Dyslipidaemia in Rheumatoid Arthritis

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Dr Tracey E Toms

School of Medicine

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ABSTRACT OF THESIS submitted by Dr Tracey E Toms for the PhD entitled **Dyslipidaemia in Rheumatoid Arthritis.** Month and Year of submission: March 2012.

Introduction: Rheumatoid arthritis (RA) is known to be associated with an increased risk of cardiovascular disease (CVD), resulting in excess mortality. Dyslipidaemia has been identified as a major CVD risk factor in the general population. Current evidence would suggest that lipid metabolism is altered in RA due to inflammation, and that use of anti-inflammatory therapy may reverse some of these changes. However, the impact of such lipid changes on CVD in RA remains unknown. Data regarding the effects of RA/drug therapy on lipid structure and function are sparse. Genetic factors are important in the pathogenesis of RA and play a central role in the regulation of lipid metabolism. To date, no studies have assessed the impact of genetic polymorphisms on lipids in RA.

The aim of this thesis is to: 1) assess the prevalence of dyslipidaemia in RA and the CVD risk this confers 2) establish the effects of inflammation on lipid levels and lipid ratios 3) assess the impact of anti-inflammatory drug therapy (anti-TNF, rituximab and intravenous glucocorticoids) on lipid levels, structure and function 4) assess the prevalence and associations of particular genetic polymorphisms (RA susceptibility and lipid metabolism regulating genes) with lipids in RA.

Methods: Data from 400 RA patients were used to address aims 1, 2 and 4 in cross-sectional studies. All patients had a clinical assessment and fasting blood taken. Blood was processed to provide data on the lipid profile, ESR, CRP and DNA was extracted for genotyping. Aim 2 and 4 also utilised a retrospective longitudinal cohort of 550 RA patients and the DNA from 400 healthy controls, respectively. Aim 3 was addressed using a longitudinal cohort including: patients due to commence anti-TNF (n=35), rituximab (n=10), intravenous glucocorticoids (n=12); 15 RA controls on stable therapy; and 40 healthy controls. Assessments and blood samples were taken at baseline, 2 weeks and 3 months.

Results: Dyslipidaemia was highly prevalent (56.8%), but undertreated in many RA patients at risk of developing CVD. Systemic inflammation associated with many of the changes in lipid levels and structure. Lipid ratios were found to be less susceptible to fluctuations due to inflammation. The use of anti-inflammatory drug therapy produced changes in lipid structure and function through both generic suppression of inflammation and drug specific mechanisms (particularly in the case of glucocorticoids). The prevalence of cholesterol ester transfer protein (CETP) and Apolipoprotein C3 genetic polymorphisms differed between RA patients and local population controls. RA susceptibility genes (HLA-DRB1-SE and TRAF1C5) and several 'lipid metabolism genes' (Apolipoprotein E, ATP-binding cassette transporter 1 (ABCA1) and CETP) were found to associate with lipid levels within the RA population.

Conclusion: Dyslipidaemia is highly prevalent in RA and currently undertreated. Dyslipidaemia in RA is regulated by numerous factors including inflammation, drug therapy and genetic factors. Further longitudinal studies are required to assess whether these findings have an impact on hard CVD endpoints.

Declaration

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

Copyright statement

Abbreviations

ABCA1: adenosine triphosphate- binding cassette transporter A1

ACAT: acyl co-enzyme A:cholesterol acyltransferase

ACE: angiotensin converting enzyme

ACR: American College of Rheumatology

AIDS: acquired immunodeficiency syndrome

ANA: anti-nuclear antibodies

ANOVA: analysis of variance

Anti-CCP: anti-cyclic citrullinated peptide

Anti-TNF: anti-tumour necrosis factor therapy

AP-HDL: acute phase high density lipoproteins

ApoA-I: apolipoprotein A-I

ApoA-II: apolipoprotein A-II

ApoB: apolipoprotein B

ApoE: apolipoprotein E

BCA: bicinchoninic acid

BMI: body mass index

CETP: cholesterol ester transfer protein

CHD: coronary heart disease

COXII: cyclooxygenase II

CRP: C reactive protein

CVD: cardiovascular disease

CyA: ciclosporin A

DAS: disease activity score

DGoH: Dudley Group of Hospitals

DMARDs: disease modifying anti-rheumatic drugs

EL: endothelial lipase

ELISA: enzyme-linked immunosorbent assay

eNOS: endothelial nitric oxide synthetase

EULAR: European League Against Rheumatism

ESR: erythrocyte sedimentation rate

FBC: full blood count

FGF: fibroblast growth factor

FRET: fluorescence resonance energy transfer

GC: glucocorticoids

GM-CSF: granulocyte-macrophage- colony stimulating factor

HAQ: health assessment questionnaire

HC: healthy controls

HCQ: hydroxychloroquine

HDL: high density lipoproteins

HIV: human immunodeficiency virus

HL: hepatic lipase

HLA: human leukocyte antigen

HMGCoA: hydroxymethylglutaryl coenzyme A reductase

HPLC: high performance liquid chromatography

HSP: heat shock protein

IDL: intermediate density lipoprotein

IL: interleukin

IFN: interferon

IR: insulin resistance

LCAT: lecithin-cholesterol acyltransferase

LDL: low density lipoproteins

LOX-1: lectin type oxidised low density lipoprotein receptor 1

Lp(a): lipoprotein (a)

IPAQ: international physical activity questionnaire

LPC: lysophosphatidylcholine

LPL: lipoprotein lipase

MAF: multidimensional assessment of fatigue scale

MCP: metacarpalphalangeal

M-CSF: macrophage-colony stimulating factor

MetS: metabolic syndrome

MMP: matrix metalloproteinase

MTP: microsomal triglyceride transfer protein

NCEP: National Cholesterol Education Programme

NF: nuclear factor

NHANES: National Health and Nutrition Examination Survey

NO: nitric oxide

NSAIDs: non steroidal anti-inflammatory drugs

OA: osteoarthritis

ONS: Office for National Statistics

OxLDL: oxidised low density lipoproteins

PAF-AH: platelet activating factor acetylhydrolase

PBS: phosphate buffer solution

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PIP: proximal interphalangeal

PLTP: phospholipid transfer protein

PON-1: paraoxonase

PPAR: peroxisome proliferator-activated receptors

PTPN22: protein tyrosine phosphtase, non receptor type 22

QoL: quality of life

OxLDL: oxidised LDL

RA: rheumatoid arthritis

RANK: receptor activator of nuclear kappa beta ligand

RASFs: rheumatoid arthritis synovial fibroblasts

RhF: rheumatoid factor

ROS: reactive oxygen species

SAA: serum amyloid A

SES: Stanford arthritis self-efficacy scale

SLE: systemic lupus erythematosus

SNP: single nucleotide polymorphism

sPLA2: secretory phospholipase A2

STAT4: signal transducer and activator of transcription 4

sTNF-R: soluble-tumour necrosis factor-receptor

TC: total cholesterol

TG: triglycerides

TGF: transforming growth factor

TMB: tetramethylbenzadine

TNF: tumour necrosis factor

TRAF1C5: tumour necrosis factor-receptor associated factor 1 and complement component 5

TSH: thyroid stimulating hormone

US: United States

VCAM: vascular cell adhesion molecule

VLDL: very low density lipoproteins

vWF: von Willebrand factor

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Lastly, but perhaps most importantly of all I would like to thank all the patients and healthy controls for giving their time to participate in this study. Without their help this research would not have been possible. I am sure there are other people who I have not acknowledged individually, but to everyone who has contributed to this thesis I would like to say a huge thankyou.

Contribution to work

The work included in this thesis is predominately all my own. However, for some aspects of the methodology there was a contribution from others. All of the routine laboratory tests e.g. full blood count, urea and electrolytes, erythrocyte sedimentation rate, C reactive protein were processed by Jackie Smith (Chief biomedical scientist). In order to process the vast number of genetic samples, the genotyping was undertaken by Jackie smith and myself. In addition, Jackie contributed to the separation of LDL via ultracentrifugation, due to the time consuming nature of the procedure. The remaining laboratory work including lipid subfraction analysis and assessment of LDL function was undertaken by myself. All statistical analyses were performed by myself, however, these were reviewed by the hospital statistician (Peter Nightingale).

CHAPTER ONE: Introduction

1.1 Introduction to Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease, which primarily affects the synovial joints. RA was first recognised in 1859 by Dr A.Garrod (1), when he described widespread joint pain and stiffness. However, almost another century passed before it was hypothesized that RA may have an autoimmune origin (2). Our understanding of the condition has advanced exponentially over the last 100 years, including our knowledge of the pathogenesis of the disease, clinical presentation, co-morbidities, and drug therapy. Current data suggests that RA affects approximately 0.8 % of the adult population in the United Kingdom (3), occurring with a female:male ratio of 3:1 and has a peak age of onset between forty and seventy years of age (4). The effects of uncontrolled inflammation in RA are far reaching and include, physical limitations secondary to joint pain and deformity, psychosocial dysfunction, and an excess mortality (5). For the purposes of this thesis RA was classified according to the 1987 American College of Rheumatology criteria (ACR) (outlined in Table 1)(6), requiring 4 out of the 7 criteria to be met for the patient to be classified as having RA. However, since starting work on this thesis the diagnostic criteria have been revised (2010 RA classification criteria) in an attempt to improve the sensitivity in early disease (7).

Table 1.1: The 1987 American College of Rheumatology criteria

	Qualifying criteria
1	Morning stiffness lasting > 1 hour before improvement
2	Arthritis involving 3 or more joints
3	Arthritis of the hand (particularly MCP, PIP and wrist joints)
4	Symmetrical distribution of joint involvement
5	Positive Rheumatoid factor
6	Rheumatoid nodules
7	Radiographic evidence of RA
MCP: metacarpalphalangeal joints, PIP: proximal interphalangeal, RA: rheumatoid arthritis	

1.2 Pathogenesis of RA

The pathogenesis of RA is likely to be multi-factorial with intense interaction between causative factors. Evolving data suggest a role for hormonal factors (8;9), infectious agents (10), genetic factors (11) and environmental factors (12). Although it is

hypothesized that these factors interact to initiate the onset of disease, it is less clear whether they have a role in disease perpetuation or whether this is a self-perpetuating process.

1.2.1 Genetic factors

The first suspicion of a potential genetic influence on the development of RA arose in the 1970's, when it was noted that many RA patients had a variation in the human leukocyte antigen (HLA) region of chromosome 6 (6q21.3) compared to the general population (13;14). With advances in genetic methodology, it is now known that specific HLA-DRB1 alleles that encode for the shared epitope (DRB1*0401, DRB1*0404, DRB1*0405 and DRB1*0408) associate with an increased susceptibility to RA in Caucasians (11;15). As it is widely accepted that the HLA genes are likely to only account for around 50% of the genetic risk (16), investigators began searching for other potential genes. The last decade has lead to several exciting discoveries in the field of genetic susceptibility to RA, including the role of mutations in the protein tyrosine phosphatase non receptor type 22 (PTPN22) (17;18), signal transducer and activator of transcription 4 (STAT4) (19;20) and tumour necrosis factor-receptor associated factor 1/complement component 5 (TRAF1-C5) genes (21). The impact of the PTPN22 mutation on the genetic risk of developing RA is second only to HLA-DRB1 (11). A single nucleotide polymorphism (SNP) in the PTPN22 gene results in the translation of a dysfunctional protein, and ultimately a reduced ability to down regulate activated T cells (22). A SNP mutation in STAT4, may alter the production of a transcription factor involved in cytokine signal induction (23). The link between a SNP in the TRAF1-C5 gene locus and the development of RA was recently established following a genome wide association study (21). Despite the identification of these and a range of other RA susceptibility genes, we remain unable to fully account for the genetic risk in RA, thus implying that further genetic mutations are still to be identified.

1.2.2 Infectious agents

There has long been speculation regarding a possible infectious trigger to the onset of RA. However, despite extensive investigation, a cause-effect relationship remains difficult to confirm or refute, as a result of the intricate interplay that exists between infection and inflammation, and also the potential time lag between exposure to the organism and the onset of symptoms. Irrespective of this, many infectious agents

including bacteria (e.g. Borrelia burgdorferi, Chlamydia) and viruses (e.g. Epstein Barr virus) mimic the symptoms of RA (24). The majority of infections tend to produce transient symptoms, but infections such as Borrelia burgdoferi can result in a form of arthritis virtually indistinguishable from RA, characterised by a chronic disease course and evidence of erosions (10). It is therefore possible that mechanisms including antigen/molecular mimicry may explain how an infective agent could initiate a chronic inflammatory process, and the clinical syndrome we recognise as RA (25;26).

1.2.3 Hormonal factors

RA predominantly affects females, with a ratio of 3:1, thus a natural assumption is that sex hormones may play a role in the pathogenesis. To raise suspicion further, remissions of RA during pregnancy and severe rebound flares of disease postpartum are well described (8;27). In addition, studies have demonstrated that the female sex hormones oestrogen and progesterone, may alter immune function by inhibiting Th1 responses (28). This offers a plausible explanation as to how RA, a Th1 driven disease, may improve during exposure to high levels of these hormones. However, there is an opposing immunological theory that RA remission during pregnancy is due to maternal immune responses to foetal paternally inherited class II HLA antigens (27;29). The exact contribution of hormones to both the onset and disease course of RA still needs to be further elucidated.

1.2.4 Environmental factors

Establishing a causal relationship between environmental factors and RA is challenging due to the presence of multiple confounding factors. Observational studies have identified a number of factors may either increase or reduce the risk of developing RA. The risk of developing RA may be increased from birth as an association between high birth weight and RA has been described (30). Conversely, other early environmental exposures, such as breast-feeding may reduce the risk (30). During a lifetime, exposure to multiple factors may add to this risk, including poor socio-economic status (31), low level of education (32), smoking (33), and geographical location (34).These factors may be particularly important in the context of gene-environment interactions, thus escalating an individuals predetermined genetic risk (35).

1.3 Pathophysiology of RA

1.3.1 The role of T cells

For many years RA has been considered as predominantly a T cell driven disease (36). The normal balance of CD8+ cytotoxic T cells and CD4+ helper cells has been shown to be disrupted in RA, with an increased ratio of CD4+:CD8+ cells (37). T cells have also been found in abundance in the synovial tissue, indicating a potential role in producing some of the clinical manifestations of RA (38). T cells become activated following interaction with an antigen-presenting cell, resulting in the production of cytokines including interleukin-2 (IL-2), interleukin-17 (IL-17) and interferon gamma (IFNγ).

1.3.2 The role of B cells

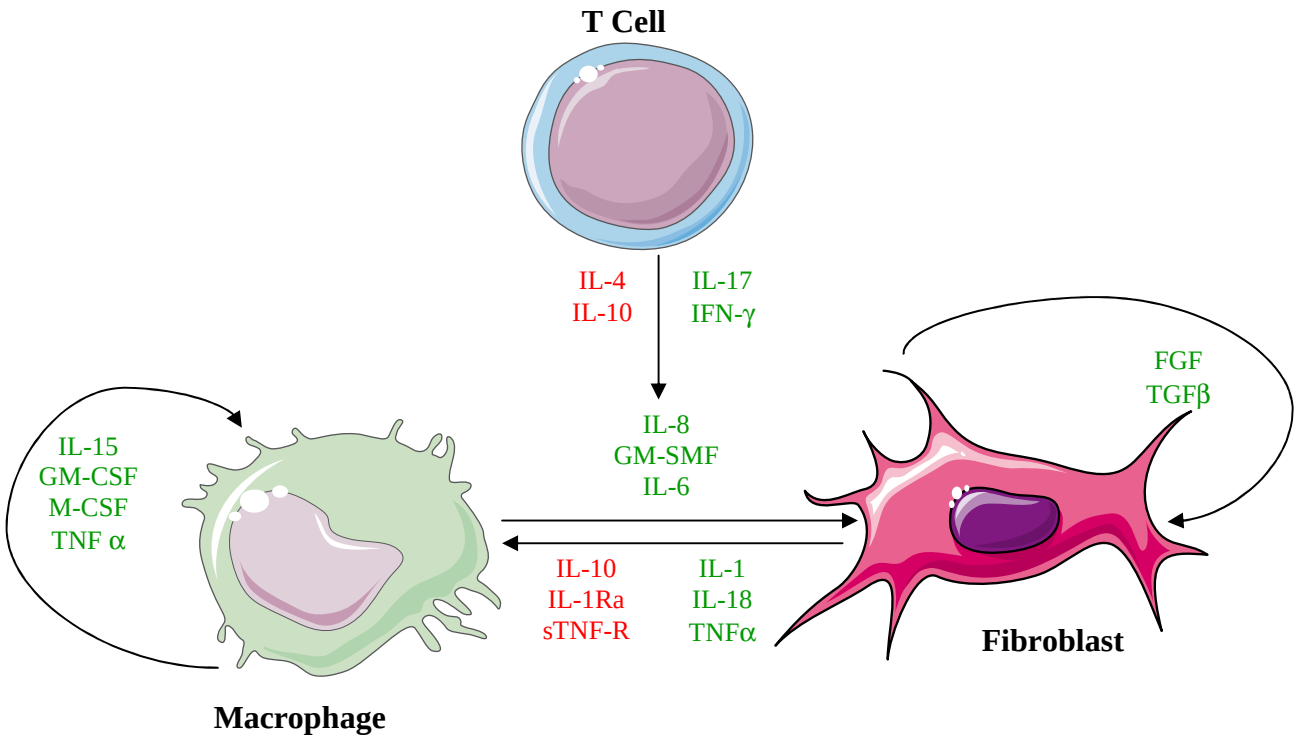
The potential contribution of B cells to the pathogenesis of RA has been revisited in recent years. Much of this interest has been sparked by the discovery that disease activity improves and remission can be achieved by B cell depletion, induced by anti-CD20 monoclonal antibody treatment (39;40). B cells are ultimately responsible for auto-antibody production, including rheumatoid factor (RhF) and anti-cyclic citrullinated peptide antibodies (anti-CCP). This occurs when activated T cells stimulate the transformation of B cells in to plasma cells. The auto-antibodies secreted by plasma cells form immune complexes which can contribute to neutrophil activation and inflammation (26).

1.3.3 The role of cytokines

Cytokines are molecular mediators for many normal biological processes. Cytokines act in a self-regulatory manner to maintain homeostasis between the actions of pro-inflammatory (e.g. tumour necrosis factor alpha (TNFα), interleukin-1 (IL-1)) and anti-inflammatory (e.g. tumour growth factor beta (TGF β) interleukin-10 (IL-10)) cytokines. The balance between the two opposing subsets of cytokines is disturbed in RA due to the up regulation of pro-inflammatory cytokines (41;42). Cytokines are produced by a variety of cells including macrophages, monocytes, T cells and fibroblasts, and have the ability to activate themselves or their neighbouring cells (43). (see **figure 1.1**). Thus it is unsurprising that they are found in abundance in not only the serum of RA patients but also the synovial fluid and tissue. The release of each cytokine

triggers a specific cascade of events, ranging from macrophage activation resulting in the release of further pro-inflammatory cytokines (e.g. interleukin-1 (IL-1), Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα)) to the clonal expansion of T cells and osteoclast activation (44). Osteoclast activation is regulated by RANK Ligand, a receptor activator of nuclear factor (NF)-κβ which is abundant on T cells and synoviocytes. The combination of the presence of these cells and certain cytokines triggers osteoclast maturation and activation, and ultimately leads to bone resorption and the development of erosions (43). Understanding the fundamental role of cytokines in RA has lead to the production of several very successful targeted therapies including anti-TNF therapy and Anakinra (IL-1 receptor antagonist).

Figure 1.1: Cytokine production and self regulation (Adapted from (43))



Green text = Positive effects, Red text = negative effects
IL: Interleukin, IFN -γ: interferon gamma, TGF β: tumour growth factor beta, TNF α: tumour necrosis factor alpha, sTNF-R: soluble-tumour necrosis factor-receptor, M-CSF: macrophage- colony stimulating factor, GM-CSF: granulocyte macrophage- colony stimulating factor, FGF: fibroblast growth factor

1.4 Clinical presentation of RA

RA is traditionally considered to primarily be a disease characterised by symmetrical synovial joint pain and swelling, and morning stiffness lasting greater than 1 hour. However, the onset of RA can vary greatly, with some patients presenting with a gradual onset of a mono-arthritis and others with an explosive polyarticular onset. In addition to articular symptoms, patients may also present with constitutional symptoms of general malaise, weakness, fever, weight loss and myalgia.

As part of the systemic nature of RA, patients often manifest features of extra-articular disease. These can be very diverse, ranging from the development of rheumatoid nodules, scleritis and cachexia to lung fibrosis, pericarditis and vasculitis. The presence of extra-articular disease has harsh implications including increased mortality (45).

Several predictors of disease severity have been identified. Factors found to associate with a worse prognosis include seropositivity (positive RhF or anti-CCP) (46), early radiographic damage (erosions) (47), multiple joint involvement at first presentation (46;48), and high levels of inflammatory markers (49). The identification of prognostic markers has helped to guide the intensity of disease management.

1.5 Drug therapy in RA

The 20th century led to the gradual introduction of several disease modifying anti-rheumatic drugs (DMARDs) for the management of RA. Many of these medications were not developed with the primary objective of managing RA, but were later coincidentally found to be effective e.g. hydroxychloroquine, intramuscular gold. Despite intense investigation the mechanism of action of many of the traditional DMARDs (including sulphasalazine, and gold) remains elusive. The discovery of glucocorticoids (GCs) in 1949 lead to such dramatic improvements in the clinical signs and symptoms of RA, that they were for several years considered as a ‘cure’(50). However, the undesirable side effect profile and loss of efficacy over time made them far from ideal for the long-term management of RA (51;52). Rapid advances in our understanding of drug pharmacology and the pathogenesis of RA have revolutionised the management, with the evolution of therapies specifically targeting components of the immune cascade e.g. anti-tumour necrosis factor therapy (anti-TNF), and anti-CD20

therapy (rituximab). Current management of RA varies slightly between clinicians, however the treatment algorithm is generally initiated with conventional DMARDs either as mono-or combination therapy. Failure to respond to two or more DMARDs will prompt the prescription of an anti-TNF agent, either in combination with a DMARD or alone. Lack of efficacy or side effects will result in either switching to an alternative anti-TNF agent or the prescription of rituximab. The management of RA has been transformed from a 'watch and wait' approach to more aggressive management strategies, including the use of drugs in combination and a rapid escalation of therapy if there is no clinical improvement (53). GCs are still used widely in the management of RA, but tend to be reserved as a rescue therapy for severe flares of disease and are now only used as a mainstay of treatment in a minority of patients.

1.6 Comorbidity in RA

Patients with RA have an excess morbidity and mortality compared to the general population (54;55), resulting in a significantly shortened lifespan of 5-10years (56). Several conditions including cardiovascular disease (CVD), lymphoproliferative disorders and osteoporosis have been found to occur in association with RA, and may account at least in part for the increased mortality.

1.7 Cardiovascular disease in RA

CVD is the leading cause of death in RA, being responsible for around a half of all RA deaths (57). CVD in RA presents in many guises including myocardial infarction, congestive cardiac failure, and pericarditis. Of these, it is the ischaemic pathologies attributable to atherosclerotic disease that are the most common and confer the greatest increase in morbidity and mortality (58;59). Atherosclerotic disease in RA is often silent or presents atypically, therefore creating difficulties in the diagnosis and management of the condition (60). RA patients also have a poorer prognosis following a myocardial infarction (MI), with significantly higher death rates reported with the initial event (61). The potential harm posed by CVD in RA has sparked an explosion of research in an attempt to identify contributing factors (traditional and novel disease specific) and methods of addressing these. To date the role of several traditional risk factors for CVD has been studied in RA, including hypertension (62;63), insulin resistance (64), and

obesity (65;66). However, in-depth data on the impact of lipid levels, structure and function on the development of atherosclerosis in RA is sparse.

1.8 Atherosclerotic plaque development: the role of inflammation and lipids

Coronary artery disease develops due to the formation and rupture of atherosclerotic plaques. The term atherosclerosis covers a spectrum of disease ranging from endothelial dysfunction and fatty streak development, through to the formation and rupture of a mature plaque. The development of atherosclerotic plaques is complex, and it is dependant on the involvement of multiple interacting factors. Inflammation has been shown to be fundamental to all stages of atherosclerotic plaque development (67), with an intense bi-directional interaction occurring between lipids and inflammation. In RA, a disease associated with a heightened inflammatory state, these processes may be accelerated.

1.8.1 Plaque initiation

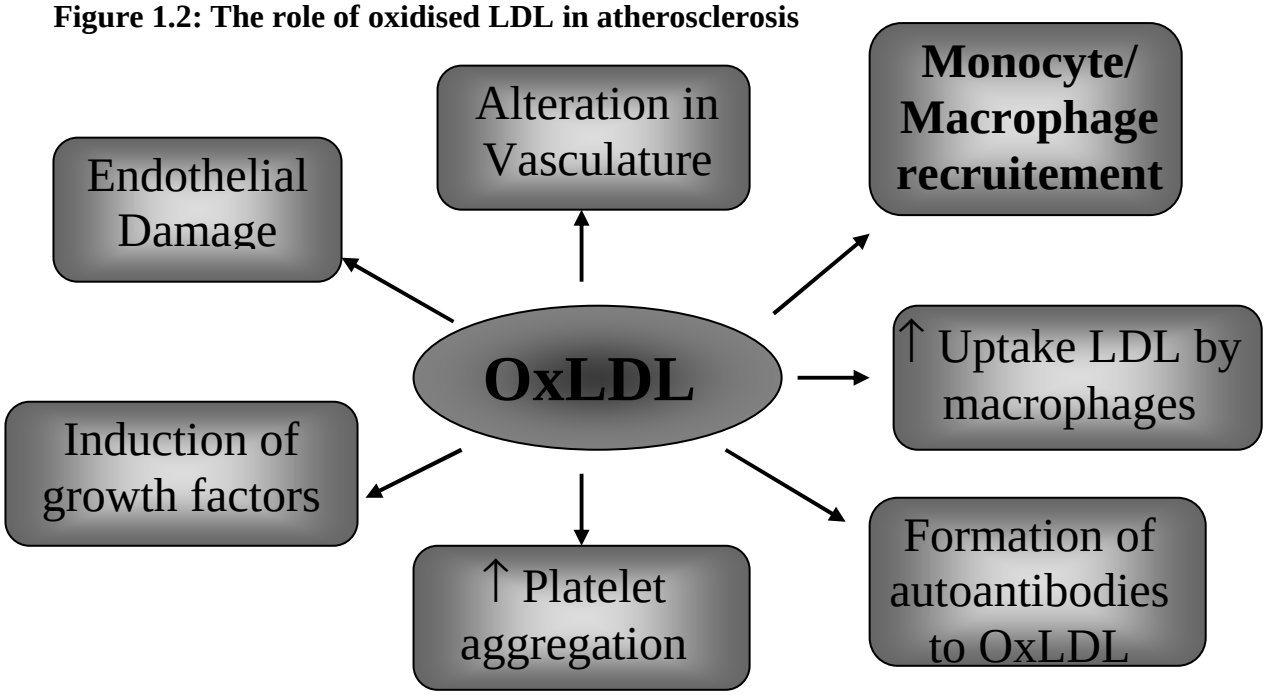
Endothelial dysfunction is the initiating step in plaque development (68). The vascular endothelium is a dynamic structure that forms a functional barrier between vessel wall and blood stream, regulates vascular tone, and controls coagulation and fibrinolysis. Healthy endothelium exerts a number of vasoprotective effects such as vasodilation, suppression of smooth muscle cell growth and inhibition of inflammatory responses, thereby helping to protect against atherosclerosis. Nitric oxide (NO) mediates many of these effects by inhibiting the oxidisation of low-density lipoproteins (LDL) and platelet aggregation, as well as opposing the effects of endothelium-derived vasoconstrictors. Prostacylin and bradykinin also act as vasodilators with beneficial vasoprotective effects. Healthy endothelium also releases potent vasoconstrictors, angiotensin II and endothelin, which produce proatherogenic effects by promoting smooth muscle cell proliferation and pro-oxidant effects on LDL. Endothelial damage occurs when the fine balance between these vasoconstrictive and vasodilatory pathways is disrupted. Although endothelial dysfunction is likely to be a multi-factorial process, the major cardiovascular risk factors such as hypercholesterolaemia, hypertension, diabetes and smoking have been implicated via their ability to increase the production of reactive oxygen species (ROS) (69). It is postulated that the increase in reactive oxygen species

may in turn reduce endothelial nitric oxide (NO) availability (70;71). Multiple lipid abnormalities have been associated with endothelial dysfunction. Hypercholesterolaemia has been shown to cause focal activation of the endothelium in medium and large arteries and has been associated with an increased number of monocytes entering the intima (72). High levels of oxidised LDL (oxLDL) may down regulate endothelial NO synthase (eNOS), thus reducing available NO and restricting coronary vasodilation (73). High levels of circulating triglycerides (TGs) may also damage the endothelium via their oxidative charge and result in disruption of the normal NO pathway (74;75). Studies in to the effects of lipoprotein (a) (Lp(a)) have shown elevated levels to be inversely correlated with small artery compliance and NO production (76;77). High levels of systemic inflammation may also disrupt endothelial homeostasis via the NO pathway, by reducing the expression of eNOS and increasing the expression of inducible NO synthase producing a net excess of NO (78).

1.8.2 Fatty streak development

Due to the increased permeability of the dysfunctional endothelium, excess LDL infiltrates the artery wall and is retained in the intima by matrix components, primarily at sites of heamodynamic strain. LDL then undergoes modification and oxidisation leading to the release of phospholipids, inducing the endothelial cells to express leukocyte adhesion molecules (79) and initiating an inflammatory response in the artery wall(80). (The contribution of oxidised LDL particles to the development of atherosclerosis is summarised in **figure 1.2**).

Figure 1.2: The role of oxidised LDL in atherosclerosis



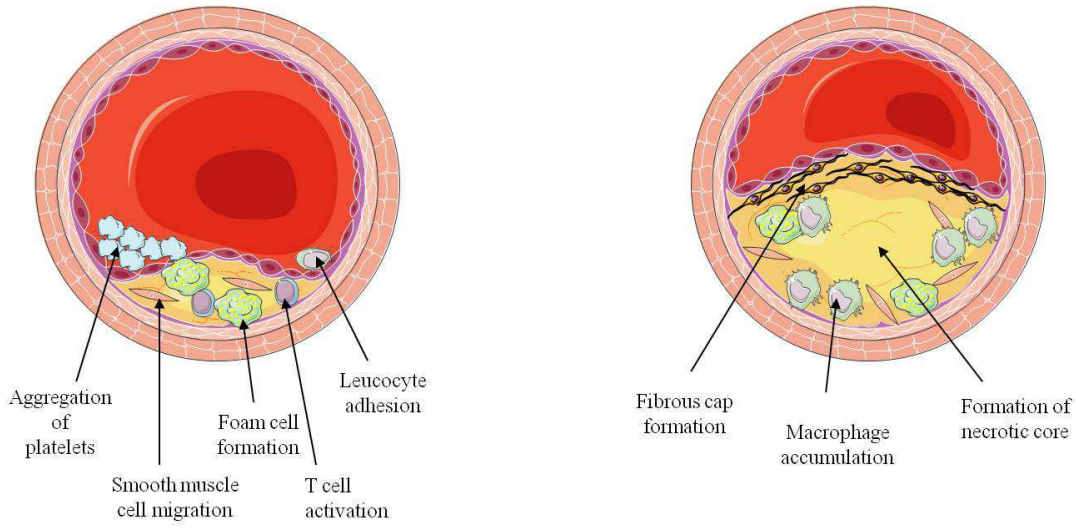
The first cells attracted to the activated endothelium are platelets (81). They adhere via glycoproteins on their surface, triggering further endothelial activation resulting in leukocyte infiltration (82). Further inflammatory cells adhere via the expressed leukocyte adhesion molecules. Of interest, vascular cell adhesion molecule (VCAM-1) is up regulated in response to hypercholesterolaemia, allowing monocytes and lymphocytes to preferentially attach (83). This initiates a cascade of chemokine production in the intima, resulting in migration of the cells across the inter-endothelial junction. Once in the sub endothelial space, monocytes are transformed to macrophages by macrophage colony stimulating factor, resulting in up-regulation of scavenger receptors and toll-like receptors (84). Subsequent incorporation of LDL via endocytosis by scavenger receptors (e.g. CD36) differentiates them further in to foam cells. Lipoproteins are also taken up by intimal smooth muscle either through the native lipoprotein receptors or a scavenger receptor. Toll-like receptors bind microbes, human heat shock protein (HSP) (85) and oxLDL (86) causing activation of macrophages, and the subsequent production of inflammatory cytokines. Smooth muscle cells also migrate into the abnormal area of artery stimulated by platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF2), and TGF-β. The accumulation of foam cells, smooth muscle cells and T cells results in the formation of a fatty streak (**Fig. 1.3a)**, the earliest recognisable lesion of atherosclerosis (87).

1.8.3 Plaque maturation

Fatty streaks can progress to form intermediate and advanced lesions (**Fig. 1.3b)** by amplification of the processes involved in formation of fatty streaks, e.g. smooth muscle migration and LDL infiltration/modification. The advanced lesions tend to form a protective fibrous cap that walls off the lesion from the lumen as a consequence of increased activity of TGF-β, PDGF, IL-1, and TNF-α, and decreased connective tissue degradation. The fibrous cap covers leukocytes, lipids and debris, which can form a necrotic core containing apoptotic cells such as smooth muscle cells and macrophages. The lipid core of the plaque is rich in tissue factor, which plays a vital role in thrombus formation upon plaque rupture (88).

Figure 1.3: The formation of a fatty streak (Fig 1.3a) and an advanced plaque (Fig 1.3b)

Fig. 1.3a **Fig.1.3b**



1.8.4 Plaque Rupture

Plaque instability and rupture occurs due to uneven thinning or erosion of the fibrous cap. Destabilisation and degradation of the fibrous cap results from the production of inflammatory cytokines, proteases, radicals, coagulation factors and vasoactive molecules from activated macrophages, T cells and mast cells (89). Matrix metalloproteinase (MMP) expression is increased in the cells resident in atherosclerotic plaques, due to the effects of cytokines, oxLDL, and haemodynamic stress. MMPs such as collagenases, elastases, and stromelysins, promote plaque instability by excessive degradation of the extracellular matrix of the fibrous cap (90), and further implicating an immune response (91). The above events lead to plaque rupture, exposing the contents of the core of the plaque (tissue factor, lipids etc) to the circulating blood. The clotting cascade is activated via the interaction of tissue factor with factor VII (92). Exposure of the highly thrombotic lipid core generates thrombi up to six times larger than exposure of other components of the arterial wall (93). As the thrombus forms and enlarges, the arterial lumen narrows and ischaemic symptoms may present.

1.9 Atherosclerosis in RA

Atherosclerosis in RA may be enhanced through several pathways. Firstly, traditional cardiovascular risk factors such as hypertension, insulin resistance and obesity may

occur more frequently in RA. However, disease specific factors such as systemic inflammation, activation of the coagulation pathway and hyperhomocysteinaemia may also confer additional cardiovascular risk. High levels of systemic inflammation have been identified as an independent risk factor for plaque development (94) and may exert this effect by increasing levels of oxidative stress, activating coagulation and secondary dyslipidaemia (78). In addition, to these factors RA patients may be genetically predisposed to the development of atherosclerosis and myocardial infarction (95).

1.10 Traditional cardiovascular risk factors in RA

Although the precise contribution of traditional risk factors to the development of atherosclerosis in RA is still a subject of some debate, current data suggests that traditional risk factors alone do not account fully for the extent of cardiovascular risk witnessed (96). However, traditional CVD risk factors may be modified in patients with RA, and remain important to identify as they can be readily treated.

1.10.1 Hypertension

Hypertension is highly prevalent in RA, with reported prevalence rates ranging from 3% (97) to 73% (98). However, due to a lack of appropriately designed studies it is still unclear whether these rates are comparable or higher than those seen in the general population (63). There are several plausible reasons why the blood pressure of RA patients may be higher than observed in the general population, including drug use (e.g. GCs and non-steroidal anti-inflammatories) (51), systemic inflammation triggering NO mediated vasoconstriction (99;100) and increased inactivity (101;102) secondary to physical disability. Although, hypertension in RA has been identified as one of the most significant independent predictors of CVD (103) it remains sub-optimally diagnosed and managed in RA (62).

1.10.2 Insulin resistance

Insulin resistance is a complex condition that arises when a given concentration of insulin cannot sustain a normal blood glucose level. In the general population insulin resistance is predominantly found in association with visceral obesity. Visceral adipose tissue is metabolically active and readily produces pro-inflammatory cytokines including TNF-α, IL-1 and IL–6. The release of pro-inflammatory cytokines is believed

to profoundly disrupt the normal actions of insulin on fat and muscle cells, thus inhibiting glucose release. RA patients appear to be particularly predisposed to this condition, ultimately contributing to atherosclerosis (104). Reasons underlying this phenomenon include the use of biologic and non biologic DMARDS, alterations in fat:lean mass ratio leading to obesity, and the high levels of circulating inflammatory mediators (105). Therefore, modifying this risk factor in RA is likely to require a multi-disciplinary approach, with increased exercise, dietary advise, and optimisation of disease activity with judicious use of GCs.

1.10.3 Obesity

It is well established that obesity is associated with an increased cardiovascular risk (106), and that certain distributions of body fat are associated with a higher risk e.g. central and visceral adiposity (107-109). Accumulation of centrally deposited adipose tissue may incite metabolic and immune responses that stimulate the development of cardiovascular risk factors such as dyslipidaemia and hypertension (110). The onset of obesity can be triggered by reduced levels of physical exercise, changes in satiety control, hormonal changes or as a result of genetic predisposition.

Many RA patients struggle to exercise regularly and even find physical daily tasks, such as dressing and washing a challenge. These restrictions are found in varying degrees throughout the RA population and can affect patients with all spectrums and stages of the disease. In addition, GCs are used widely in RA as a short-term measure to control disease flares, and in a smaller percentage as long-term salvage therapy (111). A frequently occurring side effect of GC use is weight gain, which is induced by hyperphagia and the retention of sodium and water (112). Collectively, the effects of medications, reduced exercise and systemic inflammation, may contribute to the development of the condition termed rheumatoid cachexia. This is a condition that affects approximately two thirds of RA patients, and is characterised by depletion of lean body mass and progressively increased fat mass, without associated changes in body weight (65). These observations have lead to the production of RA specific body mass index (BMI) cut offs, allowing at risk patients to be identified in a clinical setting and subsequent management to be modified (113)

1.10.4 Dyslipidaemia

Definition of dyslipidaemia

The term dyslipidaemia encompasses a broad range of abnormalities of lipoprotein metabolism resulting in the over and underproduction of lipoproteins.

Prevalence and associations of dyslipidaemia in RA

Dyslipidaemia in RA is likely to be governed by many factors, including disease activity (114), reduced physical activity secondary to pain and disability (115), and drug therapy (116). Although, collectively these factors are likely to exert a significant influence on the lipid profile of RA patients, the potential contribution of genetic factors controlling lipid metabolism has also not been addressed. This may partially explain why the prevalence of dyslipidaemia in RA varies between populations. Two studies have commented on prevalence. The first study by Dessein et al, assessed the prevalence of dyslipidaemia in 87 patients with inflammatory arthritis (117). According to the United States (US) National Cholesterol Education Program (NCEP) guidelines (118), 55% of inflammatory arthritis patients were dyslipidaemic compared to 8% of controls. Unfortunately, these figures are not specific to RA, but encompass a broad spectrum of inflammatory joint disease, (RA, spondyloarthropathies and undifferentiated inflammatory arthritis), exhibiting varying degrees of inflammation and different disease specific characteristics. The second study was carried out on 60 RA patients but was restricted to males (119), thus not reflecting the typical disease population . The investigators reported 68% of patients had serum levels of TC, high density lipoproteins (HDL) or LDL that would be considered as risk factors for the development of atherosclerosis according to NCEP criteria.

Dyslipidaemia appears to manifest in RA patients with both early (120) and advanced disease (121). While the inflammatory burden can be partially blamed for dyslipidaemia in early RA, there is also evidence to suggest that lipid profile may already be altered prior to the onset of disease. One retrospective study performed in the Netherlands on 1078 blood donors identified 79 patients who later developed RA, and compared the lipid profiles of the RA patients to controls from blood samples taken at least 10 years before the onset of RA (122). Patients who later developed RA were found to have lower levels of HDL, and higher levels of TC, TG and Apolipoprotein B (ApoB). A further population-based incident cohort study of 577 RA patients, found that TC and

LDL levels were suprssesed during the 5 years preceeding the onset of RA (123). Such observations indicate that either that alterations in the lipid profile may render people more susceptible to the future development of RA (124), or that RA patients are genetically predisposed to the development of RA related dyslipidaemia, or that the transcription of these genes is altered by persistent inflammation.

Alterations in the lipid profile in RA

In 1963, London et al (125) described a negative association between TC levels and RA disease activity, but no data were produced regarding the effects of inflammation on other lipid parameters. Nevertheless, this study acted as a springboard for future studies to evaluate the contribution of dyslipidaemia to the cardiovascular risk seen in RA. Currently, the most widely reported abnormality of the lipid profile associated with active RA is suppression of HDL levels (120;126;127). Although, a fall in TC levels has been observed (125), the drop is not as substantial as that seen in HDL levels, thus producing an increased atherogenic index (TC:HDL ratio) (120). The atherogenic index may be even higher if TC levels increase as claimed by several investigators (128). There is some controversy over the effects of inflammation on TGs and LDL. The effects of heightened disease activity on TGs is conflicting, with some studies showing a significant elevation (128) and others a reduction (127). Similar to TGs, the precise relationship between LDL and inflammation also remains unclear (128;129). Much of this controversy can be explained by small study size and differences in the populations studied.

To date, the National Health and Nutrition Examination Survey (NHANES III) is the largest study to compare lipid profiles of untreated RA patients to a control group (116). This study recruited 128 patients with RA, but only 104 patients were untreated with DMARDs or GCs and thus included in the analysis. Although all major components of the lipid profile were analysed, only HDL and Apolipoprotein A-I (ApoA-I) levels were found to be lower in the RA patients.

There is growing evidence that ratios of lipid components and apolipoproteins have a higher predictive value of first myocardial infarction than individual components of the lipid profile (130). Ratios of particular interest include: TC:HDL (discussed above), LDL:HDL and Apo B:ApoA-I. In RA, not only are all three of these ratios increased

(120), but they may offer a more reliable way of assessing the lipid profile by overcoming individual fluctuations in lipids occurring as part of disease flares.

HDL and LDL particles are categorised according to their size and density. This is clinically relevant as small dense LDL particles (LDL3) more readily infiltrate the endothelium and are more prone to oxidative change than their larger counterparts (131). Whereas larger HDL particles (HDL2) are more successful at performing reverse cholesterol transport and thus confer greater cardio-protection (132). In RA, only one small study has assessed the characteristics of lipoprotein sub fractions (133). This study, on 31 RA patients, demonstrated significantly higher levels of small dense LDL and lower levels of HDL2 compared with controls. Interestingly, there were no changes in the levels of basic components of the lipid profile, except LDL levels, which were lower in the RA group.

Lp(a) is a low density lipoprotein particle in which apolipoprotein B-100 is bound to apolipoprotein-A. Current evidence suggests Lp(a) may be a key factor in the development of atherosclerosis (134). The recognition of Lp(a) as an independent cardiovascular risk factor in the general population (135) has sparked interest into the role of this lipoprotein in RA. Levels of Lp(a) have been found to be increased in RA patients (120;128). The significant increases in Lp(a) could be purely as a direct result of inflammation or may be due to increased genetic expression (136). Irrespective of these findings, it is still unclear whether increased levels of Lp(a) accelerate atherosclerosis in RA. **Table 1.2** summarises the findings from all the available studies reporting lipid changes in untreated RA.

RA has been associated with high levels of oxidative stress (137). The increased oxidative load may contribute to articular tissue damage and perpetuates the inflammatory process (138), but may also be strongly implicated in the pathophysiology of CVD in RA (139). In RA, established data demonstrate that LDL within the synovial fluid of an inflamed rheumatoid joint is prone to oxidative modification (140). More recently it has also been reported that increased levels of oxLDL are seen in the plasma of RA patients with ischaemic heart disease (141). The trigger for LDL oxidation in RA is not fully understood, particularly as a study by Kim et al suggests that inflammation does not play a significant role (142).

In summary, RA patients with active disease develop a pro-atherogenic lipid profile with low HDL levels and raised atherogenic indices. The additional influence of the other lipid components (LDL and TG), and lipid sub fractions on cardiovascular risk in RA requires further investigation.

Table 1.2: Studies of lipid profiles in patients with untreated Rheumatoid

Study	Study design	TC	HDL	LDL	TG	Lp(a)	Apo A1	TC:HDL ratio	LDL:HDL ratio	apoB:apo A1 ratio
Georgiadis (143)	40 RA <1 yr 45 controls	↑*	↓	↑*	↑*	N/S	↓*	↑*	↑*	N/D
Georgiadis (144)	58 RA <1 yr 63 controls	↑	↓*	↑	↑	N/S	N/S	↑	↑*	N/S
Dursunoglu (128)	87 female RA 50 controls	N/D	↓*	↑	↑*	↑*	N/S	↑*	↑*	N/S
Choi (116)	104 RA age >60 4758 controls	N/D	↓	N/S	N/D	N/S	↓	↑	N/S	N/S
Yoo (114)	184 RA 161 controls	↓* females ↓males	↓females ↓* males	↑* females ↑males	↑ females ↑males	↑* females ↑* males	↓females ↓ males	↑ females ↓ males	↑ females ↑males	↑ females ↑* males
Lee (145)	21 RA 19 controls	↓	↓*	N/D	N/D	↑	N/S	N/D	N/S	N/S
Park (120)	42 RA 42 controls ?disease severity		↓*			↑*	↓*	↑*	↑*	↑*
Seriolo (146)	137 RA 78 controls	↓*	N/D	↓*	N/D	↑*	N/D	N/S	N/S	N/D
Lakator (147)	129 RA (77 GC, 52 NSAID) No diff between drugs 1374 controls	↑	↓*	↑*	↓*	N/S	N/S	N/S	N/S	N/S
Svenson (148)	48 RA 21 sero-ve SA	↓	↓*	↓*	↓*	N/S	N/S	N/S	N/S	N/S

* = significant change, N/D = no difference between RA and controls, N/S = not studied, SA = spondyloarthropathies
RA: Rheumatoid Arthritis, GC: glucocorticoids, NSAID: non steroidal anti inflammatory drugs, TC: total cholesterol, HDL: high density lipoproteins, TG: trigylcerides, LDL: low density lipoproteins, ApoA: apolipoprotein A, ApoB: apolipoprotein B

1.11 Lipids and inflammation

1.11.1 Types of lipoproteins and apolipoproteins

There are 5 main lipoproteins and 11 apolipoproteins. The function of each is described in **Table 1.3.**

Table 1.3: The function of lipoproteins and their associated apolipoproteins

Lipoproteins	**Main function**	**Associated apolipoproteins**
Chylomicrons	Transfer of dietary lipids	A-I, A-II, A-IV, B-48, C-I, C-II, C-III, E
VLDL	Predominately carries triglycerides & some amounts of cholesterol	B-100, C-I, C-II, C-III, E
IDL	Carries cholesterol esters and triglycerides	B-100, C-III, E
LDL	Carries cholesterol esters	B-100
HDL	Carries cholesterol esters from peripheral cells to liver	A-I, A-II, C-I, C-II, C-III, D, E
Apolipoproteins		
Apo (a)	Inhibitor of plasminogen activation on lipoprotein (a)	
A-I	Activator of LCAT	
A-II	Activator of HL	
A-IV	Activator for LPL and LCAT	
B-100	Ligand for LDL receptor, assists in assembly and secretion of VLDL	
B-48	Assists assembly and secretion of chylomicrons	
C-I	Activator of LCAT	
C-II	Cofactor for LPL	
C-III	Inhibits triglyceride hydrolysis by LPL and HL	
D	Likely cofactor for CETP	
E	Ligand for LDL, hepatic chylomicron and VLDL remnant receptors	
LCAT: lecithin-cholesterol acyltransferase, LPL: lipoprotein lipase, HL: hepatic lipase, VLDL: very low denisity lipoproteins, IDL: intermediate density lipoproteins, LDL: low density lipoproteins, HDL: high density lipoproteins, CETP: cholesteryl etser transfer protein		

1.11.2 Normal lipid metabolism (Fig 1.4)

Exogenous pathway

The exogenous pathway is the process by which dietary lipids are transported and metabolised. Dietary lipids and lipids excreted in bile are hydrolysed by an array of enzymes secreted into the intestinal lumen. Cholesteryl ester hydrolase triggers the cleavage of cholesteryl esters, leaving free cholesterol able to form micelles with other lipids and fat-soluble vitamins prior to being absorbed by the cells of the intestinal wall. Inside the intestinal cells, free fatty acids combine with glycerol to form TGs and free cholesterol is re-esterified by ACAT (149). The lipids are then package in to chylomicrons and secreted via the mesenteric lymph in to the blood. Chylomicrons acquire several apolipoproteins both during their formation (apoB-48) and once released in to the blood stream (apo-CII and apo E), which later perform significant regulatory roles in the metabolism of chylomicrons. For example, chylomicrons are hydrolysed to chylomicron remnants by LPL. However, this reaction is dependant on the presence of apo-CII as a cofactor for LPL. Chylomicron remnants are then removed from the circulation via hepatic uptake, assisted by a high affinity apoE ligand. Many of the surface components of the chylomicron remnants are then used for the formation of HDL particles.

Endogenous pathway

The endogenous pathway refers to the hepatic transport and metabolism of lipids. This pathway revolves around the hepatic synthesis of VLDL. VLDL is formed in the endoplasmic reticulum of liver cells following the translation of Apo B100. VLDL particles then undergo a process of maturation by acquiring triglycerides (facilitated by microsomal triglyceride transfer protein) and cholesteryl esters. The cholesterol concentration in the liver cells appears to govern the rate at which VLDL is produced. When VLDL is secreted into the plasma is contains several surface lipoproteins including apo-CII, apo-CIII, Apo-B100 and ApoE. Once in the circulation VLDL is gradually transformed in to IDL following the hydrolysis of the triglyceride rich core by LPL. IDL is then either removed from the circulation via the LDL receptor/B 100 ligand complex or undergoes remodelling by HL to produce LDL particles.

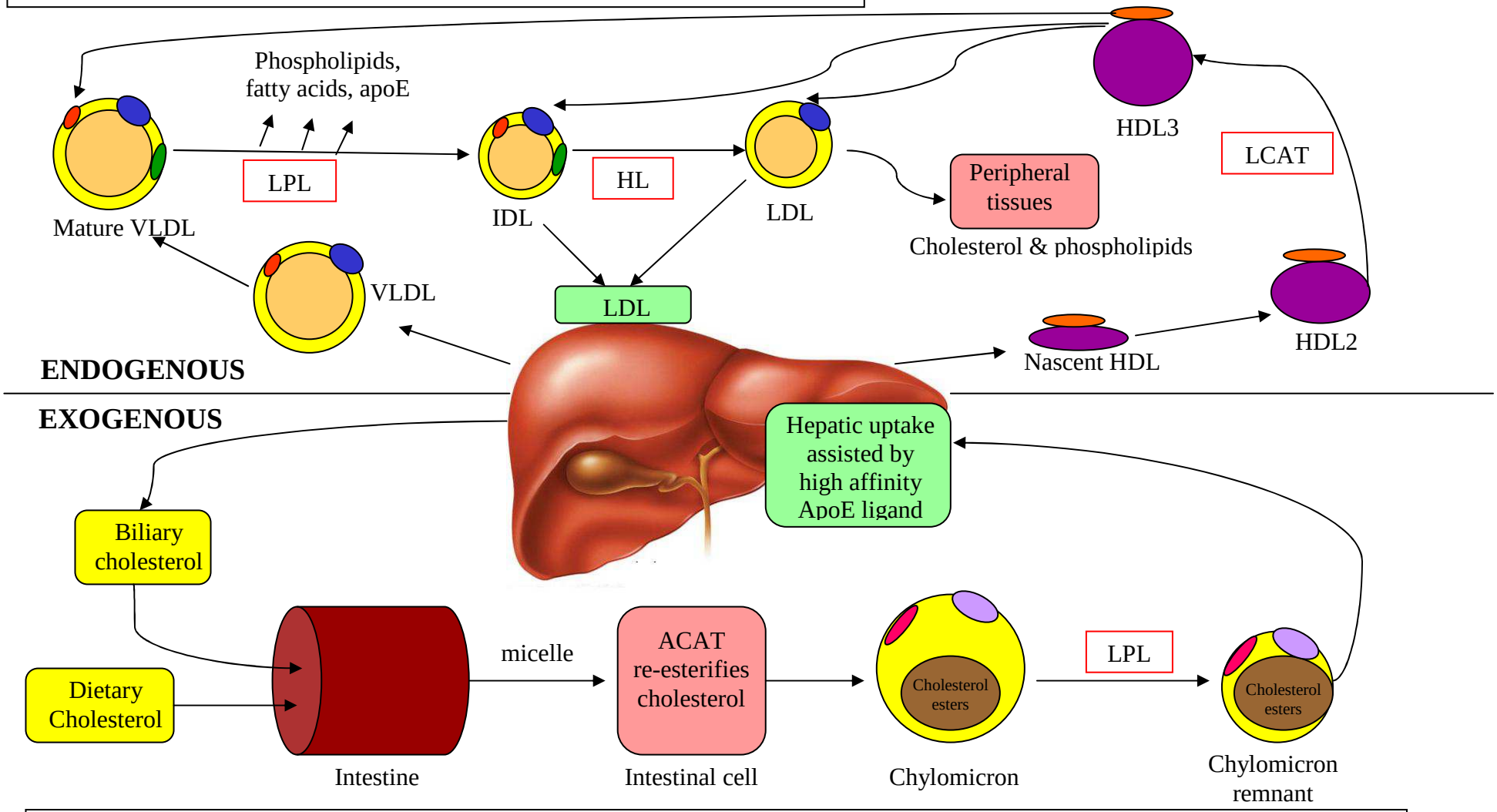
Circulating LDL can be incorporated into a variety of tissues, both for cellular storage and to contribute to production of cell membranes and hormones. The uptake of LDL in

to cells is important in the formation of atherosclerotic plaques, as LDL is readily taken up into macrophages via an unregulated scavenger receptor, ultimately leading to the production of foam cells. Some LDL will be removed from the circulation following internalisation in to hepatic cells via the LDL receptor.

Lp(a), a variant of LDL is formed when ApoA combines with LDL through an interaction with surface apo-B100 lipoproteins (150). Part of the Lp(a) structure is similar to plasminogen, thus allowing this lipoprotein to compete with plasminogen and disrupt fibrinolysis.

The formation of HDL is highly dependant on the metabolism of VLDL, IDL and LDL for the acquisition of surface components such as apolipoproteins. Nascent HDL particles are synthesized by the liver from small amounts of cholesterol and phospholipids, which are bound to Apo-AI. The apo-AI that is used for the formation of HDL can be newly synthesized by the liver or derived from the catabolism of other lipoproteins. Once released in to the circulation, nascent HDL crosses the vascular endothelium to collect cholesterol and phospholipids from peripheral cells and vascular intima cells. The accumulation of cholesterol and phospholipids is facilitated by ATP-binding cassette transporter (ABCA1) and the interaction of several surface apolipoproteins including (A-I, A-II, A-IV, C and E). This process transforms nascent HDL particles into discoidal shaped HDL2 particles, and alters the tertiary structure of the main surface apolipoprotein, ApoAI. The alterations in ApoAI structure enhance its ability to act as a co-factor for Lecithin-cholesterol acyltransferase (LCAT). Activation of LCAT triggers further esterification of free cholesterol, which is ultimately incorporated in to the HDL2 particles, transforming them further to form spherical HDL3 particles. The HDL particles ladened with cholesterol then return to the liver to deliver cholesteryl esters and remove them from the circulation. The process by which HDL collects cholesterol from peripheral cells and removes it from the circulation is called reverse cholesterol transport.

Figure 1.4: Normal lipid metabolism: endogenous and exogenous pathways



VLDL: very low density lipoproteins, IDL: intermediate density lipoproteins, LDL: low density lipoproteins, HDL: high density lipoproteins, LPL: lipoprotein lipase, HL: hepatic lipase, LCAT: lecithin:cholesterol acyltransferase, ACAT: acylCoA:cholesterol acyltransferase.

1.11.3 Inflammation mediated mechanisms controlling lipid metabolism

1.11.3.1 The effects of inflammation on HDL metabolism

HDL confers multiple cardiovascular benefits through its anti-oxidant properties and its ability to perform reverse cholesterol transport (151;152). It is now well recognised that high levels of HDL are cardio-protective, whereas low levels act as an independent cardiovascular risk factor (153-156). Studies in the general population have provided convincing evidence that increasing HDL levels through drug intervention and lifestyle modification can slow the progression of atherosclerosis and improve cardiovascular outcomes (157;158).

HDL is of particular interest in the context of active inflammation, as its levels can dramatically fall to a far greater extent than the changes seen in other components of the lipid profile. In acute inflammation, such as infection (bacterial and viral), HDL levels closely correspond to the degree of inflammatory burden, as levels are rapidly suppressed following the onset of infection and their return to normal appears to shadow the patient's recovery (159). Although the majority of studies report normalisation of HDL levels within 4 weeks of the onset of infection (159-161), one study has shown that the reduced levels persist up to 6 months post infection (162). In chronic inflammatory conditions, such as RA, HDL levels are persistently low. However, levels may still fluctuate as a consequence of alterations in the disease activity (163), drug usage (164;165), and physical activity levels (115).

The effect of Acute phase proteins of HDL metabolism

There is accumulating evidence to suggest that many of the usual anti-oxidant and anti-atherogenic properties of HDL are lost due to direct and indirect effects of inflammation. The inflammatory process triggers the synthesis of many plasma proteins by the liver (166;167). Serum amyloid A (SAA) and C reactive protein (CRP) are two of the key plasma proteins whose production is greatly enhanced during inflammation. The net increase in SAA occurs as a result of increased gene transcription (168), and leads to alterations in HDL composition and function. Large amounts of SAA become bound to HDL (mainly HDL3) following the displacement of apoA-I and apolipoprotein AII (apoA-II) (169). The composition of this remodelled acute phase HDL (AP-HDL) is also altered, becoming deplete in cholesterol esters and laden with TG, free cholesterol and fatty acids (170;171). The size of AP-HDL is larger than conventional HDL,

although the density remains comparable (172). The modification of the HDL structure to incorporate SAA directly impacts upon its ability to carry out reverse cholesterol transport, as LCAT, an enzyme responsible for the esterification of HDL, requires the presence of ApoA-I to be activated. Alongside this, the HDL/SAA structure has an increased affinity for macrophages and a reduced affinity for hepatocytes in comparison to the unmodified HDL structure (173). These changes can be partly attributed to alterations in the number of binding sites for the HDL/SAA complex, as inflammation has been shown to increase the number on macrophages and decrease the number on hepatocytes (173). However, it has been shown that SAA must constitute more than half of the HDL protein in order for cholesterol efflux to become compromised (174).

Interest has also developed around the role of two other acute phase reactants, namely secretory phospholipase A2 (sPLA2), known to possess the capacity to remodel HDL (175), and ceruloplasmin, a copper transporting protein with pro-oxidant properties (176). Inflammation induces elevations in the plasma concentrations of sPLA2 and this has been linked to smaller HDL particle size, a reduction in HDL cholesterol and apoA-I levels, and an increase in HDL and TGs in transgenic mice (177). Acute phase HDL is also susceptible to enrichment with ceruloplasmin, resulting in a reduced ability to protect LDL against oxidative modification (176;178). However, this theory is under scrutiny, as other studies conclude that ceruloplasmin exhibits anti-oxidant properties (179). The discrepancies regarding the properties of ceruloplasmin may be explained by differences in its structure, as the removal of one copper atom appears to alter its function from anti-oxidant to pro-oxidant (180).

Transferrin, a plasma protein involved in iron transport, can be found in association with HDL (181). It is thought that the usual role of this metal binding protein is to protect LDL against oxidation (181). However, during inflammation, levels of transferrin fall (182) and thus further predispose the host to a proatherogenic environment.

The effect of inflammatory mediated enzymatic change on HDL metabolism

Several enzymes fundamental to the metabolism of HDL are affected by inflammation. HL converts larger HDL particles (HDL2) in to smaller (HDL3) particles, facilitating cholesterol uptake from cells (183). This process occurs largely due to the hydrolysis of

TGs and phospholipids within HDL2. HL also plays a key role in many other aspects of lipoprotein metabolism, including assisting hepatic uptake of HDL and LDL particles by acting as a ligand (184) and aiding reverse cholesterol transport by promoting uptake of HDL cholesterol esters by the scavenger receptor B1 (185). Animal (186) and human studies (187-189) have shown the levels of HL to be reduced by the inflammatory process, thus inhibiting its normal functions and the production of a more pro-atherogenic environment.

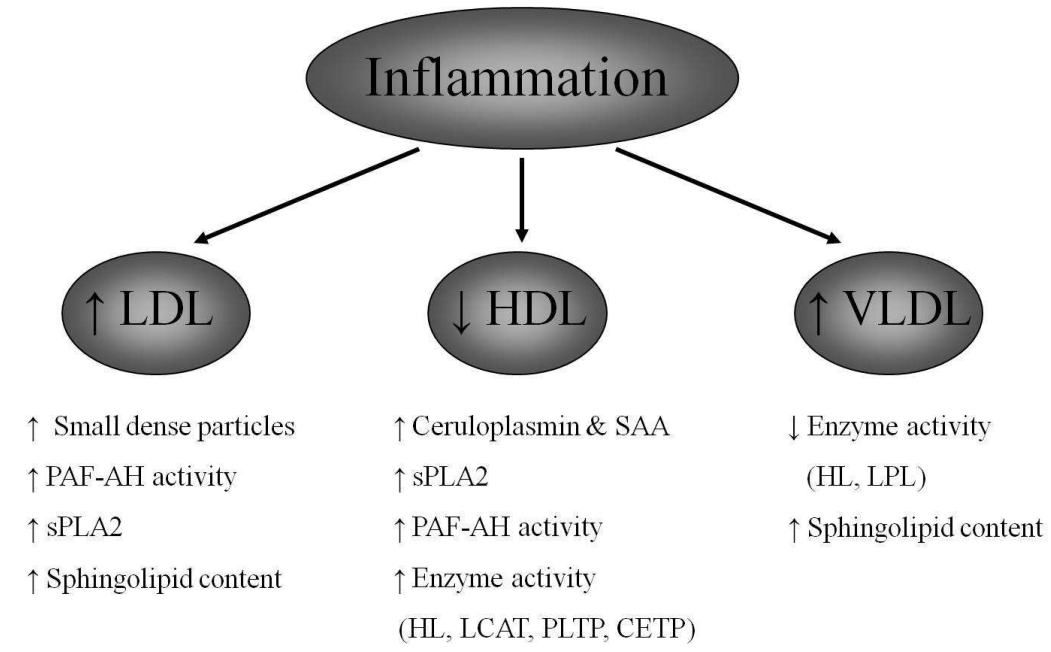
Reverse cholesterol transport is a complex cascade of events, which requires the presence of many factors in order for the whole process to be completed efficiently. Cholesterol ester transfer protein (CETP) plays a pivotal role, providing a pathway for cholesterol esters to be transferred from HDL to lipoproteins rich in apo-B, such as VLDL and LDL, allowing cholesterol to ultimately be cleared by the liver (190). Inflammation has been shown to indirectly impact upon CETP activity by reducing mRNA expression in transgenic mice (191). However, further studies in this field are required to confirm these findings in the context of chronic inflammation in humans. Phospholipid transfer protein (PLTP) is another key protein required for successful reverse cholesterol transport, whose activity is suppressed by the inflammatory process (192). The resultant limited activity of PLTP means that essential actions, such as mediating the exchange of cholesterol between TG rich particles and HDL, cannot be carried out. Thus, alterations in PLTP activity can be held partly responsible for lower HDL levels observed during inflammation (193).

Inflammation mediated variations in the enzymatic content of HDL have also been observed, including reductions of paraoxonase (PON-1) and elevated levels of platelet-activating factor acetylhydrolase (PAF-AH) (178). Deficiency of PON-1 within HDL renders them susceptible to oxidation and can ultimately convert HDL into a pro-oxidant, pro-inflammatory complex (178;194). PAF-AH is found in relation to both LDL and HDL particles. Elevated PAF-AH levels have been observed in a broad spectrum of inflammatory conditions, including the human immunodeficiency virus (HIV) (195) and RA (196). However, it remains unclear in humans whether PAF-AH activity is increased during inflammation in both HDL and LDL particles (195;197). It is postulated that if PAF-AH activity is increased within HDL particles. This may then confer mainly anti-atherogenic properties, as it may protect against the oxidation of

LDL, but also proatherogenic effects by escalating the production of lysophosphatidylcholine (LPC) (198). Occasional published reports have demonstrated a negative association between inflammatory burden and PAF-AH levels (199;200). However, such studies may lack significant power due to small study size.

It is clear from this evidence that it is not just the reduction in HDL that is an important risk factor for coronary heart disease in heightened inflammatory states such as RA, but that there are multiple other modifications in its function that may also have an additive effect (**See figure 1.5**).

Figure 1.5: The effects of inflammation of the structure, composition and function of lipids



LDL: low density lipoproteins, HDL: high density lipoproteins, VLDL: very low density lipoproteins, PAF-AH: platelet activating factor acetylhydrolase, sPLA2: secretory phospholipase A2, SAA: serum amyloid A, HL: hepatic lipase, LCAT: lecithin-cholesterol acyltransferase, PLTP: phospholipid transfer protein, CETP: cholesterol ester transfer protein. LPL: lipoprotein lipase

1.11.3.2 The effects of inflammation on TG metabolism

The relative contribution of elevated TG levels to the development of cardiovascular disease remains controversial. Although there is growing evidence that hypertriglyceridaemia associates with an increased CVD risk (153;201;202), it remains

difficult to classify it as an independent risk factor, as alterations in the levels occur in conjunction with changes in HDL levels (203).

For many decades, studies have demonstrated dramatic elevations in TG levels in response to infection, acute and chronic inflammation (160;204). Some studies have reported these changes to be specific to the infective organism (e.g. Gram negative bacteria) (204), whilst others report a significant increase irrespective of the factor triggering the immune activation (160;188). TGs are primarily transported in VLDL, therefore hypertriglyceridaemia occurs as a result of either overproduction or impaired clearance of VLDL. The inflammatory process interferes with the normal metabolism of TGs through the release of multiple cytokines and alterations in enzymatic activity.

The effects of cytokines on TG metabolism

Numerous cytokines released during inflammation may hinder TG metabolism. TNF-α is released in vast quantities during active inflammation. Elevations in TNF-α levels may disturb lipoprotein metabolism by decreasing LPL activity, reducing liver metabolism (205) and modifying the composition of lipoprotein particles (206). Studies in rats have clearly demonstrated that the administration of exogenous TNF results in doubling of baseline plasma TG levels (207). Subsequent studies in humans have produced data confirming a positive correlation between TNF-α and TG levels. However, much of this data has been generated from studies carried out on healthy subjects (208) and patients without active inflammation (209). Although studies assessing this correlation on the background of chronic inflammation are few, the available data confirm a striking positive association in patients with systemic lupus erythematosus (SLE) (210).

In RA, the advent of new biologic therapies specifically designed to target TNF-α has enabled this issue to be addressed from another angle. Interestingly, there is no convincing evidence to support the reverse association, (i.e. that as levels of TNF-α fall or TNF-α is inactivated, levels of TG also reduce). The precise effects of anti-TNF agents on TG levels are an issue of much debate. However, the vast majority of current published data indicates that treatment either does not impact upon TG levels (211-213) or may actually result in a paradoxical rise (214). These unexpected findings may be

explained by small study size, inadequate adjustment for other potential confounders or by an unknown mechanism. Further large-scale studies are required to elucidate the exact effects of anti-TNF agents on cardiovascular risk factors, such as dyslipidaemia. Over recent years, interest in the inflammatory cytokine IL-6 has escalated, due to the recognition of its atherogenic properties (215), and its potential as a therapeutic target both in cardiovascular diseases and in RA (216;217). A growing bank of evidence indicates that IL-6 levels influence lipoprotein metabolism (218), generating particular effects on the concentrations of TGs and HDL. IL-6 levels correlate positively with TG, and negatively with HDL levels (219;220). Despite the strong evidence to support a positive relationship between cytokine exposure and TG levels, there are also a few studies demonstrating a negative association (221).

Drug therapy again offers a further insight in to the relationship between IL-6 and TG levels, as lipid lowering therapies such as statins display a dual mode of action by lowering TG levels and suppressing IL-6 through their anti-inflammatory properties (222).

The effects of inflammatory mediated enzymatic change on TG metabolism

LPL is an intravascular enzyme specifically found in endothelial cells. It is a multifunctional enzyme, displaying the ability to mediate lipoprotein uptake and to catalyse the hydrolysis of TGs within circulating VLDL and chylomicrons (223). The explosive release of cytokines such as TNF-α and IL-6 occurring during inflammation, leads to a reduction in the levels of LPL and HL via down regulation of gene expression at the transcriptional level (224;225), which ultimately leads to a reduced clearance of TG-rich particles. The net result of these modifications takes some time to accrue. Thus, such changes only contribute to hypertriglycerideamia in the setting of persistent chronic inflammation (226).

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors, important in several aspects of lipid metabolism. Of particular interest is PPAR-α, regulating lipid metabolism both at the intracellular and extracellular level (227). LPL has been shown to activate PPARs through a preferential action on VLDL (228). Therefore, inflammation mediated suppression of LPL levels inhibits PPAR activation

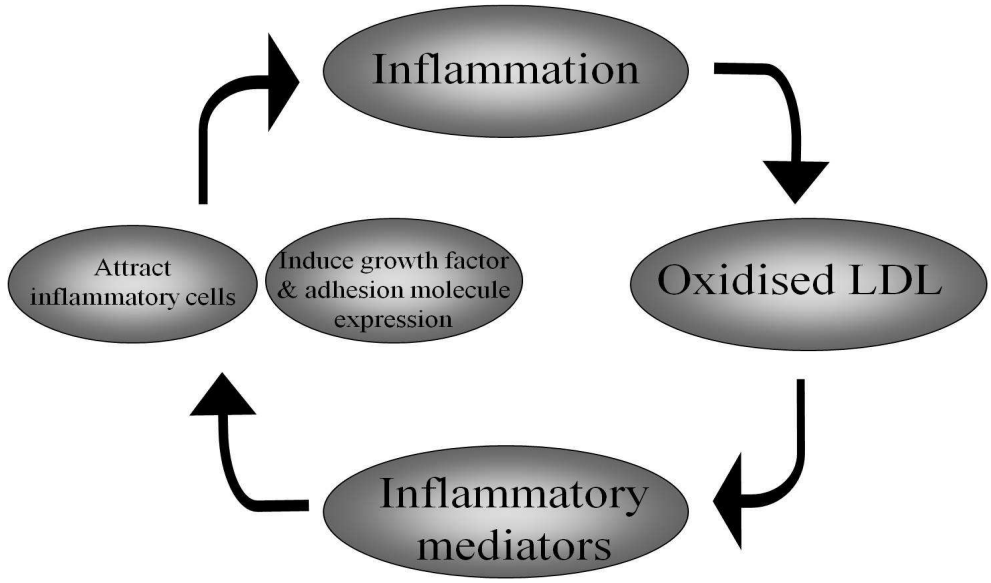
and ultimately contributes to hypertriglyceridaemia (225). The pathway of PPAR activation has long been used as a lipid lowering therapeutic target. Fibrates are PPAR-α agonists, used to treat dyslipidaemia by reducing TG levels and increasing HDL levels (229). HDL levels increase with fibrate therapy due to PPAR activated up-regulation of ApoA-I and ApoA-II gene transcription (230;231).

1.11.3.3 The effects of inflammation on LDL metabolism

Epidemiological studies have shown elevated LDL levels to be one of the strongest predictors of coronary artery disease (232). To combat this risk, guidelines have been developed, with LDL as the primary focus for lipid lowering therapy (118;233). Several large scale studies in the general population have demonstrated that intensive lipid lowering therapy confers greater cardiovascular benefits than moderate therapy (234-236). Furthermore, such studies demonstrate continued clinical benefit even when LDL levels are lowered below the current recommended treatment goals (235). Over the last 50 years a steady decline in LDL levels has been observed amongst Americans (237). These changes may have occurred secondary to an increased prescription of lipid lowering medication and an overall more aggressive approach to the management of dyslipidaemia.

Inflammation lowers LDL levels (126;238). On the surface this appears to produce a less atherogenic environment. However, by delving deeper it becomes apparent that inflammation mediated structural/oxidative changes may promote atherogenesis via the development of pro-inflammatory, atherogenic LDL particles (239;240). In fact, persistent inflammation such as that seen in RA, may fuel a vicious circle of oxidation and inflammation (**Fig 1.6)**

Figure 1.6: The vicious cycle of inflammation and oxidised LDL



Inflammatory mediated structural changes of LDL

LDL is sub-classified according to size and density. A predominance of small dense LDL particles is associated with a 3-5 times increased risk of coronary heart disease (the Quebec cardiovascular study)(241). Studies in patients affected by inflammatory disorders, including psoriatic arthritis (242), the acquired immunodeficiency syndrome (AIDS) (243) and RA (133) have demonstrated a preponderance of small dense LDL particles compared to the control groups. At present, information regarding the precise mechanisms behind these changes is limited and further research in this area is required.

Inflammatory mediated oxidative changes of LDL

During inflammation there is release of reactive oxygen species (ROS) from activated leucocytes. Elevated ROS levels overwhelm the host's usual antioxidant mechanisms, resulting in damage to cells and lipid peroxidation (244). The alterations in LDL composition have been blamed almost entirely for the accelerated oxidative modification during inflammation. In animal models, inflammation simulated by the introduction of lipopolysaccharide (a major component of the cell wall of gram negative bacteria), has been shown to increase markers of lipid peroxidation (245). Other studies carried out in human subjects with inflammatory disorders have confirmed similar elevations in markers of lipid peroxidation and an increased oxidative susceptibility of LDL (246). Conflicting results are reported by Paredes et al, who found no differences

in lipid peroxidation between patients with active RA and the control group (247). This discrepancy may be explained by the small study size, the degree of inflammatory burden or by the methods used to identify oxidative change.

The effects of acute phase proteins on LDL metabolism

The pro-atherogenic properties of LDL are enhanced by CRP and sPLA2 during inflammation (248). The inflammation mediated increase in sPLA2 activity produces small dense LDL particles that have a surface layer relatively deficient in phospholipids (249). Such changes in the outer-most layer of LDL increase its ability to interact with arterial proteoglycans, perhaps further enhancing its uptake into the arterial wall and the formation of atherosclerotic plaques. sPLA2 may also indirectly promote LDL oxidation through the production of fatty acids. Fatty acids are prone to oxidative change and may subsequently induce oxidation of LDL (250). Once oxidised, LDL is readily taken up by macrophages, and an abundance of CRP is recognised to facilitate this process and promote subsequent foam cell formation (251).

The effects of inflammatory mediated enzymatic change on LDL metabolism

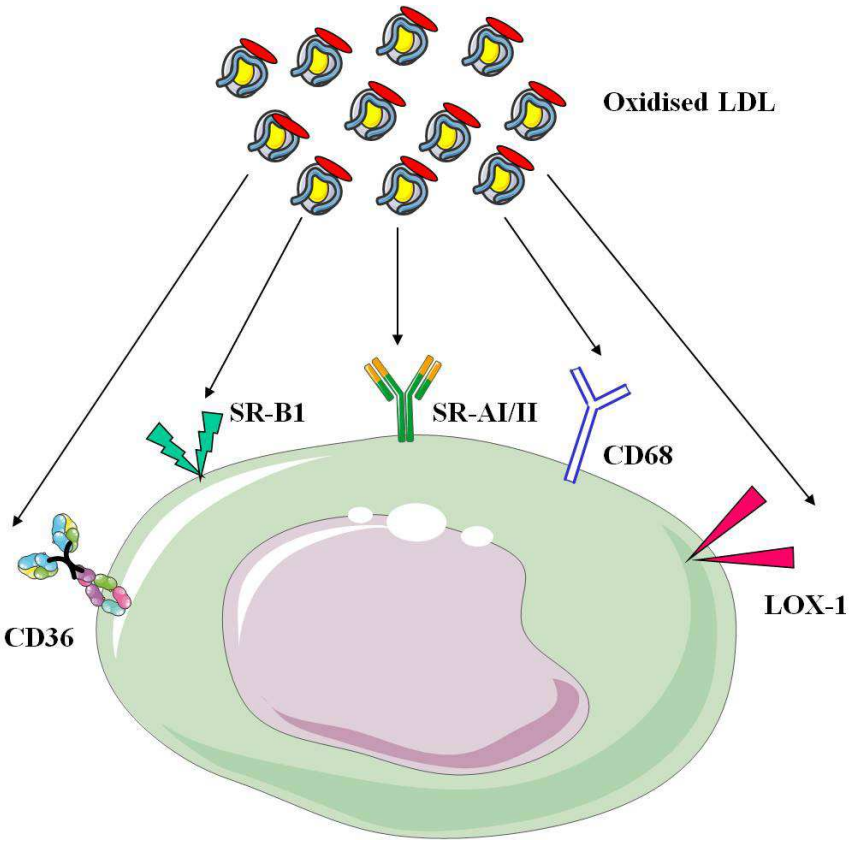
Endothelial lipase (EL) is a newly identified member of the triglyceride lipase family (252). Apart from its role in HDL homeostasis, EL may enhance lipid uptake into the vascular endothelium via its bridging function (253). Although it is widely accepted that EL is up-regulated during inflammation (254;255), the exact effect that this has on LDL is still a matter of debate. One of the earliest studies to address this issue was carried out in LDL receptor deficient mice, and demonstrated that hepatic expression of EL leads to a reduction in serum LDL levels (252). On the contrary, a later study in EL knockout mice has reported a massive increase (90%) in LDL cholesterol levels (256). Two further studies have also tried to identify the role of EL in LDL metabolism. The first failed to demonstrate any effect of EL on LDL levels in the mouse model (257), whilst the second demonstrated that EL promotes LDL uptake by macrophages (258).

LDL uptake by macrophage scavenger receptors

The uptake of LDL by macrophages signifies a pivitol step in the formation of foam cells, and subsequently atherosclerotic plaque formation. Modification of the apolipoprotein B component of LDL by oxidation, aceylation and nitration, renders it susceptible to uncontrolled uptake by scavenger receptors expressed on the surface of

macrophages, leading to foam cell formation (259). Although a number of scavenger receptors are known to mediate oxLDL uptake into macrophages (e.g scavenger receptor-AI/II, CD36, lectin-like oxidised LDL receptor-1 (LOX-1)) (260) (summarised in **figure 1.7**), the CD36 receptor appears to be one of the most important and well studied (261;262). It has been reported that patients with a genetically determined deficiency of the CD36 receptor experience a 40% reduction in the binding of oxLDL and accumulation of cholesterol ester than patients with normal CD36 expression (263). The CD36 receptor differs from the other main class of scavenger recptor (SR-AI/II) as it preferentially binds minimally oxidised LDL (264). CD36 expression on macrophages is upregulated by cellular cholesterol, LDL (both native and modified), and cytokines (e.g. IL-4) (265), and downregulated by TGF-β1 (266), HDL (267;268) and ceremides (269). The regulation of CD36 expression occurs at a transcriptional level, with PPAR-γ playing a central role in this process (270). The uptake of oxLDL in to macrophages may further perpetuate atherosclerosis by enhancing VEGF expression on macrophages and endothelial cells, which appears to be at least partially mediated by PPAR-γ activation (271).

Figure 1.7: Receptor mediated uptake of LDL in to macrophages



CD36: CD36 receptor, SR-B1: scavenger receptor B1, SR-A: scavenger receptor A, CD68: CD68 receptor, LOX1: lectin-like oxidised LDL receptor.

Inflammatory stress may accelerate foam cell formation through a number of mechanisms: 1) enhanced LDL modification e.g. oxidation (245), 2) disruption of cholesterol mediated LDL receptor feedback, thus increasing the uptake of unmodified LDL (272), 3) CRP binding to native LDL particles may enhance uptake in to macrophages by the CRP receptor CD32 (273). Interestingly, two recent studies have challenged the hypothesis that CRP/LDL complexes are important for foam cell formation (274;275). The first study explored the effects of unbound and CRP-bound enzymatically modified LDL on foam cell formation (274). This study demonstrated that the binding of CRP to enzymatically modified LDL did not lead to foam cell formation, unlike its unbound counterpart. The second study investigated the interaction of the CRP monomer, which has been implicated in atherosclerotic plaque formation, with monocytes using the U937 cell line (275). They report that the CRP monomer inhibits oxLDL-induced foam cell formation. Thus the latter two studies imply that CRP may confer anti-atherogenic effects through its effect on the behaviour of monocytes.

RA, a chronic systemic inflammatory disease with an increased CVD risk is a perfect platform for studying the effects of inflammation on foam cell formation. To date only one small scale study has investigated the uptake of LDL from RA patients into macrophages derived from the U937 monocytic cell line (276). This compared LDL modification (oxidation and nitration) and LDL uptake into macrophages in RA patients with (n=13) and without CVD (n=12) compared to osteoarthritis (OA) patients with (n=13) and without CVD (n=12). The investigators demonstrate a statistically higher level of LDL nitration but not oxidation in the patients with CVD compared to disease matched controls, and that this was associated with a trend for increased LDL uptake by U937 monocytes. The authors also suggest a potential mechanism for these findings. Homocysteine, a known CVD risk factor, was found to promote LDL nitration, thus higher serum levels of homocysteine (often found in association with systemic inflammation) may trigger LDL nitration and subsequently LDL uptake in to monocytes/macrophages. Interestingly, RA patients with CVD were found to have greater LDL uptake in to macrophages than OA patients with CVD, however this did not reach statistical signifance, perhaps as a consequence of limited power. Such a difference in LDL uptake by macrophages could be explained by differences in the underlying pathopysiology of the conditions e.g. the degree of systemic inflammation or

drug therapy etc, however, to date no studies have been conducted to examine the effects of inflammation/drug therapy.

1.12 Effects of lifestyle on the lipid profile

1.12.1 Seasonal variation

Studies have demonstrated both biological variation (i.e. normal day to day variation) (277) and cyclical seasonal variation in plasma lipid and lipoprotein levels (278;279). Within patient biological variation is in the order of 6-7% for TC and HDL, 8-10% for LDL and 20-30% for TG (277;280). The most widely reported seasonal changes are elevations in TC and LDL levels in the winter months (281), with peak levels being reached during January (279;282). Apolipoprotein levels also demonstrate seasonal fluctuations, with ApoA-I and A-II levels markedly elevated in the summer months (283). Interestingly, LDL demonstrates an increased susceptibility to oxidation during winter months (January) and a reduced susceptibility during summer months (June/July) (284). Some studies also report winter elevations in HDL and TG levels (282;285). The precise mechanisms controlling these changes remain elusive. However, several hypotheses have been put forward. These include: 1) a haemodilution effect due to secondary mobilisation of fluid from the interstitial to the intravascular compartment, due to heat acclimatization (286) occurring during the summer months as a result of a combination of increased environmental temperature and physical activity 2) seasonal variations in physical activity levels (282).

Currently, there are no studies to address the effects of biological/seasonal variance in RA patients. The lack of studies is not surprising as multiple other factors associated with disease activity display similar fluctuations, e.g morning joint stiffness, and disease activity worse in colder weather. Thus it would be difficult to distinguish between the effects of the disease itself on lipid parameters and those occurring as a result of 'normal' variation.

1.12.2 Menopause

Female sex hormones have long been shown to exert a protective effect against the development of CVD. A hormonal deficiency, such as that observed with the menopause, leads to an increased risk of CVD (287). This may be partially explained by accelerated proatherogenic changes in the lipid profile occurring independent of

increasing age (288). Postmenopausal women experience a dramatic deterioration in their lipid profile, with significant increases in LDL, TC, TG and HDL3 (288-290), with some studies reporting a worse lipid profile than age matched male counterparts (291). It has been suggested that genetic mechanisms that control individual variation of lipids, in particular HDL, may differ between premenopausal and postmenopausal women (292). Relative correction of the hormonal profile through the use of hormone replacement therapy can produce beneficial changes in the lipid profile by elevating HDL2 and reducing LDL levels (293).

In RA, postmenopausal status has been associated with significantly higher TC, TG, TC:HDL ratio, ApoB, Lp(a) and LDL:HDL ratio than those observed in premenopausal RA patients (114). Postmenopausal RA women have also been shown to have higher TG and Lp(a) and a lower TC than healthy postmenopausal women (114).

1.12.3 Physical activity

Regular long-term exercise promotes many health benefits, ranging from improved bone density to a reduction in cardiovascular risk. Much of the reduction in cardiovascular risk can be attributed to modification of conventional risk factors, including a reduction in blood pressure (294), improvement of the lipid profile (115;143) and weight reduction.

In the general population, exercise is recommended as a lifestyle change for all patients with dyslipidaemia or established cardiovascular disease (118). Supporting this recommendation are multiple studies and meta-analyses demonstrating significant improvements in the lipid profile in patients adhering to a regular exercise programme (294-296). The most significant changes observed with moderate exercise are elevations in HDL levels, lower TC:HDL ratio and lower TG levels (294;296-298). Age has been shown to influence the degree of alteration in the lipid levels seen following exercise (299). Older patients appear to lower their TC levels to a greater extent and more readily enhance their HDL levels (299). Unfortunately, the majority of exercise studies have been conducted in normolipidaemic subjects, and there is a lack of data available in dyslipidaemic patients.

Many of the improvements in the lipid profile require several months for the benefits to accrue. Although the minimum amount of exercise required to improve blood lipids is not known, the United Kingdom government recommendations suggest at least 30mins of continuous exercise 5 days a week are required for a healthy lifestyle.

As HDL is one of the key components of the lipid profile to be modified by exercise, some interest has been generated around the effects of exercise on HDL sub fractions. A large proportion of studies assessing the influence of short term exercise on HDL subfractions has shown significant increases in both HDL2 and HDL3 (300;301). The relative increases in the HDL subfractions differ between studies with some demonstrating similar increases in both HDL2 and HDL3 (301), and others demonstrating a disproportionate increase in HDL2, resulting in an increase in the HDL2/HDL3 ratio (302). These discrepancies can be explained by differences in the subjects studied (athletes vs. healthy men), the number of subjects involved, the mode and length of exercise, and advice regarding other lifestyle modifications. Evidence to support similar changes in HDL sub fractions amongst subjects adhering to longer-term exercise programmes, or in dyslipidaemic subjects is lacking.

The mechanism by which exercise mediates positive effects on HDL can be partly attributed to the alterations in the enzymes responsible for HDL metabolism (303). One study demonstrated large increases in the activity of LPL and a significant reduction in HL activity in endurance trained individuals vs. sedentary controls (304). The enhanced enzyme activity of LPL may escalate lipid transfer to HDL, and simultaneous decreases in HL activity may result in reduced clearance of HDL.

In RA, traditionally, patients and rheumatology healthcare professionals have been cautious regarding exercise, due to a widely held but unfounded fear that it would trigger aggravation of disease or damage the joints (305). However, over recent years well designed physical exercise programmes have been shown not only to be safe in RA but also to promote prolonged improvements in morning stiffness, Stanford arthritis self-efficacy scale (SES) and other disease outcomes (306;307). Although regular exercise as part of an individually designed programme is now recommended as an integral part of RA treatment, and has been included in the ACR treatment guidelines, there are no specific trials addressing the effects of exercise on CVD risk factors (e.g.

dyslipidaemia) in RA. Specific well-designed studies are required to address this issue in RA, as the inflammatory burden in these patients may have already modified conventional cardiovascular risk factors (abnormal lipid profile, changes in body fat: muscle ratio etc), and thus the beneficial effects of exercise on CVD may be even greater than those seen in the general population.

1.12.4 Obesity

The classical pattern of dyslipidaemia in overweight and obese subjects is characterised by elevated levels of TG, TC, apoB-100 and small LDL particles, and decreases in HDL levels (308;309). Insulin resistance (IR) is often found in conjunction with both obesity, and plays a key role in the development of dyslipidaemia. One of the primary obesity associated defects in lipid metabolism is the overproduction of VLDL (310). This phenomenon may be a consequence of a cascade of events occurring in the insulin resistant state, resulting in hepatic steatosis (311). Furthermore, there is a delayed clearance of VLDL particles due to a reduction of the LDL receptor activity that occurs in conjunction with IR (232). The low HDL levels seen in obese states are likely to be triggered by several mechanisms. Firstly, HDL clearance is enhanced in insulin resistant states, due to stimulation of hepatic lipase and the resultant production of smaller HDL particles (312). Secondly, transfer of apolipoproteins and phospholipids from TG–rich lipoproteins to HDL is reduced. Thirdly, the delayed clearance of TG-rich lipoproteins facilitates the CETP mediated exchange between cholesterol esters in HDL and triacylglycerols in VLDL (313).

Although rheumatoid cachexia, a condition characterised increased total fat mass nd a reduction in muscle mass, is widely recognised as a complication of the RA disease process, there are currently no studies addressing whether the presence of this condition further alters the lipid profile in RA.

1.12.5 Smoking

Smoking reduces life expectancy through multiple detrimental effects on health (314). Much of the associated morbidity and mortality can be attributed to the carcinogens contained within the cigarette smoke, resulting in an increased risk of developing cancer (especially lung cancer) (315), or the increased rates of CVD (316). The mechanism by which smoking promotes CVD still needs to be elucidated. However, it has been

suggested that smoking induced alterations in the lipid profile may contribute to this susceptibility (317). Multiple large studies confirm that smoking exposure associates with a pro-atherogenic lipid profile, with elevated TC, LDL, TG levels, and TC:HDL ratio, and an associated reduction in HDL levels (318-321). Data produced from such studies has to be interpreted with caution, as it is difficult to control for other lifestyle factors, such as weight changes, diet, alcohol consumption and physical activity. The relationship between smoking and lipid levels appears to be dose dependant (321) and readily reversible on cessation (322). Cigarette smoke is known to create a pro-oxidative state in the circulation (323), and disturb endothelial function (324). The smoking induced oxidative burden induces lipid peroxidation (325), thus further accelerating atherosclerosis.

Smoking has been linked to a 2-4 fold increased risk of developing RA (326), and may also influence the severity of the disease (327) and functional status (328). However, a recent study has produced conflicting findings suggesting that cigarette smoke does not accelerate RA disease progression. In fact, the study demonstrated that heavy smoking may be associated with reduced radiographic progression and improvement in functional scores (329). The effect of smoking in RA varies due to the length of exposure, with acute exposure resulting in immuno-stimulatory effects and chronic exposure resulting in immunosuppressive effects (330). Although it is clear that smoking may influence the development and pathogenesis of RA, no specific studies have addressed the effects of smoking on lipid metabolism in RA.

1.12.6 Alcohol

Moderate alcohol intake confers a lower risk of CVD (331-333). The reduction in CVD risk of moderate drinkers compared to those who abstain has been shown to lie between 10 and 40% (333). The cardio-protective effect of alcohol has been linked to beneficial changes in both the lipid profile (333;334) and haemostatic parameters (335;336). By far the most common and well-reported change in the lipid profile is an increase in HDL concentrations (321;333;337;338). The precise mechanisms contributing to the quantitative change in HDL are still being scrutinised. However, current proposals include: 1) increased hepatic production or increased transport rate of apoA-1 and apoA-II (339;340) 2) increased cholesterol efflux from macrophages to HDL mediated by ABCA1 (341) 3) alcohol-induced reduced activity of CETP (342). However, a recent

study failed to demonstrate any relationship between CETP activity and HDL levels amongst moderate alcohol consumers compared to abstinent controls (343). Furthermore, the study demonstrated alcohol-induced alterations in lipoprotein lipase activity.

A degree of controversy exists regarding the relative contributions of HDL subfractions, to the elevation of HDL levels seen with moderate alcohol intake. Virtually all studies demonstrate an increase in both HDL2 and HDL3 subfractions (338;344). However, the effects on the HDL2:HDL3 ratio varies between studies (344;345). One study assessing the effects of habitual alcohol consumption has demonstrated that the changes in HDL subfractions may differ between genders, with males experiencing a rise in both HDL2 and HDL3, but females only experiencing an isolated significant rise in HDL2 (338). A recent study by Schafer et al, evaluated the qualitative changes of HDL observed during exposure to varying degrees of alcohol in 279 healthy men (344). The investigators confirmed that alcohol consumption results in a shift from HDL3 to predominantly HDL2a, and a significant phospholipid enrichment of all HDL subfractions.

Hypertriglyceridaemia has long been associated with regular alcohol use (346). A meta-analysis of 42 studies has confirmed a positive relationship between moderate alcohol consumption and TG levels, reporting a 0.19mg/dl increase per gram of alcohol consumed per day (333). The impact of alcohol exposure on the other components of the lipid profile including LDL and TC levels have been addressed in comparatively fewer studies. However, the majority of these demonstrate a modest inverse correlation of both TC and LDL with alcohol (334;347). LDL particle size may be reduced in patients with alcohol induced hypertriglyceridaemia, resulting in a subsequent increased susceptibility to oxidation (348).

Epidemiological studies indicate that alcohol consumption correlates with a reduced risk of developing RA (349;350). Unfortunately, post diagnosis data to assess the effects of alcohol on disease parameters and the lipid profile are lacking. Studies to address this issue are likely to be hampered by ethical issues and safety issues regarding interactions with DMARD therapy.

1.12.7 Diet

It is well established that dietary intake has far reaching health implications. An unhealthy diet rich in saturated fats and sugars has been linked to an increased risk of CVD (351), whereas diets high in unsaturated fats and anti-oxidants such as the Mediterranean diet are linked to a reduced CVD risk (352). There are multiple individual dietary components that impact upon the lipid profile. Fish oils are rich in omega-3 polyunsaturated fatty acids which lowers plasma TG levels (353), red yeast rice has been shown to reduce TC and LDL levels (354) and olive oil raises HDL-cholesterol and reduces levels of oxLDL (355). In RA, data regarding the impact of diet on the lipid profile is sparse. One randomised study on 66 active RA patients, demonstrated that patients treated with a gluten-free vegan diet resulted in lower LDL and oxLDL levels (356).

1.13 The effects of drugs on the lipid profile in rheumatoid arthritis

The impact of recent therapeutic advances in the management in RA on cardiovascular risk is not clear. However, several recent studies suggest that therapeutic intervention and control of disease activity may reduce cardiovascular risk (143;144;357). A large cross sectional study (QUEST) of over four thousand RA patients, suggested a reduced cardiovascular risk with prolonged use of DMARDs, GCs, or anti-TNF (357). The findings from this study should be interpreted with caution as the study is limited by its cross sectional design and therefore causality can not be assumed. Despite this, a further prospective study has confirmed significant improvements in cardiovascular risk factors following treatment with methotrexate and GCs (143).

1.13.1 Glucocorticoids

GCs have long been recognised to have beneficial effects in RA (50;358). However, in current rheumatological practice GC use has been curbed due to adverse effects. They are used increasingly as a short term measure to induce a rapid reduction in disease activity whilst awaiting the effects of slower acting DMARD therapy (111). The long term use of GCs is controversial due to loss of efficacy over time, and the undesirable side effect profile (358).

For many years it has been widely assumed that GC use associates with a more atherogenic lipid profile (raised TC, TG and HDL levels) regardless of the indication for use (359). However, many of these early studies were performed on renal transplant patients. Thus they may have been confounded by other lipid influencing variables, such as the underlying disease process, co-morbid conditions and concomitant medications (360;361). Over more recent years, conflicting data has been produced from several prospective studies, indicating that GC may actually improve the lipid profile by increasing HDL and lowering the TC:HDL ratio (362;363). In 2005, a large study involving 15,004 participants demonstrated differences in the lipid profile in patients receiving GCs according to their age (364;365). In patients over the age of 60, GC use was associated with higher HDL and Apo-A1 levels, and lower TC:HDL and ApoB:ApoA-I ratios. No association was noted between GC use and HDL levels, TC levels or TC:HDL ratio amongst subjects below the age of 60. A potential limitation of this study was the difference in the indication for GC use between the age groups, and thus the underlying effects of the individual disease processes on the lipid profile. Unsurprisingly, studies assessing the effects of GCs on lipid metabolism in healthy individuals are sparse. A small study on 8 normolipidaemic healthy men treated with prednisolone for 14 days demonstrated a significant increase of VLDL-TG, VLDL-cholesterol, HDL-cholesterol, Apo A-I and ApoE, with normalisation of the lipid profile to baseline levels two weeks after discontinuation of prednisolone (365). A further study on eight normolipidaemic healthy men examined the relationship between GC exposure and HDL sub fractions (366). This demonstrated a rapid change in the lipid profile following initiation of 30mg oral prednisolone, with elevations seen in HDL within two days of commencement. Within the HDL subclasses, GC exposure induced redistribution of HDL particles, resulting in increased levels of HDL2 and a reduction of HDL3.

Currently, only 8 papers have mentioned the effects of GCs on the lipid profile in RA. However, the majority did not set out to assess this relationship as their primary objective. Thus, the number of patients included that were actually receiving GCs in the majority of studies was very small. The results of some of the studies may also have been affected by potential confounders, such as concurrent changes in DMARD therapy (367). The largest and most robust study to comment on the effect of GCs on the lipid profile was reported by Boers et al (368). This was a randomised controlled trial in

which 76 RA patients were randomised to take combination therapy of methotrexate (stopped at 40 weeks), sulphasalazine and a reducing dose of prednisolone (initially 60mg/day prednisolone, tapered in 6 weekly steps to 7.5mg/day and stopped after 28 weeks) and 79 RA patients who were allocated to take sulphasalazine alone. Both arms of the study were well matched according to age, disease duration and disease activity. The study reported a significant increase in TC and HDL levels, and a reduction in the TC:HDL ratio in both study arms. However, the greatest improvements were observed in the combination therapy arm, with the corrective effect on the lipid profile occurring far more rapidly. The lipid changes were far more significant in the combination therapy arm up until 28 weeks and then all lipid parameters returned to levels similar to those seen in the sulphasalazine only arm. Thus indicating that the changes in the lipid profile may be more specific to GC use (stopped at 28 weeks) rather than the additive effects of the 3 combination therapy drugs.

In RA, the limited available data addressing the relationship between GC exposure and the lipid profile, demonstrate a corrective effect on the altered lipid profile seen in active RA. The most widely reported changes include an increase in both TC and HDL levels (363;369;370). However, HDL appears to increase at a proportionately higher rate compared to TC, thus generating a more favourable atherogenic index (121). Much of the witnessed 'normalisation' of TC and HDL levels with GC use in RA has been attributed to the suppression of disease activity through their anti-inflammatory actions (121;368). Data produced regarding changes in TG levels is not so clear and has only been specifically looked at in 3 out of the 8 studies identified. Although two studies have shown an increase in TG levels with GC use (121;148;371), another study failed to demonstrate any change (367). This discrepancy may be attributed to differences in the populations studied including disease characteristics, drug use, and the power of the individual studies. Further large studies are required in order to fully elucidate the effects of GCs on TG levels.
All studies demonstrating the effects of GCs on the lipid profile in patients with RA are summarised in **Table 1.4**

Table 1.4: Studies assessing the impact of GC use on the lipid profile

Study	Number of patients	Other drugs used	F/U	Patient details	Lipid changes
Garcia-Gomez et al Eur J. Clin Invest 2008 (369)	65 RA on pred/methylpred 13 RA controls not on pred	DMARDs Anti-TNF therapy	N/A	Age 60 yrs DD 13 years Female 100%	Pred ↑ HDL
Hafstrom et al J. Rheum 2007 (370)	67 RA Randomised to 7.5mg pred (n=34) or no pred (n=33)	DMARDs	5 yrs	Age 52	Pred ↑ TC
Peters et al Ann rheum dis 2007 (372)	80 RA (35 on pred)	Infliximab	48 wks	Age 56 DD: 10 yrs Female 77 %	Pred ↑ TC and HDL levels and ↓ TC:HDL ratio
Dessein et al J.Rheumatol 2004 (367)	92 RA (37 previously on pred, 18 currently on pred ≤ 4mg) No controls	At enrolment 17 patients taking DMARDs,by completion 84 patients on DMARDs	6 m	Female 80% Age 56 yrs DD: 11 yrs	No changes in LDL, HDL or TGs
Boers et al Ann rheum dis 2003 (368)	155 early active RA	Patients randomised to either MTX& SLZ & pred (76) or SLZ alone (79)	56 wks	Age 50 yrs DD: 4 months Female 59%	↑ TC, HDL and ↓ TC:HDL ratio in both group but greater in combination therapy group.
Dessein et al Arthritis res 2002 (373)	79 RA (10 on pred) 39 age/sex matched OA controls (6 on pred)	Median dose of pred 5 mg	N/A	Age 52 yrs Female 83% DD: 8.5 yrs	No significant differences between RA patients taking and not taking steroids.
Wallace et al Am J med 1990 (371)	108 SLE, 47 RA (14 HCQ, 8 pred, 4 HCQ & pred, 21 neither drug)	HCQ	N/A	Age 46.6 yrs	Pred alone have ↑ TC, HDL and TG but ↓ LDL compared to those not on treatment.
Svenson et al Arch intern med 1987 (148)	33 (only 4 treated with just pred)	Pred & AZA or Pred & cyclo or Pred alone	9 mths	Age 49 yrs	↑ TC, TG, HDL and LDL in all groups including pred only arm.

TC: total cholesterol, TG: triglycerides, HDL:high-density lipoproteins, LDL: low-density lipoproteins, Pred: prednisolone, MTX: methotrexate, SLZ: sulphasalazine, methylpred: methylprednisolone, AZA: azathioprine, anti-TNF: anti-tumour nerosis factor, HCQ: hydroxychloroquine, cyclo: cyclophosphamide, OA: osteoarthritis, SLE: systemic lupus erythematosus, DD: disease duration, RA: rheumatoid arthritis, DMARDs: disease modifying anti-rheumatic drugs

1.13.2 Hydroxychloroquine

Hydroxychloroquine (HCQ) is an anti-malarial medication that also exhibits disease modifying and anti-inflammatory actions when prescribed for RA or SLE. In the 1980's, Beynen noted a reduction in cholesterol synthesis with low dose chloroquine (374). A subsequent study by Svenson et al, confirmed these initial observations and demonstrated reductions in cholesterol and TG levels in those prescribed chloroquine (148). In the 1990's Wallace et al carried out a study to assess whether HCQ exhibited similar lipid lowering properties as its parent drug, chloroquine (371). The study involved 150 patients with RA or SLE who were randomised to take HCQ alone, GCs alone, HCQ and GCs, or no drug. HCQ use resulted in a reduction in cholesterol, LDL and serum TG levels, which appeared to be independent of changes in weight and diet. Later studies demonstrate that HCQ produces a less atherogenic lipid profile, by increasing HDL levels by approximately 15% (375).

The mechanism underlying the lipid lowering effects of HCQ and chloroquine are still disputed. However, some plausible modes of action have been described in studies analysing the interaction of chloroquine. Potential reasons include: 1) via the inhibitory actions on VLDL secretion by the liver (376) 2) inhibition of cholesterol synthesis by blockage of a site distal to hydroxymethlyglutaryl coenzyme A (HMGCoA) reductase (377) 3) inhibition of proteolysis of internalise cholesterol esters leading to increased LDL receptor values (374;378).

1.13.3 Ciclosporin

Since the first reported use of ciclosporin A (CyA) for the management of RA in 1979 (379), the drug has been used widely both as monotherapy or in combination with other DMARD therapy, such as methotrexate (380;381). Chronic CyA administration has been shown to adversely affect the lipid profile. However, much of this data has been generated from small studies on transplant patients (382;383). A study of 65 post renal transplant patients demonstrated elevations in TG and Lp(a) levels, and suppressed HDL levels in those treated with CyA monotherapy compared to azathioprine and prednisolone (384). The reduction in HDL levels may be explained by the inhibitory actions of CyA on ABCA1-mediated lipid efflux (385). Despite these findings in transplant patients, further studies are required to address the potential effects of CyA on the already altered lipid profile seen in RA.

1.13.4 Gold

Gold is one of the oldest treatments for RA, and was first used in the late 1920s (386). Its use has diminished over recent years with the advent of newer more effective DMARDs and biologic therapies. Gold therapy has many adverse effects including, dermatitis, stomatitis, post injection reactions, haematuria, and proteinuria. However, not much has been reported about its effects on the lipid profile. Munro et al reported that gold use may have the net effect of producing a more atherogenic profile by increasing TG levels and reducing HDL levels (375). No further studies have directly looked at the influence of gold on lipid levels.

1.13.5 Non Steroidal anti-inflammatory drugs

NSAIDs are widely used in RA for symptom control. However, their use has been associated with an increased risk of CVD in the general population (387). Several mechanisms are thought to contribute to the increased CVD risk found in association with NSAID use, including enhanced thrombosis and elevated blood pressure (388). To date, no associations have been found between NSAIDs and the lipid profile.

1.13.6 Anti-Tumour necrosis factor

The identification of TNF-α as a key cytokine in the pathogenesis of RA has resulted in the development of specific biologic therapies designed to target TNF-α with the net effect of inhibiting its inflammatory properties (389). The introduction of anti-TNF agents in the year 2000, has revolutionised the treatment of RA with better disease control and dramatic improvements in quality of life (390). Despite the overwhelming benefits of anti-TNF therapy, a number of complications and adverse effects have been noted (391). By far the most common complication of anti-TNF therapy is infection (392). However, there is expanding evidence to suggest that these agents may also interact with other metabolic parameters, such as the lipid profile (165). At the time of commencing this PhD only three anti-TNF agents were licensed for use in RA (Infliximab, Adalimumab, and Etanercept), thus my thesis will focus on these. It is important to note that during the course of my PhD two newer ant-TNF agents have also been licensed for use (golimumab and certolizumab).

The structure and mode of action of the original three anti-TNF agents varies. Infliximab is a chimeric monoclonal antibody, adalimumab is a fully humanised monoclonal antibody and etanercept is a soluble TNF -α receptor fusion protein. The underlying characteristics of these molecules may be key to their mode of action and effects on the lipid profile.

Currently, 17 studies have attempted to address the impact of anti-TNF agents on the lipid profile. Overall, the studies recruited relatively small study populations and had a short duration of follow up, with several studies only looking at a handful of patients over a 6 week period (393-395). The largest study by Nishida et al, enrolled 97 RA patients who were prospectively followed up for 1 year (396). The study was limited by the lack of a control group, and the data it produced was very focused, reporting only changes in HDL and TC. Twelve of the seventeen studies did not include a control group for comparison.

Multiple studies have produced information on the short-term effects of anti-TNF agents on the lipid profile (397-399), but there are relatively few addressing the longer term effects (372;400). In the short term studies, duration of treatment ranged from 6 weeks to 6 months, and the majority only assessed the effects of infliximab (397;398;401). Irrespective of their duration, these studies demonstrate similar findings with a universal increase in TC, and a large proportion confirming an increase in HDL, but no overall change in the atherogenic index (TC:HDL ratio). Although TG levels were not looked at in all studies, the available data tends to show an increase in TG levels up until 6 months in patients treated with infliximab (397). Saiki et al, have shown the most convincing evidence of a relationship between anti-TNF and an increase in TG levels (402). The study compared TC and TG levels amongst 32 patients with refractory RA treated with infliximab, to 32 age and sex matched control patients with active RA treated with methotrexate over a 6month period. A significant and persistent elevation in TG levels was observed from 2 weeks in the infliximab arm, but no change was seen amongst those treated with methotrexate, thus indicating that changes in TG levels are likely to be due a drug specific mechanism rather than a ‘blanket’ anti-inflammatory effect.

Published data regarding the effects of the other two anti-TNF drugs is limited. The short term effects of etanercept and adalimumab have only been studied in two studies (213;399). However, due to study design and small number of patients enrolled, they only reported on the generic effect of anti-TNF on the lipid profile rather than the effect of the individual drugs. Seriolo et al, reported similar findings to the studies performed solely on patients receiving infliximab (increased TC and HDL) (213), whereas the other study reported no effect of anti-TNF on the lipid profile . A further study has been published addressing the effects of adalimumab on the lipid profile, during a 1 year follow up period (403). At the 1 year follow up visit, there was a significant sustained increase in HDL levels and a reduction in Lp(a) levels. The effect of etanercept and adalimumab on the lipid profile needs to be addressed in further large scale studies, particularly as differences in their molecular structure, mode of action and half life may alter how they affect the lipid profile.

The effects of anti-TNF therapy on Lp (a) levels were addressed in only four studies (213;393;399;403). Only one study demonstrated a significant change in Lp(a) levels (403), with Lp(a) levels found to decrease during the 1 year follow up peroid. Interestingly, this was the largest of the four studies (n=50 patients), therefore it is possible the negative findings reported by the other three may have been due to a lack of power. Unfortunately, two of these studies attempted to look at the effects of all three anti-TNF agents, thus making the results difficult to interpret, as Lp(a) levels may be affected differently by each TNF agent e.g. cancelling each other out in the reported results.

A summary of all studies addressing the effects of anti-TNF agents on the lipid profile in patients with RA is shown in **Table 1.5.**

1.13.7 Rituximab

Rituximab, an anti-CD20 monoclonal antibody, is used to treat patients with active RA unresponsive to DMARDs and/or one anti-TNF agent. Rituximab was originally developed for the treatment of B cell lymphoma, but has since been found to be tremendously useful in other conditions including RA, with significant reductions in disease activity and functional improvement (39;404). Although rituximab is now widely used, relatively little is known about the effects of the drug on the lipid profile, structure or function. To date, two small scale prospective studies reported the effects of

rituximab on the lipid profile (405;406). The first included six RA patients treated with rituximab, for six months (405). The investigators report minor lipid changes at 2 weeks. However, these are most likely to have been induced by the concomitant infusions of methylprednisolone (given to reduce side effects) rather than the rituximab per se. The lipid profile returned to baseline and no significant differences were reported after 6 months of treatment. However, this study had a primary objective of assessing endothelial function and not lipid parameters and therefore it was unclear whether potential confounders such as statin use were taken into consideration. In the second study, Kerekes et al demonstrated a more persistent change in lipid levels (a decrease in TC levels and an increase in HDL levels) after 16 weeks of treatment in 4 out of the 5 patients studied (406). Again these findings may be as a consequence of the IV methylprednisolone rather than the rituximab per se, but this is perhaps less likely as the effects of methylprednisolone infusions are often seen to wain after around 3 months.

Table 1.5: Studies assessing the effect of anti-tumour necrosis factor therapy agents on the lipid profile

Study	**Type of anti-TNF**	**Number of patients**	**F/U**	**Changes in lipid profile**				
				TC	**HDL**	**TC/HDL**	**TG**	**Lp(a)**
Wijbrandts et al Ann Rheum Dis 2009 (403)	**Adal**	50 RA	1 yr	N/C	↑	N/C	N/C	↓
Garces et al, Ann.Rheum.Dis 2008 (407)	**Inflix** **Etan**	30 RA, 29 AS, 6 PsA Inflix= 44 Etan= 21	1yr	↑ inflix N/C Etan	N/C inflix ↑ Etan	N/S N/S	N/S N/S	N/S N/S
Nishida et al Endocrine J 2008 (396)	**Inflix**	97	1 yr	↑	↑	N/S	N/S	N/S
Soubrier et al Joint bone spine 2008 (399)	**All**	Etan =6 Inflix =11 Adal = 12	14 wks	N/C	N/C	N/C	N/C	N/C
Oguz et al Acta Clin Belg 2007 (395)	**Inflix**	7	9 mths	N/C	N/C	N/S	N/S	N/S
Popa et al Ann Rheum Dis 2007 (165)	**Inflix**	55	55=6 mths 31=1 yr	↑	↑	↑	N/S	N/S
Peters et al Ann Rheum Dis 2007 (121)	**Inflix**	80	48 wks	↑ at 6 weeks, by 48 weeks baseline	↑ at 6 weeks, by 48 weeks baseline	↑at 6 weeks, by 48 weeks back to baseline	N/S	N/S
Saiki et al J. Rheumatol 2007 (402)	**Inflix**	43 refractory RA 32 age/sex matched RA controls on MTX 32 healthy controls	6 mths	↑ from 2 weeks	N/S	N/S	↑ from 2 weeks	N/S

Tam et al Clin Rheumatol 2007 (397)	**Inflix**	19	14 wks	↑	↑	N/C	↑	N/S
Allanore et al Clin Chim Acta 2006 (211)	**Inflix**	59 refractory RA 56 RA controls not on anti-TNF	30 wks	↑	↑	N/C	↑	N/S
Dahlqvist et al Scand J Rheumatol 2006 (400)	**Inflix** (41 also MTx, 13 other DMARD 28 Pred)	52 RA on infliximab 70 early RA controls	2 yrs	↑ initial, then ↓ by 6 month	↑initial, then ↓ by 6 month	↑	N/S	N/S
Kiortsis et al J Rheumatol 2006 (214)	**Inflix**	82 (50 = RA, 32 =AS)	6 mths	↑	N/C	N/C	↑	N/S
Seriolo et al Ann NY Acad Sci 2006 (213)	**All** (plus MTX & pred)	Etan = 16 Inflix = 14 Adal =4	48 wks	↑	↑	N/C	N/C	N/C
Spanakis et al J Rheumatol 2006 (401)	**Inflix**	60 (24=RA,26=AS, 10=PsA)	6 mths	↑ (1st month only	↑	↓ (3 months)	N/S	N/S
Vis et al J Rheumatol 2005 (398)	**Inflix**	69	6 wks	↑	↑	N/C	N/S	N/S
Irace et al Atherosclerosis 2004 (394)	**Inflix**	10	6 wks	↓	↓	N/S	N/C	N/S
Cauza et al Wien Klin Wochenschr 2002 (393)	**Inflix**	7	6 wks	N/C	↓	N/S	↑	N/C
Anti-TNF: anti- tumour necrosis factor, Inflix: Infliximab, Etan: Etanercept, Adal: adalimumab, MTX: methotrexate, Pred: prednisolone, DMARD: disease modifying anti-rheumatic drugs, RA: rheumatoid arthritis, AS:Ankylosing spondylitis, PsA: psoriatic arthritis, wks: weeks, mths: months, N/C: no change, N/S: not studied,								

1.14 Lipid Metabolism and Genetic polymorphisms

In RA, lipid parameters may be altered as a consequence of an increased prevalence of SNPs known to regulate lipid metabolism in the general population. However, it is also possible that the genes known to predispose to RA (RA susceptibility genes) could also either directly or indirectly (through their effects on inflammatory parameters) alter lipid parameters. This potential association is particularly interesting in the light of 2 studies that have demonstrated changes in the lipid profile up to 10 years before the onset of RA (122;123).

1.14.1 ATP binding cassette transporter gene (ABCA1) polymorphisms

The ATP binding cassette transporter protein is primarily involved in the transport of phospholipids and cholesterol from cells to intracellular acceptors, including lipid free ApoA1. Polymorphisms of the ABCA1 gene lead to alterations in protein function. In the most severe form, patients develop Tangiers disease, characterised by reduced HDL in heterozygotes or clinically absent HDL in homozygotes (408). To date, a number of SNPs have been identified and their association with plasma lipid levels and cardiovascular risk addressed in general population studies (409-411). Patients carrying the K allele of the rs2230806 (R219K) SNP appear to be significantly protected from developing CVD, and have been reported in a variety of studies to confer lower TG levels and higher HDL levels (412-414). Other polymorphisms including the I883M (rs4149313) and V771M (rs2066718) have been associated with an increased CVD risk (410;415;416), but demonstrate inconsistent effects on lipid parameters (417;418). R1587K (rs2230808) and E1172D (rs33918808) have also both been associated with an increased CVD risk (410;419).

1.14.2 Cholesterol Ester Transfer Protein (CETP) TaqIB polymorphism

CETP is key to the transfer of cholesterol esters and triglycerides between lipoproteins. It aids reverse cholesterol transport and HDL metabolism by transferring cholesterol esters from HDL to VLDL and LDL. Variations in CETP levels can occur as a result of a SNP in intron 1 of the CETP gene (TaqIB), located on chromosome 16. The presence of a B2 allele confers lower CETP levels than the presence of the more common B1 allele. Thus, patients homozygous for the B2 allele often have significant elevations in

their HDL-C levels compared to B1 homozygous patients (420). Based on these findings, it is rather unsurprising that the B2 allele has also been associated with a lower CVD risk in the general population (421;422). A meta-analysis of 113,833 patients has demonstrated further changes associated with inheriting the B2 allele, including lower CETP mass and activity, lower levels of LDL-C, apoB, and TGs and increased levels of ApoAI (423). Interestingly, the presence of the B2 allele may also enhance clinical benefit from statin therapy amongst patients with significant coronary artery disease, with a net effect of reducing cardiovascular events (424). The worldwide frequency of the minor allele (B2 allele) is 42%. This was reported to be identical amongst East Asian and white populations (423). The genotypic and allelic frequencies of the CETP TaqIB and the influence this may have on the lipid profile or CVD risk has not been studied in RA.

1.14.3 Apolipoprotein E (ApoE) polymorphisms

Apolipoprotein E is a major component of VLDL and chylomicrons. It is essential for the catabolism of TG rich lipoprotein constituents, and facilitates the uptake of VLDL and chylomicrons into the liver (425). ApoE may further regulate lipid metabolism via enhancing effects of LPL and HL (426). Three isoforms of ApoE exist as a result of 2 SNPs, ApoE2, ApoE3 and ApoE4. ApoE3 is considered as the parent form, occurring in more than 60% of the population (427) and the other two as mutations. ApoE2 is produced due to a base change (arginine to cysteine) at residue 158, whereas ApoE4 is the consequence of a base change (cysteine to arginine) at residue 112. Such changes alter the structure and function of the protein, resulting in a significantly reduced binding affinity of ApoE2 to the liver receptors (428), a subsequent delay in the removal of dietary fat from the blood (429) and the development of type III hyperlipidaemia (430). ApoE4 predisposes to atherosclerosis through mechanisms that are still being elucidated (427). However, some studies indicate that the lipoprotein distribution of ApoE4 differs from the parent form, and that particles containing ApoE4 are prone to catabolism, which ultimately leads to further alterations in lipoprotein metabolism with the end result of increasing LDL, TC and TG levels (431;432). ApoE polymorphisms may also be partially accountable for variations observed in atherosclerotic plaques, with differences being observed in carotid intima-media thickness (433). The prevalence and contribution of the ApoE genotypes to the development of CVD in RA have not yet been studied.

1.14.4 Lipoprotein Lipase (LPL) polymorphisms

The LPL gene is located on chromosome 8p22 and is responsible for the production of the enzyme lipoprotein lipase. The enzyme plays a key role in the mobilisation of non-esterified fatty acids and monoglycerol molecules for energy utilisation and storage by cells, via the hydrolysis of the triacylglycerol component of VLDL and chylomicrons. Thus, a deficiency of this enzyme can result in an accumulation of both plasma chylomicrons and TGs, as seen in the autosomal recessive condition –familial lipoprotein lipase deficiency (434). To date several SNPs of the LPL gene have been associated with lipid abnormalities and CVD (435). Four SNPs including rs1801177, rs268, rs328 and rs320 have been shown to significantly alter plasma levels of TG and HDL (435). Several independent studies have demonstrated an increased CVD risk with LPL mutations (rs328, rs285) (436-438), and this association has recently been confirmed in a large meta analysis (435). The HuGE association review and meta-analysis demonstrated an increased odds ratio (OR) for myocardial infarction and coronary stenosis with G188E (OR=2.8), rs1801177 (OR=1.33) and T-93G (OR=1.22). However, the remaining four SNPs (rs268, rs285, rs320, rs328) analysed were not found to associate (435). The frequency and impact of such SNPs have not been studied in RA.

1.14.5 Apolipoprotein (A1/C3/A4/A5) gene cluster

The long arm of chromosome 11 is home to the regulatory gene cluster that encodes for proteins ApoA1, ApoC3, ApoA4 and ApoA5. Interestingly, not only have the genes responsible for these proteins been found to be close in their chromosomal proximity, but the proteins produced by each gene have been found to have inter related functions involved in the metabolism of TG-rich lipoproteins and HDL (439;440), a phenomenon that may be attributed to linkage disequilibrium. ApoA1 is the main protein component of HDL and plays an important regulatory role in reverse cholesterol transport. ApoC3 is found in association with VLDL and HDL, and primarily acts as an inhibitor of LPL (441), thus slowing the rate of catabolism of triglyceride rich particles. Due to the inhibitory effects of this protein, an inverse relationship between ApoC3 levels and TG levels exists. ApoA4 is a major constituent of chylomicrons, and is thought to be involved in TG metabolism (442), along with ApoA5 (443-445). An extensive array of SNPs has been identified within the genes responsible for the production of each of these proteins, which have been linked not only to alterations in lipid metabolism, but

also to CVD. For example, around 20 SNPs of the ApoA5 gene have been identified, with around half of these found to be associated with disorders of TG metabolism (446), and others associated with excess CVD risk (443;447). For the purposes of this thesis I have decided to assess ApoC3 (rs2854116), ApoA4 (rs675), ApoA5 (rs3135506). ApoC3 (rs2854116) is associated with HDL levels, with the presence of the C allele shown to significantly reduce levels (448). The Apo A4 (rs675) is associated with an increased risk of CVD(449), and apo A5 (rs3135506) with significantly elevated TG levels (450;451).

1.14.6 RA susceptibility genes

The aetiology of RA is multifactorial, however genetic factors are known to play a central role (see section 1.2.1). Numerous genetic polymorphisms have now been identified that are known to predispose to RA (RA susceptibility genes), however, for the purposes of this thesis I will focus on only four susceptibility genes that have been shown to confer the greatest risk (HLADRB1, STAT4, TRAF1C5, and PTPN22) (13;17-19;21). To date, no studies have assessed the impact of RA susceptibility genes on the lipid profile in RA. However, several of the RA susceptibility genes may contribute to the excess CVD morbidity and mortality in RA (452;453).

1.15 Summary

RA is associated with an increased risk of cardiovascular morbidity and mortality. Dyslipidaemia is a major CVD risk factor in the general population, however, despite the increased CVD risk in RA only limited data exists assessing the impact of chronic inflammation and drug therapies on lipid levels, ratios, structure or function. Furthermore, RA is a condition with strong genetic aetiological links, thus it is possible that RA susceptibility genes also contribute to the regulation of lipid metabolism in RA, or that genetic polymorphisms known to regulate lipid metabolism in the general population are more common in RA or have an altered function.

1.16 Hypotheses

1) NCEP-defined dyslipidaemia is highly prevalent in RA and this translates into increased CVD risk
2) Lipid levels, structure and function are affected by systemic inflammation
3) The initiation of drug therapies, including GCs and biologic agents, will affect lipid levels, structure and function, through "generic" anti-inflammatory actions or drug-specific effects on lipids
4) Lipid abnormalities in RA are, in part, genetically determined

1.17 Aims and objectives

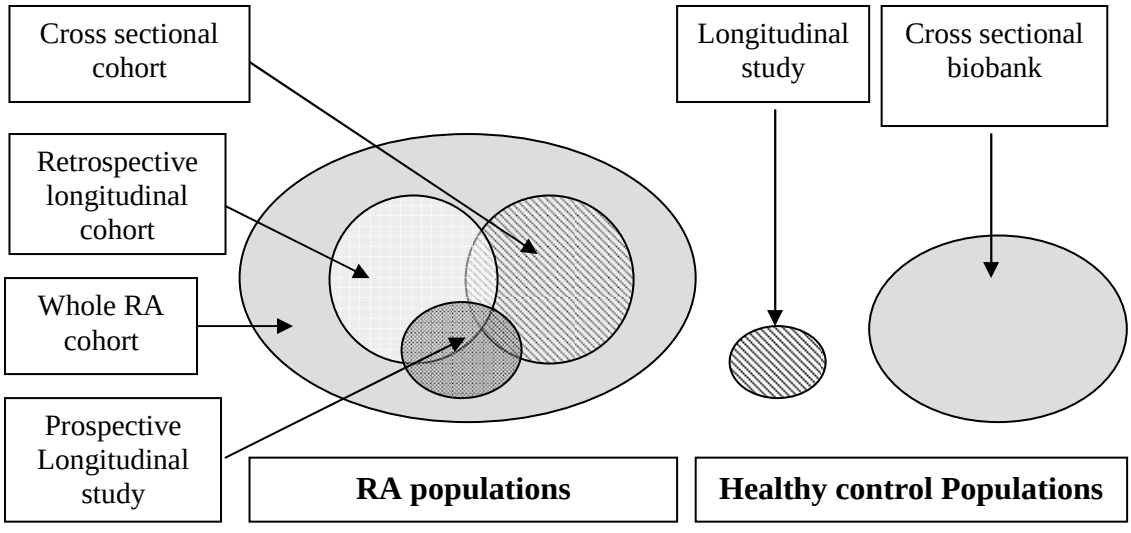
1) To assess cross-sectionally, in a large, well-characterised population of RA patients the prevalence and associations of NCEP-defined dyslipidaemia and lipid levels with demographic, anthropometric, genetic, and RA disease-related factors (including disease activity, severity, duration and therapy).
2) To assess cross-sectionally, how dyslipidaemia in RA translates into CVD risk and to establish whether this risk is managed appropriately with lipid lowering therapy.
3) To assess, in a longitudinal cohort, the magnitude, timing and persistence of changes of lipid levels, structure and function in RA patients in response to treatment with potent anti-inflammatory agents (e.g. GCs and biologic agents) and whether these are due to control of inflammation or drug-specific effects.
4) To determine whether RA susceptibility genes influence lipid levels in RA
5) To assess the prevalence and associations of specific genetic polymorphisms known to influence lipid/lipoprotein metabolism in RA patients and in local general population controls.

CHAPTER TWO: Methods

2.1 Study populations

In order to address all aims and objectives several study populations were used. Aims one and two were addressed using data from an established cross-sectional cohort of 400 RA patients. Aim one also used a previously collected biobank of samples from 437 local population healthy controls, to allow comparison of the frequency of genetic polymorphisms in healthy controls versus RA patients. Aims three and four were addressed using a newly recruited longitudinal RA cohort and healthy control population. As patients in both the cross sectional and longitudinal arms of the study were recruited from the same pool of RA patients managed at the Dudley Group NHS Foundation Trust (DGoH) there was a small degree of overlap, with some patients participating in both studies (**Fig 2.1**). In addition, in order to address all aspects of aim three in full, a further retrospective RA cohort of 550 patients was used in order to assess the directionality of lipid changes in the context of changes in systemic inflammation.

Figure 2.1: The study populations



Similar data were collected for both the cross-sectional cohort of 400 RA patients and RA patients recruited into the prospective longitudinal study (n=72), thus allowing extensive characterisation of RA and CVD status (demographic and anthropometric

characteristics, details regarding RA disease, physical function, co-morbid conditions, drug use, CVD status, metabolic status, as well as routine haematological, biochemical and immunological blood tests). The only difference in the data collected was that extra blood was taken in the longitudinal cohort to allow for more in depth lipid studies (lipoprotein structure and function- outlined below).

2.1.1 Cross-sectional population

RA Patients: The Department of Rheumatology at DGoH has a long-standing interest in the association of CVD with RA. A prospective cohort of 400 RA patients fulfilling the 1987 ACR criteria (6) was recruited from outpatient clinics between 2004 and 2006. No exclusion criteria were applied when recruiting. Patients were introduced to the study by the lead investigator, outpatient nursing or medical staff working in the department of rheumatology at DGoH, and were provided with an Ethics Committee approved patient information leaflet. Informed written consent was taken from willing participants prior to data collection via one-to-one interviews, retrospective case note analysis, self-administered questionnaires, physical examination and fasted blood sampling. DNA was collected on all patients for further analysis. A summary of baseline characteristics can be seen in **table 2.1**

All RA patients recruited in to the cross sectional study were flagged by the Office for National Statistics (ONS), thus allowing the cause of death to be identified. At the time of writing this thesis a total of 62 (15.5 %) RA patients had died.

Healthy controls: A DNA biobank of 437 local general population controls was also available and was used for comparative studies. This existing healthy control population was recruited from subjects attending for routine screening blood tests who had previously consented to anonymously donate blood for DNA extraction for a similar polymorphism study within the hospital. The only other information available on the healthy controls was age and gender.

All data produced from this study were entered in to an SPSS database to allow subsequent data analysis.

Table 2.1: Baseline characteristics of 400 cross-sectional RA patients

Variable	**RA (n=400)**
Demographics	
Age (years) median (IQR)	63.1 (55.5-69.6)
Gender (female) n(%)	292 (73.0)
Disease Characteristics	
Disease duration median (IQR)	10 (4-18)
ESR (mm/Hg) median (IQR)	21 (9.3-37)
CRP (mmol/L) median (IQR)	8 (5-20)
HAQ median (IQR)	1.5 (0.6-2.1)
DAS28 mean ± SD	4.2 ± 1.4
CVD risk factors	
Smoking status n(%) - Never - Ex -Current	 176 (44) 151 (37.8) 65 (16.3)
Diabetes n(%)	28 (7)
Hypertension n(%)	223 (56.9)
BMI (kg/m^2) mean ± SD	27.8 ± 5.0
Medications	
Methotrexate n(%)	225 (56.3)
Sulphasalazine n(%)	118 (29.5)
Hydroxychloroquine n(%)	80 (20)
Leflunomide n(%)	16 (4)
Anti-TNF n(%)	46 (11.5)
Prednisolone n(%)	131 (32.8)
Statins n(%)	83 (20.7)
Antihypertensives n(%)	171 (42.8)
IQR: interquartile range, SD: standard deviation, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, DAS: disease activity score, HAQ: health assessment questionnaire, BMI: body mass index, Anti-TNF: anti-tumour necrosis factor	

2.1.2 Prospective longitudinal study population

A new cohort of RA patients was established that was followed longitudinally, at baseline (before drug commencement), 2 weeks, and 3 months (post-treatment). RA patients (fulfilling the 1987 ACR criteria) (6) who were due to commence treatment with IV GCs, anti-TNF therapy or rituximab as part of their routine management (RA intervention group), were identified from routine rheumatology outpatient clinics and specific biologic clinics held at DGoH. In total, 12 patients receiving GCs, 35 receiving anti-TNF and 10 receiving rituximab were recruited.

Two control populations were recruited in parallel: (1) 15 RA patients on stable DMARD therapy were recruited from routine outpatient clinics (RA controls); (2) 40 healthy control (HC) subjects were recruited from hospital staff and their family members. Of the 15 RA controls only 10 attended all follow up visits, due to changes in their medication which would have confounded the results. RA patients were excluded from the study if they were participating in other intervention studies or had received GCs in the previous 3months. Patients were introduced to the study by the lead investigator, outpatient-nursing or medical staff working in rheumatology at DGoH. All potential participants were provided with a patient information leaflet, which had received prior ethical approval from the Black Country research ethics committee. Patients were given a minimum of 24 hours to consider the information prior to being contacted either directly or via telephone to establish willingness to participate. Willing participants were provided with a suitable morning appointment (8am-10am) to attend a research clinic, where informed written consent was taken prior to commencing assessments. All patients were advised to attend the appointment in a fasted state (12 hour overnight fast). Data were collected in an identical manner at each visit. Data were obtained via face-to-face interviews, retrospective case note analysis, self completed questionnaires, physical examination, fasted blood sampling. The baseline demographics of the longitudinal population are shown in **table 2.2**.

Table 2.2: Baseline demographics of longitudinal RA (RA intervention and RA controls) and HC population

	RA (n=72)	**HC (n=40)**	**P value**
Age (Years) mean ± SD	55.11 ±14.15	48.6 ± 11.35	**0.011**
Female sex n(%)	50 (69.4)	31 (77.5)	0.217
BMI (kg/m^2) mean ± SD	28.83 ± 5.64	27.53 ± 5.74	0.239
Smoking status n(%)			
Current	13 (18.6)	1 (2.5)	
Ex-smoker	24 (34.3)	4 (10.0)	**0.001**
Never	33 (47.1)	30 (75.0)	
ESR (mm/hr) median (IQR)	23.5 (10-40.5)	6 (2-9)	**<0.001**
CRP (mg/L) median (IQR)	9 (2.9-22)	1 (1-3)	**<0.001**
Diabetes n(%)	3 (4.2)	0 (0)	0.227
SBP (mmHg) mean ± SD	130.49 ± 14.8	123.09 ± 12.81	**0.014**
DBP (mmHg) mean ± SD	79.53 ± 9.59	78.16 ± 10.34	0.497
IR n(%)	35 (43.2)	10 (25)	**0.010**

IQR: interquartile range, SD: standard deviation, BMI: body mass index, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, SBP: systolic blood pressure, DBP: diastolic blood pressure, IR: insulin resistance

2.1.3 Retrospective longitudinal RA cohort

550 RA patients were identified from a departmental database of 1138 RA patients fulfilling the 1987 ACR criteria (6), following a retrospective review of all blood lipids and inflammatory markers (CRP and ESR) recorded on our electronic hospital database from February 2004 to December 2007. Patients included in this retrospective cohort had lipid profiles and simultaneous CRP and ESR assessments on at least two occasions. The number of simultaneous recording of lipids and inflammatory markers per patient ranged from 2 to 9. These data were used to analyse the impact of systemic inflammation on the individual components of the lipid profile and lipid ratios over time. The only other data available on these patients (other than lipid parameters and inflammatory markers) were age and gender.

2.2 Ethical approval

The Dudley Local Research Ethics committee granted ethical approval for both the cross-sectional and longitudinal components of this thesis in June 2004 and Feb 2008, respectively.

2.3 Clinical history

2.3.1 Demographic characteristics

Demographic details were recorded including: the patients' unique hospital number, date of birth, gender, ethnicity, and geographical area inhabited (as defined by postcode).

2.3.2 RA characteristics

Year of diagnosis: This information was taken from the medical notes and was represented by the date diagnosed to have RA by a physician rather than onset of symptoms

Extra-articular manifestations: The presence of extra-articular manifestations was established both from the clinical history and case note analysis. The features of extra-articular disease recorded included:

- Sicca symptoms
- Serositis
- Inflammatory eye disease
- Rheumatoid nodules
- Systemic vasculitis
- Nail-fold vasculitis
- Pulmonary fibrosis

2.3.3 Drug history

A full drug history was taken from all patients to include past and present medication use.

Previous DMARD use

A full history of DMARD, biologic and oral GC use was obtained from the medical notes. Due to the complexity of non-RA drug prescribing, particularly as this is predominantly co-ordinated by general practitioners, we did not attempt to record this.

Current drug use

For each patient a list of currently prescribed medications was compiled, including dosage. This included RA medications (such as non-steroidal anti-inflammatory drugs (NSAIDs), cyclo-oxygenase (COXII) inhibitors, DMARDs, biologics and oral GC use), anti-hypertensive medications (grouped into classes of drugs e.g. calcium channel blockers), lipid-lowering agents (statins and fibrates), diabetic medications, thyroid medications and "other" medications.

8.2.4 Previous cardiovascular history

Family history

All patients were asked about a family history of CVD and CVD risk factors in first-degree relatives. Although we strove to record accurate data (e.g. age of relative at first cardiac event), this information was dependant on an individual's recall of events. No attempts were made to verify this data.

Personal History

All patients were questioned and notes scrutinised to establish details of a personal history of cardiac events, including cardiac investigations (24 hour cardiac monitoring, echocardiography, nuclear cardiology, angiography/angioplasty, exercise tolerance tests), and the presence of all cardiac risk factors.

2.4 Clinical assessments

At each visit, patients underwent a number of clinical assessments. All patients had basic anthropometric measurements including:

- Height
- Weight
- Body mass index (BMI)
- Body composition (measured by a TANITA BC-418 analyser)

A range of other validated assessments were then used to record details of general well being, disease activity, degree of pain, disability and physical activity. These methods are described in more detail below.

2.4.1 Disease activity score

The complexity of RA has led to difficulties in defining 'active disease', due to the large number of clinical and laboratory variables that need to be taken into consideration. The demand for a reliable validated tool to assess disease activity increased with advances in drug therapy, both to provide an end point in the commercial trial setting, and also at a clinical level to assess response to treatment in a standardised manner. Attempts to produce a validated tool begun in the early 1990's, with a search for potential activity measures that fulfilled multiple validity checks. A method of scoring disease activity was developed, and was based on observed differences in clinical and laboratory variables amongst patients who were classified as high or low disease activity (based on blinded clinical treatment decisions e.g. low disease activity - if treatment with DMARDs remained the same or it was stopped) (454). Factors found to reliably associate with the physician's treatment decisions included number of tender joints, number of swollen joints, assessment of general health and erythrocyte sedimentation rate (ESR). A formula was then derived to give a score, with higher scores reflecting more active disease and lower scores less active disease.

The original disease activity score underwent further modification to provide a simplified version that could be incorporated into routine clinical practice (the DAS28) (455). The current DAS 28 includes a 28 joint assessment for tender and swollen joints, an ESR level and a patient completed visual analogue scale of well-being.
These factors are then added to a validated formula to produce a score:

DAS28 = 0.56 * sqrt(tender28) + 0.28 * sqrt(swollen28) + 0.70 * ln(ESR) + 0.014 * GH

Although other methods of assessing disease activity now exist e.g. ACR (456) and European league against rheumatism (EULAR) (457), I opted to use the DAS28 to assess disease activity amongst the RA patients, as DAS28 is now widely used throughout the world in RA clinical trials, and in the UK it has formed the basis of eligibility criteria for biologic therapies (458).

2.4.2 Health assessment questionnaire

The Stanford health assessment questionnaire (HAQ)(459) is a standardised validated self reported questionnaire, that is widely used in the rheumatological community to assess functional status. The questionnaire was originally designed to assess five specific outcomes related to RA: (1) disability; (2) discomfort and pain; (3) drug side effects; (4) costs of care; (5) mortality. However, completion of the original questionnaire was time consuming and impractical, thus prompting the development of a simplified shortened version of the HAQ. In 1986 Kirwan et al produced a modified version solely concentrating on disability and pain, and validated it for use in British patients with RA (459). Kirwans' version of the HAQ was used in this study to provide a score relating to the patients functional status. The score is derived from 20 questions, relating to eight aspects of daily living including dressing and grooming, arising, eating, walking, hygiene, reach, grip and errands and tasks. Each question has four possible answers based on a Likert scale ranging from 'without any difficulty' to 'unable to do'.

2.5 Classification of dyslipidaemia

Several methods of classifying dyslipidaemia have been developed. One of the earliest classification systems described by Frederickson et al (460) in 1971 was primarily based the pattern of lipoproteins on electrophoresis or ultracentrifugation (**Table 2.3**). However, this classification system has several limitations, particularly in the context of RA as it does not account for changes in HDL, and as a result its use is diminishing.

Table 2.3: Frederickson's classification of dyslipidaemia

Hyperlipoproteinaemia	Associated clinical disorder	Appearance of serum	Elevated particles	Serum TC	Serum TG
Type I	LPL deficiency ApoC-II deficiency	Creamy top layer	chylomicrons	→	↓↓
Type Iia	Familial hypercholesterolaemia, Polygenic hypercholesterolaemia, Nephrosis, Hypothyroidism, Familial combined hyperlipidaemia	Clear	LDL	↑↑	→
Type IIb	Familial combined hyperlipidaemia	Clear	LDL, VLDL	↑↑	↑
Type III	Dysbetalipoproteinaemia	Turbid	IDL	↑	↑
Type IV	Familial hypertriglyceridaemia, Familial combined hyperlipidaemia, Sporadic hypertriglceridaemia, Diabetes	Turbid	VLDL	→/↑	↑↑
Type V	Diabetes	Creamy top, turbid bottom	chylomicrons	↑	↑↑

TC: total cholesterol, TG: triglycerides, LPL: lipoprotein lipase, ApoC-II: apolipoprotein C-II, LDL: low density lipoproteins, VLDL: very low-density lipoproteins, IDL: intermediate density lipoproteins. ↑ = increased → = normal ↑↑= greatly increased →/↑= normal or increased

Although a number of other classification systems have been developed (118;461), for the purposes of this study dyslipidaemia was classified according to the more recently developed and widely used NCEP ATP III criteria (118). The NCEP criteria define dyslipidaemia as one or more of the following or taking lipid lowering therapy:

- TC ≥6.2 mmol/L
- LDL-C ≥4.13 mmol/L
- HDL-C <1.03 mmol/L
- TG ≥1.7 mmol/L

2.6 Blood sampling and storage

All blood samples were obtained following an overnight fast, between 8.30 and 10.30 am. Prior to venopuncture, patients were rested in a reclining chair. The skin was cleaned with a sterile wipe and a tourniquet applied immediately before commencement of the procedure. The blood samples were obtained by inserting a butterfly needle into a vein located in the anterior cubital fossa of the patient's arm. The needle was stabilised to minimise patient discomfort and aid the collection of multiple samples. Blood samples were then collected into vacutainer tubes and immediately taken to a single laboratory in DGoH for further processing. Routine haematological and biochemical tests were performed on the fresh samples the same day. Further blood collected in EDTA vacuette® tubes underwent centrifugation to separate the plasma and serum, prior to storage at –80^{o}C. These samples were later used for the lipid analyses (separation of lipoproteins, protein and cholesterol quantification, oxidation and nitration). Each sample was only exposed to a single freeze/thaw cycle to minimise the risk of corrupting the samples.

2.7 Laboratory quality control measures

With the exception of the functional lipid assays, all biochemical analyses were performed in a single laboratory at DGoH. The laboratory is subject to rigorous internal and external quality control performed on all analysers to ensure the highest degree of accuracy with all tests performed. Internal quality control is performed daily by laboratory staff and external quality control fortnightly by the Welsh External Quality Assurance Screen (WEQAS). The functional lipid assays were performed in a single

laboratory at Aston University, Birmingham, with similar quality control measures. Samples were transferred between laboratories on dry ice to minimise risk of thawing.

2.8 Routine blood tests performed on all patients

The routine blood tests outlined below were performed on all patients from both the cross sectional and longitudinal study arms. Individual methods for all haematological, biochemical and immunological blood tests are described in **Appendix 1: Laboratory methods.**

2.8.1 Haematology tests

These included both routine haematological variables (full blood count (FBC), erythrocyte sedimentation rate (ESR), serum ferritin, vitamin B12 and folate levels) and coagulation factors (INR, fibrinogen and von Willebrand factor (vWF)).

2.8.2 Biochemistry tests

These included: glucose, insulin, calcium, phosphate, urea, creatinine, sodium, potassium, alkaline phosphatase, alanine transferase, albumin, thyroid stimulating hormone (TSH), complement levels (C3 and C4), uric acid levels, CRP, serum amyloid A (SAA), serum angiotensin converting enzyme (ACE), iron, total iron binding capacity and homocysteine levels.

2.8.3 Immunology tests

Autoantibodies tested for included: RhF, anti-CCP, and anti-nuclear antibodies (ANA). For each of these autoantibodies the titre was recorded and the patient was classified as being ‘positive’ for the antibodies according to the local specific cut-off values.

2.9 Lipid assessments

2.9.1 The standard lipid profile

The standard lipid profile was analysed using a Vitros® chemistry system. TC, TG, and HDL were analysed using multi-layered slides, whereas apoA and ApoB were measured using a dual chamber package (both techniques are described in more detail in **appendix 1**). LDL levels were calculated using the Freidewald formula:

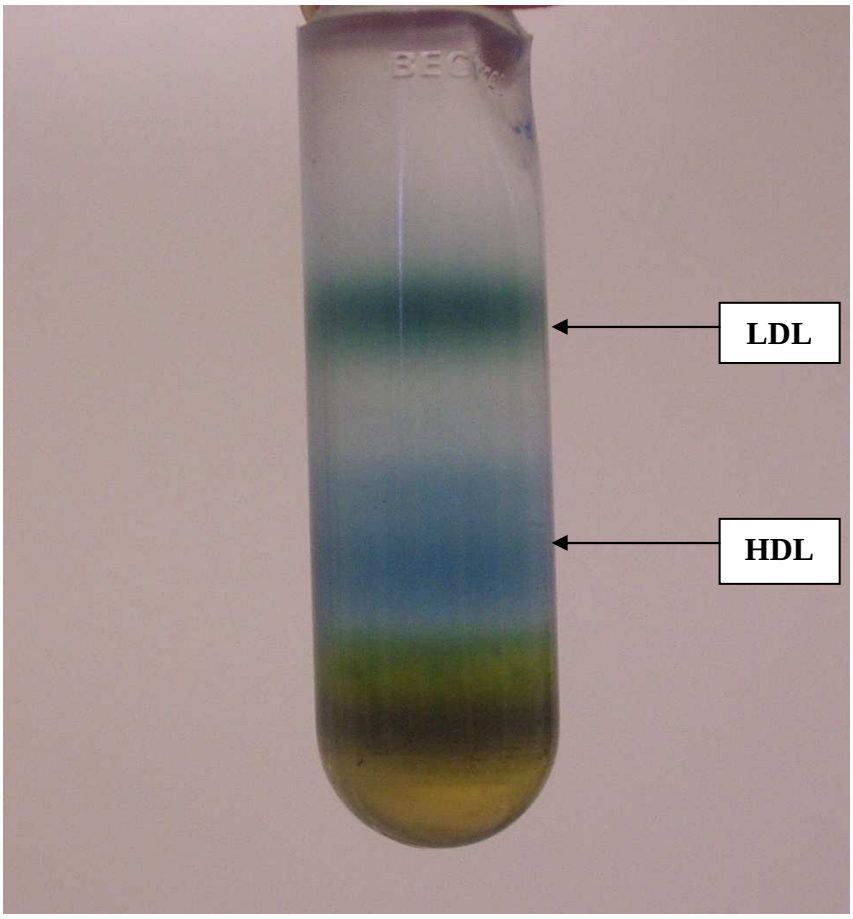
$$\mathbf{LDL = TC - HDL - (TG \div 5)}$$

Lipoprotein a levels were measured using a turbidmetric immunoassay kit. Samples were mixed with a buffer and anti-human Lp(a) antibodies. As Lp(a) joins with the antibodies, it yields an insoluble aggregate that causes increased turbidity. The degree of turbidity was then measured optically using Konelab^{TM}30 analyser and is proportional to the amount of Lp(a) in the sample.

2.9.2 Isolation of LDL

The methods used to isolate LDL were based on methods previously described by Chung et al (462). Whole blood collected in an EDTA tube was centrifuged (2000rpm) at 8^{o}C for 10 mins to separate out the plasma. Potassium bromide was then added to the plasma in order to raise the density of the solution and allow the lipid fractions to later separate into distinct bands. The potassium bromide (0.5724g) was added to each bijoux before 1.5ml of plasma was pipetted in to dissolve it completely. This solution (1.5ml) was then placed on a mixer for 10min. Following this, the potassium bromide solution (1.5ml) was pipetted into a 4.7ml centrifuge tube (optiseal) and the tube was filled to the brim with 0.15M sodium chloride (density 1.006g/ml). Once full, the tubes were sealed and placed in a TLA-110 fixed angle rotor before undergoing ultracentrifugation at 100,000rpm for 180 mins at 16^{o}C using a Beckman optima XP benchtop ultracentifuge. On completion of ultracentrifugation, two distinct suspended light yellow bands and a yellow solution at the bottom of the tube were visible. The top suspended light yellow band should be LDL and the bottom suspended band HDL (**see figure 2.2** - The lipoprotein bands have been stained with sudan black for the purpose of clarity of the figure). The LDL was collected using a sterile needle and syringe. The distribution of lipid fractions was validated using gradient gel electrophoresis.

Figure 2.2: Separation of lipids by density gradient ultracentrifugation



All lipoprotein fractions then underwent a process of desalting using Econo-Pac 10DG columns (BIO-RAD). The columns were washed with 20ml of phosphate buffered saline (PBS) prior to introducing the sample. The sample (3ml) was then introduced to the column. PBS buffer (4ml) was then pipetted into the column while the lipoprotein fractions from the column were collected into individual bijoux. These samples then immediately underwent further processing to analyse the protein content of the lipoprotein fractions (described below).

2.9.3 Protein quantification of isolated LDL

The protein content of LDL was quantified using a bicinchoninic acid protein kit assay. The bicinchoninic acid (BCA) working reagent was produced by mixing BSA with 4% copper sulphate solution in a 50:1 ratio until a uniform pale green solution was produced. Standards were prepared using 1mg/ml bovine serum albumin made up to serial dilutions of 0, 200, 400, 600, 800 and 1000 µg/ml. 10µl of each diluted standard were transferred to a 96 well plate in triplicate and 200µl of BCA working reagent

added. Samples were prepared in a similar manner, with 10µl of each sample (diluted LDL) plated in triplicate and 200µl of the BCA working reagent added. Samples and standards were then incubated for 30mins at room temperature (25^{o}C). The absorbance of each well was measured using a spectophotometeric plate reader at 562nm. The protein concentration of each sample was determined by comparing the absorbance of the unknown samples to the standard curve prepared using the bovine serum albumin standards.

2.9.4 Quantification of LDL and HDL subclasses

The separation of HDL subclasses (HDL2 and HDL3) was performed using a single precipitation method (463). This method allows the simultaneous precipitation of apoB containing lipoproteins and HDL2. A combination of heparin (1.4mg/ml), magnesium chloride (16.4mg/ml) and dextran sulphate (2mg/ml) were added to 0.3 ml of serum. These reagents were then mixed and left at room temperature (25^{o}C) for 30mins, prior to being ultracentrifuged at 10,000 rpm for 10mins at 4^{o}C. Following ultracentrifugation an aliquot of supernatant was taken for HDL3 measurement (performed on vitros chemistry machine). The measured value of HDL3 was then multiplied by 1.2 to correct for reagent dilution. The value of HDL3 was then subtracted from the total HDL level (as measured on the vitros chemistry system) to give the value of HDL2.

Although LDL can be divided in to a number of subclasses, it is the small dense type that appear to be the most clinically relevant and have been linked to atherosclerotic disease (464). Thus levels of small dense LDL (sdLDL) were quantified using the s LDL-EX "SEIKEN" test, on our konelab machine. The assay consisted of two steps. First the non-sdLDL lipoproteins were decomposed by a surfactant and sphingomyelinase. The cholesterol released from the non-sd LDL lipoproteins is then degraded to water and oxygen by the action of enzymes. In the second stage, a further surfactant releases cholesterol from sdLDL particles. This cholesterol is then subjected to enzymatic reactions, resulting in the development of a purple-red colour with the coupler in the presence of peroxidase.

2.9.5 Oxidation and nitration of LDL

The proportion of oxidised LDL was measured using an enzyme-linked immunosorbent assay (ELISA). Blood collected in EDTA tubes was centrifuged to separate the plasma fraction. Samples were diluted via a two-step dilution process to a final dilution of 6561 times, by adding 25 µl of patient sample and 2000 µl of buffer, vortex-mixing, and then repeating this dilution/mixing process. 25 µl of each standard (containing varying concentrations of human oxidised LDL), controls (containing a known amount human oxidised LDL) and diluted samples were added to the wells of ELISA plates coated with mouse monoclonal anti-oxidised LDL (mAb-4E6). Into each well 100µl of buffer was added and the plates incubated on a plate shaker for 2 hours at room temperature (25^{o}C). Each well then underwent a thorough 6 stage washing process involving aspiration of the reaction volume, refilling of wells with 350 µl of wash buffer and discarding the wash buffer solution, this process was repeated a further 5 times. After the final wash, the plate was inverted over absorbent paper and tapped gently to remove remaining fluid. A 100 µl of peroxidase conjugated mouse monoclonal anti-apoB (6 µg/ml) solution was added to each well, and the plates incubated for 1 hour at room temperature (25^{o}C) on a plate shaker. The 6 step washing process was repeated to remove all excess enzyme solution, prior to adding 200 µl of substrate 3,3',5,5'-tetramethylbenzidine (TMB), and incubating (not shaken) for 15min at room temperature (25^{o}C). A stop solution (50 µl) was then added to the wells to prevent further reactions and the ELISA plate then shaken for 15mins to ensure mixing. The optical density of the wells was read at 450 nm on a Tecan sunrise microplate reader. The results were calculated by plotting the absorbance values obtained for the calibrators (excluding calibrator 0) against the oxidised LDL concentration to construct a calibration curve. The concentrations of the controls and unknown samples were read from the calibration curve and the concentration multiplied by 6561 to correct for previous dilution.

The degree of LDL nitration was measured using an OxiSelect nitrotyrosine ELISA kit (cell biolabs, inc). This method allows the quantification of 3-nitrotyrosine in a protein sample by comparing its absorbance with that of a known nitrated bovine serum albumin (BSA) standard curve. 50µl of the patient's desalted LDL sample and nitrated BSA standards were added to a nitrated BSA preabsorbed enzyme immunoassay plate

in triplicate, and were incubated at room temperature for 10 mins. Subsequently, 50µl of an anti-nitrotyrosine antibody was added and the plate was incubated for a further hour at room temperature on a plate shaker. Each well was then washed three times with wash buffer to remove any excess antibody, prior to the addition of 100µl of a secondary antibody-enzyme conjugate to all wells. The plate was incubated for a further hour on a plate shaker at room temperature. All wells then underwent a further washing stage, before adding 100µl of the substrate solution to each well. The plate was then incubated further prior to the addition of 100µl of stop solution provided in the kit to each well, and the absorbance of each microwell being read on a spectrophotometer at 450nm.

2.9.6 Functional lipid assays

LDL oxidation lag time analysis

Oxidation of LDL *in vitro* can be measured by the formation of conjugated dienes produced as a consequence of oxidation of polyunsaturated fatty acids (PUFAs). In order to measure LDL susceptibility to oxidation, LDL samples were separated from serum and desalted (as described in section 2.9.2). The protein concentration of each sample was measured using the BCA method (previously described in section 2.9.3) and was standardised to a concentration of 50mg/ml. Subsequently, 950µl of the protein standardised patient LDL sample and 50µl of copper chloride working solution (40µM) were added to a cuvette and pipetted up and down three times. The samples were then placed on a UVIKON spectrophotometer, and absorbance analysed at 230nm for 200 minutes, with recordings taken every 5 minutes. The readings obtained from the spectrophotometer were then converted in to a graphical format, the intercept of the slope of the initial phase and the slope of the propagation phase determined the lag time.

DiIox LDL uptake by U937 cells

The human monocytic cell line U937 were maintained in RPMI 1640 media, supplemented with 10% heat inactivated fetal bovine serum (inactivated via incubation in 65^{o}C water bath for 15mins) and 1% penicillin/streptomycin. The cells were grown at 37^{o}C in a humidified 5% CO_2/95% air incubator and passaged every 3 or 4 days. A Nuebauer haemocytometer was used to identify the number of viable cells. A batch of oxLDL was made by incubating LDL with copper sulphate for 1hr. The batch of oxLDL

was then labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) at a concentration of 300mgDiI/mg oxLDL (DiIoxLDL). This process involved incubating oxLDL with DiI at 37^{o}C overnight in the dark. Excess dye was then removed through a PD10 column by adding 3mls of DiIoxLDL into a PBS pre-washed PD10 column and adding 4ml of PBS to the column. Drops of the eluted desalted sample were collected and pooled for protein determination.

The uptake of LDL (isolated from the plasma of subjects recruited to the longitudinal arm of the study) into monocytes and macrophages was studied using a competition assay (**see figure 2.3**). U937 cells were suspended in fresh 1640 RPMI media at $0.5x10^{6}$/ml and seeded 1ml per well in a 24 well plate. Each well was treated with 4µl phorbol 12-myristate 13-acetate (PMA) (100nM) for 4 hours. All LDL samples were then desalted using G25 micro-columns, and condensed from 1ml to 0.5ml in a speedvac. The protein concentration of the condensed desalted LDL samples (patient/subjects, control LDL and DiIoxLDL) was then quantified using a BCA assay (previously described in section 2.9.3). The cells were then treated with 10µg/ml of DiIoxLDL and 10µg/ml of the patient/subjects desalted LDL, and were plated in triplicate. In addition, there were several control wells per assay, including cells treated with PMA alone, cells treated with PMA and DiIoxLDL, but no competitor LDL (to assess maximal uptake) and cells treated with PMA, DiIoxLDL and control LDL to allow consistency between assays to be assessed. To reduce the effects of intra assay variability on the results, LDL extracted from one patient at all follow up time points from each arm of the longitudinal study was used per assay (e.g. per assay LDL from one patient treated with GCs, one treated with an anti-TNF agent, one treated with rituximab, one healthy control and one RA control, at all follow up time points were included). The plated cells were then left for a further 20 hours in the dark at 37^{o}C in a humidified 5% CO_2/95% air incubator to allow the competitive uptake of LDL/DiIoxLDL.

The following day the cells were harvested from the wells, and each well was washed out with 1ml of PBS prior to the cells being centrifuged at 1500 rpm for 5 mins at 20 ^{o}C. The supernatant was then removed and the cells resuspended in 6ml of fresh RPMI media. This washing process was then repeated to remove free DiIoxLDL not taken up

by the cells: further centrifugation (1500rpm for 5 mins at 20 ^{o}C), supernatant removed and cells resuspended in 3ml of fresh RPMI media. The supernatant was removed and the cells were lysed by adding 350µl of 5% triton before returning them to their original well in the 24-well plate (to ensure all cells including those adherent to the base of the well were lysed). The fluorescence of each well was then read in triplicate using a colourimetric plate reader (Molecular Devices Gemini XS microplate reader) at excitation 540nm, emission 590nm using softmax pro software. The protein concentration of each cell lysate sample was also measured in triplicate using the BCA method (previously described in section 2.9.3).

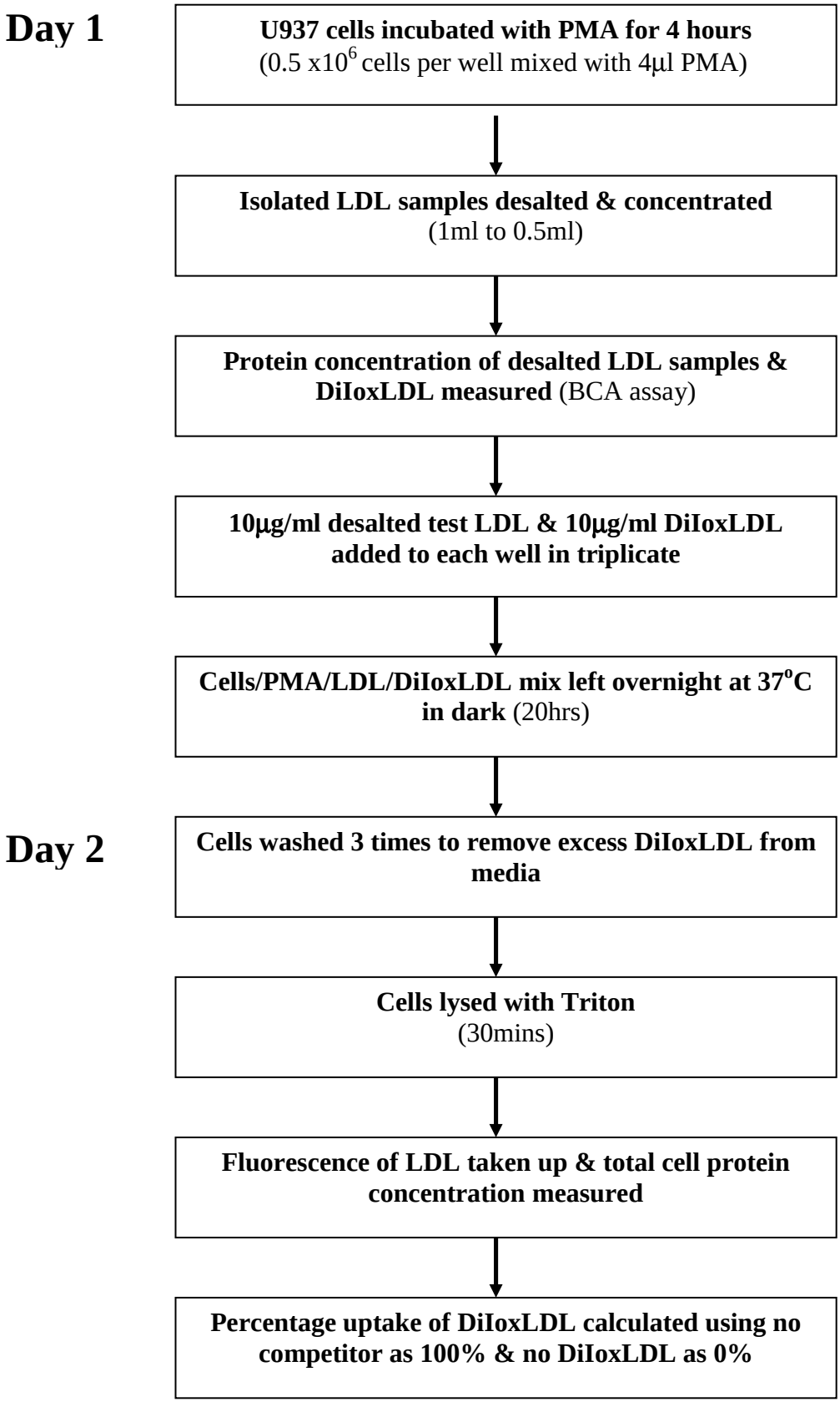
In order to exclude potential outlying values arising from either the end of assay BCA or fluorescence readings, values greater than 2 SD of the mean were excluded prior to performing the calculation outlined below to quantify the uptake of DiIoxLDL in to U937 cells:

$$\frac{\text{Av. fluorescence of sample}}{\text{Av. protein conc of sample}} \times 10 \text{ minus } \frac{\text{Av. fluorescence of cells/PMA}}{\text{Av. protein conc of cells/PMA}} \times 10$$

$$\downarrow$$

$$\frac{\text{Amount of diI labelled oxLDL (µg)}}{\text{Protein concentration (mg)}} \times 100 = \textbf{\% \textit{DiIoxLDL uptake}}$$

Figure 2.3: Flow chart summarising the protocol used to measure LDL uptake



2.9.7 Optimisation of LDL uptake assay

Although methods for assessing LDL uptake into the U937 human monocytic cell line have previously been described (276), initial analysis using this method identified the need to optimise several aspects.

Optimisation of LDL separation

LDL separation was originally based on the method described in section 8.9.2 with samples being spun for 90 mins. However, subsequent tests to examine the purity of the isolated LDL band revealed significant albumin contamination. The albumin concentration of the isolated LDL band was measured on a Vitros chemistry system (see **appendix 1**). Albumin contamination was important to observe as albumin is known to interfere with the process of LDL oxidation (465), and may have interfered with results of some of the assays using the isolated LDL e.g. LDL lag times. However, doubling the ultracentrifuge spin time from 90 mins to 180 mins at 100,000 rpm resulted in a much purer LDL band, containing either none or tiny traces of albumin. (**see table 2.4**)

Table 2.4: Optimal ultracentrifugation to reduce the albumin contamination of LDL samples

	Albumin concentration after 90 min spin	Albumin concentration after 180 min spin
Sample 1	30.2 mg/l	0.0 mg/l
Sample 2	28.6 mg/l	0.3 mg/l

2.9.7.2 Optimal length of incubation time with PMA

PMA is used *in vitro* to differentiate U937 monocytic cells. During differentiation, monocytes undergo a number of changes, including enhanced expression of CD36 (466). However, despite widespread use of PMA as a monocyte differentiating agent, PMA has been shown to be toxic to cells after prolonged exposure. Thus I aimed to establish the optimal length of time required for maximal CD36 expression without significant effects on cell longevity. In this assay, 0.5 $x10^6$ U937 cells per well were incubated with PMA for different lengths of time (4, 6, 24, 48, 72 hours). Following this the cells/PMA were incubated for a further 30mins on ice with 1µl of anti-CD36 FITC

antibody. The degree of CD36 receptor expression and cell viability were then assessed using flow cytometry. The results are summarised in **figures 2.3 and 2.4**.

Figure 2.4: Expression of CD36 receptor following incubation of U937 cells with PMA

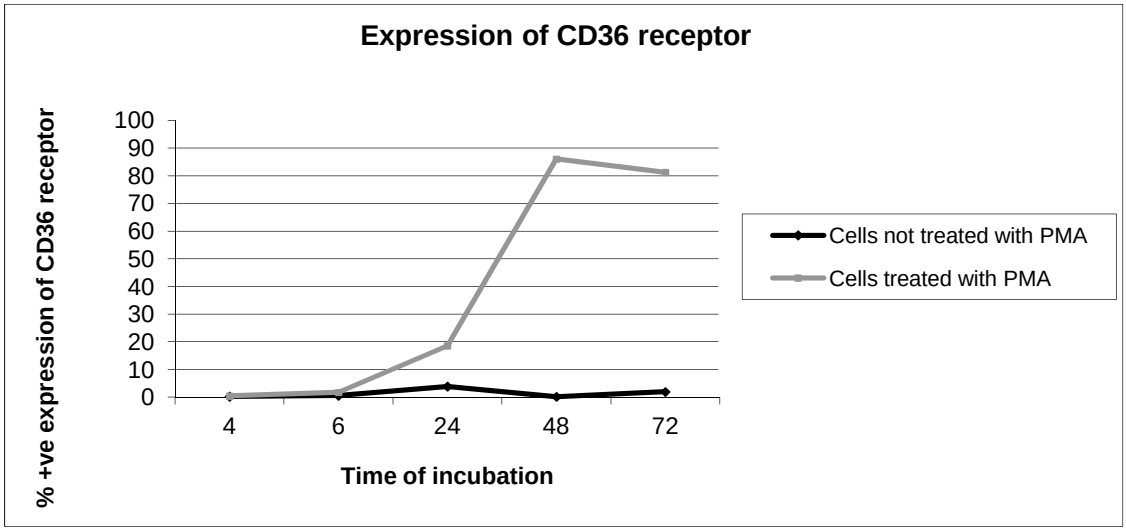
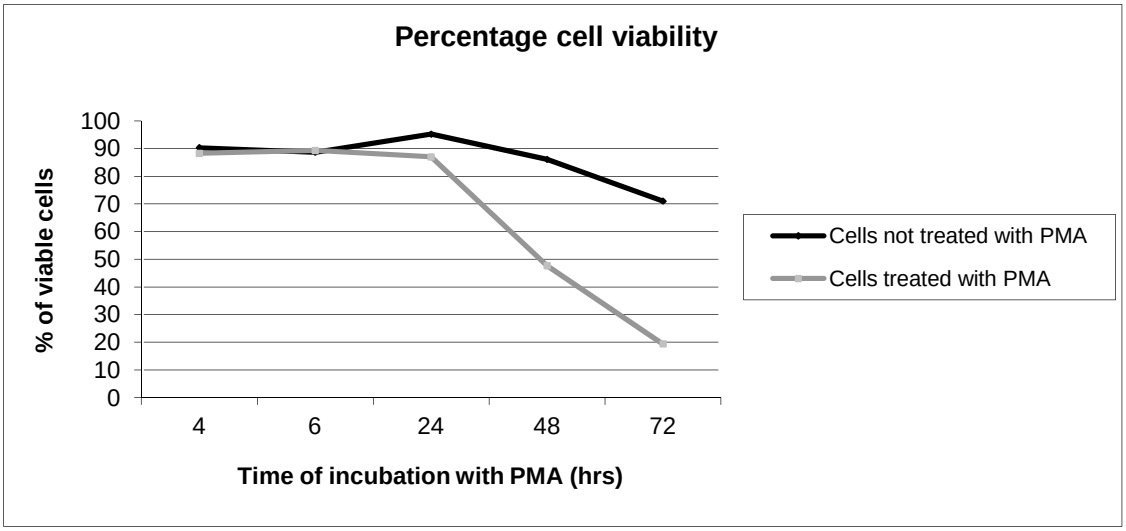


Figure 2.5 The percentage of viable cells following the incubation of U937 cells with PMA



The results demonstrate a 24 hr incubation of the cells with PMA is optimal, allowing good CD36 receptor expression without compromising cell viability.

Percentage of uptake of DiIoxLDL mediated through CD36 receptor: CD36 receptor blocking using anti-CD36 FITC antibody

Although many scavenger receptors are involved in the uptake of LDL into macrophages, the CD36 receptor is thought to dominate. In order to prove the importance of the CD36 receptor, for the purposes of this functional assay, an attempt was made to block the uptake of DiIoxLDL by blocking CD36 receptor sites with anti-CD36 FITC antibody. The two day functional assay was based on the methods described in figure 2.3. The U937 cells (0.5×10^6/ml) were treated with 5µl (per ml) of the anti-CD36 antibody prior to the addition of DiIoxLDL at different concentrations (10, 15, 20µg per ml) to the wells. Control wells were included in the assay: cells not treated with any DiIoxLDL or anti-CD36 antibody, cells treated with the anti-CD36 antibody but no DiIoxLDL and cells treated with the three concentrations of DiIoxLDL but no CD36 antibody. At the end of the assay the difference in the percentage uptake of DiIoxLDL was calculated for each concentration of DiIoxLDL (10, 15, 20µg per ml) used (**table 2.5**) e.g. (% uptake of DiIoxLDL by cells *not* treated with CD36 antibody – % uptake of DiIoxLDL by cells treated with CD36 antibody).

Table 2.5: Percentage uptake of DiIoxLDL when CD36 receptors are blocked by anti-CD36 antibodies

	Percentage of uptake blocked by anti-CD36 antibody
Cells & 10µg per ml DiIoxLDL	9.3%
Cells & 15µg per ml DiIoxLDL	31.4%
Cells & 20µg per ml DiIoxLDL	24%

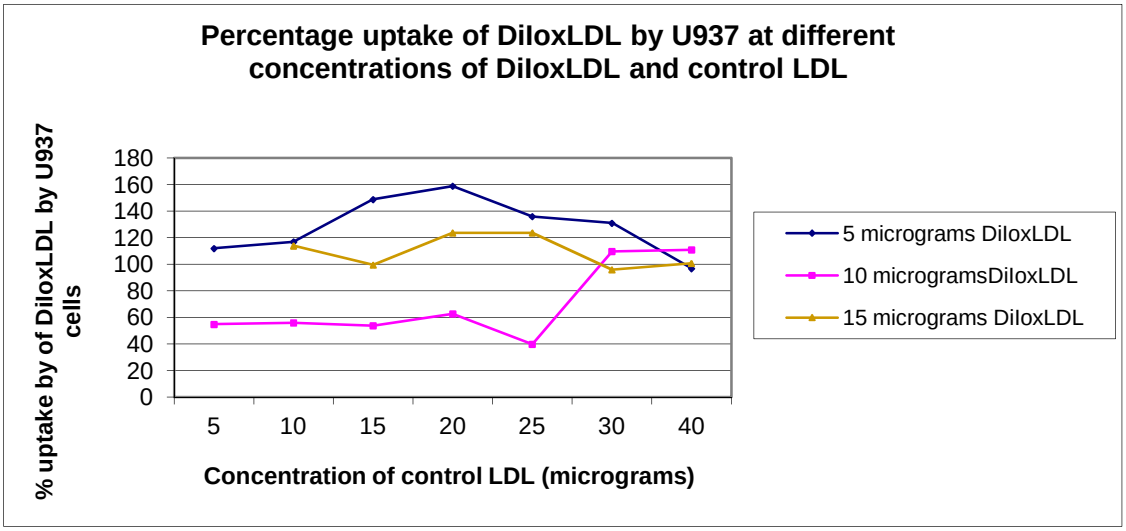
This assay demonstrates that DiIoxLDL uptake into U937 cells is at least partially mediated through the CD36 receptor. Despite this, it is possible that a lower level of DiIoxLDL uptake was not seen either because of CD36 receptor sites not being adequately blocked by the antibody e.g. a higher concentration of antibody was required or that the remaining DiIoxLDL was being taken up through an alternative route e.g. other scavenger receptors. I attempted to ensure CD36 receptor saturation by repeating the assay but using different concentrations of the anti-CD36 antibody (5, 10, 15, 20µl per ml), however, no significant differences in percentage of uptake of DiIoxLDL were

seen from those already described (results not shown). This would suggest the remaining DiIoxLDL uptake is mediated through alternative pathways including other scavenger receptors e.g. SR-AI/II, etc.

Optimising concentrations of competitors (DiOxLDL and patient LDL)

This functional assay is based on the competitive uptake of DiIoxLDL and the patients LDL via the scavenger CD36 receptor. The optimal concentration of each of the competitors was established by varying the concentrations of both DiIoxLDL and a control LDL sample. The analysis was performed according to the basic principles outlined in section 2.9.6, but in addition adopting the optimal 24hour incubation of the cells with PMA. In this assay, three different concentrations of DiIoxLDL were added (5, 10 and 15µg per ml) in combination with varying concentrations of the control LDL (5, 10, 15, 20, 25, 30, 40, 50µg per ml) to 0.5 $x10^6$ U937 cells per well. The percentage uptake of DiIoxLDL by the cells was then measured. The results are summarised in **Figure 2.6**.

Figure 2.6: The percentage uptake of DiIoxLDL by U937 cells following incubation with varying concentrations of both DiIoxLDL and control LDL



The results from this assay demonstrated that the optimal competition between the control LDL and DiIoxLDL appeared to occur when using 10µg per ml of DiIoxLDL in combination with one of the lower concentrations of the control LDL (5, 10, 15 or 20µg per ml). However, as the previous published data in RA used 10 µg/ml DiIoxLDL and

10µg/ml of the patients LDL (276), for data consistency a concentration of 10µg DiIoxLDL and 10µg patient LDL was chosen as the optimal concentration to use in future assays.

Reproducibility of the assay

Once all the assay parameters had been optimised the reproducibility of the assay was checked. The assay was performed on two separate occasions (2 days apart), thus allowing the results to be fully independent of each other. In order to maximise the reproducibility of this assay, the LDL from three healthy control patients was compared. The results from this assay are shown in **table 2.6**.

Table 2.6: The percentage uptake of DiIoxLDL: comparison in two reproducibility assays

	Percentage uptake of DiIoxLDL by U937 cells	
	Assay 1	**Assay 2**
LDL 1	83.3%	82.8%
LDL 2	76.4%	79.1%
LDL3	87.4%	79.1%

The assay was deemed to be reproducible, with only small changes in the percentage uptake detected between the two assays. The analysis was then applied to the LDL obtained from subjects recruited in to the longitudinal study (RA Intervention, RA controls and HC).

2.10 Genetic tests

2.10.1 DNA extraction

DNA was extracted from whole blood using the QuickGene-810 system. Blood collected in EDTA containing tubes were placed in an isolation vessel and the red cells lysed. A filter matrix was used to capture the white blood cells and these were lysed to release DNA, which was in turn entrapped around the fibres. Isolated DNA was released from the matrix and eluted in a collection vessel in the enclosed environment of the QuickGene 810 system, and then stored at 4^{o}C. To ensure quality control, a blank

tube was processed in an identical manner (without the addition of any blood) alongside each batch of extractions. If any DNA was found in the blank the whole DNA batch was rejected.

2.10.2 Polymerase chain reaction

In order to amplify specific regions of DNA so that sufficient DNA is available for further analysis, we will use a technique called the polymerase chain reaction (PCR). This method allows a single gene to be replicated exponentially. The process is reliant on the presence of an enzyme (DNA polymerase), and is based on technique called thermal cycling. Double stranded DNA is separated at high temperatures (DNA melting) in to single strands, which later act as templates for DNA synthesis. As the single strands of DNA are cooled, two primers, specific for the mutation of interest, anneal to the DNA strand in order to flank the mutation of interest. DNA polymerase then synthesizes a new strand of DNA complementary to the single strand DNA template that extends beyond the position of the primer-binding site on the other template. This process is then repeated by reheating the reaction mixture to induce further separation of the original and newly synthesized DNA strands, ultimately producing four binding sites to which the primers may anneal and DNA polymerase can act. The extension of the strands of newly synthesized DNA is restricted precisely to the target sequence. This cyclical process can then be repeated.

2.10.3 Roche LightCycler Real-time PCR: detection of genetic polymorphisms

All genetic polymorphisms of interest were analysed using real-time PCR performed on a Roche lightcycler. This system allows the formation of PCR products to be monitored, and offers a relatively rapid method for the detection of genetic polymorphisms. For each genetic polymorphism real-time PCR requires the presence of two individually designed primers and probes. The specific primers anneal to the heat separated single strands of DNA, identifying the DNA sequence of interest (as described above). Hybridisation probes are designed as a pair, an anchor probe and a sensor probe, and are complementary to specific regions of the DNA sequence of interest. The anchor probe is designed to hybridise to the DNA strand 1-5 base pairs away from the SNP of interest, whereas the sensor probe bridges the base pair affected by the single nucleotide polymorphism. Each probe is labelled with a dye, the anchor probe with LC red 640, and the sensor probe with flourescein. When the two probes have hybridised to the

amplified DNA fragment, the two fluorescent dyes are in close proximity to each other. This positioning is crucial for the emission of light, which is detected by the LightCycler. The flourescein dye is excited by the LightCycler's light emitting diode filtered light source, and emits green fluorescent light at a slightly longer wavelength. This emitted energy excites the closely positioned LC red 640 dye on the other probe, leading to the emission of red fluorescent light, which is then filtered and measured by the lightcycler's fluorimeter. The energy transfer between dyes is called fluorescence resonance energy transfer (FRET).

The detection of SNPs is based on the melting temperature of the hybridisation probes. A sensor probe that fully hybridises to the amplified DNA (including the nucleotide affected by the mutation) will have a higher melting temperature than a sensor probe that fails to fully hybridise to the anchor probe due to a mismatch in the region of the SNP. As probes melt away from the DNA strand, the fluorescent signal is lost. The difference in melting temperatures can be plotted in a graphical format based on the fluorescence. Patients who are heterozygous for a mutation will have two peaks visible on the melting curve graph, whereas patients who are homozygous will have just one peak. Patients who are homozygous for the mutation will have a different melting temperature from patients who are homomozygous for the wild type, which will alter the position of the peak. The detailed methods for the detection of SNPs related to alterations in lipid metabolism and CVD are discussed in chapter 6, section 6.3..

2.11 Statistical methods

All data was analysed using SPSS 18.0 for windows (SPSS Inc. Chicago, Illionis). The general approach that was taken for all statistical analyses was to identify whether each parameter was normally distributed using the Kolmogorov-Smirnov test. Data that does not follow a normal distribution was log transformed for tests that assume normal distribution e.g. linear regression. The average of normally distributed data is presented as a mean value ± standard deviation, whereas not-normally distributed data is presented as a median (25^{th} to 75^{th} percentile). Categorical data is presented as percentages.

All analyses consider a p value of <0.05 as statistically significant.

2.11.1 Cross-sectional data

Univariate tests:

1) Associations between two categorical variables were assessed using a chi-squared test.

2) Associations between a categorical and a continuous variable were analysed using a student's t test or Mann Whitney U test for normally distributed and not-normally distributed data, respectively. For analysis of categorical variables with 3 or more categories, one-way Analysis of Variance (ANOVA) was used if the continuous variable was normally distributed and Kruskal-Wallis was used if the continuous variable was not normally distributed.

3) Correlations between two continuous variables were assessed using Pearson's correlation test when comparing two normally distributed variables, whereas Spearman's correlation test was used to compare two sets of not-normally distributed data.

4) Allelic and genotypic frequencies were analysed using a chi-squared test.

Multivariate analysis:

Multivariate analyses were carried out on the cross sectional data to identify independant predictors and control for potential confounders. The techniques that were used included:

1) Binary logistic regression when the variable of interest was categorical (2 categories)
2) General linear model when the variable of interest was categorical (>3 categories e.g. genotypic associations).
3) Linear regression when the variable of interest was continuous

A potential confounder is defined as a factor known to correlate with both the dependant and independent variable, which does not act as an intermediate step in the causal pathway between the dependent and independent variable. All potential confounders were identified and adjusted for in multivariate analyses to minimise the risk of a type 1 error (false positive). For the purposes of this thesis, potential confounders included in the analyses were factors identified in the univariate analyses and factors known to exert a biological effect on the dependant and independent variable (even if not significant univariately).

2.11.2 Longitudinal data

Generalised Estimating equations were used to analyse the longitudinal data presented in this thesis. Firstly, they were used to look for longitudinal associations between individual lipid parameters and markers of systemic inflammation in a large retrospective longitudinal cohort. Secondly, they were used to examine the effects of inflammation on lipid structure and function in a smaller prosepctive longitudinal cohort. GEEs offered two distinct advantages over other methods of longitudinal data analysis. These included 1) an ability to handle an inconsistent amount of data on each patient 2) an ability to longitudinally adjust for potential confounders (e.g. CRP where the levels may significantly alter during the course of follow up).

Chapter Three: The magnitude of dyslipidaemia in RA and the CVD risk this confers

3.1 Introduction

This first results chapter will attempt to set the scene, by providing an understanding of the scale and potential clinical cardiovascular implications of dyslipidaemia in a large well characterised cross sectional RA cohort.

'Dyslipidaemia' is a broad term that encompasses a variety of changes in lipids from alterations in their levels, to changes of structure or function. However, for practical purposes dyslipidaemia has been defined by the NCEP III criteria (118) (see **table 3.1**).

Table 3.1: NCEP III definition of dyslipidaemia

One or more of the following must be present to fulfil the NCEP III definition of dyslipidaemia:
TC ≥6.2 mmol/L
LDL ≥4.13 mmol/L
HDL <1.03 mmol/L
TG ≥1.7 mmol/L
taking lipid lowering therapy

Although this definition is vital for establishing the prevalence of dyslipidaemia and is useful in an academic setting, it is not very useful as a clinical tool to guide management, as the presence of other CVD risk factors have to be taken into consideration. Current guidelines recommend the use of statins to produce a less atherogenic lipid profile in patients deemed to be at risk of CVD and in those with established CVD or cerebrovascular disease (467;468). CVD risk is assessed using risk algorithms which estimate an individual's risk of developing CVD over a given time period (in those without existing CVD) by taking into account many other CVD risk factors (e.g. age, gender, smoking status, hypertension and lipid levels).

To date, a number of risk algorithms have been developed and are used across the world to predict CVD risk. The earliest risk prediction tool was developed over 30 years ago by the Framingham Heart study investigators, the Framingham risk score (FRS) (469), allowed an individual person's 10 year CVD event probability to be expressed as a percentage. This risk stratification system has formed the basis of quantifying risk and guiding treatment for many years, and is still used widely throughout the world. However, progression of our understanding of CVD has prompted the development of newer risk scoring systems (118;469-471). The NCEP III guidelines (118) developed in 2002, perhaps offer a more comprehensive method of risk assessment, as they have been designed to incorporate the Framingham 10 year risk score as well as additional factors such as high risk conditions (e.g. type 2 diabetes) and the use of drugs (e.g. anti-hypertensives and lipid lowering agents). The NCEP guidelines have been formulated specifically to guide the management of dyslipidaemia via lifestyle modification and drug intervention. A more recently developed cardiovascular risk tool which was produced and is used in most of Europe, the Systematic Coronary Risk Evaluation (SCORE) (472), involves the use of risk tables with the main focus being total cholesterol (TC) levels or the TC:HDL ratio, and again allows a person's 10 year risk of CVD to be expressed as a percentage. However this method, unlike the FRS estimates the 10 year risk of any first fatal atherosclerotic event and not just death from coronary heart disease, and it also only estimates CVD mortality and not morbidity. Further advances in our understanding of the pathogenesis of CVD, including the recognition of C-reactive protein (CRP) as an independent CVD risk factor in the general population (473;474) have led to the development of CVD risk algorithms (475;476). The Reynolds risk score (RRS) (475) is based on the FRS but incorporates additional risk factors including levels of high sensitivity CRP and a parental history of myocardial infarction before the age of 60; thus offering the potential to account for the excess CVD risk seen in RA as a result of systemic inflammation. The Reynolds risk score calculates a person's 10 year risk of cerebrovascular events in addition to CVD morbidity and mortality. A summary of the similarities and differences of the CVD risk calculators are shown in **table 3.2.**

Table 3.2: Comparison of the CVD risk calculators: components and outcome measures of risk

	FRS	NCEP	SCORE (TC)	SCORE (TC:HDL)	RRS
Validated age (yrs)	<75	Men ≥45 Women ≥55	35-65	35-65	45-80
Smoking	√	√	√	√	√
Blood pressure					
Systolic	√	√	√	√	√
Diastolic	-	√	-	-	-
Anti-hypertensive	-	√	-	-	-
Lipids					
TC	√	-	√	√	√
HDL	√	√	-	√	√
LDL	-	-	-	-	-
TG	-	-	-	-	-
FHx CVD	-	√	-	-	√
CRP	-	-	-	-	√
Outcome measure of CVD risk	10 year risk of fatal and non-fatal CHD events	10 year risk of fatal and non-fatal CHD events	10 year risk of fatal CVD and non CHD CVD events		10 year risk of fatal and non-fatal CVD events

FRS: Framingham risk score, NCEP: national cholesterol education programme, SCORE: systematic coronary risk evaluation, RRS: Reynolds risk score, TC: total cholesterol, HDL: high density lipoproteins, LDL: low density lipoproteins, TG: trigylcerides, FHx CVD: family history of cardiovascular disease, CRP: C-reactive protein, CVD: cardiovascular disease

A recent meta-analysis has demonstrated that RA patients have an increased cardiovascular standardised mortality ratio of between 1.6-1.7 (477). As a result of this observation, it has been suggested that risk, as calculated by conventional risk stratification methods, should incorporate a multiplier (478;479). In particular, the EULAR taskforce have suggested that the use of a 1.5 risk multiplier should be reserved for RA patients fulfilling two of the following three criteria: i) a disease duration greater than 10 years ii) seropositive for rheumatoid factor or anti-cyclic citrullinated peptide (anti-CCP) antibody and iii) presence of extra-articular manifestations.

In this first results chapter I will describe: 1) the prevalence and predictors of NCEP defined dyslipidaemia amongst the cohort of 400 cross-sectional RA patients 2) how

changes in the lipid profile translate in to CVD risk in RA using the four original CVD risk algorithms (FRS, NCEP, SCORE, RRS) and also by incorporating a 1.5 multiplier in applicable patients, according to EULAR taskforce recommendations to account for excess RA risk 3) establish whether statin therapy was initiated appropriately in those deemed to be at risk of CVD.

3.2 Methods

Recruitment and baseline assessment of RA patients in to the cross sectional arm of this study has been described in the methods section (chapter 2, section 2.1). Fasting blood samples were obtained from all RA patients. Routine blood tests performed and the measurement of lipid parameters are outlined in chapter 2, section 2.7 and section 2.9.1, respectively.

Patients were classified as dyslipidaemic if they fulfilled the NCEP III criteria for dyslipidaemia (118)(**see table 3.1**).

CVD risk assessment was performed on all patients by application of the FRS (469), NCEP (118), SCORE (472) and RRS (475). Calculation of the FRS involved scoring patients according to their age, TC level, HDL-cholesterol level, systolic blood pressure, and smoking status. The scores from each of these components were then added together to give a total score and a corresponding 10-year CVD risk prediction. A 20% 10 year risk of global CVD events as calculated using the FRS is generally accepted as a cut-off for the implementation of statin therapy for primary prevention in the UK (233). This tool is only validated for use in patients below the age of 75 and therefore was only applied to patients less than 75 without a prior history of CVD or diabetes. The five step NCEP risk assessment involved classifying fasting lipid levels, identifying major risk factors for CHD (e.g. age, cigarette smoking, hypertension and family history of CVD in first degree relatives), and calculating 10 year CHD risk. This evaluation enables the determination of the risk category that establishes need for lipid lowering therapy and the LDL goal. In general, lipid lowering therapy should be commenced in all patients found to have a 10 year risk >10% for global CHD events
and an LDL-cholesterol >130 mg/dl (further details regarding risk categories and LDL treatment thresholds are summarised in **table 3.3**).

Table 3.3: NCEP risk categories/LDL levels requiring treatment with lipid lowering therapy.

Risk category	**LDL level to consider lipid lowering therapy**
High risk: CHD or CHD risk equivalent (10 year risk >20%)	2.58 mmol/L
Moderately high risk: 2 or more risk factors (10 year risk 10-20%)	3.36 mmol.L
Moderate risk: 2 or more risk factors (10 year risk<10 %)	4.13 mmol/L
Lower risk: 0-1 risk factor	4.91 mmol/L
CHD: coronary heart disease, LDL: low density lipoproteins	

Risk stratification using the SCORE criteria involves the use of specially designed and validated risk tables. The risk tables include data on age, gender, smoking, blood pressure, TC and HDL level. Individual tables have been developed for both low and high risk patients (based on TC levels) and for risk based on the TC:HDL ratio. For the purposes of this study high risk tables were used, as this study was carried out on a population based in the United Kingdom, a country classified as 'high risk' (472). In addition, analysis was restricted to tables based on the TC:HDL ratio as this is in line with the EULAR task force recommendations (478;480). Patients were classified as at risk of CVD if their 10 year risk was ≥ 5% for CVD deaths. Patients are deemed eligible for statin therapy as part of CVD risk reduction if they have a 10 year risk ≥ 5% and an LDL ≥3 mmol/L or TC ≥5 mmol/L. Patients were excluded from SCORE risk stratification if they were already deemed at high risk of CVD e.g. a prior history of CVD, diabetes mellitus, familial hypercholestrolaemia (TC ≥8 mmol/L or LDL ≥6 mmol/L), or a blood pressure ≥180/110 mmHg. The RRS was calculated using an online electronic tool (481). Patients over the age of 80 and diabetic patients were deemed to be at high risk and thus excluded from risk stratification. Details regarding the patients age, gender, systolic blood pressure, smoking status, TC level, HDL level, high sensitivity CRP level and parental history of CVD were used in the calculation of the 10 year risk. Patients with a 10 year risk ≥ 20% were classified at risk of CVD and were eligible for statin therapy.

In total, 266 patients were assessed according to FRS (limited to patients over the age of 75, without DM or prior history of CVD), 294 patients were eligible for NCEP risk stratification following the exclusion of patients with a prior history of CVD and

diabetes, 166 patients were assessed by SCORE (limited to those who are <65 years of age, without prior history of CVD, DM, familial hypercholestrolaemia and severe hypertension), and 291 patients were available to be assessed by the RRS once patients with DM, CVD and those over the age of 80 were excluded.

In accordance with the EULAR taskforce specific recommendations (478) patients with two out of three of the following: i) a disease duration ≥10 years ii) seropositive (RF positive or anti-CCP positive) iii) evidence of extra-articular disease, had their CVD risk according to each definition multiplied by 1.5. For the FRS this required a straightforward multiplication of the 10 year risk. Adaptation of the NCEP risk stratification criteria was done in a similar manner by incorporating the multiplied FRS into the 5 stage process. For SCORE and RRS, the final 10-year risk was multiplied by 1.5. The cut off levels for implementing lipid-lowering therapy remained unchanged, therefore allowing excess patients to be identified as at risk. The appropriateness of lipid-lowering prescriptions was then examined.

Statistical analysis was carried out using SPSS 18.0 (SPSS Inc, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to determine whether each parameter was normally distributed. Values were expressed as mean ± standard deviation (SD), median (interquartile range - IQR) or percentages, as appropriate**.** For the univariate analysis, Chi-squared, t-test and Mann-Whitney U tests were used to test categorical, normally and not normally distributed data, respectively.

A binary logistic regression model including age, sex, hypertension, uric acid levels, total HAQ, IR, BMI, thyroid stimulating hormone (TSH), Prednisolone, HCQ and CRP was utilised in order to evaluate which factors were independently associated with the presence of dyslipidaemia according to the NCEP criteria.

3.3 Results

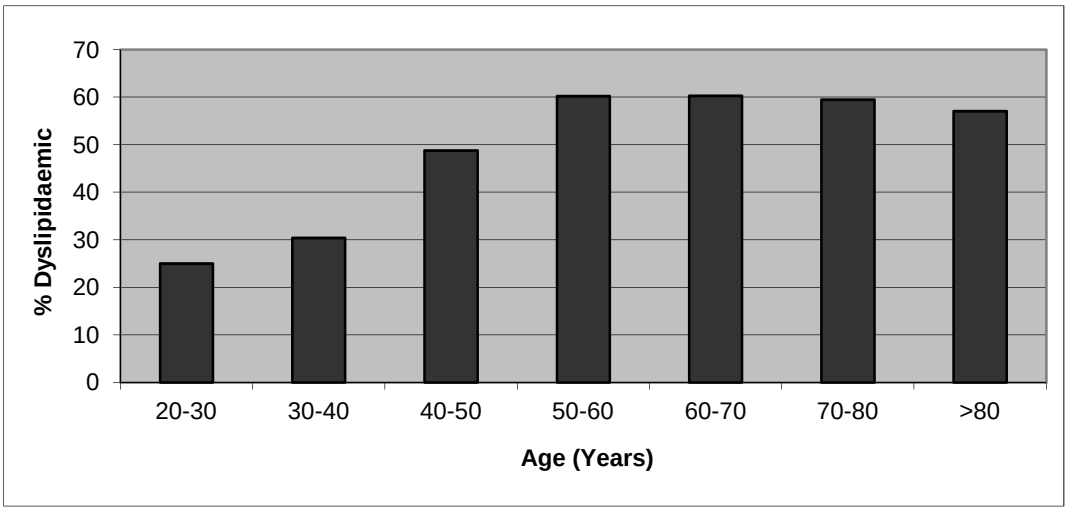
3.3.1 Basic demographics of study population

Basic demographics and clinical characteristics of the cross sectional RA population are described previously in chapter 2, section 2.1.1, table 2.1.

3.3.2 The prevalence of NCEP defined dyslipidaemia in RA

Two hundred and twenty seven (56.8%) RA patients were dyslipidaemic according to the NCEP criteria, of whom 79% were female. The prevalence of NCEP defined dyslipidaemia steadily increased up until the age of 50, where it peaked and then stabilised (see **figure 3.1**). 144 (63.4%) patients identified as being dyslipidaemic according to NCEP criteria were not on lipid lowering therapy.

Figure 3.1: The prevalence of NCEP defined dyslipidaemia by age in RA



3.3.3 Baseline comparison of dyslipidaemic and non-dyslipidaemic RA patients

Dyslipidaemic patients were significantly older than those who were not dyslipidaemic [median 63.9 (IQR: 57.0-69.8) years vs median 62.2 (IQR:51.9-68.6) years, p=0.034] and had features of more severe RA including higher HAQ score [median 1.63 (IQR: 0.75-2.25) vs median 1.38 (IQR 0.38-2), p=0.030] and higher frequency of joint replacement surgery [n=76 (33.5%) vs n=40 (23.1%), p=0.024]. The dyslipidaemic group also had significantly higher rates of hypertension [n=174 (76.7%) vs n=108 (62.4%), p=0.002] and IR [n=94 (43.5%) vs n=53 (31.5%)]. Significantly more dyslipidaemic patients were prednisolone users [n=89/227 (39.2%) vs n=42/173 (24.3%), p=0.002], whereas the opposite was the case for HCQ use [n=32/227 (14.1%) vs n=48/173 (27.7%), p=0.001]. Dyslipidaemia did not associate with inflammation (CRP, p=0.910 or ESR, p=0.180) in univariate analysis.

3.3.4 Predictors of NCEP defined dyslipidaemia in RA

In the multivariate binary regression analysis, factors found to be independent predictors of NCEP-defined dyslipidaemia included female sex and prednisolone use. Hydroxychloroquine use and higher CRP levels were found to associate with reduced likelihood of having NCEP dyslipidaemia (see **table 3.4**).

Table 3.4: Independent predictors of NCEP defined dyslipidaemia in RA

	Odds ratio (95% confidence interval)	**P value**
Age	1.0 (0.99-1.03)	0.478
Gender (female)	1.89 (1.09-3.30)	**0.024**
Hypertension	1.41 (0.84-2.37)	0.196
BMI	0.99 (0.95-1.04)	0.872
Uric acid	1.15 (0.94-1.39)	0.172
TSH	1.01 (0.94-1.09)	0.783
Insulin resistance	1.61 (0.98-2.65)	0.059
HAQ	1.16 (0.89-1.50)	0.279
Prednisolone	1.93 (1.16-3.21)	**0.011**
Hydroxychloroquine	0.49 (0.28-0.84)	**0.010**
CRP	0.99 (0.97-0.99)	**0.047**
BMI: body mass index, TSH: thyroid stimulating hormone, HAQ: health assessment questionnaire, CRP: C-reactive protein.		

3.3.5 CVD risk and statin use (see **table 3.5**)

***FRS*:** 5/266 (1.6%) had a 10 year risk of >20% and required primary prevention as per current UK guidelines. Of them, only 1 (20%) patient was receiving lipid-lowering therapy (statins/fibrates), leaving a total of 4 untreated at risk patients (80% of the at risk patients or 1.5% of the total population).

NCEP: 64/294 (21.8%) were at high risk of CVD. Of those, 58 were eligible for statin therapy on the basis of their LDL level, but only 3 (5.2%) were receiving lipid-lowering therapy, leaving 55 untreated at risk patients (94.8% of the at risk patients and 18.7% of the total population).

SCORE: Based on the TC:HDL ratio, 43/166 (25.9%) patients with a 10 year risk ≥ 5% and an LDL ≥3 mmol/L or TC ≥5 mmol/L were identified. Of these, 25 (58.1% of the at

risk population or 15.1% of the total population) were untreated and thus remained at risk.

RRS: 45/291 (15.5%) patients had a 10 year risk ≥ 20%. Of these, only 5 (11.1%) were receiving statin therapy, thus leaving 40 (88.9% of the at risk population or 13.7% of the total population) patients untreated and at risk

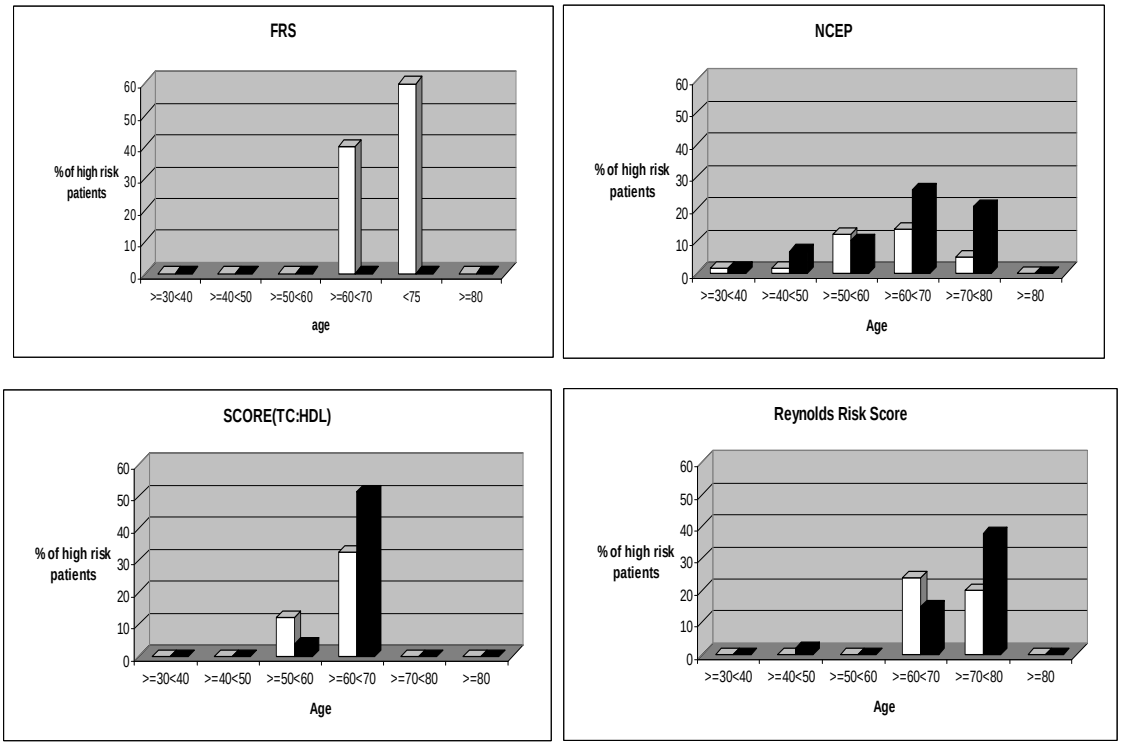
Table 3.5: Statin use amongst patients without prior CVD identified as being at risk

	Total at risk n (%)	**Number of at risk patients on statin n (%)**	**Number of untreated at risk patients n (% total population)**
FRS	5/266 (1.6)	1/5 (20)	4/266 (1.5)
NCEP	58/294 (21.7)	3/64 (5.2)	55/294 (18.7)
SCORE(TC:HDL)	43/166 (25.9)	18/43 (41.8)	25/166 (15.1)
RRS	45/291 (15.5)	5/45 (11.1)	40/291 (13.7)
FRS: Framingham Risk Score, NCEP: National Cholesterol Education Programme, SCORE: Systematic Coronary Risk Evaluation, TC:HDL: Total Cholesterol: High Density Lipoproteins, RRS: Reynolds Risk Score			

3.3.6 CVD risk according to age and gender

Patients aged 60-70 years were most likely to be identified as high risk irrespective of which one of the four CVD risk stratification methods were applied. The FRS algorithm was the only method to identify only males as high risk. The distribution of patients deemed to be at high risk of CVD with each definition according to age and gender is shown in **figure 3.2**.

Figure 3.2: The distribution of patients at high risk of CVD with each definition according to age and gender.



□ =Male, ■ = Female, FRS: Framingham Risk Score, NCEP: National Cholesterol Education Programme, SCORE(TC): Systematic Cardiovascular Risk Evaluation based on total cholesterol levels, SCORE(TC:HDL): Systematic Cardiovascular Risk Evaluation based on total cholesterol:high density lipoprotein ratio.

3.3.7 Differences between high risk patients treated with statins and those untreated

Patients identified as being at 'high risk' of CVD according to one or more methods of risk stratification (FRS/NCEP/SCORE(TC:HDL)/RRS) were grouped together to form a 'high risk population'. This identified a total of 93/299 (31.1%) at risk patients without a prior history of CVD or DM. Of these only 19 were receiving statins, leaving 74 (79.6% of the at risk population or 24.5% of the total population) untreated and at risk. The only factor that was found to significantly associate with statin prescription was older age (statin users vs non-statin users, median 66.2 (IQR:62.5-69.5) years vs median 64.2 (IQR:59.6-67.7) years, p=0.043).

3.3.8 Modification of risk stratification algorithms to account for additional RA- associated risk

The EULAR taskforce recommendations were applicable to 140/299 patients who had two of the following: a disease duration ≥10 years, were sero-positive (rheumatoid factor and/or anti- CCP) or had extra-articular disease. The numbers of additional patients identified following the application of a 1.5 multiplier are summarised in **table 3.6**.

Table 3.6: Comparison of high risk patients identified by the original risk stratification methods and by incorporating a 1.5 multiplier according to EULAR taskforce recommendations

	Original definition of risk algorithms applied to total eligible population	Original definition of risk algorithms applied to patients eligible according to EULAR taskforce recommendations	Risk algorithms multiplied by 1.5, applied to patients eligible according to EULAR taskforce recommendations
FRS	5/ 266 (1.9%)	2/131 (1.5%)	9/131 (6.9%)
NCEP	64/294 (21.7%)	28/144 (19.4%)	34/144 (23.6%)
SCORE (TC:HDL)	43/166 (25.9%)	13/73 (17.8%)	16/73 (21.9%)
RRS	45/291 (15.5%)	20/140 (14.3%)	40/140 (28.6%)

FRS: Framingham Risk Score, NCEP: National Cholesterol Education Programme guidelines, SCORE (TC:HDL ratio): Systematic COronary Risk Evaluation according to total cholesterol:high density lipoprotein ratio tables, RRS: Reynolds Risk Score. Risk multiplication (x1.5 selection): the EULAR taskforce recommendations – a 1.5 multiplier applied to all patients two out of three criteria: a disease duration ≥10 years, seropositive or evidence of extra-articular disease

In patients to whom the EULAR taskforce recommendations were applicable in the under 65 population (thus allowing comparison of all risk algorithms), just under a half (32/84) were identified as high risk according to one or more definitions. Eighteen patients (56.3%) were identified by a single risk stratification method, 7 (21.8%) by two methods, 4 (12.5%) by three methods and 3 (9.3%) by all four methods.

3.4 Discussion

In this chapter I have demonstrated that NCEP defined dyslipidaemia is highly prevalent in RA, and I have identified several factors that independently associate with the presence of NCEP defined dyslipidaemia (female sex, inflammation, prednisolone and HCQ use). Many of the predictors of dyslipidaemia are modifiable, thus the data produced in this chapter not only highlight that dyslipidaemia is highly prevalent, but provide potential strategies for addressing this potentially devastating CVD risk factor, such as good suppression of inflammation with hydroxychloroquine either alone or in combination with other disease modifying drugs. Perhaps more importantly, I have demonstrated that a significant percentage (2-26%) of RA patients in secondary care without a prior history of CVD are at high risk of developing CVD, as calculated by the FRS, NCEP, SCORE or RSS algorithms. This risk rises to 7-30% if a multiplier of 1.5 is applied to applicable patients, to reflect the additional risk conferred by having RA. Despite these worrying findings, statin use was found to be grossly sub-optimal and the reasons for this need to be addressed by the medical community**.**

In this RA population, the highest prevalence of at risk patients (25.9%) was found when applying the SCORE (TC:HDL) criteria. The other conventional methods of risk stratification (NCEP, RRS and FRS) identified a lower prevalence of 21.8%, 15.5% and 1.6%, respectively; the latter however, have a higher threshold for requiring treatment. A recent study (482) adopted a similar comparative approach in a Spanish primary care non-RA population. This reported conflicting results, with the FRS detecting the highest prevalence rates (13.5%), followed by the SCORE (11.4%) and NCEP (7.1%). Such large differences may be explained by disease specific phenomena occurring as part of RA (e.g. activity, severity, duration or therapy), as well as differences in other baseline demographic or anthropometric characteristics of the populations studied, particularly age and sex. In the present RA population, we have also seen a considerable lack of overlap between the different methods of risk stratification, with the majority of at risk patients only being identified by one or two out of the four methods. Reasons underlying this may include: (1) differences in the components of each risk stratification system. For example the SCORE risk stratification method is only applicable to patients under the age of 65, whereas FRS is applicable up to the age of 75 (2) differences in the sensitivity and specificity of each of the risk stratification methods; (3) differences in

the objective of each risk stratification method e.g. SCORE focuses on the 10 year risk of any first fatal atherosclerotic event, whereas the FRS focuses on the 10 year risk of any cardiovascular event, fatal or non-fatal; (4) differences in the application of lipid parameters for statin eligibility. This may be particularly important in an inflammatory condition such as RA, where lipid levels are often suppressed as a consequence of inflammation. Irrespective of this, these findings may have significant implications for clinical practice: most rheumatologists will choose to adopt just one method of risk stratification and thus large numbers of potentially at risk patients may remain unidentified and untreated.

There are many potential explanations for under treatment of CVD in RA. These include: lack of 'ownership' for the management of CVD risk in RA (is it the role of the primary care physician, rheumatologist or cardiologist?); the wrong perception that CVD risk is low amongst most women (who constitute the majority of RA patients); ambiguity, lack of clarity, or indeed knowledge, amongst RA specialists about risk stratification and its implications; or a perceived or actual reluctance of patients to adhere to further polypharmacy alongside their standard RA drug therapy (483). Interestingly, lipid-lowering therapy prescriptions were significantly higher in patients with shorter disease duration. This may reflect our evolving perceptions and management strategies, with patients with a relatively new onset of disease experiencing a more aggressive treatment approach for both, their RA, and associated co-morbidities.

Overall, conventional risk calculators such as the FRS, NCEP, SCORE(TC:HDL) and RRS are reliable and have a good degree of accuracy in the general population. However, such tools have never been properly validated in chronic inflammatory conditions such as RA, where CVD risk is elevated. The validity of some of the conventional CVD risk assessment tools may also be questionable in the elderly (e.g.>75 for the FRS), while both the FRS and NCEP algorithms have been shown to underestimate risk amongst women, and may miss approximately a third of at risk females (484). These problems may be particularly relevant in a condition like RA where there is a strong (3:1) female preponderance and many patients are elderly. New gender-specific prediction tools have recently been developed by the Framingham heart study researchers but they require further validation, particularly within specific populations such as RA (485).

In RA, there is likely to be a further underestimation of CVD risk by conventional calculators (with the exception of RRS), as they do not take into account the impact of inflammation. It has been hypothesized that systemic inflammation may play a more significant role in the development of CVD than traditional risk factors in RA (486). Multiple studies and recent meta-analyses suggest that the added risk RA confers is in the region of x1.5-1.7 (477), thus expert bodies, such as the EULAR task force (478) have suggested the application of a 1.5 multiplier to each risk stratification method, at least to specific patient sub-groups, to account for this. In the present population, this approach led to the identification of considerably more at risk patients, with increases ranging from 4-49%; despite this however, over half of the total population was still not at high risk, and this may argue against the "blanket" usage of statins in all RA patients. These findings clearly indicate the need for widely acceptable guidelines while RA-specific risk calculators are developed. One previous study has proposed an algorithm for risk prediction amongst patients with chronic inflammatory diseases (487) but, although it recommends minimising disease activity and GC use amongst those found to be at risk of developing CVD, it does not include inflammation as a parameter when calculating risk. While the field evolves, a pragmatic approach may be to systematically screen all RA patients using the nationally recommended risk stratification system with a x1.5 multiplier in applicable patients, as suggested by expert bodies. In addition, audit must be implemented to ensure that pre-determined treatment targets are reached and adjust therapy as necessary.

In this chapter I have described the prevalence and independent predictors of NCEP defined dyslipidaemia, and have for the first time cardiovascular risk stratified a large, well-characterised, RA population with established disease. It has highlighted the need for a more dynamic approach to managing the burden of CVD in RA, especially through appropriate prescription of lipid-lowering agents. However, it has not been without limitation. The cross-sectional nature and absence of a non-RA control group makes it impossible to show how "stable" risk stratification is, using these calculators, in the context of the changing inflammatory activity of the disease over time in a given individual. In addition, data regarding previous statin use was not available, and as a consequence I could not account for this in my analyses. Thus, some high risk patients who appear to be untreated with statin therapy, may have previously been treated with

statins but have not been able to tolerate them due to an adverse reaction. The study was performed in a single UK centre, so the findings regarding statin under-utilisation may be location-or system-specific. Most importantly, this study does not provide any evidence that systematic risk stratification and primary prevention strategies encompassing statin use would actually reduce future CVD events in RA. This needs to be addressed prospectively in studies developed specifically for the purpose.

Chapter 4: The effects of inflammation on the lipid profile and lipid ratios

4.1 Introduction

In the previous chapter I demonstrated that CRP was an independent predictor of NCEP defined dyslipidaemia. In this chapter I examine the effects of inflammation on individual lipid levels and lipid ratios further, to establish their association systemic inflammation, a factor that may be of up most importance when assessing CVD risk in RA.

As described in the previous chapter, CVD risk algorithms use individual lipid parameters as integral components when calculating CVD risk. This approach may underestimate CVD risk in RA due to suppression of individual lipid parameters by inflammation e.g. LDL. For example, current criteria (NCEP) (118) focus on LDL as the primary therapeutic target (118;233) and may therefore underestimate the presence of lipid-associated CVD risk during an inflammatory flare, due to inflammatory mediated suppression of LDL. However, amongst the general population in Europe, focus is moving away from analysis based on the individual components of the lipid profile (e.g. LDL or HDL) and has turned towards interpreting ratios of lipids (e.g. TC:HDL-cholesterol), using risk algorithms such as SCORE TC:HDL (472;478). Although, it has been suggested that lipid ratios may be less susceptible to change from current inflammatory load, robust evidence for this is sparse (488).

In this chapter I aim to assess the association of systemic inflammation and lipid levels/lipid ratios, and to find out whether lipid ratios are less susceptible than individual lipid levels to the degree of systemic inflammation. In the process of addressing these aims I will also investigate whether other factors independently predict lipid levels and lipid ratios e.g. medications, disease characteristics, in order to adjust for these in multivariate analyses.

4.2 Methods

For this chapter, I initially used the data collected on the 400 cross sectional RA patients (recruitement and data collection have been previously described in chapter 2, section 2.1) to look for associations between lipids/lipid ratios and inflammatory parameters (ESR/CRP). However, due to the limitations of the cross-sectional data (e.g. unable to comment on the directionality of any associations found), I also utilised a second retrospective longitudinal cohort of 550 RA patients (see chapter 2 section 2.1.3) to investigate the impact of systemic inflammation on the individual components of the lipid profile and lipid ratios over time.

Statistical analysis was carried out using SPSS 18.0 (SPSS Inc, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to test the normality of each parameter and values were expressed as mean ± SD, median (IQR), or percentages, as appropriate.

For the analysis of the cross-sectional data, patients receiving lipid lowering therapy were excluded due to the potential confounding effects. A linear regression model was applied to each component of the lipid profile, and each of the lipid ratios in turn, and was adjusted for potential confounders.

With respect to the retrospective longitudinal data, Generalised Estimating Equations (GEE) with adjustment for age and gender were used to confirm the association of inflammatory parameters with individual lipid components or lipid ratios. GEEs were deemed to be the most appropriate statistical methods as they allowed forthe differences in the number of simultaneous recordings of the lipid profile and inflammatory markers available for each patient. Although the data was longitudinal, time was not included as a factor in the analysis. The reported Beta (B) values reflect the predicted change in the dependent variable (e.g. TC) if the value of that predictor (CRP or ESR) is increased by one. All analyses were adjusted for multiple comparisons using a Bonferoni correction.

4.3 Results

4.3.1 Demographics of the study populations

The demographics of the cross-sectional RA population have been described in detail in chapter 2, section 2.1.1 and table 2.1.

The retrospective longitudinal population of 550 RA patients, comprised 76.0% females and had a median age of 64.4 (IQR: 58.2-70.4) years.

4.3.2 Independent predictors of individual components of the lipid profile and lipid ratios

Linear regression was performed to identify factors that independently predict lipid levels and lipid ratios. The results are summarised below and in **table 4.1 and table 4.2**.

TC: Factors found to be independently associated were female sex (B= 0.579, 95% CI: 0.257 to 0.902, p<0.001), uric acid levels (B= 0.002, 95% CI: <0.001 to 0.004, p=0.019) and HCQ use (B= -0.471, 95% CI: -0.785 to –0.158, p=0.003).

logHDL: Factors found to be negatively associated with HDL levels were IR (B= -0.039, 95% CI: -0.069 to –0.009, p=0.012) and rheumatoid factor positivity (B= -0.030, 95% CI: -0.060 to <0.001, p=0.048). An independent positive association was also found between HDL and female sex (B= 0.057, 95% CI: 0.024 to0.090, p=0.001), systolic BP (B= 0.001, 95% CI: <0.001 to 0.002, p=0.014), prednisolone use (B= 0.033, 95% CI: 0.004 to 0.063, p=0.028) and methotrexate use (B= 0.027, 95% CI: <0.001 to 0.053, p=0.047).

logTG: Factors found to independently associate with logTG were uric acid levels (B= 0.001, 95% CI: <0.001 to 0.001, p=0.002), and prednisolone use (B= 0.072, 95% CI: 0.022 to 0.121, p=0.005).

LDL: Factors found to independently associate with LDL were HCQ use (B= -0.519, 95% CI: -0.862 to –0.176, p=0.003) and IR (B= 0.413, 95% CI: 0.092 to 0.735, p=0.012).

TC:HDL: Factors found to independently associate with TC:HDL ratio included IR (B= 0.330, 95% CI: 0.057 to 0.603, p=0.018), and HCQ use (B= -0.456, 95% CI: -0.745 to –0.168, p=0.002).

LDL:HDL: Factors found to independently associate with LDL:HDL ratio included IR (B= 0.514, 95% CI: 0.243 to 0.785, p<0.001) and HCQ use (B= -0.399, 95% CI: -0.688 to –0.103, p=0.008).

Table 4.1: Independent predictors of lipid levels

	TC		logHDL		logTG		LDL	
	B (95% confidence interval)	**P value**	**B (95% confidence interval)**	**P value**	**B (95% confidence interval)**	**P value**	**B (95% confidence interval)**	**P value**
Age	0.010 (-0.001 to 0.022)	0.076	0.001 (<0.001 to 0.002)	0.117	0.001(-0.001 to 0.003)	0.145	<0.001 (-0.013 to 0.012)	0.937
Sex (female)	0.579 (0.257 to 0.902)	**<0.001**	0.057 (0.024 to 0.090)	**0.001**	0.032 (-0.022 to 0.085)	0.244	0.298 (-0.035 to 0.632)	0.079
Smoking	-0.004 (-0.175 to 0.168)	0.967	-0.003 (-0.021 to 0.014)	0.703	0.029 (<0.001 to 0.078)	0.060	0.080 (-0.107 to 0.268)	0.399
BMI	-0.027 (-0.054 to 0.001)	0.060	-0.002 (-0.005 to 0.001)	0.123	0.002 (-0.003 to 0.006)	0.474	-0.017 (-0.047 to 0.013)	0.256
Uric acid levels	0.002 (<0.001 to 0.004)	**0.019**	<0.001 (0.001 to<0.001)	0.957	0.001 (<0.001 to 0.001)	**0.002**	0.001 (<0.001 to 0.003)	0.135
IR	0.013 (-0.282 to 0.309)	0.929	-0.039 (-0.069 to -0.009)	**0.012**	0.035 (-0.015 to 0.086)	0.170	0.413 (0.092 to 0.735)	**0.012**
Systolic BP	0.003 (-0.006 to0.011)	0.512	0.001 (<0.001 to 0.002)	**0.014**	<0.001 (-0.002 to 0.001)	0.818	<0.001 (-0.009 to 0.009)	0.958
Diastolic BP	0.008 (-0.006 to 0.022)	0.243	<0.001 (-0.002 to 0.001)	0.521	0.001 (-0.001 to 0.003)	0.426	0.010 (-0.005 to 0.025)	0.198
RF positivity	-0.014 (-0.309 to 0.281)	0.925	-0.030 (-0.060 to 0.001)	**0.048**	-0.026 (-0.077 to 0.024)	0.302	-0.123 (-0.442 to 0.196)	0.448
Methotrexate	0.140 (-0.117 to 0.397)	0.285	0.027 (<0.001 to 0.053)	**0.047**	-0.017 (-0.061 to 0.027)	0.436	0.004 (-0.275 to 0.283)	0.977
HCQ	-0.471 (-0.785 to -0.158)	**0.003**	0.014 (-0.017 to 0.046)	0.375	-0.041 (-0.094 to 0.013)	0.133	-0.519 (-0.862 to -0.176)	**0.003**
Anti-TNF	-0.260 (-0.665 to 0.145)	0.207	-0.012 (-0.053 to 0.030)	0.582	-0.020 (-0.089 to 0.050)	0.579	-0.392 (-0.838 to 0.054)	0.084
Prednisolone	0.190 (-0.099 to 0.480)	0.197	0.033 (0.004 to 0.063)	**0.028**	0.072 (0.022 to 0.121)	**0.005**	0.091 (-0.219 to 0.401)	0.563

= positive association, = negative association , Abbreviations: TC: total cholesterol, logHDL: log high density lipoproteins, logTG: log trigylcerides, LDL: low density lipoproteins, BMI: body mass index, IR: insulin resistance, BP: blood pressure, RF: rheumatoid factor, HCQ: hydroxychloroquine, anti-TNF: anti-tumour necrosis factor, B= beta coefficient

Table 4.2: Independent predictors of lipid ratios

	TC: HDL ratio		LDL: HDL ratio	
	B (95% confidence interval)	**P value**	**B (95% confidence interval)**	**P value**
Age	-0.001 (-0.011 to 0.009)	0.863	-0.007 (-0.017 to 0.004)	0.216
Sex (female)	-0.103 (-0.388 to 0.181)	0.474	-0.117 (-0.398 to 0.163)	0.411
Smoking	0.068 (-0.090 to 0.226)	0.396	0.116 (-0.041 to 0.273)	0.148
BMI	0.005 (-0.020 to 0.030)	0.701	-0.004 (-0.029 to 0.021)	0.750
Uric acid levels	0.001 (<0.001 to 0.003)	0.071	0.001 (-0.001 to 0.002)	0.292
IR	0.330 (0.057 to 0.603)	**0.018**	0.514 (0.243 (0.785)	**<0.001**
Systolic BP	-0.007 (-0.015 to 0.001)	0.068	-0.006 (-0.014 to 0.001)	0.113
Diastolic BP	0.009 (-0.003 to 0.022)	0.152	0.009 (-0.004 to 0.022)	0.159
RF positivity	0.226 (-0.045 to 0.497)	0.101	0.034 (-0.234 to 0.302)	0.805
Methotrexate	-0.135 (-0.370 to 0.100)	0.258	-0.154 (-0.389 to 0.080)	0.196
HCQ	-0.456 (-0.745 to -0.168)	**0.002**	-0.399 (-0.688 to -0.103)	**0.008**
Anti-TNF	0.023 (-0.351 to 0.396)	0.905	-0.306 (-0.680 to 0.069)	0.109
Prednisolone	-0.046 (-0.312 to 0.220)	0.733	-0.036 (-0.297 to 0.225)	0.785

= positive association, = negative association , Abbreviations: TC: total cholesterol, logHDL: log high density lipoproteins, logTG: log trigylcerides, LDL: low density lipoproteins, BMI: body mass index, IR: insulin resistance, BP: blood pressure, RF: rheumatoid factor, HCQ: hydroxychloroquine, anti-TNF: anti-tumour necrosis factor, B= Beta coefficient.

4.3.3 The association of inflammation and individual lipid levels both cross-sectional data and the retrospective longitudinal data (summarised in table 4.9)

In the sections below, the cross-sectional associations of each component of the lipid profile/lipid ratios and inflammation (CRP) is assessed using a linear regression model. This is first presented as an unadjusted model and then adjusted for factors identified as potential confounders from the analysis presented above in section 4.3.2, (these included age, gender, smoking status, BMI, uric acid levels, systolic and diastolic BP, insulin resistance, methotrexate, prednisolone, anti-TNF therapy). ESR was not included in the analyses for reasons of co-linearity with CRP. Longitudinal associations of lipid levels/lipid ratios and inflammation (CRP/ESR) were assessed using GEEs adjusted for age and gender.

TC:

Cross-sectional: TC levels were significantly inversely associated with CRP in the unadjusted model (B= -0.008, 95% CI: -0.013 to -0.002, p=0.006) and this association remained following adjustment for potential confounders (B= -0.008, 95% CI: -0.013 to –0.002, p=0.006) (see **table 4.3**).

Table 4.3: The association of TC and CRP following adjustment for potential confounders

	B (95% confidence interval)	**P value**
Unadjusted	-0.008 (-0.013 to -0.002)	0.006
Model a	-0.007 (-0.013 to -0.002)	0.007
Model b	-0.007 (-0.012 to -0.001)	0.016
Model c	-0.008 (-0.013 to -0.002)	0.006
Model a: adjusted for demographics (age, gender, smoking status, body mass index) Model b: adjusted for model a plus RA specific factors (Rheumatoid factor positivity), and cardiovascular risk factors (systolic and diastolic blood pressure, insulin resistance, uric acid levels) Model c: adjusted for models a, b and medications (methotrexate, hydroxychloroquine, anti-tumour necrosis factor therapy, prednisolone)		

Retrospecctive longitudinal data: GEE (with age and sex adjustment) demonstrated a negative association between TC levels and CRP (B= -0.007, 95% CI: -0.008 to –0.005, p<0.001) and ESR (B= -0.005, 95% CI: -0.007 to –0.002, p<0.001).

HDL:

Cross-sectional: logHDL levels were significantly inversely associated with CRP in the unadjusted model (B= -0.001, 95% CI: -0.002 to <0.001, p=0.001) and this association remained following adjustment for potential confounders (B= -0.001, 95% CI: -0.001 to<0.001, p=0.007) (see **table 4.4**).

Table 4.4: The association of logHDL and CRP following adjustment for potential confounders

	B (95% confidence interval)	P value
Unadjusted	-0.001 (-0.002 to <0.001)	0.001
Model a	-0.001 (-0.001 to <0.001)	0.003
Model b	-0.001 (-0.001 to <0.001)	0.014
Model c	-0.001 (-0.001 to <0.001)	0.007
Model a: adjusted for demographics (age, gender, smoking status, body mass index) Model b: adjusted for model a plus RA specific factors (Rheumatoid factor positivity), and cardiovascular risk factors (systolic and diastolic blood pressure, insulin resistance, uric acid levels) Model c: adjusted for models a, b and medications (methotrexate, hydroxychloroquine, anti-tumour necrosis factor therapy, prednisolone)		

Retrospective longitudinal data: GEE in serial measurements confirmed an inverse association between logHDL and CRP (B= -0.001, 9% CI: -0.001 to –0.001, p<0.001) or ESR (B= -0.001, 95% CI: -0.001 to –0.001, p<0.001).

TG:

Cross-sectional: logTG was not found to associate with CRP in either the unadjusted or adjusted model (see **table 4.5**).

Table 4.5: The association of logTG and CRP following adjustment for potential confounders

	B (95% confidence interval)	P value
Unadjusted	0.000 (-0.001 to 0.001)	0.792
Model a	-0.001 (-0.002 to <0.001)	0.248
Model b	-0.001 (-0.001 to <0.001)	0.292
Model c	-0.001 (-0.002 to <0.001)	0.152
Model a: adjusted for demographics (age, gender, smoking status, body mass index) Model b: adjusted for model a plus RA specific factors (Rheumatoid factor positivity), and cardiovascular risk factors (systolic and diastolic blood pressure, insulin resistance, uric acid levels) Model c: adjusted for models a, b and medications (methotrexate, hydroxychloroquine, anti-tumour necrosis factor therapy, prednisolone)		

Retrospective longitudinal data: GEE demonstrated that CRP had a significant negative association with logTG (B= -0.001, 95% CI: -0.001 to <0.001, p=0.003), but ESR was not found to associate (p=0.860).

LDL:

Cross-sectional: A significant inverse association was observed between LDL and CRP both in the unadjusted (B= -0.006, 95% CI : -0.011 to<0.001, p=0.045) and the adjusted model (B= -0.008, 95% CI: -0.014 to –0.002, p=0.007) (see **table 4.6**).

Table 4.6: The association of LDL and CRP following adjustment for potential confounders

	B (95% confidence interval)	**P value**
Unadjusted	-0.006 (-0.011 to <0.001)	0.045
Model a	-0.007 (-0.012 to -0.001)	0.027
Model b	-0.007 (-0.013 to -0.002)	0.014
Model c	-0.008 (-0.014 to -0.002)	0.007
Model a: adjusted for demographics (age, gender, smoking status, body mass index) Model b: adjusted for model a plus RA specific factors (Rheumatoid factor positivity), and cardiovascular risk factors (systolic and diastolic blood pressure, insulin resistance, uric acid levels) Model c: adjusted for models a, b and medications (methotrexate, hydroxychloroquine, anti-tumour necrosis factor therapy, prednisolone)		

Retrospective longitudinal data: GEE confirmed an inverse association between LDL and CRP (B= -0.006, 95% CI: -0.008 to –0.003, p<0.001), but not ESR (p=0.891).

4.3.4 The association of inflammation and lipid ratios both cross-sectionally and longitudinally (summarised in table 4.9)

TC:HDL ratio:

Cross-sectional: No association was found between CRP and the TC:HDL ratio in the unadjusted (B= 0.002, 95% CI: -0.002 to 0.007, p=0.320) or adjusted model (B= <0.001, 95% CI: -0.005 to 0.005, p=0.931) (see **table 4.7**).

Table 4.7: The association of TC:HDL ratio and CRP following adjustment for potential confounders

	B (95% confidence interval)	P value
Unadjusted	0.002 (-0.002 to 0.007)	0.320
Model a	0.001 (-0.004 to 0.006)	0.637
Model b	<0.001 (-0.005 to 0.005)	0.871
Model c	<0.001 (-0.005 to 0.005)	0.931
Model a: adjusted for demographics (age, gender, smoking status, body mass index) Model b: adjusted for model a plus RA specific factors (Rheumatoid factor positivity), and cardiovascular risk factors (systolic and diastolic blood pressure, insulin resistance, uric acid levels) Model c: adjusted for models a, b and medications (methotrexate, hydroxychloroquine, anti-tumour necrosis factor therapy, prednisolone)		

Retrospective longitudinal data: GEE demonstrated a significant positive association between ESR levels and the TC:HDL ratio (B= 0.001, 95% CI: <0.001 to 0.001, p<0.001), but no association was found with CRP (p=0.478).

LDL:HDL ratio:

Cross-sectional: No association was found between CRP and the LDL:HDL ratio in either the unadjusted (B= 0.001, 95% CI: -0.003 to 0.006, p=0.557) or adjusted models (B= -0.001, 95% CI: -0.006 to 0.003, p=0.564) (see **table 4.8**).

Table 4.8: The association of LDL:HDL ratio and CRP following adjustment for potential confounders

	B (95% confidence interval)	P value
Unadjusted	0.001 (-0.003 to 0.006)	0.557
Model a	<0.001 (-0.005 to 0.005)	0.940
Model b	-0.001 (-0.006 to 0.004)	0.651
Model c	-0.001 (-0.006 to 0.003)	0.564
Model a: adjusted for demographics (age, gender, smoking status, body mass index) Model b: adjusted for model a plus RA specific factors (Rheumatoid factor positivity), and cardiovascular risk factors (systolic and diastolic blood pressure, insulin resistance, uric acid levels) Model c: adjusted for models a, b and medications (methotrexate, hydroxychloroquine, anti-tumour necrosis factor therapy, prednisolone)		

Retrospective longitudinal data: GEE demonstrated a positive correlation between ESR and LDL:HDL ratio (B= 0.001, 95% CI: 0.001 to 0.001, p<0.001), however, no significant association was found with CRP (p=0.389).

Table 4.9: A summary of factors found to be independent predictors of changes in individual lipid levels and lipid ratios

	TC	HDL	TG	LDL	TC:HDL Ratio	LDL:HDL Ratio	ApoB:ApoAI ratio
CRP	- ve	- ve		- ve			
RF positivity		- ve					
Methotrexate		+ve					
Hydroxychloroquine	-ve			- ve	- ve	-ve	
Prednisolone		+ve	+ ve				
Gender	+ ve	+ ve					
Systolic BP		+ ve					
Uric acid			+ ve				
BMI	- ve						
IR		- ve		+ ve	+ ve	+ ve	+ ve

☐ = positive association, ☐ = negative association

Results shown in this table all reached statistical significance in multivariate testing (p value <0.05). CRP: C-reactive protein, RF: rheumatoid factor, BMI: body mass index, IR: insulin resistance, TC: total cholesterol, HDL: high density lipoprotein, TG: triglycerides, LDL: low density lipoprotein, ApoB: apolipoprotein B, ApoAI: apolipoprotein AI.

4.3.5 The association of lipid parameters with DAS (used as a marker of inflammation)

The cross sectional data were analyzed replacing CRP with DAS (a composite marker of disease activity). The results from the multivariate models demonstrated that DAS was found only to significantly associate with HDL (B=0.001, 95% CI= -0.019 to -0.002, p=0.019) and ApoA (B=-0.036, 95% CI=-0.066 to –0.002, p=0.036). Other individual lipid parameters and lipid ratios were not found to associate.

4.3.6 The effects of statins on lipid levels/lipid ratios and their relationship with CRP

The cross-sectional data were analysed in all patients including those receiving statins, to assess the impact of statins on lipid levels/lipid ratios and their relationship with markers of inflammation (CRP). In the multivariate model, statins were found to be an independent predictor of TC (B= -1.209, 95% CI: -1.497 to –0.921, p<0.001), HDL (B= -0.036, 95% CI: –0.066 to –0.007, p=0.016), LDL (B= -1.169, 95% CI: –1.486 to –0.851, p<0.001), and ApoB (B= -0.251, 95% CI: –0.329 to –0.173, p<0.001) levels, as well as ApoB:ApoA ratio (B= -0.140, 95% CI: –0.202 to –0.079, p<0.001), TC:HDL ratio (B= -0.554, 95% CI: –0.820 to –0.289, P<0.001), and LDL:HDL ratio (B= -0.646, 95% CI: –0.911 to –0.381, p<0.001). The addition of statins to the model did not alter the associations of individual lipid levels or lipid ratios with CRP from those previously described. (results not shown).

4.4 Discussion

In this chapter I have demonstrated that in contrast to individual lipid components, lipid ratios in RA are much less susceptible to changes in inflammatory burden, and the use of anti-rheumatic drugs, including GCs (but not HCQ). Thus, in RA patients lipid ratios (including apoB:apoA1) appear to offer a more reliable method of identifying lipid abnormalities or the true extent of lipid-associated risk. These findings suggest that future studies are required to address and compare the predictive ability of lipid ratios versus individual components for CVD risk in a range of RA patients.

The prospective collection of data in the cross-sectional cohort of consecutive patients has minimised selection or recall bias and missing values, and has allowed adjustments

for multiple potential confounders. Although this was informative, it could not guarantee causality or prove the directionality of any of the associations found. However, the confirmation of several of the associations, particularly the inverse change of individual lipid components with systemic inflammation, in a separate, large cohort of RA patients who had serial, contemporary measurements of lipid levels, ESR and CRP provided internal support for our findings. However, it remains that the longitudinal study is limited by a lack of data on other potential confounders. Irrespective of this, the findings reported in this chapter extend prior observations from other studies(129).

When comparing the relationship between inflammation and changes in individual lipid components, versus the effects of inflammation on lipid ratios, distinct differences are seen. In the cross-sectional data, CRP had a strong inverse association with multiple individual lipid moieties including TC, HDL, and LDL levels, but not with any of the lipid ratios. An almost identical pattern was observed in the longitudinal data, with the exception of the TC:HDL ratio, which was found to positively correlate. The relationship between ESR and lipids/lipid ratios was rather different, with only limited effects noted in the longitudinal data (reduction in HDL and TC and elevations of the LDL:HDL ratio). A potential explanation for the discrepancy between the relationships seen with CRP and ESR is the difference in time taken for these two inflammatory markers to fluctuate as a consequence of changes in inflammatory burden. It is well recogonised that fluctuations in ESR levels lag well behind those seen with CRP. Thus for each inflammatory episode ESR levels will take longer to increase and will be elevated for longer.

When the analysis was extended to include a more contemporary, clinical measurement of disease activity, DAS 28, interestingly, it was found only to negatively associate with HDL and ApoA levels. The potential reasons for the discrepancy between the findings with DAS and CRP include a difference in the sensitivity of the tests (DAS28 perhaps also detecting signs of chronic disease e.g. chronic synovial thickening as well as signs of acute inflammation), and a time lag in the onset of symptoms/signs relative to the change in CRP. A longitudinal study would offer a more robust way of assessing the association of DAS with lipids and lipid ratios, however, these data were unfortunately lacking from my longitudinal data.

Despite rapid advances in drug therapy for RA, we remain reliant on GCs (GC) as rescue therapy for acute flares of disease, and as longer-term maintenance therapy in those with resistant disease. Short and long-term GC use has been associated with many adverse effects, including accelerated CV risk (51;59;489). In the present chapter, GC use was independently associated with elevations of HDL and TG, but not TC and LDL levels. A study by Boers et al, also demonstrated an increase in HDL and TC levels in the study arm treated with a combination of DMARD and oral prednisolone versus DMARD alone (368). Although GC-induced elevations in HDL would appear to be protective, GC treatment clearly has other effects, which on balance could enhance CVD risk. One such change that may help to explain this in the present study is the GC-induced increase in TG levels, a phenomenon that has been previously well described in the literature (490).

In recent years, HCQ use has expanded. This is likely at least in part to be a consequence of the NICE guidelines recommending combination DMARD therapy for early RA (490;491). The lipid lowering effects of HCQ are well recognized (371;374;375;490). HCQ suppresses TC, LDL and TG levels whilst increasing HDL levels (371;374;375;490;492). In this chapter I have confirmed many of these findings, with HCQ use associating with lower TC ($p=0.003$) and LDL ($p=0.003$) levels. Interestingly, HCQ was not found to associate with HDL or TG levels. In addition to these findings, I have also demonstrated for the first time that HCQ also lowers lipid ratios (TC:HDL and LDL:HDL); thus indicating that HCQ produces global anti-atherogenic effects on the lipid profile that may translate to a reduction in CVD risk**.**

Statins are commonly prescribed both for primary and secondary prevention of CHD. They induce a wide range of beneficial changes in the lipid profile including, potent reduction of TC, LDL and less so TG levels, whilst minimally increasing HDL levels (493;494). However, current published data on the effects of statins on HDL levels in the general population remain far from conclusive (495). I demonstrated that HDL levels were lower in those on statins, which may well be due to patients with lower HDL deemed to be at higher CVD risk and therefore more commonly prescribed statins, rather than statin-induced reduction in HDL. Interestingly, an RCT of statins in RA showed no significant change in HDL with atorvastatin (496).

In conclusion, the cross-sectional and longitudinal data presented in this chapter indicate that inflammation is key to many of the lipid changes observed in RA, and that lipid ratios are less susceptible to fluctuation as a result of changes in inflammatory markers (CRP/ESR) and corticosteroid use, although they may all be beneficially affected by HCQ. These findings suggest that lipid ratios, rather than TC or LDL alone, may be more useful for routine absolute CVD risk estimations in RA patients to facilitate decisions on prescribing lipid lowering therapy e.g. SCORE algorithm. However, this needs to be confirmed in future studies.

Chapter 5: The effects of drug-induced suppression of inflammation on lipid levels, structure and function

5.1 Introduction

The purpose of this chapter is to explore the more intricate effects of inflammation on lipid metabolism such as changes in lipid subfractions, lipid structure (nitration and oxidation) and how these translate in to lipid function with regards to how readily LDL oxidises (LDL lag times) and LDL is taken up into macrophages.

There is evidence to suggest that changes in lipid structure and function can significantly contribute to CVD risk (497-499). Several studies in the general population have attempted to quantify the CVD risk associated individual lipid subfractions (HDL and LDL) (499;500). It has been suggested that more refined analyses of lipoprotein subclasses may lead to improvements in CVD risk evaluation and the identification of therapeutic targets (497). The majority of studies demonstrate that an increase in small dense LDL (501) and a decrease in HDL2 levels is associated with an increased CVD risk (500). However, the results for HDL3 are equivocal (132;500). **Table 5.1** summarises which alterations in lipid levels, structure and function are proatherogenic.

Table 5.1: Proatherogenic changes in lipid levels, structure and function

Pro-atherogenic lipid changes
↑ TC, LDL, TG, ApoB, HDL3, SdLDL, oxLDL, nitrated LDL
↓ HDL, HDL2, ApoA, LDL lag times, DiIoxLDL uptake
TC: total cholesterol, LDL: low density lipoproteins, TG: triglycerides, SdLDL: small dense low density lipoproteins, nitrated LDL: nitrated low density lipoproteins, HDL: high density lipoproteins, ApoA: apolipoprotein A, ApoB: apolipoproteinB, DiIoxLDL: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine labelled oxidised low density lipoprotein uptake

In the context of RA, the heterogeneity of lipid sub-fractions has been studied in 4 cross-sectional studies (133;369;502;503). Three of these have demonstrated differences in the lipoprotein sub-fraction profile amongst RA patients compared to age- and sex-matched healthy controls, with lower levels of HDL2 and higher levels of small dense LDL

(SdLDL) particles (133;502;503). A further study compared the HDL sub-fraction profile of 78 RA patients on standard DMARD therapy to 65 RA patients receiving GCs at a mean dose of 5.1mg/d (369). Patients receiving GCs had higher levels of HDL2 and HDL3 compared to steroid-naïve patients. However, no longitudinal studies have assessed the impact of initiating anti-inflammatory drug therapy on the lipoprotein subfraction profile in RA.

Modifications of LDL (oxidation/nitration) have also been associated with CVD risk and inflammation has been shown to enhance such modifications through exposure to ROS. In RA, little is known about the effects of systemic inflammation or drug therapy on LDL modifications. A study by Kim et al (142) demonstrated significantly higher levels of oxLDL in 54 RA patients compared to 115 age matched controls. Interestingly, oxLDL levels were not found to associate with inflammatory markers.

The uptake of LDL into macrophages in RA patients has been examined in a small study (276). This study compared LDL uptake in RA patients with (n=13) and without CVD (n=12) to patients with OA, and the potential mechanisms by which this may occur. RA patients with CVD had increased LDL uptake compared to disease matched OA patients, but this did not reach statistical significance. The authors also found higher levels of nitrated LDL, which correlated with increased homocysteine levels. The authors therefore concluded that homocysteine may promote LDL nitration and that the nitrated LDL is then taken up more readily by macrophages. There is no current data available on the effects of inflammation or drug therapy on LDL uptake, nor is there data assessing the effect of other lipid alterations e.g. subfractions, LDL lag times on this process in RA.

In this chapter I will: (1) assess the longitudinal impact of systemic inflammation in RA on lipid subfractions (HDL2, HDL3, HDL2:HDL3 ratio and SdLDL) and lipid modifications (oxidation/nitration); (2) assess whether changes in these lipid parameters are due to global changes in systemic inflammation or a drug-specific effect; (3) assess whether changes in lipid subfractions and lipid modifications translate in to functional changes in LDL (alterations in lag times or diIoxLDL uptake by U937 cells).

5.2 Methods

The recruitment and data collection of the longitudinal cohort are described in detail in chapter 2, section 2.1.2. Baseline characteristics of RA and healthy control populations are summarised in table 2.2. The methods used to assess lipid subfractions, lipid structure (oxidation/nitration) and function (LDL lagtimes and DiIoxLDL uptake in to U937 cells) are described in chapter 2, section 2.9. The uptake of DiIoxLDL into U937 cells is inversely proportional to the uptake of the study participant's actual LDL uptake into U937 cells.

Statistical analysis was performed using SPSS 18.0 (SPSS Inc, Chicago, IL, USA). Baseline comparisons of lipid parameters were made between RA and healthy controls using a Student's t test for normally distributed variables or a Mann Whitney U test for non normally distributed variables. Univariate associations of each of the lipid parameters were assessed using students t test or Mann Whitney U for binary categorical variables, ANOVA and Kruskal Wallis for categorical variables with 3 or more groups, and Spearman's and Pearson's correlations for continuous variables. Longitudinal analyses assessing changes in lipid parameters over the treatment period were performed using GEEs. This method of analysis was chosen as it allowed adjustment of changes in inflammatory parameters (CRP) at the different time points. Each GEE model was adjusted only for CRP, as other known potential confounders did not differ significantly over the follow up period. However, in order to check that any differences observed between the groups weren't purely due to differences in baseline confounders e.g. age, a generalised linear model (GLM) was performed adjusting for potential confounders. For completeness, the analysis also repeated replacing CRP with ESR, but as this did not significantly affect the results I have just reported the CRP-adjusted results. For each lipid parameter, GEEs were performed twice, in order to look for: (1) differences between the three main study arms (RA intervention, RA controls and HC); and (2) differences between the three intervention arms (Anti-TNF, IV GCs and rituximab). All non-normally distributed variables were log transformed prior to being analysed.

5.3 Results

5.3.1 Baseline differences between RA and healthy controls

Comparisons of baseline data of the longitudinal RA (RA intervention and RA controls) and HC populations demonstrated that RA patients had significantly lower levels of HDL2 (p=0.005), HDL2:HDL3 ratio (p=0.009), shorter LDL lag times (p=0.007), but higher levels of HDL3 (p=0.006). Although no significant differences in other lipid parameterswere observed, levels of TC , HDL andApoA were lower and TGs, OxLDL and nitrated LDL higher amongst the RA group. A similar trend was found for lipid ratios with TC:HDL, LDL:HDL and ApoB:ApoA ratios all appearing higher in RA patients but without reaching statistical significance (the results are summarised in **Table 5.2**).

In a multivariate linear regression model adjusting for potential confounders (age, gender, BMI, smoking status, IR and systolic BP), only HDL2 levels (B= -0.225, 95% CI: -0.070 to - 0.380, p=0.005) were found to be significantly lower amongst RA patients.

In a subanalysis no significant differences were observed in lipid parameters when comparing RA patients who were antibody positive (RhF or anti-CCP +ve) to those who were antibody –ve, or when comparing those with a DAS28 ≥5.1 to those with a DAS28 <5.1. However, in both the unadjusted and adjusted linear regression model, RA patients with a DAS28 ≥3.2 had higher levels of nitrated LDL compared to patients with a DAS28 <3.2 (unadjusted: B= 0.599, 95% CI: 0.151 to 1.046, p=0.010, adjusted: B= 0.914, 95% CI: 0.021 to 1.807, p=0.045). In the unadjusted linear regression model, ApoA levels were lower amongst RA patients with a DAS28 ≥3.2 compared to patients with a DAS28 <3.2 (B= -0.256, 95% CI: -0.460 to-0.151, p=0.015), however this was lost following adjustment for potential confounders (B= -0.195, 95% CI: -0.555 to 0.164, p=0.280). When comparing RA patients with a CRP ≥5 mmol/L to those with an CRP <5 mmol/L levels of nitrated LDL were higher amongst those with an CRP over 5 mmol/L in both the unadjusted and adjusted linear regression models (unadjusted: B= 0.285, 95% CI: 0.054 to 0.516, p=0.016, adjusted: B= 0.397, 95% CI: 0.084 to 0.709, p=0.014).

Table 5.2: A comparison of baseline lipid levels, structural and functional parameters in RA patients (RA intervention group and RA controls) and healthy controls

	RA (n=72)	HC (n=40)	P value
Lipid levels and lipid ratios			
TC(mmol/L)	5.06 ± 1.03	5.18 ± 1.01	0.557
HDL (mmol/L)	1.49 ± 0.36	1.59 ± 0.42	0.154
LDL (mmol/L)	3.30 ± 0.93	3.36 ± 0.89	0.733
TG (mmol/L)	1.20 (0.90-1.20)	1.0 (0.70-1.33)	0.051
Apo A (g/L)	1.45 ± 0.28	1.52 ± 0.31	0.204
Apo B (g/L)	0.89 ± 0.23	0.88 ± 0.22	0.791
TC:HDL ratio (mmol/L)	3.57 ± 1.03	3.42 ± 0.81	0.416
LDL:HDL ratio (mmol/L)	2.36 ± 0.94	2.25 ± 0.74	0.511
ApoB:ApoA ratio (g/L)	0.63 ± 0.19	0.59± 0.16	0.275
Lipid subfractions			
HDL2 (mmol/L)	0.95 ± 0.35	1.15 ± 0.33	**0.005**
HDL3 (mmol/L)	0.52 ± 0.18	0.43 ± 0.13	**0.006**
HDL2:HDL3 (mmol/L)	2.15 ± 1.55	2.96 ± 1.45	**0.009**
SdLDL (mmol/L)	0.95 (0.6-1.21)	1.03 (0.75-1.34)	0.216
Lipid modifications			
OxLDL (U/L)	81.5 (45.9-112.4)	59.2 (45.75-79.9)	0.207
Nitrated LDL (μg/ml)	11.1 (3.48-18.49)	8.82 (1.36-18.5)	0.308
Functional changes			
LDL lag times (mins)	116.51± 19.01	128.0 ± 21.05	**0.007**
DiIoxLDL uptake (%)	96.6± 10.2 (n=32)	95.1 ± 4.61 (n=12)	0.604

Results are reported as mean ± standard deviation or median (25^{th}-75^{th} interquartile range).
RA: rheumatoid arthritis, HC: healthy controls, TC: total cholesterol, HDL: high density lipoproteins, LDL: low density lipoproteins, TG: triglycerides, ApoA: Apolipoprotein A, ApoB: Apolipoprotein B, oxLDL: oxidised LDL, SdLDL: small dense low density lipoproteins

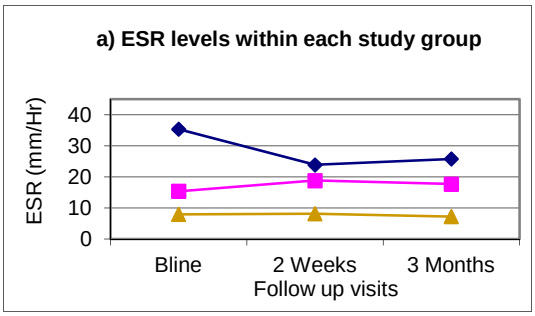
5.3.2 The longitudinal effects of anti-inflammatory treatment on inflammatory parameters (ESR/CRP) and disease activity (DAS28) **(figure 5.1)**

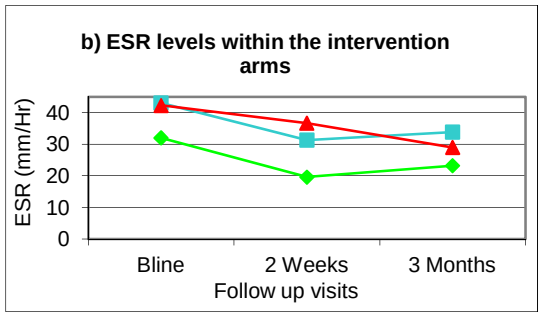
Study Groups: In the RA intervention arm (Anti-TNF, intravenous GCs or rituximab), compared to baseline, there were significantly lower levels at follow-up of: CRP (2 weeks: B= -0.327, 95% CI: -0.436 to -0.218, p<0.001, 3 months: B= -0.250, 95% CI: -0.356 to -0.144, p<0.001); ESR (2 weeks: B= -0.195, 95% CI: -0.265 to -0.125, p<0.001, 3 months: B= -0.169, 95% CI: -0.269 to -0.068, p=0.001) and DAS28 (2weeks: B= -1.354, 95% CI: -1.715 to -0.993, p<0.001, 3 months: B= -1.437, 95% CI: -1.795 to -1.078, p<0.001). CRP and ESR levels did not differ over time in the HC or RA control populations. DAS 28 was significantly higher amongst RA controls at 3 months compared to baseline (B=1.002, 95% CI: 0.436 to 1.568, p=0.001).

Intervention arms: A significant difference in the pattern of response was observed with CRP (p<0.001), ESR (p=0.003) and DAS 28 (p<0.001): Intravenous GC use produced transient falls in ESR and DAS28 at 2 weeks (B= -0.085, 95% CI: -0.168 to -0.003, p=0.043 and B= -1.749, 95% CI: -2.596 to -0.901, p<0.001, respectively) with levels returning to baseline at 3 months; Anti-TNF use resulted in lower ESR, CRP and DAS28 at 2 weeks (ESR: B= -0.239, 95% CI: -0.336 to -0.141, p<0.001, CRP: B= -0.398, 95% CI: -0.539 to -0.256, p<0.001 and DAS28: B= -1.496, 95% CI: -1.887 to -1.104, p<0.001), and at 3 months (ESR: B= -0.158, 95% CI: -0.287 to -0.029, p=0.017, CRP: B= -0.275, 95% CI: -0.422 to -0.129, p<0.001 and DAS28: B= -1.671, 95% CI: -2.064 to -1.277, p<0.001), while rituximab use resulted in lower ESR and DAS28 levels at 3 months (ESR: B= -0.343, 95% CI: -0.607 to -0.079, p=0.011, DAS28: B= -1.363, 95% CI: -2.311 to -0.415, p=0.005).

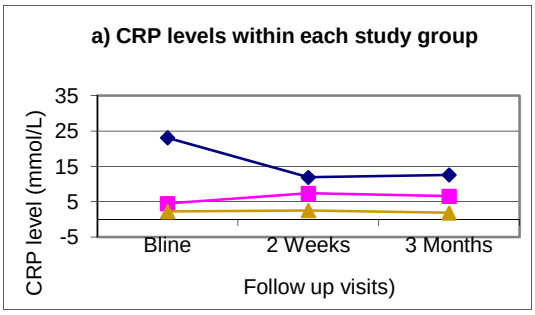
Figure 5.1: Longitudinal changes in inflammatory parameters and disease activity a) within the three study groups (RA intervention, RA controls and healthy controls) b) within the three RA intervention arms (Anti-TNF, GCs and Rituximab).

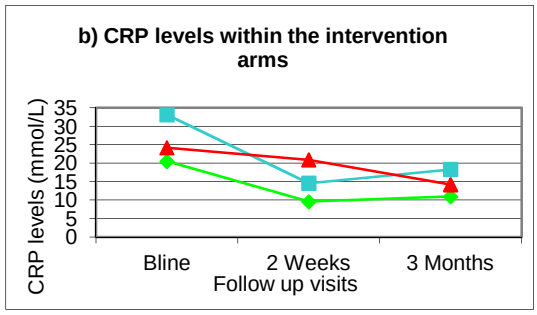
ESR



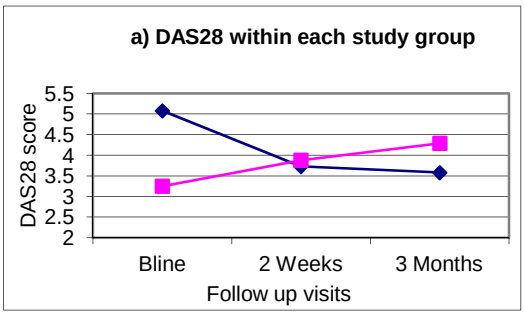


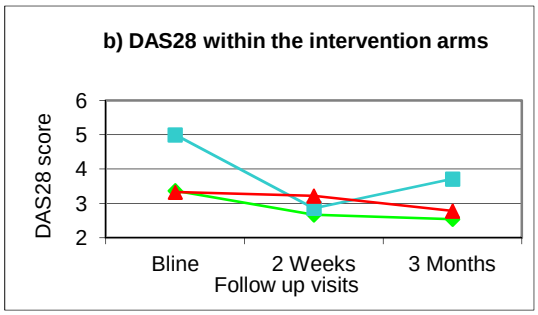
CRP





DAS28





a) ___ = Healthy controls, ___ =RA intervention, ___ = RA controls
b) ___ = IV GCs, ___ =Rituximab, ___ = Anti-tumour necrosis factor therapy

Lipid levels: **(see figure 5.2)**

LDL:

Study groups: A difference in the pattern of response over time was observed (p=0.006), with LDL levels being significantly lower at 2 weeks compared to baseline in the RA control arm, this remained following adjustment for CRP (B= -0.306, 95% CI: -0.522 to –0.090, p=0.005). No significant changes in LDL levels were seen in the RA intervention or HC arms.

Intervention arms: No overall effect of time or group and no difference in the pattern of response were observed between the groups in either the unadjusted or CRP adjusted model.

A GLM adjusted for all baseline potential confounders (age, gender, BMI, IR, smoking status, and CRP) did not identify any significant differences between the groups.

HDL:

Study groups: A difference in the pattern of response was observed between the arms (p=0.002). This association remained following adjustment for CRP (p=0.011). Within the RA intervention arm HDL levels were significantly higher at 2 weeks compared to baseline (B= 0.094, 95% CI: 0.032 to 0.156, p=0.003) but returned to baseline levels at 3 months (p=0.406). Changes in HDL levels were also observed in the RA control arm with HDL levels significantly lower at 2 weeks (B= -0.130, 95% CI: -0.256 to –0.006, p=0.040) compared to baseline. HDL levels remained stable over time amongst the HC arm.

Intervention arms: An overall effect of time irrespective of group was noted with HDL levels increasing at 2 weeks (B= 0.093, 95% CI: 0.031 to 0.155, p=0.003) and returning to baseline levels at 3 months (p=0.433). No group effect or differences in the pattern of response were observed.

A GLM adjusted for all baseline potential confounders (age, gender, BMI, IR, smoking status, and CRP) did not find any significant differences between the groups.

TC:

Study groups: A significant difference in the pattern of response between the study groups was observed (p<0.001), which remained following adjustment for CRP (p<0.001). At 2 weeks, TC levels were significantly higher in the RA intervention arm (B= 0.255, 95% CI: 0.012 to 0.408, p=0.001), and lower in the RA control arms (B= -

0.361, 95% CI: -0.576 to –0.147, p=0.001) compared to baseline values (no difference between baseline and 3 month levels in either arm). No differences were observed in TC levels over time in the HC arm

Intervention arms: A difference in the pattern of response was observed, which remained following adjustment for CRP (p<0.001), with TC levels significantly higher at 2 weeks in the rituximab (B= 0.226, 95% CI: 0.009 to 0.443, p=0.042) and IV GC arms (B=0.715, 95% CI: 0.365 to 1.065, p<0.001) compared to baseline values, both returning to baseline levels at 3 months. No significant change in TC levels were seen in the anti-TNF arm over the 3 follow up visits.

TG:

Study groups: No significant period effect, group effect or difference in the pattern of response was observed between the groups in either the unadjusted or CRP adjusted model.

Intervention arms: No significant period effect, group effect or difference in the pattern of response was observed between the treatment arms in the unadjusted and CRP adjusted models.

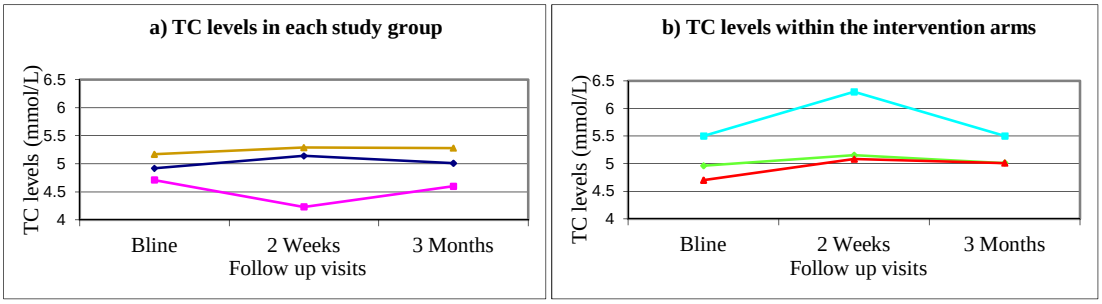
Lipid ratios: **(see figure 5.3)**

Study groups: Using a GEE model, no period effect, group effect or difference in the pattern of response was observed for any of the lipid ratios (TC:HDL ratio, LDL:HDL ratio or ApoB:ApoA) in the unadjusted or CRP adjusted model.

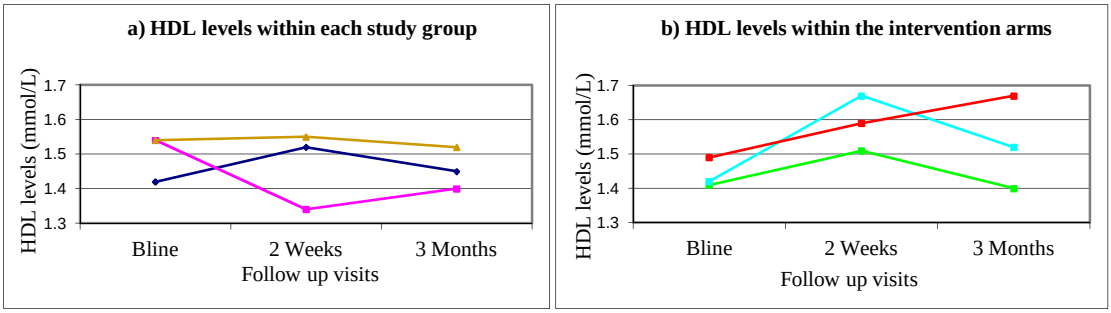
Intervention arms: No period effect, group effect or differences in the pattern of response were noted in TC:HDL ratio, LDL:HDL ratio or ApoB:ApoA ratio over time amongst the three treatment arms in either the unadjusted or CRP adjusted model.

Figure 5.2: Longitudinal changes in lipid levels a) within the three study groups (RA intervention, RA controls and healthy controls) b) within the three RA intervention arms (Anti-TNF, GCs and Rituximab)

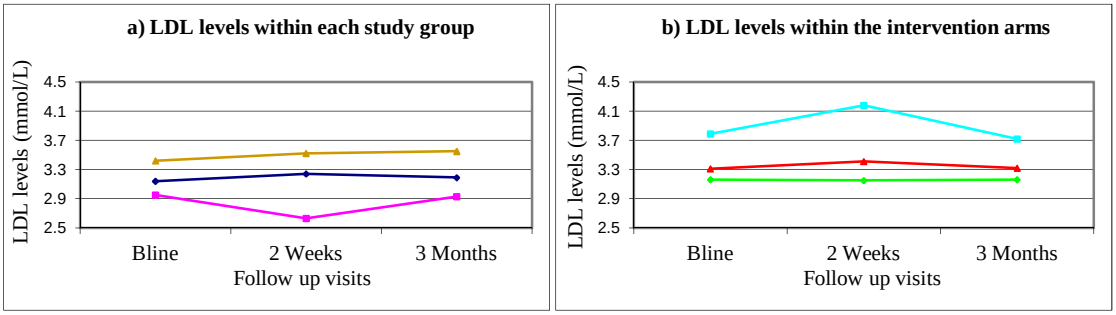
Total Cholesterol



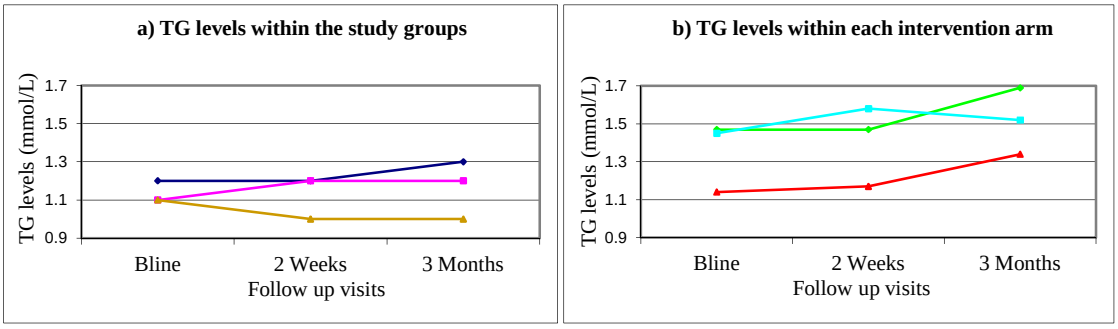
HDL



LDL



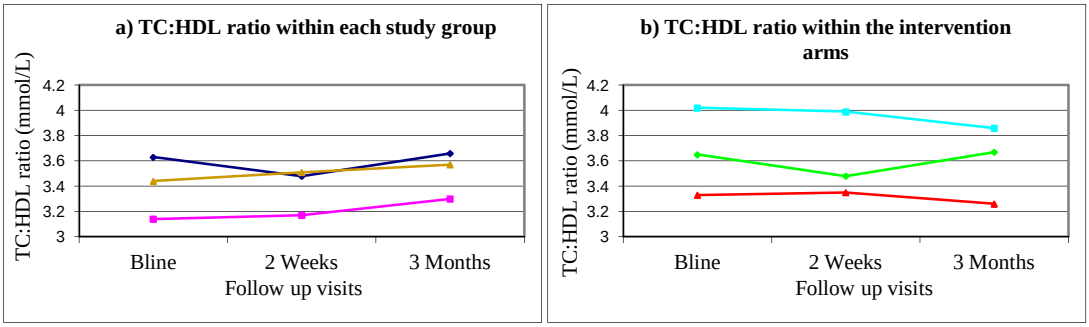
TG



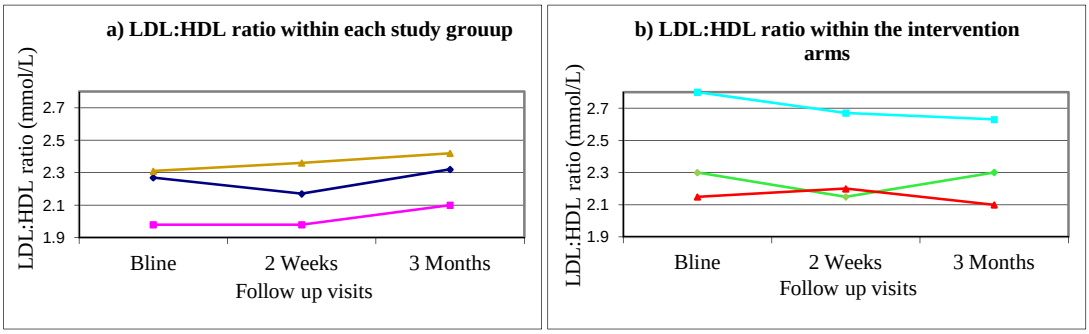
a) ___ = Healthy controls, ___ =RA intervention, ___ = RA controls
b) ___ = Intravenous glucocorticoids, ___ =Rituximab, ___ = Anti-tumour necrosis factor therapy

Figure 5.3: Longitudinal changes in lipid ratios a) within the three study groups (RA intervention, RA controls and healthy controls) b) within the three RA intervention arms (Anti-TNF, GCs and Rituximab)

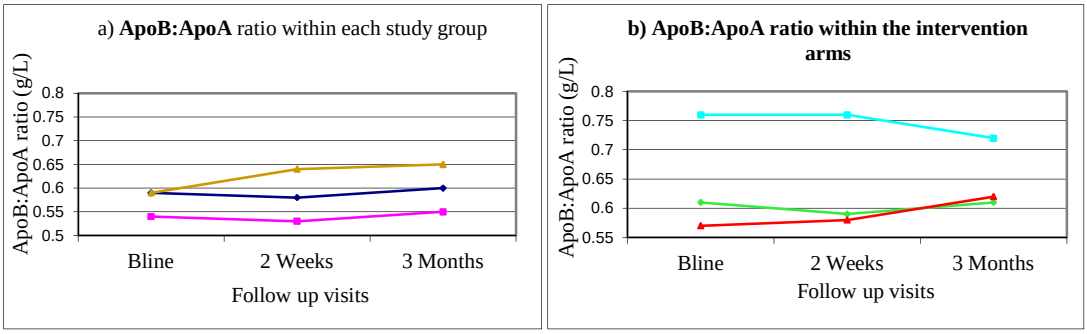
TC:HDL ratio



LDL:HDL ratio



ApoB:ApoA ratio



a) ___ = Healthy controls, ___ =RA intervention, ___ = RA controls
b) ___ = Intravenous glucocorticoids, ___ =Rituximab, ___ = Anti-tumour necrosis factor therapy

5.3.4 Longitudinal changes in lipid subfractions **(see figure 5.4)**

HDL2:

Study groups: A significant difference in the pattern of response was seen between the groups, which remained following adjustment for CRP (p=0.010), with HDL2 levels higher at 2 weeks in the RA intervention arm (B= 0.097, 95% CI: 0.029 to 0.164, p=0.005), and lower at 3 months in the HC arm (B= -0.105, 95% CI: -0.188 to –0.022, p=0.013) compared to baseline values. Levels of HDL2 remained stable amongst the RA control arm during the follow up period.

Intervention arms: A significant effect of time irrespective of the groups was seen with HDL2 increasing at 2 weeks (B= 0.101, 95% CI: 0.033 to 0.170, p=0.004 – adjusted for CRP), and returning to baseline levels at 3 months.

HDL3:

Study groups: A significant difference in the pattern of response was observed. This association remained following adjustment for CRP (p=0.001). In the RA control arm, HDL3 levels were significantly lower at both 2 weeks (B= -0.191, 95% CI: -0.308 to –0.074, p=0.001) and 3 months (B= -0.195, 95% CI: -0.303 to –0.087, p<0.001) compared to baseline. HDL3 levels did not change significantly over time in the RA intervention or HC arms.

Intervention arms: A significant difference in the pattern of response was observed with patients receiving IV GCs having significantly lower HDL3 levels at 3 months (B= -0.113, 95% CI: -0.174 to –0.051, p<0.001 – adjusted for CRP) compared to baseline. HDL3 levels amongst the other two treatment arms (Anti-TNF and rituximab) did not change during follow up.

HDL2:HDL3 ratio:

Study Groups: The pattern of response over time differed between the groups both in the unadjusted (p=0.002) and CRP adjusted models (p=0.004). HDL2:HDL3 ratio was significantly lower at 3 months compared to baseline in the HC arm (B= -0.476, 95% CI: -0.823 to -0.129, p=0.007) and higher at 3 months compared to baseline in the RA control arm (B= 0.627, 95% CI: 0.083 to 1.172, p=0.024). The HDL2:HDL3 ratio did not significantly change over time in the RA intervention arm.

Intervention arms: No significant group effect, period effect or difference in the pattern of response was observed between the three treatment arms in either the unadjusted or

CRP adjusted model. In a GLM, adjusting for other potential confounders at baseline (age, gender, BMI, IR, smoking status, and CRP) found no differences between the groups.

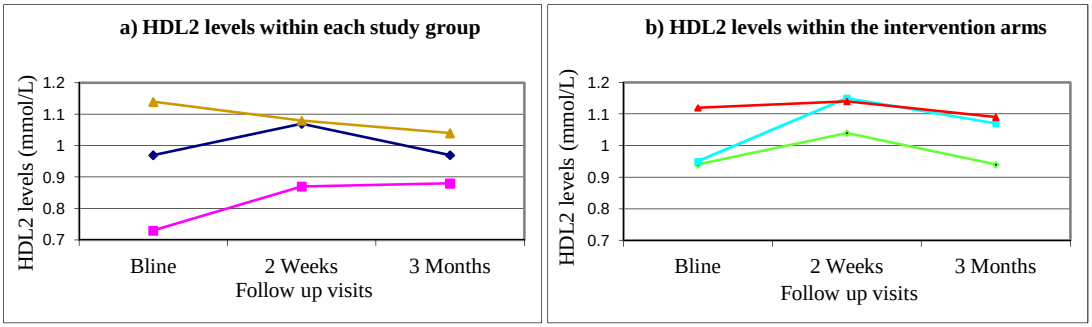
Log SdLDL:

Study groups: No significant period effect, group effect, or differences in the pattern of response were observed between the three study arms in either the unadjusted or adjusted model. Following adjustment for all potential baseline confounders (age, gender, BMI, IR, smoking status, and CRP) in a GLM no significant differences between the groups were seen.

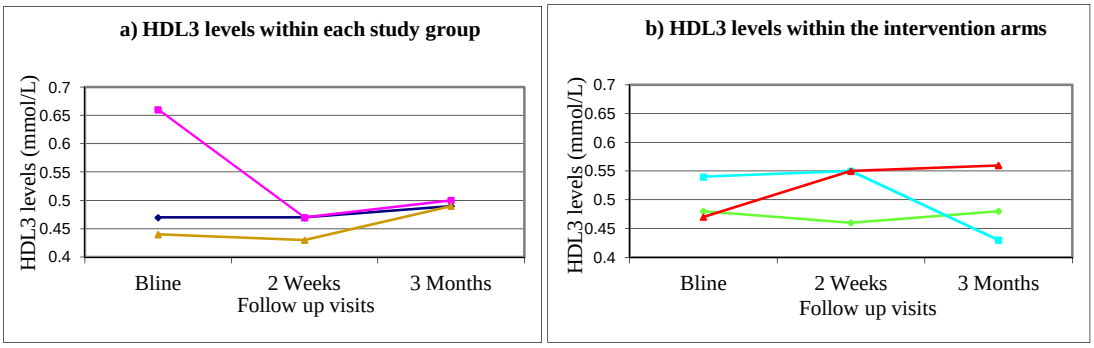
Intervention arms: A significant difference in the pattern of response was observed between the three treatment arms, which remained following adjustment for CRP (p=0.017). Patients treated with IV GCs had higher levels of SdLDL at 2 weeks (B= 0.084, 95% CI: 0.036 to 0.133, p=0.001) compared to baseline. These returned to baseline levels at 3 months. No significant differences in the levels of SdLDL were seen in the Anti-TNF or rituximab arms over the follow up period.

Figure 5.4: Longituinal changes in lipid subfractions a) within the three study groups (RA intervention, RA controls and healthy controls) b) within the three RA intervention arms (Anti-TNF, GCs and Rituximab)

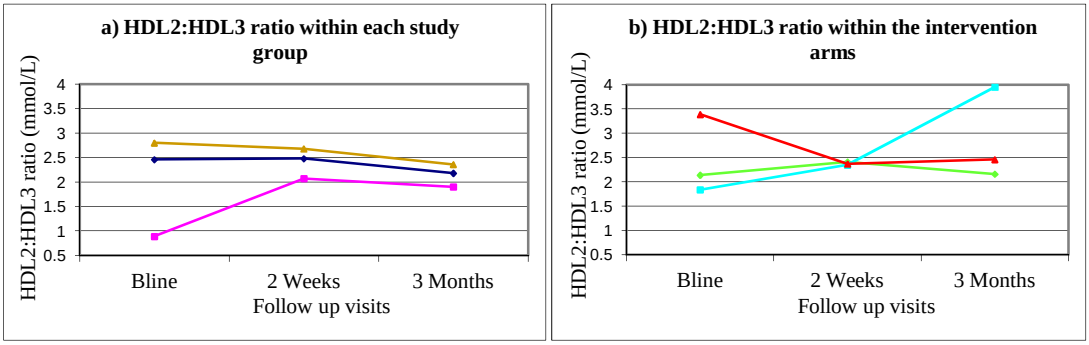
HDL2



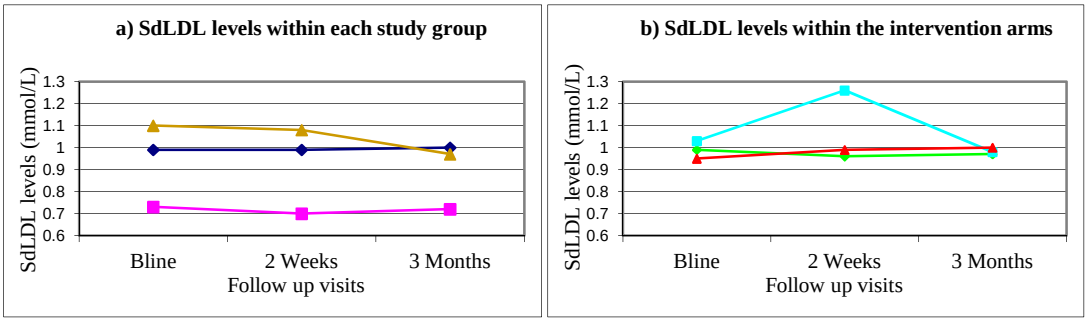
HDL3



HDL2:HDL3 ratio



logSdLDL



a) ___ = Healthy controls, ___ =RA intervention, ___ = RA controls
b) ___ = Intravenous glucocorticoids, ___ =Rituximab, ___ = Anti-tumour necrosis factor therapy

5.3.4 Longitudinal changes in LDL Modifications **(see figure 5.5)**

OxLDL:

Study groups: In both the unadjusted and CRP adjusted models a significant group effect and period effect was observed amongst the three study groups. Irrespective of group, there was a significant effect of time with OxLDL levels increasing at 2 weeks (B= 0.043, 95% CI: 0.014 to 0.071, p=0.003), but returning to baseline levels by 3 months. A group effect was noted with RA controls having significantly higher levels of oxLDL than HC subjects (B= 0.128, 95% CI: 0.053 to 0.203, p=0.001). However, following adjustment for all potential confounders at baseline (age, gender, BMI, IR, smoking status, and CRP) in a GLM no significant differences in OxLDL were observed between the groups.

Intervention arms: In the GEE model adjusted for CRP, there was a significant difference in the pattern of response over time (p<0.001), with patients receiving anti-TNF therapy (B= 0.052, 95% CI: 0.001 to 0.102, p=0.044) and rituximab (B= 0.220, 95% CI: 0.103 to 0.337, p=<0.001) found to have significantly higher levels of oxLDL at 3 months compared to baseline; whereas, patients receiving intravenous GCs had significantly lower levels of oxLDL at 3 months compared to baseline (B= -0.211, 95% CI: -0.335 to -0.087, p=0.001).

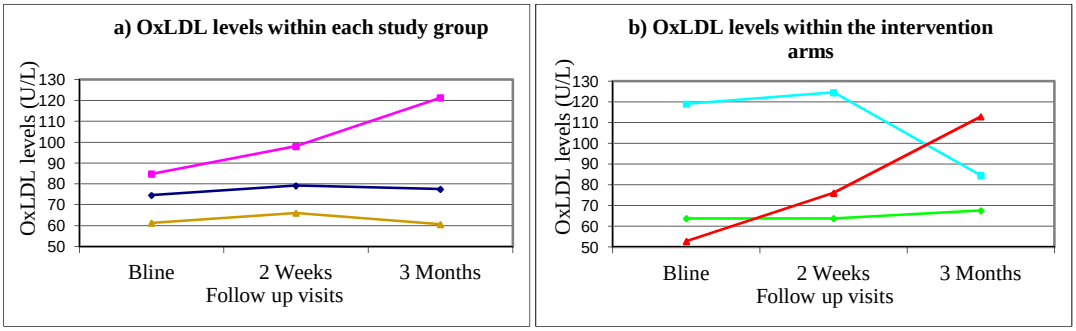
LDL nitration:

Study groups: No significant period effect, group effect or difference in the pattern of response was observed between the three study groups.

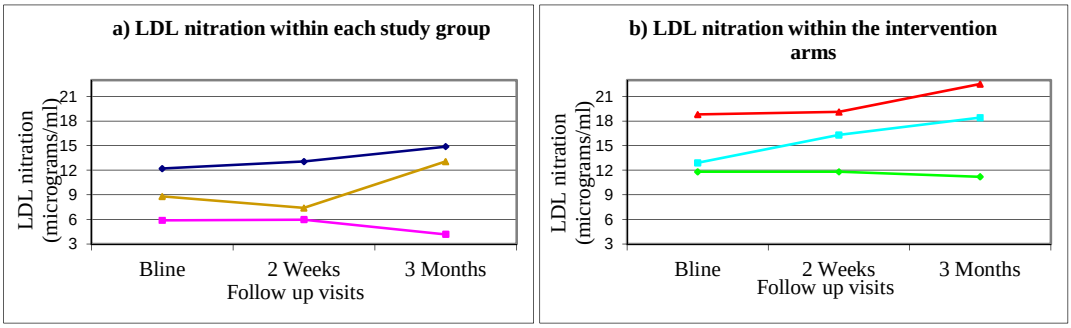
Intervention arms: In the GEE model adjusted for CRP, a significant group effect (irrespective of time) was noted with anti-TNF patients having significantly lower levels of LDL nitration compared to the rituximab arm (B= -0.26, 95% CI: -0.46 to –0.08, p=0.006). However, following adjustment for all potential baseline confounders (age, gender, BMI, IR, smoking status, and CRP) in a GLM this association was lost and no differences were observed between the groups.

Figure 5.5: Longitudinal changes in lipid modifications a) within the three study groups (RA intervention, RA controls and healthy controls) b) within the three RA intervention arms (Anti-TNF, GCs and Rituximab)

OxLDL



Nitrated LDL



a) ___ = Healthy controls, ___ =RA intervention, ___ = RA controls
b) ___ = Intravenous glucocorticoids, ___ =Rituximab, ___ = Anti-tumour necrosis factor therapy

5.3.5 Longitudinal changes in LDL function **(see figure 5.6)**

Susceptibility of LDL to oxidise (LDL lag times):

Study groups: A significant period effect was observed irrespective of group in both the unadjusted and CRP adjusted models, with lagtimes getting progressively shorter during the follow up period (adjusted model: 2 wks: B =-4.31, 95% CI: -6.94 to –1.68, p=0.001, and 3 months: B= -4.92, 95% CI: -7.61 to –2.23, p<0.001). A group effect was also observed, with lag times found to be significantly shorter following adjustment for CRP amongst both the RA Intervention arm (B= -12.18, 95% CI: -20.196 to –4.159, p=0.003) and the RA control arm (B= -13.13, 95% CI: -23.18 to –3.08, p=0.010) compared to the HC arm. The differences between RA intervention and RA controls versus HCs remained following adjustment for all other potential confounders (age, gender, BMI, IR, smoking status, and CRP) in a GLM (RA intervention vs HC: B= -12.62, 95% CI: -23.83 to -1.42, p=0.028).

Intervention arms: In the GEE model adjusted for CRP, a significant group effect was seen with patients receiving anti-TNF therapy having longer lag times than patients receiving rituximab therapy (B= 12.371, 95% CI: 7.616 to 24.213, p=0.001). No other group effects, period effect or differences in the pattern of response were observed. In a GLM adjusted for all potential confounders at baseline (age, gender, BMI, IR, smoking status, and CRP), lagtimes remained longer in patients on anti-TNF therapy compared to those on rituximab (B=15.915, 95% CI: 7.616 to 24.213, p=0.001).

DiIoxLDL uptake in to U937 cells:

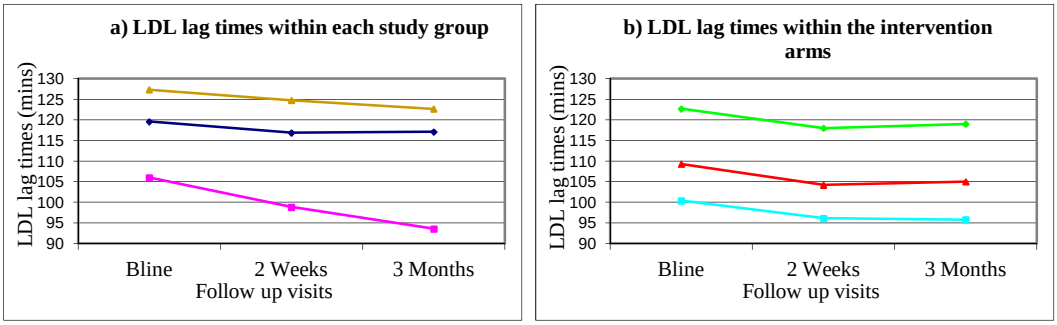
Study groups: No significant period effect, group effect, or differences in the pattern of response over time was observed between the three study groups. In a GLM adjusted for all potential confounders at baseline (age, gender, BMI, IR, smoking status, and CRP), no significant differences were found between the groups.

Intervention arms: No significant period effect, group effect or differences in the pattern of response was observed between the three treatment arms.

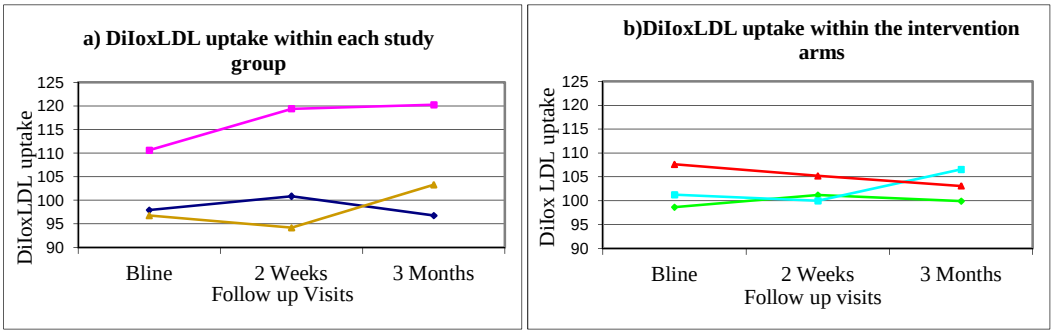
An overall summary of the longitudinal changes in lipid parameters amongst both the study groups and the intervention arms can be found in **tables 5.3 and 5.4**, respectively.

Figure 5.6: Longitudinal changes in the functional uptake of LDL by U937 cells a) within the three study groups (RA intervention, RA controls and healthy controls) b) within the three RA intervention arms (Anti-TNF, GCs and Rituximab)

LDL lagtimes



diIoxLDL uptake by U937 cells



a) ___ = Healthy controls, ___ =RA intervention, ___ = RA controls
b) ___ = Intravenous glucocorticoids, ___ =Rituximab, ___ = Anti-tumour necrosis factor therapy

Table 5.3: Summary of the longitudinal changes seen in lipid levels, structure and function within the three study groups (changes from baseline)

	RA intervention		RA controls		Healthy controls	
	2 Weeks	3 Months	2 Weeks	3 Months	2 Weeks	3 Months
Lipid levels						
LDL	↔	↔	↓	↔	↔	↔
HDL	↑	↔	↓	↔	↔	↔
TC	↑	↔	↓	↔	↔	↔
TG	↔	↔	↔	↔	↔	↔
Lipid ratios						
TC:HDL	↔	↔	↔	↔	↔	↔
LDL:HDL	↔	↔	↔	↔	↔	↔
ApoB:AopA	↔	↔	↔	↔	↔	↔
Lipid subfractions						
HDL2	↑	↔	↔	↔	↔	↓
HDL3	↔	↔	↓	↓	↔	↔
HDL2:HDL3	↔	↔	↔	↑	↔	↓
logSdLDL	↔	↔	↔	↔	↔	↔
Lipid modifications						
OxLDL	↑	↔	↑	↔	↑	↔
Nitrated LDL	↔	↔	↔	↔	↔	↔
LDL function						
LDL lagtimes	↓	↓	↓	↓	↓	↓
DiIoxLDL uptake	↔	↔	↔	↔	↔	↔

Arrows indicate direction of change: ↑= increased, ↓= decreased, ↔= no change from baseline.
Arrow colour indicates whether the change is potentially pro-atherogenic or anti-atherogenic or not known: ↑= anti-atherogenic, ↑= pro-atherogenic, ↑= unknown
RA: rheumatoid arthritis, LDL: low density lipoproteins, HDL: high density lipoproteins, TC: total cholesterol, TG: triglycerides, ApoB: apolipoprotein B, ApoA: apolipoprotein A, OxLDL: oxidised low density lipoproteins, DiIoxLDL uptake: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine labelled oxidised low density lipoprotein uptake

Table 5.4: Summary of the longitudinal changes seen in lipid levels, structure and function within the three intervention arms (changes from baseline)

	Anti-TNF		IV GCs		Rituximab	
	2 Weeks	3 Months	2 Weeks	3 Months	2 Weeks	3 Months
Lipid levels						
LDL	↔	↔	↔	↔	↔	↔
HDL	↑	↔	↑	↔	↑	↔
TC	↔	↔	↑	↔	↑	↔
TG	↔	↔	↔	↔	↔	↔
Lipid ratios						
TC:HDL	↔	↔	↔	↔	↔	↔
LDL:HDL	↔	↔	↔	↔	↔	↔
ApoB:AopA	↔	↔	↔	↔	↔	↔
Lipid subfractions						
HDL2	↑	↔	↑	↔	↑	↔
HDL3	↔	↔	↔	↓	↔	↔
HDL2:HDL3	↔	↔	↔	↔	↔	↔
logSdLDL	↔	↔	↑	↔	↔	↔
Lipid modifications						
OxLDL	↔	↑	↔	↓	↔	↑
Nitrated LDL	↔	↔	↔	↔	↔	↔
LDL function						
LDL lagtimes	↔	↔	↔	↔	↔	↔
DiIoxLDL uptake	↔	↔	↔	↔	↔	↔

Arrows indicate direction of change: ↑= increased, ↓= decreased, ↔= no change from baseline.
Arrow colour indicates whether the change is potentially pro-atherogenic or anti-atherogenic: ↑= anti-atherogenic, ↑= pro-atherogenic, ↑= unknown
Anti-TNF: anti-tumour necrosis factor, IV GCs: intravenous glucocorticoids, LDL: low density lipoproteins, HDL: high density lipoproteins, TC: total cholesterol, TG: triglycerides, ApoB: apolipoprotein B, ApoA: apolipoprotein A, OxLDL: oxidised low density lipoproteins, DiIoxLDL uptake: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine labelled oxidised low density lipoprotein uptake

5.3.6 Associations of lipid parameters with LDL function (LDL lag times and diIoxLDL uptake)

Baseline associations:

The baseline associations of individual lipid parameters with measures of LDL uptake (LDL lagtimes and diIoxLDL uptake in to U937 cells) in all subjects (RA and HC) were assessed using Pearson's coefficient. This demonstrated that HDL3 and log OxLDL levels are significantly inversely associated with lag times. Whilst a similar trend was seen with these parameters and diIoxLDL uptake by U937 cells, this did not reach statistical significance. The results are summarised in **table 5.5**.

Table 5.5: Baseline correlations of measurements of lipid levels and structure with LDL function amongst all subjects (RA and HC)

	diIoxLDL uptake into macrophages		**LDL lagtimes**	
	r value	p value	r value	p value
Lipid levels				
TC	0.088	0.569	0.072	0.470
HDL	-0.055	0.725	0.041	0.684
LDL	0.100	0.516	0.083	0.404
TG	0.089	0.566	-0.189	0.057
Lipid subfractions				
HDL2	0.033	0.830	0.180	0.071
HDL3	-0.153	0.321	-0.200	**0.044**
SdLDL	0.096	0.535	0.114	0.257
Lipid modifications				
log OxLDL	-0.061	0.697	-0.206	**0.041**
log nitrated LDL	0.165	0.296	0.021	0.837
Functional changes				
LDL lagtimes	-0.005	0.977		

▭ = significant negative association
† = p value remains significant following bonferroni correction
TC: total cholesterol, HDL: high density lipoproteins, LDL: low density lipoproteins, TG: triglycerides, SdLDL: small dense low density lipoproteins

Longitudinal associations of lipid parameters with LDL function over time

Association of lipid parameters and LDL lag times:

Study groups: In a GEE model, the effect of time on the association between LDL lag times and logSdLDL (p=0.003), oxLDL (p<0.001) and TG (p<0.001) levels was not the same within the three intervention arms (significant 3 –way interaction). For the association between logSdLDL and LDL lagtimes, persistent positive association was seen amongst the RA control arm, however this was only significant at baseline (B=21.25, 95% CI: 1.11 to 41.39, p=0.039) and 3 months (B=65.64, 95% CI: 24.22 to 107.07, p=0.002). No significant associations between logSdLDL and LDL lagtimes were observed amongst the RA intervention or HC arms. For the association between oxLDL and LDL lagtimes, the RA control arm had a significant positive association at 2 weeks (B= 48.09, 95% CI: 8.68 to 87.51, p=0.017) whereas the HC arm had a significant negative association at 2 weeks (B=-15.12, 95% CI: -27.7 to –2.34, p=0.019). No significant associations were identified in the RA intervention arm. TGs and LDL lagtimes were found to be negatively associated at all time points in both the RA intervention and RA control arms. However, this was only significant at baseline (B= -4.51, 95% CI: -8.48 to –0.54, p=0.026) and 3 months (B= -6.27, 95% CI: -11.28 to –1.26, p=0.014) in the RA intervention arm and at 2 weeks (B= -23.59, 95% CI: -36.51 to –10.68, p<0.001) in the RA control arm. These results are summarised in **table 5.6**.

Table 5.6: Summary of the 3 way interactions found between LDL lagtimes and lipid parameters between the three study groups (results shown are adjusted for CRP)

	Baseline B (95% CI)	2 Weeks B (95% CI)	3 Months B (95% CI)	P value
OxLDL				
RA Intervention	-4.21 (-18.39 to 9.61)	-5.86 (-19.96 to 8.25)	-5.07 (-16.98 to 6.84)	**<0.001**†
RA controls	7.71 (-23.9 to 39.37)	48.09 * (8.68 to 87.51)	-31.51 (-63.09 to 0.07)	
Healthy controls	-17.56 (-36.58 to 1.47)	-15.12 * (-27.7 to –2.34)	-6.09 (-41.1 to 28.96)	
LogSdLDL				
RA Intervention	-15.72 (-36.41 to 4.97)	-0.28 (-12.3 to 11.73)	4.87 (-14.16 to 23.9)	**0.003**†
RA controls	21.25 * (1.11 to 41.39)	1.98 (-41.54 to 45.5)	65.64 * (24.22 to 107.1)	
Healthy controls	0.12 (-36.84 to 37.1)	-21.69 (-45.54 to 2.27)	-0.12 (-34.9 to 34.69)	
LogHDL2				
RA Intervention	12.59 (-12.8 to 37.91)	0.76 (-38.6 to 40.16)	0.64 (-39.1 to 40.24)	0.892
RA controls	-6.56 (-45.6 to 32.47)	-8.95 (-68.1 to 50.12)	5.93 (-36.6 to 48.42)	
Healthy controls	29.19 (-1.57 to 59.96)	25.22 (-16.62 to 67.1)	31.14 (1.19 to 61.09)	
LogHDL3				
RA Intervention	-5.98 (-28.94 to 16.9)	-8.78 (-28.5 to 10.09)	6.23 (-10.5 to 22.97)	0.170
RA controls	-12.56 (-55.8 to 30.67)	13.10 (-53.4 to 79.62)	16.77 (-27.07 to 60.6)	
Healthy controls	-22.82 (-45.7 to 0.03)	20.69 (-4.07 to 45.63)	-9.89 (-35.2 to 15.42)	
Log nitrated LDL				
RA Intervention	-2.54 (-9.32 to 4.23)	-0.72 (-7.69 to 6.26)	-0.59 (-6.43 to 5.24)	0.060
RA controls	2.53 (-22.64 to 27.7)	-8.49 (-28.26 to 11.3)	7.04 (-11.92 to 25.9)	
Healthy controls	4.57 (-2.76 to 11.91)	3.49 (-3.54 to 10.52)	-2.53 (-7.67 to 2.60)	
TC				
RA Intervention	3.39 (-2.55 to 9.23)	1.68 (-3.19 to 6.55)	1.10 (-3.58 to 5.78)	0.062
RA controls	2.12 (-5.43 to 9.68)	-6.14 (-15.01 to 2.74)	2.07 (-8.52 to 12.66)	
Healthy controls	4.44 (-3.06 to 11.95)	1.17 (-5.13 to 7.47)	5.56 (-1.33 to 12.46)	

HDL				
RA Intervention	2.24 (-10.7 to 15.14)	-0.52 (-13.32 to 12.3)	2.22 (-9.63 to 14.08)	0.515
RA controls	-13.43 (-32.53 to 5.69)	-2.88 (-32.04 to 26.3)	2.71 (-29.6 to 35.02)	
Healthy controls	1.14 (-11.7 to 14.01)	-3.30 (-20.7 to 14.13)	-0.83 (-17.5 to 15.84)	
LDL				
RA Intervention	4.76 (-2.48 to 11.99)	3.00 (-0.98 to 6.98)	2.96 (-1.95 to 7.87)	0.103
RA controls	16.85 (8.06 to 25.65)	10.24 (2.15 to 18.34)	17.97 (-0.73 to 36.68)	
Healthy controls	2.69 (-7.91 to 13.29)	2.63 (-4.41 to 9.67)	6.94 (-1.25 to 15.13)	
TGs				
RA Intervention	-4.51 * (-8.48 to 0.54)	-3.28 (-9.40 to 2.83)	-6.27 * (-11.3 to –1.26)	**<0.001**†
RA controls	-4.05 (-19.7 to 11.55)	-23.59 * (-36.5 to –10.7)	-13.29 (-29.20 to 2.62)	
Healthy controls	-0.23 (-7.48 to 6.98)	4.54 (-4.66 to 13.73)	8.20 (-1.84 to 18.26)	

* = significantly different from zero
† = p value remains significant following bonferroni correction
= significant negative association, = significant positive association.
OxLDL: oxidised low density lipoproteins, SdLDL: small density low density lipoproteins, HDL: high density lipoproteins, LDL: low density lipoproteins, TGs: Triglycerides, TC: total cholesterol.

No other significant associations (2 or 3 way interactions) were observed between lognitrated LDL, logHDL2, logHDL3, TC, HDL, LDL, and lag times.

Intervention arms: In a GEE model (both unadjusted and CRP adjusted), the effect of time on the association between LDL lag times and both logHDL2 (p<0.019) and logHDL3 (p0.017) levels was not the same within the three drug therapy arms (a significant 3-way interaction). The association between logHDL2 and LDL lag times was explained by a significant positive association (B=88.21, 95% CI: 26.95 to 149.47, p=0.005) in the rituximab arm at 2 weeks, which was lost by 3 months. No significant associations were seen in the Anti-TNF or IV GC arms. The significant 3 way interaction for logHDL3 and LDL lagtimes can be explained by changes in the rituximab arm, with a significant positive association seen at 3months (B= 55.94, 95% CI: 22.93 to 88.94, p=0.001). No other significant associations (2 or 3 way interactions) were observed. These results are summarised in **table 5.7**.

Table 5.7: Details of the 3 way interactions found between LDL lagtimes and lipid parameters between the three intervention arms (results shown are adjusted for CRP)

	Baseline B (95% CI)	2 Weeks B (95% CI)	3 Months B (95% CI)	P value
OxLDL				
Anti-TNF	0.81 (-24.63 to 26.25)	-7.54 (-30.56 to 15.48)	-3.16 (-27.04 to 20.73)	0.854
IV GCs	-31.39 (-68.68 to 5.90)	-42.39 (-80.75 to –4.06)	-47.21 (-72.3 to –22.15)	
Rituximab	-41.05 (-134.1 to 51.96)	-29.75 (-104.9 to 45.42)	-27.67 (-161.9 to106.6)	
LogSdLDL				
Anti-TNF	-2.47 (-20.80 to 15.87)	-3.08 (-23.49 to 17.33)	-7.38 (-23.94 to 9.19)	0.295
IV GCs	-21.50 (-67.02 to 24.02)	-16.24 (-54.81 to 22.33)	-23.32 (-55.64 to 9.00)	
Rituximab	103.54 (-14.22 to 221.3)	58.29 (3.15 to 113.45)	8.68 (-31.32 to 48.68)	
LogHDL2				
Anti-TNF	-2.21 (-24.09 to 16.68)	-3.41 (-42.89 to 36.07)	5.01 (-26.88 to 36.91)	**0.019**
IV GCs	3.70 (-42.41 to 49.81)	-24.22 (-68.78 to 20.34)	-11.82 (-57.65 to 34.01)	
Rituximab	28.21 (-24.88 to 81.29)	88.21 * (26.95 to 149.47)	-4.88 (-59.69 to 49.94)	
LogHDL3				
Anti-TNF	0.62 (-28.11 to 29.35)	-23.86 (-49.16 to 1.44)	7.38 (-17.18 to 31.94)	**0.017**
IV GCs	9.45 (-31.76 to 50.67)	-11.71 (-65.08 to 41.65)	-13.27 (-39.17 to 12.62)	
Rituximab	-20.18 (-53.73 to 13.36)	28.63 (-38.11 to 95.37)	55.94 * (22.93 to 88.94)	
Log nitrated LDL				
Anti-TNF	-3.85 (-12.69 to 4.98)	-0.50 (-8.45 to 7.45)	-0.74 (-10.17 to 8.70)	0.193
IV GCs	-8.59 (-19.10 to 1.91)	-0.08 (-11.48 to 11.31)	-0.74 (-10.17 to 8.70)	
Rituximab	13.01 (-0.29 to 26.33)	-6.54 (-18.55 to 5.47)	-2.37 (-16.75 to 12.01)	
TC				
Anti-TNF	0.62 (-5.03 to 6.27)	-0.02 (-6.49 to 6.44)	-0.89 (-6.34 to 4.54)	0.873
IV GCs	-9.59 (-21.82 to 2.64)	-8.43 (-16.45 to –0.41)	-9.19 (-16.64 to –1.74)	

Rituximab	19.69 (-3.03 to 42.42)	16.68 (1.44 to 31.91)	10.84 (-7.77 to 29.27)	
HDL				
Anti-TNF	-3.74 (-21.27 to 13.79)	-6.32 (-25.56 to 12.93)	0.13 (-14.23 to 14.80)	0.188
IV GCs	5.63 (-21.69 to 32.95)	-7.72 (-26.51 to 11.07)	-6.60 (-27.37 to 14.17)	
Rituximab	-4.05 (-46.19 to 38.10)	26.96 (3.09 to 50.83)	26.24 (-7.16 to 59.64)	
LDL				
Anti-TNF	0.94 (-5.53 to 7.40)	0.81 (-6.42 to 7.76)	-1.63 (-8.42 to 5.15)	0.123
IV GCs	-7.84 (-18.82 to 3.15)	-5.77 (-12.45 to 0.92)	-6.79 (-13.67 to 0.09)	
Rituximab	14.88 (-9.42 to 39.16)	2.72 (-22.65 to 28.09)	-4.51 (-26.81 to17.79)	
TGs				
Anti-TNF	-1.83 (-6.67 to 3.02)	-2.93 (-12.31 to 6.46)	-5.05 (-11.78 to 1.68)	0.123
IV GCs	-9.36 (-18.87 to 0.16)	-1.04 (-7.58 to 5.51)	-7.82 (-18.29 to 2.66)	
Rituximab	5.09 (-7.04 to 17.23)	33.81 (-13.95 to 81.58)	41.52 (-0.63 to 83.68)	

▭ = significant positive association
* = significantly different from zero
† = p value remains significant following bonferroni correction
Anti-TNF: anti-tumour necrosis factor, IV GCs: intravenous glucocorticoids, OxLDL: oxidised low density lipoproteins, SdLDL: small density low density lipoproteins, HDL: high density lipoproteins, LDL: low density lipoproteins, TGs: Triglycerides, TC: total cholesterol.

Association of lipid parameters and DiIoxLDL uptake into U937 cells:

Study groups: In the unadjusted GEE model, the effect of time on the association between the uptake of diIoxLDL into U937 cells and oxLDL ($p<0.001$), lognitrated LDL ($p<0.001$), log HDL3 ($p<0.001$) and TG ($p<0.001$) levels were not the same within the three intervention arms (a significant 3-way interaction). Following adjustment for CRP, these associations remained significant, however a new 3-way interaction was unmasked between diIoxLDL uptake and LDL lagtimes (Unadjusted: $p=0.124$, CRP adjusted: $p=0.029$). The details of the three way interactions are summarised in **table 5.8.** For the association between oxLDL and diIoxLDL uptake, no significant associations were found amongst the RA intervention or HC arms at any of the 3 time points. However, RA controls had a significant negative association at 3 months (B= -79.18, 95% CI: -128.77 to –29.59, $p=0.002$). There were negative associations between lognitrated LDL and diIoxLDL uptake at all 3 time points in the HC and RA control arms. However, amongst the HC arm it was only significant at baseline (B= -9.76, 95% CI: -19.0 to –0.47, $p=0.039$), and amongst the RA control arm it was only significant at 2 weeks (B= -25.58, 95% CI: -53.3 to –4.84 $p=0.018$). No significant associations were found amongst the RA intervention arm. The RA control arm had a significant positive association between logHDL3 and diIoxLDL uptake at baseline (B= 112.49, 95% CI: 66.42 to 158.57, $p<0.001$) which changed to a significant negative association at both 2 weeks (B= -27.28, 95% CI: -44.87 to –9.97, $p= 0.002$) and 3 months (B= -41.23, 95% CI: -50.40 to –32.05, $p<0.001$), where as the HC arm had no significant associations at baseline or 2 weeks but a negative association at 3 months (B= -45.88, 95% CI: -74.51 to-17.26, $p= 0.002$). The RA control arm had a positive association between TG and diIoxLDL uptake,at all time points, which was significant at baseline (B= 8.87, 95% CI: 0.24 to 17.49, $p=0.044$) and 3 months (B= 7.91, 95% CI: 0.66 to 15.15, $p= 0.032$). The RA control and HC arms had no significant associations. The RA control arm had a negative association between LDL lagtimes and diIoxLDL uptake, at all three time points, which was only significant at baseline (B= -0.95, 95% CI: -1.62 to –0.29, $p= 0.005$).The HC and RA intervention arms had no significant associations.

Table 5.8: Summary of the 3 way interactions found between diIoxLDL uptake and lipid parameters between the three study groups (results adjusted for CRP)

	Baseline B (95% CI)	2 Weeks B (95% CI)	3 Months B (95% CI)	P value
LDL lagtimes				
RA Intervention	-0.01 (-0.51 to 0.48)	0.19 (-0.15 to 0.52)	-0.09 (-0.33 to 0.15)	**0.029**
RA controls	-0.95 * (-1.62 to –0.29)	-0.22 (-0.46 to 0.03)	-0.13 (-0.68 to 0.42)	
Healthy controls	-0.22 (-0.45 to 0.01)	0.42 (-0.21 to 1.04)	0.21 (-0.3 to 0.72)	
OxLDL				
RA Intervention	-5.54 (-21.34 to 10.2)	-3.52 (-22.3 to 15.23)	-5.99 (-27.90 to 15.9)	**<0.001**†
RA controls	22.37 (-24.9 to 69.63)	-66.04 (-132.3 to 0.19)	-79.18 * (-128.8 to– 29.6)	
Healthy controls	3.02 (-17.96 to 23.9)	10.45 (-6.06 to 27.52)	-0.94 (-24.65 to 22.7)	
logSdLDL				
RA Intervention	25.40 (7.15 to 43.64)	-14.07 (-25.9 to –2.16)	2.89 (-14.12 to 19.9)	0.185
RA controls	14.20 (-3.32 to 31.74)	29.76 (-26.11 to 85.6)	1.43 (-19.3 to 22.15)	
Healthy controls	21.09 (5.05 to 37.14)	-18.09 (-36.73 to 0.53)	-16.31 (-28.8 to –3.81)	
LogHDL2				
RA Intervention	11.06 (-43.2 to 65.36)	12.42 (-27.4 to 52.28)	2.70 (-31.4 to 36.78)	0.224
RA controls	35.98 (-1.04 to 73.00)	120.75 (59.91 to 181.6)	33.71 (8.36 to 59.07)	
Healthy controls	53.15 (-68.5 to 174.8)	17.95 (-70.7 to 106.6)	37.91 (1.00 to 74.81)	
LogHDL3				
RA Intervention	14.26 (-76.6 to 105.1)	-11.81 (-45.87 to 22.2)	-21.03 (-53.47 to 11.4)	**<0.001**†
RA controls	112.49 * (66.42 to 158.6)	-27.28 * (-44.6 to –9.97)	-41.23 * (-50.4 to –32.1)	
Healthy controls	-14.09 (-104.9 to 76.7)	26.91 (-36.27 to 90.8)	-45.88 * (-74.5 to –17.2)	
Log nitrated LDL				
RA Intervention	5.19 (-2.2 to 12.63)	-6.23 (-15.89 to 3.43)	-0.43 (-5.99 to 5.12)	**<0.001**†
RA controls	-2.59 (-21.11 to 15.9)	-28.58 * (-53.3 to –4.84)	-1.48 (-15.73 to 12.8)	
Healthy controls	-9.76 * (-19.0 to –0.47)	-11.99 (-26.39 to 2.41)	-8.00 (-18.31 to 2.31)	

TC				
RA Intervention	10.43 (3.09 to 17.76)	-7.39 (-14.5 to –0.27)	-0.09 (-8.27 to 8.09)	0.415
RA controls	27.78 (13.18 to 42.38)	15.98 (-12.92 to 44.8)	25.57 (7.43 to 43.70)	
Healthy controls	5.99 (-3.34 to 15.32)	5.09 (-6.01 to 16.21)	7.67 (-4.62 to 19.96)	
HDL				
RA Intervention	16.28 (-14.52 to 47.0)	-12.96 (-31.60 to 5.68)	-8.34 (-14.47 to 7.86)	0.404
RA controls	20.98 (-3.32 to 45.28)	47.44 (7.05 to 87.83)	21.44 (-14.4 to 57.34)	
Healthy controls	4.92 (-33.19 to 43.0)	-11.22 (-37.9 to 15.53)	-2.10 (-26.79 to 22.5)	
LDL				
RA Intervention	11.24 (2.97 to 19.56)	-6.52 (-16.77 to 3.62)	2.50 (-6.81 to 11.82)	0.311
RA controls	16.72 (0.96 to 32.49)	31.22 (-19.96 to 82.3)	21.67 (-16.57 to 59.9)	
Healthy controls	6.72 (-2.89 to 16.26)	8.25 (-2.98 to 19.49)	10.33 (-1.52 to 22.17)	
TGs				
RA Intervention	8.87 * (0.24 to 17.49)	6.75 (-1.96 to 15.45)	7.91 * (0.66 to 15.15)	**<0.001**†
RA controls	-39.88 (-96.26 to 16.5)	-34.65 (-77.12 to 7.83)	-18.57 (-62.05 to 24.9)	
Healthy controls	-7.96 (-16.92 to 1.01)	2.58 (-6.23 to 11.39)	-2.68 (-9.17 to 3.82)	

= significant negative association, = significant positive association
* = significantly different from zero
† = p value remains significant following bonferroni correction
OxLDL: oxidised low density lipoproteins, SdLDL: small density low density lipoproteins, HDL: high density lipoproteins, LDL: low density lipoproteins, TGs: Triglycerides, TC: total cholesterol.

For the other lipid parameters no significant 3 way interactions were observed. However, for logHDL2 there was a significant two way interaction. The way LDL uptake varies with logHDL2 (p=0.007) levels was significantly different between the 3 study groups irrespective of time. Details of these differences are summarised in **Table 5.9**.

Table 5.9: Summary of the significant 2 way interactions between diIoxLDL uptake and logHDL2 (results shown are adjusted for CRP)

	B (95% CI)	P value
LogHDL2		
RA intervention	8.68 (-10.11 to 27.26)	0.365
RA controls	52.32 (31.19 to 73.45)	**<0.001**
Healthy controls	21.69 (-3.23 to 46.62)	0.088
= significant positive association, HDL: high density lipoproteins		

Intervention arms: In both the unadjusted and CRP adjusted GEE models (adjusted model reported), the effect of time on the association between uptake of diIoxLDL into U937 cells and LDL lagtimes (p=0.001), lognitrated LDL (p<0.001), SdLDL (p<0.001), HDL (p=0.027), LDL (p=0.003), TC (p=0.002) and LDL (p<0.001) was not the same within the three drug therapy arms (a significant 3-way interaction). There was a significant positive association (B=0.69, 95% CI: 0.35 to 1.04, p<0.001) in the anti-TNF arm and a significant negative association in the rituximab arm (B= -0.36, 95% CI: -0.071 to -0.01, p=0.041) between LDL lag times and diIoxLDL uptake . For the association of logSdLDL and diIoxLDL uptake, a persistent positive association was seen in the rituximab arm, which became progressively stronger over time, with significant associations seen at 2 weeks (B=17.68, 95% CI: 0.14 to 35.23, p=0.042) and 3 months (B=35.59, 95% CI: 18.84 to 52.34, p<0.001). No significant associations were seen in the Anti-TNF or IV GC arms. Several significant negative associations were seen between lognitrated LDL and diIoxLDL uptake, but the timing of these differed across the treatment groups. A significant negative association was seen at baseline (B= -12.39, 95% CI: -19.06 to-5.73, p<0.001) in the anti-TNF arm, but this was lost at 2 weeks and 3 months. The IV GC arm developed a significant negative association at 2 weeks (B= -15.46, 95% CI: -21.87 to -9.04, p<0.001) which persisted at 3 months (B= -5.15, 95% CI: -9.96 to -0.34, p=0.036). However, the rituximab arm did not develop a significant negative association until 3 months (B=-40.84, 95% CI: -65.53 to -16.1, p=0.001). A significant positive association was seen at 3 months in the rituximab arm between TC and diIoxLDL uptake, (B=16.30, 95% CI: 2.78 to 29.82, p=0.018). The opposite was found for HDL and diIoxLDL uptake with a significant negative association (B=-50.42, 95% CI: -83.13 to -17.02, p=0.003) seen at 3 months in the rituximab arm. The 3 way

interaction involving TG and diIoxLDL uptake is explained by a significant negative association at 2 weeks (B= -57.82, 95% CI: -88.52 to -27.2, p=0.007) in the rituximab arm. Details of where the differences lie are summarised in **table 5.10**.

Table 5.10: Details of the 3 way interactions found between diIoxLDL uptake and lipid parameters between the three intervention arms (results shown are adjusted for CRP)

	Baseline B (95% CI)	**2 Weeks B (95% CI)**	**3 Months B (95% CI)**	**P value**
LDL lagtimes				
Anti-TNF	0.69 * (0.35 to 1.04)	0.17 (-0.17 to 0.50)	-0.08 (-0.31 to 0.15)	**0.001**†
IV GCs	-0.12 (-0.55 to 0.32)	-0.10 (-0.65 to 0.44)	0.04 (-0.23 to 0.30)	
Rituximab	-0.36 * (-0.71 to –0.01)	-0.06 (-0.23 to 0.12)	-0.14 (-0.39 to 0.12)	
OxLDL				
Anti-TNF	-17.79 (-39.34 to 3.77)	-14.81 (-31.69 to 2.06)	-29.17 (-53.88 to –4.46)	0.075
IV GCs	-15.09 (-53.43 to 23.24)	7.86 (-15.52 to 31.24)	-2.27 (-14.69 to 10.16)	
Rituximab	11.87 (-34.25 to 57.98)	-15.27 (-75.40 to 44.86)	-58.94 (-205.2 to 87.34)	
LogSdLDL				
Anti-TNF	0.14 (-11.86 to 12.15)	-3.27 (-12.83 to 6.28)	-7.23 (-15.39 to 0.92)	**<0.001**†
IV GCs	-10.97 (-24.39 to 2.44)	-6.32 (-18.03 to 5.39)	-7.09 (-23.91 to 9.72)	
Rituximab	1.25 (-10.39 to 12.91)	17.68 * (0.142 to 35.23)	35.59 * (18.84 to 52.34)	
LogHDL2				
Anti-TNF	4.01 (-21.54 to 29.57)	31.03 (-7.49 to 69.55)	7.92 (-11.29 to 27.14)	0.060
IV GCs	-5.49 (-76.89 to 65.91)	8.47 (-44.11 to 61.04)	10.69 (-46.44 to 67.83)	
Rituximab	-2.51 (-62.85 to 57.84)	-0.75 (-47.81 to 46.32)	-52.87 (-152.8 to 47.10)	
LogHDL3				
Anti-TNF	45.08 (-11.54 to 101.7)	34.89 (-26.57 to 96.15)	12.16 (-31.74 to 56.06)	0.229
IV GCs	4.74 (-44.79 to 54.79)	8.64 (-38.85 to 56.13)	-10.54 (-34.09 to 13.00)	
Rituximab	39.91 (8.32 to 71.49)	24.16 (-9.69 to 58.02)	-17.53 (-57.15 to 22.15)	

Log nitrated LDL				
Anti-TNF	-12.39 * (-19.06 to –5.73)	-4.54 (-11.01 to 1.95)	1.28 (-5.66 to 8.23)	**<0.001**†
IV GCs	2.69 (-3.09 to 8.48)	-15.46 * (-21.87 to –9.04)	-5.15 * (-9.96 to –0.34)	
Rituximab	9.56 (-32.82 to 51.94)	-1.25 (-11.07 to 8.58)	-40.84 * (-65.53 to –16.1)	
TC				
Anti-TNF	5.40 (-3.74 to 14.55)	4.45 (-3.32 to 12.22)	2.44 (-5.92 to 10.81)	**0.002**†
IV GCs	-3.49 (-10.06 to 3.07)	-0.85 (-5.53 to 3.83)	-0.35 (-5.29 to 4.59)	
Rituximab	3.06 (-7.59 to 13.72)	4.41 (-7.88 to 16.69)	16.30 * (2.78 to 29.82)	
HDL				
Anti-TNF	9.19 (-14.15 to 35.52)	15.50 (-2.52 to 33.53)	1.04 (-13.04 to 15.13)	**0.027**
IV GCs	1.48 (-31.10 to 34.07)	2.48 (-16.11 to 21.07)	3.29 (-15.32 to 21.91)	
Rituximab	-16.67 (-47.64 to 14.29)	-14.21 (-35.32 to 6.91)	-50.42 * (-83.1 to -17.02)	
LDL				
Anti-TNF	2.58 (-5.75 to 10.90)	4.15 (-3.52 to 11.82)	0.52 (-7.75 to 8.79)	**0.003**†
IV GCs	-1.69 (-8.39 to 5.03)	0.56 (-4.69 to 5.81)	1.23 (-4.04 to 6.50)	
Rituximab	-5.97** (-21.88 to 9.95)	-6.11** (-26.15 to 13.93)	5.39** (-15.29 to 26.07)	
TGs				
Anti-TNF	2.36 (-4.76 to 9.47)	0.18 (-8.62 to 8.97)	-0.62 (-6.28 to 5.05)	**0.002**†
IV GCs	-5.83 (-25.74 to 14.08)	-1.65 (-14.68 to 11.37)	-2.34 (-15.62 to 10.94)	
Rituximab	-23.32 (-50.02 to3.38)	-57.82 * (-88.52 to –27.1)	27.93 (-25.59 to 81.46)	

= significant negative association, = significant positive association
* = significantly different from 0, ** = significant difference between timepoints
† = p value remains significant following bonferroni correction
Anti-TNF: anti-tumour necrosis factor, IV GCs: intravenous glucocorticoids, OxLDL: oxidised low density lipoproteins, SdLDL: small density low density lipoproteins, HDL: high density lipoproteins, LDL: low density lipoproteins, TGs: Triglycerides, TC: total cholesterol.

A significant 2 way interaction was also observed for logHDL3 (p=0.020), with the way diIoxLDL uptake varied with logHDL3 levels found to be significantly different over time irrespective of group. The associations between diIoxLDL uptake and logHDL3 levels was positive at baseline (B= 48.36, 95% CI: -20.68 to 117.39, p=0.170) and 2

weeks (B= 6.97, 95% CI: -25.05 to 39.02, p=0.669) but negative at the 3 month (B= -1.71, 95% CI: -30.03 to 26.61, p=0.906) follow up time point. Although none of the individual Beta values was significantly different from zero, it is likely the significance of the 2–way interaction is due to the association at baseline being considerably greater than the association at 2 weeks and 3 months.

A summary of the longitudinal associations found between individual lipid parameters and LDL function across the 3 study groups and 3 intervention arms would be: 1) Overall the changes in individual lipid parameters do not seem to have much effect on LDL function 2) the associations between individual lipid parameters and LDL function appear to be subject to gross fluctuations, particularly amongst the RA control arm, perhaps suggesting an influence of disease specific factors 3) there do appear to be some differences in the longitudinal associations of individual lipid parameters and LDL function between the different anti-inflammatory drug therapies, with the rituximab arm found to account for the majority of changes observed.

5.4 Discussion

In this chapter I have demonstrated that lipoproteins are altered in RA resulting in a pro-atherogenic lipoprotein subfraction profile (increased HDL3 and reduced HDL2) and pro-atherogenic LDL modifications (e.g. increased oxLDL), which can influence LDL function (e.g. DiIoxLDL upatake). In addition, I have demonstrated that intervention with drug therapy can produce profound effects on lipid levels, structure and function. These effects appear to be predominately mediated by generic anti-inflammatory effects (e.g. changes in nitrated LDL) but drug-specific mechanisms (e.g. GC-specific) may also play a role. Interestingly, the majority of changes in lipid parameters with anti-inflammatory treatment appeared to be transient, with levels returning to baseline values by 3 months. In the IV GC arm, such changes are easily explained by the duration of the drug effect. However, for the rituximab and anti-TNF arms this is less easily explained. It is possible that the methylprednisolone infusion given concurrently with the rituximab infusion may trigger some transient changes, however this would not explain the findings in the anti-TNF arm. These findings are in contrast to previously published 1 year data on the effects of anti-inflammatory therapy on lipid levels in RA which would support persistent changes in lipid levels at 1 year post initiation of anti-TNF (504). However, during the course of a year multiple changes in disease activity, drug therapy,

autoantibody status, physical activity etc may have ensued and these may not have been adequately controlled for in the methodology of such studies. Thus, it may be that the results produced from 1 year data are less reliable than short term data in this context.

Interestingly, current evidence would suggest that anti-TNF therapy may be cardioprotective and significantly reduce the incidence of CVD in RA (505;506). TNF blockade has been shown to suppress the expression of adhesion molecules associated with atherosclerosis (507) and improve endothelial dysfunction (508). In this chapter anti-TNF use was found to produce an overall anti-atherogenic profile (increased HDL and HDL2). These observations are consistent with, and add to the findings of several recent meta-analyses (504;509). The overall findings were that there was a significant increase in HDL and TC levels, but no change in LDL or ApoA1 levels during long term treatment with TNF inhibitors. Such improvements in the lipid profile with TNF therapy may confer improvements in CVD risk through enhanced reverse cholesterol transport, restoration of the balance of pro and anti-atherogenic HDL, and anti-oxidant effects.

Many of the changes observed in lipid levels, structure and function were similar across the three drug therapy arms (anti-TNF, rituximab and IV GCs). However, the IV GC arm did produce some additional changes that were not observed in the other arms e.g. suppression of HDL3 and oxLDL and an increase in logSdLDL. Overall, patients receiving IV GCs appeared to develop a more pro-atherogenic lipid profile than the other drug therapies (elevation of TC and sdLDL, and suppression of HDL3 and oxLDL levels). This observation would be consistent with previous studies performed in the general population, which have demonstrated that changes in lipid levels (elevated TC, HDL and TG) occur as a consequence of corticosteroid administration (359). The drug specific effects of GCs on the lipid profile may be mediated through increased plasma insulin levels, increased lipid production in the liver and impaired lipid catabolism (510). Interestingly, unlike rituximab and anti-TNF, IV GC use produced a paradoxical decrease in the levels of oxLDL at 3 months. This finding was unexpected as in the general population GC use has been associated with an increase in oxLDL, which is thought to be mediated by indirect stimulation of eNOS and the release of NO (511). The paradoxical association of IV GCs with oxLDL in this study could be due to either a disease-specific phenomenon or a type I statistical error. Furthermore, the reduction in oxLDL did not translate into improvements in LDL function (lagtimes or uptake). The

combination of pro and anti-atherogenic changes in lipid structure produced by treatment with IV GCs, as well as limitations in the study design (e.g no CVD endpoints) make it impossible to quantify the relative contributions of steroid-induced lipid changes on CVD risk in RA. In addition, the clinical effects of IV GCs on both disease activity and lipid parameters appear short lived (approximately 3 months duration), thus in order to assess the impact of steroids on lipid associated CVD risk in RA further large scale studies of RA patients receiving oral prednisolone are required.

Patients in the rituximab arm were found to have shorter lag times than those in the anti-TNF therapy arm, both at baseline (prior to initiation of treatment) and throughout the treatment period (following adjustment for potential confounders). This finding may be explained by differences in: 1) underlying RA pathology e.g. anti-CCP status, 2) genetic factors 3) previous drug therapy e.g. in line with NICE guidance (512) most rituximab patients will have previously received and failed to respond to an anti-TNF drug (albeit 12 weeks prior to study entry). In addition, several other interesting associations were seen in the rituximab arm, including a positive association between logSdLDL and diIoxLDL uptake into U937 cells, which strengthened during the follow up period, and a persistent negative association (only significant at 3 months) between HDL and diIoxLDL uptake in to U937 cells at all time points. Thus, it appears that as levels of SdLDL increase/HDL decrease the amount of patient LDL taken up into U937 cells decreases (opposite to DiIoxLDL uptake). These findings are contradictory to published data (501;513) in the general population, where increased levels of sdLDL and decreases in HDL levels have been shown to be pro-atherogenic. One potential explanation could be that other modifications of these lipids could affect their function. For example, RA patients have previously been shown to have increased levels of pro-atherogenic HDL (514),thus lower levels of HDL would also confer lower levels of pro-atherogenic HDL, which in turn may prove to be protective against the uptake of LDL.

A number of 3 way interactions were observed between lipid parameters and LDL function (LDL lag times and DiIoxLDL uptake). However, the vast majority of these were due to fluctuations within the RA control arm. This finding can be explained by: 1) biological variation due to RA specific factors e.g. disease activity 2) insufficient study size. Although possible, a methological factor is unlikely to explain these observations as fluctuations in the RA control arm were found when assessing associations for both

functional assays (LDL lagtimes and DiIoxLDL uptake) and similar erratic fluctuations were not seen in the other study arms, in particular the HC arm. Interestingly, the comparison of the 3 intervention arms (anti-TNF vs IV GCs vs rituximab), the majority of 3 way interactions observed were due to changes in the rituximab arm. This finding would suggest a possible drug specific effect. However, the rituximab arm did include the least number of patients and therefore it is possible that some of these findings may be due to a lack of statistical power.

The present study has several strengths including its longitudinal design; the use of two control populations (RA controls on stable DMARD therapy and HCs) allowing examination of the natural course of lipid/lipoprotein changes; and systematic, detailed, prospective characterisation of subjects minimising missing data. However, although this is a large study in the context of RA and lipoprotein sub-fractions, it still has limited power, and does not assess the impact of changes in lipid parameters on hard CVD endpoints in RA. Due to the number of analyses performed the bonferroni correction was used to minimise the risk of reporting a type 1 error. However, it is worth noting that use of the bonferroni correction may actually result in an ‘over correction’, as some of the dependent variables are positively correlated and thus are not truly independent.

In summary, although suppression of inflammation ‘corrects’ many of the lipid changes observed in RA e.g. increases lipid levels, a ‘blanket’ approach to treatment may not be the most appropriate due to significant differences between the classes of drugs, with some producing an overall more pro-atherogenic lipid profile.

Chapter 6: Associations or genetic polymorphisms with lipid levels and lipid ratios in RA

6.1 Introduction

In this chapter I am going to address the hypothesis that RA patients are genetically predisposed to alterations in their lipid profile. Firstly, I will assess whether the genes known to predispose patients to RA (RA susceptibility genes) are important for the regulation of lipid metabolism. Secondly, I will study in patients with RA, the prevalence and associations of specific genetic polymorphisms occurring within genes known to play an important role in lipid metabolism in the general population (ApoE, ABCA1, CETP, LPL, Apoc3, A4, A5).

6.2 RA susceptibility genes

Despite a number of RA susceptibility genes having been identified (see chapter 1, section 1.2.1), their effects on CVD or CVD risk factors in RA remain relatively unknown. To date, a few studies have demonstrated an association between several of the RA susceptibility genes and CVD in RA (515;516), but none has assessed the effects of these genes on lipid levels (or indeed other classical CVD risk factors) in RA patients. There is now expanding evidence to suggest a potential genetic link between RA and lipid levels, with a number of studies demonstrating that abnormalities in the lipid profile may predate the onset of RA (122;123). For the purposes of this thesis, I examined the associations of four major RA susceptibility genes – STAT4, TRAF1/C5, PTPN22 and HLA DRB1-SE with the lipid profile.

6.2.1 Methods

The recruitment and baseline data collection of the 400 cross sectional RA patients are outlined in chapter 2, section 2.1.1. RA susceptibility genes were only genotyped in the RA population, as the prevalence in the healthy control population would be very low due to these genes being associated with disease

The Roche LightCycler 2.0 system (2007c) was used to identify the SNPs of STAT4 (rs7574865), TRAF1/C5 (rs3761847) and PTPN22 (rs2476601) using real-time

polymerase chain reaction (PCR) and melting curve analysis (further details are provided in chapter 2, section 2.10). These four susceptibility genes were chosen for analysis over the array of other RA susceptibility genes as they have been shown to contribute the most to the genetic susceptibility of RA (11). The risk alleles for each of the RA susceptibility genes were: STAT4 rs7574865 (T allele), TRAF1C5 rs3761847 (G allele), PTNP22 rs2476601 (T allele), and one or more copies of the HLADRB1-SE. Probes and primers used are described in detail in **appendix 2**, section 10.1.

The genotyping of the HLADRB1-SE (HLA-SE) was performed using reverse line assay sequence-specific oligonulceotide probes with Dynal RELI sequence specific oligonucleotide strip detection reagent kit (http://www.dynalbiotech.com/). Assay results were interpreted using the pattern matching program provided by Dynal (Invitrogen, Paisley, UK). The following alleles were classified as shared epitope positive: DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *0413, *0416, *1001 and *1402 (15).

Of the 400 RA patients, 394 patients were genotyped for STAT4, 397 for PTPN22, 387 for TRAF1C5 and 355 patients for the HLA-SE.

Statistical analysis

The Kolmogorov-Smirnov test was used to test the normality of each parameter. Comparisons were performed by ANOVA, Kruskal-Wallis, and Chi-square test for normally distributed, non-normally distributed and categorical variables, respectively. Values were expressed as mean ± standard deviation (SD), median (25^{th}-75^{th} percentile) or percentages, as appropriate. The independence of the associations of lipid parameters with the genotypes was established using a multivariate generalised linear model, whereas a linear regression model was used to establish associations between alleles. All multivariate models were adjusted for multiple comparisons. For all lipid-associated analyses, patients on lipid lowering therapy (statins/fibrates) were excluded. All genotype frequencies were found to be in Hardy-Weinberg equilibrium.

Power calculations

Table 6.1: The minimum change in lipid levels which could be detected with 80% power and 5% significance.

	TRAF1C5	PTPN22	HLA-DRB1-SE	STAT4
The difference in lipid levels detectable at 80% power with 5% significance				
TC (mmol/L)	0.245	1.367	0.059	0.234
LDL (mmol/L)	0.250	1.395	0.405	0.239
ApoA (g/L)	0.091	0.508	0.147	0.087
ApoB (g/L)	0.061	0.340	0.098	0.058
LDL:HDL ratio	0.204	1.137	0.330	0.194
The percentage difference in lipid levels detectable at 80% with 5% significance				
logTG (%)	9.6	66.7	15.9	9.1
logHDL (%)	6.2	38.4	9.9	5.7
logTC:HDL (%)	5.9	38.0	9.9	5.7
logApoA:ApoB (%)	7.6	51.4	12.7	7.4
TC: total cholesterol, LDL: low density lipoproteins, ApoA: apolipoproteinA, ApoB:apolipoproteinB, TG: triglycerides, HDL: high density lipoproteins				

6.2.2 Results

Baseline characteristics across the genotypes *(see* ***appendix 3****, tables 11.1 t0 11.4)*

No significant differences in age, gender, disease activity (DAS28, ESR, CRP) or disease severity (HAQ scores) were found between patients with the genotypes for PTPN22, STAT4 or TRAF1/C5. However, having one or more copies of the HLA DRB1-SE associated with significantly higher levels in CRP and ESR. In addition, there were some significant variations in RA characteristics: RF positivity ($p<0.001$) and anti-CCP positivity ($p<0.001$) were higher and disease duration longer ($p=0.021$) amongst RA patients with one or more copies of the HLA DRB1-SE compared to those with no copies of the HLA DRB1-SE; anti-CCP positivity was higher amongst RA patients either heterozygous or homozygous for the TRAF1/C5 G allele ($p=0.018$) compared to those homozygous for the A allele.

Association of RA susceptibility genes with lipid levels

Two out of the four RA susceptibility genes (HLA DRB1–SE, and TRAF1/C5) examined were found to associate with individual lipid levels, but not with NCEP-defined dyslpidaemia (following, in all cases, adjustment for potential confounders including age, gender, CRP, medications and other significant associations identified in the univariate analysis specifically for each gene (see **appendix 3**, tables 1 to 4). Although associations were observed across the three genotypes, the associations were much stronger when comparing the allelic effects (e.g. AA versus G allele (GA or GG)). Comparisons of lipid levels across the three genotypes in an unadjusted general linear model demonstrated a significant association between TRAF1/C5 and TC levels, with patients homozygous (GG) (B= 0.351, 95% CI: -0.702 to 0.001, p=0.050) and heterozygous (AG) (B= -0.349, 95% CI: -0.634 to -0.064, p=0.017) for the minor allele having lower levels of TC compared to those harbouring the AA genotype. These associations remained following adjustment for potential confounders (GG vs AA: B= -0.418, 95% CI: -0.765 to -0.072, p= 0.018, AG vs AA: B= -0.357, 95% CI: -0.633 to -0.081, p=0.012). The comparison of allelic effects using a linear regression model strengthened these associations, and demonstrated a number of other significant associations between lipid parameters and RA susceptibility genes (see **table 6.2**). In both the unadjusted and adjusted linear regression models, patients with one or more copies of the HLA DRB1-SE had significantly lower levels of ApoA than patients with no copies of the HLA DRB1 SE (unadjusted: B= -0.139, 95% CI: -0.262 to -0.017, p=0.026) , Adjusted: B= -0.145, 95% CI: -0.284 to –0.006, p=0.041). Patients heterozygous or homozygous for the G allele of TRAF1/C5 had significantly lower TC levels (Unadjusted: B= -0.350, 95% CI: -0.617 to -0.082, p=0.011, Adjusted: B= -0.338 (-0.609 to –0.068, p=0.014), LDL levels (Unadjusted: B= -0.328, 95% CI: -0.605 to -0.051, Adjusted: B= -0.359, 95% CI: -0.650 to –0.069, p=0.016) and ApoB levels (Unadjusted: B= -0.080, 95% CI: -0.150 to -0.011, p= 0.023, Adjusted: B= -0.110, 95% CI: -0.182 to –0.038, p=0.003) than patients homozygous for the A allele.

Associations of RA susceptibility genes with cardiovascular outcomes

In a binary regression model (unadjusted and adjusted for confounders), no associations between any of the RA susceptibility gene SNPs and a history of either CVD or deaths occurring from CVD were observed.

Table 6.2: Multivariate analyses of RA susceptibility genes and lipid parameters

	B (95% Confidence Interval)	P value
STAT4	***T allele (TT and TG) versus GG***	
TC	0.094 (-0.158 to 0.346)	0.465
logHDL	-0.008 (-0.034 to 0.018)	0.551
LDL	0.015 (-0.256 to 0.287)	0.911
logTG	0.005 (-0.040 to 0.050)	0.816
ApoA	-0.034 (-0.137 to 0.068)	0.512
ApoB	0.015 (-0.053 to 0.083)	0.664
TRAF1C5	***G allele (GA/GG) versus AA***	
TC	-0.338 (-0.609 to –0.068	**0.014**
logHDL	-0.002 (-0.031 to 0.027)	0.913
LDL	-0.359 (-0.650 to –0.069)	**0.016**
logTG	-0.047 (-0.095 to 0.003)	0.060
ApoA	-0.110 (-0.221 to 0.001)	0.056
ApoB	-0.110 (-0.182 to –0.038	**0.003**
HLA DRB1–SE	**No copies vs one or two copies SE**	
TC	0.146 (-0.196 to 0.488)	0.401
logHDL	-0.023 (-0.059 to 0.013)	0.209
LDL	0.100 (0.268 to 0.469)	0.592
ApoA	-0.145 (-0.284 to –0.006)	**0.041**
ApoB	0.045 (-0.046 to 0.136)	0.330
PTPN22	***A allele (AA/AG) versus GG***	
TC	0.026 (-0.234 to 0.286)	0.844
logHDL	-0.019 (-0.047 to 0.010)	0.197
LDL	0.096 (-0.187 to 0.379)	0.504
logTG	0.040 (-0.007 to 0.086)	0.095
ApoA	0.013 (-0.094 to 0.120)	0.817
ApoB	0.026 (-0.044 to 0.097)	0.465

☐ = negative association
STAT4 adjusted for age, gender, CRP, medications, hypertension and IR
TRAF1C5 adjusted for age, gender, CRP, medications, anti-CCP positivity and anti-hypertensive use
HLADRB1-SE adjusted for age, gender, CRP, medications, anti-CCP positivity and disease duration.
PTPN22 adjusted for age, gender, CRP, medications
Abbreviations: Lp(a): lipoprotein (a), TC: total cholesterol, LDL: low density lipoproteins, HDL: high density lipoproteins, ApoB: apolipoprotein B, ApoA: Apolipoprotein A, HLA DRB1-SE: human leukocyte antigen – shared epitope, SE: shared epitope, anti-CCP: anti-cyclic citrullinated peptide, CRP: C-reactive protein

6.2.3 Discussion

These results suggest a genetic link between RA and lipid parameters independent of inflammation and other RA-specific factors. The potential importance of these findings is far reaching, both for our overall understanding of lipid metabolism and CVD in RA, but also for the identification and prevention of CVD in individual RA patients.

Patients harbouring the AA genotype for TRAF1/C5 or one or more copies of the SE appear to be at most lipid-associated risk of CVD, with these genotypes associating with pro-atherogenic changes in the lipid profile e.g. the HLA DRB1-SE associated with increased ApoB:ApoA and TC:HDL ratios. Thus the identification of patients harbouring these genetic polymorphisms may aid screening and aggressive management of lipid associated CVD risk in RA.

Two papers have reported that changes in the lipid profile occur many years prior to the onset of RA (122;123). The first study, demonstrated that blood donors who later developed RA (n=79) had significantly higher levels of TC, TGs, and ApoB, but lower HDL levels than matched controls (n=1071) (123). The second study demonstrated that TC and LDL levels were significantly lower during the 5 years prior to the onset of RA in a large population-based incident cohort (577 RA patients and 540 non-RA controls) (122). The changes observed in the lipid profile prior to the onset of RA could be the result of either sub-clinical inflammation, genetic predisposition or a range of other unknown factors. Interestingly, the study by Van Halm et al (122) attempted to assess whether inflammatory parameters could account for the magnitude of lipid changes observed. However, they demonstrated that only a very small percentage of the difference in lipid levels between RA patients and controls could be explained by changes in CRP e.g. only 3.6% of the difference in HDL levels between the groups could be explained by CRP concentrations. A further population-based, prospective, nested case-control study failed to demonstrate any difference in lipid levels (TC,HDL, LDL, TG) between patients who developed inflammatory polyarthritis and controls (517). The results of this study may differ from the results of the previous two studies due to differences in the populations studied (e.g. RA versus inflammatory polyarthritis), size of the population studied and differences in frequency of genetic polymorphisms e.g. susceptibility genes.

To date there are limited data available on the wider CVD effects of RA susceptibility genes. Current evidence would suggest that patients with two copies of the HLA DRB1-SE (particularly the HLA-DRB1*01/04 combination) have increased all cause and CVD mortality (452;515). In addition, the HLA-DRB1*0404 allele is associated with decreased endothelium-dependent vasodilatation (518). Although several studies have failed to demonstrate an association between polymorphisms of TRAF1/C5 with CVD mortality in RA (453;519), a further susceptibility variant at the CCL21 locus has been shown to associate with CVD mortality in patients with inflammatory polyarthritis (516). Within the present study, I did not observe any associations between the susceptibility genes and either co-morbid CVD or deaths occurring from CVD. However, due to power limitations and study design I am unable to draw firm conclusions as to the role of RA susceptibility genes on CVD morbidity and mortality. Further specifically designed studies are required to examine this further.

Despite these advances, the pathological mechanisms that may link susceptibility genes with CVD in RA remain poorly understood. Perhaps the most obvious mechanism is mediation through an inflammatory pathway, as certain RA susceptibility genes (especially HLA DRB1-SE) associate with more severe, erosive disease (520;521). However, it is possible that RA susceptibility genes could mediate/partially mediate their effects on CVD in RA through both direct and indirect effects on traditional CVD risk factors. This is the first study to have specifically assessed whether RA susceptibility genes associate with one of the key traditional CVD risk factors (dyslipidaemia/lipid parameters). The observation that two out of the four RA susceptibility genes examined associate with individual lipid levels is interesting. However, the diversity of genetic effects on the lipid profile e.g HLA-DRB1-SE only affects ApoA levels, whilst TRAF1C5 affects TC, LDL and ApoB levels, would suggest that each susceptibility gene acts independently through specific mechanisms rather than through generic effects on the inflammatory process. This thought is supported by the observation that adjustment for inflammation (CRP) in the multivariate models did not influence the strength of association between each of the susceptibility genes and lipid levels. Factors other than inflammation may be important, for example enzymes involved in lipid metabolism. A recent study by Palmino-Morales et al (522) failed to demonstrate an association of PTPN22, STAT4 and TRAF1C5 polymorphisms with cardiovascular risk in RA. Although an important study, it is not without limitation, with the authors failing to

consider the major susceptibility gene, HLA DRB1-SE. It is clear that to fully establish whether RA susceptibility genes are important determinants of CVD in RA, much larger studies with hard end points are required.

The extensive characterisation of a large cross-sectional RA population has enabled me to perform an in depth study of the associations of the RA susceptibility genes, something not always feasible with large genome wide association studies. However, the study has limitations. Firstly, the cross sectional design precludes firm conclusions on the directionality or causality of the associations observed. Secondly, the sample size provided just enough power for most of the differences found. Thirdly, the absence of a normal control group precludes any conclusions about the potential association of these genes with lipid levels in the general population. Unfortunately analysis of the Welcome Trust Case Consortium did not offer a means of confirming our findings or overcoming some of the above limitations, due to the lack of stored clinically relevant data e.g. lipid levels and inflammatory markers (523). In addition, despite adjustment for inflammation in the multivariate analyses, a direct link between lipid levels and the susceptibility genes cannot be assumed.

In summary, I have demonstrated significant associations between several RA susceptibility genes and lipid parameters in patients with RA. These findings may have important implications for both the screening for and management of CVD risk in such patients. Further large-scale studies are required to confirm these findings and establish the underlying mechanisms, both in RA and in the general population.

6.3 Lipid metabolism genes

In the general population, a number of genes have been identified that are fundamental to the regulation of lipid metabolism, including Apolipoprotein E, ABCA1, CETP taq1B, LPL, and the Apolipoprotein C3,A4,A5 gene cluster. To date no data exist regarding the prevalence or effect of these genes on lipid parameters in RA patients.

6.3.1 General methods

The recruitment and baseline assessments of 400 cross-sectional RA patients and 400 healthy controls are described in the methods section (see chapter 2, section 2.1.1).

The Roche LightCycler 2.0 system (2007c) was used to identify the SNPs (ABCA1: rs2230808, rs2066715, rs33918808, rs2066718, rs2066714, CETPtaq1b: rs708272, LPL: rs268, ApoC3, A4, A5 gene cluster: ApoC3 : rs2854116, ApoA4: rs675, ApoA5: rs3135506, ApoE: rs7412 and rs429358) using real time PCR and melting curve analysis (further details are described in chapter 2, section 2.10). The probes and primers for each of these, along with further details of their melting curves etc are given in details in **appendix 2**. These SNPs were chosen due to their effects on lipid parameters and CVD risk.

Statistical methods

See RA susceptibility genes methods section (section 6.2.1)

6.3.2 Specific methods for Apo E

Two ApoE SNPs were identified simultaneously (rs7412 and rs429358) using the Roche LightCycler® 480 System and a Roche ApoE mutation detection kit (cat no.03004716001). By combining the melting curve analysis from the genotyping of codon 112 and codon 158 the allelic set-up of the analysed samples was determined. This was dependant on the amino acids encoded for e.g. E2 (cysteine at 112/cysteine at 158), E3 (cysteine 112, arginine 158), E4 (arginine at 112 and arginine and 158) (see **table 6.3**)

Table 6.3: The assignment of genotypes following the combination of the melting curves from rs7412 (ApoE2) and rs429358 (ApoE4)

Genotype of codon 112	Genotype of codon 158	Allelic set up
TGC/TGC	TGC/TGC	E2/E2
TGC/TGC	CGC/CGC	E3/E3
CGC/CGC	CGC/CGC	E4/E4
TGC/TGC	CGC/TGC	E2/E3
CGC/TGC	CGC/TGC	E2/E4
CGC/TGC	CGC/CGC	E3/E4

When analysing the association of ApoE genotypes with lipid levels the E2E4 genotype was excluded due to its potential to exert mixed phenotypic effects relating to the E2 and E4 allele.

Power calculations:

Table 6.4: The range of minor allele frequency in the RA population that could be detected as being significantly different (p<0.05) from the general population with 80% power.

	Assumed prevalence of the minor allele in general population	**RA prevalence that would be significant at p<0.05 and power >80%**
ABCA		
rs33918808	3.3 %	< 0.4% or > 8.2%
rs2066718	1.3%	> 4.9%
rs2230808	20%	< 12.4% or > 28.8%
rs2066715	9%	< 3.8% or > 15.8%
rs2066714	13.6	<8.2 % or > 19.0%
CETP taq1B		
rs708272	41.3%	< 31.5% or > 51.5%
LPL		
rs268	1.7%	> 5.7%
Apo C3, A4, A5 cluster		
rs2854116	37.5%	< 27.9% or > 47.6%
rs675	20%	< 12.4% or > 28.8%
rs3135506	5.8%	< 1.7% or > 11.7%
ApoE		
rs429358	14.9%	< 8.3% or > 22.9%
rs7412	8%	< 3.2% or > 14.5%
ABCA1: ATP binding cassette transporter, CETP: cholesterol ester transfer protein, Apo: apolipoprotein, LPL: lipoprotein lipase		

6.3.3 Results

Genotypic and allelic frequencies of lipid metabolism genes amongst RA and HC

The genotypic and allelic frequencies of each of the polymorphisms known to regulate lipid metabolism in the general population are outlined in **tables 6.5 and 6.6**, respectively. All genotypic frequencies were in Hardy-Weinberg Equilibrium. The genotypic frequencies of the CETP taq1B polymorphism were found to differ between RA and HCs in both the unadjusted and adjusted (age and gender) binary regression model. Following adjustment, the B1B1 and B1B2 genotypes were significantly lower in the RA population (B= 0.636, 95% CI: 0.417 to 0.968, p=0.035 and B= 0.619, 95% CI: 0.423 to 0.906, p=0.014, respectively) compared to HC. Although the allelic frequencies did not differ statistically, a trend was noted (p=0.067) for RA patients to have a lower frequency of the B1 allele. In addition, genotypic frequencies of the ApoC3 polymorphism were significantly different between RA and HC (p=0.037 see **table 6.5**). In a binary regression model, adjusted for age and gender, RA patients were less likely to harbour the CT genotype compared to HC (OR= 0.718, 95% CI: 0.513 to 0.992, p=0.044). The allelic frequencies of the ApoC3 polymorphism were also significantly different between RA and HCs, with the C allele being less frequent in patients with RA (see **table 6.6**)

Table 6.5: comparison of genotype frequencies in patients with RA and healthy controls

	Genotypes						P value
ABCA1							
rs2230808 n(%)	**GG**		**AG**		**AA**		
HC	235 (55.6)		150 (35.5)		38 (9.0)		0.320
RA	222 (57.1)		143 (36.8)		24 (6.2)		
rs2066715 n(%)	**CC**		**CT**		**TT**		
HC	386 (89.1)		43 (9.9)		4 (0.9)		0.163
RA	337(85.5)		55 (14.0)		2 (0.5)		
rs33918808 n(%)	**GG**		**GC**		**CC**		
HC	409 (93.6)		28 (6.4)		0 (0)		0.057
RA	383 (96.5)		14 (3.5)		0 (0)		
rs2066718 n(%)	**GG**		**GA**		**AA**		
HC	412 (94.5)		23 (5.3)		1 (0.2)		0.304
RA	381 (96.5)		14 (3.5)		0 (0)		
rs2066714 n(%)	**AA**		**AG**		**GG**		
HC	327 (74.5)		105 (23.9)		7 (1.6)		0.559
RA	303 (76.3)		85 (21.4)		9 (2.3)		
CETP							
rs708272 n(%)	**B1B1**		**B1B2**		**B2B2**		
HC	127 (53.8)		221 (55.8)		92 (44.9)		**0.036**
RA	109 (46.2)		175 (44.2)		113 (55.1)		
LPL							
rs268 n(%)	**AA**		**AG**		**GG**		
HC	424 (96.8)		14 (3.2)		0 (0)		0.949
RA	384 (96.7)		13 (3.3)		0 (0)		
Apo,C3,A4,A5							
rs2854116 n(%)	**CC**		**CT**		**TT**		
HC	77 (17.8)		192 (44.3)		163 (37.6)		**0.037**
RA	60 (15.2)		150 (38.1)		184 (46.7)		
rs675 n(%)	**AA**		**AT**		**TT**		
HC	20 (4.6)		135 (31.3)		276 (64.0)		0.492
RA	13 (3.3)		118 (29.7)		266 (67.0)		
rs3135506 n(%)	**CC**		**CG**		**GG**		
HC	1 (0.2)		50 (11.9)		368 (87.6)		0.501
RA	3 (0.8)		42 (10.7)		348 (88.5)		
Apo E (rs7412 and rs429358)							
n (%)	**E2E2**	**E2E3**	**E2E4**	**E3E3**	**E3E4**	**E4E4**	
HC	5 (1.2)	48 (11.4)	6 (1.4)	262 (62.4)	87 (20.7)	12 (2.9)	
RA	3 (0.8)	46 (11.9)	7 (1.8)	235 (60.7)	88 (22.7)	8 (2.1)	0.908

RA: Rheumatoid arthritis, HC: healthy controls, ABCA1: ATP- binding cassette transporter, CETP: cholesterol ester transport protein, LPL: lipoprotein lipase, Apo C3, A4, A5: apolipoprotein C3, A4, A5, ApoE: apolipoprotein E

Table 6.6 Allelic frequencies in patients with RA and healthy controls

Polymorphism	Alleles			P value
ABCA1				
rs2230808 n(%)	**G allele**	**A allele**		
RA	587 (75.4)	191 (24.6)		0.319
HC	620 (73.3)	226 (26.7)		
rs2066715 n (%)	**C allele**	**T allele**		
RA	729 (92.5)	59 (7.5)		0.139
HC	815 (94.1)	51 (5.9)		
rs33918808 n (%)	**G allele**	**C allele**		
RA	780 (98.2)	14 (1.8)		0.061
HC	846 (96.8)	28 (3.2)		
rs2066718 n (%)	**G allele**	**A allele**		
RA	776 (98.2)	14 (1.8)		0.141
HC	847 (97.1)	25 (2.9)		
rs2066714 n (%)	**A allele**	**G allele**		
RA	691 (87.0)	103 (13.0)		0.727
HC	759 (86.4)	119 (13.6)		
CETP				
rs708272 n (%)	**B1 allele**	**B2 allele**		
RA	393 (49.5)	401 (50.5)		0.067
HC	475 (54.0)	405 (46.0)		
LPL				
rs268 n (%)	**A allele**	**G allele**		
RA	781 (98.4)	13 (1.6)		0.950
HC	862 (98.4)	14 (1.6)		
ApoC3,A4, A5				
rs2854116 n (%)	**C allele**	**T allele**		
RA	270 (34.3)	518 (65.7)		**0.015**
HC	346 (40.0)	518 (60.0)		
rs675 n (%)	**A allele**	**T allele**		
RA	144 (18.1)	650 (81.9)		0.264
HC	175 (20.3)	687 (79.7)		
rs3135506 n (%)	**C allele**	**G allele**		
RA	48 (6.1)	738 (93.9)		0.934
HC	52 (6.2)	786 (93.8)		
ApoE (rs7412 and rs429358)				
n (%)	**E2**	**E3**	**E4**	
RA	59 (7.6)	604 (78.0)	111 (14.4)	0.894
HC	64 (7.6)	659 (78.5)	117 (13.9)	

RA: Rheumatoid arthritis, HC: healthy controls, ABCA1: ATP- binding cassette transporter, CETP: cholesterol ester transport protein, LPL: lipoprotein lipase, Apo C3, A4, A5: apolipoprotein C3, A4, A5, ApoE: apolipoprotein E

Associations of SNPs occurring in the ApoE, ABCA1, CETP taq1B, LPL and apolipoprotein A1,C3,A4,A5 gene cluster with the lipid profile in patients with RA

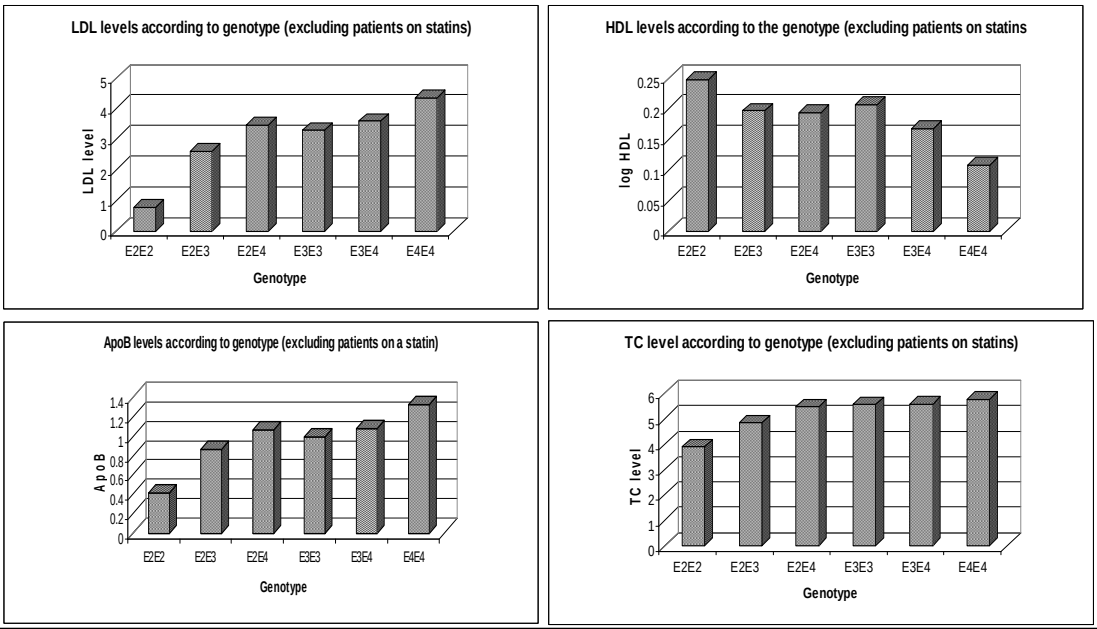
The associations of each of the SNPs studied with the lipid profile in RA are described below and summarised in **table 6.8.** Further tables demonstrating univariate differences in demographics, disease characteristics and lipid parameters across the genotypes for each genetic polymorphism studied are attached in **appendix 3**, tables 5 to 15. For all analyses a standardised set of potential confounders was adjusted for, including age, gender, BMI, smoking status, rheumatoid factor, CRP, methotrexate, sulphasalazine, HCQ, anti-TNF or prednisolone use.

ApoE rs7412 and rs429358:

The differences in demographics and clinical characteristics across the ApoE genotypes are summarised in **Appendix 3**, table 15.

Differences in lipid levels according to the ApoE allele present are shown in **Figure 6.1**.

Figure 6.1: Lipid levels across the ApoE genotypes in RA patients



LDL: low-density lipoproteins, HDL: high density lipoproteins, TC: total cholesterol, ApoB: apolipoprotein B.

In a general linear model, TC (B -0.77, 95% CI: -1.13 to -0.41, p<0.001), LDL (B= -0.76, 95% CI: -1.13 to -0.39, p<0.001) and ApoB (B= -0.15, 95% CI: -0.25 to -0.06, p=0.004) levels were lower in patients with the E2 allele (E2E3, E2E2) compared to those with the parent E3 allele (E3E3). ApoB (B= 0.10, 95% CI: 0.02 to 0.18, p=0.011) and LDL (B= 0.36, 95% CI: 0.05 to 0.67, p=0.022) levels were significantly higher in patients with the E4 compared to E3 allele (p=0.034), whilst logHDL (B= -0.04, 95% CI: -0.08 to -0.01, p=0.012) was significantly lower in patients with the E4 compared to the E3 allele. These associations remained following adjustment for potential confounders including age, gender, smoking status, BMI, rheumatoid factor positivity, methotrexate, sulphasalazine, HCQ, anti-TNF and prednisolone use. (See **table 6.7**).

In an unadjusted binary regression analysis, NCEP defined dyslipidaemia twice as common in patients with the E4 allele (E3E4, E4E4) (OR= 1.97, 95% CI: 1.11 to 3.49, p=0.021) and significantly less common in patients with the E2 (E2E2, E2,E3) allele (OR=0.231, 95% CI: 0.101 to 0.529, p=0.001) compared to those with the E3 (E3E3) allele. This association remained following adjustment for potential confounders (see **table 6.7**).

In a binary regression model (excluding patients on lipid lowering therapy), the prevalence of a past history of CVD did not differ across the genotypes in either the unadjusted (E2 vs E3: OR= 1.13, 95% CI: 0.40 to 3.21, p=0.816, E4 vs E3: OR= 0.98, 95% CI: 0.39 to 2.45, 0.977) or adjusted (E2 vs E3: OR= 1.14, 95% CI: 0.36 to 3.6, p=0.821, E4 vs E3: OR= 1.74, 95% CI: 0.63 to 4.78, p=0.286) models. However, if high-risk patients (those receiving lipid-lowering therapy) were included in the analysis, a trend was observed in the unadjusted model with patients with the E2 allele less likely to have a history of CVD and those with the E4 allele more likely to have a history of CVD. Following adjustment for potential confounders, patients with the E4 allele were significantly more likely to have a history of CVD (E4 vs E3: OR=1.93, 95% CI: 1.04 to 3.59, p=0.039). Although, CVD did not significantly differ between the E2 and E3 alleles, there was a trend for patients with the E2 allele to be less likely to have a history of CVD (See **table 6.7**).

Table 6.7: Associations of ApoE genotypes with lipids, CVD and NCEP dyslipidaemia in patients with RA

	E2 (E2E2, E2E3)		E4 (E3E4, E4E4)	
	B (95% CI)	**P value**	**B (95% CI)**	**P value**
TC				
Unadjusted	-0.77 (-1.13 to -0.41)	**<0.001**	0.03 (-0.27 to 0.33)	0.853
Adjusted	-0.69 (-1.07 to -0.33)	**<0.001**	0.05 (-0.25 to 0.35)	0.737
LDL				
Unadjusted	-0.76 (-1.13 to -0.39)	**<0.001**	0.36 (0.05 to 0.67)	**0.022**
Adjusted	-0.73 (-1.13 to -0.034)	**<0.001**	0.42 (0.10 to 0.74)	**0.009**
logHDL				
Unadjusted	-0.01 (-0.05 to 0.03)	0.723	-0.04 (-0.08 to -0.01)	**0.012**
Adjusted	0.01 (-0.03 to 0.05)	0.708	-0.05 (-0.08 to -0.02)	**0.001**
logTG				
Unadjusted	0.01 (-0.06 to 0.07)	0.839	0.05 (-0.010 to 0.10)	0.081
Adjusted	0.01 (-0.06 to 0.08)	0.747	0.05 (-0.03 to 0.11)	0.062
ApoA				
Unadjusted	0.02 (-0.13 to 0.17)	0.774	-0.08 (-0.20 to 0.05)	0.218
Adjusted	0.04 (-0.11 to 0.19)	0.576	-0.09 (-0.21 to 0.03)	0.138
ApoB				
Unadjusted	-0.15 (-0.25 to -0.06)	**0.001**	0.10 (0.02 to 0.18)	**0.011**
Adjusted	-0.17 (-0.26 to -0.07)	**0.001**	0.11 (0.03 to 0.19)	**0.009**
	Odds ratio (95% CI)	**P value**	**Odds ratio (95% CI)**	**P value**
Hx CVD				
Unadjusted	0.76 (0.33 to 1.73)	0.513	1.52 (0.88 to 2.63)	0.130
Adjusted	0.59 (0.23 to 1.54)	0.284	1.93 (1.04 to 3.59)	**0.039**
NCEP dyslipid				
Unadjusted	0.30 (0.15 to 0.59)	**<0.001**	1.83 (1.09 to 3.05)	**0.020**
Adjusted	0.21 (0.09 to 0.46)	**<0.001**	1.86 (1.06 to 3.28)	**0.031**

□ = positive association, □ = negative association. Adjusted for age, gender, body mass index, smoking status, rheumatoid factor positivity, CRP, methotrexate, sulphasalazine, hydroxychloroquine, prednisolone, anti-tumour necrosis factor therapy. All analyses (except NCEP dyslipidaemia, and Hx CVD) excluded patients on lipid lowering therapy. Abbreviations: TC: total cholesterol, HDL: high density lipoproteins, LDL: low density lipoproteins, TG: triglycerides, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Hx CVD: history of cardiovascular disease, NCEP dyslipid: national cholesterol education programme defined dyslipidaemia, CI: confidence interval.

ABCA1:

The differences in demographics and clinical characteristics across each of the genotypes for each of the polymorphisms studied are summarised in **Appendix 3**, tables 5 to 14.

rs2230808: Lipid levels did not differ across the genotypes in either the unadjusted or adjusted general linear model. Allelic analysis using a linear regression model also didn't demonstrate any significant associations between lipid levels and the minor allele (A) in either the unadjusted or adjusted models. In binary regression analysis no differences in the prevalence of NCEP defined dyslipidaemia, prevalent CVD or death from CVD were observed across the genotypes in either the unadjusted or adjusted models.

rs2066715: No significant differences in lipid levels (TC, HDL, LDL, TG, ApoA, ApoB), the prevalence of NCEP defined dyslipidaemia, the presence of CVD or the number of CVD deaths were observed across the genotypes or alleles in the unadjusted or adjusted models.

rs33918808: No significant differences were observed in the levels of lipid parameters (TC, HDL, LDL, TG, ApoA, ApoB) across the genotypes in either the unadjusted or adjusted analysis. However, in the unadjusted binary regression model, there was a trend for RA patients with the GG to have a lower prevalence of past CVD than those with the GC genotype. (OR=0.273, 95% CI: 0.072 to 1.035, p=0.056) (associations between GG and CC could not be tested for as no patients had the CC genotype). This association became significant following adjustment for potential confounders (OR= 0.232, 95% CI: 0.064 to 0.836, p=0.025). No significant differences were observed in the prevalence of NCEP defined dyslipidaemia or deaths relating to CVD across the genotypes in either the unadjusted or adjusted binary regression model.

rs2066718: No significant differences were observed across the genotypes/alleles in any of the lipid levels (TC, HDL, LDL, TG, ApoA, ApoB), the presence of NCEP defined dyslipidaemia, the presence of CVD, or the number of CVD deaths.

rs2066714: RA patients homozygous for the minor allele (GG) had higher levels of ApoB (B= 0.361, 95% CI: 0.129 to 0.594, p=0.002) and TC (B= 1.336, 95% CI: 0.436 to 2.236, p=0.004) model compared to patients harbouring the AA genotype in the

unadjusted general linear. These associations remained following adjustment for potential confounders (ApoB: B= 0.381, 95% CI: 0.144 to 0.618, p=0.002 and TC: B=1.240, 95% CI: 0.376 to 2.104, p=0.005). There were no significant allelic effects in a linear regression model adjusted for potential confounders . No differences in the prevalence of NCEP defined dyslipidaemia, CVD or CVD deaths were observed across the genotypes.

CETP taq1B (rs708272):

In an unadjusted general linear model, patients with the B1B1 genotype had significantly lower levels of HDL compared to those with the B2B2 genotype (B= -0.038, 95% CI: -0.074 to -0.001, p=0.043. This association strengthened following adjustment for potential confounders (B= -0.152, 95% CI: -0.279 to -0.025, p=0.020). Other components of the lipid profile (TC, LDL, TG, ApoA and ApoB) did not differ across the genotypes following adjustment for potential confounders. Amongst the RA cohort, no association was found between the genotypes and the prevalence of NCEP dyslipidaemia, prevalent CVD or deaths from CVD.

LPL (rs268):

No significant differences in lipid levels, the prevalence of CVD or deaths from CVD were observed across the genotypes.

ApoC3 (rs2854116): ApoA levels were significantly lower amongst patients with the TT genotype compared to CC or CT genotypes (1.57 g/L (SD 0.41 g/L) vs 1.74 g/L (SD 0.52 g/L) and 1.71 g/L (SD 0.44 g/L) respectively, p=0.013). Following adjustments for potential confounders ApoA levels remained significantly lower amongst patients with the TT genotype compared to those with the CT genotype (TT vs CT: B= -0.140, 95% CI: -0.245 to -0.034, p=0.010), but no significant differences were found in ApoA levels between the TT and CC genotypes. In univariate analysis, deaths from CVD differed across the genotypes (p=0.043) (see **appendix 3**, table 12), but this association was lost following adjustment for potential confounders. There were no other significant differences in lipid levels, NCEP defined dyslipidaemia, or the prevalence of CVD across the genotypes.

ApoA4 (rs675): RA patients with the TT genotype had lower levels of ApoA compared to those with the AA or AT genotype (1.59 g/L (SD 0.41 g/L) vs 1.71 g/L (SD 0.65 g/L) and 1.75 g/L (SD 0.49 g/L), respectively, p=0.013). However, this association was lost following adjustment for potential confounders. No significant differences were observed in the prevalence of NCEP defined dyslipidaemia, prevalent CVD or CVD deaths across the genotypes.

ApoA5(rs3135506): TG levels were higher amongst patients with the CG genotype compared to the CC and GG genotypes (1.4 mmol/L (IQR: 1.1-2.1 mmol/L) vs 0.8 mmol/L (IQR: 0.6-0.9 mmol/L) and 1.1 mmol/L (IQR: 0.9-1.55 mmol/L) respectively, p=0.016) and this association remained following adjustment for potential confounders (B=0.084, 95% CI: 0.012 to 0.155, p=0.022). No significant differences were observed in the prevalence of NCEP defined dyslipidaemia, CVD or CVD related death across the genotypes.

The associations of each polymorphism with lipid levels and CVD prevalence are summarised in **table 6.8**.

Table 6.8: A summary of the associations between polymorphisms of lipid metabolism genes and the lipid profile/ CVD prevalence in RA (following adjustment for potential confounders).

	logHDL	LDL	logTG	TC	ApoB	ApoA	Hx CVD	NCEP dyslipid
ABCA1								
rs2230808								
rs2066715								
rs33918808							↓	
rs2066718								
rs2066714				↑	↑			
CETP								
rs708272	↓							
LPL								
Rs268								
ApoC3A4A5								
rs2854116						↓		
rs675								
rs3135506								
ApoE								
E2		↓		↓	↓			↓
E4	↓	↑			↑		↑	↑

= proatherogenic changes, = antiatherogenic changes

HDL: high density lipoproteins, LDL: low density lipoproteins, TG: trigylcerides, TC: total cholesterol, ApoB: apolipoprotein B, ApoA: apolipoprotein A, Hx CVD: history of cardiovascular disease, NCEP dyslipid: national cholesterol education programme defined dyslipidaemia.

6.4 Discussion

In the second section of this chapter, I have demonstrated that the prevalence of most (with the exception of CETP taq1B and ApoC3) genetic polymorphisms occurring within lipid metabolism genes is similar in RA patients and HCs. In addition, I have demonstrated that the phenotypic effects of these genetic polymorphisms appear to mirror those seen in the general population. Thus, at a first glance it would appear that most of the genetic polymorphisms considered in the second part of this chapter are unlikely to contribute significantly to the pattern of dyslipidaemia specifically observed in RA. However this may not be the case, as limitations in study design (e.g. power) and the collective ability of these and other genetic polymorphisms (not studied) to pool their effects, may be sufficient to contribute to dyslipidaemia in RA: this cannot be accurately investigated within the boundaries of this thesis. Thus although these findings are useful, further large scale specifically designed genetic studies in RA are required to address this fully.

One of the most interesting findings in this chapter was the difference in CETP taq1B genotype frequencies between RA patients and HCs. RA patients had a lower prevalence of the B1B1 genotype and a higher prevalence of the B2B2 compared to HCs. This difference in prevalence may be explained by either an increase in the prevalence of the B2B2 amongst the RA population or a decrease in the HC population. However, existing evidence would appear support the former as the worldwide prevalence of the B2 allele has been reported to be 42% (423), which is similar to the allelic prevalence observed in the HCs (46%). Despite this data being reassuring, such populations cannot be used as direct comparator to establish whether the HCs used in this thesis are a 'true' representation of the general population, due to differences in geographical location etc. Thus further large scale genetic studies are required to confirm or refute these findings.

In the general population, the B2B2 genotype has been associated with higher HDL levels (420), and some studies claim it is protective against CVD (421;422) whilst others claim that it associates with a paradoxical rise in CVD risk (524). In this chapter, I demonstrate similar phenotypic effects of the CETP1taq1b genotypes amongst the cross-sectional RA population with the B2B2 genotype associating with the highest HDL levels and B1B1 the lowest HDL levels. The B2B2 genotype was found to be more

prevalent in RA, thus suggesting a beneficial rather than detrimental role of this genetic polymorphism on lipid metabolism in RA. However, as the B2B2 genotype may confer a paradoxical rise in CVD risk despite the increase in HDL levels, it is still plausible that higher prevalence of the B2B2 genotype seen within this cross-sectional RA population may contribute to the CVD risk observed in RA. Interestingly, I did not find a significant association of the CETPtaq1B genotypes and CVD. This is likely to be a reflection of the power of the study rather than the actual absence of an association, as only 86 patients (21.5%) had a history of CVD.

ApoE genotypes are strongly linked to lipid levels in RA. The effects of the genotypes are again similar to those seen in the general population, with the E2 allele associating with low levels of TC and LDL and the E4 allele associating with high levels of TC and LDL. Interestingly, one study has reported that the effects of ApoE on regression of atherosclerosis occur independently of changes in lipid levels (525). Irrespective of this, RA patients harbouring the E4 allele are twice as likely to have dyslipidaemia than those with the E3 allele, thus potentially increasing their CVD risk. In the general population, it is well described that the E4 allele increases CVD risk (526); whether the E2 allele is 'protective' is less clear (427). ApoE genotypes did not significantly associate with prevalent CVD in this RA cohort, probably due to insufficient power, but a trend was observed ($p=0.074$). It is still feasible that polymorphisms of the ApoE gene contribute to atherosclerotic plaque formation in RA, through inflammation-mediated suppression of gene transcription. At the level of the atherosclerotic plaque, ApoE is primarily produced by macrophages and it exerts anti-atherogenic properties by facilitating reverse cholesterol transport. Interestingly, a recent study using lipopolysaccharide (LPS) treatment to mimic inflammatory stress demonstrated that LPS represses ApoE gene expression in macrophages through its effects on inflammatory signalling pathways (527). Such mechanisms are likely to be escalated in RA as a consequence of high levels of systemic inflammation, and this should be addressed in specifically designed studies. The results in the second part of this chapter demonstrate that some genetic polymorphisms known to regulate lipid metabolism in the general population are more common in RA and are important in the regulation of lipid metabolism in RA patients. It is possible that these genes may have an impact on CVD risk in RA. However, much larger studies are required to establish the precise role of these genes and others (not studied in this thesis) on dyslipidaemia and CVD risk in RA.

Chapter 7: Discussion

7.1 General discussion

Much of the work presented in this thesis has already been discussed in the individual results chapters (see chapters 3, 4, 5 and 6). The aim of this chapter is to bring all of these findings together in order to highlight the overall conclusions of the thesis, the impact these findings confer and how they may lead on to future research.

The work described in this thesis has expanded our understanding of dyslipidaemia in RA. Dyslipidaemia has been shown to be highly prevalent in RA, but undertreated amongst patients deemed to be at a high risk of developing CVD. In addition, virtually all lipid parameters have been shown to be altered in RA, including lipid levels, lipid structure and lipid function. These changes have been shown to be governed by multiple interacting factors including systemic inflammation, anti-inflammatory drug therapy, and genetic factors.

This study has highlighted deficiencies in the methods available for quantifying CVD risk in RA. Although the EULAR task force have recommended that conventional risk algorithms (e.g. FRS) are adjusted by a 1.5 multiplier, to account for the excess CVD risk in RA, this remains a far from perfect method for risk quantification. Firstly, there are vast discrepancies in the proportion of patients identified as being ‘at risk of CVD’ between the conventional cardiovascular risk algorithms and this is further exaggerated when the 1.5 multiplier is applied. Secondly, this approach has never been validated in an RA population. Ultimately, we should work towards the development of an RA specific and RA validated CVD risk algorithm. The work presented in this thesis would also support an algorithm based on lipid ratios rather than individual lipid levels, as this may represent a more accurate reflection of CVD risk at any given time point, due to lipid ratios being relatively less affected by inflammatory fluctuations.

Prior to starting the work described in this thesis certain aspects of dyslipidaemia (e.g. alterations in lipid levels) in RA were already well recognised and reported (see chapter 1, section 1.10.4) (120;128;148). These lipid changes were thought to be mediated primarily through fluctuations in the inflammatory burden (128;129). I have demonstrated that lipids are widely affected in terms of their overall plasma levels, size,

structure and function, by the processes occurring in RA (see chapter 5). Whilst the work described in this thesis would support the theory that inflammation is fundamental to many of these lipid changes, I have also demonstrated that other factors including individual drug therapies and genetic factors also contribute. Based on these findings, our approach to the management of dyslipidaemia in RA may require a more targeted approach rather than just a blanket suppression of inflammation.

Summary of key findings:

1) Dyslipidaemia is highly prevalent in RA but undertreated amongst those most at risk of developing CVD.
2) Lipid levels, structure and function are altered in RA, giving rise to an overall proatherogenic lipid profile.
3) Inflammation is fundamental to many of the lipid changes observed in RA.
4) Anti-inflammatory drug therapy produces alterations in lipid parameters both through a 'blanket suppression of inflammation but also through drug specific mechanisms.
5) Genetic factors are important in the regulation of lipid metabolism in RA.
    a) The presence of certain RA susceptibility genes (HLA DRB1-SE, TRAF1C5) associate with alterations in the lipid profile
    b) There is an increased prevalence of several genetic polymorphisms occurring within the lipid metabolism genes

7.2 Novel findings

One of the most novel findings reported here is the association of genetic polymorphisms occurring between two of the RA susceptibility genes (HLA DRB1-SE and TRAF1C5) and lipid levels (see chapter 6, section 13.2). This finding may help to explain why lipid parameters are altered years before the onset of RA (122;123) and offers a potential mechanism by which RA susceptibility genes may also contribute to CVD risk in RA (515;516). As this is the first time these associations have been described it is important that the findings are replicated in other populations, and subsequent work is carried out to establish the mechanisms underlying these findings. In addition, I have demonstrated for the first time that the prevalence of some genetic polymorphisms occurring in the lipid metabolism genes (e.g. CETP taq1B) may differ between RA patients and HC (see

Chapter 6, section13.3). These findings are important as they may not only contribute to lipid abnormalities in RA, but they may also offer a link to CVD in RA both through direct (non lipid associated) and indirect (lipid associated) pathways.

Another novel finding relates to LDL function (see Chapter 5). Despite LDL function having been assessed in one previous study in RA (276), the findings presented in this thesis are unique, due to differences in study design and the hypotheses being addressed. In addition, LDL function has been addressed here using two complementary methods (LDL lagtimes and DiIoxLDL uptake into U937 cells). In this study, I demonstrated that LDL function is altered in RA and may be influenced by changes in CRP levels and also a number of other lipid parameters. To the best of my knowledge, LDL lagtimes have not been previously studied in RA. LDL lag times have been found to be significantly shorter amongst the general population with coronary artery disease (528) and have been shown to be modified by other disease processes e.g. glycaemic status (529). Fatty acids with three or more double bonds have been identified as one of the most important predictors of LDL lag times, with an inverse association being observed (530). However, in the general population there is also evidence suggesting an atheroprotective role of fatty acid (e.g.omega 3) supplementation (531). The effects of omega-3 fatty acids on pain and disease activity in RA has been studied previously, with some studies demonstrating a possible mild beneficial effect (532). As fatty acids are often considered to be 'harmless' and possibly even beneficial, physicians and patients may be tempted to use them as an adjunctive therapy. However, in RA they may in fact contribute to CVD via their effects on LDL oxidisability. Further research is required in RA to assess the overall risks:benefit ratio of taking omega 3 fatty acid supplementation.

7.3 Implications for changes in treatment or interventions

In Chapter 3 I highlighted several deficiencies in the current management of dyslipidaemia and CVD risk in RA. In the future, rheumatologists need to adopt a more systematic approach to screening for CVD risk in RA e.g annual review clinics, and ensure that all CVD risk factors are optimally managed amongst those patients identified as being at an increased risk, e.g. prescribing a statin. The use of statins for the primary prevention of CVD in RA is currently being investigated in a multi-centre trial (533). The results of this trial may help to further guide our treatment of CVD in RA. One of

the most striking conclusions arising from this thesis was the need for an 'RA specific CVD risk algorithm'. Although two of the current CVD calculators attempt to account for RA or CRP (QRISK2 (470) and Reynolds risk score (481), respectively), neither was produced or validated in an RA population. Thus current guidelines suggest we adjust conventional CVD risk calculators (e.g. FRS) for the excess CVD risk observed in RA (x1.5) (478), but this approach is far from ideal.

The work presented in this thesis has highlighted several drug specific effects on the lipid profile. In the cross sectional study (see chapter 4), HCQ use was associated with 'improvements' in the lipid profile, whilst in both the cross sectional and longitudinal study (see chapter 5) steroid use (oral prednisolone or IV GCs) associated with a more pro-atherogenic lipid/lipoprotein subfraction profile. Thus, based on these findings (and previous supporting published work (371;492)), HCQ should be prescribed more widely and steroids with more caution amongst RA patients deemed to be at risk of CVD.

7.4 Strengths and weaknesses

The strengths and weaknesses of the study populations used and relevant methodology have been discussed in detail in the individual result chapters. However, as a whole the work described in the thesis has a number of other strengths and weaknesses. One of the major strengths of this thesis is the targeted and structured approach adopted in order to address a range of hypotheses. A further strength is the systematic data collection and processing. As lipid levels are known to fluctuate as a result of multiple environmental factors e.g. time of day, dietary intake, all fasting blood samples were collected early in the morning. All blood samples were spun within an hour of collection and stored at-80^{o}C, to minimise the effects of storage on lipid parameters (especially LDL oxidation/nitration). All laboratory assays used validated commercial kits. Data processing and recording of data were audited to ensure no errors and a hospital statistician reviewed all statistical analyses. Despite the systematic approach to data collection and processing, due to reasons beyond my control, there was a degree of missing data. However, this was dealt with in a consistent manner in order to not introduce bias.

As part of the methodology, a number of complementary study populations were utilised, allowing associations to be established and their directionality to be assessed. Although this approach was the most appropriate to address the hypotheses posed in this thesis, it does confer a number of weaknesses. Despite all attempts being made to recruit control populations that were well matched according to age and sex, to that of the RA patients, unfortunately the longitudinal healthy control arm was significantly younger than the longitudinal RA patients. As age is known to strongly influence lipid metabolism and CVD risk, attempts were made to compensate for this discrepancy via statistical adjustment. A further weakness of the study populations was the limited data available on the anonymised genetic biobank of healthy controls, thus limiting the analysis I could carry out. Patient selection and thus 'confounding by indication' may have also introduced bias as RA patients were not randomised to the longitudinal treatment arms. However, a randomised controlled trial to overcome this was beyond the scope of this thesis. Furthermore, no data was collected on alcohol consumption and menopausal status, and therefore I was unable to adjust for the effects of these factors on lipid metabolism. In addition, for the longitudinal study, the sample size and duration of follow up may have been a significant limitation. A larger sample size may have confirmed further associations (not found to be significant in the current work due to a lack of power) and minimised the background 'noise' caused by gross fluctuations in lipid parameters in the RA control arm. A longer follow-up period would have allowed us to confirm whether many of the lipid changes are indeed transient as the work in this thesis would suggest.

7.5 Implications for future research

This thesis has allowed me to systematically answer many questions relating to dyslipidaemia in RA - but in the process it has generated many more. Some hypotheses have been generated as a direct extension of the work presented in this thesis, whereas others branch out in a new direction. Since starting this thesis much has changed in the field of rheumatology from new diagnostic criteria (7;492) to the availability of new anti-inflammatory drugs e.g Golimumab, Certolizumab, Tocilizumab, Abatacept. The development of new drugs opens new research avenues. In line with the work carried out in this thesis, it would be interesting to extend the longitudinal cohort to include some of these newer drug therapies (particularly those with a different mode of action) to examine

their effects on lipid levels, structure and function. Tocilizumab, an IL-6 antagonist, may be particularly interesting to examine in the context of dyslipidaemia and CVD risk. Current data arising from clinical trials (492;534) has demonstrated profound effects of treatment with tociluzimab on lipid levels, which has largely been attributed to rapid suppression of CRP levels. To date, no data has been published on the effects of tociluzimab on other lipid parameters such as structure or function and the implications these may have on CVD risk in RA. I also think that, in light of the pro-atherogenic effects IV GC administration had on the lipid levels and structure in this thesis, further work is required to assess the impact of long-term oral prednisolone use on lipid metabolism and CVD. For all drugs, both new and old, further research is required to establish their effects on all CVD risk factors e.g. hypertension and obesity, as well as CVD outcomes.

RA is a complex condition with a spectrum of disease, and therefore it is reasonable to hypothesize that lipids and other CVD risk factors may be affected differently amongst different subgroups of patients. For example, it would be interesting to look at whether lipids are also modified by citrullination, by comparing lipid parameters in patients who are positive for anti-CCP antibodies to a group of patients who are anti-CCP negative.

The CVD effects of genetic polymorphisms occurring within the RA susceptibility genes require further examination. Do RA susceptibility genes associate with other CVD risk factors e.g. hypertension? Do RA susceptibility genes affect vascular function? What are the individual/collective effects of other RA susceptibility genes (not studied in this thesis) on both dyslipidaemia and other CVD risk factors?

Based on the findings in this thesis, the next step would be to refine and expand the longitudinal arm of the study. Alongside the expansion of existing drug therapy/control arms, additional arms should be included to examine the drug effects of some of the newer biologic therapies e.g. tocilizumab, and oral prednisolone. Expansion of the study, would allow a number of other lipid parameters to be investigated including changes in enzymes fundamental to lipid metabolism, functional changes in HDL, and other lipid modifications such as glycation or citrullination. Although in the context of this study it would be difficult to look at hard CVD endpoints, surrogate measures of vascular function e.g. flow mediated dilatation, and laser Doppler could be measured.

In conclusion, the work described in this thesis has significantly broadened and added to our understanding of dyslipidaemia in RA, demonstrating both the scale and complexity of the problem. However, it also highlights several deficiencies in our under understanding which need to be explored through future research.

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Appendix 1: Laboratory methods

Biochemistry tests

The majority of biochemical tests including, TC, glucose, calcium, phosphate, urea, creatitine, sodium, potassium, alkaline phosphatase, alanine transferase and albumin were measured using dry slides in a Vitros® 5,1 FS chemistry system (www.orthoclinical.com). A small amount of serum/plasma obtained from the patient was placed on to a slide containing a multi-layered analytical element coated on a polyester support. Each biochemical test required the use of a specific microslide, with appropriate imbedded reagents. The sample permeates through the layers of the slide until it reaches the reagent layer containing either a dye or a chemical with which it reacts. Reactions that occur in the reagent layer produce methods of quantifying the biochemical parameter of interest e.g. TC via the detection of specific wavelengths using reflective spectophotometry. For example, a dye within the reagent layer can bind to the biochemical parameter of interest that requires measurement. Bound dye can be distinguished from free dye due to a change in reflective wavelength. The concenetration of the biochemical parameter being measured is then established using reflective spectrophotometry. The serum/plasma concentration of an indivual biochemical parameter is equal to the concentration of bound dye detected. Although the measurement of vurtually all biochemical parameters are based on relective spectophotometry, slight variations of the method exist in order to accommodate the different chemical properties of each substance. An example of this would be measuring enzymes such as ALT. Such measurements are not reliant on the incorporation of dye, and are actually measured according the wavelength of the reaction product. For ALT, the rate of oxidation of NADH is measured according to a change in the reflection density. See below:

$$\text{Alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{ALT P-5-P}} \text{pyruvate} + \text{glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{lactate} + NAD^{+}$$

Sodium and potassium concentrations are also measured using a slightly modified method. This method requires the use of multilayers electrode slides containing two ion-selective electrodes. A small amount of the patients sample is added to one side of the

slide and a small amount of Vitros reference fluid to the other side. Both fluids then migrate towards the center of the paper bridge, producing a stable liquid junction which connects the reference electrode to the sample indicator electrode. Each electrode produces an electrical potential in response to the activity of the ions applied to it and the potential difference between the electrodes measured. The potential difference is proportional to the concentration of ions being measured.

ApoB, ApoA, HDL and CRP were measured using dual chamber kits on the Vitros® chemistry system. The wet chemistry system requires the sample to be mixed with the reagents in a cuvet. Changes in the samples opacity/turbidity are then measured using reflective spectrophotometry. For apoA and ApoB the addition of the specific reagent leads to an immunochemical reaction yielding antigen and antibody complexes. The presence of these complexes results in an increased turbidity of the sample. Sample turbidity is then measured spectrophotometrically. The concentration of ApoA is derived from this, as it is directly proportional to level of turbidity.
HDL and CRP were also processed as wet sample on the Vitros ® chemistry system using a wako kit (418-72495) and (419-22016) produced by alpha laboratories.

Insulin was measured using a kit (PIL5KIN-7) and will be analysed using an immulite 2500 analyser. The method is based on a competitive chemiluminescent enzyme immunoassay. Antibody coated polystyrene beads was dispensed into reaction tubes. The samples were added to the reaction tubes and incubated with an alkaline phosphatase labelled reagent. The beads were then be separated from the sample by centrifugation. The beads were washed to remove residual unbound phosphatase label. Dioxetane substrate was then be added to react with the phosphatase label bound to the bead. This reaction emitted light which was quantified using a photo multiplier tube. The amount of emitted light is proportional to the amount of insulin in the sample.

Thyroid stimulating hormone (TSH) was measured using a two-site immunoenzymometric assay (AIA-PACK TSH 3rd-Gen), and was analysed using a Tosoh AIA 1800. TSH present in the sample is bound with monoclonal antibody immobilised on magnetic beads and monoclonal antibody conjugated with bovine alkaline phosphatase in the AIA-PACK. The magnetic beads are washed to remove unbound enzyme-labelled monoclonal antibody and are then incubated with a fourogenic

substrate. The amount of enzyme conjugated with the monoclonal antibody that binds to the beads is proportional to the TSH concentration in the sample.

Serum Angiotensin Converting Enzyme (ACE*)*: A kit (KK-ACKX) produced by Buhlmann laboratories was used. The method is reliant on ACE in the specimen mediating the cleavage of a synthetic substrate (hippuric acid attached to a dipeptide moiety) to produce and amino-acid derivative and a dipeptide. The kinetics of this reaction were then measured spectrophometrically. One unit of enzyme activity is defined as the amount of enzyme required to release one μmol of hippuric acid per minute of serum at 37^{o}C.

Serum Iron, Total iron binding capacity (TIBC) and complement (C3 and C4) was measured using immunoturbidimetry methods. This involved the use of Labmedics assay kits and a ThermoTrace method kit, which were later be processed on a KoneLab™30 analyser. In order to measure serum iron it needs to be released from its carrier protein. This process is induced through the addition of a guanidine buffer to the serum sample. Ascorbic acid is then used to reduce ferric ion to its ferrous state. The ferrous iron then can bind to ferene S, producing a blue colour, which is then read spectophotometrically. Serum TIBC was calculated by adding the amount of serum iron to the amount of serum unsaturated iron binding capacity (UIBC). UIBC is measured adding a known excess of ferrous ions to the sample. These will bind to unsaturated sites on transferrin, and any remaining ions were then measured using the ferrozine reaction. This reaction creates a purple complex that can be measured spectophotometrically at 560nm. The difference between the amount of unbound iron and the total amount added to the sample is equivalent to the quantity bound to transferrin, the UIBC. The measurement of complement levels is based on immunoprecipitation enhanced by polyetheylene glycol at 340nm. Specific anti-serum will be added in excess to buffered samples. The increase in immunoprecipation will then be recorded when the reaction has reached its end-point. The change in absorbance is proportional to the amount of C3 or C4 in solution.

Serum amyloid A (SAA) was measured using an ELISA method, using a kit (EL10015) that is produced by Abazyme, LLC. This method is identical to that previously described for the dectection of oxidised LDL, except the plate is pre-coated with monoclonal

antibody that is specific for SAA. Thus, when the samples are added to the wells SAA in the sample will bind and become immobilised by the antibody pre-costed on the wells.

Homocysteine levels were measured using an IMMULITE 2500 solid phase, competitive chemiluminescent enzyme immunoassay. Serum from patient/subjects was incubated with S-adenosyl-L-homocysteine hydrolase (SAH) and dithiothreitol for 30 mins, resulting in the release of bound homocysteine, before transferring to a second reaction tube conataining a SAH-coated polystyrene bead and an alkaline phosphatase-labelled antibody specific for SAH.

Haematological methods

Full blood counts was analysed on the ADOVA® 120 Haematology system, produced by Bayer Healthcare

Vitamin B12, folate and ferritin

Vitamin B12, folate and ferritin were all analysed using an chemi-illuminescence method, which was carried out on a ACS 180 analyser, produced by Bayer healthcare.

ESR was measured on a starrsed compact from mechatronics

INR was measured by quantifying optical density based on clot formation. This was performed on a IL ACL Futura Advance system.

Appendix 2: Probes, primers and melting curves for each genetic polymorphism

Probes and primers for each genetic polymorphism

STAT4 rs7574865:

The sensor probe is specific for the G allele

Forward primer: 5′-TACGGATGTCTTTGAAGGTAG-3′

Reverse primer: 5′-CTTTATAATTTCTTTCT-3′

Sensor probe: 5′-AGATAACCACTATTCACATTTT-3′-FLU

Anchor probe: 5′-LCRED640-CCAACTTTTCATACTTTTACTGCATACACAC-PH.

TRAF1C5 rs3761847:

The sensor probe is specific for the T allele

Forward primer: 5′ -ACTCCCTTTTAACTGTGTACCCCATA-3′

Reverse primer: 5′-GCTTAGCCTCTCTGTGCCTCAG-3′

Sensor probe: 5′-TCTCCCCTCCAGCCTCAA-3′-FLU

Anchor probe: 5′-LCRED640-ACCACCCTCTCTCTACCTGCTCATTCCCA-PH.

PTPN22 ***rs2476601***:

The sensor probe is specific for the A allele

Forward primer: 5′ -GCCTCAATGAACTCCTCAAAC-3′

Reverse primer: 5′-CTGATAATGTTGCTTCAACGGA-3′

Sensor probe 5′-CAGGTGTCCATACAGGAAGTG-3′-FLU

Anchor probe: 5′-LCRED640-GGGGATTTCATCATCTATCCTTGGAGCAGTTG-PH.

ABCA1 rs2230808:

The sensor probe is specific for the C allele

Forward primer: 5’-CTCTTTTCTGTTGTGAATGC-3’

Reverse primer: 5’ –AACAGTCACAACTGAGC-3’

Sensor probe – 5’-CTTGACATTATTTCTGGTGTCCAG—FL

Anchor probe: 5’-LC640-CCTGTCATAAATCTTCCCAAGCTGTTG--PH

ABCA1 rs2066715:

The sensor probe is specific for the C allele

Forward primer: 5'-GGAAAGACAGCCTCAATGTA-3'

Reverse primer: 5' –TTTCTCACAGAGCCTGCT-3'

Sensor – 5'-TCATGGAGACCGAAGTGGTG--FL

Anchor probe: 5'-LC640-GATTGAAGCCATCTTCCTCCACAGGA--PH

ABCA1 rs33918808:

The sensor probe is specific for the C allele

Forward primer: 5'-GCTTTTTCCTTTAGTTCTCACACAA-3'

Reverse primer: 5' –GGGGAAGCTCAGGCACCA-3'

Sensor probe – 5'-CGACCATGACAGTGACACGCT—FL

Anchor probe: 5'-LC640-ACCATCGGTAAGGACTCTGGGGTTT—P

ABCA1 rs2066718:

The sensor probe is specific for the A allele

Forward primer: 5'-TGCATGAAATGCTTCCAGGTATT-3'

Reverse primer: 5' –AGTGCTTGAAGTTTCTCCAGTGA-3'

Sensor probe – 5'-TGGCCTACCAAAGGAGAAACTG—FL

Anchor probe : 5'-LC640-CTGCAGCAGAGCGAGTACTTCGTTCCAAC—PH

ABCA1 rs2066714:

The sensor probe specific for G allele

Forward primer: 5'-GAATTCCCAGGCCCTGGTA-3'

Reverse primer: 5'-GTTAGCAGAGGCAGCAGCACTAG-3'

Sensor Probe: 5'-CAACCAGAAGAGAATGTCAGAAAGT-FL

Anchor Probe: 5'-LC640-GTGCTGTTGACCTCCTGCTCTTTCTT-PH

CETP (Taq1B) rs708272 :

The sensor probe is specific for the C allele.

Forward primer: 5'-TCTTTTCATGGACACCCACTATG-3'

Reverse primer: 5' –CCCCAACACCAAATATACACCA-3'

Sensor – 5'-AACCCTAACTCGAACCCTAGTGATTCT—FL

Anchor probe: 5'-LC640-TCGCAGACAAACACAAATCCCTATACCTGG-PH

ApoC3 rs2854116:

The sensor probe is specific for the C allele with additional T mismatch

Forward primer: 5’-CTGGGTGAGCAGCACTCG-3’

Reverse primer: 5’ –GGTGAGGGGCTTCTTCAGACT-3’

Sensor – 5’-CTTTACTCCAAACACCCCCCA--FL

Sensor + mismatch – 5’-CTTTACTCCAAACACCTCCCA--FL

Anchor probe: 5’-LC640-CCCAAGCCACCCACTTGTTCTCAAGT—PH

ApoA4 rs675:

The sensor probe is specific for the A allele

Forward primer: 5’-AACAGCTCAGGCAGAAACTG-3’

Reverse primer: 5’ –CTGCTGCTGTTCCTGCTGTT-3’

Sensor – 5’-GAGAGCCAGGACAAGTCTCTC--FL

Anchor probe: 5’-LC640-CCCTCCCTGAGCTGGAGCAACA—PH

ApoA5 rs3135506:

The sensor probe is specific for the C allele

Forward primer: 5’-CAGCAGAGGCAGGTCATC-3’

Reverse primer: 5’ –TTCTTTCAGGTGGGTCTCCGAC-3’

Sensor – 5’-GTGGCCCAAAACGCTGTGG--FL

Anchor probe: 5’-LC640-AGGGACTAGGTAATCAGGGCCTGGCT—PH

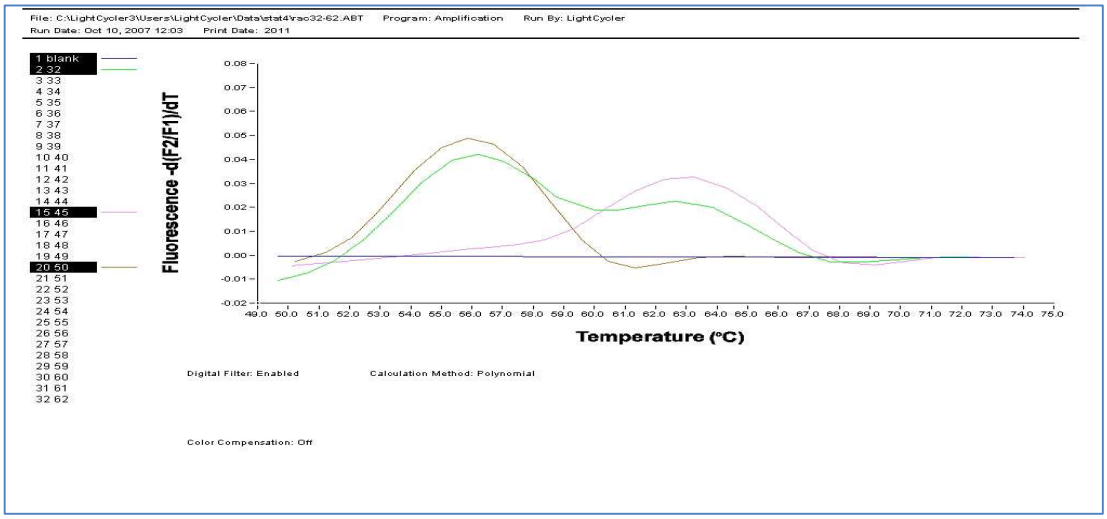
LPL rs268:

A simple probe kit was used (tibmolbiol)

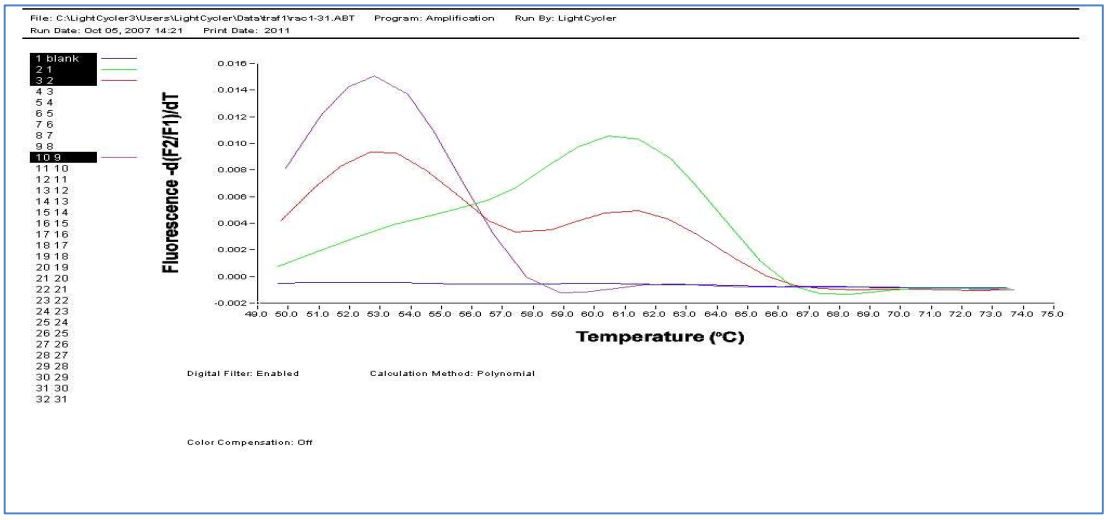
Melting curves for each genetic polymorphism

The melting curves for each genetic polymorphism are shown below. The sensor probe produces the peak at the highest temperature (red peak on graph at highest temp) as it is able to fully hybridise to the anchor probe. In contrast a mismatch in the region of the SNP would mean the sensor probe would not fully hybridise and would therefore produce a melting curve peak at a lower temperature (red peak on graph at lower temp). Heterozygozotes would therefore have two melting peaks (shown on the graphs below in green). Further details explaining the formation of melting curves and their interpretation can be found in chapter 2, section 2.9.

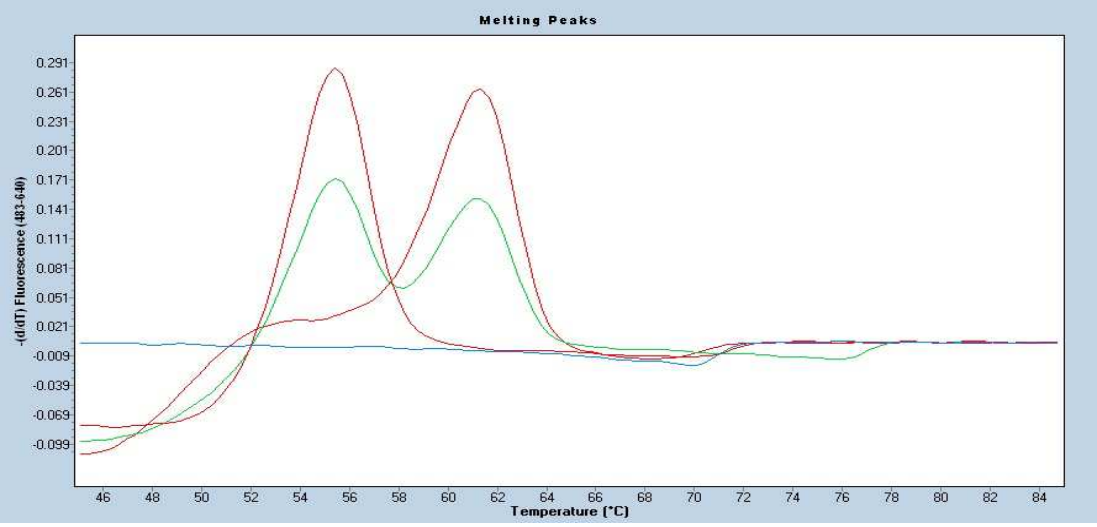
STAT4 rs7574865:



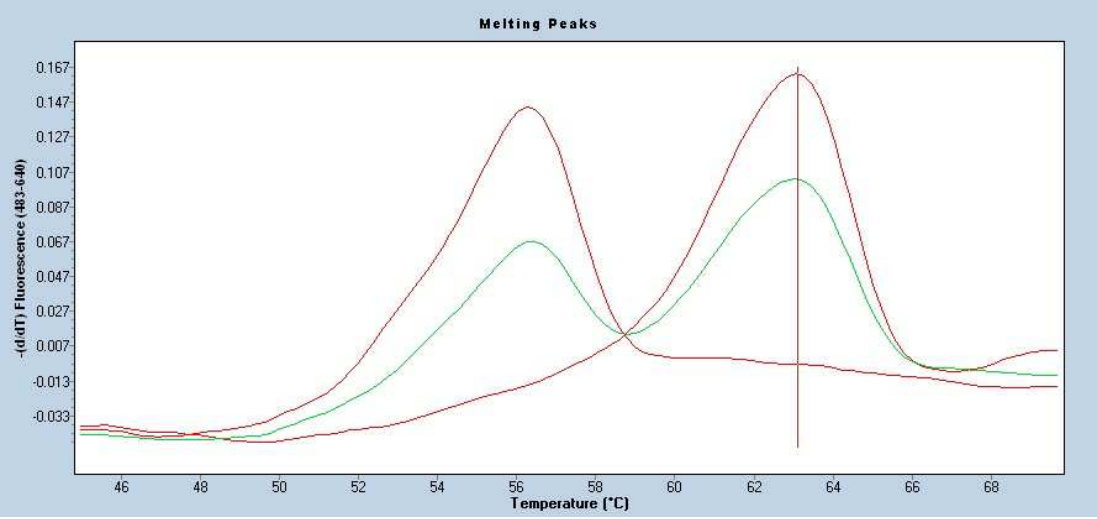
TRAF1C5 rs3761847:



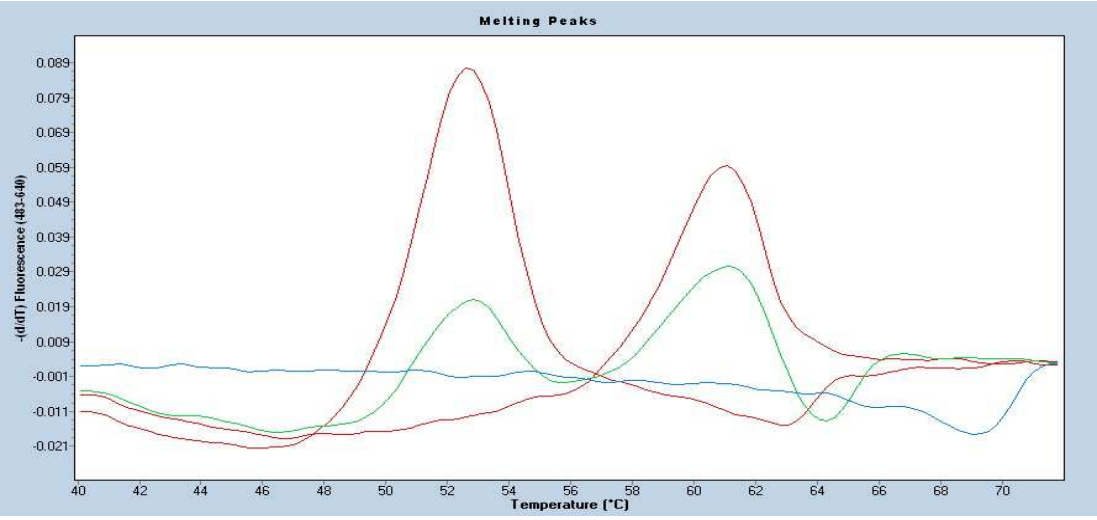
PTPN22 rs2476601:



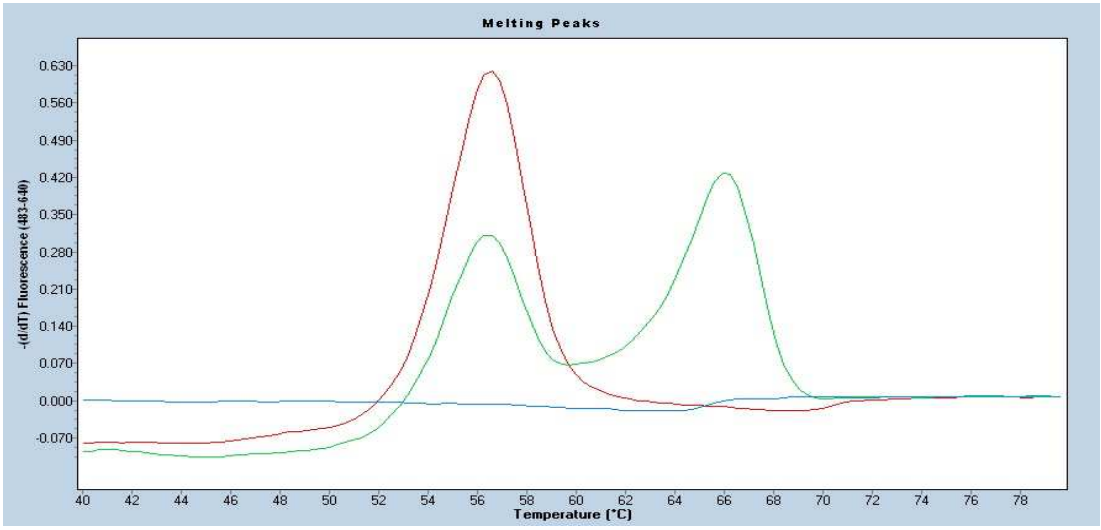
ABCA1 rs2230808:



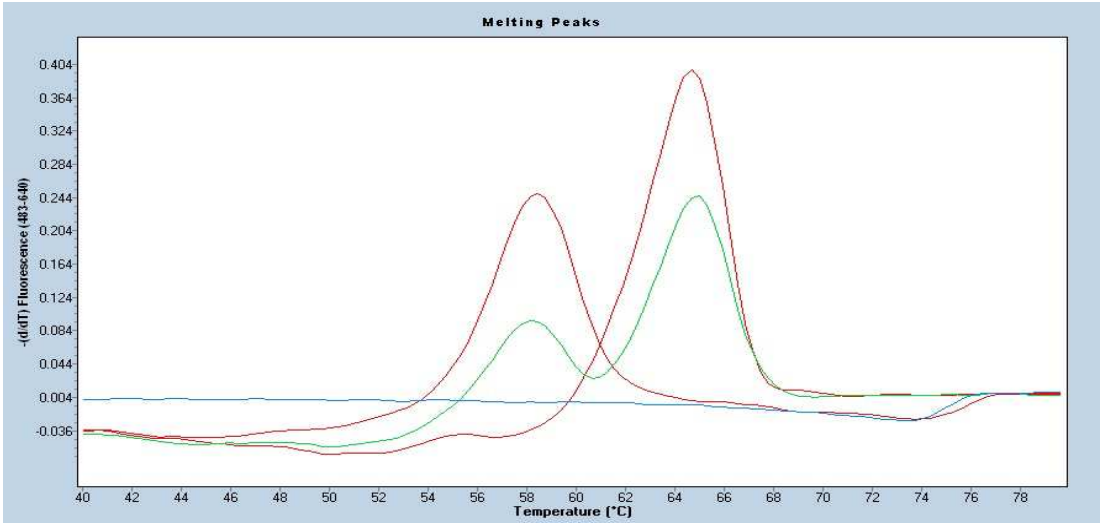
ABCA1 2066715:



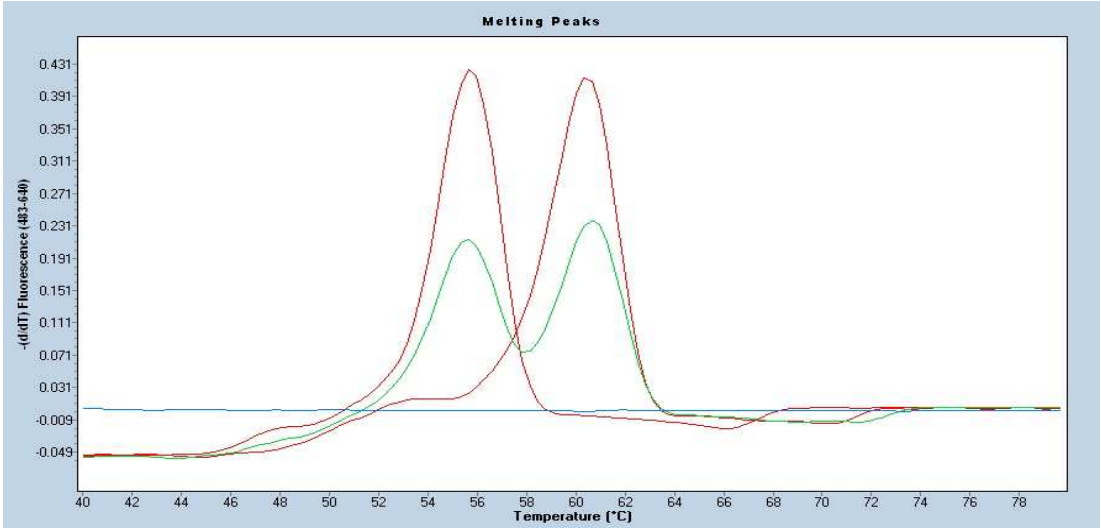
ABCA1 rs33918808:



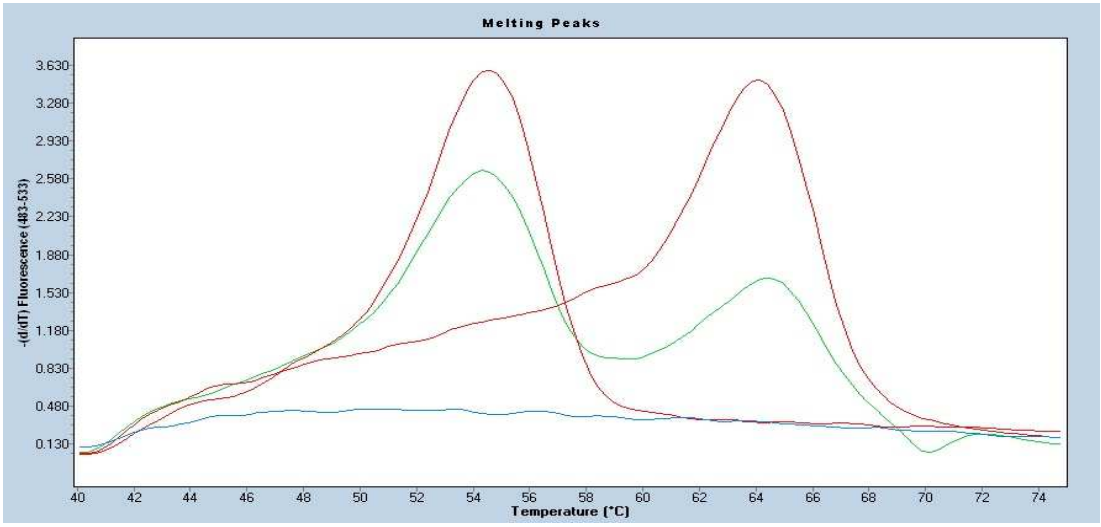
ABCA1 rs2066718:



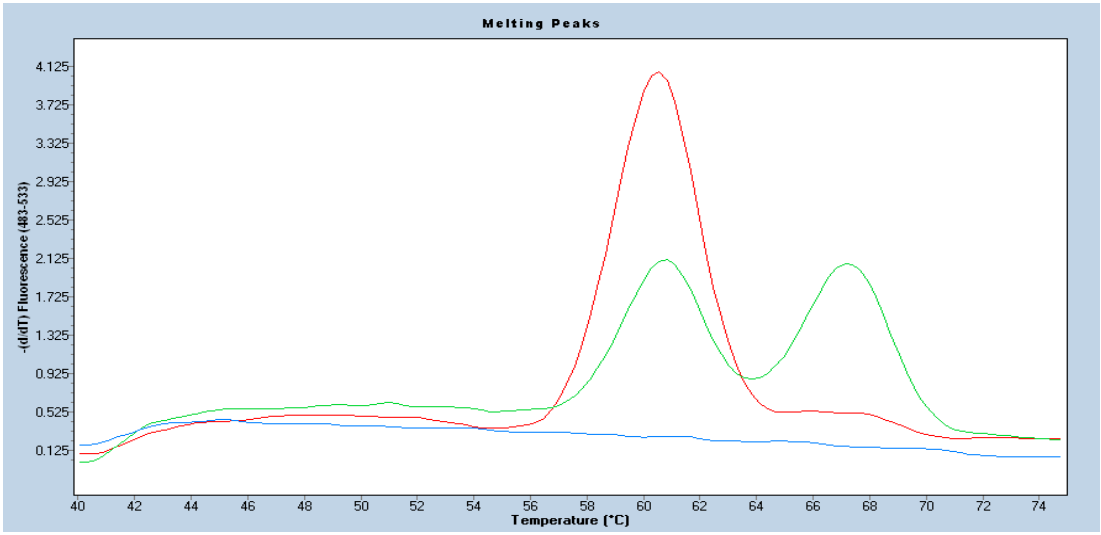
ABCA1 rs2066714:



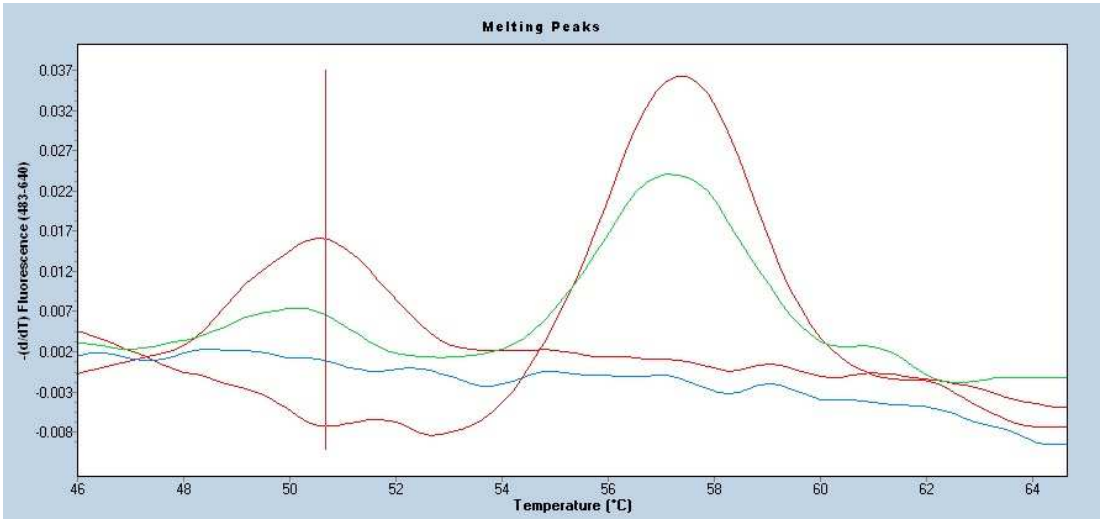
CETP taq1b rs708272 :



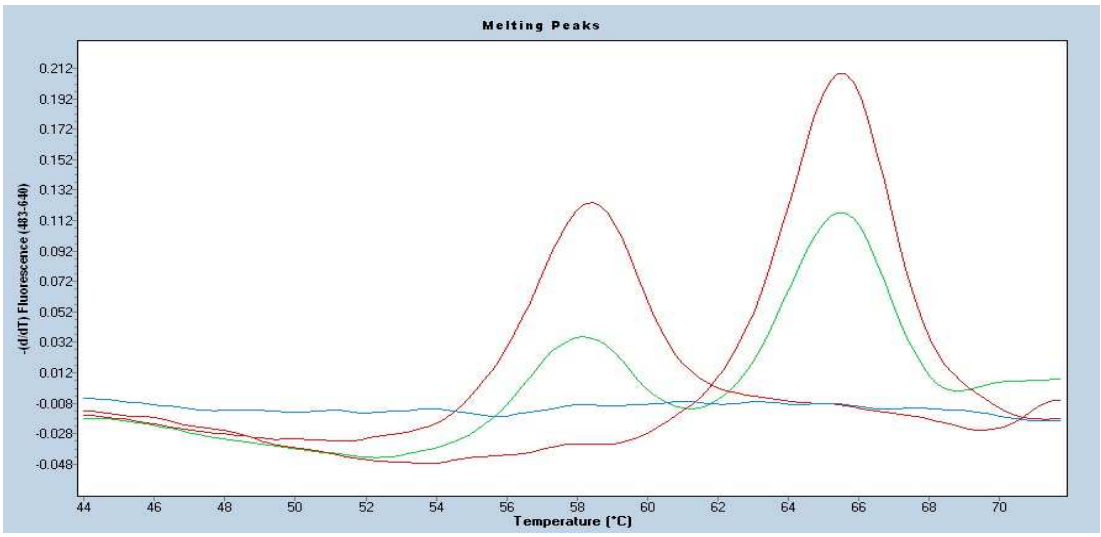
LPL rs268:



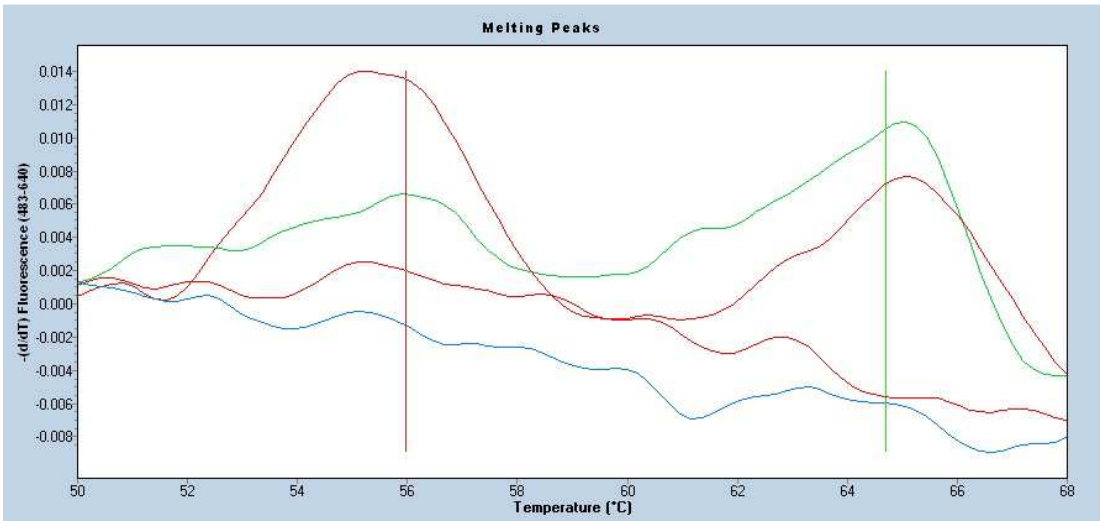
ApoC3 rs2854116:



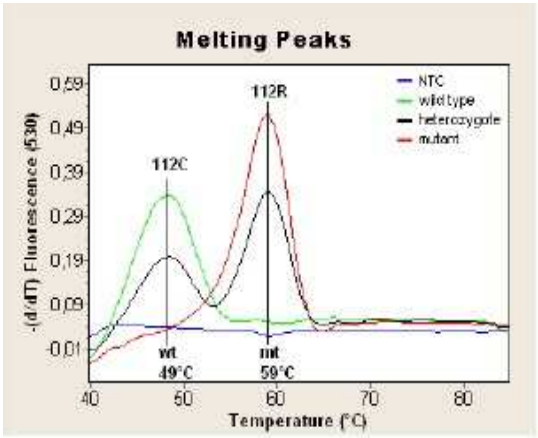
ApoA4 rs675:

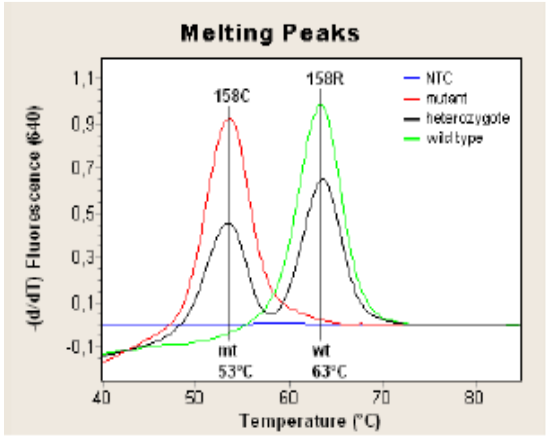


ApoA5 rs3135506:



ApoE rs7412 and rs429358:





Appendix 3: Descriptive tables for each genetic polymorphism

Table 1: Demographic, clinical and laboratory characteristics of the cohort according to the TRAF1C5 genotypes

	AA (N=121)	AG (N=190)	GG (N=76)	P value
General demographics				
Age (years)	62.59 (53.81-69.14)	62.8 (53.5-69.9)	65.54 (59.2-69.66)	0.086
Sex female n(%)	99 (80.5)	137 (70.6)	53 (67.9)	0.079
Smoking status n(%)				
Never	59 (48.8)	81 (42.6)	33 (43.4)	0.202
Ex-smoker	38 (31.4)	76 (40)	35 (46.1)	
Current	24 (19.8)	33 (17.4)	8 (10.5)	
RA characteristics				
General characteristics				
RF positive n(%)	83 (69.7)	147 (76.6)	62 (82.7)	0.114
antiCCP positive n(%)	69 (57.5)	130 (70.7)	56 (74.7)	0.018
Disease duration (yrs)	9 (3.5-18)	10 (4-17)	12 (6-19.25)	
Disease activity				
CRP (mg/L)	7 (4-20)	9 (5-18)	9 (5-21.5)	0.332
ESR	19 (9-36)	20 (10-36.5)	26 (10-39.5)	0.231
DAS 28	4.2 ± 1.37	4.18 ± 1.36	4.38 ± 1.57	0.571
Disease severity				
HAQ	1.38 (0.63-2.0)	1.63 (0.47-2.3)	1.75 (0.88-2.16)	0.349
EAD n(%)	82 (66.7)	124 (63.9)	59 (75.6)	0.176
Joint replacement surgery n(%)	39 (31.7)	52 (26.8)	24 (30.8)	0.605
Medication				
Methotrexate n(%)	75 (61.0)	104 (53.6)	41 (52.6)	0.360
Sulphasalazine n(%)	33 (26.8)	63 (32.5)	22 (28.2)	0.529
Hydroxychloroquine n(%)	23 (18.7)	35 (18.0)	22 (28.2)	0.148
Anti-TNF n(%)	17 (13.8)	22 (11.3)	7 (9.0)	0.570
Leflunomide n (%)	6 (4.9)	7 (3.6)	3 (3.8)	0.851
Prednisolone n(%)	41 (33.3)	65 (33.5	24 (30.8)	0.903
NSAIDs/COX II n(%)	35 (28.5)	55 (28.4)	20 (25.6)	0.889
Anti-hypertensives n(%)	42 (34.1)	94 (48.5)	32 (41.0)	0.041
Statin/fibrate n(%)	27 (22.0)	41 (21.1)	13 (16.7)	0.634
Lipid Profile				
Total cholesterol (mmol/L)	5.44 ± 1.3	5.17 ± 1.09	5.08 ± 1.13	0.054
HDL (mmol/L)	1.6 (1.3-1.9)	1.83 (1.5-2.17)	1.8 (1.6-2.1)	0.095
LDL (mmol/L)	3.23 ± 1.3	2.98 ± 1.13	2.95 ± 1.18	0.153
Triglycerides (mmol/L)	1.2 (1.0-1.6)	1.25 (1.0-1.7)	1.2 (0.9-1.5)	0.613
ApoA (g/L)	1.7 ± 0.46	1.6 ± 0.43	1.57 ± 0.39	0.091
ApoB (g/L)	0.99 ± 0.33	0.94 ± 0.26	0.93 ±0.29	0.160
Comorbidities				
Cardiovascular disease n(%)	23 (18.7)	42 (21.6)	19 (24.4)	0.623
Hypertension n(%)	27 (22)	41 (21.1)	13 (16.7)	0.634
Systolic BP n(%)	142 (125-152.3)	140 (127.5-156.5)	144 (128.5-154.3)	0.852
Diastolic BP n(%)	79.2 ± 11.68	78.57 ± 11.36	79.28 ± 10.44	0.843
Insulin resistance n(%)	38 (32.2)	80 (42.8)	26 (34.7)	0.146
Obesity (BMI)	27.46 ± 4.6	27.72 ± 5.3	28.18 ± 5.03	0.627
NCEP defined dyslipidaemia n(%)	78 (63.4)	106 (54.6)	41 (52.6)	0.209
Deaths from CVD n(%)	5 (5.2)	2 (1.3)	2 (3.1)	0.198

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin.

Table 2: Demographic, clinical and laboratory characteristics of the cohort according to the STAT4 genotypes

	GG (N=226)	GT (N=144)	TT (N=24)	P value
General demographics				
Age (years)	64.1 (55.48-69.83)	63.0 (54.2-69.3)	60.2 (54.7-66.98)	0.631
Sex female n(%)	167 (73.9)	106 (73.6)	16 (66.7)	0.745
Smoking status n(%)				
Never	98 (44.1)	64 (45.7)	11 (45.8)	0.657
Ex-smoker	82 (36.9)	55 (39.3)	11(45.8)	
Current	42 (18.9)	21 (15)	2 (8.3)	
RA characteristics				
General characteristics				
RF positive n(%)	168 (76)	105 (75)	18 (75)	0.974
antiCCP positive n(%)	138 (63.9)	101 (73.2)	16 (66.7)	0.190
Disease duration (yrs)	10 (4-19)	11 (4-17)	9 (3-14)	0.521
Disease activity				
CRP (mg/L)	9 (5-20)	8 4.5-19)	7 (4.5-21)	0.969
ESR	21 (10-38)	20 (8.5-33.5)	20.5 (12.5-29.0)	0.828
DAS 28	4.28 ± 1.4	4.19 ± 1.39	3.95 ± 1.47	0.533
Disease severity				
HAQ	1.5 (0.5-2.13)	1.63 (0.88-2.13)	1.5 (0.81-2.13)	0.380
EAD n(%)	148 (65.5)	98 (68.1)	18 (75)	0.606
Joint replacement surgery n(%)	66 (29.2)	43 (29.9)	6 (25)	0.889
Medication				
Methotrexate n(%)	132 (58.4)	77 (53.5)	12 (50)	0.534
Sulphasalazine n(%)	66 (29.2)	44 (30.6)	7 (29.2)	0.961
Hydroxychloroquine n(%)	46 (20.4)	26 (18.1)	6 (25)	0.695
Anti-TNF n(%)	26 (11.5)	18 (12.5)	2 (8.3)	0.835
Leflunomide n (%)	9 (4)	6 (4.2)	1 (4.2)	0.996
Prednisolone n(%)	80 (35.4)	45 (31.3)	5 (20.8)	0.302
NSAIDs/COX II n(%)	63 (27.9)	42 (29.2)	6 (25)	0.905
Anti-hypertensives n(%)	102 (45.1)	57 (39.6)	8 (33.3)	0.374
Statin/fibrate n(%)	43 (19)	33 (22.9)	6 (25)	0.583
Lipid Profile				
Total cholesterol (mmol/L)	5.23 ± 1.12	5.23 ± 1.28	5.2 ± 1.11	0.992
HDL (mmol/L)	1.6 (1.3-1.8)	1.6 (1.3-1.9)	1.4 (1.2-1.65)	0.335
LDL (mmol/L)	3.07 ± 1.19	2.99 ± 1.24	3.27 ± 1.04	0.551
Triglycerides (mmol/L)	1.3 (0.9-1.6)	1.2 (1.0-1.6)	1.2 (1.0-1.65)	0.873
ApoA (g/L)	1.64 ± 0.46	1.63 ± 0.41	1.64 ±0.39	0.966
ApoB (g/L)	0.96 ± 0.28	0.95 ± 0.32	0.98 ± 0.28	0.845
Comorbidities				
Cardiovascular disease n(%)	49 (21.7)	32 (22.2)	3 (12.5)	0.549
Hypertension n(%)	169 (74.8)	96 (66.7)	12 (50)	**0.020**
Systolic BP n(%)	144 (130-156)	140 (127-151)	132 (122-144)	0.059
Diastolic BP n(%)	79.28 ± 11.35	77.94 ± 11.44	79.88 ± 8.88	0.482
Insulin resistance n(%)	90 (41.3)	44 (32.1)	9 (37.5)	0.222
Obesity (BMI)	27.84 ± 5.0	27.47 ± 5.2	28.22 ± 4.18	0.708
NCEP defined dyslipidaemia n(%)	130 (57.5)	81 (56.3)	15 (62.5)	0.846
Deaths from CVD n(%)	8 (4.4)	1 (0.9)	0 (0)	0.170

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin.

Table 3: Demographic, clinical and laboratory characteristics of the cohort according to the PTPN22 genotypes

	AA (N=6)	GA (N=119)	GG (N=272)	P value
General demographics				
Age (years)	65.4 (43.3-74.5)	63.0 (55.1-69.6)	63.1 (55.1-69.2)	0.758
Sex female n(%)	4 (66.7)	88 (73.9)	199 (73.2)	0.922
Smoking status n(%)				
Never	3 (50)	49 (43.4)	123 (45.6)	0.658
Ex-smoker	1 (16.7)	47 (41.6)	101 (37.4)	
Current	2 (33.3)	17 (15)	46 (17)	
RA characteristics				
General characteristics				
RF positive n(%)	4 (66.7)	85 (73.9)	205 (76.8)	0.728
Anti CCP positive n(%)	4 (66.7)	73 (65.2)	180 (68.4)	0.826
Disease duration (yrs)	12 (3.5-15)	9(4-16.5)	11 (4-19)	0.616
Disease activity				
CRP (mg/L)	8 (5.5-16.0)	8 (4-17.5)	9 (5-20)	0.692
ESR	18 (9.5-33.5)	26 (10-42.5)	20 (9-34)	0.250
DAS 28	3.92 ± 1.16	4.4 ± 1.52	4.16 ± 1.35	0.253
Disease severity				
HAQ	1.38 (0.63-2.19)	1.5 (0.38-2.13)	1.63 (0.63-2.13)	0.922
EAD n(%)	3 (50)	76 (63.9)	188 (69.1)	0.394
Joint replacement surgery n(%)	1 (16.7)	35 (29.4)	79 (29.0)	0.797
Medication				
Methotrexate n(%)	4 (66.7)	65 (54.6)	153 (56.3)	0.829
Sulphasalazine n(%)	1 (16.70	30 (25.2)	87 (32)	0.314
Hydroxychloroquine n(%)	1 (16.7)	23 (19.3)	56 (20.6)	0.938
Anti-TNF n(%)	0 (0)	19 (16.0)	27 (9.9)	0.154
Leflunomide n (%)	0 (0)	7 (5.9)	9 (3.3)	0.433
Prednisolone n(%)	1 (16.7)	47 (39.5)	82 (30.1)	0.135
NSAIDs/COX II n(%)	4 (66.7)	30 (25.2)	71 (28.3)	0.085
Anti-hypertensives n(%)	2 (33.3)	55 (46.2)	112 (41.2)	0.585
Statin/fibrate n(%)	1 (16.7)	30 (25.2)	51 (18.8)	0.338
Lipid Profile				
Total cholesterol (mmol/L)	5.0 ± 0.47	5.2 ± 1.04	5.3 ± 1.24	0.746
HDL (mmol/L)	1.5 (1.15-1.7)	1.6 (1.3-1.75)	1.6 (1.3-1.9)	0.434
LDL (mmol/L)	2.9 ± 1.33	3.0 ± 1.16	3.0 ± 1.22	0.961
Triglycerides (mmol/L)	1.2 (0.7-1.45)	1.3 (1.0-1.6)	1.2 (0.9-1.6)	0.753
ApoA (g/L)	1.5 ± 0.65	1.6 ± 0.43	1.6 ± 0.43	0.745
ApoB (g/L)	0.91± 0.29	0.95 ± 0.29	0.95 ± 0.3	0.925
Comorbidities				
Cardiovascular disease n(%)	1 (16.7)	27 (22.7)	57 (21.0)	0.892
Hypertension n(%)	3 (50)	86 (72.3)	191 (70.2)	0.496
Systolic BP n(%)	125 (115-150.5)	141 (130-157.5)	140 (127-153)	0.462
Diastolic BP n(%)	71.83 ± 6.71	79.99 ± 11.04	78.57 ± 11.35	0.154
Insulin resistance n(%)	2 (33.3)	45 (39.1)	98 (37.5)	0.932
Obesity (BMI)	25.68 ± 4.17	27.74 ± 4.79	27.8 ± 5.15	0.596
NCEP defined dyslipidaemia n(%)	3 (50.0)	69 (58.0)	154 (56.6)	0.913
Deaths from CVD n(%)	0 (0)	2 (2.2)	7 (3.2)	0.842

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate
RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin.

Table 4: Demographic, clinical and laboratory characteristics across the shared epitope

	No copies of SE (N=96)	One copy of SE (N=181)	Two copies of SE (N=78)	P value
General demographics				
Age (years)	61.7 (50.8-67.9)	65.0 (57.6-71.7)	63.6 (55.6-69.1)	**0.019**
Sex female n(%)	71 (74)	130 (71.8)	58 (74.4)	0.885
Smoking status n(%)				
Never	41 (43.2)	77 (43.8)	38 (49.4)	0.564
Ex-smoker	39 (35.8)	70 (39.8)	30 (39.0)	
Current	20 (21.1)	29 (16.5)	9 (11.7)	
RA characteristics				
General characteristics				
RF positive n(%)	53 (58.2)	143 (79.9)	64 (83.1)	**<0.001**
antiCCP positive n(%)	32 (36.0)	131 (74.0)	64 (86.5)	**<0.001**
Disease duration (yrs)	7 (3-15.8)	11 (5-18)	12 (5-22)	**0.021**
Disease activity				
CRP (mg/L)	7 (5-14)	10 (5-25)	10 (5.5-17)	**0.014**
ESR	16 (7.3-32)	23 (10-40)	22 (14.5-40.5)	**0.013**
DAS 28	4.11 1.48	4.3 1.4	4.3 1.34	0.502
Disease severity				
HAQ	1.37 (0.5-2.12)	1.63 (0.63-2.25)	1.38 (0.69-2.5)	0.274
EAD n(%)	23 (74.0)	54 (29.8)	29 (37.2)	0.166
Joint replacement surgery n(%)	64 (66.7)	123 (68.0)	54 (69.2)	0.937
Medication				
Methotrexate n(%)	48 (50.0)	101 (55.8)	48 (61.5)	0.311
Sulphasalazine n(%)	23 (24.0)	60 (33.1)	23 (29.5)	0.281
Hydroxychloroquine n(%)	17 (17.7)	37 (20.4)	14 (17.9)	0.820
Anti-TNF n(%)	5 (5.2)	24 (13.3)	8 (10.3)	0.113
Leflunomide n (%)	7 (7.3)	5 (2.8)	3 (3.8)	0.200
Prednisolone n(%)	26 (27.1)	71 (39.2)	23 (29.5)	0.084
NSAIDs/COX II n(%)				
Anti-hypertensives n(%)	36 (37.5)	82 (45.3)	34 (43.6)	0.453
Statin/fibrate n(%)	24 (25.0)	32 (17.7)	17 (21.8)	0.341
Lipid Profile				
Total cholesterol (mmol/L)	5.15 1.13	5.26 1.14	5.39 1.34	0.410
HDL (mmol/L)	1.5 (1.3-1.8)	1.6 (1.3-1.9)	1.5 (1.3-1.8)	0.929
LDL (mmol/L)	3.12 1.19	3.07-1.14	3.12 1.37	0.925
Triglycerides (mmol/L)	1.2 (0.9-1.7)	1.2 (1.0-1.8)	1.2 (1.0-1.6)	0.531
ApoA (g/L)	1.71 0.49	1.62 0.44	1.63 0.41	0.240
ApoB (g/L)	0.94 0.28	0.96 0.28	0.98 0.34	0.647
Comorbidities				
Cardiovascular disease n(%)	18 (18.8)	38 (21.0)	20 (25.6)	0.535
Hypertension n(%)	60 (62.5)	137 (75.7)	57 (73.1)	0.065
Systolic BP n(%)	135 (122.8-150)	144 (131.3-157)	140 (123-152.5)	**0.028**
Diastolic BP n(%)	76 52 10.55	80.03 11.13	79.32 12.24	**0.045**
Insulin resistance n(%)	31 (38.4)	70 (39.8)	30 (40.5)	0.605
Obesity (BMI)	28.0 4.8	28.0 5.15	26.9 4.9	0.252
NCEP defined dyslipidaemia n(%)	54 (56.3)	108 (59.7)	42 (53.8)	0.659
Deaths from CVD n(%)	1 (1.4)	7 (4.7)	1 (1.6)	0.312

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

SE: shared epitope, RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin.

Table 5: Demographic, clinical and laboratory characteristics across the ABCA rs2230808 genotypes

	GG (N=222)	AG (N=143)	AA (N=24)	P value
General demographics				
Age (years)	62.9 (53.8-69.6)	60.9 (52.7-68.2)	58.9 (50.5-66.9)	0.472
Sex female n(%)	165 (74.3)	103 (72)	18 (75)	0.876
Smoking status n(%)				
Never	93 (42.7)	66 (47.5)	13 (54.2)	0.681
Ex-smoker	87 (39.9)	54 (38.8)	7 (29.2)	
Current	38 (17.4)	19 (13.7)	4 (16.7)	
RA characteristics				
General characteristics				
RF positive n(%)	165 (75.7)	106 (76.3)	17 (73.9)	0.969
Anti-CCP positive n(%)	146 (68.5)	92 (67.6)	13 (54.2)	0.361
Disease duration (yrs)	10 (4-20)	10.5 (4-16.3)	10.5 (5-18.8)	0.973
Disease activity				
CRP (mg/L)	9 (5-20)	8 (4-21)	9 (5-16.8)	0.879
ESR	21 (10-36)	21 (9-38)	15.5 (7.3-34)	0.445
DAS 28	4.3± 1.4	4.2± 1.4	4.0± 1.5	0.742
Disease severity				
HAQ	1.5 (0.5-2.3)	1.6 (1.0-2.1)	1.2 (0.4-1.8)	0.242
EAD n(%)	149 (67.1)	97 (67.8)	18 (75.0)	0.734
Joint replacement surgery n(%)	67 (30.2)	41 (28.7)	7 (29.2)	0.953
Medication				
Methotrexate n(%)	127 (57.2)	79 (55.2)	12 (50.0)	0.773
Sulphasalazine n(%)	61 (27.5)	46 (32.2)	9 (37.5)	0.441
Hydroxychloroquine n(%)	41 (18.5)	29 (20.3)	8 (33.3)	0.224
Anti-TNF n(%)	25 (11.3)	18 (12.6)	2 (8.3)	0.814
Leflunomide n (%)	11 (5.0)	4 (2.8)	1 (4.2)	0.598
Prednisolone n(%)	71 (32.0)	53 (37.1)	5 (20.8)	0.251
NSAIDs/COX II n(%)	64 (28.9)	40 (28.0)	7 (29.2)	0.795
Anti-hypertensives n(%)	98 (44.1)	61 (42.7)	6 (25.0)	0.196
Statin/fibrate n(%)	46 (20.7)	31 (21.7)	4 (16.7)	0.854
Lipid Profile				
Total cholesterol (mmol/L)	5.6± 1.1	5.4± 1.2	5.1± 0.9	0.182
HDL (mmol/L)	1.6 (1.3-1.9)	1.7 (1.3-1.9)	1.7 (1.2-1.8)	0.848
LDL (mmol/L)	3.4± 1.2	3.1±0.9	3.0 ±1.13	0.234
Triglycerides (mmol/L)	1.3 (1.0-1.8)	1.1 (0.9-1.5)	1.1 (0.9-1.4)	**0.023**
ApoA (g/L)	1.7± 0.4	1.6± 0.4	1.6± 0.5	0.696
ApoB (g/L)	1.0 ±0.3	1.0± 0.3	0.9± 0.3	0.083
Comorbidities				
Cardiovascular disease n(%)	46 (20.7)	33 (23.1)	4 (16.7)	0.733
Hypertension n(%)	169 (72.1)	103 (72.0)	12 (50.0)	0.071
Insulin resistance n(%)	87 (40.8)	54 (39.1)	2 (8.7)	**0.010**
Obesity (BMI)	26.7 (24.2-30.4)	27.6 (25-31.9)	28.6 (23.6-32.6)	0.753
NCEP defined dyslipidaemia n(%)*	132 (59.5)	83 (58.0)	9 (37.5)	0.117
Deaths from CVD n(%)	9 (4.1)	5 (3.5)	0 (0)	0.597

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 6: Demographic, clinical and laboratory characteristics across the ABCA rs2066715 genotypes

	CC (N=337)	CT (N=55)	TT (N=2)	P value
General demographics				
Age (years)	62.3 (52.7-69.1)	59.8 (50.6-67.2)	60.4	0.593
Sex female n(%)	250 (74.2)	37 (67.3)	2 (100)	0.390
Smoking status n(%)				
Never	153 (46.4)	21 (38.9)	1 (50.0)	0.704
Ex-smoker	125 (37.9)	21 (38.9)	1 (50.0)	
Current	52 (15.8)	12 (22.2)	0 (0)	
RA characteristics				
General characteristics				
RF positive n(%)	243 (74.1)	47 (85.5)	1 (50.0)	0.135
Anti-CCP positive n(%)	215 (66.2)	40 (76.9)	0 (0)	0.108
Disease duration (yrs)	10 (4-16)	13 (5-20)	11	0.973
Disease activity				
CRP (mg/L)	9 (5-20)	8 (4-22.5)	9	0.930
ESR	19 (9-35)	21 (7-40)	18	0.850
DAS 28	4.2± 1.4	4.3± 1.3	4.0	0.749
Disease severity				
HAQ	1.5 (0.5-2.1)	1.6 (0.9-1.9)	1.5	0.642
EAD n(%)	221 (67.4)	35 (63.6)	2 (100)	0.526
Joint replacement surgery n(%)	98 (29.1)	17 (30.9)	0 (0)	0.636
Medication				
Methotrexate n(%)	180 (53.4)	37 (67.3)	2 (100)	0.071
Sulphasalazine n(%)	100 (29.7)	17 (30.9)	0 (0)	0.643
Hydroxychloroquine n(%)	65 (19.3)	12 (20.3)	8 (33.3)	0.224
Anti-TNF n(%)	25 (11.3)	18 (21.8)	1 (50)	0.510
Leflunomide n (%)	15 (4.5)	1 (1.8)	0 (0)	0.629
Prednisolone n(%)	108 (68.0)	22 (40.0)	0 (0)	0.310
NSAIDs/COX II n(%)	90 (26.7)	19 (34.6)	1 (50.0)	0.089
Anti-hypertensives n(%)	141 (41.8)	24 (43.6)	2 (100)	0.247
Statin/fibrate n(%)	71 (21.1)	9 (16.4)	1 (50.0)	0.426
Lipid Profile				
Total cholesterol (mmol/L)	5.4± 1.0	5.7± 1.5	5.9	0.189
HDL (mmol/L)	1.6 (1.3-1.8)	1.7 (1.3-2.0)	1.7	0.779
LDL (mmol/L)	3.2± 1.1	3.4±1.3	3.9	0.654
Triglycerides (mmol/L)	1.2 (0.9-1.6)	1.4 (0.9-1.8)	1.2	0.417
ApoA (g/L)	1.6± 0.4	1.7± 0.5	1.4	0.698
ApoB (g/L)	1.0 ±0.3	1.0± 0.4	1.0	0.301
Comorbidities				
Cardiovascular disease n(%)	72 (21.4)	11 (20.0)	1 (50.0)	0.595
Hypertension n(%)	234 (69.4)	42 (76.4)	2 (100)	0.381
Insulin resistance n(%)	120 (36.8)	22 (43.1)	1 (50.0)	0.644
Obesity (BMI)	26.7 (24.2-30.6)	28.5 (24.5-31.9)	28.6	0.841
NCEP defined dyslipidaemia n(%)*	190 (56.4)	34 (61.8)	1 (50.0)	0.736
Deaths from CVD n(%)	10 (3)	3 (5.5)	1 (50)	0.125

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 7: Demographic, clinical and laboratory characteristics across the ABCA rs33918808 genotypes

	GG (N=383)	GC (N=14)	CC (N=0)	P value
General demographics				
Age (years)	63.0 (54.4-69.2)	60.4 (47.4-68.9)	-	0.300
Sex female n(%)	282 (73.6)	9 (64.3)	-	0.438
Smoking status n(%)			-	
Never	170 (45.2)	5 (38.5)		0.836
Ex-smoker	143 (38.0)	6 (46.2)		
Current	63 (16.8)	2 (15.4)		
RA characteristics				
General characteristics				
RF positive n(%)	283 (75.7)	11 (78.6)	-	0.803
Anti-CCP positive n(%)	250 (67.9)	7 (53.8)	-	0.287
Disease duration (yrs)	10 (4-17)	7.5 (2-13)	-	0.270
Disease activity				
CRP (mg/L)	8 (5-18)	22 (4.8-29.3)	-	0.106
ESR	20 (9-35)	20.5 (7.8-38.3)	-	0.943
DAS 28	4.2± 1.4	4.3± 1.7	-	0.865
Disease severity				
HAQ	1.5 (0.6-2.1)	1.6 (0.5-2.1)	-	0.880
EAD n(%)	258 (67.4)	9 (64.3)	-	0.810
Joint replacement surgery n(%)	115 (30.0)	0 (0)	-	**0.015**
Medication				
Methotrexate n(%)	216 (56.4)	6 (42.9)	-	0.316
Sulphasalazine n(%)	113 (29.5)	5 (35.7)	-	0.618
Hydroxychloroquine n(%)	74 (19.3)	6 (42.9)	-	**0.031**
Anti-TNF n(%)	45 (11.7)	1 (7.1)	-	0.597
Leflunomide n (%)	16 (4.2)	0 (0)	-	0.435
Prednisolone n(%)	125 (32.6)	5 (35.7)	-	0.810
NSAIDs/COX II n(%)	108 (28.2)	3 (21.4)	-	0.654
Anti-hypertensives n(%)	164 (42.8)	5 (35.7)	-	0.597
Statin/fibrate n(%)	77 (20.1)	5 (35.7)	-	0.156
Lipid Profile				
Total cholesterol (mmol/L)	5.5± 1.1	5.1± 1.1	-	0.381
HDL (mmol/L)	1.6 (1.3-1.9)	1.3 (1.2-1.7)	-	0.162
LDL (mmol/L)	3.3± 1.1	2.7±1.2	-	0.160
Triglycerides (mmol/L)	1.2 (0.9-1.6)	1.0 (0.7-1.7)	-	0.236
ApoA (g/L)	1.7± 0.4	1.4± 0.4	-	0.085
ApoB (g/L)	1.0 ±0.3	0.8± 0.2	-	0.087
Comorbidities				
Cardiovascular disease n(%)	79 (20.6)	6 (42.9)	-	**0.046**
Hypertension n(%)	270 (70.5)	10 (71.4)	-	0.940
Insulin resistance n(%)	138 (37.5)	7 (50.0)	-	0.344
Obesity (BMI)	27.0 (24.0-30.9)	28.0 (26.2-32.2)	-	0.290
NCEP defined dyslipidaemia n(%)*	219 (57.2)	7 (50)	-	0.594
Deaths from CVD n(%)	13 (3.4)	1 (7.1)	-	0.455

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 8: Demographic, clinical and laboratory characteristics across the ABCA rs2066718 genotypes

	GG (N=372)	GA (N=14)	AA (N=0)	P value
General demographics				
Age (years)	63.0 (54.7-69.3)	61.6 (49.3-68.2)	-	0.389
Sex female n(%)	279 (73.2)	10 (71.4)	-	0.881
Smoking status n(%)			-	
Never	165 (44.1)	9 (69.2)		0.162
Ex-smoker	147 (39.3)	2 (15.4)		
Current	62 (16.6)	2 (15.4)		
RA characteristics				
General characteristics				
RF positive n(%)	282 (75.8)	11 (78.6)	-	0.812
Anti-CCP positive n(%)	245 (67.1)	10 (71.4)	-	0.736
Disease duration (yrs)	10 (4-17)	8 (5.5-16.0)	-	0.927
Disease activity				
CRP (mg/L)	8 (5-20)	7 (4.5-14.5)	-	0.523
ESR	20 (9-36)	20 (12.5-30.5)	-	0.769
DAS 28	4.2± 1.4	4.3± 1.7	-	0.867
Disease severity				
HAQ	1.5 (0.6-2.1)	1.0 (0.6-2.1)	-	0.271
EAD n(%)	254 (66.7)	11 (78.6)	-	0.352
Joint replacement surgery n(%)	111 (29.1)	3 (21.4)	-	0.532
Medication				
Methotrexate n(%)	216 (56.7)	5 (35.7)	-	0.120
Sulphasalazine n(%)	115 (30.2)	3 (21.4)	-	0.482
Hydroxychloroquine n(%)	76 (19.9)	4 (28.6)	-	0.430
Anti-TNF n(%)	43 (11.3)	2 (14.3)	-	0.729
Leflunomide n (%)	16 (4.2)	0 (0)	-	0.434
Prednisolone n(%)	127 (33.3)	3 (21.4)	-	0.352
NSAIDs/COX II n(%)	107 (28.0)	4 (28.5)	-	0.892
Anti-hypertensives n(%)	165 (43.3)	4 (28.6)	-	0.274
Statin/fibrate n(%)	80 (21.0)	2 (14.3)	-	0.534
Lipid Profile				
Total cholesterol (mmol/L)	5.5± 1.1	5.0± 0.7	-	0.163
HDL (mmol/L)	1.6 (1.3-1.9)	1.4 (1.3-1.5)	-	0.625
LDL (mmol/L)	3.2± 1.2	3.1±0.9	-	0.325
Triglycerides (mmol/L)	1.2 (0.9-1.6)	1.1 (1.0-1.6)	-	0.793
ApoA (g/L)	1.7± 0.4	1.2± 0.4	-	0.640
ApoB (g/L)	1.0 ±0.3	0.9± 0.2	-	0.187
Comorbidities				
Cardiovascular disease n(%)	84 (22.0)	1 (7.1)	-	0.183
Hypertension n(%)	268 (70.3)	11 (78.6)	-	0.507
Insulin resistance	134 (36.5)	9 (69.2)	-	**0.017**
Obesity (BMI)	27.0 (24.4-30.9)	29.0 (26.7-34.9)	-	0.275
NCEP defined dyslipidaemia n(%)*	220 (57.7)	6 (42.9)	-	0.269
Deaths from CVD n(%)	14 (3.7)	0 (0)	-	0.465

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 9: Demographic, clinical and laboratory characteristics across the ABCA rs2066714 genotypes

	AA (N=303)	AG (N=85)	GG (N=9)	P value
General demographics				
Age (years)	63.6 (56.0-69.3)	61.1 (49.8-69.2)	62.7 (51.3-65.1)	0.151
Sex female n(%)	220 (72.6)	62 (72.9)	9 (100)	0.187
Smoking status n(%)	140 (47.3)	29 (34.5)	6 (66.7)	
Never	114 (38.5)	32 (38.1)	3 (33.3)	**0.019**
Ex-smoker	42 (14.2)	23 (27.4)	0 (0)	
Current				
RA characteristics				
General characteristics				
RF positive n(%)	225 (76.5)	61 (71.8)	8 (88.9)	0.432
Anti-CCP positive n(%)	195 (66.8)	56 (69.1)	6 (75.0)	0.830
Disease duration (yrs)	10 (4-17)	9 (4-17)	24.5 (16.8-31.8)	**0.047**
Disease activity				
CRP (mg/L)	9 (5-20)	8 (4.8-18)	7 (5.3-15.5)	0.880
ESR	20 (9-35)	21.5 (9-38)	27 (16-35.8)	0.609
DAS 28	4.2± 1.4	4.3± 1.4	4.8± 1.4	0.512
Disease severity				
HAQ	1.5 (0.6-2.3)	1.5 (0.7-1.9)	1.5 (0.2-2.4)	0.343
EAD n(%)	205 (67.7)	54 (63.5)	8 (88.9)	0.291
Joint replacement surgery n(%)	89 (29.4)	22 (25.9)	4 (44.4)	0.481
Medication				
Methotrexate n(%)	166 (54.8)	49 (57.6)	7 (77.8)	0.367
Sulphasalazine n(%)	94 (31.0)	23 (27.1)	1 (11.1)	0.363
Hydroxychloroquine n(%)	56 (18.5)	22 (25.9)	2 (22.2)	0.319
Anti-TNF n(%)	32 (10.6)	14 (16.5)	0 (0)	0.176
Leflunomide n (%)	12 (4.0)	3 (3.5)	1 (11.1)	0.542
Prednisolone n(%)	97 (32.0)	31 (36.5)	2 (22.6)	0.588
NSAIDs/COX II n(%)	82 (27.1)	24 (28.3)	5 (55.5)	0.224
Anti-hypertensives n(%)	131 (43.2)	33 (38.8)	5 (55.6)	0.559
Statin/fibrate n(%)	66 (21.8)	13 (15.3)	3 (33.3)	0.271
Lipid Profile				
Total cholesterol (mmol/L)	5.4± 1.0	5.5± 1.2	6.7± 2.9	**0.011**
HDL (mmol/L)	1.6 (1.3-1.9)	1.6 (1.3-1.9)	1.7 (1.6-1.9)	0.717
LDL (mmol/L)	3.2± 1.1	3.3±1.3	4.2 ±2.4	0.088
Triglycerides (mmol/L)	1.2 (0.9-1.6)	1.3 (0.9-1.6)	1.4 (0.9-2.1)	0.677
ApoA (g/L)	1.7± 0.5	1.6± 0.4	1.6± 0.3	0.994
ApoB (mmol/L)	1.0 ±0.3	1.0± 0.3	1.3± 0.8	**0.007**
Comorbidities				
Cardiovascular disease	66 (21.8)	16 (18.8)	3 (33.3)	0.570
Hypertension n(%)	211 (69.6)	61 (71.8)	8 (88.9)	0.441
Insulin resistance	110 (37.7)	29 (35.4)	6 (75.0)	0.086
Obesity (BMI)	27.2 (24.6-30.9)	27.3 (24-30.9)	26 (23.7-33.3)	0.766
NCEP defined dyslipidaemia n(%)*	176 (58.1)	45 (52.9)	5 (55.6)	0.696
Deaths from CVD n(%)	10 (3.3)	3 (3.5)	1 (11.1)	0.457

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 10: Demographic, clinical and laboratory characteristics across the CETP taq1B rs708272 genotypes

	B1B1 (N=109)	B1B2 (N=175)	B2B2 (N=113)	P value
General demographics				
Age (years)	64.1 (57.6-68.5)	62.2 (53.0-69.6)	63.0 (53.8-69.6)	0.578
Sex female n(%)	72 (66.1)	132 (75.4)	87 (77.0)	0.128
Smoking status n(%)				
Never	44 (41.5)	81 (47.1)	50 (45.0)	0.894
Ex-smoker	43 (40.6)	62 (36.0)	44 (39.6)	
Current	19 (17.9)	29 (16.9)	17 (15.3)	
RA characteristics				
General characteristics				
RF positive n(%)	78 (72.9)	137 (80.1)	79 (71.8)	0.204
Anti-CCP positive n(%)	65 (63.1)	120 (71.9)	72 (64.9)	0.259
Disease duration (yrs)	10 (4-20)	10 (4-18)	9 (3-15)	0.400
Disease activity				
CRP (mg/L)	8 (5-17.5)	9 (5-20)	8 (5-17)	0.485
ESR	18 (9-37)	21.5 (10.3-36.5)	20 (8-36)	0.357
DAS 28	4.2± 1.3	4.3± 1.3	4.2± 1.6	0.844
Disease severity				
HAQ	1.5 (0.6-2.3)	1.5 (0.7-1.9)	1.5 (0.2-2.4)	0.824
EAD n(%)	82 (75.2)	106 (60.6)	79 (69.9)	**0.029**
Joint replacement surgery n(%)	36 (33.0)	48 (27.4)	31 (27.4)	0.548
Medication				
Methotrexate n(%)	61 (56.0)	103 (58.9)	58 (51.3)	0.454
Sulphasalazine n(%)	32 (29.4)	51 (29.1)	35 (31.0)	0.942
Hydroxychloroquine n(%)	25 (22.9)	37 (21.9)	18 (15.9)	0.380
Anti-TNF n(%)	10 (9.2)	24 (13.7)	12 (10.0)	0.473
Leflunomide n (%)	2 (1.8)	9 (5.1)	5 (4.4)	0.375
Prednisolone n(%)	46 (42.2)	50 (28.6)	34 (30.1)	**0.046**
NSAIDs/COX II n(%)	32 (29.4)	48 (27.4)	31 (27.4)	0.930
Anti-hypertensives n(%)	51 (46.8)	68 (38.9)	50 (44.2)	0.385
Statin/fibrate n(%)	26 (23.9)	35 (20.0)	21 (18.6)	0.600
Lipid Profile				
Total cholesterol (mmol/L)	5.6± 1.3	5.3± 1.1	5.5± 1.0	0.302
HDL (mmol/L)	1.5 (1.3-1.8)	1.6 (1.3-1.9)	1.7 (1.4-1.9)	0.058
LDL (mmol/L)	3.4± 1.3	3.2±1.2	3.2 ±1.0	0.211
Triglycerides (mmol/L)	1.4 (1-2)	1.1 (0.9-1.5)	1.2 (1-1.6)	**0.033**
ApoA (g/L)	1.6± 0.5	1.7± 0.4	1.7± 0.4	0.360
ApoB (g/L)	1.0 ±0.3	1.0± 0.3	1.0± 0.3	0.248
Comorbidities				
Cardiovascular disease n(%)	27 (24.8)	38 (21.7)	20 (21.4)	0.435
Hypertension n(%)	85 (78.0)	117 (66.9)	78 (69.0)	0.124
Insulin resistance n(%)	46 (42.2)	63 (37.7)	36 (34.0)	0.459
Obesity (BMI)	27.0 (24.1-30.3)	27.1 (24.6-30.7)	27.3 (24.3-31.4)	0.943
NCEP defined dyslipidaemia n(%)*	68 (62.4)	94 (53.7)	64 (56.6)	0.356
Death from CVD n(%)	6 (5.5)	5 (2.9)	3 (2.7)	0.420

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 11: Demographic, clinical and laboratory characteristics across the LPL rs268 genotypes

	AA (N=384)	AG (N=13)	GG (N=0)	P value
General demographics				
Age (years)	63.0 (55.2-69.3)	60.3 (52.1-73.0)	-	0.932
Sex female n(%)	280 (72.9)	10 (76.9)	-	0.749
Smoking status n(%)				
Never	169 (44.9)	6 (46.2)	-	0.991
Ex-smoker	144 (38.3)	5 (38.5)		
Current	63 (16.8)	2 (15.4)		
RA characteristics				
General characteristics				
RF positive n(%)	284 (75.7)	9 (69.2)	-	0.592
Anti-CCP positive n(%)	248 (67.2)	8 (66.7)	-	0.969
Disease duration (yrs)	10 (4-18)	4 (1-8.5)	-	**0.005**
Disease activity				
CRP (mg/L)	8 (5-19)	7 (5-24)	-	0.876
ESR	20 (9-36)	22 (14-45.5)	-	0.612
DAS 28	4.2± 1.4	3.9± 1.3	-	0.371
Disease severity				
HAQ	1.5 (0.6-2.1)	1.0 (0.3-2.1)	-	0.520
EAD n(%)	262 (68.2)	6 (46.2)	-	0.095
Joint replacement surgery n(%)	111 (28.9)	4 (30.8)	-	0.884
Medication				
Methotrexate n(%)	215 (56.0)	8 (61.5)	-	0.692
Sulphasalazine n(%)	115 (29.9)	2 (15.4)	-	0.257
Hydroxychloroquine n(%)	78 (20.3)	2 (15.4)	-	0.663
Anti-TNF n(%)	44 (11.5)	2 (15.4)	-	0.664
Leflunomide n (%)	16 (4.2)	0 (0)	-	0.452
Prednisolone n(%)	128 (33.3)	3 (23.1)	-	0.439
NSAIDs/COX II n(%)	107 (27.9)	3 (23.1)	-	0.704
Anti-hypertensives n(%)	165 (43.0)	5 (38.5)	-	0.747
Statin/fibrate n(%)	80 (20.8)	3 (23.1)	-	0.845
Lipid Profile				
Total cholesterol (mmol/L)	5.4± 1.1	5.6± 1.3	-	0.624
HDL (mmol/L)	1.6 (1.3-1.9)	1.4 (1.2-1.7)	-	0.186
LDL (mmol/L)	3.2± 1.1	3.5±1.3	-	0.484
Triglycerides (mmol/L)	1.2 (0.9-1.6)	1.4 (1.1-1.7)	-	0.261
ApoA (g/L)	1.7± 0.4	1.6± 0.4	-	0.922
ApoB (g/L)	1.0 ±0.3	1.1± 0.3	-	0.380
Comorbidities				
Cardiovascular disease n(%)	83 (21.6)	2 (15.4)	-	0.590
Hypertension n(%)	272 (70.8)	8 (61.5)	-	0.470
Insulin resistance n(%)	143 (38.6)	3 (25.0)	-	0.338
Obesity (BMI)	27.0 (24.4-30.8)	28.7 (25.2-32)	-	0.233
NCEP defined dyslipidaemia n(%)*	219 (57.0)	8 (61.5)	-	0.747
Deaths from CVD n(%)	14 (3.6)	0 (0)	-	0.483

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 12: Demographic, clinical and laboratory characteristics across the ApoC3 rs2854116 genotypes

	CC (N=60)	CT (N=150)	TT (N=184)	P value
General demographics				
Age (years)	64.6 (56.0-70.8)	62.4 (52.6-69.2)	63.0 (55.3-69.1)	0.298
Sex female n(%)	40 (66.7)	113 (75.3)	137 (74.5)	0.409
Smoking status n(%)				
Never	22 (37.3)	70 (47.3)	81 (45.3)	0.661
Ex-smoker	26 (44.1)	52 (35.1)	71 (39.7)	
Current	11 (18.6)	26 (17.6)	27 (15.1)	
RA characteristics				
General characteristics				
RF positive n(%)	43 (76.8)	110 (74.3)	139 (76.8)	0.859
Anti-CCP positive n(%)	39 (67.2)	93 (66.0)	123 (68.7)	0.879
Disease duration (yrs)	11.5 (7-18.8)	9.5 (4-17)	9 (4-17.5)	0.394
Disease activity				
CRP (mg/L)	8.5 (5-20)	9.5 (5-21)	8 (4-16)	0.305
ESR	22.5 (10.5-41.8)	21 (10.0-34.0)	19 (9-36)	0.515
DAS 28	4.2± 1.3	4.2± 1.4	4.3± 1.5	0.792
Disease severity				
HAQ	1.6 (0.8-2.2)	1.6 (0.5-2.1)	1.5 (0.6-2.1)	0.891
EAD n(%)	45 (75.0)	105 (70.0)	115 (62.5)	0.133
Joint replacement surgery n(%)	20 (33.0)	43 (28.7)	52 (28.3)	0.743
Medication				
Methotrexate n(%)	33 (55.0)	76 (50.7)	110 (59.8)	0.248
Sulphasalazine n(%)	16 (26.7)	48 (32.0)	53 (28.8)	0.699
Hydroxychloroquine n(%)	19 (31.7)	24 (16.0)	35 (19.0)	**0.034**
Anti-TNF n(%)	8 (13.3)	16 (10.7)	22 (12.0)	0.851
Leflunomide n (%)	4 (6.7)	7 (4.7)	5 (2.7)	0.361
Prednisolone n(%)	26 (43.3)	37 (24.7)	67 (36.4)	**0.014**
NSAIDs/COX II n(%)	8 (13.3)	43 (28.7)	59 (32.1)	**0.019**
Anti-hypertensives n(%)	25 (41.7)	61 (40.7)	82 (44.6)	0.763
Statin/fibrate n(%)	12 (20.0)	34 (22.7)	36 (19.6)	0.775
Lipid Profile				
Total cholesterol (mmol/L)	5.3± 1.0	5.5± 1.1	5.4± 1.2	0.343
HDL (mmol/L)	1.7 (1.3-1.9)	1.6 (1.3-1.9)	1.6 (1.3-1.9)	0.284
LDL (mmol/L)	2.9± 1.0	3.4±1.1	3.2 ±1.2	0.123
Triglycerides (mmol/L)	1.2 (1-1.5)	1.1 (0.9-1.6)	1.2 (0.9-1.6)	0.844
ApoA (g/L)	1.7± 0.5	1.7± 0.4	1.6± 0.4	**0.013**
ApoB (g/L)	0.9 ±0.3	1.0± 0.3	1.0± 0.3	0.203
Comorbidities				
Cardiovascular disease n(%)	13 (21.7)	32 (21.3)	38 (20.7)	0.981
Hypertension n(%)	50 (83.3)	103 (69.7)	125 (67.9)	0.061
Insulin resistance n(%)	20 (34.5)	56 (38.9)	68 (38.4)	0.833
Obesity (BMI)	26.4 (24.9-30.3)	27.3 (24.1-31.7)	27.0 (24.4-30.7)	0.726
NCEP defined dyslipidaemia n(%)*	29 (48.3)	91 (60.7)	104 (56.5)	0.263
Death from CVD n(%)	4 (6.7)	1 (0.7)	9 (4.9)	**0.043**

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 13: Demographic, clinical and laboratory characteristics across the ApoA4 rs675 genotypes

	AA (N=13)	AT (N=118)	TT (N=266)	P value
General demographics				
Age (years)	64.2 (53.4-68.6)	62.9 (54.3-69.5)	63.0 (55.4-69.2)	0.725
Sex female n(%)	7 (53.8)	91 (77.1)	193 (72.6)	0.177
Smoking status n(%)				
Never	5 (38.5)	55 (47.4)	115 (44.2)	0.528
Ex-smoker	4 (30.8)	40 (34.5)	105 (40.4)	
Current	4 (30.8)	21 (18.1)	40 (15.4)	
RA characteristics				
General characteristics				
RF positive n(%)	11 (84.6)	83 (72.8)	200 (76.6)	0.548
Anti-CCP positive n(%)	9 (69.2)	71 (64.0)	177 (68.9)	0.647
Disease duration (yrs)	8 (5.5-18.5)	10 (5-16)	10 (4-18)	0.950
Disease activity				
CRP (mg/L)	8 (6-23)	9 (5-20)	8 (4-18)	0.640
ESR	19 (8.5-70.5)	20 (10.0-33.5)	20 (9-38)	0.599
DAS 28	4.8± 1.5	4.1± 1.3	4.3± 1.4	0.129
Disease severity				
HAQ	1.8 (0.7-2.2)	1.5 (0.4-2.1)	1.6 (0.7-2.3)	0.209
EAD n(%)	10 (76.9)	82 (69.5)	175 (65.8)	0.583
Joint replacement surgery n(%)	3 (23.1)	37 (31.4)	75 (28.2)	0.732
Medication				
Methotrexate n(%)	5 (38.5)	67 (56.8)	150 (56.4)	0.435
Sulphasalazine n(%)	5 (38.5)	42 (35.6)	71 (26.7)	0.166
Hydroxychloroquine n(%)	5 (38.5)	24 (20.3)	51 (19.2)	0.238
Anti-TNF n(%)	1 (7.7)	11 (9.3)	34 (12.8)	0.562
Leflunomide n (%)	0 (0)	3 (2.5)	13 (4.9)	0.422
Prednisolone n(%)	5 (38.5)	40 (33.9)	85 (32.0)	0.844
NSAIDs/COX II n(%)	3 (23.1)	28 (23.7)	80 (30.1)	0.408
Anti-hypertensives n(%)	6 (46.2)	39 (33.1)	124 (46.6)	**0.045**
Statin/fibrate n(%)	5 (38.5)	20 (16.9)	57 (21.4)	0.165
Lipid Profile				
Total cholesterol (mmol/L)	4.8± 0.6	5.5± 1.1	5.4± 1.1	0.176
HDL (mmol/L)	1.6 (1.4-1.8)	1.6 (1.4-1.9)	1.6 (1.2-1.8)	0.290
LDL (mmol/L)	2.9 ± 0.9	3.3 ±1.1	3.3 ±1.2	0.721
Triglycerides (mmol/L)	1.1 0.9-1.5)	1.1 (0.9-1.5)	1.2 (0.9-1.6)	0.308
ApoA (g/L)	1.7 ± 0.6	1.8 ± 0.5	1.6 ± 0.4	**0.013**
ApoB (g/L)	0.9 ±0.1	1.0± 0.3	1.0± 0.3	0.611
Comorbidities				
Cardiovascular disease n(%)	5 (38.5)	21 (17.8)	59 (22.2)	0.196
Hypertension n(%)	11 (84.6)	85 (72.0)	184 (69.2)	0.448
Insulin resistance n(%)	4 (30.8)	40 (36.0)	101 (39.1)	0.736
Obesity (BMI)	29.7 (25.3-32.1)	26.7 (24.1-30.9)	27.1 (24.4-30.7)	0.561
NCEP defined dyslipidaemia n(%)*	7 (53.8)	64 (54.2)	155 (58.3)	0.743
Death from CVD n(%)	0 (0)	4 (3.7)	10 (3.8)	0.769

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 14: Demographic, clinical and laboratory characteristics across the ApoA5 rs31335006 genotypes

	CC (N=3)	CG (N=42)	GG (N=348)	P value
General demographics				
Age (years)	35.0	61.6 (54.0-69.3)	63.1 (55.1-69.5)	0.450
Sex female n(%)	3 (100)	26 (61.9)	258 (74.1)	0.138
Smoking status n(%)				
Never	1 (33.3)	21 (52.5)	152 (44.4)	0.702
Ex-smoker	2 (66.7)	13 (32.5)	132 (38.6)	
Current	0 (0)	6 (15.0)	58 (17.0)	
RA characteristics				
General characteristics				
RF positive n(%)	3 (100)	29 (72.5)	258 (75.8)	0.556
Anti-CCP positive n(%)	3 (100)	27 (64.3)	224 (67.5)	0.441
Disease duration (yrs)	3 (1-5)	9 (4.8-17.3)	10 (4-18)	0.355
Disease activity				
CRP (mg/L)	3 (3-5)	7 (5-13.3)	8 (5-20)	0.187
ESR	22 (7-25)	18.5 (8-29.5)	20 (10-37)	0.293
DAS 28	4.1± 0.7	4.3± 1.4	4.2± 1.4	0.864
Disease severity				
HAQ	0.5 (0.3-1.3)	1.9 (0.7-2.3)	1.5 (0.6-2.1)	0.160
EAD n(%)	1 (33.3)	30 (71.4)	234 (67.2)	0.387
Joint replacement surgery n(%)	0 (0)	13 (31.0)	100 (28.7)	0.519
Medication				
Methotrexate n(%)	3 (100)	25 (59.5)	192 (55.2)	0.264
Sulphasalazine n(%)	1 (33.3)	15 (35.7)	99 (28.4)	0.613
Hydroxychloroquine n(%)	0 (0)	10 (23.8)	69 (19.8)	0.568
Anti-TNF n(%)	1 (3.3)	2 (4.8)	42 (12.1)	0.183
Leflunomide n (%)	0 (0)	0 (0)	15 (4.3)	0.365
Prednisolone n(%)	0 (0)	17 (40.5)	111 (31.9)	0.257
NSAIDs/COX II n(%)	0 (0)	11 (26.2)	99 (28.4)	0.530
Anti-hypertensives n(%)	1 (33.3)	16 (38.1)	150 (43.1)	0.783
Statin/fibrate n(%)	0 (0)	6 (14.3)	75 (21.6)	0.369
Lipid Profile				
Total cholesterol (mmol/L)	5.7± 1.6	5.5± 1.1	5.4± 1.1	0.811
HDL (mmol/L)	1.6 (1.4-1.8)	1.6 (1.4-1.9)	1.6 (1.2-1.8)	0.119
LDL (mmol/L)	3.9 ± 1.6	3.3 ±1.2	3.2 ±1.1	0.524
Triglycerides (mmol/L)	0.8 (0.6-1.0)	1.4 (1.1-2.1)	1.1 (0.9-1.6)	**0.016**
ApoA (g/L)	1.7 ± 0.3	1.6 ± 0.4	1.7 ± 0.5	0.854
ApoB (g/L)	1.2 ±0.6	1.0± 0.3	1.0± 0.3	0.376
Comorbidities				
Cardiovascular disease n(%)	0 (0)	10 (23.8)	72 (20.7)	0.601
Hypertension n(%)	2 (66.7)	30 (71.4)	244 (70.1)	0.976
Insulin resistance n(%)	0 (0)	16 (40.0)	127 (37.9)	0.385
Obesity (BMI)	24.4 (19.8-27.0)	26.1 (23.1-29.6)	27.1 (24.6-30.8)	0.169
NCEP defined dyslipidaemia n(%)*	3 (100)	28 (66.7)	192 (55.2)	0.115
Death from CVD n(%)	0 (0)	1 (2.4)	13 (3.7)	0.856

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate
RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Table 15: Demographic, clinical and laboratory characteristics across the ApoE genotypes

	E2 allele (E2E2, E2E3) (n= 48)	E3 allele (E3E3) (n=230)	E4 allele (E3E4, E4E4) (n= 94)	P value
General demographics				
Age (years)	63.2 (57-72.6)	62.5 (53.8-69.1)	58 (51.6-66.1)	0.061
Female sex n(%)	33 (67.3)	175 (74.5)	68 (70.8)	0.537
Smoking status n(%)				
Never	21 (43.8)	107 (46.5)	39 (41.5)	0.943
Ex-smoker	19 (39.6)	87 (37.8)	38 (40.4)	
Current	8 (16.7)	36 (15.7)	17 (18.1)	
RA characteristics				
General characteristics				
RF positive n(%)	40 (87)	168 (72.7)	71 (75.5)	0.124
Anti-CCP positive n(%)	35 (74.5)	150 (66.7)	63 (68.5)	0.578
Disease duration (years)	10 (3.5-17.5)	11 (5-17)	10 (4-16)	0.838
Disease Activity				
CRP (mg/L)	14 (6.5-23.5)	10 (5-23)	7 (4-11)	**0.001**
ESR (mm/hour)	19 (10-36.5)	21 (9-37)	13 (5-26)	**0.007**
DAS 28	4.3 ± 1.4	4.3 ± 1.4	3.8± 1.5	**0.023**
Disease severity				
HAQ (0-3)	1.5 (0.3-2.1)	1.5 (0.5-2.1)	1.4 (0.34-2.)	0.502
EAD n(%)	32 (65.3)	153 (65.1)	69 (71.9)	0.480
Joint replacement surgery n(%)	15 (30.6)	72 (30.6)	25 (26.0)	0.695
Medications				
Methotrexate n (%)	19 (38.8)	137 (58.3)	57 (59.4)	**0.033**
Sulphasalazine n(%)	15 (30.6)	68 (28.9)	33 (34.4)	0.622
Hydroxychloroquine n(%)	12 (24.5)	44 (18.7)	20 (20.8)	0.638
Anti-TNF n(%)	5 (10.2)	26 (11.1)	12 (12.5)	0.901
Leflunomide n(%)	3 (6.1)	6 (2.6)	6 (6.3)	0.206
Prednisolone n(%)	17 (34.7)	76 (32.3)	34 (35.4)	0.848
NSAIDs/COXII n(%)	13 (26.5)	67 (28.5)	28 (29.2)	0.945
Anti-hypertensives n(%)	20 (40.8)	101 (43.0)	42 (43.8)	0.944
Statin/fibrates n(%)	6 (12.2)	43 (18.3)	28 (29.2)	**0.027**
Lipids				
Total cholesterol (mmol/L)	4.8 ± 0.9	5.6 ± 1.0	5.6 ± 1.4	**<0.001**
HDL (mmol/L)	1.6 (1.2-1.9)	1.6 (1.3-1.9)	1.5 (1.2-1.8)	0.060
LDL (mmol/L)	2.52 ± 1.0	3.3 ± 1.1	3.6 ± 1.3	**<0.001**
Triglycerides (mmol/L)	1.2 (1-1.4)	1.2 (0.9-1.6)	1.3 (1-1.8)	0.278
Apo A (g/L)	1.7 ± 0.4	1.7 ± 0.5	1.6 ± 0.4	0.397
Apo B (g/L)	0.8 ± 0.2	1.0 ± 0.3	1.1 ±0.3	**<0.001**
Co-morbidities				
Cardiovascular disease n(%)	8 (16.3)	48 (20.4)	27 (28.1)	0.186
Insulin resistance n(%)	15 (33.3)	88 (38.3)	36 (39.1)	0.790
Hypertension n(%)	32 (65.3)	170 (72.3)	67 (69.8)	0.597
Obesity (BMI)	26.1 (24.1-30.1)	27 (24.2-30.9)	26.5 (23.6-29.3)	0.739
NCEP defined dyslipidaemia n(%)*	14 (28.6)	134 (57.0)	68 (70.8)	**<0.001**
Deaths from CVD n(%)	2 (4.1)	7 (3)	5 (5.2)	0.613

Results expressed as percentages, median (25-75th percentile values) or mean ± SD as appropriate

RF: Rheumatoid factor, Anti-CCP: Anti-Cyclic Citrullinated Peptide, CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, EAD: Extra-Articular Disease, NSAIDs/COX II: non steroidal anti-inflammatory drugs/ cyclooxygenase II inhibitors, TNF: Tumor Necrosis Factor, BP: blood pressure, HDL: High Density Lipoprotein, LDL: low density lipoprotein, ApoA: apolipoprotein A, ApoB: apolipoprotein B, Lp (a): lipoprotein (a), BMI: Body Mass Index, NCEP: national cholesterol education program, * =total cholesterol ≥6.2 or triglycerides ≥ 1.7 mmol/L or LDL ≥4.13 mmol/L or HDL <1.03 mmol/L or on a statin

Appendix 4: Publications arising from this thesis

Much of the work arising from this thesis has now been published in peer review journals. These include:

1) Dyslipidaemia in Rhauematoid Arthritis: the role of inflammation, drugs, lifestyle and genetic factors. Toms TE, Symmons DP, Kitas GD. Curr Vasc Pharmacol. 2010 May 1;8(3):301-26
2) Statin use in rheumatoid arthritis in relation to actual cardiovascular risk: evidence for substantial undertreatment of lipid-associated cardiovascular risk? Toms TE, Panoulas VF, Douglas KM, Griffiths H, Sattar N, Smith JP, Symmons DP, Nightingale P, Metsios GS, Kitas GD. Ann Rheum Dis. 2010 Apr;69(4):683-8
3) Are lipid ratios less susceptible to change with systemic inflammation than individual lipid components in patients with rheumatoid arthritis? Toms TE, Panoulas VF, Douglas KM, Nightingale P, Smith JP, Griffiths H, Sattar N, Symmons DP, Kitas GD.Angiology. 2011 Feb;62(2):167-75.
4) Rheumatoid arthritis susceptibility genes associate with lipid levels in patients with rheumatoid arthritis. Toms TE, Panoulas VF, Smith JP, Douglas KM, Metsios GS, Stavropoulos-Kalinoglou A, Kitas GD. Ann Rheum Dis. 2011 Jun;70(6):1025-32.
5) Apolipoprotein E gene polymorphisms are strong predictors of inflammation and dyslipidemia in rheumatoid arthritis. Toms TE, Smith JP, Panoulas VF, Blackmore H, Douglas KM, Kitas GD. J Rheumatol. 2012 Feb;39(2):218-25

For all papers arising from this thesis please see the CD enclosed inside the back cover of the thesis.