen mostly in the arterial wall underlying the plaque, in the fibrous cap and some in the plaque.

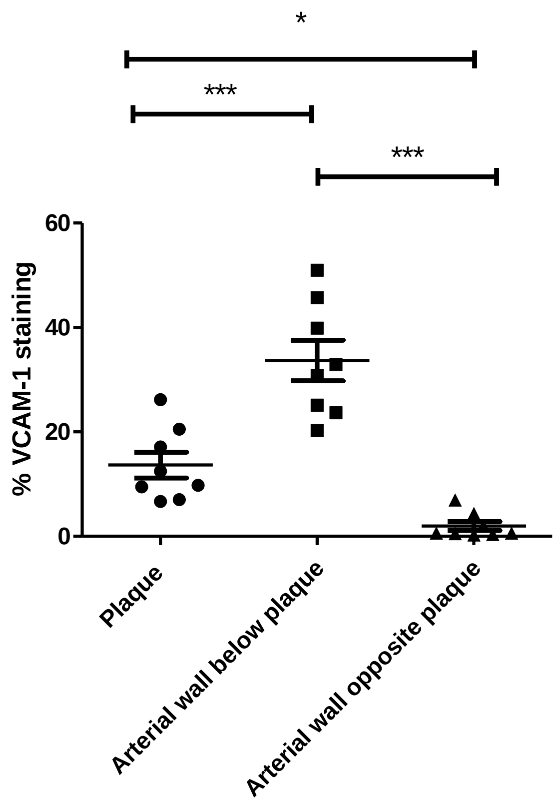

Figure 45. Comparison of percentage VCAM-1 staining in different areas of the brachiocephalic artery. Mean percentage staining for VCAM-1 was significantly higher in the artery wall underlying plaque (33.7 + 11.0 %), then in the plaque itself (13.65 + 7.0 %) and lowest in the arterial wall opposite the plaque (2.0 + 2.5 %) , *p < 0.05, *** p < 0.0001.

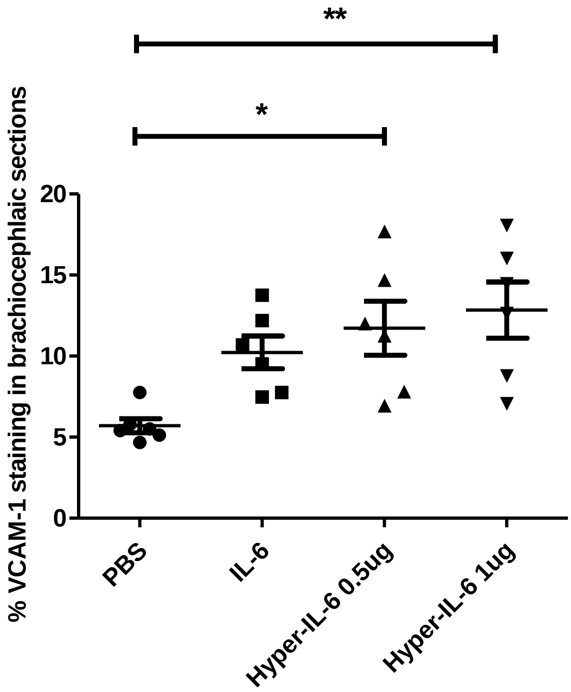

Figure 46. Percentage VCAM-1 staining in brachiocephalic artery and plaque in ApoE$^{-/-}$ mice aged 16 weeks and fed a high fat diet for 8 weeks and administered either IP PBS, IL-6, Hyper-IL-6 0.5 µg or Hyper-IL-6 1 µg. Percentage staining for VCAM-1 significantly higher in mice administered Hyper IL-6 0.5 µg and Hyper-IL-6 1 µg (mean percentage staining 11.7 $\pm$ 4.07 % and 12.8 $\pm$ 4.24 % respectively) compared to mice administered PBS (mean 5.7 $\pm$ 1.1 %) but not mice administered IL-6 (mean 10.2 $\pm$ 2.5 %).

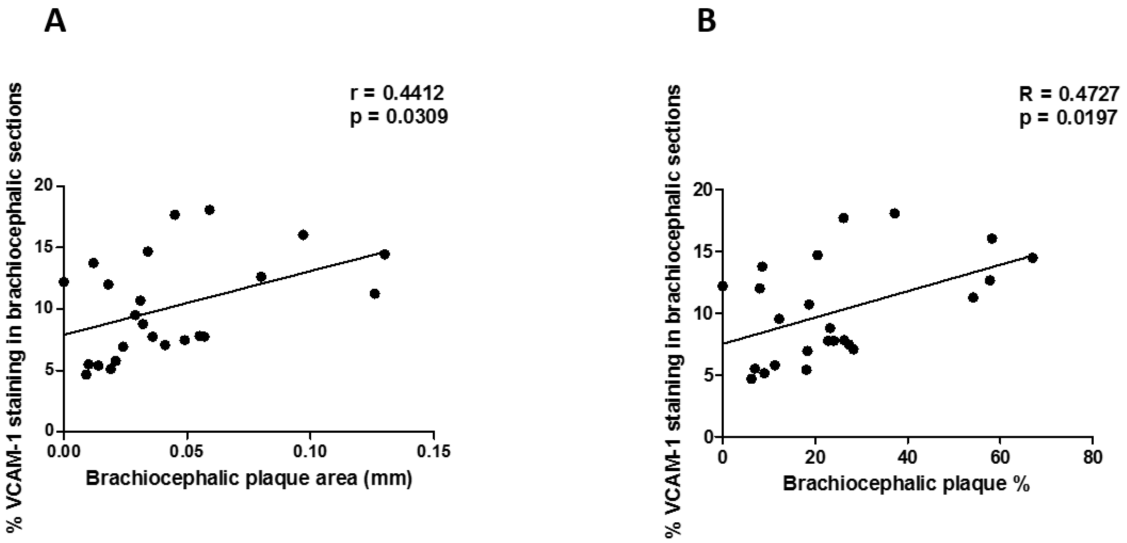

Figure 47. Significant positive correlation between percentage VCAM-1 staining in the brachiocephalic artery of 16 week old ApoE$^{-/-}$ mice fed high fat diet for 8 weeks and (A) brachiocephalic plaque area R = 0.44, *p* = 0.03 and (B) brachiocephalic plaque percentage R = 0.47, *p* = 0.02

4.4.7 Fibrous cap thickness

There was no significant difference the in average fibrous cap thickness between the groups; mean fibrous cap thickness for PBS (5.08 ± 1.4 μm), IL-6 (2.86 ± 0.6 μm), Hyper-IL-6 0.5 μg (4.69 ± 0.7 μm) and Hyper-IL-6 1 μg (10.5 ± 3.6 μm) (Figure 48A). There was no significant difference in mean thickness of the widest part of the fibrous cap between groups; PBS (7.37 ± 2.0 μm), IL-6 (4.17 ± 1.3 μm), Hyper-IL-6 0.5μg (5.05 ± 0.9 μm), Hyper-IL-6 1μg (10.54 ± 2.6 μm) (Figure 48B). Mice administered Hyper-IL-6 1 μg had significantly greater mean fibrous cap thickness at the thinnest part of the fibrous cap (5.21 ± 0.9 μm) compared to mice administered IL-6 (1.94 ± 0.5 μm), p = 0.04 (Figure 48C).

There were significant positive correlations between the mean fibrous cap thickness and mean brachiocephalic plaque size (R = 0.57, p = 0.0034) and brachiocephalic plaque percentage (R = 0.61, p = 0.0015) (Figure 49). There was a significant negative correlation between mean fibrous cap thickness and weight gain (Figure 50).

Figure 51 shows examples of EVG staining of the fibrous cap. It was also noted that some large plaques also had fibrous cap internally and a necrotic core. (Figure 52).

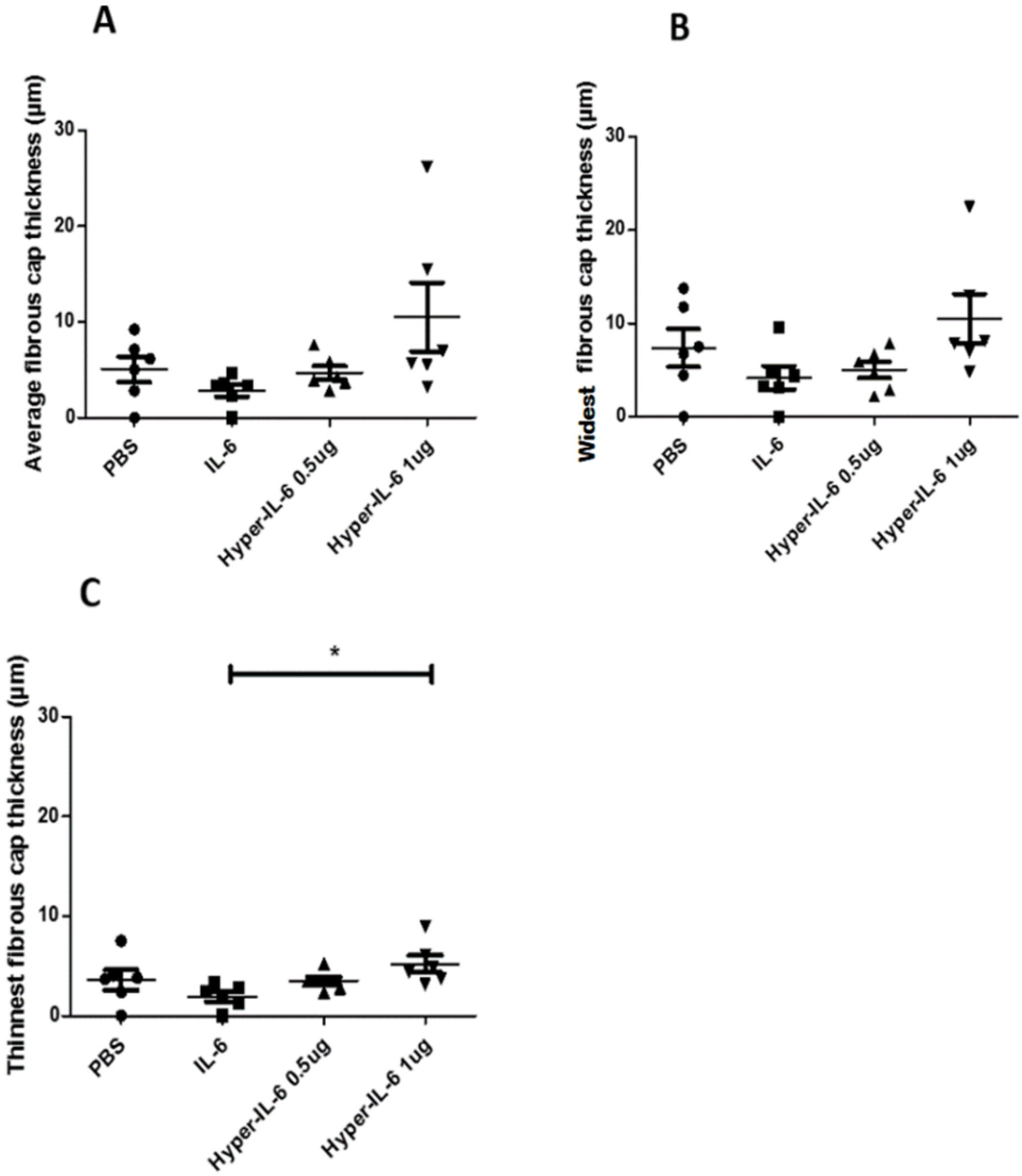

Figure 48. Fibrous cap thickness measurements in ApoE$^{-/-}$ mice fed high fat diet for 8 weeks and administered either PBS, IL-6, Hyper-IL6 0.5µg or Hyper-IL-6 1µg. (A) No significant difference the mean fibrous cap thickness between PBS (5.08 $\pm$ 1.4 µm), IL-6 (2.86 $\pm$ 0.6 µm), Hyper-IL-6 0.5µg (4.69 $\pm$ 0.7 µm), Hyper-IL-6 1µg (10.5 $\pm$ 3.6 µm) . (B) No significant difference in mean thickness of the widest part of the fibrous cap between groups; PBS (7.37 $\pm$ 2.0 µm), IL-6 (4.17 $\pm$ 1.3 µm), Hyper-IL-6 0.5µg (5.05 $\pm$ 0.9 µm), Hyper-IL-6 1µg (10.54 $\pm$ 2.6 µm). (C) Mean thinnest fibrous cap thickness was significantly greater in mice administered Hyper-IL-6 1µg (5.21 $\pm$ 0.9 µm) compared to mice administered IL-6 (1.94 $\pm$ 0.5 µm), * *p* = 0.04.

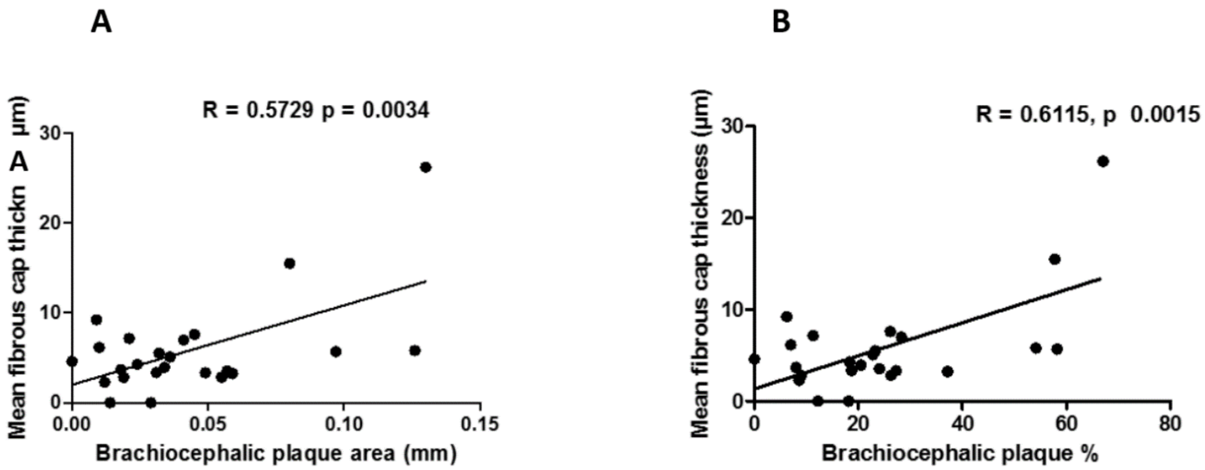

Figure 49. Significant positive correlation between mean brachiocephalic fibrous cap thickness and (A) brachiocephalic plaque area, R = 0.57, *p* = 0.003 and (B) brachiocephalic plaque percentage, R = 0.61 *p* = 0.0015 in ApoE$^{-/-}$ mice aged 16 weeks fed a high fat diet for 8 weeks.

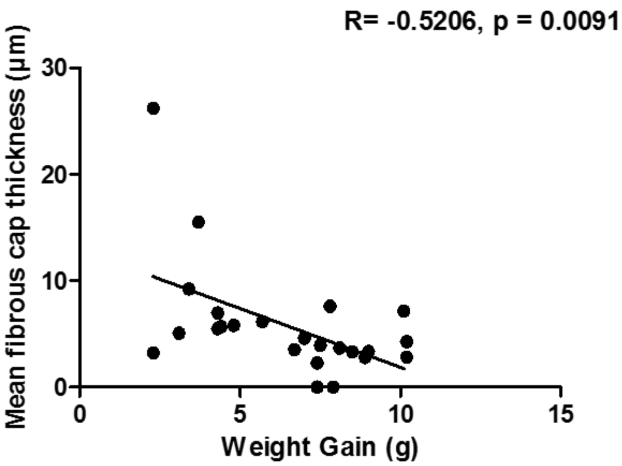

Figure 50. Significant negative correlation between weight gain and mean brachiocephalic fibrous cap thickness in 16 week old ApoE$^{-/-}$ mice fed high fat diet for 8 weeks. R = -0.52, p = 0.009

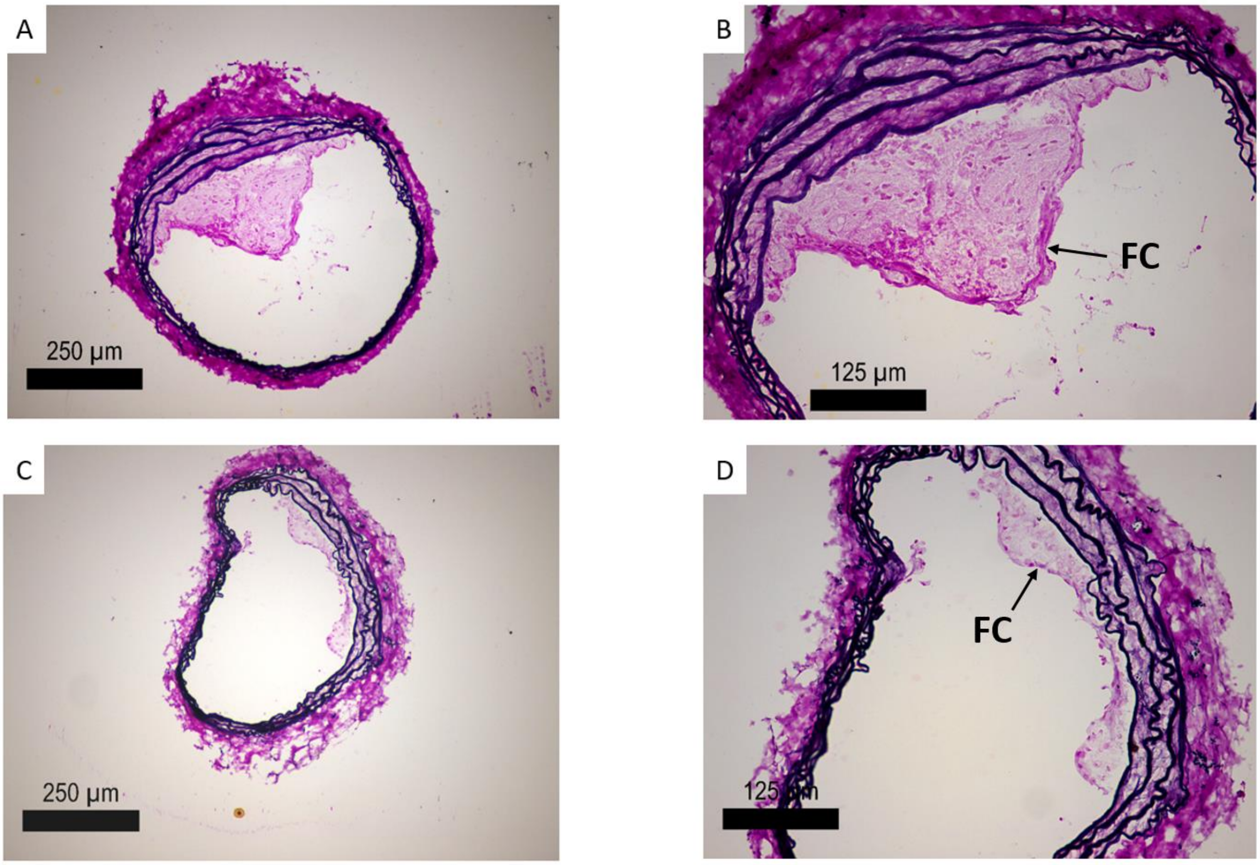

Figure 51. Brachiocephalic artery from ApoE$^{-/-}$ mice aged 16 weeks, fed high fat diet for 8 weeks. Arteries stained with EGV. (A) Example of larger plaque with thicker fibrous cap at X 10 and (B) X 20 magnification. (C) Example of smaller plaque with thinner fibrous cap at X 10 and (D) X 20 magnification. FC = fibrous cap

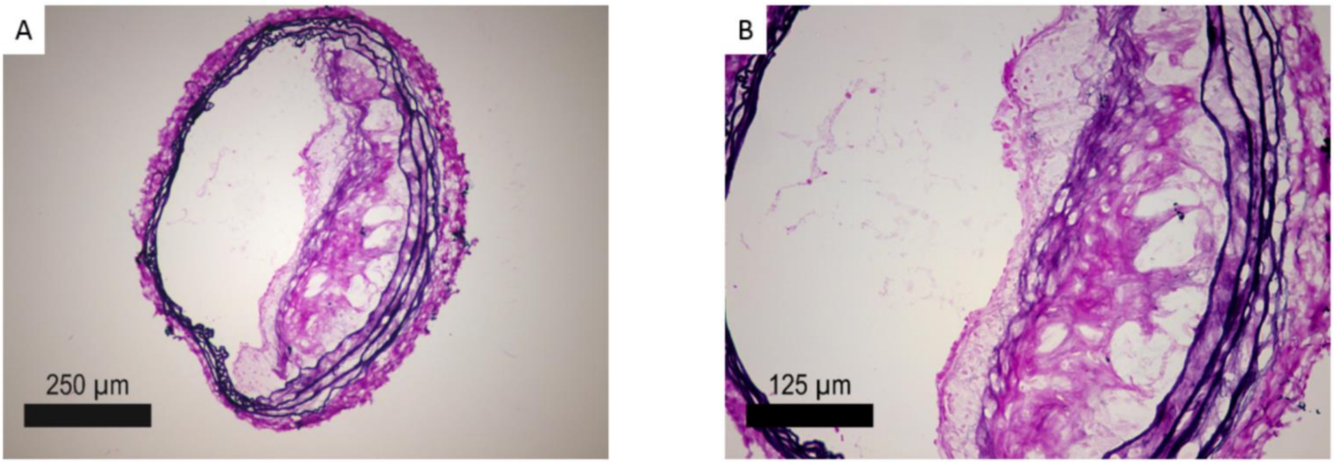

Figure 52. Brachiocephalic artery from ApoE$^{-/-}$ mouse aged 16 week fed high fat diet for 8 weeks. (A) Example of a complex plaque with internal fibrous material with the plaque at X 10 magnification and at (B) X 20 magnification

4.4.8 Lipid levels

There was no significant difference in total cholesterol (Figure 53A), HDL (B), LDL (C), triglycerides (D), free fatty acids (E) or cholesterol: HDL ratio (F) between mice administered PBS, IL-6, or Hyper-IL6. Mean lipid levels are shown in Table 25.

	PBS Mean ± SEM	**IL-6** Mean ± SEM	**Hyper-IL-6 0.5 µg** Mean ± SEM	**Hyper-IL-6 1µg** Mean ± SEM
Total cholesterol (mmol/L)	22.3 ± 2.6	27.0 ± 1.8	25.9 ± 1.4	27.2 ± 2.1
HDL (mmol/L)	1.7 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2.0 + 0.1
LDL(mmol/L)	16.7 ± 1.8	20.0 ± 1.2	19.1 ± 0.9	20.6 + 1.5
Triglycerides (mmol/L)	1.7 ± 0.3	1.8 ± 0.2	1.8 ± 0.2	1.7 + 0.2
Free fatty acids (mmol/L)	0.73 ± 0.05	0.93 ± 0.09	0.92 ± 0.05	0.90 ± 0.03
Cholesterol:HDL ratio	12.6 ± 0.5	14.3 ± 0.8	13.5 ± 0.6	13.5 ± 0.6

Table 25. Mean lipid levels of ApoE$^{-/-}$ mice fed high fat diet for 8 weeks from age 8 weeks and administered either PBS, IL-6, Hyper-IL-6 0.5 µg or 1 µg. no significant differences between the groups.

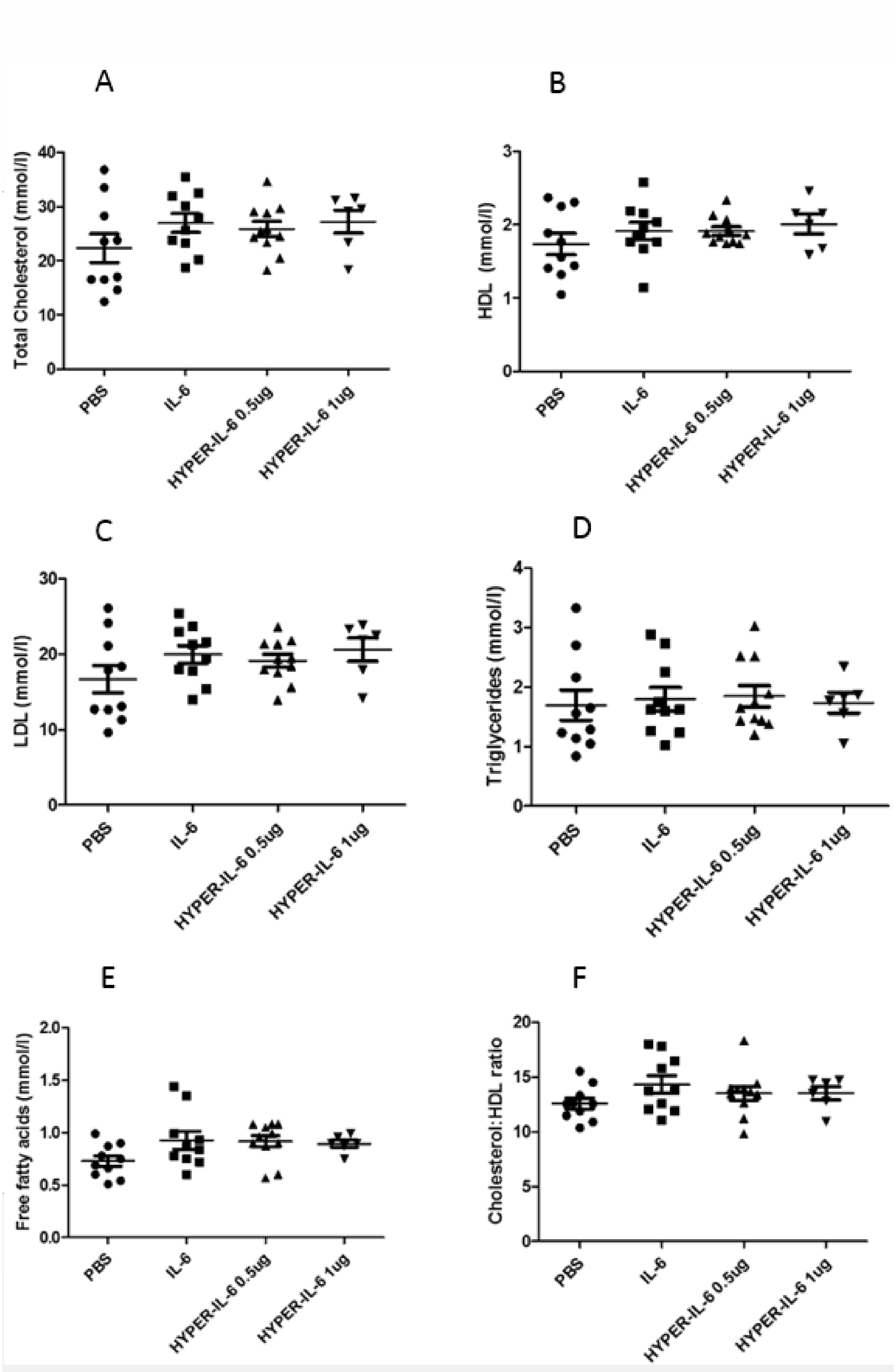

Figure 53. No significant difference in (A) total cholesterol, (B) HDL, (C) LDL, (D) triglycerides, (E) free fatty acids or (F) Cholesterol: HDL ratio in 18-week-old mice fed high fat diet for 8 weeks and administered either PBS, IL-6, Hyper-IL-6 0.5 µg or Hyper-IL-6 1 µg IP twice weekly for 8 weeks.

4.4.9 No significant difference in serum VCAM-1 or CCL-2 between mice administered PBS, IL-6 or Hyper-IL-6

There was no significant difference in serum VCAM-1 levels across the groups (Figure 54). Mean serum VCAM-1 for mice administered PBS (4617 $\pm$ 1393 ng/ml), IL-6 (4622 $\pm$ 983 ng/ml), Hyper IL-6 0.5 µg (4648 $\pm$ 1338 ng/ml), Hyper-IL-6 1 µg (4563 $\pm$ 1618 ng/ml), p = 0.999. There was no significant correlation between serum VCAM-1 and brachiocephalic plaque area or brachiocephalic percentage plaque (Figure 55) or significant correlation between VCAM-1 level and lipid levels. There was no significant correlation between serum VCAM-1 and brachiocephalic percentage staining of VCAM-1.

There was no significant difference in serum CCL2 levels across the groups (Figure 56). Mean serum CCL2 for mice administered PBS (124 $\pm$ 41 pg/ml), IL-6 (149 $\pm$ 77 pg/ml), Hyper-IL-6 0.5 µg (88 $\pm$ 40 pg/ml), Hyper-IL-6 1 µg (101 $\pm$ 20 pg/ml) p = 0.051. There was a significant negative correlation between serum CCL2 and brachial plaque area (r = -0.3348, p = 0.030) and brachial percentage plaque (R= -0.362, p = 0.024) (Figure 57). There was a significant positive correlation between serum VCAM-1 and serum CCL2, r = 0.355, p = 0.027. There was a significant negative correlation between serum CCL2 levels and serum triglyceride levels (r= -0.0343, p = 0.041) (Figure 58).

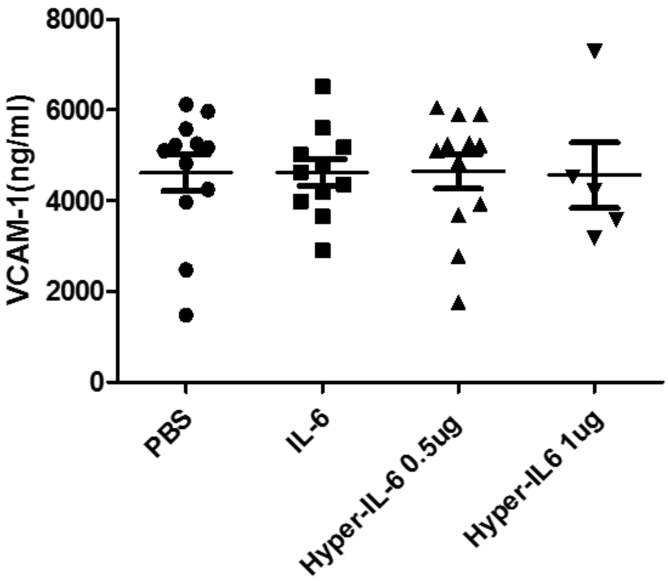

Figure 54. No significant difference in serum VCAM-1 between the groups. Mean serum VCAM-1 for PBS (4617 ± 1393 ng/ml), IL-6 (4622 ± 983 ng/ml), Hyper IL-6 0.5 µg (4648 ± 1338 ng/ml), Hyper-IL-6 1 µg (4563 ± 1618 ng/ml), *p* = 0.999

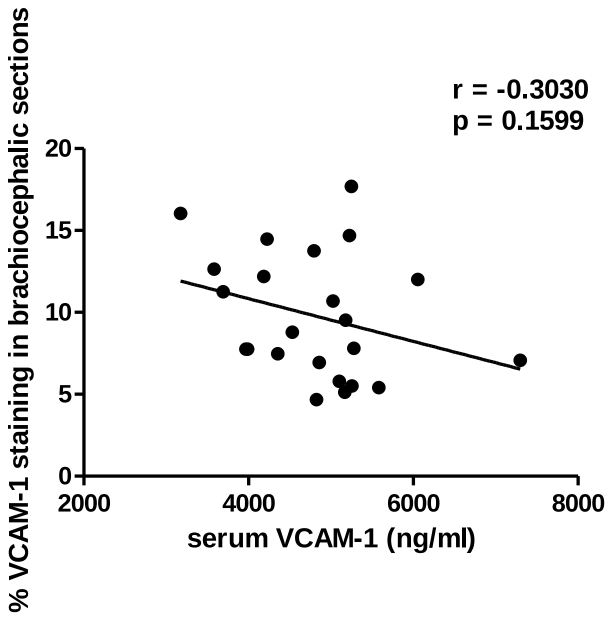

Figure 55. No significant correlation between serum VCAM-1 and percentage staining for VCAM-1 in the brachiocephalic artery in 16 weeks old ApoE$^{-/-}$ mice fed high fat diet for 8 weeks

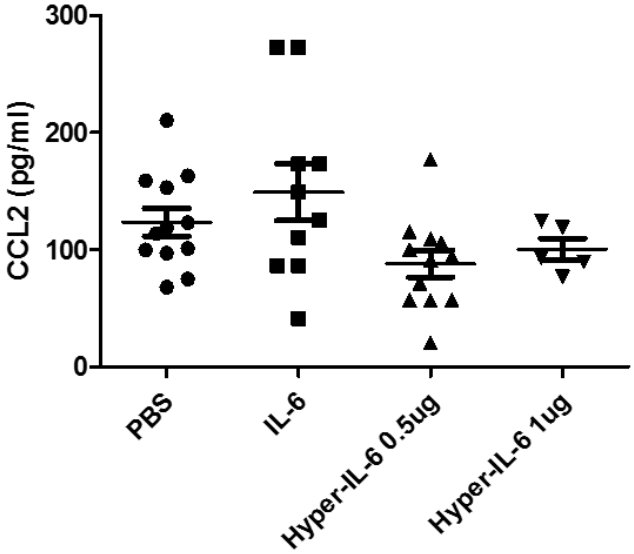

Figure 56. No significant difference in serum CCL2 between the groups. Mean serum CCL2 for ApoE$^{-/-}$ mice administered PBS (124 ± 41 pg/ml), IL-6 (149 ± 77 pg/ml), Hyper-IL-6 0.5 µg (88 ± 40 pg/ml), Hyper-IL-6 1 µg (101 ± 20 pg/ml) *p* = 0.051

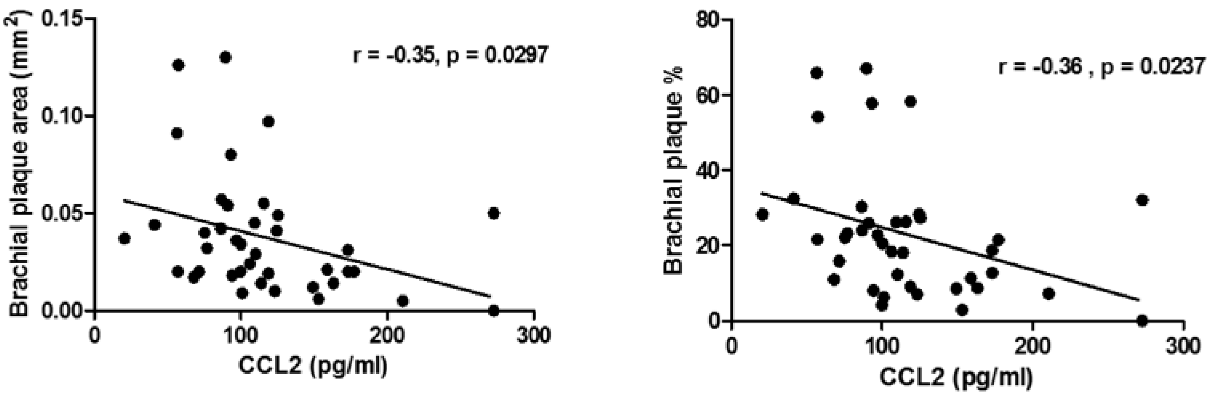

Figure 57. Significant negative correlation between serum CCL2 and brachiocephalic plaque area (R = -0.35, *p* = 0.03) and brachiocephalic plaque percentage (R = -0.36, *p* = 0.024) in ApoE$^{-/-}$ mice

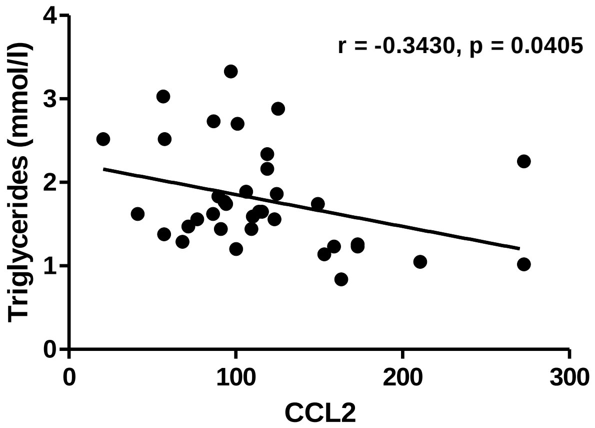

Figure 58. Significant negative correlation between serum CCL2 and serum triglycerides, r = -0.3430, *p* = 0.00405 in ApoE$^{-/-}$ mice

4.5 Discussion

We have shown that in our facility 18 week old ApoE$^{-/-}$ mice develop plaques in the brachiocephalic artery and the aortic root after 8 weeks of high fat feeding. The size of plaque seen in the aortic root is comparable with other centres under similar conditions (Jones et al, 2014). No information on plaque size in the brachiocephalic artery was found in the literature for the conditions used in this study. In this chapter, mice were housed in conventional units which were not pathogen free. This was chosen to reflect the environment of RA patients.

Mice administered Hyper-IL-6 1 µg had significantly reduced weight gain compared with those administered either PBS, IL-6 or Hyper-IL-6 0.5 µg. This could be due to systemic inflammation; in RA, chronic inflammation and reduced physical activity leads to muscle wasting but maintenance of the fat mass (Masuko, 2014). This is termed rheumatoid cachexia. It is proposed that cytokines such as TNF-α and IL-6 play pivotal roles here by activating NF-κB and leading to an increase in muscle proteolysis via the ubiquitin-proteasome pathway (Morley, 2006). Thus, the lower weight gain seen in mice administered Hyper-IL-6 in this experiment may be due to a reduction in muscle mass. To investigate this further it would be interesting to measure the body composition of these mice at the time of sacrifice. Weight gain correlated negatively with brachiocephalic plaque percentage, so those mice with more weight gain had less atherosclerosis in the brachiocephalic artery. This perhaps reflects the role of inflammation on both parameters, high levels of inflammation are known to reduce weight gain and increase atherosclerosis. RA patients treated with Tocilizumab show both reduced levels of inflammation (as reflected by disease activity and CRP), and weight gain (Hirabayashi et al, 2010). Tocilizumab blocks both IL-6 classical and trans-signalling. Thus, IL-6- driven inflammatory pathways seem to play a role in body mass in both atherosclerosis-prone mice and in patients with RA.

Here, for the first time, we demonstrate that enhanced IL-6 trans-signaling using Hyper-IL-6 increases atherosclerosis in an animal model. In the brachiocephalic artery Hyper-IL-6 significantly increased plaque percentage compared to PBS and IL-6. IL-6 did not increase plaque size compared to PBS. Therefore, in this model, IL-6 trans-signaling, rather than IL-6 classical signalling increased atherosclerosis. Our finding of no increase in plaque size when IL-6 was administered is conflicting to some reports in the literature. A previous study in ApoE$^{-/-}$ mice found that IL-6 administration increased atherosclerotic lesion size in the aortic sinus (Huber et al, 1999). The dose used in this study was 5000u weekly, less than the dose in this chapter. However, in this study mice were fed high fat diet earlier than in this thesis, starting at 3 weeks of age for 6 weeks. Another study found that in a mouse model, serum cholesterol levels and atherosclerotic lesion formation were significantly increased in ApoE$^{-/-}$-IL-6$^{-/-}$ mice compared with ApoE$^{-/-}$ and wild-type (WT) mice (Schieffer et al, 2004). Plaques of ApoE$^{-/-}$-IL-6$^{-/-}$ mice showed significantly reduced transcript and protein levels of matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1, collagen I and V, and lysyl oxidase. Recruitment of macrophages and leukocytes into the atherosclerotic lesion was significantly reduced in ApoE$^{-/-}$-IL-6$^{-/-}$ mice. The transcript and serum protein levels of IL-10 were significantly reduced. Thus, from the literature, the role of IL-6 in atherosclerosis appears complex, with increased atherosclerosis in above physiological levels of IL-6, but also in the complete absence of IL-6. For the first time, the work in this chapter shows that IL-6 trans-signaling, rather than IL-6 classical signaling, increases atherosclerosis in a mouse model.

There was no difference in plaque size in the aortic root in those administered Hyper-IL-6 (both doses) or IL-6 compared to PBS. A possible reason for the significant increase in plaque size in the brachiocephalic artery with Hyper-IL-6 but not in the aortic root may be that plaque develops in the aortic root before the brachiocephalic artery in $ApoE^{-/-}$ mice . In one study, at 10 weeks in $ApoE^{-/-}$ mice fed a high fat diet, atherosclerotic lesions were identified in 100% of all aortic roots and in 57% of brachiocephalic arteries (McAteer et al, 2004). Perhaps if mice had been sacrificed at an earlier age or fed a high fat diet for a shorter period, there may have been a difference in plaque size in the aortic root. In another study which compared plaque size in the aortic root with brachiocephalic artery, variables such as diet and strain of mouse used had greater effects on plaque size in the brachiocephalic artery than the aortic root (Teupser et al, 2003). Therefore, in the timeframe used in this thesis, the lesions in brachiocephalic artery may have been more sensitive to drug administration as they were likely to have been at earlier stages of atherosclerosis than those in the aortic root.

From this work the brachiocephalic artery appears to be a useful site for quantification of atherosclerosis for the time point used in this work. However, using a cross sectional method has drawbacks, mainly that this does not show the volume of plaque. Although sections were taken at the same relative points in mice, it is possible that a short plaque could have been missed, or in fact the end of a plaque may have been sectioned, rather than its centre. However, the cross sectional method has the advantage that other parameters can be measured, such a fibrous cap thickness and immunohistochemistry, which cannot be used with the en face method.

The increase in plaque size in mice administered Hyper-IL-6 was associated with increase in expression of VCAM-1 in the artery of those administered Hyper-IL-6. There was no difference in VCAM-1 expression between those administered IL-6 and PBS. This suggests that IL-6 trans-signaling, rather than classical signalling, increases the expression of VCAM-1 in the vessel wall. VCAM-1 mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium. VCAM-1 is of critical importance in the development of atherosclerosis, and VCAM-1 has been shown to be rapidly induced by proatherosclerotic conditions (O'Brien et al, 1993). The ligand for VCAM-1 is Very Late Antigen Activation-4 (VLA-4), also called $\alpha_4\beta_1$ integrin. Studies have shown that recruitment of peritoneal macrophages into atherosclerotic lesion of ApoE$^{-/-}$ mice can be inhibited by an α4 blocking antibody (Ley and Huo, 2001). Studies have attempted to investigate the effect of complete absence of VCAM-1 by using VCAM-1$^{-/-}$ mice. However, this has been very difficult as no or very few mice homozygous for the null allele are born; it appears this is necessary for placental development (Gurtner et al, 1995). Another study has disrupted its fourth Ig domain, producing the murine VCAM-1^{D4D} allele (Cybulsky et al, 2001). VCAM-1$^{D4D/D4D}$ mice had significantly reduced atherosclerotic lesions, compared to wild type mice and ICAM-1$^{-/-}$ mice.

In this chapter, we have shown that plaque size in the brachiocephalic artery correlates with arterial VCAM-1 expression. We have found that VCAM-1 staining in the brachiocephalic artery was highly prevalent in the artery wall underlying plaque. This is in agreement with the literature, O'Brien et al (1993) found high VCAM-1 staining in inflammatory infiltrate at the base of plaques and also in areas of neovascularization. This suggests that adhesion of monocytes, mediated by VCAM-1, and subsequent infiltration of plaque occurs from the vasa vasorum of the arterial adventitia and media.

Interestingly, there was no difference in serum VCAM-1 in mice administered Hyper-IL-6 or IL-6 compared to PBS. This is unexpected, as VCAM-1 expression was higher in the brachiocephalic artery in mice administered Hyper-IL-6. Serum levels of VCAM-1 were high in all groups, in fact mean levels in ApoE$^{-/-}$ mice were up to 4 times higher than serum levels seen in RA patients in Chapter 5 and approximately 1.5 times those seen in mice with CIA in Chapter 3. We have shown that in patients with RA, serum VCAM-1 is higher in those with high CV risk. What is not well defined is the relationship between soluble VCAM-1 and VCAM-1 on the endothelial cell surface. In this chapter there was no significant correlation between serum VCAM-1 and expression of VCAM-1 in the brachiocephalic artery. In vitro, soluble VCAM-1 levels correlate with surface expression (Kjaergaard et al, 2013), however the relationship between the two in vivo, and the effect of soluble VCAM-1 appears to be context dependant. It has been proposed previously that soluble VCAM-1 could act as a competitive inhibitor of ligand binding (Rose et al, 2000); if there is an abundance of serum soluble VCAM-1 then this could bind to VLA-4 on monocytes, preventing their adhesion to cell surface VCAM-1 on the endothelium. Of note, Kitani et al (1996) found that in RA synovial fluid, the binding of soluble VCAM-1 to T cells inhibited their activation.

There was no significant difference in serum CCL2 in mice administered IL-6, Hyper-IL-6 or PBS. There was a significant negative correlation between serum CCL2 and brachiocephalic plaque size. This is unexpected given that CCL2 has been shown to correlate with CIMT in RA patients (Södergren et al, 2010) and elevated serum CCL2 levels are found in patients with coronary artery disease or increased coronary risk factors (Martinovic et al, 2005). It would be interesting to measure CCL2 expression the brachiocephalic artery but this was not possible for this thesis due to time constraints.

There was no difference in mean fibrous cap thickness in mice administered Hyper-IL-6, IL-6 or PBS. Mice administered Hyper-IL-6 1 µg had greater mean fibrous cap thickness at the thinnest point of the cap compared to PBS and IL-6. This may be explained by the fact that there was also a positive correlation between fibrous cap thickness and plaque size, and plaque size was greater in those administered Hyper-IL-6. To investigate this further mice could be grouped into those with similar sized plaques i.e. small, medium and large plaques, and then mean fibrous cap thickness compared within these plaque-size subgroups for PBS, IL-6 and Hyper-IL-6 administered mice. However, numbers in these experiments were too small to make this comparison and very few plaques in the PBS groups were similar size to those administered Hyper-IL-6.

Interestingly, there was no difference in the lipid profiles of mice administered IL-6 or Hyper-IL-6 compared with PBS. It has previously been shown that IL-6 plays a role in lipid metabolism, though there are variable reports in the literature. For example in healthy volunteers, administration of IL-6 increased total cholesterol (Lyngso et al, 2002). However in middle-aged and old rhesus monkeys (Ettinger et al, 1995), and cancer patients (Veldhuis et al, 1995) IL-6 administration reduced total cholesterol levels. In patients with myocardial infarction (Brugada et al, 1996), those who have undergone major surgery (Akgun et al, 1998) and those on haemodialysis (Bologa et al, 1998), serum IL-6 levels correlate negatively with total cholesterol levels. In patients with renal disease undergoing haemodialysis low IL-6 levels have been shown to be the strongest predictor of mortality (Bologa et al, 1998). Overall, it appears that the effect of IL-6 on lipid levels depends somewhat on the inflammatory state of the patient or animal. Perhaps in the ApoE$^{-/-}$ mice used in this thesis, lipids levels (mostly LDL cholesterol) are already considerably raised so that the effect of additional inflammation in negligible. The absence of difference in lipid levels in mice administered Hyper-IL-6 compared to PBS is in keeping with previous work in LDLR$^{-/-}$ mice, where blockade of IL-6 trans-signaling, by administration of sgp130Fc, reduced atherosclerosis but did not affect serum lipid levels (Schuett et al, 2012).

IL-6 increases VLDLR expression in several tissues (Hashizume et al, 2009) and this decreases triglyceride levels. Interestingly, in an in vitro study, IL-6 and sIL-6R significantly induced expression of VLDLR mRNA in VSMC but IL-6 or sIL-6R alone and TNF-α did not do so (Hashizume et al, 2009). In the same study IL-6 injection into mice increased the expression of VLDLR in heart, adipose tissue and liver and decreased TC and TG levels. This suggests that IL-6 trans-signaling in vitro, rather than IL-6 classical signaling, increases VLDLR expression. In the in-vitro part of this study, the administration of IL-6 may have increased both classical and trans-signaling, as there would have been sIL-6R in the circulation to bind to the IL-6, and therefore it is unclear which signaling mechanism is influencing this increase in VLDLR expression.

Dyslipidaemia is commonly observed in patients with active RA. They have lower total cholesterol, HDL and LDL than people without RA (Robertson et al, 2013). When inflammation is supressed in these patients, for example with the use of DMARDs or biological agents, these lipid fractions increase. These changes in lipid profile can differ depending on the drugs used. It has been shown that IL-6 blockade using Tocilizumab increases serum total cholesterol, HDL, LDL and triglyceride levels in RA patients (Choy et al, 2014). Importantly LDL:HDL and Total cholestrerol:HDL ratios remain relatively stable. These ratios have been shown to be more closely associated with CV risk than individual lipid measures (Natarajan et al, 2003).

Previous research found that ApoE$^{-/-}$ mice were resistant to CIA (Asquith et al, 2010). In this study, ApoE$^{-/-}$ mice did not develop articular inflammation, but did develop anti-collagen antibody responses that are similar to those observed in C57BL/6 wild type mice that developed CIA. The authors concluded that the inflammatory burden of polyarticular disease may be crucial for vascular disease acceleration. However, it is possible that as a result of the deletion of the ApoE gene, there are changes to serum proteins which may influence CIA development. In this study the authors found significantly lower IL-17 levels in the sera of ApoE$^{-/-}$ mice than in the sera of wild type C57BL/6 mice after CIA induction. Previous studies have shown that IL-17 plays a crucial role in CIA development (Nakae et al, 2003). Another possible reason for the lack of CIA development in ApoE$^{-/-}$ mice may be that hyperlipidaemia supressed CIA development. However, in the same study, high fat diet did not change arthritis severity compared to normal chow diet in wild type C57BL/6 mice. In addition, previous work has shown that in DBA-1 mice immunised with CIA, injection of ^{3}H-cholesterol was associated with greater severity of CIA (Hamer et al, 2002). To further investigate the role of lipids in CIA development, CIA could be induced in other models, such as the LDLR$^{-/-}$ mouse.

4.6 Conclusion

Here, for the first time, we have shown that IL-6 trans-signalling using Hyper-IL-6 increases plaque size in ApoE$^{-/-}$ mice. This accelerated atherosclerosis was not seen with IL-6 alone. This increase in plaque size with Hyper-IL-6 may be partly driven by the increased arterial and plaque expression of VCAM-1 in those administered Hyper-IL-6. IL-6 trans-signaling did not effect fibrous cap thickness or lipid levels.

5 Chapter 5: The role of sIL6R-regulated CVD Candidate Proteins (SCCPs) in progression of subclinical atherosclerosis in patients with early RA and in cardiovascular risk in established RA.

5.1 Introduction

It has been shown that rapid progression of CVD occurs soon after RA onset (Kerola et al, 2012). Cardiovascular risk scores, even with modification as recommended by EULAR, underestimate CVD risk in RA patients (Rosales-Alexander et al, 2014). A prospective study of patients with early RA (classified as symptoms of less than 12 months) followed up 442 patients over 5 years (Innala et al, 2011). At 5 years, 10.9% of patients had experienced a new cardiovascular event, 12 of these 48 events were fatal.

Using cardiovascular events as an outcome for exploratory studies in this thesis is not appropriate, since the required sample size is prohibitively large. Several validated non-invasive imaging techniques are now available as surrogate markers for determining subclinical atherosclerosis in RA and were recently reviewed by Kerekes et al (2012). Of these, ultrasonographic assessment of carotid intima media thickness (CIMT) and the presence of plaques have been identified as most useful for stratifying RA patients with high CV risk. In a recent study, approximately 60% of patients identified as having moderate cardiovascular risk, according to the modified EULAR cardiovascular SCORE, had evidence of carotid plaques and/or CIMT >0.90 mm (Corrales et al, 2013) (both considered factors indicative of poor cardiovascular prognosis in the general population). Furthermore, the proportion of patients identified as having high or very high cardiovascular risk increased from 9.2% with the modified EULAR SCORE to 47.7% with additional carotid ultrasound results (Corrales et al, 2013). However, the feasibility of performing these ultrasound assessments within rheumatology clinics or in partnership with specialist cardiology clinics remains to be established. In particular, most cardiology departments do not have capacity to scan over 1000 RA patients annually. In this study, CIMT thickness and CIMT progression over time were used as a surrogate marker of CVD and CVD progression. If SCCPs are associated with progression of CVD then they could be used to identify high-risk patients; these patients could then be selected for more intensive investigation and treatment. This project will examine this hypothesis in a longitudinal study of patients with early RA using CIMT as a surrogate of CVD. In this chapter, CIMT > 0.90 mm and/or carotid plaques were used as the gold standard test for subclinical atherosclerosis and high CV risk (Corrales et al, 2013). However, this is a surrogate marker for CVD, thus there are limitations in applying this to other or additional CVD pathologies that RA patients can experience. Wald et al (2009) found that neither carotid plaque nor CIMT has a CVD screening performance that is sufficiently discriminatory between affected and unaffected individuals to be a worthwhile screening test in the general population.

Due to inherent problems with recruitment and retainment of patients over a prolonged period of follow up, it was decided that both a cross sectional study in patients with established RA and a prospective study in patients with early RA would be performed. This enabled greater patient numbers to be recruited. In addition, differences in SCCPs could be assessed in those who had a history of CVD versus those that did not.

5.2 Hypothesis and Aims

Hypothesis: SCCPs are associated with cardiovascular risk in patients with established RA and progression of subclinical atherosclerosis in patients with early RA.

In order to address this hypothesis the following aims were identified:

1. To investigate whether SCCPs are associated with RA disease activity in patients with established RA.
2. To investigate whether SCCPs are associated with cardiovascular risk in patients with established RA.
3. To investigate whether SCCPs are associated with previous CVD in established RA.
4. To investigate whether SCCPs are associated with RA disease activity and with progression of disease activity in early RA.
5. To investigate whether SCCPs are associated with presence and progression of subclinical atherosclerosis, using CIMT, in early RA.

5.3 Methods

5.3.1 Established RA recruitment and data collected

Patients with established rheumatoid arthritis (duration greater than 1 year) were recruited from general rheumatology clinics in University Hospital of Wales (UHW). Research Ethics Committee for Wales ref no 12/WA/0045. After written consent was gained the following was collected:

Data collected:

- Gender
- Age
- Height
- Ethnicity
- Postcode
- RA disease duration
- Concomitant diseases
- Family history of cardiovascular disease or stroke
- History of chronic kidney disease (also checked by looking at renal function on the UHW clinical portal)

- History of atrial fibrillation
- Smoking status
- Alcohol intake
- Medications
- History of cardiovascular disease (ischaemic heart disease including angina and acute coronary syndrome, cerebrovascular accident or transient ischaemic attack, peripheral vascular disease)

Data measured:

- Blood pressure
- Weight
- Height
- DAS28
- As part of routine care ESR, CRP and lipid profile were requested via the laboratories at UHW.

5.3.2 Early Arthritis Study recruitment and data collected

Patients with early RA, defined by ACR/EULAR 2010 criteria (Aletaha et al 2010) and symptoms of less than 6 months were identified from rheumatology clinics and referral letters to the rheumatology department at UHW. Patients were assessed 6-monthly for 12 months. Ethics and R&D approval was in place for a larger study: Inflammation and Immune Regulation in Early Inflammatory Arthritis (Research Ethics Committee for Wales ref no 11/WA/0326). An amendment to the protocol was successfully made to add carotid ultrasonography to assessments to determine CIMT and presence of plaques.

Inclusion criteria

- Age 18 or over
- Able and willing to give written consent and comply with the requirements of the study protocol
- Duration of symptoms 4 weeks to 6 months
- Synovitis in at least one joint
- Meets criteria of 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative (Aletaha et al, 2010).

Exclusion criteria

- Definite other autoimmune rheumatic disease e.g. SLE, MCTD, scleroderma, polymyositis.
- Functional class IV, defined by the ACR Classification of Functional Status in RA
- Previous treatment with any biological agent.
- Treatment with intravenous gamma globulin, plasmapheresis or Prosorba TM column within the past 6 months.
- Current other inflammatory joint disease (e.g. gout, reactive arthritis, Lyme disease).
- Previous cardiovascular disease (ischaemic heart disease including angina and acute coronary syndrome, cerebrovascular accident or transient ischaemic attack, peripheral vascular disease)
- Concomitant diabetes mellitus

Patients that fulfilled inclusion criteria were assessed in the Clinical Research Facility in UHW.

Data collected:

- Gender
- Age

- Ethnicity
- Postcode
- Symptom duration
- Concomitant diseases
- Extra articular features i.e. rheumatoid nodules, eye, lung, cardiac or skin symptoms, Sjogren's syndrome, neuropathy, vasculitis, back pain
- Family history of cardiovascular disease or stroke
- History of chronic kidney disease (also checked by looking at renal function on the UHW clinical portal)
- History of atrial fibrillation
- Smoking status
- Alcohol intake
- Employment status
- Medications
- Patient Visual Analogue Scale for arthritis severity

Data measured:

- Height
- Weight
- Body mass index
- Blood pressure was measured in both arms while sitting, after the patient had rested for at least 30 minutes
- Tender joint count
- Swollen joint count
- US 7 and 10 score
- CIMT and presence of plaque using carotid US

5.3.3 Assessment of cardiovascular risk

CV risk was calculated for each patient using the QRISK2, Framingham and SCORE algorithms. These risk algorithms are described in section 1.8, including the variables each uses to calculate risk.

5.3.4 Calculation of DAS28

From the tender and swollen joint counts, ESR and CRP, the DAS-28(ESR) (Prevoo et al 1995) and DAS-28(CRP) (Fransen et al 2003) were calculated using the standard formula. Patients were grouped by DAS28 (Prevoo et al, 1995) into severity, see Table 26.

DAS28 Score	DAS28 group
< 2.6	Remission
2.6 – 3.2	Mild
3.2 - 5.1	Moderate
> 5.1	Severe

Table 26. Stratification of disease activity by DAS28 score

5.3.5 Musculoskeletal Ultrasound (US) assessments

Ultrasound was performed of patients' hands and feet, by two rheumatology registrars trained in musculoskeletal ultrasound (RD and CR). Images or clips were stored and later scored blind by both RD and CR using 7-joint ultrasound (Backhaus et al, 2009) (Figure 59) and 10-joint ultrasound (Larché et al, 2010) (Figure 60) scores. The US10 score was adapted from Larché et al (2010), to include semi quantitative measures of synovial thickness and vascularity. The original score also includes a quantitative measure of vascularity, the authors using an automated measurement of the number of colour pixels in a region of interest (ROI). However, we did not have access to this software and therefore this was omitted.

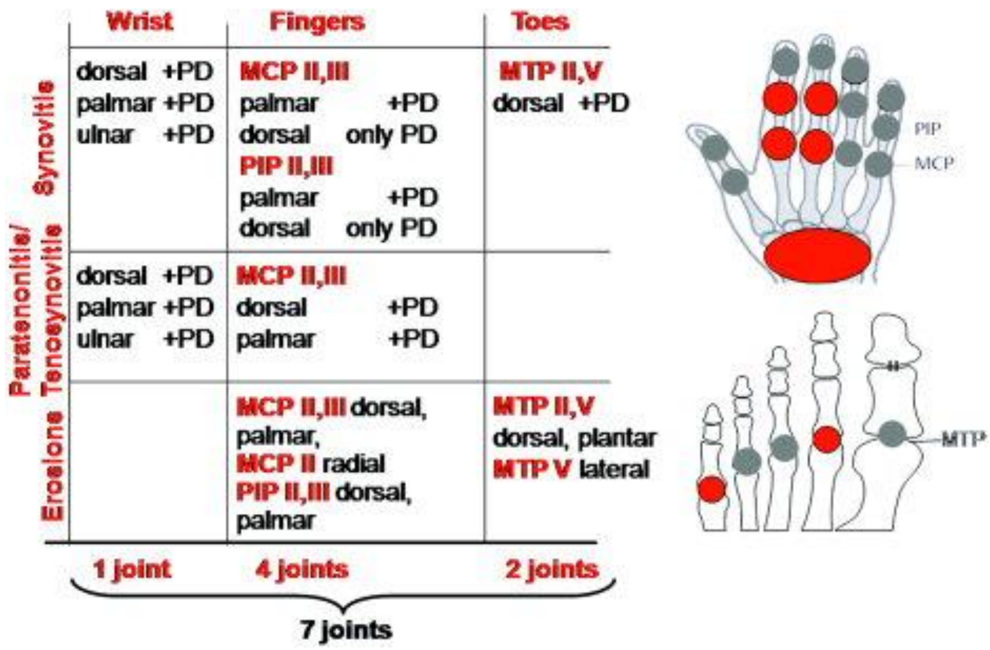

Figure 59. US7 score. Gray-scale ultrasound and power Doppler (PD) US performed for synovitis, tenosynovitis/paratenonitis, and erosions from the dorsal, palmar, and ulnar aspects of the wrist, metacarpophalangeal (MCP), proximal interphalangeal (PIP), and metatarsophalangeal (MTP) joints. From Backhaus et al, 2009.

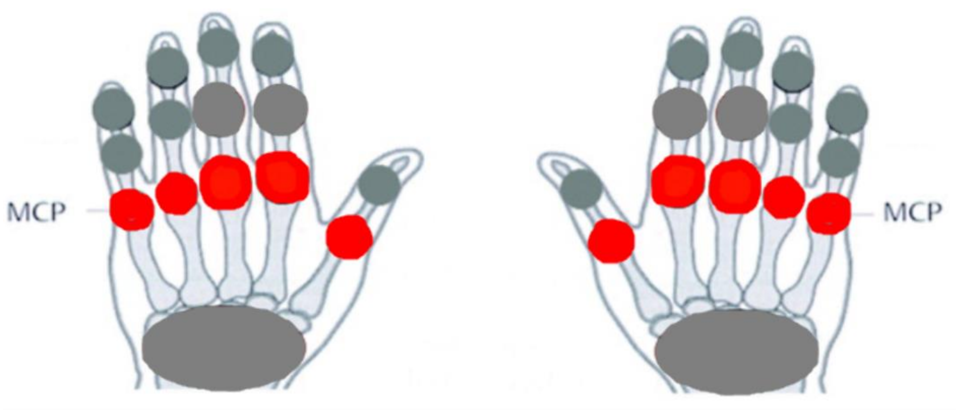

Figure 60. US 10 score. Gray-scale ultrasound and power Doppler US performed for synovial thickening and power Doppler activity for 10 MCP joints. Adapted from Larché et al, 2010.

5.3.6 Training in carotid ultrasound

I was trained in carotid ultrasound by a senior vascular scientist with a strong track record in study set up, training and project management of vascular studies. I had 40 hours of training over several weeks and 2 meetings per month to cover data review, quality control and trouble shooting.

Reproducibility of scanning

To ensure reproducibility of images 5 healthy volunteers were scanned twice, approximately a week apart. Each scan was analysed. These were checked by a senior vascular scientist for:

- Correctly labelled images
- Images recorded in the correct order – i.e. right carotid first, starting with the cross sectional loop.
- Image set up appropriately – suitable gain, focus point in appropriate position.
- Correct zoom
- Image quality - intima visible on both walls, presence of an analysable area, straight image
- Similarity of two images

Reproducibly of analysis

To ensure reproducibility of analysis 10 images were analysed twice, 1 week apart. The following were checked by a trained vascular scientist:

- Calibration

- Appropriate choice of waveforms (3 consecutive waveforms, all end-diastolic diameters and systolic diameters within 0.1 mm of each other)

- Size of the analysis box

- Location from the carotid bifurcation

- Correct placement of detection lines

  - Coefficient of variation

5.3.7 Assessment of CIMT using carotid ultrasound

Patients were positioned supine after resting for at least 30 minutes. The left and right common carotid arteries were imaged longitudinally 1 cm proximal to the carotid bifurcation. Images were focused on the posterior (far) wall of the artery and then magnified. Several ten-second cine loops were recorded in DICOM format and then downloaded for offline analysis.

5.3.8 Analysis of CIMT

An automated carotid analyser (Carotid Analyzer, Medical Imaging Applications, Iowa City, IA) was used to measure CIMT. Three end-diastolic frames were selected and CIMT measured, defined as the interface between lumen-intima and media-adventitia, for both right and left carotid arteries. The mean of the three end-diastolic frames was calculated, and then the mean of the left and right-sided readings was calculated. The internal and external carotid arteries and bifurcation were also scanned and presence or absence of plaque was noted.

CIMT > 0.90 mm and/or carotid plaques were used as the gold standard test for subclinical atherosclerosis and high CV risk (Corrales et al, 2013). These patients were classed as 'US positive'. Those patients who did not have plaque or CIMT > 0.9 mm were 'US negative', Table 27. Plaque was defined as a focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness of $\geq$ 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface (Touboul et al, 2004).

Carotid US positive	Carotid US negative
Plaque present or CIMT > 0.9 mm	No plaque and CIMT < 0.9 mm

Table 27. Definition of carotid US positive and US negative patients.

5.3.8.1 Definition of rapid progressor

A previous study by Södergren et al (2010) in 27 patients with early RA found that mean increase in CIMT at 18 months was 0.05 + 0.15mm (mean $\pm$ SD). For this study, rapid progression was defined as increase in CIMT >0.05 mm and patients were defined as per Table 28.

Non-rapid Progressor	Rapid Progressor
Increase in CIMT < 0.05mm	Increase in CIMT > 0.05mm

Table 28. Definition of non-rapid and rapid progressor according to change in CIMT

5.3.9 Measurement of carotid distensibility

The Carotid Analyzer software was used as above. Three consecutive frames were selected and the end-diastolic and systolic diameters were recorded for left and right carotid arteries. The mean of these readings was calculated. Right and left arm blood pressure was recorded and the respective pulse pressure calculated. The following equation was used to calculate the distensibility coefficient (DC) in 10^{-3} kPa^{-1} (from Dijk et al, 2005):

$$((2 \text{ X } \Delta D/ D_d)/ PP) \text{ X } 1000$$

ΔD = Mean carotid distension. (Systolic diameter- end diastolic diameter)

D_d = End-diastolic diameter

PP= Pulse pressure (in kPa)

5.3.10 Assessment of cardiovascular risk status

For each patient the SCORE, QRISK2, ACC/AHA and Framingham 10 year risk of cardiovascular event was calculated. Thresholds have previously been set by risk algorithm authors to define 'high risk' patients as per Table 29. If the patient's 10-year risk of cardiovascular event was greater than 10% using the QRISK2 then a letter was sent to the patient's general practitioner to inform them, along with information about the patient's blood pressure, lipid profile and BMI. Current NICE guidelines state that people with a greater than 10% ten year risk of cardiovascular disease should be offered atorvastatin 20 mg for the primary prevention of CVD (NICE guideline CG181, 2014). The patient was informed about their risk status and advised to make an appointment with their GP to discuss this further.

Variable	Threshold used to define 'high risk'
SCORE (Perk et al 2012)	> 5%
QRISK2 (NICE guideline CG181)	>10%
ACC/AHA (Goff et al 2013)	>7.5%
Framingham (National Cholesterol Education Program 2012)	> 20%

Table 29. Cardiovascular risk scores and thresholds used to define 'high risk' for each score.

5.3.11 Blood sampling

Blood was sent to the laboratory at UHW for measurement of rheumatoid factor, anti-CCP antibodies, lipid profile (Total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides), ESR and CRP. Anti CCP and Rheumatoid factor groups were included as there is data that citrullination is found in plaques. Also Pratt et al (2012) identified IL-6 mediated STAT-3 signalling in CD4 T cells during the earliest clinical phase of RA, and was most prominent in anti-CCP negative patients who developed RA. At the same time, blood was also taken for SCCP measurement. Blood was taken into Vacutainer® serum separation tubes (BD) and then centrifuged at 1500rpm for 5 minutes at 4°C. Serum was aliquoted and frozen at -70°C.

5.3.12 ELISA

Levels of IL-6, sIL-6R, IL-6/sIL-6R complex and SCCPS were measured using duoset ELISA development kits from RnD Systems (Abingdon) in accordance with manufacturer's instructions. See Table 8 in general methods section for protocol for ELISA on human serum. All ELISAs were read in a plate reader at OD 450 nm.

Standards and samples were assayed in duplicate. See Table 30 for concentrations used in each ELISA.

Protein	Capture antibody working concentration	Detection antibody working concentration	High standard concentration
IL-6	2 µg/mL	50 ng/mL	600 pg/mL
sIL-6R	2 µg/mL	100 ng/ml	1000 pg/ml
Il-6/sIL-6R complex	2 µg/mL	0.5 µg/mL	5000 pg/mL
VCAM-1	2 µg/mL	200 ng/ml	1000 pg/ml
CCL2	1 ug/ml	50 ng/ml	1000 pg/ml
Tissue Factor	4 ug/ml	200 ng/ml	500 pg/ml
CXCL8	4 ug/ml	20 ng/ml	2000 pg/ml
M-CSF	2 µg/mL	200 ng/ml	1000 pg/ml
CXCL9	1 µg/mL	200 ng/mL	4000 pg/mL
Thrombomodulin	2 µg/mL	1 µg/mL	2000 pg/mL
MMP-1	2 µg/mL	100 ng/mL	4000 pg/mL

Table 30. Antibody and standard concentrations for SCCPs used in ELISA for human serum

5.3.12.1 Statistics

Statistics used were dependent on the data analysed. Where two groups were compared the paired means student's t-test was used. Where multiple groups were compared, a one-way ANOVA and post hoc Bonferroni test were performed. All results were expressed as the mean $\pm$ SEM. Binary logistic regression was used to examine the effect of multiple variables on particular outcomes. Differences that were considered significant were $p < 0.05$.

Sample size estimation. A previous study showed that in early RA patients CIMT increased by 0.05 $\pm$ 0.15 mm. For this study, rapid progression will be defined as increase in CIMT >0.05mm/year. In the absence of a biomarker, 50% of patients will have CIMT increase of at least 0.05mm. We expect SCCPs to improve the proportion of predicted patients by 50%. A sample size of 77 patients will give 80% power to detect an increase in the proportion of correctly predicted patients with an increase of CIMT >0.05 mm from 50% to 75%. Allowing 10% lost to follow up, we aim to recruit 85 patients.

5.4 Results

5.4.1 Established rheumatoid arthritis

182 patients with established RA were recruited, of which 67% were female. Patient characteristics are shown in Table 31. 27% of patients were in remission, 16% had mild disease activity, 35% had moderate disease activity and 22% had severe disease activity defined by the DAS28 score (Figure 61). 49% were taking Methotrexate and 10% were taking Tocilizumab. Mean SCCP levels and comparison with those in the literature for RA are shown in Table 32.

Variable	Mean
Age (years)	60 $\pm$ 1.2
Disease Duration (years)	13.1 $\pm$ 1.0
Rheumatoid factor positive (%)	63.9
Anti-CCP antibody Positive (%)	66.3
CRP	11.9 $\pm$ 2.1

ESR (mm/hr)	22 ± 1.7
DAS28	3.6 ± 0.2
Systolic BP (mmHg)	129 ± 2.2
Cholesterol : HDL ratio	3.8 ± 0.1
QRISK2 (%)	16 ± 1.5
Framingham (%)	13 ± 0.9

Table 31. Demographics details for cross sectional study of 182 patients with established RA.

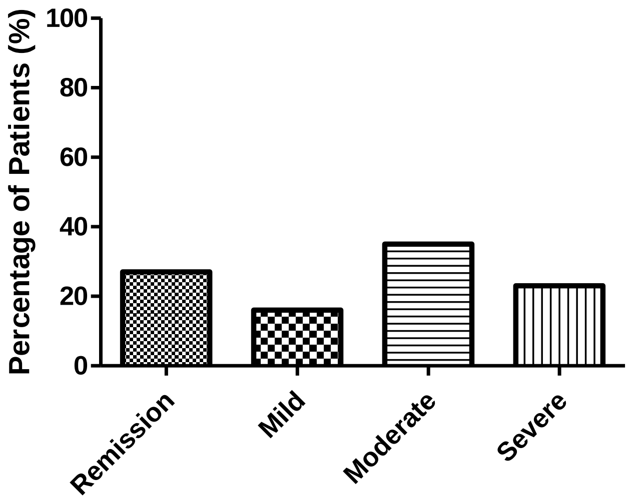

Figure 61. DAS28 group in patients with established RA. 27% of patients were in remission, 16% had mild disease activity, 35% had moderate disease activity and 22 % had severe disease activity.

SCCP	Mean in this study	Value in RA in literature
IL-6 (pg/ml)	19.8 + 2.9	1.5-234 (Robak et al 1998) (range)
sIL-6R (ng/ml)	126 + 46	153.9 + 56.9 (Kohno et al 1998)
IL-6/sIL-6R complex (pg/ml)	348 + 201	6800-8900l (De Benedetti et al 1994) (range in systemic JIA, RA unknown)
CCL2 (pg/ml)	199 + 14	<0.08 – 179 (Green et al 2003) (range)
CXCL8 (pg/ml)	40 + 8	17 – 49 (Slavić et al 2005) (range)
CXCL9 (ng/ml)	0.8 + 0.1	3.1 (1.2–4.9) (Kaun et al 2010) median and IQR
MCSF (pg/ml)	4.3 + 1.8	1.32 + 0.50 (Kawaji et al 1995) (mean)
MMP-1 (ng/ml)	5.9 + 0.4	41.8 (Green et al 2003) (mean)
Thrombomodulin (ng/ml)	4.8 + 0.1	5.9 + 2.2 (Abdul-Moniem et al 2009) (mean)
Tissue Factor (pg/ml)	29 + 7	unknown
VCAM-1 (ng/ml)	855 + 53	475 ± 258 (Navarro-Hernández et al 2009) (mean)

Table 32. Mean SCCP levels in patients with established RA in cross sectional study and in the literature

5.4.1.1 Association of SCCPS with disease activity in established RA

There was a significant positive correlation between DAS28 and VCAM-1 (Figure 62) but not with other SCCPs. There was no significant difference between DAS28 groups (remission, mild, moderate or severe) when IL-6, sIL-6R, IL-6/sIL-6R complex, CRP, IL-8, MCSF, CCL2, CXCL9, thrombomodulin, MMP-1, or tissue factor were compared. ESR was significantly higher in severe RA groups compared to moderate, mild and remission groups and VCAM-1 was significantly higher in severe RA compared to moderate RA (Figure 63).

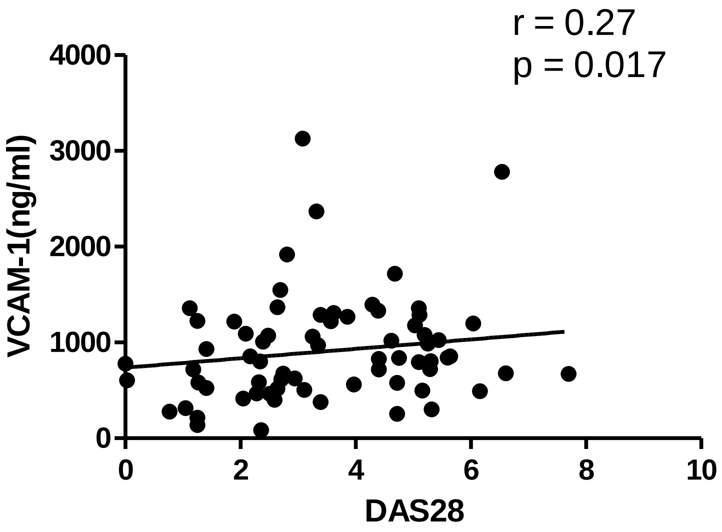

Figure 62. Significant positive correlation between VCAM-1 and DAS28 in patients with established RA, r = 0.27, p = 0.017

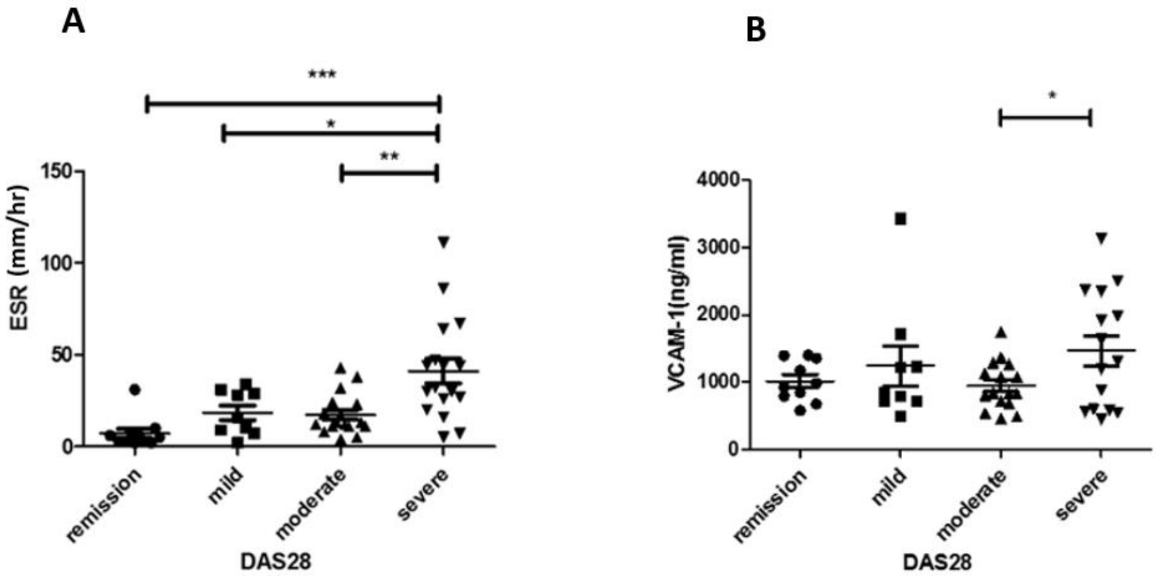

Figure 63. Significant difference in ESR and VCAM-1 between DAS28 groups. (A) ESR was significantly lower in RA remission (7.9 + 9.1 mm/hr), compared to mild (8.2 + 10.5 mm/hr), moderate (18.5 + 12.2 mm/hr) and severe RA (40.3 + 26.4 mm/hr), *p* = 0.000. (B) VCAM-1 was significantly lower in moderate RA (780 + 412ng/ml) compared to severe RA (1349 + 880 ng/ml), *p* = 0.023. Mean VCAM-1 for those in remission was 825 + 358 ng/ml and 955 + 865 ng/ml in mild RA.

5.4.1.2 Association of SCCPS with cardiovascular risk in established RA

There was a significant positive correlation between the QRISK2 score and serum MMP-1 (r = 0.35, p = 0.0014), thrombomodulin (r = 0.32, p = 0.0034) and VCAM-1 (r = 0.32, p = 0.0025) (Figure 64). There was a significant positive correlation between the Framingham risk score and serum MMP-1 (r = 0.32, p = 0.0026) and thrombomodulin (r = 0.31, p = 0.0034) (Figure 65, Table 32). There was a significant correlation with age and MMP-1 (r = 0.25, p = 0.013), thrombomodulin (r = 0.318, p = 0.001) and VCAM-1 (r = 0.27, p = 0.002). The QRISK2 and Framingham scores were significantly moderately correlated, r = 0.7, p < 0.0001 (Figure 66). Patients were classified according to the QRISK2 as having a low risk (< 10%) or high risk (> 10%) of cardiovascular event over the next 10 years. 52% of patients were high risk according to the QRISK2. Those classified as low risk had a significantly higher CRP than those at high risk (19.7 ± 34.8 mg/L versus 8.2 ± 8.5 mg/L), Table 33. Those classified as low risk had significantly lower serum thrombomodulin (4.6 ± 1.2 ng/ml versus 5.4 ± 1.6 ng/ml) and VCAM-1 (977 ± 600 ng/ml versus 1247 ± 289 ng/ml) (Figure 67) than those classified as high risk (Table 34).

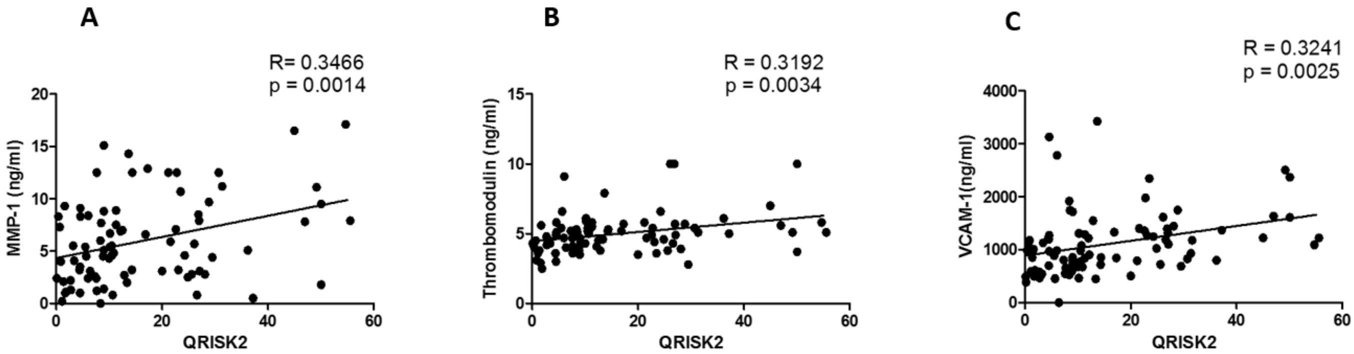

Figure 64. Significant positive correlations between QRISK2 and (A) MMP-1 (r = 0.35, p = 0.0014), (B) Thrombomodulin (r = 0.32, p = 0.0034) and (C) VCAM-1 (R = 0.32, p = 0.0025).

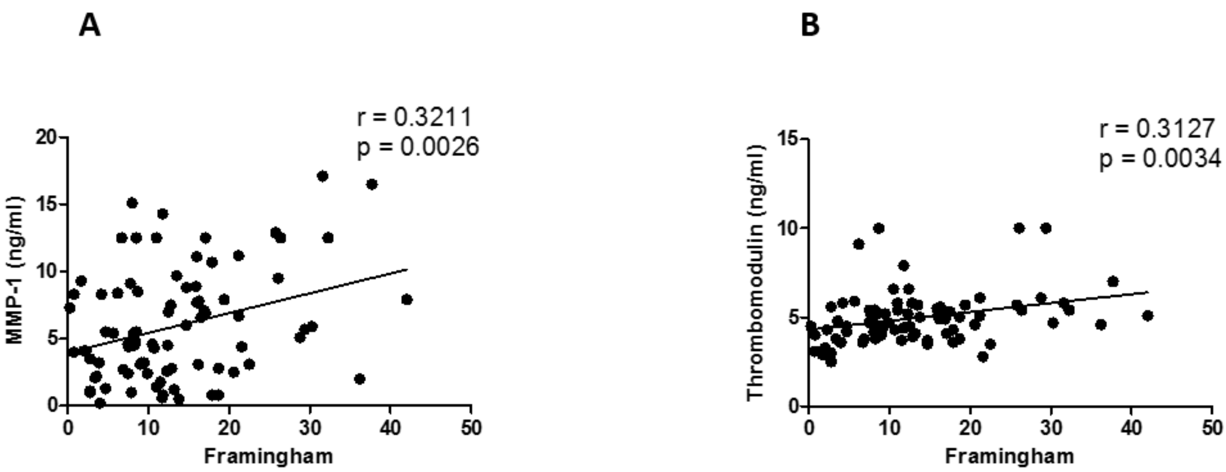

Figure 65. Significant positive correlation between the Framingham risk score and (A) serum MMP-1 (r = 0.32, *p* = 0.0026) and (B) serum thrombomodulin (r = 0.31, *p* = 0.0034)

Variable	Correlation with QRISK2 (r value)	p value	Correlation with Framingham (R value)	p Value
CRP (mg/L)	-0.139	ns	-0.107	ns
ESR (mm/hr)	0.211	ns	0.101	ns
IL-6 (pg/ml)	-0.078	ns	0.004	ns
sIL-6R (ng/ml)	0.119	ns	0.128	ns
IL-6/sIL-6R complex (pg/ml)	-0.063	ns	-0.021	ns
CCL2 (pg/ml)	0.204	ns	0.098	ns
CXCL8 (pg/ml)	0.113	ns	0.069	ns
CXCL9 (ng/ml)	0.183	ns	0.054	ns
MCSF (pg/ml)	0.111	ns	0.171	ns
MMP-1 (ng/ml)	0.347	**0.001**	0.321	**0.003**
Thrombomodulin (ng/ml)	0.319	**0.001**	0.313	**0.003**
Tissue Factor (pg/ml)	-0.084	ns	-0.241	ns
VCAM-1 (ng/ml)	0.324	**0.002**	0.134	ns
QRISK2	1		0.698	**0.000**
Framingham	0.698	**0.000**	1	
Chol/HDL ratio	-0.0076	ns	0.315	**0.008**
Age (years)	0.879	**0.000**	0.637	**0.000**
Disease Duration (yrs)	0.054	ns	0.068	ns
DAS-28	0.09	ns	0.011	ns
BP (mmHg)	0.496	**0.000**	0.588	**0.000**

Table 33. Correlation between SCCPs and CV risk variables with QRISK2 and Framingham scores

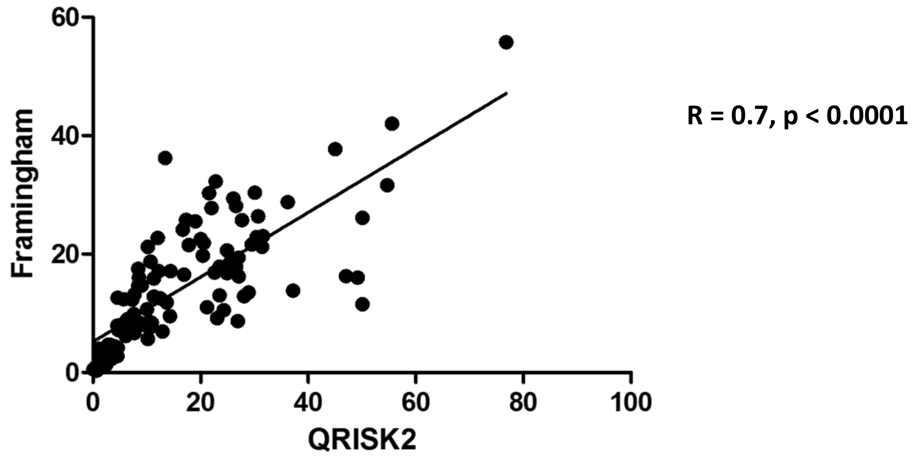

Figure 66. Significant positive correlation between the QRISK2 and the Framingham score in patients with established RA, r = 0.7, *p* < 0.0001

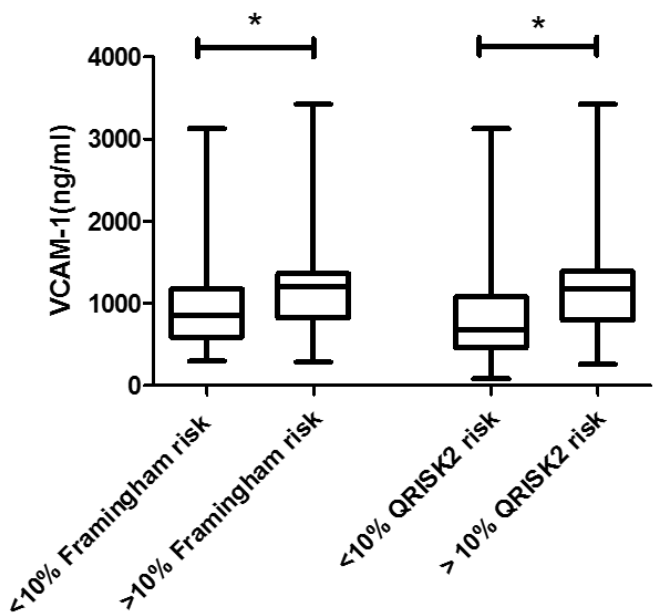

Figure 67. Serum VCAM-1 was significantly higher in those with a > 10% CV risk over the next 10 years according to both the QRISK2 and the Framingham score, compared to those with < 10% risk in patients with established RA. * *p* < 0.05

Variable	Low risk QRISK2	High risk QRISK2	P value
CRP (mg/L)	19.7 + 34.8	8.2 + 8.5	**0.046**
ESR (mm/hr)	21.5 + 17.4	23.7 + 21.9	ns
IL-6 (pg/ml)	24.0 + 46.8	16.2 + 29.1	ns
SIL-6R (ng/ml)	123 + 51	113 + 42	ns
Ratio IL-6 : sIL-6r	0.12 + 0.2	0.15 + 0.3	ns
IL-6/sIL-6R complex (pg/ml)	239.7 ± 521	519 ± 2630	ns
CCL2 (pg/ml)	258 +134	242 + 146	ns
CXCL8 (pg/ml)	27 + 28	42 + 70	ns
CXCL9 (ng/ml)	0.78 +1.0	1.0 + 1.0	ns
MCSF (pg/ml)	2.3 + 6.3	6.7 + 23.6	ns
MMP-1 (ng/ml)	5.3 + 4.1	6.7 + 4.1	ns
Thrombomodulin (ng/ml)	4.6 + 1.2	5.4 + 1.6	**0.009**
Tissue Factor (pg/ml)	50.1 + 83.7	46.8 + 81.7	ns
VCAM-1 (ng/ml)	977 + 600	1247 + 289	**0.039**

Table 34. Differences in SCCPs in those classified as low risk versus high risk of CVD according to the QRISK2

5.4.1.3 Association of SCCPs with history of cardiovascular disease in established RA

Twelve patients (9.6%) had previous CVD. Of these, seven (58.3%) were female. In those patients with a history of CV disease, mean serum CRP (3.9 ± 5.7 mg/L versus 13.3 ± 24.5 mg/L), ESR (20 ± 13.2 mm/hr versus 22 ± 19.6 mm/hr), IL-6 (5.1 ± 10.6 pg/ml versus 22 ± 39.8 pg/ml), sIL-6R (107 ± 22 versus 128 ± 53), MCSF (0 ± 0 pg/ml versus 4.8 ± 18.0 pg/ml) and tissue factor (0 ± 0 pg/ml versus 50 ± 82 pg/ml) were significantly lower than those with no history of CVD (Figure 68 and Table 35). All patients with a history of CVD had undectable levels of MSCF and tissue factor. There was no significant difference in serum VCAM-1 levels in those with history of CVD (1029 ± 607 ng/ml) compared to those with no history of CVD (947 ± 442 ng/ml). A further analysis of the subgroup of patients with previous CVD revealed that most were on secondary prevention for CVD; 92% of patients with CVD were taking a statin, 80% were taking an antihypertensive.

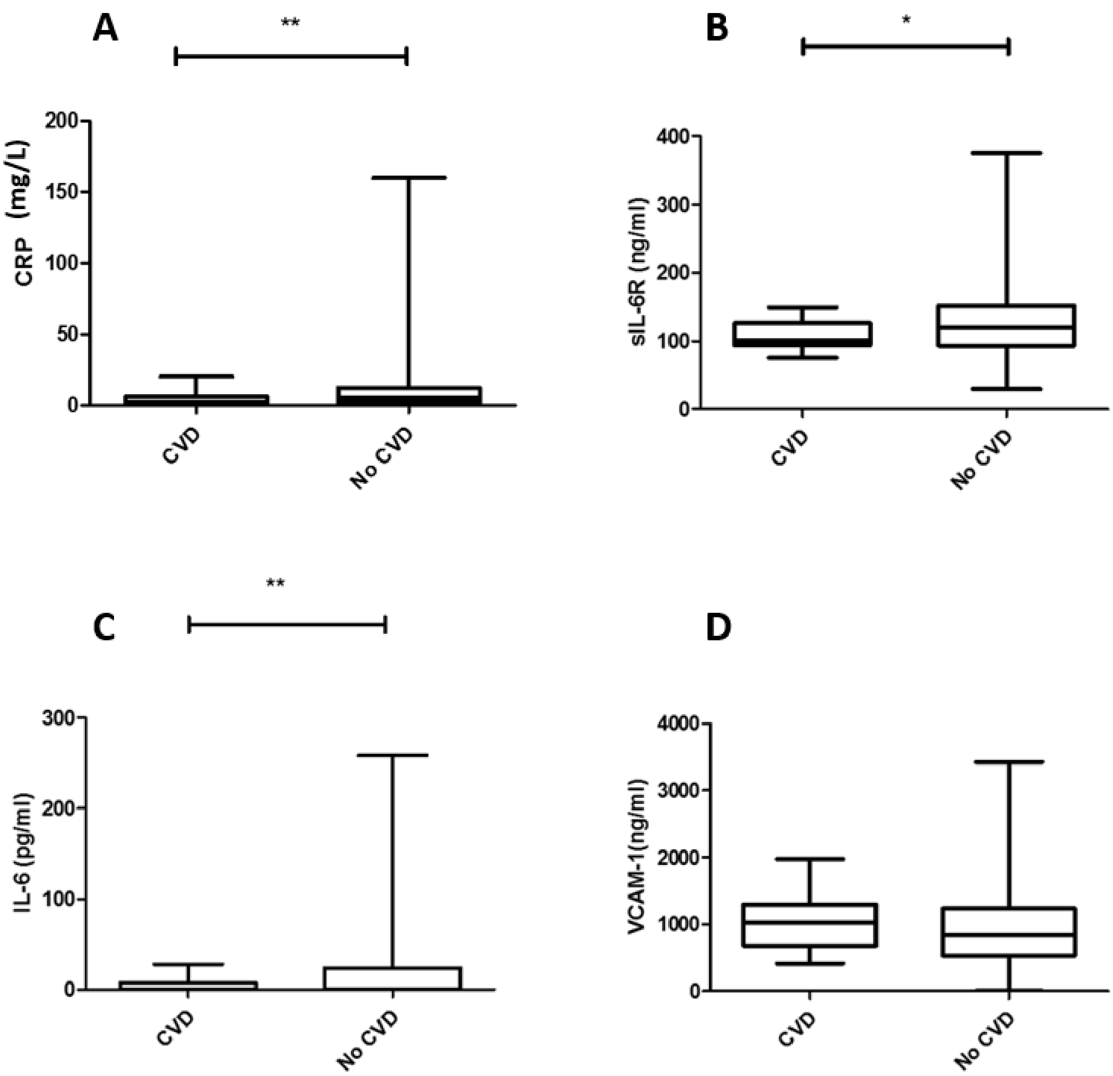

Figure 68. Significantly lower (A) CRP (3.9 + 5.7 mg/L versus 13 + 24.5 mg/L, *p* = 0.002), (B) sIL-6R (107 + 22 ng/ml versus 128 + 53 ng/ml *p* = 0.015 (C) IL-6 (5.1 + 10.6 pg/ml versus 22 + 39.8 pg/ml, *p* = 0.009) in those with history of CVD compared to those without history of CVD. (D) No significant difference in serum VCAM-1 levels in patients with history of CVD (1029 + 607 ng/ml) compared to those with no history of CVD (947 + 442 ng/ml).

Variable	History of CVD n = 12	No history of CVD n = 113	P value
CRP (mg/L)	3.9 ± 5.7	13 ± 24.5	ns
ESR (mm/hr)	20 ± 13.2	22 ± 19.6	**0.002**
IL-6 (pg/ml)	5.1 ± 10.6	22 ± 39.8	**0.009**
Sil-6R (ng/ml)	107 ± 22	128 ± 53	**0.015**
IL-6/sIL-6R complex (pg/ml)	144.6 ± 51.5	251.4 ± 140.0	**0.14**
CCL2 (pg/ml)	243 ± 93	196 ± 112	ns
CXCL8 (pg/ml)	91 ± 164	36 ± 56	ns
CXCL9 (ng/ml)	0.924 + 0.94	0.91 ± 1.01	ns
MCSF (pg/ml)	0 ± 0	4.8 ± 18.0	**0.019**
MMP-1 (ng/ml)	5.0 ± 5.2	5.9 ± 4.1	ns
Thrombomodulin (ng/ml)	4.5 ± 0.7	5 ± 1.5	ns
Tissue Factor (pg/ml)	0 ± 0	50 ± 82	**0.00**
VCAM-1 (ng/ml)	1029 ± 442	947 ± 607	ns
QRISK2 score (%)	19 ± 5.2	16 ± 15	ns
Framingham score (%)	18 ± 8	13 ± 9	ns
Chol/HDL ratio	5.4 ± 1.8	3.7 ± 0.9	ns
Age (years)	61 ± 10	60 ± 14	ns
Disease Duration (years)	12.8 ± 6.5	13.4 ± 11.2	ns

Table 35. Levels of SCCPs in those with a history of cardiovascular disease and those without in patients with established RA

5.4.1.4 Lower CRP in those taking statin

As all patients who had CVD were taking a statin, a subgroup analysis was performed to investigate whether there was any difference in SCCP levels in those taking or not taking a statin. Mean serum CRP was significantly lower in those patients taking a statin (CRP 7.0 ± 1.5 mg/L) compared to those not taking a statin (13.5 ± 2.7 mg/L), *p* = 0.037 (Figure 69). There was no significant difference in SCCPs or disease activity in those taking statins and those not, Table 36.

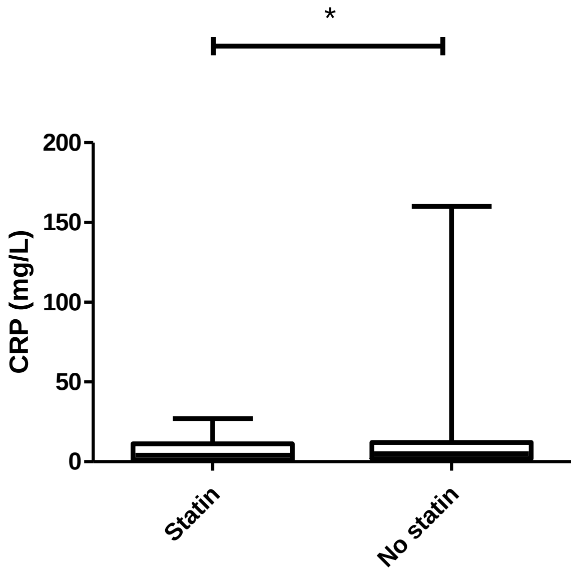

Figure 69. Mean serum CRP levels significantly lower in patients taking a statin (7.0 + 1.5) than those not taking a statin (13.5 + 2.7) *p* = 0.037 in patients with established RA

Variable	Statin (n = 36)	No Statin (n = 105)	P value
CRP (mg/L)	7.1 + 8.2	13.6 + 26.2	**0.039**
ESR (mm/hr)	18.3 + 13.6	22.5 + 19.4	ns
IL-6 (pg/ml)	21.8 + 31.9	19.3 + 40.1	ns
Sil-6R (ng/ml)	132.4 + 69	124.2 + 44	ns
Ratio IL-6 : sIL-6R	0.16 + 0.3	0.13 + 0.2	ns
IL-6/sIL-6R complex (pg/ml)	130.5 + 212.5	491.5 + 2345	ns
CCL2 (pg/ml)	227 + 123	255 + 142	ns
CXCL8 (pg/ml)	76 + 129	30 + 33	ns
CXCL9 (ng/ml)	1.29 + 1.3	0.76 + 0.88	ns
MCSF (pg/ml)	9.5 + 30.5	2.8 + 10.1	ns
MMP-1 (ng/ml)	5.0 + 4.1	6.0 + 1.2	ns
Thrombomodulin (ng/ml)	5.4 + 1.7	4.8 + 1.3	ns
Tissue Factor (pg/ml)	28.7 + 75	33.0 + 68	ns
VCAM-1 (ng/ml)	1012 + 409	919 + 625	ns
DAS28	3.57 + 1.43	3.60 + 1.76	ns

Table 36. Differences in SCCPs and other variables in those taking and not taking a statin in patients with established RA.

5.4.1.5 Effect of methotrexate and tocilizumab on SCCPs

There was no difference in SCCPs or DAS28 between those taking methotrexate and those not taking methotrexate (Table 37). Those patients on tocilizumab had significantly lower DAS28 (2.5 $\pm$ 0.4 versus 3.8 $\pm$ 0.2) (Figure 70) and serum ESR (8.7 $\pm$ 3 mm/hr versus 24.3 $\pm$ 1 mm/hr) than those not on tocilizumab (Figure 71, Table 38). IL-6 was significantly higher in those taking tocilizumab than those not (845 $\pm$ 27 pg/ml versus 14 $\pm$ 3 pg/ml, p = 0.034) (Figure 72). There was no difference in CRP between patients taking tocilizumab (mean CRP 11.3 $\pm$ 6.3 mg/L) and patients not taking tocilizumab (mean CRP 12.0 $\pm$ 2.2 mg/L).

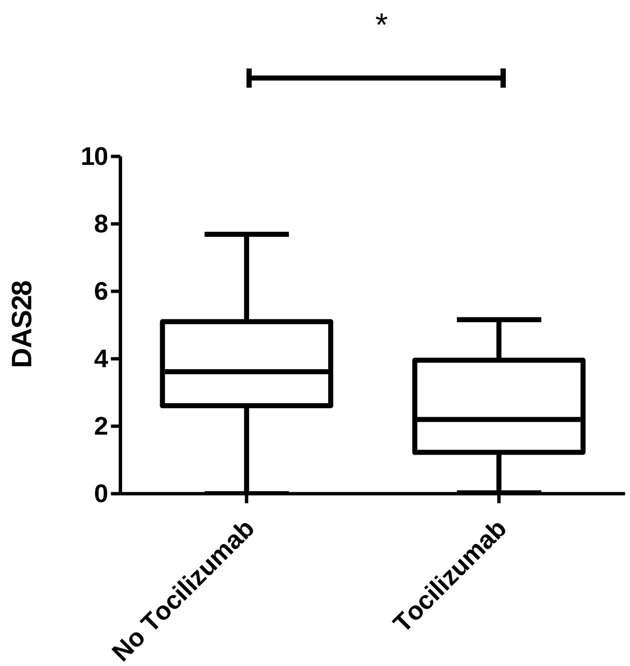

Figure 70. DAS28 significantly higher in established RA patients not taking tocilizumab than those taking tocilizumab (3.8 $\pm$ 0.2 versus 2.5 $\pm$ 0.4) *p* = 0.017

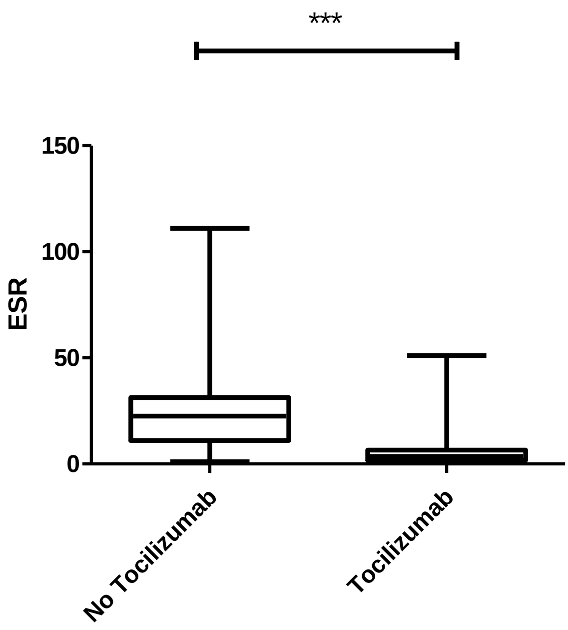

Figure 71. Serum ESR levels significantly higher in established RA patients not taking tocilizumab than those taking tocilizumab (24 + 2 mm/hr versus 9 + 4 mm/hr) p < 0.000

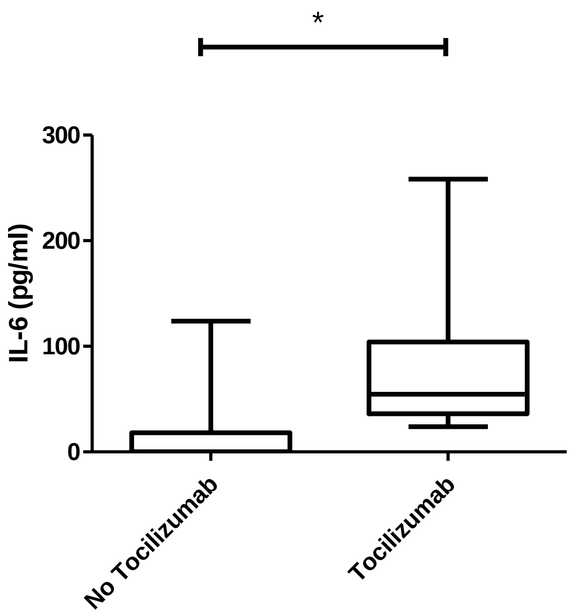

Figure 72. Serum IL-6 levels significantly higher in established RA patients taking tocilizumab than those not taking tocilizumab (845 + 27 versus 14 + 3 pg/ml, *p* = 0.034).

Variable	No Methotrexate (n = 67)	Methotrexate (n = 75)	P value
CRP (mg/L)	16 ± 24	20 ± 26	0.259
ESR (mm/hr)	25 ± 20	20 ± 15	0.227
IL-6 (pg/ml)	23 ± 44	16 ± 31	0.362
SIL-6R (ng/ml)	119 ± 51	116 ± 43	0.728
Ratio IL-6 : sIL-6R	0.13 ± 0.2	0.14 ± 0.3	0.884
IL-6/sIL-6R complex (pg/ml)	217 ± 493	541 ± 2736	0.490
CCL2 (pg/ml)	256 ± 139	236 ± 137	0.479
CXCL8 (pg/ml)	32 ± 34	48 ± 94	0.317
CXCL9 (ng/ml)	0.98 ± 1.00	0.83 ± 1.01	0.493
MCSF (pg/ml)	2.5 ± 6.7	6.2 ± 23.2	0.312
MMP-1 (ng/ml)	5.9 ± 4.1	5.7 ± 4.2	0.822
Thrombomodulin (ng/ml)	4.99 ± 1.6	4.9 ± 1.2	0.854
Tissue Factor (pg/ml)	40.3 ± 83	24.5 ± 56	0.340
VCAM-1 (ng/ml)	1047 ± 491	1184 ± 673	0.268
DAS28	3.65 ± 1.61	3.54 ± 1.74	0.78
Cholesterol : HDL ratio	3.84 ± 1.1	3.76 ± 1.1	0.76

Table 37. SCCP and other variables in established RA patients taking methotrexate versus those not taking methotrexate

Variable	No Tocilizumab (n = 123)	Tocilizumab (n = 19)	P value
CRP (mg/L)	12 ± 2	11 ± 6	0.924
ESR (mm/hr)	24 ± 2	9 ± 3	**0.000**
IL-6 (pg/ml)	14 ± 3	85 ± 27	**0.034**
SIL-6R (ng/ml)	124 ± 5	148 ± 16	0.172
IL-6/sIL-6R complex (pg/ml)	359 ± 253	564 ± 323	0.625
CCL2 (pg/ml)	194 ± 14	258 ± 30	0.107
CXCL8 (pg/ml)	39 ± 8	55 ± 22	0.526
CXCL9 (ng/ml)	0.8 ± .1	1.3 ± 0.5	0.368
MCSF (pg/ml)	4.1 ± 1.7	8.0 ± 4.7	0.459
MMP-1 (ng/ml)	6.1 ± 0.4	4.1 ± 1.5	0.241
Thrombomodulin (ng/ml)	4.7 ± 0.1	6.0 ± 0.6	0.073
Tissue Factor (pg/ml)	29.6 ± 7.3	27.0 ± 13.7	0.872
VCAM-1 (ng/ml)	968 ± 60	877 ± 95	0.424
DAS28	3.8 ± 0.2	2.5 ± 0.4	**0.017**
Cholesterol : HDL ratio	3.7 ± 0.1	4.0 ± 0.4	0.567

Table 38. SCCP and other variables in established RA patients taking tocilizumab versus those not taking tocilizumab

5.4.1.6 Effect of Rheumatoid Factor and anti–CCP status on SCCPs

SIL-6R and MMP-1 were significantly higher in patients that were rheumatoid factor positive compared to those that were negative (Table 39). There was no significant difference between other SCCPs. Those patients that were anti-CCP positive had significantly lower CRP than those that were negative. There was no difference in other SCCPs (Table 40).

Variable	RF positive (n = 78)	RF negative (n = 44)	P value
CRP (mg/L)	9.8 ± 18.4	15.4 ± 30.4	0.284
ESR (mm/hr)	20.3 ± 14.9	23.4 ± 21.4	0.401
IL-6 (pg/ml)	21.5 ± 24.2	18.2 ± 33.5	0.687
SIL-6R (ng/ml)	137 ± 55	103 ± 39	**0.002**
Ratio IL-6 : sIL-6r	0.15 ± 0.04	0.12 ± 0.04	0.699
IL-6/sIL-6R complex (pg/ml)	602 ± 2577	99 ± 290	0.223
CCL2 (pg/ml)	250 ± 142	245 ± 129	0.877
CXCL8 (pg/ml)	45 ± 90	35 ± 36	0.493
CXCL9 (ng/ml)	0.82 ± 0.95	0.88 ± 1.07	0.789
MCSF (pg/ml)	6.5 ± 22.7	1.9 ± 4.8	0.182
MMP-1 (ng/ml)	7.0 ± 4.6	4.7 ± 3.5	**0.008**
Thrombomodulin (ng/ml)	4.8 ± 1.4	4.7 ± 1.5	0.884
Tissue Factor (pg/ml)	18.9 ± 48.3	55 .5 ± 95.2	0.071
VCAM-1 (ng/ml)	943 ± 560	936 ± 649	0.955
DAS28	3.65 ± 1.71	3.39 ± 1.54	0.496

Table 39. SCCP and DAS28 levels in rheumatoid factor positive and negative established RA patients

Variable	Anti-CCP positive (n = 55)	Anti-CCP negative (n = 28)	P value
CRP	8.4 ± 12.6	27.3 ± 42.9	**0.036**
ESR	18.3 ± 14.7	27.5 ± 23.6	0.074
IL-6 (pg/ml)	15.3 ± 26.3	26.5 ± 57.8	0.367
Sil-6R (ng/ml)	135 ± 60	111 ± 52	0.071
Ratio IL-6 : sIL-6r	0.16 ± 0.06	0.10 ± 0.03	0.312
IL-6/sIL-6R complex (pg/ml)	525 ± 2734	60 ± 150	0.315
CCL2 (pg/ml)	260 ± 149	236 ± 131	0.483
CXCL8 (pg/ml)	32 ± 37	30 ± 30	0.751
CXCL9 (ng/ml)	0.82 ± 0.92	0.87 ± 0.99	0.836
MCSF (pg/ml)	3.86 ± 15.01	1.97 ± 5.51	0.463
MMP-1 (ng/ml)	5.74 ± 4.29	5.99 ± 4.33	0.820

Thrombomodulin (ng/ml)	4.96 ± 1.27	4.68 ± 1.34	0.411
Tissue Factor (pg/ml)	10 ± 23	48 ± 84	0.075
VCAM-1 (ng/ml)	989 ± 528	1170 ± 697	0.236
DAS28	3.7 ± 1.52	3.88 ± 1.59	0.748

Table 40. SCCPs and DAS28 levels in anti-CCP positive and negative established RA patients.

5.4.2 Early Arthritis Study (EAS) results

Forty-five patients were recruited. The baseline characteristics of the participants are presented in Table 41. 35 patients were followed up at 6 months, and 23 patients at 12 months. 91% were Caucasian, 4% Indian, 2% Pakistani, and 2% Czech. Figure 73 shows RA treatment at baseline, 6 months and 12 months. At baseline 63% were taking any DMARD, 58% of patients were taking methotrexate, 30% were taking two DMARDs, 26% were taking corticosteroids and 9% were taking two DMARDs plus corticosteroids (Figure 73). At 6 months, one patient and at 12 months, 2 patients were taking a biological drug (Tocilizumab). 52% of patients were not taking NSAIDs, 26% were taking occasional NSAIDs and 22% were taking regular NSAIDS.

Variable	Mean
Age (years)	56.1 ± 2.2
Female Sex (%)	75
Disease Duration (months)	4.0 ± 0.2
Rheumatoid factor positive (%)	59
Anti-CCP antibody positive (%)	75
CRP (mg/L)	11 ± 2
ESR (mm/hr)	24 ± 3

DAS28	3.87 ± 0.20
Extra articular features (%)	15
Systolic BP (mmHg)	136 ± 3.2
QRISK2 (%)	16.9 ± 2.5
Smoking (%) **Never smoked** **Former** **Current**	 39.5 34.9 25.6
BMI	27.3 ± 0.9
HbA1c	40.2 ± 1
Family history of CVD < 60 years (%)	18

Table 41. Baseline characteristics of patients recruited in the early arthritis study.

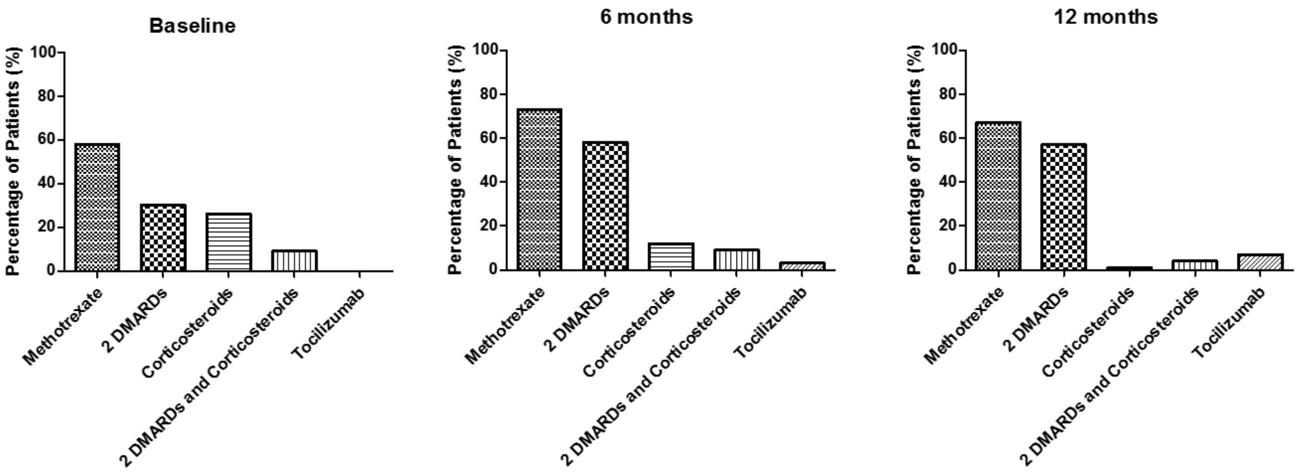

Figure 73. Medication for RA taken by early RA patients at (A) baseline, (B) 6 months and (C) 12 months

5.4.2.1 Association of SCCPs with disease activity in early RA

Patients were grouped by DAS28 into severity, as in Table 26, Section 5.3.4. At baseline 18% were in remission, 43% had moderate disease activity, 18% had mild disease activity and 20% had severe disease activity (Figure 74). There was no significant correlation between baseline DAS28 and baseline IL-6, sIL-6R or SCCPs. To further assess the relationship between IL-6 and sIL-6R in this cohort, patients were grouped according to these serum cytokine concentrations, as per table 42. The mean serum level was calculated for IL-6 and sIL-6R and those below the mean were 'low' and those above the mean were 'high'.

	Group 1	Group 2	Group 3	Group 4
IL-6	Low	High	Low	High
sIL-6R	Low	Low	High	High

Table 42. Patients with early RA grouped according to IL-6 and sIL-6R level.

As expected, those in DAS28 remission at baseline had significantly lower ESR (10 $\pm$ 3 mm/hr) than those not in remission (27 $\pm$ 3 mm/hr). However, there was no significant difference in CRP or IL-6 between these groups (Figure 75). There were significant positive correlations between DAS28 and ESR and CRP (Figure 76). There was a significant positive correlation between DAS28 and US7 score (Figure 77). There was no correlation between the DAS28 and US10 score. There were no significant correlations between either the US7 score or US10 score and SCCPs. There was no significant difference in CRP, ESR or SCCPs in those taking methotrexate and those not taking methotrexate. CXCL9 was significantly lower in those taking corticosteroids at baseline versus those not taking corticosteroids (119 $\pm$ 48 pg/ml) versus 922 $\pm$ 169 pg/ml), Figure 78. There was no difference in CRP, ESR or other SCCPs in these groups.

Mean SCCPs in early and established RA are shown in Table 43. In early RA, mean IL-6, sIL-6R, CXCL8, thrombomodulin and VCAM-1 were significantly lower than established RA. MMP-1 and tissue factor were significantly higher in early RA than established RA. There was no significant difference in CCL2, CXCL9 or MSCF between early and established RA.

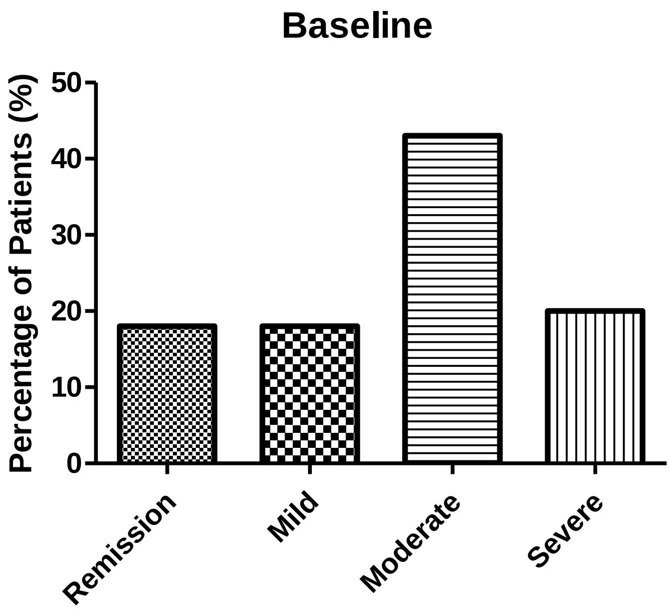

Figure 74. Percentage of patients in DAS28 categories at baseline. 18% of patients were in remission, 18% had mild disease activity, 43% had moderate disease activity and 20% had severe disease activity.

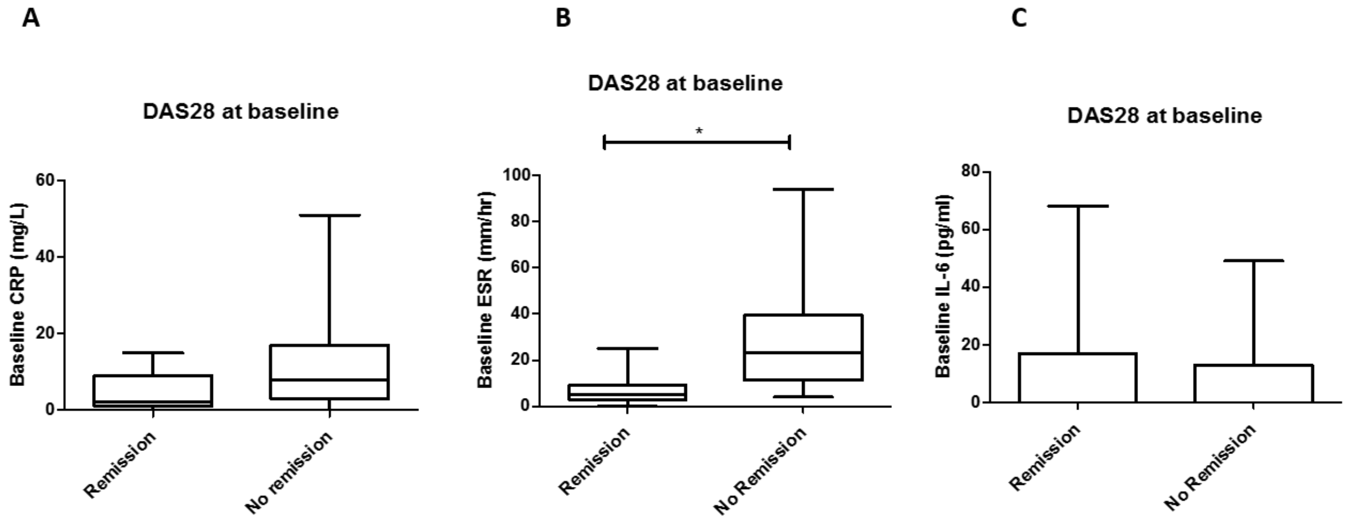

Figure 75 (A) No significant difference in baseline CRP in those in DAS28 remission (5 + 2 mg/L) and those not (35 + 2 mg/L). (B) Baseline ESR significantly lower in those in DAS28 remission (10 + 3 mm/hr) compared to those not in remission (27 + 3 mm/hr), *p* < 0.05. (D) No significant difference in baseline IL-6 in those in remission (11 + 11 pg/ml) and those not at baseline (29 + 7 pg/ml).

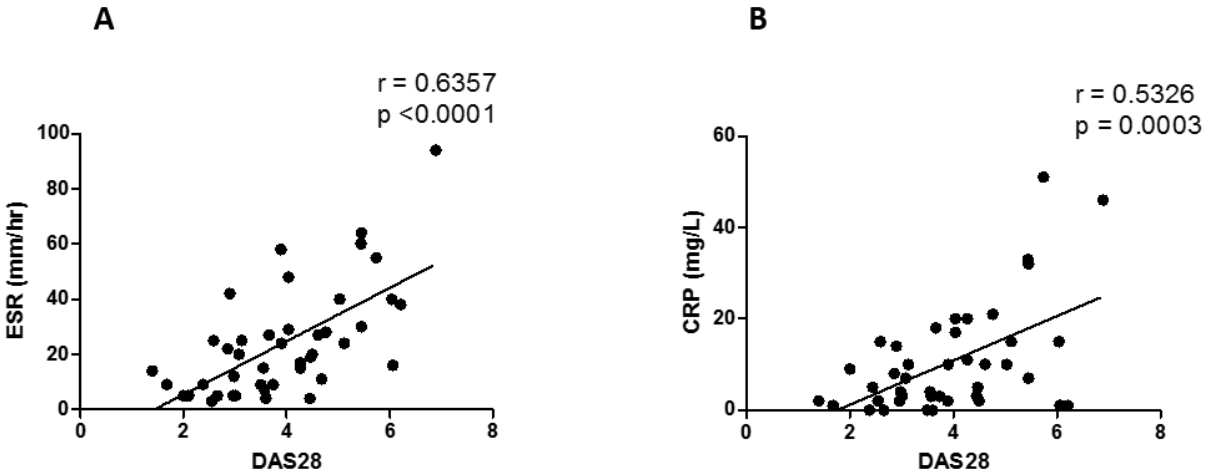

Figure 76. Significant positive correlation between baseline DAS28 and baseline ESR (r= 0.64, *p* <0.0001) and CRP (r= 0.53, *p* = 0.0003) in patients with early RA.

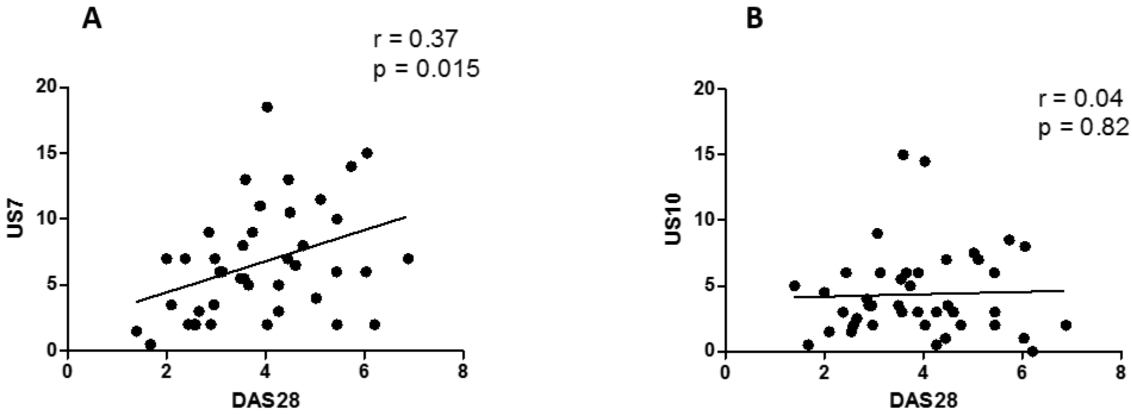

Figure 77. (A) Significant positive correlation between DAS28 and US7 score at baseline in patients with early RA, r = 0.37, *p* = 0.015. (B) No correlation between DAS28 and US10 score at baseline in patients with early RA, r =0.04, *p* = 0.82.

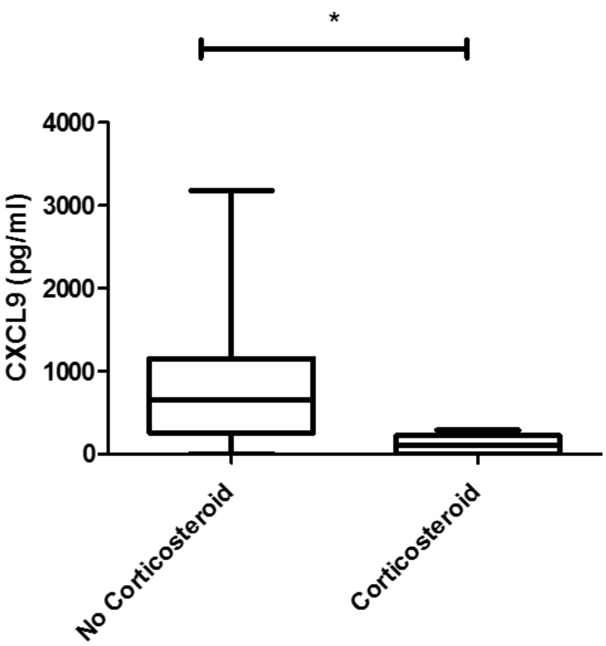

Figure 78. Baseline CXCL-9 significantly lower in patients with early RA taking corticosteroids (119 $\pm$ 48 pg/ml) compared to those not taking corticosteroids (922 $\pm$ 169 pg/ml), *p* < 0.05

SCCP	Mean in EAS	Mean in established RA	P value
IL-6 (pg/ml)	7.7 ± 2.5	19.8 ± 2.9	**<0.0001**
sIL-6R (ng/ml)	95 ± 7	126 + 46	**0.0002**
IL-6/sIL-6R complex (pg/ml)	364 ± 101	348 ± 201	ns
CCL2 (pg/ml)	209 ± 21	199 ± 14	ns
CXCL8 (pg/ml)	2 ± 2	40 ± 8	**0.0059**
CXCL9 (ng/ml)	788 ± 144	826 ± 92	ns
MCSF (pg/ml)	15 ± 13	4.3 ± 1.8	ns
MMP-1 (ng/ml)	11 ± 1.5	5.9 ± 0.4	**P<0.0001**
Thrombomodulin (ng/ml)	4.3 ± 0.1	4.8 ± 0.1	**P<0.0001**
Tissue Factor (pg/ml)	50 ± 25	29 ± 7	**P<0.0001**
VCAM-1 (ng/ml)	708 + 60	855 ± 53	**0.0115**

Table 43. Differences in serum SCCPs in early and established RA

5.4.2.2 Disease activity and US10 score significantly reduced over time in patients with early RA

DAS28 significantly reduced over time and was significantly lower at 12 months (2.75 ± 0.39) and 6 months (3.02 ± 0.22) compared to baseline (3.87 ± 0.20), (Figure 79). At 6 months, those in DAS28 remission (DAS28 < 2.6) had significantly lower baseline CRP, ESR and IL-6 compared to those not in remission at 6 months (Figure 81). There was no significant difference in baseline CRP, ESR, IL-6 or other baseline SCCPs in those in remission versus those not in remission at 12 months. There was a significant reduction in mean US10 score at 12 months (2.3 ± 0.7) compared to baseline (4.4 ±

0.5) (Figure 82). There was no significant reduction in US7 score over time. Table 44 summarises change in DAS28, US7 and US10 scores over time. There was a moderate positive correlation between baseline IL-6 and US7 score at 12 months (r=0.57, p = 0.013) and strong positive correlation between baseline IL-6 and US10 score at 12 months (r = 0.61, p = 0.0078) (Figure 83). There was no other significant correlation between baseline SCCPs and US scores at 12 months.

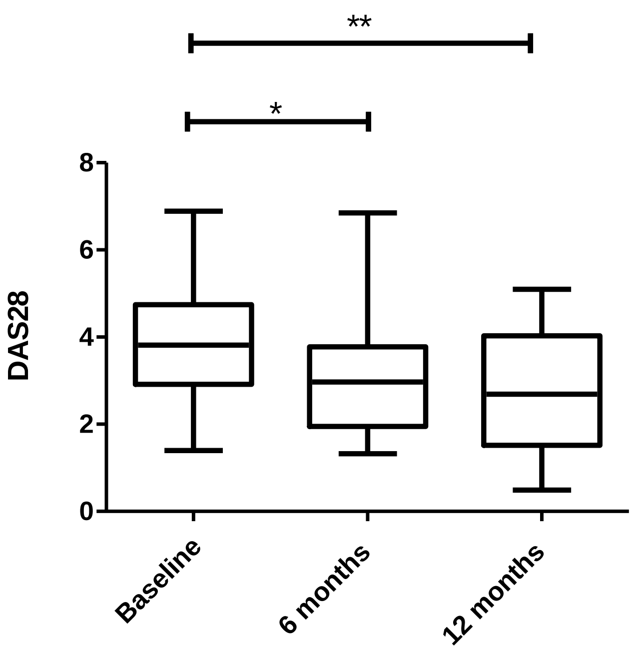

Figure 79. Mean DAS28 was significantly lower at 6 months (3.02 ± 0.22) and 12 months (2.75 ± 0.39) than baseline (3.87 ± 0.20) in patients with early RA * p < 0.05, ** p < 0.01.

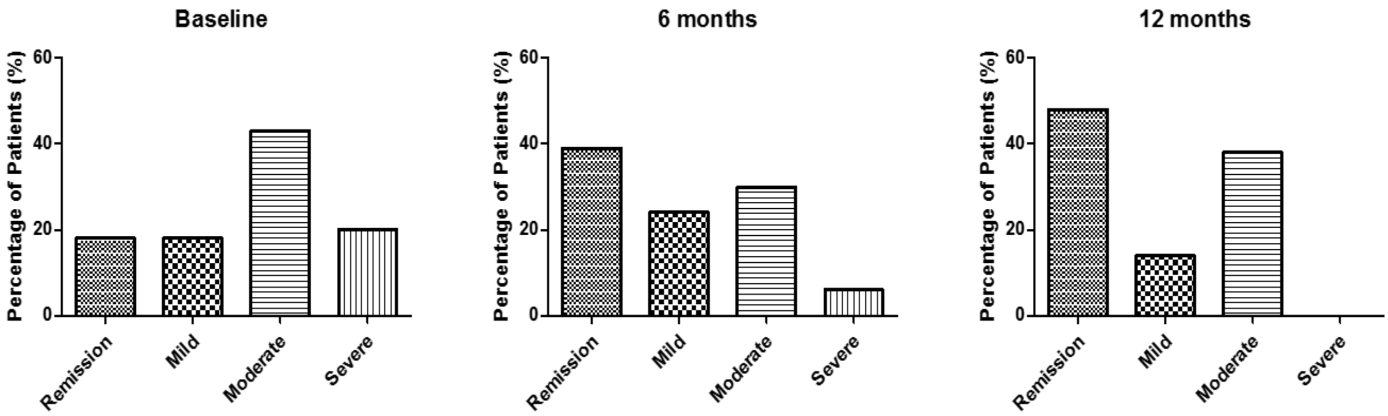

Figure 80. Percentage of patients in DAS28 groups at baseline, 6 month and 12 months

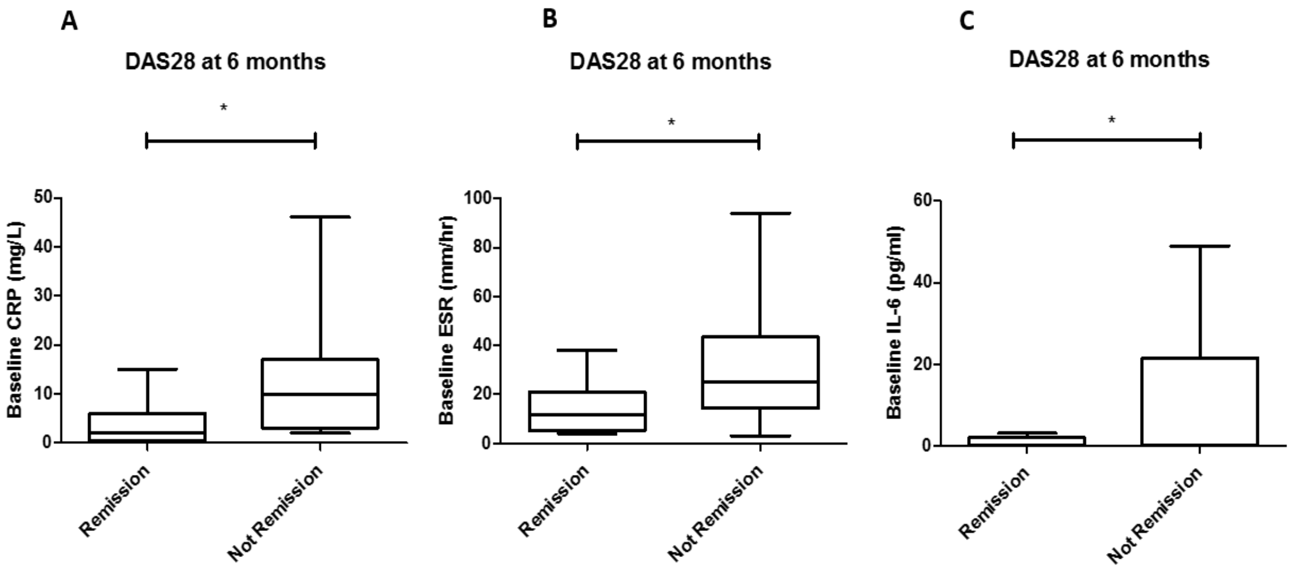

Figure 81. Significantly lower baseline CRP, ESR and IL-6 in patients with early RA in remission at 6 months compared to those not in remission at 6 months. (A) Mean baseline CRP in remission was 3.8 ± 1.2 mg/L versus 12.1 ± 2.6 mg/L. (B) Mean baseline ESR in remission was 14.5 ± 3 mm/hr versus 30.7 ± 6 mm/hr. (C) Mean IL-6 in remission was 0.9 ± 0.4 pg/ml versus 10.7 ± 3.8 pg/ml, * *p* < 0.05.

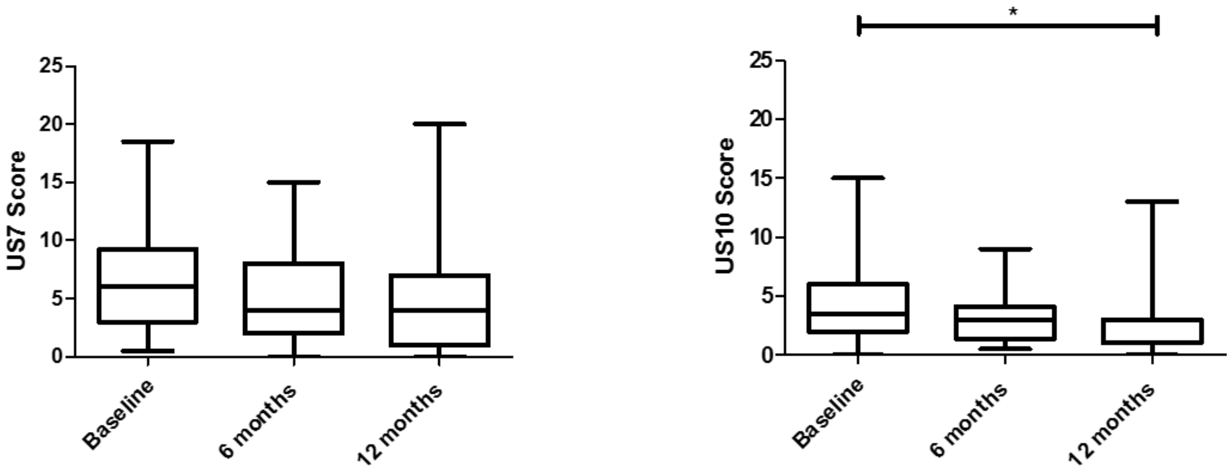

Figure 82. Ultrasound scores over time in patients with early RA. (A) No significant change in US7 score over time. (B) Significant reduction in US10 score at 12 months (2.3 ± 0.7) compared to baseline (4.4 ± 0.5), *p* < 0.05

Variable	Baseline n = 44	6 months n = 35	12 months n = 23	P
DAS28	3.87 ± 0.20	3.02 ± 0.22	2.75 ± 0.39	0.0016
US7 Score	6.7 ± 0.6	5.1 ± 0.7	5.5 ± 1.2	ns
US10 Score	4.4 ± 0.5	3.2 ± 0.4	2.3 ± 0.7	0.03

Table 44. Mean DAS28 and US 7 and US 10 scores at baseline, 6 and 12 months in patients with early RA.

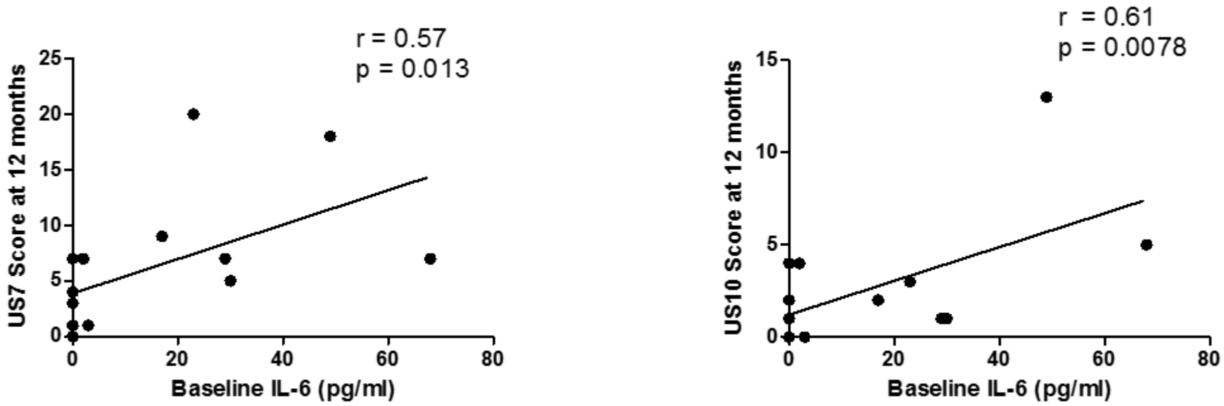

Figure 83. Significant positive correlation between baseline IL-6 level and US7 (r = 0.57, *p* < 0.05) and US10 score at 12 months (r = 0.61, *p* < 0.01) in patients with early RA.

5.4.2.3 Baseline CV risk scores and correlations with CIMT

A summary of the CV risk scores and carotid ultrasound findings of patients at baseline is shown in Table 45. Table 46 summarises the percentage of EAS patients classified as high risk according to each CV risk algorithm**.** 58% of patients had a QRISK2 greater than 10% and according to NICE guidelines, should be offered a statin. Consequently, these patients and their General Practitioners were informed of this, if not already on a statin. Of these patients with > 10% risk who returned at 6 months, 29% were newly prescribed a statin, 50% were already on a statin and 21% should have been offered at statin but were not prescribed one. Table 47 shows the sensitivity and specificity of the risk scores in carotid US positive patients. The QRISK2 score had the highest sensitivity and the SCORE had the highest specificity.

All risks scores had a significant positive correlation with CIMT (Figure 84). The strongest correlation was with the Framingham risk score.

Variable	Mean
QRISK2	17.2 ± 2.5 %
SCORE	2.1 ± 0.4 %
Framingham	14.5 ± 2.2 %
ACC/AHA	13.8 ± 2.8 %
% patients with CIMT > 0.9mm	21.6
Carotid plaque (% patients with plaque)	29.7
% patients classified as carotid US positive	40.5
% patients classified as carotid US negative	59.5

Table 45. CV risk scores and carotid US characteristics of EAS patients at baseline

Variable	Threshold used to define 'high risk'	Percentage EAS patients classified as high risk (%)
SCORE	> 5%	8
QRISK2	>10%	54
ACC/AHA	>7.5%	43
Framingham	> 20%	35

Table 46. Cardiovascular risk scores, high CV risk definition according to algorithm used and percentage of patients with early RA defined as high risk.

Variable	US positive sensitivity (CI)	US positive specificity
QRISK2 > 10%	87 (60-98%)	68 (45-86%)
Framingham > 20%	53 (27-79%)	77 (55-92%)
SCORE > 5%	27 (8-55%)	95 (77-99.8%)
ACC/AHA > 7.5%	66 (38-88%)	94 (70-99.8%)

Table 47. Sensitivity and specificity of cardiovascular risk scores in predicting carotid US positive patients in patients with early RA.

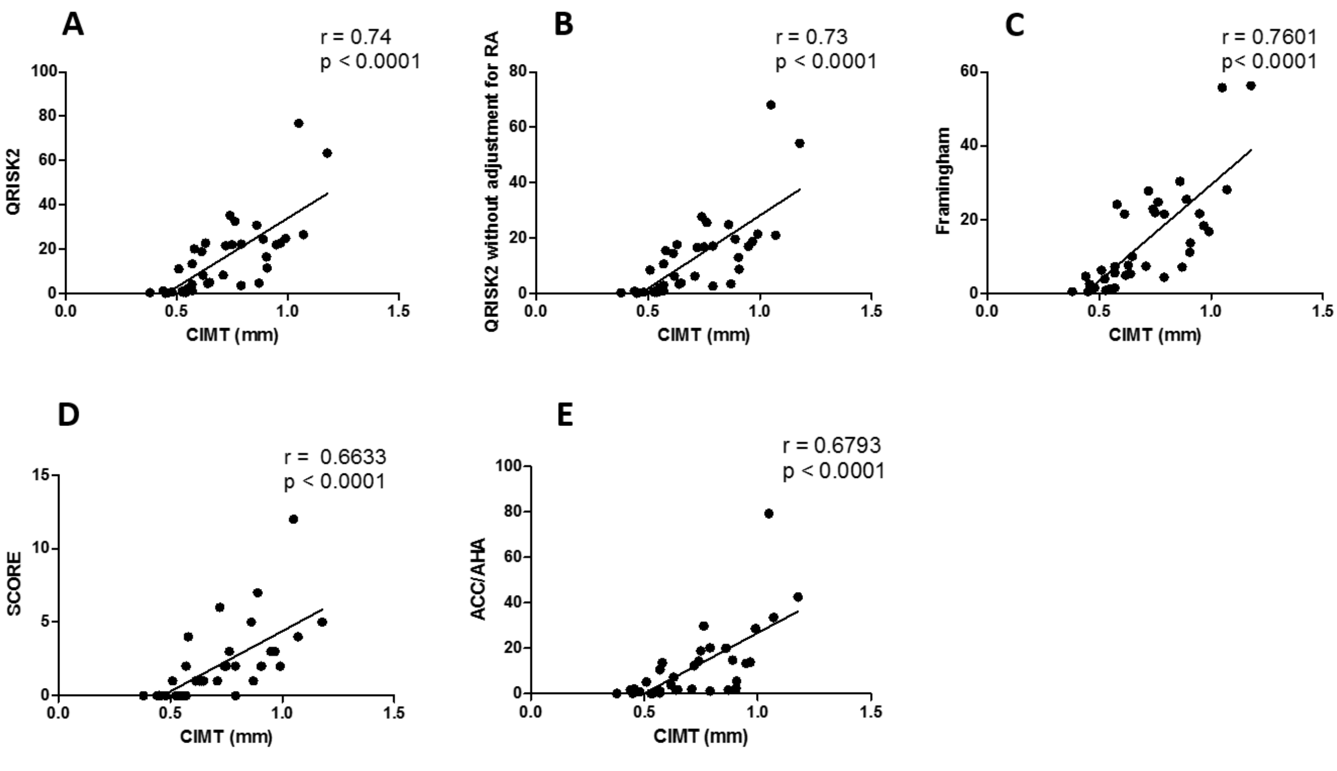

Figure 84. Significant positive correlation between cardiovascular risk scores and CIMT in patients with early RA at baseline. Significant positive correlation between CIMT and (A) QRISK2 r = 0.74, *p* < 0.0001 (B) QRISK2 without adjustment for RA, r = 0.73, *p* < 0.0001 (C) Framingham r = 0.76 *p* < 0.0001, (D) SCORE r = 0.66, *p* < 0.0001 and (E) ACC/AHA, r = 0.68, *p* < 0.0001

5.4.2.4 Association between CIMT and serum lipid levels and HbA1c

There was a significant positive correlation between total cholesterol: HDL ratio (r = 0.44, *p* < 0.01), total cholesterol (r = 0.38, *p* < 0.05) and LDL cholesterol (r = 0.47, *p* < 0.01) and CIMT at baseline. There was no significant correlation between HDL cholesterol and CIMT. (Figure 85). There was a significant positive correlation between CIMT and HbA1c (r = 0.40, p = 0.04), Figure 86.

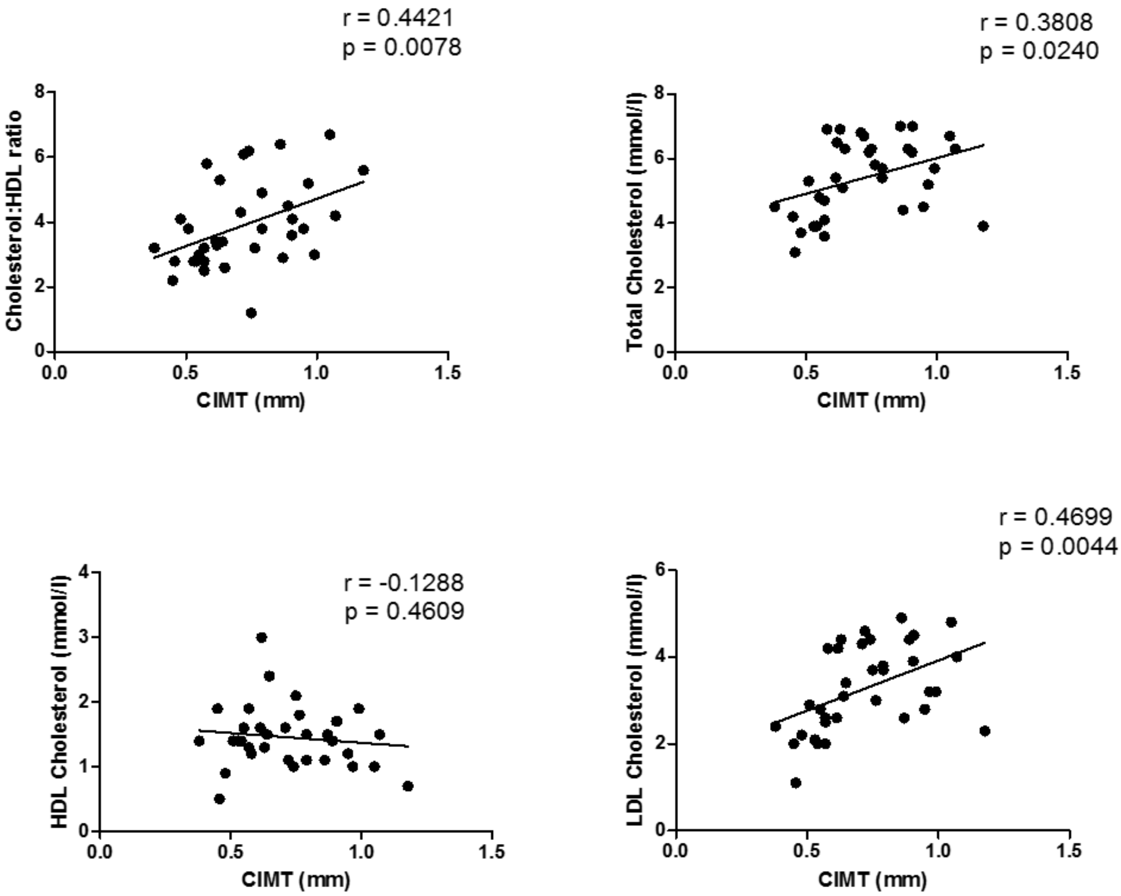

Figure 85. Significant positive correlation between total cholesterol:HDL ratio (r = 0.44, p < 0.01), total cholesterol (r = 0.38, p < 0.05) and LDL cholesterol (r = 0.47, p < 0.01) and CIMT in patients with early RA. No significant correlation between HDL cholesterol and CIMT (r = -0.12, *p* =0.46).

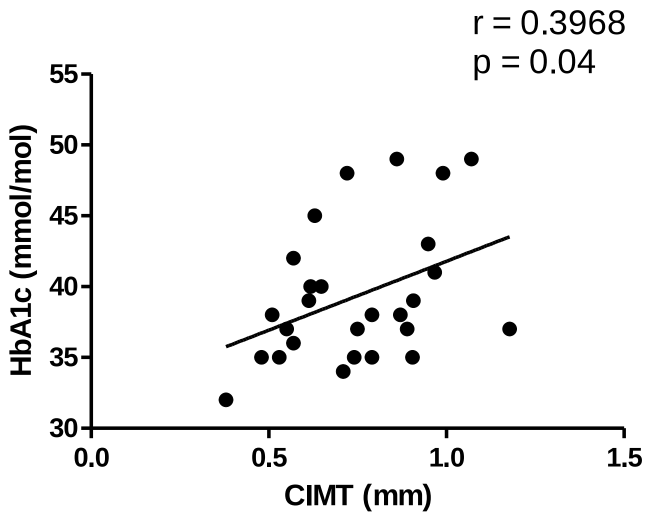

Figure 86. Significant positive correlation between CIMT and HbA1c in patients with early RA, r = 0.397, *p* = 0.04

5.4.2.5 Correlation of cardiovascular risk scores with distensibility coefficient

There was a significant negative correlation between the distensibility coefficient and all cardiovascular risk scores at baseline (Figure 87). There were also significant negative correlations between the distensibility coefficient and CIMT, total cholesterol and LDL cholesterol at baseline (Figure 88) but no significant correlation between distensibility coefficient and CRP, ESR, SCCPs or DAS28.

There was a significant negative correlation between baseline CXCL9 and distensibility coefficient at 12 months (Figure 89).

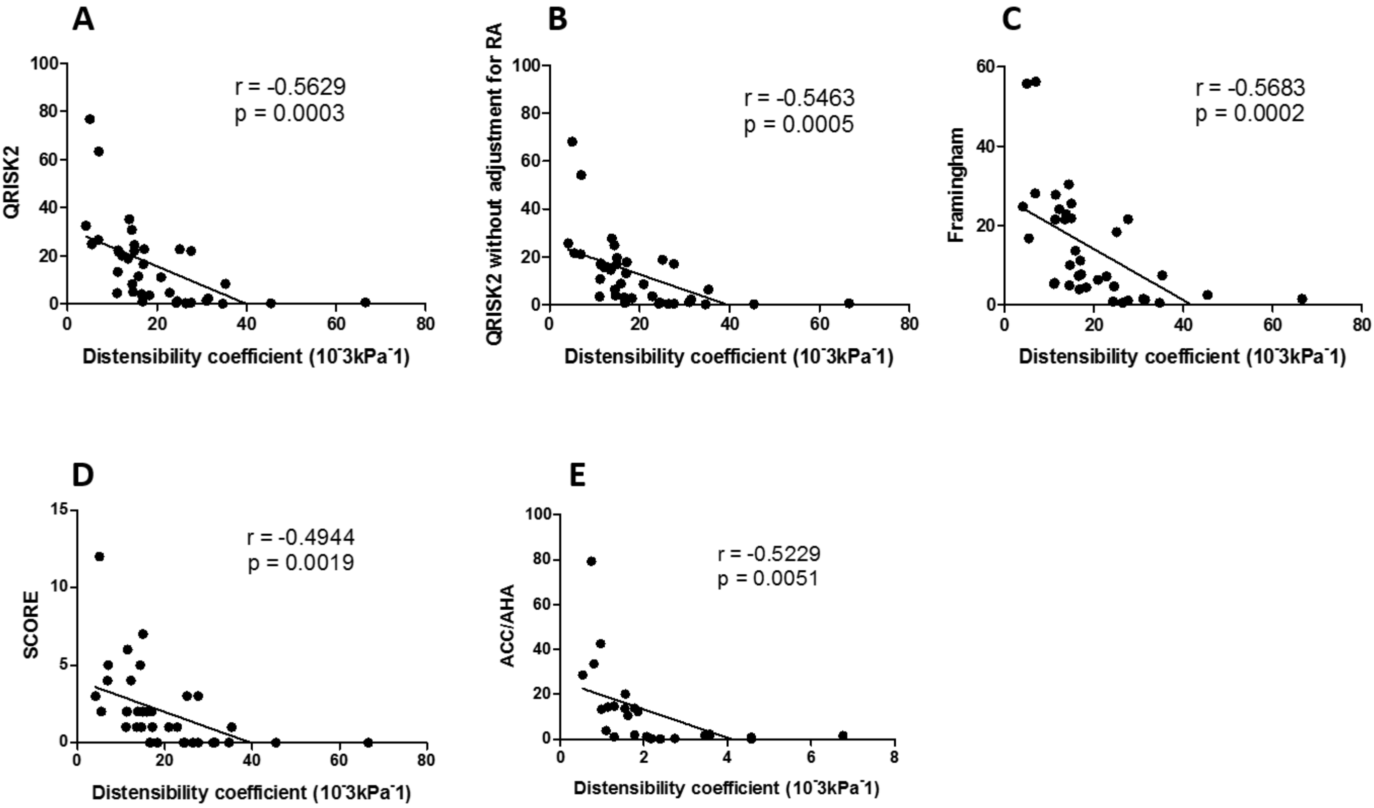

Figure 87. Significant negative correlations between distensibility coefficient and cardiovascular risk scores in patients with early RA. Significant negative correlation between distensibility coefficient and (A) QRISK2 r =-0.56, *p* < 0.0003, (B) QRISK2 without adjustment for RA, r = -0.55 p = 0.0005, (C) Framingham r = -0.59, *p* = 0.0002, (D) SCORE r= -0.49, *p* = 0.0019 and (E) ACC/AHA r= -0.52 *p* = 0.0051.

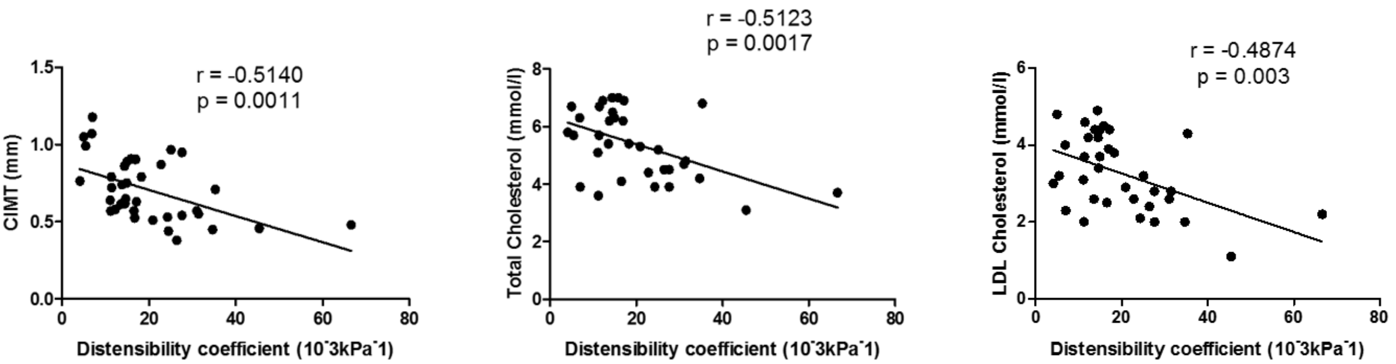

Figure 88. Significant negative correlations between distensibility coefficient and CIMT (r = -0.51, *p* = 0.0011), Total cholesterol (r = -0.51, *p* = 0.0017) and LDL cholesterol (r = -0.49, *p* = 0.003) in patients with early RA.

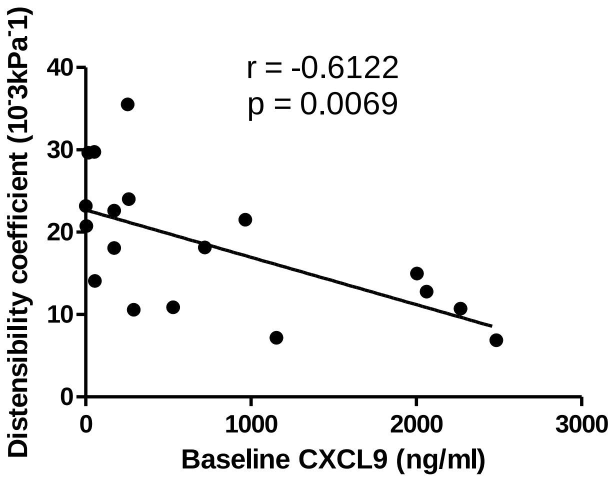

Figure 89. Significant negative correlation between baseline CXCL9 and distensibility coefficient at 12 months (r = -0.61, p = 0.0069).

5.4.2.6 Differences in traditional risk factors and SCCPs in carotid US positive and negative patients

41% of patients were carotid US positive. These patients had significantly higher age, BMI, systolic BP, total cholesterol, cholesterol: HDL ratio, LDL cholesterol, QRISK2, Framingham, SCORE, ACCAHA, thrombomodulin and CXCL9 than US negative patients (Table 48). Multivariate logistic regression analysis including age, BMI, systolic BP, total cholesterol, cholesterol:HDL ratio, LDL cholesterol, ESR and CXCL9 as covariates found that total cholesterol, LDL cholesterol, age, BMI, systolic BP, thrombomodulin and CXCL9 were significant independent variables.

Baseline variable	US negative n = 22	US positive n = 15	P value
Age (years)	49 ± 3	64 ± 2	**0.000**
BMI	24.6 ± 0.8	30.8 ± 1.8	**0.005**
Systolic BP (mm	129 ± 3	143 ± 5	**0.030**
Total Cholesterol (mmol/L)	5.0 ± 0.3	5.9 ± 0.2	**0.011**
Cholesterol: HDL ratio	3.5 ± 0.3	4.4 ± 0.3	**0.048**
LDL cholesterol (mmol/L)	2.9 ± 0.2	3.8 ± 0.2	**0.002**
HDL cholesterol (mmol/L)	1.5 ± 0.1	1.4 ± 0.1	0.4080
Triglycerides (mmol/L)	1.4 ± 0.2	1.6 ± 0.1	0.419
Arterial distention coefficient	23 ± 3	16 ± 2	0.071

HbA1c	39.2 + 1	39.5 + 1.3	0.857
QRISK2	8.3 + 2	26.9 + 5	**0.003**
Framingham	8.8 + 2	22.2 + 4	**0.007**
SCORE	1.2 + 0.4	3.2 + 0.8	**0.028**
ACC/AHA	5.4 + 1.8	19.9 + 5.3	**0.018**
Disease duration (months)	4.1 + 0.2	3.9 + 0.3	0.645
DAS28	3.8 + 0.3	4.0 + 0.3	0.663
CRP (mg/L)	8 + 2	14 + 4	0.212
ESR (mm/hr)	19 + 3	32 + 7	0.086
IL-6 (pg/ml)	6.8 + 4	11.7 + 15	0.432
sIL-6R (ng/ml)	86 + 9	105 + 14	0.299
IL-6/sIL-6R complex (pg/ml)	280 + 99	371 + 174	0.653
CCL2 (pg/ml)	201 + 18	219 + 56	0.765
CXCL8 (pg/ml)	5.2 + 5.2	1.4 + 1.4	0.494
CXCL9 (ng/ml)	20 + 129	1122 + 284	**0.043**
MCSF (pg/ml)	31.2 + 31	8.4 + 8.4	0.494
MMP-1 (ng/ml)	12 + 2	11 + 4	0.810
Thrombomodulin (ng/ml)	4.2 + 0.1	4.7 + 0.2	**0.017**
Tissue Factor (pg/ml)	80 + 60	29 + 26	0.449
VCAM-1 (ng/ml)	657 + 95	834 + 93	0.193

Table 48. Difference in baseline variables and SCCPs in carotid US positive and US negative patients.

5.4.2.7 No significant difference in CIMT at 12 months in patients with early RA

There was no significant difference in CIMT at 12 months (0.706 + 0.048 mm) compared to 6 months (0.729 + 0.041 mm) or baseline (0.708 + 0.033 mm) (Figure 90). Table 49 summarises the change in variables at baseline, 6 and 12 months.

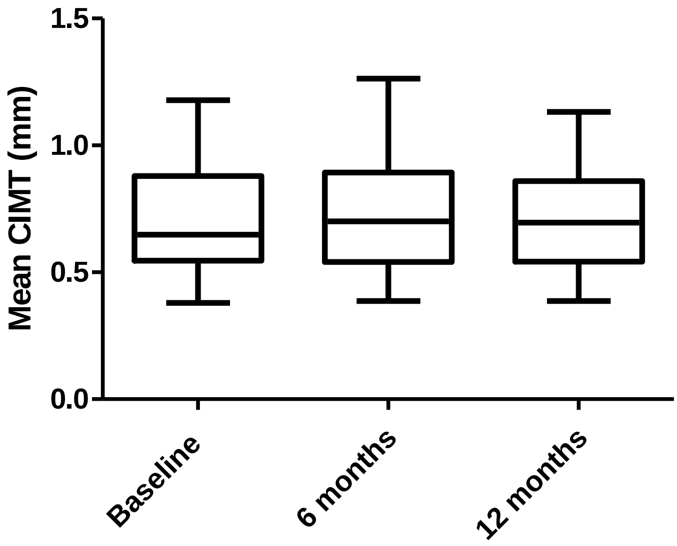

Figure 90. No significant difference in mean CIMT at baseline (0.708 ± 0.033 mm), 6 months (0.729 ± 0.041 mm) and 12 months (0.706 ± 0.048 mm) in patients with early RA.

Variable	Baseline n = 44	6 months n = 35	12 months n = 18
CIMT (mm)	0.708 ± 0.033	0.729 ± 0.041	0.706 ± 0.048
Systolic BP (mmHg)	136 ± 3.2	132 ± 3.8	132 ± 4
QRISK2 (%)	16.9 ± 2.5	14.1 ± 3.1	12.4 ± 2.8
Distensibility coefficient (10^{-3} kPa^{-1})	19.8 ± 2.0	22.3 ± 2.6	18.3 ± 1.7
Percentage taking statin	12 %	24 %	19 %
Cholesterol: HDL ratio	3.8 ± 0.2	4.0 ± 0.3	3.7 ± 0.3
BMI	27.3 ± 0.9	27.1 ± 1.3	27.0 ± 1.5

Table 49. Summary of characteristics at baseline, 6 and 12 months in patients with early RA.

5.4.2.8 Baseline disease activity correlates with change in CIMT at 6 months in patients with early RA

There were significant positive correlations between baseline DAS28, baseline CRP, baseline ESR and baseline QRISK2 and change in CIMT at 6 months (Figure 91). There was a significant difference in change in CIMT at 6 months (but not 12 months) in those in different IL-6 and sIL-6R groups (Figure 92), with the largest increase in those who had high IL-6 but low sIL-6R.

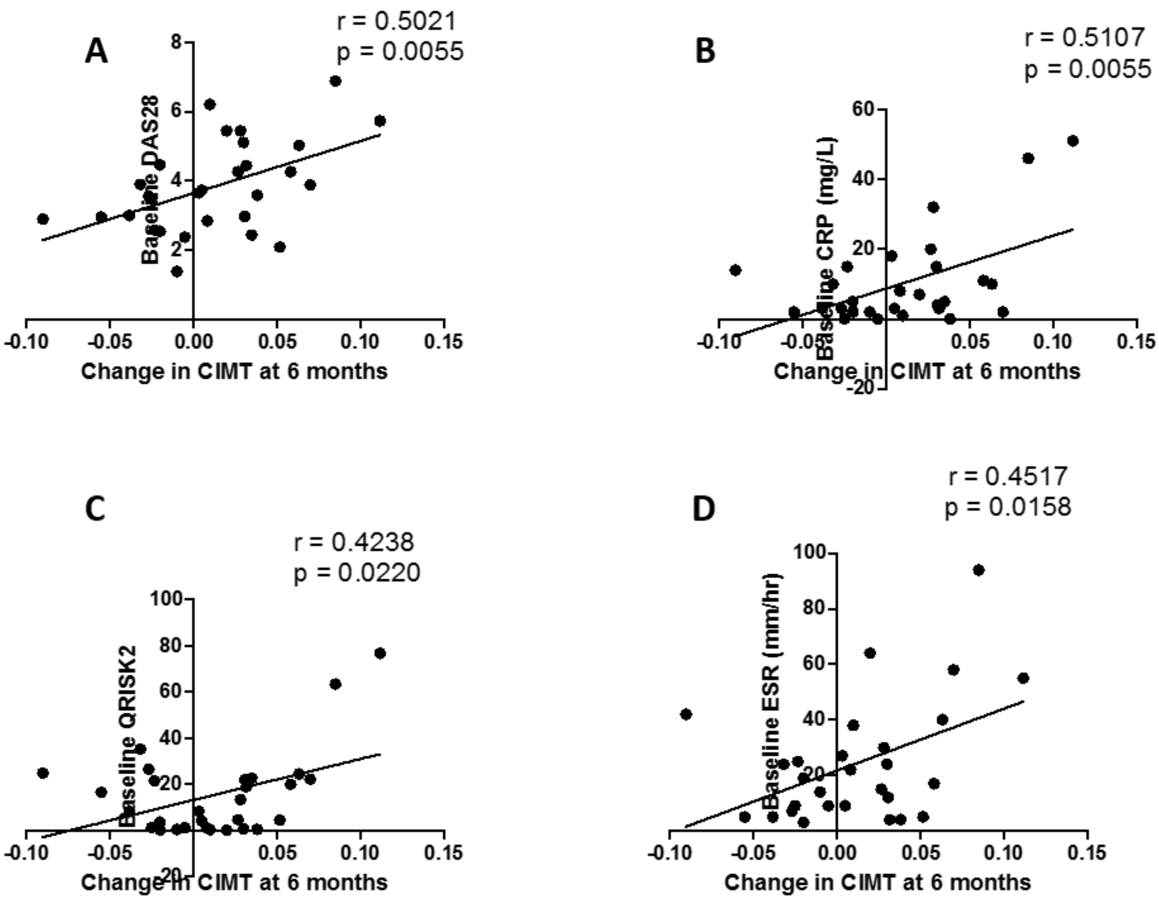

Figure 91. Significant positive correlation between change in CIMT at 6 months and (A) Baseline DAS28 r = 0.50, *p* = 0.006, (B) Baseline CRP r = 0.51, *p* = 0.006, (C) Baseline QRISK2 r = 0.42, *p* = 0.02, (D) Baseline ESR r = 0.45, *p* = 0.016.

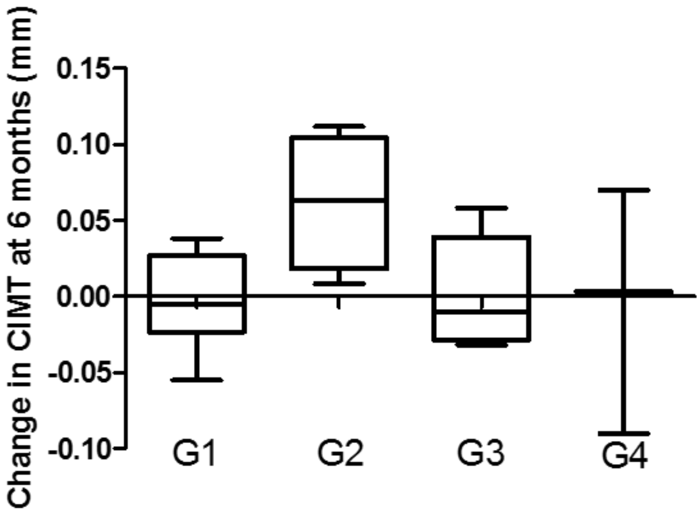

Figure 92. Significant difference in change in CIMT at 6 months in patients with early RA in different IL-6/sIL-6R groups. G1 (IL-6 low and sIL-6R low), G2 (IL-6 high and sIL-6R low), G3 (IL-6 low and sIL-6R high), G4 (IL-6 high and sIL-6R high). Largest increase in CIMT at 6 months in patients with a high IL-6 and low sIL-6R at baseline

5.4.2.9 Rapid progressors at 12 months

Rapid progressors were defined as those who had an increase > 0.05mm in CIMT. 3 of 18 patients (17%) were classified as rapid progressors at 12 months. There was no significant difference in baseline BP, HDL cholesterol, triglycerides, CIMT, Age, BMI, disease duration, DAS28, CRP, IL-6, sIL-6R, IL-6/sIL-6R complex, CCL-2, CXCL8, CXCL9, Tissue factor, MCSF, Thrombomodulin and MMP-1 in rapid versus non-rapid progressors at 12 months (Table 50). Baseline total cholesterol, cholesterol**:**HDL ratio, LDL cholesterol, HbA1C and VCAM-1 were significantly higher in rapid progressors compared to non-rapid progressors (Figure 93). Baseline QRISK2, Framingham and SCORE were significantly higher in rapid progressors but ACC/AHA was not. Arterial distension coefficient was significantly lower in rapid progressors versus non-rapid progressors (Figure 93E). There was no significant difference in change in CIMT or change in distensibility coefficient in smokers at baseline versus non-smokers at baseline. Multivariate logistic regression analysis including baseline cholesterol, cholesterol:HDL ratio, age, VCAM-1 and HbA1c as covariates found that total cholesterol,, triglycerides and HbA1c were significant independent factor while VCAM-1 just failed to reach statistical significance at *p*=0.055.

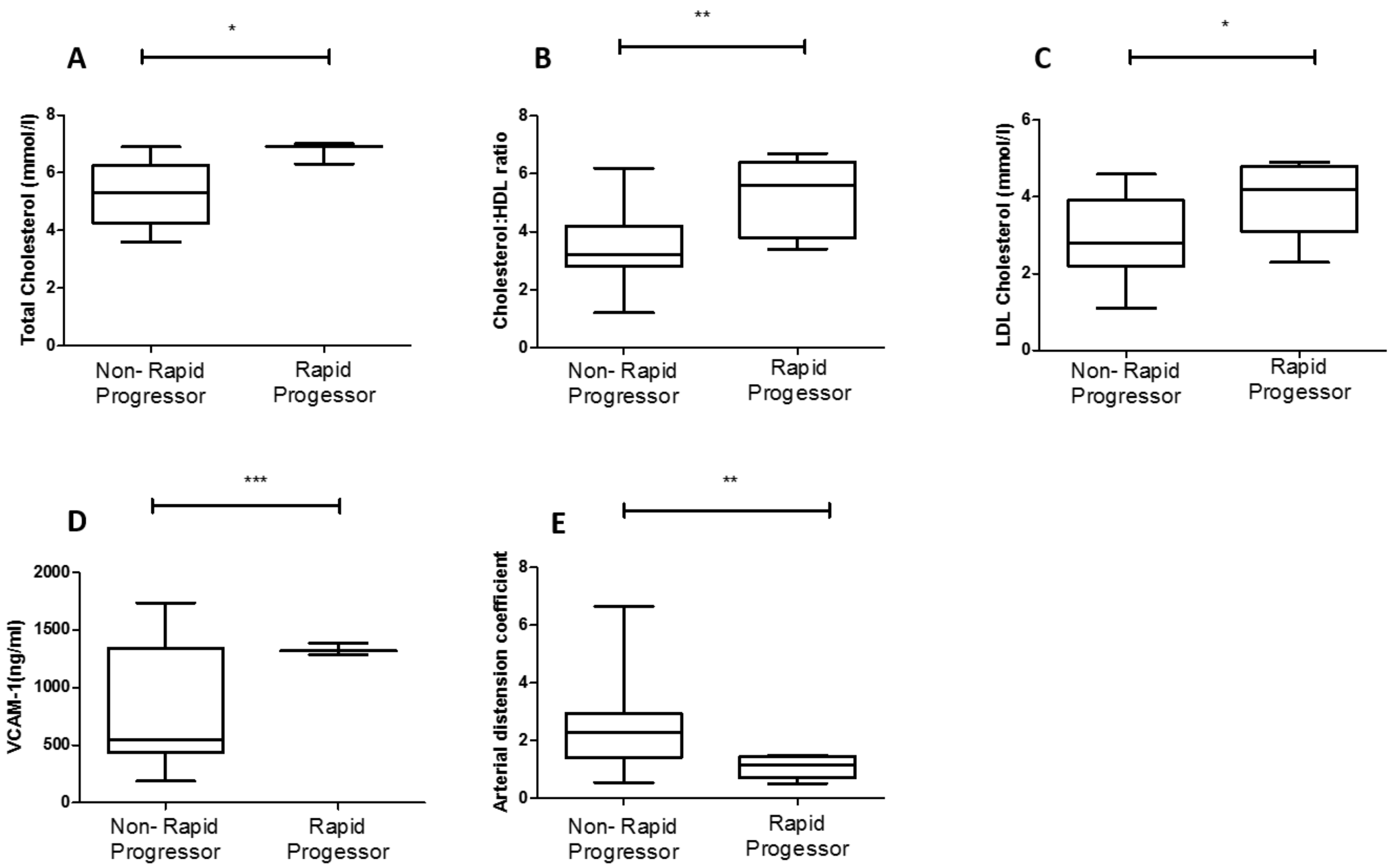

Figure 93. Significantly higher baseline (A) Total cholesterol (6.7 + 0.2 versus 5.2 + 0.3 mmol/L) , (B) Cholesterol: HDL ratio (3.7 + 0.3 versus 5.5 + 0.7), (C) LDL cholesterol 3.1 + 0.3 mmol/L versus 4.4 + 0.3 mmol/L) (D) VCAM-1 (734 + 126 ng/ml versus 1328 + 28 ng/ml) in rapid progressors compared to non-rapid progressors. (E) Significantly lower arterial distension coefficient in rapid progressors (18.8 + 1.8) compared to non-rapid progressors (11.2 + 2.2). *p < 0.05, ** p < 0.01, *** p < 0.001

Baseline value	**Non-rapid Progressor (n= 15)**	**Rapid Progressor (n = 3)**	**P value**
CRP (mg/L)	9.5 + 2.5	5 + 3.1	0.30
ESR (mm/hr)	22.4 + 3.8	11 + 3.0	0.2
Systolic BP (mm	127 + 4	148 + 10	0.176
Total Cholesterol (mmol/L)	5.2 + 0.3	6.7 + 0.2	**0.002**
Cholesterol: HDL ratio	3.7 + 0.3	5.5 + 0.7	**0.004**
LDL cholesterol (mmol/L)	3.1 + 0.3	4.4 + 0.3	**0.018**
HDL cholesterol (mmol/L)	1.5 + 0.1	1.3 + 0.1	0.19
Triglycerides (mmol/L)	1.3 + 0.2	2.4 + 0.4	0.08
Arterial distention coefficient	18.8 + 1.8	11.2 + 2.2	**0.04**

HbA1c (mmol/mol)	38.7 ± 1.4	47.7 ± 1.3	**0.002**
Baseline CIMT (mm)	0.64 ± 0.05	0.84 ± 0.14	0.30
Age (years)	52.9 ± 3.5	67.3 ± 4.0	**0.038**
BMI	27.4 ± 2.0	27.4 ± 2.3	0.995
Disease duration (months)	4.0 ± 0.3	4.5 ± 0.3	0.26
DAS28	3.7 ± 0.3	3.2 ± 0.8	0.56
QRISK2 (%)	10.5 ± 2.9	25.8 ± 3.1	**0.01**
Framingham (%)	8 ± 2	20 ± 3	**0.00**
SCORE (%)	1.1 ± 0.5	4.3 ± 0.6	**0.00**
ACC/AHA (%)	6.5 ± 2.2	22.4 ± 2.9	0.10
IL-6 (pg/ml)	9 ± 4	23 ± 23	0.61
SIL-6R (ng/ml)	96 ± 8	120 ± 37	0.59
IL-6/sIL-6R complex (pg/ml)	383 ± 172	167 ± 167	0.39
VCAM-1 (ng/ml)	734 ± 126	1328 ± 28	**0.000**
CCL2 (pg/ml)	181 ± 25	197 ± 80	0.78
CXCL8 (pg/ml)	1.55 ± 1.55	0 ± 0	0.35
CXCL9 (ng/ml)	843 ± 287	658 ± 258	0.65
Tissue factor (pg/ml)	37 ± 28	221 ± 383	0.49
MCSF (pg/ml)	9.3 ± 9.3	0 ± 0	0.35
Thrombomodulin (ng/ml)	4.2 ± 0.2	4.4 ± 0.4	0.72
MMP-1 (ng/ml)	12.5 ± 2.5	14.1 ± 5.1	0.78

Table 50. Baseline variables in rapid and non-rapid progressors in early RA patients

5.4.2.10 Disease activity and CIMT in seropositive versus seronegative patients

There was no significant difference in DAS28 at 12 months in either RF or anti-CCP positive or negative patients. There was no significant difference in CIMT at 0, 6 and 12 months, or change in CIMT at 12 months in seropositive and seronegative patients, Table 51.

	RF negative	RF positive	p value	Anti-CCP negative	Anti-CCP positive	p value
DAS28 0 months	3.7 ± 0.3	4.0 ± 0.3	0.5	3.8 ± 0.5	3.9 ± 0.2	0.8
DAS28 6 months	3.2 ± 0.4	2.9 ± 0.3	0.7	2.7 ± 0.3	3.1 ± 0.3	0.4
DAS28 12 months	2.7 ± 0.6	2.7 ± 0.47	0.9	3.1 ± 0.8	2.7 ± 0.45	0.7
Change in DAS28 at 12 months	-0.87 ± 0.5	- 0.93 ± 0.5	0.9	-0.55 ± 0.94	- 0.99 ± 0.42	0.4
CIMT 0 months (mm)	0.77 + 0.04	0.66 + 0.05	0.1	0.822 + 0.07	0.67 + 0.19	0.07
CIMT 6 months (mm)	0.81 + 0.05	0.66 +0.05	0.06	0.87 + 0.10	0.69 + 0.04	0.17
CIMT 12 months (mm)	0.76 +0.07	0.66 + 0.07	0.3	0.73 + 0.14	0.70 + 0.05	0.84
Change in CIMT at 12 months (mm)	0.03 + 0.02	0.01 + 0.01	0.4	0.03 + 0.02	0.02 + 0.01	0.80

Table 51. Effect of RF and anti-CCP status on change in DAS28 and CIMT over time

5.4.2.11 Effect of medication at baseline on change in DAS28 and CIMT

Mean change in DAS28 at 12 months (but not 6 months) was greater in those taking methotrexate at baseline (-1.52 ± 0.40) compared to those not taking methotrexate at baseline (0.31 ± 0.63), *p* = 0.03 (Figure 94A). There was no significant difference in change in CIMT at 12 months in those taking methotrexate at baseline versus those not taking methotrexate at baseline. Patients taking corticosteroids at baseline had a significantly greater change in DAS28 at 12 months (-2.2 $\pm$ 0.7) (but not at 6 months) compared to those not taking corticosteroids at baseline (-0.39 $\pm$ 0.4), p = 0.047 (Figure 94B). There was no difference in change in CIMT at 12 months in those taking or not taking corticosteroids at baseline. There was no difference in change in CIMT or distensibility coefficient in those taking NSAIDS at baseline versus those not taking NSAIDS at baseline.

At 6 months 1 patient was prescribed tocilizumab. There were no patients taking other biologic drugs e.g. anti-TNF. At 12 months 2 patients were prescribed tocilizumab. Those on tocilizumab at 12 months had a greater reduction in DAS28 at 12 months (-2.62 $\pm$ 0.3) compared to those not taking tocilizumab (-0.80 $\pm$ 0.4), p = 0.010. Those taking tocilizumab had an increase in cholesterol/HDL ratio at 12 months (0.6 $\pm$ 0.1) compared to those not taking tocilizumab (-0.24 $\pm$ 0.4), p = 0.045. There was no difference in change in CIMT in these patients.

There was no significant difference in DAS28 or CIMT at 12 months or change in these variables in those taking or not taking statin at baseline. At 6 months, 4 patients were prescribed a statin that was ‘new’, i.e. statin introduced since baseline. As expected, these patients had a greater reduction in cholesterol: HDL ratio at 6 months than those who were not newly prescribed a statin (Figure 95). There was no significant difference in disease activity or change in CIMT in those newly prescribed a statin. At 12 months, a further 2 patients were newly prescribed a statin compared to baseline. There was no significant difference in change in cholesterol: HDL ratio, CIMT or DAS28 at 12 months compared to baseline in those newly prescribed a statin compared to those not newly prescribed a statin.

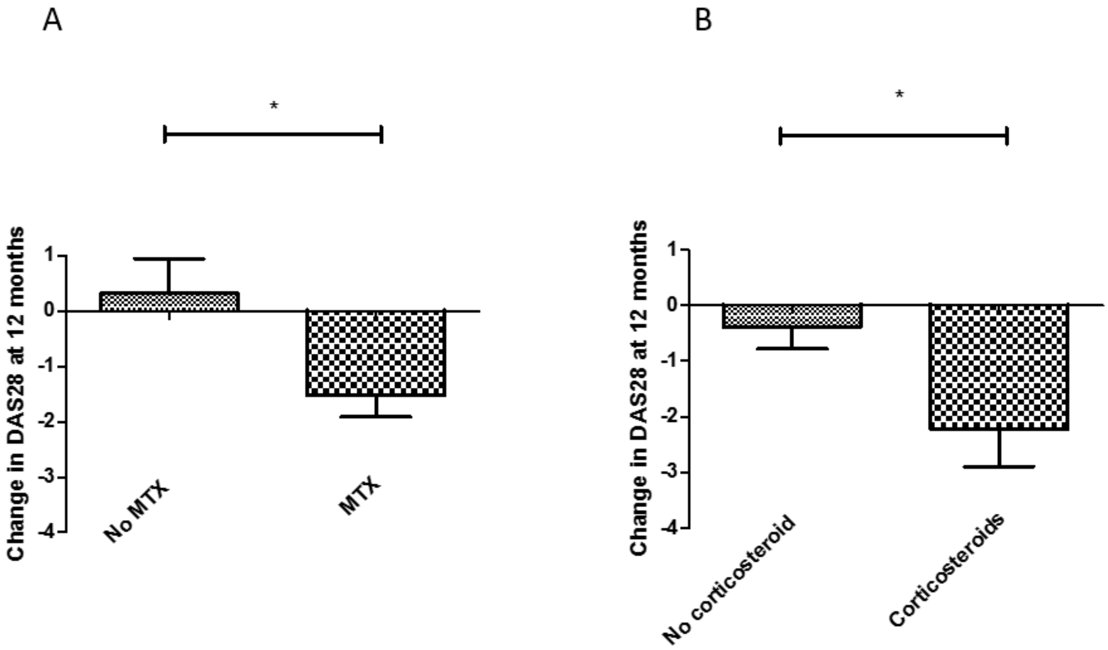

Figure 94. (A) Patients with early RA taking methotrexate at baseline had a significantly greater change in DAS28 at 12 months (-1.52 + 0.4) compared to those not taking methotrexate at baseline (0.31 + 0.63), *p* = 0.03. (B) Patients taking corticosteroids at baseline had a significantly greater change in DAS28 at 12 months (-2.2 + 0.7) compared to those not taking corticosteroids at baseline (-0.39 + 0.4), p = 0.047

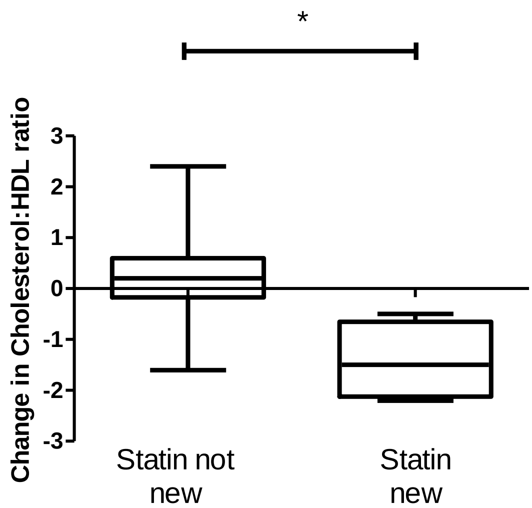

Figure 95. Significantly greater reduction in mean cholesterol: HDL ratio at 6 months in those prescribed a new statin at 6 months (-1.4 + 0.4) compared to those not newly prescribed a statin (0.2 + 0.2) in patients with early RA * *p* < 0.05.

5.5 Discussion

5.5.1 Established RA study

The patient cohort was similar to those seen in the general population in the UK, in terms of age, gender and levels of inflammatory markers (Humphreys et al 2012). There was a significant but weak positive correlation between serum VCAM-1 and DAS28. This supports results from Chapter 3; there was a positive correlation between arthritis severity in mice and serum VCAM-1. Other studies in RA patients have shown correlations between serum VCAM-1 and ESR, CRP, number of swollen joints (Klimiuk et al 2002), and DAS28 (Navarro-Hernández et al 2009). As expected, ESR was highest in those with severe RA according to the DAS28. Those with severe RA also had higher VCAM-1 levels.

There were significant positive correlations between the QRISK2 and serum MMP-1, thrombomodulin and VCAM-1. The Framingham risk score correlated positively with MMP-1 and thrombomodulin. In the general population, elevated MMP-1 was found to predict the presence of advanced CVD (Hwang et al 2009). However, thrombomodulin was shown to be reduced in CVD (Wei et al 2011) and its expression was reduced in atherosclerotic lesions compared to control arteries (Lasik et al 2001). In this thesis thrombomodulin (and VCAM-1 and MMP-1) correlated significantly but weakly with age, which may account partly for the discrepancy between my data and the literature on thrombomodulin.

Over 50% of patients in this cohort were classified as high risk of having a CV event according to the QRISK2. This observation is supported by published literature that show RA patients have increased atherosclerosis in early RA (Hannawi et al 2007). Interestingly, in this thesis, those classified as low CV risk had a significantly higher CRP than those at high risk. This contradicts epidemiological studies which report that circulating CRP is associated with higher risk of CVD in the general population (Ridker and Cook 2004) and in RA (Graf et al 2009). However, in this thesis, of those high risk patients, 34% were on a statin, while only 9% of low risk patients were taking a statin. It is known that statins reduce the CRP (Zakynthinos and Pappa 2009), which may partly account for the difference seen here. Patients with a low CV risk had significantly lower serum thrombomodulin. Studies of serum thrombomodulin in CV disease are conflicting: some found that soluble thrombomodulin showed a strong, inverse association with incident coronary heart disease (Salomaa et al 1999) and others that raised soluble thrombomodulin was predictive of the progression of atherosclerosis in patients with ischaemic heart disease. Another study reported that thrombomodulin expression reduced in atherosclerotic lesions (Lasik et al 2001). The role of thrombomodulin in predicting risk of CVD in RA or the general population is unclear in the light of such conflicting data.

Patients classified as high CV risk had significantly higher VCAM-1 than those classified as low CV risk. A previous study found that VCAM-1 was higher in those who go on to develop CV events in the general population (Schmidt et al 2008). However, several other studies of the general population found VCAM-1 did not provide significant improvement in CVD risk assessment beyond conventional CVD risk factors (Kunutsor et al 2017) and was not significantly different in apparently healthy individuals that went on to develop CVD compared to those that did not (Luc et al 2003). However, these studies were in a non-RA population. It may be that in RA patients, atherosclerosis pathogenesis differs slightly to the general population. For example, previous studies have shown that RA patients have more inflammation in both their aorta (Greenberg et al 2012) and in atherosclerotic plaques (Aubry et al 2007) compared with non-RA controls.

In those patients with established RA and a history of CV disease serum CRP, ESR and sIL-6R, IL-6/sIL-6R complex, MCSF and Tissue factor were significantly lower than those with no history of CVD. There was no difference in VCAM-1 between these groups. These findings were unexpected because inflammation is a factor acknowledged for increasing CV risk. However, as these patients were known to have a history of CV disease most were on secondary prevention for CVD; 92% were taking a statin and 80% were taking an antihypertensive. Those taking a statin had a lower CRP. Other SCCPs were unchanged. It may also be the case that patients with a prior history of CVD are treated more aggressively by their rheumatologists for RA, due to the increasingly well-recognised association of inflammation and CVD.

There was no difference in SCCPs, IL-6, sIL-6R, IL-6/sIL-6R complex, CRP or ESR in those taking or not taking methotrexate. Serum IL-6 was higher in those prescribed Tocilizumab. This has been shown in previous studies (Nishimoto et al 2008). It is thought that this increase in serum IL-6 is due to the binding of Tocilizumab to sIL-6R; therefore reducing the availability sIL-6R for IL-6 to bind to**.** Thus, more unbound IL-6 is detectable in the serum (Nishimoto et al 2008). Interestingly, CRP was not lower in those taking Tocilizumab. This is at odds with the literature whereby CRP is significantly reduced by Tocilizumab (Genovese et al 2008). However, in order to meet the criteria for Tocilizumab treatment, patients had by definition severe disease activity, and therefore likely higher CRP to begin with.

SIL-6R and MMP-1 were significantly higher in those that were rheumatoid factor positive compared to those that were negative. A previous study did not show an association with sIL-6R level and rheumatoid factor positivity (Nishina et al 2013). It could be hypothesised that IL-6 and sIL-6R levels are higher in rheumatoid factor positive patients as IL-6 stimulates B cells to differentiate into plasma cells to produce immunoglobulins (Hirano et al 1988). There was no significant difference between other SCCPs. Those patients who were anti-CCP positive had significantly lower CRP than those who were negative. There was no difference in other SCCPS.

5.5.2 Early arthritis study

75% of those recruited were female, in line with female prevalence of RA in the general population (Humphreys et al 2012). Mean disease duration at the baseline visit, defined as the time from which the patient first developed symptoms of RA, was 4 months. However, there was a variable time between first diagnosis with RA and time recruited to the study. Therefore some patients were seen before DMARD therapy was initiated, others after. This could have had a bearing on level of inflammation and progression of subclinical atherosclerosis before the patient was assessed. 52% of patients were followed for 12 months. Those who were lost to follow up included those who were uncontactable, had moved away, or did not reach the 12 month visit by the end of the study. Recruitment was lower than hoped; a large factor in this was that many patients were not eligible for recruitment as they had had symptoms of RA for greater than 6 months.

Current UK NICE guidelines for RA state that patients should be offered a combination of DMARDs including methotrexate and short term glucocorticoids as first line treatment. This is the current practice in the early arthritis clinic in UHW. However, due to patient co-morbidities and patient choice this was not always possible. Only 9% of patients at baseline were taking this combination therapy but information about what was offered to patients in clinic was missing. Most patients at baseline were taking methotrexate.

There was no significant correlation with baseline DAS28 and IL-6, sIL-6R or SCCPs. In the cross sectional study there was a significant positive correlation between DAS28 and VCAM-1, perhaps reflecting greater patient numbers. There were also no significant correlations with baseline ultrasound scores and baseline SCCPs. There was a significant correlation between the DAS28 and US7 score but not US10 score. Interestingly, the US7 joint score has less joints in common with the DAS28 (5 joints in common) than the US10 score, which has 8 joints in common. However, the US7 score includes more variables; tenosynovitis, paratenonitis, erosions, synovial thickening and vascularity, whereas the US10 score includes synovial vascularity and thickening only. Perhaps these extra markers included in the US7 reflect more closely the RA disease process, which may be reflected by the ESR and the patient VAS, both of which form part of the DAS28. Of note, the original US10 score from Larché et al (2010) also includes a quantitative measure of vascularity which we did not include in this study as the software was not available.

Disease activity significantly reduced at month 6 and 12. This was to be expected given treatment with DMARDs. At baseline there was no significant difference in CRP or IL-6 in those in DAS28 remission versus those not in remission. Those in DAS28 remission had significantly lower ESR, to be expected given that the ESR forms part of the DAS28. Interestingly, at 6 months those in DAS28 remission had significantly lower baseline CRP, ESR and IL-6. This may simply reflect the increased numbers of those in remission at 6 months compared to baseline (14 patients in remission at 6 months versus 9 at baseline). In addition, baseline IL-6 correlated significantly with US7 and US10 score at 12 months. These observations suggest the importance of inflammation at baseline in propagating the inflammatory response.

There was no difference in CRP, ESR, IL-6 or SCCPs at baseline in those in remission at 12 months versus those not in remission. Baseline ESR, CRP and SCCPs were not significantly different in patients taking methotrexate versus those not taking methotrexate at baseline. CXCL9 at baseline was lower in those taking corticosteroids, other SCCPs, ESR and CRP were not different. In a study of patients with Adult Onset Stills disease, CXCL-9 fell significantly after treatment with corticosteroids (Han et al 2017). It may be expected that those taking corticosteroids would have lower CRP and ESR. However, those patients prescribed corticosteroids by a physician were likely to have had higher disease activity.

54% patients had a greater than 10% risk of CV event over 10 years. This was similar to the rate found in the cross sectional study. These patients were advised to see their GP and a letter sent to the GP explaining this. Of these, only half were then subsequently started on a statin. Although no formal questionnaire was used to assess patients' understanding of why no statin was prescribed, some stated that they discussed it with their GP and did not feel it was necessary, some did not visit their GP and some were not sure. The NICE guidelines were updated in 2014 to reduce the threshold for statin consideration from QRISK2 > 20% risk of CVD over 10 years, to > 10% risk CVD over 10 years. Some GPs may not have been aware of the change in guidelines.

CIMT > 0.90mm or the presence of plaques are predictors of CV events in RA as well as the general population (Gonzalez-Juanatey et al 2009). This definition was used to classify patients as carotid US positive in this study. A significant proportion (41%) of patients in this study were carotid US positive. Carotid US positive patients had several traditional risk factors which were significantly higher than carotid US negative patients: age, BMI, systolic BP, total cholesterol, cholesterol:HDL ratio and LDL cholesterol. The QRISK2, Framingham, SCORE, ACCAHA were all significantly higher in carotid US positive patients. Of the SCCPs, only CXCL9 was higher in US positive patients. A previous study in the Yonsei Cardiovascular Genome Centre cohort measured CXCL9 and CIMT in 164 apparently healthy patients (Yu et al 2015) Of note, patients with cardiovascular disease, inflammatory disease and use of inflammatory medications were excluded. The authors found that CXCL-9 correlated significantly with CIMT after adjusting for confounding factors such as age, diabetes and smoking status, and medication. Patients with plaque on carotid US had significantly higher CXCL-9 than those without plaque. However, CXCL9 did not correlate with change in CIMT over the 2 year follow up. This is similar to the findings of this thesis. CXCL9 is a T cell chemoattractant which is produced by dendritic cells, B cells and macrophages and is induced by IFN-γ (Rosenblum et al 2010). T cells contribute to atherosclerosis by propagating inflammation and plaque growth (Robertson and Hansson 2006).

All cardiovascular risk scores had a strong correlation with CIMT. Interestingly, the Framingham risk score had a stronger correlation than the QRISK2, which is the only risk score to include RA as an independent risk factor for CVD. There was a very similar correlation between CIMT and QRISK2 value ($r = 0.74$) and CIMT and QRISK2 value without adjustment for RA ($r = 0.73$) in patients with early RA. Of the CV risk scores, QRISK2 had the highest sensitivity but lowest specificity to detect carotid US positive patients. The SCORE had the lowest sensitivity but highest specificity to detect carotid US positive patients. Although the Framingham risk score had a strong correlation with CIMT, it did not perform well in sensitivity for US positive patients. This is likely to be due to the cut off used to define 'high risk' patients of > 20%. Another study by Ozen et al, 2016 found that the ACC/AHA score better identified high risk patients (using the same definition of carotid US positive patients as this thesis) compared to the QRISK2 and SCORE. They did not examine the Framingham risk score. However, this study was performed in patients with established RA in Turkey. Of note, the QRISK2 includes the Townsend deprivation index using a UK postcode, and such a measure would not have been included in the Turkish study.

There was no significant difference in CIMT at 6 and 12 months compared to baseline. There was a trend for CIMT to increase at month 6 and then reduce at month 12 compared to baseline. Similarly, there was a trend for arterial stiffness and cholesterol:HDL ratio to increase at 6 months and then reduce at 12 months, again this was not significant. There are variable reports in the literature regarding changes in CIMT over time in RA patients. In a study of early RA patients in India, CIMT decreased significantly at 12 months from baseline, by an average of 0.07mm (Guin et al 2013). However, in this study RA patients had significantly higher DAS28 at baseline (5.61) and after 1 year (4.01) than in this thesis. In the study by Södergren et al (2010), mean CIMT increased by 0.05mm over 18 months, a longer period of time. The baseline mean DAS28 was 3.62, slightly lower than this thesis.

This study found a moderate correlation between CIMT and cholesterol:HDL ratio and LDL cholesterol and a weak correlation between CIMT and total cholesterol. CV risk scores correlated more strongly with CIMT than with arterial distensibility. Distensibility coefficients were inversely associated with all cardiovascular risk scores, CIMT, total cholesterol and LDL cholesterol. Studies in animals have shown changes in arterial distensibility in the early stages of atherosclerosis (Hironaka et al 1997). In humans, reduced distensibility is associated with CV risk factors (Dart et al 1991) and can predict those at increased risk of future CV disease in healthy individuals (Mattace-Raso et al 2006). In Chapter 3 we found reduced aortic constriction in mice with CIA. There was a moderate inverse relationship between baseline CXCL9 and distensibility coefficient at 12 months. This finding has not been shown previously. Previous studies have shown that patients with acute coronary syndromes, those who developed events (death, nonfatal acute MI, and refractory unstable angina) had significantly higher CXCL9 than those who did not develop events (Dusi et al 2016). Studies have also shown increased expression of CXCL9 in human atherosclerotic plaques (Mach et al 1999).

There was a significant relationship between change in CIMT at 6 months and baseline disease activity measured by DAS28, CRP and ESR. This suggests that inflammation is contributing to subclinical atherosclerosis progression in early RA. A study in the Netherlands found that while baseline DAS28 in patients with early RA was not significantly different in those who had a CV event at 10 years versus those who did not, time-averaged DAS28 did have a significant effect on the risk of CVD (Arts et al 2014). In a prospective study in patients with inflammatory polyarthritis, CRP at baseline was found to predict death due to CVD (Goodson et al 2005). In another study of patients with longstanding RA, CRP correlated directly with the presence of atherosclerosis, as measured by CIMT, over an extended follow-up (Gonzalez-Gay et al 2005). In this thesis there was no difference in seropositivity and change in CIMT or DAS28 at 6 or 12 months. Interestingly, we found that increase in CIMT at 6 months was highest in those with high IL-6 and low sIL-6R level. The relationship between IL-6 and sIL-6R is complex and it is difficult to know whether this state would favour IL-6 classical or trans-signaling. Physiologically, there is more sIL-6R available in the serum than IL-6. IL-6 binds to sIL-6R and this complex is immediately neutralized by sgp130. Therefore, the sIL-6R and sgp130 constitute a buffer in the blood for circulating IL-6, which will only act systemically once the capacity of the buffer is exceeded (Calabrese and Rose-John 2014). Thus in this group of patients with high IL-6 and low sIL-6R, IL-6 trans-signaling may be more prominent. There did not seem to be a relationship between the complex of IL-6/sIL-6R and IL-6 and sIL-6R individually. We found the IL-6/sIL-6R complex to be more unstable than other proteins detected by ELISA, for example more variation in levels measured by ELISA using the same sample at different time points. It may be more susceptible to environmental factors such as change in temperature. Also, IL-6/sIL-6R complex binds to sgp130 and therefore there may be more IL-6/sIL-6R complex in serum than detected by ELISA.

Rapid progressors in this study had significantly higher baseline HbA1c, total cholesterol, cholesterol:HDL ratio, LDL cholesterol and VCAM-1. In a study by Arts et al RA patients who developed a CV event had significantly higher baseline (at the time of RA diagnosis) total cholesterol: HDL ratio (but not total cholesterol or LDL cholesterol) (Arts et al 2015). A complicating factor here is the lipid paradox; inflammation reduces total cholesterol, HDL cholesterol and LDL cholesterol in patients with RA (Gonzalez-Gay et al 2005). When inflammation improves, serum cholesterol improves. This phenomenon was seen with tocilizumab treatment in this thesis and other studies have consistently shown that tocilizumab is associated with increased lipid levels in the context of decreased inflammatory markers (Ito and Takagi 2010, Emery et al 2008). The study by Arts et al (2015) also found significantly higher DAS28, ESR and CRP at baseline in those who developed CV events. Several of these variables, which are higher in rapid progressors (HbA1c and lipid levels) form part of the 'metabolic syndrome'. The metabolic syndrome is defined most recently by the International Diabetes Foundation criteria (2005) (Zimmet et al 2005). This includes obesity, impaired fasting glucose, dyslipidaemia and hypertension. Central obesity is a prerequisite for this definition, which I did not measure. In this thesis, change in CIMT and change in distensibility over 12 months was not associated with smoking at baseline or ethnicity. Also, surprisingly, several of the traditional risk factors such as age, BP, BMI and serum triglyceride levels were not significantly higher in rapid progressors compared to non-rapid progressors. A prospective 5-year study of patients with early RA (classified as symptoms of less than 12 months) found that at 5 years, 10.9% of patients had experienced a new cardiovascular event, 12 of 48 events were fatal. An increase in the hazard rate of a new CVE during follow up was predicted by some traditional risk factors: treated hypertension, higher triglyceride level, diabetes mellitus, greater age at disease onset, being male, having had a previous CV event, but also with higher cumulative disease activity and progression of extra articular disease. In this study, ESR at baseline had an unfavourable prognostic significance for a new CV event when evaluated together with CV risk factors and DMARD treatment (Innala et al 2011).

This thesis found rapid progressors had significantly higher VCAM-1 than non-rapid progressors. This is a novel finding in RA patients. In the general population VCAM-1 has been associated with CV disease. As previously mentioned, there are conflicting data in the literature regarding VCAM-1. In a six year observational study men with CV events during follow-up had higher baseline median serum ICAM-I and VCAM-I than those without CV events (Schmidt et al 2008). When the median serum ICAM-I and VCAM-1 were used as a cut off, those with levels above the cut-off value had an increased risk of having a plaque in the femoral artery. Another study found a correlation between serum ICAM-1 and VCAM-1 and carotid IMT in the general population (Kondo et al 2005). However, a prospective cohort study found no association between elevated VCAM-1 and risk of future acute coronary event or angina pectoris in apparently healthy men aged 50-59 years over a 5 year follow up (Luc et al 2003). Studies have shown that VCAM-1 is rapidly induced by proatherosclerotic conditions, including early lesions. A study of 24 atherosclerotic plaques found that all contained some VCAM-1, compared to 45% of control segments (O'Brien et al 1993). The majority of VCAM-1 was found in areas of neovascularization and inflammatory infiltrate in the base of plaques compared to endothelial cells at the arterial lumen in control segments and in plaques. Most VCAM-1 was expressed by subsets of plaque smooth muscle cells and macrophages.

There was no difference in change in CIMT at 1 year and methotrexate, corticosteroids or NSAID use. A prospective study of patients with early RA found that treatment with COX-2 inhibitors was significantly predictive of a new CV event at 5 year follow up (Innala et al 2011). Another prospective study over 3 years of patients with established RA found no difference in CVD risk for patients taking NSAIDs but patients with RA who developed CVD were significantly more often treated with COX-2 inhibitors (Peters et al 2009). Another study found that in RA patients with no history of CVD, corticosteroid use was associated with a 78% increased risk if CV death compared to those who did not receive corticosteroids (Maradit-Kremers et al 2005b). However, in RA patients with a previous history of CVD, the risk of CV death was lower in those who received corticosteroids compared to those who did not. There is evidence that patients with RA have evidence of more unstable plaque and more inflammation within plaques, compared to controls (Aubry et al 2007). Perhaps, in this setting, corticosteroids play a role in plaque stabilisation.

A few drawbacks of the present studies should be mentioned. There are inherent drawbacks with one-off measurements of SCCPs; they may change over a matter of days and can be altered by other medical conditions and intercurrent illness such as infection. To control for diurnal variation in several of the serum markers measured, e.g. IL-6, patients were assessed in the morning between the hours of 9am and 11am. However, there is still some variability within this timeframe. In addition, there was a variable duration of RA at the time of baseline assessment. However, there were no correlations between disease duration and disease activity or atherosclerosis. We did not record physical activity levels of patients and this may also contribute to CV risk.

There are several limitations to the methods used in this chapter. These are non-controlled observational cohorts and disease is heterogenous. Also, patients were treated with different DMARDs, and some were treated with corticosteroids or NSAIDs. Thus, the sample may not be representative of the general RA population. In hindsight, the MSK US assessments (US7 and US10 scores) probably do not add much to information risk of CVD disease or subclinical atherosclerosis and the time used to perform these procedures may have been better used on other measurements of CV risk such as aortic pulse wave velocity. The definition of a rapid progressor (CIMT increase > 0.05mm) was made using results from Sodergren et al in the RA population. This is a fairly arbitrary cut off and is taken from a cohort of RA patients, not from the evidence base in the general population.

5.6 Conclusions

1. Over 50% of patients with established RA and early RA (at the time of diagnosis) are classified as being high risk of CVD according to the QRISK2.
2. VCAM-1 correlated with disease activity and cardiovascular risk in patients with established RA.
3. Several traditional CV risk factors predict CIMT progression. Inflammation from disease onset in CV susceptible individuals may accelerate atherosclerosis. In early RA baseline disease activity correlated significantly with change in CIMT at 6 months.
4. Rapid progressors had significantly higher serum VCAM-1 at baseline than non-rapid progressors.

6 General discussion

Cardiovascular mortality in patients with RA is up to 50% higher than the general population (Gabriel 2008). Although it is well established that incidence of CVD is increased in RA, the precise cause is unclear. There is increasing recognition that systemic inflammation is a major driver of this increased CV risk (Gabriel 2008, del Rincon et al 2001, Arts et al 2014). IL-6 has been implicated in CVD in the general population but its role in CVD in RA is not well defined. Of the two modes of IL-6 signaling, there is increasing evidence that trans-signaling is pro-inflammatory whereas classical signaling has important regenerative or anti-inflammatory effects. In chapter 3, I show that IL-6 trans-signaling is implicated in vascular dysfunction in CIA. In chapter 4, I show that IL-6 trans-signaling leads to accelerated atherosclerosis in a susceptible animal model. In chapter 5, I show that VCAM-1, regulated by IL-6 trans-signaling, is associated with progression of subclinical atherosclerosis in patients with early RA.

Prior to the work carried out in this thesis it was known that CIA was associated with aortic contractile dysfunction. The work in this thesis validates this finding but, for the first time, I show that selective blockade of IL-6 trans-signaling, using intravenous sgp130Fc, reduces arthritis severity and restores the vascular dysfunction associated with CIA. This improvement in vascular function may be due to the effect of IL-6 trans-signaling on macrophage recruitment into the vessel wall and surrounding adipose tissue. Previous work by Williams et al (2016) found mice with CIA have increased macrophages in the aorta and perivascular adipose tissue (PVAT). Another study by Kraakman et al (2015) found IL-6 trans-signaling recruited macrophages to adipose tissue in high fat diet-induced obesity in mice and that blocking IL-6 trans-signaling with sgp130Fc in these mice prevented macrophage accumulation in adipose tissue. The improvement in vascular function in mice treated with sgp130Fc in this thesis was associated with a reduction in serum CCL2 and VCAM-1. CCL2 recruits monocytes to sites of inflammation; these then differentiate into macrophages (Yang et al 2014). VCAM-1 induces immune-cell recruitment within vessel walls. Previous work using the Biomap® system found that CCL2 and VCAM-1 were regulated by IL-6 trans-signaling (Tan et al 2013). Thus, sgp130Fc may restore vascular function by decreasing CCL2 and VCAM-1-driven recruitment of macrophages into the aorta and PVAT. To further explore this hypothesis I could examine the effect of blockade of IL-6 trans-signaling on VCAM-1, CCL2 and macrophage expression in the aorta and PVAT in CIA.

Also for the first time, I have shown that IL-6 trans-signaling using Hyper-IL-6 increases plaque size in ApoE$^{-/-}$ mice. This accelerated atherosclerosis was not observed with IL-6 alone. There is conflicting evidence of the role of IL-6 in atherosclerosis in the literature. A previous study in ApoE$^{-/-}$ mice reported that IL-6 administration increased atherosclerotic lesion size in the aortic sinus (Huber at al 1999). However, another study found that serum cholesterol levels and atherosclerotic lesion formation were significantly increased in ApoE$^{-/-}$-IL-6$^{-/-}$ mice compared with ApoE$^{-/-}$ and wild-type mice (Schieffer et al 2004). Previous work in LDLr$^{-/-}$ mice showed that blockade of IL-6 trans-signaling, using sgp130Fc, reduced atherosclerosis (Schuett et al 2011). Overall, the role of IL-6 in atherosclerosis is complex; while a potent pro-inflammatory cytokine, IL-6 can have anti-atherosclerotic effects, and its opposing effects may be explained in part by its two modes of signaling. For the first time, the work in this chapter shows that IL-6 trans-signaling, rather than IL-6 classical signaling, increases atherosclerosis in a mouse model. This increase in plaque size with Hyper-IL-6 may be partly driven by the increased arterial and plaque expression of VCAM-1 in those administered Hyper-IL-6. This increase in VCAM-1 was not seen with IL-6 administration. Plaque size in the brachiocephalic artery correlated with arterial VCAM-1 expression but not serum VCAM-1. The increase in plaque with Hyper-IL-6 was not accompanied by an increase in serum lipid levels. This is similar to findings in LDLr$^{-/-}$ mice, whereby sgp130Fc reduced atherosclerosis but did not affect serum lipid levels (Schuett et al 2011). In RA patients, IL-6 blockade using Tocilizumab increases serum total cholesterol, HDL, LDL and triglyceride levels (Choy et al 2014). In the ApoE$^{-/-}$ model used in this thesis lipid levels were considerably higher than in RA patients (total cholesterol approximately 4 times higher in ApoE$^{-/-}$ mice than in RA patients), so that any increase in lipid levels with additional inflammation may be negligible.

In chapter 5, I found that over half of patients with established RA were classified as high risk of CVD over the next 10 years. These patients had significantly higher VCAM-1 than those classified as low CV risk. This is a novel finding in RA. In the general population, there is conflicting evidence of the role of VCAM-1 in predicting those at high risk of CV events. Schmidt et al (2008) reported higher VCAM-1 in those who developed CV events in the general population. However, other studies of the general population found VCAM-1 did not provide significant improvement in CVD risk assessment beyond conventional CVD risk factors (Kunutsor et al 2017, Malik et al 2001) and was not significantly different in apparently healthy individuals that went on to develop CVD compared to those that did not (Luc et al 2003). These studies in the general population examined patients longitudinally and measured CV outcomes. To examine whether the finding of higher VCAM-1 in patients classified as high CV risk in this thesis is in fact predictive of CV events, these patients could be followed-up over the next 10 years and CV events recorded.

In chapter 5, patients with early RA in DAS28 remission at 6 months had significantly lower baseline CRP, ESR and IL-6. In addition, baseline IL-6 correlated significantly with US7 and US10 scores at 12 months. A previous study reported a positive association between baseline IL-6 and radiographic progression of RA over 4 years (Klein-Wieringa et al 2011). To my knowledge, this is the first time that baseline IL-6 has been shown to be positively associated with ultrasound scores at 12 months. These observations suggest the importance of inflammation at baseline in propagating the inflammatory response. I did not correlate SCCPs at baseline with radiologic damage at 12 months using established scoring systems such as the Sharp/van der Heijde scoring (SHS) method (van der Heijde 2000). This could be an area for future work, as radiographic damage and joint deformity are major causes of disability for RA patients.

It is known that in early RA, CV risk is increased. I have confirmed the high prevalence (41%) of subclinical atherosclerosis in patients with early RA. Carotid US positive patients had several traditional risk factors which were significantly higher than carotid US negative patients: age, BMI, systolic BP, total cholesterol, cholesterol/HDL ratio and LDL cholesterol. Ozen et al found that carotid US positive RA patients had higher cholesterol:HDL ratio and age, but not LDL cholesterol or BMI (Ozen et al 2016). A novel finding of this chapter is that carotid US positive patients had higher CXCL9. This has been found previously in the general population (Yu et al 2015) but not in RA patients. Previous work using the Biomap® system found that CXCL9 is regulated by IL-6 trans-signaling. CXCL9 is a T cell chemoattractant; these cells contribute to atherosclerosis by propagating inflammation and plaque growth (Robertson and Hansson 2006). CXCL9 was not associated with progression of atherosclerosis over 12 months in this thesis but it would be interesting to follow these patients up over an extended period and examine the association between baseline CXCL9 and CV events.

I have confirmed previous findings that some traditional risk factors (HbA1c, total cholesterol, cholesterol:HDL ratio and LDL cholesterol) predict CIMT progression in early RA. However, I also show the novel finding that baseline serum VCAM-1 is elevated in RA patients who become rapid progressors in terms of subclinical atherosclerosis. This adds weight to the finding in this thesis in the cross sectional study of patients with established RA that serum VCAM-1 was higher in those classified as high CV risk. I also show that baseline disease activity correlates with change in change in CIMT at 6 months. Taken together, these findings suggest that in RA, inflammation from disease onset may accelerate atherosclerosis in susceptible individuals, which confirms the data in $ApoE^{-/-}$ in Chapter 4. This proposed interaction between pre-existing CV risk factors and inflammation, autoimmunity and dyslipidaemia at the onset of RA, or even before RA becomes apparent, is summarised in Figure 96.

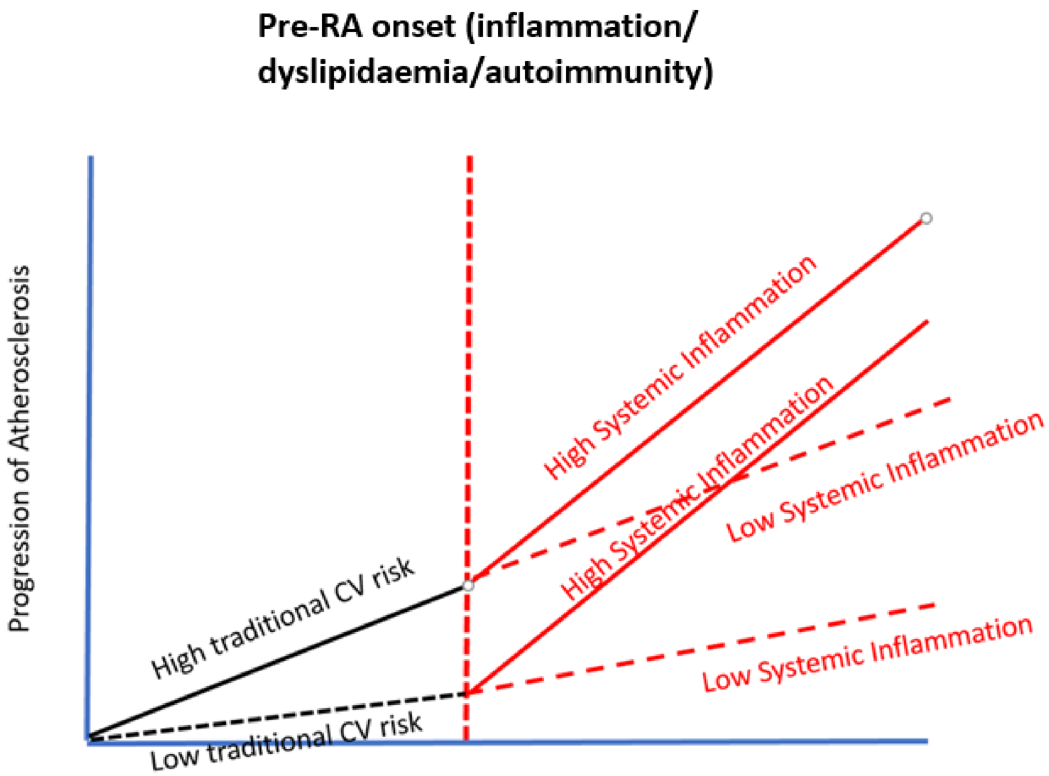

Figure 96. Summary of hypothesis of the effect of inflammation at RA onset on progression of atherosclerosis

In all three results chapters, VCAM-1 was consistently associated with disease states. In chapter 3, restoration of vascular function in mice treated with sgp130Fc was associated with reduced serum VCAM-1. In chapter 4, the increase in plaque size seen in ApoE$^{-/-}$ mice with Hyper-IL-6 was associated with increased arterial and plaque expression of VCAM-1, which correlated with VCAM-1 arterial expression but not serum VCAM-1. In chapter 3, serum VCAM-1 correlated with disease activity and cardiovascular risk in established RA, and in early RA baseline VCAM-1 was higher in patients who developed rapid progression of subclinical atherosclerosis. What remains to be elucidated is whether serum VCAM-1 can predict CV events in RA patients. It is also important to mention that inclusion of a biomarker in a risk model requires detailed knowledge of the biomarker level in the general population. In addition, these consistent findings of association of VCAM-1 in disease states prompt consideration about the potential role of blockade of VCAM-1 in the treatment of RA and atherosclerosis.

The relationship between cell surface VCAM-1 and soluble serum VCAM-1 is complex and appears to be context dependent. In vitro, soluble VCAM-1 levels correlate with surface expression (Kjaergaard et al 2013). Of note in this thesis, there was no significant correlation between VCAM-1 expression in atherosclerotic plaque in ApoE$^{-/-}$ mice and serum VCAM-1. In RA patients, VCAM-1 synovial membrane expression does not correlate with serum or synovial fluid levels (Mulherin et al 1996). Circulating soluble VCAM-1 results either from alternating splicing of mRNA or proteolysis of the membrane-bound protein form. The ligand for VCAM-1 is VLA-4 (Navarro-Hernández et al 2009). Soluble VCAM-1 may act as a competitive inhibitor of ligand binding (Rose et al 2000). Of note, Kitani et al found that in RA synovial fluid, the binding of soluble VCAM-1 to T cells inhibited their activation (Kitani et al 1996).

In view of its role in both RA and atherosclerosis, blockade of VCAM-1 may be beneficial in these diseases. Park et al (2013) found anti-VCAM-1 antibodies attenuated atherosclerosis in ApoE$^{-/-}$ mice. Carter et al found that neutralizing monoclonal antibody to VCAM-1 resulted in reduced severity, but not incidence of CIA in mice (Carter et al 2002). However, studies in patients with atherosclerosis have not been successful so far. A study in patients with ACS found that succinobucol (an agent which blocks VCAM-1 expression and has anti-oxidant effects) was not associated with a difference in the primary endpoint of CV death, cardiac arrest, MI, stroke, unstable angina, or coronary revascularization compared with placebo at 2-year follow-up (Tardif et al 2008). Also, compared with placebo, succinobucol increased LDL cholesterol and systolic blood pressure, and decreased HDL cholesterol and HbA1c. Natalizumab is a recombinant, humanized antibody which binds to VLA-4 and blocks its interaction with VCAM-1. Natalizumab is licensed for treatment of highly active relapsing remitting multiple sclerosis (Torkildsen et al 2016) and refractory Crohn's disease (Sandborn et al 2005). However, inhibition of the VLA-4/VCAM-1 interaction affects the cellular immune response and there have been reports of progressive multifocal leukoencephalopathy (Ho et al 2017) and sarcoidosis (Parisinos et al 2011) in patients treated with Natalizumab.

Other drugs that have recently been approved for the treatment of RA are the JAK inhibitors baricitinib and tofacitinib. Baricitinib inhibits JAK1 and JAK2 and tofacitinib inhibits JAK1 and JAK3. IL-6 signals through the JAK/STAT pathway, specifically JAK1 and JAK2. Therefore both these drugs inhibit the action of IL-6, but also many other cytokines such as IFN-γ, IL-8 and IL-23 (Cutolo et al 2013). Both drugs have shown efficacy in RA in trials and have the benefit of being oral drugs, but side effects include serious infections, deranged liver function tests, neutropenia and increased serum lipid levels (Dougados et al 2017, Cutolo et al 2013). Similar to tocilizumab, despite the increase in lipids levels, so far there does not seem to be an increase in CV events (Weinblatt et al 2017, Charles-Schoeman et al 2016), although long terms effects remain to be elucidated. These drugs block downstream activities of both IL-6 classical and trans-signaling. Selective blockade of IL-6 trans-signaling may offer an advantage over these drugs as this strategy offers a reduction in inflammation whilst allowing the regenerative and homeostatic effects of IL-6 classical signaling to continue.

Overall, IL-6 trans-signaling appears to play a pivitol role in vascular dysfunction and atherosclerosis in mouse models. In humans, proteins regulated by IL-6 trans-signaling are associated with progression of subclinical atherosclerosis in early RA. These findings suggest that blockade of IL-6 trans-signaling may be beneficial to patients with RA, and perhaps for atherosclerosis in the general population. Sgp130Fc successfully underwent phase I clinical trials in 2014 and is currently under the name Olamkicept in phase II clinical trials for use in treatment of inflammatory bowel disease (Rose-John 2017), this could also have therapeutic applicability in the management of rheumatoid arthritis.

Directions for future work

To further explore the mechanisms of improved vascular function with IL-6 trans-signalling blockade in CIA I could examine VCAM-1, CCL2 and macrophage expression in the aorta and PVAT. Another area of investigation would be to examine the effect of VCAM-1 blockade effect on vascular dysfunction in CIA. Another strategy would be to examine the effect of soluble VCAM-1 on atherosclerosis in the ApoE$^{-/-}$ and CIA model to determine if soluble VCAM-1 regulates inflammation. It would also be interesting to examine plaques from RA patients at autopsy and measure VCAM-1, CCL2, IL-6 and IL-6R and compare with those from the general population.

As discussed, patients in both the cross sectional study and early RA study could be followed up over an extended period, perhaps 5 years, and onset of CVD or CV events measured. The association of SCCPs, particularly VCAM-1 and CXCL9 with CV outcomes could be examined. I could also examine joint radiological damage at 5 years and correlate with baseline SCCPs.

The relationship between serum IL-6, sIL-6R, the complex of IL-6/sIL-6R and sgp130 is still not fully elucidated and there are likely to be complex mechanisms at play in RA patients, which change throughout the course of the disease. Serial measurements of these proteins over time and their response to treatment would be interesting to examine.

Another area for future study, which has increasing recognition in the literature, is ‘pre-RA’. It is known that even before RA diagnosis, patients have increased levels of inflammation and dyslipidaemia. To study these patients is inherently difficult, but studies on groups that are at increased risk of developing RA, such as those with a strong family history, would be of value. Measures of subclinical atherosclerosis and measurement of SCCPs could be performed, and a longitudinal study examining RA and atherosclerosis development undertaken.

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