The Role of Interleukin-6 in the Metabolism of the ApoB-Containing Lipoproteins in Rheumatoid Arthritis

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To Jen, Cammy & Sophie

- For your love, attention, and for keeping my feet (very firmly) on the ground

Declaration

The work presented within this thesis was conducted during my time as a Clinical Research Fellow at the University of Glasgow, and as Specialty Registrar in Rheumatology / General (Internal) Medicine at NHS Tayside and NHS Ayrshire & Arran. The text contained within is entirely my own work, with assistance provided by others in the following areas:

Some of the text in Chapter 1 relating to lipid changes in RA and the use of tocilizumab in clinical therapeutics is derived from review manuscripts published previously which I have written or contributed to. These manuscripts are cited in the "Publications" section of this thesis. The text is drawn only from the sections of manuscript for which I was the primary author, and I hereby acknowledge the input and oversight of co-authors Dr Mike Peters, Professor Naveed Sattar, Professor Iain McInnes and Dr Stefan Siebert.

The MEASURE study was designed by F-Hoffman la Roche in conjunction with Professors Iain McInnes and Naveed Sattar. NMR outcomes, including quantification of GlycA, were provided by LabCorp (formerly LipoScience), Raleigh, North Carolina, USA. I performed all statistical analyses and graphical data presentations in Chapter 2.

The KALIBRA study was designed by Professors Iain McInnes, Naveed Sattar, Chris Packard and Muriel Caslake, all of the University of Glasgow, and Drs Duncan Porter and David McCarey of NHS Greater Glasgow & Clyde. Dr McCarey and Professor Caslake were the co-Principle Investigators. I generated the application for ethical approval under supervision of Drs McCarey and Porter, and attended the subsequent regional ethics committee meeting myself. The paper case report form (CRF) was drafted by me and amended by staff of the Robertson Institute for Biostatistics at the University of Glasgow. I performed screening of and gained consent from all prospective participants, and performed all clinical assessments including DAS28 scoring. Blood sampling for kinetic studies was performed jointly by me and nursing staff at the Clinical Research Facility in the Western Infirmary Glasgow. Kinetic data were generated by Professors Caslake and Packard, and subsequent statistical analysis was carried out between myself and the Robertson Centre for Biostatistics.

I undertook and analysed all in vitro work described in chapter 4.

The text contained within this thesis has not been submitted in any form to any other University or Institute.

Jamie Robertson

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Abstracts

Robertson J, Dale J, Sattar N, Porter N
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Robertson J, Porter D, Sattar N, Packard C, Caslake M, McInnes I, McCarey D.
Interleukin-6 Blockade Raises LDL Via Reduced Catabolism Rather Than Via Increased Synthesis – A Cytokine-Specific Mechanism For Cholesterol Changes In Rheumatoid Arthritis
Poster presentation – European League Against Rheumatism Meeting, June 2016

Summary

Background

Patients with rheumatoid arthritis (RA) carry an increased risk of cardiovascular disease and cardiovascular death compared to age- and sex-matched controls. This risk appears to be related to cumulative inflammatory burden, and can be at least partially ameliorated by successful treatment of the disease with conventional or biologic disease-modifying anti-rheumatic drugs (DMARDS). However, RA patients typically exhibit reduced serum levels of cholesterol, which can be increased following DMARD therapy; this is in contrast to the general population, where serum cholesterol is directly proportional to cardiovascular risk. The magnitude and nature of this increase varies between therapeutic agents. Blockade of interleukin-6 (IL-6) signalling with the drug tocilizumab conveys perhaps the most profound lipid changes, leading to average increases in LDL-cholesterol (LDL-c) of around 20% as well as changes in HDL-cholesterol (HDL-c) and triglycerides.

The mechanisms behind this so-called "lipid paradox", and its impact on cardiovascular outcomes following RA therapy, are not fully understood. Animal studies have shown that hypercatabolism of LDL can lead to reduced circulating LDL-c, possibly due to increased consumption by the reticulo-endothelial system. A deeper understanding of the lipid paradox, and its implications for cardiovascular risk, is vital to allow physicians to provide optimal management of both articular RA and its cardiovascular manifestations. Using IL-6 blockade as a molecular tool, I attempted to unravel the physiological processes underlying the lipid changes observed in RA, and to understand what these changes might mean in term of cardiovascular risk for patients.

Objectives

1) To investigate lipid changes in patients treated with tocilizumab as assessed by nuclear magnetic resonance (NMR) spectroscopy

2) To determine whether increased LDL-c following IL-6 blockade is due to increased production or reduced catabolism of LDL

3) To explore the lipid-handling behaviour of macrophages in response to IL-6

Methods: The MEASURE study was a placebo-controlled, randomised controlled trial evaluating NMR lipid profiles in patients with severe active RA following treatment with tocilizumab or placebo, with the placebo group switching to open-label tocilizumab after 24 weeks. Results to week 12 have been published previously. Data on all patients to 52 weeks using an updated NMR platform were evaluated, and changes in lipid values were correlated with markers of disease activity, including the acute phase markers C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), and the composite clinical score DAS28. NMR analysis also yielded the novel marker GlycA, a composite NMR signal reflecting a measure of the acute phase response. GlycA levels were compared to other markers of disease activity, and GlycA's utility as a marker of future response to therapy and of persistent disease activity in those with normal ESR or CRP was assessed.

The KALIBRA study analysed the kinetics of the apoB-containing lipoproteins before and after IL-6 blockade. Patients with severe active RA (defined as DAS28 ≥5.1) and who were eligible for tocilizumab therapy underwent kinetic modelling of VLDL, IDL and LDL at baseline and again following at least three months' treatment with tocilizumab. The primary outcome measure was the fractional catabolic rate of LDL, though LDL production rate was also assessed as well as a variety of other lipid parameters. Changes in LDL production and catabolic rates were correlated with serum LDL-cholesterol ester content and with measures of disease activity.

To provide information on the cellular processes underlying kinetic changes, macrophages were generated in vitro from the THP-1 monocyte cell line or from healthy human donor monocytes. These cells were then exposed to IL-6 and assessed for signs of response, including phosphorylation of STAT3 and production of TNF-α. Macrophage lipid loading (and subsequent foam cell formation) following stimulation with 10ng/ml IL-6 for 24 hours was assessed by staining with oil red O and fluorimetry following culture with fluorescently-labelled oxidised LDL. Altered expression of genes involved in lipid metabolism, including surface receptors for both native and oxidised LDL, was assessed at the

RNA level using quantitiative polymerase chain reaction (qPCR) and Taqman Low-Density Array (TLDA) plates, with fold change of ≥2 considered significant. Outcomes from these experiments were then validated at the protein level by flow cytometry.

Results

The MEASURE study showed no change in small LDL particles with tocilizumab therapy. Increases were seen in large LDL, small HDL and some VLDL particles in the tocilizumab group, with change seen by the earliest assessment timepoint of 2 weeks. At 52 weeks follow-up, the same changes were observed in placebo patients who had switched to open-label tocilizumab. Greater increases in large LDL and small HDL tended to associate with falls in CRP, but less so with disease activity as measures by CDAI.

GlycA levels correlated significantly at baseline with CRP ($r=0.70$, $p<0.001$) and ESR ($r=0.44$, $p<0.001$) but not with CDAI, and fell in a similarly precipitous manner following IL-6 blockade. Neither baseline (area under curve =0.60) or week 2 (AUC=0.53) GlycA levels were effective at predicting response at week 24 as measured by CDAI. In treated patients, with CRP<5mg/l, GlycA did not associate with persistent clinical disease activity.

In KALIBRA, 12 patients were recruited of whom complete data was available for 11. As expected, significant increases in mean serum LDL-c (2.90 v 3.40mmol/L, $p=0.014$) and HDL-c (1.23 v 1.52 mmol/L, $p=0.006$) were observed after treatment. IL-6 blockade led to a reduction in median LDL fractional catabolic rate (FCR) from 0.53 to 0.27 pools/day, ($p=0.006$) with median reduction of 30%, and the change in LDL FCR correlated tightly with that of serum LDL cholesterol ester content ($r=-0.74$, $p=0.011$). LDL FCR correlated at baseline with CRP ($r=0.74$ $p=0.012$) but not CDAI ($r=0.04$, $p=0.91$). The degree of change in CRP with treatment showed a trend to association with change in FCR ($r=0.46$, $p=0.15$) and LDL cholesterol ester ($r=-0.43$, $p=0.18$). LDL production rate did not increase, and in fact fell (median 763.8 v 442 mg/kg/day, $p=0.002$). No changes were seen in the activity of lipoprotein lipase (LPL), hepatic lipase (HL), cholesterol-ester transfer protein (CETP) or PCSK9.

Human monocyte-derived macrophages (HMDM) displayed altered TNF-α production and increased STAT3 phosphorylation in response to IL-6; THP-1 macrophages did not, and appeared to lose their IL-6 receptor in the process of differentiation from monocytes. Neither cell type displayed increased uptake of oxidised LDL following culture with IL-6. TLDA analysis showed altered expression of scavenger receptors with an increase in the "macrophage receptor with collagenous structure" (MARCO) and a reduction in LOX-1 in HMDM, though this was not observed at the protein level on flow cytometry.

Conclusions

IL-6 blockade in RA elevates numbers of large LDL and small HDL particles, but not the most pro-atherogenic small LDL particles. These changes are maintained up to 52 weeks follow-up, and seem to be more prominent in subjects with greater reductions in the acute phase response. The KALIBRA study showed that the elevation in LDL is almost entirely due to a reduction in LDL fractional catabolic rate, from a baseline state of hypercatabolism in severe active disease to values approximating the population average after treatment. Greater changes in FCR were associated with greater reductions in acute phase reactants, regardless of RA activity as assessed clinically by CDAI. Lipid changes did not appear to be explicable by changes in activity of lipolytic enzymes, CETP or PCSK9. IL-6 does not appear to exert its lipidaemic effects via augmented macrophage lipid metabolism or increased foam cell formation. These findings are consistent with a normalisation of a pathological, IL-6 driven state of hypercatabolism leading to LDL-c increases following IL-6 blockade, with hepatocytes as the possible main effector cell type. This suggests that LDL-c elevations observed during treatment for RA may not be pro-atherogenic or contribute to increased CVD risk, though confirmation of this hypothesis is required with trials reporting clinical cardiovascular outcomes.

1 Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune disease of unknown aetiology which manifests primarily as an inflammatory polyarthritis. Over the course of the disease, the initial symptoms of joint pain, stiffness and swelling are succeeded by the accumulation of irreversible joint damage and deformity. The resultant disability of this common disease imposes a considerable burden on both the individual and society as a whole. This burden can, however, be reduced by effective use of medications such as corticosteroids and disease-modifying anti-rheumatic drugs, and by attention to relevant co-morbidity.

1.1.1 Epidemiology

A 2002 primary care-based study in Norfolk using the 1987 American College of Rheumatology (ACR) criteria for RA reported prevalence of 0.81%, with around two thirds of patients female (1). If this figure were extrapolated to the rest of the country, this would give a total RA population in the year 2000 of 386,600 across the United Kingdom. Prevalence seems to have fallen somewhat through the 20th century (2, 3). Disease onset is most common in the 6th-7th decades (4), though can occur at any age over 16 (before this point, a seropositive inflammatory arthritis would by convention be diagnosed as juvenile idiopathic arthritis). Similar figures have been found in the USA (5), again with a reduction in prevalence since the 1960s (2, 6), although one recent study from Minnesota reported an increase in prevalence (0.62% v 0.72%) from 1995 to 2005 (7). This last study reported incidence of 40.9 cases per 100,000 population annually. Whilst these figures are derived from Caucasian populations, prevalence in other ethnic groups can be significantly higher (6.8% in Chippewa native Americans (8)) or lower (0.2% in Japan (9), 0.26% in China (10) and 0.3% in British Afro-Caribbeans (11)).

1.1.2 Clinical Features

Rheumatoid arthritis classically presents as a symmetrical, small joint polyarthritis, most commonly affecting the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints. Most other synovial joints can also be affected. Inflammation in joints causes pain and stiffness, sometimes with

erythema, heat and swelling (this latter due to effusion or synovial hyperplasia), and ultimately loss of function. Symptoms tend to be worst after inactivity or on waking, receding to some degree with activity. RA can run a somewhat relapsing-remitting course, but symptoms are often persistent. Over time, irreversible damage will result in the form of cartilage loss and bony erosions; accumulation of structural damage leads to deformities such as ulnar deviation of the fingers, MCP subluxation, and swan-neck or boutonniere deformities. Loss or limitation of employment is common in RA, and the total cost of RA in the UK (including work-related disability) is estimated at between £3.8 and £4.8 billion per year in the UK (12).

Extra-articular manifestations may affect varied tissues including skin (rheumatoid nodules, vasculitis), lung (bronchiolitis obliterans, pleural effusion), nerve (peripheral and compression neuropathies, fatigue), bone (osteoporosis), eye (sicca symptoms, scleritis), kidney (AA amyloidosis), blood (anaemia, neutropenia and Felty's syndrome), heart (pericarditis, myocarditis, and nodules causing conduction defects) and vasculature (atheromatous disease and deep vein thrombosis) (13). RA is associated with increased rates of malignancy, including lymphoma (over three-fold compared to the non-RA population) as well as some solid tumours such as lung cancer (14). Perhaps most profoundly for the physician, RA is associated with increased mortality, a concept which will be explored in later sections.

1.1.3 Diagnosis

There is no single diagnostic test for RA. Plain radiographs can identify erosions, though these typically require years of inflammation to become apparent. Ultrasound scanning (USS) and magnetic resonance imaging (MRI) are able to illustrate active synovitis long before erosive damage has accrued, and are especially helpful in detecting "subclinical" synovitis (15). Importantly, they can also exclude inflammation as a cause of arthralgia, thus avoiding futile and potentially harmful drug therapy (16). Blood may be tested for elevated acute phase reactants such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) but these are normal in up to half of patients at presentation. Autoantibodies, particularly rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA), are more useful. Rheumatoid factor

refers to a variety of immunoglobulin molecules directed against the Fc component of human IgG; IgM rheumatoid factors are most common, but they may also exist in IgG and IgA forms. These were first identified in RA in the 1940s, and are present in around two-thirds of RA patients as well as other conditions such as Sjogren's syndrome and cryoglobulinaemias, and about 5% of healthy over-70s. RF is associated with the presence of extra-articular features and more severe arthritis. ACPA are present in 70-90% of patients, and, like RF, confer a worse prognosis. Modern assays, however, carry sensitivities of around 95% (17). ACPAs are raised against citrullinated proteins such as fibrinogen and vimentin, and are potentially pathogenic (17).

The 2010 American College of Rheumatology (ACR) diagnostic criteria for RA are outlined in Table 1. These can be used in day-to-day practice but are more commonly used for research purposes. The target population for these criteria are patients who have at least one joint with definite clinical synovitis (swelling) without a better alternative explanation; the criteria for RA are met if the sum of the scores from categories A-D is ≥6.

	Score
A. Joint involvement*	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Table 1 - ACR/EULAR classification criteria for rheumatoid arthritis. 'Large joints' refers to shoulders, elbows, knees, hips, and ankles. 'Small joints' refers to MCP joints, PIP joints, 2nd-5th MTP joints, thumb interphalangeal joints and wrists.

1.1.4 Aetiology

The underlying cause of RA remains unknown, but is thought to be due to an environmental "trigger" in a genetically susceptible individual. Twin studies demonstrate a genetic component to the disease, with concordance rates of around 10-15% in monozygotic twins and 3-7% in dizygotic twins (18, 19). These studies conclude that genetic factors account for roughly 60% of disease risk (19). Genome-wide association studies (GWAS) implicate numerous genes

involved in different branches of immune regulation, especially pathways related to T-cell activation and nuclear factor κB (NFκB) (20). The best understood genetic association is with the HLA-DRB1 locus. Possession of the DRB1 allele on its own increases disease risk, but susceptibility is particularly increased with certain haplotypes which share a common five amino-acid sequence termed the "shared epitope". HLA genes code for the major histocompatibility complex II (MHC-II) molecule which presents antigen to T-cells, and the shared epitope can bind citrullinated peptides more strongly than native sequences (21). This has led to the hypothesis that altered antigen binding, antigen presentation or T-cell selection may cause the initial "breach of tolerance" that leads to chronic inflammation. Notably, whilst most shared epitope patients are ACPA positive, ACPA-negative RA seems to involve different HLA alleles, suggesting that ACPA positive and negative RA should perhaps be treated as separate disease entities (22). Risk may also be modulated by epigenetic factors, including histone modification and DNA methylation (23).

Environmental risk factors are also recognised. The most prominent is tobacco smoking: this was first observed in the 1980s (24), and subsequently shown to increase the risk of seropositive RA in several observational studies (25). This increased risk may persist for over 10 years after smoking cessation (26). Interestingly, smoking can act synergistically with the shared epitope to markedly increase ACPA production (27) and RA risk (28, 29), though smoking also seems to increase risk in seronegative patients with the shared epitope (30). It has been suggested that smoking may increase severity of RA, but this may simply be a result of confounding by ACPA status (31).

Various infectious agents, including parvovirus, Epstein-Barr virus, cytomegalovirus, *Escherichia coli* and *Porphyromonas gingivalis*, have been posited as having a causative role in RA. *P. gingivalis* is a common cause of chronic inflammatory periodontitis (32), and expresses peptidyl argenine deaminase IV (PAD4) enzymes which can promote protein citrullination (33). Molecular mimicry has been suggested as a possible mechanism of disease, but this is controversial (34). Discrete infectious agents aside, it is also increasingly recognised that the microbiome as a whole can alter the host's systemic

inflammatory response, and research is ongoing into the roles of microbiota in gut, mouth and lung (35).

1.1.5 Pathophysiology

Synovitis, the signature lesion of inflammatory arthritis, occurs when leukocytes migrate into the synovial membrane and fluid. A variety of immune and inflammatory pathways have been identified in the joint which may contribute to the initiation or perpetuation of synovitis and joint destruction.

1.1.5.1 Cell migration

Infiltration of leukocytes into the joint depends on the activation of endothelial cells in synovial capillaries, where expression of adhesion molecules and chemokines is increased. Actively inflamed synovium in both early and established disease demonstrates increased vascularity on ultrasound or magnetic resonance imaging. This is reflected histologically by increased angiogenesis (36) and inadequate lymphangiogenesis (37) which may be induced by local cytokines or hypoxia.

1.1.5.2 Adaptive immunity

Evidence of a role for adaptive immunity in RA is seen in the contribution of autoantibodies and genes including HLA to disease risk, and the clinical efficacy of B-cell depletion and T-cell co-stimulatory blockade (discussed later). T-cells, including autoreactive T-cells against citrullinated peptides, abound in the synovitic joint, as do B-cells and their plasma-cell descendents. Indeed, synovial T-cell-B-cell aggregates are common, with lymphoid follicle and germinal centre development occasionally seen within the joint (38). An array of factors that may drive this process are also detectable in the joint, including a proliferation-inducing ligand (APRIL), B-lymphocyte stimulator (BLyS)(38), IL-12, IL-15, IL-23, and various chemokines (39). The success of anti-CD20 therapy, which depletes B cells but spares plasma cells, implies a pathogenic role for humoral immunity beyond autoantibody production alone, possibly involving cytokine production or antigen presentation.

Despite these factors, therapies aimed specifically at targeting T-cells (such as cyclosporine-A or monoclonal antibodies against CD4) have shown little or no clinical efficacy (40). More recently, the role of Th-17 cells, which can produce IL-17 family members as well as cytokines such as TNF-α, has been explored. IL-17 can synergise with TNF-α to activate fibroblasts. However, whilst IL-17 blockade is efficacious in ankylosing spondylitis and psoriatic arthritis (41, 42), results in RA have been disappointing (43, 44). An alternative target may be CD25 and Foxp3-expressing "regulatory" T cells ("T-regs"). These cells are capable of suppressing inflammation and re-establishing homeostasis via cytokines including TGF-β and IL-10, and are functionally impaired in RA (45).

1.1.5.3 Innate immunity

Several innate immune cells are present in the inflamed joint. Macrophages, the archetypal cell of chronic inflammation, are derived from circulating monocytes which rely on macrophage colony-stimulating factor (M-CSF) or macrophage-granulocyte colony-stimulating factor (GM-CSF) for migration into and maturation in the synovium. They are primarily activated by receptors which recognise pathogen- and damage-associated molecular patterns (PAMPs and DAMPS, respectively) which are commonly present on bacteria and viruses, but can also be affected by cytokines, immune complexes, oxidised lipoproteins and direct interaction with T-cells. Macrophages are highly capable of phagocytosis, ingesting and destroying pathogens, toxic particles or waste materials. Other cells present include neutrophils (short-lived cells which produce prostaglandins and reactive oxygen species) and mast cells (which can synthesise vasoactive peptides as well as cytokines and chemokines). These cells produce a variety of pro-inflammatory cytokines which drive leukocyte recruitment and survival, endothelial activation, angiogenesis and pain; the most significant of these are discussed later in the context of therapeutic targeting.

1.1.5.4 Bone Erosion

The influx of leukocytes and their behaviour described above has a profound impact on fibroblast-like synoviocytes (FLS), the endogenous stromal cell that constitutes the local tissue cellular phenotype. The healthy synovial membrane

is composed of a thin layer of these cells on a basement membrane, secreting small amounts of viscous synovial fluid to lubricate the joint. In RA, these cells demonstrate both proliferation and resistance to apoptosis, resulting in a massively enlarged membrane, often generating large quantities of fluid (detectable clinically as an effusion). Pro-inflammatory cytokines, amongst other pathways, probably support this behaviour; the recruitment of new mesenchymal cells may also contribute (46).

The resulting volume of tissue containing leukocyte-rich synovium is known as pannus, which manifests as an advancing tissue front which invades and erodes cartilage and bone. FLS synthesise matrix metalloproteinases (MMPs) and other enzymes, which degrade collagen and alter its biomechanical properties. IL-1, TNF and IL-17A drive chondrocytes to apoptosis, retarding the ability of cartilage to regenerate. A variety of other cytokines, including receptor activator of Nf-κB ligand (RANKL), TNF-α, IL-1 and IL-6, promote osteoclast differentiation and invasion into bone beside the articular cartilage (47); osteoclasts enzymatically destroy mineralised bone in discrete pits which are visible on plain radiography as erosions. Bone marrow inflammation may also occur, forming a focus of tissue promoting immune dysfunction (48). Unlike synovitis, these bone and cartilage changes are essentially irreversible, and lead to lifelong loss of joint function and disability.

1.1.6 Drug treatments for rheumatoid arthritis

Most current treatments in RA are aimed at suppressing the inflammatory response to reduce synovitis, improve symptoms and prevent erosions. These can be broadly divided into steroid therapy, conventional disease-modifying anti-rheumatic drugs (DMARDS), biologic drugs and JAK inhibitors.

1.1.6.1 Corticosteroids

Corticosteroids were identified in the 1940s as agents which could profoundly and rapidly improve joint inflammation; a cine-film of a previously bedridden RA patient walking independently was hailed as a “modern miracle” and landed Kendall, Reichstein and Hench the 1950 Nobel Prize in Physiology or Medicine. More recently, the Utrecht (49) and CAMERA II (50) trials demonstrated the

ability of 10mg/day prednisolone to both reduce disease activity and reduce radiological disease progression. This relatively low dose is important, as long-term corticosteroid use carries a litany of potential side effects, including osteoporosis, infection, insulin resistance, weight gain, and cardiovascular disease. These effects appear to be dose-dependent (51, 52), though the possibility of confounding by indication is hotly debated (53). Some centres (including my own) attempt to minimise these risks by using intra-muscular (IM) depot or intra-articular (IA) delivery.

1.1.6.2 DMARDS

Conventional DMARDS also aim to reduce inflammation and disability, without corticosteroid-related harms. Methotrexate (MTX), a dihydrofolate reductase inhibitor, is perhaps the most commonly used. Its mode of action in RA is unclear but probably involves reduced availability of intracellular adenosine to leukocytes; folate supplementation improves adherence without reducing efficacy (54). Oral administration of 10-20mg weekly is usual but IM or subcutaneous (SC) preparations can have increased efficacy and tolerance, and may avert unnecessary and expensive switch to biologic drugs in some patients (55). Common side effects include nausea, headache, mouth ulcers and infection; neutropenia, marrow suppression, transaminitis and pneumonitis are rarer. MTX is highly teratogenic and must be avoided during pregnancy or breastfeeding. Monthly blood monitoring is recommended for the first year of treatment (56).

Sulfasalazine (SSZ) was the first DMARD synthesised specifically for treatment of RA by the addition of a sulfapyridine group to 5-aminosalicylic acid, and was shown in controlled trials to be as effective as gold (57) and penicillamine (58). The side effect profile may be slightly better than MTX, with headache and GI disturbance the most common, and the drug may cause transient oligospermia and therefore reduced fertility in men. Monthly monitoring of full blood count, renal and liver function is recommended initially, and quarterly thereafter (56). A third agent is the antimalarial hydroxychloroquine (HCQ). HCQ tends to be well tolerated, though retinopathy is a rare but serious complication; annual review by an optician is recommended. Both SSZ and HCQ have multiple anti-inflammatory and possibly antimicrobial effects, though their exact mechanisms

of action in RA are unknown. Other DMARDS used less frequently include leflunomide, penicillamine and gold salts. A systematic review has shown these agents to have roughly equivalent efficacy (59) but methotrexate remains the most commonly used first-line agent in our centre.

1.1.6.3 "Biologics"

Biologic medications are so names as they mimic naturally-occurring substances in the human body. Most biologics are monoclonal antibodies, and can be divided into several categories.

Cytokine blockade: Tumour necrosis factor alpha (TNFα) is a potent pro-inflammatory cytokine produced by macrophages, dendritic cells and T-cells in response to an assortment of molecular patterns on microbes – for example, bacterial lipopolysaccharide (LPS) binding to toll-like receptor (TLR4) and signalling via the NFκB pathway. TNFα can drive inflammation by activating endothelial cells and neutrophils, and has other systemic effects such as fever via the hypothalamus. In vitro work in the 1980s implicated TNFα in cartilage destruction (60), and inhibition of TNFα in mouse models improved joint swelling and erosive progression (61). These results were replicated in several large human studies with Infliximab, a chimeric monoclonal antibody (Mab) against TNFα (62). Today, five TNF blockers are licensed for use in RA: infliximab; the fully human Mabs adalimumab and golimumab; certulizumab pegol, a humanised antigen-binding fragment of antibody conjugated to polyethylene glycol; and etanercept, a soluble TNF-receptor fusion protein. These drugs are efficacious in many patients but must be given with methotrexate for full benefit. Risks of therapy include infection (particularly tuberculosis), cardiac failure, allergic reactions and possibly melanoma (63), though other malignancies do not seem to be increased (64). Other attempts at cytokine blockade, including IL-1 (65) and IL-17 (43), have been less successful and are not recommended for use in clinical practice (66); targeting of GM-CSF may hold more promise (67). IL-6 blockade will be discussed in depth later in the manuscript.

B-cell depletion: Rituximab is a chimeric antibody which binds to the CD20 molecule on B cells. This induces depletion of B cells in peripheral blood (68) but allows reconstitution as CD20 is not present on pro-B cells. Administration

of two infusions two weeks apart improves disease activity, with results still apparent after 48 weeks in some patients (69). Efficacy seems significantly better in rheumatoid factor-positive patients (70). Infusion reactions are common, and hypogammaglobulinaemia with repeat infusions may lead to opportunistic infection(70). An alternative method of depleting B-cells by targeting B-lymphocyte stimulator (BLyS, also known as BAFF) has failed to consistently demonstrate efficacy in RA (71).

Co-stimulatory blockade: Abatacept is a fusion protein of recombinant cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and the Fc portion of human IgG1. By binding to CD80/86 on antigen-presenting cells, and thus preventing ligation of CD28 on the T-cell membrane, this drug prevents the co-stimulatory signal required to activate T-cells. Abatacept is superior to placebo (72) and non-inferior to adalimumab (73) in RA.

1.1.6.4 JAK inhibition

Many cytokine receptors (including IL-6R) transduce their signal via janus kinases (JAKs), named after the Roman deity Janus for their two phosphate-binding domains. Receptor ligation triggers phosphorylation of the intracellular part of the receptor by JAK; this then recruits signal transduction and activation of transcription (STAT) proteins which are themselves phosphorylated by JAK. Phosophorylated STAT (pSTAT) then migrates to the nucleus to act as a transcription factor for cytokine-dependent genes. Three JAKs (numbered 1-3) have thus far been identified. Inhibition of JAK activity as therapy for RA has two main conceivable advantages: use of a biological “bottleneck” to block signalling of multiple cytokines with one agent, overcoming cytokine redundancy and obtaining a greater chance of clinical efficacy; and the ability to deliver these small molecules orally, as opposed to the parenteral administration required for large proteins such as Mabs. Tofacitinib is a JAK1/3 inhibitor which has shown efficacy greater than MTX (74) and similar to adalimumab (75) in RA; it is licensed for use in the United States of America but is not currently approved by the European Medicines Agency due to concerns over its side effect profile. Baricitinib, a selective JAK1/2 inhibitor, has demonstrated efficacy in a phase III trial (76), and other JAK inhibitors with different specificities are being developed (77).

1.1.7 Treatment strategies

Modern treatment of RA typically follows a "treat-to-target" strategy aimed at achieving low disease activity or remission, especially in early RA (78). Various regimens have been employed toward this goal, including step-up (78, 79), parallel (80) and step-down (81) combination DMARDs. All these approaches seem superior to sequential DMARD monotherapy at reducing disease activity and preventing radiographic progression, but it is unclear which is the most effective (82-84), and about a third of patients can achieve good control with a single DMARD (84, 85). Due to the side-effect profiles and costs of biologics (around £9,000 per year per patient), prescription in the UK is restricted to patients who have a 28-joint disease activity score (DAS28) greater than 5.1 (reflecting severe active disease) and have failed two conventional DMARDS (12). Initial therapy with TNF blockers in a step-down approach achieves high rates of low disease activity, but many patients still either do not respond or remain reliant on their biologic drug (86). In keeping with international guidelines (66) our unit prefers initial methotrexate therapy with bridging intramuscular or intra-articular corticosteroids, stepping up to combination DMARD therapy and then to biologic drugs if low disease activity is not reached.

Early initiation of treatment has been shown to improve treatment response (87) and functional status (88), and improve long-term radiological outcomes (89). In one study, even a short delay in treatment (>4 months) was associated with worse disease activity 2 years later (80). Current guidance (66) emphasises the importance of early intervention, as well as treating to target with regular, frequent clinical review, and use of the multi-disciplinary team including physiotherapists, occupational therapists and specialist nurses.

1.1.8 IL-6 in rheumatoid arthritis

A variety of cytokines have been implicated in RA pathogenesis, particularly TNF-α as outlined above. In recent years considerable attention has been paid to another pro-inflammatory cytokine, interleukin 6 (IL-6), both in understanding its pathogenic role in RA and also its potential as a therapeutic target for various inflammatory or autoimmune disorders. IL-6 is found at high concentrations in the serum (90), synovial tissue (91) and synovial fluid (92) of RA patients. First

identified as a driver of B-lymphocyte proliferation, it was originally named B-cell stimulatory factor 2. In the intervening three decades, it has become apparent that IL-6 has a myriad of immunological and inflammatory functions, and its therapeutic blockade is now a valued treatment option in active RA.

1.1.8.1 IL-6 molecular biology and signalling

IL-6 is a 21 - 28kDa glycoprotein encoded by the IL6 gene on chromosome 7. In human plasma, it is normally present at a concentration of 5-10pg/ml, though this can increase hugely in infection or inflammation; in severe sepsis, it may approach 1μg/ml (though this is in the context of illness which may not be survivable). It exerts its influence by ligating the IL-6 receptor (IL-6R) protein, which can be found on the membranes of hepatocytes, vascular endothelial cells, and some leukocyte populations (including megakaryocytes, B cells, CD4+ T cells, neutrophils, monocytes and macrophages). IL-6R lacks a transmembrane domain, but on ligation will recruit the gp130 protein, which does span the cell membrane and can bind janus kinases (JAKs) on its intracellular aspect. Gp130 then dimerises, phosphorylating and activating JAKs and subsequently the transcription factor signal transduction and activator of transcription 3 (STAT3). Phosphorylated STAT3 (pSTAT3) then continues an intracellular signalling cascade which leads to altered gene expression. The above process, referred to as “cis-” or “classical” signalling, occurs in cells carrying membrane-bound IL-6R (mIL-6R). However, IL-6R also exists in a soluble form (sIL-6R), generated either by alternative splicing or (more commonly) by shedding of mIL-6R by the membrane-bound protease ‘a disintegrin and metalloprotease 17’ (ADAM17). The complex of IL-6 and sIL-6R can then bind gp130 (which is expressed ubiquitously), and thus transduce a signal. This “alternative” form of IL-6 signalling, or “trans-” signalling, means that IL-6 can transduce a signal in virtually any cell in vivo (93) Most intriguingly, evidence is emerging that cis- and trans-signalling may generate anti- and pro-inflammatory responses respectively (94). SIL-6R may also have an inhibitory impact on IL-6 signalling, as the IL-6/sIL-6R complex can also be sequestered by circulating soluble gp130, preventing ligation of membrane-bound gp130 or mIL-6R (93).

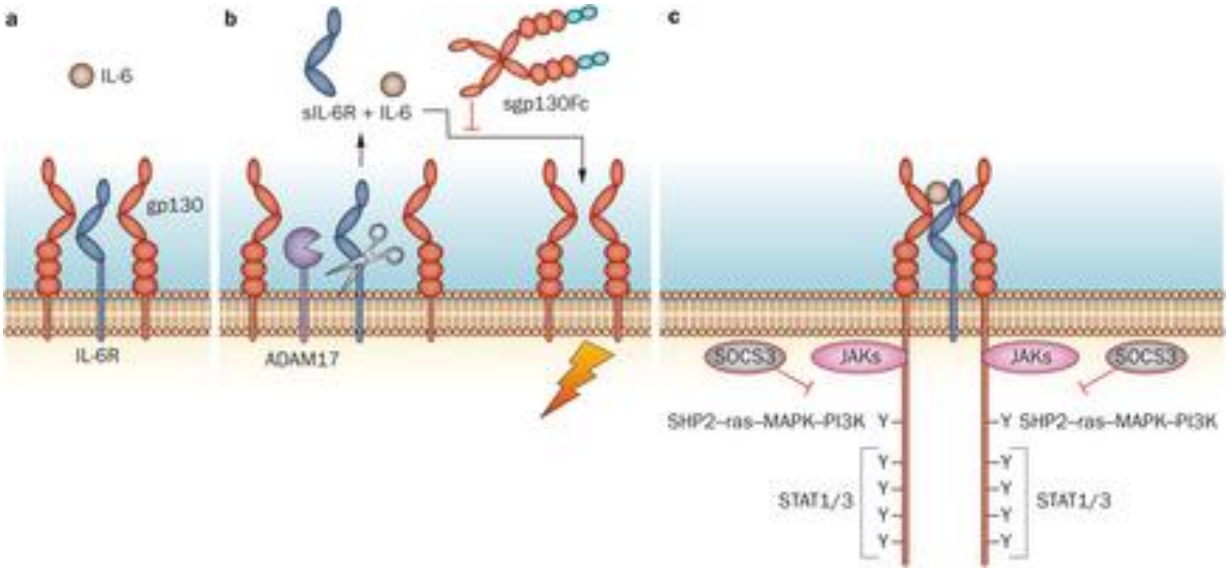


Figure 1 - IL-6 ligates (a) membrane-bound IL-6R or (b) sIL-6R, in this case generated via cleavage of IL-6R by ADAM17. The IL-6/IL-6R complex binds to gp130, with subsequent activaiton of the JAK/STAT pathway. From: Calabrese et al. *Nat Rev Rheumatol 2014*; 10(12):720-7

1.1.8.2 Role of IL-6 in RA Pathophysiology

In vitro experiments have shown that IL-6 can induce several features of inflammation, including production of chemokines and leukocyte adhesion molecules (95), demargination of neutrophils (96), angiogenesis (97) and proliferation of fibroblast-like synoviocytes (98). Il-6 can also drive bone damage by stimulating production of RANKL (which induces osteoclastogenesis) (98), and matrix metalloproteinases (which degrade cartilage) (99). Beyond the joint, IL-6 is responsible for the liver's acute phase response – a term for the collective expression of various proteins in response to infectious or inflammatory insult, which can be quantified in clinical practice by serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) or plasma viscosity. IL-6 also drives the phenomenon of anaemia of chronic disease by inducing expression of hepcidin by the liver, which impairs release of iron from body stores and thus reduces the availability of iron for erythrocyte precursors in the bone marrow. This leads to a characteristic normochromic, normocytic anaemia with normal (or elevated) serum ferritin but reduced serum iron and transferrin saturation. IL-6 also plays a role in the generation of autoimmunity

in mouse models of arthritis, with co-stimulation of IL-6 and TGF-ß required for generation of Th-17 cells in vitro (100).

1.1.8.3 Tocilizumab and IL-6 blockade in RA

The above data suggested that selective blockade of IL-6 could be a viable therapeutic strategy. To this end, tocilizumab is a humanised monoclonal antibody directed against both soluble and membrane-bound forms of the IL-6R; its epitope is the cytokine binding site on the IL-6R, making it in effect a competitive antagonist of IL-6.

An extensive phase III programme has demonstrated tocilizumab's efficacy in RA in DMARD-naive patients (101), inadequate responders to DMARDS (102-106), and patients who have not responded to with anti-TNF drugs (107-109). At a standard dose of 8mg/kg in combination with conventional DMARDS, tocilizumab led to ACR20 responses of approximately 60% by 24 weeks (102, 105). Doses of 4mg/kg typically gave more modest responses which were nevertheless superior to placebo (103, 108). Onset of action was rapid, with falls in mean tender and swollen joint counts observed after 4 weeks (110), and drug efficacy was maintained in long-term follow-up studies of up to 5 years (111, 112). Improvements in functioning and independence, as assessed by the Health Assessment Questionnaire (HAQ) were also observed (102, 105, 106, 108). Efficacy in patients who had failed anti-TNF was less impressive than for anti-TNF-naive patients, but remained superior to placebo (107, 108, 110). Several studies also demonstrated the ability of tocilizumab to reduce radiographic progression (103, 104, 113); this reduction was observed even in the presence of persistent synovitis or high disease activity (114), a feature also described with anti-TNF agents and rituximab. Whilst tocilizumab was initially licensed for intravenous (IV) delivery, a subcutaneous formulation has been approved on the basis of two RCTs which demonstrated non-inferiority of subcutaneous tocilizumab 162mg weekly compared to IV (115, 116).

In contrast to the other biologic agents licensed for the treatment of RA, tocilizumab appears effective as monotherapy, with only small additional benefit attained from combination with methotrexate (109, 113, 117). One trial did, however, suggest superiority of combination therapy for the higher

response targets of ACR70 (37% vs. 16%) and DAS28 remission (34% vs. 17%) (117) suggesting that concomitant DMARD use is probably best practice in order to obtain good disease control or remission in as many patients as possible. In addition, several studies demonstrated significantly greater efficacy of tocilizumab monotherapy compared to conventional DMARD monotherapy (101, 104, 111). This stands in contrast to studies of anti-TNF agents which have consistently shown significant improvement in efficacy when these agents are used in combination with MTX, and which have struggled to demonstrate superiority of anti-TNF agents over DMARDS when used as monotherapy (118). This contrast is best exemplified by the ADACTA study, a head-to-head comparison of tocilizumab 8mg/kg monotherapy against adalimumab monotherapy which demonstrated superiority of tocilizumab for ACR50 (47% vs. 28%), EULAR response (78% vs. 55%) and DAS28 remission rates (40% vs. 11%) (119). These findings were not significantly altered by use of scoring systems which did not rely on the acute phase response. Studies also demonstrated that the ability of tocilizumab monotherapy to reduce radiographic progression (104, 113). These findings are highly relevant to clinical practice as up to 30% of RA patients on biologics are not prescribed MTX despite international guidelines (120).

1.1.8.4 Toxicity of tocilizumab

The most common adverse event attributed to tocilizumab is infection. A serious infection rate of 47 per 1000 patient years was reported in a meta-analysis of pooled clinical trial data (121), with similar rates reported in phase IV post-marketing studies (111, 122, 123). These figures are comparable to those seen with TNF inhibitors. Bacterial pneumonia was the most commonly reported infection. The meta-analysis detected 7 cases of TB in 1,870 patients treated with 8mg/kg tocilizumab (121) with pre-treatment screening for TB recommended in treatment guidelines. However, the biological role of IL-6 in tuberculosis is unclear, and all the large phase III studies screened for latent TB prior to enrolment. Use of live or attenuated vaccinations is not recommended, though vaccination against influenza remains effective (124).

In pooled clinical trial data, tocilizumab increased mean haemoglobin by around 1g/dl within 6 weeks (in keeping with IL-6 driving anaemia of chronic disease),

but was associated with dose-related falls in mean neutrophil and platelet counts (121), probably due to neutrophil margination and reduced platelet production respectively. Liver transaminase level increases were similar levels in patients treated with MTX monotherapy and tocilizumab monotherapy. The percentage of patients with alanine transaminase (ALT) levels >3 times the upper limit of normal was 1.9% with tocilizumab monotherapy, 3.7% with MTX monotherapy and 5.7% with combination tocilizumab and DMARD (121). A significant proportion of these patients were able to continue on a reduced dose of tocilizumab. EULAR guidelines recommend blood tests every 4-8 weeks for 6 months, and 3-monthly thereafter. Treatment should be terminated if transaminases are elevated >5x the upper limit of normal (ULN) or persistently >3x ULN; elevations 1-3x ULN can be managed with dose reduction to 4mg/kg or treatment interruption (125). Treatment should also be terminated if neutrophil count falls <500/mm^3, and interrupted at counts of 500-1,000/mm^3 (125).

Other reported adverse events include bowel perforation, predominantly in patients with previous diverticulitis or on concomitant corticosteroid or NSAID therapy (126), and one reported case of leukoencephalopathy (127). Data are limited on pregnancy are limited; recent guidelines from the British Society of Rheumatology advise stopping treatment three months before conception, but note that “exposure early in the first trimester is unlikely to be harmful” (128). No increased risk of malignancy has yet been detected in clinical trials or long-term follow-up studies (111, 121); however, large-scale observational studies addressing this issue are currently lacking.

1.1.8.5 Tociluzmab efficacy in other inflammatory conditions

Given IL-6’s role in Th-17 cell generation, tocilizumab has been used experimentally in seronegative spondyloarthritides. The BUILDER studies (129) aimed to evaluate tocilizumab’s efficacy in ankylosing spondylitis. Unfortunately, analysis of preliminary results showed no indication of efficacy, and the studies were terminated. Case reports have suggested efficacy of tocilizumab in some patients with psoriatic arthritis (130, 131). More interestingly, a recent phase II study (132) reported promising results with tocilizumab being used to treat giant cell arteritis (GCA), a large vessel vasculitis

which commonly demonstrates significant elevations in ESR and CRP. 85% of patients treated with tocilizumab reached disease remission at 12 weeks (compared to 40% on placebo), with no disease relapses recorded after 52 weeks of follow-up; the tocilizumab group also required significantly smaller cumulative doses of corticosteroids. Similarly impressive results have been obtained in the related condition of polymyalgia rheumatica (133) and in the rare lymphoproliferative disorder Castleman's disease, where B-cell proliferation is driven by abnormal production and signalling of IL-6 (134).

1.2 Cardiovascular disease

Cardiovascular disease (CVD) describes a process of progressive blood vessel dysfunction and occlusion which results in tissue damage and subsequent patient disability or death. Accumulation of lipid plaques ("atheroma") in arterial walls, a state known as atherosclerosis, leads to restriction of blood flow and tissue ischaemia; depending on the vessels involved, this may manifest clinically as angina (coronary arteries supplying the myocardium, also known as coronary heart disease [CHD]), transient ischaemic attack (carotid or cerebral arteries supplying the brain) or intermittent claudication (femoral and more distal arteries supplying the legs). Plaque rupture and subsequent thrombus formation can occlude the vessel entirely and cause tissue infarction, manifesting clinically as myocardial infarction, stroke or critical limb ischaemia. Prompt restoration of lumen patency by pharmacologic thrombolysis or percutaneous angioplasty may salvage ischaemic tissue and prevent disability or death.

The scale of the global CVD burden is intimidating. With 17.5 million deaths in 2012 (equating to 31% of all deaths), it is the largest single cause of death worldwide (135). In the UK, CHD is the single largest cause of death with around 73,000 people dying from this disease every year; this equates to roughly one victim every seven minutes. Most of these deaths result from one of the 175,000 myocardial infarctions that occur annually in the UK. Stroke, meanwhile, causes 40,000 deaths per year. When combined with related conditions including atrial fibrillation, cardiomyopathy and heart failure, CVD costs the UK economy an estimated £19 billion per year (136). Of note,

however, the number of deaths from CHD has halved in the last fifty years, due to advances in both disease prevention and treatment (137).

Scotland has an especially poor record of cardiovascular health with over 7,000 deaths from CHD annually. Whilst mortality rates have improved over the past fifty years, they remain 30-40% higher than in England (137), and treating and preventing CVD is a priority for the Scottish Government (138). Scotland has high levels of smoking, obesity and social deprivation, all of which impact CVD prevalence and mortality (139) and which are taken into account by the Scottish ASSIGN CVD risk calculation algorithm.

1.2.1 Pathophysiology of cardiovascular disease

1.2.1.1 Blood vessel anatomy

To understand the pathophysiology of atherosclerosis, it is necessary to outline the anatomy of a typical artery. All but the smallest vessels are composed of three layers: the tunica intima; tunica media; and tunica adventitia. The innermost tunica intima immediately surrounds the blood-containing lumen and consists of a single layer of endothelial cells on a thin membrane of type IV collagen, laminin and proteoglycans. Vascular endothelial cells together form a highly functional tissue which interacts with blood and the rest of the vessel wall, regulating nutrient exchange, vascular tone and coagulation. An internal elastic lamina lies between the intima and the tunica media. The media is a layer of smooth muscle cells which can alter vessel tone to maintain local blood pressure and flow, resting on an external elastic lamina. The outermost tunica adventitia is a loose arrangement of connective tissue, fibroblasts, nerves and capillaries which supply the vessel wall.

1.2.1.2 Atheroma formation

The pathogenesis of an atheromatous plaque is increasingly well understood, and may be described in four stages (Figure 2).

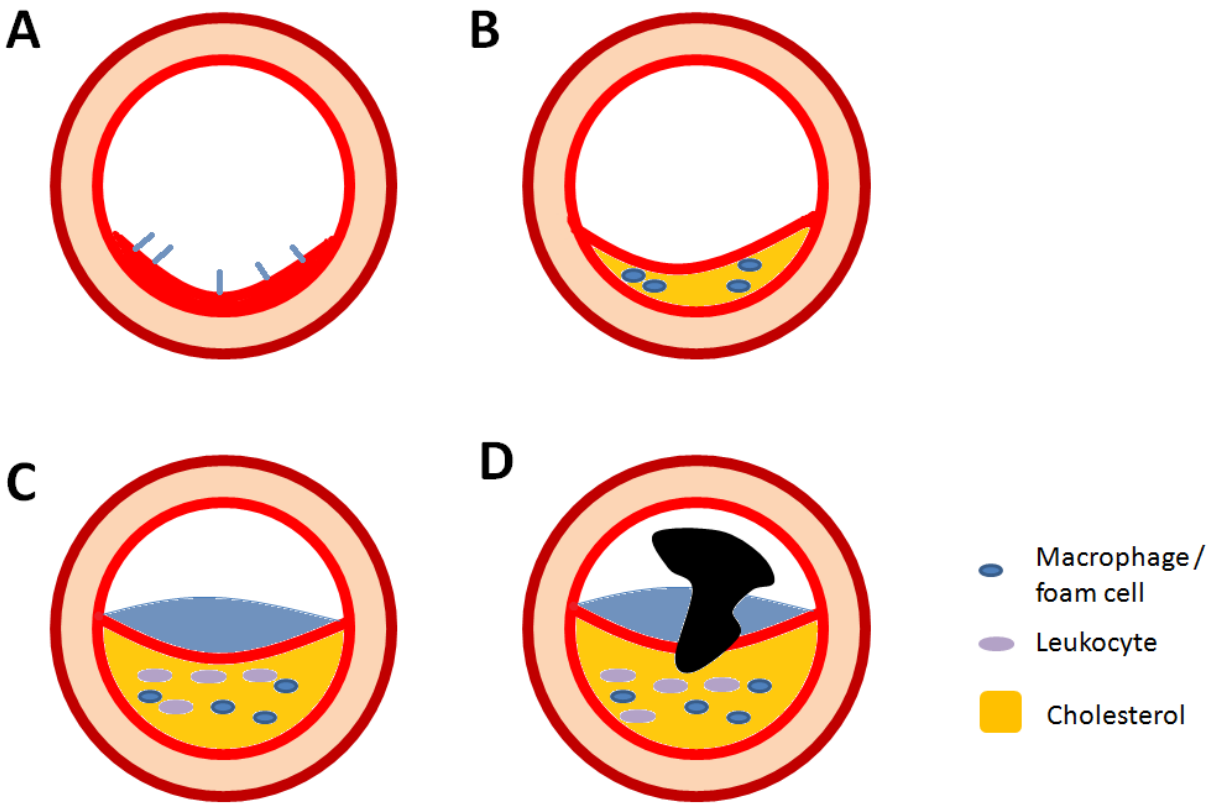


Figure 2 - Stages of atheroma formation. A: Endothelial activation (adhesion molecules denoted in blue). B: Fatty streak formation with foam cells. C: A mature lesion (fibrous cap denoted in gray). D: Plaque rupture (thrombus denoted in black). (author's own design)

1 - Endothelial activation and the initial lesion: In most models of atherosclerosis, the disease process is initiated by activation of the vascular endothelium. Activated endothelial cells upregulate adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) and chemokines including monocyte chemotactic protein 1 (MCP-1), macrophage colony-stimulating factor (M-CSF) and IL-8, permitting recruitment of circulating leukocytes (particularly monocytes) into the tunica intima (140-142). Endothelial activation seems to be driven predominantly by complex, disturbed blood flow patterns and the resultant wall stresses; arterial segments with disturbed flow (eg. carotid bifurcation, aortic arch) are more prone to atheroma formation, whilst uniform shear stresses lead to endothelial expression of atheroprotective genes including endothelial nitric oxide synthase (eNOS) and cyclooxygenase 2 (COX-2)(143). Suppression of these genes, particularly eNOS, can manifest as endothelial dysfunction, a phenotype characterised by

diminished production of nitric oxide (with reduced vasodilatory and anti-oxidant capacity) and a pro-coagulant state. Perhaps most importantly, dysfunctional endothelium becomes more permeable to apoB-containing lipoproteins, especially small dense low density lipoprotein (LDL) particles which are prone to oxidative modification; these particles can themselves activate endothelium and thus potentiate the disease process (144). Of note, TNFα can also upregulate VCAM-1 (145) and pro-thrombotic factors (146) and reduce eNOS and COX-2 activity (147).

2 - The fatty streak: Monocytes recruited to the tunica intima differentiate into tissue macrophages. These macrophages ingest oxidised LDL (oxLDL) and other lipoproteins via scavenger receptors, a family of cell surface molecules used to clear pathogens or apoptotic cell debris (148). Scavenger receptors play a key role in atherosclerosis as oxidative modification of the apoB molecule on LDL can prevent it from binding to the LDL receptor and being cleared in the conventional manner (148). The resulting lipid-laden macrophages are known as foam cells, from the microscopic appearance of their lipid droplets; accumulation of these cells leads to the first visible form of atheroma, termed the "fatty streak". The importance of LDL-cholesterol (LDL-c) as a driver of this process will be explored later.

3 - The mature stable lesion: Over time, the immature "fatty streak" lesion is modified by migration of smooth muscle cells (SMCs) from the media to the intima. Driven by endothelial cells, macrophages and T-lymphocytes (149), SMCs proliferate and produce extracellular matrix molecules to form a fibrous cap over the lesion. Underlying the cap is a lipid-rich core of LDL, free cholesterol and apoptotic and necrotic macrophages; a rich milieu of lymphocytes, dendritic cells and mast cells also accumulates, in a pattern remarkably similar to a synovitic joint. In this environment, macrophages may secrete extracellular matrix-degrading enzymes, and the stability of the mature plaque is reliant on the balance between these and the viability and matrix products of SMCs (150).

4 - *Plaque rupture & thrombosis:* Stable atheromatous plaques may be asymptomatic, and significant clinical manifestations of atherosclerosis are

usually due to plaque rupture and thrombus formation. Macrophages again seem critical to this event: macrophages of a "pro-inflammatory" or M1 phenotype, predominate at the rupture-prone shoulder region of the plaque, with both M1 and M2 macrophages observed around the fibrous cap (151). Exposure of subendothelial connective tissue or the necrotic core triggers platelet aggregation and activation of the coagulation cascade; this leads to lumen occlusion and downstream tissue ischaemia and infarction. Circulation can be restored, tissue saved and mortality avoided by prompt administration of antiplatelet or anti-thrombolytic drugs (152), or by percutaneous angioplasty (153).

1.3 Cholesterol and lipoprotein metabolism

Several types of lipid, and the lipoprotein structures in which they are transported around the body, play roles in the development of atherosclerosis. Most prominent amongst these is cholesterol.

Cholesterol is a lipid with numerous key roles in mammalian biology. It is a key component of the cell membrane, and is also a precursor molecule for bile acids, steroid hormones and vitamin D. Its structure is shown in Figure 3 – it is a planar molecule composed almost entirely of carbon and hydrogen, with a molecular weight of 386Da.

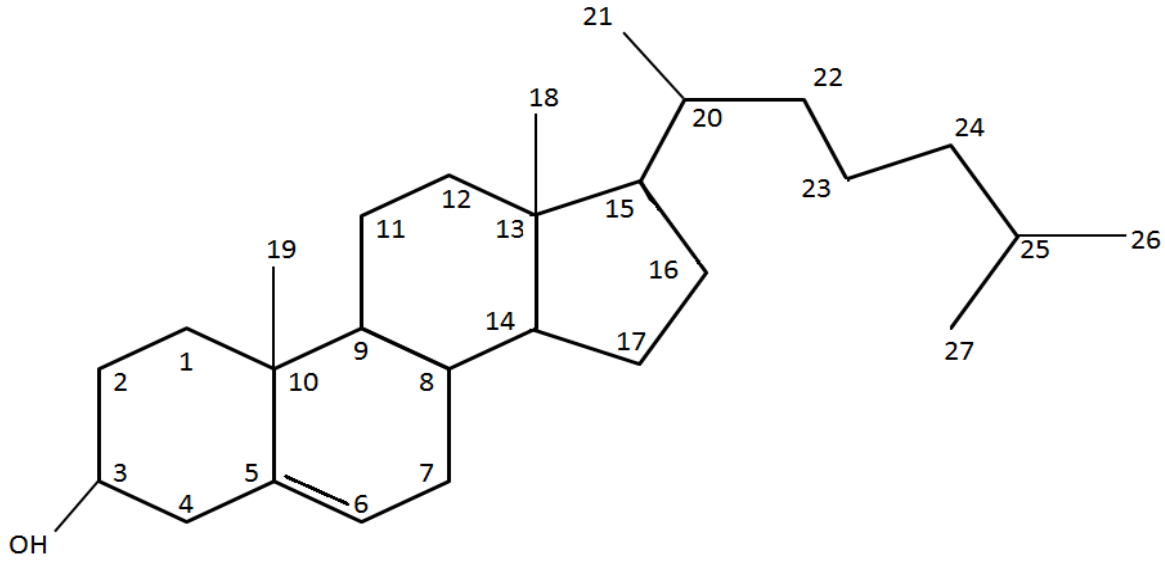


Figure 3 - Structure of cholesterol, with carbon atoms numbered 1 – 27. Adapted from "Medical Biochemistry" 2nd Ed., Baynes & Dominiczak, Elsevier Mosby (Philadephia).

A typical Western daily diet contains around 500mg (1.2mmol) of cholesterol, predominantly in meat, eggs and dairy products. Normally, approximately half of this is absorbed by the gut. However, cholesterol may also be synthesised de novo by human cells. The majority of cholesterol synthesis occurs in hepatocytes, though gut, adrenal glands and gonads also contribute. Beginning with the molecule acetyl CoA (itself derived from fatty acids or amino acids), this process is lengthy with numerous intermediate molecules and crucial enzymes. However, the rate-limiting step occurs early on with the conversion of HMG CoA to mevalonate by the enzyme HMG CoA reductase (Figure 4). Statins, inhibitors of HMG CoA reductase, typically reduce total serum cholesterol by around 20%, and this translates to a relative risk reduction in cardiovascular mortality of 25-30% (153).

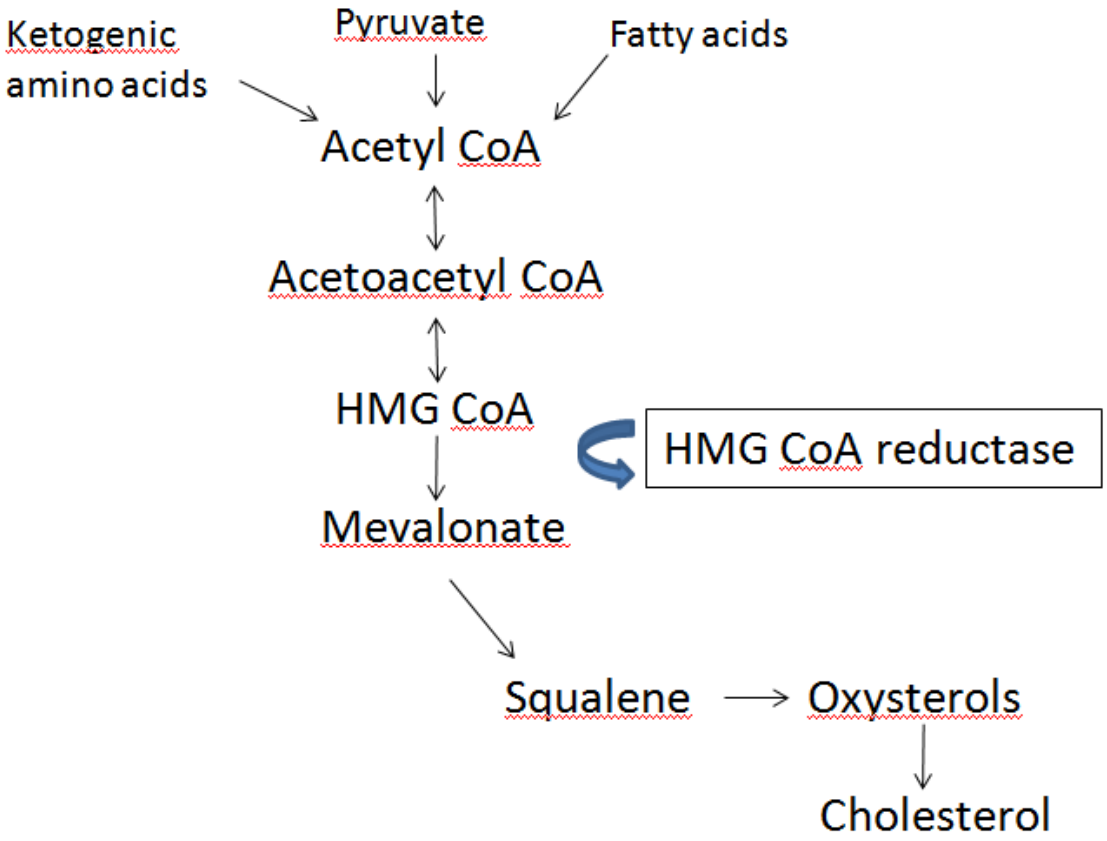


Figure 4 - Abridged schematic of cholesterol biosynthesis (author's original design)

Free cholesterol accounts for around 30% of circulating cholesterol. The remainder is bound to various long chain fatty acids to make cholesterol esters. Neither free nor esterified cholesterol are particularly soluble in water, and so cholesterol in serum is almost entirely transported within specialised molecular structures called lipoproteins.

1.3.1 Lipoprotein structure

Lipoproteins are roughly spherical particles measuring 5 to 1,200 microns in diameter. They consist of a phospholipid monolayer membrane which holds proteins called apoproteins embedded within. This allows the lipoprotein to be very soluble in water whilst bearing large quantities of poorly-soluble content internally, mainly triglyceride and cholesterol. Lipoproteins exist on a spectrum of size and density. The largest and least dense are chylomicrons, followed by: very low-density lipoprotein type 1 (VLDL1) or type 2 (VLDL2); remnant particles following VLDL metabolism, including intermediate-density lipoprotein (IDL); low-density lipoprotein (LDL); and high-density lipoprotein (HDL). The largest and least-dense particles contain large amounts of triglyceride, with TG content reduced in smaller, denser particles. LDL and HDL are triglyceride-poor and cholesterol-rich.

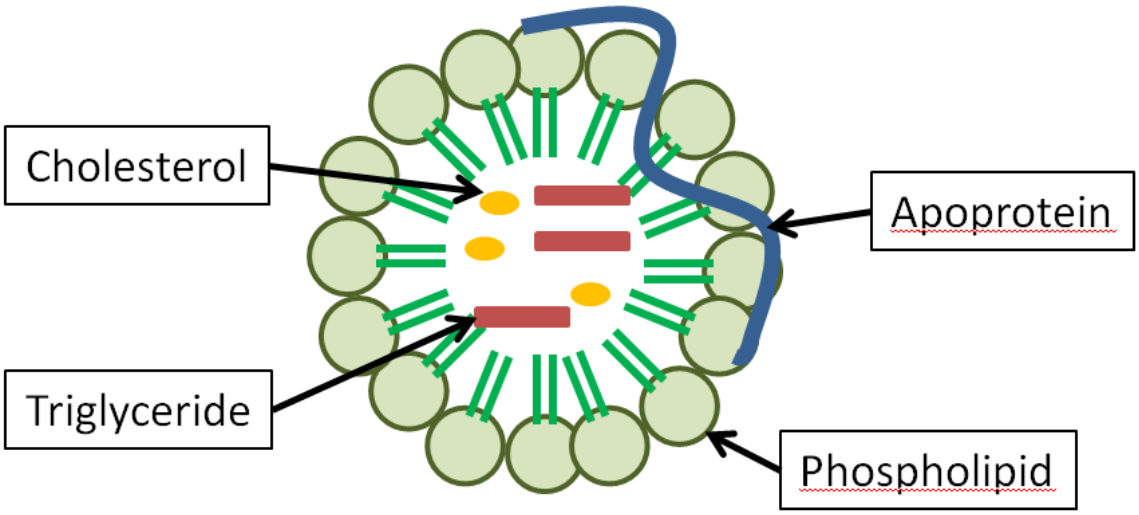


Figure 5 - Schematic structure of a lipoprotein particle. The external coating consists of a phospholipid monolayer bearing at least one apoprotein. Triglycerides and cholesterol (largely cholesterol esters) are present in the core. Adapted from "Medical Biochemistry" 2nd Ed., Baynes & Dominiczak, Elsevier Mosby (Philadephia).

Apoproteins, whilst making up only a small proportion of lipoprotein particle mass, are vitally important as they can bind with receptors on cell membranes and activate or inhibit enzymes which direct the metabolic fate of the particle. Apoproteins A (AI and AII) are present on HDL, and allow HDL to bind to hepatocytes for cholesterol transfer; multiple copies of apoAI or apoAII can be present on each HDL particle. Apoprotein B, in the form of apoB100, is present on VLDL, IDL and LDL, and binds to the LDL receptor (LDLr). Each particle contains only one apoB100 protein. For the purposes of this thesis, "apoB" will refer to this apoB100 molecule; a shortened form of apoB, apoB48, is present on chylomicrons, but cannot bind to the LDLr. ApoE is present on remnant particles and also binds to the LDL receptor, whilst apoCI, CII and CIII may be transferred between different lipoprotein subclasses and act as a regulator for enzymes such as lipoprotein lipase (LPL) and cholesterol ester transfer protein (CETP). One unusual lipoprotein particle of import is known as lipoprotein (a), or Lp(a). This is essentially an LDL particle (containing one apoB100 molecule) linked to a glycoprotein of between 200 and 800kDa called apo(a). Lp(a) has been found to a strong independent predictor of cardiovascular disease (154).

1.3.2 Lipoprotein receptors

In recent years it has become apparent that there exists a family of molecules derived from or related to the LDLr, and some of these play notable roles in lipid metabolism. The VLDL receptor (VLDLr) is structurally similar to the LDLr but can bind to a broader repertoire of apoE allelic variants in concert with lipases; hence, VLDLr preferentially metabolises triglyceride-rich VLDL and remnants in tissues such as heart, skeletal muscle, liver, adipose tissue and macrophages (155). Another functionally prominent molecule is the LDL receptor-related protein (LRP-1), a large protein which binds apoE (but not LDL), and binds remnant lipoproteins in conjunction with LDLr.

Separate from the LDLr family, but of key importance in cholesterol-rich lipoprotein metabolism are a broad range of cell-surface molecules known as the scavenger receptors. These receptors are present on leukocytes, especially macrophages, and can act to trigger the immune response by binding to a variety of pathogen-associated molecular patterns (PAMPS) or bacterial products such as lipopolysaccharide (LPS). However, most scavenger receptors are also capable of binding and internalising LDL which has been modified by acetylation or oxidation (156). This is of clinical relevance as oxidised LDL (oxLDL) may undergo conformational change in the apoB molecule rendering it unable to bind to the LDLr; it then instead becomes prone to removal by macrophages in, amongst other places, the subendothelial space, leading to foam cell formation. Peritoneal macrophages from ApoE -/- mice with multiple scavenger-receptor knockouts demonstrated reduced foam cell formation (156), although further studies using this animal model have not demonstrated reduction in atherosclerotic burden (157, 158). Probable targets are outlined in Table 2, though it is likely that this represents incomplete knowledge as well as an element of redundancy in this broad family of molecules. As oxidation can occur in virtually any inflammatory environment, it stands to reason that the immune system would have multiple mechanisms for clearing such epitopes effectively before they cause tissue damage or generate autoimmune responses. Scavenger receptor B-1 can also bind HDL, and has been proposed as a candidate for the putative hepatic HDL receptor.

Scavenger receptor	Lipoprotein binding	Pathogen recognition
SR-AI/II	AcLDL, oxLDL	Listeria monocytogenes, Staphycoccus aureus, Neiserria meningitides, Streptococcus pyogenes, Group B Streptococcus
MARCO	AcLDL	LPS, Streptococcus pneumonia, Neiserria meningitides
CD36	AcLDL, oxLDL	Microbial diacylglycerides, Mycoplasma pneumonia, Staphylococcus aureus
SR-B1	AcLDL, oxLDL, native LDL, native HDL	Hepatitis C receptor
CD68	OxLDL	Unknown
LOX-1	OxLDL	Unknown
CD163	None	Streptococcus mutans, Staphylococcus aureus, E. coli
FEEL-1	AcLDL	Staphylococcus aureus, E. coli
Feel-2	AcLDL	Staphylococcus aureus, E. coli
SCARA5	none	Staphylococcus aureus, E. coli

Table 2 - Major known scavenger receptors and their lipid or pathogenic ligands. AcLDL = acetylated low-density lipoprotein. oxLDL = oxidised low-density lipoprotein. LPS = lipopolysaccharide. From Greaves & Gordon *J Lipid Res* 2009; 50 Suppl:S282-6.

1.3.3 Lipoprotein function & cholesterol transport

The major pathways of cholesterol (and triglyceride) are outlined in Figure 6.

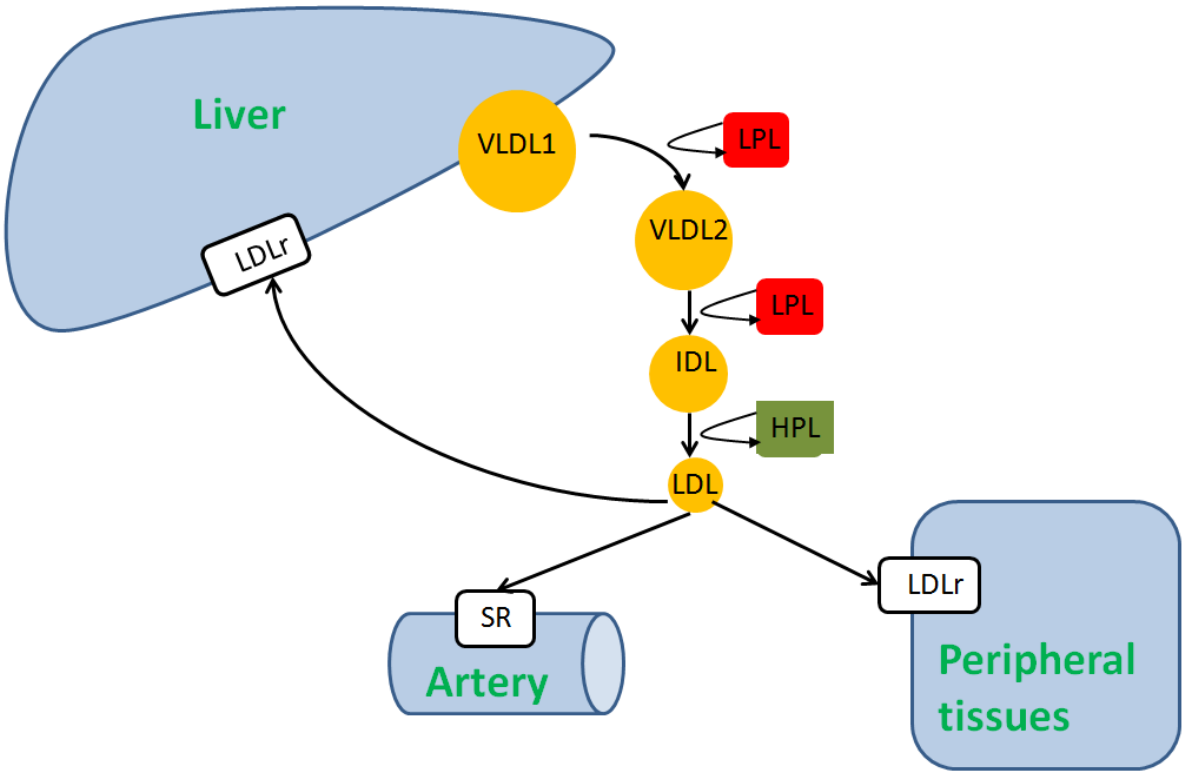


Figure 6 - Metabolism of the apoB-containing lipoproteins. VLDL = very low density lipoprotein. IDL = intermediate density lipoprotein. LDL= low density lipoprotein. LPL = lipoprotein lipase. HPL = hepatic lipase. LDLr = LDL receptor. SR = scavenger receptor. (Author's own design)

Following a meal, chylomicrons are synthesised by enterocytes in the small bowel, and carry dietary triglyceride and cholesterol to peripheral tissues. Chylomicrons are hydrolysed by lipoprotein lipase (LPL), losing some triglyceride into recipient cells, and the resulting remnant particles are taken up by the liver via LDLr & LRP-1. These are processed and provided with an apoB100 molecule, and released into the circulation as triglyceride-rich VLDL1. VLDL1 itself relinquishes triglycerides through the hydrolytic action of LPL, and so is sequentially modified to VLDL2 and then IDL. By this point, the particle has lost all apoproteins except apoB100. IDL may then be taken up by the liver via LDLr, as the particle's reduction in size leads to conformational change in apoB which permits effective binding to LDLr. Alternatively, it may be further transformed

in LDL by hepatic lipase (HPL) bound to hepatic sinusoidal cells. LDL particles hold little triglyceride but proportionally large quantities of cholesterol, and are the major carriers of cholesterol in blood. LDL may be taken up by liver or peripheral tissues via LDLr ("receptor-dependent" uptake), though excess or modified LDL may preferentially be consumed by phagocytic cells in the reticulo-endothelial system or vessel wall, probably via scavenger receptors.

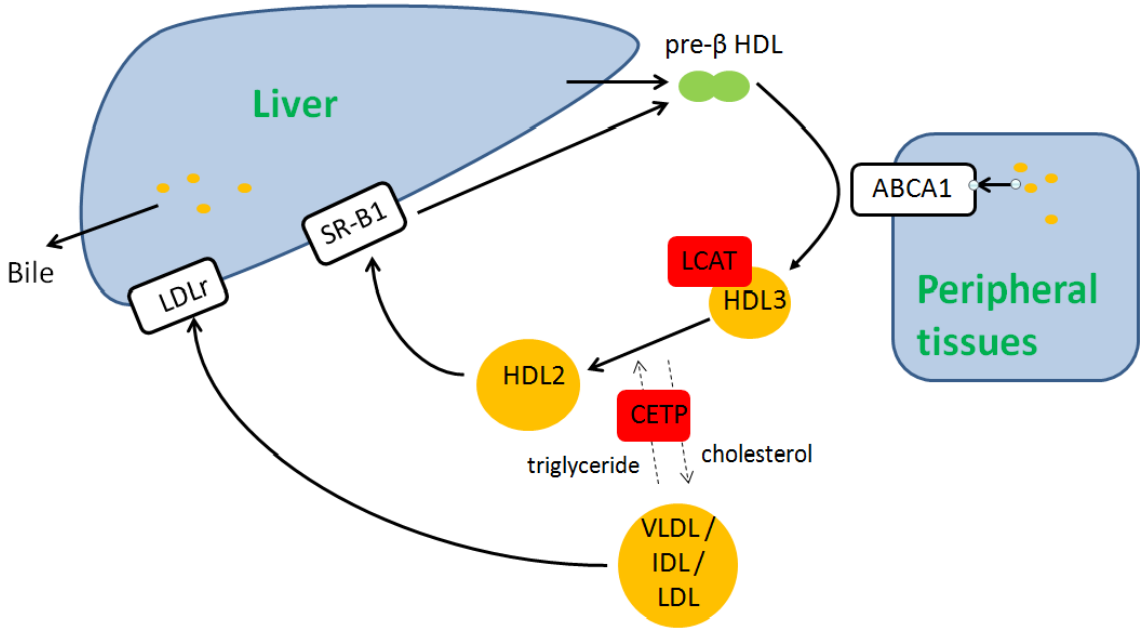


Figure 7 - Reverse cholesterol transport and the life cycle of HDL. LCAT = lecithin cholesterol acetyltransferase. CETP = cholesterol ester transfer protein. SR-B1 = scavenger receptor B1. ABCA1 = ATP-binding cassette A1. (Author's own design)

HDL stands distinct from the other lipoproteins as it has its own metabolic cycle known as reverse cholesterol transport, outlined in Figure 7. This is the mechanism whereby excess cholesterol is exported from cells; humans cannot metabolise the cholesterol ring, and instead must transport it to the liver for excretion in bile. HDL in its nascent phase ("pre-β HDL") exists as a discoidal, lipid-poor particle bearing apoAI synthesised by the liver. It is able to accept free cholesterol exported from peripheral cells by ATP-binding cassette transporters, predominantly ABCA1. Though other active members of this family exist, ABCA1 appears to be the rate limiting protein involved. Genetic defects in ABCA1 cause the rare Tangier disease, characterised by accumulation

of cholesterol in peripheral tissues and extremely low levels of HDL-c. Free cholesterol within HDL is esterified by the lecithin-cholesterol acetyltransferease (LCAT) enzyme which is bound to the particle; the particle then becomes spherical and is known as HDL3. HDL3 then exchanges cholesterol esters with apoB-containing lipoproteins in return for triglycerides under the action of cholesterol ester transfer protein (CETP), increases in size and becomes HDL2. HDL2 can then bind to the class B scavenger receptor (SR-B1) on hepatocytes, transfer cholesterol into the cell, and shrink back into a pre-ß HDL particle.

1.3.4 Regulation of intracellular cholesterol levels

Accumulation of excess intracellular cholesterol can be highly toxic to cells, and so intracellular cholesterol levels are tightly controlled. The major regulatory proteins involved are the sterol regulatory element binding proteins (SREBP) and the liver X receptors (LXR). SREBPs act to increase intracellular cholesterol levels, whilst LXRs perform the opposite function.

SREBP exists in two isoforms; SREBP1 is mainly involved in fatty acid biosynthesis, whilst SREBP2 regulates cholesterol. When a cell is cholesterol replete, SREBP is inactive, bound to the endoplasmic reticulum by a complex including oxysterols (intermediate molecules in cholesterol synthesis which are now recognised to have metabolic functions of their own) and the SREBP cleavage activation protein, or SCAP. When cellular cholesterol stores run low, the lack of oxysterols liberate the SCAP/SREBP complex that then travels to the golgi apparatus. Here, SREBP is cleaved by proteolytic enzymes, and can travel to the nucleus where it acts as a transcription factor to increase expression of the LDLr and HMGCoA reductase. This increases cellular cholesterol levels both by uptake from the serum and by de novo synthesis.

LXRs also exist in two forms. LXRα is particularly involved in cholesterol regulation, and is expressed in gut, adipose tissue and liver. LXRß is expressed more broadly, and has roles in nervous system and immune system function. In conditions of excess intracellular cholesterol, oxysterols bind to and activate LXRα or LXRß. This allows LXR to form a heterodimer with its partner retinoic acid receptor (RXR). This heterodimer can then act as a transcription factor, binding to a genetic locus called the LXR response element. Expression of

cholesterol export proteins such as ABCA1 and ABCG1 is upregulated (allowing for increased cholesterol efflux to HDL) whilst LDLr expression is repressed (reducing cholesterol intake from LDL).

Three other protein families warrant mention in regards to cholesterol metabolism. Firstly, the peroxisome proliferation activating receptors (PPARs) are nuclear receptors which, like LXR, form heterodimers with RXR, and can then act as transcription factors by binding to PPAR-response elements on gene promoters. PPARα is present in liver, skeletal muscle, brown adipose tissue and macrophages. On ligation, PPARα increases expression of LPL and other enzymes involved in fatty acid uptake and oxidation, and reduces expression of apoCIII. This removal of triglyceride from the lipoprotein pool leads to increased clearance and reduced synthesis of VLDL, and an increase in average LDL particle size with reduction in overall LDL concentration [FRUCHART2009]. Serum HDL also increases due to increased hepatic synthesis of apoAI and AII, and through increased cholesterol efflux to HDL from macrophages in an ABCA1-dependent manner (159). Fibrates, drugs which act as PPARα agonists, have been used for many years as a treatment for hypertriglycerideaemia. PPARß/δ and PPARγ are involved in adipocyte proliferation and glucose metabolism respectively; the latter is the target for the thiazoladinedione drugs, used to lower blood glucose levels in type 2 diabetes mellitus.

Secondly, the forkhead transcription factors FoxO1, FoxO3a and FoxO4 are implicated in a wide range of cellular processes including the cell cycle, cell survival, adipocyte differentiation and insulin signalling (160); indeed, FoxO1 knockout in mice leads to embryonic lethality. Activation of FoxO1 stimulates gluconeogenesis and reduces pancreatic insulin production (161), and has been shown to inhibit expression of SREBPs (162). Knockout of other FoxO molecules in ApoE-/- mice leads to increased liver triglyceride content, progressing to steatosis in the context of a high-fat diet, elevated serum levels of IL-6, and increased atherosclerosis (163).

Finally, much attention has recently been paid to the proprotein convertase subtilisin/kexin (PCSK) family, particularly PCSK9. In hepatocytes, PCSK9 binds to and degrades the LDLr, thus reducing hepatic uptake of LDL. A phase III trial

of evolocumab, a monoclonal antibody directed against PCSK9, reduced circulating LDL-c by over 50% (164), and also appears to reduce serum Lp(a) by a similar mechanism (165).

1.3.5 Lipoproteins and CVD risk

In the general population, several strands of evidence have firmly established that high serum LDL-c and low serum HDL-c levels are associated with increased risk of cardiovascular disease (CVD). Cholesterol is abundant in the atherosclerotic plaque, and familial hypercholesterolaemia—a genetic condition that results in elevated serum levels of LDL—leads to a greatly increased CVD risk from the age of 30. Furthermore, statins, drugs which block cholesterol synthesis, lower both LDL-c and CVD risk (166-168); a meta-analysis of five large RCTs demonstrated mean reductions in TC and LDL-c of 20% and 28% respectively, with subsequent reduction of relative risk of major coronary events of 34% in primary prevention and 30% in secondary prevention (169). Regression analysis indicated that for every 1% reduction in LDL-c, we would expect a 1% reduction in mortality from CHD (170). Consequently, statins are recommended for primary and secondary prevention of myocardial infarction (MI) and ischaemic stroke (171). All instruments for calculation of CVD risk, such as SCORE (Systematic Coronary Risk Evaluation; Europe), ASSIGN (assessing cardiovascular risk using Scottish Intercollegiate Guidelines Network [SIGN] guidelines; Scotland), QRISK® (UK) and the Framingham Heart Study risk calculators (USA) make use of the total cholesterol to HDL-c ratio (termed the 'atherogenic index') in some way.

A comprehensive review of the literature was published by the Emerging Risk Factors Collaboration (ERFC), which gathered data relating to risk of CVD from 68 long-term prospective studies involving over 350,000 patients (172). 'Non-HDL' cholesterol (as a surrogate for LDL-c) correlated strongly with coronary heart disease (CHD), and an inverse correlation was seen between HDL-c and CHD, both in a log-linear pattern (Figure 8); these relationships were independent of each other. However, after adjusting for serum cholesterol levels, no association of serum triglycerides with CHD was observed, a finding which goes against conventional wisdom. Similar, but much less pronounced, relationships were shown for ischaemic stroke.

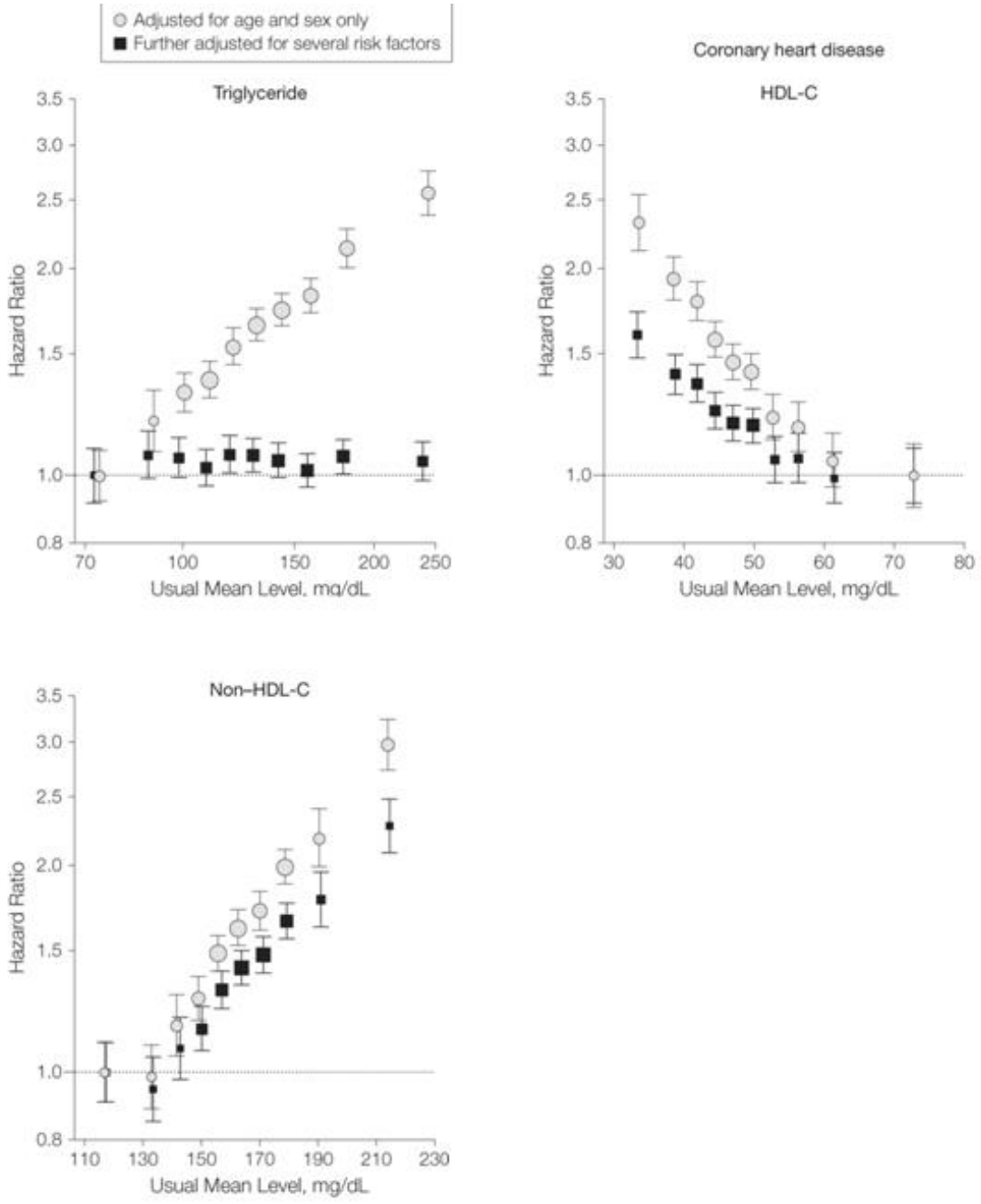


Figure 8 - Hazard ratios for coronary heart disease across quantiles of TG, HDLc, and non–HDLc levels, based on 302,430 non-RA patients from 60 studies. Adapted from Emerging Risk Factors Collaboration *JAMA* 2009; 302:1993-2000

Despite the strong associations described in the literature, some evidence has emerged that questions the causality within the relationship between HDL-c and atherogenesis. In 2012, Voight *et al*. reviewed case–control studies of single nucleotide polymorphisms (SNPs) known to affect serum lipoprotein levels. They found that, although SNPs that correlated with high LDL-c levels conferred an increased risk of MI, only 6 of 15 SNPs associated with higher serum HDL-c levels were associated with MI risk, and all 6 SNPs were also associated with other effects on either triglycerides or LDL-c (173). Moreover, a Mendelian randomization was performed using the *LIPG* gene encoding endothelial lipase (an enzyme that hydrolyzes HDL) in which 2.6% of the population carry a SNP that elevates serum HDL-c without affecting any other risk factor for CVD (174). A meta-analysis of six prospective cohort studies revealed no association between MI risk and the increased HDL-c levels associated with this SNP (173). These data, together with the lack of beneficial effect on CVD risk of cholesterol ester transfer protein (CETP) inhibitors, which inhibit reverse cholesterol transport and thus increase HDL levels (175) suggest that whilst HDL-c is a valuable biomarker in atherosclerosis, it might not be a causative agent. These studies do not, however, negate the potential for HDL particles modified as a result of inflammatory processes to be causally related to atherogenesis. Further studies are needed to examine this potential.

1.4 Cardiovascular disease in RA

The risk of cardiovascular death in patients with RA is increased by around 50% compared to age- and sex-matched controls, with individual observational studies generating standardized mortality ratios (SMRs) from 1 to 3 (176, 177). One Dutch cross-sectional study found RA conferring a risk of cardiovascular disease similar to type 2 diabetes mellitus (178), a finding replicated in a much larger Danish database analysis (179). This increased risk is only partially explained by conventional risk factors (180, 181) Whilst cardiovascular mortality has declined significantly over recent decades in the general population (137), a meta-analysis of 17 studies conducted over a period of 50 years showed no change in standardized mortality ratio (SMR) for CVD over time (182), though none of the studies included covered the “treat-to-target” era. A prospective cohort study of 2,519 patients recruited into the Norfolk Arthritis

Register between 1990 and 2004, and followed for up to 7 years, found that CVD mortality did fall over time but only in line with changes also seen in the general population (183). Relative risks of incident CVD (184) or CVD-mortality (177) seem to be slightly higher for myocardial infarction than for ischaemic stroke. One meta-analysis showed that inception cohorts, which tend to include patients with less than 2 years of symptoms, generated lower SMRs than cohorts including those with established disease (mean SMR 1.2 v 1.9) (185). Some studies have found increased prevalence of surrogates for CVD in early RA, including endothelial dysfunction (186), increased carotid intima-media thickness and presence of carotid plaque (187).

Supporting the paradigm of atherosclerosis as an inflammatory condition, increasing RA disease activity appears to associate with a worsening cardiovascular phenotype. Inflammatory markers or clinical disease scores such as DAS28 can predict increasing carotid intima-media thickness (188), incident CVD (189-191), and CVD mortality (192, 193). Whilst many of these studies use single timepoint measurements of disease activity, a Swedish case-control study found RA patients with CVD to have higher time-averaged ESR than RA controls without CVD (194). Similar outcomes have been found for time-averaged DAS28 in an early-RA inception cohorts (189, 195) and CDAI in a large US-based registry (191). This latter analysis suggested that a 10 point reduction in time-averaged CDAI was associated with a 21% reduction in risk of MACE (a composite outcome of MI, stroke of CV death). An analysis of the South Korean KARRA cohort (196) showed that cumulative inflammatory burden, as measured by “area under the curve” ESR, predicted the presence of carotid plaque in a manner which was synergistic with conventional risk factors. This implies that reduction of CVD risk in these patients requires both adequate disease control and management of conventional risk factors, as is the case in the non-RA population.

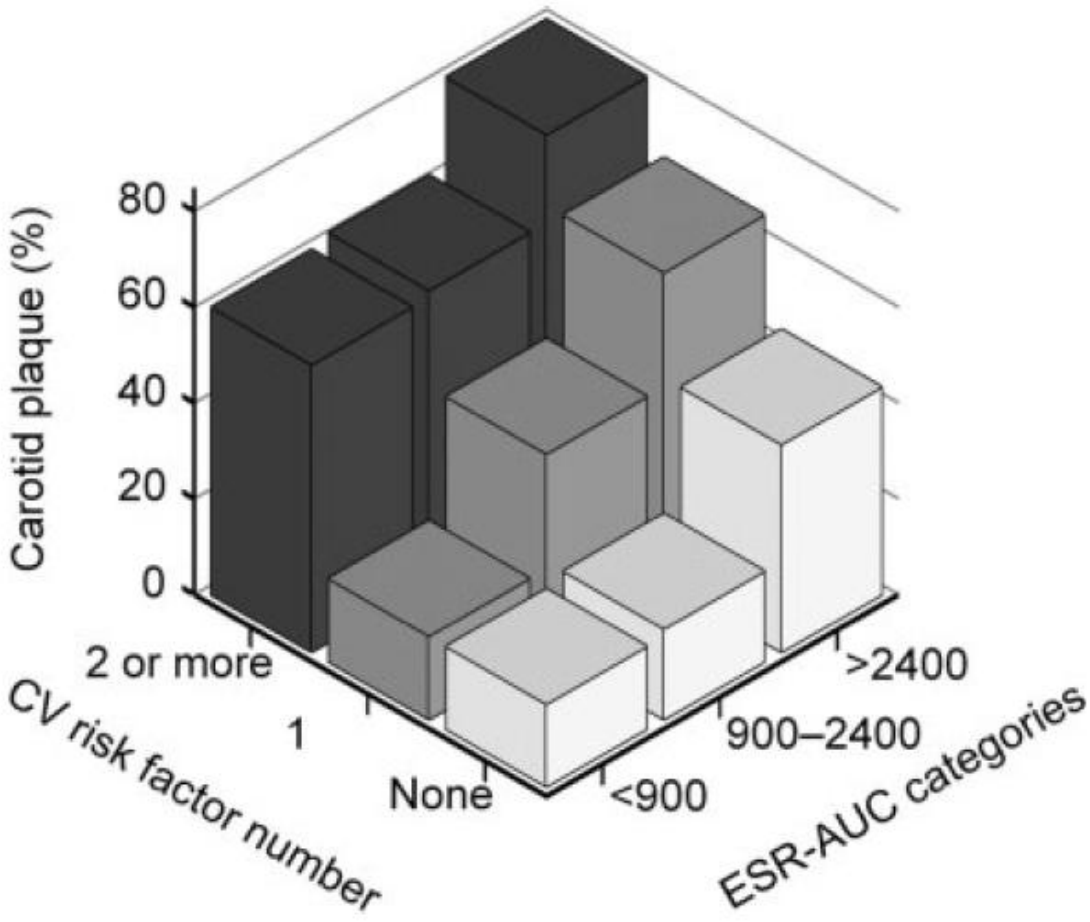


Figure 9 - Percentage of RA patients with detectable atherosclerotic plaque on carotid artery ultrasound, as stratified by the number of conventional CV risk factors and tertile of ESR-AUC (ESR area-under-curve). Adapted from Im et al. *Rheumatology* 2015; 54(5):808-15.

Based on the above findings, current EULAR guidelines (197) recommend multiplying estimated CVD risk by 1.5 when the patient has two of the following three clinical features: seropositivity for RF or ACPA; disease duration of >10 years; or severe disease with extra-articular manifestations. Recent data using the SCORE algorithm suggest, however, that this practice may still underestimate CVD risk in many patients. In one Spanish cohort (198) of RA patients who were calculated to be low risk for CVD (SCORE of zero), 24% had ultrasound evidence of carotid artery plaque. Additionally, in a Dutch RA inception cohort, use of the adapted SCORE calculator (i.e. with the CV risk multiplied by 1.5) tended to underestimate the observed rate of incident cardiovascular disease, especially in those with the lowest SCORE result (199).

1.4.1 Effect of DMARDS on CVD risk

In accord with the hypothesis of inflammation driving cardiovascular disease, therapeutic suppression of RA disease activity has been shown to ameliorate CVD risk. Most data concerning conventional DMARDS in this regard is derived from studies of methotrexate, with relatively little on other agents such as Sulfasalazine or hydroxychloroquine. The effect of methotrexate therapy on CVD risk was first studied in a cohort of 1,240 RA patients, followed up for an average of 6 years (200). The 588 RA patients receiving methotrexate demonstrated a 70% reduction in CV mortality compared to those not receiving methotrexate; no similar effect was found with other DMARDS, though few events were seen with these agents, and confidence intervals were wide. A meta-analysis (201) suggested a 21% reduction in risk for total CVD (n of studies=10) and 18% reduction in risk for MI (n=5).

1.4.2 Effect of anti-TNF therapy on CVD risk

The advent of biologic therapies has been accompanied by a large number of studies examining the effect of these drugs on cardiovascular disease, with reference to a number of clinical or surrogate endpoints. As the first biologics to enter clinical practice, the TNF inhibitors have been subjected to the greatest scrutiny. Roubille (202) conducted a systematic literature of observational studies and RCTs dating from 2005 onwards examining the effect of anti-TNF on different CVD outcomes. A total of 28 studies were analysed, with over 230,000 subjects included. Reductions in relative risk were found for MI (RR 0.59, [95% C.I. 0.36-0.97] n=6), stroke (RR 0.57 [0.35-0.92] n=6) and MACE (RR 0.30 [0.15-0.57] n=4), with a non-significant reduction in risk for congestive cardiac failure (RR 0.75 [0.49-1.15], n=7). In contrast, corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDS) increased the risk of cardiovascular events. Of note, results were not uniform, and not all studies demonstrated reductions in risk with therapy. However, is likely to be because not all patients who received anti-TNF displayed a clinical response to it. One study drawing data from the British Society for Rheumatology Biologics Registry (BSR-BR) stratified

7,515 patients receiving anti-TNF depending on clinical response (response was defined as a fall in DAS28 of >1.2 after 6 months, or a fall of >0.6 which resulted in a DAS28 of ≤5.1) (203). Responders in the registry had a myocardial infarction incidence of 3.5/1,000 patient years, compared to 9.4/1,000 patient years in non-responders, leading to an incidence rate ratio of 0.38 for responders relative to non-responders. This lends further weight to the concept of inflammatory burden being a driver behind atherogenesis.

1.4.3 Effect of tocilizumab on CVD risk

Relatively little data exists regarding CVD risk with other biologics. This is partly because of the difficulty inherent in detecting significant changes in outcomes which are not especially common. Whilst nationwide prevalence of cardiovascular disease is high, an individual's absolute risk may be low, requiring very large numbers of study recruits followed for a long time to generate enough events to confidently pronounce a statistically significant difference between groups. The process of atherogenesis is also kinetically modest, and so the effects of a therapeutic intervention on atheroma formation (and thus CV events) may take several years to become apparent. A 6-months randomised controlled trial, such as may commonly be used for new RA drugs, may therefore be a poor tool for evaluating CVD risk, and so cohort studies using clinic databases or disease registries (with large patient numbers and long periods of follow-up) are often preferred. Pooled data from extension phases of tocilizumab RCTs (121), with mean treatment duration of 2.4 years, failed to demonstrate excess CVD risk from treatment. A more recent post-hoc analysis of 5 RCTs and their extension studies (204) identified 50 incidences of MACE in 14,683 patient years of follow-up (median duration of follow-up 4.5 years). A multivariate analysis indicated that baseline DAS28 and TC/HDL-c ratio were independently associated with MACE. A greater reduction in DAS28 by 24 weeks was associated with a smaller incidence of MACE, though no association was seen for changes in serum lipids or ESR. Unfortunately, the nature of the analysis meant no comparison of MACE risk in placebo-treated patients was possible.

There is, however, evidence in favour of IL-6 blockade from large-scale genetic studies. Two consortia (205, 206) performed Mendelian randomizations, studying the effect of a single nucleotide polymorphism (SNP) (Asp358Ala) which increases proteolytic cleavage of mIL-6R and subsequent loss of IL-6R from the surface of leukocytes and hepatocytes. This leads to reduced IL-6 signalling on these cells, and subsequently higher serum IL-6 and lower CRP and fibrinogen. Possession of this SNP led to per-allele reductions in CVD risk of 5% and 3.4% respectively. Whilst these effects appear modest, this is likely to be a result of the much lower levels of IL-6 signalling in these populations compared to patients with RA, and the correspondingly greater reduction in IL-6 signalling following tocilizumab which one would see in RA. Additionally, the very large numbers of patients and events in these analyses permit a strongly statistically significant result. Extrapolation of these results to tocilizumab is not without risk, however, as the Asp358Ala SNP reduces only cis-signalling, whilst tocilizumab blocks both soluble and membrane-bound forms of the IL-6R and thus also blocks trans-signalling. Trans-signalling blockade may therefore lead to clinical consequences not seen in the Asp358Ala population.

1.4.4 Effects of other agents on CVD risk

Relatively little data exists on the effects of other biologic therapeutics on CVD risk in RA. The only source for information regarding CVD risk of other biologic agents appears to be a retrospective interrogation of the US Medicare database, which provides medical insurance cover for more than 90% of US residents over 65 years of age (207). With almost 75,000 patient years of follow-up, this study suggested that anti-TNF drugs were associated with an increased risk of MI compared to abatacept, and that tocilizumab had the lowest risk of a composite outcome of acute MI or coronary revascularization. Tocilizumab was in fact associated with a high risk of MI in biologic naïve patients, though this had broad confidence intervals due to low numbers (only 11 instances of MI in the biologic-naïve population). Whilst the analysis adjusted for a variety of cardiovascular risk factors, confounding by “contraindication” (i.e. tocilizumab being avoided in patients at high risk of CVD due to its perceived worsening of lipid profiles) is a risk in this retrospective analysis. One other prospective observational study (208) assessed markers of CVD risk (but not cardiovascular events) in 36 patients

commencing abatacept, rituximab and tocilizumab. Arterial stiffness, as measured by pulse wave velocity, was reduced at 3 months by tocilizumab and 12 months by rituximab.

1.4.5 Potential mechanisms behind CVD in RA

There are several proposed mechanisms for how high-grade inflammation, as seen in RA, may contribute to accelerated atherogenesis (209). Inflammation can drive endothelial dysfunction and upregulation of adhesion molecules and platelet-activating agents in the vasculature, leading to a pro-thrombotic state and encouraging leukocyte migration into the intima. Pro-inflammatory cytokines can also increase pro-oxidative stress, and thus oxidation of lipoproteins, and act on skeletal muscle or adipose tissue to increase insulin resistance. As noted above, some of these parameters can be measured and used as surrogates for clinical CVD, and have been found to be prevalent in even early RA. The extensive literature behind these mechanisms is beyond the scope of this thesis, but one crucial component to atherogenic effects of inflammation is its effects on lipid profiles, and how they may relate to the development of cardiovascular disease.

1.5 Lipids and CVD in RA - the “lipid paradox”

Several studies have attempted to compare lipid profiles in patients with RA to those of controls (210-216). Although not all of these studies report consistent results, it is generally accepted that active RA leads to a fall in both LDL- and HDL-cholesterol levels (217, 218), This ‘lipid paradox’ phenomenon - the reduction in levels of serum lipids in a disease associated with increased CVD risks – is also seen in other autoimmune inflammatory diseases and sepsis, in which greater inflammatory burden is associated with lower levels of circulating lipids. Consistent with these findings are reports from numerous studies of increases in serum lipid fractions with successful anti-inflammatory treatment, which will be discussed further in a subsequent section. Nevertheless, some studies have obtained conflicting results regarding total cholesterol, HDL-c, LDL-c and triglyceride levels in RA; the inconsistencies might be explained by the small numbers included in the cohorts studied (usually less than 100 patients), as

well as the considerable demographic and disease-related heterogeneity between studies (for example, early versus advanced disease, active versus quiescent disease or male versus female). In addition, few placebo-controlled studies exist in this area, and on many occasions data was gathered retrospectively.

1.5.1 Does dyslipidaemia in precede clinical RA?

The findings described above raise the question of whether it is possible to discern a 'pre-rheumatoid' serum lipid pattern, which might prove useful in early diagnosis of the disease and/or defining the risk of CVD in RA. To address this question, van Halm *et al*. examined lipid profiles in samples from 79 blood donors who later developed clinical RA; on average, these patients had 4% higher total cholesterol, 9% lower HDL-c, AND 17% higher trigylceride levels compared with control samples matched for storage time, and donor age and gender (219). This lipid profile was apparent up to 10 years before the development of RA symptoms. More recently, the Rochester Epidemiology Project medical records linkage system was used to identify the lipid profiles of 577 patients with RA, from 5 years before until 5 years after diagnosis (220). Despite having lower rates of statin use compared with the control population, the patients with RA displayed a mean reduction in total cholesterol (10%) and LDL-c (17%) in the 5 years preceding diagnosis; no statistically significant change in HDL-c and triglyceride levels was observed, and thus the total cholesterol to HDL ratio progressively fell in patients with RA during the 5 years before diagnosis.

1.5.2 Using lipid profiles to predict CVD in RA

Given the seemingly paradoxical fall in LDL-c levels in a condition that associates with an increase in CVD mortality, the question arises: do levels of serum lipids predict CVD risk in RA, as they do in the normal population? Surprisingly, until very recently this question had not been widely studied. In 2010 an analysis of the AMORIS cohort failed to demonstrate a significant relationship between TC and development of CVD (221). The following year, however, a retrospective cohort study my Myasoedova et al. involving 651 patients investigated the associations between inflammatory markers, lipids and CVD risk. This paper confirmed that the risk of developing CVD was increased in patients with a

higher inflammatory burden, with a lower total cholesterol to HDL-c ratio associated with risk (222). Most interestingly, in contrast with the linear association seen in individuals without RA, the relationship between total cholesterol and CVD risk was represented by a U-shaped curve in patients with RA, with risk increasing at total cholesterol levels below 4 mmol/l. LDL-c displayed a similar but non-statistically significant relationship (Figure 10). In both values the confidence intervals were wide, but exploratory analysis showed that in patients with ESR >25mm/hr, the risk of incident CVD was higher for patients with LDLc <1.5mmol/L compared to those with LDLc >2mmol/L (Figure 11).

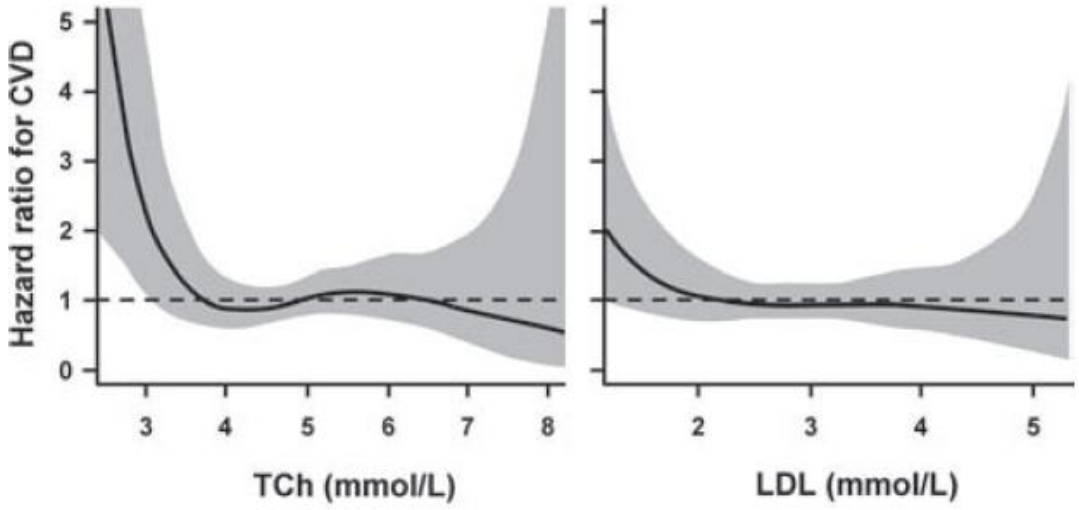


Figure 10 - Hazard ratio for incident cardiovascular disease (shadow = 95% confidence interval) at different serum levels of total cholesterol and LDL-cholesterol. From Myasoedova et al . *Ann Rheum Dis* 2010; 70:482-487

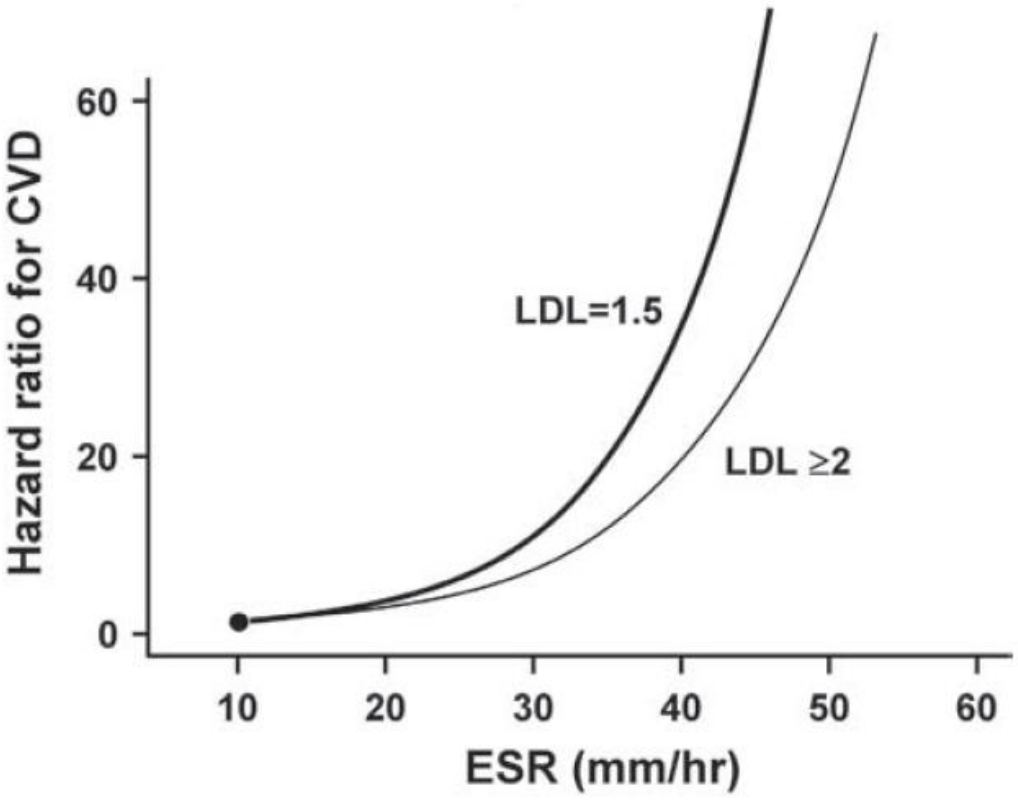


Figure 11 - Hazard ratio for incident cardiovascular disease in patients with low LDL-c (thick line) or high LDL-c (thin line) at different levels of ESR. From Myasoedova et al. *Ann Rheum Dis* 2010; 70:482-487

Subsequent, larger studies have replicated and refined these observations. Zhang el al also conducted a retrospective cohort study, though from a large US commercial healthcare plan, with complete data on over 20,000 patients with 366 myocardial infarctions. This showed a U-shaped relationship between LDL-c and MI, with a linear inverse relationship between HDL-c and MI (223). Liao et al looked at data from another commercial database in the USA, enrolling 16,085 RA patients and a total of 32,000 patient years of follow-up. Here the end-point was "major adverse cardiac event" (MACE), a composite of myocardial infarction, coronary artery bypass graft, coronary revascularization or stroke. A U-shaped curve was demonstrated between MACE and LDL-c, though statistical analysis failed to yield a significantly increased odds ratio for all but the highest quintile of LDL-c (224). Most recently, Navarro-Millan et al used data from the National Veteran's Health Administration cohort to identify more than 37,000 RA patients (90% of whom were male, with mean age of 63 years), with 896 MIs and 122 cardiovascular deaths. In this study, a U-shaped curve was again

demonstrated for the association between LDL-c and MI (190). Again, however, very broad confidence intervals were displayed, and no relationship was seen between LDL-c and cardiovascular death. Reassuringly, and in keeping with previous results, there was an inverse relationship between HDL-c and MI, and a log-linear relationship between CRP and MI. In these studies, statistical significance is rarely met, and confidence intervals tend to be broad. Nevertheless, the reproducibility of this data and its consistency with what is already known about lipidaemic changes in inflammation make it seem likely that there is at least a subset of RA patients who have significant inflammatory burden which leads to both elevated CVD risk and lower LDL-c levels. Intriguingly, these findings are mirrored in end-stage renal failure, which commonly manifests as a chronic inflammatory state. In a prospective study of 800 patients starting dialysis, those displaying signs of inflammation or malnutrition (C-reactive protein [CRP] ≥ 10 mg/l, hypoalbuminaemia [serum albumin < 36 mg/l] or serum IL-6 ≥ 3.09 pg/ml) displayed higher CVD risk with reducing serum levels of total cholesterol (225). Importantly, the opposite and anticipated relationship is seen in patients without these signs of inflammation, underlining the confounding effect of inflammation on CVD risk.

1.5.3 The lipid paradox—potential mechanisms

Many factors could influence lipid metabolism and increase cardiovascular risk in RA. These influences could include shared risk factors (such as smoking), genetic pathways, effects of therapeutics (corticosteroids, for example), or might reflect specific molecular interactions driven by the inflammatory process itself. The molecular factors that potentially drive the interaction between inflammatory disease and altered lipid metabolism are not entirely understood; however, several credible mechanistic pathways have come to the fore in recent years. Logically, in evolutionary terms, immune defence, which requires high levels of both leukocyte cell division and death, protein synthesis, cell motility and host tissue responses to damage and repair, should be integrated with metabolic function, in order that appropriate energy and endocrine homeostasis is maintained at a time of ‘high demand’. Thus, cytokines might have direct effects on lipid metabolism, in the same way we know an acute inflammatory response alters carbohydrate metabolism and insulin resistance, and engenders a

hypercoagulable state. It has been demonstrated in a cohort of post-operative patients that serum IL-6 levels correlated inversely with TC and LDL-c (226) whilst administration of exogenous IL-6 reduces serum TC and alters VLDL subclasses in humans (227, 228). TNF-α and bacterial lipopolysaccharide (LPS) can achieve similar results (229). Whilst this may confer survival advantage in the acute setting, such responses could become maladaptive in the context of *chronic* high-grade inflammation, such as seen in RA.

Inflammation might also promote changes in the levels and composition of lipoprotein subfractions, which are not detectable using standard laboratory lipid profiling. Small, dense LDL particles - detectable on NMR spectroscopy - have been shown to cross the endothelium more easily and are more prone to oxidative damage than larger, less dense particles (230). Elevated levels of these small LDL particles have been observed in severe active RA (216), although these findings remain controversial (217). Conversely, small, dense HDL particles isolated from healthy individuals have been found to be effective at protecting LDL from atherogenic oxidation (and subsequent uptake by macrophages) .[KONTUSH2003] Furthermore, serum levels of these small particles are diminished in RA (216), a finding which has been associated with coronary calcification in one report (217).

HDL from patients with RA has been observed to have impaired antioxidant and reverse cholesterol transport capacity, correlated with disease activity, compared with HDL-c from healthy individuals (231). Interestingly, these functions of HDL were inversely correlated with serum myeloperoxidase activity, suggesting that the inflammation in RA might lead to oxidative modification of lipids that alters their properties and activities. Moreover, a 2012 cross-sectional study described low levels of the cardioprotective HDL-2 lipid subfraction in patients with RA (232). The changes observed in HDL function can be so marked that a distinction is made between 'proinflammatory' and 'anti-inflammatory' HDL based on their antioxidative capacity. A comprehensive proteomic analysis (233) demonstrated the proinflammatory HDL phenotype to be more prevalent with increasing RA disease activity and was associated with increased levels of molecules such as apolipoprotein J, fibrinogen, haptoglobin, serum amyloid A (SAA) and several complement factors,

and reduced levels of serum paraoxonase-1 (a hydrolytic enzyme which might protect LDL from oxidation (234), compared with patients with anti-inflammatory HDL. As a consequence, in RA, the ability of HDL particles to protect LDL particles from oxidation might be impaired in the inflammatory state.

Additionally, a variety of other lipoprotein-associated molecules might be altered in RA and, as a result, contribute to accelerated atherogenesis. Lipoprotein(a) (Lp(a)), has been shown to be elevated in the serum of patients with RA in comparison with control individuals (235). Furthermore, in the PROCARDIS study (154), Lp(a) was strongly and independently associated with CVD, a finding supported by a meta-analysis. Indeed, Lp(a) is probably a causal factor in CVD, as suggested by the observation that single nucleotide polymorphisms (SNPs) linked to higher Lp(a) lipoprotein concentrations are predictive of CVD (154). In RA, group IIA phospholipase A2 (sPLA2-IIA) was positively and negatively correlated with plasma levels of small, dense LDL and small HDL particles, respectively, and has been linked to the increased uptake of small, dense LDL in tissues (216). Furthermore, platelet-activating factor acetylhydrolase (PAF-AH), which circulates in complex with LDL and HDL, might have a variety of proatherogenic functions, including impairment of reverse cholesterol transport (236). Thus, all these molecules are upregulated in inflammation or the acute-phase response and could contribute to CVD risk.

1.5.4 Lipid modulatory effects of antirheumatic therapy

Many data informing inflammation–lipid interactions have emerged since the advent of biologic therapies in the past decade. The increased research focusing on this association in part reflects increased interest and recognition of the clinical importance of this phenomenon, but is especially a consequence of the availability of these new therapeutic agents; biologic agents can be thought of as highly specific ‘molecular scalpels’, which offer unprecedented opportunity for molecular dissection of interactions between the immune system and metabolic processes in humans. Such studies thereby provide a window into the biological mechanisms underlying the lipid paradox and offer clues as how best to improve the clinical outlook for patients.

1.5.4.1 Conventional DMARDs and corticosteroids

A number of studies have addressed the effects of conventional DMARDs on lipid profiles. Park *et al*. (237) performed a 1-year prospective cohort study that enrolled 42 DMARD-naive patients with RA. In the 27 individuals (64%) who met the American College of Rheumatology (ACR) criteria for 20% improvement (ACR20), mean serum HDL-c increased by 21%, a change not seen in ACR20 non-responders. Furthermore, in ACR20 responders, LDL-c, triglycerides and Lp(a) remained unchanged, and the LDL-c:HDL-c ratio decreased by 13%. The 12-month change in serum CRP levels inversely correlated with change in serum HDL-c ($r = -0.38$). Interestingly, no difference in 12-month lipid levels was observed between corticosteroid users (prednisolone <10 mg/day) and non-users, suggesting that these changes were brought about by DMARDs reducing rheumatoid disease activity. Boers and co-workers (238) analysed data from the Dutch Combinatietherapie Bij Reumatoide Artritis (Combination Therapy in Early RA; COBRA) study, which randomized 134 patients newly diagnosed with RA to receive sulfasalazine (2 g/day) as a monotherapy or in combination with methotrexate (7.5 mg/week), together with a high but rapidly tapered dose of prednisolone (starting at 60 mg/day and decreasing to 7.5 mg/day over 7 weeks). Serum HDL-c levels increased by a remarkable 50% in both treatment groups, but this elevation was achieved much quicker in steroid users compared to non-users (16 and 40 weeks, respectively). Despite a parallel increase in total cholesterol, the total cholesterol:HDL-c ratio fell. The same study assessed data from a separate cohort of patients with established RA, and found that serum HDL-c levels were 25% lower in those with active disease compared with those in remission. Georgiadis *et al*. (239) treated 58 DMARD-naive patients with a steady dose of methotrexate (mean 15 mg/week) together with prednisolone (initially 7.5 mg/day, then tapered according to response), and compared them to 63 healthy controls. In keeping with the findings of Boers and colleagues, after 1 year of treatment, elevations in total cholesterol and HDL-c levels were seen, although the total cholesterol to HDL-c ratio fell. Again, no change was observed in serum LDL-c, and a strong inverse relationship between CRP and HDL-c was observed. These findings have been complimented by the suggestion that methotrexate could impair foam cell formation in THP-1 macrophages by promoting cholesterol efflux (240). In addition, Munro *et al*.

(241) showed hydroxychloroquine to be associated with a reduced total cholesterol to HDL-c ratio when compared with intramuscular gold, a finding which was later replicated in a larger patient sample (242).

Recently, post-hoc analyses of two studies have looked at lipid changes in the context of a more aggressive, "treat-to-target" strategy. This is an important step as it reflects current best practice in clinical rheumatology, and subsequently more profound changes in disease activity may produce correspondingly larger changes in lipid profile. The largest dataset yet available is derived from the Treatment of Early Rheumatoid Arthritis (TEAR) study, which was set up to compare different treatment strategies in early RA. 755 patients, 90% of whom had RA duration of less than one year and 75% of whom were DMARD-naïve, were randomized to: methotrexate (MTX) monotherapy, escalating to MTX + Sulfasalazine + hydroxychloroquine ("triple therapy" if low disease activity was not achieved after 6 months; MTX monotherapy escalating to MTX+ etanercept; triple therapy from baseline; and MTX + etanercept from baseline. 459 participants had bloods taken for lipid analysis at baseline and 24 weeks (providing, essentially, three treatment groups, as the two "step-up" arms were on MTX monotherapy for the duration of this part of the study). Significant elevations from baseline in TC, HDL-c and LDL-c were seen in all three groups, with no clear differences between the treatment groups. No differences in lipid effects were seen between patients who achieved DAS28 <3.2 and those who did not; change in DAS28 correlated with change in LDL-c, but this effect did not persist in a multivariate analysis. However, change in LDL-c was significantly associated with change in CRP in multivariate analysis (243). A recent paper reported on these patients after two years of follow-up (244). At 48 weeks, cholesterol levels remained significantly higher than at baseline, and decreases in CRP, ESR and DAS28 all significantly associated with increases in TC, LDL-c and HDL-c after adjusting for conventional risk factors. Groups were generally similar in their lipid profiles, though the magnitude of increase in LDL-c was less in the triple-therapy group; indeed, patients who escalated from MTX to triple therapy displayed reductions in LDL-c, perhaps due to the inclusion of hydroxychloroquine as per the results of Munro (241) and Morris (242).

The Targeting Synovitis in Early RA (TASER) study was another RCT (16), where 111 patients with newly diagnosed, DMARD-naïve RA followed a "treat-to-target" protocol for 18 months as follows: MTX monotherapy; triple therapy; MTX + etanercept. Oral corticosteroids were discouraged but bridging IA or IM steroid was given liberally. The patients were randomized to standard treatment (with disease activity assessed clinically) or additional assessment of synovitis with doppler ultrasound scanning (USS); in this second group, patients with a low DAS28 but active disease on USS had their treatment escalated as above. During the analysis of the study, I had the opportunity to perform a post-hoc analysis of lipid profiles in this cohort. After exclusion of patients taking statins, etanercept or oral steroid, a total of 48 patients had lipid data at baseline and 18 months. In contrast to TEAR, no change was seen in TC or LDL-c. However, HDL-c increased by an average of 22%, with a subsequent fall in mean TC/HDL-c ratio. LDL-c levels at baseline correlated modestly with CRP (r=-0.36). A preliminary version of this analysis was presented at the EULAR 2013 congress in poster form, which can be found in Appendix B. These studies allow us to conclude that conventional DMARD therapy is capable of increasing HDL-c, though the evidence for LDL-c being influenced by DMARDS is less consistent.

1.5.4.2 TNF-α blockade

A wealth of data is now available concerning the effects of TNF inhibition, particularly from national registries for biologic agents. Despite initial concern about potentially serious adverse effects of these potent anti-inflammatory agents, evidence now demonstrates a substantial mortality benefit for patients who respond clinically to them (203). A number of studies have investigated lipid profiles after treatment of RA with biologic anti-TNF therapy. In 2011, van Sijl *et al* (245) published a meta-analysis of 15 studies, involving a total of over 700 patients with RA, with disease duration ranging from 1 to 20 years, treated between 2004 and 2010. Most patients received infliximab, and the majority of studies included patients on concurrent methotrexate and corticosteroids. Using multivariate linear mixed models, van Sijl and colleagues estimated lipid levels at various time points from baseline until 6 months after cessation of therapy, and found that 5 of the 15 studies observed considerable increases in serum levels of TC and, generally, serum triglyceride levels also increased.

Furthermore, 9 of 14 studies that included assessment of serum HDL-c levels showed marked increases in serum HDL-c. Taken together, these data resulted in a summary estimate that a 10% increase in total cholesterol and a 7% increase in HDL-c occur within 6 months of TNF-blockade. Interestingly, serum HDL-c levels seemed to rise rapidly and plateau by 6 weeks, whereas total serum cholesterol continued to rise steadily for up to 6 months in studies with this level of follow-up (Figure 12). A more recent meta-analysis, (246) which included data from some different studies, also showed increases in total cholesterol and HDL-c levels, without change in serum LDL-c concentrations or the total cholesterol to HDL-c ratio.

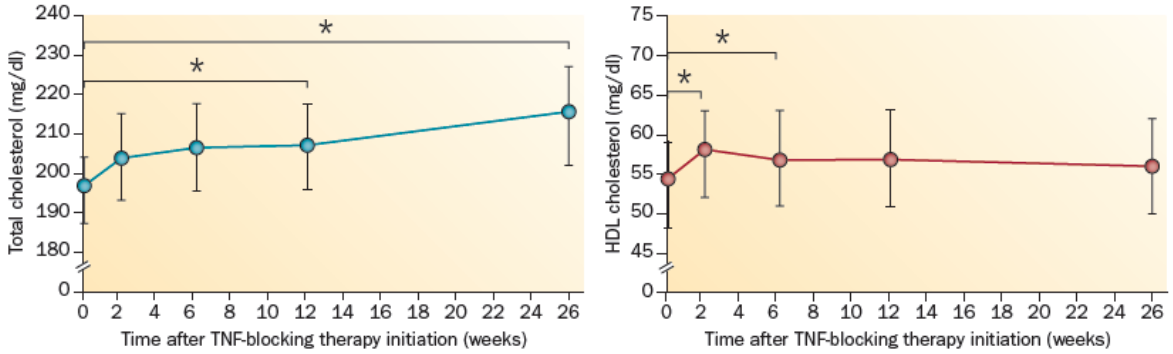


Figure 12 - Change in serum TC (left) and HDL-c (right) through 26 weeks of treatment with anti-TNF agents. Data derived from a meta-analysis of 15 studies. Graphic adapted from Robertson et al. *Nat Rev Rheumatol* 2013;9(9):513-23

Although insufficient data was available to reliably assess changes in LDL-c or apolipoproteins in meta-analyses, several short-term studies have reported rapid increases in serum LDL-c or apoB levels after treatment with anti-TNF agents (247-249). In addition, etanercept substantially reduced the amount of SAA within HDL particles over the course of a 3-month cohort study comprising patients with ankylosing spondylitis (250). Moreover, infliximab therapy is capable of improving paraoxonase-1 activity, and thus the antioxidative capacity of HDL, regardless of serum HDL-c concentrations (251). Evidence from a randomized trial suggests that TNF inhibition also leads to a dose-dependent reduction in serum Lp(a) concentrations in psoriatic arthritis (252).

1.5.4.3 IL-6 blockade

Tocilizumab is currently the only licensed agent for IL-6 blockade in clinical practice. I conducted a literature search which revealed eight clinical trials of tocilizumab which reported lipid changes (Table 1); elevations in serum total cholesterol, HDL-c, LDL-c and triglyceride levels (if measured) were universally reported (101, 102, 104-106, 110, 117, 123, 253). Notably, effects on the atherogenic index were inconsistent, but increases in LDL-c levels by around 15-20% were seen in multiple studies, which is consistent with IL-6-driven lipid changes observed *in vivo* and *in vitro* described earlier, although more detailed mechanistic studies would be of value.

Tocilizumab therapy consistently had greater effects on lipid profiles than DMARDs such as methotrexate; however, in a head-to-head comparison, combination therapy of tocilizumab with methotrexate produced a numerically greater rise in total cholesterol levels than tocilizumab monotherapy (101). Furthermore, while the directional change in levels of lipids is consistent across therapies which block IL-6R or TNF pathways, the magnitude of LDL-c elevation seems greater with the former, as reported in the ADACTA head to head study (253). ADACTA also identified reductions in HDL-associated serum amyloid A (SAA), secretory phosopholipase A2 (sPLA2), and Lp(a) which were significantly greater following tocilizumab compared with adalimumab. Of interest, two parallel genetic studies examined a SNP associated with IL-6R blockade, and thus lower IL-6R signalling in the general population (205, 206). Although these studies did not show association of the SNP with lipid changes (probably as the study population had normal levels of inflammation, and so lipids were not altered by higher levels of systemic inflammation), both studies reported a lower risk of CHD in carriers of the IL-6R associated SNP.

Study name	Study design	Lipd changes observed in tocilizumab groups
CHARISMA (2006)	16 week, 359 patients Placebo and 7 treatment arms	TC, TG and HDL-c increased in all treatment groups. TC:HDL-c ratio increased in 8 mg/kg tocilizumab.
SAMURAI (2007)	52 week, 366 patients Tocilizumab monotherapy or DMARDs	TC, TG and LDL-c increased in 38%, 17% and 26% of patients respectively. HDL-c increased to above ULN in 24% of patients; no change in TC:HDL-c ratio.
OPTION (2008)	24 week, 623 patients Tocilizumab 4mg/kg or 8mg/kg, or placebo	8mg/kg: TC, HDL-c and LDL-c increased by 0.9 mmol/l (18%), 0.1 mmol/l (7%) and 0.6 mmol/l (20%), respectively
TOWARD (2008)	24 week, 1,220 patients Tocilizumab 8mg/kg or placebo	TC, HDL-c and LDL-c increased by 0.8 mmol/l, 0.1 mmol/l and 0.5 mmol/l, respectively TC:HDL-c ratio increased by >30% in 12% tocilizumab group & 7% of placebo group
AMBITION (2010)	24 week, 673 patients Tocilizumab 8mg/kg monotherapy or MTX	Increase TC to >240 mg/dl in 13.2% and 0.4%, with no change in 24% and 46.8%, for tocilizumab and MTX groups LDL-c increased to >160 mg/dl in 3.1% and 0%, with no change in 11% and 22%, for tocilizumab and MTX groups
REACTION (2011)	24 week, 229 patients study of tocilizumab ± MTX	As monotherapy, TC increased 12.9mg/dl (7%); with MTX, TC increased 17.7 mg/dl (9%)
ADACTA (2016)	8 week, 324 patients tocilizumab 8mg/kg or adalimumab	TC, HDL-c and LDL-c increased by 0.8 mmol/l, 0.1 mmol/l and 0.5 mmol/l respectively for tocilizumab; all changes significantly greater than adalimumab group.

Table 3 - Overview of key phase III/IV tocilizumab studies reporting lipid outcomes

Whilst the above studies assessed changes in serum levels of cholesterol subfractions, novel methods of analysis have emerged which convey much more information about lipid profiles in vivo. Nuclear magnetic resonance spectroscopy (NMR) is one such technique which uses variations in magnetic properties of different atomic nuclei to quantify molecules or particles, and has been widely used in analysis of lipoproteins. The MEASURE study was a double-blind RCT evaluating the effects of tocilizumab on serum lipids and associated molecules as measured by NMR. 132 patients were randomised to tocilizumab or placebo, with all participants converting to open-label tocilizumab after 16 or 24 weeks. The primary outcome data of MEASURE (254) showed that 12 weeks of treatment with tocilizumab did not lead to significant increases in either small, dense LDL particles or oxidized LDL (particles typically suspected of being pro-atherogenic); instead, elevated LDL-c was driven by increases in larger LDL particles. In addition, tocilizumab led to reductions of greater than 30% in a variety of pro-atherogenic or pro-thrombotic factors including Lp(a), HDL-associated SAA, sPLA2, fibrinogen and D-dimers; paraoxonase, an anti-oxidant thought to be atheroprotective, increased following tocilizumab. Tocilizumab led to improvements in many surrogates for cardiovascular risk, and conferred a more anti-inflammatory phenotype to HDL, suggesting that it may be capable or reducing cardiovascular risk. This hypothesis, however, remains to be tested in long-term cardiovascular outcome studies.

1.5.4.4 Other biologic agents

Limited data are available on the lipidaemic effects of the other biologic agents used in the treatment of RA. My literature search identified a small number of papers reporting the effects of treatment with rituximab. A small pilot study (255) with 5 RA patients reported that after 16 weeks' treatment, mean total cholesterol levels fell by 8.5% and HDL-c levels increased by an average of 35%, whereas LDL-c concentrations increased in 2 patients but fell in the other 3, with an overall mean reduction of 3%. Interestingly, the results of a different small study (256) suggested HDL composition might also be favourably affected by rituximab therapy, with evidence for a reduction in HDL-associated SAA. A recent study from Russia treated 55 RA patients with rituximab and stratified them according to clinical response (257). The patients who responded

clinically demonstrated increased of 23% in HDL-c, and reduction in the CVD surrogates of arterial stiffness and CIMT.

1.5.4.5 JAK inhibition

Phase III trials of tofacitinib in patients with RA, a dual JAK1–JAK3 inhibitor, have shown serum levels of LDL-c and HDL-c to be elevated by up to around 21% and 14% within 12 months, respectively (258). These increases were substantially higher than the increases seen after treatment of patients with RA using the anti-TNF antibody adalimumab (Figure 13) in the ORAL Standard head-to-head comparison trial, despite similar ACR20, ACR50 and ACR70 responses (75).

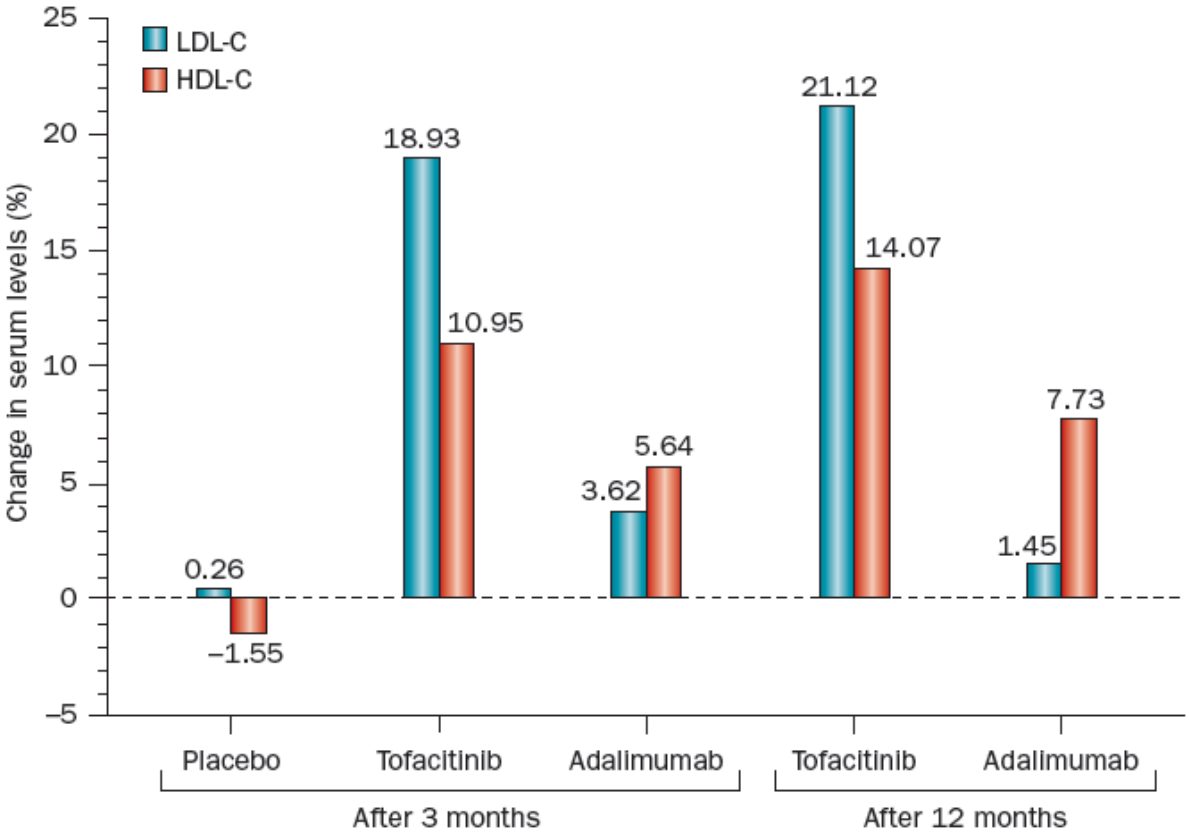


Figure 13 - Effects on LDL-c and HDL-c following treatment of active RA with adalimumab, tofacitinib or placebo in a head-to-head RCT. Adapted from Robertson et al. *Nat Rev Rheumatol* 2013;9(9):513-23

Similar results have been obtained from a phase II trial of tofacitinib in patients with active ulcerative colitis, with LDL-c and HDL-c levels both increasing dose-dependently (259). An earlier dose-ranging study of tofacitinib in RA supported the observations described above and demonstrated changes in levels of lipids within 2 weeks of commencing therapy, with levels reaching a plateau after 6-8 weeks (103). Preliminary results of phase II trials of other jakinibs, however, have been inconsistent; as such careful observation of cohorts will be mandatory, with addition data evaluated as they emerge. Similarly as new kinase inhibitor studies (for example, regarding targeting of SYK) arise, examination of the potential effects of such agents on lipid biochemistry, and thereby the potential to modify ongoing vascular risk in patients with chronic inflammatory diseases, will be important.

These findings suggest that, whereas suppression of inflammation partially underlies the increases in lipid levels, factors specific to different treatment modalities also operate and influence the degree to which lipid profiles change. Such therapy-specific factors could comprise as yet poorly defined pathways independent of those responsible for the primary disease modifying effects, which are thus implicated in driving lipid changes.(Figure 14)

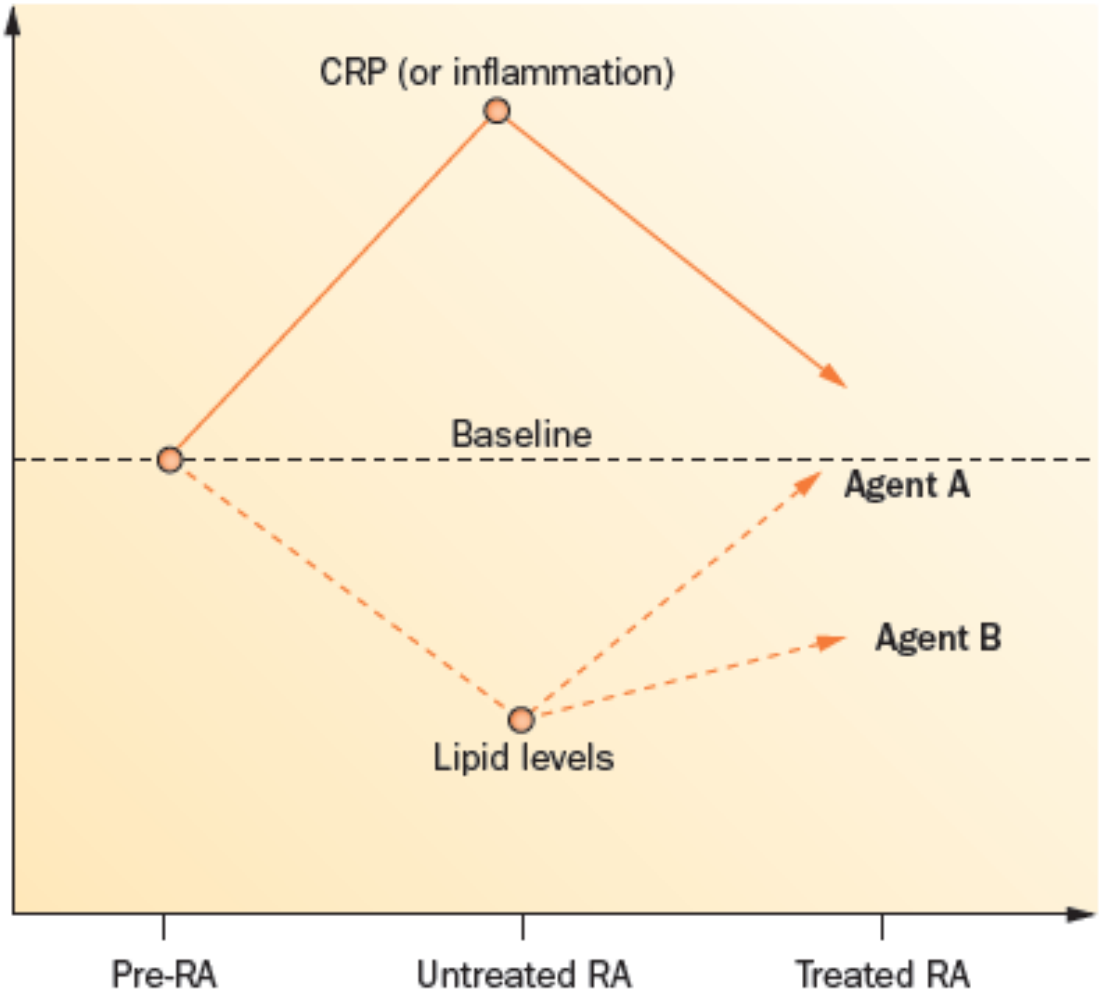


Figure 14 - Schematic of lipid changes in RA over time, with a broad correlation between disease activity and lipids which is reversed to some degree by different disease-modifying therapeutics. Adapted from Robertson et al. *Nat Rev Rheumatol* 2013;9(9):513-23

1.5.5 Serum lipids and predicting CVD risk in RA – ongoing and future research

Despite the advances in knowledge of the lipid–inflammation paradigm that we have described, substantial gaps in our understanding remain, which invite further study. Although the lipid changes seen after treatment of RA might reflect the variable 'correction' of an inflammatory dyslipidaemia, long-term mortality data are not yet available for many drugs, including biologic agents, and thus the cardiovascular effects of these agents cannot be confirmed. This point is particularly relevant for tocilizumab and the JAK inhibitors, which seem to have the most profound effects on LDL-c levels. The TRACE-RA study (Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Rheumatoid

Arthritis), which aimed to investigate this issue, was terminated early for futility owing to the low numbers of cardiovascular events being recorded. However, a specific CVD endpoint study is currently underway: ENTRACTE will compare tocilizumab with etanercept in RA patients with RA who have risk factors for CVD (ClinicalTrials.gov identifier NCT01331837). The results of this study will usefully inform on several important issues pertinent to cardiovascular disease in RA. Nevertheless, such outcomes will be some time in coming and, in the interim, mechanistic studies in humans that integrate interventions in immune pathways with detailed metabolic examination of lipid biochemistry will be informative, especially as rodent models are poor surrogates for investigation of such interactions. This approach will benefit understanding not only in the context of inflammatory disease but potentially also in evaluating mechanisms of risk attribution in the 'normal' population. Similarly the clinical use of lipid measurements in RA to predict CVD risk requires further development. At present, the most useful predictor of CVD seems to be the total cholesterol to HDL-c ratio; this value either does not change or only modestly 'improves' with successful anti-inflammatory therapy owing to concomitant rises in both total cholesterol and HDL-c levels in serum, with the latter sometimes being proportionally greater.

1.6 Objectives

1. To analyse the long-term effects of IL-6 blockade on lipoproteins, and their relationship with disease activity, from analysis of data from the MEASURE study.

2. To analyse the utility of a novel NMR-derived biomarker, GlycA, for assessing disease activity and changes in serum lipoproteins in RA following tocilizumab therapy.

3. To provide mechanistic explanations of the findings of MEASURE by conducting KALIBRA - a basic science study in a cohort of human subjects with RA, examining the effects of tocilizumab on the kinetics of LDL.

4. To provide mechanistic explanations of the findings of KALIBRA at the cellular & molecular level using in vitro techniques.

1.7 Hypotheses

1. Changes in serum lipoproteins in IL-6 blockade are a result of reductions in RA disease activity, as quantified by clinical, biochemical and NMR-derived measures.

2. Elevations in serum LDL-c following IL-6 blockade are a result of reduced catabolism of LDL by macrophage foam cells.

2 The MEASURE study

2.1 Methods

MEASURE was a randomised, multi-centre, placebo-controlled trial, comparing changes in lipid profiles following tocilizumab or placebo in patients with active RA despite methotrexate. The study was performed in a double-blind manner for 24 weeks, with open-label tocilizumab therapy given to all participants thereafter. The results of this trial after 12 weeks of therapy have already been published (254). The data I had available to analyse for my thesis was different to that already published in three aspects. Firstly, results were available up to 52 weeks, which allowed me to see if changes observed in the first 12 weeks were maintained over a longer follow-up period. Secondly, results were generated from an updated NMR platform patented by LipoScience, who performed the original sample analysis. This in theory would make the results more accurate and reliable. Lastly, clinical and laboratory measures of disease activity were provided for each patient at each timepoint, including a novel NMR-based quantification of the acute phase response called GlycA.

A description of the methods of the MEASURE study is available in the McInnes et al. 2013 manuscript. For completeness, a summary of the methods is presented here, with particular emphasis on the analyses novel to my thesis.

2.1.1 Patients

132 patients with active RA (according to ACR criteria) of more than 6 months duration were enrolled. Patients had active disease despite stable MTX therapy, requiring swollen joint count (SJC) and tender joint count (TJC) both ≥6, plus either CRP >10mg/L or ESR >28m/hr. MTX was continued during the study, but initiation of lipid-lowering, glucose-lowering or antihypertensive drugs, or a change in dose of these drugs, were prohibited in-study and within 12 weeks of baseline measurements. Glucocorticoids were permitted at doses ≤10mg/day; dose alterations were prohibited. Patients who had failed a TNF-inhibitor 6 months before baseline, or failed two TNF-inhibitors at any time, were excluded.

2.1.2 Procedures

The study was conducted at 34 sites across the UK, USA and Canada. Patients were randomly assigned by a voice response system, in a double-blind manner and in a 1:1 ratio, to TCZ 8mg/kg or placebo intravenously every 4 weeks with continuing stable doses of oral MTX. Patients were then offered open label TCZ at 24 weeks, or at 16 weeks for those patients who had not reached 20% improvement in SJC or TJC by that point. Blinded clinical assessments and blood sampling were performed at baseline and weeks 1, 2, 4, 8, 12, 16, 20 and 24. Open label assessments and sampling were performed every 12 weeks until week 104.

2.1.3 Patient allocation

Patient numbers through the study are illustrated in

Figure 15. Sixty-three patients were randomised to placebo+MTX and sixty-nine to TCZ+MTX. One patient was misrandomised to placebo, and two in the TCZ+MTX group were excluded from my analysis (one with no baseline data and one with baseline data only). Due to patients withdrawing consent, sixty and sixty-three patients in the two groups were included in the week 24 analysis; at week 52, the groups had fifty-three and fifty-five patients respectively.

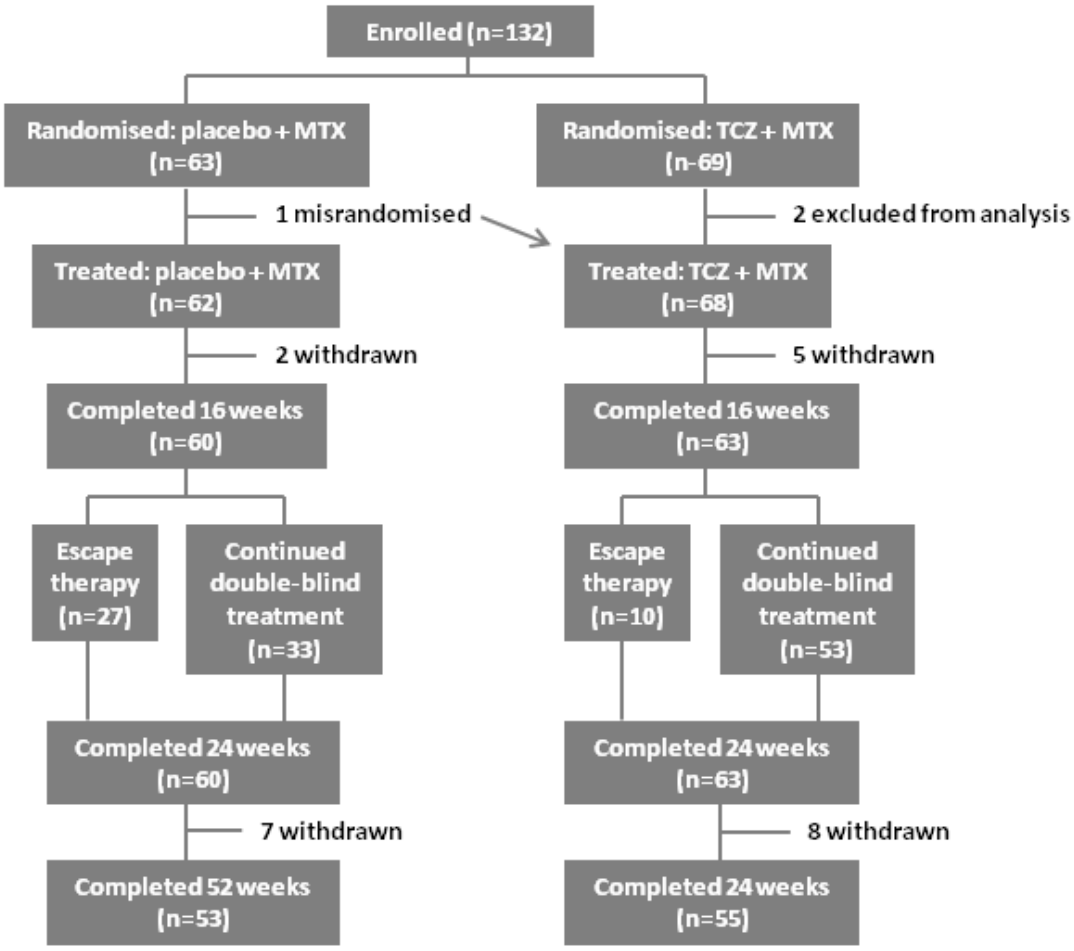


Figure 15 - Patient allocation and flow through the MEASURE study to week 52.

2.1.4 Assays

Commercial assays were used to measure high sensitivity C-reactive protein (CRP) and ESR (Covance Laboratories, Indiana, USA). Serum lipid subclasses and the GlycA signal were quantified by nuclear magnetic resonance (LipoScience, North Carolina, USA). Further biomarkers not discussed in this thesis were also measured and detailed in print (254).

2.1.5 GlycA

GlycA is a signal observed with NMR analysis of serum that can be numericalised to yield a serum 'level' that is amenable to reliable and reproducible quantification for clinical purposes. It is generated by N-acetyl-methyl groups on enzymatically glycosylated proteins, mainly α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin, and has been shown to reflect systemic inflammation in two large healthy cohorts (260, 261) and one RA cohort (262). As the GlycA signal is a composite score generated from the carbohydrate side-chains of several circulating acute phase proteins, it exhibits less biological variability than CRP and is not subject to the same confounding issues with pregnancy, anaemia, serum protein concentrations, and abnormal red blood cell shape or size as is ESR. It thus may offer substantial advantages over existing measures that are established in clinical practice.

2.1.6 Statistical analysis

The primary outcome measure was the change in small LDL particle number following TCZ therapy compared to placebo at 12 weeks. For this study I analysed the secondary endpoints of changes in lipoprotein particles at 24 and 52 weeks. Values were displayed as mean ± 95% confidence interval for normally-distributed data, or median ± IQR for non-parametric data. Between-group comparisons were performed using student's t-test or Wilcoxon Rank Sum test. Changes from baseline were assessed using paired t-test or Wilcoxon matched-pairs test. Correlation coefficients were calculated by Spearman's r value as many of the data were non-parametric. Normality of data was assessed by comparing population mean and median values, and inspecting values plotted on a histogram. As in the original manuscript, for data up to and including week 24, the analysis used last-observation-carried-forward only in samples taken before escape therapy (i.e. week 12 values carried forward to week 24 in those who received escape therapy). No other data was imputed. This meant that patients with missing values were excluded, and so different timepoints had different sample sizes as described in "Patient Allocation" above; in effect, the majority of my analysis was conducted on a per-protocol basis.

The original power calculations estimated that a sample size of 120 patients (60 per arm) would be able to detect a difference of 30% in small LDL particles at week 12 in patients treated with TCZ compared to placebo.

2.2 Results

2.2.1 Lipid particle changes

A series of figures demonstrate the changes observed in lipid parameters over 52 weeks of the study. In the TCZ+MTX group, levels of large VLDL/chylomicrons and small VLDL particles (the latter making up more than half of the total VLDL/chylomicron mass) both increased significantly from baseline by the week 2 assessment (Figure 16). The degree of change was significantly different from that in the placebo+MTX group (Figure 17). By week 52, the placebo group "caught up" with change in large VLDL but a significant difference remained between the groups for small VLDL (Figure 17).

Total LDL particles increased with treatment, driven by rises in IDL and large LDL particles (Figure 18). Again, these changes were apparent at the week 2 assessment. Small LDL particles did not change. The between-groups differences were significant for large LDL but not IDL (Figure 19). Notably, whilst there seems to be no clear difference in total or large LDL in Figure 18 at week 24, this is because the placebo+MTX group had higher median levels at baseline; hence Figure 19 illustrates the difference in percentage change from baseline. In all parameters, there was no difference in percentage change between the groups at 52 weeks.

Total HDL particles increased in the TCZ+MTX group, again with the bulk of the change seen by week 2, driven solely by an increase in small HDL particles (Figure 20). This represented a significant difference in percentage change from baseline compared to the placebo+MTX group. Once again, the placebo+MTX group displayed a "catch-up" pattern after switching to open label treatment at week 24, and no significant difference was seen between the groups at week 52 (Figure 21).

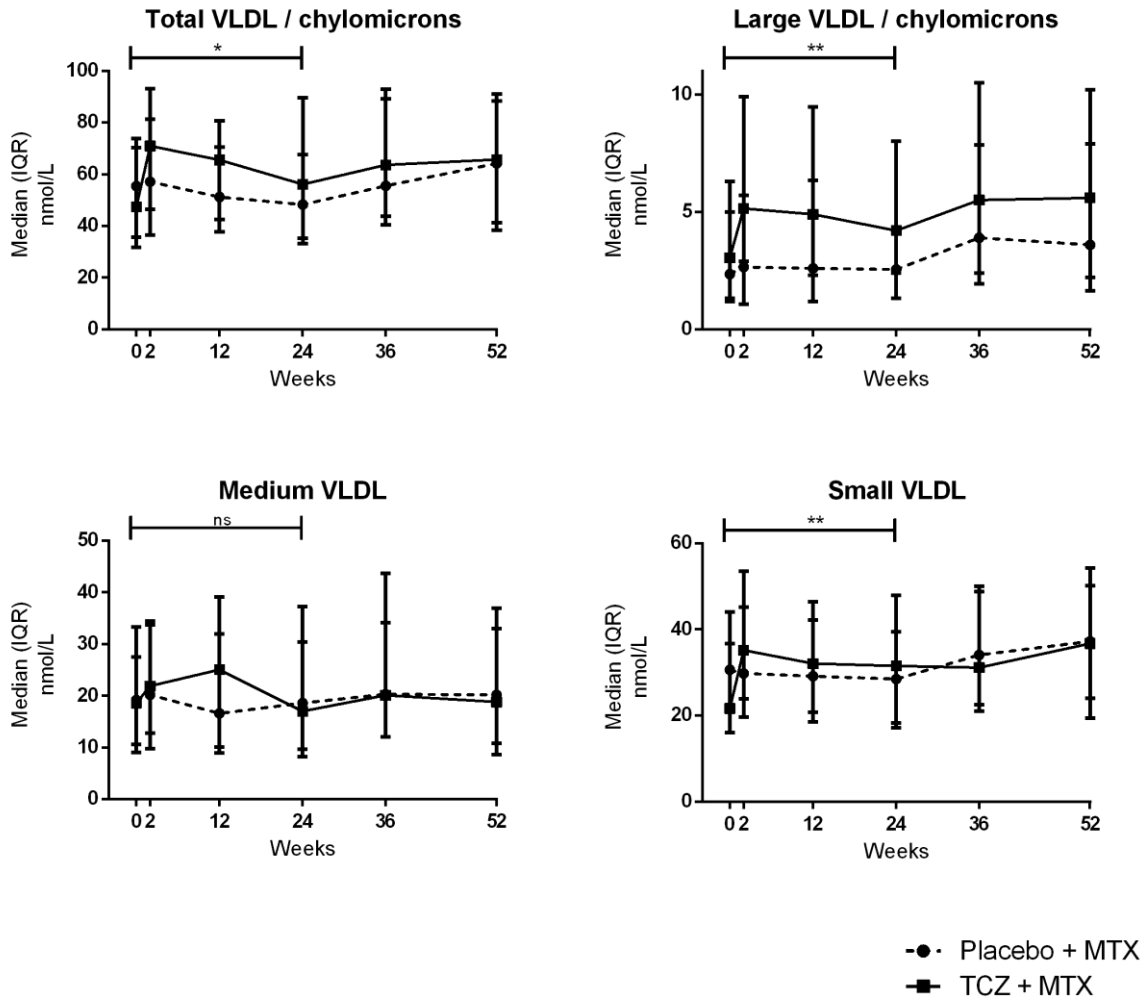


Figure 16 - Absolute values of VLDL/chylomicron particles from baseline to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 in TCZ+MTX group compared to baseline.

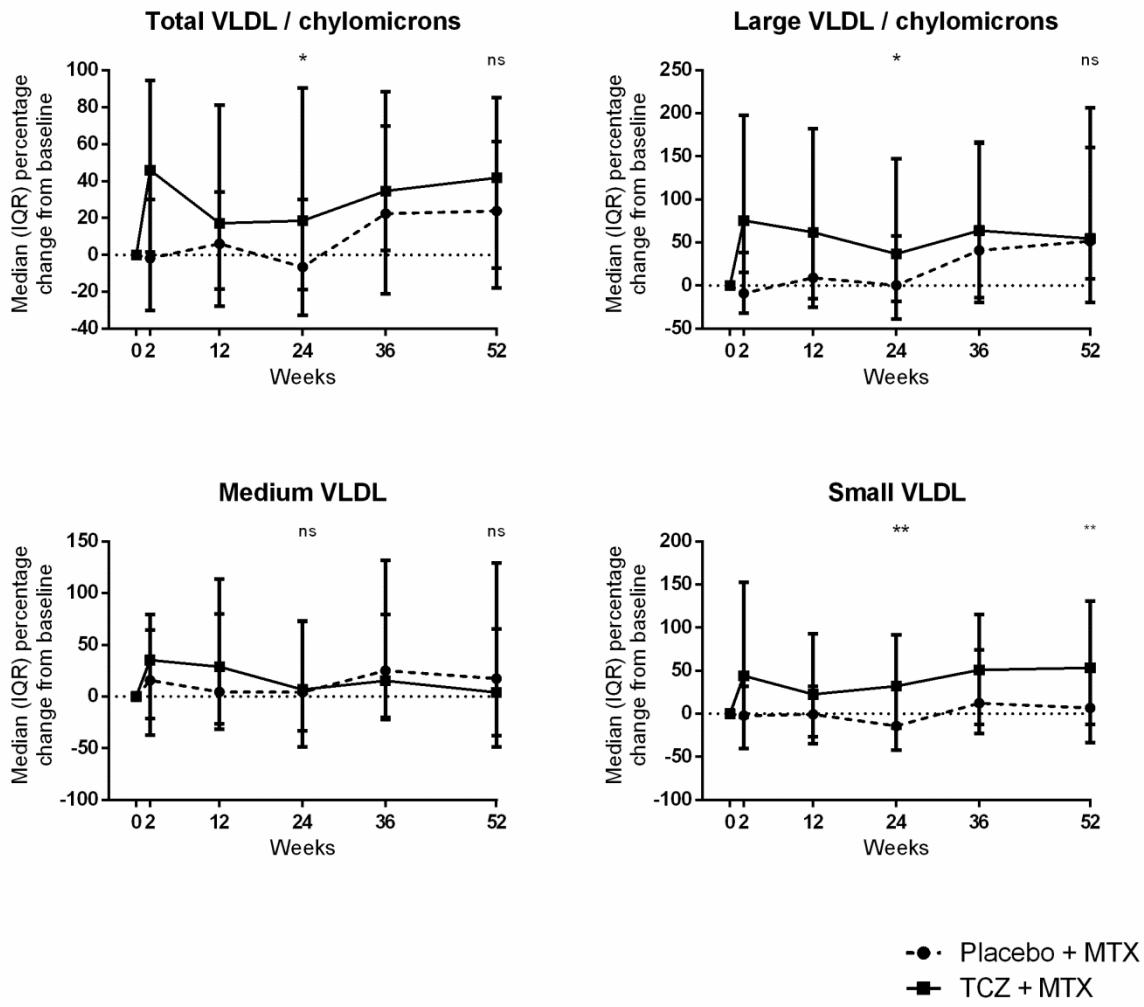


Figure 17 - Percentage change from baseline in VLDL/chylomicron particles to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 between groups.

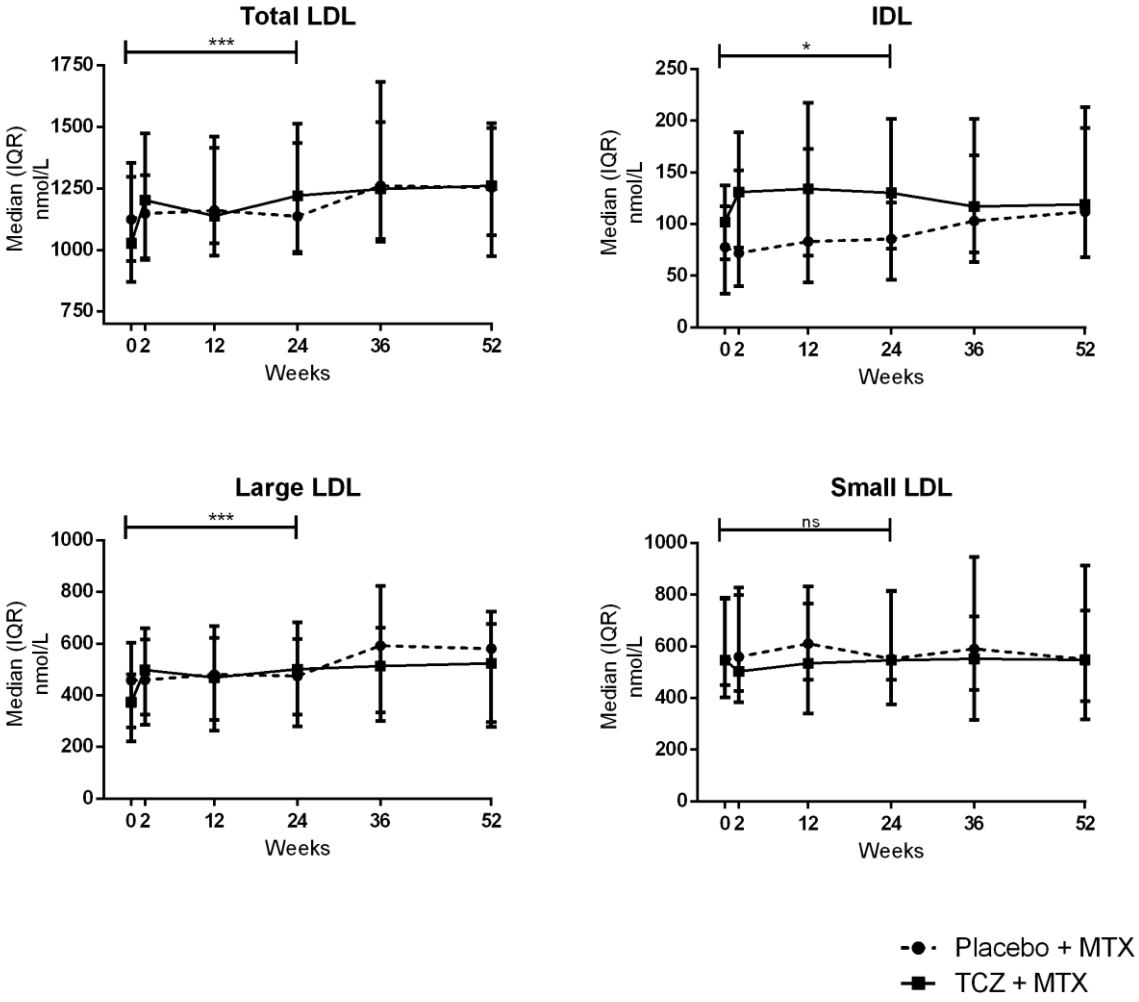


Figure 18 - Absolute values of LDL particles from baseline to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 in TCZ+MTX group compared to baseline.

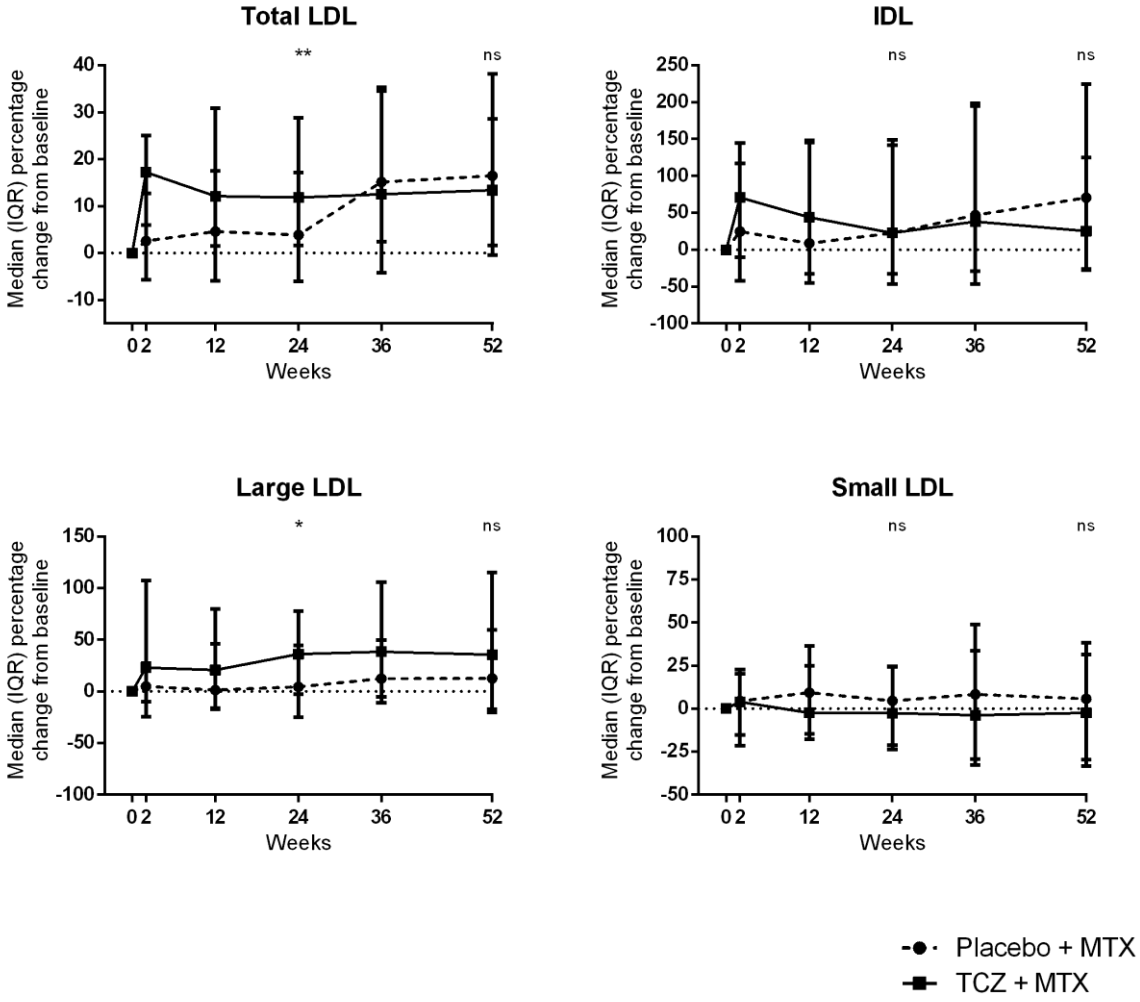


Figure 19 - Percentage change from baseline in LDL particles to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 between groups.

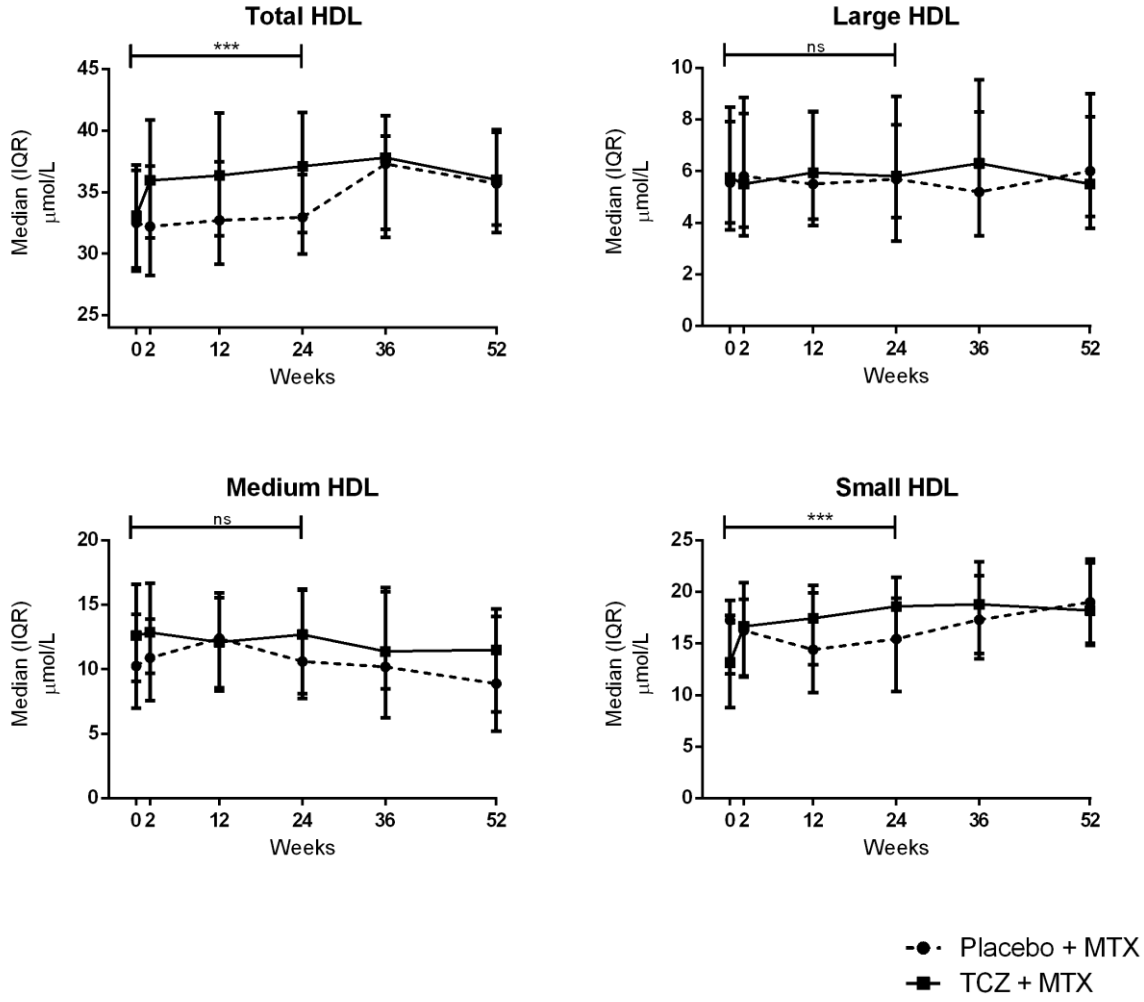


Figure 20 - Absolute values of HDL particles from baseline to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 in TCZ+MTX group compared to baseline.

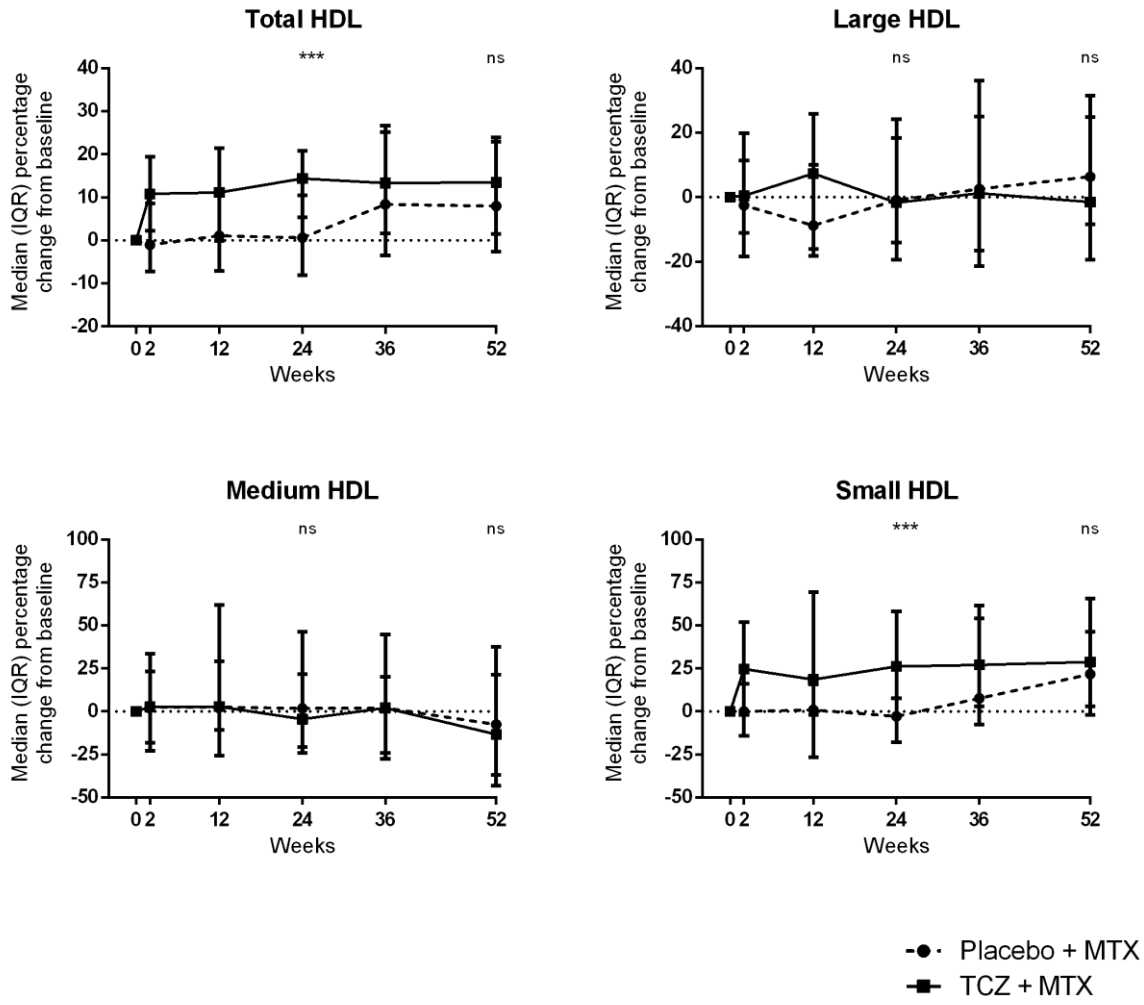


Figure 21 - Percentage change from baseline in HDL particles to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 between groups.

Mean VLDL particle size increased with treatment, with changes evident by week 2 (Figure 22). However, this change was only just statistically significant, and the degree of change from baseline was numerically but not significantly greater in the TCZ+MTX group (Figure 23). Mean LDL size increased numerically (in keeping with the observation that large LDL particle number increased - Figure 22) but neither this increase nor the difference in change between the two groups reached statistical significance (Figure 23). Mean HDL size fell significantly by week 24 in the TCZ+MTX group, again with an apparent change by the week 2 assessment (Figure 22). HDL size in the TCZ+MTX group fell by more than the placebo+MTX group, but the difference in change from baseline between the groups was no longer significant at week 52 (Figure 23), again

indicating “catch up” by the placebo+MTX group switching to open-label treatment.

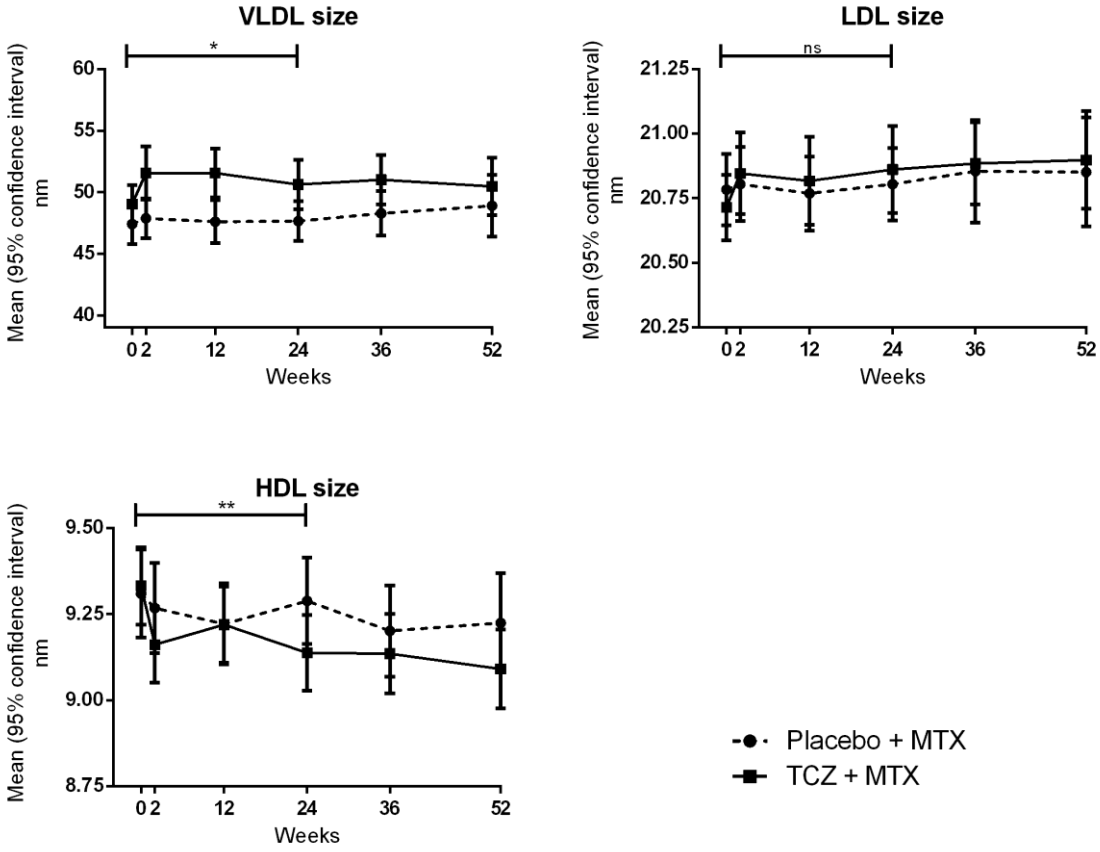


Figure 22 - Absolute values of mean lipoprotein particle size from baseline to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 in TCZ+MTX group compared to baseline.

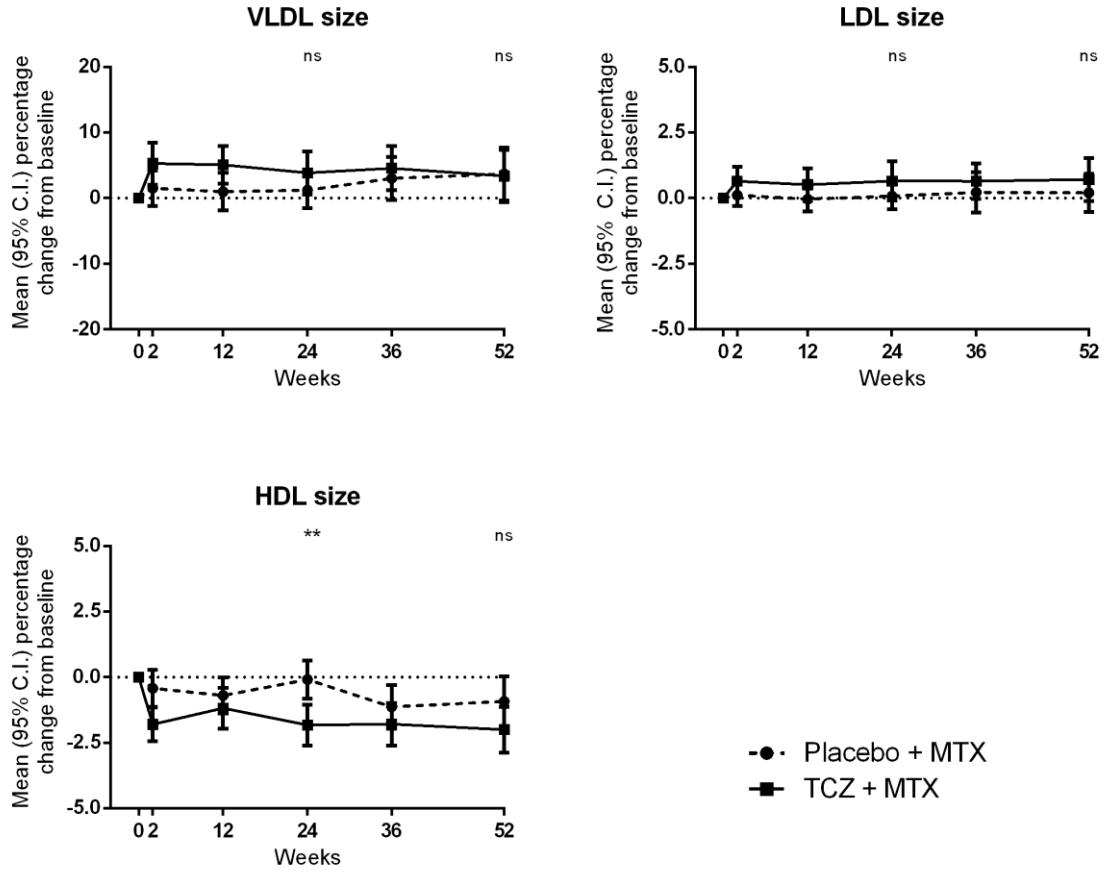


Figure 23 - Percentage change from baseline in mean lipoprotein particle size to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 between groups

2.2.2 GlycA & clinical response

I used access to GlycA values and clinical data from each timepoint in two ways. I first assessed the performance of GlycA as a measure of RA disease activity, and then moved on to exploring relationships between measures of disease activity and the lipid values I have described above.

2.2.2.1 Baseline parameters

Data on age, sex and measures of disease activity at baseline in the placebo and treatment groups are shown in

Table 4. There were no significant differences between the groups in any parameter. The cohort generally had high levels of disease activity, particularly illustrated by the high mean DAS28 and CDAI scores. However, inflammatory

markers at baseline were more varied. 34 patients in total had CRP <5mg/L at baseline, with 11 patients having ESR <18mm/hr at baseline (the "normal" cut-off ranges used by laboratories in our centre). No defined normal ranges yet exist for GlycA. Akinkuole et al (260), described a large healthy cohort with a median (IQR) GlycA value of 369μmol/L (327 - 416). Only one other RA cohort (n=166) has been analysed so far for GlycA (262). In this group, median (IQR) GlycA was found to be 398μmol/L (348 – 473). Compared to the MEASURE cohort, this group had lower disease activity, as reflected in a median (IQR) CRP of 4.0mg/L (1.2 – 11) and median (IQR) ESR 15mm/hr (7 – 36); median DAS28-CRP was 3.09, with median DAS28-ESR 3.86.

	Placebo + MTX (n=62)	TCZ + MTX (n=68)	*p* for difference
Age, years (mean (range))	56 (25-72)	56 (35-76)	0.92
Female, n (%)	46 (74)	57 (84)	0.176
CRP, mg/L	8.7 (3.9-18.1)	9.3 (5.1-26.9)	0.25
ESR, mm/hr	38 (26.3-58)	38 (28-58)	0.64
GlycA, μmol/L (mean (SD))	476 (117)	484 (107)	0.68
SJC (28 joints)	10 (8-16)	11 (7-16)	0.93
TJC (28 joints)	16 (10-23)	16 (12-23)	0.86
Patient global VAS, mm	63 (49-74)	63 (47-81)	0.48
DAS28-CRP (mean (SD))	5.80 (0.95)	5.91 (1.06)	0.53
DAS28-ESR (mean (SD))	6.54 (1.01)	6.62 (1.05)	0.66
CDAI (mean (SD))	41.0 (14.1)	41.4 (14.4)	0.86

Table 4 - Patient demographics and parameters of disease activity at baseline visit. Data presented as median (IQR) unless otherwise indicated.

To assess GlycA's function as a biomarker of RA disease activity, I combined the baseline data of all 130 participants into one cohort and correlated GlycA levels at baseline with other measures of disease activity. These are outlined in

Table 5. GlycA levels correlated strongly with CRP, modestly with ESR, and not at all with joint counts or CDAI. Correlation with DAS28 scores was statistically significant but numerically very modest.

	GlycA	
	r	*P*
CRP	0.70	*<0.0001*
ESR	0.44	*<0.0001*
SJC	-0.04	*0.69*
TJC	-0.11	*0.20*
VAS	0.35	*<0.0001*
DAS28-CRP	0.28	*0.002*
DAS28-ESR	0.20	*0.025*
CDAI	0.01	*0.88*

Table 5 - Correlation between GlycA and markers of disease severity in whole cohort at baseline assessment (n=130) using Spearman's coefficient.

It could be argued that GlycA's poor correlation with joint counts reduces its utility as a marker of disease activity. For context,

Table 6 shows that CRP also correlated only moderately with ESR, and that neither acute phase marker correlated at all with joint counts, patient's visual analogue scale or CDAI. Patterns of correlation between GlycA and acute phase markers or composite disease activity scores are illustrated in Figure 24.

CRP versus	r	*p*	**ESR versus**	r	*p*
ESR	0.36	*<0.0001*			
SJC	0.07	*0.42*	SJC	0.14	*0.12*
TJC	-0.01	*0.89*	TJC	0.05	*0.61*
VAS	0.29	*<0.001*	VAS	0.19	*0.03*
CDAI	0.12	*0.19*	CDAI	0.13	*0.14*

Table 6 - Correlation between CRP or ESR and markers of disease severity in whole cohort at baseline assessment (n=130) using Spearman's coefficient.

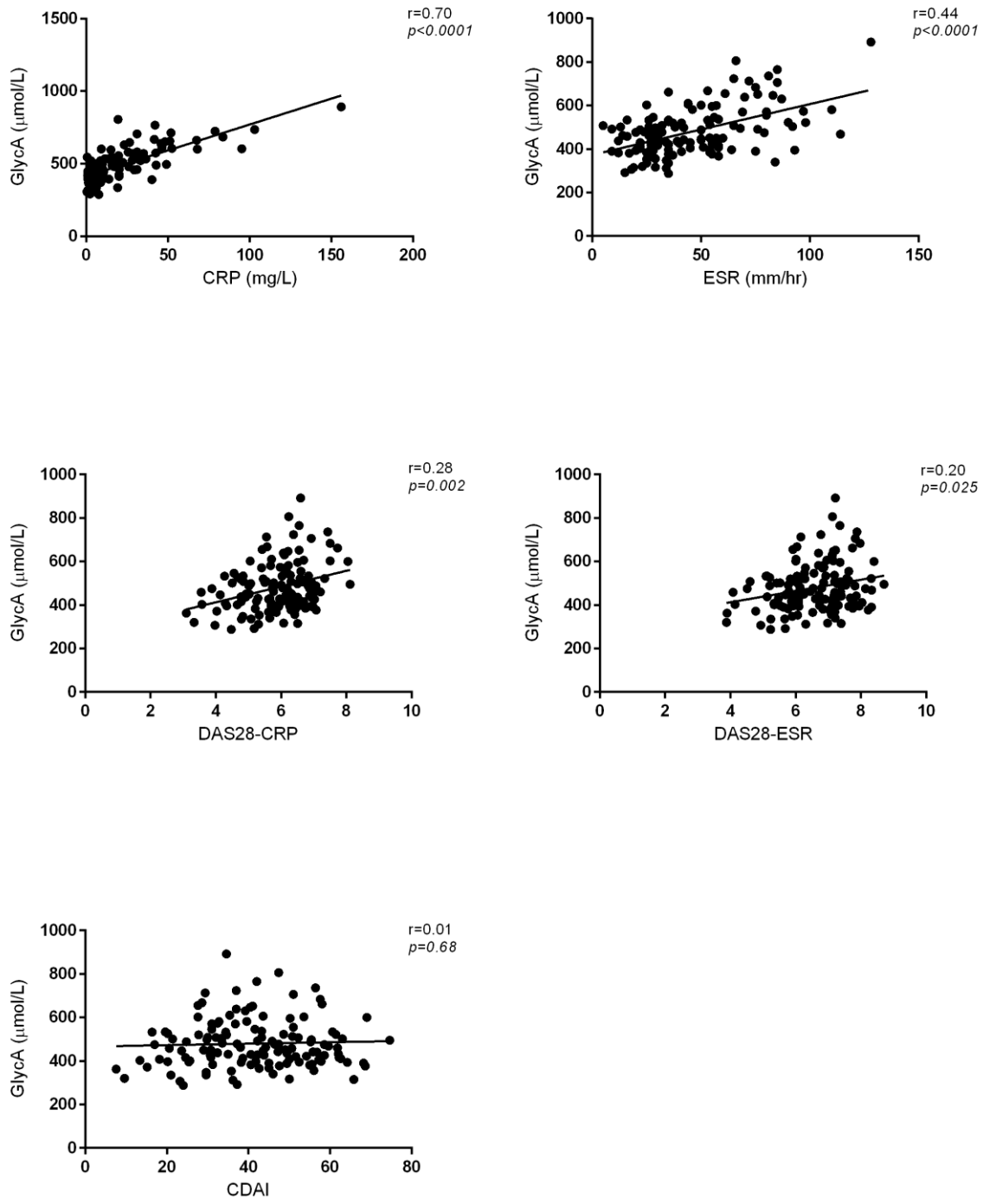


Figure 24 - Spearman's correlation coefficients between GlycA and measures of disease activity at baseline (n=130).

2.2.2.2 Longitudinal behaviour of GlycA

One advantage of a placebo controlled study was that a subset of patients (the placebo+MTX group) was followed up for several weeks with no active change in

their treatment. In effect, this allowed the above observations in active RA to be replicated by repeating correlations in this group using data taken at 2 and 12 weeks after baseline.

Table 7 shows that the strength of GlycA's correlation with CRP and ESR remained approximately steady throughout 12 weeks of placebo treatment. The same was true for DAS28-CRP. At week 12 GlycA's association with swollen joint count became statistically significant but remained numerically modest; the same is true for DAS28-ESR at week 2, an association which fell and lost significance by week 12.

	Week 0		**Week 2**		**Week 12**	
	r	*p*	r	*p*	r	*p*
CRP	0.71	*<0.0001*	0.78	*<0.0001*	0.71	*<0.0001*
ESR	0.40	*0.002*	0.58	*<0.0001*	0.47	*<0.001*
SJC	0.03	*0.81*	0.23	*0.074*	0.31	*0.016*
TJC	-0.20	*0.12*	0.08	*0.51*	0.20	*0.12*
VAS	0.22	*0.081*	0.16	*0.21*	0.09	*0.51*
DAS28CRP	0.23	*0.070*	0.37	*0.004*	0.38	*0.003*
DAS28ESR	0.11	*0.38*	0.34	*0.007*	0.12	*0.37*

Table 7 - Spearman's correlation coefficient between GlycA and other measures of disease activity in the placebo group (n=62) at baseline, week 2 and week 12.

2.2.2.3 Baseline GlycA as a predictor of clinical response

To test our hypothesis that GlycA would serve as a useful predictor of treatment response, receiver-operating characteristic (ROC) curves were generated. Using data from the TCZ + MTX group (n62) I used CRP, ESR and GlycA measures at baseline and after two weeks of tocilizumab therapy as predictors of clinical response. Since DAS28 scores (and therefore EULAR response rates) are frequently skewed by large changes in ESR or CRP with tocilizumab, for the purposes of this analysis I divided participants depending on whether or not they

reached a state of low disease activity, as measured by a CDAI of <10, at week 24.

Figure 25 shows that neither baseline (AUC = 0.60) or week 2 (AUC = 0.53) measures of GlycA functioned as a good predictor of CDAI status at week 24. CRP and ESR returned similar results. A baseline GlycA of <430μmol/L predicted reaching CDAI low disease activity with sensitivity of 56% and specificity 70% (likelihood ratio 1.888). This compares to a baseline CRP of <6.1mg/L predicting low CDAI with sensitivity of 63% and specificity 81% (likelihood ratio 3.264) and baseline ESR of <24mm/hr predicting low CDAI with sensitivity of 25% and specificity 85% (likelihood ratio 1.679). I concluded that GlycA was superior to ESR but less effective than CRP at predicting clinical response in tocilizumab-treated RA patients. However, none of these measures were particularly effective, and no baseline or week 2 measures returned sensitivity and specificity values strong enough to be considered useful in clinical practice.

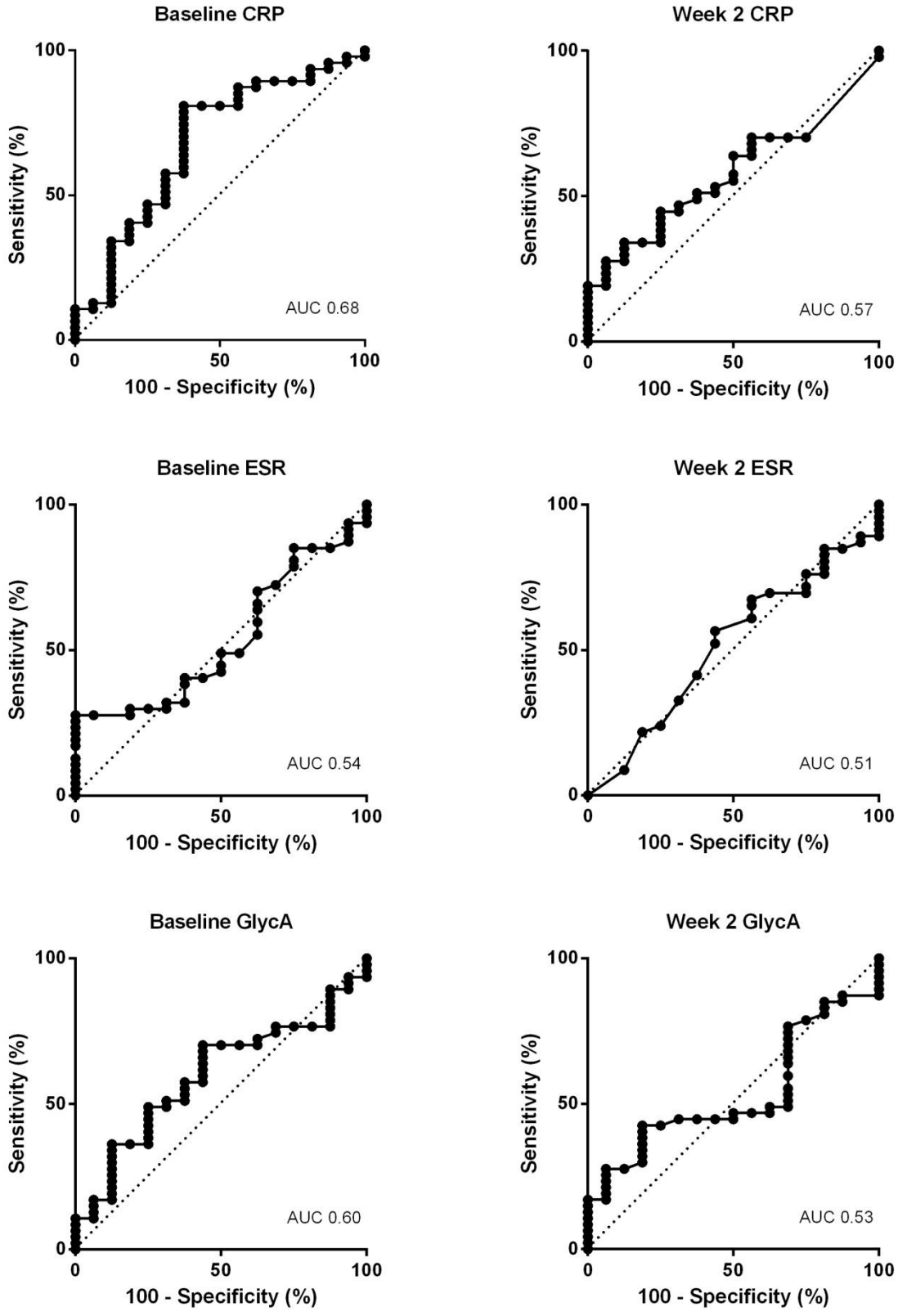


Figure 25 - ROC curves for ability of CRP, ESR and GlycA levels at baseline (left) and week 2 (right) to predict attainment of CDAI low disease activity in the TCZ+MTX group (n=62).

Despite the strong association between GlycA and CRP, there remained several patients with discordant results for these two measures (i.e. one value high and one low). I hypothesised that GlycA could provide extra information on disease burden in these patients. To test this, I analysed baseline data from patients with CRP <5mg/L and stratified them into those with low or high GlycA. For a cut-off GlycA point, I chose 416μmol/L as this was the upper quartile of values in a healthy, non-RA cohort. This yielded 34 patients, 19 of whom had "low" GlycA (median 368μmol/L, IQR 317–398) and 15 with "high" GlycA (median 447μmol/L, IQR 430-498). There were no differences between the groups in baseline DAS28-CRP, DAS28-ESR or CDAI score, indicating that GlycA seemed to add little to CRP when ascertaining disease activity (Figure 26).

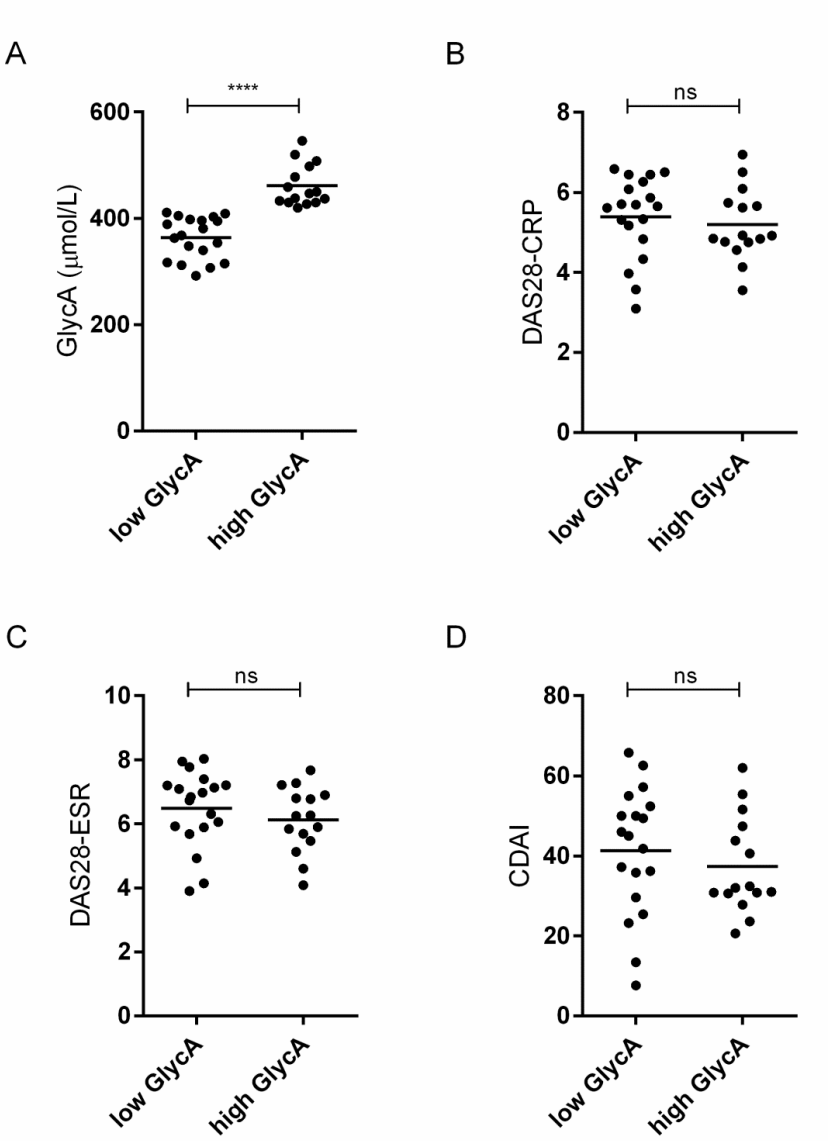


Figure 26 - Baseline values of (A) GlycA, (B) DAS28-CRP, (C) DAS28-ESR and (D) CDAI in patients with CRP<5md/L and low or high GlycA. P value calculated by Mann-Whitney U test.

2.2.2.4 Change in disease activity markers with treatment

Changes in levels of GlycA, acute phase reactants, clinical measures of disease activity and composite disease activity scores are outlined below. Figure 27 shows absolute values from baseline to week 52. Figure 28 shows the percentage change or value change from baseline to week 52, with asterisks denoting statistical significance for between-group differences at 24 and 52 weeks. Values are presented as mean ±95% C.I. or median ± IQR, depending on normality of data. P values were generated using student's t-test or Mann-Whitney U test depending on normality of data.

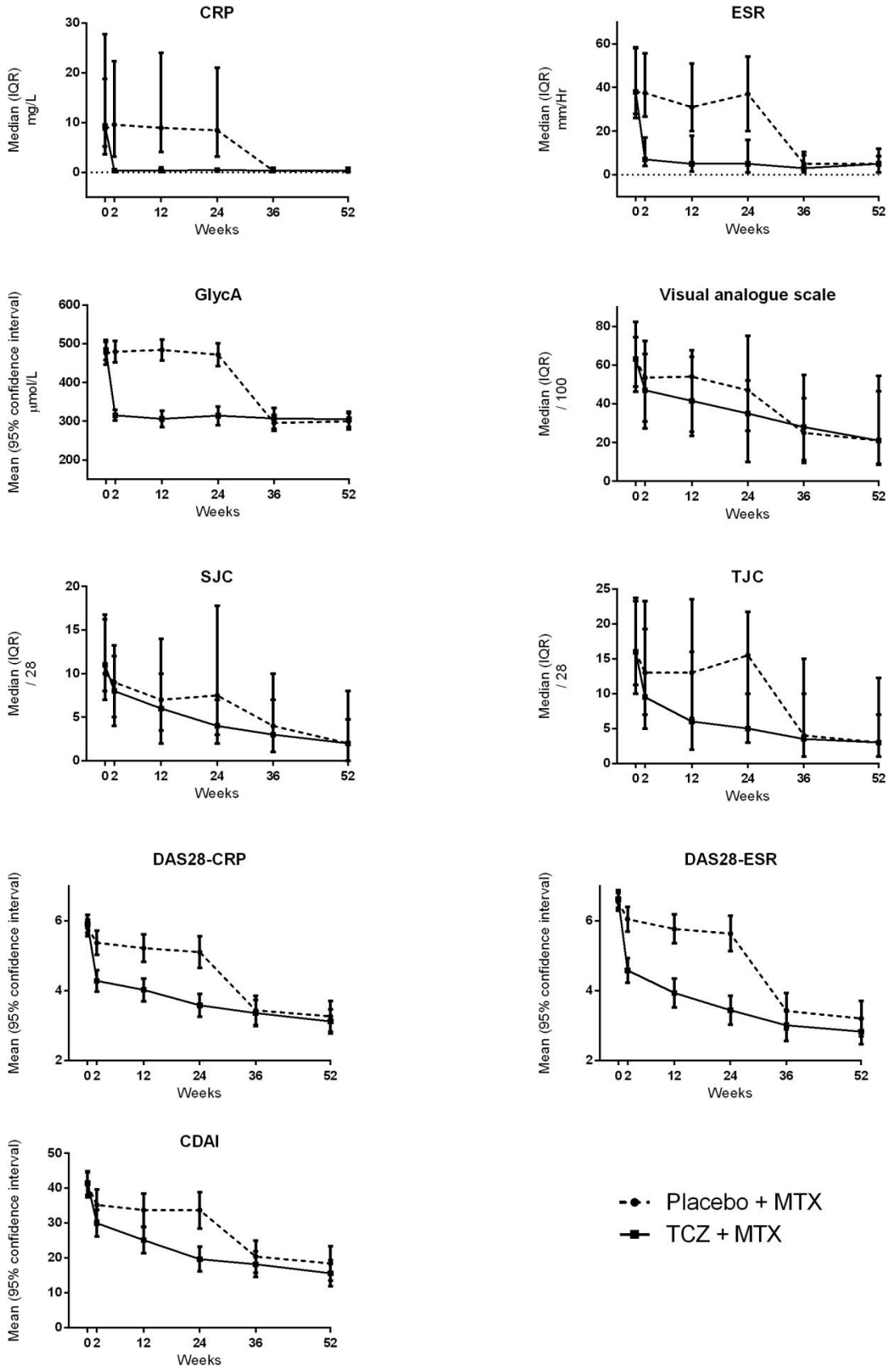


Figure 27 - Measures of disease activity from baseline to week 52.

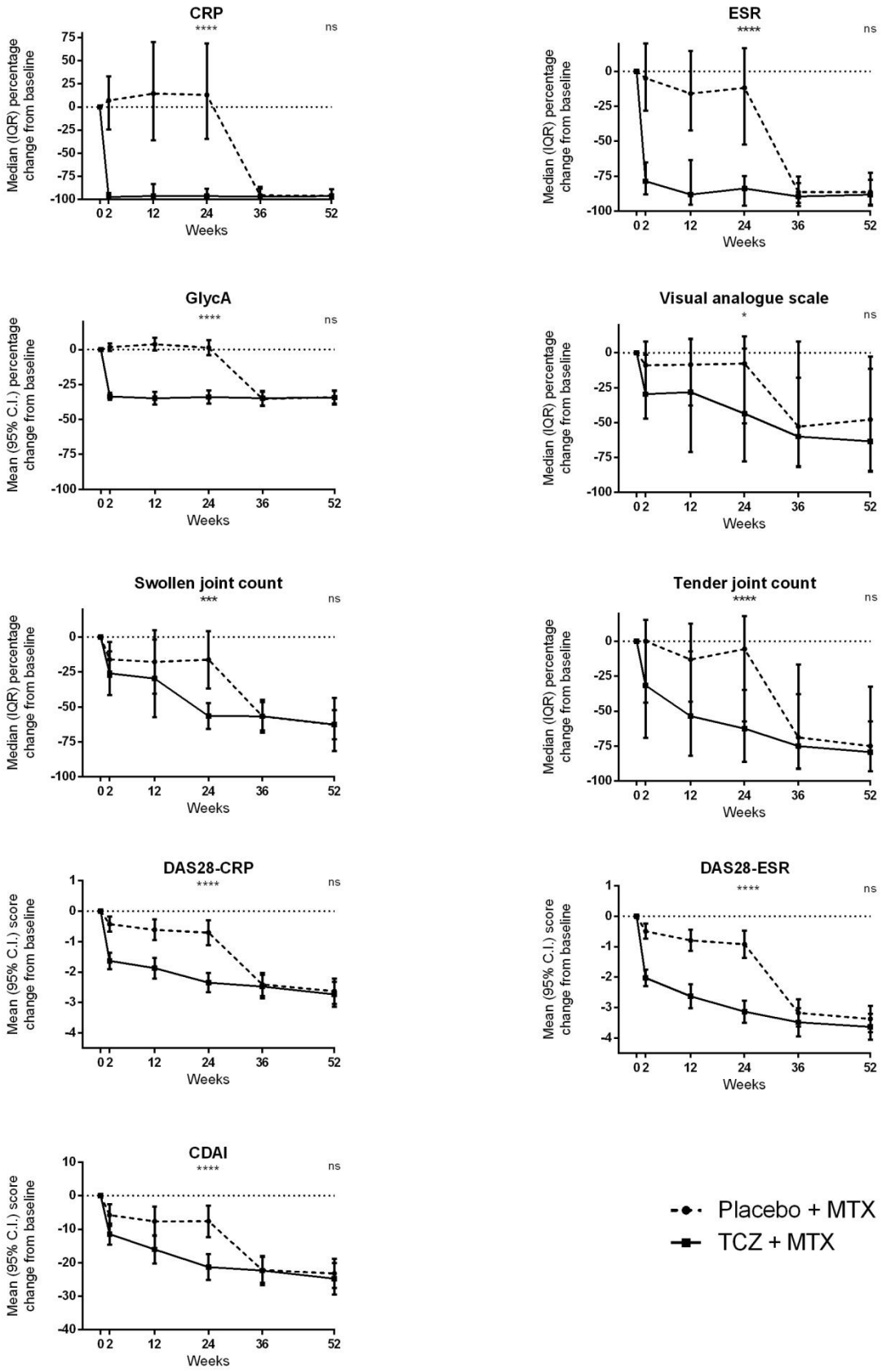


Figure 28 - Change in measures of disease activity from baseline to week 52. * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 between groups.

As expected, CRP and ESR fell profoundly and rapidly in the TCZ+MTX group, reaching median values of 0.34mg/L and 7mm/hr by week 2, and staying at around this level for the duration of the study. In a similar manner, median GlycA in the TCZ+MTX group fell from 464μmol/L to 315μmol/L at week 2, before plateauing at 290μmol/L by week 12. As one would expect, joint counts, VAS and CDAI scores fell more gradually, with the lines of DAS28 scores sitting somewhere in the middle. Significant differences between the groups in the degree of change from baseline were present in all parameters at week 24.

2.2.3 Relationships between clinical and lipid measures

I next set out to see if there were any discernible relationships between our observed changes in lipoproteins and those in disease activity, at baseline, and at week 24. GraphPad Prism assists this process by allowing generation of correlation matrices: tables which lay out r values between different variables in a form which allows rapid presentation of quite a large amount of data. The p values for each calculation are laid out in a separate, identically-formatted table. This allows the user to scan for relationships, either individually or in patterns, which can then be interrogated further by e.g. scatter plot. It is this technique which I will make use of in the following pages, as it allows the results of numerous correlation calculations to be summarised relatively cogently. Rather than simply duplicate every result in the form of a scatter plot, before correlations were calculated I decided that scatter plots would be generated at each timepoint for: small LDL particles (the primary outcome measure of the study); large LDL particles (as the LDL component that appeared to be most affected by tocilizumab therapy); and any other particles which appeared to have strong relationships in the correlation matrices.

2.2.3.1 Relationships at baseline

Spearman's r values between acute phase markers (GlycA, CRP and ESR) and clinical disease scores (DAS28 and CDAI) in the whole cohort at baseline are outlined in Table 8 and Table 9. Small LDL particle number did not associate with any clinical measures (Figure 29). A significant but modest inverse correlation was seen between large LDL particle number and all clinical measures apart from ESR. On reviewing the scatter plots, there did appear to

be generally lower lipoprotein levels in those with high CRP or GlycA (Figure 30). On the other hand, I did not think there was any clear visible pattern of relationship between large LDL and DAS28 or CDAI scores, despite the p values being below 0.05. More impressive associations were observed with small HDL particle number, where the strongest correlation was seen with CRP ($r=-0.47$, $p<0.0001$)(Figure 31). Again, the scatter plots of composite disease scores were much less convincing that those of CRP or GlycA. Of note, r and p values for GlycA tended to be quite similar to those of CRP throughout the correlation matrix.

r values	VLDL				LDL				HDL			
	Total	Large	Medium	Small	Total	IDL	Large	Small	Total	Large	Medium	Small
GlycA	-0.01	0.00	-0.05	0.03	-0.08	-0.12	-0.28	0.14	-0.25	-0.15	0.19	-0.31
CRP	-0.14	-0.09	-0.18	-0.06	-0.14	-0.21	-0.27	0.12	-0.44	-0.17	0.14	-0.47
ESR	0.04	-0.05	-0.10	0.13	0.00	0.04	-0.11	0.04	-0.22	-0.07	0.10	-0.23
DAS28-CRP	-0.03	0.09	-0.02	-0.04	-0.21	-0.11	-0.26	-0.02	-0.16	0.06	0.16	-0.35
DAS28-ESR	0.01	0.08	-0.02	0.02	-0.16	-0.02	-0.19	-0.06	-0.06	0.13	0.16	-0.26
CDAI	0.01	0.12	0.04	-0.04	-0.17	-0.06	-0.18	-0.05	-0.01	0.13	0.11	-0.19

p values	VLDL				LDL				HDL			
	Total	Large	Medium	Small	Total	IDL	Large	Small	Total	Large	Medium	Small
GlycA	0.93	0.97	0.59	0.76	0.36	0.16	0.001	0.11	0.004	0.078	0.027	<0.001
CRP	0.11	0.28	0.037	0.50	0.11	0.017	0.002	0.17	<0.0001	0.058	0.11	<0.0001
ESR	0.66	0.61	0.25	0.13	0.96	0.65	0.23	0.61	0.011	0.45	0.28	0.010
DAS28-CRP	0.75	0.30	0.79	0.61	0.017	0.23	0.003	0.83	0.073	0.49	0.067	<0.0001
DAS28-ESR	0.89	0.35	0.82	0.84	0.069	0.79	0.033	0.47	0.51	0.15	0.074	0.003
CDAI	0.94	0.16	0.69	0.68	0.053	0.46	0.042	0.54	0.91	0.15	0.21	0.030

Table 8 - Spearman's r value (top) and p value (bottom) for measures of disease activity and lipid NMR values at baseline in whole cohort (n=130). Green = p<0.05. Red = p<0.01

values	Particle size		
	VLDL	LDL	HDL
GlycA	0	-0.15	0.08
CRP	-0.06	-0.17	0.06
ESR	-0.11	-0.07	0.08
DAS28-CRP	0.11	-0.11	0.18
DAS28-ESR	0.07	-0.06	0.21
CDAI	0.13	-0.06	0.17

p values	Particle size		
	VLDL	LDL	HDL
GlycA	0.97	0.084	0.35
CRP	0.53	0.061	0.5
ESR	0.22	0.42	0.34
DAS28-CRP	0.23	0.19	0.035
DAS28-ESR	0.41	0.49	0.017
CDAI	0.13	0.48	0.05

Table 9 - Spearman's r value (top) and p value (bottom) for measures of disease activity and mean lipid particle size at baseline in whole cohort (n=130). Green = p<0.05. Red = p<0.01

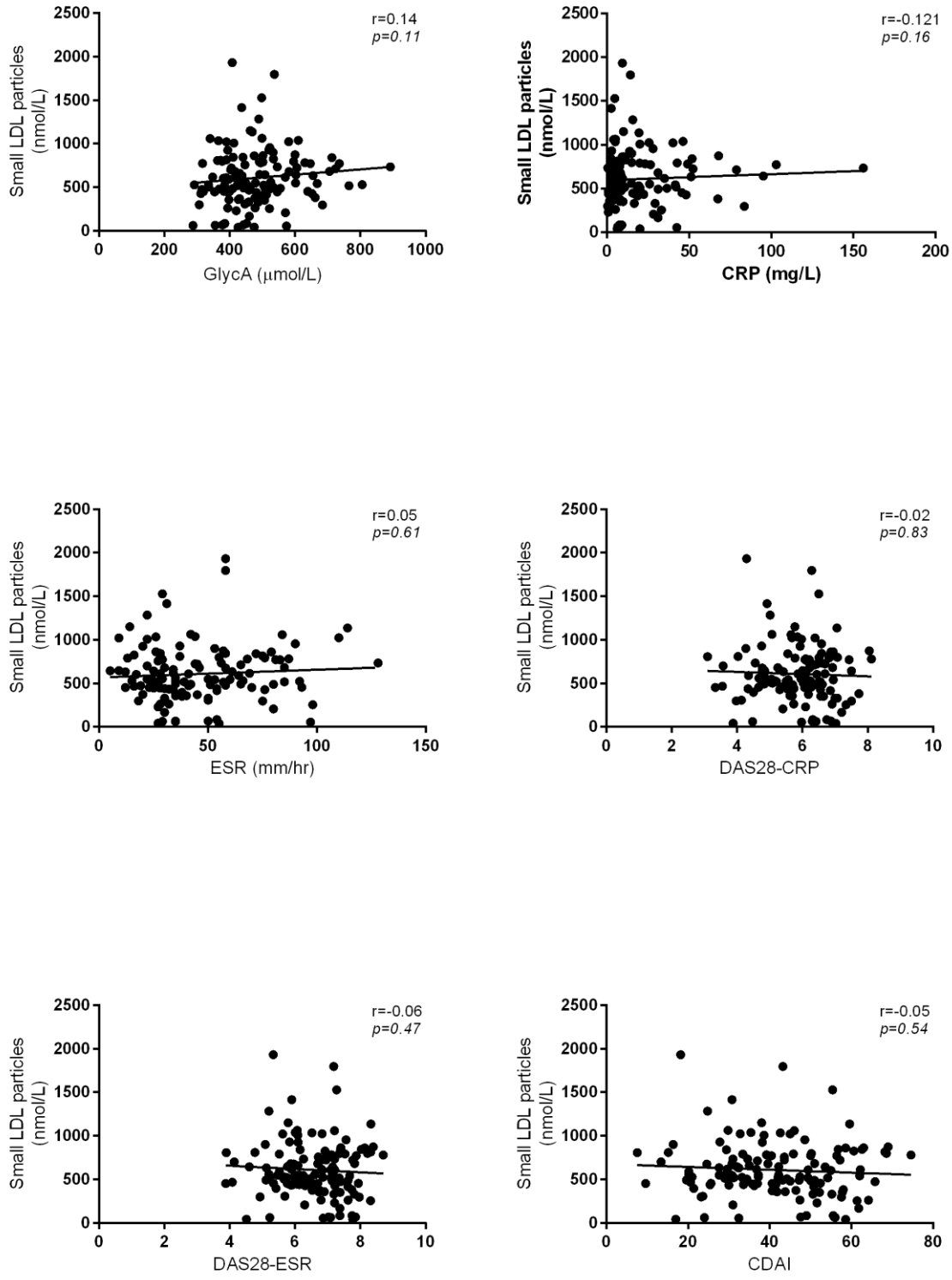


Figure 29 - Spearman's correlation coefficients between small LDL particles and measures of disease activity at baseline (n=130).

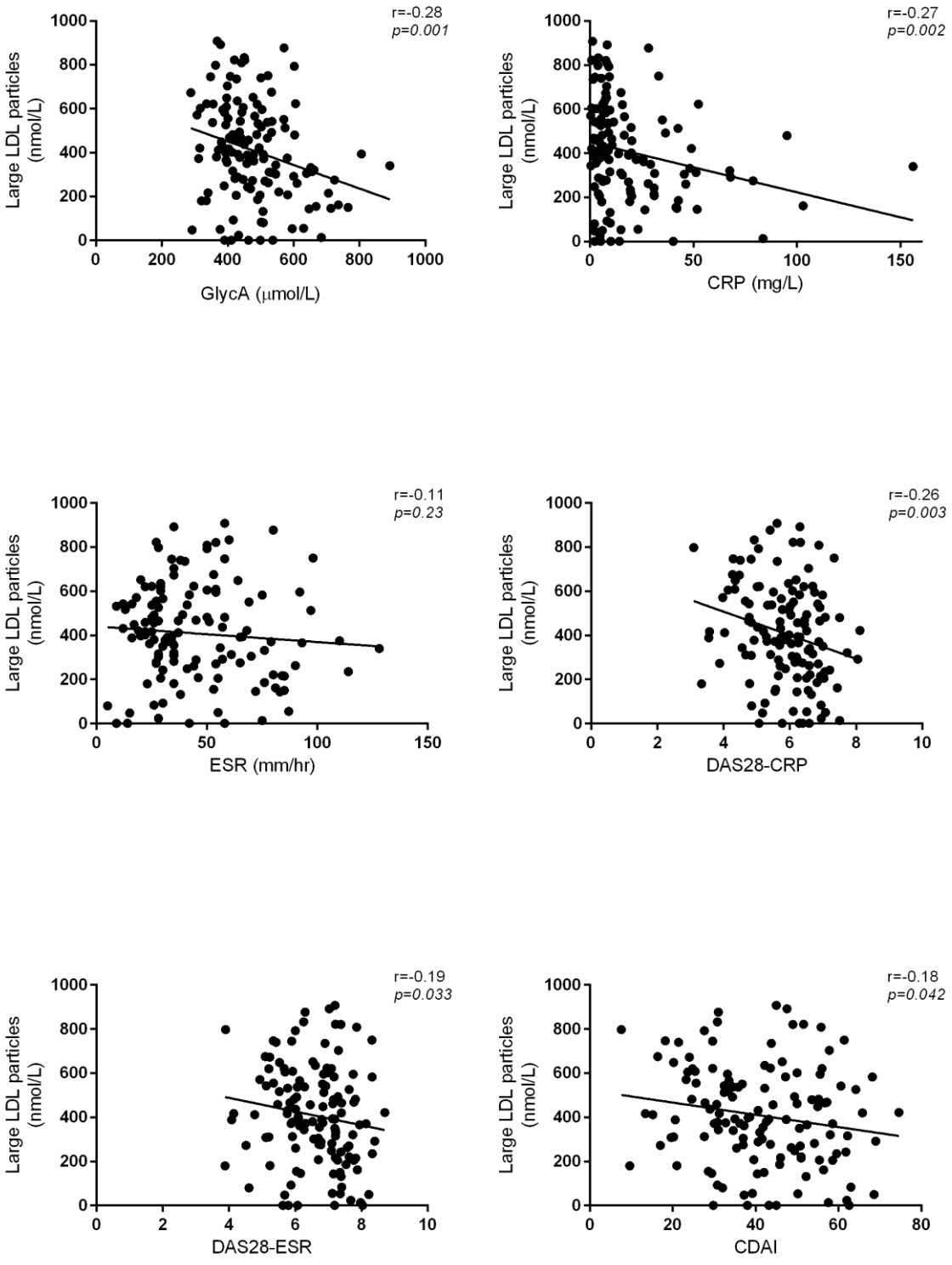


Figure 30 - - Spearman's correlation coefficients between large LDL particles and measures of disease activity at baseline (n=130).

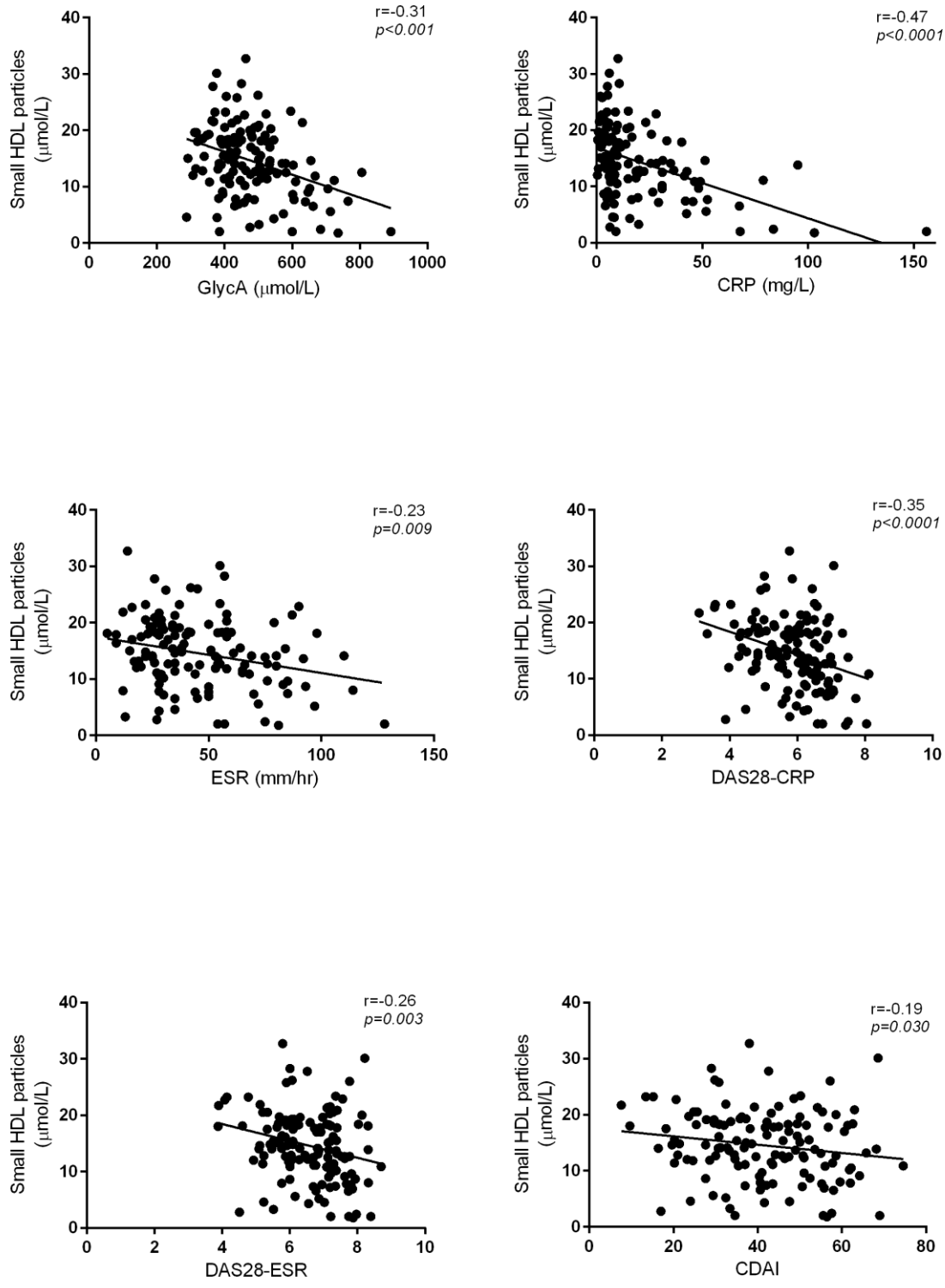


Figure 31 - Spearman's correlation coefficients between small HDL particles and measures of disease activity at baseline (n=130).

2.2.3.2 Correlations at week 24

Reflecting the analysis performed at baseline (above), the same correlation matrix-based approach was performed for the TCZ+MTX group (n=62) at week 24. R and p values are shown in

Table 10 and Table 11. In contrast to calculations at baseline, significant positive correlations were seen between small LDL particle number and GlycA (r=0.36, p=0.004) and ESR (r=0.32, p=0.012) though not CRP (r=0.12, p=0.36) or composite disease activity scores. Scatter plots in Figure 32 for GlycA and ESR showed the majority of patients had low values for both particle number and inflammatory markers; I was not personally convinced of a strong relationship in either graph. None of the relationships with large LDL particles appeared significant (Figure 33).

In contrast to the baseline results, at week 24 small HDL particle number seemed to correlate more strongly with DAS28 and CDAI scores. Review of the scatter plots in Figure 34 suggested a real relationship with composite scores but with significant variation around the regression line. Whilst CRP showed a significant relationship (r=0.30, p=0.020), conclusions must be drawn cautiously as only a few participants had elevated CRP by this point. Nevertheless, the few that did have elevated CRP tended to have lower small HDL particle numbers.

r values	VLDL				LDL				HDL			
	Total	Large	Medium	Small	Total	IDL	Large	Small	Total	Large	Medium	Small
GlycA	0.18	0.14	0.30	0.07	0.22	0.09	-0.18	0.36	0.13	-0.10	0.16	0.10
CRP	-0.10	0.00	-0.02	-0.17	0.00	-0.05	0.01	0.12	-0.08	0.12	0.15	-0.30
ESR	0.05	0.04	0.09	0.02	0.14	0.00	-0.15	0.32	-0.09	-0.03	0.02	-0.06
DAS28-CRP	-0.13	0.04	-0.07	-0.16	-0.12	-0.16	0.04	-0.08	-0.22	0.12	0.08	-0.50
DAS28-ESR	0.01	0.07	0.04	-0.01	-0.04	-0.09	-0.08	0.06	-0.12	0.07	0.13	-0.38
CDAI	0.03	0.19	0.05	-0.02	-0.10	-0.01	-0.01	-0.09	-0.11	0.09	0.06	-0.40

p values	VLDL				LDL				HDL			
	Total	Large	Medium	Small	Total	IDL	Large	Small	Total	Large	Medium	Small
GlycA	0.16	0.29	0.017	0.61	0.078	0.50	0.16	0.004	0.32	0.45	0.20	0.42
CRP	0.45	0.99	0.89	0.20	0.97	0.70	0.94	0.36	0.56	0.35	0.25	0.020
ESR	0.72	0.74	0.49	0.89	0.27	0.98	0.23	0.012	0.49	0.84	0.89	0.64
DAS28-CRP	0.30	0.73	0.60	0.21	0.34	0.23	0.74	0.55	0.08	0.37	0.54	<0.0001
DAS28-ESR	0.94	0.56	0.78	0.95	0.76	0.50	0.53	0.62	0.33	0.56	0.31	0.002
CDAI	0.80	0.14	0.69	0.90	0.44	0.94	0.93	0.51	0.38	0.47	0.62	0.001

Table 10 - Spearman's r value (top) and p value (bottom) for measures of disease activity and lipid NMR values at week 24 in TCZ+MTX group (n=62). Green = p<0.05. Red = p<0.01

r values	Particle size		
	VLDL	LDL	HDL
GlycA	0.2	-0.31	-0.08
CRP	0.08	-0.06	0.16
ESR	0.08	-0.2	0.03
DAS28-CRP	0.08	0.05	0.24
DAS28-ESR	0.04	-0.04	0.2
CDAI	0.1	0.04	0.2

p values	Particle size		
	VLDL	LDL	HDL
GlycA	0.12	0.013	0.51
CRP	0.56	0.67	0.22
ESR	0.52	0.11	0.82
DAS28-CRP	0.53	0.71	0.06
DAS28-ESR	0.78	0.78	0.11
CDAI	0.43	0.74	0.11

Table 11 - Spearman's r value (top) and p value (bottom) for measures of disease activity and mean particle size at week 24 in TCZ+MTX group (n=62). Green = p<0.05. Red = p<0.01

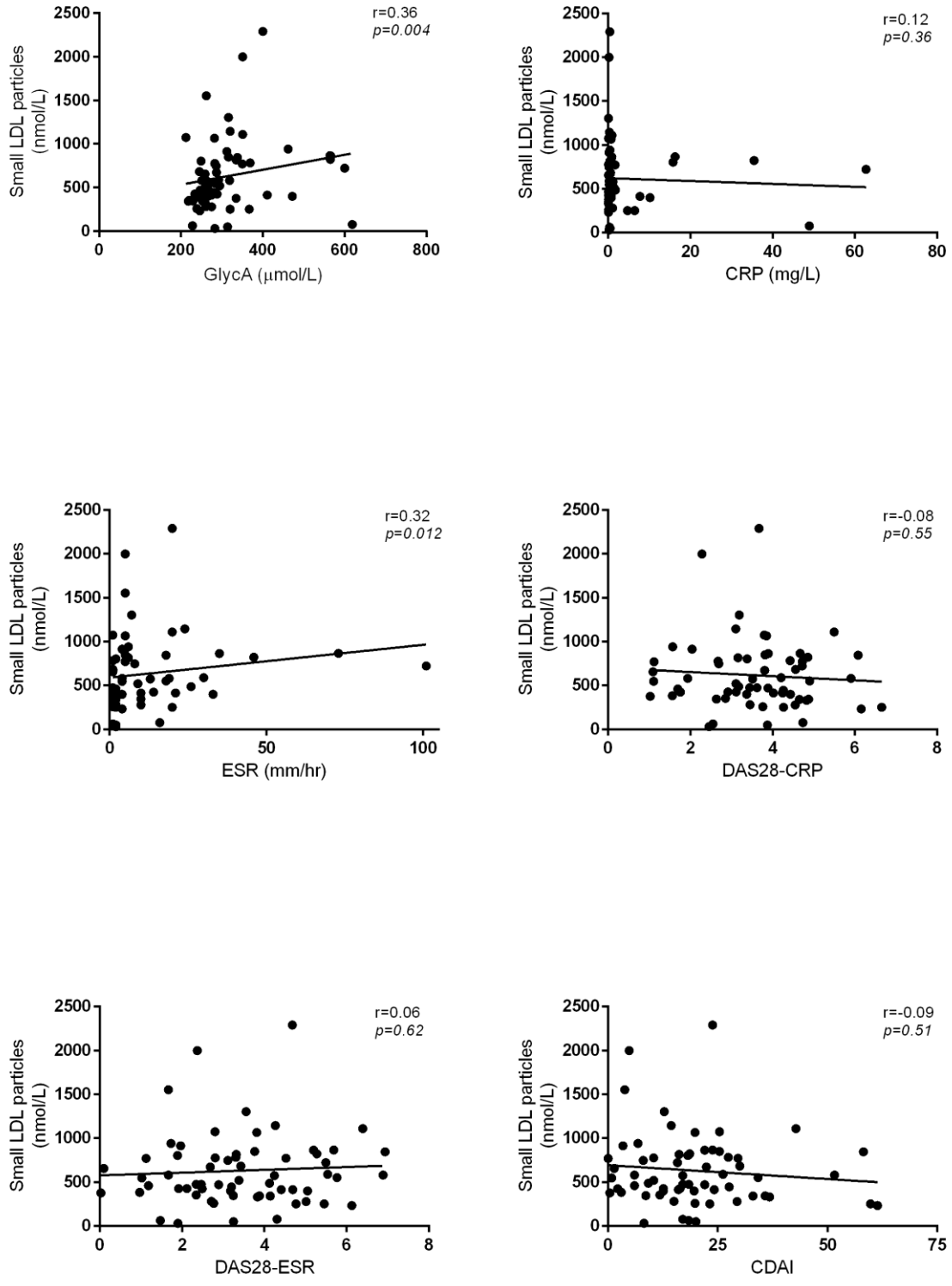


Figure 32 - Spearman's correlation coefficients between small LDL particles and measures of disease activity at week 24 (n=62).

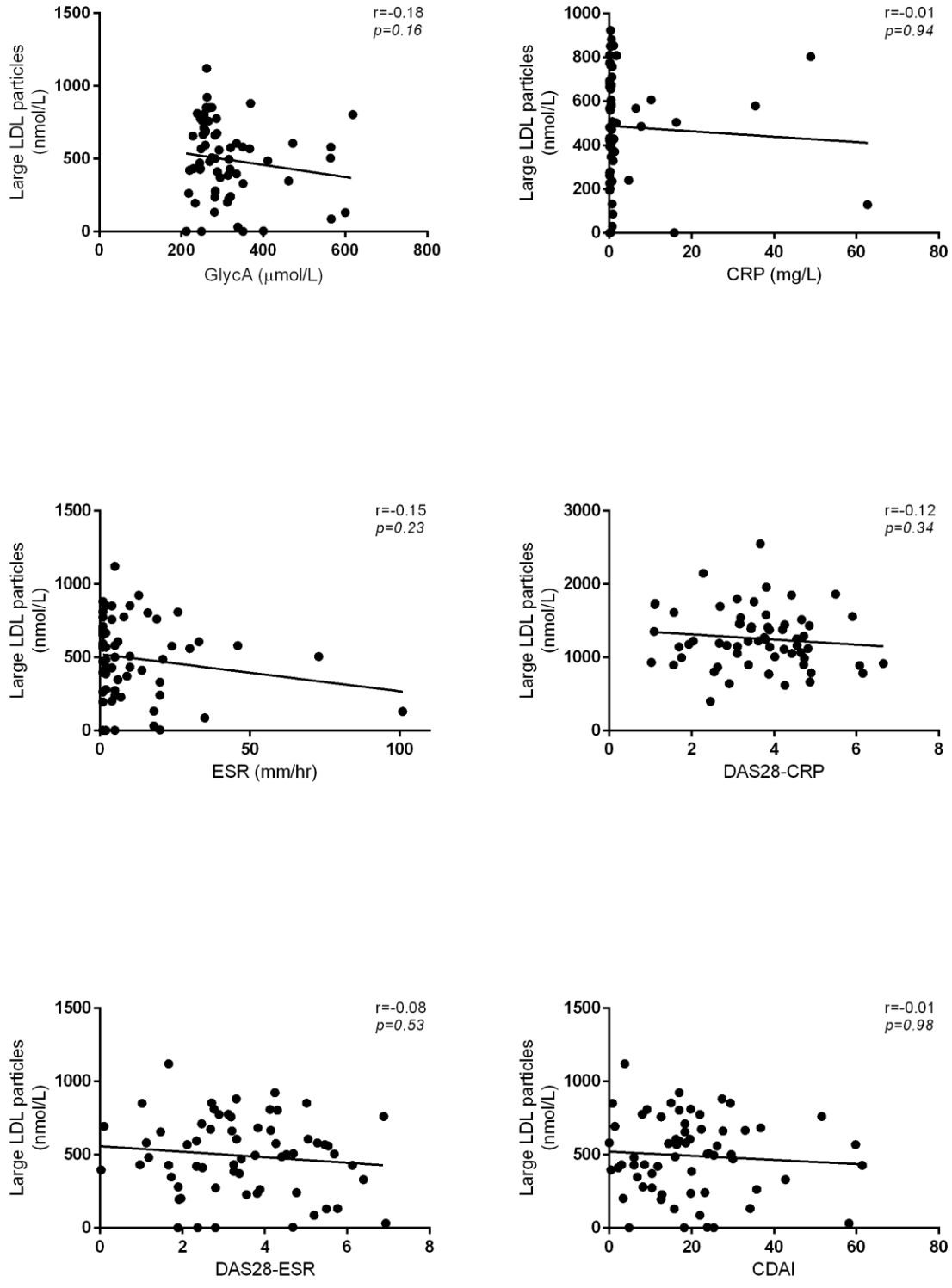


Figure 33 - Spearman's correlation coefficients between large LDL particles and measures of disease activity at week 24 (n=62).

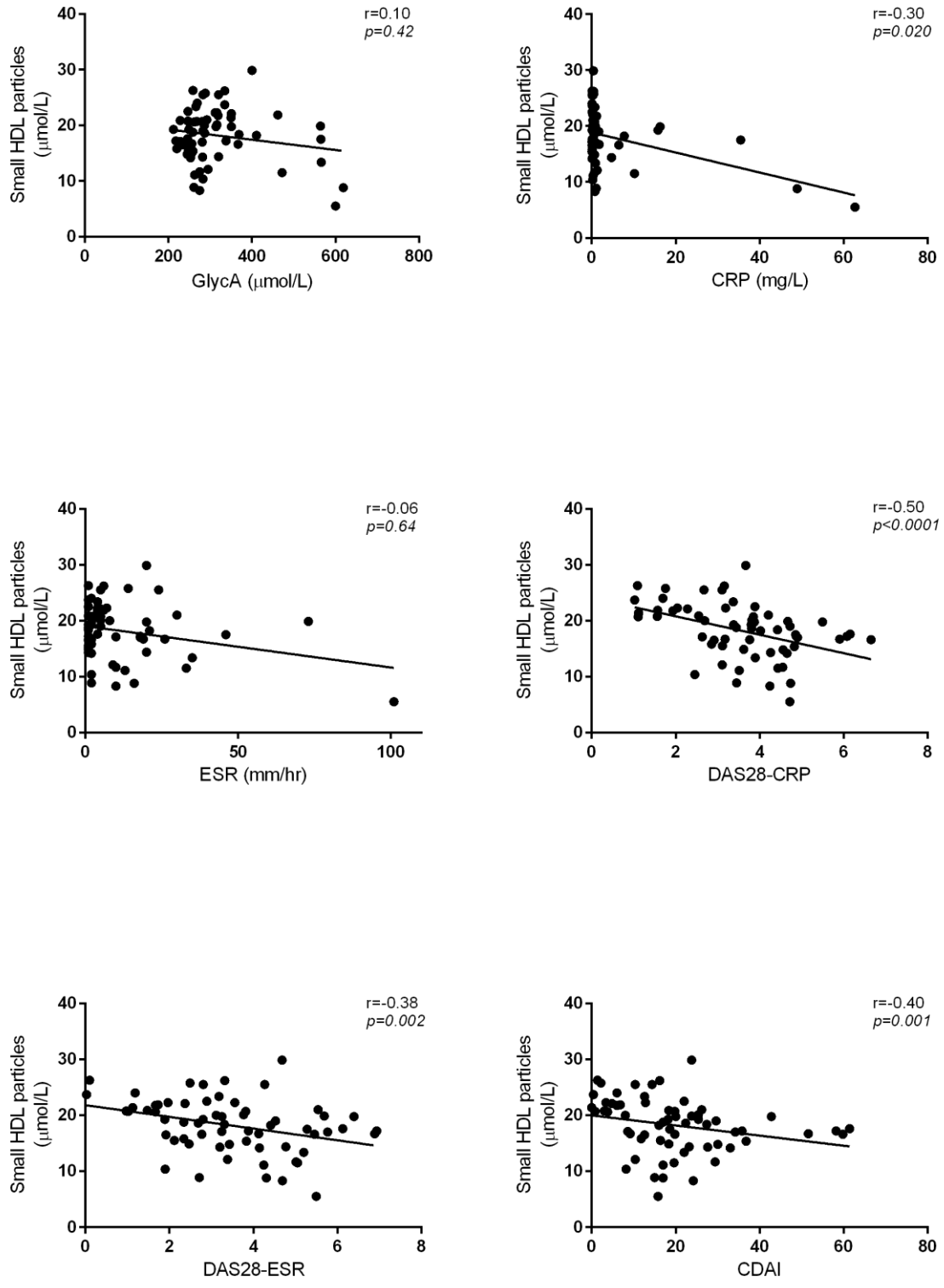


Figure 34 - Spearman's correlation coefficients between small HDL particles and measures of disease activity at week 24 (n=62).

2.2.3.3 Correlations in degree of change at week 24

Table 12 and Table 13 show a correlation matrix between change from baseline in disease activity measures and change from baseline in lipid parameters at week 24 in the TCZ+MTX group. Small LDL particles appeared to fall in participants with large falls in GlycA, though the statistical values were modest ($r=0.29$, $p=0.019$) and no such relationship was seen for CRP, ESR or composite scores (Figure 35).

Large LDL particle change correlated inversely with GlycA and ESR; no significant pattern was reached with CRP but the scatter plots for all three parameters appear to show increases in large LDL particle number in participants with large falls in their inflammatory markers (Figure 36). The scatter plot led me to suspect that a relationship did exist between CRP and large LDL; to investigate this further I plotted change in large LDL in the top and bottom quartiles of CRP change. Figure 37 shows that the degree of change in large LDL was no different in subjects with large fall in CRP (lowest quartile) compared to subjects with a modest fall or rise in CRP (top quartile). No relationships were apparent between large LDL particles and composite disease scores.

Small HDL particle levels tended to increase in participants with greater falls in disease markers. Correlations for small HDL particles were significant for CRP and both DAS28 scores; non-significant numerical trends existed in other parameters. Review of the scatter plots (Figure 38) confirmed a general trend of particle increase in those patients with greatest falls in CRP and ESR, but no clear relationship with CDAI.

Large HDL particle change also showed clear inverse correlation with CRP, ESR and GlycA; Figure 39 clearly shows falls in large HDL associating with increasing inflammatory markers, and elevations in those with the greatest fall in inflammatory markers. Figure 39 also reflects the complete absence of relationship between CDAI or DAS28 and large HDL particle change.

r values	VLDL				LDL				HDL			
	Total	Large	Medium	Small	Total	IDL	Large	Small	Total	Large	Medium	Small
GlycA	-0.12	0.01	-0.08	-0.08	-0.02	-0.13	-0.35	0.29	-0.28	-0.33	0.05	-0.22
CRP	-0.21	-0.24	-0.15	-0.11	0.05	-0.04	-0.18	0.19	-0.34	-0.33	0.08	-0.31
ESR	-0.02	0.27	-0.09	0.00	0.06	0.23	-0.26	0.14	-0.27	-0.37	0.07	-0.24
DAS28-CRP	-0.26	-0.19	-0.14	-0.23	0.06	-0.06	0.00	0.06	-0.25	-0.03	0.13	-0.28
DAS28-ESR	-0.28	-0.13	-0.16	-0.22	0.10	-0.07	-0.13	0.19	-0.14	-0.09	0.19	-0.28
CDAI	-0.17	-0.12	-0.10	-0.15	0.00	0.04	0.01	-0.04	-0.18	0.01	0.09	-0.17

p values	VLDL				LDL				HDL			
	Total	Large	Medium	Small	Total	IDL	Large	Small	Total	Large	Medium	Small
GlycA	0.34	0.91	0.51	0.51	0.90	0.30	0.005	0.019	0.025	0.008	0.72	0.082
CRP	0.098	0.058	0.24	0.37	0.72	0.75	0.16	0.13	0.007	0.010	0.55	0.013
ESR	0.89	0.032	0.48	0.98	0.64	0.069	0.037	0.28	0.031	0.003	0.58	0.060
DAS28-CRP	0.044	0.13	0.29	0.0716	0.67	0.65	1.00	0.63	0.049	0.81	0.31	0.030
DAS28-ESR	0.029	0.31	0.21	0.087	0.43	0.57	0.33	0.14	0.27	0.46	0.13	0.027
CDAI	0.19	0.34	0.44	0.25	0.99	0.75	0.94	0.76	0.15	0.94	0.49	0.17

Table 12 - Spearman's r value (top) and p value (bottom) for change from baseline in measures of disease activity and change in lipid NMR values at week 24 in TCZ+MTX group (n=62). Green = p<0.05. Red = p<0.01

r values	Particle size		
	VLDL	LDL	HDL
GlycA	0.14	-0.41	-0.06
CRP	-0.03	-0.18	-0.09
ESR	0.11	-0.15	-0.12
DAS28-CRP	0.02	0.03	0.07
DAS28-ESR	0.03	-0.05	0.04
CDAI	0	0.04	0.08

p values	Particle size		
	VLDL	LDL	HDL
GlycA	0.29	0.001	0.63
CRP	0.84	0.16	0.5
ESR	0.4	0.26	0.36
DAS28-CRP	0.88	0.81	0.56
DAS28-ESR	0.84	0.72	0.78
CDAI	0.99	0.73	0.52

Table 13 - Spearman's r value (top) and p value (bottom) for change from baseline in measures of disease activity and change in mean particle size at week 24 in TCZ+MTX group (n=62). Green = p<0.05. Red = p<0.01

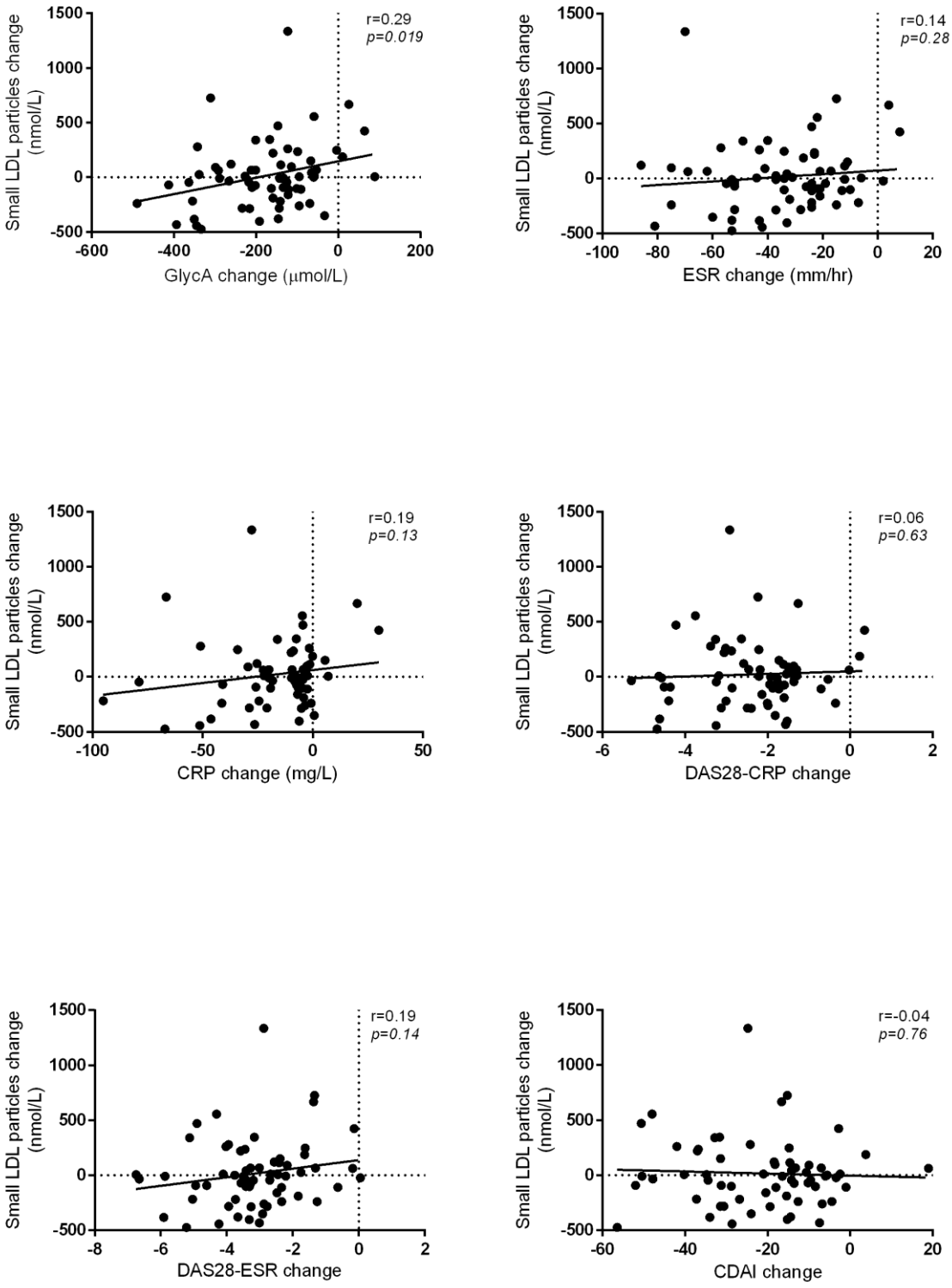


Figure 35 -Spearman's correlation coefficients between changes in small LDL particles and measures of disease activity at week 24 (n=62).

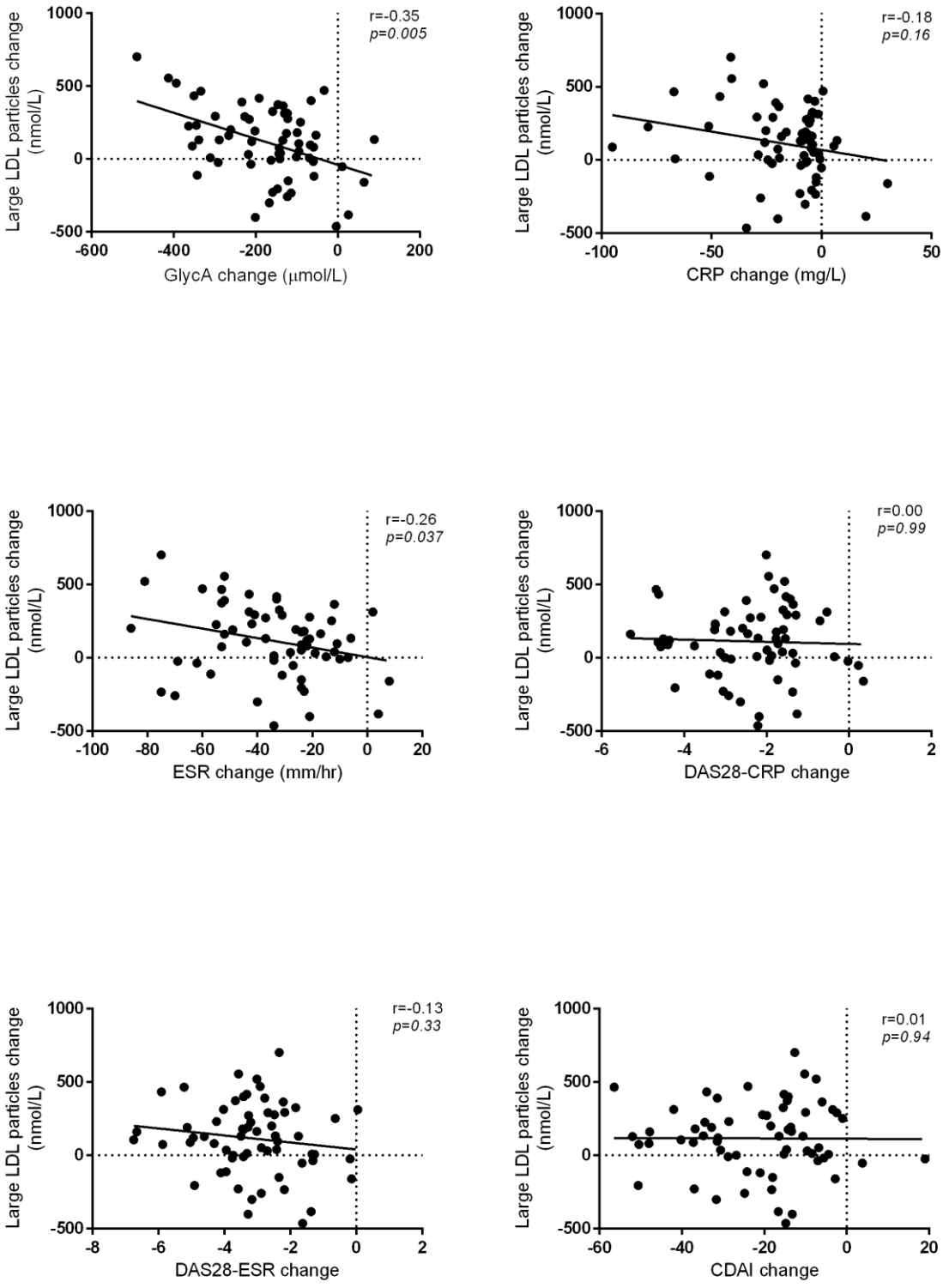


Figure 36 - Spearman's correlation coefficients between changes in large LDL particles and measures of disease activity at week 24 (n=62).

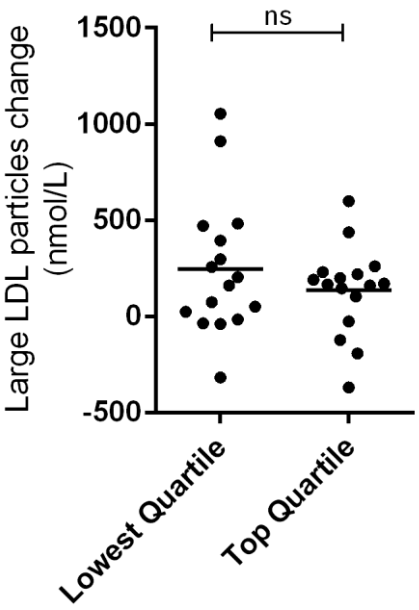


Figure 37 - Change in large LDL particle number at week 24 in top and bottom quartiles of CRP change. Analysis by Mann-Whitney U test.

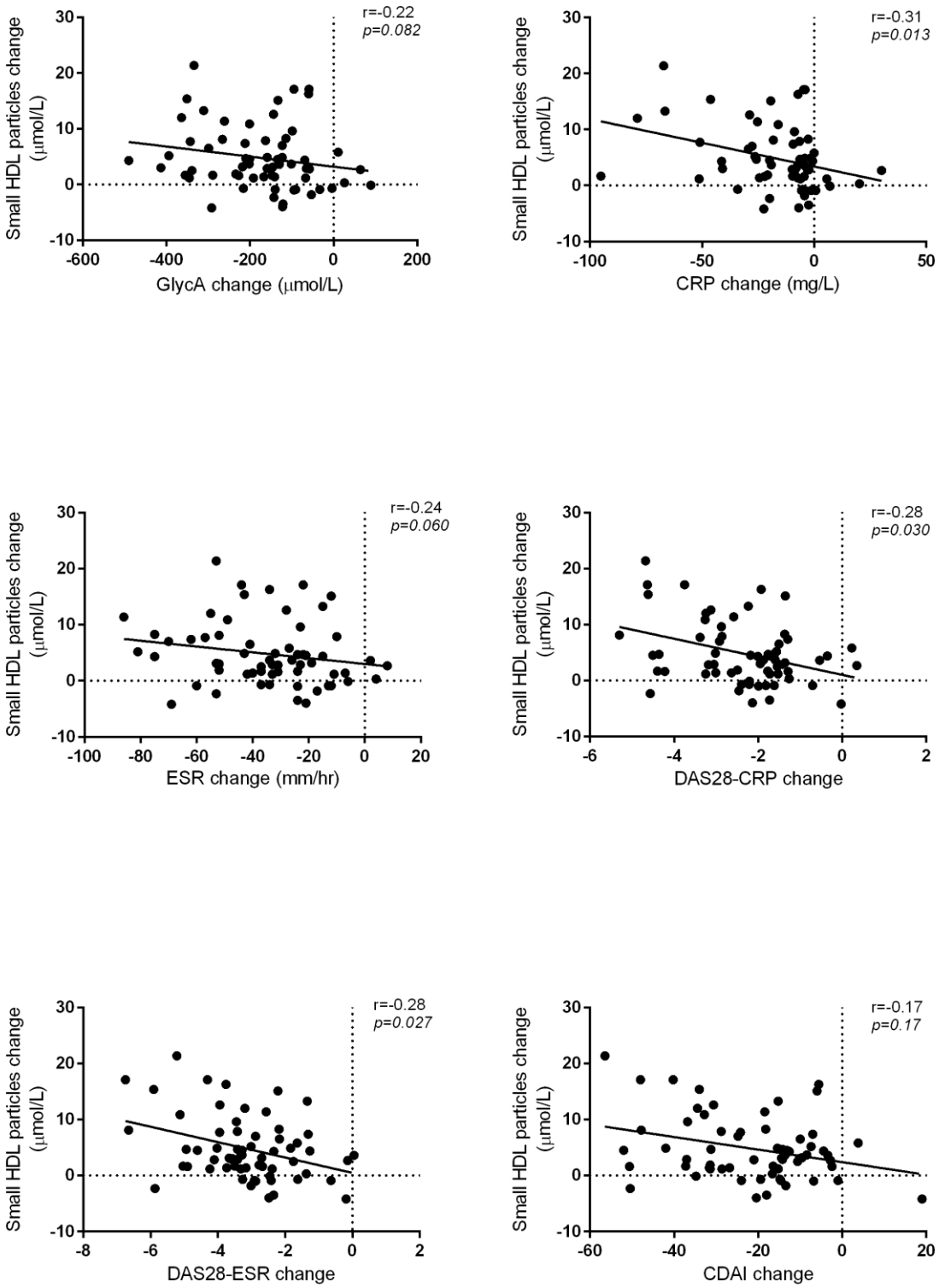


Figure 38 - Spearman's correlation coefficients between changes in small HDL particles and measures of disease activity at week 24 (n=62).

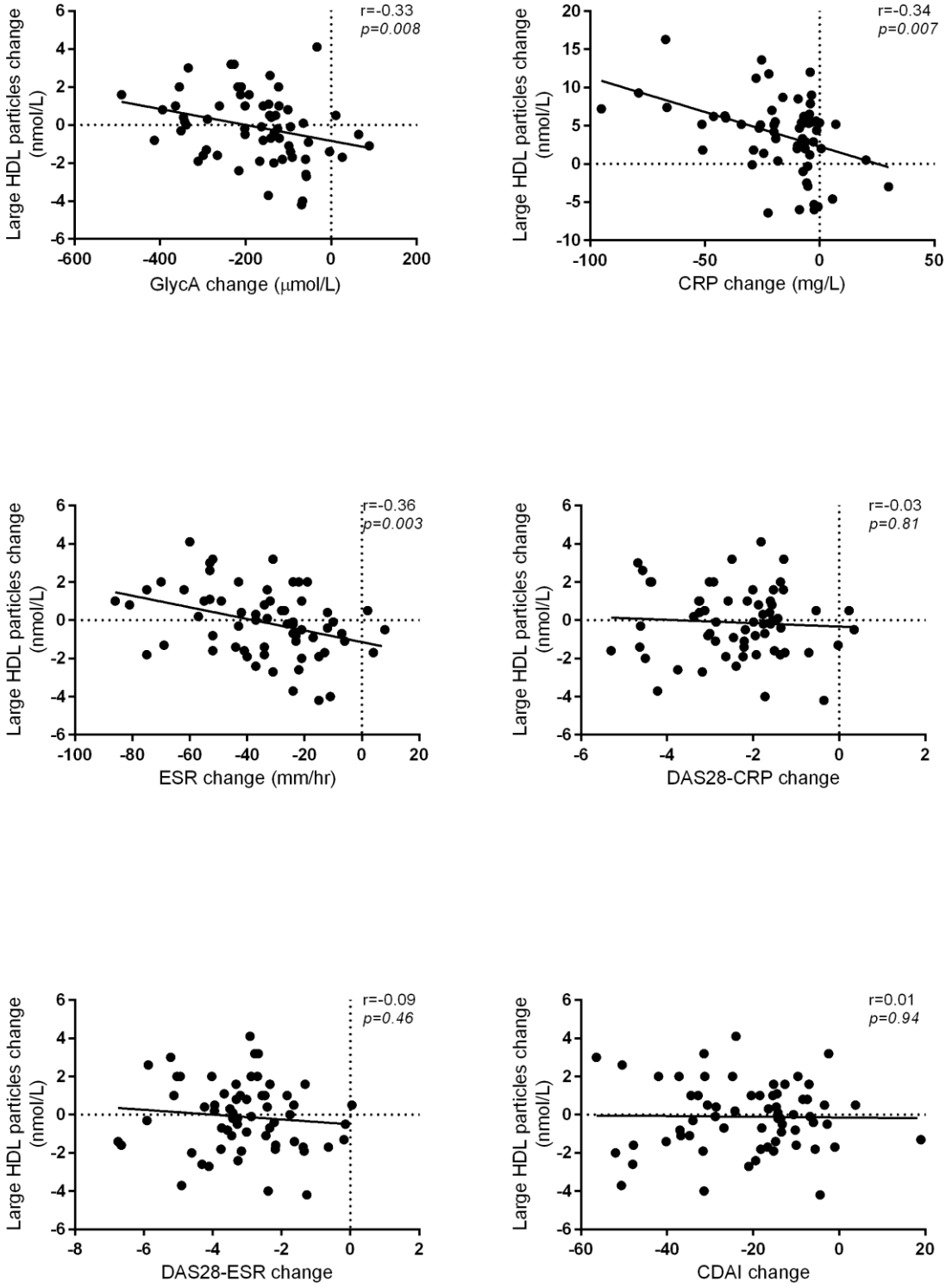


Figure 39 - Spearman's correlation coefficients between changes in large HDL particles and measures of disease activity at week 24 (n=62).

2.3 Discussion

2.3.1 Lipoprotein changes

The changes in lipoproteins seen in the TCZ+MTX group were similar to those previously published, with some exceptions. For the primary outcome measure, small LDL particle number, I found no difference between the groups at 24 weeks. This mirrors the findings of the original analysis, and is reassuring on clinical grounds given that small, dense LDL particles are believed to be the most pro-atherogenic. I also found a significant increase in large LDL particles; the original analysis reported a median increase of 19%, but also found a 13% increase in the placebo+MTX group that led to no significant difference between the groups. I believe that my own findings are more believable given the evidence of LDL-c increases in tocilizumab phase III trials.

Whilst small LDL particle number is generally accepted to be a marker of CVD risk, the clinical relevance of an increase in large LDL is not as clear. This is despite them carrying more cholesterol mass than small LDL particles, and thus contributing more to measured LDL-c. The largest prospective study to look at this issue (263) performed NMR analysis on serum from 27,673 healthy women in the Women's Health Study. They were then followed up for 11 years for incident CVD (n=1,015). After adjustment for known risk factors, large LDL particle number did not predict incident CVD (HR of top quintile compared to bottom quintile 0.86, 95% CI 0.72-1.03, p=0.21) whilst small LDL particle number did (HR of top quintile compared to bottom quintile 1.76, 95% CI 1.41−2.18, p<0.001). Other, smaller studies have generated similar results (264-266), including one in RA patients (217). However, one cross-sectional study of 5,538 subjects from the MESA study (267) suggested a positive association between large LDL particles and carotid IMT after adjusting for small LDL particle number, given that the two measures tend to inversely correlate. Another study of 656 Alaskan Inuit suggested a contribution of large LDL to carotid plaque score, but not carotid IMT (268).

Despite the balance of the literature as detailed above, some caution is required before stating that tocilizumab's LDL-c increase is not pro-atherogenic. This is for two reasons. Firstly, it is not at all clear that NMR subparticle quantification

is superior to traditional lipidaemic risk factors for CVD risk quantification. Indeed, in the Women's Health Study, LDL particle number's ability to predict incident CVD became non-significant when added to two models which adjusted for (1) LDL-c, HDL-c and triglyceride, or (2) the total/HDL-c ratio. Secondly, the trials above were not performed in the context of a high-grade inflammatory state such as RA, which of course generates counter-intuitive relationships between serum cholesterol and CVD risk. My personal view is that these findings are both mechanistically explanatory and clinically reassuring. The ultimate solution to this conundrum would of course be a study on RA patients with NMR lipoprotein quantification before and after tocilizumab with long-term follow up for CVD outcomes. Whilst it would be interesting to use MEASURE for this purpose, the relatively small patient numbers (and thus likely low numbers of CVD events) make it unlikely that the MEASURE cohort could be effectively used in this manner.

I found no change in medium-sized VLDL, whilst the original analysis found an increase of 58% from baseline at 12 weeks. The magnitude of change was also different; for example, large VLDL increased by median 62% at week 12 in the TCZ+MTX group in my analysis, compared to 206% in the original. This is likely related to the use of a newer NMR platform at LipoScience for generating lipoprotein values. The clinical significance of these findings is uncertain. Large VLDL particle number correlated strongly to serum TG and all VLDL particle sizes predicted increasing CVD risk with increasing concentration in the Women's Health Study (263). This may simply reflect the TG-induced increase in small dense LDL, though triglycerides did also increase following treatment in MEASURE (254). In the Women's Health Study no further analysis was performed on VLDL to adjust for other lipid measures.

The significant increase in small HDL particles replicates the original analysis, though I failed to find any decrease in medium HDL. RA patients have lower levels of small HDL particles than controls (216, 217); our observed increase can be seen as a normalisation of abnormally low levels. Some studies indicate a protective association between small HDL on NMR and CVD, including one in RA (217), though others do not(263, 266). Perhaps more important than the quantity of HDL particles is their makeup and function, as detailed in the

original report. Unfortunately however I did not have access to any other HDL-related parameters, such as SAA or paraoxonase.

In all lipoprotein parameters, there was no difference in change from baseline between the TCZ+MTX groups and placebo+MTX at 52 weeks, demonstrating that the placebo+MTX group underwent the same lipoprotein changes as the active treatment group following 24 weeks of open-label tocilizumab. This can reassure investigators that the changes observed in the TCZ+MTX group were due to tocilizumab rather than an unknown confounder between the groups. It also makes it unlikely that any additional lipoprotein changes occur beyond 24 weeks of treatment, although as previous graphs have demonstrated the majority of changes appeared to take place by the week 2 assessment. These observations are a reassuring validation of the integrity of the study and the original publication. The one exception to the above is small VLDL particles; although the numerical values were identical at week 52, the two groups had different baseline values. I believe this is likely a non-significant finding, as with thirty different parameters being analysed there is a possibility that one will be different purely by chance given an alpha of 0.05.

2.3.2 GlycA as a marker of inflammation / RA activity

Our results from analysis of the cohort at baseline show GlycA to be a reliable indicator of the acute phase response. It correlated well with CRP and more modestly with ESR, and maintained these associations in repeated measures over 12 weeks in the placebo+MTX group. Similarly, GlycA values in our cohort were higher than those seen in a healthy cohort, and an RA cohort with less severe disease and lower ESR and CRP. However, there was no relationship found with CDAI score. It could be argued that the weakness of these associations is evidence against GlycA's utility as a biomarker for inflammation. I believe this is unfair, as I have demonstrated that ESR and CRP also correlate very poorly with CDAI, and with each other. Indeed, it is for this very reason that the clinician performs multiple measures and compares and contrasts them, or uses them together in a composite score such as DAS28. GlycA was also unable to act as a marker for ongoing disease activity in those patients with low CRP. It would be interesting to see how well GlycA and other markers reflected disease activity as measured by another objective, quantitative marker of inflammatory

burden such as MRI or USS, as this may provide evidence of further utility as a biomarker. Further studies would be required to evaluate this possibility.

There remains a strong appetite amongst rheumatology researchers for a biomarker which can predict response to a given therapy. Unfortunately, GlycA was unable to convincingly do so, though ESR and CRP did not fare much better. The ability of GlycA to predict radiographic progression could not be evaluated in this study as no such data were available.

The sharp fall in GlycA following the start of tocilizumab treatment was similar to that seen with ESR and CRP, with median levels below that of a non-RA cohort. This potential "supra-normalisation" implies that, like CRP, the GlycA signal is exquisitely dependent on IL-6. This is perhaps surprising as other IL-6 related peptides such as fibrinogen and CRP can be glycosylated but make negligible contributions to the overall GlycA signal (261). Moreover, glycan processing is currently understood to be influenced by various inflammatory signals, including TNFα (269) and endothelial dysfunction (270). On the other hand, the proteins whose N-acetyl-methyl groups generate the GlycA signal are well recognised as acute-phase proteins, and will inevitably be upregulated by IL-6. GlycA, like CRP or ESR, should therefore be interpreted with caution in the context of IL-6 blockade. It may, however, be useful in quantifying disease activity in other conditions associated with excessive IL-6 signalling, and research is ongoing into GlycA's behaviour in treatment-naive RA and vasculitis (M. Klearman, personal communication). Moreover, it has been reported that GlycA has clinical associations with cardiovascular disease outcomes, for example ischemic stroke, independent of CRP and traditional cardiovascular risk factors. These observations suggest that the two inflammatory markers be differentially regulated and may provide overlapping but distinct clinical information for patient evaluation.

2.3.3 Clinical response in the cohort

Of note, some steady improvement in clinical parameters was seen even beyond 24 weeks. I have certainly had personal experience in my clinical career of meeting RA patients on biologic agents who describe prolonged, gradual improvements in their condition beyond 6 months of therapy. Alternatively, the

phenomenon may have been in part due to the lack of imputed data in my analysis, as the seven patients who dropped out between weeks 24 and 52 had a mean week 24 CDAI of 23.4, compared to mean 19.2 in those that went on to complete 52 weeks; hence, their dropping out of the study probably contributed to lower week 36 and 52 median values. Perhaps the simplest explanation is that from week 24, patients in the TCZ+MTX group moved from a blinded treatment regimen to open-label tocilizumab, and the knowledge of their current therapy generated a placebo effect.

Reassuringly, the placebo + MTX group generally demonstrated little or no change in GlycA or other measures of disease activity until changing to open label tocilizumab at week 24. From this point on, the curves tended to mirror the changes seen in the TCZ+MTX group, and there were no significant differences between the groups in the degree of change from baseline in any parameters at week 52. One unexpected feature was the improvement in swollen joint count seen in the placebo+MTX group by week 2, and maintained until the week 24 crossover point. This was responsible for the falls in DAS28-CRP, DAS28-ESR and CDAI seen in this group during the placebo phase of the trial. However, the change in SJC was much more pronounced in the TCZ+MTX group, leading to a statistically significant difference between the groups at 24 weeks.

2.3.4 Lipoprotein correlations

High levels of CRP (typically greater than around 40mg/l) and GlycA seemed to drive down large LDL particle number at baseline, and strong responses to treatment (as measured by acute phase reactants) tended to lead to an increase in these particles. The same seemed to be true of small HDL and CRP, ESR and GlycA, with low particle numbers in patients with high inflammatory markers. Large HDL particles also tended to increase in those with the largest falls in inflammatory markers after 24 weeks. These observations support the hypothesis of IL-6 being a driver of lipid change in both the HDL and LDL compartments, rather than cholesterol elevations being an “off-target” effect of the drug.

Nevertheless, correlations between inflammatory and lipoprotein markers were generally modest, and were more commonly seen with acute phase reactants than CDAI or DAS28. IL-6, as tocilizumab's profound and rapid changes in inflammatory markers demonstrate, is the main driver of the acute phase response; this could lead us to conclude that IL-6 contributes more to dyslipidaemia in RA than other molecular or cellular components of the disease. This view is supported by the LDL changes seen with tocilizumab, which are of greater magnitude and consistency than seen in TNF-α blockade or conventional DMARDS. Alternatively, part of the reason may simply be the greater reproducibility of biochemical tests compared to joint counts and visual analogue scale scores, which are subjective and prone to inter- and intra-individual variation.

3 The KALIBRA study

3.1 Rationale

The previous chapters have shown that there is abundant evidence of tocilizumab causing elevations in LDL-c, whilst the MEASURE study showed that this increase is predominantly in large LDL particles. This increase appears to associate more with the acute phase response than joint counts or other clinical measures of disease activity.

The mechanisms behind these observations, however, remain unclear. One possibility is that tocilizumab increases production of LDL or one of the "upstream" apoB-containing lipoproteins by the liver. This would potentially provide more substrate for atherogenesis, and so may increase cardiovascular risk in an already high-risk patient population. Alternatively, the changes may be due to reduced catabolism of LDL, in the form of reduced consumption of LDL particles by peripheral tissues. This hypothesis of increased LDL catabolism in certain patients has its origin in studies of hypertriglyceridemic patients in the 1980s. These studies showed a "bell-shaped curve" relationship between serum LDL-c and triglycerides. As serum triglyceride levels rise to around 2mmol/L (i.e. towards the upper limit of the normal range), serum LDL levels steadily increase. This appears to be because these LDL particles (which are derived from triglyceride-rich VLDL) themselves contain increased triglyceride. This results in conformational changes in the apoB molecule, making it less able to bind to the LDL receptor (LDLr). However, at triglyceride levels above 4mmol/L, LDL levels begin to fall, because the LDL particles become abnormally small and more susceptible to removal by mechanisms independent of the LDLr. However, even more pertinently to our study, work in our centre with rabbits showed that suppressing reticuloendothelial lipoprotein uptake with IV ethyl oleate slowed catabolism of LDL which had been altered to resist LDLr-dependent uptake (271). Given the shared role of macrophages in the reticuloendothelial system, atherogenesis and inflammatory arthritis, it stands to reason that a similar scenario of altered LDL clearance may be at work in RA and, by extrapolation, following IL-6 blockade. This is clinically important, as if tocilizumab reduces the fractional catabolic rate of LDL by the reticuloendothelial system, this would suggest a potential athero-protective role for IL-6 blockade in RA. Such changes would be in keeping with the recognised

effects of systemic inflammation to suppress circulating levels of lipids, and the apparent ‘normalisation’ of several lipid parameters observed in MEASURE.

We therefore hypothesised that IL-6 blockade with tocilizumab would reduce the removal rate of LDL-c, leading to qualitative and quantitative changes in LDL particles and in apoB levels.

3.2 Methods

3.2.1 Patient recruitment

KALIBRA was a multi-centre mechanistic study conducted in Glasgow. Patients were recruited from three centres (Glasgow Royal Infirmary, Stobhill Hospital and Gartnavel General Hospital), whilst all study procedures took place in the Glasgow Clinical Research Facility (CRF) at the Western Infirmary, Glasgow. Patients were identified in RA clinics by consultant or trainee rheumatologists in or when attending for screening for biologic therapy by specialist nurses. Posters advertising the study were placed in RA clinic rooms and day wards (Appendix C). In addition, I was able to look through details of patients who were scheduled to come for biologics screening, and so identify potential candidates, or exclude ineligible participants, in advance.

Patients were required to meet the following inclusion criteria. These ensured our cohort reflected the patients who would receive tocilizumab in routine clinical practice:

- Diagnosed rheumatoid arthritis according to ACR 2010 criteria
- Eligible for tocilizumab therapy according to NICE guidelines, i.e.
- DAS28≥5.1 in keeping with severe active disease
- Failure to tolerate or respond to two or more conventional DMARDs including methotrexate.
- Age 25 to 75
- Able to provide written informed consent
- Absence of contraindication to biologic therapy
- Suitable for tocilizumab therapy in the opinion of the consultant rheumatologist most directly responsible for their care

Exclusion criteria ensured that subjects did not have co-morbidities which might interfere with cholesterol metabolism and thus make interpretation of lipid parameters difficult:

- Known familial dyslipidaemia
- ApoE 2/2 homozygosity
- Diabetes mellitus
- Fasting total cholesterol >6.5mmol/L, or fasting triglycerides >3mmol/L
- Treatment with any known lipid-lowering therapy, including statins, fibrates, bile-acid sequestrants or ezetimibe.
- Pregnancy
- Untreated hypothyroidism, or recent (6 weeks) change in thyroxine dose
- Known hypersensitivity or contraindication to heparin
- Concomitant use of oral glucocorticoids at a steady dose was permitted. Intramuscular steroid, a common method of steroid delivery in our area, was not permitted during the study. Intra-articular steroids were permitted in exceptional circumstances (i.e. if refusal would cause unacceptable patient suffering or would result in withdrawal from the study) at a maximum cumulative dose of 40mg triamcinolone.
- Concomitant use of DMARDS, NSAIDs or other analgesia at a steady dose was permitted.

All patients were compensated for their time with £200 for participating in the study. Travel expenses in the form of bus fares or taxi receipts were also reimbursed. Patients were introduced to the study verbally by myself or a Rheumatology specialist nurse, and provided with an information leaflet (Appendix D). After at least 48 hours, the subject would discuss the study with

me in person or over the telephone. If the subject wished to participate, a screening visit was arranged where written informed consent was obtained. The consent form is shown in Appendix E. At the screening visit, the patient was assessed for inclusion and exclusion criteria. This included blood samples being drawn for inflammatory markers, lipids, fasting glucose and ApoE genetic testing.

3.2.2 Procedures

After screening, a four-week period was allowed if necessary for washout of previous biologic drugs such as anti-TNF agents. The first 5-day kinetic study then took place, followed by a period of at least ten weeks to allow a minimum of three infusions of TCZ 8mg/kg. The second kinetic study was carried out two weeks after the third TCZ infusion. If the dosing schedule was interrupted, a further three consecutive infusions of TCZ were required before performing the second kinetic study.

The labour-intensive nature of lipoprotein isolation, and the sample-processing capacity of our biochemistry lab, meant that one kinetic study could take place every two weeks. Coupled with the timescale described above, this meant that recruitment was carefully staggered; patients were enrolled in “blocks” of no more than five at a time. This was because by the time the fifth participant had completed their first kinetic study, ten weeks had passed since the enrolment of the first participant, who was then due their second kinetic study.

3.2.3 Kinetic blood sampling rationale

Kinetic studies are conducted using a stable deuterated isotope of the amino acid leucine (5,5,5-d3 leucine), a component of many proteins including apoB. When this is administered IV, d3-leucine is rapidly taken up by hepatocytes. A substantial quantity is incorporated rapidly into newly-synthesised apoB molecules in a manner indistinguishable from native leucine. The labelled apoB is used in the synthesis of VLDL-1 from chylomicron remnants, and remains with that particle as it is metabolised by lipoprotein lipase and hepatic lipase to VLDL-2, IDL and finally LDL. At serial timepoints after administration, serum samples can be taken and mass spectrometry used to quantify the percentage of

apoB enrichment in d3-leucine. Graphing the tracer/tracee ratio against time for different particles can yield a graph similar to Figure 40 below.

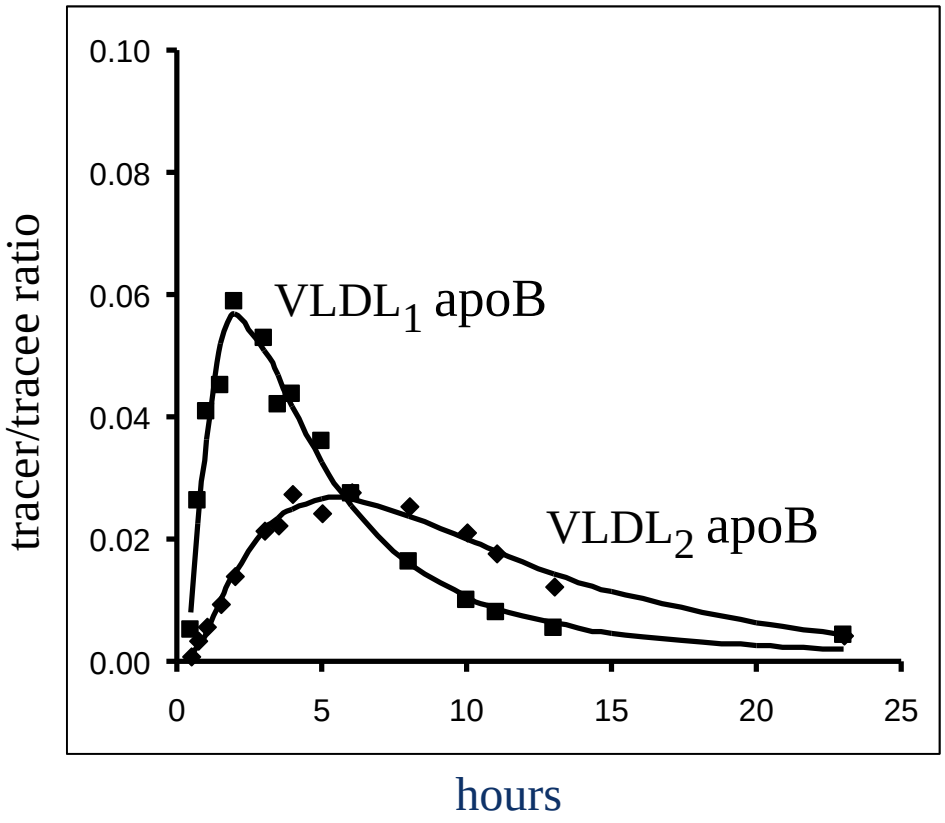


Figure 40 - Example curves of labelled apoB in generation of VLDL-1 and transfer to VLDL-2.

This example graph shows rapid incorporation of labelled apoB into VLDL-1, peaking at around two hours. This peak then falls off as VLDL-1 is cleared and metabolised by LPL to VLDL-2, as reflected by the appearance of a VLDL-2 apoB peak at around 5 hours. Levels of labelled VLDL-2 apoB will themselves fall in the same manner, and similar curves may be plotted for the downstream particles IDL and LDL, each with distinctive rates of rise (as new particles are formed incorporating the labelled apoB) and fall (as they are metabolised by lipolysis or cleared from the circulation). Mathematical modelling can then be used to calculate the synthetic and catabolic rates of the apoB-containing lipoproteins; this process is outlines later.

3.2.4 D3-Leucine production

Manufacture of the d3-leucine solution for the study was carried out by Tayside Pharmaceuticals (Dundee, UK) on behalf of NHS Greater Glasgow & Clyde. Leucine was suspended in glass vials of 45ml 0.9% sodium chloride at a concentration of 10mg/ml, and was stored in the dark at 0 – 5^{o}C in the pharmacy unit of the Western Infirmary, Glasgow.

3.2.5 Tocilizumab supply

All tocilizumab used in the study was graciously provided without charge by Roche Products Ltd, under an agreement with NHS Greater Glasgow & Clyde. This was stored and reconstituted in the sterile products unit of Glasgow Royal Infirmary. On completion of the study, patients who demonstrated a clinical response to the drug and who wished to continue were switched to NHS stock in either IV infusion or subcutaneous form, depending on the patient's preference.

3.2.6 Kinetic blood sampling timetable

On day 1, the patient was admitted to the Clinical Research Facility (CRF) having fasted overnight. At 08:00, an 18G or 20G cannula was inserted in the antecubital fossa and baseline blood samples were taken. 5mg/kg of d3-leucine was then administered as an IV bolus through a cannula in the other arm. The patient remained in a semi-recumbent position throughout the day except for natural breaks. Blood samples were taken in EDTA vacutainers through the cannula at the following timepoints: 2min, 5 min, 10min, 15min, 20min, 30min, 45min, 1h, 1.5h, 2h, 3h, 3.5h, 4h, 5h, 6h, 8h, 10h, 11h, and 13h. The patient was fasted throughout the day, and encouraged to drink plenty of water. Following the 10h sample, the patient was provided with a low-calorie (1,000kcal) meal. The patient was allowed home after the 13h sample.

Further fasting samples were taken on day 2 (24h, 36h), day 3 (48h), day 4 (72h) and day 5 (96h). After the day 5 sample was taken, unfractionated heparin 70IU/kg was administered as an IV bolus. After 12 minutes, blood samples were taken through the cannula for hepatic lipase and lipoprotein lipase activity measurements, and stored on ice for transportation to the university

biochemistry laboratory. It was calculated that for each kinetic study a total of 266ml of blood would be drawn over the course of one week, with most of that volume coming in the first 12 hours.

Three patients lived several miles away from the CRF, but in relatively close proximity to my own home. These patients agreed to have blood samples on Tuesday – Thursday taken at their home address by me, with the samples being refrigerated and transferred the following day to our centre's laboratory.

3.2.7 Assessments

Day 1 assessments for all patients included swollen and tender joint counts, patient's global assessment by visual analogue scale (VAS) and physician's global assessment by VAS, all of which I performed myself. Blood samples were taken for FBC, CRP and ESR, which were processed in standard fashion at NHS GGC laboratories. Samples were also taken for betaquant (direct measurement of TC, VLDL-c, LDL-c, HDL-c and TG), CETP activity, apolipoproteins, lipoprotein fraction composition, Lp(a) and insulin.

3.2.8 Lipoprotein sample handling and processing

Measurement of blood cholesterol, triglyceride and lipoprotein parameters were all performed by the University of Glasgow Centre for Vascular Biochemistry, in the Western Infirmary Glasgow, under supervision of Prof. Caslake. Plasma from kinetic study blood samples was isolated by centrifugation. Density-gradient ultracentrifugation was then used to isolate the apoB-containing fractions – VLDL-1, VLDL-2, IDL and LDL. ApoB from each fraction was precipitated, delipidated and measured by gas chromatography mass spectrometry (GCMS) to ascertain the proportion of tracer in apoB at each timepoint.

3.2.9 Pooling of serum for subfraction analysis

Serum samples were taken on day 1 of each kinetic study for Beta-quantification (Betaquant); this is the gold standard technique of directly measuring LDL-c by ultracentrifugation. Values for total cholesterol, VLDL-c, HDL-c and triglycerides are also generated by this technique.

However, it is recognised that there can be significant intra-individual variation in serum lipid levels throughout the 96 hours that the kinetic studies span, despite the patients fasting for at least 8 hours before each sample. It was therefore not appropriate to use day 1 serum samples (readings reflecting one timepoint) for the lipid subfraction values which were to be used in generating kinetic data (readings spanning numerous timepoints across five days). Lipid subfraction and some apolipoprotein values were instead obtained by taking small volumes of leftover serum from kinetic timepoint samples and pooling them. For each kinetic study in each patient, three serum pools were constructed: pool A was composed of samples from the first day; pool B from the second and third days; and pool C from the final two days. Subfractions were then isolated from each pool, and the mean of the three values was used in subsequent calculations. Specific calculation methods are discussed where relevant in the results section.

3.2.10 Data recording

Demographic data, clinical data, routine haematology and biochemistry assessments, and kinetic study sampling times were recorded on a paper case report form, generated as a collaborative effort between myself and the Robertson Centre for Biostatistics, University of Glasgow (Appendix X). The form also included data on concomitant medications, adverse events, and dates of tocilizumab infusions. During the study the case report forms were stored in the CRF in a locked filing cabinet, in a room only accessible by electronic pass. As each participant completed the study, their case report form was redacted of patient-identifiable information and passed to the Robertson Centre for secure storage. Photocopies of the sheets containing kinetic study sampling times were forwarded to Prof. Caslake for use in kinetic modelling.

3.2.11 Statistical analysis and compartmental modelling

The primary outcome measure was the change in fractional catabolic rate of LDL. Secondary outcome measures included serum lipoprotein compositions and levels of other proteins and particles as detailed above. As much of the data was non-parametrically distributed (as determined by visual inspection of histograms and comparing sample means to medians), differences between

baseline and treatment were usually assessed by the Wilcoxon matched-pairs test. Correlations were all measured by Spearman's correlation coefficient. Raw data was stored on MS Excel, with statistical analyses and graphs generated on GraphPad Prism 6.

Tracer/tracee ratios for apoB, together with apoB pool sizes, in each fraction are used to derive kinetic parameters in SAAMII modelling software (SAAM Institute, Seattle). Some years ago, [DEMANT 1996] our unit generated a multi-compartmental model for apoB metabolism, illustrated by Figure 41 below.

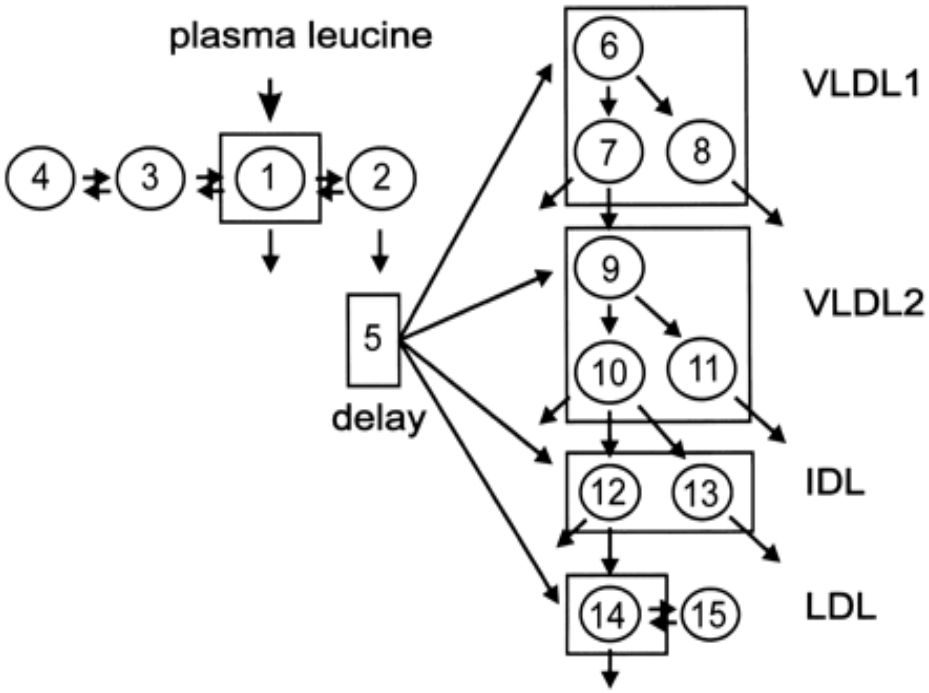


Figure 41 - Schematic of the compartmental model of apoB synthesis and transfer down the delipidation cascade.

Compartment 1 represents plasma leucine which is in equilibrium with an intracellular compartment (2). As leucine is also taken up by other protein-synthesising cells / pathways, compartments 3 and 4 are used to denote the uptake and release of leucine by protein pools in the body that have slow turnover rates. Intracellular compartment 2 feeds directly into compartment 5 which represents apoB synthesis and incorporation into lipoproteins in

hepatocytes. VLDL-1 is present in compartments 6-8; VLDL-2 in compartments 9-11; IDL in compartments 12 and 13; and LDL in compartment 14. Whilst the majority of newly formed apoB is used for production of VLDL-1 from chylomicron remnants, previous similar kinetic studies have demonstrated that small amounts of apoB may be incorporated into any of the four lipoprotein classes. [PACKARD2000] There is a delipidation cascade, driven by lipoprotein lipase and hepatic lipase, from compartment 6 to 7 to 9 to 10 to 12 to 14. Compartments 8, 11 and 13 represent "remnant" populations generated enzymatically, which are slowly cleared from the system without progressing further down the delipidation cascade. From compartment 14, LDL-associated apoB may transfer to compartment 15 (representing extravascular LDL) or be excreted. Using this model, rates of transfer from one fraction to the next can be calculated, as well as rates of de novo synthesis (between compartment 5 and 6, 9, 12 or 14) and catabolic rate of LDL.

3.2.12 Determination of sample size

The primary outcome measure for KALIBRA was the fractional catabolic rate (FCR) of LDL-related apoB. In normal subjects, the FCR is around 0.3 pools per day. In those with increased catabolism due to hypertriglyceridemia in a previous study, the FCR is around 0.5 pools per day; this corresponded to a large decrease in LDL-c of around 1.5mmol/l.[PACKARD2000] In contrast, a study of lipid kinetics using ciprofibrate showed an increase in mean LDL catabolic rate from 0.32 to 0.38 pools per day, in the context of a 22% fall in LDL-c. This is broadly similar to the rises of around 20% in LDL-c seen in the tocilizumab phase III programme, and reflects a biologically significant change in FCR.

Using these figures, we used a conservative change in FCR of around 0.05 pools per day to determine power. A sample size of 15 subjects allows us to detect a difference in fractional catabolic rate of 0.05 with SD 0.05 at 90% power and alpha error at 5%. This sample size is typical for kinetic trials of this type, and was felt to be achievable given the RA population available between the three recruitment sites. Nevertheless, we realised that this could be an ambitious target given the stringent inclusion and exclusion criteria, and a study protocol which was potentially very inconvenient for patients. We were therefore aware that that a sample size of 15 may be unattainable in the time available to carry

out the study, and so set an informal target of at least 10, and ideally 12, recruits. The consensus of the investigators was that a sample size smaller than 10 was unlikely to generate statistically significant data, and any "positive" results would be treated with scepticism on peer review.

3.2.13 Ethical approval

Ethical approval for the study was sought from the West of Scotland Regional Ethics Service (WosRES) Research Ethics Committee (REC) 3, reference 12/WS/0171. A meeting of the REC was attended by me on 26th July 2012, and provided a provisional opinion on 3rd August. Questions and concerns articulated in the provisional opinion included: Amending the patient information sheet to include details on tocilizumab and heparin, the number of blood tests being performed, and the fact that samples would be stored for future research; how samples and confidential information would be stored; how adverse events would be documented; and concern from lay members of the panel on the quantity of blood being taken during the kinetic studies. These were responded to in writing on 23rd August 2012, and REC approval was obtained on 4th September 2012.

3.3 Results – Clinical and Kinetic Outcomes

3.3.1 Demographics

Patients were recruited from summer 2013 through to winter 2014. The final kinetic study was performed in February 2015, as in Table 14.

Study #	Source	Screening	1st Kinetic study	2nd Kinetic study
KAL001	GRI	22/07/2013	29/07/2013	04/11/2013
KAL002	GGH	25/07/2013	12/08/2013	24/02/2014
KAL003	ST	27/08/2013	09/09/2013	18/11/2013
KAL004	GGH	12/09/2013	23/09/2013	09/12/2013
KAL005	GRI	29/01/2014	17/03/2014	09/06/2014
KAL006	GRI	03/03/2014	31/03/2014	23/06/2014
KAL007	GRI	18/03/2014	14/04/2014	18/08/2014
KAL008	GGH	19/09/2014	22/09/2014	08/12/2014
KAL009	GGH	01/10/2014	13/10/2014	12/01/2015
KAL010	ST	09/10/2014	27/10/2014	19/01/2015
KAL011	GRI	30/10/2014	10/11/2014	02/02/2015
KAL012	GRI	30/10/2014	24/11/2014	23/02/2015

Table 14 - Recruitment sites and dates for KALIBRA participants. GRI = Glasgow Royal Infirmary. GGH = Gartnavel General Hospital. ST = Stobhill Hospital.

Demographic details of the patient cohort are shown in

Table 15. In keeping with observed patterns in our RA clinics, the majority were female and seropositive for either RF or ACPA, with a mean age of 55. Only four patients took concomitant MTX. This is probably because patients intolerant of MTX were prescribed TCZ instead of anti-TNF; some patients were instructed specifically for TCZ by their consultant, whilst others were referred simply for “biologics screening” with no drug specified, and TCZ was decided as the drug of choice when I reviewed their details in the biologics clinic. Two patients were on “triple therapy” (MTX, SSZ & HCQ) and two were on no

conventional DMARDS at all. Three patients had previously failed or not tolerated at least one anti-TNF drug.

Four adverse events were documented. One patient developed grade 2 neutropenia; neutrophil counts recovered spontaneously and re-challenge with full-dose TCZ was successful. Three patients contracted infections. One patient required hospitalisation for an abscess on her back following one dose of TCZ, but recommenced her drug after incision & drainage and successful treatment with antibiotics. A severe adverse event (SAE) form was completed and the data shared with Roche as per their internal protocols. One patient developed shingles; suspension of TCZ treatment was not required.

KAL008 developed a paronychia after three doses of TCZ, requiring removal of her fingernail and suspension of TCZ. As she was without treatment for several weeks, we were unable to re-treat her with TCZ and perform kinetic studies within the available time window, and she was withdrawn from the study. However, this was not classed as a SAE given that hospital admission was not required and no risk to life or long-term health resulted from the infection. As no post-treatment data was collected for this patient, all analyses hereafter are carried out on a “per-protocol” basis using data from the remaining 11 patients.

Study #	Age (y)	Sex	Weight (kg)	BMI	Systolic BP (mmHg)	Prednisolone (mg/d)	NSAID (Y/ N)	MTX (mg/wk)	SSZ (mg/d)	HCQ (mg/d)	LEF (mg/d)	Previous Biologic?	RF / ACPA + (Y/N)
KAL001	49	F	63	23.4	138	1	Y	7.5				Y	Y
KAL002	58	F	78	29.0	137		Y						Y
KAL003	46	M	88	26.3	161		Y			200		Y	Y
KAL004	65	M	78	26.7	121			10	4000	200		Y	
KAL005	61	F	60	24.3	129	7.5				200			Y
KAL006	50	F	63	29.2	113								Y
KAL007	32	F	62	23.9	105		Y		2500				Y
KAL008	28	F	60	23.4	123			20					
KAL009	57	F	74	29.6	149		Y			400	10		Y
KAL010	51	F	48	17.2	146		Y		2000	300			Y
KAL011	60	F	69	26.3	156		Y	12.5	2500	400			Y
KAL012	42	F	59	19.3	103		Y		3000	400			Y
Mean / %	49.9	83% F	66.8	24.9	132	4.25	67%	12.5	2800	300	10	25%	83%

Table 15 - Demographic data of KALIBRA cohort at baseline.

3.3.2 Clinical outcomes

Changes in markers of disease activity after TCZ are shown in Figure 42. At baseline, mean DAS28-CRP and DAS28-ESR were 5.16 and 5.61 respectively. Mean CDAI was 29.9. As is typical for IL-6 blockade, CRP and ESR fell markedly; at the time of the second kinetic study, all patients had CRP of 2mg/l or less, and ESR of 10mm/hr or less. Tender and swollen joint counts also improved, but to a lesser extent. Mean DAS28-CRP and DAS28-ESR were 2.24 and 1.81 respectively. Mean CDAI was 8, with six patients reaching low disease activity (CDAI ≤10), and one reaching CDAI remission (CDAI ≤2.8).

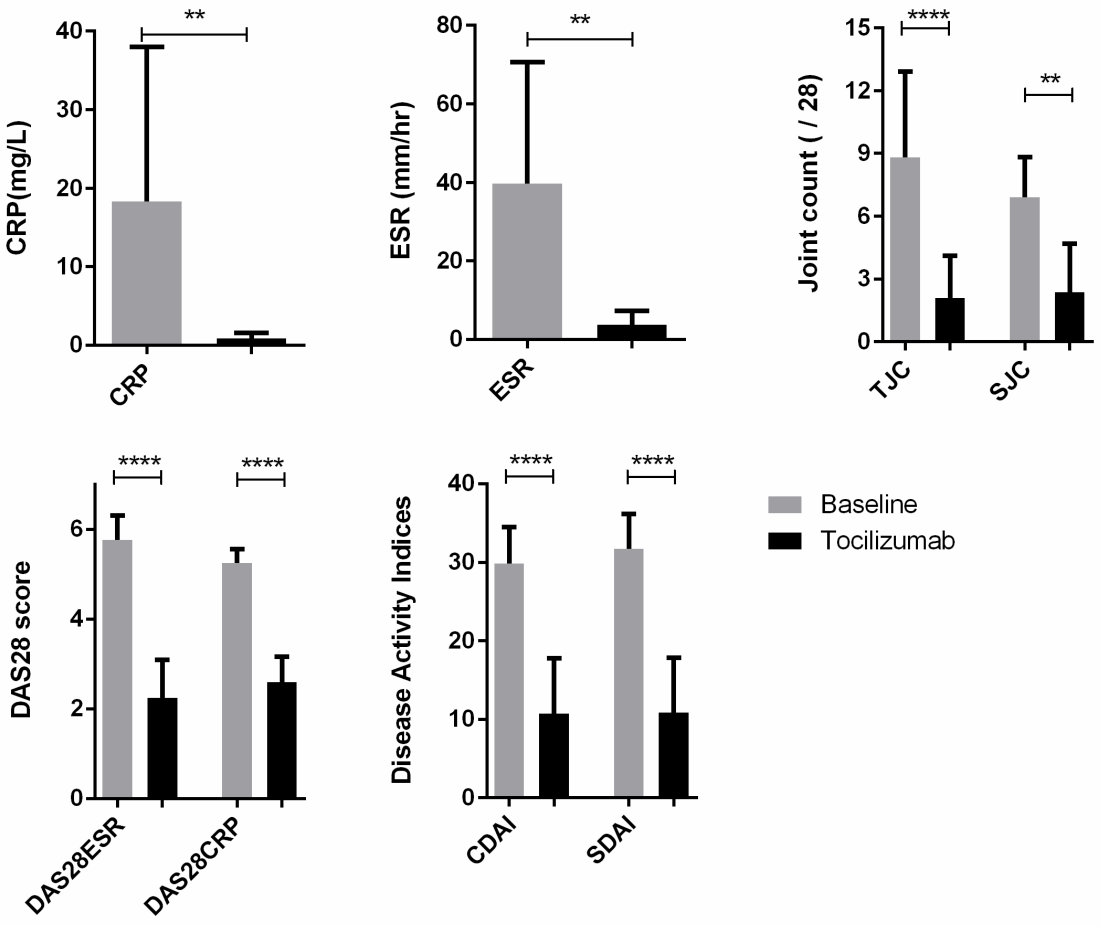


Figure 42 - Measures of disease activity pre- and post-treatment, presented as means ± SD. * = p<0.05. ** = p<0.01 *** = p<0.001 **** = p<0.0001 by paired t-test.

3.3.3 Serum cholesterol by betaquant

Serum cholesterol levels were first analysed by betaquant. This is the standard industry method of measuring serum LDL-c and uses a combination of ultracentrifugation and precipitation; the value given for LDL-c also includes cholesterol within IDL and Lp(a). Betaquant values are shown in

Table 16. In accordance with previous studies, total cholesterol, HDL-c and LDL-c all increased. A non-significant rise in TG was noted. The TC / HDL-c ratio did not change.

	Baseline		Treatment		
	Mean	95% C.I.	Mean	95% CI	*p*
TC	**4.81**	**4.30 – 5.32**	**5.66**	**4.91 – 6.42**	***0.003***
VLDL-c	0.69	0.57 – 0.83	0.75	0.57 – 0.93	*0.56*
LDL-c	**2.90**	**2.46 – 3.34**	**3.40**	**2.72 – 4.09**	***0.014***
HDL-c	**1.23**	**1.07 – 1.39**	**1.52**	**1.39 – 1.64**	***0.006***
TG	0.93	0.74 – 1.12	1.12	0.84 – 1.41	*0.21*
TC/HDL-c	4.08	3.55 – 4.61	3.81	3.17 – 4.46	*0.18*

Table 16 - Serum lipids as measured by betaquant at baseline and after treatment. All values in mmol/L except TC/HDL-c ratio. Analysis by paired t-test.

3.3.4 Lipoprotein particle composition

Before running mathematical models to generate kinetic data, it was first necessary to analyse the composition of VLDL-1, VLDL-2, IDL and LDL. This is because particle composition is required to calculate apoB pool sizes for each subfraction; pool sizes are then required for the kinetic modelling stage. Each particle contains phospholipid (mostly in the particle membrane); protein in the form of ApoB; free cholesterol; cholesterol ester; and triglyceride.

Changes in the mass and composition of VLDL-1, VLDL-2 and IDL are summarised in Table 17. VLDL-1 mass increased, driven by significant increases in all

particle constituents except protein, and especially triglyceride. The proportions of the constituents did not change, though there was a non-significant (p=0.076) fall in proportional protein content. For VLDL-2 and IDL, no changes were observed in particle mass, individual constituent levels or constituent proportions. Figure 43 shows the mean percentage composition of VLDL-1, VLDL-2 and IDL, demonstrating the progressive loss of triglyceride and proportional increase in cholesterol within the particles as they travel down the lipolytic pathway.

	Mass		Protein		Free cholesterol	
	Baseline	Treatment	Baseline	Treatment	Baseline	Treatment
VLDL-1	24.8	44.9 *p=0.009*	2.6	3.6 *p=0.13*	1.5	2.5 *p=0.056*
VLDL-2	24.4	31.2 *p=0.28*	3.7	4.9 *p=0.24*	2.1	3.4 *p=0.24*
IDL	47.7	59.1 *p=0.19*	8.8	11.2 *p=0.38*	4.1	5.8 *p=0.13*

	Cholesterol ester		Triglyceride		Phospholipid	
	Baseline	Treatment	Baseline	Treatment	Baseline	Treatment
VLDL-1	3.1	6.5 *p=0.002*	15.6	24.4 *p=0.012*	3.5	6.8 *p=0.030*
VLDL-2	4.3	7.2 *p=0.21*	10.1	11.6 *p=0.24*	4.7	6.5 *p=0.28*
IDL	14.9	20.4 *p=0.15*	6.1	6.2 *p=0.95*	8.3	11.2 *p=0.090*

Table 17 - Median lipoprotein masses and constituents at baseline and after treatment. N=11. P values generated by Wilcoxon matched-pairs test.

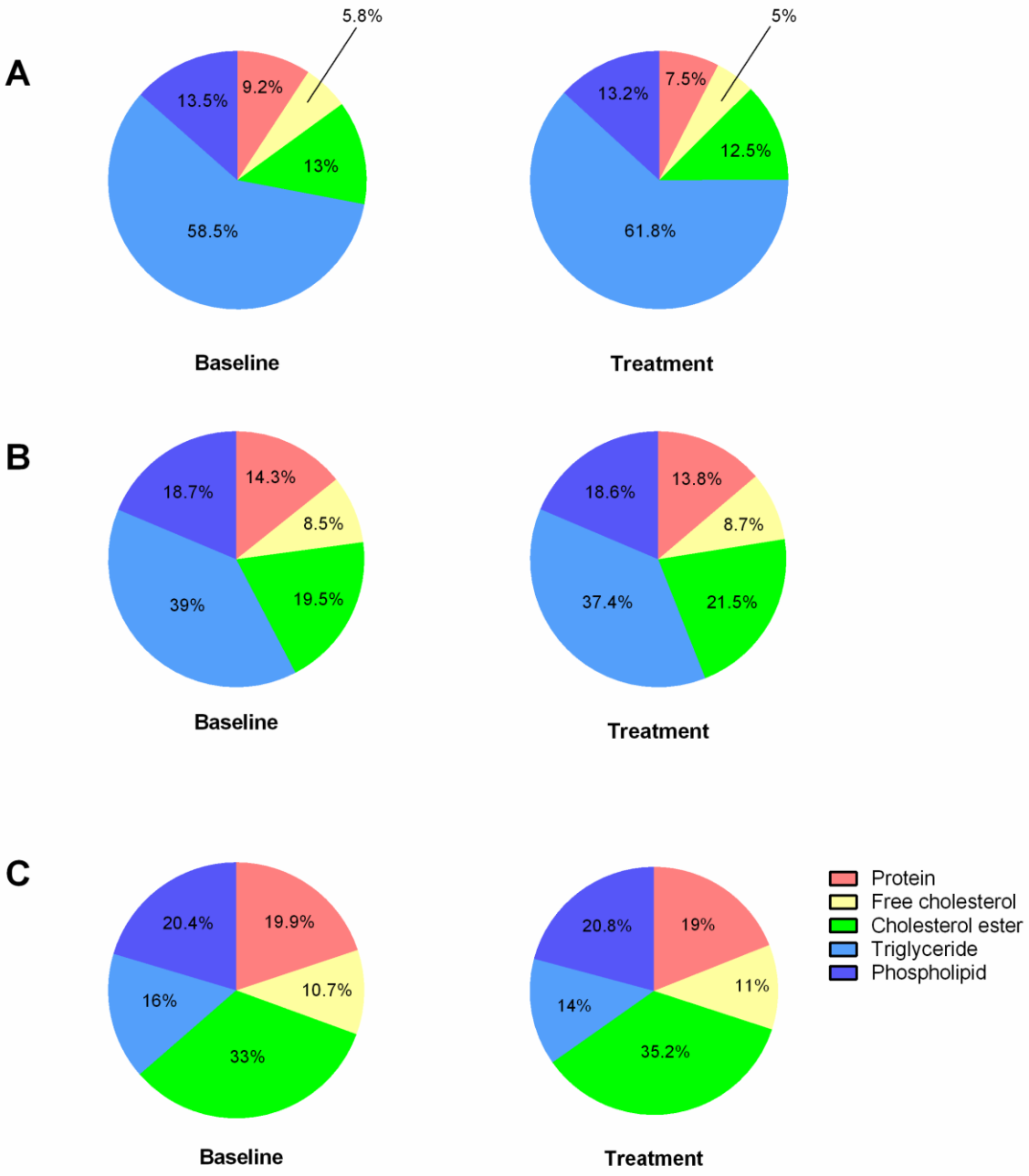


Figure 43 - Particle compositions before and after TCZ in (A) VLDL-1, (B) VLDL-2 and (C) IDL.

To my surprise, LDL mass did not increase significantly. Free cholesterol increased modestly but no significant change was seen in cholesterol ester, which made up nearly 40% of the particle's mass (Figure 44). Similar results were seen when looking at the proportion of LDL that each constituent made up (Figure 45). Figure 46 demonstrates constituent proportions in LDL.

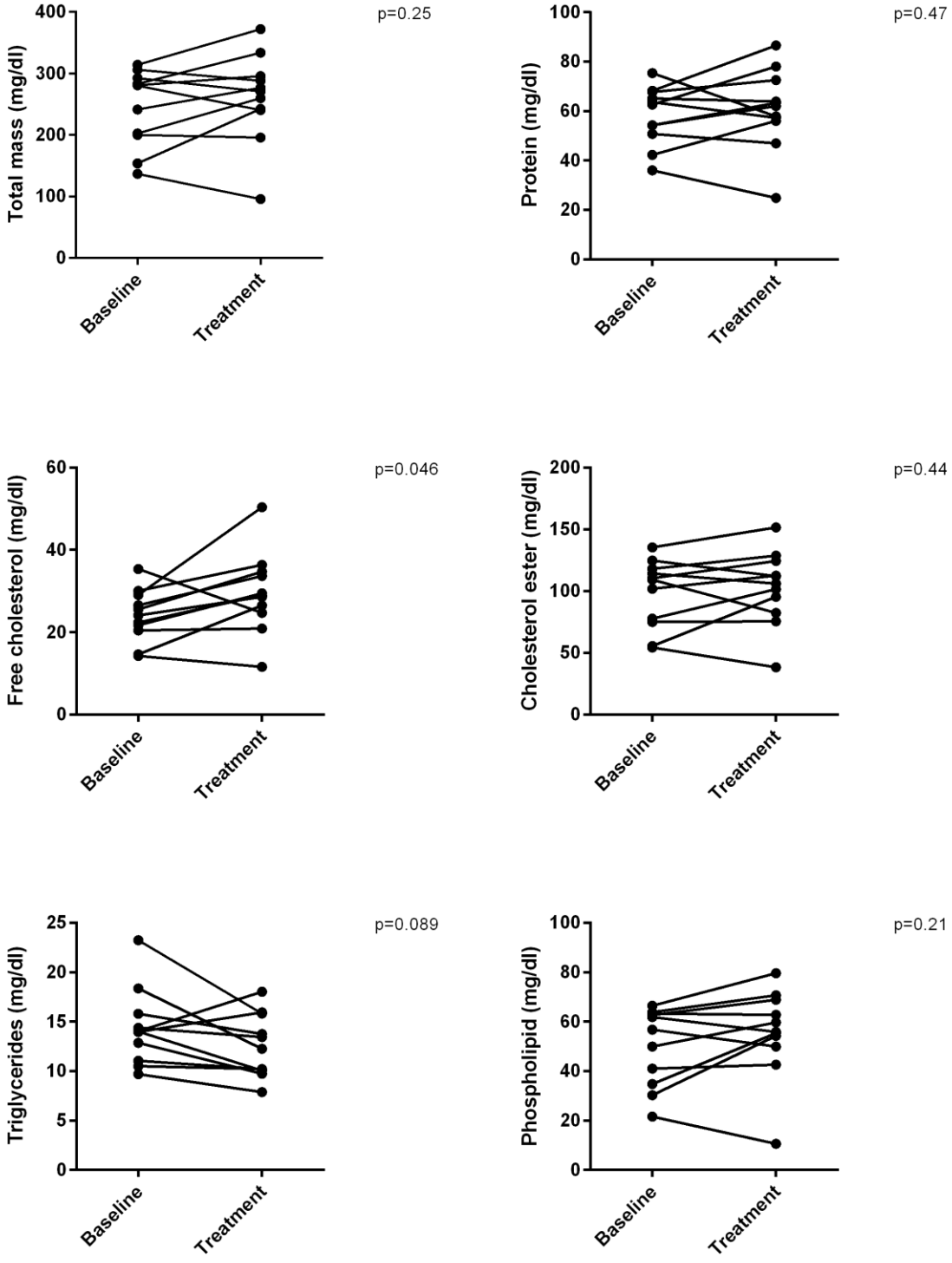


Figure 44 - LDL mass and constituents at baseline and after treatment, as analysed by Wilcoxon matched-pairs test.

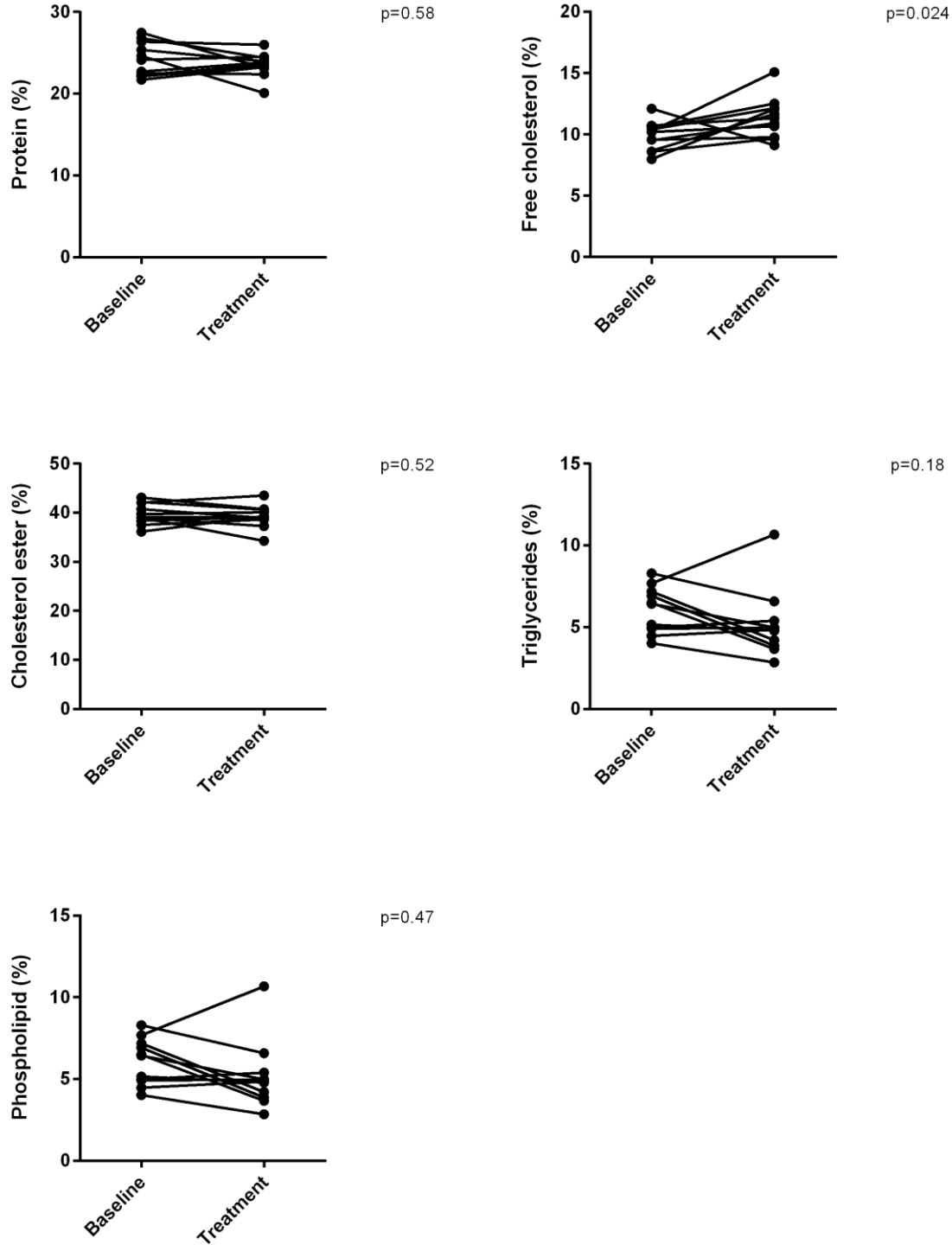


Figure 45 - Percentage composition of LDL particles, as analysed by Wilcoxon matched-pairs text.

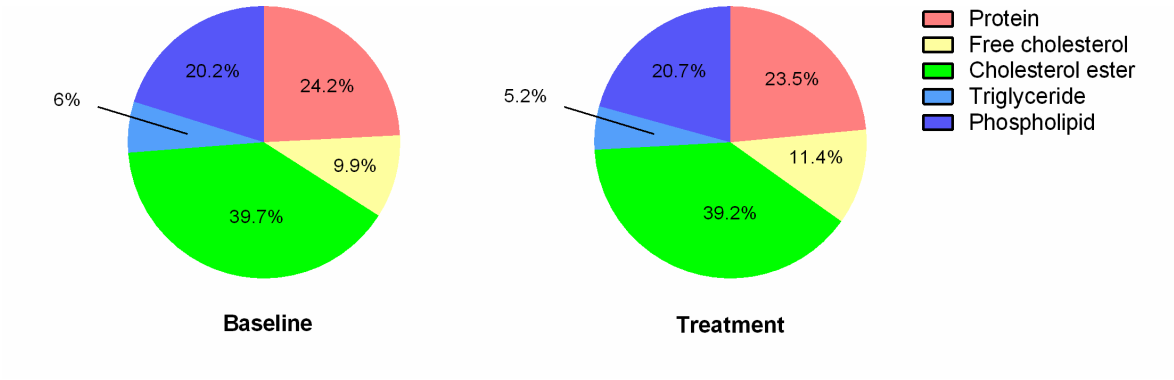


Figure 46 - Particle composition before and after TCZ in LDL.

The plots in Figure 44 show that, although median LDL-cholesterol ester (LDL-CE) content did not change, individual responses varied significantly. Stratifying patients by their response showed four patients whose LDL-CE fell, and seven who accumulated LDL-CE. On interrogating their clinical parameters, three of the four patients with falls in LDL-CE had low acute phase response at baseline, translating to very modest changes in CRP and ESR, despite having similar magnitudes of CDAI change (Table 18).

	Change LDL-CE (mg/dl)	Baseline CRP (mg/L)	ESR (mm/Hr)	Change CRP (mg/L)	ESR (mm/Hr)	Change CDAI
KAL012	-26.78	1	5	-0.5	6	-19.5
KAL007	-15.99	55	102	-54.5	-101	-24
KAL010	-12.58	2	20	-1.5	-18	-28.5
KAL011	-8.15	1	5	-0.5	-3	-29
KAL001	0.54	22	33	-20	-31	-21.4
KAL002	10.73	2.8	17	-2.6	-15	-7.5
KAL006	10.75	5	27	-3	-25	-13
KAL004	14.07	29	55	-28.7	-45	-7
KAL003	16.21	12	70	-11.8	-68	-16.9
KAL005	23.83	21	31	-19	-26	-26
KAL009	39.70	51	71	-50.5	-69	-17.5

Table 18 - All participants sorted by change in LDL-cholesterol ester, with baseline and change clinical parameters.

Plotting the change in LDL-CE with change in CRP or ESR showed a trend to inverse association. Of note, KAL007 appeared to be acting as an outlier (Figure 47, KAL007 circled in red). With exclusion of KAL007, the associations with CRP and ESR become statistically significant ($r=-0.7964$, $p=0.0075$ and $r=-0.8182$, $p=0.0058$ respectively). No association with CDAI change was seen with or without inclusion of KAL007.

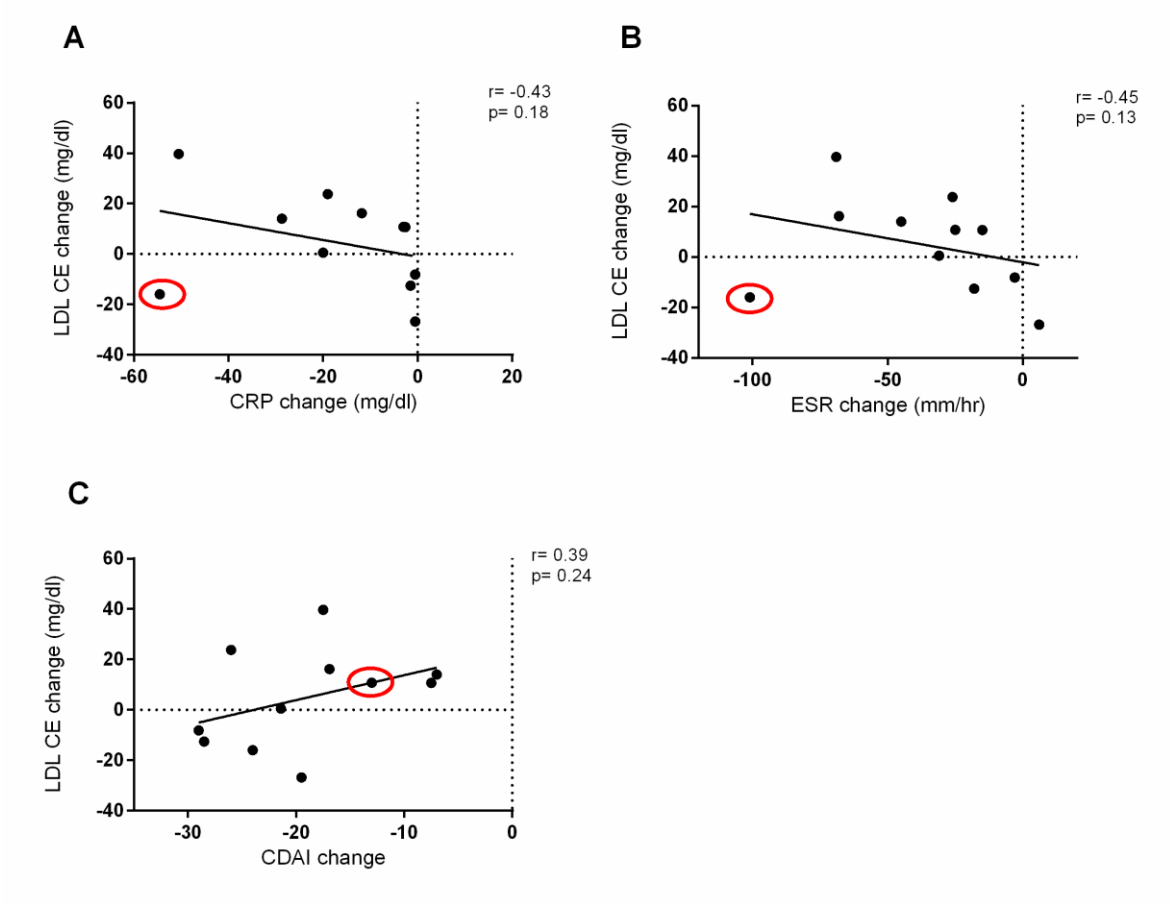


Figure 47 - Scatterplots of relationship between change in LDL-associated cholesterol ester and: (A) change in CRP; (B) change in ESR; (C) change in CDAI. Subject KAL007 is circled in red. R and p values calculated by Spearman's correlation coefficient.

Stratifying the cohort according to their baseline CRP showed a trend towards greater increase in LDL-CE in those with CRP≥5mg/l compared to those with baseline CRP<5mg/l. This difference became statistically significant on exclusion of KAL007 (Figure 48).

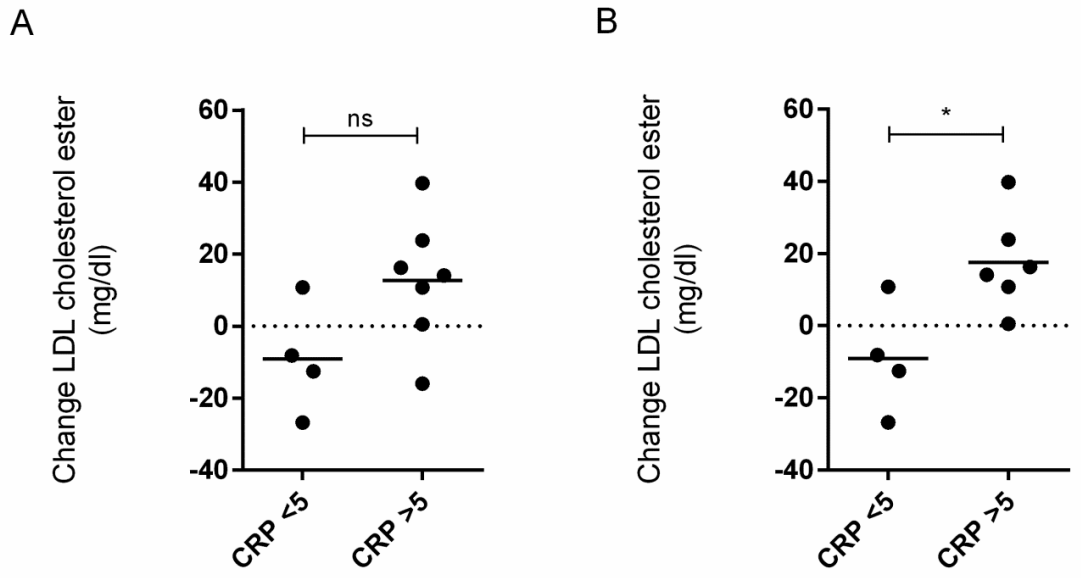


Figure 48 - Change in LDL cholesterol ester depending on baseline CRP. (A) All patients included. (B) Excluding KAL007. Analysis by student's t-test.

To investigate the applicability of these observations to more conventional methods of serum cholesterol measurement, I repeated the above analyses with LDL-c as measured by Betaquant on day 1 of each kinetic study. On exclusion of KAL007, trends towards associations were observed with CRP and ESR, though statistical significance was not reached (Figure 49). Stratifying the cohort by baseline CRP showed a non-significant difference in change in LDL-c between the groups (mean increase 0.36 v 0.75mmol/L, p=0.14)(Figure 50).

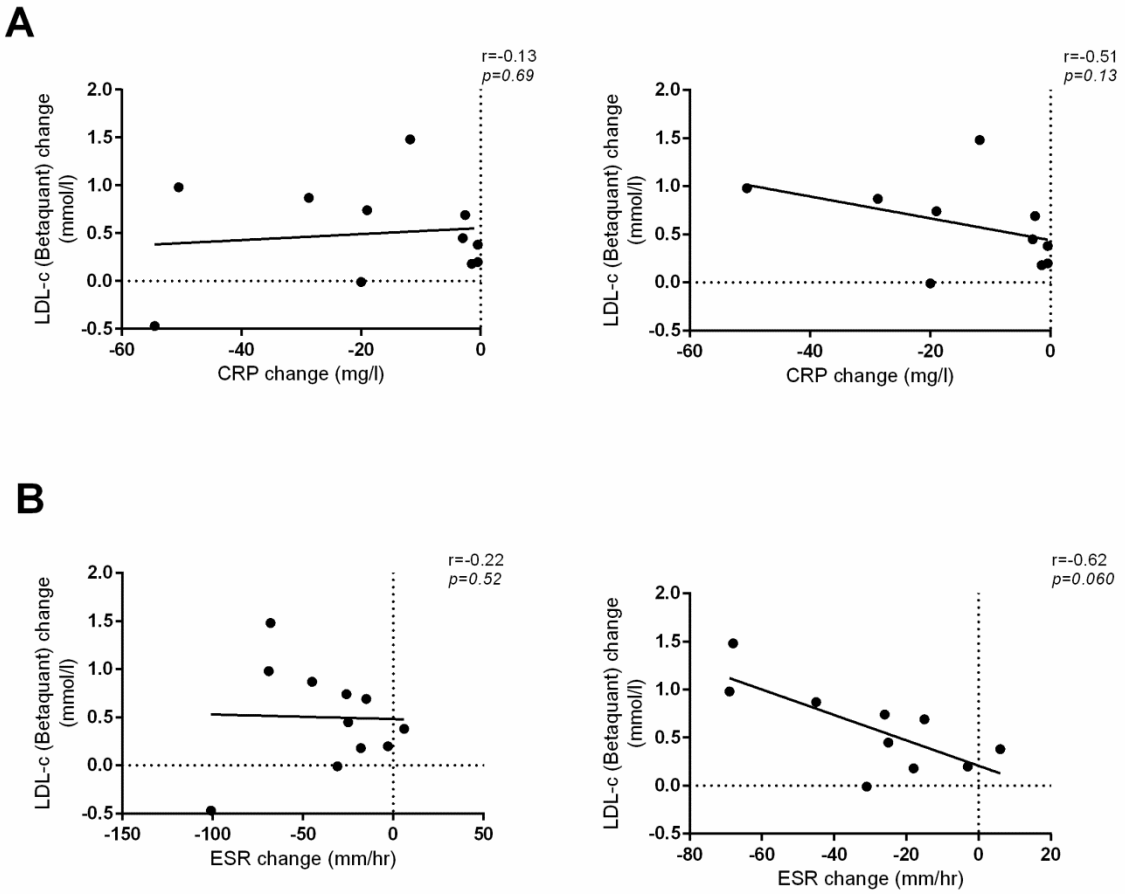


Figure 49 - Scatterplots of relationship between change in LDL-cholesterol and: (A) change in CRP; (B) change in ESR. Plots are shown with (left) and without (right) inclusion of patient KAL007. R and p values calculated by Spearman's correlation coefficient.

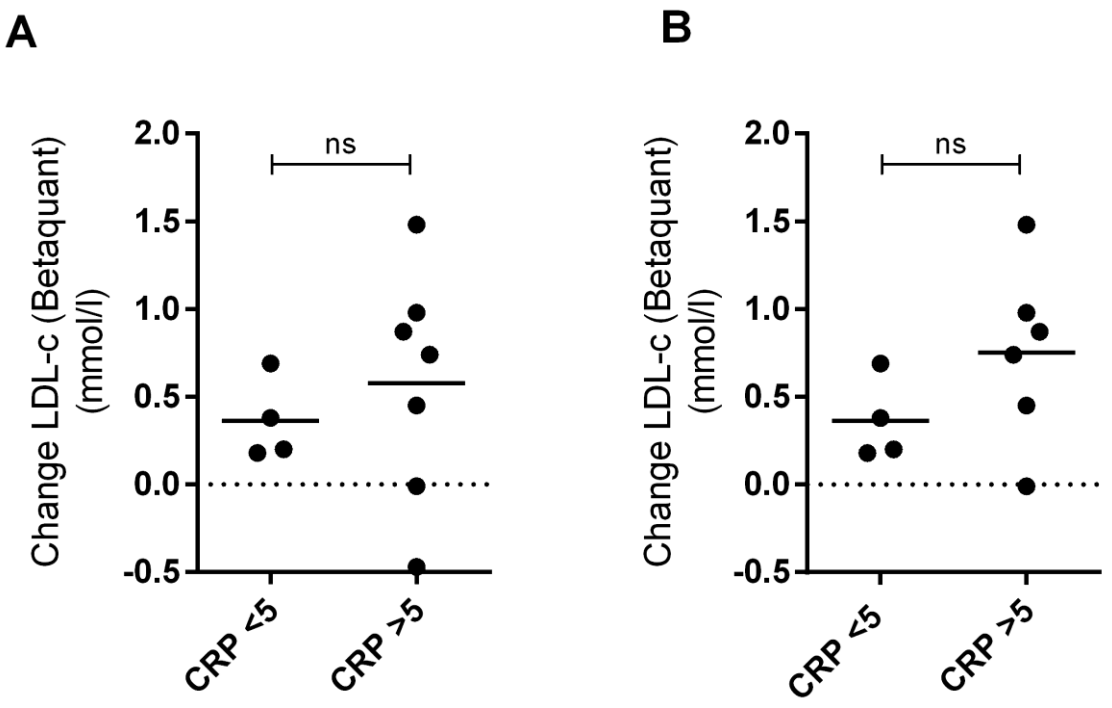


Figure 50- Change in LDL cholesterol ester depending on baseline CRP. (A) All patients included. (B) Excluding KAL007. Analysis by student's t-test with Welch's correction.

3.3.5 Lipoprotein Kinetics

Appendix F contains a summary of kinetic modelling outcomes for all lipoproteins at baseline, after treatment, and in a normal population based on data held on file within out unit. Parameters which changed significantly after tocilizumab are highlighted in red.

Figure 51 illustrates changes in median total LDL production rate (PR – the sum of transfer of apoB from IDL and de novo LDL-associated apoB synthesis from the liver) and fractional catabolic rate (FCR). Unexpectedly, and out of keeping with the general pattern of increase in LDL-c, PR fell significantly following tocilizumab treatment (763.8 v 442.0 mg/kg/day, p=0.002). Median FCR, the primary outcome measure of the study, fell from 0.53 to 0.27 pools/day (p=0.006). This equates to a median fall from baseline of 30%, as seen in Table 19. A graph showing pre-and post-treatment LDL kinetics, using subject KAL004 as an example, can be found in Appendix G. This clearly shows a slower increase in the tracer/tracee ratio following d3-leucine administration

(reflecting reduced production rate), followed by an elongated, shallow reduction in the ratio (reflecting reduced catabolic rate).

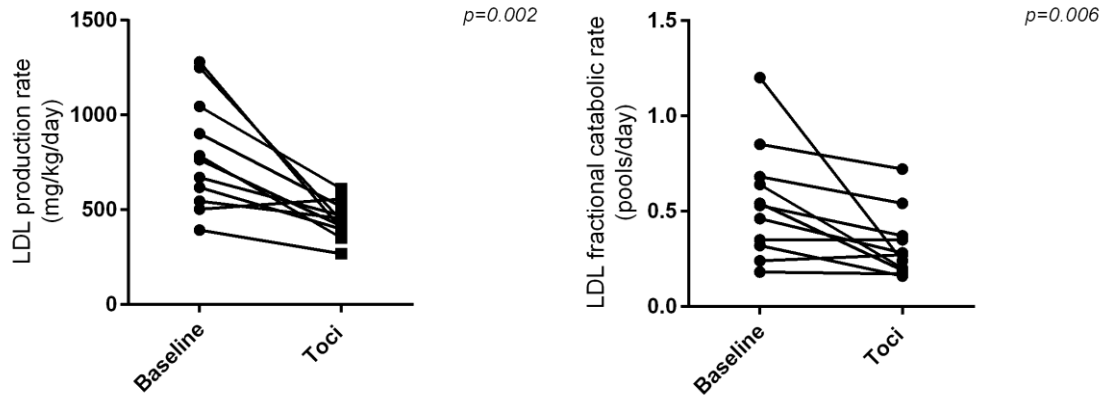


Figure 51 - Production rate and fractional catabolic rate of LDL. Analysis by Wilcoxon matched-pairs test.

	Production rate (mg/kg/day)			
	Baseline	Treatment	Value change	% change
Mean	796.5	443.9	-352.6	-38.4
SD	295.7	95.3	287.6	22.3
Median	763.8	442.0	-348.8	-41.7
p		***0.002***		

	Fractional catabolic rate (pools/day)			
	Baseline	Treatment	Value change	% change
Mean	0.54	0.32	-0.23	-33.08
SD	0.29	0.17	0.28	30.05
Median	0.53	0.27	-0.16	-30.19
p		***0.006***		

Table 19 - Production rate and fractional catabolic rate of LDL. P value generated by Wilcoxon matched-pairs test

At baseline, LDL FCR correlated positively with CRP (r=0.74, p=0.012) though non-significantly with ESR (r=0.54, p=0.091). No relationship was observed with CDAI (r=0.04, p=0.91) (Figure 52).

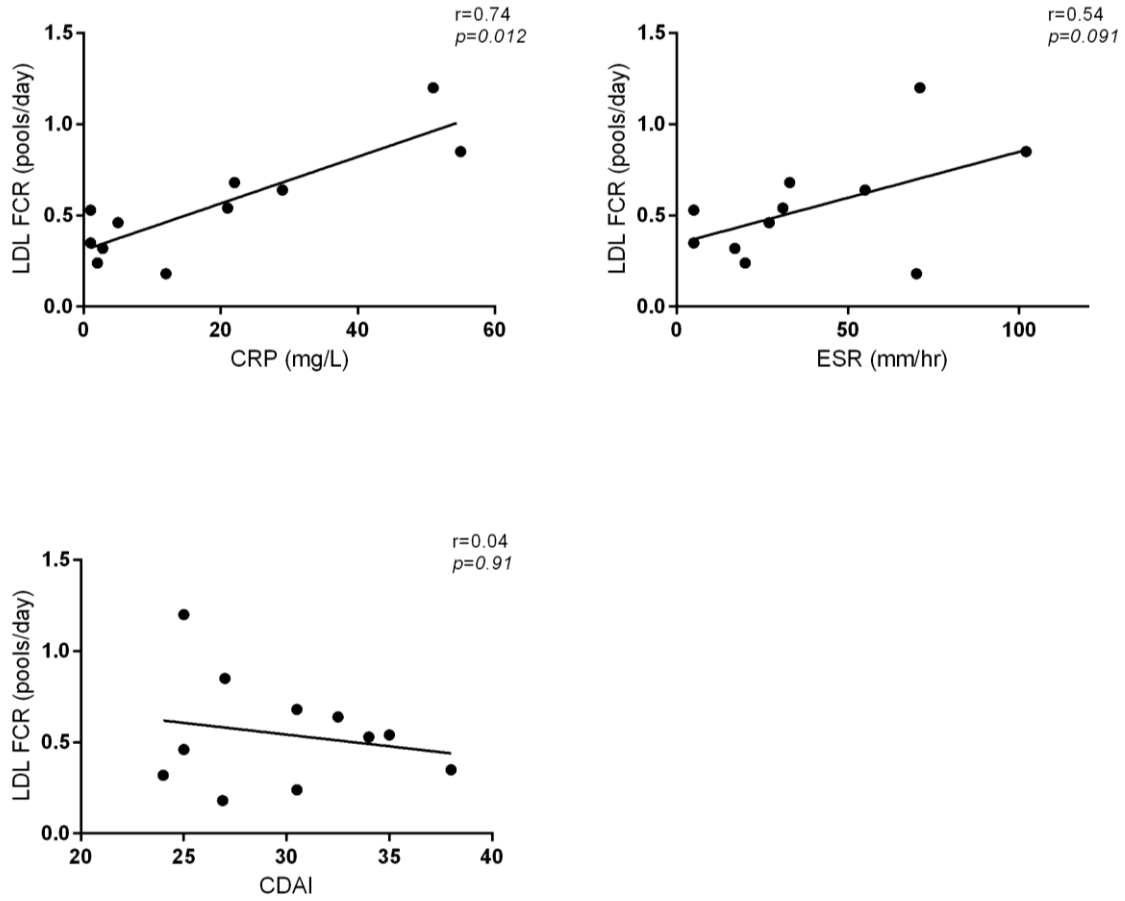


Figure 52 - Correlation of LDL FCR with measures of disease activity at baseline by Spearman's r.

The degree of change in FCR did not correlate at all with the change in disease markers initially, but on removal of participant KAL007 there was a significant relationship with CRP (r=0.68, p=0.035) and a non-significant visual trend towards correlation with ESR (r=0.50, p=0.15). Again, no relationship was observed with CDAI (Figure 53).

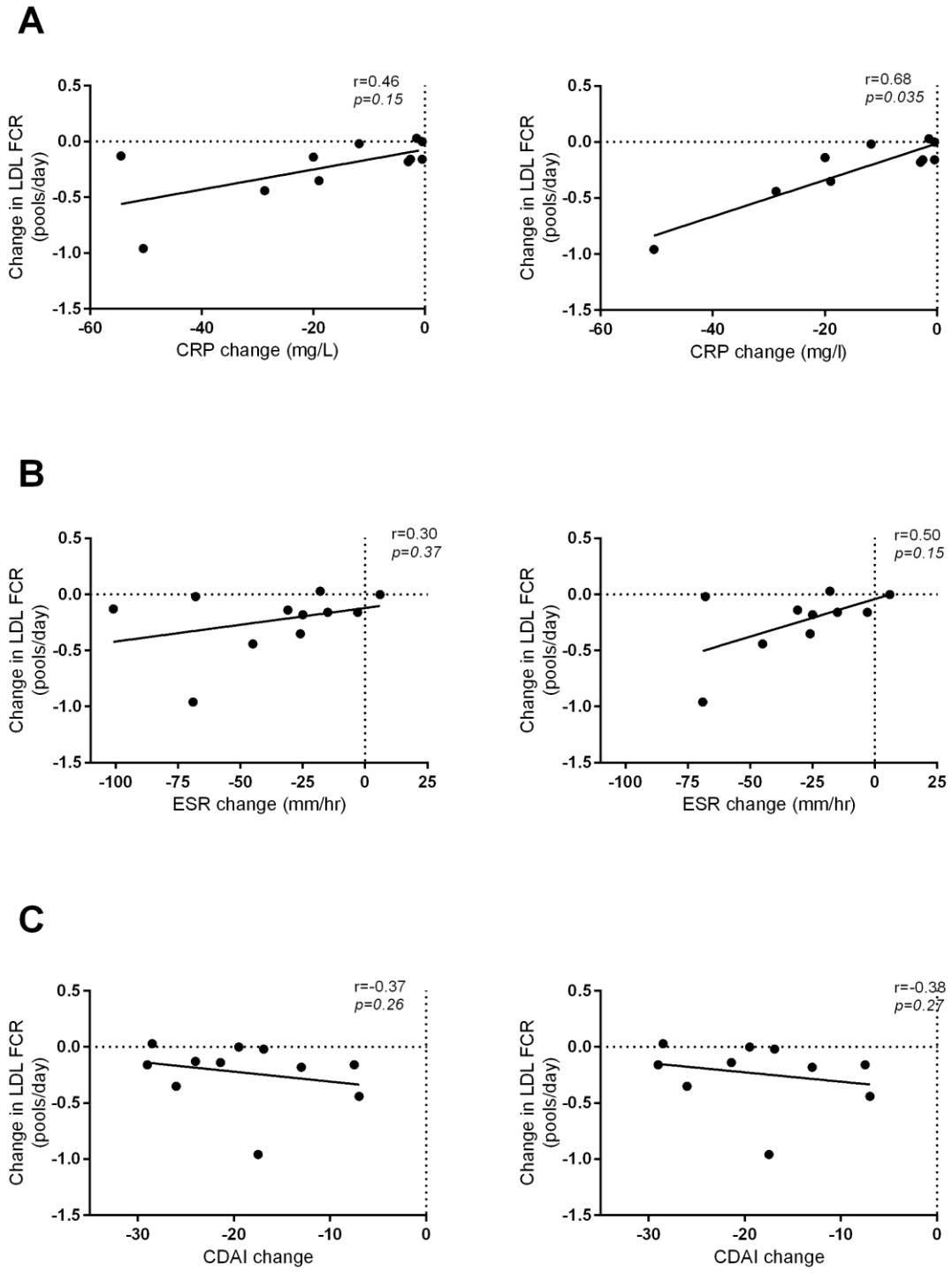


Figure 53 – Spearman's correlation of change in LDL FCR with change in (A) CRP, (B) ESR and (C) CDAI. Left: whole cohort; right: excluding KAL007

LDL production rate did not correlate with LDL cholesterol as measured either by day 1 betaquant or LDL particle cholesterol ester content (LDL-CE). This was the case at baseline, post-treatment and with degree of change (Table 20).

		LDL-CE		LDL Betaquant	
		Spearman's r	*p*	Spearman's r	*p*
LDL production rate	**Baseline**				
	all inclusive	-0.29	*0.39*	-0.23	*0.50*
	exc KAL007	-0.49	*0.16*	-0.44	*0.120*
	Degree of change				
	all inclusive	-0.37	*0.26*	-0.37	*0.26*
	exc KAL007	-0.32	*0.37*	-0.29	*0.43*
	Treatment				
	all inclusive	-0.08	*0.82*	-0.105	*0.88*
	exc KAL007	0.01	*1.00*	0.16	*0.66*

Table 20- Correlations between LDL production rate and LDL cholesterol ester content or LDL-c betaquant

However, LDL fractional catabolic rate correlated strongly with both measures of LDL cholesterol content at baseline (Figure 54). The degree of change in FCR correlated strongly with LDL-CE, though less so with betaquant values (Figure 55), and following treatment the FCR was a strong predictor of both LDL-CE and LDL-c by betaquant (Figure 56). Excluding subject KAL007 made no difference to the pattern or magnitude of correlations.

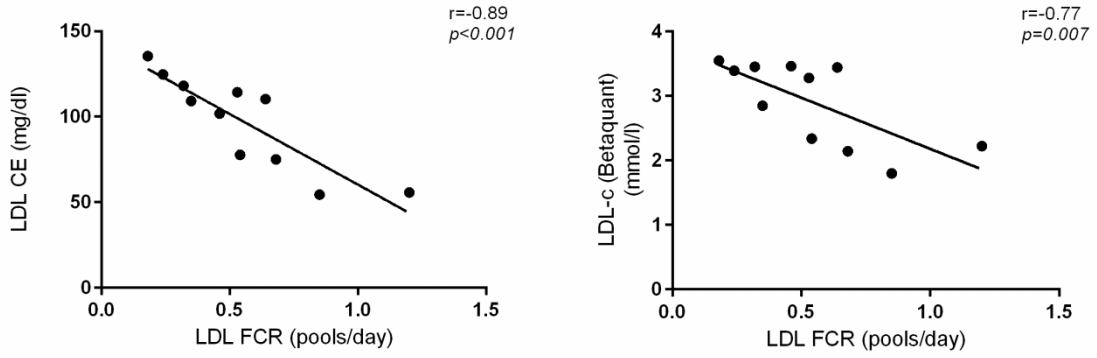


Figure 54 – Spearman's correlation between LDL catabolic rate and serum LDL cholesterol levels at baseline

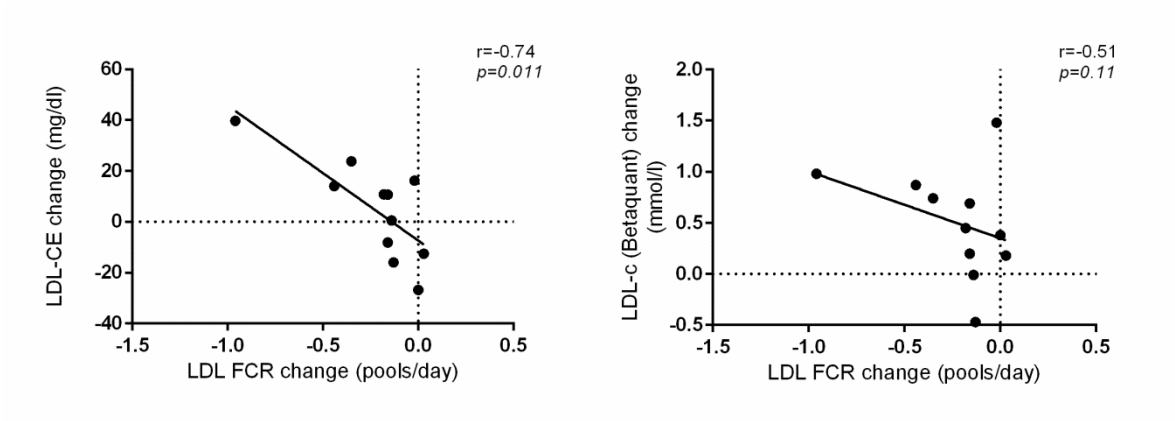


Figure 55 - Spearman's correlation between change in LDL catabolic rate and serum LDL cholesterol levels.

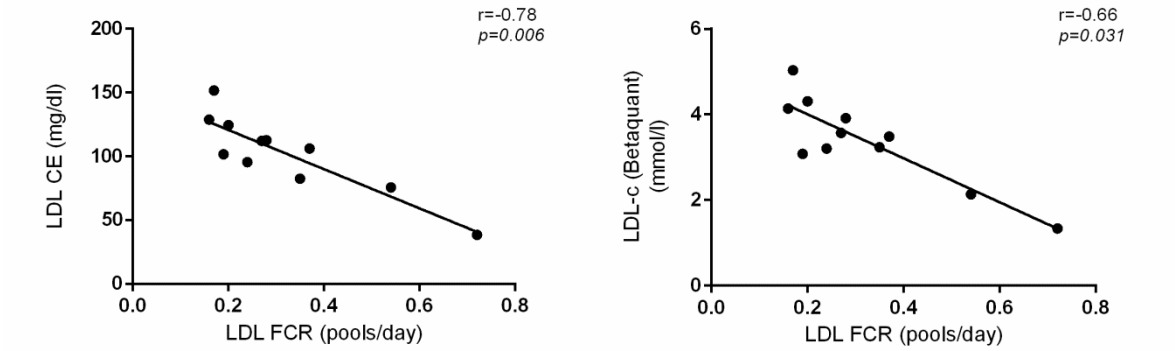


Figure 56 – Spearman's correlation post-treatment between LDL fractional catabolic rate and serum LDL cholesterol levels.

3.4 Results – Secondary Outcome Measures

3.4.1 LDL Subfractions

LDL can be divided into subfractions (titled I, II and III) depending on their density; LDLI are the least dense, while LDLIII have the greatest density. No significant changes were observed in any subfraction quantity (Figure 57).

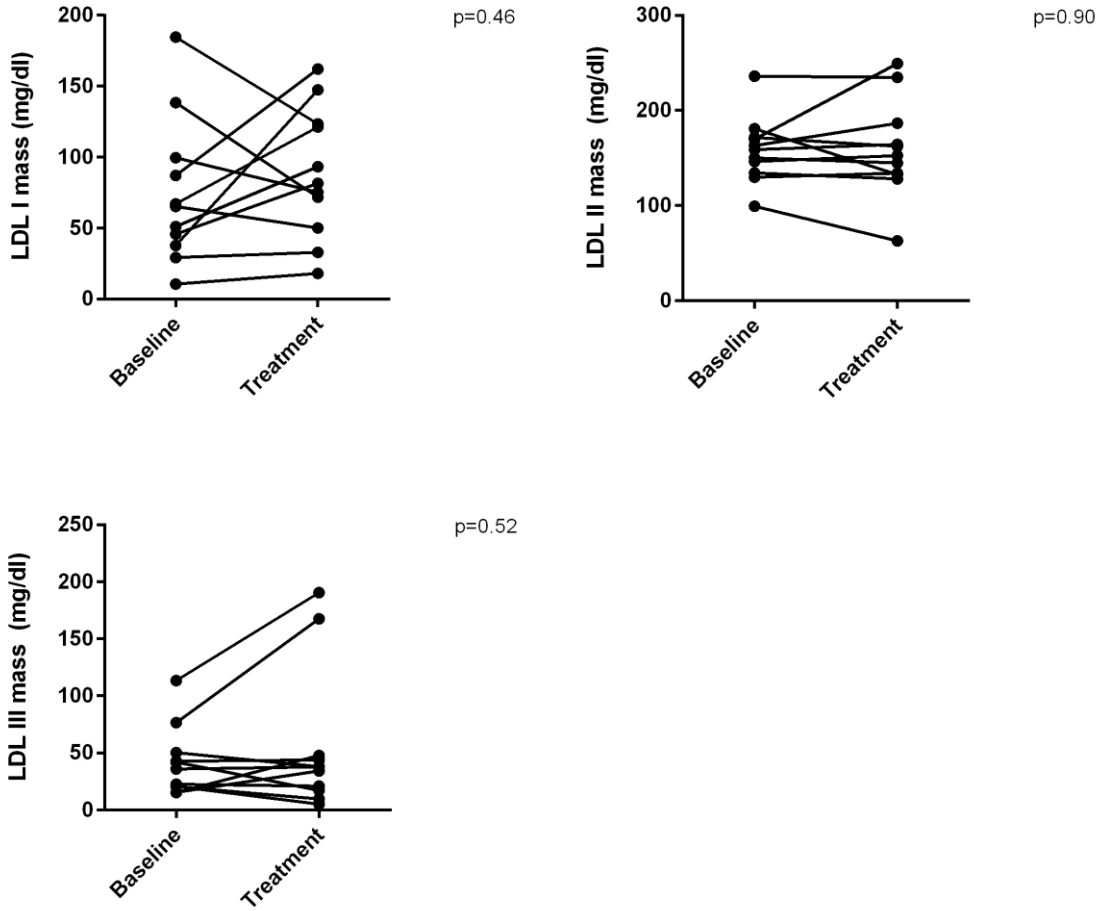


Figure 57 - LDL subfraction levels at baseline and after treatment, as analysed by Wilcoxon matched-pairs test.

Stratification of the change in LDL subfraction mass by baseline CRP (CRP<5mg/l versus CRP≥5mg/l) showed no difference in any subfraction, with or without exclusion of KAL007's data (Figure 58). Similarly, Spearman's correlation coefficient showed no association between change in LDL I, II or III and change in CRP (r=-0.15, p=0.65; r=0.07, p=0.84; r=-0.09, p=0.79 respectively) or ESR (r=-0.14, p=0.69; r=-0.09, p=0.80; r=-0.10, p=0.78 respectively).

However, change in LDL III associated with change in CDAI ($r=0.79$, $p=0.006$), whilst LDL I and II did not (Figure 59). In this case, patients with a fall in CDAI score of less than 20 points tended to increase their LDL III levels; those with greater falls in CDAI either lost LDL III or remained stable. This observation was unexpected, given that a fall of 10 points in CDAI is still clinically significant, in contrast to the doubtful biological significance of a CRP fall of <5mg/dl.

Stratifying the patients depending on their final CDAI score showed that those who reached CDAI low disease activity (CDAI <10 after treatment) had on average no change in LDL III mass; in the one patient who reached CDAI remission (CDAI <2.8 after treatment), LDL III fell by 12.1mg/dl. In patients with CDAI ≥10 after treatment, LDL III mass increased by mean 38mg/dl. Comparing the groups with Welch's corrected t-test did not reach significance ($p=0.095$ for LDL III), as shown in Figure 60. Welch's corrected t-test was used on this occasion as both parameters were normally distributed on this occasion, though the magnitudes of distribution were different.

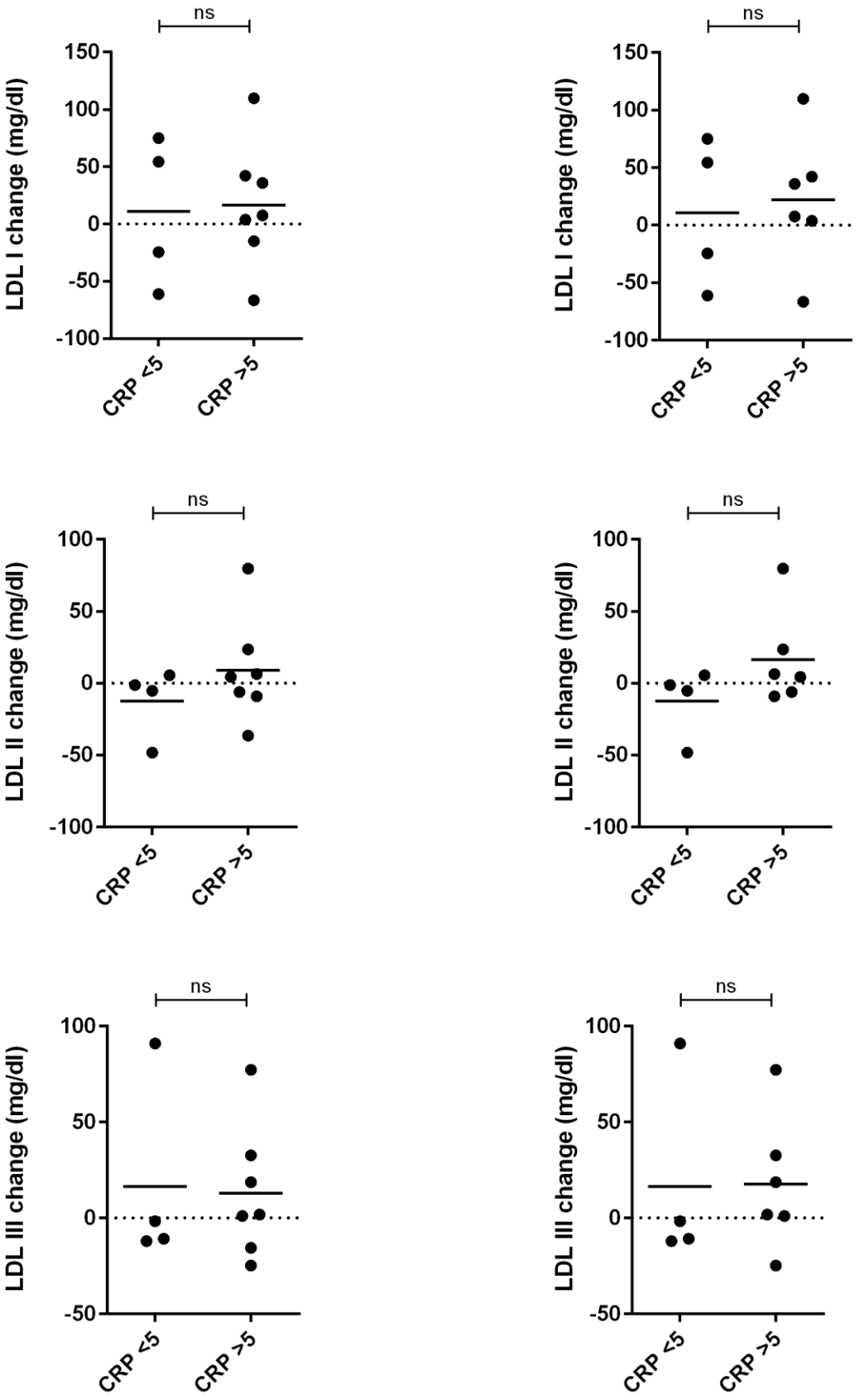


Figure 58 - Change in serum LDL subfraction mass, stratified by baseline CRP, with data from KAL007 included (left) or excluded (right). P value generated by Mann-Whitney test.

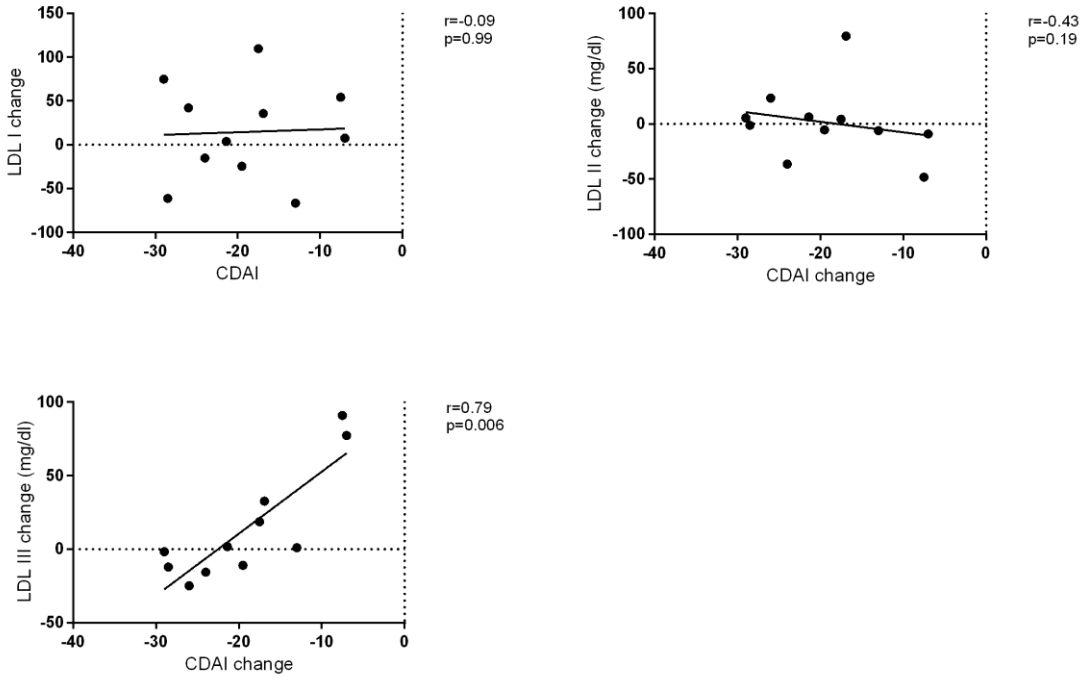


Figure 59 - Spearman's correlation coefficient between change in LDL subfraction mass and change in CDAI.

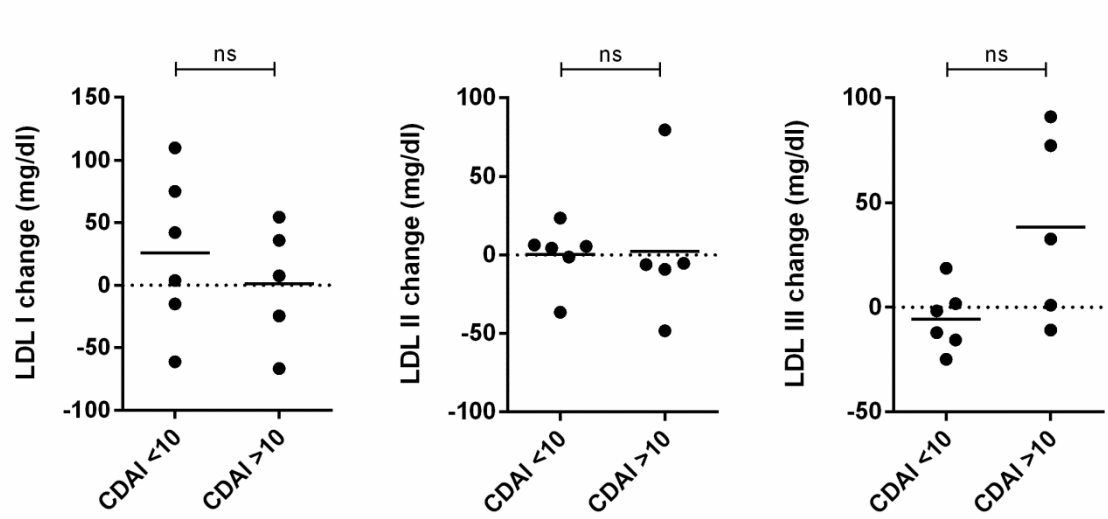


Figure 60 - Change in LDL subfractions depending on post-treatment CDAI score. Analysis by Welch's corrected t-test.

Determination of LDL I, II and III mass also permitted calculation of the proportion of total LDL mass made up of each of these subfractions. Figure 61 shows changes in the percentage of total LDL mass made up by the respective subfractions. These broadly reflect the patterns of absolute value change shown in Figure 57 above, with no statistically significant differences seen after

treatment. Given that patients who demonstrated value increase in a given subfraction invariably also demonstrated increase in the proportion of that subfraction, no further analysis was performed on this parameter.

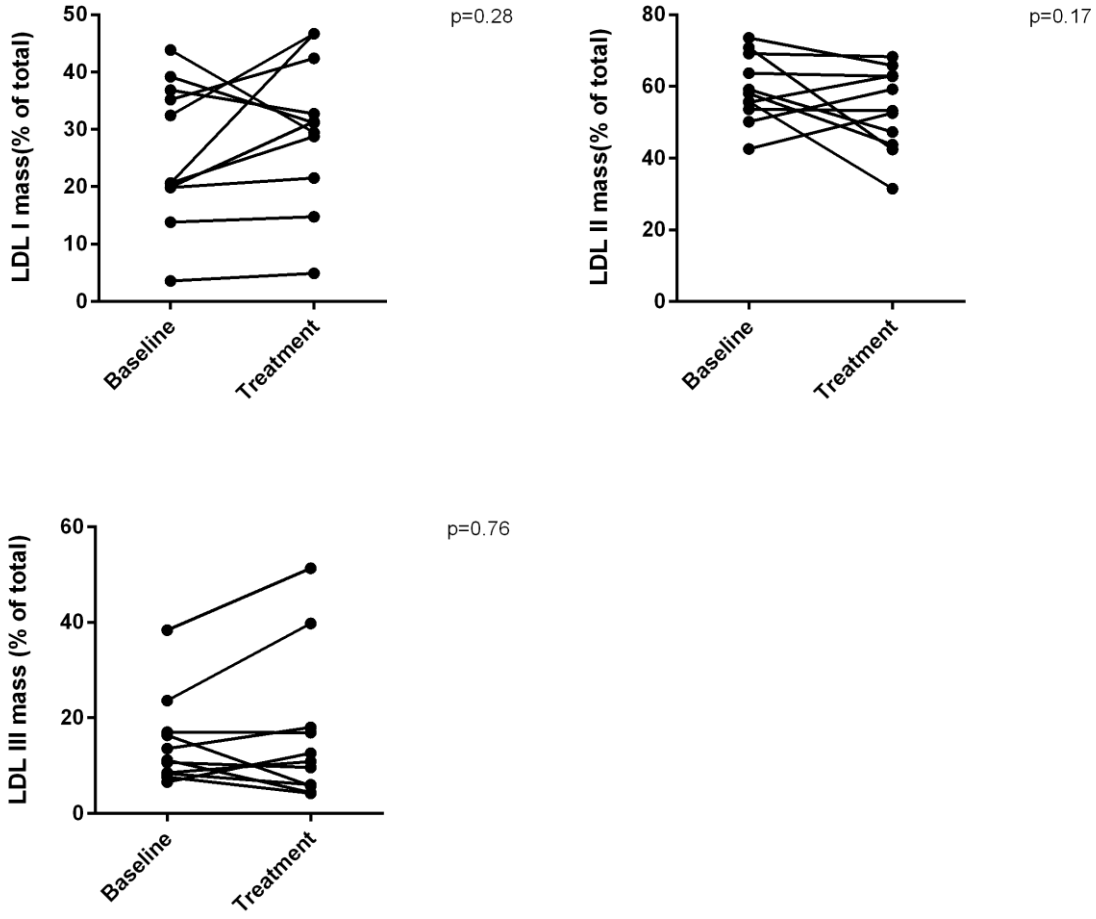


Figure 61 - Percentage of total LDL mass made up by each subfraction. P value generated by Wilcoxon matched-pairs test.

3.4.2 LDL Particle Diameter

Peak LDL particle diameter and mean LDL particle diameter were ascertained. The average value of both measurements did not change, with individual peak and mean particle sizes rising or falling by less than 1% (Figure 62).

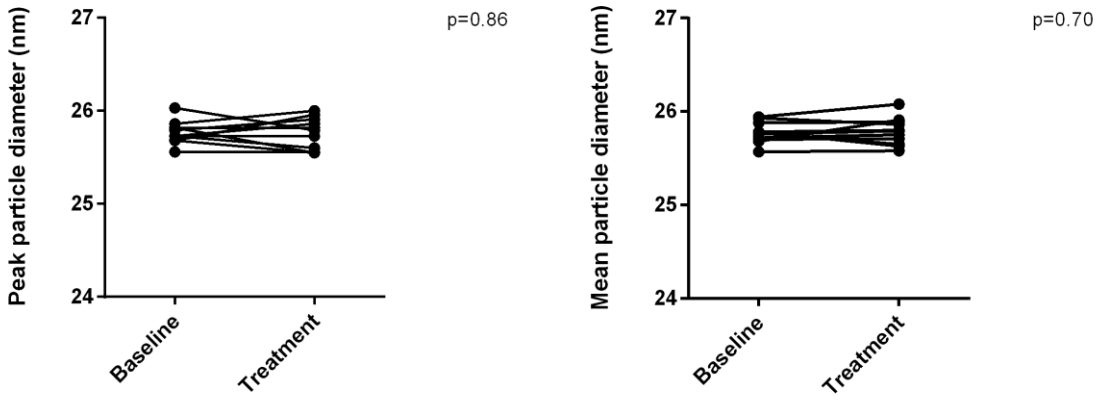


Figure 62 - Peak (left) and mean (right) LDL particle diameter. P value generated by Wilcoxon matched-pairs test.

3.4.3 Lipoprotein(a)

Lipoprotein(a) (Lp(a)) serum levels varied markedly at baseline, but fell in all patients after treatment (Figure 63). At baseline, Lp(a) levels associated positively but very modestly with CRP (r=0.62, p=0.045) and ESR (r=0.53, p=0.095). A n inverse relationship was observed with CDAI (r=0.58, p=0.063) but on examining the scatterplot this did not seem as clear cut as the strong r value would suggest (Figure 64). Similar, non-significant relationships were seen between change in Lp(a) and change in CRP (r=0.51, p=0.11), ESR (r=0.44, p=0.18) and CDAI (r=-0.54, p=0.094), as seen in Figure 65.

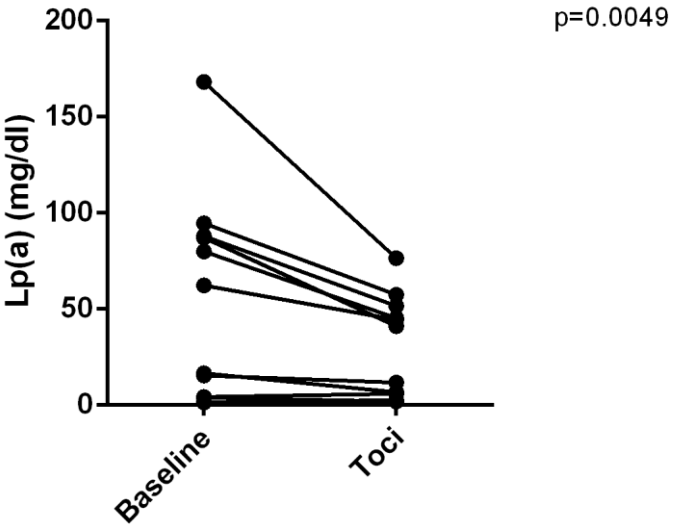


Figure 63 - Serum levels of Lipoprotein(a). P value generated by Wilcoxon matched-pairs test.

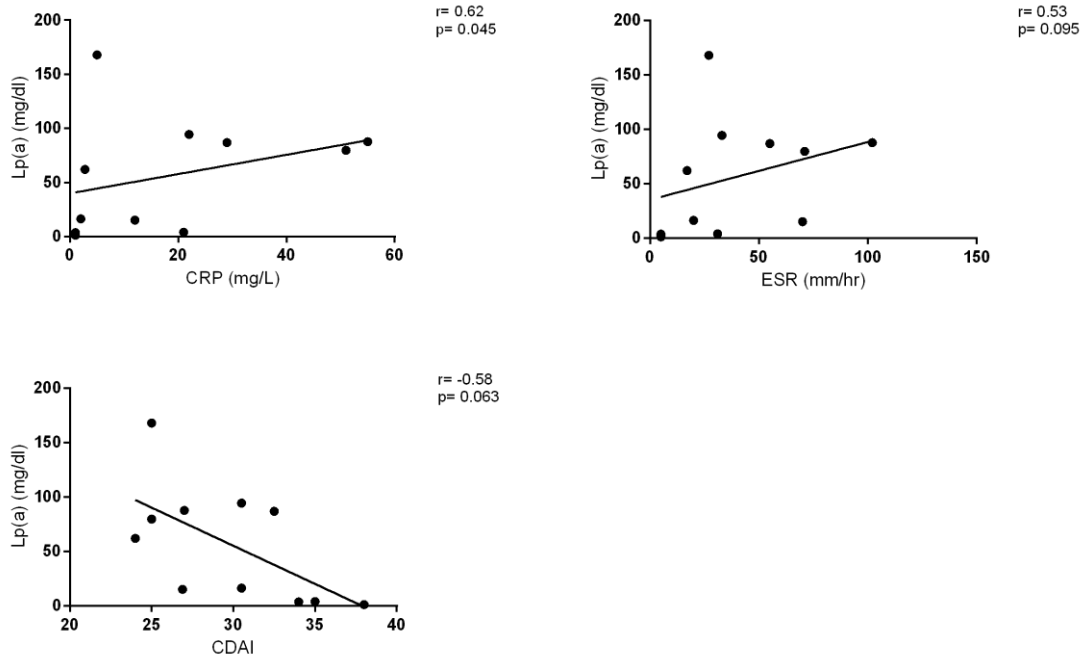


Figure 64 - Spearman's correlation coefficient between baseline Lp(a) and baseline CRP, ESR and CDAI.

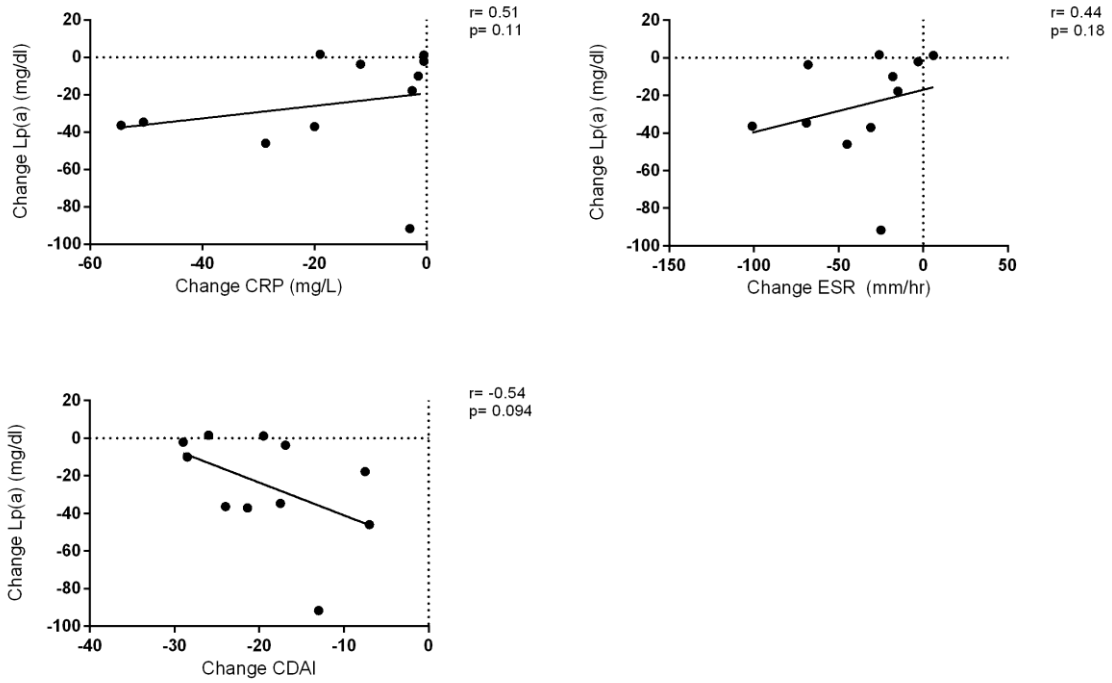


Figure 65 - Spearman's correlation coefficient between change Lp(a) and change CRP, ESR and CDAI.

3.4.4 Apolipoproteins

Serum levels of apolipoproteins at day 1 of each kinetic study, as measured by immunoturbidimetry, are shown in Table 21 below. Significant increases were seen in the HDL-associated ApoAI and ApoAII, in keeping with observed increases in HDL-c.

	Baseline		Treatment		Change (%)		
	Median	IQR	Median	IQR	Median	*p*	
ApoAI	115.4	100.7 - 124.2	148.5	141.7 - 174.4	28.5	*<0.001*	***
ApoAII	25.0	20.3 - 28.1	35.3	32.0 -38.4	41.3	*<0.001*	***
ApoB	76.0	51.6 - 78.2	103.1	92.3 - 118.3	54.1	*0.002*	**
ApoCII	1.4	0.5 - 2.9	3.6	2.3 - 4.7	100.0	*0.042*	*
ApoCIII	5.1	4.0 - 8.3	8.1	5.9 - 11.7	28.5	*0.22*	ns

Table 21. Median serum levels of apolipoproteins at baseline and after treatment. All values mg/dl unless specified.

A correlation matrix of apolipoproteins with clinical response is shown in Table 22 below. Changes in ApoAI and AII, both of which are components of HDL, associated with change in acute phase reactants but not CDAI. ApoB and Apo CII did not associate with clinical measures. On exclusion of data for KAL007, their relationships with CRP and ESR strengthened but remained non-significant.

	ApoAI	ApoAII	ApoB	ApoCII	ApoCIII
Whole cohort					
CRP	-0.61	-0.77	-0.08	-0.16	-0.10
ESR	-0.70	-0.80	-0.20	-0.24	-0.22
CDAI	0.35	0.27	0.40	0.58	0.45
Excluding KAL007					
CRP	-0.50	-0.84	-0.44	-0.45	-0.09
ESR	-0.64	-0.87	-0.60	-0.57	-0.22
CDAI	0.44	0.31	0.38	0.56	0.50

Table 22 - R values describing relationships between change in markers of disease activity and change in serum apolipoprotein levels. R values are calculated by Spearman's correlation coefficient. Green indicates p<0.05. Red indicates p<0.01.

Scatterplots of ApoB (a component of the primary outcome measure) with clinical parameters are shown in Figure 66.

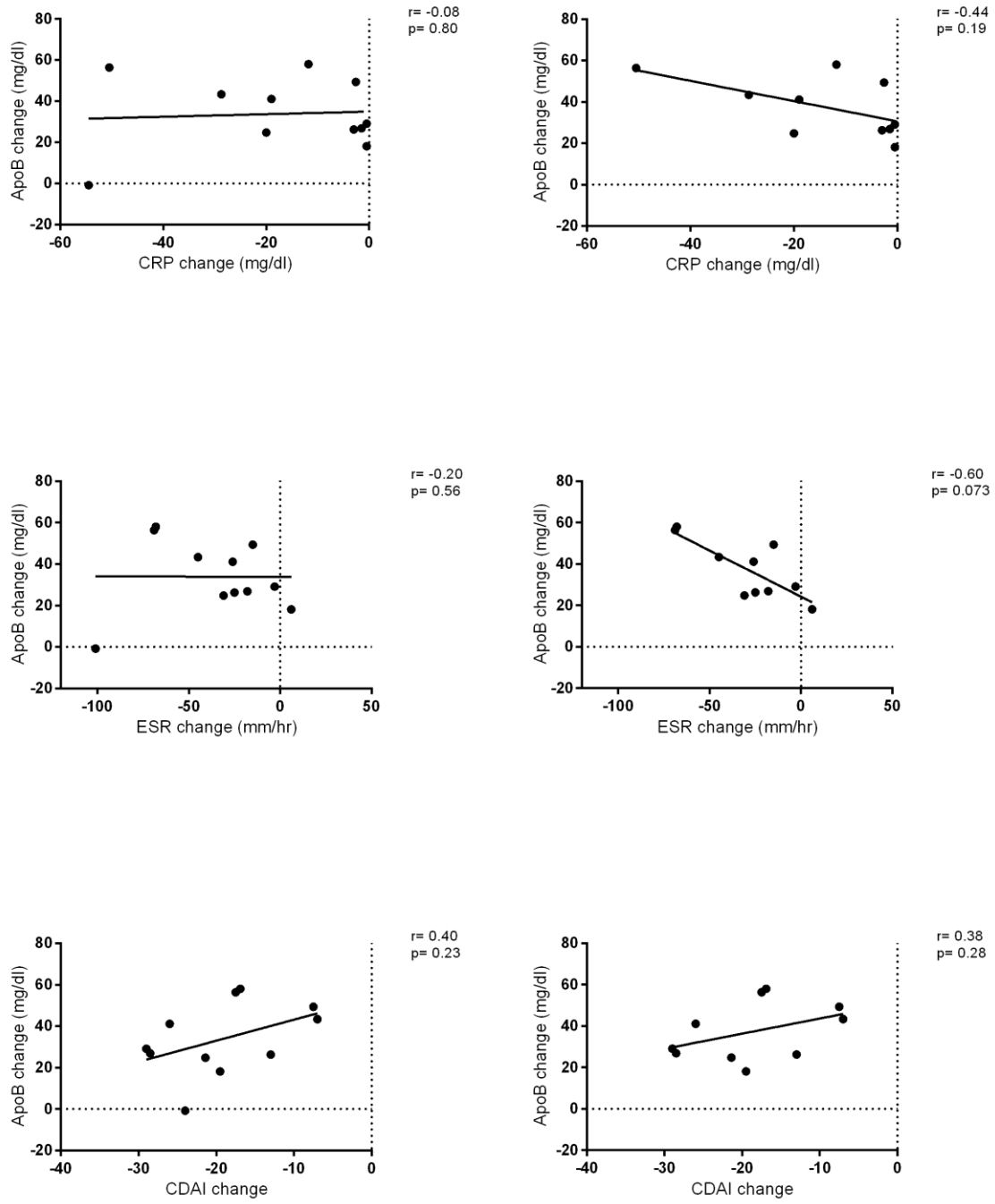


Figure 66 - Spearman's correlation coefficient between change in serum apoB and change CRP, ESR and CDAI, including (left) and excluding (right) participant KAL007.

3.4.5 Enzyme activity

CETP activity is displayed in Figure 67. Activity rose in all participants apart from three; KAL007 reduced by 17.6pmol/µl/hr, equivalent to 32% of baseline value. CETP activity fell by 2pmol/µl/hr and 3.6pmol/µl/hr in the other two

subjects. Median change in CETP activity was an increase of 6.2pmol/µl/hr, or 11% from baseline. Excluding KAL007 gives p=0.019 for change from baseline.

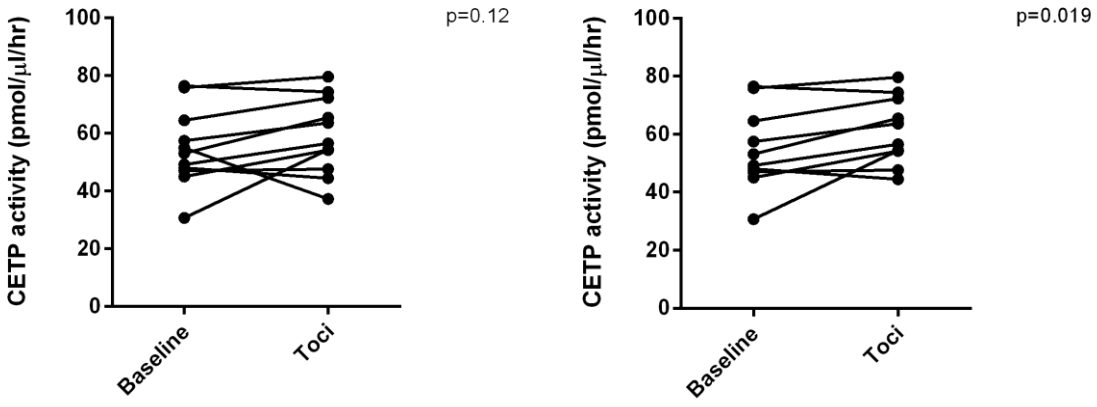


Figure 67 - CETP activity with (left) and without (right) participant KAL007. P value generated by Wilcoxon matched-pairs test.

No significant changes were seen in average lipoprotein lipase or hepatic lipase activity (Figure 68), despite median increases of 19% and 9% respectively. The visible outlier in the graphs below is KAL006, who had increases of 314% in LPL activity and 31% in HL activity. Exclusion of this patient did not change the outcome of the test.

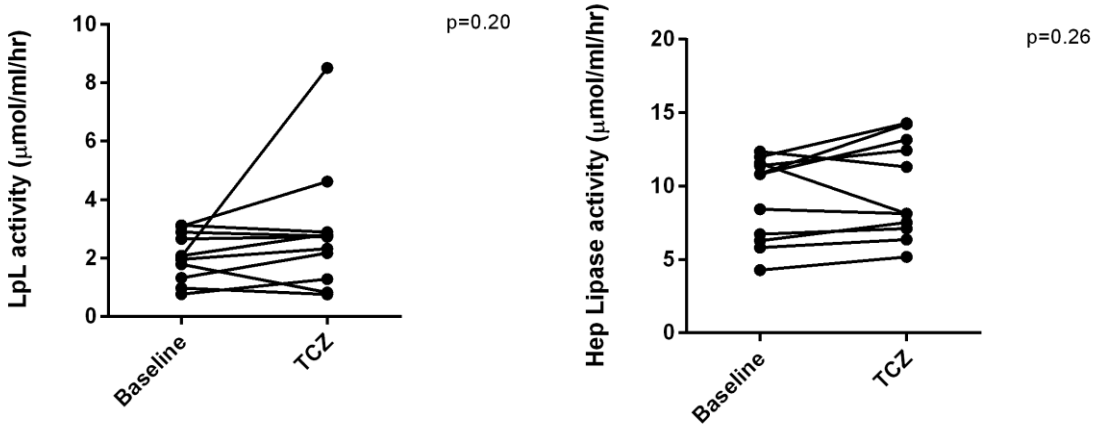


Figure 68 - Lipoprotein lipase (left) and hepatic lipase (right) activity. P value generated by Wilcoxon matched-pairs test.

No significant associations were found between changes in any enzyme and changes in CRP, ESR or CDAI (Table 23). Similarly, segregating patients by their

baseline CRP, as I have done with previous analyses, did not yield any significant differences in change in LPL or HL activity.

	Cholesterol ester transfer protein	Lipoprotein lipase	Hepatic lipase
CRP	-0.14	-0.15	0.07
ESR	-0.25	-0.22	0.15
CDAI	0.28	0.26	0.07

Table 23 - R values describing relationships between change in markers of disease activity and change in enzyme activity. R values are calculated by Spearman's correlation coefficient.

CETP is known to increase LDL cholesterol ester content by transferring cholesterol esters from VLDL-1 and HDL particles in exchange for triglycerides. This knowledge was reassuringly confirmed by the observation that changes in CETP activity associated positively (though not reaching statistical significance) with change in LDL-CE content and inversely with change in HDL-c. These data are demonstrated in Figure 69.

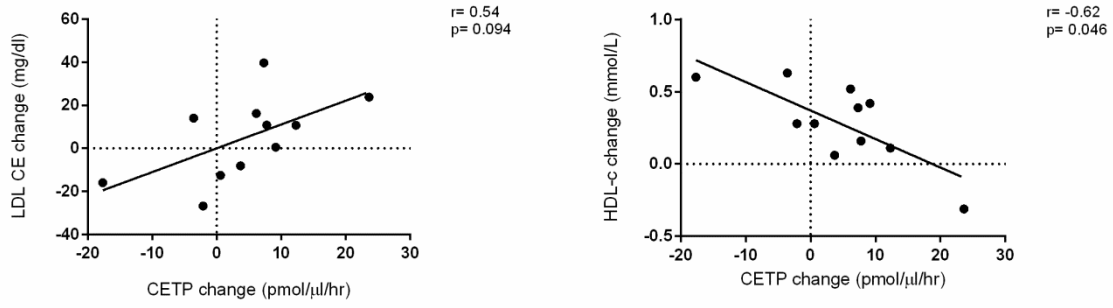


Figure 69 - Spearman's correlation coefficient between change in CETP activity and (left) change in LDL-cholesterol ester, and (right) change in HDL-cholesterol as measured by betaquant.

3.4.6 PCSK9

PCSK9 activity analysis was performed in light of its role in regulating LDLr activity and recent interest in PCSK9 inhibitors in clinical practice, as outlined in the introduction. Serum PCSK9 levels were elevated by mean of 23% and median 7% after treatment, but this did not reach statistical significance (Figure 70).

Alterations in PCSK9 could potentially alter LDL catabolism by regulating the availability of the LDL receptor. However, no correlations were found between PCSK9 levels and LCL FCR or serum LDL-c levels (Figure 71).

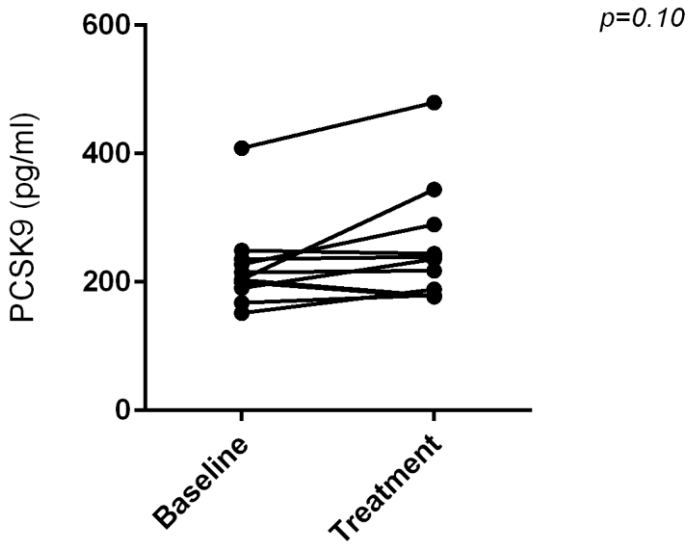


Figure 70 - Serum PCSK9 levels. P value generated by Wilxocon matched-pairs test.

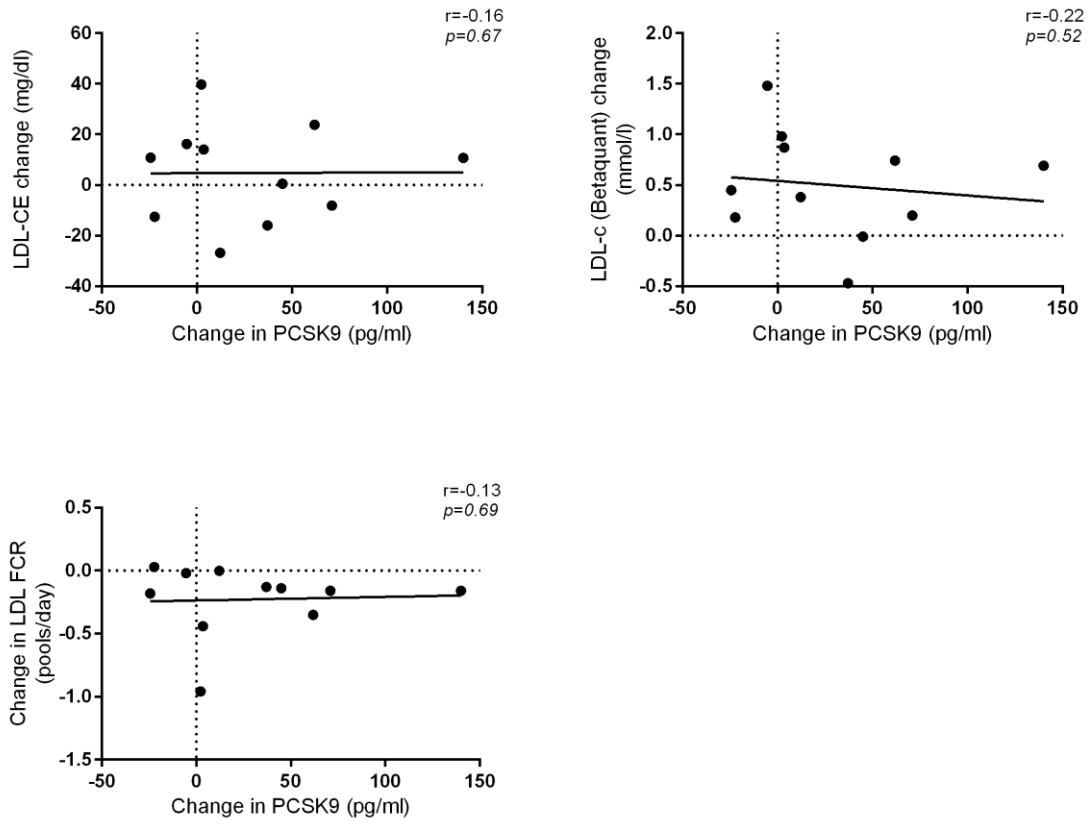


Figure 71 - Spearman's correlation coefficient of serum PCSK9 with LDL-cholesterol and fractional catabolic rate.

3.4.7 HDL particle analysis

High density lipoproteins were analysed for particle size in the same manner as LDL, as described earlier in this chapter. No significant changes were seen in mean or peak particle size (Figure 72). Mean particle size analysis revealed one outlier who demonstrated a large increase of around 25%. Removing this outlier from the analysis (KAL011) lowered the p value to 0.027, but I was unable to identify any clinical or other laboratory reason to exclude them; I believe it is safest to assume that no difference in these parameters can be demonstrated by KALIBRA's data.

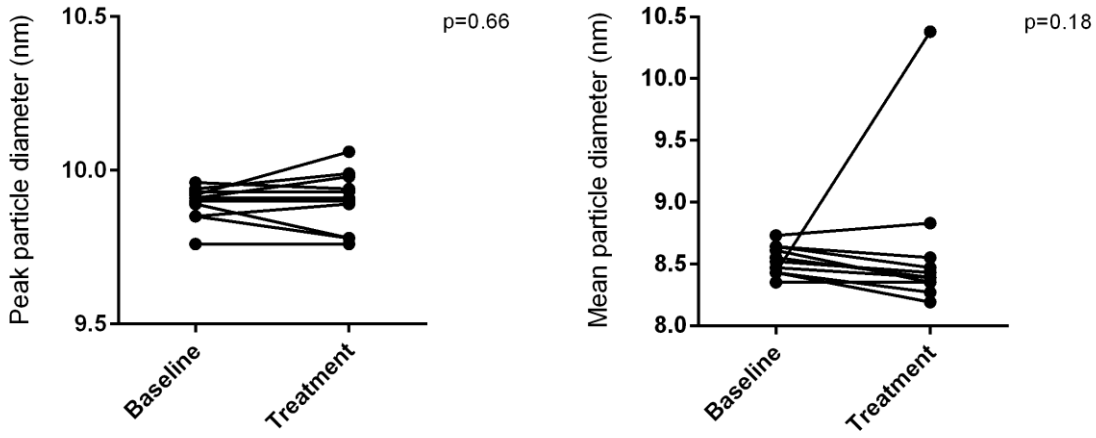


Figure 72 - Peak (left) and mean (right) HDL particle diameter. P value generated by paired t-tests.

Particle analysis was used to determine what proportion of serum HDL was made up of each of 5 subparticles, graded on basis of density. A trend was seen of HD2a being replaced by HDL2b; however, all the changes seen were of low magnitude, and only the fall in HDL2a was statistically significant. These results are summarised in Table 24.

	Baseline		Treatment		Change (% of mass)		
	Mean	95% C.I.	Mean	95% C.I.	Mean	*p*	
HDL2b	31.1	28.1 – 34.0	31.7	28.4 – 35.9	1.1	*0.37*	
HDL2a	27.4	26.1 – 28.7	25.7	24.7 – 26.6	-1.7	*0.005*	**
HDL3a	25.2	24.2 – 26.1	24.7	23.2 – 26.2	-0.5	*0.53*	
HDL3b	10.4	9.5 – 11.3	10.9	10.1 – 11.6	0.5	*0.14*	
HDL3c	6.0	4.7 – 7.2	6.5	5.4 – 7.7	0.6	*0.083*	

Table 24 - Changes in proportion of serum HDL subparticles. P values generated by paired t-tests.

3.5 Discussion

3.5.1 Conclusions from outcome data

The primary outcome measure of the KALIBRA study was the fractional catabolic rate of LDL, which fell by a median of 30% (or 0.16 pools/day) following tocilizumab therapy. Individual changes in FCR varied widely. The largest decrease was a fall of 80% from baseline. The median baseline (i.e. in severe active RA) FCR of 0.53 pools/day is close to that measured in hypertriglycerideaemic patients by our own centre [PACKARD 2000]. Similarly, the median post-treatment FCR of 0.27 pools/day compares well with an expected "normal population" value of 0.3 pools/day, as observed in Packard et al.'s study (272). In contrast, LDL production rates did not increase, but rather fell significantly from baseline. This change, however, was too small to offset the dominant kinetic effect of reduced catabolism. FCR correlated with CRP and ESR at baseline, but not CDAI. A similar pattern of association was displayed when analysing the degree of change in FCR and inflammatory parameters.

Reassuringly, serum cholesterol levels changed in a manner consistent with existing tocilizumab literature, with increases observed in TC, LDL-c and HDL-c but no change in the atherogenic index (the TC/HDL-c ratio), lending external validity to our results. LDL cholesterol ester content (LDL-CE), as measured by pooled samples throughout the course of the week-long kinetic studies, increased from baseline in those subjects who had elevated CRP and ESR at baseline (and thus large falls in these reactants with tocilizumab therapy). Despite apparent correlations between the changes in LDL-CE and acute phase reactants, no association was seen with disease activity as quantified clinically by CDAI. LDL-c as measured by betaquant seemed to behave in a similar manner but often without reaching statistical significance. The LDL FCR was a strong determinant of LDL-c both before and after tocilizumab treatment. In contrast, LDL production rate did not correlate significantly with LDL-c at any stage.

Taken together, these findings allow some conclusions to be drawn. Firstly, they imply that elevations in serum LDL-c following tocilizumab therapy are a

direct result of changes in the LDL fractional catabolic rate, a confirmation of our original hypothesis. The magnitude of these changes suggests that this fall in FCR is more likely a "normalisation", rather than a clear pathological or off-target drug effect; this further leads to the likelihood that the reductions in serum cholesterol observed in severe, active RA are a result of increased catabolism, not reduced production.

Secondly, the changes in LDL FCR seen in RA, and thus changes in serum LDL-c, are a specific result of alterations in hepatic IL-6 signalling, rather than reductions in inflammatory "disease activity" (i.e. synovitis). This conclusion is further supported by the different lipidaemic effects that have been observed in past years between IL-6 blockade and anti-TNF despite the two therapies delivering broadly similar reductions in clinical disease activity, although admittedly no kinetic studies exist for anti-TNF therapy. This leads me to suspect that the target tissue ultimately responsible for LDL-c changes is the liver. Given that the liver is the only organ capable of directly excreting unwanted cholesterol from the body (in the form of bile salts), it would seem to be a reasonable target for any system aiming to drastically alter the catabolic rate of LDL. However, the ubiquity of the gp130 component of the IL-6 receptor on cell membranes means IL-6 is capable of transducing a signal in other tissues such as vascular endothelium, macrophages or indeed any other peripheral cells capable of taking up LDL particles; the kinetic assays employed in KALIBRA cannot differentiate between different sites of LDL breakdown. Inflammation driving increased uptake by such other cells makes evolutionary sense; in times of insult or stress, increased IL-6 would stimulate increased LDL consumption (not excretion) as a way of directing fuel and synthetic substrates to cells that have greatest need of them. Whilst this mechanism might provide a survival advantage in an *acute* scenario (such as infection), it could prove maladaptive in the pathological state of *chronic*, high-grade inflammation that is RA. Potential cellular mechanisms for underlying my observed kinetic changes are the subject of a later chapter in this thesis.

I had the opportunity analyse a wide variety of secondary outcomes from the KALIBRA data. Fewer changes in the composition of the other apoB-containing lipoproteins were observed; coupled with the lack of change in lipase activity,

this implies that tocilizumab's effects are predominantly confined to LDL. No significant changes were observed in LDL particle size or subfraction proportion.

The reduction in serum Lp(a) is clinically relevant as this particle is recognised as pro-atherogenic, and thus our findings imply at least one other potentially atheroprotective function for IL-6 blockade. This replicates the findings of the MEASURE (254) and ADACTA (253) trials. In contrast to LDL-c and kinetic changes, no clear association was observed with inflammatory markers; this might suggest that a distinct mechanism or target tissue lies behind inflammatory manipulation of Lp(a). KALIBRA was not designed or powered to explore changes in Lp(a), however, and further studies are required to determine the precise mechanisms behind this change.

The increases in apoAI, apoAII and apoB reflect increased levels of the lipoproteins which contain them. Coupled with the absence of change in LDL particle size, this indicates that LDL-c increases due to increased numbers of LDL particles in the circulation; again, this fits consistently with the concept of reduced particle catabolism (as opposed to another mechanism such as altered cholesterol flux between particles). The biochemical and clinical significance of the changes seen in other apoproteins (particularly CII and CIII) and HDL are outwith the remit of this thesis but would potentially be a fertile area for future study.

What do these findings mean for these patients' cardiovascular risk? In the absence of clinical cardiovascular endpoints, conclusions must be drawn cautiously. The fact that our patients return from a hypercatabolic state to a roughly normal LDL FCR fits with largely a normalisation of function, rather than novel pathology. This view is supported by the correlation of LDL FCR and LDL-c with acute phase reactants; i.e. lipid changes are in proportion to reversal of (pathological) elevated IL-6 signalling, a key feature of the high-grade inflammatory state of RA. The unchanged atherogenic index, increase in HDL-c and reduction in Lp(a) are also reassuring, suggesting neutral or even cardioprotective changes. An alternative possibility could be that increased LDL catabolism is a protective evolutionary adaptation to inflammation (and its subsequent increase in cardiovascular risk conferred by endothelial dysfunction,

platelet aggregation and other physiological changes) which attempts to reduce cardiovascular disease by safely sequestering LDL-c or excreting it from the body, and thus reducing the amount of serum LDL-c available for uptake into atheroma. This position may be supported by our observation that CETP activity increases post-tociliuzmab; this would drive cholesterol ester from HDL to LDL, which can then bind to LDLr on hepatocytes and then be excreted. Accepting this view would, however, require us to believe that the significant increased CV risk seen in RA occurs *in spite of*, rather than because of, the profound cholesterol changes which define the lipid paradox. Given that TC and LDL-c have been shown to alter to some extent even with drugs such as methotrexate and anti-TNF (which reduce CVD risk), I find this view difficult to accept.

3.5.2 Comparison with Tofacitinib kinetics

Only one other study has been published analysing lipid kinetics in RA. Charles-Schoeman et al. (273) recently performed kinetic studies on a cohort of 33 RA patients and 31 controls. RA patients were assessed at baseline and following 6 weeks of treatment with tofacitinib, a JAK inhibitor recently approved for use in treating RA in the USA which has been shown to increase HDL-c and LDL-c following 4-6 weeks of treatment. LDL-associated apoB and HDL apoAI production and catabolic rates were measured using stable isotope leucine, given here in a 20-hour continuous infusion with blood samples taken at baseline and 16, 18 and 20 hours. Cholesterol production and catabolic rates were measured using a 22-hour continuous infusion of 3,4-$^{13}C_2$ –labelled free cholesterol. A three-compartment mathematical model was used to calculate kinetic parameters. The study showed an increase in mean LDL-c of 15% (p=0.0002) and in mean HDL-c of 14% (p=0.0001), both of which represent normalisation to levels seen in the control group. Similar changes were seen in serum apoAI and apoB. In contrast to the KALIBRA cohort, RA patients at baseline had an LDL-associated apoB FCR which was not significantly different from healthy controls (1.61%/hour v 1.50%/hour, equivalent to 0.38 pools/day v 0.36 pools/day, p=0.27) and did not significantly change with tofacitinib. No significant changes were seen in HDL-associated apoAI production or catabolic rates. However, baseline cholesterol ester FCR was greater in RA than in

controls, and fell towards “normal” levels after treatment. The change in cholesterol ester FCR correlated inversely with the change in HDL-c (r=0.42, p=0.018) and in large HDL particle numbers as assessed by NMR (r=0.54, p=0.002). Given the change in cholesterol ester metabolism without any change in apoAI or apoB catabolism, the authors conclude that RA patients have increased selective cholesterol ester uptake by scavenger receptor B1 in the liver; the subsequent reduction in HDL-c then leads to lower levels of LDL-c as there is less cholesterol ester in HDL for CETP to transfer to LDL particles (although it should be noted that the group found no change in CETP mass or activity).

Why did the Charles-Schoeman study come to such a different conclusion regarding apoB catabolism from KALIBRA? I believe there are some features of this study which restrict direct comparisons to KALIBRA’s findings. Firstly, tofacitinib blocks the JAK/STAT signalling pathway; as mentioned previously, IL-6 signals via activation of STAT3, and so tofacitinib will affect similar intracellular changes to tocilizumab. However, the large number of cytokines which transduce signals using JAKs means that some of tofacitinib’s effects will be due to blockade of other cytokines, not just IL-6. Secondly, the mathematical model used differs from our own, using a more recent model which is simplified and is not able to provide detailed information on non-LDL lipoproteins in the apoB delipidation cascade. Thirdly, the baseline patient demographics are not clearly delineated and so the cohort may not be comparable to my own. Entry criteria for disease severity consist of >4 tender and swollen joints, and ESR > 28mm/hour or CRP >7mg/L; baseline TJC and SJC are given (thought not out of what total count), but no other clinical data are presented at baseline or post-treatment. Composite scores such as DAS28 or CDAI are not mentioned at any point. It is also not stated how many, if any, patients were DMARD- or biologic-naive, limiting our judgement of how treatment resistant this group’s disease might have been. Finally, and most concerningly, the study does report significant increases in apoB and apoAI following tofacitinib (rising to the levels seen in the control group) despite no change in their production or catabolic rates. This does not seem biologically possible. Potential explanations include: (a) underpowering of the study so that

it was unable to detect a genuine change in LDL FCR; or (b) wide margins of error in the laboratory techniques used to calculate reported values.

3.5.3 KALIBRA – strengths and weaknesses

KALIBRA had some notable strengths which should be documented in defence of our findings. Many of my initial results, whilst not novel, are in accordance with what is already known about tocilizumab's behaviour. For example, increases in TC, LDL-c and HDL-c are well documented as detailed in the introduction. The rapid reduction in acute phase reactants, with clinically relevant but more modest reductions in joint counts and clinical composite scores such as CDAI, is in keeping with the phase III trial literature and our own clinical experience. Whilst all patients responded clinically to the drug, residual disease was present in most, with only one patient reaching ACR/Boolean remission – a finding perhaps not surprising given that all patients started from a baseline DAS28 of >5.1, and that several had previously failed at least one previous biologic drug. Moreover, the demographics of the cohort are I believe quite representative of a typical RA population in the UK (78, 82), with the possible exception that only three of our patients were prescribed methotrexate. These observations are reassuring for three reasons. Firstly, they replicate the earlier findings of other groups, and lend credence to the validity of our novel findings. Secondly, had some patients not responded to tocilizumab therapy, our observed average changes in inflammatory and lipid parameters would likely have been smaller and less likely to reach statistical significance. Lastly, the cohort's demographics, disease severity, drug history and magnitude of clinical responses reflect "real life" practice in tocilizumab prescribing, increasing the likelihood that our observed lipid changes accurately reflect those which occur in the typical clinic population. KALIBRA's results also demonstrate a reassuringly strong degree of internal consistency, with changes in lipids, apoproteins and lipoprotein kinetics all contributing to a consistent picture of inflammation-driven hypercatabolism which is reversible by tocilizumab.

The relatively small cohort size of this study also brought some advantages. It allowed each patient to be analysed in depth, with adequate numbers of blood

samples over an appropriate timespan and a wide variety of secondary outcome measures obtained. The volume of data generated has been such that much remains to be analysed, such as the kinetics of VLDL1, VLDL2 and IDL, and changes in HDL-related parameters. Nevertheless, the use of three separate Rheumatology outpatient departments in the Greater Glasgow area allowed me to draw on a reasonably sized pool of patients as I attempted to recruit the required number of subjects. Additionally, the single-centre nature of the kinetic studies allowed all key assessments to be performed by the same individual. For example, all clinical assessments including DAS28 and CDAI scoring were performed by me; one named research nurse in the Glasgow CRF performed phlebotomy; all sample processing was performed by the same small team of biochemists; and data analysis for mathematical modelling was performed by Professors Caslake and Packard. By allowing different staff members to repeatedly practice the same techniques, and by reducing the risk of inter-operator variability, I suspect this pattern of working promoted consistency and efficiency in all stages of data collection and preparation, and improved the overall reliability of our outputs. What's more, I can personally testify that the familiarity and confidence engendered in our own skills and those of the rest of the team by this method of working encouraged good working relationships and a strong sense of teamwork within the unit. As an increasingly busy research fellow, this was hugely important to my enjoyment of and enthusiasm for the project!

Some criticisms could be aimed at the way the study was conducted. The most apparent one is the small size of the cohort, with only 11 patients (short of our stated target of 15) completing the study. In the end, our observed changes in FCR were sufficiently strong and consistent that even this small number of subjects allowed us to reach statistical significance for our primary outcome measure, and some of our secondary measures. Nevertheless, I firmly believe that many of my analyses reflect genuine changes in biology but do not meet statistical significance because of having so few numbers, and issue which I will reflect further on later. From the outset, it was recognised that recruitment would be the largest barrier to successfully completing the study, as is the case in all clinical trials and studies. For the participants, the study was intense, with a 13-hour stay in hospital; only one low-calorie meal in a 21-hour period;

repeated venopuncture; and recurrent early starts (difficult in those with an inflammatory arthritis, whose pain and stiffness is typically worst on waking). Despite drawing on three centres for recruitment, our patient pool was limited by several factors. In Glasgow, most physicians typically use tocilizumab as a second- or third-line biologic behind anti-TNF and possibly Rituximab. To counter this, I actively identified patients who were booked to see a biologics specialist nurse for screening before commencing a biologic, and gave information on the study to any who would be eligible regardless of which biologic they were being considered for; if they were due to start anti-TNF but were a good candidate for KALIBRA, I discussed tocilizumab therapy with them and sought permission from their consultant Rheumatologist to enrol them into the study. Another limiting factor was our desire to exclude patients with diabetes mellitus or who were taking statins, both scenarios which are extremely common in the west of Scotland. At one point, around half the biologics patients that I had identified as potentially suitable for inclusion in KALIBRA were excluded solely because of either diabetes or statin use.

A second potential criticism of the study is the lack of blinding. Typically any study comparing two sets of results in human subjects benefits from blinding of either the subjects (to minimise the placebo effect of a treatment) or the assessors (to minimise bias in analysis and interpretation of results). This was not felt to be practical for several reasons. The “crossover” nature of the study, where values in the same participants are compared before and after treatment, was not amenable to blinding as by definition all patients began from a position of being “pre-treatment”. Blinding of laboratory staff was also not feasible as the intensive nature of sample processing meant only one sample at a time could be handled, and the study number and stage of treatment of each sample could easily be determined by the time of month or year the sample was received at. A “crossover” style study did help in that each patient acted as their own comparator, allowing paired statistical tests and removing the need for a separate healthy control group. It could be argued that a control group would have been desirable (this was the method used by Charles-Schoeman et al.) but our laboratory sample processing capacity limited the number of participants we could enrol. A group may have helped us to determine whether changes in lipids or kinetic rates were pathological changes (going away from

healthy population averages) or normalisation (returning towards healthy population averages). However, the values we obtained for FCR were similar to those seen in the normal population and hypertriglycerideaemic patients in previous studies, as documented above, and so addition of a control group would have added little to our knowledge.

3.5.4 Dilemmas

3.5.4.1 Subject KAL007 as a statistical outlier

Some of the analyses, especially correlation calculations, were affected by the presence of KAL007 as a clear statistical "outlier". In contrast to the other subjects, this patient had a large change in their acute phase response but relatively small changes in LDL-c and FCR. Even using the non-parametric Spearman's r (which uses ranking to adjust for extreme values), correlations in several domains did not reach statistical significance despite scatterplots seeming (to my eyes) to show a strong relationship. Some of these r values then became statistically significant on exclusion of KAL007. This problem is particularly acute in a small study such as this, where one patient makes up 9% of the cohort; inclusion or exclusion of only one patient can thus have particularly profound effects on average values. Exclusion of patients in this manner is fraught with ethical difficulties. It is a potential route to confirmation bias, as I could be accused of deliberately and selectively choosing to omit a reading which did not agree with my pre-determined conclusion; following the data on its own terms could arguably only be done by including the whole cohort. Additionally, despite my own extensive discussions with and assessment of KAL007, and interrogations of their past medical notes, there was nothing in their clinical history or examination which would set them apart from the rest of the cohort. The only possible finding of relevance I could determine was that KAL007 fell pregnant shortly after the conclusion of her second kinetic study. However, she claimed to have been using barrier (not hormonal) contraception during the trial, and confirmed that she had menstruated after completing the kinetic study, making pregnancy during the sampling period unlikely. The most likely explanation appears to be the fact that KAL007 was the only participant completing the study who was a premenopausal female. It

is well recognised that LDL metabolism is drastically different pre- and post-menopause, with oestrogen capable of influencing LDLr expression amongst other effects. KAL008 was also premenopausal, and it would have been most interesting to compare and contrast her results with KAL007's had she not been forced to withdraw from the study.

I discussed KAL007's inclusion at length with Profs. Mcinnes, Sattar, Caslake and Packard throughout the analysis period. We recognised that such quandaries are the result of "real world" data, which rarely fits neatly with the preconceived ideas of the researcher. Indeed, a trial with 11 subjects which reached statistical significance in all outcomes would be suspected of biased data manipulation at best and outright fraud at worst. My own view is that many of the correlations are biologically relevant and genuine despite not having a $p < 0.05$. If the statistical outlier KAL007 had not been enrolled in the study, or if data from a larger cohort were available, I suspect that, for example, the association between change in betaquant LDL-c and FCR would be more clear-cut. One could also argue that, as a woman in her 30s, KAL007 is a "clinical" outlier in a disease population which has a peak incidence in the 6^{th} decade of life; as such, excluding her from the analysis would not be unreasonable if she is not representative of the majority of RA patients one would encounter in a clinic. Overall, I believe that in these circumstances it is not sufficient to dismiss a result or association as "non-significant" by simply quoting a p-value in a table, something major clinical journals also recognise. Accordingly, if there is doubt over the relevance of an association, I have displayed the values graphically, with and without KAL007. This suppresses allegations of bias, and allows the reader to draw their own conclusions.

3.5.4.2 LDL-CE versus Betaquant

Two different measures of LDL-cholesterol are used in this analysis. LDL-c by Betaquant, a standard technique for measuring serum LDL-c, was taken on day 1 of each kinetic study, in the same venepuncture as CRP and ESR and on the same morning of clinical assessment. An alternative measure is the cholesterol ester content of LDL particles as calculated by using pooled samples throughout the 5-day kinetic study (during which serum lipids were not in steady-state). LDL-CE is a reliable, directly-measured quantification of LDL-c, which carries minimal

statistical link to FCR. The use of LDL-CE carries two main advantages over Betaquant. Firstly, it can be viewed as a more reliable "average" value for a naturally fluctuating LDL-c, and may be preferable to the Betaquant sample which only gives a value at one timepoint. Secondly, the Betaquant technique also includes IDL-cholesterol and Lp(a). These latter two substances are unchanged and reduced, respectively, by tocilizumab therapy, in contrast to the general trend of increase for LDL-CE. This probably explains why the Betaquant LDL-c does not correlate as cleanly with kinetic and inflammatory parameters, and reduces its utility as a mechanistic tool.

The major drawback of LDL-CE for quantifying LDL-c is that it is not a technique which is easy or widely available, and never will be given the repeated blood sampling and intensive laboratory resources required. Therefore, whilst it may reflect cholesterol *biology* more accurately than betaquant sampling, it does not reflect *clinical* practice or the LDL-c values the physician or scientist is likely to encounter in RA patients, and will not equate cholesterol values generated in most other studies or cohorts. It is thus hazardous to directly compare these values to the findings of other groups who measure LDL-c by other means, such as Betaquant or the Freidwald equation. For clarity, I have generally provided results for both measures of LDL-c side by side.

3.5.4.3 Measuring disease activity

Most studies, even those involving tocilizumab, use DAS28 as the first-choice composite measure of RA disease activity. In contrast, I chose the clinical disease activity index (CDAI) as a way of quantifying synovitis. CDAI consists only of tender and swollen joint counts, and patient and physician global assessment scores, and is a validated (274) measure of disease activity. I did this because most other disease activity scores include acute phase reactants in their calculations, and can report misleadingly low levels of disease activity following IL-6 blockade due to supra-normalisation of the acute phase response even when persistent synovitis is apparent on clinical examination. DAS28 is particularly susceptible to this phenomenon, as it includes the natural log of CRP or ESR; thus, small falls at low acute phase reactant levels will have a disproportionately large effect on the change in DAS28. By way of illustration, a fall in CRP from 60mg/l to 25mlg/l (ln 4.1 to 3.2) has less impact on DAS28

than a fall from 10mg/l to 3mg/l (ln2.3 to 1.1). CDAI does not include any biochemical component and I felt it was more useful as a surrogate for synovitic burden in the context of tocilizumab therapy.

4 Interleukin-6 and Foam Cell Formation In Vitro

4.1 Rationale

Having demonstrated the effects of IL-6 blockade on LDL kinetics, I aimed to investigate the cellular mechanisms which may underlie these observations. As outlined in the introduction to this thesis, IL-6's biological functions are legion; it may demonstrate a variety of pro-inflammatory, and sometimes anti-inflammatory, properties in vivo and in vitro. More pertinently, IL-6 has also been directly implicated in lipid metabolism beyond the observations of decreased cholesterol in RA patients. Serum IL-6 levels correlated inversely with TC and LDL-c in a cohort of postoperative patients (226) whilst exogenous IL-6 reduces serum TC and alters VLDL subclasses in humans (227, 228). TNF-α and bacterial lipopolysaccharide (LPS) can achieve similar results (229).

Prior studies have examined the effects of IL-6 in vitro using a variety of cellular systems in an attempt to explain observed in vivo effects. IL-6 has been shown to upregulate the LDL receptor (LDLr) in human HepG2 cells, a hepatocyte cell line (275), and decrease apoB excretion from these same cells (276). IL-6 induced expression of the scavenger receptors SR-A, CD36 and CD68 (but not LOX-1) in human aortic endothelial cells (277). The same group also looked at macrophages generated from the THP-1 monocyte cell line and found IL-6 was capable of inducing expression of SR-A, but not SR-B, LOX or CD36, and facilitated cholesterol uptake and macrophage transformation into foam cells (278). Frisdal's group, in contrast, found IL-6 retarded foam cell formation by upregulating ABCA1 expression in THP-1 macrophages and human monocyte-derived macrophages (HMDM) (279), though this seems to be contradicted by more recent findings of impaired cholesterol efflux in RA (280). IL-6 has also been shown to increase production of intracellular cholesterol-regulating molecules, including HMGCoA reductase and SREBP in THP-1 macrophages (281). These findings are in contrast to studies in rodents, which have significantly different lipid physiology to primates. Compared to humans, rodents normally demonstrate high HDL-c, low LDL-c and very little atherosclerosis (229). Mouse models of atherogenesis are used quite frequently in cardiovascular research, but require genetic manipulation by LDLr or APO-E in conjunction with a high fat diet. Rodents also seem to respond differently to IL-6. Administration of exogenous IL-6 increased serum TC and TG (282), and whilst IL-6-driven foam

cell production has been observed in mouse cells, this is in the context of upregulating VLDLR, SR-A and CD36, not LDLr (283, 284).

Macrophages are key cells in both the pathogenesis of rheumatoid arthritis and atherogenesis, and are the effector cells of the reticuloendothelial system which previous animal data have implicated in LDL hypercatabolism (as noted in Chapter 3). I hypothesised that tocilizumab's reduction in LDL catabolism is a result of reduced IL-6 signalling in macrophages, and their subsequent reduced uptake of LDL via scavenger receptors. To investigate this possibility, I performed a series of experiments on macrophages in vitro. Firstly, I aimed to confirm that IL-6 was capable of transducing a signal in the macrophage and changing its biology as a positive control. Secondly, I measured the effects of IL-6 on macrophage lipid uptake and their transformation into foam cells. Thirdly, I investigated the effects of IL-6 on the expression of a variety of membrane-bound and intracellular proteins involved in cholesterol uptake, export and metabolism. Finally, I attempted to replicate my observations in patients with RA, using blood samples taken from the KALIBRA patient cohort before and after tocilizumab therapy.

4.2 Methods

4.2.1 THP-1 Macrophage Generation

THP-1 cells are a monocyte cell line originally derived from a patient with monocytic leukaemia (285). They have for many years been used as models of macrophages following differentiation with Vitamin D3 or phorbol 12-myristate 13-acetate (PMA). In an attempt to mirror the phenotype of primary human macrophages as closely as possible, two different THP-1 maturation protocols were used. The "original" technique is well-established and has been extensively studied. The "alternative" technique, as described by Daigneualt et al (286), may lead to generation of cells which more closely resemble primary human monocyte-derived macrophages [HMDM] in appearance and behaviour.

4.2.2 "Original" THP-1 macrophage generation

Proliferating THP-1 monocytes were maintained in 250ml vented flasks at 37°C incubation. Cells were split and re-seeded twice a week at $2x10^6$ cells in 25ml complete medium (RPMI 1640 (Invitrogen) supplemented with 10% foetal calf serum, 1% Penicillin/Streptomycin and 1% L-glutamine). On the day of differentiation, cells were counted, resuspended at $2.5x10^5$ cells/ml in complete RPMI supplemented with 50ng/ml PMA, and plated in a 24-well tissue culture plate at 1ml/well. Cells were incubated at 37°C for 72 hours before stimulation.

4.2.3 "Alternative" THP-1 macrophage generation

THP-1 monocytes which were maintained in culture as above were counted and suspended at $2.5x10^5$ cells/ml in complete RPMI supplemented with 5ng/ml PMA. These were then seeded at 1ml/well in a 24-well tissue culture plate and incubated at 37°C for 24 hours. At this point medium was aspirated and replaced with fresh complete RPMI, and the cells were incubated for a further 5 days before stimulation.

4.2.4 CD14 isolation

Healthy human donor blood samples were obtained in the form of "buffy coats" (the fraction of a whole blood donation following gradient centrifugation that contains leukocytes and platelets) graciously provided by the Scottish National Blood Transfusion Service via the Western Infirmary, Glasgow. From each donor, under sterile conditions buffy coat (typically 10-20ml) was diluted 1:1 in wash medium (500ml sterile PBS with 5ml Penicillin/Streptomycin). This was then carefully layered with a dropper onto 4ml Histopaque in a 15ml centrifuge tube, to a total volume of 14ml, and spun at 2100rpm for 20 minutes. From each tube, a dropper was used to remove the visible interphase of peripheral blood mononuclear cells lying immediately above the histopaque layer into a 50ml centrifuge tube, and this was washed in 50ml wash medium at 1800rpm for 10 minutes. Cells were resuspended in 50ml wash medium, filtered through a 70µm cell strainer into a fresh tube, and counted. They were then spun at 1500rpm at 4^{o}C for 10 minutes and resuspended at 10^7 cells / 80µl in MACS buffer (500ml sterile PBS with 10ml foetal calf serum and 5ml Penicillin/Streptomycin) in a 15ml centifuge tube on ice. CD14+ monocytes were then isolated from PBMCs using positive cell selection on an AutoMACS Pro (Miltenyi Biotech) according to the manufacturer's instructions.

4.2.5 Primary HMDM generation

CD14+ monocytes, isolated from donor blood as above, were suspended in 10ml RPMI and counted. They were then spun at 1,500RPM for 10 minutes and then resuspended at $2.5x10^5$ cells/ml in complete RPMI supplemented with 50ng/ml M-CSF (Peprotech). Cells were then seeded in 24 well plates at a 1ml/well. The medium was replaced with fresh complete RPMI + 50ng/ml M-CSF after three days, and cells were used after a total of 6 days differentiation.

4.2.6 TNF production by macrophages

Differentiated THP-1 macrophages or HMDMs in 24-well plates were "primed" by providing them with fresh complete RPMI supplemented with recombinant human IL-6 (Peprotech) at 1, 10 or 20ng/ml, recombinant soluble human IL6-Receptor (Peprotech) at 100ng/ml, or combinations thereof. After 24 hours

culture at 37°C, medium was aspirated and replaced with fresh complete RPMI supplemented with lipopolysaccharide (LPS, Sigma-Aldrich) at varying doses for a further 24 hours. Supernatants were aspirated into 1.5ml microcentrifuge tubes, spun at 1,500 RPM for 5 minutes to remove any cells in suspension, and decanted into clean tubes. Samples were stored at -20°C. Prior to use, the samples were thawed at room temperature and vortexed. Supernatants of cells exposed to LPS were diluted 1:10 with RPMI due to the high concentrations of TNF-α induced by LPS.

4.2.7 TNF-α ELISA

Supernatant TNF-α levels were measured by Human TNF- α CytoSet ELISA (Invitrogen). A 96-well ELISA plate was coated overnight with 100µl/well of capture antibody diluted in phosphate buffered saline (PBS, Sigma Aldrich) to a concentration of 2µg/ml. The next morning the plate was washed with PBS/0.1% Tween (Sigma-Aldrich) then blocked for one hour at room temperature with 200µL PBS/0.5% BSA (Sigma-Aldrich) per well. After washing, TNF-α standards were prepared in complete RPMI and added at 100µl/well using serial dilutions to generate a seven-point standard curve, with the top standard at a concentration of 2,000pg/ml. Supernatant samples were added at 100µl/well, and 50µl of detection antibody diluted to 0.32µg/ml in PBS/0.5% BSA was added to all wells. The Plate was left on a rocker at room temperature for 2 hours, and washed three times with PBS/0.1% Tween. A working Streptavidin-HRP solution was prepared by diluting stock solution 1/625 in PBS/0.5% BSA, and 100µl was added to each well for 30 minutes on a rocker at room temperature. The plate was washed five times, and 100µl TMB substrate solution (R&D Systems) was added to each well for 5-10 minutes in the dark. Reactions were stopped by adding 100µl Stop Solution (2N H_2SO_4). TNF-α concentrations were determined according to absorbance at 450nm measured on a Dynex MRX TCii plate reader (Dynex Technologies, Chantilly VA, USA) with reference to the standard curve.

4.2.8 Foam cell formation and visualisation

THP-1 macrophages or HMDM were incubated for 24 hours at 37° with fresh complete RPMI alone, 10ng/ml recombinant human TNFα (Peprotech), 10ng/ml

IL6 or IL6 + 10µg/ml tocilizumab. Where relevant, tocilizumab was added to wells 30 minutes before application of IL6. Cells were then gently washed in PBS and incubated with varying concentrations of oxLDL (BTI Inc, Ward Mill USA) for a variety of durations, depending on the requirements of the experiment. Specific parameters are detailed where relevant in the results section. Adherent cells were fixed in their wells and stained with oil red O (Sigma Aldrich) to demonstrate lipid uptake. Two different staining protocols were used to allow cross validation of observations.

The first technique was based on recommendations from the manufacturer of oxLDL. A stock solution of 0.4% oil red O in was made by adding 200µg oil red O powder to 50ml of 100% isopropanol and filtering through a 0.2µm syringe filter. A working solution was made by diluting 60:40 with distilled water, and filtering; this was suitable for use for 4 hours, and was discarded afterwards. Cells in the wells of the culture plate were washed twice with PBS, fixed with 250µl 3% paraformaldehyde for 5 minutes at room temperature, and washed twice again with PBS. Cells were covered briefly with oil red O working solution then rinsed quickly twice with distilled water. Cells were then visualised under light microscopy.

The second technique was derived from an optimised staining protocol derived by Xu et al (287). Stock and working solutions of oil red O were prepared as above. Cells in the wells of the culture plate were fixed in 10% phosphate buffered formalin for 10 minutes, rinsed for 1 minute in PBS and rinsed for 15 seconds in 60% isopropanol to facilitate staining. Cells were then stained with oil red O working solution for 1 minute in darkness at 37^{o}C. The cells were destained by adding 60% isopropanol for 15 seconds, then washing three times with PBS for 3 minutes each. Cells were then visualised under light microscopy.

4.2.9 DiI-oxLDL uptake assay

HMDMs were primed for 24 hours at 37^{o} with fresh complete RPMI alone, 10ng/ml recombinant human TNFα (Peprotech), 10ng/ml IL6 or IL6 + 10µg/ml tocilizumab. Where relevant, tocilizumab was added to wells 30 minutes before application of IL6. Cells were then incubated for 4 hours with 100µg/ml DiI-labelled oxLDL (Intracel, Frederick, USA) then washed 5 times with PBS. 300µl

100% isopropanol was added to each well and plates were placed on a rocker for 15 minutes at room temperature. Isopropanol was then aspirated and analysed on a BMG Fluostar Optima with excitation/emission values of 540/590nm. Each sample was read at 3 different gain levels, and the values which were highest without going over the machine's upper limit of detection were used. All conditions were generated in duplicate for each donor with the mean of the duplicate values used for analysis.

4.2.10 STAT3 phosphorylation

Macrophages were detached from culture wells by adding 1ml of non-enzymatic cell dissociation solution (Sigma), incubating at 37°C for 20 minutes, and gently agitatating with a pipette. Cells were resuspended in PBS at $5x10^5$ cells/ml. $2.5x10^5$ cells in 0.5ml were added to FACS tubes containing: no stimulant; 10ng/ml IL6; 100ng/ml sIL6R; IL6 + sIL6R; or IL6 + 10µg/ml tocilizumab (kindly provided by Center for Rheumatic Diseases, Glasgow Royal Infirmary, UK). The cells were incubated a water bath at 37°C for 15 minutes. At this point cells were washed in ice-cold PBS, fixed, permeablised and stained with 2.5µl pSTAT3 intracellular antibody (BD Biosciences) in the dark for 30 minutes. Cells were washed with Cellwash or PBS between each step before resuspending in 250µl PBS with 0.5% bovine serum albumin (BSA, Sigma-Aldrich). Fluorescence was immediately measured using an AutoMacs Pro (Miltenyi, Cologne, Germany) and analysed on FlowJo V10 software.

4.2.11 Flow cytometry antibody conjugation

In preparation for flow cytometry staining, anti-human MARCO antibodies (Thermo Scientific, Rockford, IL, USA) were conjugated using the Lightning-Link PE Tandem Conjugation Kit (Innovia Biosciences, Cambridge, UK) according to the manufacturer's instructions. Briefly, 3µl of LL-modifier was added to 30µl of antibody in a 200µl microcentrifuge tube and mixed gently. 11µl of this mix was pipetted directly onto a liophylised mixture provided in a glass vial containing the PE ligand, mixed gently by pipetting, and left overnight at room temperature in the dark. 1µl of LL-quencher was added to each vial and left at 4°C for thirty minutes before use.

4.2.12 Flow cytometry analysis of cell surface molecules

THP-1 macrophages and HMDMs were cultured as previously described. Following 24 hours stimulation with 10ng/ml IL-6 or complete RPMI alone, supernatant was removed and cells were incubated at 37^{o}C with 1ml non-enzymatic Cell Dissociation Solution (Sigma) in each well for 45 minutes. Non-adherence was confirmed visually by light microscopy; adherent cells were loosened by gentle agitation using the pipette, and the suspension was transferred to a centrifuge tube. Cells were counted, spun at 1,500RPM for 10 minutes and resuspended in FACS buffer (PBS + 0.5% bovine serum albumin) at 5x10^{6} cells/ml. For each experimental condition, 5x10^{5} cells (ie. 100µl) were decanted into a FACS tube. Cells were then incubated with 5µl Trustain Fc block (BioLegend, San Diego, USA) for 10 minutes at room temperature. Fluorescent antibody or isotype was added at 2.5µl per tube, and cells were incubated on ice for 15-20 minutes in the dark. After washing twice with FACS buffer at 350g for 5 minutes, cells were resuspended in 500µl buffer. Fluorescence was immediately measured using an AutoMacs Pro (Miltenyi, Cologne, Germany) and analysed on FlowJo V10 software.

4.2.13 RNA isolation from in vitro macrophages

Primary human CD14+ monocytes were isolated and differentiated into macrophages as previously described. For transcript analysis, cells were exposed for 24 hours to 10ng/ml IL-6 or RPMI alone, with two wells of 2.5x10^{5} cells each per condition.

After 24 hours stimulation the cell supernatant was aspirated and cells lysed with 350µl QIAzol Lysis Reagent in the well. This was aspirated, incubated at room temperature for 5 minutes to allow complete cell lysis, and stored at -20^{o}C in a 1.5ml eppendorf tube; as two wells were combined for each condition from each donor, this resulted in each condition having RNA from a total of 5x10^{5} cells in 700µl QIAzol ready for processing in a miRNeasy kit (Qiagen). After thawing at room temperature, 140µl chloroform was added to each tube and centrifuged at 12,000g for 15 minutes. 200µl of the upper aqueous phase was carefully transferred to a new tube and 525µl of 100% ethanol added. The sample was spun in an RNeasy Mini column at 8,000g for 15 seconds; 200µl buffer RWT was

added to the column and spun again at the same settings. A DNase digest was then performed by making a master mix of 10µl DNase stock I solution and 70µl Buffer RDD for each sample, placing 80µl of this mix directly onto the spin column membrane, and leaving the column on the benchtop at room temperature for 15 minutes. The digest was halted by adding 300µl buffer RWT and the column spun at 8,000g for 15s. 500µl buffer RPE was added and spun at 8,000g for 15s; this was repeated with a further 500µl buffer RPE for 2 minutes. The RNA now held within the spin column was eluted by adding 30µl directly to the membrane and centrifuging at 8,000g for 1 minute. This 30µl eluate was pipetted back onto the membrane and spun again using the same collection tube.

4.2.14 cDNA synthesis

Purity and concentration of RNA were measured by NanoDrop spectrophotometry (Thermo Scientific), and reverse transcription performed using AffinityScript Multi-Temperature cDNA synthesis kit (Aligent, California, USA) and the Veriti 96-well thermal cycler (Applied Biosystems) RNA from each sample was transferred into a 200µl PCR tube. The required quantity of RNA was calculated so that 12 µl of the sample with the lowest concentration of RNA was used; for other samples, a lower volume of sample was thus be required to obtain the same quantity of RNA, and the volume was made up to 12 µl with RNase-free water (Life Technologies). 3µl of random primers was added to each tube and the reaction was incubated for 65^{o}C for 5 minutes to denature the RNA, then left at room temperature for 10 minutes to allow primers to anneal. A master mix was prepared on ice of:

- 2µl AffinityScript RT Buffer
- 0.8µl dNTP mix
- 0.5µl RNase Block Ribonuclease Inhibitor

for each sample; 3.3µl of mix was added to each tube followed by1µl of AffinityScript Multiple Temperature RT. The tubes were briefly centrifuged to eliminate air bubbles and then incubated at 25^{o}C for 10 minutes to extend the

primers, then 42-55^{o}C for one hour. The reaction was terminated by incubating at 70^{o}C for 15 minutes. Samples were then placed on ice for immediate use, or stored at -20^{o}C.

4.2.15 RT-qPCR by SYBR

Before extended gene analysis, samples were first assessed for suitability by real time PCR using SYBR Green reagents. A 96-well PCR plate was loaded in triplicate with 2µl cDNA from each sample. To each well was added:

- 10µl SYBR Select Master Mix (Life Technologies)
- 1µl forward and reverse primers for GAPDH or SOCS3 (Integrated DNA Technologies, Coralville, USA)
- 7µl RNase-free water (Life Technologies)

For convenience and pipetting accuracy, these were prepared in a master mix beforehand. The plate was spun at 350g for at least 10 seconds to ensure each reaction mix was at the bottom of the well. Amplification was then quantified on a 7900HT Fast Real-Time PCR System (Life Technologies) as detailed below.

4.2.16 RT-qPCR by TLDA

Gene expression was quantified using TaqMan Low Density Array cards (Applied Biosystems). The array is a 384 well microfluidic card, fed by one of eight loading ports. Each well is pre-loaded with primers for a specified gene, and cards can be custom-designed according to the needs of the researcher. In my case, cards were set up to accommodate four samples, allowing 32 genes to be analysed in triplicate for each sample. Appendix H lists the genes included in this study, and the corresponding protein products and their roles.

The card was removed from storage at 4oC and allowed to settle at room temperature for 15 minutes in the dark. After defrosting cDNA on ice and gently vortexing, a master mix was made in a 1.5ml microcentrifuge tube of:

- 70µl cDNA

- 30µl RNase-free water

- 100µl TaqMan Universal Master Mix II (Applied Biosystems)

This mix was gently vortexed, spun briefly to eliminate air bubbles, and carefully added to the card. The mix was equally divided between two loading reservoirs (i.e. 100µl into each port) to allow analysis of four samples per card as above. The card was twice centrifuged at 1,200RPM for 1 minute at room temperature. The card was then sealed to prevent samples leaking between wells. Amplification was then quantified on a 7900HT Fast Real-Time PCR System (Life Technologies) by thermal cycling as follows: 50°C for 2 minutes; 95°C for 10 minutes; then forty cycles of 97°C for 30 seconds; and 59.7°C for one minute.

4.2.17 RT-qPCR analysis

The comparative threshold (C_T) method was used to quantify gene expression with RQ Manager (Applied Biosystems). The CT value indicates the PCR cycle, from 0 to 40, at which the amplified gene is deemed as being detected by reaching a defined threshold of fluorescence. Mean C_T values for all 32 genes in every sample from the three replicate values were calculated, including GAPDH as a control gene. The ΔC_T value (i.e. the expression of target gene relative to that of GAPDH) was then calculated as:

$$\Delta C_T = C_T \text{ (target gene)} - C_T \text{ (GAPDH)}.$$

The change in gene expression between stimulated and unstimulated cells for each donor was calculated as:

$$\Delta\Delta C_T = \Delta C_T \text{ (stimulated sample)} - \Delta C_T \text{ (unstimulated sample)}$$

Gene expression could then be expressed as fold change relative to unstimulated cells using the formula:

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

For down-regulated genes, the negative inverse of the result was used to demonstrate a negative value.

4.2.18 RNA isolation from KALIBRA blood samples

Whole blood samples provided for the KALIBRA study were processed in the Centre for Vascular Biochemistry at the University of Glasgow, removing serum for lipoprotein analysis. This left small volumes (around 2ml each) of blood from each patient rich in erythrocytes and leukocytes, which were stored at -20°C. Some of these samples, pre- and post-tocilizumab, were used to obtain RNA for gene expression analysis.

Blood samples were thawed at room temperature, and transferred to PAXgene tubes (Qiagen). Tubes were left at room temperature for 2 hours, then spun at 3,000g for 10 minutes. Supernatant was decanted, 4ml RNAse-free water was added, and the tubes were vortexed and spun at 3,000g for 10 minutes. Supernatant was removed, 350µl BR1 buffer was added, and tubes were vortexed. Samples were pipette into a 1.5ml microcentrifuge tube, to which 300µl BR2 buffer and 40µl proteinase K were added. The tubes were vortexed for 5 seconds and incubated at 55°C for 10 minutes in a shaker-incubator at 500RPM. Lysate was transferred to a PAXgene shredder spin column in a 2ml microcentrifuge tube, and spun for 3 minutes at 8,000g. The supernatant was transferred to a fresh 1.5ml microcentrifuge tube and 350µl 100% ethanol was added. The tubes were vortexed and spun briefly. 700µl of this sample was pipette into a PAXgene RNA spin column in a 2ml processing tube, and spun for 1 minute at 8,000g. This process was repeated in the same spin column with any remaining sample. 350µl BR3 buffer was added to the spin column, which was spun for 1 minute at 8,000g. A DNAse digest was then performed by adding a pre-prepared mix of 10µl DNase I stock solution and 70µl RDD buffer directly to the spin column membrane, and leaving at room temperature for 15 minutes. 350µl BR3 buffer was then added and the tubes spun for 1 minute at 8,000g. This process was repeated twice with 500µl BR4 buffer, spinning for 3 minutes at the final stage. The spin column was spun again for 1 minute at 8,000g in a clean 2ml processing tube, then transferred to a 1.5ml microcentrifuge tube. 40µl BR5 buffer was pipette directly onto the spin column membrane, and this was spun for 1 minute at 8,000g; this step was performed twice. The resulting

eluate was incubated in a shaker-incubator for 5 minutes at 65°C, then immediately placed on ice. The resulting RNA was analysed for quantity and purity by NanoDrop spectrophotometry as described above.

4.3 Results

4.3.1 TNFα assay

In THP-1 macrophages generated by the "original" method, a dose-ranging assay showed a strong TNFα response following 24 hours of culture with 50ng/ml LPS, and a discernible elevation from baseline with 5ng/ml LPS (Figure 73). In these cells, IL-6 alone did not provoke any secretion of TNFα at any dose. Priming of cells with IL-6 with or without exogenous sIL-6R failed to augment LPS-driven TNFα production (Figure 74). This conflicts with previously published data which showed IL-6 potentiating the ability of LPS to provoke TNFα production by THP-1 macrophages (288). I repeated the experiment in macrophages generated by the "alternative" method, using lower levels of PMA and LPS in case this had overwhelmed the cells' TNFα production capability. Again, no increase in TNF production was seen with IL6 or sIL6R supplementation, though a slight reduction of TNF production was seen when stimulated with 1ng/ml LPS (Figure 74).

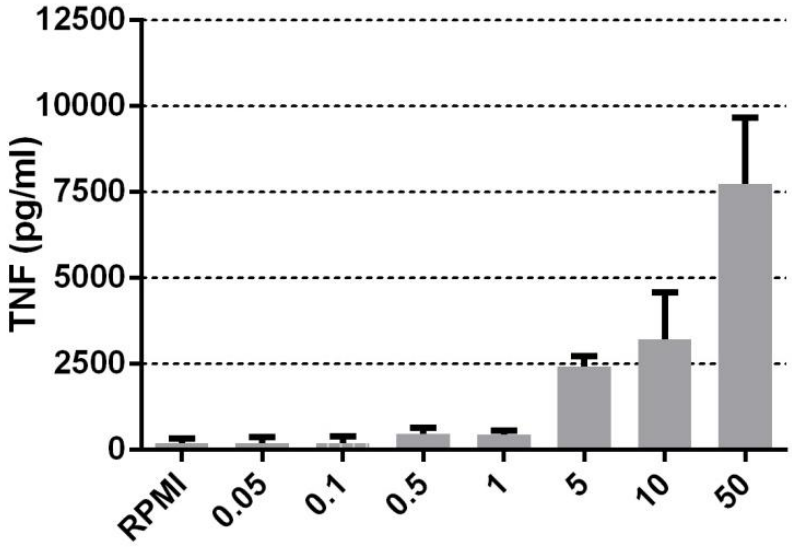


Figure 73 – TNF-α production in THP-1 macrophages following culture for 24 hours with ascending concentrations of LPS (ng/ml). Mean of 2 repetitions with different cell generations ± SD.

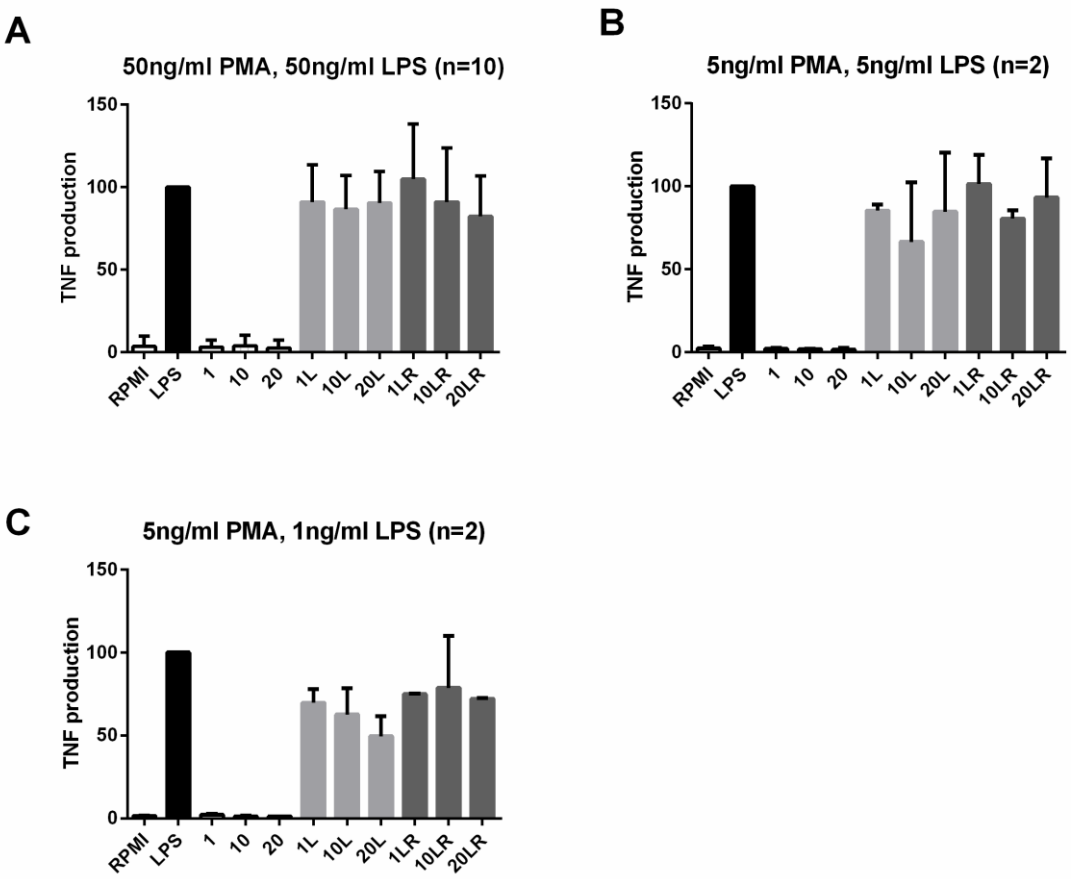


Figure 74 - TNF production in THP-1 macrophages on culture with LPS with or without IL-6, as percentage of response seen with cells exposed to LPS alone. (A) "Original" method of differentiation. (B) "Alternative" method of differentiation. Values presented as mean ± SD. N = number of repetitions using different cell generations. Black bar: LPS alone. White bars: IL-6 alone at 1, 10 or 20ng/ml. Light grey bars: IL-6 + LPS. Dark grey bars: IL-6 + LPS + sIL6R (100ng/ml).

In HMDM, a dose-ranging assay gave a similar dose-response curve as in THP-1 macrophages, albeit with greater magnitude of production at each dose of LPS (Figure 75). Priming with IL6 at all doses attenuated TNFα production following culture with 5ng/ml LPS but did not abrogate it completely (Figure 76), in keeping with previously published data (289).

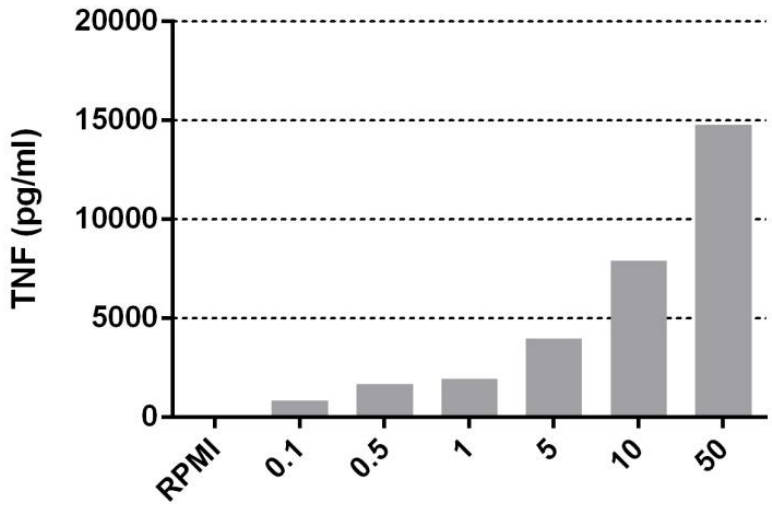


Figure 75 - TNFα production in HMDM on culture for 2 hours ascending concentrations of LPS (ng/ml). Donor n = 1

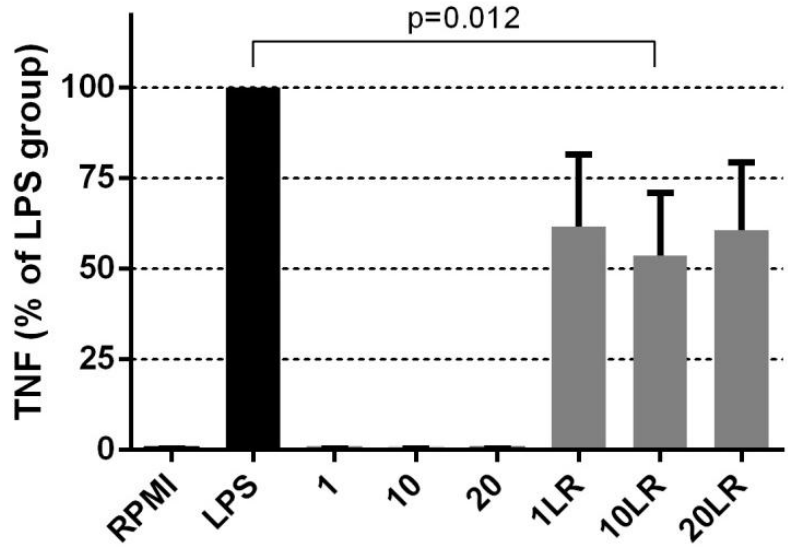


Figure 76 – TNF-α production in HMDM on culture for 24 hours with LPS with or without IL-6, as percentage of response seen with cells exposed to LPS alone. Mean±SD; analysed by t-test, donor n=6. Black bar = LPS alone. White bars (not visible due to TNF-α production being below limit of detection): IL-6 alone at 1, 10 or 20ng/ml. Grey bars: IL-6 + LPS + sIL-6R (100ng/ml).

4.3.2 Foam cell formation quantified by Oil Red O staining

I first attempted to replicate the results described in Hashizume et al. (278), where incubation of THP-1 macrophages with IL-6 increased foam cell formation. Initially however I carried out my own dose-ranging experiment to find out how much oxLDL was required to generate positive staining; 100µg/ml was used in

the above paper, which I was keen to reduce if possible given that oxLDL is (a) toxic to macrophages, and (b) expensive. THP-1 macrophages generated using the “original" protocol were therefore activated with 50ng/ml LPS for 24 hours, then incubated for 48 hours with oxLDL at 1, 10 or 50µg/ml. The intensity of staining increased incrementally with increasing concentration of oxLDL (Figure 77), leading me to conclude that 50µg/ml was an appropriate concentration of oxLDL for foam cell generation.

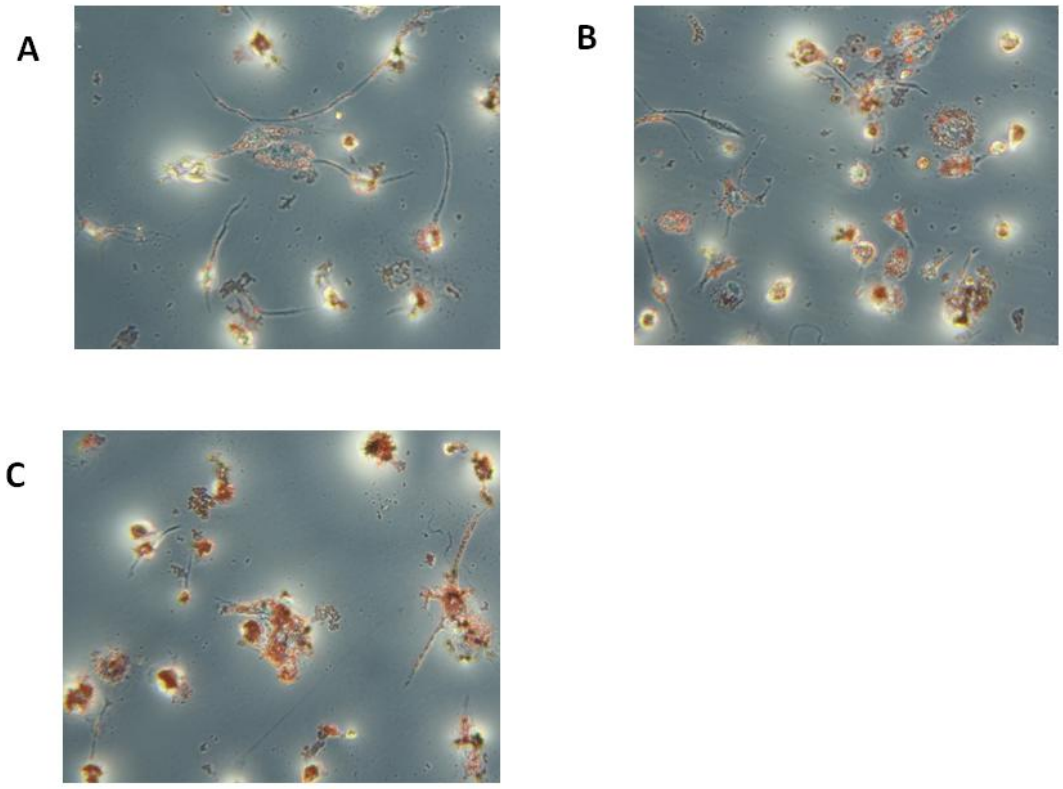


Figure 77 - Oil red O staining of THP-1 macrophage foam cells cultured for 48 hours with oxLDL at (A) 1mcg/ml, (B) 10mcg/ml and (C) 50mcg/ml. 10x magnification Images representative of two experiments.

However, I noted that even cells with minimal oxLDL loading showed significant lipid staining, in contrast to previous literature. To counter this I attempted to culture cells for the final 24 hours in serum-free medium, in case oil-red O staining simply reflected uptake of lipid from the foetal calf serum present in complete RPMI. Unfortunately this led to significant amounts of cell death and loss of adherence to the well, and thus uninterpretable results. In future, use of specifically designed serum free media would obviate this problem.

These experiments were repeated using the “alternative” method of THP-1 macrophage generation as discussed above (286). Results from these experiments were conflicting. On one attempt, there was a subjective increase in frequency and intensity of oil red O staining following IL-6 priming, and this was ameliorated by pre-culture with tocilizumab (Figure 78). However, attempts to replicate this were unsuccessful, with no observable difference between stimulated and unstimulated cells (Figure 78). Once again, even cells cultured only in RPMI without exogenous cytokine or oxLDL supplementation stained heavily (Figure 79).

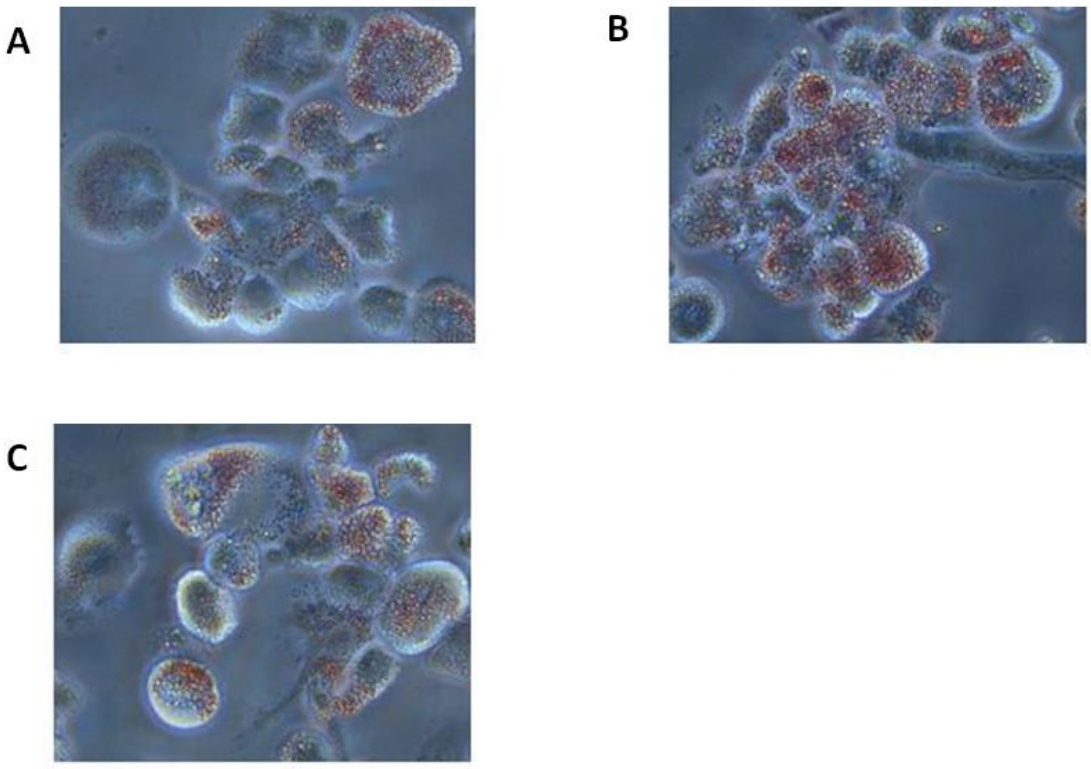


Figure 78 - Oil red O staining of THP-1 macrophage foam cells cultured for 48 hours with 50 mcg/ml oxLDL and (A) RPMI alone (B) 10ng/ml IL-6 (C) IL-6 + 100ng/ml tocilizumab. 20x magnification. Images representative of one experiment.

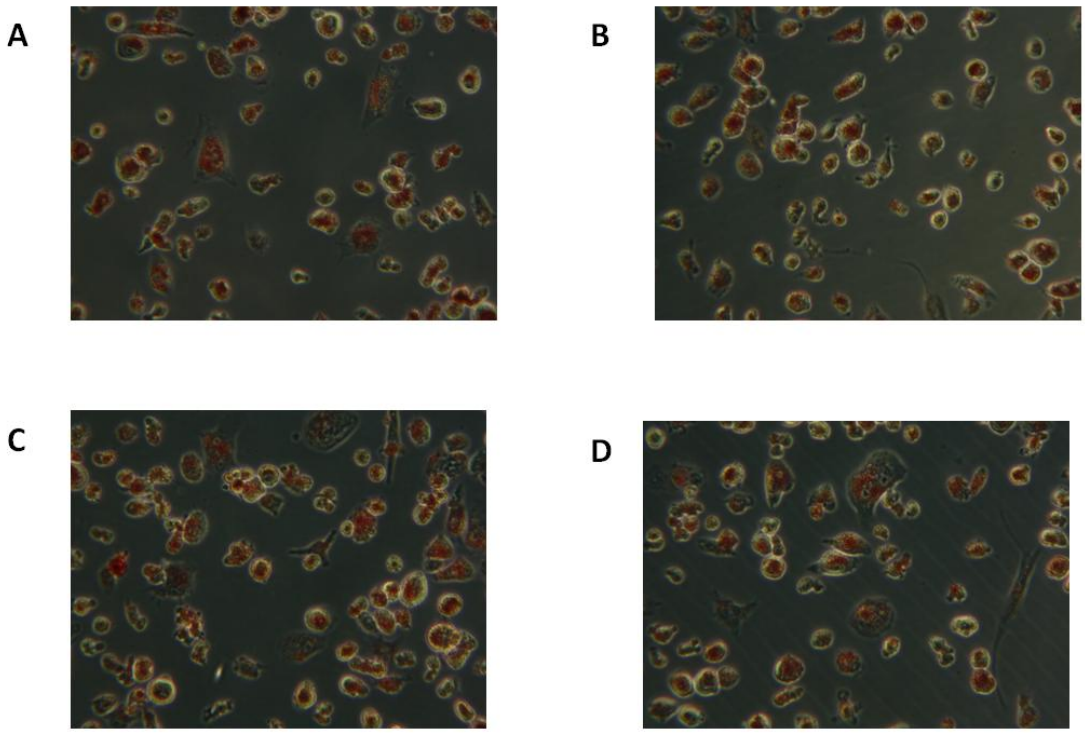


Figure 79 - Oil red O staining of THP-1 macrophage foam cells cultured for 48 hours with (A) RPMI alone (B) 50mcg/ml oxLDL, (C) oxLDL + 10ng/ml IL-6 (D) oxLDL + IL-6 + 100ng/ml tocilizumab. Images obtained from the same experiment.

4.3.3 Foam cell formation quantified by DiI-oxLDL

DiI-labelled oxLDL was sequentially diluted in RPMI and directly analysed via fluorimetry, creating a standard curve (Figure 80) demonstrating its ability to generate increasing levels of fluorescence at increasing concentrations.

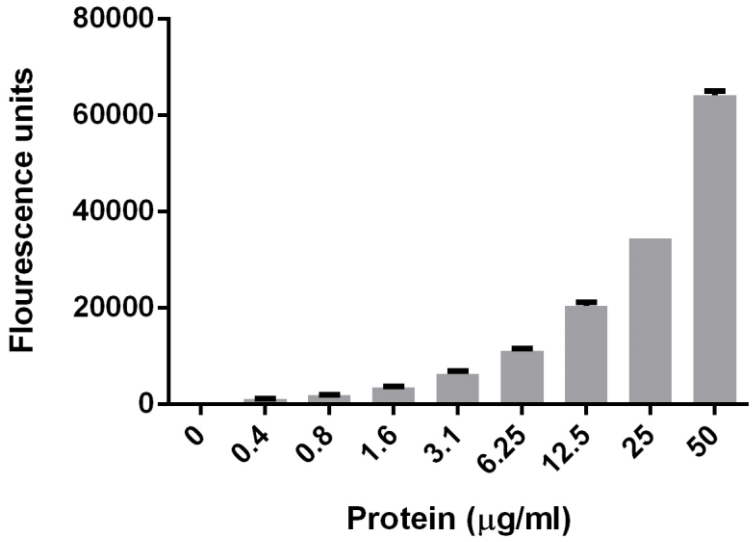


Figure 80 - Fluorescence of DiI-oxLDL at increasing concentrations in a 96-well plate. Bars represent mean±SD of three wells for each condition in one experiment.

Given the difficulties I encountered in replicating the findings of previous groups with oxLDL culture, dose-ranging studies were then carried out to ascertain the optimum concentration and duration of culture of THP-1 macrophages with DiI-oxLDL. Figure 81 shows the results of this experiment, with detectable fluorescence at culture concentrations of 10μg/ml oxLDL and above. Fluorescence was detectable from the earliest timepoint of 30 minutes of culture; this increased with longer periods of culture before plateauing (or at least having a slower rate of increase) beyond two hours. Figure 82 shows these results in bar chart form for concentrations of 10μg/ml or 50μg/ml oxLDL. After one hour of culture in these conditions, whilst a discernable increase of

fluorescence from baseline was detected, there was still capacity for further increase of oxLDL uptake following cell stimulation. Given the significant expense of purchasing DiI-labelled oxLDL, I decided to conduct all further experiments by culturing cells in 10µg/ml DiI-oxLDL for one hour before analysing.

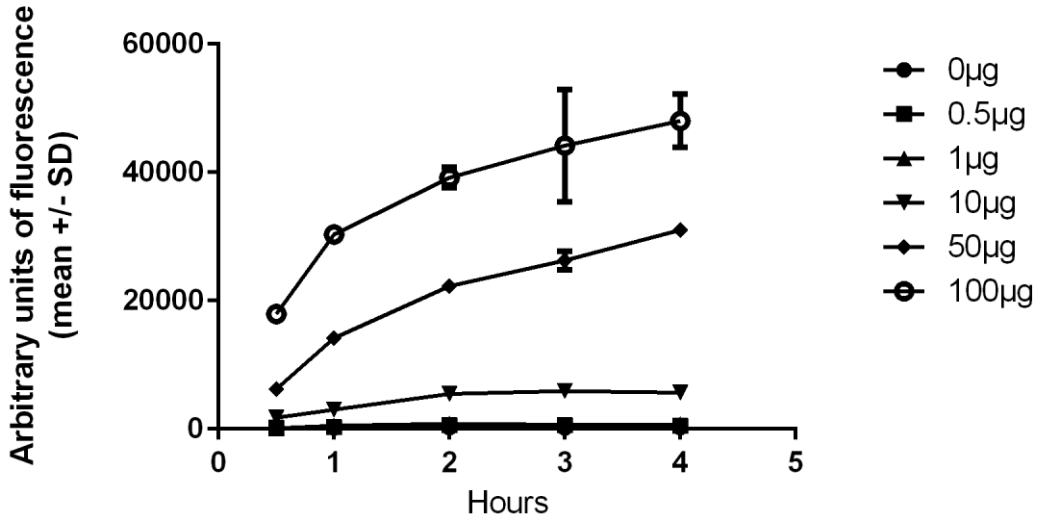


Figure 81 - Fluoresence of THP-1 macrophages following culture with DiI-labelled oxLDL at varying times and concentrations. Points represent mean±SD of three wells for each condition in one experiment.

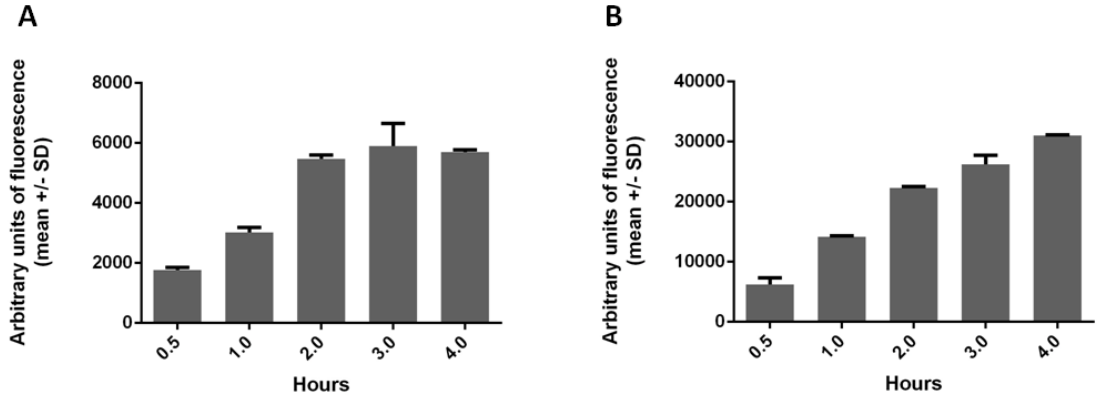


Figure 82 - Fluorescence of THP-1 macrophages following culture for varying times with (A) 10mgc/ml or (B) 50mcg/ml DiI-labelled oxLDL. Points represent mean±SD of three wells for each condition in one experiment.

Using these culture conditions, no significant differences were seen in oxLDL uptake following cell stimulation with IL-6 in either THP-1 macrophages or HMDMs from 5 healthy donors (Figure 83). In an attempt to demonstrate the

reliability of my results I also included cells primed instead with 100ng GM-CSF. This was prompted by previous (unpublished) data obtained in our lab which suggested GM-CSF was capable of retarding oxLDL uptake into macrophages. However, in my experiments this effect was not observed. I also repeated foam cell quantification using these culture condition and staining with oil red O as per Xu's technique, as detailed in the methods section. Again, no clear difference in staining was seen following IL-6 or tocilizumab exposure in THP-1 macrophages (Figure 84) or HMDM (Figure 85).

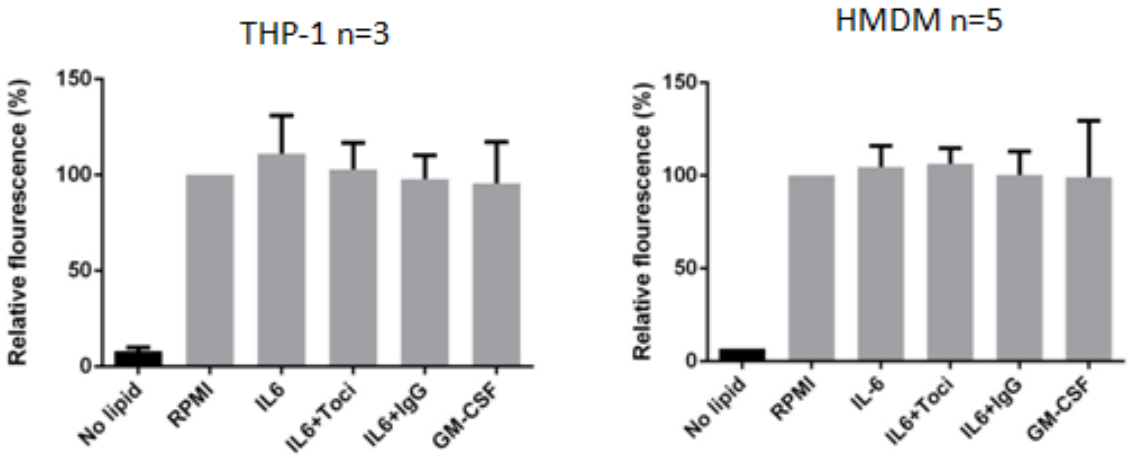


Figure 83 - Fluorescence of macrophages following culture with DiI-oxLDL, expressed as % of fluorescence of cells cultured with DiI-oxLDL and RPMI alone. Bars represent mean ± 95% C.I. of three wells for each condition in three or five experiments.

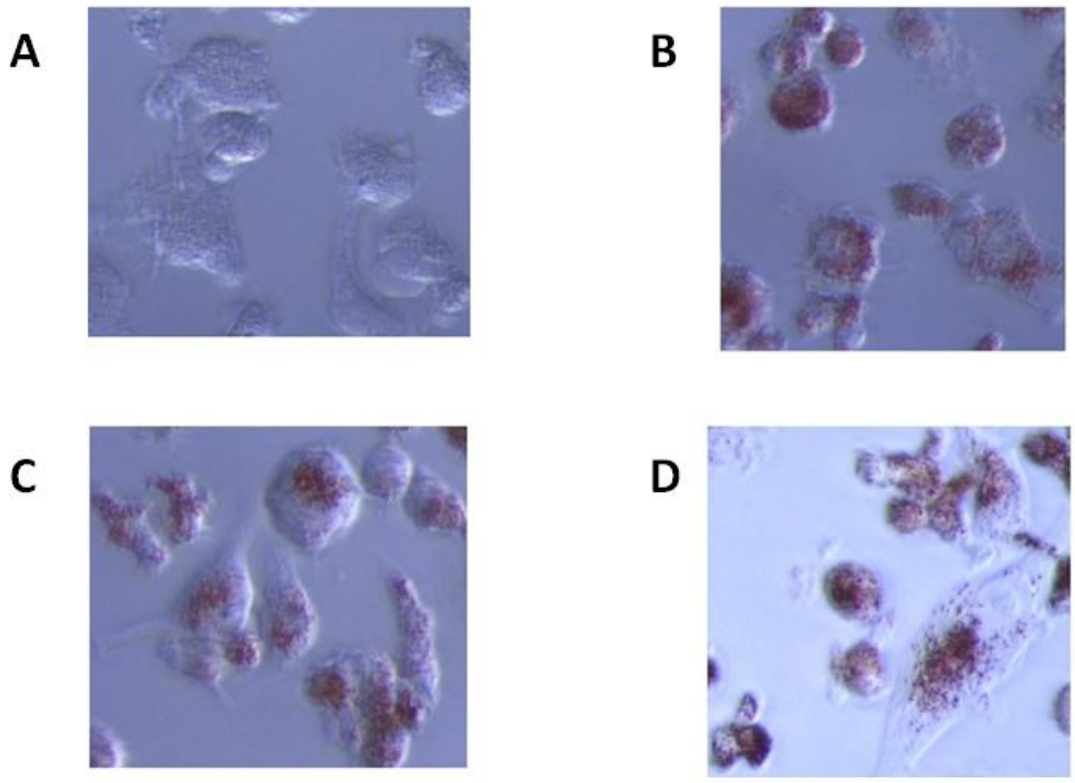


Figure 84 – Oil red O staining of THP-1 macrophage foam cells. (A) unstained (B) unstimulated (C) IL-6 10ng/ml (D) IL-6 + Tocilizumab 100µg/ml. Images obtained from one experiment.

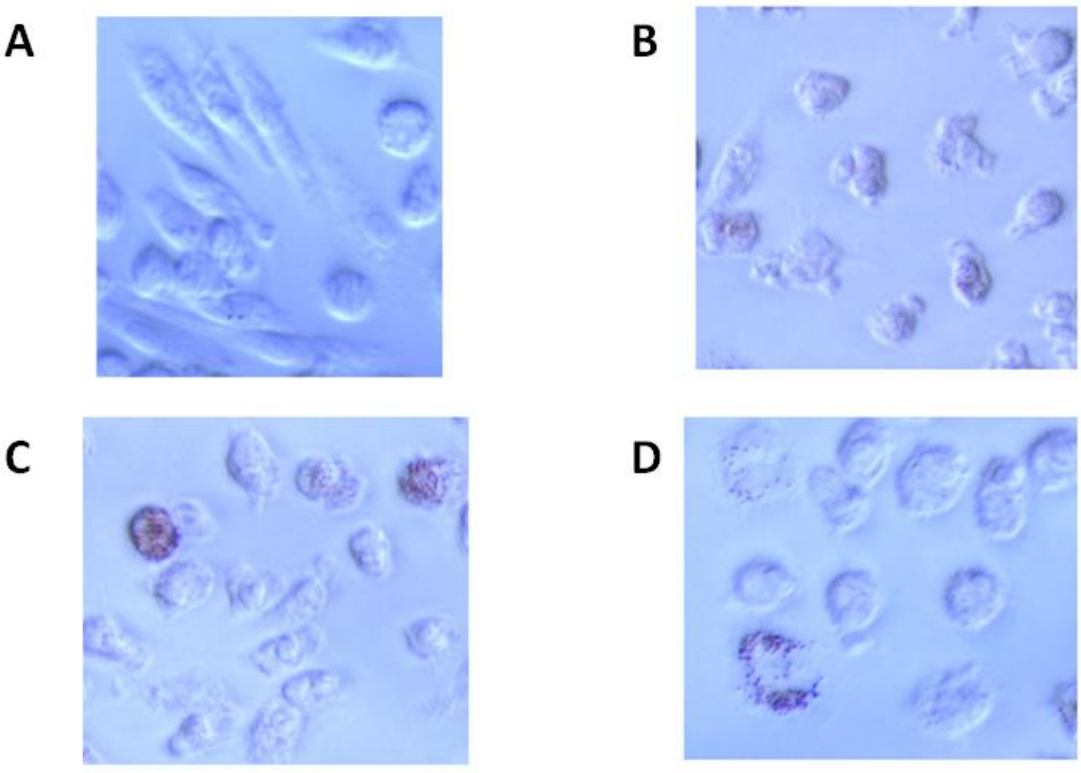


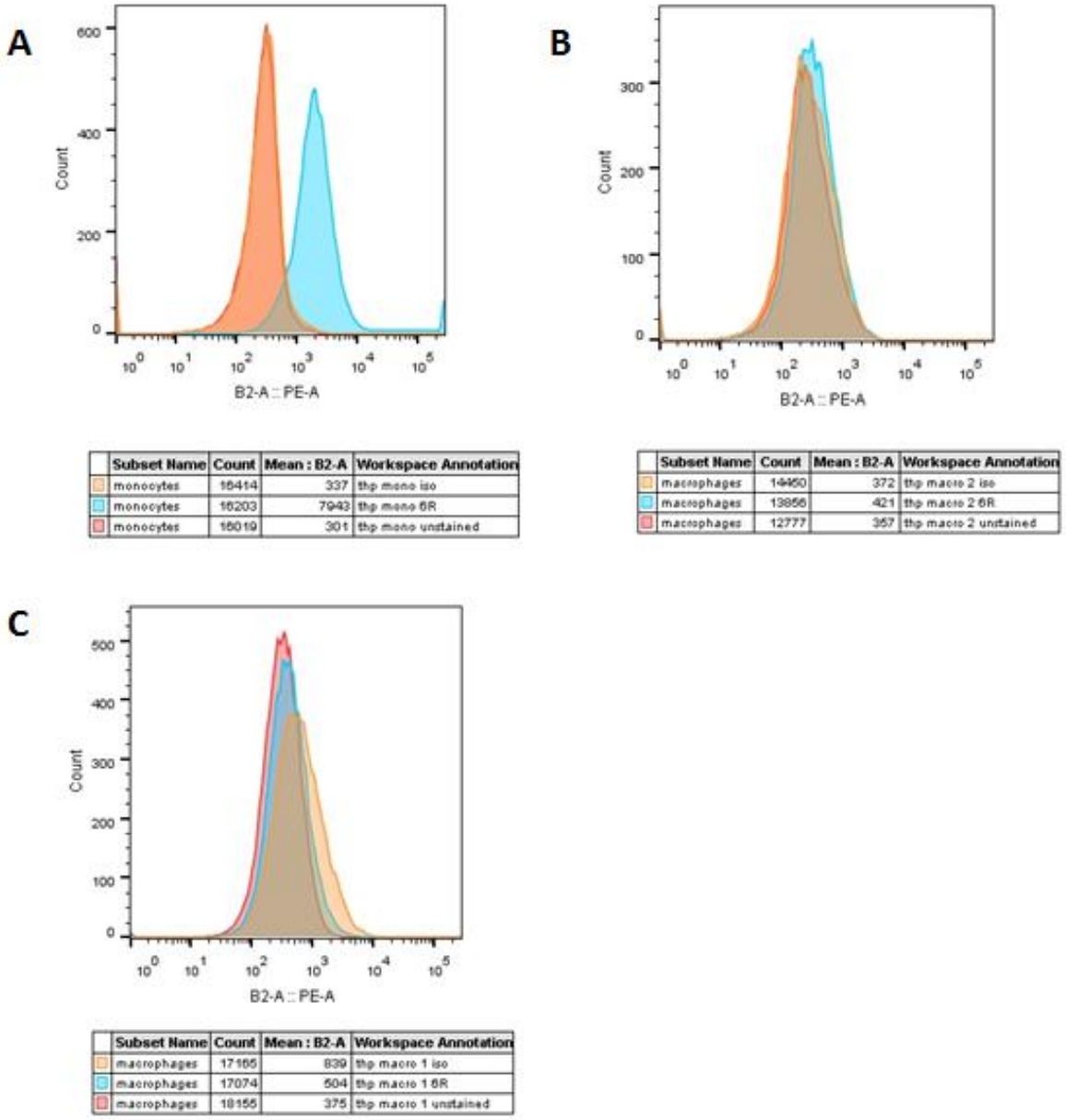
Figure 85 - Oil red O staining of HMDM foam cells. (A) unstained (B) unstimulated (C) IL-6 10ng/ml (D) IL-6 + Tocilizumab 100µg/ml. Images obtained from one experiment.

4.3.4 STAT3 phosphorylation

The difficulties I encountered in demonstrating responses to IL-6 in different cells, especially THP-1 macrophages, raised the possibility that no active IL-6 signalling was taking place in these cells. To address this possibility, I quantified the presence of membrane-bound IL-6 receptor (IL-6R) on monocytes and macrophages, and the presence of intracellular phosphorylated STAT3 (pSTAT3) after exposure of the cells to recombinant IL-6.

Cell surface staining of THP-1 cells demonstrated the presence of membrane-bound IL-6R on monocytes, but not on macrophages generated by either the "original" or "alternative" differentiation methods (Figure 86). In accordance with this observation, IL-6 generated detectable increases in pSTAT3 in THP-1 monocytes, but not macrophages (Figure 87), and this effect was blocked completely by pre-incubation of cells with tocilizumab (Figure 87).

Flow cytometry did, however, demonstrate the presence of IL-6R on CD14+ monocytes and HMDM. This experiment was performed with 3 donors for each cell type; Figure 88 shows representative FACS plots from one donor for each cell type. Unfortunately, time pressures in the laboratory during my fellowship meant I was unable to demonstrate pSTAT3 signalling in human CD14+ monocytes or HMDM. I deprioritised this because I had demonstrated both the presence of membrane-bound IL-6R and the effect of IL-6 administration on TNFα production on these cells.

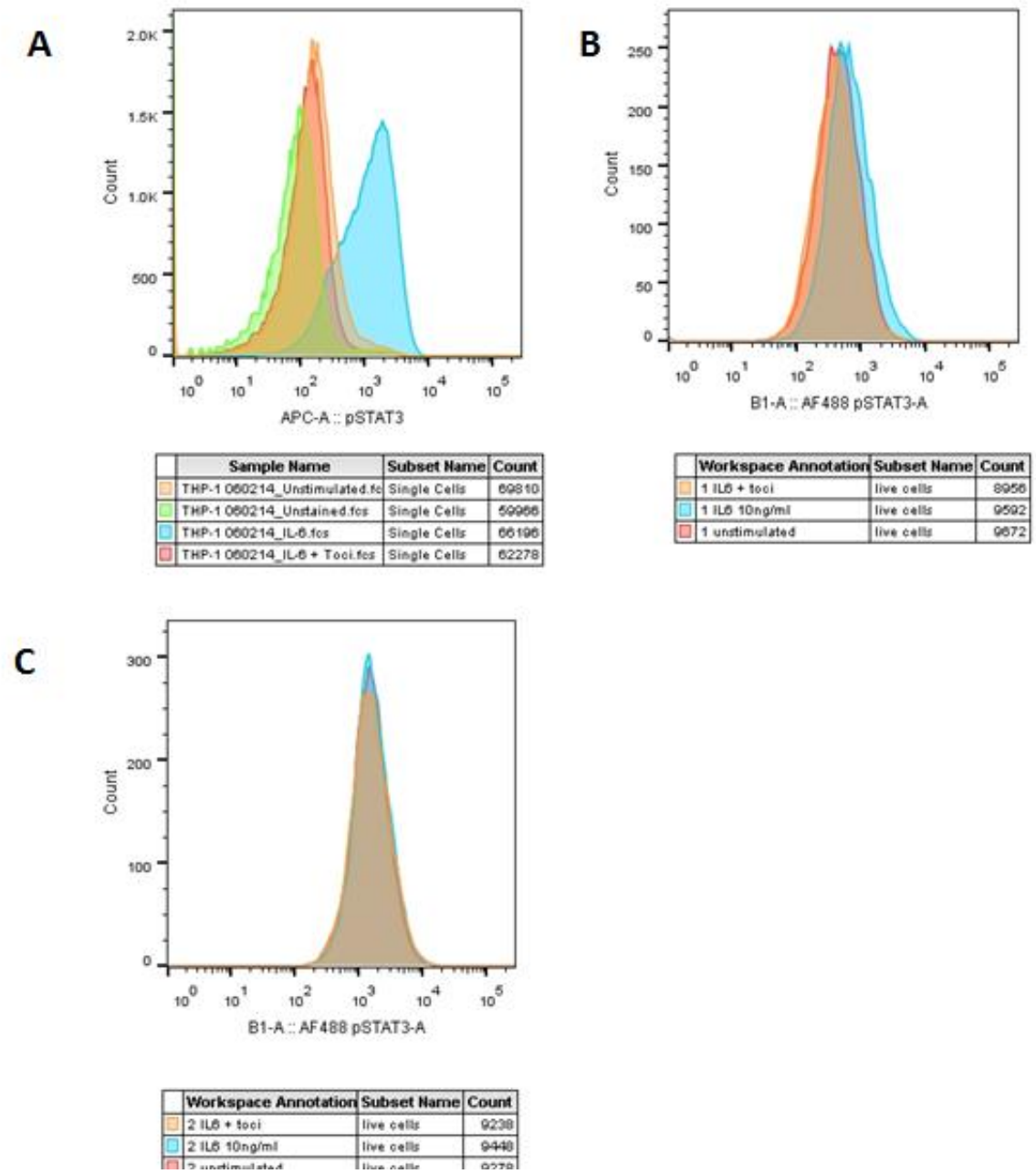


	Subset Name	Count	Mean : B2-A	Workspace Annotation
	monocytes	16414	337	thp mono iso
	monocytes	16203	7943	thp mono 6R
	monocytes	16019	301	thp mono unstained

	Subset Name	Count	Mean : B2-A	Workspace Annotation
	macrophages	14450	372	thp macro 2 iso
	macrophages	13856	421	thp macro 2 6R
	macrophages	12777	357	thp macro 2 unstained

	Subset Name	Count	Mean : B2-A	Workspace Annotation
	macrophages	17165	839	thp macro 1 iso
	macrophages	17074	504	thp macro 1 6R
	macrophages	18155	375	thp macro 1 unstained

Figure 86 - Cell surface staining for membrane-bound IL-6R in (A) THP-1 monocytes, (B) "original" THP-1 macrophages and (C) "alternative" THP-1 macrophages. Red: unstained. Blue: IL-6R. Orange - isotype.



	Sample Name	Subset Name	Count
	THP-1 060214_Unstimulated.fc	Single Cells	69810
	THP-1 060214_Unstained.fcs	Single Cells	59966
	THP-1 060214_IL-6.fcs	Single Cells	66196
	THP-1 060214_IL-6 + Toci.fcs	Single Cells	62278

	Workspace Annotation	Subset Name	Count
	1 IL6 + toci	live cells	8956
	1 IL6 10ng/ml	live cells	9592
	1 unstimulated	live cells	9672

	Workspace Annotation	Subset Name	Count
	2 IL6 + toci	live cells	9238
	2 IL6 10ng/ml	live cells	9448

Figure 87 - Intracellular staining for phosphorylated STAT3 in (A) THP-1 monocytes, (B) "original" THP-1 macrophages and (C) "alternative" THP-1 macrophages. Green: unstained. Orange: unstimulated. Blue: IL-6 10ng/ml. Red: IL-6 10ng/ml plus tocilzumab 10µg/ml.

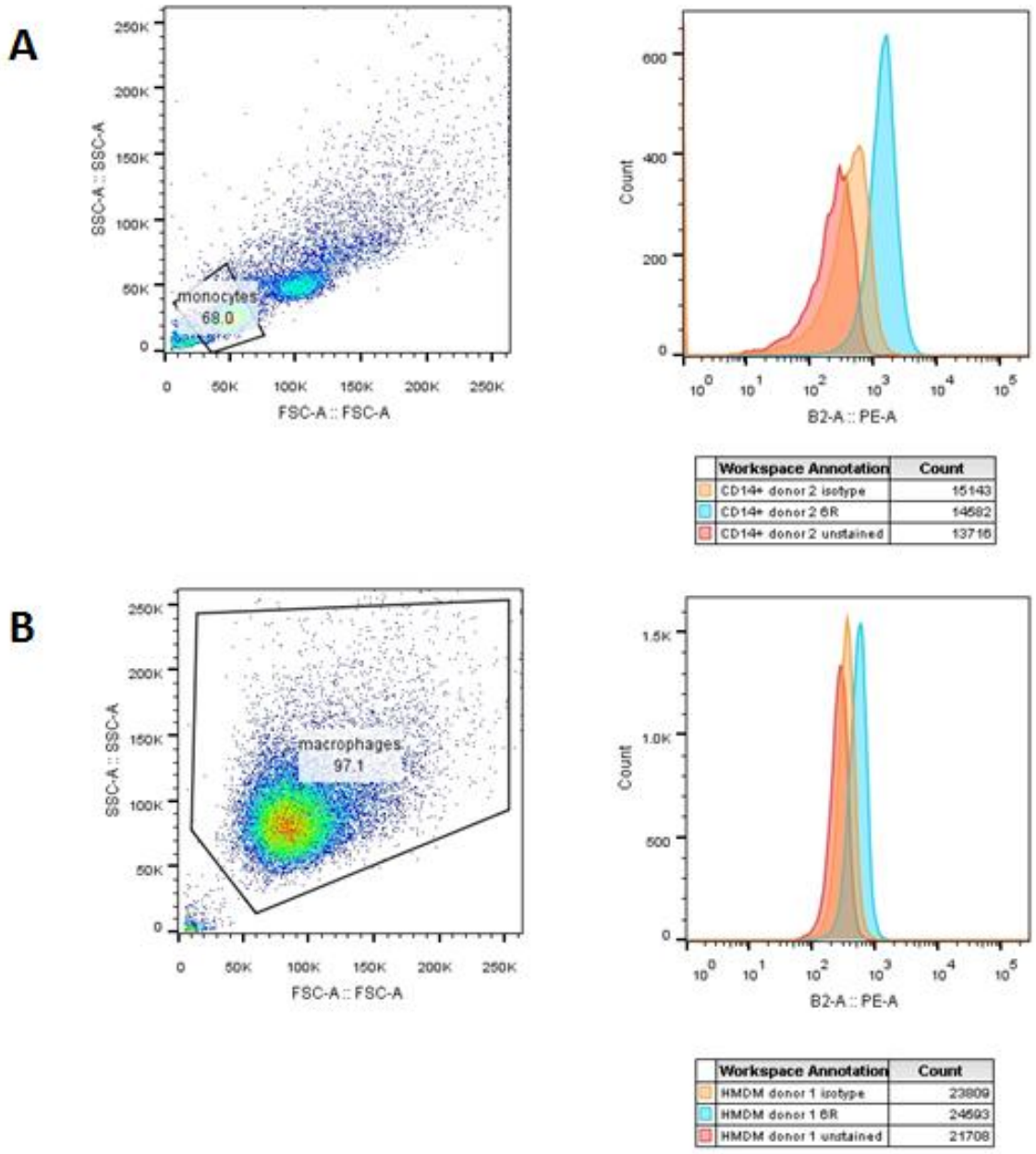


Figure 88 - Cell surface staining for membrane-bound IL-6R in (A) human primary CD14+ monocytes and (B) human monocyte-derived macrophages (HMDM). Red: unstained. Blue: IL-6R. Orange: isotype.

4.3.5 QPCR analysis of lipid-processing molecules in HMDM

HMDM were generated using monocytes from a total of 13 healthy donors. RNA was then harvested from HMDM following exposed to either complete RPMI or IL-6 10ng/ml for 24 hours as detailed in the methods section. SOCS3 expression was measured by SYBR qPCR as shown in Table 25. Using SOCS3 upregulation as a marker for successful IL-6 signalling, the eight donors with the greatest fold increase in SOCS3 RNA were chosen for TLDA analysis. The exception to this was donor #1, where there was a significant discrepancy in GAPDH expression

between unstimulated and stimulated cells, and which was therefore excluded. Unfortunately due to equipment error, the TLDA card failed to generate data for donors 9 or 10, and so full TLDA data was available for 6 donors.

Donor	Fold change		Donor	Fold change
1	4.00		8	2.85 *
2	1.70		9	2.19 *
3	3.16 *		10	1.51 *
4	0.27		11	0.78
5	1.44		12	11.25 *
6	4.63 *		13	5.40 *
7	5.20 *			

Table 25 - Fold change in SOCS3 RNA expression in HMDM from different donors following stimulation with 10ng/ml IL-6 compared to unstimulated cells Samples chosen for TLDA analysis are identified by asterisk (*).

TLDA analysis confirmed the increased expression of SOCS3 following IL-6 stimulation in these donors, with mean fold change 4.23. Of the cell surface molecules analysed at mRNA level, only the class A scavenger receptor MARCO (also known as macrophage receptor with collagenous structure) increased, demonstrating a mean fold change of 3.53 (Figure 89). The biological significance of this finding was unclear, however, as several of the donors demonstrated CT values >30 both pre- and post stimulation with IL-6 (Table 26). This implies that even after a several-fold increase, the quantity of mRNA (and thus quantity of functional protein resulting from gene expression) could still be very low and unable to drive any significant change in cell phenotype. Additionally, the nature of qPCR is such that at very high CT values (and thus very low levels of RNA) the technique becomes inherently less reliable. In contrast, expression of the scavenger receptor LOX-1 appeared to fall slightly (mean fold change 0.61 - Figure 89).

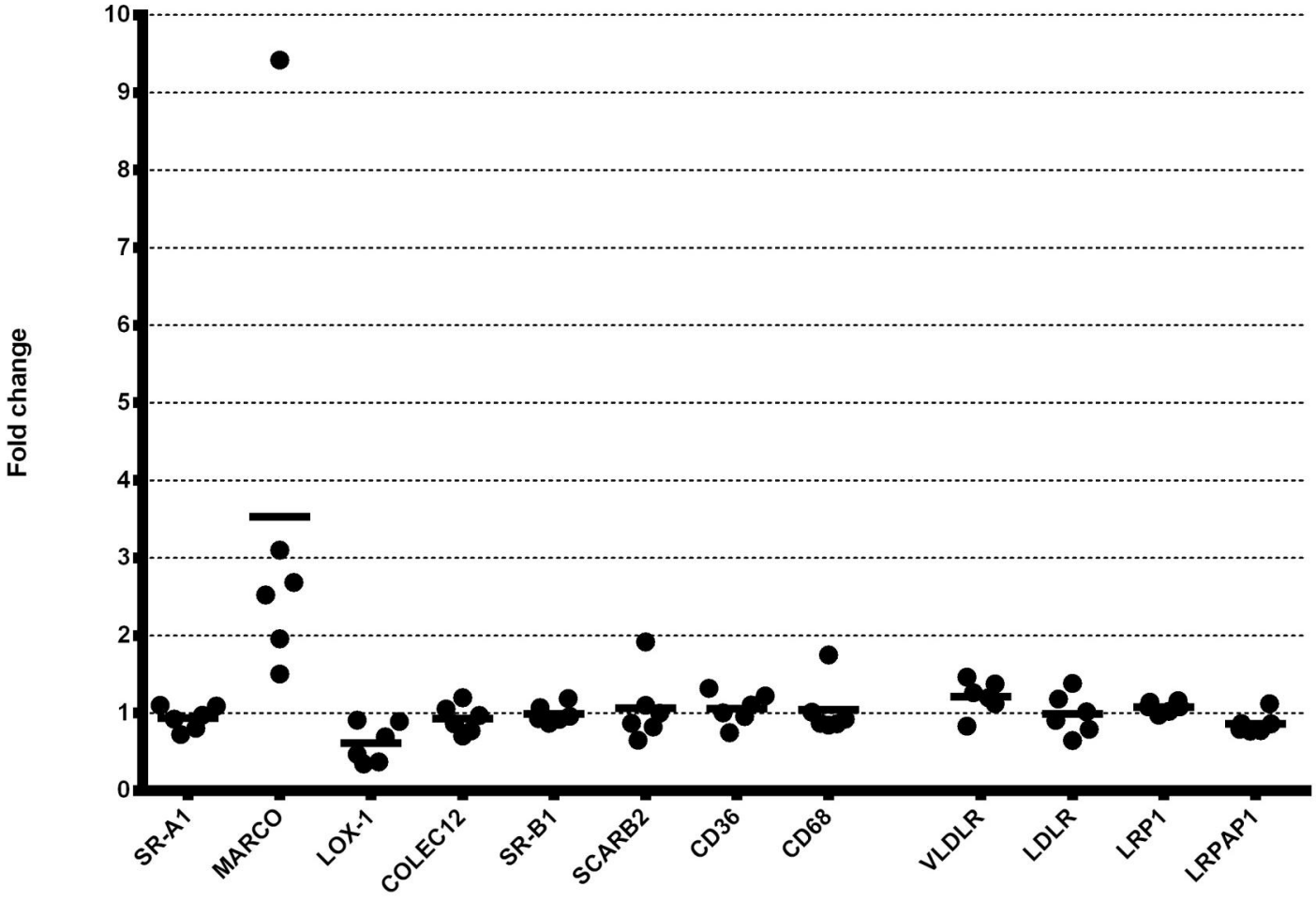


Figure 89 - Fold change in gene expression for lipid receptors in IL-6-exposed HMDM compared to unstimulated cells from same donor. N of donors=6

Donor #	MARCO CT		GAPDH CT	
	Unstimulated	Stimulated	Unstimulated	Stimulated
3	35.35	34.71	24.93	25.92
6	32.02	30.77	22.25	22.34
7	33.89	33.12	22.70	22.89
8	34.62	34.03	22.50	22.51
12	35.57	34.24	21.35	21.45
13	35.30	31.89	23.11	22.93

Table 26 - CT values for MARCO and GAPDH gene expression in HMDM cultured with 10ng/ml IL-6 ("Stimulated") or RPMI alone ("unstimulated") for 24 hours.

No clear change was seen in expression of cholesterol export proteins (Figure 90) or intracellular molecules known to have roles in lipid metabolism (Figure 91). Most intracellular proteins demonstrate one "outlier" which had a large fold increase in RNA production. On interrogation of the data, these outlying values were all obtained from one donor (#3).

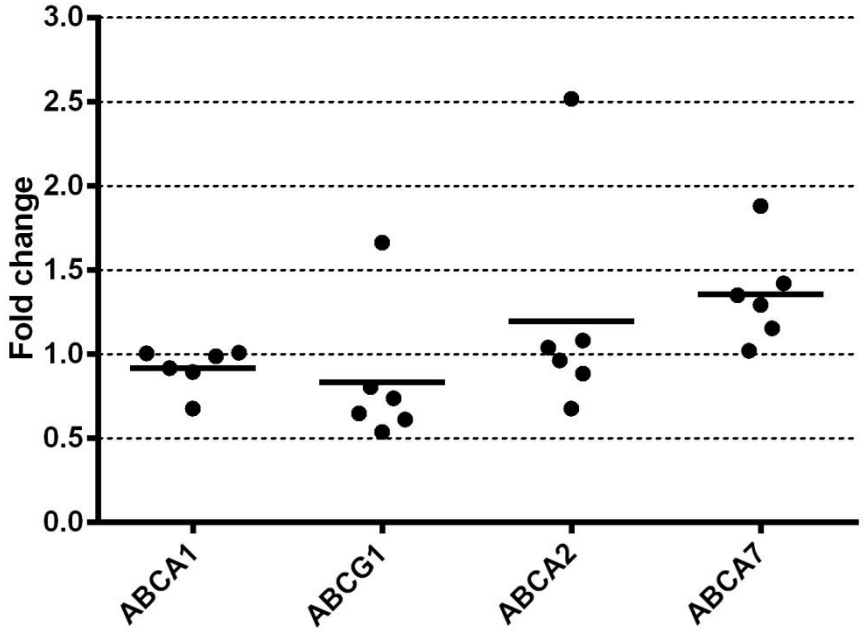


Figure 90 - Fold change in gene expression for cholesterol export proteins in IL-6-exposed HMDM compared to unstimulated cells from same donor. N of donors=6

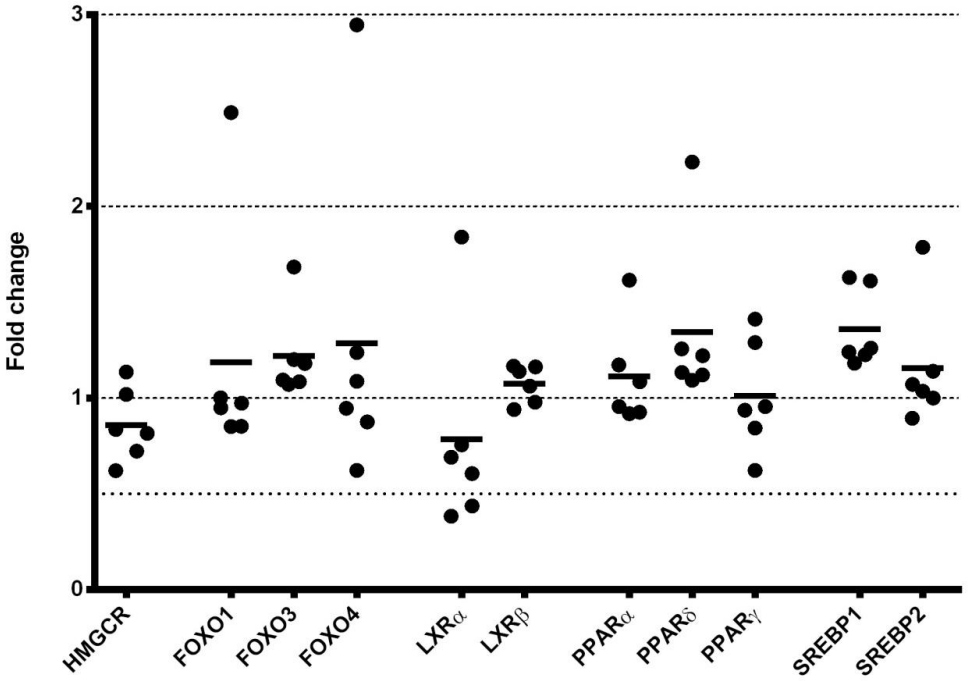


Figure 91 - Fold change in gene expression for intracellular mediators of lipid metabolism in IL-6-exposed HMDM compared to unstimulated cells from same donor. N of donors=6

4.3.6 MARCO and LOX-1 protein quantification in HMDM

I next sought to validate the findings of TLDA analysis by quantifying MARCO and LOX-1 expression at the protein level following macrophage stimulation with IL-6. HMDM were generated from 5 healthy donors, exposed to 10ng/ml IL-6 or RPMI for 24 hours, and stained for MARCO or LOX-1 by flow cytometry. No change was found in mean fluorescence intensity of staining antibody for MARCO (Figure 92). Mean fluorescence intensity of staining for LOX fell non-significantly (Figure 92). Figure 93 and Figure 94 show representative flow cytometry graphs for MARCO and LOX-1 respectively.

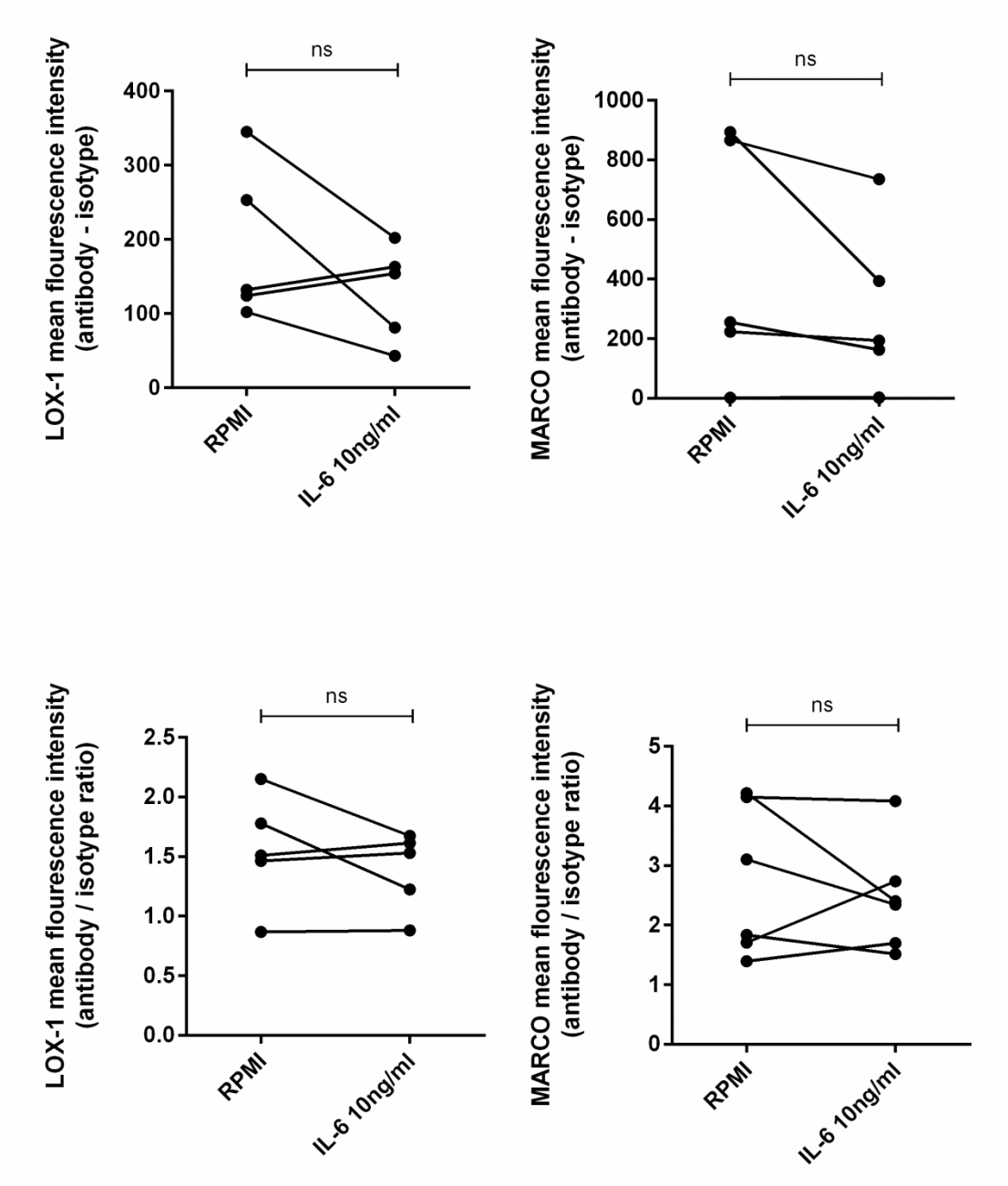


Figure 92 - Change in expression of LOX-1 and MARCO as determined by (a) mean fluorescence intensity of primary antibody minus that of isotype; (b) ratio of mean fluorescence intensity of primary antibody / isotype. Analysis by Wilcoxon matched-pairs test.

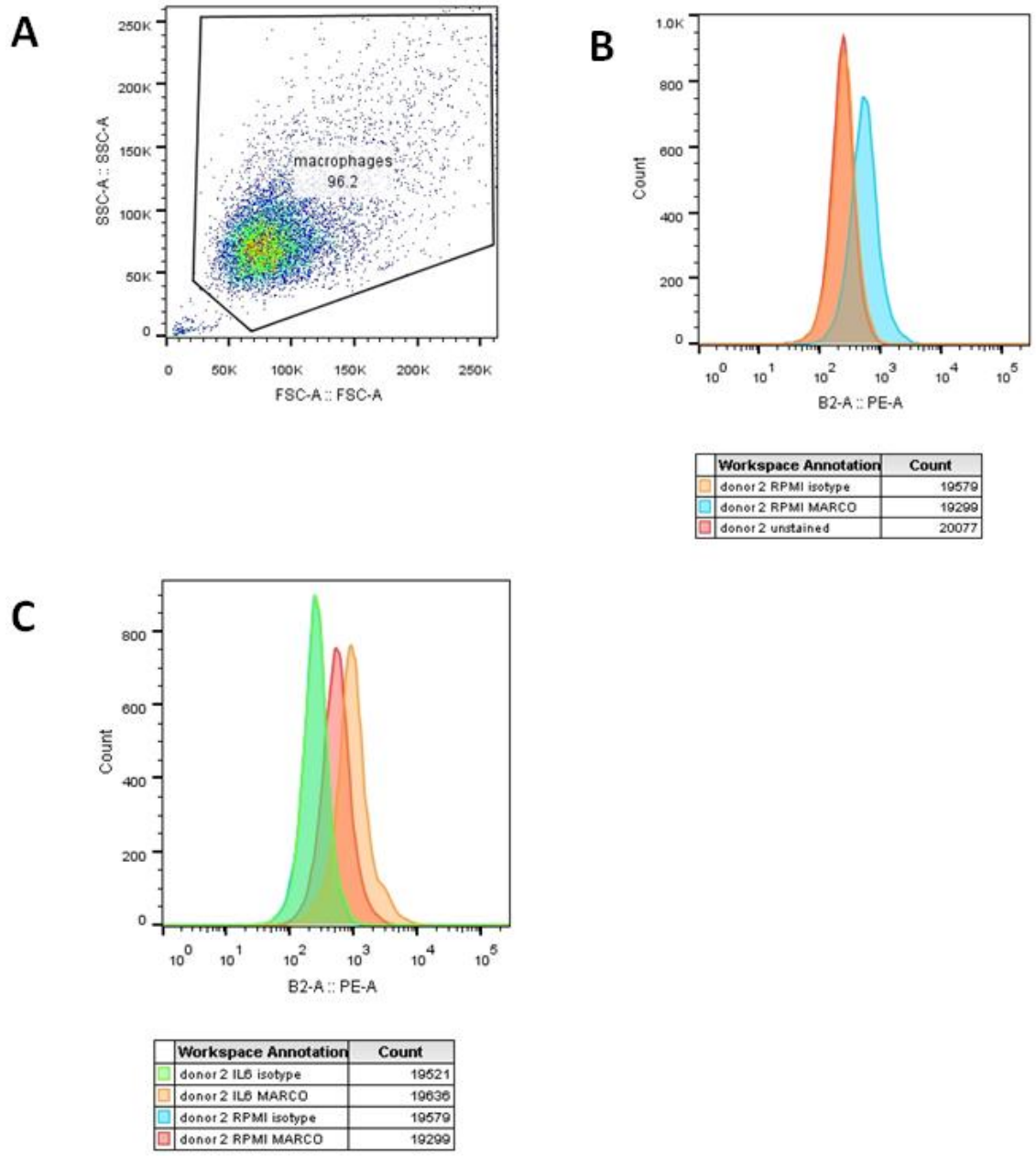


Figure 93 - Flow cytometric analysis of MARCO expression on HMDM from one donor. (A) FACS plot of selected cells. (B) MARCO present in detectable levels on cell surface. (C) MARCO expression increased on this donor following IL-6 stimulation.

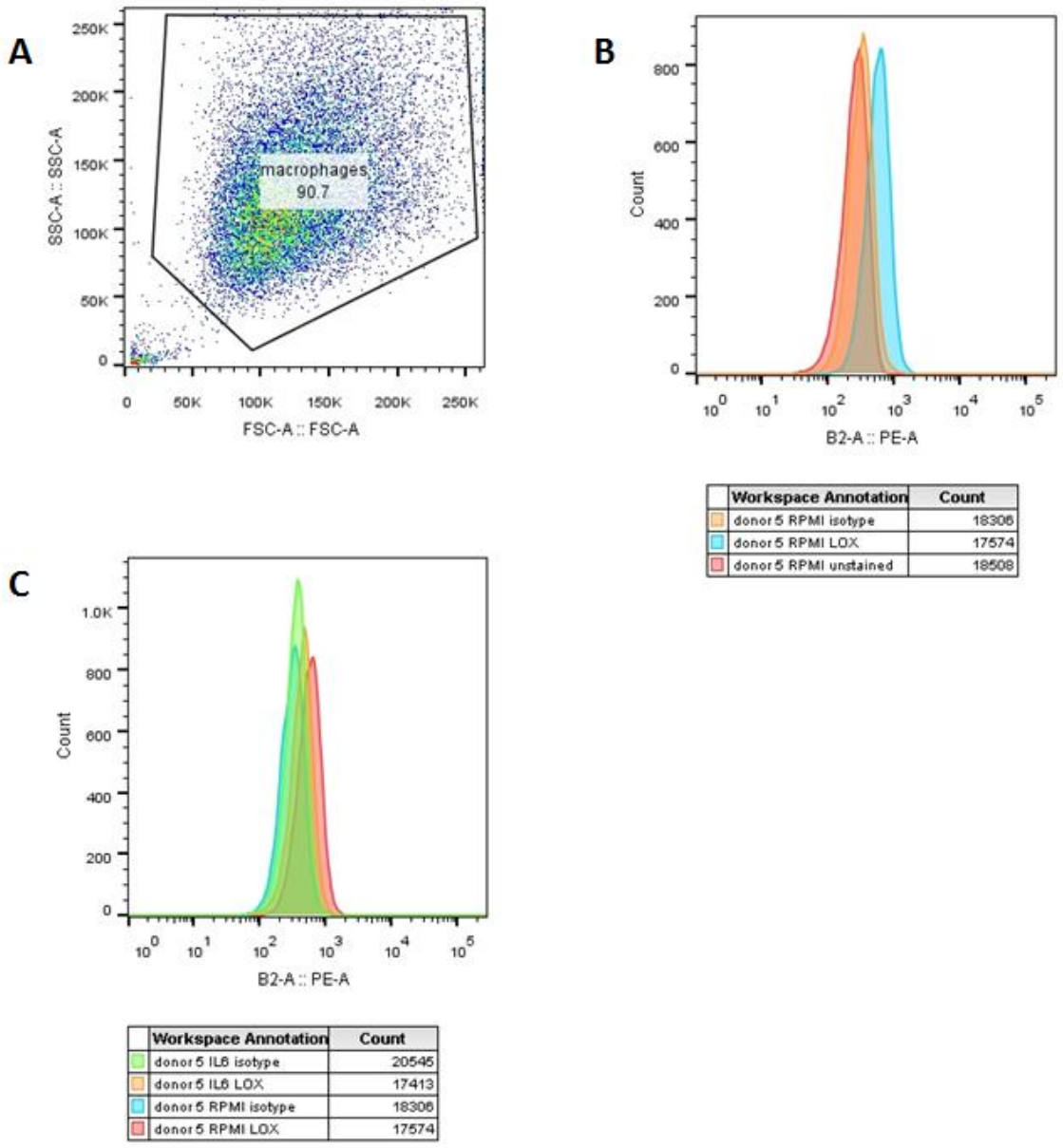


Figure 94 - Flow cytometric analysis of LOX-1 expression on HMDM from one donor. (A) FACS plot of selected cells. (B) LOX-1 present in detectable levels on cell surface. (C) No change in LOX-1 expression on this donor following IL-6 stimulation.

4.3.7 Gene expression in the KALIBRA cohort

With the above data available, I was keen to try to replicate these findings in RA derived cells. Monocytes present in RA blood have been shown to behave differently to those derived from healthy donors (Data from our centre currently being submitted for publication). It is therefore possible that HMDM derived from RA monocytes could behave differently from non-RA cells in regards to lipid loading and gene expression, and indeed may show changes in phenotype

between active and treated RA. It would therefore have been interesting to obtain CD14+ monocytes from RA patients before and after tocilizumab therapy, differentiate them into HMDM, and analyse their gene expression using my remaining TLDA plates. Unfortunately, samples from the KALIBRA cohort had been stored in a manner not conducive to cell survival. Instead, I attempted to observe genetic changes in the overall leukocyte population; this was the closest analogous technique I could conceive of to my previous in-vitro experiments.

On thawing, it became apparent the samples were overtly haemolysed. I initially attempted to obtain RNA from two samples using a miRNeasy kit as above. However, during processing the samples developed large globules of precipitate which partly disappeared on vortexing. Analysis by nanodrop showed extremely low concentrations of quantity RNA, compared to concentrations consistently >30ng/µl from HMDM. Additionally, the quality of RNA was poor, precluding further analysis; this was particularly the case at the 260/230 ratio, possibly reflecting contamination with EDTA as a result of the sampling process.

	Sample 1	Sample 2
260/280	1.20	1.17
260/230	0.40	0.19
Concentration (ng/µl)	4.7	3.1

Table 27 - NanoDrop analysis of quantity and quality of RNA isolated from KALIBRA samples by miRNeasy kit.

Subsequently, samples were analysed using PAXgene tubes. This appeared to yield RNA at reasonable quality at around 40ng/µl. However, when performing SYBC qPCR for GAPDH expression, CT values for five separate samples were uniformly greater than 34, and thus unlikely to yield reliable results. At this point, I chose to abandon further analysis of the KALIBRA samples.

4.4 Discussion

4.4.1 THP-1 macrophage biology

Despite numerous attempts with different differentiation and culture conditions, I was unable to demonstrate any effect of IL-6 on the behaviour of THP-1 macrophages. This conflicts with previous literature, where other groups have claimed that IL-6 can potentiate TNF-α production (288), reduce uptake of acetylated LDL (290), increase uptake of oxLDL (278), and stimulate cholesterol export (279). One manuscript showed that the chemokine CCR-1 was capable of inducing pSTAT3 on THP-1 macrophages, an effect ameliorated by anti-IL-6 antibody (291), thus providing indirect evidence of IL-6R ligation leading to STAT3 phosphorylation in these cells. This conclusion was confirmed very recently (292) by direct observation of IL-6 inducible pSTAT3 by Western blot in both THP-1 monocytes and macrophages, albeit using different differentiation techniques and a higher concentration of IL-6 (100ng/ml). In sharp contrast to these findings, I did not find the IL-6 receptor present on the membrane of differentiated THP-1 macrophages. This would explain why, in my experiments, exogenous IL-6 had no effect on STAT3 phosphorylation, TNF-α production or foam cell formation. A possible explanation may lie in the wide variety of protocols for the culture and differentiation of these cells which have evolved over the last two decades, though a lack of experimental reproducibility would be especially disappointing given that a cell line (rather than randomly selected human donors) is being used. In hindsight, I may have been able to demonstrate effects by the addition of exogenous soluble IL6-R to the cell culture, thus permitting STAT3 phosphorylation via trans-signalling. However, this would not resolve the above conflicts with previous literature.

It must be recognised that the THP-1 cell line acts as a macrophage *model*, not a *replacement*, for HMDM. These cells lack the variation in response inherent in using cells from different human donors; a THP-1 experiment thus provides only a very narrow window on what in reality would be a wide spectrum of biological responses. THP-1 cells, being derived from a leukaemia cell origin, also appear to be very metabolically active, as seen by their high levels of oil red O staining even when differentiated with low levels of PMA and in the absence of exogenous cytokine or oxLDL; this again contrasts with the much more subtle

staining of HMDM, and makes me more suspicious of their applicability to in vivo human biology. Because of these considerations, if I were to obtain conflicting data from THP-1 macrophages and HMDM, I would feel the latter are most likely to represent human biology.

4.4.2 HMDM and foam cell formation

Subsequent in vitro experiments using primary cells generated important positive results regarding the role of IL-6 in human macrophage behaviour. I demonstrated that the IL-6 receptor is present on human CD14+ monocytes and HMDM, and that IL-6 signalling can be detected in these cells by staining for pSTAT3 and by observing inhibition of LPS-induced TNF-α production (as documented in existing literature (289, 293)). These foundation findings are reassuring, and make me more confident that subsequent results with these cells are robust. Despite this, IL-6 did not demonstrate an ability to increase oxLDL uptake by HMDM, as measured by two separate research methods. This conflicts with both my original hypothesis and the findings of other researchers as detailed above. Previous unpublished work in our lab by another doctoral student also showed no increase in oil red O staining in HMDM exposed to IL-6; however, this researcher did find a significant reduction in lipid uptake following addition of GM-CSF, which I again failed to replicate. I believe my results to be reliable as they were achieved after demonstrating successful IL-6 signalling and persisted despite a variety of culture conditions, but this clearly prompts speculation as to why my results clash with those of others. Like the THP-1 experiments, previous groups have used different techniques for HMDM differentiation that may affect final HMDM phenotype. Liao (290) seeded the entire buffy coat onto polystyrene culture dishes, retained those cells adherent after 1 hour of culture, and differentiated them with RPMI supplemented with 10% pooled human serum. Frisdal (279) obtained PBMCs through an unclear method and differentiated them with M-CSF for 10 days. Hashizume (278), whose paper was one of the first I read on this topic and which encouraged me to generate the main hypothesis of this chapter, only studied THP-1 macrophages. The protocol I used for HMDM generation is one that is used routinely in our lab for a variety of experiments evaluating the activation and behaviour of macrophages under inflammatory stimuli, usually in the context of RA. In these parallel experiments they behave in a consistent manner albeit

recognising that individual donors offer variable magnitude and occasional qualitative responses.

4.4.3 The limits of oil red O

Another simple (but powerful) explanation for conflicting results may lie in the subjectivity of oil red O staining. I found that the images generated by staining were highly dependent on factors such as: the intensity of light used on the microscope; the number of seconds the stain was applied for; the intensity of cell washing; and even the area of the well photographed. Most importantly, small images suitable for a thesis or publication only show a tiny fraction of all the cells in a well. In an "unblinded" experiment it would be very easy for the researcher to choose to photograph cells from a well which had taken up a little more or less stain. Some authors claim to have "measured" or "quantified" oil red O staining, but do not give detail as to how this was performed. I therefore suggest that results of oil red O experiments should be interpreted cautiously. This is also the reason I was keen to replicate my findings using an alternative, *quantitative* technique, namely using fluorescently-labelled oxLDL.

4.4.4 Scavenger receptors

No consistent, significant changes were observed in expression of lipoprotein receptors, scavenger receptors or cholesterol export proteins in HMDM after IL-6 exposure – again, in contrast to earlier studies. The increase in production of MARCO following IL-6 stimulation found at the mRNA level is unlikely to be a relevant finding: it was not confirmed at the protein level by flow cytometry in most donors; it was detected at very high CT values, where the reliability of qPCR diminishes; and in any case it was clearly insufficient to exert phenotypic change as determined by foam cell formation.

The disconnect between changes in gene expression at the mRNA and protein levels may also be because donors chosen for TAQMAN analysis were those who had demonstrated the greatest increases in SOCS3 expression after IL-6 stimulation, and thus had more profound IL-6 signalling. However, no such selection was performed for donors undergoing flow cytometry. This leads on to another potential criticism of my experimental setup. SOCS3 can be

regarded as an *anti*-IL-6 protein, as it binds to cytokine receptors or Janus kinases and acts as a regulator in a negative feedback loop to inhibit the intracellular effects of IL-6 signalling. One could argue then that the cells with greater SOCS3 expression were in fact those who would be *least* likely to show phenotypic changes typical of an inflammatory state, as these cells were those where continuing IL-6 signalling was most successfully inhibited. If I had analysed cells from donors with no elevation in SOCS3 mRNA, would more relevant changes in expression of other genes have been observed?

Nevertheless, SOCS3 is well recognised as a downstream marker of IL-6R ligation. Perhaps a more effective approach would have been for me to accompany all the above phenotypic experiments with concomitant pSTAT3 quantification by flow cytometry on cells from every donor, only continuing further experimentation on donors with demonstrable increases in pSTAT3 after IL-6 exposure. Whilst planning my experiments originally I thought this would be unnecessary and labour-intensive; however, in retrospect, and with appropriate experiment planning, it may have been both feasible and worthwhile. If I were to revisit these experiments, I would also attempt alternative culture conditions including higher concentrations of IL-6 (20ng/ml as in Laio, 50ng/ml as in Frisdal et al, or 100ng/ml as in Kiedar et al) and explore the effects of supplementation with IL-6R to encourage trans-signalling. Given the relatively high CT values seen in some of my results, qPCR experiments may also benefit from having increased numbers of cells, and so more available mRNA, for each condition.

4.4.5 Extrapolation and replication in RA patients

Frustratingly, no data could be gleaned from stored samples from the KALIBRA cohort, as such an experiment had not been planned at KALIBRA's inception and so samples were not stored in such a way as to preserve viable leukocytes. As my in vitro work was an attempt to mimic the behaviour of macrophages in the reticuloendothelial system, or arterial wall, reproducing my experimental methods in vivo is challenging. One could assess gene expression in circulating CD14+ monocytes before and after tocilizumab therapy, though it would seem that tissue macrophages are more likely to take up oxLDL than undifferentiated monocytes. It is theoretically possible that RA monocytes post-tocilizumab may be sufficiently altered in vivo as to persistently display altered phenotype even

after differentiation into macrophages, though I have no reason to believe this is the case. Then again, in the RA patient, IL-6 would be present before, during and after differentiation of monocytes; this was one factor I did not control for in vitro, as I added IL-6 only after cells had fully matured into macrophages.

In summary, in contrast to the findings of previous researchers, I found that culture with IL-6 has no effect on foam cell formation or expression of scavenger receptors in either THP-1 macrophages or HMDM. Based on these findings, at least in my experimental systems I cannot support the notion that tocilizumab alters LDL catabolism as a result of reduced IL-6 signalling in macrophages in the atheromatous plaque or the reticuloendothelial system. I recognise however the limitations in the analysis I have performed in vitro and in future experiments would wish to re-examine this hypothesis with refined methodologies.

5 Final Discussion and future study

5.1 Summary of conclusions

Herein I draw together some final conclusions based on my observations described thus far.

Patients with severe active RA display hypercatabolism of LDL, which can be reduced to a value approximating normal values following tocilizumab treatment. This reduction in LDL fractional catabolic rate is largely responsible for the increase in LDL-cholesterol seen following tocilizumab.

Changes in LDL catabolic rate, and LDL-cholesterol, associate tightly with changes in acute phase response, but not CDAI. This raises the possibility that hepatic IL-6 signalling, rather than the burden of synovitis, is the prime driver of reduced LDL in RA. These may be related, but there are complexities to the relationship that bear further observation, and that challenge overly simplistic interpretations of current inflammation – lipid level – clinical response paradigms.

In the MEASURE study, changes in NMR lipid profile previously described after 12 weeks of tocilizumab therapy – namely, increased large LDL particles, unchanged small LDL particles and increased small HDL particles – are maintained at 52 weeks, and have been replicated in the placebo group of the study after they switched to open-label tocilizumab. These changes are driven by inflammation (as measured by CRP and ESR).

The “normalisation” of LDL catabolism and the pattern of lipoprotein changes observed in these studies suggest that IL-6 blockade may be cardioprotective in patients with RA despite elevations in serum LDL-c.

The novel NMR-based biomarker GlycA is highly reliant on IL-6 signalling and, commensurate with this, behaves in a manner similar to CRP. GlycA was unable to predict clinical response in those exposed to tocilizumab, and provided little extra information on clinical response over and above decisions built on the use of CRP or ESR.

Elevations in serum LDL-c do not appear to be due to abrogation of IL-6 signalling on macrophages and reduction in subsequent lipid uptake and foam cell formation. However, the absence of IL-6-related effects on foam cell formation or scavenger receptor expression on macrophages stand in contrast to previous studies, and require further attempts at replication.

5.2 Application to clinical practice

One of the driving factors behind investigating the role of IL-6 blockade in RA dyslipidaemia was the possibility that this treatment could increase the CVD risk of an already higher-risk population by increasing LDL-c levels. This has led to reluctance on the part of some clinicians to prescribe tocilizumab, especially in those with a history of hyperlipidaemia or pre-existing cardiovascular disease. The work in this thesis has generated several strands of evidence to ameliorate some of these concerns, particularly the following observations following IL-6 blockade, namely: elevations of large, rather than small, LDL particles on NMR; reduced levels of the proatherogenic Lp(a); normalisation of LDL fractional catabolic rate; reduction, not elevation, in the production rate of LDL (though this is not reflected in serum cholesterol levels as it is overcome by the reduced catabolic rate that leads to a net increase in LDL-c); stability of the total cholesterol / HDL-c ratio; increased levels of HDL-c and small HDL particles – though the significance of these changes in predicting CVD risk is not as certain as once thought, and a matter of considerable debate.

These findings can be added to existing evidence of IL-6 contributing to CVD risk from Mendelian randomisation based approaches, and epidemiological data showing high-sensitivity CRP as a predictor of cardiovascular disease in the non-RA population. Taken together, I propose that these results should support clinicians to be more accepting of use of IL-6 blockade as a therapeutic tool in RA patients who are at high risk of cardiovascular disease. Additionally, if we as clinicians are able to reassure our patients that our medications are safe (or even reduce CVD risk) this may well increase patients' compliance with their therapy and thus improve clinical outcomes. Nevertheless, this is a conclusion which can be implied only indirectly from these mechanistic studies. Trials using hard cardiovascular clinical outcomes are required, and such studies are relatively lacking at present but are ongoing. Until their endpoints are achieved

(or not as the case may be), our mechanism based approaches provide temporary relief and information to drive prescribing.

The profound changes in lipid metabolism outlined here should also act to remind clinicians of the increased CVD risk that RA patients carry, and prompt us to conduct CVD risk assessment before and after treatment. In addition to tight disease control, control of conventional risk factors remains critical. For example, both RCTs and cohort studies have demonstrated that RA patients derive similar reductions in serum LDL-c to non-RA subjects from commencing statin therapy (294, 295). In a separate population-based longitudinal study, cessation of statin therapy in RA patients was associated with a 60% increase in cardiovascular death, also in a manner similar to that observed in the non-RA population (296).

On a personal note, I believe that the clinical significance of the questions which prompted these studies, and the results gained from them, should also prompt clinicians to encourage enrolment of our patients into clinical trials or biologics registries as a way of improving the care we provide. My experience of KALIBRA was that, at the end of the study, the feedback I received from participants about their experience was almost universally positive. This was despite a fairly arduous protocol which demanded significant amounts of time and effort from participants. Common themes of feedback included being able to “give something back” to the health service that has assisted them so far; the perception that they were receiving closer attention and more personalised and precise care as study participants (helped in part, no doubt, by being provided with the business card and mobile phone number of a rheumatology registrar); a sense of “getting some control back” over their disease, and taking an positive, pro-active role in their own treatment; and the knowledge that their effort would improve treatment for future patients with RA – which generally still included themselves, given the chronic nature of the disease .

5.3 Avenues for future study

Whilst the results in this thesis have progressed our understanding of the lipid paradox in RA, much of this complex area of biology remains unclear. I suggest

there are a number of ways in which this research could be successfully taken forward in the future.

NMR analysis, as performed in MEASURE, offers an opportunity for detailed analysis of lipid profiles. NMR data, with some clinical data, is currently available for the OPTION study, a large phase III RCT of 623 RA patients randomised to tocilizumab 8mg/kg, tocilizumab 4mg/kg, or placebo for 24 weeks. This would offer the chance to replicate the findings of MEASURE in a larger cohort. Similar analysis may also be available on other RA cohorts, including TaSER. TaSER provides a change to compare and contrast lipid profiles with the tocilizumab cohorts, as its patients had lower average disease activity, much shorter duration of disease, were almost exclusively treatment naive at enrolment, and were treated largely with conventional DMARDS. The nature of the "treat-to-target" strategy in TaSER also differentiates this from the less aggressive treatment protocols of OPTION and MEASURE, and reflects modern best practice in early RA. NMR would therefore be useful both from a mechanistic perspective (helping us untangle which lipid changes are reflective of "disease control" generally rather than IL-6 blockade specifically) but also in terms of informing clinicians on the effects of the current gold-standard treatment for early RA. Lipid changes in TaSER could also be examined for their relationships with the detailed clinical assessments available for all patients at each timepoint. As TaSER contains ultrasound and radiographic changes as endpoints, we can also evaluate GlycA's performance in predicting disease progression and radiological response, and compare its utility as a biomarker with that of CRP and ESR.

As alluded to above, assessment of cardiovascular outcomes in patients receiving tocilizumab therapy remains a priority. In Glasgow we have increasing numbers of patients using tocilizumab but at present no way of accessing data from them on a city-wide basis. In any case, it is unlikely that we would be able to have enough patients on the drug to generate a cohort large enough to successfully examine CVD event rates, though using surrogates such as CIMT or presence of plaque or coronary artery calcification may be easier. Even here, however, reliable outcomes are far from guaranteed; assessment of arterial stiffness by quantification of pulse wave velocity was a secondary outcome of MEASURE, but technical difficulties precluded a successful analysis.[MCINNES2013] Two,

perhaps more profitable, avenues are currently available. The first is the British Society for Rheumatology Biologic Registers (BSRBR), a series of prospective cohorts to which patients commencing biologic drugs are recruited from across the UK. The RA register has been interrogated previously for cardiovascular outcomes following anti-TNF therapy (as discussed in chapter 1) and may provide a fertile source of investigation for patients receiving tocilizumab. The second involves accessing national health databases for information on RA patients with cardiovascular outcomes. The service for accessing secondary care patient data in Scotland is provided by the Farr Institute Scotland. This allows access to anonymised clinical data through a secure system known as a "Safe Haven", one of which is hosted by NHS GGC. Hosted data includes the Scottish Morbidity Record, a combination of national datasets derived from inpatient and outpatient hospital consultations since 1997. A similar system for general practice, known as SPIRE (Scottish Primary Care Information Resource) is currently under development. Either of these routes may conceivably be used to assess CVD events associated with tocilizumab, with or without accessory data on lipid levels.

Beyond KALIBRA, only one other published study has evaluated LDL kinetics in RA patients (273). Performing a similar study on alternative therapies for RA (e.g. anti-TNF, or conventional DMARDS) may shed light on the different mechanisms underlying lipid changes in specific therapies. However, as should be apparent from chapter 3 of this thesis, kinetic studies are difficult to perform, and a fresh kinetic study on other RA therapeutics seems less appealing because (a) LDL changes are much less pronounced, and so statistically significant data will be harder to obtain, and (b) both methotrexate and anti-TNF agents have proven CVD benefits, and so less clinical concern over potential elevations in LDL-c. I do, however, have access to the remainder of the kinetic data from KALIBRA which has not been looked at in detail, specifically the kinetics of VLDL and IDL, and some data on the behaviour of HDL. Analysis of this data may shed further light on the mechanisms underscoring altered LDL metabolism. In addition, the presence of the only pre-menopausal female participant in KALIBRA as an apparent outlier in regards to the relationship between lipid changes and disease control raises the intriguing possibility that this subset of patients may exhibit significantly different lipidaemic responses to IL-6 inhibition. This in turn may

have implications for cardiovascular risk, and indeed for prescribing decisions in these patients. As we enter the era of "personalised medicine", evaluating this phenomenon could be critical for ensuring the most appropriate treatment for each patient.

As discussed extensively in chapter 4, I am uncertain of how robust my findings regarding macrophage lipid uptake are. Further attempts at replication, perhaps using alternative methods of macrophage generation, would seem reasonable. One technique that I was unable to properly perform was the examination of gene expression in monocytes or HMDM isolated from RA patients pre- and post-tocilizumab. Given the possibility of the liver being a target organ in IL-6 induced dyslipidaemia, similar work on gene expression could also be performed on hepatocytes (for example the hep-G2 cell line) as has been done in one published manuscript previously. The above techniques require cell culture with an accompanying number of variables; a less precise, but more straightforward, alternative would be to perform a whole-blood transcriptomic analysis using a technique such as the PAXGene tube. This technique causes leukocyte lysis and RNA stabilisation at the point of phlebotomy, potentially allowing for more robust transcriptome data at the cost of reduced information on the specific cell lineages being affected. PAXGene samples may be used in a "hypothesis-generating" manner through microarray analysis (e.g. Affimetrix), but also permit hypothesis testing by analysing pre-chosen genes of interest as in my experiments on TLDA plates.

Undertaking this PhD has been an enormous challenge, but a very rewarding one, and I consider it a privilege to have been a clinical research fellow in a time of such exciting discovery in our discipline. I sincerely hope that my findings are not just of academic interest to researchers in the field, but become relevant to both patients and physicians as together we strive to improve all aspects of our patient care. I firmly believe that the experience I have gained over these years in trial design, data analysis, and translational research are valuable ones which will shape me as I go forward in my career as a rheumatologist, and that my collaborations with other clinicians and scientists will equip me to perform such research more effectively in the future.

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Appendix A – Abbreviations

ACR	American College of Rheumatology
ACPA	Anti-Citrullinated Protein Antibody
AUC	Area under the curve
CCP	Cyclic citrullinated peptide
CDAI	Clinical Disease Activity Index
CETP	Cholesterol Ester Transfer Protein
CRP	C-reactive protein
DAS28	28 joint Disease Activity Score
DMARD	Disease Modifying Anti-Rheumatic Drug
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FBC	Full blood count
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCQ	Hydroxychloroquine
HDL	High-density lipoprotein
HDL-c	High-density lipoprotein cholesterol
HMDM	Human monocyte-derived macrophages
HPL	Hepatic lipase
IL-6	Interleukin 6
LCAT	Lecithin-cholesterol acetyltransferase
LDL	Low-density lipoprotein
LDL-c	Low-density lipoprotein cholesterol
LDL-CE	Low-density lipoprotein cholesterol ester
LFT	Liver function tests
LPL	Lipoprotein lipase
MTX	Methotrexate
NHSGGC	NHS Greater Glasgow and Clyde
OA	Osteoarthritis
ORO	Oil-red O stain
oxLDL	Oxidised low-density lipoprotein
PBMC	Peripheral blood mononuclear cells
PCSK9	Proprotein convertase subtilisin/kexin type 9
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROC	Receiver-Operating Characteristics
RPMI	Roswell Park Memorial Institute medium
SSZ	Sulfasalazine
SDAI	Simplified Disease Activity Index
SNP	Single nucleotide polymorphism
TNF-α	Tumour Necrosis Factor-α
U&E	Urea and electrolytes
ULN	Upper limit of normal
VLDL	Very low-density lipoprotein

Appendix B – Poster presentation of lipid changes in the TaSER study

FRI0142

Aggressive DMARD Therapy Elevates HDL-Cholesterol And Lowers The Atherogenic Index In The TaSER Study

J Robertson[1], J Dale[2], N Sattar[1] & D Porter[2]
1. University of Glasgow 2. Gartnavel General Hospital

Introduction

In patients with RA elevations in total, HDL and LDL cholesterol, with variable changes in TC to HDL-c ratio, have been reported after biologic drugs therapy. In contrast, lipidaemic data for conventional DMARDS is relatively scant. The Targeting Synovitis in Early Rheumatoid Arthritis (TaSER) study (NCT00920478) is an ongoing RCT in early RA comparing a standard DAS28-driven DMARD escalation strategy with one driven by musculoskeletal ultrasound (MSUS). All patients were treated in a stepwise fashion with DMARDS and/or etanercept as dictated by a standardised escalation protocol.

For this presentation we analysed the relationship between changes in lipid profile and disease activity in the context of an intensive "treat-to-target" strategy.

Methods

Newly diagnosed patients with RA were randomised between standard (DAS28) and MSUS-assisted assessment groups, with 3-monthly assessments of DAS and CRP. Non-fasting lipid profiles were obtained at baseline and after the 18-month treatment period in 34 patients who were not taking cholesterol-lowering or biologic drugs.

We analysed (a) the changes in lipid profile after 18 months of anti-inflammatory treatment; and (b) the correlation between lipids and measures of inflammation. Changes in mean measures of inflammation and lipids were analysed using paired t-test, with non-parametric data log transformed prior to analysis. Correlations between the lipid and inflammatory parameters were analysed using Pearson's coefficient and Spearman's rank coefficient depending on normality of data.

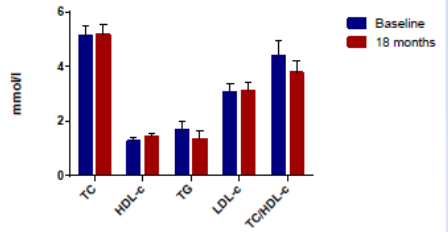
Results

Significant falls in mean DAS (4.44 v 1.75, $p < 0.001$) and CRP (44mg/l v 11mg/l, $p < 0.001$) were seen after treatment. Triglycerides and the TC/HDL-c ratio also fell, with a rise in HDL-c and a numerical but not statistical rise in TC (Table 1; Figure 1).

Significant correlations were seen between baseline CRP v TC ($r = -0.55$, $p<0.001$) and CRP v LDL-c ($r = -0.54$, $p < 0.001$); and change in CRP v TC ($r = -0.35$, $p=0.04$) and CRP v HDL-c ($r = -0.39$, $p=0.02$)(Figure 2). DAS did not correlate with any lipid values (Figure 3).

	Baseline	18 months	Change (% change)	*p*
Total cholesterol	5.11	5.17	0.06 (1%)	0.73
HDL-c	1.27	1.45	0.18 (14%)	<0.001
Triglycerides	1.68	1.34	-0.34 (-20%)	0.002
LDL-c	3.07	3.10	0.03 (1%)	0.83
TC/HDL-c	4.39	3.78	-0.61 (-14%)	<0.001
DAS	4.44	1.75	-2.69	<0.001
CRP *(mg/L)*	44	11	-33	<0.001

Figure 1. Serum lipid levels from baseline to 18 months



Conclusion

Aggressive use of conventional DMARDS in RA resulted in a fall in serum triglycerides and the atherogenic index, driven predominantly by an increase in serum HDL-c of 14%; this compares with documented increases in HDL-c of around 7% following anti-TNF treatment. CRP reduction over 18 months correlated with the rise in total and HDL-cholesterol. Given the importance of TC:HDL-c ratio in CVD risk prediction, these results add to the paradigm that lipid profiles are potentially favourably modified upon inflammation suppression.

Figure 2. Correlation of change in CRP v change in HDL-c

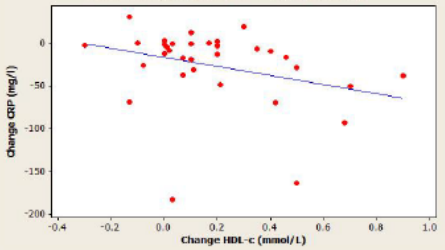
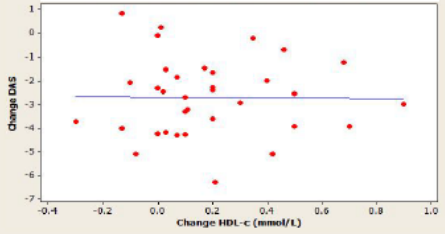


Figure 3. Correlation of change in DAS v change in HDL-c



Appendix C – KALIBRA recruitment poster

University of Glasgow

NHS Greater Glasgow and Clyde

KALIBRA

Kinetics of the **A**po B-containing **Li**poproteins in **I**L-6 **B**lockade for **R**heumatoid **A**rthritis

Objective	To analyse the effects of Tocilizumab therapy on the levels and structure of VLDL, IDL and LDL and their atherogenic potential in Rheumatoid Arthritis
Site	Glasgow Royal Infirmary, Gartnavel General Hospital & Stobhill Hospital
Patient Involvement	Attending the Clinical Research Facility (WIG) for fasting blood tests for one week on two occasions 10 weeks apart.

Inclusion Criteria

- Rheumatoid Arthritis (2010 criteria)
- Eligible for biologic therapy
- Able to comply with study protocol
- Male or female, aged 25 – 75

Exclusion Criteria

- Untreated hypothyroidism or recent change in thyroxine dose
- Known familial dyslipidaemia or apoE2 homozygosity
- Diabetes Mellitus
- Treatment with any lipid-lowering therapy
- Pregnancy
- Fasting triglycerides >3mmol/l or total cholesterol >6.5mmol/l
- Heparin hypersensitvity

If you have a patient who you plan to start treating with Tocilizumab and who would be interested in participating, please contact Dr Jamie Robertson at

jamie.robertson@glasgow.ac.uk

Appendix D – KALIBRA patient information leaflet

NHS
Greater Glasgow and Clyde

Contacts: Dr David McCarey (0141 211 4965)
Dr Duncan Porter (0141 211 3262)
Dr Jamie Robertson (07828 039 945)

PATIENT INFORMATION SHEET

KALIBRA - the relationship between IL6 and the kinetics of the Apo B – containing Lipoproteins in people with chronic inflammatory joint disease.

Invitation

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. Please feel free to talk to others about the study if you wish, and ask us if there is anything that is not clear.

Introduction

Patients with rheumatoid arthritis (RA) have inflammation in their joints causing pain, stiffness and difficulty in moving. In addition, patients with RA also have an increased risk of heart disease (causing angina or heart attacks) and stroke. This is thought to be caused by the effects of inflammation on lipoproteins: substances that contain cholesterol and transport it around the body. RA patients have low blood levels of lipoproteins, and their structure differs from healthy individuals without RA. However, some treatments for RA (such as tocilizumab) may be able to correct this.

What is the purpose of this research?

The purpose of this research study is to discover if reductions in inflammation can restore lipoprotein structure to normal, and how it does this, by taking blood samples before and after treatment with tocilizumab.

Why have I been invited to take part?

Your arthritis has not been well controlled on second line therapy, and you and your doctors have decided to try using a tocilizumab to improve your symptoms.

Do I have to take part?

No. It is up to you to decide whether you want to take part in the study. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive. Data collected up until then would be used but no further information would be collected. Your participation in the trial may not be of direct benefit to you (though all patients in the trial will be offered active treatment with the aim of achieving the best possible control of their arthritis), but may help in the development of treatment for the benefit of future patients. If you do take part in this trial, your GP will be informed of your participation and the treatment you receive.

Which treatments are being used in the study?

Everyone who takes part in the study receives tocilizumab, a drug that works to block the function of a protein in the blood named IL6. You will be treated in the same way as patients who do not take part in the study, using the same dose.

IL6 is produced by the body and causes inflammation, which helps to fight infection but is also the cause of the symptoms of arthritis. In arthritis patients, the levels of IL6 are high and blocking its action can improve pain and stiffness in the joints. On the other hand, it can increase the risk of infections. Tocilizumab is given once a month by injection into a vein, and involves attending the Rheumatology unit for a few hours on one day a month.

Are there any risks involved in taking part?

We do not anticipate anyone being put at risk by having blood taken, or by going through any other procedures in this trial, though participating in the trial does involve a commitment to attending hospital quite frequently as detailed later in this information sheet.

Tocilizumab has some potential side-effects. Commonly, patients taking tocilizumab may find they develop upper respiratory tract infections (minor coughs or colds) more often. All patients on tocilizumab should have blood tests monthly at their GP. Less common side effects are detailed in the patient information leaflet for tocilizumab, which you will be provided with.

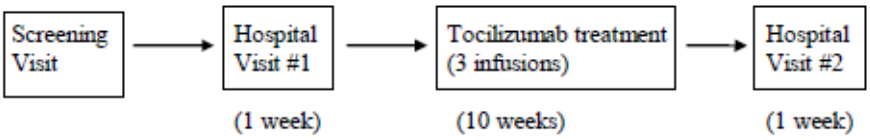
Patients will also receive two morning injections of heparin, a blood-thinning agent which allows us to measure specific proteins in blood. Heparin can increase a person's risk of bleeding, and rarely can cause allergic reactions or changes in the full blood count. However, your body can remove heparin very rapidly, and there will be virtually no trace of heparin in your blood by the afternoon.

What will happen to me if I take part?

Before you start treatment we will test you to make sure that you do not have hepatitis or evidence of previous exposure to TB. This will involve an examination, chest X-ray and some blood tests. You will be provided with the Arthritis Research UK information sheets on tocilizumab. We may need to wait for a period of 4 weeks to allow your body to "wash out" any previous medications which could interfere with the study. When you attend a "screening visit" at clinic to ensure you are eligible to take part, we will ask you to sign a consent form if you wish to take part.

Before treatment, you will receive an injection of a labeled amino acid called d3-leucine. This is completely safe (there is **NO** radioactivity – the label is called a 'stable isotope') and the body incorporates the d3-leucine into lipoprotein molecues in your body. Because the leucine is labeled we can study how the body makes, transports and uses up the lipoprotein in you. After the injection, we will take blood samples over the following 5 days (described below). After this, tocilizumab therapy will start. The

sequence of injection and blood tests will be repeated after 10 weeks of tocilizumab therapy to see if the way the body handles its lipoprotein has been affected by the tocilizumab treatment. From the first injection to the last, your participation in the trial will last a total of 12 weeks. The overall course of your participation will run as follows:



What will happen at each visit to the hospital?

You will attend the Clinical Research Facility in the Western Infirmary at 07:30am, without breakfast, where a small plastic line will be inserted using a needle into a vein. The d3-leucine injection will be given through this, and blood samples will be taken via a second line a total of 19 times between 8am and 9pm. At around 6pm you will be provided with a low-calorie meal, at which point (if you wish) you may return home with 2 blood tests at home that evening rather than in hospital. The following day, further blood samples will be obtained at 09:30am and 9:30 pm. Then, each morning for the following 3 days, a further blood sample must be obtained at 09:30. These tests can be done at home, but the final sample (on "day 5") must be performed at the Research Facility as it will involve an injection of heparin into the vein lasting around 15 minutes. The morning tests at home should be after only a light breakfast of tea (without milk) and toast, but throughout the day in hospital you will be encouraged to drink plenty of fluid. The times of blood tests are summarized below; those marked with an asterisk (*) may be taken at home:

Day 1: 0800; 0805; 0810; 0815; 0820; 0830; 0845; 0900; 0930; 1000; 1100; 1130; 1200; 1300; 1400; 1600; 1800; *meal provided*; 1900*; 2100*

Day 2: 0930*; 21:30*

Day 3: 0930*

Day 4: 0930*

Day 5: 0930

As noted, this whole process occurs twice – once before, and once after, treatment with tocilizumab. Each process will require taking 266ml blood over the course of 5 days, meaning each participant will give a total of under 1 pint of blood.

What should I do if I feel unwell?

Because all biologic drugs are associated with an increased risk of infection, it is important that if you develop any signs of infection (such as fever, pain passing your urine, cough with green spit) you should contact the hospital immediately for advice. You will be monitored closely during the trial. Patients who develop side effects to tocilizumab may stop the drug and withdraw from the trial at any time.

What happens at the end of the trial?

After the study, all patients will be assessed by a doctor to see how well their RA has responded to tocilizumab. If the patient wishes, and their joints have improved appropriately, they can continue with the drug as would any other patient who had been prescribed it.

Can I become pregnant or breast feed during the study?

You must not take part if you are breast feeding, pregnant, planning to become pregnant or are not using a reliable method of contraception. If appropriate we will advise you about contraception before you decide whether to take part in the trial, and a pregnancy test will be performed in women of child-bearing potential before starting treatment.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. You will be given a study number which will be used to identify study samples, and only members of the research team will have access to this information which will be kept on a file and stored in a secure place at the Western Infirmary, Glasgow. All samples will only be identified by the study number, therefore analyses will be carried out anonymously. Any information about you which leaves the

hospital or the clinical research facility will have your name and address removed so that you cannot be recognised from it.

What will happen to any samples I give?

You will donate blood samples for research purposes. Some analyses of these samples will be done straight away while others will be done at a later stage when we collect more samples from other patients. We will also store some of your samples for up to 10 years for use in other studies which will require to be reviewed and approved by the Ethics Committee if you agree to this. The samples will be treated as a "gift"; this means you will not be entitled to any future financial reimbursement related to their use. Future studies may also require access to your medical records, but again this would be subject to approval by the Ethics Committee.

Travel expenses

You can claim back any travel expenses associated with attending the hospital for the research, and participants will be reimbursed for their time.

What should I do if I am not happy with my care?

In the event that something goes wrong and you are harmed during the research, and this is due to someone's negligence, then you may have grounds for a legal action for compensation but you may have to pay your legal costs. There are no special arrangements for compensation in this study, and the normal National Health Service complaints and compensation scheme will still be available to you.

Private Health Insurance

If you have private medical insurance, you should check with your insurer whether participating in this study will affect your cover.

Who has reviewed this study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the West of Scotland (1) Research Ethics Committee.

Who is funding the research?

The research is being supported by funding from Roche, the pharmaceutical company who manufacture tocilizumab.

Can I find out the results of the research?

If you would like to know the final results of the study then a copy of the journal article will be sent to you on request. As a matter of routine we will also inform your GP of the results.

Who can I speak to if I want further information?

If you would like to speak to another health professional who is not directly involved in the study, please phone Sister Joan Roberts (telephone number 0141 211 3057). If you have any problems, or queries, contact Dr David McCarey, (telephone number 0141 211 4965) or Dr Jamie Robertson (telephone number 07828 039 945, email Jamie.robertson@glasgow.ac.uk)

Appendix E – KALIBRA patient consent form

Consent Form

Study Title: ***The relationship between IL6 and the kinetics of the Apo B – containing Lipoproteins in people with chronic inflammatory joint disease.***

Name of Researcher:

Please initial each statement

1. I confirm that I have read and understood the information sheet (version . . . .) for the above study and any questions I have asked have been answered to my satisfaction ----------

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason, and without my medical care or legal rights being affected ----------

3. I understand that sections of my medical notes may be looked at by responsible individuals who are either working on this study or from regulatory authorities where it is related to my taking part in this study. I give permission for these individuals to have access to my medical records ----------

4. I understand that my General Practitioner will be informed that I have agreed to participate in this study ----------

5. I understand that blood samples may be stored for future tests related to this study for 10 years ----------

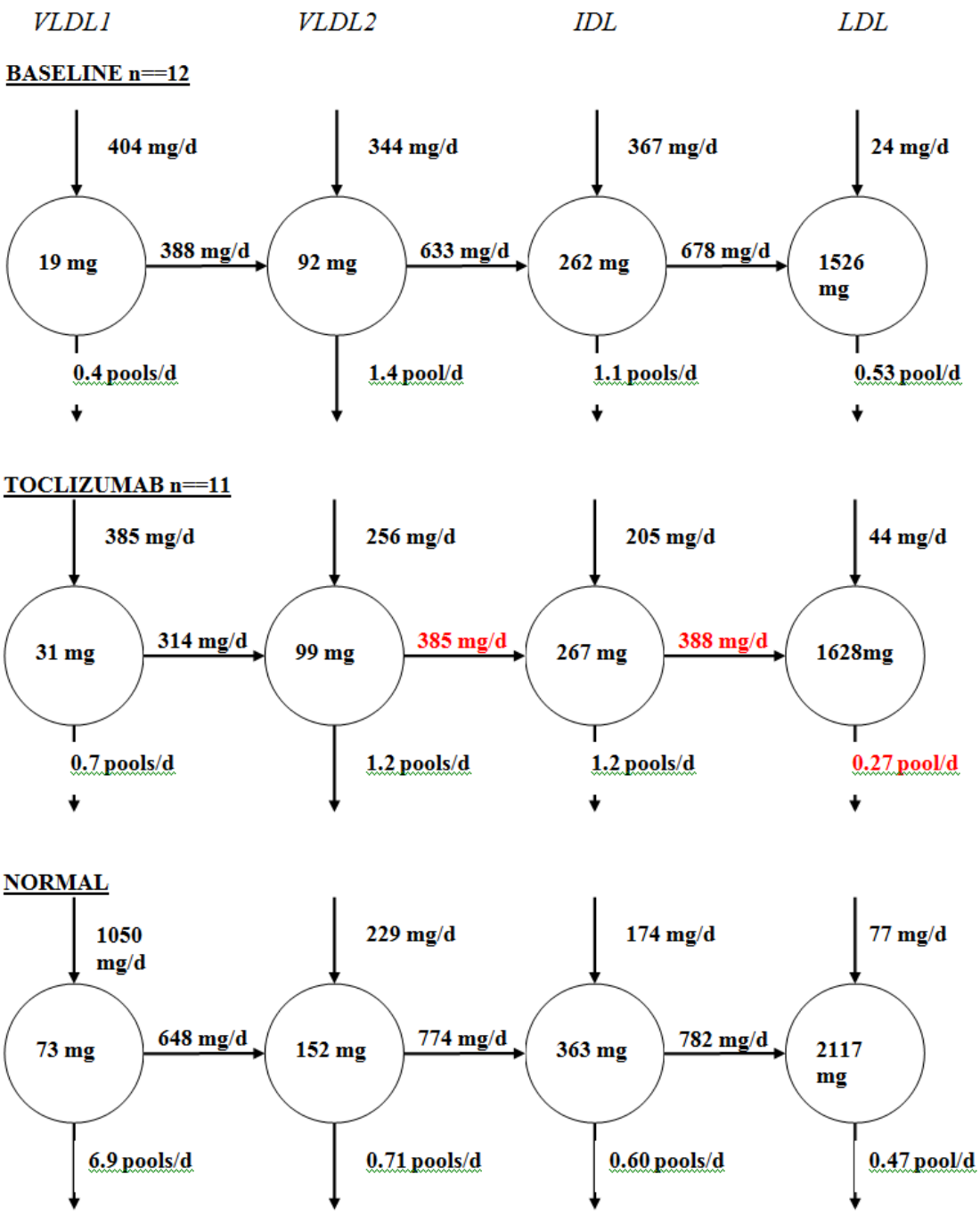
6. I agree to take part in the above study ----------

Signature of Participant	**Print Name**	**Date**
Signature of Person taking Consent	**Print Name**	**Date**
Signature of Researcher	**Print Name**	**Date**

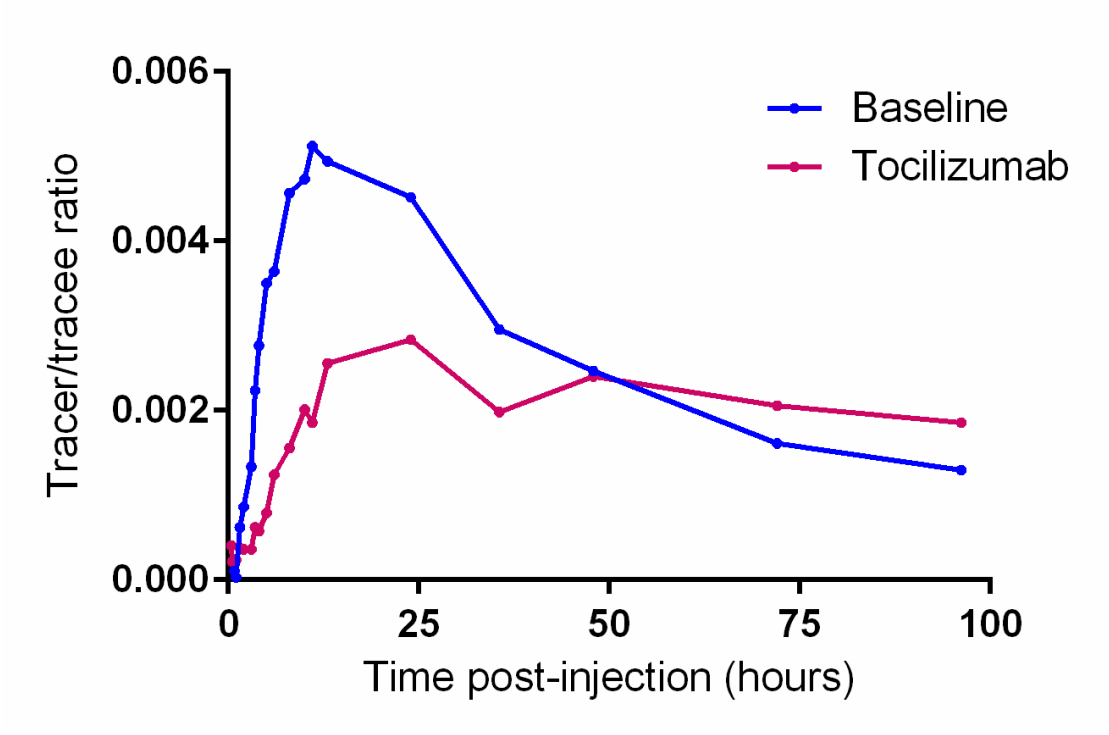
Copy for case record/CRF/patient (delete as appropriate)

GN11RH469 apoB kinetics study consent form v2

Appendix F – Kinetic overview of apoB-containing lipoproteins



Appendix G – LDL kinetic graph of subject KAL004



Appendix H – Genes studied in TLDA analysis

Gene Symbol	Protein Symbol	Protein name
Lipoprotein and scavenger receptors		
LDLR	LDLr	Low-density lipoprotein receptor
VLDLR	VLDLr	Very low-density lipoprotein receptor
LRP1	LRP-1	LDL-receptor related protein 1
LRP2	LRP-2	LDL-receptor related protein 2
LRPAP1	LRPAP	LRP-associated protein
SCARA1	SR-A1 (MSR1)	Scavenger receptor A1 (Macrophage scavenger receptor)
SCARA2	MARCO	Macrophage receptor with collagenous structure
SCARA3	SR-A3	Scavenger receptor A3
COLEC12	SR-A4	Scavenger receptor A4
SCARA5	SR-A5	Scavenger receptor A5
SCARB1	SR-B1	Scavenger receptor B1
SCARB2	SR-B2	Scavenger receptor B2
SCARB3	CD36	Cluster of differentiation 36
OLR1	LOX-1	Lectin-type oxidised LDL receptor 1
CD68	CD68	Cluster of differentiation 68
Cholesterol efflux proteins		
ABCA1	ABCA1	ATP-binding cassette A1
ABCA2	ABCA2	ATP-binding cassette A2
ABCA7	ABCA7	ATP-binding cassette A7
ABCG1	ABCG1	ATP-binding cassette G1
Intracellular proteins		
NR1H3	LXRα	Liver X receptor α
NR1H2	LXRß	Liver X receptor ß
FOXO1	FoxO1	Forkhead box O1
FOXO3a	FoxO3a	Forkhead box 03a
FOXO4	FoxO4	Forkhead box 04
PPARA	PPARα	Peroxisome proliferation activating receptor α
PPARD	PPARδ	Peroxisome proliferation activating receptor δ
PPARG	PPARγ	Peroxisome proliferation activating receptor ß
SREBF1	SREBP1	Sterol regulatory element binding protein 1
SREBF2	SREBP2	Sterol regulatory element binding protein 2
SCAP	SCAP	SREBP cleavage activation protein
HMGCR	HMGCOaR	HMG-coenzyme A reductase
SOCS3	SOCS	Suppressor of cytokine signalling 3
GAPDH	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase