Cardiovascular disease in Autoimmune Rheumatic Diseases

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Declaration

I, Jyoti Bakshi, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

SLE and Rheumatoid Arthritis both carry an early and increased risk of cardiovascular diseases (CVD). I explored the following linked areas that may contribute to the increased risk;

Immunological markers- Apolipoprotein A1 (ApoA1) is the main constituent of high density lipoproteins (HDL), which have an atheroprotective role. Using ELISA, there were no differences in serum levels of IgG anti-apoA-1 antibodies in SLE CVD patients compared to SLE controls. I studied invariant natural killer cells *(i*NKT) responses from serum samples of RA CVD, matched RA controls and healthy PBMCs using flow cytometry. *i*NKT cells are known to play a role in both autoimmunity and atherosclerosis. No differences in the RA cohort were found.

Carotid vascular ultrasound studies -Using a cohort of previously scanned 100 patients with no history of CVD, I re-scanned 69% of this cohort on average 5 years later and found that 9% developed new plaque, 26% had plaque progression (increase in plaque number) and almost all patients who developed CVD events had plaque at baseline (6/7). Age >52 years and a systolic BP>133 mmol/ mg were independent predictors of plaque progression. Novel ultrasound measures such as total plaque area and plaque echolucency may be better predictors of CVD risk in SLE, but further studies are required.

Metabolomics- I found that various subclasses and sizes of very low density lipoprotein (VLDL) concentrations were significantly higher in SLE vs HC. I have identified five individual VLDL metabolites whose levels could be measured to

distinguish SLE-P from SLE-NP. Significantly, higher concentrations in of HDL particles was found in HC vs RA.

Traditional CVD risk factors- I explored the feasibility of *assessing traditional risk factors*, by designing a simple one-page protocol. Using this protocol in the SLE population at UCLH at the time, 77% (309/400) of patients in the total cohort were captured over a 9-month period.

Contribution to Chapters

I tested all of my anti-ApoA1 ELISA samples in the case-control group and most of the samples in the Vascular Ultrasound Cohort. I sex/age matched the RA samples from our collaborators and tested the RA samples in my iNKT studies. I conceptualised the idea of looking at RA to build on the previous work in SLE by my supervisor Professor Jury's group.

In the metabolomics chapter, I analysed the data for the SLE samples which had been previously sent and wanted to look at the metabolomics of my RA cohort to see if there were differences between the SLE cohort. I selected all the RA samples for metabolomic studies and analysed the raw data.

I contacted and recruited the patients for the follow up vascular ultrasound study from the original cohort and analysed the follow up data. The vascular ultrasound scans were performed by the same ultrasonographer Dr Maura Griffin, who also performed the scans in the original cohort.

I formulated the idea of how well we assess and manage CVD risk factors in the SLE clinic and designed and implemented the CVD risk protocol.

Impact statement

There is currently a significant academic interest in the resolution of the mechanisms of increased cardiovascular risk in SLE. The data generated in my thesis may lead to the development of potential immunological biomarkers linking inflammation with atherosclerosis in patients with SLE, which would be of interest to basic immunologists studying the properties of autoantibodies and invariant natural killer T cells (*i*NKT). The study of *i*NKT cells is an emerging field and most information on the effects of these cells on atherosclerosis comes from mouse models. Thus, my work will be beneficial to *i*NKT researchers.

This research will encourage imaging researchers looking at the characteristics of atherosclerotic plaque in patients with autoimmune diseases. My database of imaging data from 69 patients with SLE contains detailed information about sensitive outcome measures such as echogenicity and total plaque area, which have not been reported by other groups working on SLE. Thus, my results on clinical and immunological factors associated with these outcomes will advance this field.

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Abbreviations

2D: two-dimension
37°C: 37 degrees Celsius
3D: three-dimension
ACR: American college of Rheumatology
ACS: acute coronary syndrome
AHA: American Heart Association
ANA: antinuclear antibodies
Anti-ApoA1: anti-apolipoprotein 1 antibodies
ACPA: anti-citrullinated protein antibodies
Anti-dsDNA: anti-double stranded DNA antibodies
AU: absorbance units
BILAG: British Isles Lupus Assessment Group index
C: Caucasian
C3: complement fraction 3
CAD: coronary artery disease
CCA: common carotid artery
CFA: common femoral artery
CIMT: carotid intimal medial thickness
CNS: central nervous system
CRP: C-reactive protein
CT: computed tomography
CVD: Cardiovascular disease
DAS28:Disease Activity Score of 28 joints
DMARDs: Disease modifying antirheumatic drugs
EBV: Epstein-Barr virus
ECA: external carotid artery
ENA: anti-extractable nuclear antigens antibodies
ESR: Erythrocyte Sedimentation Rate
GSM: grey scale median
HC: healthy control
h: hour

HDL: high-density lipoprotein

HCQ: hydroxychloroquine

HRP: horse radish peroxidase

*i*NKT: Invariant Natural killer cells

ICA: internal carotid artery

IDL: intermediate-density lipoprotein

IL1: interleukin 1

IMT: intima-media thickness

IQR: interquartile range

iTCR: invariant T-Cell receptor

LDL: low-density lipoprotein

MGV: mean grey value

MHC: major histocompatibility complex

MI: myocardial infarction

min: minutes

MRI: magnetic resonance imaging

NO: nitric oxide

NOS: nitric oxide synthetase

NSAIDs: non-steroidal anti-inflammatory drugs

OD: optical density

Paraoxonase 1: PON1

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffer solution

PC: positive control

PDGF: platelet-derived growth factor

RA: rheumatoid arthritis

RA-P: rheumatoid arthritis plaque patient

RA-NP: rheumatoid arthritis non plaque patient

RF: Rheumatoid factor

RT: room temperature

SLE: Systemic Lupus Erythematosus

SLICC: Systemic Lupus International Collaborative Clinics

TC: total cholesterol

TG: triglycerides

TNFα: tumour necrosis factor alpha

UCLH: University College London Hospital

US: ultrasound

UV: ultraviolet

VLDL: very low-density lipoprotein

VEGF: vascular endothelial growth fact

1 INTRODUCTION

Autoimmune rheumatic diseases (ARDs) are characterized by defects in the immune system, whereby the usual barriers of protection are disrupted resulting in the body attacking its own tissue. Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA are more prevalent in women, but SLE has a higher sex ratio (9:1 compared to RA, which is 3-5:1). SLE and RA in addition carry an increased risk of cardiovascular diseases (CVD). CVD is defined by the world health organization as a group of disorders of the heart and blood vessels, which includes coronary heart disease (CHD), peripheral vascular disease (PVD) and cerebrovascular disease (CVD).

1.1.1 Systemic Lupus Erythematosus pathophysiology

SLE is a multi-system ARD characterized by deposition of autoantibodies and immune complexes, leading to widespread tissue damage. The exact pathophysiology remains unknown, but is thought to be the consequence of a complex interplay of genetic, immunoregulatory and environmental factors.

The concordance rate for SLE is 25% amongst monozygotic twins and approximately 2% amongst dizygotic twins (Sullivan 2000, Niewold 2015), strongly suggestive of a genetic contribution. More than 60 genetic associations with SLE have been identified through genomic wide association studies and candidate gene approaches (Gatto, Zen et al. 2013, Mohan and Putterman 2015). The genes identified include those involved in the transcription of proteins, which regulate apoptosis, cytokine and immunoglobulin production and antigen presentation.

The most common genetic predisposition in lupus is found at the major histocompatibility (MHC) locus that contains genes for antigen presenting molecules, but also genes for some complement components and cytokines. HLA-A1, B8 and DR3 have been linked to SLE (Delgado-Vega, Sánchez et al. 2010). Genetic factors with the strongest risk for lupus are homozygous deficiencies of the complement components C1q (required for clearance of apoptotic waste), C2 or C4 (required for elimination of autoreactive B cells) (Walport 2002, Niewold 2015). Fortunately, each of these is relatively rare in the population. The reason for the increased prevalence of SLE in females remains unclear; gender differences may be related to genetic contributions on the X and Y chromosome rather than due to sex hormones. In

castrated mice of both genders who have genetic manipulations of their chromosomes, the presence of two X chromosomes increases the severity of SLE (Tsokos 2011).

Ultraviolet radiation (UV) and infectious triggers have been associated with the development of SLE. UV light is well known to exacerbate skin disease and the presence of a photosensitive rash. Pathologically, UVB is a potent inducer of apoptosis. Intermediate to high exposure to ultraviolet B has been associated with autoantigen release and cytokine production and seems to be dose dependent (Caricchio, McPhie et al. 2003). An antecedent viral-like illness may occur at the onset of lupus or immediately before a flare. Epstein-Barr virus (EBV) infections may be important since temporal associations between the onset of SLE and flares have been observed. Relationships between SLE and viral EBV DNA have been reported from peripheral blood (Yu, Wu et al. 2005). Development of antibodies to these molecules may occur through molecular mimicry, causing autoimmunity. However, given that a large percentage of the healthy population are infected by EBV, it remains unclear whether this observed association is causative.

1.1.2 **Cellular dysregulation and cytokine disruption in SLE**

The key stages in the development of lupus include a breach of tolerance in the adaptive immune system (B and T cells), amplification of autoimmunity through dysregulation of the immune system and end-organ damage (Marian and Anolik 2012). There are numerous immune defects in lupus recognized amongst T and B-lymphocytes, natural killer cells and antigen presenting cells (APCs). T-cells are central to the development of lupus. The breakdown of immunological tolerance through the activation of self-reactive autoaggressive T cells and defects in T cell suppression are important in disease pathogenesis (Tsokos 2011, Gatto, Zen et al. 2013). T-cell help is crucial for the production of autoantibodies in lupus. T-cells express receptors, which interact best with a given antigen presented in a complex with a MHC molecule on the surface of the APC. A second molecular interaction between the T-cell and APC is required through co-stimulation. The effect on the T cells depends on the interaction with other molecules on the surface of the two cells, which can either lead to activation (causing cytokine release and B-cell help) or suppression of T-cell activity (Rahman and Isenberg 2008, Alexander, Radbruch et al. 2015, Sharabi and Tsokos 2020).

An immune response is triggered in lupus to antigens normally buried inside the cell, which are exposed on superficial surface blebs that are formed as part of the normal process of apoptosis. The blebs consisting of cellular material are exposed on the surface of dying cells during apoptosis. Nucleosomes, Ro, La and anionic phospholipids are some of the exposed antigens (Alexander, Radbruch et al. 2015). Antibodies to these antigens are commonly found in SLE patients.

The cytokine profile in SLE changes with different disease phenotypes and disease severity. Interleukin-10 (IL-10) serum levels have been consistently shown to be high in SLE patients. IL-10 stimulates polyclonal B-cells, encouraging continued antibody production (Rahman and Isenberg 2008). Serum levels of interferon-alpha (INF-α) are elevated in SLE patients with active disease (Crow 2014) . INF-α can rapidly mobilise innate immune cells and genetic studies have shown a pathogenic role of excessive INF-α signalling in SLE (Niewold 2011). Fragments of RNA and DNA from dead cells are normally taken up by phagocytes; however, due to the failure of efficient clearance of apoptotic material in SLE, these fragments can bind to specific Toll-like receptors (TLRs). The subsequent activation of TLR signalling leads to increased and sometimes chronic production of INF-α and other pro-inflammatory cytokines (Thanou and Merrill 2014). INF-α activates many components of the immune system including myeloid cells. Myeloid cell cells may over produce BLyS, which promotes B-cell maturation and survival. The development of nephritis was found to be accelerated in lupus prone mice continuously exposed to interferon-alpha from a young age compared to controls (Mathian, Weinberg et al. 2005, Crow 2014).

The B-lymphocyte stimulator (BLyS), a member of the TNF-ligand superfamily promotes B-cell survival, proliferation and immunoglobulin production. Increased levels of BLyS have been found in SLE patients (Rahman and Isenberg 2008, Alexander, Radbruch et al. 2015, Morawski and Bolland 2017).

1.1.3 Defects in apoptosis in SLE

Defects in apoptosis, resulting in abnormal programmed cell death have been strongly associated with the development of SLE (Yu, Wu et al. 2005, Mistry and Kaplan 2017). Early apoptotic cells express cell surface proteins that promote circulating immune cells to engulf these cells and express signals to attract macrophages and dendritic cells. In addition to abnormal apoptosis, there is an impairment of "waste disposal' in SLE. C1q and antiphospholipid antibodies enhance opsonization and clearance and therefore a deficiency of C1q (and C2 and C4) may impair phagocytosis and delay clearance (Gatto M, 2013).

Many of the clinical manifestations of lupus are a result of immune complex deposition in tissue. Immune complexes are formed as antinuclear antibodies binding to nuclear material, which is not promptly cleared because the Fc and complement receptors are numerically and functionally deficient (Gatto M, 2013, (Mistry and Kaplan 2017). Figure 1 summarises the pathogenesis of SLE.

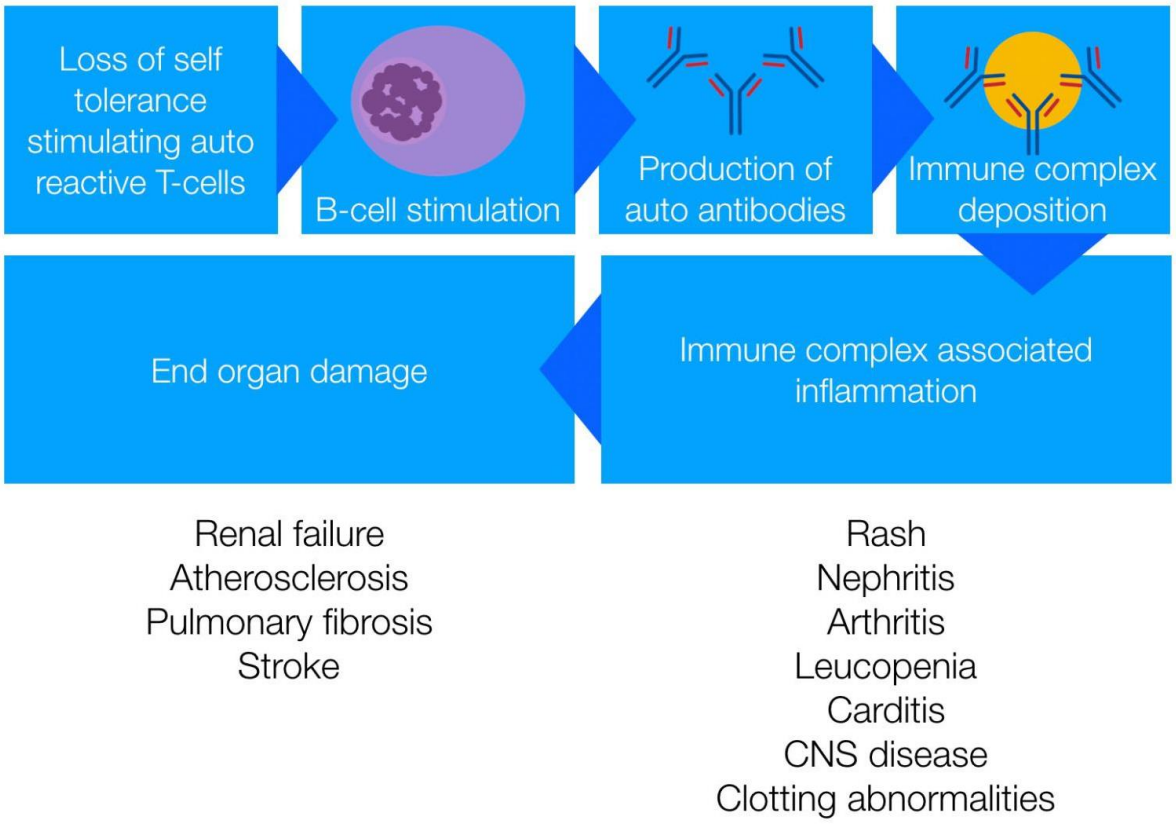


FIGURE 1 - PATHOGENESIS OF SLE

Genetic and environmental stimuli induce loss of self-tolerance causing auto-reactive T-cells to stimulate B-cells, resulting in the production of autoantibodies. Autoantibodies form immune complexes, which are not cleared resulting in end organ damage and inflammation via complement activation.

1.1.4 Epidemiology, clinical features and classification criteria of SLE

The term “lupus” (Latin for wolf) was first used in the middle ages to describe erosive skin lesions evocative of a wolf’s bite. In 1846, the Viennese physician Ferdinand von Hebra (1816-1880) introduced the butterfly metaphor to describe the malar rash. SLE was first recognized as a systemic disease with visceral manifestations by Moriz Kaposi (1837-1902) and further established by Osler in Baltimore and Jadassohn in Vienna (Scofield and Oates 2009). SLE is therefore a complex autoimmune multi-systems disease with a broad spectrum of clinical presentations encompassing almost all organs and tissues. Patients may present with constitutional symptoms (fever, fatigue arthralgia and weight loss).

Several sets of classification criteria have been developed for SLE, which are useful for categorising patients for the inclusion into research studies. The thee most commonly used ones are the 1997 ACR criteria, 2012 SLICC criteria and 2019 EULAR/ACR criteria. I will discuss each one these in turn in the section below.

The 1997 American College of Rheumatology (ACR) revised classification criteria (Hochberg 1997) are internationally used to diagnose SLE and include a constellation of clinical symptoms, haematological and immunological abnormalities (table 1). This classification criteria, which was modified from an earlier set (Tan et al 1982) was subsequently established by cluster analysis, mainly in academic centres and in Caucasian patients (Petri and Magder 2004). When tested against other rheumatic diseases, these criteria have a sensitivity of 94% and specificity of

approximately 96 percent. SLE is diagnosed if 4 or more of the 11 criteria are present (Hochberg 1997). There are however obvious limitations to this criteria set. Examples include the duplication of correlated cutaneous features (such as malar rash and photosensitivity), the lack of inclusion of other cutaneous manifestations and neurological manifestations, lack of inclusion of markers of disease activity such as low serum complement and patients with biopsy proven nephritis may not meet the criteria (Petri, Orbai et al. 2012).

With these limitations of the ACR criteria in mind, in 2012 the Systemic Lupus International Collaboration Clinics (SLICC) proposed the 2012 SLICC criteria. SLE classification by these criteria (table 2) requires either that a patient satisfy at least 4 of 17 items, including at least 1 of the 11 clinical criteria and one of the six immunologic criteria, or that the patient has biopsy-proven nephritis compatible with SLE in the presence of ANA or anti-double-stranded DNA (anti-dsDNA) antibodies. The SLICC criteria were validated in 690 patients and had greater sensitivity but lower specificity than the 1997 ACR classification criteria (sensitivity of 97% versus 83% and specificity of 84% versus 96%, respectively) (Petri, Orbai et al. 2012). Therefore, despite the improved sensitivity compared with the ACR criteria, the SLICC criteria might delay the diagnosis of SLE in a significant number of patients, and some patients might not be classified at all (Petri, Orbai et al. 2012).

The European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) combined to develop another classification criteria set for SLE to improve detection of early- or new-onset SLE, as well as improve the sensitivity and specificity compared with previous criteria (table 3). The criteria rely on a scoring

system for clinical and laboratory domains and a positive ANA by immunofluorescence as an entry criteria. Additive criteria consist of seven clinical (constitutional, hematologic, neuropsychiatric, mucocutaneous, serosal, musculoskeletal and renal) and three immunologic (antiphospholipid antibodies, complement proteins, SLE-specific antibodies) categories, each of which are weighted from two to ten. Patients are classified as having SLE with a score of 10 or more points. In the validation cohort, which included patients with early disease, the EULAR/ACR criteria had a sensitivity of 96% and a specificity of 93%, compared with the 97% sensitivity and 84% specificity of the Systemic Lupus International Collaborating Clinics (SLICC) criteria and the 83% sensitivity and 93% specificity of the ACR criteria (Aringer, Costenbader et al. 2019). Its limitation is the lack of recognition of ANA negative SLE and the time take to derive the score.

Thus, the 2019 EULAR/SLE classification criteria has the equal highest specificity and the second highest sensitivity compared to the other classification criteria sets. Whilst these criteria are useful for research purposes, diagnosis is often based upon judgement of an experienced clinician who recognizes characteristic constellations of symptoms and signs in the setting of supportive serologic studies after excluding alternative diagnoses.

System	Description
Skin	
Malar rash	Malar Rash/butterfly rash- over the cheeks and nose
Discoid rash	Thick, disc like rash that scars-usually sun exposed areas
Photosensitivity	Rash that occurs after being exposed to UVA or B
Oral ulcers	Recurrent oral or nasal ulceration
Systemic	
Arthritis	Non-erosive arthritis involving two or more peripheral joints, characterised by tenderness or swelling
Serositis	Inflammation of the pleura or the heart
Renal disorder	Persistent proteinuria > 0.5/ day or > than 3+ if quantification or abnormal urine sediment under microscope
Neurologic disorder	Seizures or psychosis
Laboratory criteria	
Haematological	Haemolytic Anaemias/ low white cell counts/ low platelets
	Either a positive lupus anticoagulant preparation, anti-
	Anti-DNA – Antibody to native DNA in abnormal titer OR
Immunologic disorder	Anti-Sm – Presence of antibody to Sm nuclear antigen OR
	Positive antiphospholipid antibody on:

	An abnormal serum level of IgG or IgM anticardiolipin antibodies OR A positive test result for lupus anticoagulant using a standard method OR
Positive Anti-nuclear antibody	A false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test

TABLE 1 ACR CRITERIA FOR DIAGNOSIS OF SLE (MODIFIED FROM (HOCHBERG 1997)).

Four out of the eleven is considered as meeting the classification criteria for a diagnosis of SLE.

Clinical Criteria	Immunological Criteria
Acute Cutaneous Lupus	1. ANA
Chronic cutaneous Lupus	2. Anti-DNA
Oral or nasal ulcers	3. Anti-Sm
Non-scarring alopecia	4. Anti-phospholipid Ab
Arthritis	5. Low complement
Serositis	6. Direct Coombs test
Renal	
Neurologic	
Haemolytic anaemia	
Leukopenia	
Thrombocytopenia	

TABLE 2 - SLICC CLASSIFICATION CRITERIA FOR SLE (PETRI, ORBAI ET AL. 2012)

Requirements 4 or more (at least one clinical or one immunological feature) or biopsy proven nephritis with positive ANA or anti-DNA.

Entry criterion

Antinuclear antibodies (ANA) at a titer of ≥1:80 on HEp-2 cells or an equivalent positive test (ever)

↓

If absent, do not classify as SLE
If present, apply additive criteria

↓

Additive criteria

Do not count a criterion if there is a more likely explanation than SLE.
Occurrence of a criterion on at least one occasion is sufficient.
SLE classification requires at least one clinical criterion and ≥10 points.
Criteria need not occur simultaneously.
Within each domain, only the highest weighted criterion is counted toward the total score§.

Clinical domains and criteria	Weight	Immunology domains and criteria	Weight
Constitutional		***Antiphospholipid antibodies***	
Fever	2	Anti-cardiolipin antibodies OR	
Hematologic		Anti-β2GP1 antibodies OR	
Leukopenia	3	Lupus anticoagulant	2
Thrombocytopenia	4	***Complement proteins***	
Autoimmune hemolysis	4	Low C3 OR low C4	3
Neuropsychiatric		Low C3 AND low C4	4
Delirium	2	***SLE-specific antibodies***	
Psychosis	3	Anti-dsDNA antibody* OR	
Seizure	5	Anti-Smith antibody	6
Mucocutaneous			
Non-scarring alopecia	2		
Oral ulcers	2		
Subacute cutaneous OR discoid lupus	4		
Acute cutaneous lupus	6		
Serosal			
Pleural or pericardial effusion	5		
Acute pericarditis	6		
Musculoskeletal			
Joint involvement	6		
Renal			
Proteinuria >0.5g/24h	4		
Renal biopsy Class II or V lupus nephritis	8		
Renal biopsy Class III or IV lupus nephritis	10		

Total score:

↓

Classify as Systemic Lupus Erythematosus with a score of 10 or more if entry criterion fulfilled.

TABLE 3 - EULAR/ACR 2019 CLASSIFICATION CRITERIA FOR SLE (ARINGER, COSTENBADER ET AL. 2019)

The serological hallmark of SLE is the presence of autoantibodies directed against many nuclear, cytoplasmic and plasma membrane antigens. At least 95% of SLE patients have anti-nuclear antibodies (ANA). Antibodies to double stranded DNA are present in 60-70% of SLE patients and are highly specific for lupus and often correlate with disease activity; especially in lupus patients with nephritis. Many other antibodies that have been identified in lupus (Alexander, Radbruch et al. 2015).

1.1.5 Treatment and Prognosis of SLE

The survival rate of patients with SLE has improved significantly over the last 5 decades, from less than 50% at 5 years in 1955 to 95% more recently (Petri 2002, Vilas-Boas, Bakshi et al. 2015). Early mortality, within 5 years, is predominantly due to organ failure or overwhelming infections, both of which can be altered by early diagnosis and treatment. The mortality risk is fivefold when compared to the normal population in the late stages, largely attributed to cardiovascular disease from accelerated atherosclerosis, the leading cause of death for SLE patients (Petri 2002).

The introduction of steroid therapy in the 1950s revolutionized the treatment of SLE and remains the mainstay of treatment for all, but the mildest forms of disease. However, there are multiple side effects associated with long-term use of steroids (e.g. increased infection risk, mood changes, diabetes, hypertension and osteoporosis).

Hydroxychloroquine is used commonly in SLE and has immunomodulatory effects; it increases lysosomal pH and interferes with antigen processing. Hydroxychloroquine is recommended by the European League in Rheumatism (EULAR) for mild disease and has been shown to be very effective in the management of mucocutaneous disease, serositis and fatigue (Bertsias et al, 2008). In the Lupus in Minority populations: Nature versus Nurture (LUMINA) trail, a case-control study of >600 patients showed the use of hydroxychloroquine not only reduced flares but also reported reduced mortality. Hydroxychloroquine had a protective effect on survival (OR 0.128 (95% CI 0.054 to 0.301) for hydroxychloroquine alone (Alarcón, McGwin et

al. 2007) Hydroxychloroquine has also been shown to be protective in renal lupus (Pons-Estel, Alarcón et al. 2009).

Disease modifying agents are used as steroid sparing agents and these include Azathioprine, methotrexate (mainly used for skin and joint disease), cyclosporine (CYC), mycophenolate mofetil (MMF) (used for induction and maintenance therapy in lupus nephritis) and tacrolimus (used for treatment of lupus nephritis and cutaneous lupus) (Xiong and Lahita 2014, Bakshi, Segura et al. 2018)**.**

Patients with significant major organ involvement are often treated with high dose intravenous methylprednisolone, and immunosuppressant therapies (including cyclophosphamide (CYC) and MMF). Cyclophosphamide-based regimens have shown long-term efficacy in SLE nephritis (Gelber, Christopher-Stine et al. 2004, Bakshi, Segura et al. 2018) but this drug in its original dose regime (proposed by the National Institute of Health giving 1 gm every month intravenously (IV) for 6 months then every 3 months for 2 years (Klippel 1998) may cause infertility and bone marrow suppression.

However, The Euro Lupus regime (Houssiau, Vasconcelos et al. 2002) utilising 500mg IV x6 at fortnightly intervals was shown to be as effective and much safer than the original regime (Petri, Brodsky et al. 2010). Many studies have shown that MMF is effective in induction and maintenance of lupus nephritis and is not associated with ovarian toxicity like high dose CYC (Ong, Hooi et al. 2005, Appel, Contreras et al. 2009, Xiong and Lahita 2014).

When conventional treatments failure to control disease, then biological therapies, targeting B cells, which are recognised as central in disease pathogenesis, may be considered.

Rituximab is a chimeric monoclonal antibody against CD20, a surface molecule present on pre-B and mature B-cells. CD20 is not expressed on stem cells, pro-B cells or plasma cells (Mok 2010). Anti-CD20 activity results in B cell depletion through complement-dependent cytotoxicity, antibody-dependent cellular toxicity and stimulation of the apoptotic pathway. Rituximab is also effective in the treatment of rheumatoid arthritis (Edwards, Szczepanski et al. 2004) and is frequently used off-label in the treatment of SLE patients with refractory disease (Edwards, Szczepanski et al. 2004, Xiong and Lahita 2014).

Rituximab has been used in many open-label studies that strongly support its clinical efficacy in inducing remission in SLE patients (Lan, Han et al. 2012, Bakshi, Segura et al. 2018). Unfortunately, two published randomized control trials of the efficacy of RTX in the treatment of moderate to severe active lupus (EXPLORER trail) and lupus nephritis (LUNAR trial) have failed to show superiority over standard treatment and will be discussed in greater detail below.

In the EXPLORER trial (n=257 patients in North America), patients were randomized to receive rituximab (1g) or placebo (on days 1, 15, 168, and 182). Background immunosuppressants were continued and prednisolone was added and tapered. There was no significant difference between the rituximab and placebo groups in terms of the primary and secondary endpoints. The primary end-points were

recorded as achieving a major clinical response (MCR), a partial clinical response (PCR) or no clinical response at week 52 assessed using each of the eight BILAG index organ system scores. Interestingly in a post hoc analysis of subgroups, a beneficial effect of rituximab on the primary end point was observed in Afro-Caribbean and Hispanic subgroups in which 20% achieved MCR and 13.8% PCR, compared to the placebo group in which MCR was achieved in 9.4% and PCR in 6.3% ($p = 0.041$). Although no significant clinical improvements were found with rituximab treatment, those treated with the drug had significant improvements in serology- reduced anti dsDNA antibody ($p = 0.006$), increased C3 ($p = 0.0029$) and C4 levels ($p = 0.0045$) at the completion of the trial (Merrill, Neuwelt et al. 2010).

In the LUNAR trial (n=144) patients were randomised to receive either rituximab (1g IV) or placebo (days 1, 15, 168 and 182) (Rovin, Furie et al. 2012). MMF therapy (3g/day) but no other immunosuppressive drugs were allowed during the study and IV methylprednisolone (1g) was given within the first 3 days of the trial. The primary end point was defined as a complete, partial or no renal response at week 52. This study did not show any difference between the treatment and placebo groups for either primary or secondary end points. Although not significant, there was a trend to an improved response rate in Afro-Caribbean subjects treated with rituximab. A statistically significant serological improvement in the treatment group was found with anti dsDNA antibodies falling ($p = 0.007$) and C3 levels rising ($p = 0.03$) (Rovin, Furie et al. 2012).

The negative results from the EXPLORER and LUNAR trials may be attributed to a number of study limitations. For example, the concomitant use of high doses of

steroids and immunosuppressive therapies may have masked the full effectiveness of rituximab. Perhaps a larger study with more patients could have shown statistically significant differences between groups – for example compared to the belimumab studies where positive results were found in trials. The recent demonstration that rituximab is better than cyclosporine in the treatment of membranous nephropathy (Fervenza, Appel et al. 2019) and its recommendation in both the ACR (Hahn, McMahon et al. 2012) and EULAR (Fanouriakis, Kostopoulou et al. 2019) guidelines for the treatment of lupus nephritis. Thus, this strongly supports the view that it does have a part to play in the management of lupus including nephritis. The Rituxilup study, was an open labelled controlled multi-centre trial that looked at whether the addition of Rituximab to MMF therapy could be useful in treating a new flare of lupus nephritis and whether it could be used as a steroid sparing agent compared to a conventional regimen (MMF and oral prednisolone). This trial showed that lupus nephritis could be treated with rituximab effectively with a marked reduction in steroid use (Condon, Ashby et al. 2013).

The trial data for belimumab will be discussed in detail below.

The positive experience of rituximab from open studies and in clinics was demonstrable in patients who had mostly failed conventional therapy. Rituximab was used successfully in combination with cyclophosphamide (CYC) in open-label trials, but in both of the RCTs for rituximab, CYC was not used, potentially negating any synergistic effect of both treatments being used together. The influence from steroid tapering may also have played an important role. Therefore, trials designed in the future that extend beyond 12 months may have a better success at demonstrating

the effect of rituximab if steroids are reduced to low levels at 6 months (Reddy, Jayne et al. 2013). In 2016, NHS England approved the use of rituximab for SLE.

The biological drug approved by the US FDA for the treatment of SLE (belimumab) acts on B cell maturation and survival pathways. Belimumab (10mg/kg every four weeks), a fully humanized monoclonal antibody that blocks a B cell activating factor known as BLyS, is the first targeted biological approved agent for SLE. Since 2012, it has been restricted to those patients with antibody positive SLE who have active skin and joint disease despite treatment with conventional drugs. The efficacy of belimumab has been evaluated in two large multi-centre RCTs (>800 patients) at separate geographic sites in patients with active SLE (BLISS-52 [n=865] and BLISS-76 [n=819]) (Furie, Petri et al. 2011, Navarra, Guzman et al. 2011). The addition of belimumab to standard of care was significantly shown to reduce disease activity, flare rates and prednisolone use. These trials were performed in patients with active disease and the majority of patients entered with active arthritis/ and or a rash (Furie, Petri et al. 2011, Navarra, Guzman et al. 2011). Patients were randomised to receive standard of care plus intravenous infusions of placebo or belimumab at 1mg/kg or 10mg/kg (at 2 and 4 weeks, and then every month until 48 weeks). To be defined as a responder the patient had to show an improvement in SLEDAI by at least 4 points, no worsening in BILAG and no worsening in physician global assessment. Both of these RCTs met the primary endpoints for efficacy. There was significant but modest response in achieving a response in patients receiving belimumab 10mg/kg over placebo at 52 weeks; in BLISS-52 58% achieved this in the belimumab 10mg/kg group vs 44% in placebo group (p=0.0006), and in BLISS-76, 43% belimumab vs 33% placebo (p=0.017). The fact that both the treatment groups received corticosteroids

and immunosuppression meant that there was a large response in the placebo group. Unlike LUNAR and EXPLORER, however this did not prevent detection of a significant difference between treatment groups. The reasons for this could be explained by the larger number of patients studied in the BLISS trials and/or because of the use of the composite responder index (which might be more sensitive to improvements than either SLEDAI or BILAG alone). More recently, there has been a third successful phase 111 RCT study of belimumab (n=677 patients from 49 centres in China, Japan and South Korea) which reported that belimumab (intravenous 10 mg/kg) significantly improved disease activity scores and reduced prednisolone use compared to placebo (Zhang, Bae et al. 2018).

1.1.6 SLE disease activity definition and disease activity scores

There is no one biomarker in SLE to measure disease activity. In clinic rheumatologists routinely measure complement (C3 and perhaps C4), anti-dsDNA and inflammatory markers (such as ESR) to measure disease activity, but they are not always abnormal during disease flares. Disease activity scores have therefore been designed to help clinicians to assess disease activity scores in SLE. There are many of these scoring systems that have been validated and probably the best known are the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and British Isles Lupus Assessment Group (BILAG). The BILAG scoring system is the most comprehensive and the only transitional scoring index (Murphy, Yee et al. 2016) . In contrast to other scoring systems, the BILAG scores disease activity in distinct organ systems separately and is the scoring system used at UCLH. For the purpose of my thesis, I will focus on the BILAG scoring system.

British Isles Lupus Assessment Group (BILAG) score

The BILAG score is based on the physician's **"**intention to treat" (Gordon, Sutcliffe et al. 2003). The systems included in the score were originally general, mucocutaneous, neurological, musculoskeletal, cardiorespiratory, vasculitis, renal and haematological. In order to calculate the score for each system, clinical features for each system are scored whether they are new, worse, the same or improving in the last 4 weeks compared to previously and have to be ascribed to SLE activity rather a symptom due to damage; a concomitant disease or a treatment side effect. Scores can be generated manually but are best derived from the British Lupus Integrated Prospective System (BLIPS), which is a purpose-designed software to generate scores. Basic

haematology and assessment of renal function are incorporated into the scoring (Isenberg and Gordon 2000).

Each organ system receives a score between A-E. Scoring of an A signifies severely active disease requiring treatment with at least 20mg of prednisolone daily of immunosuppressive therapy. A score of B implies active disease but treatment with a lower degree of immunosuppression such as NSAIDS, hydroxychloroquine or daily steroids of less than 20mg. A score of C means low disease activity requiring little treatment, D grade implies a system that is inactive but which had been previously affected and E grade means the system has never been active. A severe flare of lupus has been defined as a new score of A in any system and a moderate flare has been defined as a score of B in any system which previously scored D or E. Scores ranging from A-E are converted numerically (A=9, B=3, C=1, D=0, E=0 originally (subsequently A=12, B= 5, C=1; D/E= 0)) and combined to give a global score, which is known as the classical BILAG score (Gordon, Sutcliffe et al. 2003).

In 2004, the new version of BILAG (2004) was produced in which A=12, B=5, C=1, D=0, E=0. BILAG 2004 was more comprehensive than the classical version with nine systems instead of eight, renal disease was better captured and features that were clearly in reality damage items such as avascular necrosis were removed (Murphy, Yee et al. 2016).

My analysis of BILAG scores is for samples collected after 2004. I will refer to the BILAG 2004 for the purpose of my thesis.

1.2 Rheumatoid Arthritis (RA)

RA is an autoimmune disease that primarily affects the joints, but can cause multi-system complications including cardiovascular disease. Below I have briefly summarized the pathogenesis, diagnosis, clinical features and treatment of RA.

1.2.1 Pathogenesis of RA

The pathogenesis of RA too, is a complex interplay of genetic and environmental events. The disease however, unlike SLE, starts in the synovial membrane, where there is a breakdown of immune tolerance and synovial inflammation. The possible pathogenesis of RA is summarized in Figure 2 (Komatsu and Takayanagi 2012).

Genetics and Environmental Factors

The concordance rate for identical twins for RA has been found to be between 12-15% implicating genetic factors. The human leukocyte antigen (HLA) and major histocompatibility (MHC) genes are the most important susceptibility genes thought to contribute to the risk of developing RA. Genes that include T cell signalling genes and cytokine promoters are also thought to contribute to the risk of RA. As with SLE, it is the interplay of genetics and environmental factors that initiate RA. Epigenetic influences, such as abnormal DNA or expression of microRNAs, can also increase pro-inflammatory gene expression (Bottini and Firestein 2013, Firestein 2018, Firestein 2018).

The most well studied environmental contributor described is cigarette smoking. Combined with genetic factors, smoking can increase the risk of RA by 20-40 fold (Lundstrom, Kallberg et al. 2009). Cigarette smoking acts at mucosal surfaces where it induces peptidyl arginine deiminase (PAD) expression in alveolar macrophages. The amino acid arginine is converted to citrulline in the airway by PAD, resulting in the production of a neoantigen, which is recognised by the adaptive immune system (Makrygiannakis, Hermansson et al. 2008, Choy 2012). In RA, the immune system reacts to these neoantigens resulting in the production of anti-citrullinated protein antibodies (ACPAs.). Other mucosal surfaces such as the gut microbiome with bacterial species that express PADs in the mouth have been potentially implicated in arthritis susceptibility (Scher, Sczesnak et al. 2013).

Autoantibodies and the immune system in RA

Rheumatoid factors (RFs) are immunoglobulins (Ig) reactive against epitopes on the Fc portion of IgG. The presence of RF and ACPAs confers a worse disease prognosis with severe symptoms and erosive disease. ACPAs themselves can be pathogenic, either by activating macrophages or by activating osteoclasts via immune complex formation and Fc-receptor engagement thus promoting bone loss. RF is more directly involved in mechanisms of macrophage activation and induction of cytokine activation than ACPAs (Smolen, Aletaha et al. 2016). Both RF and ACPAs have been found to be present more than 10 years before the onset of clinical symptoms a period known as pre-RA An unknown “second hit” (possibly trauma or local inflammation) is thought to trigger clinical disease (Choy 2012).

Once the generalized abnormal immune response has become established, plasma cells derived from B cells produce RF and ACPAs of IgG and IgM isotype in large quantities. Macrophages in turn are activated via the Fc receptor and complement binding leading to intense inflammation. Inflammation in the synovium, is linked to oedema and vasodilation and entry of activated T-cells (Th1 and Th17 subset, with deficiency of Th2 and regulatory T cells (T-regs). Synovial macrophages and dendritic cells function as antigen-presenting cells by expressing MHC class II molecules establishing an immune reaction in tissue (Boldt, Goeldner et al. 2012).

Early on in the disease new synovial blood vessels form, which are accompanied by the transudation of fluid and the transmigration of inflammatory cells into the synovial fluid. Tumour necrosis factor (TNF) driven cytokine production activates endothelial cells to produce adhesion molecules, which activates sticking of leukocytes facilitating diapedesis and extravasation into the synovium (Smolen, Aletaha et al. 2016).

Like antibodies, levels of multiple cytokines gradually increase in the years before RA symptoms occur. The inflammatory milieu of the synovium is made up of cytokines and chemokines that attract and accumulate immune cells notably activated T- and B cells, monocytes and macrophages from activated fibroblasts, in the joint space. TNF, one of the key cytokines in RA, induces granulocyte macrophage colony-stimulating factor (GM-CSF) production by antigen-presenting cells; enhances proliferation of T cells; increases proliferation and differentiation of B cells; induces expression of adhesion molecules on endothelium; generates

expression of collagenase, matrix metalloproteinase, and prostaglandins by synovial cells.

Bone destruction is promoted by synovial fibroblasts taking an inflammatory phenotype, chondrocyte catabolism and synovial osteoclastogenesis. By signalling through RANKL and RANK, cytokines trigger osteoclast production, which degrades bone tissue. Cartilage damage occurs by the catabolic effects of cytokines on chondrocytes and by stimulation of matrix metalloproteinases and other enzymes (Smolen, Aletaha et al. 2016).

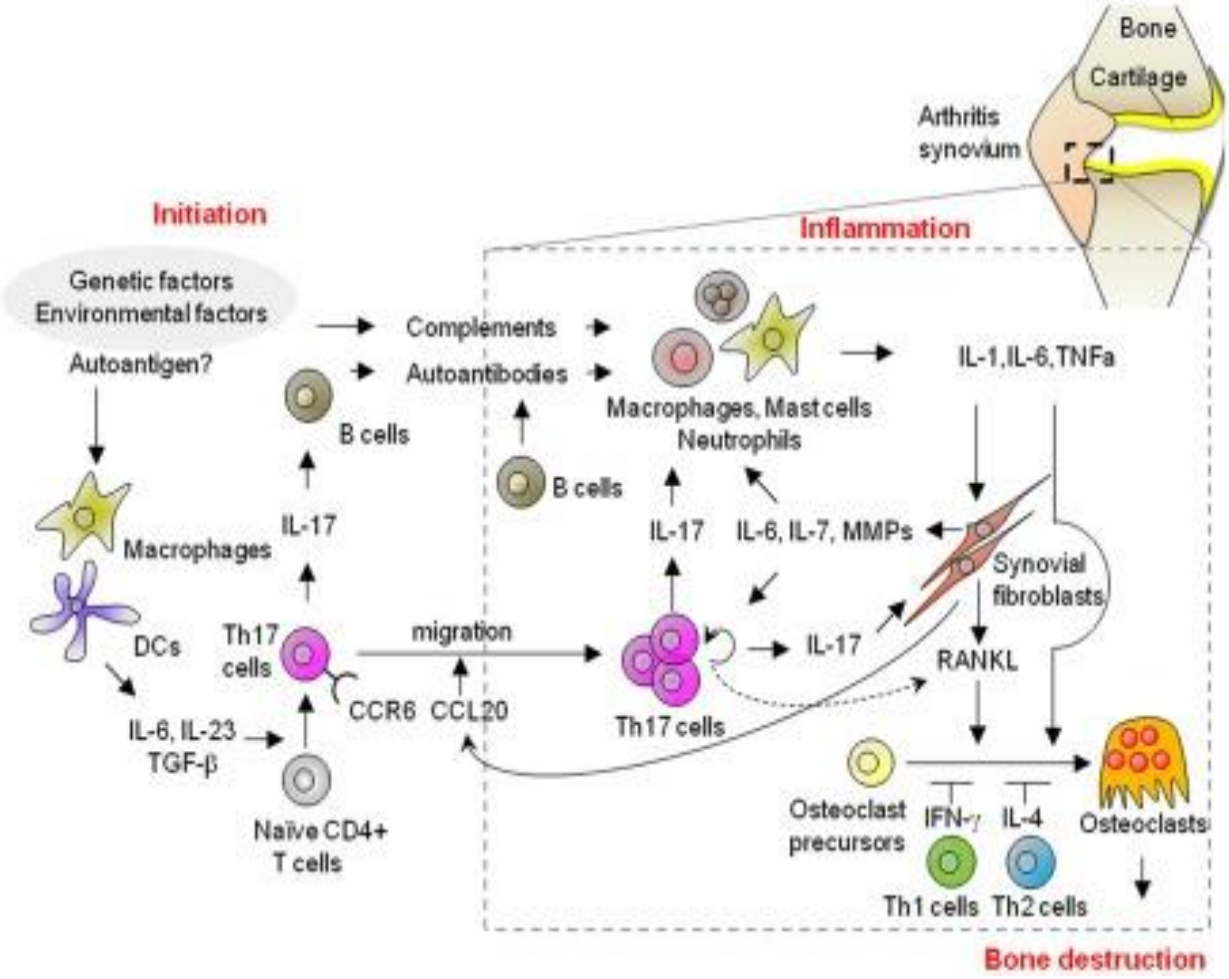


FIGURE 2 - POSSIBLE MECHANISMS OF PATHOGENESIS OF RA.

A variety of different cell populations contributes to the development of RA. Genetic and environmental factors trigger an abnormal immune response to self-antigens in the synovium. Th17 contributes in the initiation, inflammatory and bone destructive phases through the autoantibody production as well as activation of innate immunity, triggering the release of cytokines such as IL-6 and TNF-alpha which activate synovial fibroblasts causing inflammation and bone destruction.

CCL20: chemokine ligand 20; CCR6: chemokine receptor Type 6; DC: dendritic cell; IFN-γ: interferon gamma; IL: interleukin; MMP: matrix metalloproteinases; RANKL: receptor activator of nuclear factor kappa-B ligand; Tfh: T follicular helper; TGF-β:

transforming growth factor-beta; Th: T helper; TNF-α: tumour necrosis factor-alpha.

(Komatsu and Takayanagi 2012)

1.2.2 RA epidemiology, clinical features and classification criteria

RA is affects women 2-3 times more than men and the onset is usually during the third or fourth decade (Smolen, Aletaha et al. 2016). RA affects between 0.5 and 1% of adults in the developed world with between 5 and 50 per 100,000 people developing the condition each year. RA is a chronic disease, and although rarely, a spontaneous remission may occur, the natural course without treatment is one of persistent symptoms, waxing and waning in intensity, and a progressive deterioration of joint structures leading to deformations and disability (Smolen, Aletaha et al. 2016)
.

RA typically presents as a small joint polyarthritis with inflammation affecting the synovial membrane. The small joints (hands, wrists, and feet) are most commonly affected causing stiffness and swelling, but larger joints such as the shoulder and knees can be affected. The atlantoaxial joint in the cervical spine may also be affected. Early morning stiffness lasting for longer than an hour can be a salient, predominant symptom. Synovitis in the joints can lead to tethering of tissue with loss of movement and joint erosions causing deformity and loss of function. RA can cause systemic inflammation affecting other organs such as the skin, lungs, blood and cardiovascular system (Majithia and Geraci 2007).

RA is diagnosed based on clinical history and presentation, blood tests and imaging. Rheumatoid factor (RF) and anti-citrullinated protein antibodies (anti-CCP) are routinely tested when a diagnosis is suspected. RF is positive in 75-85% of people with a sensitivity of 72% and specificity of 80%, where as anti-CCP antibodies have a higher specificity of 95-96%, but a lower sensitivity of 53-71% for RA (Braschi,

Shojania et al. 2016). Inflammatory markers such as the ESR and CRP are typically elevated and are used to assess disease activity. X-rays of the hands and feet are performed at baseline and may be normal during the early stages of disease. In more advanced disease, erosions and subluxation can be observed. Early synovitis can be seen on ultrasound and can be used where there is diagnostic uncertainty or to assess response to treatment (Sharif, Sharif et al. 2018).

In 2010, the American College of Rheumatology and European League of Associations of Rheumatology published joint classification criteria for RA as an update to the 1998 guidelines (figure 3)**.** These are classification criteria to identify disease with a high likelihood of developing a chronic form. A score of six or greater unequivocally classifies a person with a diagnosis of rheumatoid arthritis (Aletaha, Neogi et al. 2010).

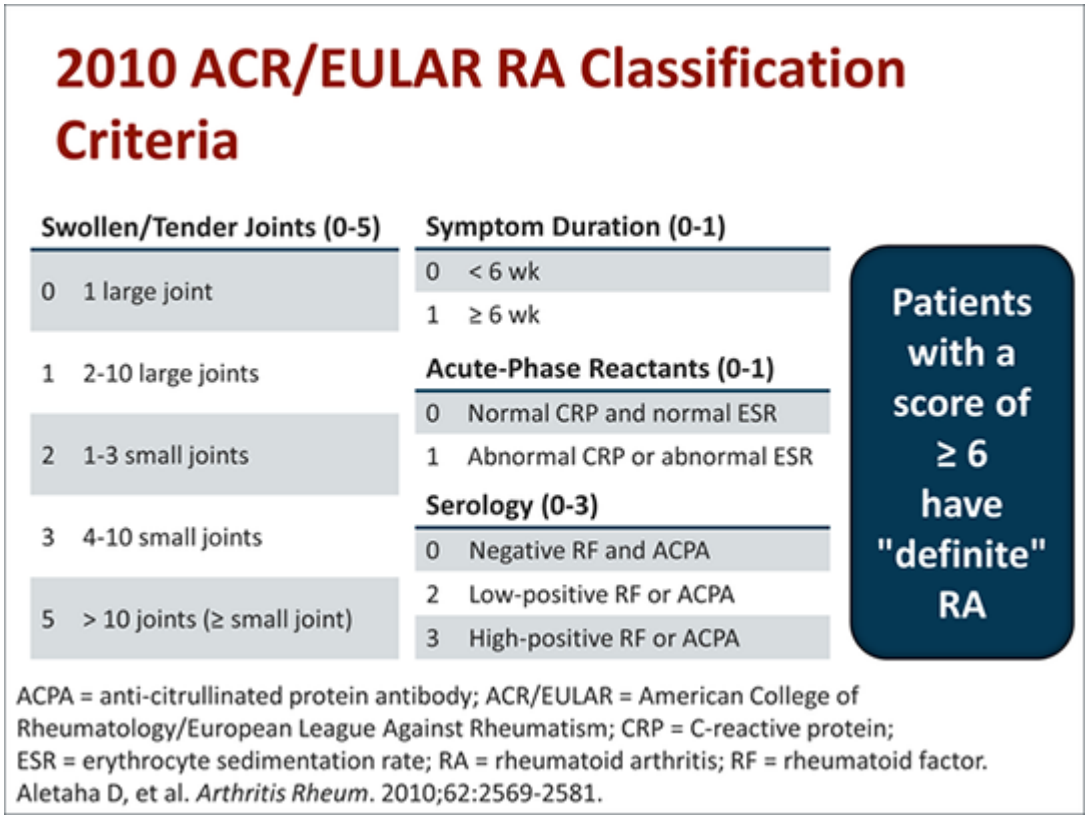


FIGURE 3 - 2010 ACR/EULAR RA CLASSIFICATION CRITERIA FOR RA

1.2.3 **Management and prognosis of RA**

A multi-disciplinary approach to treatment is taken with physiotherapy, occupational therapy and pharmacotherapy. Disease modifying antirheumatic drugs (DMARDs) are used as first line treatment. They have been found to improve symptoms, decrease joint damage, and improve overall functional abilities. DMARDs should be started early in the disease as they result in disease remission in approximately half of people and improved outcomes overall (Singh, Saag et al. 2016). The following drugs are considered as DMARDs: methotrexate, hydroxychloroquine, sulfasalazine, leflunomide and cyclosporin. In accordance with NICE guidelines, first line treatment is with DMARD monotherapy (oral methotrexate, leflunomide or sulfasalazine) as soon as possible, ideally within 3 months of onset of symptoms. Hydroxychloroquine may be used alone in mild or palindromic disease. Biological agents should generally only be used if methotrexate and other conventional agents are not effective after a trial of 6 months. Biological DMARD agents used to treat rheumatoid arthritis include: tumor necrosis factor alpha (TNFα) blockers such as infliximab; monoclonal antibodies against CD20 (rituximab), interleukin- 6 blockers (tocilizumab), T cell co-stimulation blocker such as abatacept and Janus Kinase (JAK) inhibitors. They are often used in combination with DMARDs. Glucocorticosteroids can be used in the short term and at the lowest dose (5- 7.5mgm) possible for flare-ups and while waiting for slow-onset drugs to take effect. NSAIDs reduce both pain and stiffness in those with RA but do not affect the underlying disease (Sharif, Sharif et al. 2018).

1.2.4 **Disease Activity Scores in RA**

The Disease Activity Score of 28 joints (DAS28) is widely used to capture RA disease activity and response to treatment. The clinician assesses tenderness and swelling in 28 joints. The ESR or CRP are measured and the affected person makes a subjective assessment of disease activity during the preceding 7 days on a scale between 0 and 100, where 0 is "no activity" and 100 is "highest activity possible". With these parameters, DAS28 is calculated using online calculators. The score has been extensively validated for use in clinical trials and is widely used in clinical practice High disease activity are indicated by a DAS28 >5.1, moderate activity by DAS28 score of >3.2 to 5.1, low disease activity by a score in the 2.6 to 3.2 range. A cut-off point for "remission" has also been proposed (DAS28 <2.6) (Prevoo, van 't Hof et al. 1995, Sharif, Sharif et al. 2018).

1.3 Atherosclerosis and Cardiovascular Disease

1.3.1 Pathogenesis of Atherosclerosis

The term “atherosclerosis” derives from the Greek “athero” meaning wax and “sclerotic” meaning hard. It is a chronic inflammatory disease, affecting the large and medium-sized arteries characterized by endothelial dysfunction, vascular inflammation and accumulation of lipids, cholesterol, calcium and cellular debris within the intima of the vessel wall. Atherosclerosis begins in childhood with the development of fatty streaks with the lesions advancing with age (Hansson 2005).

The widely accepted model for the pathogenesis of atherosclerosis from animal and human studies describes repeated cycles of inflammatory cell recruitment and activation, LDL accumulation and oxidization and fibrosis forming a lesion with a core of lipid and necrotic tissue covered by a fibrous cap- an advanced, complicated lesion. Different types of inflammatory cells have been found within atherosclerosis-prone aortas (Hansson and Libby 2006, Galkina and Ley 2007). When the artery reaches its maximum ability to compensate by dilatation the lesion may encroach the lumen and alter blood supply (Croce and Libby 2007). Although this theory is widely accepted, only a few among many plaques within individuals become thrombosis prone leading to a cardiovascular event (Casscells, Naghavi et al. 2003). The reasons for this may be explained by the presence of risk factors and abnormal flow. These factors will be discussed in more detail below.

The response-to-injury hypothesis was an early model for the possible initiation of atherosclerosis. In this model, it was thought that the process was initiated by an

overt injury to the intimal endothelial lining, induced by a noxious substance (e.g. cigarette smoke constituents, oxidized cholesterol) or an altered haemodynamic force (blood flow disturbance due to hypertension). Platelet adhesion and the localized release of platelet derived growth factors from focal endothelial desquamation was thought to trigger migration and proliferation of medial smooth muscle cells, generating a fibromuscular plaque (Methe and Weis 2007, Gimbrone and Garcia-Cardena 2016). However, examination of early fatty streak lesions in diet induced animal models failed to demonstrate overt intimal injury or platelet adhesion therefore the response to injury hypothesis alone could not alone account for the development of atherosclerosis (Gimbrone and Garcia-Cardena 2016).

Although the evidence supports involvement of the immune system in a systemic response to hyperlipidemia, atherosclerotic lesions have been observed in a non-random pattern. They form in very specific areas of the arterial tree with lesion-prone areas having disturbed laminar flow patterns such as at arterial branch points in contrast to areas with laminar flow (Garin and Berk 2006, Jongstra-Bilen, Haidari et al. 2006). A possible mechanism for this could be due to the effect of disturbed flow on endothelial cells (EC).

Exposure to disturbed flow has been shown to induce EC turnover and increase oxidative stress and senescence (Warboys, de Luca et al. 2014). Activated by disturbed flow, ECs have been found to elevate the expression of adhesion molecules and chemokines through gene regulation, which increases leucocyte recruitment (Passerini, Polacek et al. 2004, Gimbrone and Garcia-Cardena 2016).

Therefore, the role of endothelial cells appears to be important in the pathophysiology of atherosclerosis. This will be explored in more detail below.

Endothelial cells can become activated in response to certain agonists such as bacterial products, modified lipids, pro-inflammatory cytokines (IL-1 and TNF) as well as biochemical stimulation by disturbed blood flow (Pober and Sessa 2007). The result of this response is the activation of transcription factors such as NFκB, resulting in the expression of cell surface expression of endothelial leucocyte adhesion molecules (e.g. VCAM-1 and E-selectin), pro-coagulant factors (e.g. tissue factor) and secreted chemokines (e.g. interleukin-8) generating a “pro-inflammatory endothelial phenotype”. Endothelial cells have been shown in animal models to express leukocyte adhesion molecules (in particular VCAM1) in arteries as part of the initial vascular response to cholesterol accumulation (Hansson and Libby 2006). The expression of these factors fosters the recruitment of monocytes and T-cells. Circulating monocytes differentiate into macrophages and internalize oxidized lipoproteins to become foam cells (hallmark of early fatty lesions). Cytokines and growth factors released from activated endothelium and macrophages act on neighbouring smooth muscle cells to induce proliferation and synthesis of extracellular matrix components contributing to the fibromuscular plaque (Hansson 2001).

Platelets are also important mediators in the recruitment and activation of inflammatory cells. Von Willebrand factor is released in response to endothelial damage causing platelet recruitment at the plaque site. This interaction leads to platelet activation and production of vasoactive molecules such as thromboxane A2,

adenosine and thrombin as well as other proinflammatory mediators (Croce and Libby 2007).

1.3.2 Plaque rupture and clinical symptoms

Atherosclerotic plaques can be classified into two broad categories- stable and unstable plaque**.** Stable plaques are rich in extra cellular matrix mainly collagen and smooth muscle cells and tend to be asymptomatic. Unstable (also called vulnerable plaques) tend to be rich in macrophages and foam cells, and usually have a weak fibrous cap that is prone to rupture (Finn, Nakano et al. 2010). Proteolytic enzymes produced by activated macrophages within the necrotic core cause a decrease in smooth muscle cells and type 1 and 2 (collagen fibres, resulting in the thinning of the fibrous cap (Crea and Libby 2017).

When the fibrous cap ruptures, it exposes the lipid core which contains thrombogenic material such as collagen that can induce the formation of thrombus in the lumen, occluding arteries and thus blood flow. Thrombi can also detach and occlude smaller arteries downstream causing thromboemboli (Didangelos A, 2010).

CVD therefore has a heterogeneous clinical presentation dependent on the presence of risk factors, underlying histology of plaques and their location and progression of atherosclerosis in the vessel wall. Clinical symptoms can range from asymptomatic disease to chronic disease (stable angina and chronic peripheral ischaemia) and major events like acute coronary syndromes (Virmani, Ladich et al. 2006, Finn, Nakano et al. 2010).

Traditional modifiable risk factors for CVD–hypercholestrolemia, hypertension, diabetes and smoking have been well recognised (Patel, Winkel et al. 2015). Non-modifiable risk factors include age and sex. Risk score calculators are commonly used in the general population for primary prevention. These include the widely used Framingham risk score (Anderson, Odell et al. 1991, Wilson, D'Agostino et al. 1998) but others have also been developed, such as the ATP III hard CHD risk score (2002) and QRISK score (Hippisley-Cox, Coupland et al. 2007). Although these equations identify high-risk groups, only 40% of CVD events that occur will occur in this group. The remaining events occurs in a large group of the population classified as low risk (van Staa, Gulliford et al. 2014, Steinl and Kaufmann 2015). The different risk score calculators also exclude various emerging, genetic and otherwise unknown risk factors and are mainly driven by age and gender.

Preclinical atherosclerosis in the form of asymptomatic plaques develops slowly over many years. The early medical treatment and controlling of modifiable risk factors has been shown to reduce cardiovascular events in both symptomatic and asymptomatic patient. Patients with subclinical atherosclerotic disease would ideally be identified before any events occur, thus enabling primary prevention strategies and minimizing mortality and morbidity. Ten thousand asymptomatic (non-diabetic, normotensive, total cholesterol of <5.2 mmol/L) participants underwent carotid and femoral artery ultrasound scans and were followed up for ten years, in the CAFES-CAVE study. The average annual rate in those individuals was 0.1% compared with 5% (mainly MI) in those who had at least one plaque (Incandela, Nicolaides et al. 2002). Thus the presence of plaque, places an individual at high risk. Non-invasive imaging techniques can therefore be used to detect the presence, estimate the

extent, and evaluate the clinical consequences of atherosclerotic vascular damage. Carotid US has been shown to be a sensitive, non-invasive, reproducible imaging study to detect atherosclerosis and predict its clinical complications and therefore help to improve prediction and treatment decisions in asymptomatic patients who are considered low or moderate risk by traditional risk factor scores (Johnsen, Mathiesen et al. 2007, Piepoli, Hoes et al. 2016).

1.4 Cardiovascular disease in SLE

Whilst the increased risk of CVD in SLE is known, its cause has yet to be fully elucidated. I will explore in the following sections the evidence for this increased risk and the contribution of traditional risk factors and disease related factors.

1.4.1 Evidence for the increased risk of CVD in SLE

Urowitz et al highlighted the importance of atherosclerotic disease in SLE by reporting a biomodal peak in mortality. The first peak was associated with disease activity and infection and the second with CVD (Abu-Shakra, Urowitz et al. 1995). Various early lupus cohorts reported the prevalence of MI and angina to lie between 6-10% (Tronto-10%, Pittsburg-6.7% and Baltimore -8.3%) (Petri, Perez-Gutthann et al. 1992, Abu-Shakra, Urowitz et al. 1995, Manzi, Meilahn et al. 1997). Additionally, the average ages of the first CVD event reported from these cohorts were similar at 48-49 years. Further evidence of this risk was emerging from epidemiological studies. Manzi et al looked at the age specific- cardiovascular incidence rates in 498 SLE women compared with women of a similar age in the Framingham off-spring study (Manzi, Meilahn et al. 1997). They reported that more myocardial infarctions occurred in all of the SLE patient age groups compared with controls. In the control group, no events were recorded below the age of 34. There were 498 women studied, of which 33 (6.6%) developed CVD compared with 36 controls (1.6%). The presence of SLE in women between the ages of 35-44 was reported to increase the risk of CVD by 50 times. Although this study was large, it had several limitations. It was a retrospective design and not all events were confirmed by medical records due to lack of availability. Additionally, the Framingham control group were not

strictly matched adequately to the lupus group as they were all Caucasian, whereas the lupus group had other ethnic groups (Manzi, Meilahn et al. 1997, Elliott, Manzi et al. 2007).

In a more recent large multinational study (23 centres, 9547 patients), the investigators compared the mortality in a SLE group with geographically appropriate age, sex and calendar- year period matched general population rates. There were 1255 deaths in the SLE cohort, and of these 313 were due to CVD (126 due to cardiac causes and 21 due to stroke), for a rate of 4.1 events per 1,000 person-years (Bernatsky, Boivin et al. 2006). Standardized mortality ratio (SMR; ratio of deaths observed to deaths expected) estimates were calculated for all deaths and by cause. The overall (all-cause) SMR estimate was 2.4 (95% CI 2.3–2.5). For death due to circulatory disease, the SMR was 1.7 (95% CI 1.5–1.9). Female sex, younger age, or SLE duration <1 year, all had particularly high SMR estimates, including for circulatory disease. This large, multi-center study confirms findings from previous smaller cohort studies that SLE is associated with an increased risk of CVD, which develops at a younger age compared to the general population. Overall, the data suggests that patients with SLE have between a 5-10-fold increased risk of developing CVD compared to age and sex-matched controls (Manzi, Meilahn et al. 1997, Bruce 2005) and this increased risk is particularly evident in younger women.

The prevalence of sub-clinical atherosclerosis is also considerably raised in patients with SLE. Studies from multiple centres world –wide, using different imaging techniques such as vascular ultrasound (Manzi, Selzer et al. 1999, Roman, Shanker et al. 2003, Farzaneh-Far, Roman et al. 2006) and electron beam tomography

(Asanuma, Oeser et al. 2003) have consistently shown that patients with SLE have a significantly higher prevalence of atherosclerotic plaque than healthy controls. The rates of carotid plaque detected by carotid ultrasound in SLE patients has been reported as 30-40% (Manzi, Selzer et al. 1999, Roman, Shanker et al. 2003, Farzaneh-Far, Roman et al. 2006). Carotid intima–media thickness (CIMT) can be assessed by B or M mode ultrasound at the carotid artery level and is considered an early, sensitive marker of atherosclerosis (Boulos, Gardin et al. 2016)

In a longitudinal study by Manzi et al in Pittsburg, 217 female SLE patients compared to 104 matched controls were followed up for ten years, with a carotid ultrasound at baseline and at follow up on average 4.19 years later. The prevalence of plaque was reported as 31% at baseline and 40% at follow up. The progression of carotid plaque occurred in 27% vs 10% in the control group (Thompson, Sutton-Tyrrell et al. 2008) while carotid intima-media thickness (IMT) increased by a mean of 0.011mm/year vs 0.008 mm/year in the controls (Thompson, Sutton-Tyrrell et al. 2008). A limitation of this study was the lack of ethnic diversity- 89% of the patients were white and predominantly tertiary care center referral patients, and therefore not representative of all SLE patients. Roman et al also studied the prevalence of subclinical atherosclerosis in 197 patients with SLE compared to with 197 matched controls. Carotid plaque was reported in 37% of SLE patients vs 15% controls $p<0.001$. In this study compared to the Pittsburg study, the case mix was more diverse with 55% of the population being white (Roman, Shanker et al. 2003). A limitation of the study was the difficulty in quantifying severity of disease and different treatments as this was only collected at time of scan, and therefore it is hard to

determine if the population is fully representative of SLE populations (Roman, Shanker et al. 2003).

Kao et al studied 392 women with SLE followed for a mean of 8 years and showed that higher IMT or presence of plaque at baseline predicted development of CAD or stroke over the next 10 years in multivariable analysis (Kao, Lertratanakul et al. 2013). For every 0.05 mm increase in baseline IMT the risk of CVD increased by a factor of 1.35 (95% CI 1.12-1.64) and the presence of plaque at baseline increased this risk by a factor of 4.26 (95%CI 1.23 to 14.83) (Kao, Lertratanakul et al. 2013). Among the limitations of this study, the SLE patients were predominantly Caucasian (77%) and most had mild disease (Kao, Lertratanakul et al. 2013).

Ahmad et al, looked at subclinical atherosclerosis in 200 SLE patients compared with 100 controls, using B-mode ultrasound, and assessed the strength of the association between traditional risk factors, in a study from the North-West of England (Ahmad, Shelmerdine et al. 2007). They reported a significant increase in the prevalence of plaques in SLE patients at a younger age (<55 years had more plaque, 21% vs 3%, p<0.01) and a lower carotid IMT compared with controls. SLE patients also had more plaque in the internal carotid artery (11% *vs* 4%; $P < 0.05$). Traditional risk factor models were more strongly associated with controls than SLE patients were, (area under Receiver Operator Characteristic curves (AUC ROC) = 0.90 *vs* 0.76; $P < 0.01$). Using a model with only SLE factors age at diagnosis, disease duration, previous arterial events, higher neutrophil count, azathioprine use ever and aCL/LAC positivity ever were more strongly associated with SLE patients (AUC ROC 0.87, p<0.01). The final model in SLE included age and cigarette pack-years smoking as well as

azathioprine exposure ever, antiphospholipid antibodies (APLA) and previous arterial events (AUC ROC = 0.88). What this study adds uniquely is that perhaps characterising a SLE phenotype using SLE related factors rather than traditional risk factors may help to predict plaque. Limitations of this study include the lack of a multi-ethnic cohort as all the women were Caucasian of British, white ancestry and the cross sectional design (Ahmad, Shelmerdine et al. 2007).

A recent study from the North-West of England by Haque et al, assessed 200 patients by carotid ultrasound at baseline and 124 patients were followed up with a second scan assessment (median follow up of 5.8 years) (Haque, Skeoch et al. 2018). They reported new plaque development in 26% (n=32) and plaque progression in 41% (n=52). Older age (OR 1.13; 95% CI 1.06 -1.20), aCL (OR 3.36; 1.27 -10.40) and anti-Ro (OR 0.31; 0.11 -0.86) antibodies were found to be associated with plaque progression. A positive aCL remained independently associated with plaque progression (OR 3.14; 1.10 to 9.01) in the multi-variate analysis. In a multivariable analysis, lower systolic blood pressure, lower triglycerides and metabolic syndrome all remained independently associated with CIMT progression. The event rate for CVD was 7.2% (8 patients) over 5.8 years follow up and independent factors for CVD events reported were higher triglycerides (OR 3.61; 1.23- 10.56), ever exposure to cyclophosphamide (OR 16.7; 1.46- 63.5) and the SDI score (OR 9.62; 1.46- 123) on multi-variable analysis (Haque, Skeoch et al. 2018). Limitations of this study include the lack of ethnic diversity in the cohort (all patients were of white British ancestry and CVD event data was patient recorded.

In a recent meta-analysis, the prevalence of subclinical atherosclerotic disease assessed by carotid ultrasound (used to detect carotid intimal medial thickness or carotid plaque presence) was found to be higher in SLE patients compared with controls. Eighty studies were looked at in the meta-analysis (6085 SLE patients and 4794 controls) with data on CIMT (4814 cases and 3773 controls) and 44 studies reporting on the prevalence of carotid plaques (4417 cases and 3528 controls). Compared with controls, SLE patients showed a higher CIMT (WMD: 0.07 mm; 95%CI: 0.06-0.09; $P < 0.001$), and 2.5 fold increased prevalence of carotid plaques (95%CI: 2.02-2.97; $P < 0.001$). In SLE patients, traditional cardiovascular risk factors (age, HDL and triglycerides) and lupus related risk factors (as expressed by duration, ESR,SLEDAI and steroids) had a significant influence on CIMT and steroids and triglyceride had significant influence on the prevalence of carotid plaques shown by regression models (Wu, Liu et al. 2016).

1.4.2 Contribution of traditional risk factors to SLE CVD

Conventional risk factors that contribute to CVD risk in the general population are common in SLE and have been shown to contribute towards CVD risk in SLE.
In a large multi-center study of the Systemic Lupus International Collaborative Clinics (SLICC) inception cohort, Urowitz *et al* found that among 1249 patients followed for a mean of 8 years there were 31 atherosclerotic events. In univariate, analysis all the factors significantly associated with increased risk of atherosclerosis were generic, rather than SLE-specific. These included male gender, increased age, smoking, hypertension and family history of CVD but in multi-variable analysis, only non-modifiable risk factors – age and male gender – remained significant (Urowitz, Gladman et al. 2010). A limitation of this study was that events were only measured in patients with inactive disease, not patients with active SLE, and thus making it less likely for SLE-specific factors to be predictive of atherosclerosis.

Hypercholesterolemia is found in 34-51% of patients with SLE (Wajed, Ahmad et al. 2004, Bernatsky, Boivin et al. 2006). The SLE pattern of dyslipidemias has been characterized by low concentrations of HDL and high concentrations of triglycerides, total cholesterol, and LDL (Borba and Bonfa 1997, Wilhelm and Major 2012).Raised total cholesterol is predictive of future CHD in SLE (Bruce, Urowitz et al. 1999). The increased prevalence of dyslipidemia in SLE may be due to both steroid therapy and from disease-related pathogenic mechanisms, such as increased cytokine release (e.g., TNF-alpha and IL-6), affecting the balance between pro- and antiatherogenic lipoproteins (Tselios, Koumaras et al. 2016). Data from the Systemic Lupus International Collaborating Clinics' cohort (>900 SLE patients), showed that the

prevalence of hypercholesterolemia was 36% at diagnosis and 60%, 3 years later (Urowitz, Gladman et al. 2007). Additionally, Hypercholesterolemia was significantly associated with CV events (OR = 4.4, 95%CI 1.51–13.99) (Urowitz, Gladman et al. 2007, Urowitz, Gladman et al. 2016).

An increased risk of CHD has been observed in the presence of hypertension in SLE and subclinical atherosclerosis. SLE patients are more likely to be hypertensive than the general population due to steroid therapy use (Bruce, Urowitz et al. 2003) and the presence of renal impairment (Wajed, Ahmad et al. 2004). Bruce et al reported a 2.59 RR (95%CI 1.79–3.75) of hypertension in women with SLE (Bruce, Urowitz et al. 2003). In the Systemic Lupus International Collaborating Clinics' cohort, the presence of hypertension was an independent risk factor for CVD in SLE (OR 5.0; 95%CI 1.3–18.2) (Urowitz, Gladman et al. 2007, Urowitz, Gladman et al. 2016). In a multivariate analysis, hypertension was associated with atherosclerosis by means of higher carotid intima-media thickness in SLE patients (Doria, Shoenfeld et al. 2003).

The presence of the metabolic syndrome (associated with dyslipidaemia, hypertension and insulin resistance) has been found to be more frequent in SLE patients compared with controls (32.4% versus 10.9%; **p**< 0.001) and associated to an increased risk of atherosclerosis (Chung, Avalos et al. 2007, Sabio, Vargas-Hitos et al. 2009).

Most studies show that at least 10% of SLE patients continue to smoke (Roman, Shanker et al. 2003). Five to seven percent of patients with SLE develop diabetes

and patients with SLE are more likely to develop diabetes than the general population (Wajed, Ahmad et al. 2004).

Renal disease is common in SLE patients. Renal disease is independently associated with an increased CVD risk and also contributes through the increased risk of hypertension and diabetes. Nearly 50% of deaths in patients with renal lupus are attributed to CVD (Appel GB et al 1994).

However, traditional CVD risk factors only account for some of the CVD risk in SLE. Studies have shown that traditional CVD risk calculators such as Framingham grossly underestimate CVD risk in lupus (Roman, Shanker et al. 2003, Bessant, Hingorani et al. 2004). After adjusting for Framingham risk factors, it has been reported that patients still had a 7-10 fold increased risk of CVD (Esdaile, Abrahamowicz et al. 2001). Therefore, traditional risk scores underestimate CVD risk in lupus patients and do not fully account for risk after adjustment.

The QRISK3 score was recently published and validated as a cardiovascular risk disease algorithm. Nearly 364,000 incident cases of cardiovascular disease were identified in the derivation cohort during follow-up arising from 50.8 million person years of observation. The QRISK3 compared with QRISK2 has the addition of SLE, corticosteroid use, chronic kidney disease (including stage 3) and severe mental health illness. (Hippisley-Cox, Coupland et al. 2017).The QRISK3 model has limitations in that the study excluded patients who were on statins at baseline- thus potentially excluding a group of patients that may be at higher risk. Figure 4 summarizes the multi-factorial risk of CVD in SLE.

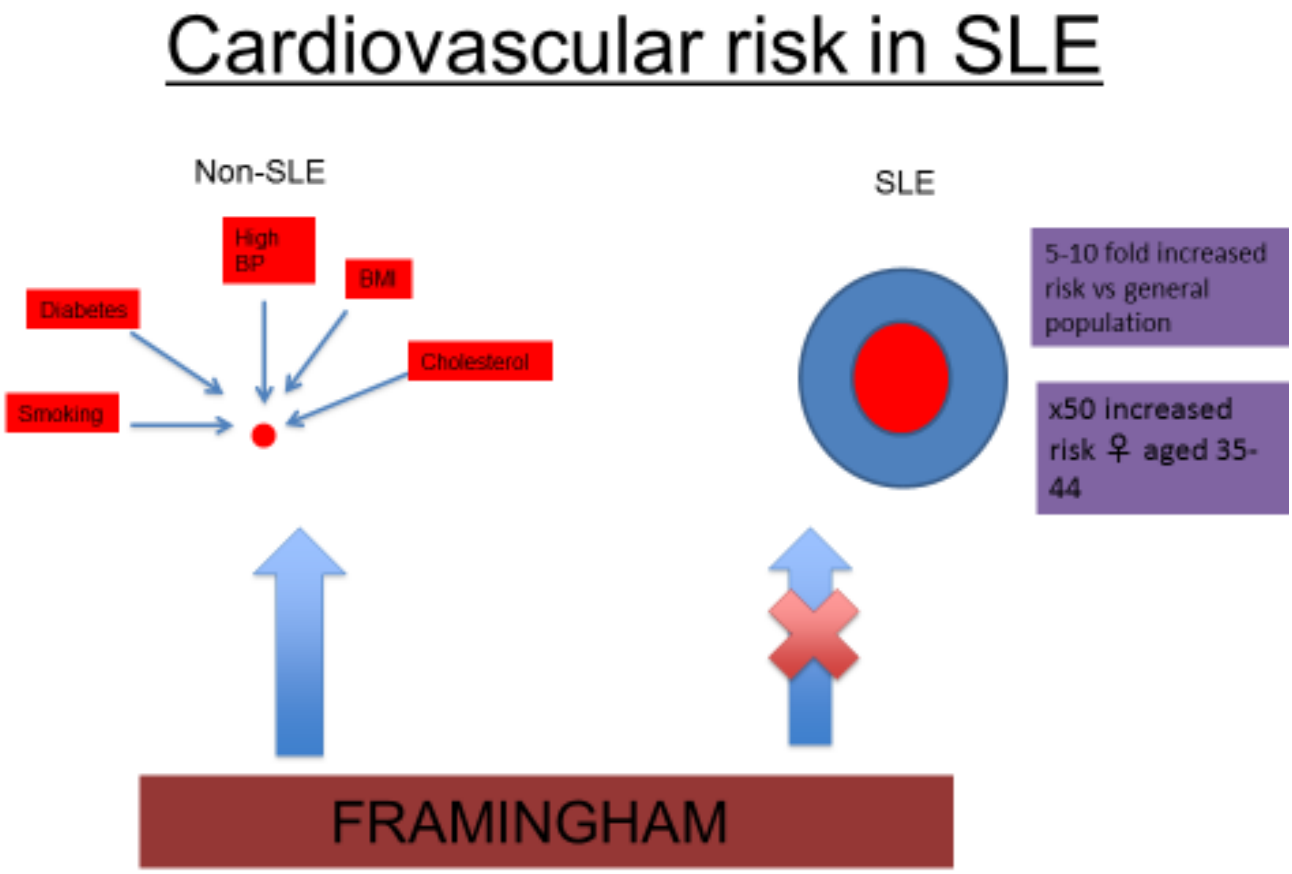


FIGURE 4 - CVD RISK IN SLE

The increased risk of CVD in SLE is thought to be multi-factorial driven by traditional CVD risk factors that occur in the general population, but in addition by immunological factors linked to the disease itself. The small dot represents a healthy individual without SLE who has an overall smaller risk of CVD compared to the large dot, representing the CVD risk of a lady with SLE. Red depicts the risk of CVD from traditional CVD risk factors and blue from the risk of immunological/disease related

markers represented in blue. The patient with SLE has an overall greater risk of CVD compared to a healthy individual, with a greater contribution of traditional CVD risk factors, but in additional risk of immunological and disease related factors.

1.4.3 SLE disease related factors

Several large cohort studies have reported the presence of a greater number of traditional CVD risk factors such as hypertension, diabetes, smoking, sedentary lifestyles in SLE than matched controls (Roman MJ et al 2003; Asanuma A et al 2003) . In the Toronto Lupus cohort, patients with CVD events had a greater number of these risk factors than those patients without events (Urowitz MB et al 2007). In SLE patients, the number of atherosclerotic plaques was associated with age ($p < 0.001$), smoking ($p = 0.016$) and hypertension ($p = 0.029$) (Tektonidou, Kravvariti et al. 2017). However, as reported by Esdaile et al, even after accounting for these traditional risk factors, SLE patients had a 10-fold relative risk of non-fatal MI and 17-fold relative risk of death from CHD (Esdaile JM et al, 2001). Therefore, the disease itself, as well as therapy used may contribute to the increased risk.

Corticosteroids are thought to carry an increased CVD risk attributed by their well-known adverse metabolic effects such as hypertension, diabetes, dyslipidaemia and obesity. Ever use of steroids or longer duration of use have been associated with an increased risk of CVD in SLE (Elliott and Manzi 2009). Tektonidiou et al showed that the number of atherosclerotic plaques was significantly associated with cumulative corticosteroid dose ($p = 0.007$). For each gram of cumulative prednisolone equivalent dose used over the patient's disease course there was a 4% increase in the expected number of atherosclerotic plaques in SLE patients (Tektonidou, Kravvariti et al. 2017) . Patients with higher cumulative doses of steroids perhaps represent a phenotype of patients with a higher inflammatory burden. Thus, if corticosteroids increase risk of atherosclerosis directly or causally through the increased risk of traditional CVD risk factors remains to be determined (Elliot JR et al. 2009).

The use of Hydroxychloroquine may have cardioprotective benefits in SLE. Studies have shown lipid lowering effects (TC, LDL and TG) (Wallace DJ et al 1990) and lower mean glucose levels (Petri 1996, Elliott and Manzi 2009). A potential mechanism underlying the beneficial effect of anti-malarials on dyslipidaemia may be represented by upregulation of LDL receptors with an enhancement of the plasma removal of this lipoprotein (Sachet, Borba et al. 2007). HCQ may also reduce the development of diabetes in RA patients (Penn S et al 2008). In a prospective study looking at the effect of HCQ on lipoproteins, the authors reported lower LDL, VLDL and triglyceride concentrations ($p < 0.001$) and higher HDL concentrations **(**$p <$ 0.001) in patients treated with HCQ after correction for the confounding effect of other variables(Sachet, Borba et al. 2007). SLE patients on HCQ have been found to have less carotid plaque than those not on HCQ (Roman MJ et al, 2003). Roman et al reported a borderline-independent effect of current or former treatment with HCQ in SLE patients (adjusted OR 0.49; 95%CI 0.21–1.12; $p= 0.09$) in reducing plaque burden, on carotid ultrasound (Roman MJ et al, 2003). However, other studies have not shown a beneficial effect of HCQ use in reducing atherosclerosis (Maksimowicz-McKinnon, Magder et al. 2006, Ahmad, Shelmerdine et al. 2007, Kiani, Vogel-Claussen et al. 2012). Overall, the current evidence is inconclusive.

Thus, it is widely established that lupus patients have an increased CVD risk, which unlike the general population occurs at a much younger age. This results in both a significant impact on disease mortality and morbidity and has an impact on the psychosocial wellbeing of young women and their families. Despite knowing about the increased risk, the factors that contribute to this increased risk have yet to be fully elucidated and therefore there are no standard guidelines to help

rheumatologists manage this risk effectively. Therefore, CVD in lupus clearly represents an important need that requires addressing to help counter the significant burden associated with CVD in SLE.

1.5 CVD risk in RA

The increased CVD risk RA will be explored in the following sections looking at the evidence suggesting an increased risk and the role of traditional risk factors in contributing to this risk.

1.5.1 Evidence of increased CVD risk in RA

CAD mortality has been reported to be 50% higher in patients with RA than in the general population in a meta-analysis of 24 observational studies comparing 111,758 patients (Avina-Zubieta, Choi et al. 2008). In a population of 1010, British patients with RA followed for a median of 11.4 years. There were 470 deaths, of which 48% of deaths reported in women and 44% of deaths in men were due to cardiovascular disease. The standardised mortality ratio (SMR) for cardiovascular disease was raised in both sexes at 1.36 (95% CI 1.05-1.75) in men and 1.93 (95% CI 1.65-2.26) in women (Gerli and Goodson 2005).

Both standard CVD risk factors and markers of RA severity (such as increased disease activity, nodules, erosions and seropositivity) contributed to risk of developing CVD from a registry of more than 10,000 RA patients followed for a median of 22 months (Solomon, Kremer et al. 2010). In a Swedish study of 6864 patients treated with biologic agents, the incidence of acute coronary syndromes (ACS) was 2.5 to 3 times higher in patients with moderate or poor response to treatment than in non-RA controls. However, patients with good response to treatment had the same rate of ACS as people without RA (Ljung, Rantapaa-

Dahlqvist et al. 2016). This suggests that controlling disease activity reduces the risk of CVD in RA.

1.5.2 Contribution of traditional risk factors in RA CVD

Traditional risk factors have been shown to contribute partly to the increased risk of CVD in RA. In the Nurses' Health Study (a prospective cohort study of 114,342 women) participating researchers found > 2-fold higher risk of myocardial infarction in women with RA compared to non-RA even after adjusting for cardiovascular risk factors (Solomon, Karlson et al. 2003). In this study, no significant differences between clinical risk factors for CVD were seen between women with and without RA (Solomon, Curhan et al. 2004). Other studies where both men and women have been included suggest traditional risk factors are more prevalent in those with longstanding disease (Chung, Oeser et al. 2006). Therefore, traditional risk factors may contribute to some, but not all, of the risk of increased CVD in RA.

The presence of the metabolic syndrome (defined as the presence of abdominal obesity, hyperglycemia, dyslipidemia and hypertension) is associated with a higher risk of CVD in the general population. In a study by Chung et al, the prevalence of the metabolic syndrome was shown to be increased in patients with RA and these patients were also found to have higher coronary calcification scores (Chung, Oeser et al. 2005, Chung, Oeser et al. 2008). The rates of smoking are also reported to be higher in RA patients compared to the general population. Being defined as a current or an ex-smoker is about 1.5 times higher in RA than the general population (Boyer, Gourraud et al. 2011) and a meta-analysis confirmed the association of smoking with the CV risk in RA (Baghdadi, Woodman et al. 2015). The evidence regarding prevalence of hypertension in RA appears conflicting; a meta-analysis of seven studies reported no significant differences between RA and controls (Boyer,

Gourraud et al. 2011), whilst others indicate a higher prevalence (Panoulas, Douglas et al. 2007, Choi, Park et al. 2018).

Dyslipidemia is common in RA affecting between 55–65% of patients (Toms, Smith et al. 2012). The role of lipids in the development of CVD in patients with RA is thought to have paradoxical effect compared to that of the general population. Lower lipid levels have been found to be associated with an increased CV risk in RA (Semb, Kvien et al. 2010) and levels of LDL, high density lipoprotein (HDL), and total cholesterol (TC) are inversely correlated with markers of chronic inflammation (Myasoedova, Crowson et al. 2011). A possible mechanism may be that inflammation promotes consumption or reduces synthesis of lipoproteins and functional and structural changes of these molecules may occur during periods of high disease activity (Carpentier and Scruel, 2002). Impaired HDL function and increased proinflammatory HDL have been reported in about 20% of RA patients (Hahn *et al.* 2008). Altered HDL function reduces its ability to cause cholesterol efflux contributing to plaque development (Charles-Schoeman, Lee et al. 2012).

The apolipoprotein-related mortality risk (AMORIS) study evaluated the prognostic significance of total cholesterol (TC) and TG (triglycerides) for CV events among RA and non-RA patients (Semb, Kvien et al. 2010). This study examined 480 406 people (including 1779 with RA, of whom 214 had an acute myocardial infarction (AMI) and 165 an ischaemic stroke (IS)). RA patients had a 1.6 times higher rate if AMI and IS than non-RA patients. Subjects were followed for a mean of 11.8 (range 7-17) years to explore the predictive value of lipids in people with and without RA in Sweden. Both total cholesterol (TC) and triglycerides (TG) were significantly lower in RA vs non-RA patients. The lower total cholesterol in RA (5.66 vs 6 mmol/l, $p<0.00001$)

could be explained by the findings of the sub-group analysis of men (n=163) which revealed a lower HDL (4.30 vs 4.60 mmol/l) (p=0.0006) and LDL levels (3.68 vs 3.91 mmol/l) (p=0.003) in RA vs non-RA, respectively). Although there was a statistically significant predictive value for higher TC (p<0.0001) and TG (p<0.0001) for non-RA patients in AMI and IS, the results for RA patients were inconsistent (Semb, Kvien et al. 2010). Higher TC was nearly significantly predictive for AMI (p=0.07) and significantly predictive for future IS in RA (p=0.02). With higher TG there was no significant predictive value for an AMI (p=0.29), however it was weakly related to IS (p=0.06) in RA.

Predictions based on standard risk factors underestimate risk of CVD in patients with RA, just as they do in SLE (Arts, Popa et al. 2016). Therefore, in 2009 the EULAR task force advocated the use of a 1.5 multiplication factor for these risk prediction models when certain RA disease characteristics were present (Peters, Symmons et al. 2010). This will be discussed in more detail in section 1.6.2.

In conclusion, traditional risk factors for CVD fail to fully account for the increased CVD risk in RA. Other possible mediators related to inflammation may well play a role and I will explore possible immunological mediators in a later section.

1.6 **Management of CVD Risk**

Identification of immunological factors may in the future enable us to identify the sub-group of patients who are at a higher risk of CVD to enable risk stratification and more stringent control of disease activity and traditional CVD risk factors. In the two sections that follow, I will explore management strategies for SLE and RA separately.

1.6.1 **Management of CVD risk in SLE**

There have been no randomised controlled trials to date to guide clinicians to the best practice of managing CVD risk in SLE. At present, management of traditional risk factors and SLE-related factors (such as disease activity) are the best strategy for modifying CVD risk. SLE subjects should be screened annually for cardiac risk factors- a Boston cohort showed that only 26% of patients had four cardiac risk factors assessed annually (Urowitz, Ibanez et al. 2007). As part of my thesis I therefore, chose to explore if conventional risk factors were being recorded and managed adequately in our large cohort of SLE patients at UCLH and by improving this could lead to the identification of high-risk groups.

Table 4 indicates how the traditional CVD risk factors should be addressed and monitored in SLE patients. An approach to management can be taken by having a target-based approach. As SLE is a very high risk condition for CVD it can be viewed as a “coronary heart disease equivalent” and therefore targets used for conditions such as Diabetes should also be used in SLE (Wajed, Ahmad et al. 2004).

Interestingly, the European Society of Cardiology and European Atherosclerosis Society (ESC/EAS) guidelines for lipid lowering therapy recommend estimation of total cardiovascular risk and do not make a distinction between symptomatic and asymptomatic patients. This is because extensive atherosclerosis can be present in asymptomatic individuals. They indicate that the presence carotid bifurcation and/or femoral plaques on ultrasound identify a group at very high-risk for cardiovascular events. The presence of such plaques should be considered a risk modifier in individuals at low to moderate risk as calculated using conventional risk factors. The guidelines recommend aggressive risk factor modification and LDL reduction by at least 50% compared with baseline or a target of <1.4 mmol/L (<55mg/dL). This should be achieved by using a statin plus ezetimibe. PCSK9 inhibitors should be considered if necessary. Such therapy should reduce the overall cardiovascular risk by 50% (Mach, Baigent et al. 2019).

Risk Factor	*Prevalence in SLE*	*Frequency of assessment*	*Target and treatment options*
Smoking	17-21% of patients with SLE smoke.	Annually	Stop smoking (in all patients) Referral to cessation clinics, specialist nurse, drug treatments e.g. nicotine replacement therapy
Cholesterol	Hypercholesterolemia 34-51% Typically low HDL and raised triglycerides (from inflammatory disease +/- steroids)	Annual fasting lipids namely total, HDL, LDL, and triglycerides.	Adult Treatment Panel 111 guidelines –LDL cholesterol key lipid parameter; 1. LDL cholesterol <2.6mmol/l no action review annually 2. LDL 2.6-3.4mmol/l therapeutic lifestyle changes- diet and weight reduction 3. LDL >3.4mmol/L or still >2.6 despite lifestyle modification - drug therapy e.g. statin
Body Mass Index	Frequently seen in truncal distribution in SLE	Annually	>25kg/m^2 (over weight): Consider steroid dose adjustment, lifestyle modification, exercise programmes, dietary education, and alcohol intake reduction. Monitor over set period. If above fail then drug treatment as recommended by NICE
Diabetes Mellitus	5-7%	Screening at every visit as part of SLE assessment with a urine sample	DM diagnosed in presence of fasting glucose ≥ 7 moll/l or random blood glucose or ≥11mmol/l.

		Random glucose- annually at least Monitor those on high dose steroids more closely	Referral to specialist in diabetes for all patients with diabetes.
Blood pressure	Common in SLE	At every clinic visit and at least annually	1.Ideal target <130/80 mmHg 2. >140/90 review closely and lifestyle measures offered. Steroid treatment reviewed and renal function monitored. 3. If > 140/90 despite measures in 2-drug treatment based on age and comorbidities. Start ACE Inhibitors in all with renal lupus. 4. Review BP every 3 months after starting treatment and ideally be kept <130/80.

TABLE 4- ASSESSMENT OF TRADITIONAL CVD RISK FACTORS IN SLE (BAKSHI, SEGURA ET AL. 2018)

1.6.2 **Management of CVD risk in RA**

Framingham model-based CV risk calculators currently used for the general population have been shown to underestimate CVD risk in RA as discussed previously. EULAR recommends the use of CVD calculators such as Framingham in calculating the 10 year risk of CVD in RA patients by multiplying by a factor of 1.5 (Peters et al. 2010). This view is based on the reasoning that other factors associated with an increased risk are important. Other factors in RA are important for increasing CVD risk such as prolonged disease duration, patients who are seropositive or anti-CCP positive and the presence of severe extra-articular disease. Therefore, it is recommended that the derived CV risk estimate should be multiplied by 1.5 if at least two of the following criteria are present: disease duration of more than 10 years, RF and/or anti-CCP positivity, presence of severe extra-articular manifestations. A conservative approach of 1.5 rather than 2 was taken on the basis that the evidence of RA related factors come from a meta-analysis of mean standardised mortality risk ratio, which included observational studies (where there was not adequate adjustment for factors such as social class or physical activity) and expert opinion (Ward 2001, Peters, Symmons et al. 2010).

Several studies have highlighted that these predictive scores still underestimate risk even after multiplication (Corrales et al. 2014, Karpouzas et al. 2013). EULAR recommends aggressive management of traditional risk factors and optimization of anti-inflammatory and immunomodulatory therapy to achieve effective disease control to prevent CVD.

Data suggests that methotrexate may decrease CVD risk and outcomes (Micha et al. 2011). The exact mechanism of this suppressive effect remains unclear but maybe

related to its effect on reducing systemic inflammation (Roubille et al. 2015). TNF-α antagonists may also exhibit a cardioprotective effect (Barnabe et al. 2011). EULAR also recommends where possible to use the lowest dose of steroid required to maintain disease control (Agca, Heslinga et al. 2017) .

Glucose screening is essential due to the high prevalence of insulin resistance in RA. EULAR recommend that non-fasting total cholesterol and high-density lipoprotein (LDL) cholesterol should be measured and treated appropriately to further mitigate CVD risk. There are no specific guidelines for RA patients and EULAR recommends national guidelines regarding the medical management of hypertension and hypercholesterolemia with anti-hypertensives and statins should be followed (Perk et al. 2012; Innala et al. 2011). The 2015 EULAR guidelines support the suggestion that carotid ultrasound screening of subclinical carotid artery plaques in patients with RA should be considered by rheumatologists to further mitigate any increased CVD risk imparted by the presence of carotid atherosclerosis

In conclusion, the excess CVD risk in both RA and SLE patients is not fully understood or adequately predicted by CVD risk calculators. Future studies need to study the impact of traditional CVD risk factors and immunological markers that may determine more appropriate management strategies - pharmacological or not.

1.6.3 Immune mediators RA and SLE CVD

In the following section, the possible mechanisms involved in the pathogenesis of CVD in RA and SLE will be explored, focusing on immunological markers that I have studied for my thesis.

1.6.4 Role of inflammation

As discussed in the previous sections, both SLE and RA are characterised by immune dysfunction. Central to the pathogenesis of atherosclerosis is inflammation and lipid dysfunction. Thus, it has been proposed that the immune system contributes to development of CVD in patients with SLE and RA.

SLE is characterized by lipid defects and chronic inflammation. The pathogenesis of atherosclerosis in SLE is not fully understood and is likely to be multi-factorial from a combination of increased disease activity and presence of autoantibodies. Possible mechanisms include;

-immune complex deposition which may stimulate the accumulation of cholesterol in atherosclerotic plaques (Kabakov, Tertov et al. 1992).

- dysfunctional proinflammatory high-density lipoprotein (HDL) cholesterol, which is commonly present among SLE patients, may accelerate low-density lipoprotein oxidation and atherosclerosis (McMahon, Grossman et al. 2009).

- apolipoprotein (ApoA1) is the main constituent of HDL. Anti-HDL and/or anti-apoA-1 antibodies could interfere with the protective functions of HDL and could thus promote development of CVD. Anti-apoA-1 antibodies have been associated with acute coronary syndromes in the general population (Vuilleumier, Reber et al. 2004).

Chronic inflammation and disease severity have been shown to be established risk factors for CV disease risk in RA. Inflammation that is occurring in the synovium in RA due to abnormal immune responses involving T-cells and dendritic cells also occurs in the inflamed atherosclerotic plaque and also occurs at the vascular wall due to circulating cytokines and CRP that are released in RA. RA patients who have positive RF, ACPA and raised CRP have been found to be at greater risk of CVD (Farragher, Goodson et al. 2008, Liang, Kremers et al. 2009, Rho, Chung et al. 2009). TNF-alpha and IL-6, which are produced in RA because of systemic inflammation, contribute to endothelial dysfunction, which occurs in early atherosclerosis (Choy, 2012). A hypercoagulable state (due to increased levels of factors such as fibrinogen and, von Willebrand factor, plasminogen activator inhibitor-1, and/or other acute phase reactants that correlate with ESR) may also contribute to the pathogenesis of CVD in RA (Wallberg-Jonsson, Cederfelt et al. 2000). Anti-apoA-1 antibodies have been associated with acute coronary syndromes in patients with rheumatoid arthritis (Vuilleumier, Rossier et al. 2010).

Endothelial dysfunction in RA may be improved with anti-TNF therapy, as suggested by a recent systematic review (Ursini, Leporini et al. 2017). A significant improvement in endothelial function following anti-TNF-α treatment ($p < 0.0001$) was found using pooled-analysis of 20 randomized clinical studies. However, interpretation may be limited due to the presence of heterogeneity and evidence of

possible publication bias. Only four out of the 20 were from RCTS, with the vast majority being small observational studies and 12/20 were rated as low quality studies. In addition, endothelial function measurement technique was a significant contributor to heterogeneity (Ursini, Leporini et al. 2017).

Inflammatory cytokines that are typically elevated in RA (IL-6, IL-18, and TNF-alpha) have been associated with cardiovascular disease (Kaptoge, Seshasai et al. 2014). Inflammatory markers such as ESR and CRP have been shown to be associated with intimal media thickness, a surrogate for atherosclerotic disease (Kaptoge, Seshasai et al. 2014) (Gonzalez-Gay, Gonzalez-Juanatey et al. 2005). Pro-atherogenic HDL may also contribute to the increased CVD risk as a consequence of RA associated inflammation (Gonzalez-Gay, Gonzalez-Juanatey et al. 2005). Inflammation thus significantly contributes to CVD risk in patients with RA in addition to traditional CVD risk factors.

Possible immune markers that I have studied include antibodies to ApoA1 and specialized T-cells called invariant natural killer cells (iNKT cells). These will be discussed in more detail in the following sections.

1.6.5 **Anti-apoA-1 antibodies**

As discussed previously, inflammation and altered lipid metabolism are integral to the pathogenesis of atherosclerosis. Several studies have shown that SLE patients have an altered more atherogenic lipid profile- commonly reduced HDL and raised triglycerides**.** HDL has long been established to have an atheroprotective role and an inverse correlation between plasma HDL levels and CVD in the general population is recognized (Grundy, 2004).

ApoA1 is the main component of HDL. The enzyme paraoxonase 1 (PON1) is transported with HDL and is stabilised by apoA-1 allowing it to play a role in preventing lipid peroxidation and oxidised LDL formation (Vuilleumier, Reber et al. 2004). HDL loses its anti-oxidant capacity during acute phase responses, causing increased production of oxidised LDL. Normal HDL also mediates reverse cholesterol transport, removing cholesterol from the arterial wall. Therefore, it has been proposed that in a chronic inflammatory disease, continued generation of a pro-inflammatory or “dysfunctional” HDL increases the risk of atherosclerosis (McMahon, Grossman et al. 2009).

The atheroprotective role of apoA-1 has been demonstrated in non-SLE patients, namely patients with elevated IgG anti-apoA-1 levels are associated with increased risk of developing major cardiovascular events in post-myocardial infarct patients (Vuilleumier, Rossier et al. 2010, Pagano, Satta et al. 2012)**.** In a single centre prospective study of 221 patients hospitalised for an acute MI completing a 1-year follow up, those patients who had high anti-apoA-1 IgG at baseline had a worse cardiovascular-event free survival at one year than those who tested negative at

baseline**.** The patients who tested positive for anti-apoA-1 IgG were also found to have a higher basal heart rate on discharge (marker of poorer prognosis) and a higher proinflammatory cytokine profile possibly associated with atherosclerotic plaque vulnerability (Pagano, Satta et al. 2012).

Higher anti-apoA-1 IgG antibodies have been reported in Rheumatoid Arthritis patients. In a longitudinal prospective study involving 133 RA patients followed-up for a median duration of 9 years, RA patients who were anti-Apo A-1 IgG positive were shown to have higher oxidised LDL levels and were associated with a pro-inflammatory cytokine profile compared to those who were negative. Anti-Apo A-1 IgG positivity was associated with higher median circulating levels of interleukin-8 (IL-8) and MMP-9, two inflammatory mediators associated with atherogenesis, but did not remain significant after Bonferroni correction (Vuilleumier, Rossier et al. 2010, Finckh, Courvoisier et al. 2012)

Both animal and in vitro studies support an atherogenic role of anti-apoaA-1 IgG in CVD. Macrophages derived from human monocytes were found to produce pro-inflammatory molecules such as IL-8, IL-6, TNF-a, MMP-9 and MCP-1 in the presence of IgG anti-apoA-1. A possible mechanism was demonstrated by Pagano et al who showed that the pro-inflammatory effect of anti-apoA-1 IgG was mediated by interaction with the toll-like receptor (TLR)-2/CD14 complex, due to molecular mimicry (Pagano S et al, 2012). IgG anti-apoA-1 have also been associated with markers of increased plaque vulnerability such as intra-plaque macrophage, neutrophil and matrix metalloproteinase content in humans and mice (Pagano, Carbone et al. 2016).

The presence of IgG anti-apoA-1 in the sera of patients with SLE has been reported by different research groups (Delago A et al, 2002; Batuca JR et al, 2006; O'Neill SG et al, 2010). ApoA-1 may be a key antigen for anti-HDL as a strong correlation between titres of anti-apoA-1 and anti-HDL has been reported (O'Neill SG et al, 2010). However, although the two antibodies are correlated not all patients positive for anti-apoA-1 are positive for anti-HDL and vice versa (Vuilleumier N et al, 2014). Therefore, there may well be other targets on the HDL molecule that are not yet known.

Raised anti-apoA-1 levels have been associated with both current and persistent disease activity in both prospective and retrospective analyses and in longitudinal and cross-sectional studies in SLE (O'Neill, Giles et al. 2010). In a larger more recent study by Croca et al, 499 SLE patients were assessed for the presence of IgG anti-apoA-1 in early samples. Twenty-seven percent (135) were positive for anti-bodies in early samples, but no association was found between early positivity and the development of CVD. Forty-nine patients who had IgG anti-apoA-1 levels measured longitudinally were found to have significantly higher antibody levels in the presence of active disease (p=0.01), higher doses of steroid (p= 0.002) and those not taking HCQ (p=0.003) in a univariable analysis. A limitation of this study for CVD outcomes was that CVD was defined by reviewing the notes and not by harder end points such as troponin rise or imaging studies (Croca, Bassett et al. 2015).

The exact mechanism of the increased CVD in SLE or RA remains unknown. However, IgG anti-apoA-1 antibodies may act as biomarkers to help identify patients who are at higher risk. A relationship between anti-HDL and anti-apoA-1 in SLE patients and atherosclerosis has not yet been demonstrated. In my thesis, one of my

goals is to explore the possibility that such a relationship exists in a matched cohort of SLE patients with and without CVD.

1.6.6 **Invariant Natural Killer cells (*i*NKT cells)**

Invariant Natural Killer T-cells (*i*NKT) are a small population of specialised immune cells that constitute less than 0.1% of peripheral blood mononuclear cells. They are thought to be important in a broad range of immune responses and are unique in that they respond to lipid antigens presented by the CD1d molecule on antigen presenting cells (Berzins et al, 2011).

Natural killer T (NKT) cells are a subset of lymphocytes that possess features of both T-cells and natural killer cells. These features enable them to orchestrate exclusively a multitude of innate and adaptive immune responses, with the specificity of T cells and rapid nature of NK cells. Similar to conventional T cells NKT cells express an αβ TCR, yet instead of responding to peptide antigen in the context of MHC class I or II, NKT cells have evolved to recognize and respond to lipid antigens in the context of CD1d (Berzins, Smyth et al. 2011). Two types of NKT cells exist in humans, characterized based on their αβ TCR expression. Type I or *i*NKT cells, are the predominant class (approximately 80%) in humans and express an invariant T cell receptor (iTCR) formed of an alpha chain comprised of Vα24 and Jα18 and a beta chain comprised of Vβ11 (Godfrey, Stankovic et al. 2010). They are homologous to the semi-invariant TCR in mice formed of Vα14, Jα18 and Vβ8.2/Vβ7/Vβ2 chains (Major, Singh et al. 2006). In contrast, type II NKT cells express diverse αβ TCRs yet relatively little is known about them due to their lack of distinct surface markers or functional characteristics. As a result, far fewer studies have looked at the role of type II NKT cells in disease, particularly in humans (Berzins, Smyth et al. 2011). Here, the focus of my project is the type I or *i*NKT cells and their role in SLE and

atherosclerosis. Figure 5 shows the structure *i*NKT cell and activation by lipid antigens.

*i*NKT cells rapidly downregulate their iTCR expression upon activation, whilst secreting copious amounts of both pro- and anti-inflammatory cytokines. *i*NKT cell activation is predominantly mediated through CD1d-mediated lipid antigen presentation, although other mechanisms have also been published including cytokines. CD1d is a member of the CD1 family of glycoproteins (Girardi and Zajonc 2012). They are expressed by a range of immune cells including monocytes, macrophages, B cells, dendritic cells (DCs) and thymocytes (but not mature T cells), as well as cells outside of the hematopoietic system e.g. epithelial and vascular smooth muscle cells (Canchis, Bhan et al. 1993). The CD1 molecule is structurally homologous to major histocompatibility complex (MHC) class I molecules.

During lipid antigen processing and presentation, CD1d undergoes internalization and vesicular trafficking to endosomal and lysosomal compartments. Lipids either endogenous to the cell or obtained from exogenous sources through endocytosis are exchanged for ligands on the CD1d molecule. CD1d then returns to the cell surface to present the lipid to the iTCR present on *i*NKT cells (Brigl, Bry et al. 2003).

Indirect activation of *i*NKT cells can occur through cytokines such as IL-12, which may be produced following TLR stimulation on APCs. Activation of *i*NKT cells by IL-12 appears to be more effective in combination with small amounts of either IL-18 or type I IFN, and results in the secretion of mainly IFN-γ (Brigl M, et al 2003). Activation of *i*NKT cells can also occur through their NK receptors CD161 and NKG2D; even in the

absence of CD1d (Joshi SK et al 2013). CD161 recognizes lectin-like transcript 1 (LLT1), a marker present on activated leukocytes and DCs, whilst NKG2D recognizes ligands associated with MHC class-I, which are altered due to cellular stress (Joshi and Lang 2013).

*i*NKT cells rapidly proliferate and secrete a range of cytokines and chemokines, after activation. Immune cell transactivation results either through cytokine and chemokine production, or through contact-dependent mechanisms. Reciprocal signals provided by the *i*NKT cell can also increase CD40, CD80 and CD86 expression on the APC, and in the case of B cells, can provide stimuli for class switching, expansion and memory cell formation. *i*NKT cells exhibit poor memory and secondary responsiveness due to the fact that they become anergic following activation, through downregulation of the iTCR (Major, Singh et al. 2006). In humans, *i*NKT cells are either CD4 or CD8 positive whereas in mice most cells are CD4 single positive, with a small population expressing CD8α alone (Godfrey, MacDonald et al. 2004).

The innate ability of *i*NKT cells to respond in a proinflammatory versus a tolerogenic manner is governed by a number of factors including the lipid antigen being presented, the APC involved, and the association between CD1d and lipid rafts.

The importance of antigen structure in determining *i*NKT cell responses was demonstrated using a synthetic α-GalCer analogue (*i*NKT cell agonist) naphthylurea-α-GalCer (NU-α-GalCer) (Aspeslagh, Li et al. 2011)). Whilst α-GalCer and NU-αGalCer were both shown to induce IFN-γ and IL-4 secretion, α-GalCer was found to stimulate greater amounts of IL-4, whereas NU-α-GalCer predominantly stimulated

IFN-γ secretion (Venken, Decruy et al. 2013). Different cell types may determine the extent of *i*NKT cell cytokine production and transactivation of other immune cells in vivo through. These include differences in expression of CD1d between B cells, monocytes and dendritic cell subsets, which may each have different influences on the *i*NKT cell response (Arora, Baena et al. 2014)

Lipid rafts are highly ordered areas of the plasma membrane. They consist of glycosphingolipids, protein receptors and cholesterol. Cholesterol is the glue holding the raft together, and differences in composition determine fluidity of cell membranes. These specialised membrane microdomains compartmentalise cellular processes by serving as organising centers for the assembly of signaling molecules, influencing membrane fluidity and membrane protein trafficking, and regulating neurotransmission and receptor trafficking membranes (Ikonen 2008).

SLE patients have been shown to have differences in their lipid raft composition, influencing the position of key signalling molecules such as lymphocyte-specific protein tyrosine kinase (Lck), CD45 in T-cells and many others. Increased levels of glycosphingolipids and cholesterol in the membrane of lymphocytes have been found (Jury, Kabouridis et al. 2004). Dysregulation of the signalling molecules in SLE increases cell susceptibility to prolonged activation and hyper-responsiveness (Simons and Toomre 2000).

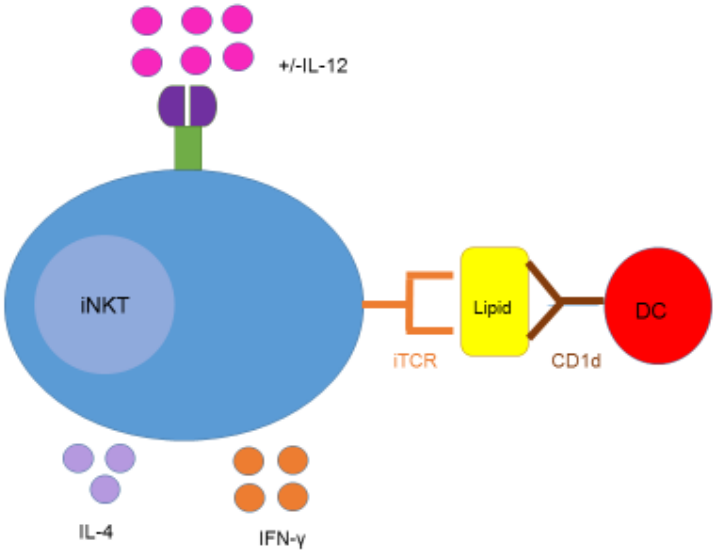


FIGURE 5- ACTIVATION OF /NKT CELLS

iNKT cells can be activated either through presentation of endogenous or exogenous lipid antigens via CD1d, as well as through IL-12. Endogenous lipid presentation is the presentation of self-lipids, which may be altered through changes in lipid metabolism during times of cellular stress, and is associated with IFN-γ secretion. Alternatively, exogenous antigen can be endocytosed and presented directly, leading to both IFN-γ and IL-4 secretion. Additionally, stimulation of TLRs on antigen presenting cells can result in the production of IL-12, which induces IFN-γ secretion by *iNKT* cells (Berzins, Smyth et al. 2011). DC=dendritic cell; IL-4=Interleukin 4; IL-12=Interleukin 12; IFN-γ =Interferon Gamma; iTCR=invariant T-cell receptor; *iNKT*= Invariant Natural Killer T-cell.; CD-1d= Cluster of differentiation 1d.

1.6.7 *i*NKT cells in autoimmune disease and atherosclerosis

*i*NKT cells are known to play a role in both autoimmunity and atherosclerosis. Mouse models that are deficient in *i*NKT cells have been shown to have a predisposition to autoimmune disease and cancers (Smyth, Thia et al. 2000). Various mouse models have been examined looking at *i*NKT cells in SLE. Mouse models with strains that have a genetic predisposition towards SLE (MRL/lpr and NZB/W F1) have shown defects in iNKT cell frequency and function compared to non-SLE mice. MRL/lpr mice, which have a defective point mutation in Fas spontaneously develop inflammatory lesions affecting the skin and kidneys with marked lymphoproliferation and autoantibody production. CD1d-deficient MRL/lpr mice show exacerbated skin lesions (Zhou, Fujio et al. 2004). In another study using NZB/W F1 (BWF1) mice, CD1d deficiency accelerates the onset and progression of nephritis (Forestier, Molano et al. 2005).

In humans, quantification of Vα24Jα18 mRNA expression indicated reduced numbers of *i*NKT cells in both the peripheral blood and synovium of SLE patients compared to healthy controls (Sumida 1998). There is a reduction in both the proportions and absolute numbers of type I and type II NKT cells, with $CD161^+$ cells within the $V\alpha24^+CD4^-CD8^+$ and $V\alpha24^+CD4^-CD8^-$ *i*NKT cell subpopulations having been reported as being reduced in SLE patients compared to healthy controls shown by flow cytometry (Takahashi, Chiba et al. 2002, Mitsuo, Morimoto et al. 2006, Cho, Kee et al. 2011). This reduction in *i*NKT cells in SLE patients was not observed in relatives of SLE patients, implying that the *i*NKT cell deficiency observed was disease-associated and not genetic (Green, Kennell et al. 2007). The reduction in

*i*NKT number has also been shown to be independent of drug treatment in SLE patients (Wither, Cai et al. 2008, Cho, Kee et al. 2011).

As well as the reduction in number of *i*NKT cells in SLE, functional impairment has been demonstrated. This has been shown by the poor responsiveness of *i*NKT to α-GalCer (Cho, Kee et al. 2011, Bosma, Abdel-Gadir et al. 2012). In one study by Cho et al the median proliferation index following α-GalCer was significantly lower in SLE patients than in healthy controls (median 7.5 versus 28.7, $p<0.001$) and impaired IL-4 and IFN-γ mRNA expression and cytokine secretion were also observed in SLE patients compared to healthy controls (Cho, Kee et al. 2011) .

These defects are thought to arise from poor functionality, rather than a defect in the ability of CD1d-mediated antigen presentation. Similar levels of CD1d expression (percentages of $CD1d^+$ PBMCs and monocytes) were observed in both SLE patients and healthy controls. This result is supported by the lack of *i*NKT cell proliferation seen when APCs from healthy controls were co-cultured with *i*NKT cells from SLE patients (Cho, Kee et al. 2011).

The poor *i*NKT cell function in SLE patients may be due to the observed finding that *i*NKT cells from SLE patients are more susceptible to apoptosis compared to those from healthy controls which was seen following a 7-day incubation with α-GalCer (Cho, Kee et al. 2011). A mouse model using β-GalCer (with a 12 carbon acyl chain), demonstrated reduced *i*NKT cell cytokine secretion in C57BL/6 mice following *i*NKT cell stimulation with α-GalCer and also diminished *i*NKT cell numbers in the liver and spleen (Ortaldo, Young et al. 2004, Morshed, Takahashi et al. 2009). Interestingly, intraperitoneal β-GalCer treatment in mice was found to improve SLE disease activity

as measured by proteinuria, IgG-anti-dsDNA and rate of survival (Morshed, Takahashi et al. 2009). Such findings suggest that this reduction in *i*NKT cells seen in SLE is more than just a defect in cell death, but also one concerned with the recognition of lipid antigens, which play a role in the pathogenesis of the disease.

There is evidence suggesting that IgG autoantibody production by B- cells may promote autoimmune disease in mouse models (Forestier, Molano et al. 2005), through a mechanism subsequently shown to be dependent upon CD1d and CD40 expression in vivo (Takahashi and Strober 2008). Wermeling and colleagues observed that specific knockout of *i*NKT cells (using Jα18$^{-/-}$ mice) resulted in autoantibody production by B cells following injection with apoptotic cells (Wermeling, Lind et al. 2010). Similarly, Yang et al. demonstrated that the *i*NKT cell ligand α-GalCer could suppress autoantibody production both in vivo and in vitro in mice (Yang, Wen et al. 2011). Overall, these studies highlight the regulatory role of *i*NKT cells, which enables them to protect against the inflammatory effects in SLE, when present in adequate numbers.

1.6.8 *i*NKT cells in atherosclerosis

*i*NKT cell numbers have generally been reported to be increased in mouse models of atherosclerosis. When apoE$^{-/-}$ mice, which are extremely susceptible to atherosclerosis, are crossed with CD1d$^{-/-}$ mice which lack *i*NKT cells, there is a significant reduction in the extent of atherosclerosis compared to apoE-/- controls (Major, Wilson et al. 2004, Nakai, Iwabuchi et al. 2004, Tupin, Nicoletti et al. 2004). Knockout mice that lack *i*NKT cells develop significantly less atherosclerosis when fed an atherogenic diet or when crossed with atherosclerosis-prone apolipoprotein E knockout mice compared to control mice that have normal *i*NKT T cells (Tupin, Nicoletti et al. 2004). *i*NKT cells occur in subsets that can be distinguished by their cell surface markers. On this basis CD4+ *iNKT* cells are more pro-atherogenic than CD8+ cells (To, Agrotis et al. 2009).

Few studies have been carried out on *i*NKT cells in humans. CD1d expression was restricted to foam cells and areas containing T lymphocytes and DCs suggested a role for CD1d-mediated lipid antigen presentation to *i*NKT cells in atherosclerosis (Melián, Geng et al. 1999, Bobryshev and Lord 2005). Using immunohistochemical techniques, the co-accumulation of dendritic cells and NKT cells was reported in carotid artery specimens in rupture-prone shoulders of human plaques to a greater extent than in stable plaques (Bobryshev and Lord 2005). Liu et al found a significantly reduced number of *iNKT* cells in the blood from 40 AMI patients (from blood samples taken after coronary stenting) compared to healthy controls and that they were moderately correlated with serum lipids (TC ($r = 0.32$, $p < 0.05$) and LDL ($r = 0.33$, $p < 0.05$) (Liu, Lǔ et al. 2011). Kyriakakis et al, used arterial tissue microarrays to isolate *i*NKT and

$CD1d^+$ cells from human arterial tissue (Kyriakakis, Cavallari et al. 2010). *i*NKT cells within the plaque comprised of up to 3% of total infiltrating T cells, and were scarce in the peripheral blood of patients who have had a cardiovascular event. It may be that the reduction seen in *i*NKT cells within the peripheral blood is due to accumulation within the plaque sites, although it may also be as a consequence of increased *i*NKT cell activation and downregulation of the iTCR following stimulation (Kyriakakis, Cavallari et al. 2010, Liu, Lǔ et al. 2011).

The role of *i*NKT cells in SLE and atherosclerosis in combination has rarely been studied. The role of these cells in SLE patients with atherosclerosis appears confusing due to their conflicting roles in both diseases.

Human studies have suggested that *i*NKT cells may have a protective role in the early stages of atherosclerosis by promoting atherosclerosis resolution via increased IL-10 production (van Puijvelde, van Wanrooij et al. 2009). Smith et al investigated the role of *i*NKT cells in the development of subclinical atherosclerosis in 100 patients with SLE who had no previous history of CVD using vascular ultrasound to quantify the presence of plaque and its composition. In this SLE patient cohort, 36% had subclinical plaque, supporting previous studies (Roman MJ et al, 2003). SLE patients with pre-clinical plaque (SLE-P) had a distinct anti-inflammatory *i*NKT cell profile characterized by increased activation and IL-4 production, which correlated with serum lipid expression levels and altered lipoprotein composition. *i*NKT cells differentiated in the presence of serum from SLE-P patients induced the polarization of M2-like macrophages (anti-inflammatory) *in vitro*. This protective *i*NKT cell phenotype was lost in SLE patients who had cardiovascular disease. SLE-CV

patients, had a different *i*NKT cell phenotype, characterized by low peripheral blood frequency, increased $CD8^+$ phenotype, reduced CD69 expression and low expression of IL-4 and IFN-ɣ compared to SLE-P and SLE-NP patients. This was associated with increased expression of pro-inflammatory monocytes ($CD14^{++}CD16^+$) and reduced M2-like monocyte frequency. The results support an athero-protective role for *i*NKT cells driven by serum lipids during the early stages of atherosclerosis, which was lost or overwhelmed during the development of clinical atherosclerosis (Smith E et al, 2016).

Smith et al also tested to see whether dyslipidemia, a risk factor for atherosclerosis, was associated with the altered *i*NKT cell activation in SLE-P patients. Serum lipid composition analysed using proton nuclear magnetic resonance spectroscopy revealed elevated concentrations of VLDL but not LDL or HDL particles in SLE-P compared to SLE-NP patients. The proportions of free cholesterol, cholesterol esters, phospholipids and triglycerides carried by lipoprotein subclasses (particularly in VLDL lipoproteins) as well as other serum metabolomic components were significantly different in SLE-P compared to SLE-NP patients. In comparison, serum lipid measures quantified in the clinical laboratory as part of routine patient management were within normal clinical ranges (Smith E et al, 2016).

In conclusion, the above results suggest that *i*NKT cells may have a unique phenotype during different stages of atherosclerosis and that this may be driven by dyslipidemias.

1.7 **Metabolomics**

Metabolomics involves the quantitative analysis of low molecular weight metabolites in biological systems. Nuclear Magnetic Resonance (NMR) is a widely used platform for metabolomics analysis as it is rapid, has high reproducibility and requires minimal sample preparation when compared to other platforms such as mass spectrometry (Guleria, Pratap et al. 2016).

Lipids are central to driving atherosclerosis and hypercholesterolaemia is found in 34-51% of patients with SLE (Wajed, Ahmad et al. 2004). Various fractions of lipoproteins can be distinguished in blood on account of their size. These fractions include High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL). Dyslipidemias are present in over 70% of cases of premature CHD (Wajed, Ahmad et al. 2004). Elevated plasma concentrations of LDL and VLDL can induce the development of atherosclerosis in the absence of other risk factors (Skalen, Gustafsson et al. 2002). In contrast, HDL has anti-atherogenic properties that include macrophage cholesterol efflux, anti-oxidation and protection against thrombosis (Rosenson, Brewer et al. 2012). Conversely, McMahon and colleagues have demonstrated the existence of a subpopulation of pro-inflammatory HDL in patients with SLE and rheumatoid arthritis that promote atherosclerosis and could be a biomarker for increased risk of developing CVD (Hahn, Grossman et al. 2008, McMahon, Grossman et al. 2009).

The “lupus pattern” of dyslipidemias recognised by Borba and Bonfa (Borba and Bonfa 1997) was characterised by elevated levels of VLDL and triglycerides and low

HDL levels. In addition, development of CVD in women with SLE has been found to be associated with smaller sub-fractions of LDL(Nuttall, Heaton et al. 2003). However, these abnormalities while detectable in routine lipid screens available in clinical practice, fail to account fully for the increased risk of CVD in patients with SLE (Borba and Bonfa 1997). Many SLE patients with normal lipid profiles on standard assays also go on to have CVD. Therefore, it may well be that there are more sensitive and specific lipid profiles that need to be delineated in order to identify high CVD risk patients in SLE cohorts. These profiles can be obtained using NMR. NMR can be used to measure metabolites present at concentrations above 10micromoles/l and is particularly suitable for lipoproteins due to their particle structure (Soininen, Kangas et al. 2015). It can be used to study lipoprotein subclasses in more detail and to relate the distribution of different lipids between the subclasses to the risk of CVD.

Previous groups have reported on use of metabolomics to compare patients with SLE, healthy controls and patients with rheumatoid arthritis (Ouyang, Dai et al. 2011, Wu, Xie et al. 2012, Guleria, Pratap et al. 2016) and one of these groups sub-classified their SLE population into those with and without nephritis. They reported that patients with nephritis could be distinguished from SLE patients, because LN patients had elevated LDLA and VLDL lipoproteins but decreased levels of acetate (Guleria, Pratap et al. 2016).

To date, no one has used metabolomics to compare detailed lipoprotein profiles in patients with SLE with and without sub-clinical atherosclerotic plaque. I therefore used the Brainshake NMR metabolomics platform (Kettunen, Tukiainen et al. 2012)

(see methods section) to measure over 200 different lipid and amino acid metabolites in the serum of patients with SLE.

1.8 **Subclinical CVD in SLE**

In my thesis, I have explored the role of subclinical CVD in SLE with carotid ultrasound. SLE patients have been shown to have a high prevalence of subclinical disease. Data from several studies have reported the prevalence of carotid plaque on ultrasound as being 30-40% of patients with SLE (Roman MJ et al, 1999; Manzi S et al 1999; Farzaneh-Far A et al 2006). The presence of common carotid artery intima-media thickening and discrete, non-obstructive carotid atherosclerotic plaques has been shown to be independently associated with increased cardiovascular risk in several longitudinal studies (O'Leary, Polak et al. 1999, Johnsen, Mathiesen et al. 2007, Baber, Mehran et al. 2015).

1.8.1 Role of vascular ultrasound in subclinical disease

The notion of subclinical disease has therefore encouraged the use of non-invasive imaging techniques such as carotid ultrasound (US) and electron beam computed tomography (EB-CT) to assess the extent of atherosclerosis. EULAR recommendations state that carotid US may be considered as part of CVD risk evaluation in RA (Agca, Heslinga et al. 2017) and several European and international recommendations have incorporated vascular US to optimise CVD prediction (Piepoli, Hoes et al. 2016, Cosentino, Grant et al. 2019).

Ultrasound techniques aiming to quantify the atherosclerosis-related burden have also been developed for the purpose of risk stratification. One-dimensional (B-mode) ultrasound measurement of IMT and plaque thickness (Ebrahim, Papacosta et al. 1999, Lorenz, von Kegler et al. 2006, Simon, Megnien et al. 2010), 2D plaque area and 3D measurement of plaque and vessel wall volume. The measurement of both plaque area and volume has greatly increased the predictive value of vascular ultrasound scanning for cardiovascular events among patients free of CVD at baseline. A correlation was found between carotid plaque and IMT thickening with the occurrence of both stroke and myocardial infarction (Bots, Hoes et al. 1997, O'Leary, Polak et al. 1999, Spence, Eliasziw et al. 2002, Johnsen, Mathiesen et al. 2007, Lorenz, Markus et al. 2007). Thus highlighting the utility and consistency of using non-invasive measurements to assess risk factors based on vessel wall biology.

B-mode carotid ultrasound is a rapid and convenient imaging modality, which can provide a direct marker of atherosclerotic disease (carotid plaque) and atherosclerotic risk (by measuring carotid intima-media thickness (IMT)). EB-CT provides coronary artery calcification scores that correlate with invasive coronary angiography findings in a non-invasive manner. Compared with matched controls, more lupus patients have been found to have carotid plaque (37.1% vs 15.2%) (Roman MJ et al, 2003) as well as coronary artery calcification (31% vs. 9%) (Asanuma Y et al 2003).

Progression of carotid plaque in SLE patients has also been investigated. In a study that followed 217 female patients for ten years, progression of carotid plaque (defined as an increase in plaque thickness and/or number of plaques) occurred in 27% vs 10% in healthy controls. Carotid IMT increased by a mean of 0.011mm/year (Thompson T et al, 2008). Importantly, increases in IMT or the presence of plaque is a predictor for an increased risk of cardiovascular events. In 392 women with SLE followed for a mean of 8 years, they showed that higher IMT or presence of plaque at baseline predicted development of CAD or stroke over the next 10 years in multivariable analysis. For every 0.05 mm increase in baseline IMT the risk of CVD increased by a factor of 1.35 (95% CI 1.12-1.64) and the presence of plaque at baseline increased this risk by a factor of 4.26 (95%CI 1.23 to 14.83) (Kao, Lertratanakul et al. 2013).

CT angiography, which provides more accurate and sensitive information, compared to US and allows a virtual histology of plaques, is an alternative method to US.

However it is limited by concerns of contrast and radiation exposure (Croca and Rahman 2012)

Even though the relative risk for CVD in SLE is high, the absolute risk for individual SLE patient remains low, which makes research difficult as actual events, invariably take many years to develop. In a large multi-center study of 1249 patients recruited within 15 months of the diagnosis of SLE and followed for up to 8 years, CVD was only found in 31 patients (Urowitz MB et al 2010). However, the study had a very stringent definition in recording CVD due to atherosclerosis as cases in whom active lupus was excluded. In fact, 97 vascular events recorded, 50 of which were attributed to active lupus rather than CVD. This may also explain why only age and gender were found to be significantly associated in a multi-variable analysis.

US could therefore be used in the future to identify the presence of CVD in patients with high levels of traditional risk factors alongside immunological factors to risk stratify patients to enable more stringent management of traditional cardiovascular risk factors.

In RA studies looking at subclinical atherosclerosis have shown that the presence of carotid plaque at baseline was associated with an increased risk of CVD in RA using carotid ultrasound compared to age/sex/ethnicity and traditional risk factors (Roman, Moeller et al. 2006, Salmon and Roman 2008, Evans, Escalante et al. 2011). Estimates of prevalence of subclinical atherosclerosis in RA range from 20-40% from carotid US studies (Del Rincon, Williams et al. 2003, Roman, Moeller et al. 2006). In a meta-analysis of 35 carotid studies reporting on the prevalence of plaque (2,859

RA patients and 2,303 controls), as compared to controls, RA patients showed an increased prevalence of carotid plaques (OR: 3.61; 95 %CI: 2.65, 4.93; p<0.00001) (Ambrosino, Lupoli et al. 2015).

In a large study by Evans et al of over 600 patients, the presence of plaque in both internal carotid arteries nearly quadrupled the ACS incidence compared to patients without carotid plaque (Evans, Escalante et al. 2011). A small area of my thesis looks at immunological markers and metabolomics of RA patients with subclinical disease.

1.8.2 SLE vascular ultrasound study at University college hospital (UCLH)

Between 2011 and 2013 Croca et al, carried out vascular ultrasound studies in 100 patients with SLE, who had no known history of previous CVD (Smith E et al, 2016). Of these 100 subjects, 95% were women with a mean age of 45.2 years (SD 12.4; range 20-66). Patients had ultrasound scans of both common carotid and common femoral artery bifurcations with measurements of IMT, plaque thickness, area and plaque echogenicity. The total plaque area (TPA) was also calculated. Previously published vascular ultrasound studies in SLE have been confined mainly to the carotid bifurcations and limited to measuring IMT and presence of plaque (Manzi S et al, 1999; Roman MJ et al 2003, Farzaneh-Far A et al 2006). Only a handful of SLE studies have reported on femoral plaque (Kisiel, Kruszewski et al. 2015, Tektonidou, Kravvariti et al. 2017, Theodorou, Nezos et al. 2018). Figure 6 shows a cross sectional IMT view.

Femoral plaques are also associated with CVD (Khoury Z, et al 1997). Combined scanning of the carotid and femoral arteries therefore increases the sensitivity of detecting plaque and gives a better picture of the overall plaque burden.

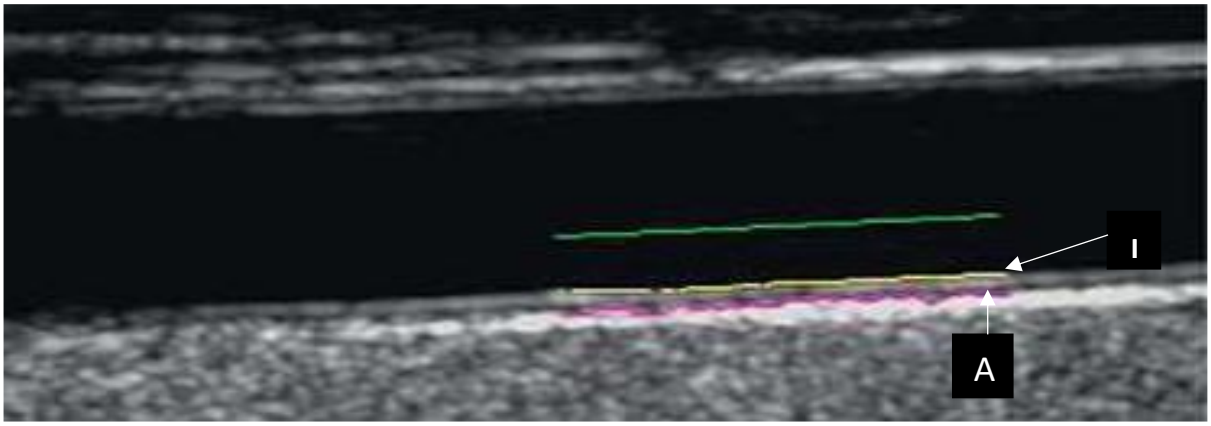
Measurement of TPA has advantages over IMT because it includes assessment of longitudinal spread of atheroma along the vessel wall as well as its extension within the lumen. Being two-dimensional (see figure 7), TPA is a more sensitive index of change than IMT (Johnsen, Mathiesen et al. 2007). Population based studies such as the Tromso study of 6226 Norwegians demonstrated that the highest tertile for

TPA was a strong predictor of myocardial infarction, particularly in women (age-adjusted RR = 4.75 compared to women with no plaque). Plaque echogenicity is also important because more echolucent plaques have a higher content of inflammatory material and some with a thin fibrous cap are thought to be more likely to rupture causing CVD events. The Tromso study showed that women with the most echolucent plaques had 2.87-fold higher risk of developing MI compared to those with no plaque (Johnsen, Mathiesen et al. 2007).

In the study by Rahman et al, a total of 100 patients were recruited. Fifty-six were Caucasian, 25 Afro-Caribbean, 11 Asian and the remaining 8 came from other ethnic backgrounds (Chinese or mixed race). Of this cohort two had thickened IMT in the common carotid artery (CCA) and 36 had plaque. This included 14 with carotid plaque only, 7 with femoral plaque only and 15 with both. Fifteen had plaque in at least three sites. A number of different demographic, clinical and serological factors including disease activity, measured by the British Isles Lupus Assessment Group (BILAG) index were assessed. Increasing age was associated with presence of plaque and increased IMT. It was reported that raised levels were associated with presence of plaque and patients taking prednisolone had higher TPA. Whist, the majority of plaques were found to be echolucent this parameter was not associated with therapy or increased disease activity (Croca, Griffin et al. 2020).

Five years on a follow-up study of these patients was undertaken to identify factors associated with progression or regression of plaque and other parameters such as TPA, IMT and echogenicity. This work is presented here in my thesis.

A)



B)

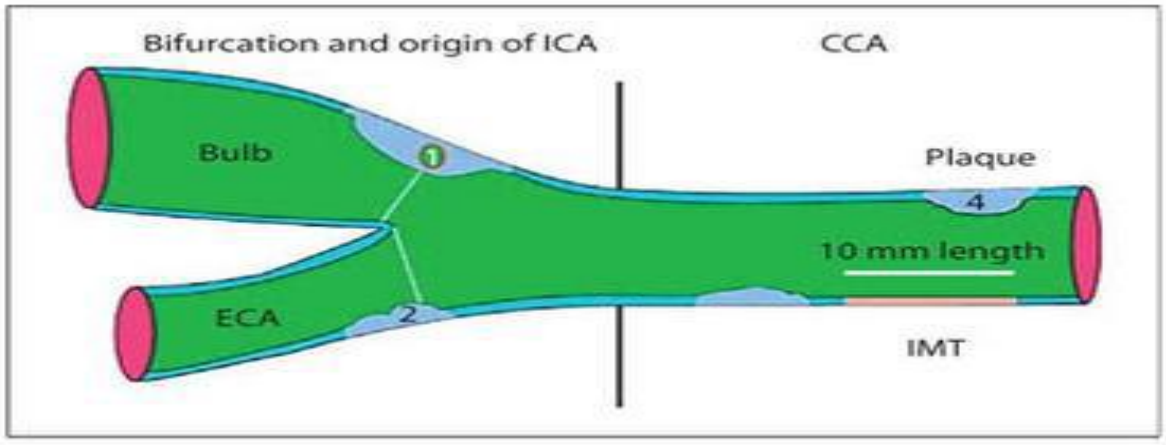


FIGURE 6 - CROSS SECTION OF A COMMON CAROTID ARTERY IN B-MODE LONGITUDINAL VIEW

IMT is measured as the distance between lumen-intima and media-adventitia interfaces. Schematic diagram below represents IMT location in common carotid artery (CCA). Where I=intima, A=adventitia, ECA=external carotid artery and ICA=internal carotid artery. Modified from: https://www.escardio.org/Journals/E-Journal-of-Cardiology-Practice.

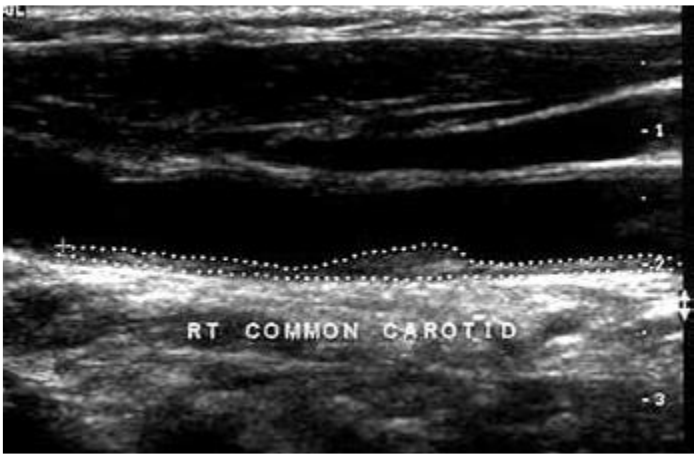


FIGURE 7 - MEASUREMENT OF CAROTID PLAQUE AREA

Plaque is measured in a longitudinal view in the plane in which the plaque is maximal. The image is frozen and magnified on the screen and a cursor was traced around the perimeter of the cross section. The microprocessor in the duplex scanner displays the cross-sectional area of the plaque (Perez, Garcia et al. 2016).

1.9 **Aims of thesis**

Audit Study

What is the frequency of traditional CVD risk factors in our SLE cohort at UCLH?

How good are we are at recording traditional CVD risk factors in our SLE cohort at UCLH?

How good are we at managing traditional CVD risk factors in our SLE cohort at UCLH?

ELISA Chapter

Are anti-apoA1 IgG antibodies associated with clinical outcomes such as cardiovascular disease and subclinical CVD disease in SLE?

Do the levels of anti-ApoA1 remain constant or do they vary over time?

Are anti-apoA1 levels influenced by ethnicity, disease activity, immunological profile and treatment?

***i*NKT chapter**

What is the role of *i*NKT cells in subclinical CVD in RA?

Can the serum from RA patients induce an *i*NKT cell response in healthy serum and what are the phenotypes of these cells?

Is *i*NKT frequency in RA influenced by ethnicity, disease activity, immunological profile and treatment?

Metabolomics chapter

Are there differences in the metabolomic profile between SLE patients and healthy controls?

Are there differences between the metabolomic profiles between RA patients and healthy controls?

Are there differences between the metabolomic profiles between SLE plaque vs non-plaque patients?

Are there differences between the metabolomic profiles between RA plaque and non-plaque patients?

Are there differences between the metabolomic profile between RA and SLE?

Vascular Ultrasound chapter

Are there differences in clinical and demographic data between patients who were lost to follow up and those who had a second vascular ultrasound scan?

What were the rates of progression of IMT and plaque in the cohort who were rescanned?

Were there any clinical or demographic differences in the plaque vs non-plaque group?

Are there any clinical or demographic features that are predictive of plaque?

Are there any factors that are predictive of scanning outcomes?

2 MATERIALS AND METHODS

2.1.1 Patient selection

For the most part of my research project, patients were recruited from the weekly SLE clinic at UCLH. The clinic takes place every Monday morning and typically, 30-40 patients with SLE attend. Patients are usually seen between two and four times per year and there are currently over 400 patients under active follow-up. Ethics approval for our research on factors affecting cardiovascular disease in patients with SLE was obtained (Joint UCL/UCLH Committee of the Ethics of Human Research reference number 06/Q0505/79) and these patients in the lupus clinic gave informed consent to take part in these studies.

Comprehensive demographic and clinical characterization was obtained for all patients, including autoantibody profile, and disease activity profile. Treatment regimens were obtained from patient records.

Between 2011 and 2013 carotid and femoral vascular ultrasound, scans of 100 patients with SLE who had no previous history of CVD were carried out at UCLH. Thirty-six patients were found to have plaque whereas 64 had no plaque.

Plaque was defined as “a focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding intima- medial thickness value or demonstrates a thickness >1.2 mm as measured from the media-adventitia interface to the intima-lumen interface” (Ebrahim, Papacosta et al. 1999, Griffin, Nicolaides et

al. 2002). Patients who had at least one region fulfilling this description were included in the group with plaque (SLE-P) and all others were designated SLE-NP (no plaque).

From this cohort of 100 patients, I recruited and rescanned 69 patients over a 5-year period, and will describe them in the scanning section below. During this thesis, I have tested both cohorts from the initial scans and the follow up scans.

A smaller part of my research looked at patients with RA whose serum samples, which were obtained from Professor Choy's group at the University College hospital in Cardiff. I was sent 13 serum samples from these RA patients, which were age/sex/ethnicity, matched (8 plaque samples and 5 non-plaque samples). Where possible, demographic and clinical information was obtained for this cohort although it was somewhat limited due to lack of access to patient records and indirect access to medical information via my collaborator.

I will provide a more detailed discussion of the cohorts in the relevant sections below.

2.2 Audit Study

2.2.1 Patient selection

Patients were captured from the weekly SLE clinic at UCLH as described previously. The total cohort of SLE patients was approximately 400 at the time of capturing of the audit data of which 309 patients were captured over a nine -month period.

Lupus CVD questionnaire

A one-page protocol was designed to capture information on age, sex, ethnicity, BMI (body mass index), smoking status, blood pressure (BP) measurements and lipid levels (figure 8). BMI was calculated from the weight and height recorded by the nursing staff at the clinic visit. BP was measured on the day and patients were asked about their smoking status (current, never or previous). Lipid levels were assessed from measurements taken from the clinic visit or if they had been measured within the last 12 months. The protocol was filled in by clinicians for every SLE patient who attended a weekly SLE clinic for a period of 9 months. If BP was ≥140/90 (elevated as defined by NICE), the measurement was repeated. If both readings were above this, 24 hour blood pressure monitoring was carried out.

Based on previous published data in SLE (Wajed, Ahmad et al. 2004), patients over 40 with a serum LDL of greater than 2.6 mmol/l were targeted and offered treatment with dietary measurement and/ or statin via liaison with the patient's GP. A LUPUS UK leaflet for diet was given to all patients and a locally designed 'stop-smoking'

leaflet with the option of referral to smoking cessation clinic was given to all current smokers.

1)Date of clinic:

2)Patient label or details Hospital Number:

DOB:

Gender: M / F

Height:

Weight:

3) Ethnicity details (please tick)

Caucasian

Black African/Caribbean

Asian

Oriental

Other (please specify)

4) Blood pressure - please record ─ / ─ mm/mg

a) Is the patient on anti-hypertensives? Yes No

If the BP **≥140 and or ≥ 90 mm/Hg** please re-check

b) Indicate repeated BP reading ─/─ mm/mg

5) Smoking status (please tick)

Current

Previous

Never

If current please refer using attached smoking cessation form

If not referred please state why:

Please give current smokers UCLH smoking leaflet

Yes No

6) Diet and Lifestyle

Has the patient received a Lupus UK 'Lupus and Healthy Eating leaflet?

Yes No Declined

7)Lipids

Tested at UCLH in last 12 months? Yes No

If No please measure today

a) Was *LDL>2.6 mmol/L* in the last 12 months and patient *over 40*?

Yes No

if a) was *Yes* please write to GP suggesting treatment in clinic letter

FIGURE 8 - CVD QUESTIONNAIRE

This questionnaire was filled out at one visit by myself / one of the other clinicians seeing the lupus patients in the SLE clinic at UCLH. Data on BMI, lipids (checked in last 12 months), blood pressure at visit (repeated if initial BP reading systolic **≥140 and/ or diastolic ≥ 90 mm/Hg**) and smoking status were recorded over a 9 month period. If LDL levels >2.6 mmol/L and the patient was over 40, clinicians were encouraged to write to the patients' GP to initiate treatment with diet/lifestyle modifications. A diet/lifestyle leaflet was given to all patients, a smoking cessation leaflet, and the option to visit smoking cessation to current smokers.

2.3 ELISA (Anti-apoA1)

2.3.1 Patient selection

Sera from all patients followed up in the UCLH SLE clinic have been routinely collected. All sera are stored at -80°C in a dedicated biobank for research purposes. All SLE patients were recruited from a pool of more than 400 patients with SLE who have been attending the clinic.

ELISAs were tested in two groups of SLE patients: a case-control group and the follow up vascular ultrasound group.

2.3.2 Case- control group

Sera from SLE patients with CVD events in the last 10 years and SLE controls (without a history of CVD matched for age, gender, ethnicity and disease duration using patient records from the Rheumatology department at UCLH) were tested measuring the levels of IgG anti-apoA1. Sera were tested from two points in time for each patient where possible.

2.3.3 Vascular ultrasound group

Forty-nine patients from the follow up vascular ultrasound cohort were tested with the help of my colleagues FF and VNR, as they had completed the follow up scan at

the time of testing. At this point in time, all 69 patients had not been re-scanned, and only 49 samples were available for testing. This cohort was further divided into 3 groups based on the interval changes between the 1st and 2nd ultrasound scans over a 5-year period.

The first group were the non-plaque group (NP) who did not develop plaque in either scan (n=29 patients).

The median of total plaque area (TPA) increase per year was 3.29 cm^2 (calculated by the TPA at second scan –TPA at baseline= TPA Difference. TPA Difference/years of follow up= TPA increase per year) and was a cut-off point used to define the remaining groups of slow plaque growth (SP) (n=7 patients) and the rapid plaque (RP) growth group (n=13).

For each group, measured IgG anti-apoA1 levels in serum samples from three time points were measured: first available stored sample in our biobank (pre-2000), at the time of the first scan and at the time of the second scan to see if antibody levels change with time.

2.3.4 **ELISA for measuring anti-apoA1 IgG antibodies**

All steps were carried out at 37°C except where specified. A Nunc maxisorp 96 well ELISA plate was divided in half. The test side was coated with 50µL of 15µg/mL apoA1 (Sigma A0722) in 70% ethanol and the control side was coated with ethanol alone. After incubation for 90 minutes uncovered, the plates were washed three times with PBS-0.1% Tween (PBST), emptied and blocked with 100µL per well of 2% BSA-PBS. The plates were covered and incubated at 4°C overnight. The following day, serum samples were thawed at room temperature and diluted 1:50 in 1% BSA-PBS and tested in duplicate. In the first row of each side of the plate, a 7-point dilution of the positive control, or standard, was performed starting at 1:25 dilution. The positive control was selected after testing 20 SLE serum samples previously identified as having high serum IgG anti-apoA1. Each sample was loaded in duplicates one on the test side and one on the control side. The plate was incubated for one hour, and then emptied and washed three times with PBS- 0.1% Tween. Following this, 50µL per well of goat anti-human IgG-alkaline phosphatase conjugate (Sigma A3150) diluted 1:1000 in 1% BSA-PBS was added at room temperature for one hour and washed with PBS three times. Finally, 50µL of alkaline phosphatase substrate (KLP 50-80-00) was added to each well.

Optical density (OD) at 405nm was recorded after 90 minutes. The net OD for each sample was calculated by subtracting the OD generated in the control well from that in the matching test well to exclude effects of non-specific background binding. The mean net OD from the duplicate samples was converted to absorbance units (AU) by

comparison to the standard curve of OD for the serial dilutions of the positive control sample on each plate. One hundred AU is defined as the OD given by a 1:50 dilution of the positive control sample. This assay is reproducible with intra and inter-plate coefficient of variation of <10%.

A healthy control cohort of 20 samples (samples were obtained from UCL donors who were age and sex matched) was tested using the methods above to establish a cut off for positivity for the anti-apoA1 ELISA. This was calculated to be 17 AU, by using the 97.5th centile and +/- 3 standard deviations from the mean.

2.4 iNKT cells in Rheumatoid Arthritis

2.4.1 Patients and controls

Professor Ernest Choy and his team scanned RA patients at the University of Cardiff. Forty-five patients were recruited and all scanned at baseline. Thirty-five patients were followed up at 6 months, and 23 patients at 12 months. Patients were positioned supine and the left and right common carotid arteries were imaged (using an Acuson X300 machine) longitudinally, 1 cm proximal to the carotid bifurcation. Images were focused on the posterior (far) wall of the artery and then magnified. Several ten-second loops were recorded in DICOM (digital imaging and communications in medicine) format and then downloaded for offline analysis. An automated carotid analyser (Carotid Analyzer, Medical Imaging Applications, Iowa City, IA) was used to measure CIMT. Three end-diastolic frames were selected and CIMT measured, defined as the interface between lumen-intima and media-adventitia, for both right and left carotid arteries. The mean of the three end-diastolic frames was calculated, and then the mean of the left and right-sided readings was calculated. The internal and external carotid arteries and bifurcation were also scanned and presence or absence of plaque was noted (Corrales, Parra et al. 2013).

Carotid intima-media thickness (CIMT) of greater than 0.90 mm and/or carotid plaques were used as the gold standard test for subclinical atherosclerosis and high CV risk (Corrales, Parra et al. 2013). Plaque was defined as a focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness of > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface (Touboul, Hennerici et al. 2004) .

Thirteen age/disease duration/sex matched serum samples from RA patients (8 plaque and 5 non-plaque) patients were tested. Serum samples were obtained from healthy donors at UCL who were age/sex and ethnicity matched to serum samples.

Previous work shows that peripheral blood mononuclear cell (PBMC) stimulation with αGalCer (activates *i*NKT cells) and IL-2 (survival factor for T-cells) leads to an exponential expansion of *i*NKT cells after 7–14 days (Watarai, Nakagawa et al. 2008). Healthy PBMCs were obtained from healthy UCL donors. Screening of different healthy donors, enabled two healthy donors (one female and one male) to be identified and used on the basis of yielding a sufficient PBMC number (>50,000) and their ability to proliferate in the presence of IL-2 and αGal-Cer.

2.4.2 Peripheral blood mononuclear cell (PBMC) isolation, storage and thawing

Density gradient centrifugation was used to isolate PBMCs from heparinised blood obtained from healthy donors. Whole blood was centrifuged at 400g for 10 minutes at room temperature with minimum acceleration and brake settings to separate cellular components from plasma layer. The plasma (upper phase) was removed and aliquoted for long- term storage. The cellular fraction of the whole blood (lower layer) was topped up with incomplete Roswell Park Memorial Institute medium (RPMI) 1640 medium (Sigma) returning the volume to 50mL. Blood was diluted 1:1 in medium, before gently layering 33 mL of blood onto SepMate tubes with 15mL of ficoll. The layered tubes were centrifuged at 1200g for 10 minutes at room temperature. The tubes were gently inverted and the plasma/PBMC upper fraction poured into sterile labelled falcon tubes (see figure 9). The cell suspension was topped up to 50ml with medium and centrifuged at 400g for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 20ml of medium.

Live cell concentration was determined with an ADAM automatic cell counter. Lastly, cells were centrifuged (400g, 4°C, 10 minutes) aliquoted into cryovials, at a concentration of $1x10^6$ PBMCs/ml freezing medium containing fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO). Vials were placed into Nalgene™ Mr Frosty freezing containers containing isopropanol in order to achieve a freezing rate of -1°C/minute before transfer to -80°C freezer for storage. For long-term storage samples were transferred to liquid nitrogen storage (-180°C).

For each experiment cells were thawed by pre-warming 20ml complete RPMI (RPMI-1640 medium; 10% FCS, 1% penicillin/streptomycin [Pen/Strep]) per sample to 37°C in a water bath. A sterile pasteur pipette was used to thaw each vial individually before diluting in pre-warmed complete RPMI, and washed to remove traces of DMSO.

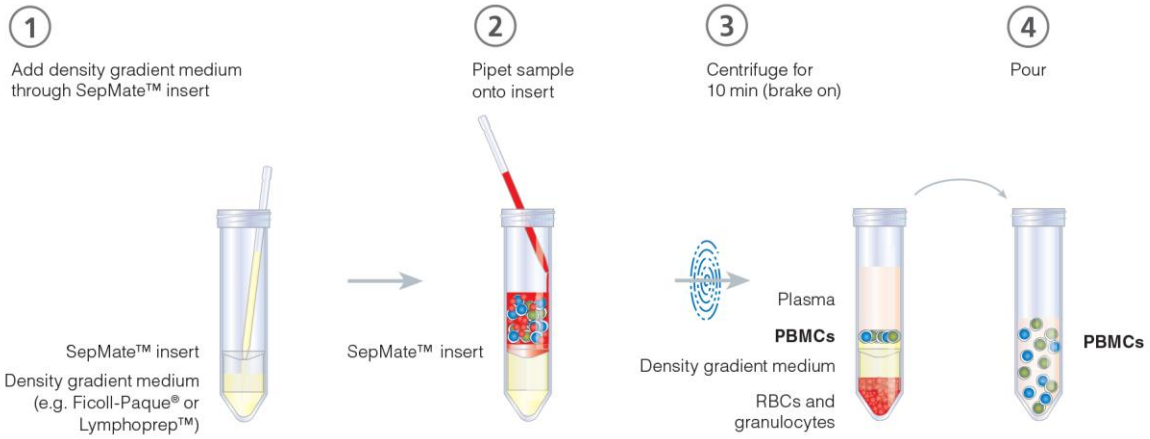


FIGURE 9- ISOLATION OF PBMCS USING FICOLL- PAQUE PLUS DENSITY GRADIENT CENTRIFUGATION.

Illustration of the layering of blood before and after centrifugation (www.stemcell.com/media/images/brand/sepmate/sepmate-protocol.png).

2.4.3 Cell surface staining and culture for *i*NKT assessment

Multi-parameter flow cytometry was used to carry out immunophenotyping of *i*NKT cells. At day 0, healthy donor PBMCs were plated with no serum. Cell staining was carried out in a 96 well plate at a concentration of $1x10^6$ PBMCs per well. Cells were stained with Live/dead (50µl/well diluted 1:200 in PBS) for 20 minutes at 4ºC before washing in PBS. Cells were then washed and stained with a surface marker using the antibodies indicated in table 5. Panels were designed for use on LSR UV Fortessa X20 and all antibodies were titrated for optimal dilution.

For the surface marker panel, staining was carried out in 50µl brilliant stain buffer containing BSA and 0.09% sodium azide. A mix was prepared containing the following antibodies, which were diluted accordingly. iTCR-PE (1:10) and CD3-APC/efluor780 (1:50) which are well known markers for identifying *i*NKT cells, with subsets CD4-BV605 (1:1000) and CD8-BV705 (1:100) (Watarai, Nakagawa et al. 2008). Other markers used included activation markers CD69-APC (1:25) and PD-1-PE/Cy7 (1:25), as well as NK cell marker CD161-BV421 (1:25) (Major, Singh et al. 2006). Cells were incubated at 4°C in the dark for 20 minutes to enable binding. Cells were then washed in 100ml cell staining buffer and centrifuged at 800g for 2 minutes at 4°C, before fixing with 200µl 2% paraformaldehyde (PFA) and incubating in the dark at RT for 20 minutes. Finally, cells were washed and resuspended in 200µl FACS buffer ready for analysis. For each sample, $1\text{-}5x10^6$ cells were acquired by flow cytometry using LSR UV Fortessa X20, and analysed using FlowJo version 10.

In addition to day 0 staining, PBMCs were plated for a 7- day culture, with three different serum conditions (HC, RA- NP and RA-P) and stained with the same markers above as day 0. Each experiment was repeated twice with the same healthy PBMC's, using the same serum conditions. During the 7- day incubation, the plate was checked by visual inspection to ensure viability of cells (looking for any colour changes and clumping of cells) and viability was checked with the live/dead stain in the flow cytometer.

Surface marker	Fluorochrome	Clone	Isotype	Company	Concentration in FACS Buffer
Invariant T cell receptor (Vα24Jα18)	PE	6B11	Mouse IgG1	BD Bioscience	3:50
CD3	BV786		Mouse IgG1		1.5:50
CD4	BUV395		Mouse IgG2b	BioLegend	1.5:50
CD8	BV421		Mouse IgG1	BioLegend	2:50
CD69	BV510		Mouse IgG1	eBioscience	2:50
CD161	BV605		Mouse IgG1	BioLegend	2.5:50
PD-1 (CD279)	PE/Cy7		Mouse IgG1	BioLegend	2:50
Live/dead	Yellow (emission at 405nm)	N/A	N/A	Life Technologies	1:200 (PBS)

TABLE 5- ***CELL SURFACE ANTIBODIES AND MARKERS***

2.5 **Metabolomics**

Metabolomics analysis of serum samples from healthy, SLE, and RA cohorts were performed by Professor Mika Ala-Korpela at the University of Eastern Finland using a biomarker analysis platform (https://nightingalehealth.com (Soininen, Kangas et al. 2015)). Two hundred and thirty three metabolites were analysed. The metabolite quantification was performed using hydrogen nuclear magnetic resonance (HNMR) using two spectral windows LIPO and LMWV to give information on lipoprotein subclass distribution and low molecular weight metabolites including systemic amino acids, urea and creatinine. Models were cross-validated against NMR-independent lipoprotein and lipid data, and verified to lie within a 10% limit of the training data set or else were rejected from lipoprotein subclass analysis (Kettunen, Tukiainen et al. 2012).

The lipoprotein subclasses detected by NMR were classified as follows; extremely large VLDL and chylomicrons (XXL-VLDL), very large VLDL (XL-VLDL), large VLDL (L-VLDL), medium VLDL (M-VLDL), small VLDL (S-VLDL), very small VLDL (XS-VLDL), intermediate density lipoproteins (IDL), large/medium/small low density lipoprotein (L-LDL, M-LDL, S-LDL), medium density lipoprotein (MDL), very large/large/medium/ small high density lipoprotein (XL-HDL, L-HDL, M-HDL, S-HDL). Data were generated on concentrations of lipid within each lipoprotein subclass (mmol/l) and these subgroups were –total lipid (L), phospholipids (PL), total cholesterol (C), cholesterol esters (CE), free cholesterol (FC) and triglycerides (TG). Distribution of these lipids was also expressed as a ratio or percentage of total lipid content for each lipoprotein subclass. For example, L-LDL-PL (%) means the percentage of lipids that were phospholipids in in large LDL.

2.5.1 SLE Cohort

Serum samples from 86 patients that were available at the time from the initial cohort of patients who had a vascular ultrasound (4 males and 82 females) were sent for metabolomics analysis (33 with plaque -30 females and 3 males, mean age = 53) and 10 healthy controls (9 females, 1 male, mean age 50).

2.5.2 RA Cohort

The 13 RA samples with plaque and non-plaque (as described in the iNKT methods) were also sent for metabolomics analysis and analysed to look at potential differences between the metabolites in the plaque vs non-plaque group. I also looked at differences between RA and the same HC as the SLE cohort.

2.5.3 Statistical analysis

Pairwise comparisons (HC versus all SLE or SLE-NP versus SLE-P) were made using an unpaired two-tailed student's t-test, without assuming equal variance in Microsoft Excel. P-values were corrected for multiple testing using the two-stage linear step-up procedure of in GraphPad Prism 7.1 (La Jolla, USA) with a false discovery rate (FDR) of 1%. The top twenty metabolite hits were selected with an unadjusted p-value of less than 0.05. Receiver Operator curves were used to determine the ability of tests based on individual metabolite levels to distinguish between SLE-P and SLE.

The 13 RA samples were also analysed using pairwise comparisons (P versus NP) using an unpaired two-tailed student's t-test, without assuming equal variance. P-values were corrected for multiple testing using the two-stage linear step-up procedure of in GraphPad Prism 7.1 (La Jolla, USA) with a FDR of 1%.

2.6 Vascular Ultrasound

2.6.1 Patient selection

Of the original 100 patients recruited, 69 patients agreed to return for a follow up scan on average 5 years after the original scan. The technique I have outlined below is based on my observations and explanations whilst attending the vascular ultrasound scanning sessions. These follow-up scans were performed by the same experienced vascular scientist Dr Maura Griffin who had carried out the previous baseline scans over 5 years, with my predecessor. Strict scanning protocols were observed for both baseline and follow-up visits using the same high-end ultrasonic machine and system settings. The technique and analysis software remained constant throughout.

2.6.2 Vascular ultrasound hardware and software specifications

The Philips iU22 ultrasound system was used with a linear array L9-3 MHz and a volume linear array transducer VL 13-5 MHz for 2D imaging. Intima-media thickness (IMT) measurements were performed on-screen using the QLAB IMT plug-in. This technology uses an intelligent edge detection algorithm to make accurate reproducible measurements across a selected region of IMT.

In addition, a stand-alone QLAB Advanced Quantification Software® package - version 7.1 (Philips Ultrasound, Bothell, USA) was used on a separate laptop/PC. All ultrasound images were stored as either single or multi-frame DICOM sequences.

2.6.3 Vascular ultrasound protocol

The stages in the vascular ultrasound protocol were as follows:
Patients were examined in the supine position with their neck and groin area exposed and tissue paper placed around the areas of interest to protect clothing from the ultrasonic gel.

Each arterial bifurcation was examined sequentially starting from - ***1) Left carotid 2) Right carotid 3) Right common femoral 4) Left common femoral*.**

Carotid Artery assessment - the whole length of the common carotid artery (CCA) from its proximal segment to the carotid bifurcation, the external (ECA) and internal carotid (ICA) arteries were examined by sweeping the neck transversely in the 2D mode then retracting back from the carotid bifurcation to the proximal CCA in colour mode. This technique allowed for a quick overview of the carotid system to detect any gross changes (i.e. plaque presence) within the arterial wall.

Intima-media Thickness (IMT) measurement - the probe was then positioned mid-section of the CCA and from the transverse rotated 90° to obtain a longitudinal section (LS). Minor adjustments to the transducer were made in order to visualise a straight segment of the CCA clearly identifying the layers of the arterial wall. The posterior wall of the artery is the focus of the most accurate measurements of IMT and therefore this was consistently used. The characteristic thicker bright white (echogenic) line represents the adventitia and the thin less echogenic line anterior to the adventitia represents the intima. The layer of blackness between the adventitia

and intima is known as the media. The combined measurement from the intima to the adventitia is commonly referred to as the intima-media thickness (IMT).
The B-mode image was enlarged and then frozen to allow a region of interest to be selected and the IMT QLAB software employed to make the IMT measurements. After IMT was measured, the image was unfrozen and a short video of the CCA was taken for arterial wall motion analysis. A video of the CCA in transverse view was also recorded.

Carotid bulb assessment – The carotid bulb was defined as a widening of the most distal segment of the common carotid artery at its main branch point into the ECA and ICA. Both longitudinal and transverse views of the carotid bifurcation were taken and in the absence of atherosclerotic plaque, the bulb origin IMT was measured using on screen callipers and recorded.

Colour and power Doppler images of the carotid bifurcation were taken to confirm or exclude the presence of any filling defects that would suggest the presence of a type 1 – completely black (echolucent) plaque. Type 2 to type 5 plaques are visible on ultrasound to the well-trained naked eye. However, a small percentage of completely echolucent plaques with no visible fibrous cap/outline can be missed on ultrasound if colour flow is not used.

Assessment of Common Femoral Artery (CFA) - The probe was placed transversely in the groin area. After identifying the CFA, the artery was followed in transverse until its bifurcation. The probe was then repositioned to visualise the whole CFA in LS to its division into the superficial femoral (SFA) and profunda femoris (PFA)

arteries. For the same reasons expressed above both colour and power Doppler images were obtained to exclude the presence of any atherosclerotic plaques.

Atherosclerotic Plaque assessment – where plaque was present, colour and Power Doppler images were obtained to ensure that the outline of the plaque was accurately assessed. Plaque thickness was measured at the point of maximum thickness on both longitudinal and transverse sections using on-screen callipers and the mean value was obtained (variability <1%).

Supine blood pressure (BP) was measured in the right upper arm using an automated BP reader (Omron M6 HEM-700-E). Three measurements were taken and the first discarded. The average systolic and diastolic BP value were calculated using the second and third readings. Pulse pressure was calculated as the difference between systolic and diastolic BP. Mean BP value was calculated for each patient using:

Mean BP= Diastolic BP + 1/3(systolic BP – diastolic BP).

2.6.4 **2D plaque analysis**

"The Plaque Texture Analysis" software version 4.5 (Life Q Ltd) was used to assess plaque area and texture (Elatrozy T 1998, Griffin M 2007, Makris, Lavida et al. 2011). As previously discussed in the introduction, the measurement of GSM is a way of assessing plaque echodensity to enable evaluation of plaque vulnerability. GSM is one of most widely used marker to help quantify plaque vulnerability. As GSM was measured in the first cohort it was again measured in the follow-up ultrasound scans thereby keeping my data consistent. A low GSM is associated with the presence of dark structures in the plaque, corresponding to increased lipids and presence of haemorrhage. The process of image normalisation enables the results of different operators and different ultrasound machines to be compared and ensure clinical relevance (Griffin, Kyriacou et al. 2013).

Each plaque image prior to analysis underwent normalization to ensure that all images had homogenous settings. In normalisation, blood and adventia are the two reference points used and the brightness of all the pixels in the image can be represented on a linear scale. Black and white images obtained by B-mode are composed of a spectrum of pixels with different values on a computer grey scale which usually ranges from 0 to 255, 0=absolute black, 255= absolute white. Blood (black) is defined as zero (absolute echolucence) and adventitia (white) as the maximum value (absolute echogenicity). These pixels appear with varying frequencies creating a histogram, with a skewed distribution. The median value of the grey scale is used as the measurement of the overall echodensity for any given image and therefore defined as grey scale median (GSM). The resolution of the

image (number of pixels per mm) is also adjusted to a standard value of 20 pixels per mm (Griffin, Nicolaides et al. 2007) .

The process of normalization has been validated by several large reproducibility studies (Sabetai, Tegos et al. 2000, Tegos, Sabetai et al. 2000, Seo, Watanabe et al. 2006, Griffin, Nicolaides et al. 2007).

All plaques identified were analysed for 2D plaque measurements regardless of their location and size. The 'plaque texture analysis software' as mentioned previously was used to measure the GSM. Lower values correspond to more echolucent i.e. lipid rich plaques thus eliminating the subjective component. It provides semi-automated measurements of distance (such as IMT or plaque length/thickness) or area (plaque area) in mm or mm^2 based on longitudinal images of each plaque (Elatrozy T 1998, Griffin M 2007, Makris, Lavida et al. 2011).

2.6.5 Inter-observer variability

The inter and intra-observer variability was found to be <10% from the initial scans which had been performed by Dr Maura Griffin and my predecessor Sara Croca. All follow-up scans performed by Dr Maura Griffin were done using the same ultrasound system employing the methods as described above.

2.6.6 **Statistical analysis**

Statistical analysis was performed with IBM® SPSS® statistics version 22. Significance was set at 0.05.

For continuous numerical variables, I compared groups (plaque and non-plaque) using a T-test if the variable was normally distributed or a Mann-Whitney U test if the distribution was not normal. For categorical variables, I used a Pearson's chi-squared test.

I used logistic regression to investigate factors associated with the presence of plaque. For continuous numerical variables showing a significant result, I constructed ROC curves to identify a cut-off for each variable. Then I tested that cut-off with logistic regression, to find its odds ratio. Due to the limited number of patients with plaque, only the four variables with higher odds ratio on univariable logistic regression were then included in the multivariable model.

In the 31 patients with plaque, linear regression was used to identify determinants of TPA, TPL, TPT and GSM. The variables with a significant result in univariable linear regression were tested in a multivariable model. The final model was chosen according to the values of the adjusted r^2 and F. The same method was used to investigate factors influencing the IMT in all 69 patients.

3 RESULTS

3.1 **Clinical Audit**

Over a 9-month period we applied a standardised data collection and CVD risk management protocol in consultations with 309 patients seen for routine appointments in our lupus clinic - 94% female, mean age 47 years, and ethnicity (figure 10).

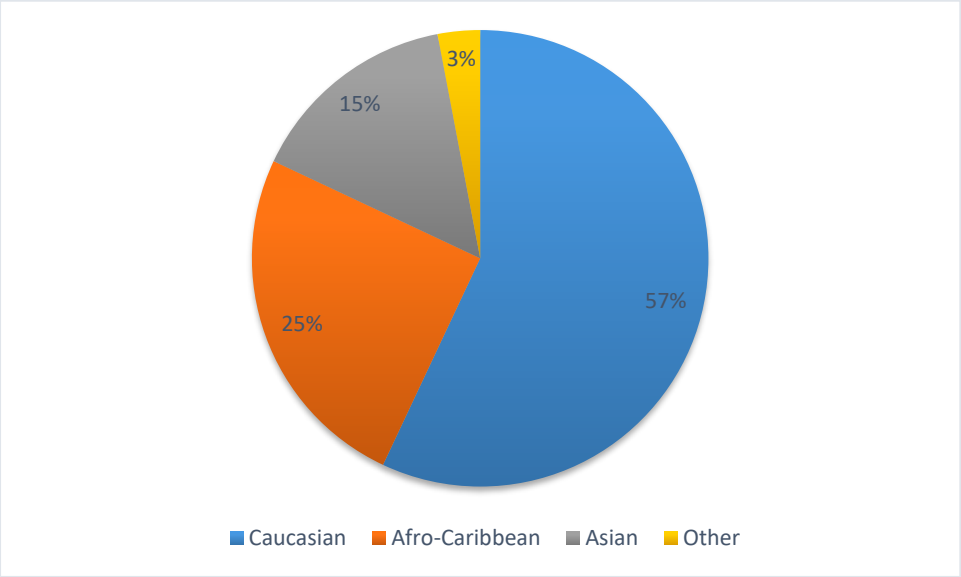


FIGURE 10- PIE CHART SHOWING BREAKDOWN OF ETHNICITY

Three hundred and nine patients were assessed for CVD risk factors in our UCLH SLE cohort; 57% (n=176) Caucasian, 25% (n=78) Afro-Caribbean, 15% (n=45) Asian and 3% (n=10) other.

3.1.1 Blood pressure

I defined hypertension as systolic pressure >140mmHg and/or diastolic pressure >80mmHg. If either value was exceeded the measurement was repeated. If still high, the patient was referred for 24-hour blood pressure recording. Of 309 patients, 32 were referred for 24-hour blood pressure, of whom six were confirmed to be hypertensive and had anti-hypertensive treatment started or modified. Thus, only 2% of patients had under-treated hypertension whereas 48% were already on anti-hypertensives.

3.1.2 Smoking

Thirty patients (10%) were current smokers. All were given a leaflet entitled "Smoking and Lupus" (designed with input from patients) and offered referral to smoking cessation services. Only one patient accepted referral and stopped smoking. Of the others, 17 were long-term persistent smokers (>10 years). Further attempts to reduce smoking are thus likely to impact a maximum of 12/309 patients (4%).

3.1.3 Lipids

Based on previous work, I aimed to measure lipid profile in every patient and to recommend dietary or drug treatment to the family practitioner for patients over 40 with low density lipoprotein (LDL) > 2.6mmol/l. Of the 309 patients seen, 253 had

lipid measurements of whom 64 were over 40 with LDL > 2.6mmol/l (Wajed, Ahmad et al. 2004). However, in only 18 patients was there any evidence that the family practitioner had been contacted and only six started statins.

3.1.4 Clustering of risk factors in obese patients

Body mass index was calculated for all patients. Seventy-six were overweight (25-30kg/m^2) and 62 were obese (BMI >30kg/m^2). Fifty-two per cent of obese patients were on steroids for more than 3 years (dose>3mg/day). Hypertension and raised LDL showed higher prevalence in the obese group than in the whole population (n=309), as shown in figure 11.

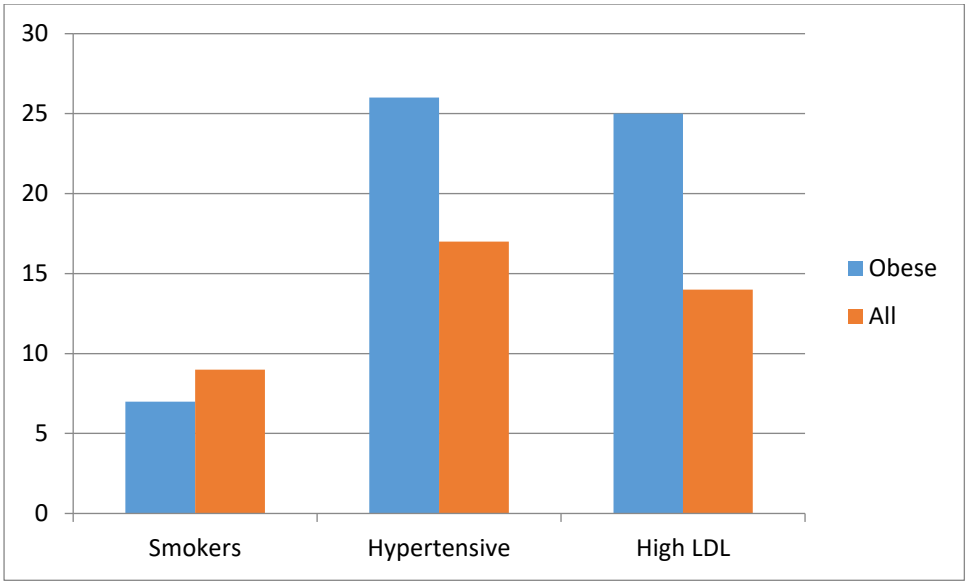


FIGURE 11- PERCENTAGE OF PATIENTS WITH CVD RISK FACTORS (OBESE VS ALL)

3.1.5 Discussion

The increased risk of developing cardiovascular disease (CVD) in patients with SLE is due to both conventional and immunological risk factors, as discussed in the background section of this thesis (Esdaile, Abrahamowicz et al. 2001, Bruce 2005). Lupus specialists must address modifiable conventional risk factors to minimise CVD risk in patients with SLE (Elliott, Manzi et al. 2007). It is not clear how best this can be done and how much can be achieved.

In several cohorts, the total number of traditional risk factors has been shown to be increased in women with SLE compared to matched controls (Asanuma, Oeser et al. 2003, Roman, Shanker et al. 2003, Urowitz, Ibanez et al. 2007). As discussed on page 64 traditional risk factors fail to account fully for the increased risk of CVD risk in SLE and some SLE cohorts with fewer CVD risk factors still develop CVD.

Traditional risk factors are still important to manage and the role of other factors such as immunological markers have yet to be ascertained. There are no standard guidelines to help clinicians manage these risk factors in SLE.

I investigated to review the screening and management of traditional CVD risk factors in our clinic at UCLH. I anticipated that that there would be some deficiencies due to a lack of awareness or support of the impact of CVD risk in SLE and due to the prioritisation in clinics of more acute clinical issues with limited time in for clinicians to address CVD risk factors.

I therefore explored the feasibility of assessing traditional risk factors, by designing a simple one-page protocol. I found that CVD risk could be addressed in 77% of patients seen over a 9-month period. This is reinforced by a questionnaire study by Rahman et al, which canvased the opinions of 32 North American and European lupus clinician on how to manage traditional CVD risk factors in SLE. This study found that overall there was a general support for the idea of assertive management of CVD risk factors. All respondents said they encourage their patients to stop smoking, 74% said they would try and keep blood pressure between 120/80 and 140/80 mmHg and 55% would prescribe a statin to patients with high cholesterol (Rahman, Bessant et al. 2006).

The reasons why this project managed to capture such a high percentage (77%) compared too much lower ones reported in other cohorts, may be explained by the fact that my study was not blind. Clinicians were educated and actively asked for participation and engagement into the project after I presented the data and my questionnaire at a departmental meeting prior to commencement. Furthermore, the presence of a questionnaire at the front of patient's notes promoted clinicians to complete the forms. In addition, UCLH is a specialist territory centre for SLE and therefore has a set of specialists that are already engaged and aware of CVD risk in SLE.

I found that it was most challenging to have an impact on smoking behaviour, as smoking was an established long-term habit for many patients. In fact as many as 17-21% of patients have been reported to smoke (O'Neill, Pego-Reigosa et al. 2009),

which is higher than the national average for women in the UK at 13% as reported by the office of national statistics.

Only 2% of patients had untreated hypertension and nearly 50% of the cohort were on existing anti-hypertensives. Hypertension is clearly being effectively addressed and managed at UCLH and this could be partly reflected by our monthly multi-disciplinary clinics with the nephrologists. In all patients with previous renal lupus, we aim for diastolic pressure below 80mmHg. This may explain the low level of untreated/undertreated hypertension in this patient group.

Conversely, high LDL levels were a clear unmet need, for which better interaction with family practitioners is required. Although 82% (253/309) of the cohort's lipids were checked, 25% (64/253) of these patients were above the age of 40 with LDL levels > 2.6mmol/l, and should have had treatment recommendations to their family practitioners. Clinicians only wrote to family practitioners suggesting treatment in 28% (18/64) of these cases, and 9% (6/64) were prescribed statins. If the treating rheumatologist had taken ownership of prescribing of the statin, perhaps this may have resulted with a higher percentage of patients receiving a statin as reflected by Rahman et al's study. In this study, 55% of rheumatologists reported they would prescribe a statin with high cholesterol (Rahman, Bessant et al. 2006). The low prescribing rates may also be a reflection of the scarcity of lipid-lowering therapy clinical trials in SLE (Elliott, Manzi et al. 2007) and the reluctance to prescribe statins to women of child-bearing age.

Patients with BMI >30kg/m^2 may be an identifiable higher risk group in whom CVD risk could be further prioritised. Future immunological studies could be focused in this group to see if they could be further risk stratified based on their immunology.

To conclude, I tested that one can assess traditional CVD risk factors in the SLE clinic at UCLH which would be of benefit to patients. Conventional risk factors such as high blood pressure are managed well; however, the management of patients with abnormal cholesterol needs more attention.

3.2 **Anti-apoA1 ELISA**

3.2.1 **Case-controls**

Table 6 shows the characteristics of the 20 matched CVD cases and controls that I identified from our SLE cohort at UCLH. The majority of case-control samples tested were female and the median age of cases and controls was 54.5 years, with a median disease duration of 23 years (range 1-36 years) for the cases and 21 years (range 3-34 years) for the controls. The majority of patients were Caucasian (70%). The remaining were 15% Afro-Caribbean and 15% Asian. This matches the local population around UCLH, which is majority Caucasian.

There were a number of unattainable samples and so not all pre and post samples were tested and therefore I tested as many samples that were available at the time. In total 11 pre-event cases and controls were tested due to limited sample available. For the post event cases (n=18) and controls (n=19) were tested, therefore one pair was not matched.

Case/Control	Age	Sex	DD (years)	E	Event	Medications (from sample date)	CVS risk factors
Case 1	55	F	22	C	MI	MTX, AZA, Prednisolone 9mg	
Control 1	54	F	25	C		HCQ	Type 1 DM
Case 2	62	F	35	C	MI	MMF, Prednisolone 5mg, Statin	
Control 2	62	F	30	C		Prednisolone 2.5mg	
Case 3	46	M	23	C	MI	Prednisolone 5mg, HCQ	
Control 3	49	M	19	C		AZA and HCQ	
Case 4	60	F	36	AC	IHD		HTN
Control 4	58	F	32	AC		Prednisolone 7.5mg	HTN
Case 5	51	F	34	C	MI	HCQ, Statin	RC
Control 5	51	F	32	C		HCQ	HTN
Case 6	36	F	9	C	CVA	HCQ, MMF, Prednisolone 3mg	HTN
Control 6	36	F	6	C		HCQ	
Case 7	71	F	34	C	IHD	HCQ	HTN
Control 7	68	F	34	C		HCQ	
Case 8	41	F	13	AC	CVA	HCQ, Prednisolone 7mg	HTN
Control 8	42	F	12	AC		HCQ, Prednisolone 5mg	
Case 9	61	F	31	A	IHD	AZA, prednisolone 3mg	
Control 9	65	F	30	A		AZA, prednisolone 5mg	HTN
Case 10	69	M	33	C	MI	Nil	HTN
Control 10	67	M	24	C		HCQ	

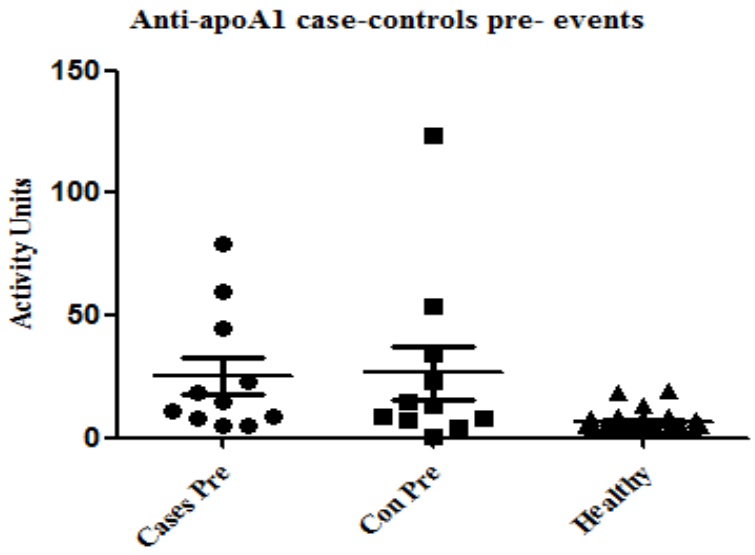
Case 11	58	F	12	AC	CVA	AZA, HCQ	
Control 11	61	F	17	AC		AZA, HCQ, Prednisolone 2.5mg	
Case 12	41	F	8	C	CVA	Rituximab, HCQ, Cyclophosphamide,	
Control 12	43	F	5	C		HCQ	
Case 13	54	M	12	C	CVA	AZA, HCQ, *Statin*	
Control 13	55	M	17	C		AZA, HCQ, Prednisolone 2.5mg	
Case 14	68	F	24	A	CVA	MTX, HCQ, Prednisolone 5mg	HTN
Control 14	68	F	25	A		Prednisolone 1mg	HTN
Case 15	37	F	23	C	CVA	MMF, HCQ, Prednisolone 5mg	
Control 15	38	F	23	C		Rituximab, HCQ. Prednisolone 5mg	
Case 16	41	F	22	C	CVA	AZA	
Control 16	42	F	22	C		MMF, HCQ, Prednisolone 5mg	Previous LN
Case 17	26	F	14	C	CVA	MMF	
Control 17	26	F	13	C		HCQ	
Case 18	57	M	40	C	MI	Prednisolone 5mg,Statin	
Control 18	55	M	17	C		Prednisolone 5mg	
Case 19	75	F	23	C	MI	HCQ, Statin	Type 2 DM, RC
Control 19	77	F	20	C		HCQ, Statin	RC
Case 20	20	F	1	A	MI	AZA, HCQ, Prednisolone 5mg	
Control 20	16	F	3	A		AZA, HCQ	

TABLE 6 - SLE *CASE-CONTROL CHARACTERISTICS*

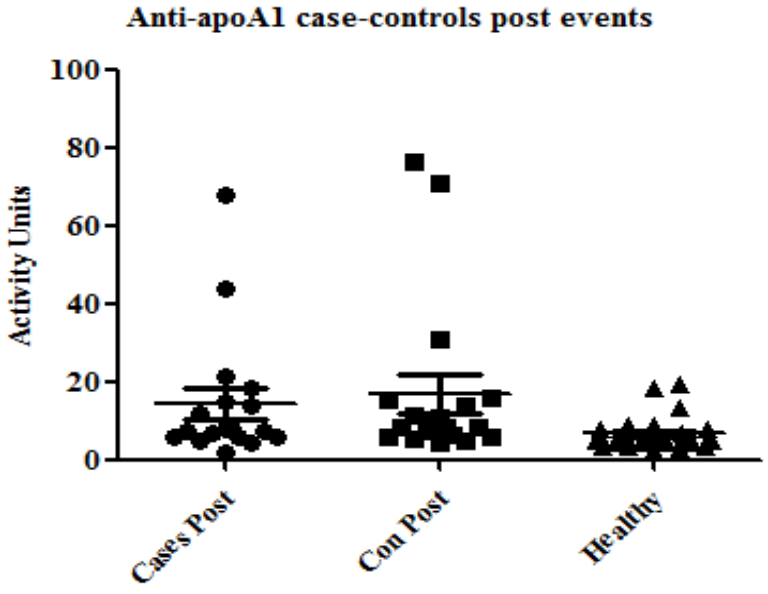
Twenty SLE CVD cases were age/sex/ethnicity and disease duration matched with 20 SLE patients without CVD using clinical records. MI=Myocardial Infarction, CVA=cerebrovascular accident.

DD=disease duration, E= ethnicity, IHD= ischaemic heart disease, Methotrexate =MTX, Azathioprine =AZA, Mycophenolate Mofetil =MMF, HCQ=hydroxychloroquine, C=Caucasian, AC= Afro-Caribbean, A=Asian. HTN=Hypertension, RC= Raised Cholesterol and DM=Diabetes Mellitus

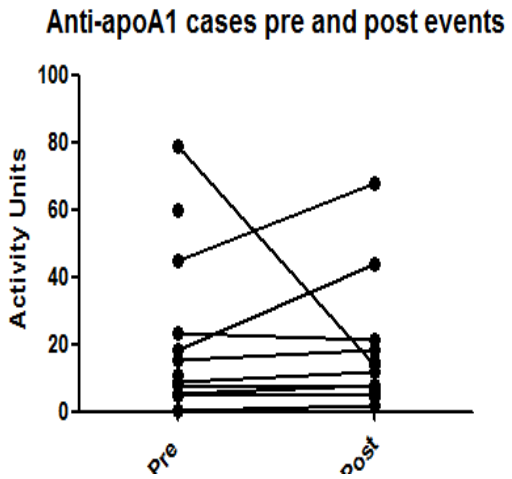
Figure 12A) shows activity units for the anti-apoA1 in the cases and control pre events. There is no statistically significant difference between the two groups (p=0.62). Similarly, in B) there is no difference in activity units between cases and controls post events (p=0.57). C) shows no statistically significant differences in the activity units in the cases pre and post events (p=0.28) and shows that in general, there isn't much change in titre from pre to post in individual cases.


Anti-apoA1 case-controls pre- events
Activity Units
150
100
50
0
Cases Pre
Con Pre
Healthy

A)


Anti-apoA1 case-controls post events
Activity Units
100
80
60
40
20
0
Cases Post
Con Post
Healthy

B)



C)

FIGURE 12- ANTI-APOA1 CASES AND CONTROLS

Comparison of anti-apoA1 activity units plotted against 3A) case (n=11) and controls (n=11) pre event; 3B) post event cases (n=18) and controls (n=19); 3C) line charts with pre and post events for cases. Each dot or square represents one patient. There were no significant differences between anti-apoaA1 levels between cases pre and post event**.**

3.2.2 Vascular Ultrasound Cohort

The 49 patients tested in the vascular ultrasound anti-apoA1 ELISA are described in table 7. The majority of patients were female (47 female vs 2 male). Ethnicity and anti-apoA1 levels were not associated (p= 0.09, ANOVA Kruskal-Wallis test). There was no significant correlation between anti-apoA1 levels and age (r= 0.10, p= 0.50).

Group	Sex	Ethnicity	Age (1st scan)	Disease Duration	Scans Interval (years)
NP	F	C	29	16	6
NP	F	C	51	8	6
NP	F	C	56	21	6
NP	F	A	40	21	6
NP	F	AC	37	18	5
NP	F	C	40	14	5
NP	F	AC	56	27	6
NP	F	C	52	22	6
NP	F	AC	31	7	5
NP	F	C	39	17	6
NP	F	AC	30	11	5
NP	F	C	57	16	5
NP	F	C	48	30	5
NP	F	C	51	10	5
NP	F	AC	45	9	5
NP	F	C	45	22	5
NP	F	C	43	13	5
NP	F	C	42	4	5
NP	F	C	37	6	6
NP	F	O	28	17	5
NP	F	AC	35	4	4
NP	F	A	60	4	4
NP	F	C	33	7	4
NP	F	A	36	7	5
NP	F	A	47	27	4
NP	F	AC	32	3	4
NP	F	C	36	9	4

NP	F	C	40	8	4
NP	F	O	33	15	5
RP	F	O	45	12	6
RP	F	C	42	21	6
RP	F	AC	50	3	6
RP	M	AC	33	17	6
RP	F	AC	59	35	6
RP	F	C	50	16	6
RP	F	C	45	13	5
RP	F	AC	51	13	5
RP	F	C	56	7	5
RP	F	A	54	25	5
RP	F	C	51	16	4
RP	M	C	49	14	4
RP	F	AC	39	5	4
SP	F	C	53	21	6
SP	F	C	48	30	5
SP	F	C	52	13	7
SP	F	C	61	34	5
SP	F	C	49	32	6
SP	F	C	51	21	5
SP	F	C	47	5	5
		Median	**45**	**14**	**5**

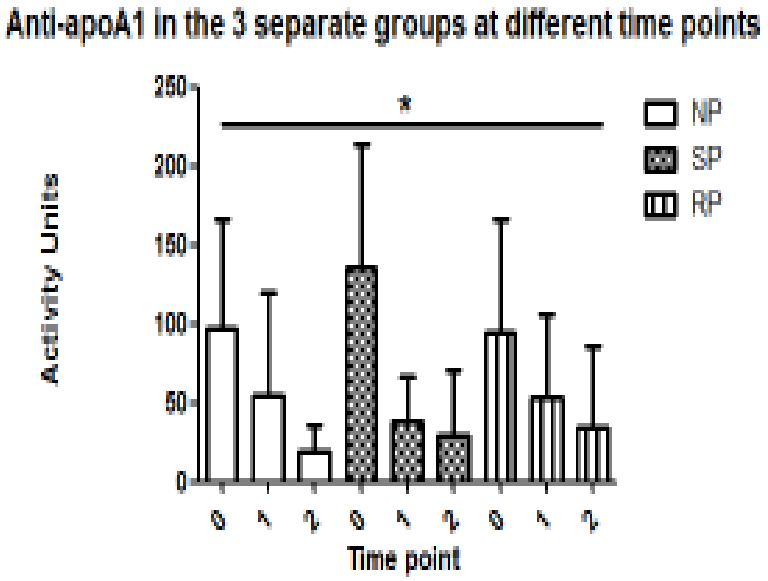
TABLE 7 VASCULAR ULTRASOUND COHORT

Forty-nine patients from the vascular ultrasound cohort were tested for anti-apoA1 antibodies at time point 0, 1 and 2. Age and disease duration correspond to the time of the first scan. Abbreviations: C=Caucasian, A=Asian, AC= Afro-Caribbean,

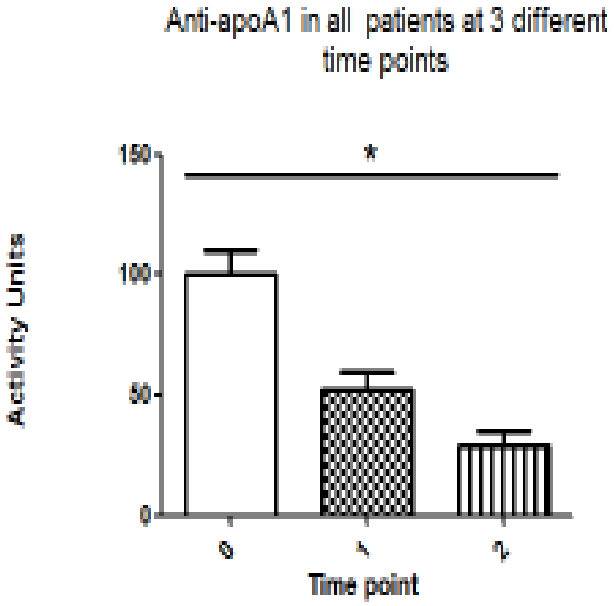
O=Other, DD= disease duration in years at time of first scan. NP= No-Plaque, RP=Rapid Plaque, SP= Slow Plaque growth.

3.2.3 Anti-apoA1 levels decline over time in all groups

Comparing median anti-apoA1 titres between the three time points there was a reduction in titres over time which was significant (ANOVA, Kruskal-wallis test, $p<0.001$) (figure 13). However, comparison between anti-apoA1 titres in the 3 different groups based on plaque status at each time point did not reach significance, time point 0 (ANOVA $p=0.482$), time point 1 (ANOVA, $p=0.800$) and time point 2 ($p=0.305$) (B).


Anti-apoA1 in the 3 separate groups at different time points
Activity Units
250
200
150
100
50
0
*
NP
SP
RP
Time point

A)



B)

Figure 13 –anti-apoa1 levels in the ultrasound cohort groups

A) Anti-apoA1 levels show a decline in the ultrasound cohort in all three groups over time (NP= non-plaque patients, SP=slow progressors, RP=rapid progressors) B) When the patients are combined, this trend in decline in anti-apoA1 levels is reflected over time. . Mean ±SE.

The decline in anti-apoA1 antibodies was specific to anti-apoA1 as when I plotted the same patient's anti-dsDNA antibody levels (which had been taken from patient records at UCLH closest to each time point) over the same three time points the levels did not reduce in a similar fashion (figure 14).

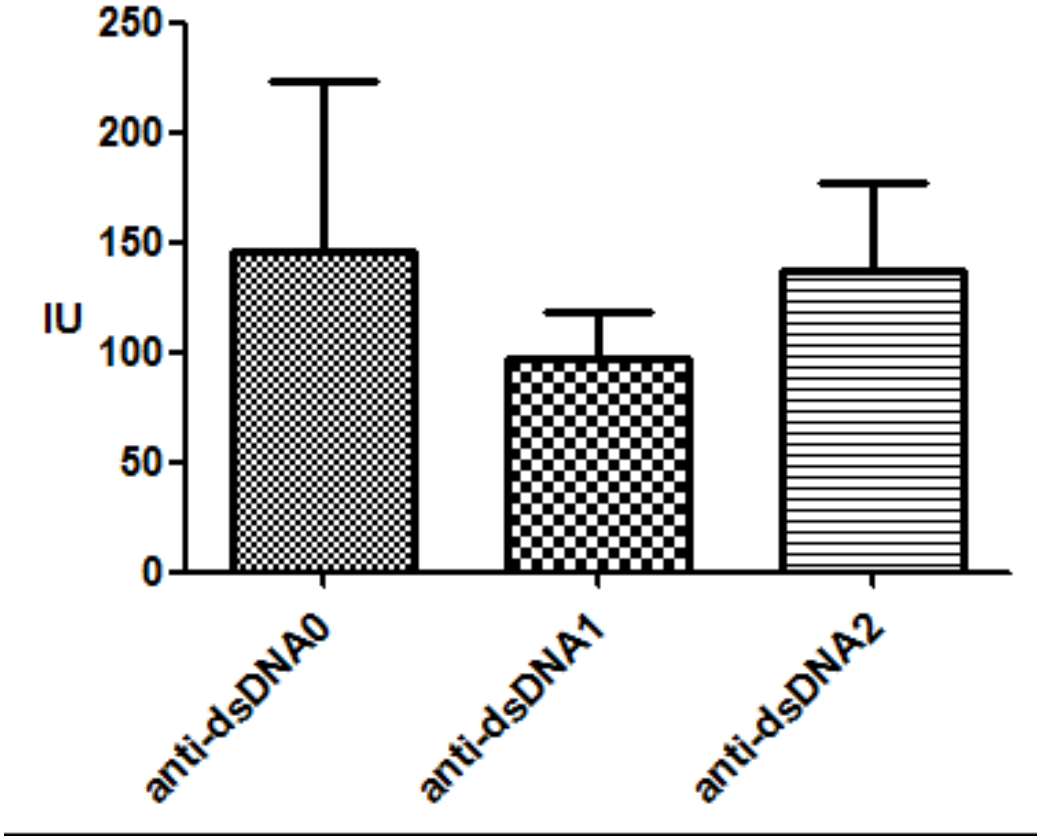


FIGURE 14- ANTI-DS DNA TITERS IN THE ULTRASOUND COHORT

Anti-dsDNA titers from the vascular cohort at the three time points (0, 1 and 2 on x-axis) do not show a decline in titres over time like anti-apoA1 titres. . Mean ±SE.

3.2.4 **Medication use**

I wanted to see if there was a reason to explain the decline in anti-apoA1 levels over time and wanted to see if use of medications could be an explanation. To do this, I collected data on four different types of treatment: statins, immunosuppressants, hydroxychloroquine (HCQ), prednisolone and rituximab, and separated patients according to whether they received a given drug at the time of the scan (within a year). As there were incomplete data for time point 0 (as this predated the computerised records), only time points 1 and 2 were looked at.

Median anti-apoA1 values of patients receiving each drug and patients not receiving that drug are shown in table 8 for both time points 1 and 2. The association of each drug used at each time point with anti-apoA1 levels is also shown. Overall, there was a trend for higher median anti-apoA1 levels in patients not taking these medications versus those who did, and use of HCQ at time point 1, rituximab at time point 1 and prednisolone at time point 2 were significantly associated with lower levels of anti-apoA1 ($p<0.0001$, $p=0.0134$ and $p=0.0094$ respectively).

Drug use Number of patients using drug at time point 1 and 2	**Time point 1**		**Time point 2**	
	Median AU	**p value**	**Median AU**	**p value**
Statin- YES Time point 1- 4 Time point 2- 3	30	p=0.648	11	P=0.06
Statin –NO Time point 1-45 Time point 2-46	34		17	
Immunosuppressant YES Time point 1-22 Time point 2-20	24	p=0.587	16	p=0.787
Immunosuppressant NO Time point 1-27 Time point 2-29	44		17	
Hydroxychloroquine YES Time point 1-31 Time point 2-33	22	**p<0.0001***	17.01	p=0.1476
Hydroxychloroquine NO Time point 1-18 Time point 2-16	54		15.42	
Rituximab YES (use in 1 year before or after time point) Time point 1-18 Time point 2-2	24	**p=0.0134***	6	Insufficient numbers in the Rituximab group
Rituximab NO (use in 1 year before or after time point) Time point 1-31 Time point 2-47	45		16	
Prednisolone YES Time point 1-31 Time point 2-29	24.97	p=0.2807	16.27	**p=0.0094***
Prednisolone NO Time point 1-18 Time point 2-20	44.96		17.41	

TABLE 8- DRUG ASSOCIATIONS AND ANTI-APOA1 LEVELS AT TIME POINT 1 AND 2

Forty-nine patients were tested from the ultrasound cohort to see if the use of a drug was associated with anti-apoA1 levels. P- values (using a Mann-Whitney test) are shown and median values of anti-apoA1 levels for patients who were on the drug and those who were not on the drug at each time point is shown.

I further analysed to see whether anti-apoA1 levels correlated with persistent disease activity (as defined by presence of BILAG score A or B in any system on two consecutive occasions 2 years prior to scan date), disease activity markers (anti dsDNA antibodies and complement) and lipids. There were no significant correlations with any of these parameters as shown in table 9 at time point 1 and 2.

	Statistical analysis- Spearman correlation	
	p –value	**r value**
Anti-dsDNA- 1	p=0.417	r= 0.119
Anti-dsDNA- 2	p=0.162	r= 0.210
C3-1	p= 0.847	r= -0.028
C3-2	p=0.622	r= -0.075
Total Cholesterol-1	p=0. 916	r= 0.01
Total Cholesterol-2	p=0.717	r=0.062
Triglycerides-1	p=0. 896	r= -0.019
Triglycerides-2	p=0. 067	r=0.304
HDL-1	p=0.208	r=-0.183
HDL-2	p=0.508	r= -0.112
LDL-1	p=0.429	r=0.116
LDL-2	p=0.610	r=0.087
Cholesterol:HDL ratio-1	p=0.185	r=0.192
Cholesterol:HDL ratio-2	p=0.112	r=0.266

TABLE 9- CORRELATIONS *OF ANTI-APOA1 WITH DISEASE ACTIVITY MARKERS, LIPIDS AND PREDNISOLONE DOSE USING SPEARMAN CORRELATIONS.*

1= time point 1 and 2= time point 2.

3.2.5 Analysis of Plaque versus Non-plaque group

I analysed to see if a difference could be observed when the cohort was split into those with plaque compared to those without plaque. In non-plaque patients, rituximab use was associated with significantly lower levels of anti-apoA1 (p=0.032) – median levels of anti-apoA1 were higher in patients not given rituximab (45 AU) compared to those for whom it had been prescribed (24 AU). In plaque patients, no significant associations were found between rituximab use and anti-apoA1 levels (p=0.50). HCQ use was not associated with anti-apoA1 in either the non-plaque (p=0.114) or plaque group (p=0.142).

3.2.6 Linear Regression Analysis results

In univariate linear regression analysis, treatment with rituximab showed a significant negative association with IgG anti-apoA1 levels (regression coefficient B= - 36.84, p=0.017). The effect was still significant when the model was adjusted for persistently active disease and also when adjusted for age and ethnicity, using multivariable regression analyses. Treatment with hydroxychloroquine did not show a significant association by linear regression. Lipid levels, anti-dsDNA antibodies and other treatments did not show associations with anti-apoA1 levels.

3.2.7 **Discussion**

In this study I looked at anti-apoA1 levels in SLE patients who had events compared to those without CVD events. The cases and controls were matched closely in age, sex, ethnicity and disease duration and where possible on medications. I have shown that there was no difference in anti-apoA1 levels in patients who have suffered a CVD event from those patients who were event free. This is corroborated by other investigators. O'Neil et al (O'Neill, Giles et al. 2010) looked at both of these antibodies in a group of 24 patients who had suffered from CVD events and found no difference between SLE patient who had an event compared to those who had not. Croca et al studied 499 SLE patients over a mean follow up of 12.1 years, found that the presence of anti-apoA1 antibodies in early disease (from 1 year of diagnosis) did not associate with increased CVD or mortality in SLE patients (8% of the cohort developed CVD). She and her colleagues reported an association between anti-apoA1 and disease activity, as have other investigators (Shoenfeld, Szyper-Kravitz et al. 2007, Batuca, Ames et al. 2009, O'Neill, Giles et al. 2010).

A possible reason for my lack of positive correlations may be that anti-apoA1 antibodies are not as important in SLE compared to other autoimmune diseases and populations. The association of anti-apoA1 antibodies in RA, diabetes and acute coronary syndromes have been reported in the literature (Vuilleumier, Reber et al. 2004, Vuilleumier, Rossier et al. 2010, Pagano, Satta et al. 2012). Baseline anti-apoA1 positivity was strongly predictive of CVD in a population of 133 Swiss RA patients followed up for a median of 9 years, after adjustment for standard CVD risk factors (Vuilleumier, Rossier et al. 2010). A possible explanation may be due to the

different cytokine environment in RA compared to SLE. RA patients tested positive for anti-apoA-1 antibodies were found to have higher levels of interleukin-8 and MMP-5 (Vuilleumier, Bas et al. 2010). The latter are two inflammatory mediators known to be associated with atherogenesis and atherosclerotic plaque vulnerability in humans (Schwartz, Galis et al. 2007). In additional, in the RA and general populations where associations between anti-apoA1 antibodies have been shown (Vuilleumier, Reber et al. 2004, Vuilleumier, Rossier et al. 2010, Pagano, Satta et al. 2012) the populations were older, predominately male and had a higher incidence of CVD events over time – which is in contrast to my study of SLE populations.

Therefore larger studies over longer periods of time may will be needed to identify predictive factors for CVD in SLE.

One of the limitations of my CVD events study was the small number of samples used. The total number of case-controls were only 20 -due to the small relative risk of developing CVD in SLE, the overall pool of possible event patients was small. Secondly, there were missing data for several of the pre event samples. The reason for this is that over half of the cases out of the 20 had events more than 10 years prior to testing. Therefore, finding samples retrospectively for pre-events was difficult because of the chronicity.

Another limitation was that when a sample was found to match the event date there was a restriction due to availability of the closest sample to that date- with median interval of 4 years from the date of event, compared to O'Neil et al's study where the sample was analysed within 1 year of the event (O'Neill, Giles et al. 2010). Patient

samples are obtained from clinic visits with a wide variation of follow appointments between patients. Therefore, the antibody levels may not be truly reflective of the levels that were present before and after the event and may have been also affected by factors such as disease activity at the time the sample was taken. This would also create difficulty in interpreting results as the antibody levels have been measured at different time points for each sample creating heterogeneity. The use of multiple serum samples pre and post events may have helped to show more statistically meaningful data.

My second study, which looked at the vascular ultrasound cohort group, is the first as far as I am aware to report a significant reduction of anti-apoA1 levels in SLE patients (with plaque and non-plaque) over time. This may be due to consumption of antibody over time although the reason for this is not clear from my study.

The use of hydroxychloroquine and rituximab were the only drugs that were associated with anti-apoA1 levels in all of the vascular cohort who were scanned and when the group was split into plaque and non-plaque, in only the non-plaque group. Fall in anti-dsDNA antibody levels post treatment with Rituximab has been previously reported (Cambridge, Isenberg et al. 2008). This effect did not occur with other antibodies such as anti-Sm and anti-Ro. However, these finding were not consistent across the time points and treatment with hydroxychloroquine did not show an independent association with IgG anti-apoA1 by linear regression.

My study is unique as I looked at anti-apoA1 longitudinally in both CVD patients and vascular ultrasound patients with SLE. My assays showed in the vascular ultrasound

group that there was a significant decline in anti-apoA1 titres over time however no significant differences between the plaque and non -plaque groups were found and similarly in the SLE CVD vs non-CVD groups. Therefore perhaps unlike other autoimmune diseases such as RA, there does not seem to be a link between IgG anti-apoA1 and CVD in SLE.

3.3 iNKT cells in Rheumatoid Arthritis

RA patients have a significantly increased risk of cardiovascular disease compared to the general population; however, the mechanisms underpinning the development of atherosclerosis in these patients remain uncertain.

In collaboration with Professor Ernest Choy at the University of Cardiff, I investigated the role of iNKT cells in plaque and non-plaque RA patients asymptomatic for CVD. Patients had carotid US scans carried out by our collaborators at the University of Cardiff and serum samples were sent to UCL. Thirteen RA serum samples patients (eight RA-P matched by age/sex/ethnicity to 5-RA NP) were tested with age/sex/ethnicity matched healthy controls on healthy PBMCs, to investigate an iNKT response.

3.3.1 Subclinical plaque in RA patients associations

I examined a cohort of 13 RA patients that had not previously suffered a cardiovascular event and had received ultrasound scans to determine the presence of preclinical atherosclerotic plaque in the right and left carotid and femoral arteries (methods section describes patient selection criteria and details of plaque analysis). Patients were categorised as having plaque or no plaque based on data from ultrasound scans. There were eight RA-P and five RA-NP. Some of the RA-P samples had duplicated matched RA-NP controls (table 10).

No significant differences in demographic or clinical parameters (serological, therapy and disease activity) was identified between RA-P and RA-NP patients assessed by statistical analysis using SPSS (table 11). All of the cohort were Caucasian and there were 11 females and 2 males.

S	E	A	S	DD	Plaque	DAS 28	I	Drug	Steroid	RF	ACPA	HTN	HCL	SS
1 *13	C	49	F	10.3	Y	3.07	Y	MTX 20mg	Y (2.5mg)	<10	700	N	N	1
2 *13	C	54	F	11	Y	2.97	N	Ibuprofen prn	N	<10	24	N	N	0
3 *11	C	61	F	9	Y	4.73	Y	MTX 20mg, SSZ 1g	N	<10	22	Y	N	2
4 *10	C	74	F	11	Y	3.01	N		N	<10	20	N	N	0
5 *12	C	62	M	9	Y	4.08	Y	MTX 15mg,	N	769	756	N	N	1
6 *9	C	63	F	11.5	Y	4.12	N	Co-codamol Tramadol	N	<10	13	Y	N	1
7 *10	C	80	F	13.4	Y	2.87	Y	MTX 20mg	Y(5mg)	208	52	Y	N	0
8*9	C	69	F	13.2	Y	2.45	Y	MTX 20mg	N	163	1000	Y	N	1
9	C	64	F	9	N	3.34	Y	MTX 20mg, SSZ 1g	N	<10	26	N	N	0
10	C	76	F	16	N	4.09	Y	MTX 15mg,	N	693	352	Y	N	0
11	C	51	F	14	N	1.32	Y	MTX 20mg	N	230	364	N	N	2
12	C	64	M	7.9	N	2.89	Y	MTX 20mg	N	<10	14	N	N	0
13	C	57	F	10	N	3.06	Y	MTX 20mg	Y(3mg)	376	94	Y	Y	1

TABLE 10- ***DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF RA PATIENTS WITH PLAQUE AND NON-PLAQUE.***

Thirteen RA patients asymptomatic for CVD had been scanned for atherosclerosis. Eight were RA-P and five were RA-NP control (*) patients. Drug treatment is from the time of scanning. Antibody status is defined as ever positive. S=Sex; E=ethnicity; C=Caucasian; A=Age at scan; DD=disease duration at scan (months); DAS 28 = disease activity score at scan; I= immunosuppression; MTX=methotrexate; SSZ=sulphasalazine; RF= Rheumatoid factor titres; ACPA= Anti-citrullinated protein titres; HTN= Hypertension HCL=hypercholesterolemia; SS= Smoking status- 0 = never; 1= previous; 2 = current.

	Healthy donors (n=8)	RA-NP (n=5)	RA-P (n=8)	P value (P vs NP)
Demographics				
Age at scan, mean ± SD	58 (45-70)	62.5 (51-76)	64 (49-80)	0.781
Disease duration at scan, mean ± SD	NA	11 (3.46)	11(1.66)	0.850
Gender: Female: Male	6:2	4:1	7:1	
Ethnicity: Caucasian (%)	8 (100)	5 (100)	8 (100)	NA
Blood pressure at time of scan(mmHg)				
Systolic mean ± SD	NA	130 (14)	137 (25)	0.545
Diastolic mean ± SD		77(11)	69 (16)	0.341
Cardiovascular risk factors				
Body Mass Index median (range)	NA	23 (23-29)	26 (21-50)	0.232
History of Hypertension (Y:N)	NA	2:3	4:4	
History of Hypocholesteraemia (Y:N)	NA	1:4	0:8	0.220
History of Diabetes (Y:N)	NA	0	0	NA
Ever smokers (%)	1	2 (40)	5 (63)	0.187

Treatments at time of Scan				
Hydroxychloroquine (%)	NA	3 (60)	3 (38)	0.524
Prednisolone use at scan (%)	NA	1 (20)	2 (25%)	1
Immunosuppressant use	NA	5 (100)	5 (63)	0.285
DAS-28 score at time of scan **median (range)**	NA	3.06 (1.32-4.09)	3.04 (2.45-4.73)	0.391
Serology (ever positive)				
Rheumatoid Factor **positive**	NA	3 (60)	3 (38)	0.222
Inflammatory markers at time of scan				
CRP (mg/dL), mean ± SD		4 (0.7)	6.63 (7.11)	0.334
ESR (mm/h), mean ± SD		15.8 (15)	20 (19)	0.673
Lipid profile at time of scan (mmol/L)				
Total cholesterol, mean ± SD		4.92±1.06	4.84±0.57	0.751

HDL, mean ± SD		1.34±0.22	1.43±0.55	0.734
LDL, mean ± SD		2.92±0.78	2.81±0.63	0.711
Total cholesterol/ HDL ratio, mean ± SD		3.72±1.32	3.70±1.10	0.791
Triglycerides, mean ± SD		1.22±0.48	1.31±0.66	0.880
CIMT, mean ± SD	NA	0.721±0.4	0.842±0.1	0.151

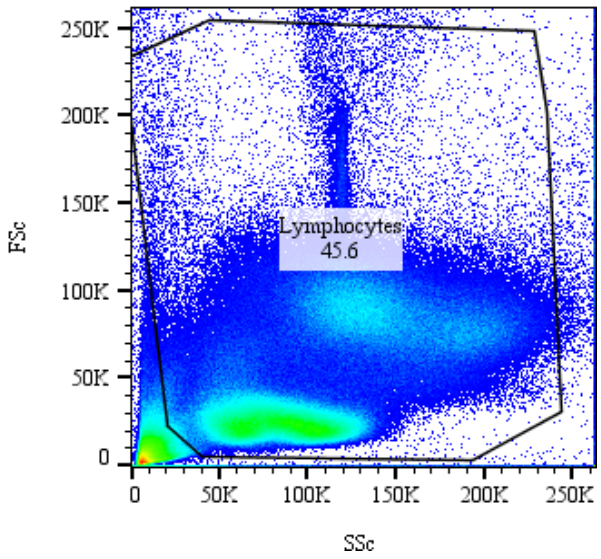
TABLE 11- CLINICAL AND DEMOGRAPHIC INFORMATION OF SERUM FROM 8 RA-P, 5 RA-NP PATIENTS AND 8 HEALTHY CONTROLS

Comparison between RA-P and RA-NP was analysed by parametric or non-parametric tests as appropriate with *p=<0.05. HC= healthy control; RA-P= Rheumatoid Arthritis Plaque patients; RA-N= Rheumatoid Arthritis Non- Plaque patients; DAS-28= Disease Activity Score; ACPA= anti-citrullinated peptide antibodies.

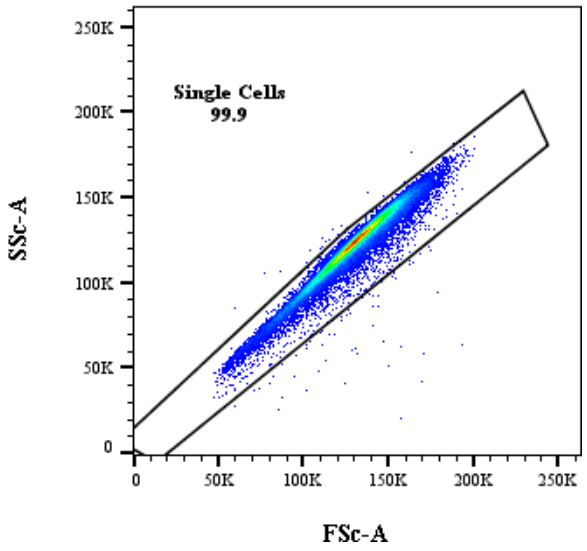
3.3.2 PBMC exposed to RA serum for 7 days do not have altered percentage of *i*NKT cells compared to PBMC exposed to HC serum

Peripheral blood *i*NKT cell expression levels were assessed by flow cytometry, according to the gating strategy shown in figure 15. Lymphocytes were gated based on size and granularity (figure 15A), subsequently only single cells (figure 15B) were selected for further analysis. After gating for live cells (figure 15C), double positive cells for iTCR and $CD3^+$ were defined as *i*NKT cells (figure 15D) (Smith, Croca et al. 2016). The *i*NKT cell population size was quantified by cells, which were positive for both CD3 and the iTCR. $CD3^+iTCR^+$ populations were gated according to fluorescent minus one (FMO) controls (figure 16).

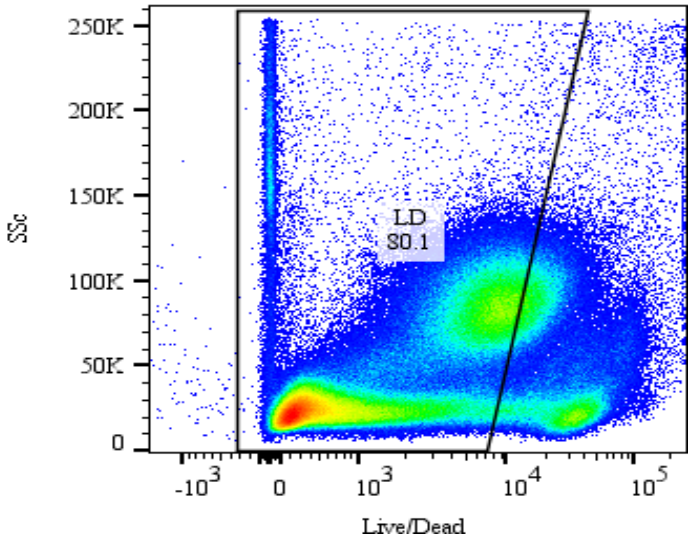
In order to determine if *i*NKT cell frequency could be influenced by RA serum, *i*NKT cell numbers were assessed at baseline (day 0) and after 7 day culture with serum from patients with RA-P and RA-NP and healthy controls. There was a trend towards reduced *i*NKT cell frequency in healthy PBMCs cultured in RA patient serum (both RA-P and RA-NP) compared to healthy control serum after 7 day culture (figure 17 representative chart from one experiment); however this was not significant (HC D7 vs RA-P p=0.27 and HC D7 vs RA-NP p=0.53). Similarly there was no significant difference in *i*NKT frequency in D0 vs RA-NP (p=0.56) and RA=P (p=0.34), between D0 and HC D7 (p=0.85), and RA-P compared to RA- NP (p=0.50). When total RA was compared to HC-D7 there was no significant difference (p=0.31) (figure 18).



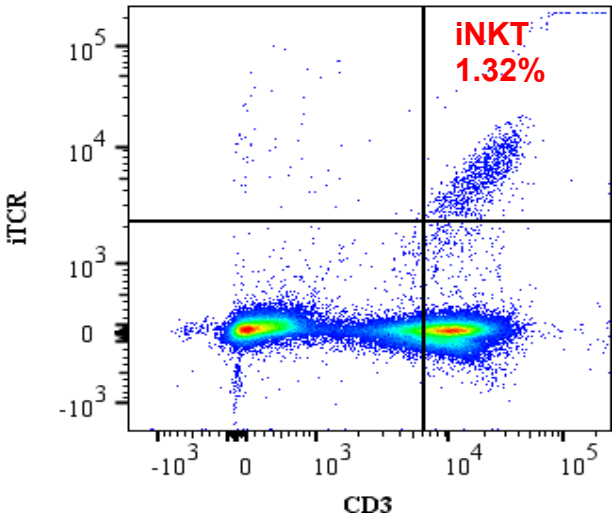
A) Lymphocytes



B) Single cells



C) Live/Dead



D) *i*NKT gate

FIGURE 15- PBMCS FROM HEALTHY DONORS WERE ASSESSED FOR *I*NKT CELL FREQUENCY BY FLOW CYTOMETRY USING ANTIBODIES TO CD3$^+$ AND INVARIANT T CELL RECEPTOR (ITCR).

Gating strategy for *i*NKT cells showing gating on lymphocytes (A), followed by single cells (B) as determined by forward scatter height and area, then live cells (C) which

were negative for live/dead stain. After gating for live cells, double positive cells for iTCR and $CD3^+$ were defined as *i*NKT cells (figure 3.3D). FSc= forward side scatter, SSc-A= side scatter area; FSc-A= forward side scatter area; iTCR=invariant T-cell receptor.

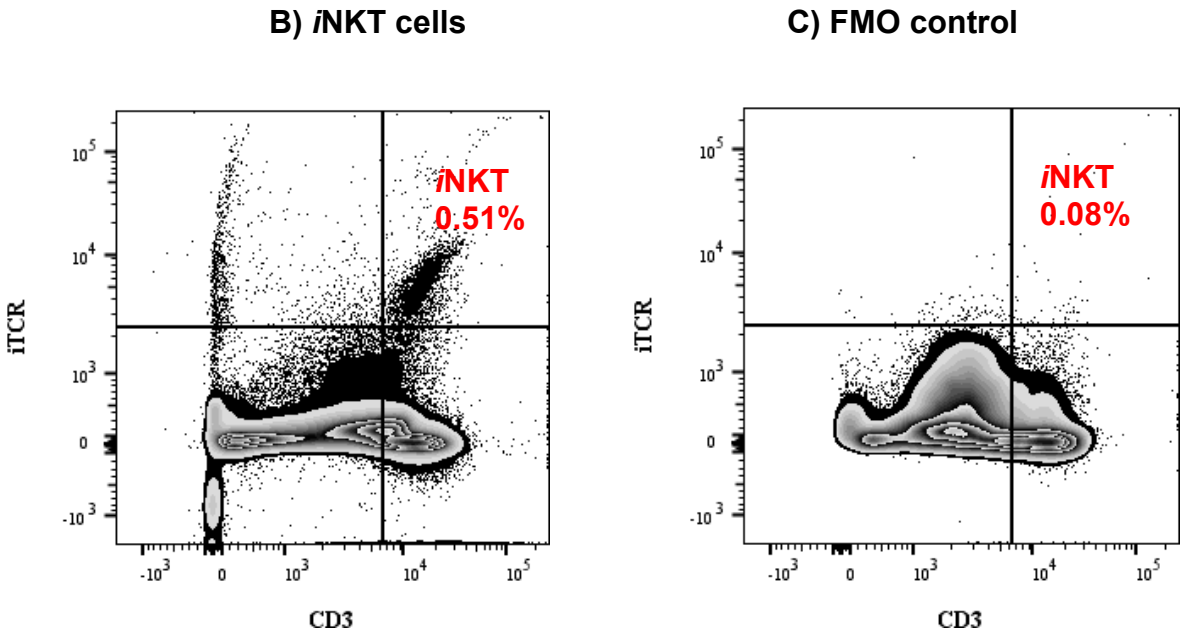
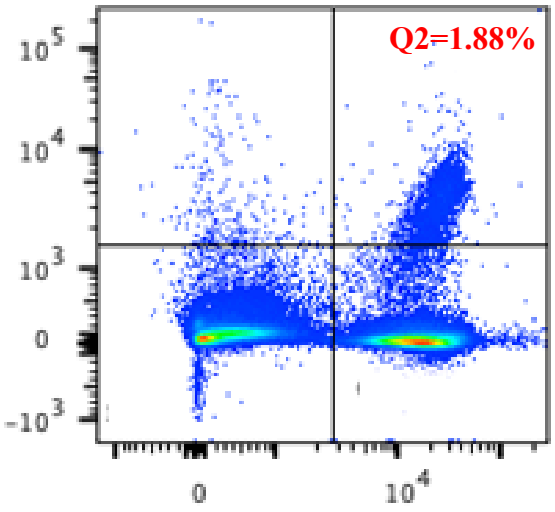
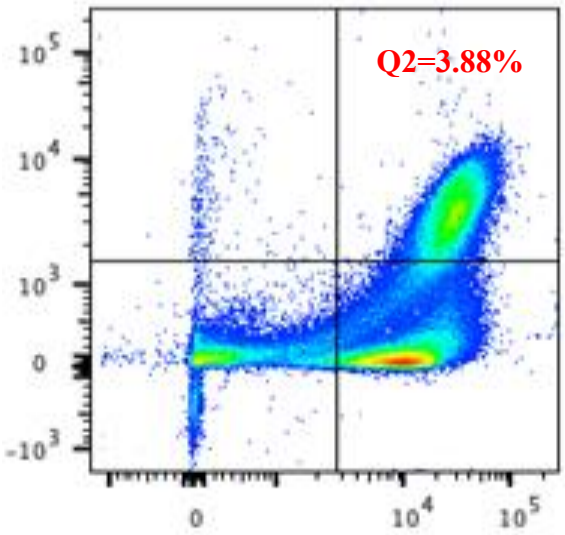


FIGURE 16- THE *i*NKT CELL POPULATION SIZE WAS QUANTIFIED BY CELLS WHICH WERE POSITIVE FOR BOTH CD3 AND THE ITCR USING PBMCS FROM HEALTHY DONORS

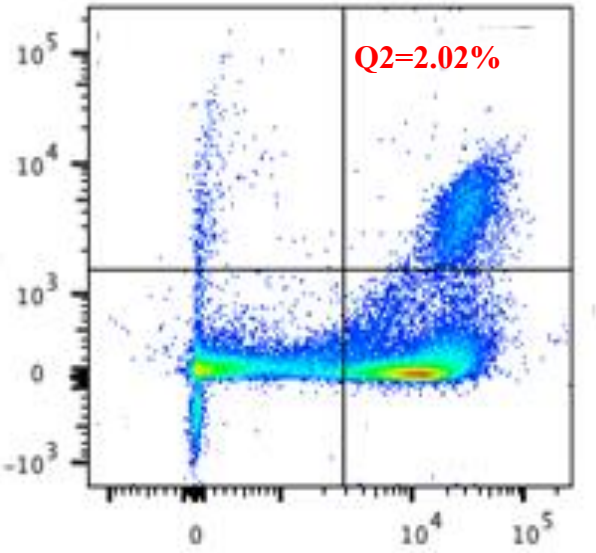
$CD3^+iTCR^+$ (B) populations were gated according to fluorescent minus one (FMO) controls (C). iTCR= invariant T-cell receptor.



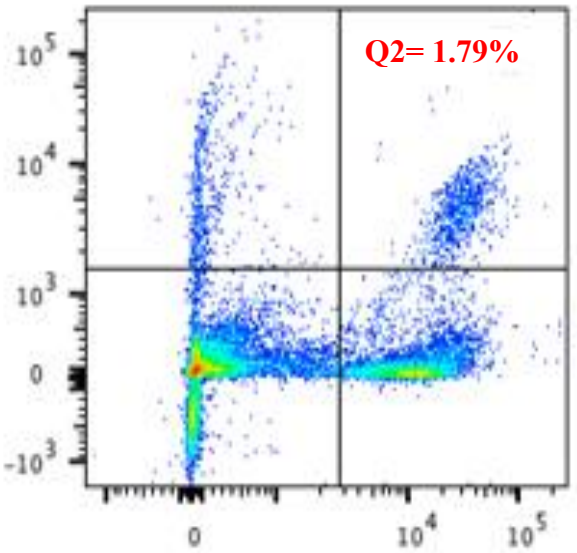
A) D0



B) HC-D7



C) RA-NP- D7

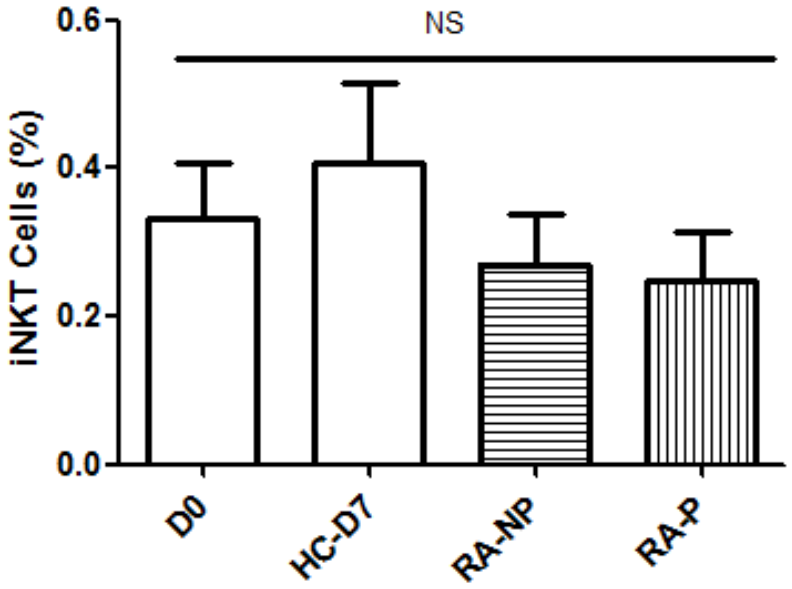


D) RA-P D7

FIGURE 17- REPRESENTATIVE FLOW CYTOMETRY FOR *I*NKT CELL FREQUENCY AT DAY 0 AND 7 DAY CULTURE (HC=3, RA-P=3, RA-NP=3)

In order to determine if *i*NKT expression was induced by RA serum, *i*NKT cell frequencies were assessed at baseline (day 0) and after 7-day culture of healthy PMBCs (n=3) with serum from patients with RA-P (n=3) and RA-NP (n=3) and healthy controls and IL-2. A *representative flow cytometry* dot plots from *one experiment* showing percentage *i*NKT cell frequency at day 0 (A) and day 7 plus HC serum (B); and healthy control PBMC plus RA-NP serum (C) and RA-P serum (D) day 7. Where the upper right quadrant represents the CD3+ *i*NKT cell populations (Q2). The x-axis represents CD3 and the y-axis represents iTCR. HC= healthy control, iTCR= invariant T-cell receptor; RA-P= Rheumatoid Arthritis -Plaque; RA-NP= Rheumatoid Arthritis -non-plaque. D0=day 0; D7-day 7.

A)


NS
0.6
0.4
0.2
0.0
iNKT Cells (%)
D0
HC-D7
RA-NP
RA-P

(B)

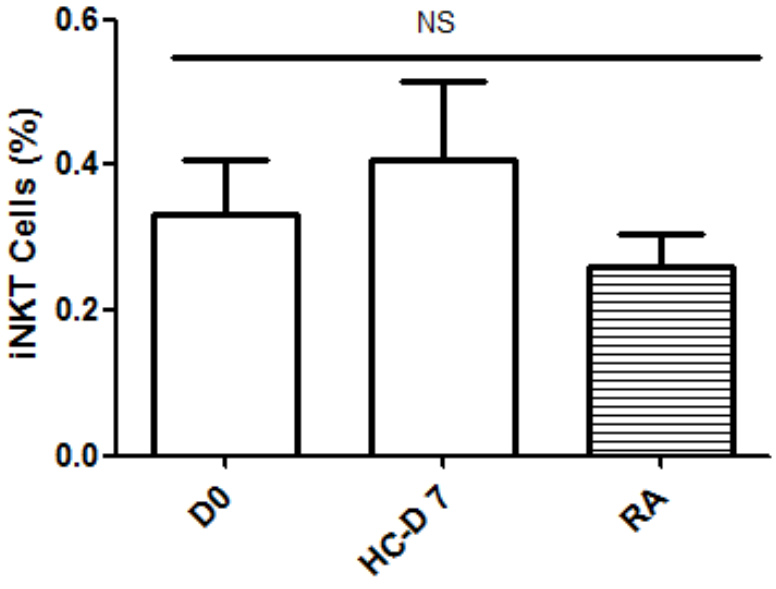

NS
0.6
0.4
0.2
0.0
iNKT Cells (%)
D0
HC-D 7
RA

FIGURE 18- BAR CHART OF *i*NKT CELL FREQUENCY

A) *i*NKT cell percentages were assessed at baseline (day 0) and after 7-day culture with serum from patients with RA-P (n=8) and RA-NP (n=5) and healthy controls (n=8) represented by bar charts. B) Compared D0, healthy controls and all RA D7 (RA-NP and RA-P). T-tests were used with p= <0.05 as significant. Mean ±SE. NS= not significant. D0= day 0; HC D7= healthy control day 7; RA-P= Rheumatoid Arthritis Plaque patient; RA-NP= Rheumatoid Arthritis non-plaque patient.

3.3.3 Clinical parameters

I wanted to determine whether *i*NKT cell proliferation in PBMC exposed to serum from RA-P and RA-NP patients was attributable to factors other than the presence of plaque; therefore *i*NKT frequency was correlated with age, disease duration, BMI, diagnosis of hypertension, anti-bodies (RF and ACPA), patient medications, DAS (disease activity) scores and serum lipids. A Spearman's (nonparametric data) or Pearson's correlation (parametric data) were used for analysis (table 12). *i*NKT frequency did not show any correlations to age, disease duration, disease activity, anti-body titres, ESR/CRP and serum lipids of the patients from whom serum was derived at the time of scan. Using T-tests, there was no significant difference in *i*NKT frequency and drug treatment use (hydroxychloroquine p=1.00 and immunosuppressants p=0.81 respectively), being a smoker at the time of scan (p=0.21), history of hypertension (p=0.23) and RF positivity (p=0.21).

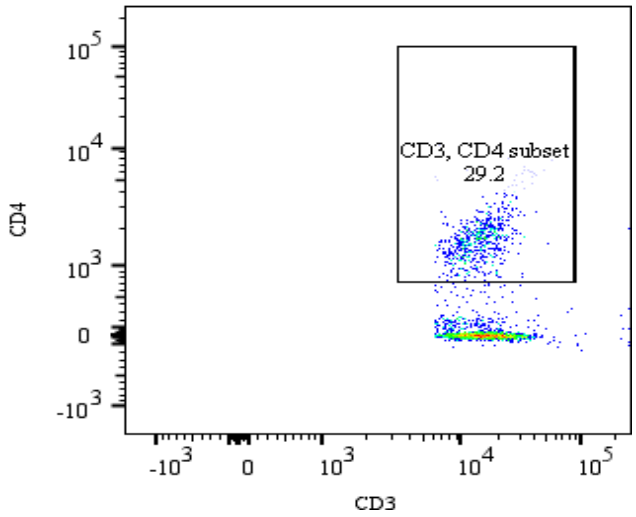
	Statistical analysis- p and r value (Pearson* or Spearman** correlation)
Age	p=0.55 r= -0.18*
Disease Duration (years)	p=0.27 r= -0.33*
RF titres	p=0.70 r=0.09*
ACPA titres	p=0.70 r=0.08*
DAS-28 score	p=0.26 r=0.39*
CRP	p=0.17 r=0.43*
ESR	p=0.18 r=0.39*
Body Mass Index	p=0.67 r=0.13*
Total Cholesterol	p=0.53 r=0.19*
HDL	p=0.21 r= -0.37*
LDL	p=0.52 r=0.20*
Triglycerides	p=0.07 r=0.54*
Cholesterol:HDL ratio	p=0.30 r=0.31*
Average CIMT (mm)	p=0.66 r=-0.13*
ACPA titres	p=0.70 r=-0.12**

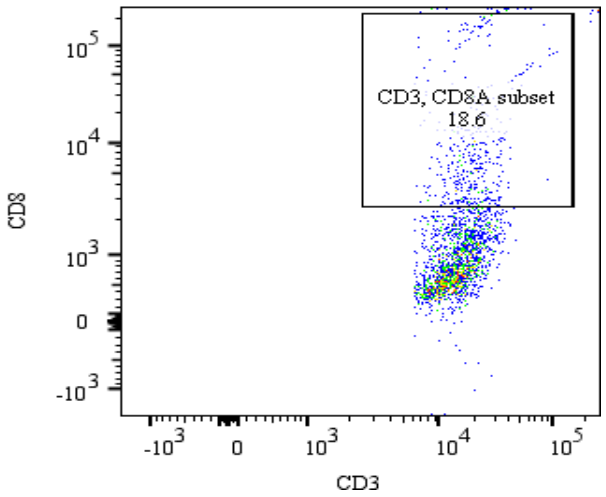
TABLE 12- CORRELATIONS TO *i*NKT FREQUENCY

Clinical information from the time of scan (excluding RF and ACPA titres) from RA-P (n=8) and RA-NP (n=5) was assessed in relation to *i*NKT frequency. *i*NKT frequency did not correlate significantly with age, disease duration, BMI, RF/ACPA titres, DAS-28 score and serum lipids using either a Spearman* or Pearson correlation**, p=0.05. DAS-28= Disease Activity Score; ACPA= anti-citrullinated peptide antibodies; HCQ =hydroxychloroquine.

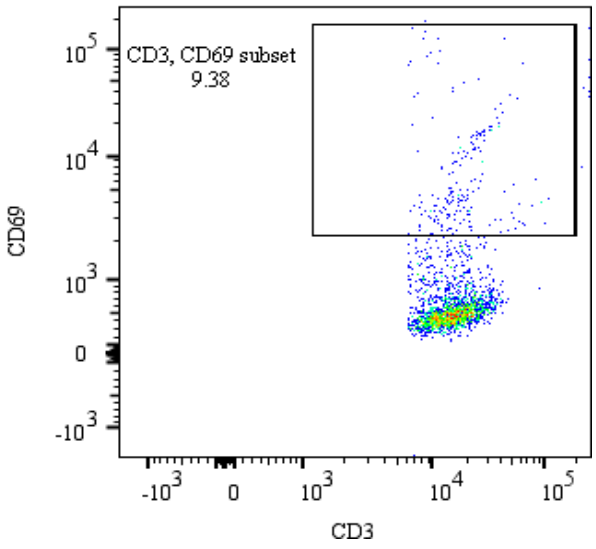
3.3.4 iNKT cell CD4/CD8+ populations and activation markers

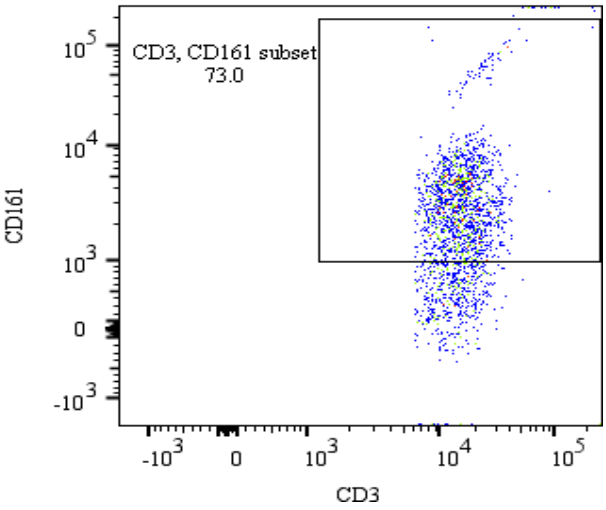
I investigated whether serum from RA patients with preclinical plaque could influence *i*NKT cell phenotype. After a 7 day serum culture different healthy controls $CD4^+$ and $CD8^+$ *i*NKT cell populations were gated using the distribution of these subsets in $CD3^+iTCR^+$ T cells. In order to assess whether *i*NKT cells were differentially activated or functionally altered in RA patients with preclinical plaque, I investigated the expression of surface markers on *i*NKT cells by flow cytometry by staining for $CD69^+$, $CD161+^+$ and $PD\text{-}1^+$. Gates were set on healthy donor $CD3^+iTCR^+$ T -cells with reference to fluorescence minus one (FMO) controls (gating strategy shown in figure 19).

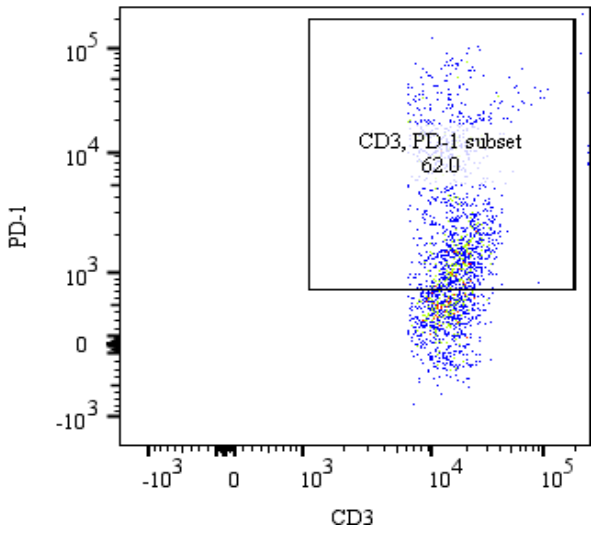

CD3, CD4 subset
29.2
CD4
CD3


CD3, CD8A subset
18.6
CD8
CD3

A)


CD3, CD69 subset
9.38
CD69
CD3


CD3, CD161 subset
73.0
CD161
CD3



B)

Figure 19- iNKT Cell Phenotype

iNKT cell phenotype was assessed in PBMC exposed to serum from RA-P (n=8), RA-NP (n=5) and healthy controls (n=8). Analysis of iNKT cell phenotype was performed using flow cytometry. CD3, iTCR, CD4 and CD8 labelling was used to identify iNKT cell subsets. A) Gating strategy for $CD4^+$ and $CD8^+$ iNKT cell populations using the distribution of these subsets in $CD3^+iTCR^+$T cells is shown. B) iNKT cell phenotype was examined on PBMCs from healthy donors, which were stained for CD3, iTCR, CD69, PD-1, and CD161. Gating strategy for CD69, CD161 and PD-1 respectively using the distribution of these subsets in $CD3^+iTCR^+$ cells is shown.

3.3.5 CD4$^+$ and CD8$^+$ *i*NKT cells and surface markers results in RA patients versus Healthy Controls

Table 13 summarises the results of the expression of *i*NKT cells, and surface marker expression in RA vs HC. The significant results are highlighted in italics. No significant differences were found between RA- P vs RA-NP.

There was a significant increase in CD8$^+$ *i*NKT cells in HC (figure 20A) and D0 vs all RA patients at day 7 (p=0.04 and p=0.02 respectively (figure 20B).

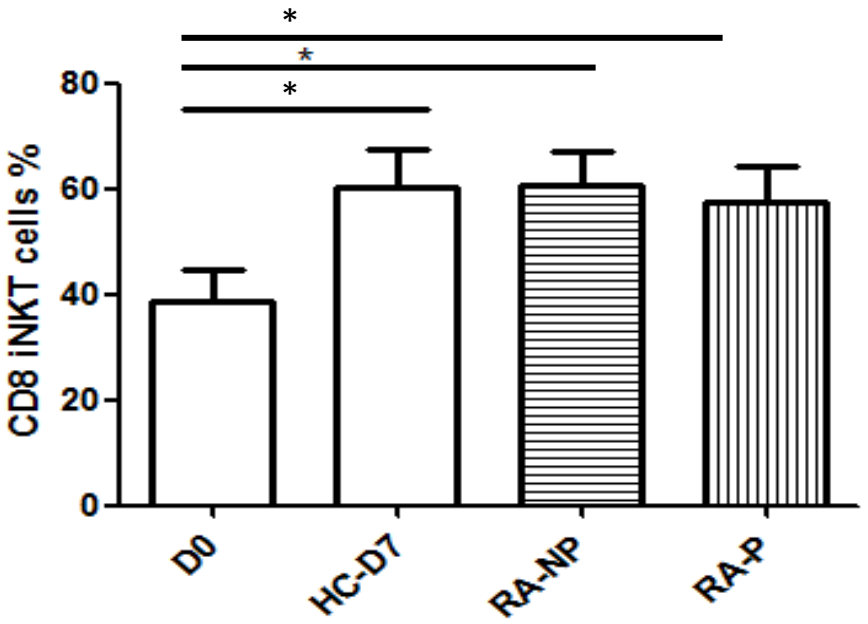
A significant increase in the percentage of CD69$^+$ *i*NKT cells in HC (p=0.013), in D0 vs RA-P (p=0.03) and D0 vs RA-NP (p=0.013) (figure 21A) and D0 vs all RA patients (p=0.0089) (figure 21B). The mean fluorescence intensity (MFI) as measured by the geometric mean is a marker of how positive the marker is; the higher the MFI the more positive the marker is. I assessed the geometric mean for CD69$^+$ given there were significant differences for this marker. The GM of CD69$^+$ *i*NKT cells was also significantly increased in all RA vs D0 (p=0.037) (figure 21C).

Figure 22 shows there was no significant differences CD4$^+$, CD161$^+$ or PD-1$^+$ *i*NKT cells in HC and RA patients.

	D0 (n=13)	HC-D7 (n=8)	**P** HCD7 vs D0	All RA (n=13)	**P** All RA vs D0	RA-NP (n=5)	RA-P (n=8)	**P** RA-P vs RA-NP
*i*NKT frequency (%)	0.33	0.41	0.85	0.26	0.34	0.27	0.25	0.50
$CD4^+$ *i*NKT (%)	58	67	0.27	64	0.31	64	63	0.84
$CD8^+$ *i*NKT (%)	39	60	**0.04**	59	***0.02***	61	57	0.75
$CD69^+$ *i*NKT (%)	58	83	**0.013**	80	***0.0089***	80	80	0.95
$CD161^+$ *i*NKT (%)	58	59	0.66	57	0.73	55	58	0.70
$PD1^+$ *i*NKT (%)	76	68	0.28	67	0.15	68	66	0.82
GM of $CD69^+$ *i*NKT	4209	8109.87	0.17	9615	***0.037***	9524	9706	0.96

TABLE 13- SUMMARY OF *I*NKT AND ACTIVATION MARKER FREQUENCY

iNKT percentage and phenotype were assessed at baseline (day 0) and after 7 day culture with serum from patients with RA-P (8) and RA-NP (5) and healthy controls (8). T-tests were used with p= <0.05 as significant. D0= day 0; HC D7= healthy control day 7; RA-P= Rheumatoid Arthritis Plaque patient; RA-NP= Rheumatoid Arthritis non-plaque patient; All RA = RA-P and RA-NP.



A)

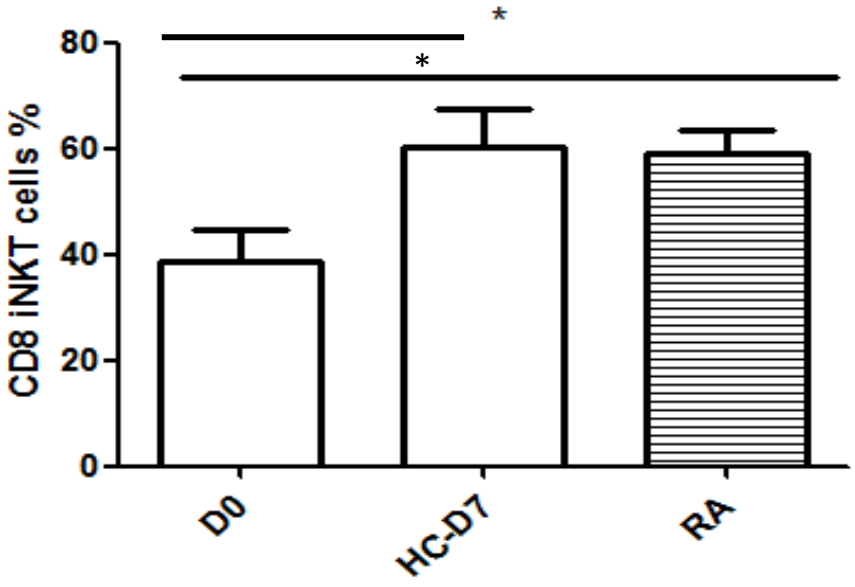
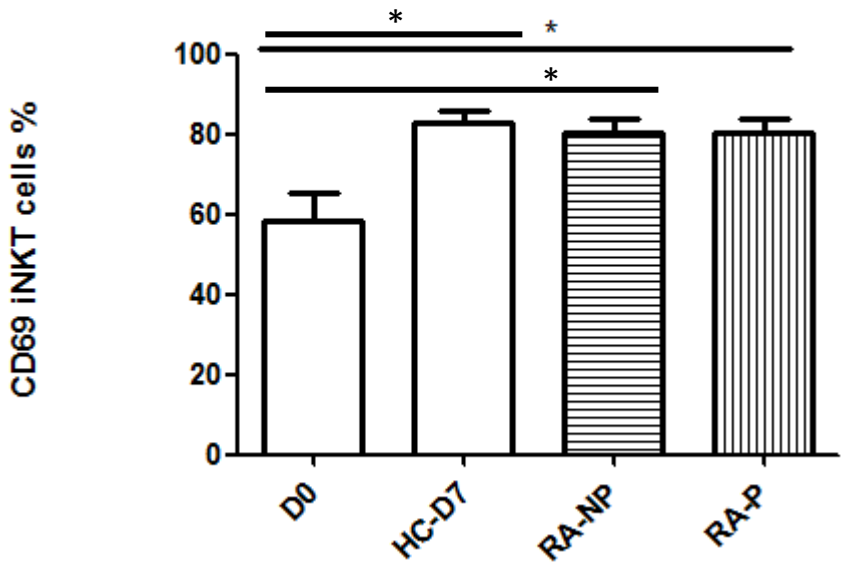
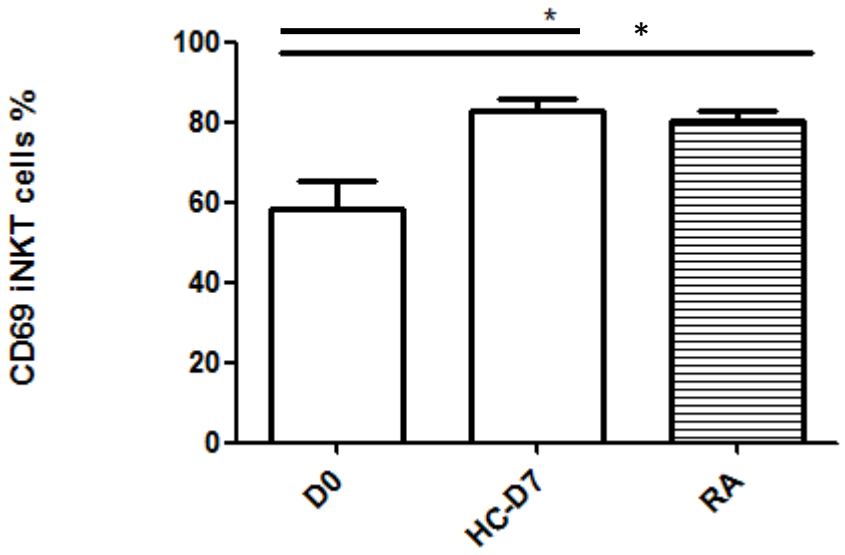


FIGURE 20- BAR CHARTS OF FREQUENCY OF CD8+ /NKT CELLS

The percentage of A) CD8+ *i*NKT cells assessed at baseline (day 0) and after 7 day culture with serum from patients with RA-P (n=8) and RA-NP (n=5) and healthy controls (n=8), tested twice with the same healthy PBMCs. B) Shows HC vs all RA (which includes RA-P and RA-NP). T-tests were used with p= <0.05 as significant. Mean ±SE. NS= not significant. D0= Day 0; HC D7= healthy control day 7; RA-P= Rheumatoid Arthritis Plaque patient; RA-NP= Rheumatoid Arthritis non-plaque patient. Blue line shows significance between D0 and RA- NP and RA-P patients.


CD69 iNKT cells %
100
80
60
40
20
0
*
*
*
D0
HC-D7
RA-NP
RA-P

A)


CD69 iNKT cells %
100
80
60
40
20
0
*
*
D0
HC-D7
RA

B)

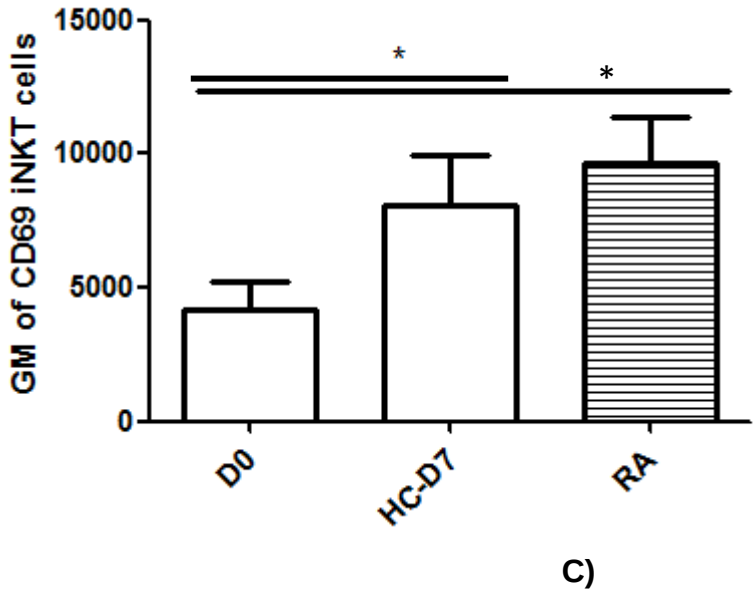
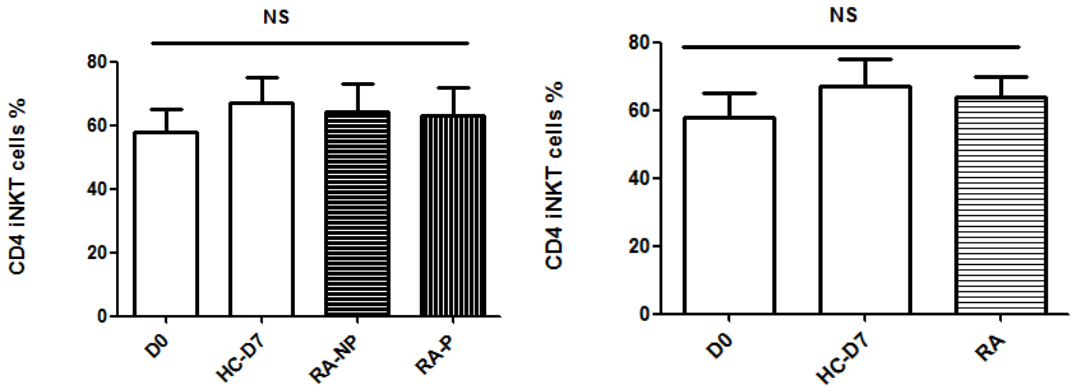
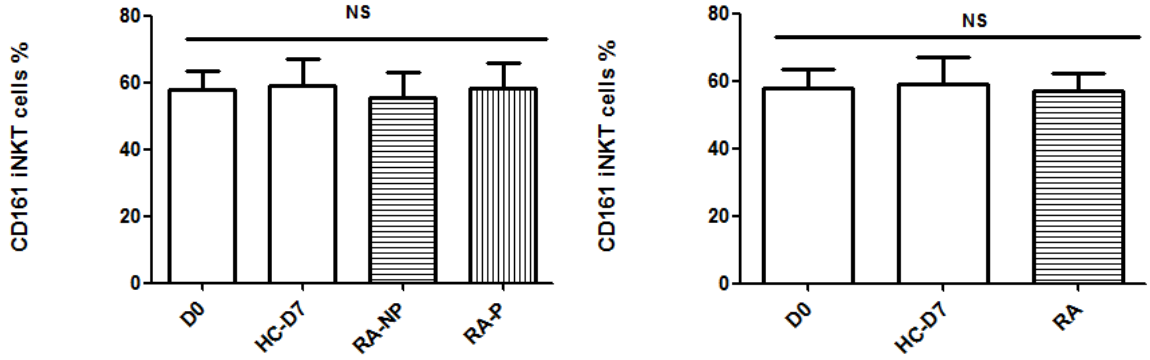


FIGURE 21- BAR CHARTS OF CD69+ INKT CELLS

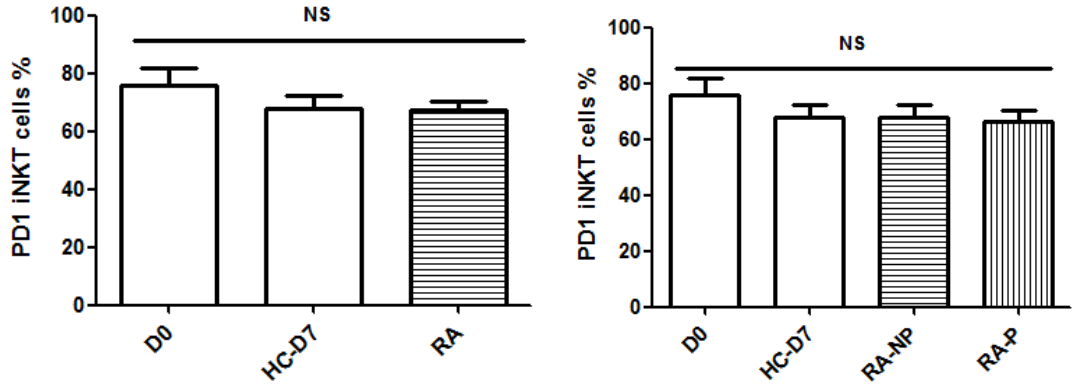
The percentage of A) $CD69^+$ iNKT cells assessed at baseline (day 0) and after 7 day culture with serum from patients with RA-P (n=8) and RA-NP (n=5) and healthy controls (n=8) tested twice with the same healthy PBMCs. B) Shows HC vs all RA (which includes RA-P and RA-NP). C) GM of $CD69^+$ iNKT in HC vs all RA. T-tests were used with p= <0.05 as significant. Mean ±SE. NS= not significant. D0= Day 0; HC D7= healthy control day 7; RA-P= Rheumatoid Arthritis Plaque patient; RA-NP= Rheumatoid Arthritis non-plaque patient; all RA= RA-P and RA-NP; GM=geometric mean. Blue line shows significance between D0 and RA- NP and RA-P patients.



A)



B)



C)

FIGURE 22- FREQUENCY OF CD4+, CD161+ AND PD1+ *i*NKT CELLS

The percentage of A) $CD4^+$ B) $CD161^+$ C) $PD1^+$ *i*NKT cells in HC vs RA-NP and RA-P and all RA assessed at baseline (day 0) and after 7 day culture with serum from patients with RA-P (n=8) and RA-NP (n=5) and non -autologous healthy controls (n=8), tested twice with the same healthy PBMCs. T-tests were used with p= <0.05 as significant. Mean ±SE. NS= not significant. D0= Day 0; HC D7= healthy control day 7; RA-P= Rheumatoid Arthritis Plaque patient; RA-NP= Rheumatoid Arthritis Non-Plaque patient; all RA= RA-P and RA-NP.

3.3.6 Discussion

The increased CVD risk in patients with RA or SLE cannot be fully explained by traditional risk factors. It is therefore believed that the immune system is also contributing to the increased CVD risk so that when either RA or SLE are active and causing inflammation, this also promotes atherosclerosis. Furthermore, RA is about 10 times more common than SLE so extending the previous research by my supervisor Professor Jury and her group looking at *i*NKT cells in SLE and atherosclerosis, I studied a group of RA patients. I matched RA patients with preclinical atherosclerosis to RA patients with no plaque (8 plaque and 5 patients with non-plaque) using serum samples from a scanned cohort obtained from a collaborator in Wales to investigate the role of *i*NKT cells in RA in patients with preclinical CVD. This is the first study of its kind as far as I am aware.

Smith et al (Smith, Croca et al. 2016), reported that SLE plaque patients (SLE-P), had an increased *i*NKT cell frequency compared to SLE non-plaque (SLE-NP). The *i*NKT cells from plaque patients were found to adopt an anti-inflammatory, anti-atherogenic phenotype and were able to induce polarisation of monocytes into the anti-inflammatory M2 phenotype. However, in patients with SLE who had already developed clinical cardiovascular disease (SLE-CVD), this protective phenotype was lost. The increased *i*NKT cell frequency in SLE-P compared to SLE-NP patients suggested that *i*NKT cell expansion could occur as a protective response during early plaque development (Smith, Croca et al. 2016).

My data did not show a statistically significant change in *i*NKT cell frequency in healthy PBMCs induced by RA serum from a seven -day culture. This is unlikely to be explained by the small numbers in my study as Smith et al reported a significantly increased *i*NKT cell frequency in SLE-P patients vs HC and SLE-NP with 5 patients in each group, in a similar experimental method to mine measuring *i*NKT cell response to serum (Smith, Croca et al. 2016).

Other studies which have indicated that peripheral *i*NKT frequency is reduced in RA patients (Kojo, Adachi et al. 2001, van der Vliet, von Blomberg et al. 2001, Linsen, Thewissen et al. 2005). Although these conclusions were based in these studies on the analysis of as few as 100, 000 cells per sample. Since the *i*NKT cell population in peripheral blood is relatively rare- reported as 0.05-0.1% of the peripheral mononuclear cells, accurate analysis can only be derived by analysing a sufficient number of cells (Berzins, Smyth et al. 2011). However, reassuringly, a study by Tudhope S et al (Tudhope, von Delwig et al. 2010), also reported the same finding and appropriately analysed at least 500 000 lymphocyte gated events, and I used the latter number as a minimum number in my experimental work. Furthermore, there are many studies in the literature, which corroborate reduced *i*NKT cell frequency in the context of autoimmune disease e.g. type 1 diabetes, multiple sclerosis, SLE, Sjogren's and others (Swann, Coquet et al. 2007, Balato, Unutmaz et al. 2009, Berzins, Smyth et al. 2011). In SLE, various methods have been used to confirm this association of reduced *i*NKT cell frequency for example by quantification of Vα24Jα18 mRNA expression (iTCR receptor) which showed reduced numbers of *i*NKT cells in both the peripheral blood and synovium of SLE patients compared to healthy controls (Sumida 1998). In addition, studies using flow cytometry have found

a reduction in both the proportions and absolute numbers of *i*NKT cells and *i*NKT cell subpopulations in SLE patients compared to healthy controls (Takahashi, Chiba et al. 2002, Mitsuo, Morimoto et al. 2006, Cho, Kee et al. 2011).

My data for this RA group did not show any significant difference in *i*NKT expression between RA-P and RA-NP groups and did not suggest an altered phenotype in terms of a shift towards $CD4^+$. $CD4^+$ *i*NKT cells have also been previously been shown to be more atherogenic in mice (To, Agrotis et al. 2009). Smith et al examined found that $CD4^+$ *i*NKT cell subsets were elevated in SLE-P patients and $CD8^+$ *i*NKT cells were increased in the SLE-CV patients compared to healthy controls. However, their experiments involved testing *i*NKT cell marker expression from peripheral blood and therefore cannot be directly compared to my data. Adoptive transfer of $CD4^+$ NKT cells into T and B cell-deficient mice in a study by Li et al. confirmed that NKT cells directly contribute to atherogenesis (Li, To et al. 2015). They found that the mechanisms employed by $CD4^+$ NKT cells in atherosclerosis were perforin and granzyme-B dependent, and contributed to multiple characteristics of atherosclerosis including necrotic core formation and increased VCAM-1 expression (Li, To et al. 2015).

My findings, of a significantly increased percentage of $CD8^+$ *i*NKT cells and CD69 ($p=0.0089$) in all RA patients ($p=0.02$) was most likely to be an effect of time in culture, rather than due to RA, as the values for HC D7 and RA D7 are almost the same (table 14).

There are limited data on $CD8^+$ *i*NKT cells, although it is thought that these cells might play a similar role to $CD8^+$ T cells through their ability to mediate cytotoxic functions (Wen, Kim et al. 2015). In addition, these cells produce IFN-γ but not IL-4 upon antigenic stimulation in mice (Lee, Hong et al. 2009, Lee, Hong et al. 2009).

Smith et al showed that the expression of CD69, an early activation marker, was raised in iNKT cells from SLE-P patients, but not SLE-NP or SLE-CV patients compared to healthy donors. The role of CD69 in atherosclerosis remains unknown; whilst CD69 knockout mice are protected from atherosclerosis (Gómez, Sanz-González et al. 2009), enhanced expression of CD69 has been observed within unstable carotid plaque specimens in humans (Rohm, Atiskova et al. 2015). The mechanisms for these conflicting results are not clear. Similar conflicting observations have been seen in SLE where CD69 expression is associated with increased IFN-γ production by NK cells (Hervier, Beziat et al. 2011), whilst CD69 expression on $CD8^+$ T cells was found to induce apoptosis of other immune cell types (Rus, Zernetkina et al. 2005).

Although limited, there are some data from humans looking at atherosclerosis and iNKT cell expression. The presence of CD1d expression within human atherosclerotic lesions indicates a possible role for *i*NKT cells in plaque progression (Melián, Geng et al. 1999). I did not measure *i*NKT cells directly and there may for my results not represent a true and accurate reflection of *i*NKT frequency. Studies investigating atherosclerosis in humans have observed that *i*NKT cells within the plaque are abundant and comprise up to 3% of total infiltrating T cells, yet are scarce in the peripheral blood of patients who have had a cardiovascular event (Kyriakakis, Cavallari et al. 2010, Liu, Lǔ et al. 2011). This observation emphasises the importance of assessing *i*NKT numbers longitudinally over disease course may provide more accurate and meaningful data.

The other possible reasons for the difference in my findings in subclinical RA compared to SLE could be explained by the differences in the populations studied. My RA population compared to that of Smith et al (Smith, Croca et al. 2016) SLE group were older (median age 63 vs 55 in the SLE group), had more men (15% vs 3% in SLE) and were all Caucasian. Age has been shown to effect *i*NKT cell frequency (with reduced expression over time) (Berzins, Smyth et al. 2011). Differences could also be explained by the different cytokine milieu (interferon alpha predominantly in SLE compared to TNF alpha in RA) in both diseases and the fact that SLE is more often associated with dyslipidemias. Lipids are key antigens that activate *i*NKT cells. It may also be that different lipid subclasses are important in activating *i*NKT frequency cells in different diseases- in my study of RA patients serum triglycerides were close to showing a positive correlation with *i*NKT frequency.

A limitation of my study is that *i*NKT cells frequency was not directly measured from patients' blood, as I was not able to obtain blood samples to isolate PBMCs from our collaborators. I had therefore to infer that factors from the serum in patients could affect *i*NKT cell expression. In the SLE cohort (Smith et al) PBMCs were directly isolated from patients and therefore more accurately reflect what is going on in vitro. Furthermore, studies in mice indicated that characteristic NKT cells from the blood cannot be directly extrapolated to the entire *i*NKT cell pool- further analysis of *i*NKT cells from blood, thymus, spleen and liver of individual human donors are required (Berzins, Smyth et al. 2011).

To conclude, I have shown from my preliminary study that *i*NKT expression in RA patients with subclinical CVD differs from that in SLE patients. I was not able to identify

a reduced expression of *i*NKT cells in RA, as reported in the literature or identify a difference between RA-P and RA-NP. To understand whether there are differences between *i*NKT expressions in preclinical atherosclerosis in RA, further studies from multi centres assessing different stages of atherosclerosis are required.

3.4 Metabolomics

3.4.1 Healthy Controls vs SLE

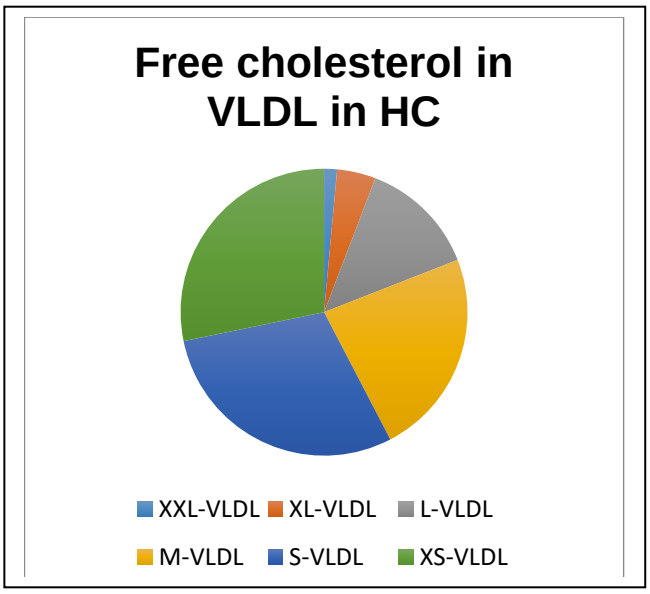
Table 14 shows the top 20 most significant values ($p<0.05$) of lipid metabolites whose levels differed in this study for healthy controls compared to patients with SLE. These remained significant at FDR 1%. Sixty percent of these metabolites (12/20) arose from the VLDL subclass and various different sizes of VLDL were represented ranging from small to very large. For 10 of these 12 VLDL metabolites, the values were higher in patients with SLE than HC. The two exceptions were both related to triglycerides (percentage of triglyceride in very large and medium VLDL were lower in SLE than HC). Figure 23 shows the pattern of distribution of free cholesterol, esterified cholesterol and triglycerides across the different sizes of VLDL, comparing patients with SLE and HC. In particular, there is a shift of triglycerides and free cholesterol (but not esterified cholesterol) into the smaller VLDL in patients with SLE.

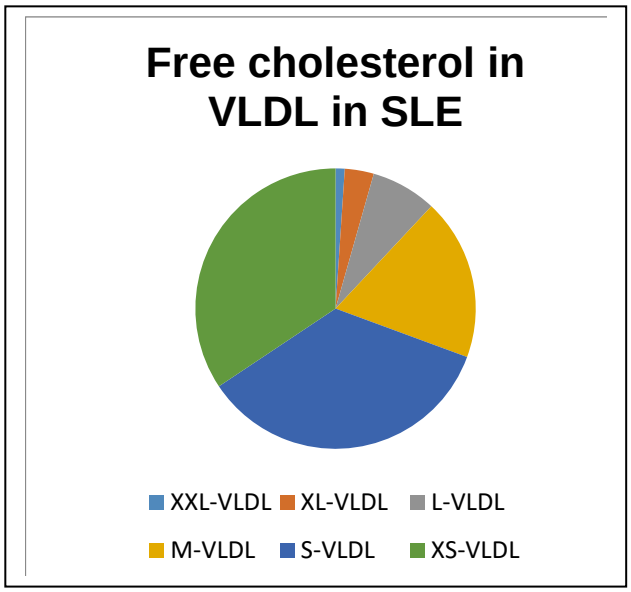
The findings were different for HDL. Six HDL metabolites differed between SLE and HC, but all of them belonged to a single size (M-HDL). In distinction to findings for VLDL, values were higher in HC compared to SLE for 5/6 M-HDL metabolites. Again, the single exception was related to triglyceride with percentage of triglycerides in M-HDL being higher in SLE compared to HC. Figure 24 shows the lipid content of M-HDL was lower for phospholipids, esterified and free cholesterol in patients with SLE than in HC.

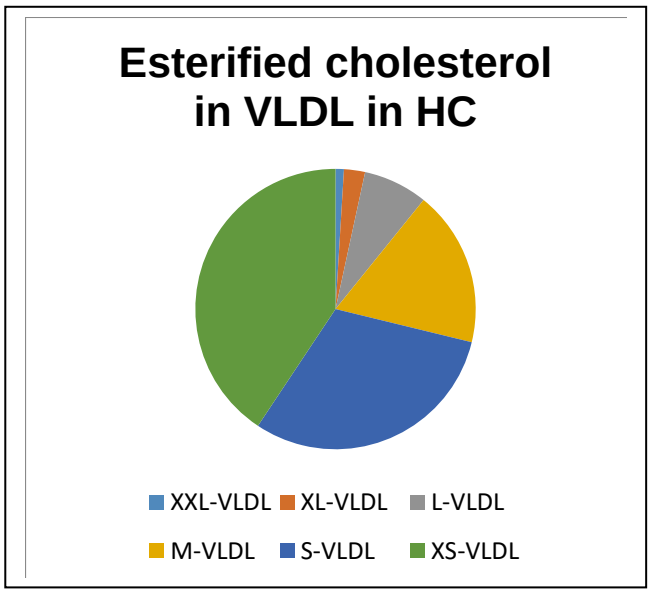
Metabolite	HC Vs SLE (p-value)	HC Mean mmol/l	SLE Mean mmol/l
Cholesterol esters to total lipids ratio in large VLDL*	2.04E-11	13.70	24.28
Total cholesterol to total lipids ratio in very large VLDL *	2.11E-09	24.19	35.78
Total cholesterol to total lipids ratio in medium HDL	3.20E-09	47.08	35.22
Total cholesterol to total lipids ratio in large VLDL *	2.44E-08	24.92	34.96
Acetate	1.30E-07	0.04	0.072
Triglycerides to total lipids ratio in very large VLDL *	2.74E-07	59.76	48.06
Cholesterol esters to total lipids ratio in medium HDL	3.98E-07	38.27	29.07
Free cholesterol to total lipids ratio in medium HDL	5.48E-07	8.81	6.14
Cholesterol esters to total lipids ratio in medium VLDL *	1.71E-06	17.4	23.64
Glutamine	9.04E-06	0.54	0.34
Cholesterol esters to total lipids ratio in small VLDL *	2.54E-05	26.84	32.263
Cholesterol esters to total lipids ratio in chylomicrons and extremely large VLDL *	4.01E-05	9.33	13.74
Total cholesterol to total lipids ratio in small VLDL *	9.67E-05	41.09	46.15
Cholesterol esters in medium HDL	0.00012	0.34	0.15
Total cholesterol to total lipids ratio in medium VLDL *	0.00015	29.18	34.82
Total cholesterol in medium HDL	0.00019	0.42	0.18
Cholesterol esters to total lipids ratio in very large VLDL *	0.00023	13.22	20.72
Free cholesterol to total lipids ratio in very large VLDL *	0.00025	10.98	15.06
Triglycerides to total lipids ratio in medium HDL	0.00026	5.29	13.25
Triglycerides to total lipids ratio in medium VLDL *	0.00029	50.92	44.31

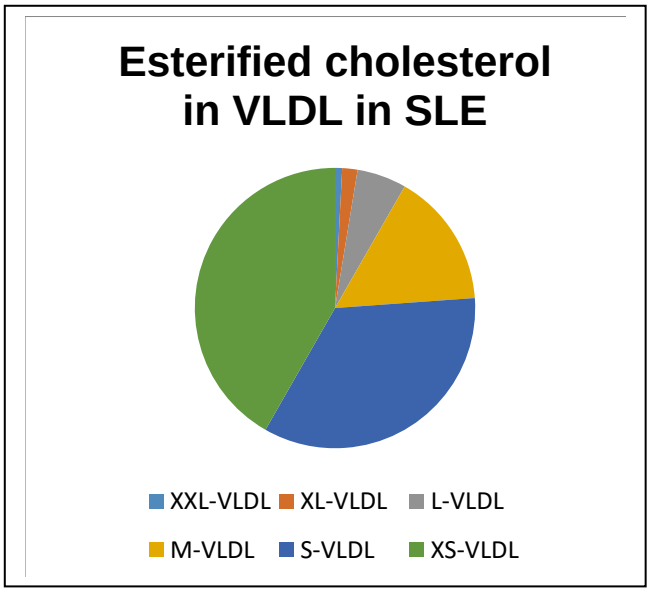
TABLE 14- ***THE TOP 20 METABOLITES SHOWING THE MOST SIGNIFICANT DIFFERENCES IN LEVEL BETWEEN PATIENTS WITH SLE (N=86) AND HC (N=10)***

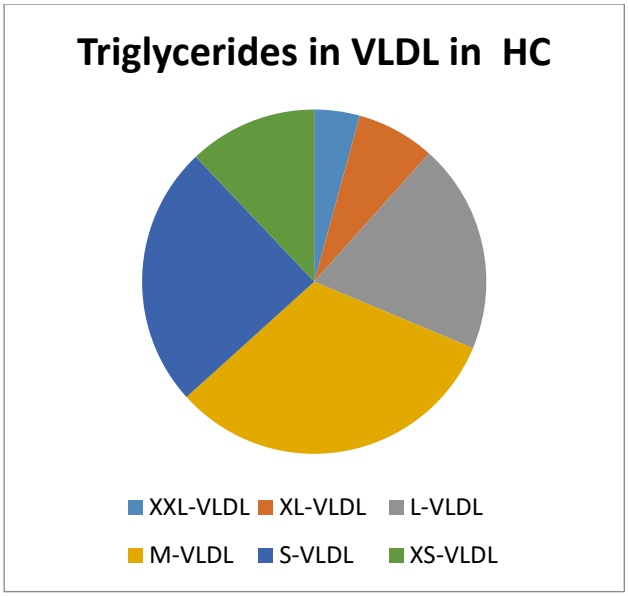
Values for the VLDL subclasses were predominantly higher in SLE compared with HC. Conversely to findings for VLDL, values were higher in HC compared to SLE for 5/6 M-HDL metabolites. They remained significant at FDR of 1%.


Free cholesterol in VLDL in HC
XXL-VLDL
XL-VLDL
L-VLDL
M-VLDL
S-VLDL
XS-VLDL


Free cholesterol in VLDL in SLE
XXL-VLDL
XL-VLDL
L-VLDL
M-VLDL
S-VLDL
XS-VLDL


Esterified cholesterol in VLDL in HC
XXL-VLDL
XL-VLDL
L-VLDL
M-VLDL
S-VLDL
XS-VLDL


Esterified cholesterol in VLDL in SLE
XXL-VLDL
XL-VLDL
L-VLDL
M-VLDL
S-VLDL
XS-VLDL



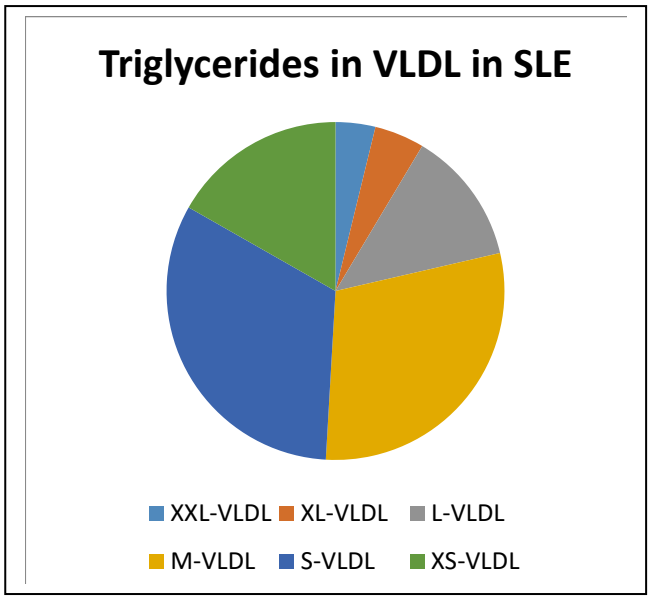


FIGURE 23- THE DISTRIBUTION OF FREE CHOLESTEROL, ESTERIFIED CHOLESTEROL AND TRIGLYCERIDES ACROSS THE DIFFERENT SIZES OF VLDL, COMPARING PATIENTS WITH SLE AND HC.

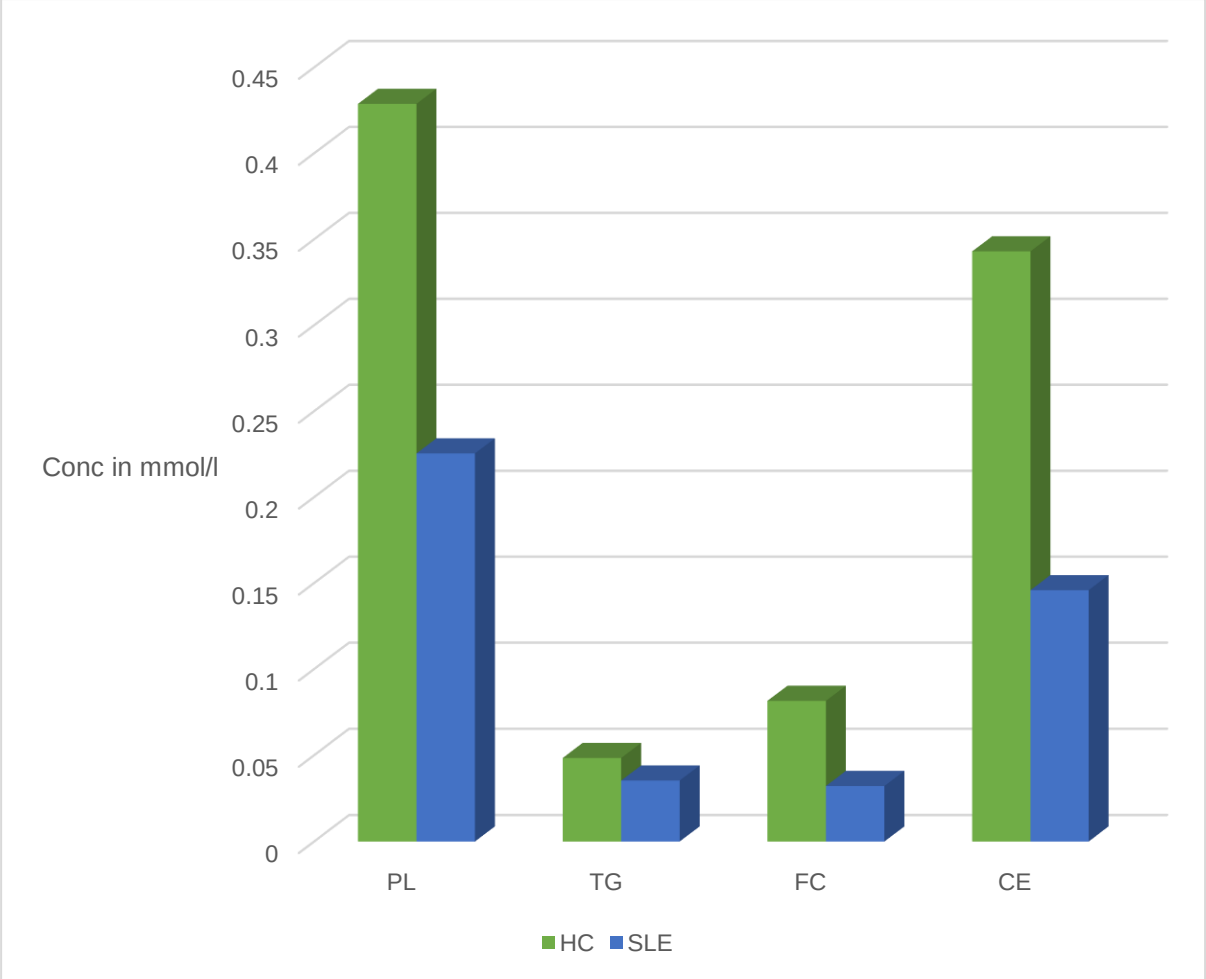


FIGURE 24- DISTRIBUTION OF LIPIDS IN MEDIUM HDL IN HC COMPARED TO SLE

The bar chart shows reduced lipids in medium HDL in patients with SLE compared to HC. Statistical analysis with T-tests: PL-p=0.0069*, TG- p=0.08, FC- p=0.0011*, CE-p=0.00012*. P=PL=phospholipids, TG=triglycerides; FC=Free Cholesterol and CE=cholesterol esters

3.4.2 Comparison of SLE-P and SLE-NP patients

Comparison of clinical, serological and treatment variables between the plaque (N=33) and non-plaque patients (N=53) is shown in table . Significant differences are seen in age ($p<0.001$), disease duration ($p=0.003$) and gender ($p=0.025$) between SLE-P vs SLE-NP, with SLE-P patients being older and having a longer disease duration at the time of the scan. Mean Cholesterol/HDL ratio and triglycerides were higher in SLE-P vs SLE-NP which were significant ($p=0.006$ and $p=0.002$ respectively).

Table 16 shows the top hits for metabolites that were significantly different between the SLE-P and SLE-NP groups. Several differences were observed between SLE-NP and SLE-P patients with a significance of $p<0.05$, although none of the metabolites reached significance according to the FDR correction.

Of 20 metabolites in this table, 9 related to VLDL, whereas (in contrast to the SLE versus HC comparison) none related to HDL. The SLE-P group had significantly higher concentrations of 6/9 VLDL subclasses compared with the SLE-NP group. This is consistent with previous findings showing that patients in the SLE-P group had consistently higher mean concentrations of all forms of VLDL compared with SLE-NP patients, which was not observed for other lipoprotein classes including IDL, LDL and HDL particles (Smith, Croca et al. 2016).

	SLE-P	SLE-NP	P-value
Total number of patients	33	53	
Demographics			
Gender (%)	30F:3M	52F:1M	**0.025**
Female	30 (91)	52 (98)	
Male	3 (9)	1 (2)	
Mean Disease duration at scan years (SD)	20 (11.0)	13 (7.07)	**0.03**
Mean Age at 1st scan (SD)	53 (9.3)	40 (11)	**<0.001**
Ethnicity (%)			
Caucasian	20 (61)	27 (51)	0.707
Afro-Caribbean	8 (24)	15 (28)	
Asian	2 (6)	6 (11)	
Other	3 (9)	5 (10)	
Current Smokers at time of scan (%)	6 (18)	5 (9)	0.154
History of Diabetes (%)	1(3)	0	0.400
History of hypertension (%)	6 (18)	8 (15)	0.20
Blood Pressure (mmHg) scan day			
Mean Systolic (SD)	132 (15)	124 (14)	0.006
Mean Diastolic (SD)	76 (11)	76 (9.5)	0.774
Treatment regimen at scan			
Statins (%)	5 (15)	6 (11)	0.605
ACE inhibitors (%)	11(33)	21 (39)	0.557

Aspirin (%)	5 (15)	5 (10)	0.421
Hydroxychloroquine (%)	21 (64)	35 (66)	0.820
Mean Prednisolone dose at scan (mg)	5	5	0.290
Immunosuppressant use (%)	13 (39)	23 (43)	0.719
B-cell depletion (%)	10 (30)	18 (34)	0.725
Bloods on day of scan			
Median anti-dsDNA antibodies IU/L (IQR)	13 (111)	33 (87)	0.404
Mean C3 g/L (SD)	1.03 (0.22)	0.99 (0.25)	0.640
Mean ESR mm/h (SD)	24 (23)	19 (15)	0.245
Mean CRP mg/d (SD)	3 (4)	2 (2)	0.091
Lipid profile (mmol/L) day of scan			
Mean Total Cholesterol (SD)	5.1 (0.94)	4.7 (1.09)	0.079
Mean Triglycerides (SD)	1.32 (0.51)	0.9 (0.42)	**0.002**
Mean HDL (SD)	1.55 (0.57)	1.65 (0.52)	0.266
Mean LDL (SD)	2.87 (0.81)	2.6 (0.89)	0.05
Mean Cholesterol/HDL ratio	3.42 (0.91)	2.85 (0.78)	**0.006**
Historical serological profile (ever positive) (%)			
Anti-cardiolipin abs positive	6 (18)	4 (8)	0.122
Anti-B2 antibodies positive	1 (3)	2 (4)	0.878
Lupus anticoagulant positive	**6 (18)**	**3 (6)**	**0.04**
Mean BILAG score at scan (standard deviation)	4 (6.51)	5 (10-38)	0.387

TABLE 15 - ***CLINICAL AND DEMOGRAPHIC FEATURES (FROM THE TIME OF THE 1ST SCAN)***

SLE (n=86) patients fulfilling the revised ACR classification for SLE from the University College Hospital London lupus cohort who were scanned to determine the presence of plaque (n=33) and who were assessed by metabolomics. SLE P= SLE plaque patients; SLE-NP =SLE non-plaque patients.

Metabolite	SLE NP vs P p-value <0.05	SLE-NP mean mmol/l	SLE-P mean mmol/l
Free cholesterol to total lipids ratio in medium VLDL *	0.0011	10.89	11.64
Free cholesterol to total lipids ratio in large VLDL *	0.0023	9.89	11.97
Glycine	0.0027	0.30	0.27
Phospholipids to total lipids ratio in chylomicrons and extremely large VLDL *	0.0040	9.35	10.86
Triglycerides to total lipids ratio in very small VLDL*	0.010	17.29	19.63
Mean diameter for LDL particles	0.013	24.06	23.89
Ratio of omega-6 fatty acids to total fatty acids	0.016	33.64	32.11
Triglycerides to total lipids ratio in IDL	0.017	10.41	12.38
Ratio of triglycerides to phosphoglycerides	0.02	0.51	0.62
Ratio of 22:6 docosahexaenoic acid to total fatty acids	0.021	1.23	1.45
Ratio of monounsaturated fatty acids to total fatty acids	0.031	22.87	24.36
Triglycerides to total lipids ratio in large LDL	0.032	9.50	11.34
Cholesterol esters to total lipids ratio in IDL	0.034	46.71	44.40
Cholesterol esters to total lipids ratio in very small VLDL	0.036	43.69	41.19
Glycoprotein acetyls, mainly a1-acid glycoprotein	0.039	1.42	1.53
Total cholesterol to total lipids ratio in IDL	0.04	63.69	60.98
Triglycerides in very small VLDL *	0.04	0.09	0.11
Phospholipids in chylomicrons and extremely large VLDL *	0.04	0.0031	0.004
Cholesterol esters to total lipids ratio in large VLDL	0.05	25.54	22.24
Total cholesterol to total lipids ratio in very small VLDL	0.05	58.24	55.65

TABLE 16- THE TOP 20 METABOLITES SHOWING THE MOST SIGNIFICANT DIFFERENCES IN LEVEL BETWEEN SLE-P AND SLE-NP PATIENTS

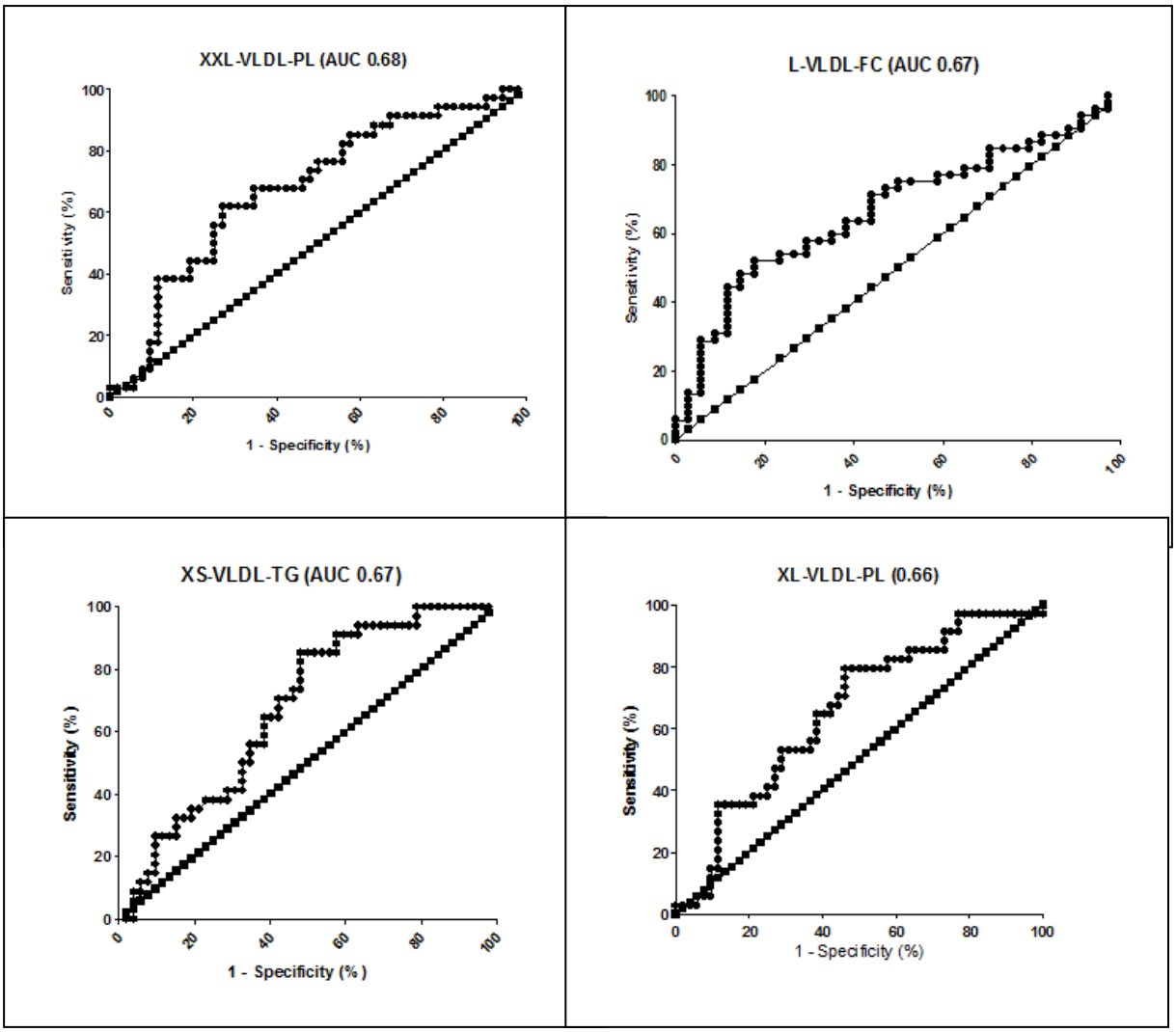
Significant differences were observed in VLDL subclasses (9/20).

Receiver operator curve (ROC) analysis

ROC analysis is a method for showing the value of an individual test for distinguishing two groups, in this case SLE-P and SLE-NP. The curve plots sensitivity against (1-specificity) for each cut-off value of the test. The area under the curve (AUC) is a measure of how good the test is at distinguishing the groups. An AUC of 0.5 (represented by the x=y line in the centre of each graph) would be a test that did not distinguish the groups better than chance alone whereas an AUC of 1.0 would be a test with perfect sensitivity and specificity.

I carried out further analyses of these data by selecting the lipoprotein metabolites which had significant p values <0.05 and analysed these metabolites with receiver operator curves (ROC). The highest AUC values for VLDL metabolites were XXL-VLDL-PL (0.68), L-VLDL-FC (0.67), XS-VLDL-TG (0.67), XL-VLDL-PL (0.66) and M-VLDL-FC (0.65). These five ROC curves are shown in figure 25.

I then combined the metabolites with the highest sensitivity and highest specificity to design a composite test such that a patient is positive if XXL-VLDL-PL > 0.0030 mmol/l and M-VLDL-FC > 0.04 mmol/l. This composite test distinguished SLE-P from SLE-NP with sensitivity 64%, specificity 79%, positive predictive value 65% and negative predictive value 76%.


XXL-VLDL-PL (AUC 0.68)
Sensitivity (%)
1 - Specificity (%)
L-VLDL-FC (AUC 0.67)
Sensitivity (%)
1 - Specificity (%)
XS-VLDL-TG (AUC 0.67)
Sensitivity (%)
1 - Specificity (%)
XL-VLDL-PL (0.66)
Sensitivity (%)
1 - Specificity (%)

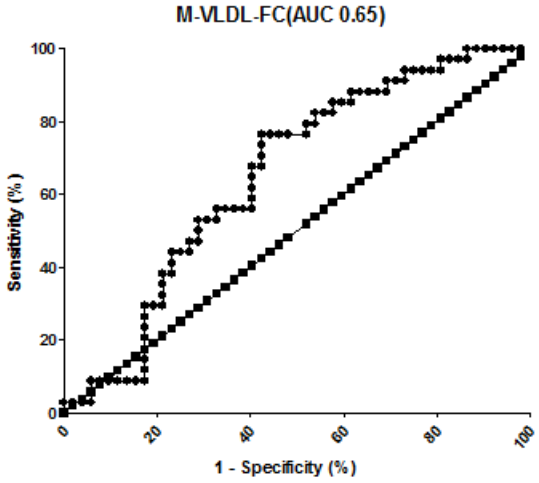

M-VLDL-FC(AUC 0.65)
Sensitivity (%)
1 - Specificity (%)

FIGURE 25 -ROC CURVES GENERATED FROM THE METABOLITES WHICH HAD SIGNIFICANT P VALUES <0.05.

The top five AUC were for VLDL metabolites, which were XXL-VLDL-PL (0.68), L-VLDL-FC (0.67), XS-VLDL-TG (0.67), XL-VLDL-PL (0.66) and M-VLDL-FC (0.65).

3.4.3 RA Cohort

RA vs Healthy Controls

Table 17 shows the top 15 hits for metabolites which were significantly different between the RA- and HC groups ($p<0.05$) and remained significant at FDR of 1% correction. Nine out of the fifteen were in the HDL particle (5/9 in small HDL and 4/9 in very large HDL), three in IDL and three in very small VLDL subclasses. In general, mean levels of the metabolites were higher in HC vs RA patients with the exception of 4 subclasses (cholesterol esters to total lipids ratio in small HDL, total cholesterol to total lipids ratio in small HDL, cholesterol esters in small HDL and phospholipids to total lipids ratio in IDL). All of these subclasses had a form of cholesterol group in the subclass (except for phospholipids to total lipids ratio in small HDL and phospholipids to total lipids ratio in IDL),

RA-P vs RA-NP

Analysis of the RA metabolomics using t-test analysis did not show any significant lipid markers to differentiate RA P vs RA NP ($p>0.05$) and therefore was not tested using FDR.

.

Metabolite	HC Vs RA (p-value)	HC mean (mmol/l)	RA Mean (mmol/l)
Mean diameter for HDL particles	0.0086	10.21	9.81
Free cholesterol to total lipids ratio in small HDL	0.0022	11.76	10.10
Cholesterol esters to total lipids ratio in small HDL	**0.00013**	***19.67***	***31.53***
Total cholesterol to total lipids ratio in small HDL	***8.59E-5***	***31.43***	***41.68***
Phospholipids to total lipids ratio in small HDL	8.34E-5	63.40	53.09
Cholesterol esters in small HDL	**4.16E-5**	***0.19***	***0.33***
Free cholesterol in very large HDL	0.0028	0.087	0.045
Cholesterol esters in very large HDL	0.00017	0.27	0.15
Total cholesterol in very large HDL	0.00039	0.36	0.20
Cholesterol esters to total lipids ratio in IDL	4.52E-5	46.75	46.92
Total cholesterol to total lipids ratio in IDL	0.00023	63.77	60.37
Phospholipids to total lipids ratio in IDL	**1.37E-5**	**26.03**	**27.50**
Cholesterol esters to total lipids ratio in very small VLDL	2.86E-6	40.11	32.33
Total cholesterol to total lipids ratio in very small VLDL	1.64E-6	55.33	47.96
Cholesterol esters in very small VLDL	0.0058	0.23	0.16

TABLE 17- THE TOP 15 METABOLITES SHOWING THE MOST SIGNIFICANT DIFFERENCES IN LEVEL BETWEEN PATIENTS WITH RA (13) AND HC (N=10) WHICH WERE ALSO SIGNIFICANT AT FDR<1%.

HC: healthy control, RA: Rheumatoid Arthritis

3.4.4 **Discussion**

I carried out a metabolomics analysis of samples from 86 patients with SLE and 13 patients with RA focusing particularly on lipoprotein profiles. I found significant differences in various sizes of VLDL and in one size of HDL between patients with SLE and HC. Since all the patients had undergone detailed carotid and femoral ultrasound scans, I was able to compare those with and without atherosclerotic plaque. I found a number of VLDL metabolites that differed significantly between the SLE-NP and SLE-P groups and showed how they could be combined, to make a putative test to distinguish these groups with sensitivity of 64% and specificity of 79%.

Lauridsen et al investigated the metabolomics profiles of RA patients using 1H-NMR spectra of human plasma samples (Lauridsen, 2010). They confirmed the inflammatory state of RA, with higher lactate, acetylated glycoproteins similar to findings reported in RA synovial fluid, probably as a consequence of the increase of anaerobic metabolism in the inflamed joints. In addition, elevated concentrations of cholesterol and low levels of HDL were found in RA patients compared to controls, consistent with previous studies (Dursunoğlu, Evrengül et al. 2005, Georgiadis, Papavasiliou et al. 2006), and together such a lipid profile may help to explain the increased risk of CVD in RA patients. In my cohort, 60% of the top 15 significant metabolites were in the HDL subclasses and generally mean levels of the metabolites were higher in HC vs RA patients.

I was unable to find any significant differences between lipoprotein profiles and RA-P and RA-NP groups. This could be attributed to the fact that I had a very small sample of patients (only 5 plaque patients in the RA group compared to 33 in the SLE group). The RA cohort were also older (SLE-P 53 and SLE NP 40 vs RA-P 64 and RA-NP 62) and had more males (RA 15% vs SLE 5%) compared to the SLE cohort. In addition, all my patients in the RA cohort were on established treatment (mainly DMARDs) for many years. In the Lauridsen et al study, they reported that the metabolomics profiles of RA patients with active disease approached the profile of patients in remission after starting therapy (Lauridsen, Bliddal et al. 2010).

In a study by Freedman *et al* in men (Freedman, Otvos et al. 1998), the presence of either high levels of large VLDL particles or small HDL measured by NMR spectroscopy increased risk of extensive CAD by 3-4 times, whereas elevated levels of both increased the risk by 15 times (Freedman, Otvos et al. 1998). Blake and colleagues used NMR Spectroscopy to compare baseline LDL profiles between 130 women (mean age 60 years) from the Women's Health Study who developed CVD within a mean of three years from baseline and 130 age-matched controls. LDL particle size was lower and median baseline levels of LDL were higher among women who developed CVD events compared to those who did not (Blake, Otvos et al. 2002). Wurtz and colleagues (Wurtz, Soininen et al. 2011, Wurtz, Raiko et al. 2012) have studied a large population of young Finns, who underwent metabolomics analysis in 2001 and carotid ultrasound in 2001 and 2007. Thus, they were able to identify metabolites that predicted development of atherosclerosis over a six-year period. These included low M-HDL, high LDL-cholesterol, high tyrosine and low docosahexaenoic acid (Wurtz, Raiko et al. 2012), Using self-organising map analysis on the same dataset these authors were able to identify 6 different metabolic

phenotypes of which three were associated with high carotid intima-media thickness (Wurtz, Soininen et al. 2011). One of these three (designated phenotype A) included high VLDL and low HDL levels, similar to the phenotype identified previously in SLE – though it should be noted that phenotype A of Wurtz *et al* comprised 74% men whereas 90% of patients with SLE are women (Wurtz, Soininen et al. 2011).

There have been relatively few metabolomics studies in patients with SLE and cardiovascular risk. In a recent study, Robinson et al studied serum metabolomics using NMR in 31 Juvenile SLE (JSLE) patients to try to identify patients with the highest CVD risk (Robinson, George et al. 2018). They identified three groups based on lipoprotein concentrations and diameters –Group-1 (reduced serum HDL and elevated VLDL/IDL/LDL particles); Group2 (elevated HDL and reduced VLDL/IDL/LDL particles) and; Group-3 (low serum levels of HDL and VLDL/LDL/IDL particles). Further analysis showed that Group-1 had a significantly elevated ApoB: ApoA1 ratio compared to Groups-2 and 3 ($p=<0.0001$). Thus, the findings of this paper suggests that SLE patients could be stratified for CVD risk with the use of serum metabolomics analysis.

Wu et al used mass spectroscopy rather than NMR to compare 20 patients with SLE and 9 healthy controls (Wu, Xie et al. 2012). They identified >100 metabolites that differed between the groups, but all were individual molecules rather than lipoprotein particles. Their cohort included only one patient with cardiovascular disease(Wu, Xie et al. 2012). Using NMR, Ouyong *et al* showed that the metabolomes of 64 patients with SLE, 30 with rheumatoid arthritis and 34 healthy controls could be distinguished and noted raised VLDL and LDL and reduced HDL in patients with SLE (Ouyang, Dai

et al. 2011). However, they did not report on subclasses of lipoproteins and neither Wu nor Ouyong had information on vascular scanning of their patients. Guleria *et al* showed that metabolomics could be used to compare different clinical subgroups within a cohort of lupus patients, as I have done here for SLE-P versus SLE-NP patients. Using NMR they compared the metabolomes of 22 patients with lupus nephritis, 40 with lupus but no nephritis and 30 healthy controls. Interestingly they found lower VLDL and LDL in patients with SLE than controls, though higher in the nephritis patients. The study was done in India so ethnicity may have played a role in these results, which are out of keeping with other cohorts.

My results are in keeping with most previous data showing that VLDL and HDL profiles differ in patients with SLE compared to healthy people. I have also shown that the VLDL (but not HDL) profile may be important in determining which patients with SLE go on to develop atherosclerosis and this links to previous data showing that VLDL from SLE-P patients could influence the phenotype of NKT cells and monocytes (Smith, Croca et al. 2016).

Although HDL have generally been felt to have a protective function against CVD (Rosenson, Brewer et al. 2012, Chei, Yamagishi et al. 2013) several authors have stressed that there are different subclasses of HDL which may serve different functions. As well as the demonstration of pro-inflammatory HDL by McMahon *et a l* (McMahon, Grossman et al. 2006), a large Japanese population study compared 241 patients who had suffered strokes with age and sex-matched controls and showed that small and medium HDL were associated with protection against stroke but large HDL were not (Chei, Yamagishi et al. 2013). In this study, I showed that differences

between patients with SLE and HC were confined to the M-HDL subclass, which appears to carry much less lipid in patients with SLE.

A limitation of my study was the small number of healthy controls for the SLE cohort. Future studies with larger healthy controls that are age/sex and ethnicity match may help tease further differences.

From my analysis, I have identified five individual VLDL metabolites whose levels could be measured to distinguish SLE-P from SLE-NP with AUC of 0.65 to 0.68 on ROC analysis. For comparison AUC for rheumatoid factor and anti-cyclic citrullinated peptide positivity in rheumatoid arthritis were 0.6 and 0.68 respectively in one study (Chen, Li et al. 2013) and both those tests are in common clinical use. However, it will be important to validate my results in a separate cohort of patients. A composite score of metabolomics with conventional risk factors may be the best way to assess CVD risk in patients with SLE, as in the type of extended risk model proposed by Wurtz *et al* (Wurtz, Raiko et al. 2012).

3.5 Vascular Ultrasound

3.5.1 Lost to follow up

From the original cohort of 100 patients who had base line scans, 69 patients were contactable and agreed to a follow up scan. Thirty-one patients did not participate in the follow up study. The reasons why these 31 patients were lost to follow-up are summarised in table 18.

A comparison of baseline characteristics between patients who were re-scanned and those who were lost to follow up is shown in table 19. Those who did not return for scans did not differ significantly with respect to SLE features, therapy exposures or CVD risk factors when compared to those who were followed up.

Reason	Number of patients (%)
Died	3 (10)
Cause of death	
Cancer	2
Other	1
Contacted but declined second scan	21 (68)
Uncontactable – lost to follow-up/moved away	7 (22)

TABLE 18- ***REASONS WHY THE 31 PATIENTS FROM THE BASELINE COHORT OF 100 SLE PATIENTS DID NOT PARTICIPATE IN RE-SCANNING.***

	Patients with SLE lost to follow up N=31 baseline	**Patients with SLE followed up N= 69 baseline**	**P value**
Demographics			
Gender N (%)			
Female	30 (97)	65 (94)	0.585
Male	1 (3)	4 (6)	
Ethnicity N (%)			
Caucasian	15 (48)	41(59)	0.740
Afro-Caribbean	8 (26)	17 (25)	
Asian	4 (13)	7 (10)	
Other	4 (13)	4 (6)	
Age at scan, mean ± SD	44±15	46±11	0.476
Disease duration at scan, mean ± SD	16 ± 11	16± 9	0.892
Current smoker	2 (7)	9 (13)	0.255
Diabetes, N (%)	0	1 (1)	1.00
History of lupus nephritis, N (%)	12 (39)	17 (25)	0.703
Blood Pressure (mmHg) measured at time of scan			
Systolic, mean ± SD	127 ±15	126±16	0.835
Diastolic, mean ± SD	77±9	75±11	0.544
Disease activity			
Global BILAG score at scan, median (IQR)	2 (8)	2 (7)	0.256
Persistently active disease, N (%)	17 (55)	31 (45)	0.430

Lipid profile (mmol/L) within year of scan			
Total cholesterol, mean ± SD (NR <5.20)	4.9±1.19	4.8±1.02	0.491
HDL, mean ± SD (NR >1)	1.59±0.53	1.71±0.52	0.321
LDL, mean ± SD (NR <2)	2.78±0.88	2.54±0.88	0.207
Total cholesterol/ HDL ratio, mean ± SD	3.25±0.91	2.97±0.86	0.145
Triglycerides, mean ± SD (NR <2.2)	1.18±0.52	1.09±0.45	0.382
Treatment regimen at scan			
Hydroxychloroquine, N (%)	20 (65)	45 (65)	0.946
Immunosuppression, N (%)	15 (48)	30 (43)	0.648
Prednisolone dose (mg), median (IQR)	5 (7)	4.5 (5)	0.224
B-cell depletion ever, N (%)	9 (29)	22 (32)	0.776
ACE inhibitors, N (%)	9 (29)	26 (37)	0.402
Aspirin, N (%)	5 (16)	9 (13)	0.681
Statins, N (%)	7(23)	6 (9)	0.06
Blood results at time of scan			
Homocysteine* (µmol/L mean ± SD)	12.6± 3.1	15.9±16.2	0.064
ESR (mm/h), median (IQR)	13 (19)	15 (18)	0.13
CRP (mg/dL), median (IQR)	1.1(4)	1.7(3)	0.56
C3 (g/l), mean ± SD	1.0±0.24	1.01±0.22	0.30
Anti-dsDNA (IU/L), median (IQR)	26 (128)	24 (65)	0.47

Anti-ApoA1 IgG (AU), median (IQR)	61 (171)	67 (142)	0.96
Historical serological profile (ever positive)			
Anti-C1q*, N (%)	7 (23)	14 (20)	0.64
Anti-cardiolipin (IgG and/or IgM), N (%)	10 (32)	21 (30)	0.89
Anti-β2 GP1, N (%)	3 (10)	2 (3)	0.14
Lupus anticoagulant, N (%)	14 (45)	25 (36)	0.43
Anti-ENA, N (%)	17 (55)	41 (59)	0.67
Anti-Ro, N (%)	12 (39)	30 (43)	0.66
Anti-La, N (%)	12 (39)	5 (7)	0.88
Anti-Sm, N (%)	12 (39)	6 (9)	0.81
Anti-RNP, N (%)	9 (29)	24 (35)	0.57

TABLE 19- ***COMPARISON OF BASE LINE CHARACTERISTICS OF PATIENTS WITH SLE WHO WERE RE-SCANNED (N=69) AND THOSE LOST TO FOLLOW UP (N=31).***

Prednisolone dose defined as dose on the day of scan. Persistent disease activity is defined as A or ≥1 B in any BILAG system on at least 2/4 occasions from the most recent assessments closest to the scan date (Croca, Bassett et al. 2015).

Follow up Cohort Characterisation

Ninety-four percent of the patients who were re-scanned were women and the mean age was 46 years (SD 11). The mean disease duration at the second scan was 16 years (SD 9). Forty-one patients were Caucasian, 17 Afro-Caribbean, 7 Asian and four had other backgrounds (mixed race or Chinese). The median time between scans was 5 years (SD 0.7).

Overall, patients had a broad spectrum of organ involvement, most commonly skin, joints and kidney. Biopsy proven lupus nephritis was present in 28 patients. Eleven patients were smokers at the time of the scan and 14 had been smokers at some point in their life. Only three had diabetes and 20 patients had a formal diagnosis of hypertension.

At the time of the scan, each patient had their blood pressure recorded as previously described in the Methods chapter. The mean BP was 130/80 mm/Hg.
From the 69 patients re-scanned, 38 patients had no plaque and 31 had plaque. Of the plaque patients, 10 patients had carotid plaque only, 9 had exclusively femoral, and 12 both femoral and carotid plaques. Seven patients had plaques in all four territories.

3.5.2 Progression of atherosclerosis and IMT over time

Table 20 summarises changes in carotid plaque and CIMT over time. At baseline, 25/69 (36%) of patients had at least one plaque and by follow up (median 5 years) this had increased to 31/69 (45%) patients. Fifty-five percent (38/69) had no plaque at either time point (1st and 2nd scans). New plaque was observed in 9% (6/69). Of those who had plaque at the second scan, 58% (18/31) had an increase in number of plaques and 42% (13/31) had no change in the number of plaques.

The median of total plaque area (TPA) was 3.29 cm^2 (calculated by the TPA at second scan –TPA at baseline= TPA Difference. TPA Difference/ years of follow up=TPA increase per year). Therefore, half the plaques will be on either side of median. Thirty-one patients with plaque can be divided into those who had slow growth of plaque per year 16/31 (52%) and 15/31(48%) with rapid growth.

The mean number of plaque sites increased from 0.85 at baseline to 1.14 at follow up. Median change in IMT per year was 0.0008cm/year.

Baseline plaque prevalence (%)	25/69 (36)
Follow up plaque prevalence (%)	31/69 (45)
New plaque onset at follow up (%)	6/69 (9)
Follow up no change in plaque site number compared with baseline (%)	13/69 (19)
Follow up increase in plaque site number compared with baseline (%)	18/69 (26)
Baseline number of plaque sites –mean (SD)	0.85 (1.30)
Follow up number of plaque sites- mean (SD)	1.14 (1.47)
Baseline CIMT median (IQR) cm	0.055 (0.01)
Follow up CIMT- median (IQR) cm	0.06 (0.0137)
Baseline IMT median (IQR) cm	0.071 (0.0461)
IMT change per year- median (SD) cm/year	0.0008 (0.012)
CIMT change per year- median (SD) cm/year	0.012 (0.01)

TABLE 20-**RATE OF CAROTID INTIMA–MEDIA THICKNESS (CIMT) (WHICH INCLUDES IMT IN BULBS AND CAROTIDS) AND PLAQUE PROGRESSION IN THE 69 SLE WHO WERE RE-SCANNED.**

3.5.3 Plaque vs Non-Plaque

Table 21 compares the 38 patients with no plaque to the 31 patients who had plaque with respect to demographics, treatment regimens, disease activity, blood results and immunological markers. Plaque patients were significantly older with a mean age of 57 years (SD 7) compared to non-plaque patients 46 years (SD 10, p<0.001) and had longer disease durations 20 years (SD 10) vs 14 years (SD 9) (p= <0.01). Mean systolic BP at the time of scan was significantly raised in plaque patients 139 (SD 12) vs 123 (SD 14) mmg/Hg in the non-plaque group (p <0.01). Although the systolic BP for plaque patients is high, it falls within the normal range <140mmg/Hg.

There were no significant differences between serum lipids in plaque vs non-plaque groups.

When comparing treatment regimens between plaque and non-plaque patients, there were no significant differences between current use of hydroxychloroquine, immunosuppressants, prednisolone, ACE-inhibitors, statins and ever use of B-cell depletion agents. Aspirin use was significantly different, being prescribed in more plaque patients (23%) compared to non-plaque patients (5%), p=0.03.

Mean complement levels were significantly raised in plaque patients (1.14mg/dl) vs non-plaque (0.97mg/dl), p=0.03, but these values are within normal range (≥0.9g/L). Median anti-dsDNA antibody levels were significantly raised in non-plaque patients (34 vs 7 IU/L), p=0.03. However, as with complement levels these levels were within

normal range (<50 IU/L). No significant differences were observed between inflammatory markers (median ESR or CRP) levels between groups.

Historical positivity of anti-La antibodies appeared to be protective for the development of plaque with a significantly greater number of non-plaque patients testing positive (26%) compared to plaque patients (3%), p=0.009. There were no significant differences in the presence of anti-phospholipid antibodies between groups.

	Plaque (*n*= 31)	No plaque (*n*= 38)	P
Demographics			
Sex, N (F : M)	28 : 3	37 : 1	0.21
Ethnicity, N (%)			
Asian	2 (6)	5 (13)	0.49
Afro-Caribbean	8 (26)	8 (21)	
Caucasian	20 (65)	21 (55)	
Other	1 (3)	4 (11)	
Age at scan, mean ± SD	57 ± 7	46 ± 10	**<0.001***
Disease duration at scan, mean ± SD	20 ± 10	14 ± 9	**<0.001***
Current smoker (%)	6 (20)	5 (14)	0.11
Ever smoker (%)	8 (26)	6 (16)	0.56
Diabetes, N (%)	3 (10)	0 (0)	0.14
History of lupus nephritis, N (%)	12 (38)	16 (42)	0.63
Blood Pressure (mmHg) at time of scan			
Systolic, mean ± SD	139 ± 12	123 ± 14	**<0.01***
Diastolic, mean ± SD	75 ± 10	76 ± 10	0.08
Disease activity scores (within 6 months of scan)			
Global BILAG score at scan, median (IQR)	1 (8)	1 (2)	0.12
Persistently active disease N (%)-	1 (3)	1 (3)	0.26
Activity within the last two years, N (%) presence any BILAG A or B score in the previous 2 years before scan	17 (55)	19 (50)	0.82
Damage score at scan, median (IQR)	1 (2)	0 (1)	0.05
Lipid profile (mmol/L) (within a year of scan)			
Total cholesterol, mean ± SD	5.1± 1.02	4.6. ± 0.9	0.15
HDL, mean ± SD	1.8 ± 0.6	1.6 ± 0.4	0.097
LDL, mean ± SD	2.7 ± 0.8	2.5 ± 0.7	0.69
Total cholesterol/ HDL ratio, mean ± SD	2.9 ± 0.8	2.5 ± 0.6	0.15

Triglycerides, mean ± SD	1.4 ± 0.8	1.2 ± 0.6	0.30
Treatment regimen at scan			
Hydroxychloroquine, N (%)	17 (55)	26 (68)	0.25
Immunosuppression, N (%)	9 (36)	16 (42)	0.26
Azathioprine, N (%)	5 (14)	12 (32)	0.53
MMF, N (%)	6 (17)	14 (37)	0.53
Others, N (%)	2 (6)	8 (21)	0.27
Prednisolone, N (%)	16 (52)	24 (63)	0.33
Prednisolone>5mg, N (%)	6 (17)	18 (47)	0.20
Prednisolone dose (mg), median (IQR)	4.1 (5.0)	5.0 (6.8)	0.34
B cell depletion **ever** (%)	2 (6)	0 (0)	0.11
ACE inhibitors, N (%)	14 (45)	11 (29)	0.16
Aspirin, N (%)	7 (23)	2 (5)	**0.029***
Statins, N (%)	3 (10)	3 (8)	0.79
Blood results (within 3 months of scan)			
Homocysteine* (µmol/L), median (IQR)	15 (5)	16 (6)	0.29
ESR (mm/h), median (IQR)	8 (17)	15 (20)	0.13
CRP (mg/dL), median (IQR)	1.7 (3.9)	1.4 (3.1)	0.56
C3 (g/l), mean ± SD	1.14 ± 0.25	0.97 ± 0.25	**0.025***
Anti-dsDNA (IU/L), median (IQR)	7 (39)	34 (145)	**0.029***
Anti-ApoA1 IgG (AU), median (IQR)	21(16)	36 (14)	0.22
Historical serological profile (ever positive)			
Anti-C1q*, N (%)	4 (13)	10 (26)	0.32
Anti-cardiolipin (IgG and/or IgM), N (%)	11 (36)	14 (38)	0.84
Anti-β2 GP1, N (%)	1 (3.2)	1 (3.9)	0.93
Lupus anticoagulant, N (%)	4 (13)	7 (18)	0.53
Anti-ENA, N (%)	16 (52)	24 (63)	0.30
Anti-RO, N (%)	11 (36)	20 (65)	0.15
Anti-La, N (%)	1 (3)	10 (26)	**0.009***
Anti-Sm, N(%)	4(13)	5 (13)	0.98
Anti-RNP, N (%)	10 (32)	12 (32)	0.95

TABLE 21- *COMPARISON OF DEMOGRAPHIC, CLINICAL AND SEROLOGICAL FEATURES BETWEEN PATIENTS WITH AND WITHOUT PLAQUE.*

*Homocysteine levels were available only for 36% (n=13) of patients with plaque and 45% (n=29) of patients without plaque at baseline. Data on anti-C1q is available only for 28 patients with plaque and 48 patients without plaque.

Persistent disease activity is defined as A or ≥1 B in any BILAG system on at least 2/4 occasions from the most recent assessments closest to the scan date.

The mean common carotid IMT (0.065 vs 0.055 cm) and mean IMT (0.130 vs 0.08 cm) were significantly raised in the plaque vs non-plaque group ($p<0.001$). Table 22 also shows the mean of scanning outcomes of the plaque group. These variables were analysed with demographic and clinical information for linear regressions and will be discussed in the next section.

	Plaque (*n*= 36)	No plaque (*n*= 64)	P value
Scanning outcomes			
CIMT, mean ± SD (cm)	0.07± 0.01	0.06 ± 0.007	**<0.001***
IMT, mean ± SD (cm)	0.13 ± 0.056	0.08 ± 0.034	**<0.001***
IMT change/year , mean ± SD (cm)	0.0009±0.001	0.0009±0.001	0.905
Mean Carotid GSM, mean ± SD	65± 29	NA	NA
GSM, mean ± SD	66± 14	NA	NA
Mean Femoral GSM, , mean ± SD	64± 22	NA	NA
Total plaque area (mm^2), median (IQR)	114 (54)	NA	NA
Increase in total plaque area/year (mm^2), median (IQR)	6.5 (26)	NA	NA
Overall plaque thickness (cm), mean ± SD	0.631±0.36	NA	NA
Increase in total plaque thickness/year - mean ± SD (cm)	0.38±0.02	NA	NA
Total plaque length, mean± SD (cm)	3.78± 2.14	NA	NA

TABLE 22- RATES OF INTIMA-MEDIA THICKNESS IN PLAQUE VS NON-PLAQUE GROUP AND SCANNING OUTCOMES IN THE PLAQUE GROUP.

CIMT= Carotid Intima Media Thickness: GSM= Grey Scale Media

3.5.4 **Predictors of plaque**

In a univariable logistic regression analysis of all of the demographic and clinical factors analysed in table 23, the determinants of plaque were found to be age at scan, disease duration, systolic BP, C3 and ever positive anti-La. ROC curves were generated for cut offs for age, disease duration, systolic BP and total cholesterol. An AUC of >0.70 was used to determine sensitivities and specificities. Total Cholesterol and C3 AUC were <0.7 and therefore excluded as cut-offs did not reach significance. Cut offs were identified (with sensitivity and specificity of >65% for presence of plaque) for age-greater than 52 years, disease duration- greater than 15 years and systolic BP>133mmg/Hg. In a multi-variable analysis, age at scan>52 years (OR 10.41, CI 2.66-40.80) and systolic BP>133 (OR 5.26, CI 1.396 – 19.862) remained independent predictors of plaque (table 24).

Logistic regression	Odds ratio	95% CI	p value
Age at scan	1.139	1.066-1.217	**<0.001**
Age>52	**14.580**	**4.40-48.35**	**<0.001**
Disease duration at scan	1.084	1.026-1.147	**0.04**
Disease duration at scan >15.5 years	**2.689**	**1.016-7.103**	**0.04**
Systolic BP at scan	1.111	1.053-1.173	<0.001
Systolic BP>133	**8.10**	**2.75-23.86**	**<0.001**
Total Cholesterol	**1.935**	**1.108-3.80**	**0.02**
LDL	2.373	.317-17.787	0.400
Triglycerides	1.402	0.587-3.350	0.447
Total cholesterol/ HDL ratio	1.371	0.626-3.001	0.430
HDL	2.872	0.982-8.399	0.054
CRP	1.026	0.916-1.149	0.658
ESR	0.967	0.933-1.003	0.072
Low C3	**0.136**	**0.027-0.682**	**0.02**
Raised dsDNA antibodies	0.321	0.103-1	0.054
Ever smoker	1.722	0.527-5.633	0.369
Ever positive anti-La	**5.55**	**1.11-27.65**	**0.036**
Ever positive anti-RO	1.417	0.540-3.717	0.479
Ever positive anti-Sm	1.931	0.522-7.141	0.324
Ever positive anti-RNP	1.742	0.633-4.795	0.282
Ever positive anti-C1q	0.105	0.088-1.256	0.333
Ever positive anti- β2 GP1	1.065	0.064-17.767	0.965
Ever positive anti-cardiolipin	1.80	0.636-5.092	0.268
Ever positive Lupus anticoagulant	0.441	0.156-2.246	0.592
Anti-ApoA1 IgG	1.001	0.999-1.003	0.499
Homocysteine	1.080	0.936-1.245	0.294
Treatment with hydroxychloroquine	0.617	0.231-1.645	0.335
Treatment with immunosuppressants	0.195	0.187-1.408	0.514
B-cell depletion ***ever***	1.620	0.585-4.489	0.354

Use of Aspirin	5.104	0.975-26.173	0.054
Use of Statin	5.104	0.975-26.713	0.054
Prednisolone use at	1.172	0.22-0.6216	0.852
scan	0.467	0.178-1.233	0.121
Prednisolone dose	0.347	0.069-1.742	0.325
>5mg			
History of Lupus	1.270	0.483-3.30	0.628
Nephritis			
Activity within the last	0.895	0.339-2.360	0.822
two years			
Median Global BILAG	1.104	0.975-1.249	0.119
score			

TABLE 23-UNIVARIABLE LOGISTIC REGRESSION ANALYSIS OF DEMOGRAPHIC, CLINICAL AND SEROLOGICAL DATA GENERATED INDEPENDENT PREDICTORS OF PLAQUE

Cut-offs for age, disease duration and systolic BP were generated using ROC curves with AUC >0.7. Where $p<0.05$ is significant. OR = odds ratio; CI= Confidence Interval. Odds ratio defined by every one-unit increase in independent variable.

Blood results from 3 months of scan and lipids results are from within a year of scan.

Treatments defined as use at time of scan, excluding B-cell depletion.

Activity within the last two years defined as presence any BILAG A or B score in the previous 2 years before scan. BILAG Score calculated using the formula A=12, B=8, C=1, D=0, E=0.

Diabetes excluded in this analysis as all patients were in the plaque group.

Multiple logistic regression	**Odds ratio**	**95% CI**	**p value**
Age at scan > 52	**13.374**	**3.277 –54.592**	**<0.001**
Systolic BP>133	**6.763**	**1.821 – 25.114**	**0.004**
Disease Duration >15.5 years	1.188	0.327 –4.316	0.793
Ever positive anti-LA	2.280	0.379-13.725	0.368

TABLE 24- MULTI-VARIABLE ANALYSIS FOR PREDICTORS OF PLAQUE

The four variables with the highest odds ratio from univariate analysis were included in the multi-variable analysis. This final model shows that age>52 years and systolic BP>133 mm/hg remained significant independent predictors of plaque.

Where $p<0.05$ is significant. OR= odds ratio; CI= Confidence Interval.

3.5.5 Regression analysis of scanning outcomes

A correlation analysis was carried out for IMT/GSM/TPT/TPL/TPA area with the demographic and clinical data for patients with plaque (scanning outcomes were not included). Factors that were significantly correlated were further analysed in a regression analysis described below for scanning outcomes.

3.5.6 Intimal Media Thickness (IMT)

For IMT, data from all 69 patients (plaque and non-plaque patients) were analysed. Table 25 shows the determinants of mean common carotid IMT from univariate analysis which were age at scan, diastolic BP, systolic BP, low C3, total cholesterol and LDL. In the final model using a multi-variable analysis, age and systolic BP at the time of scan were independent predictors of mean common carotid IMT ($p<0.001$) shown in table 26.

In a linear regression, factors that were independent predictors of the mean IMT (**mean of IMT** measurements at **common carotids and bulb region**) are shown in table 27. Age at scan was the only variable statistically significant in the univariate analysis and in the only independent predictor of mean IMT shown in table 28.

Univariable linear regression	**Beta**
	p
	value
Disease duration	0.336
	0.05
Age at scan	**0.555**
	<0.001
Systolic BP at scan	**0.575**
	<0.001
Diastolic BP at scan	**0.499**
	<0.001
Ever anti-La positive	0.161
	0.185
Ever anti-RO positive	-0.111
	0.370
Ever anti-RNP positive	0.013
	0.913
Ever anti-Sm positive	0.027
	0.824
Raised anti-dsDNA antibodies	-0.112
	0.346
Low C3	**-2.75**
	0.031
ESR	0.015
	0.902
CRP	0.204
	0.096
Ever anti-β2 GP1 positive	0.028
	0.823
Ever Lupus Anticoagulant	-0.184
Positive	0.134
Ever anti-cardiolipin antibodies	0.08
Positive	0.517
Ever anti-C1q positive	-0.185
	0.208
Homocysteine	0.502
	0.500
Anti-ApoA1 IgG	0.177
	0.251

Total Cholesterol	**0.310**
	0.021
Triglycerides	0.023
	0.868
LDL	**0.407**
	0.021
HDL	0.031
	0.823
Cholesterol/HDL ratio	0.315
	0.019
Treatment with immunosuppressants	-0.052
	0.669
Treatment with hydroxychloroquine	-.306
	0.011
B-cell depletion ever	0.016
	0.894
Use of Aspirin	0.071
	0.567
Use of Statins	-0.078
	0.524
History of Lupus Nephritis	-0.118
	0.334
Diabetes	0.211
	0.082
Prednisolone use	-0.185
	0.129
Prednisolone dose >5mg	0.088
	0.598
Ever smoker	0.112
	0.360
Activity within the last two years	0.173
	0.164
Median Global BILAG score	0.056
	0.657

TABLE 25- DETERMINANTS OF MEAN COMMON CAROTID IMT (WHEN INCLUDING ALSO PATIENTS WITHOUT PLAQUE) IN THE UNIVARIATE ANALYSIS

The units expressed for mean CCIMT here are in cm.

Blood results from 3 months of scan and lipids results are from within a year of scan.

Treatments defined as use at time of scan, excluding B-cell depletion.

Activity within the last 2 years defined as presence any BILAG A or B score in the previous 2 years before scan. BILAG Score calculated using the formula A=12, B=8, C=1, D=0, E=0.

Multiple linear regression	Beta	P	r^2 (adjusted) = 0.434 F = 27.805 P=<0.001
Age at scan	0.379	<0.001	
Systolic BP	0.421	<0.001	

TABLE 26- **MULTIVARIABLE ANALYSIS FOR DETERMINANTS OF MEAN COMMON CAROTID IMT (EXPRESSED IN CM)**

Age and Systolic BP remain independent predictors for mean CCIMT. This final model with two fits better (with the highest r^2 and F values) than a model including more independent variables.

Univariable linear regression	**Beta**
	p value
Disease duration	0.055
	0.585
Age at scan	**0.396**
	0.001
Systolic BP at scan	0.122
	0.381
Diastolic BP at scan	0.056
	0.645
Ever anti-La positive	0.056
	0.509
Ever anti-RO positive	-0.111
	0.370
Ever anti-RNP positive	-0.143
	0.159
Ever anti-Sm positive	-0.028
	0.780
Raised anti-dsDNA antibodies	-0.184
	0.105
Low C3	-0.145
	0.203
ESR	-0.048
	0.651
CRP	-0.088
	0.411
Ever anti-β2 GP1 positive	-0.104
	0.319
Ever Lupus Anticoagulant	-0.068
Positive	0.506
Ever anti-cardiolipin antibodies	0.018
Positive	0.870
Ever anti-C1q positive	-0.034
	0.771
Anti-ApoA1 IgG	0.022
	0.879
Homocysteine	0.202
	0.200
Total Cholesterol	0.102

	0.404
Triglycerides	-0.069
	0.572
LDL	0.108
	0.377
HDL	0.135
	0.267
Cholesterol/HDL ratio	0.222
	0.855
Treatment with immunosuppressants	-0.058
	0.578
Treatment with hydroxychloroquine	-0.048
	0.649
B-cell depletion ever	0.006
	0.957
Use of Aspirin	-0.101
	0.335
Use of Statins	-0.107
	0.303
Diabetes	0.176
	0.078
Prednisolone use	0.184
	0.666
Prednisolone dose >5mg	0.113
	0.474
Ever smoker	0.053
	0.598
History of Lupus Nephritis	-0.059
	0.564
Activity within the last two years	0.210
	0.091
Median Global BILAG score	0.000
	1.000

TABLE 27- DETERMINANTS OF MEAN IMT (WHEN INCLUDING ALSO PATIENTS WITHOUT PLAQUE) EXPRESSED IN MM

Blood results are from within 3 months of scan and lipids from within 12 months of scan.

Treatments are defined at the time of scan, excluding B-cell depletion.

Activity within the last two years - presence of any BILAG A or B score in the previous 2 years before scan. BILAG Score calculated using the formula A=12, B=8, C=1, D=0, E=0.

Univariable linear regression	Beta	P	r^2 (adjusted) = 0.144 F = 12.616 P =0.001
Age at scan	0.396	**0.001**	

TABLE 28- UNIVARIABLE LINEAR REGRESSION FOR DETERMINANTS OF IMT (EXPRESSED IN MM).

Age at scan is the only independent predictor.

3.5.7 **Total Plaque Area (TPA)**

Considering the 31 patients who were found to have plaque in at least one site, the mean TPA was 72.56 mm^2 (SD 56; range 10-240). The mean total plaque area increase per year was 4.37 mm^2 (SD 4.5; range -5-15).

Total cholesterol, LDL, HDL and triglycerides were determinants of TPA in the univariable analysis as shown by table 29. In the multivariable analysis in table 30, Total Cholesterol remained an independent predictor of TPA.

Univariable linear regression	**Beta** **p value**
Disease duration	-0.61 0.745
Age at scan	0.082 0.662
Systolic BP at scan	-0.172 0.355
Diastolic BP at scan	0.096 0.609
Ever anti-La positive	0.175 0.347
Ever anti-RNP positive	0.294 0.109
Ever anti-RO positive	-0.059 0.753
Ever anti-Sm positive	0.041 0.826
ESR	0.123 0.511
CRP	-0.164 0.386
Raised dsDNA antibodies	-0.125 0.516
Low C3	-0.009 0.963
Ever Lupus Anticoagulant positive	0.013 0.947
Ever anti-β2 GP1 positive	-0.120 0.519
Ever anti-cardiolipin Positive	0.175 0.347
Ever anti-C1q positive	-0.269 0.265
Anti-ApoA1 IgG	-0.082 0.745
Homocysteine	-0.051 0.864
Total Cholesterol	**-0.660** **0.01**

Triglycerides	**0.442**
	0.035
LDL	**-0.452**
	0.031
HDL	**-0.515**
	0.012
Cholesterol/HDL ratio	0.035
	0.874
Use of Statins	0.186
	0.316
Use of Aspirin	-0.070
	0.713
Treatment with	-0.047
immunosuppressants	0.802
B-cell depletion ever	0.283
	0.123
Treatment with	0.290
hydroxychloroquine	0.113
History of Lupus Nephritis	-0.032
	0.863
Diabetes	0.286
	0.119
Prednisolone use	0.268
	0.145
Prednisolone dose >5mg	0.311
	0.240
Ever smoker	0.249
	0.177
Activity within the last two	0.188
years	0.311
BILAG Score at scan	0.128
	0.502

TABLE 29- LINEAR REGRESSION OF DETERMINANTS OF TOTAL PLAQUE AREA (EXCLUDES PATIENTS WITHOUT PLAQUE). TPA MEASURED IN MM^2

Blood results are from within 3 months and lipids from within a year of scan.

Treatments are defined at the time of scan, excluding B-cell depletion.

Activity within the last two years defined as presence of any BILAG A or B score in the previous 2 years before scan. BILAG Score calculated using the formula A=12, B=8, C=1, D=0, E=0.

Multiple linear regression	Beta	P	r^2 (adjusted) = 0.406
HDL	-0.194	0.351	F = 8.513
Total Cholesterol	-0.514	**0.014**	P=0.002

TABLE 30- MULTI-VARIABLE ANALYSIS OF THE DETERMINANT OF TPA

Total Cholesterol remains an independent factor for TPA. This final model with two fits better (with the highest r^2 and F values) than a model including more independent variables.

The fact that TC has a negative relationship with TPA in the linear regression may be due to the influence of HDL in TC. Looking at the graph in figure 26 there were six outliers (with TC>5.5 mmol/L) who may be influencing the result. The median HDL levels of the outliers was 2.2 compared to 1.7 in the rest of the patients whose TC was <5.5 mmol/L. The median age of the outlier patients was 52 years and they were all females. The non-outlier patients (n=13), had a median age of 55 years, and there were two males in the group. None of the patients in the outlier group were taking statins and two patients in the non-outlier group were taking statins.

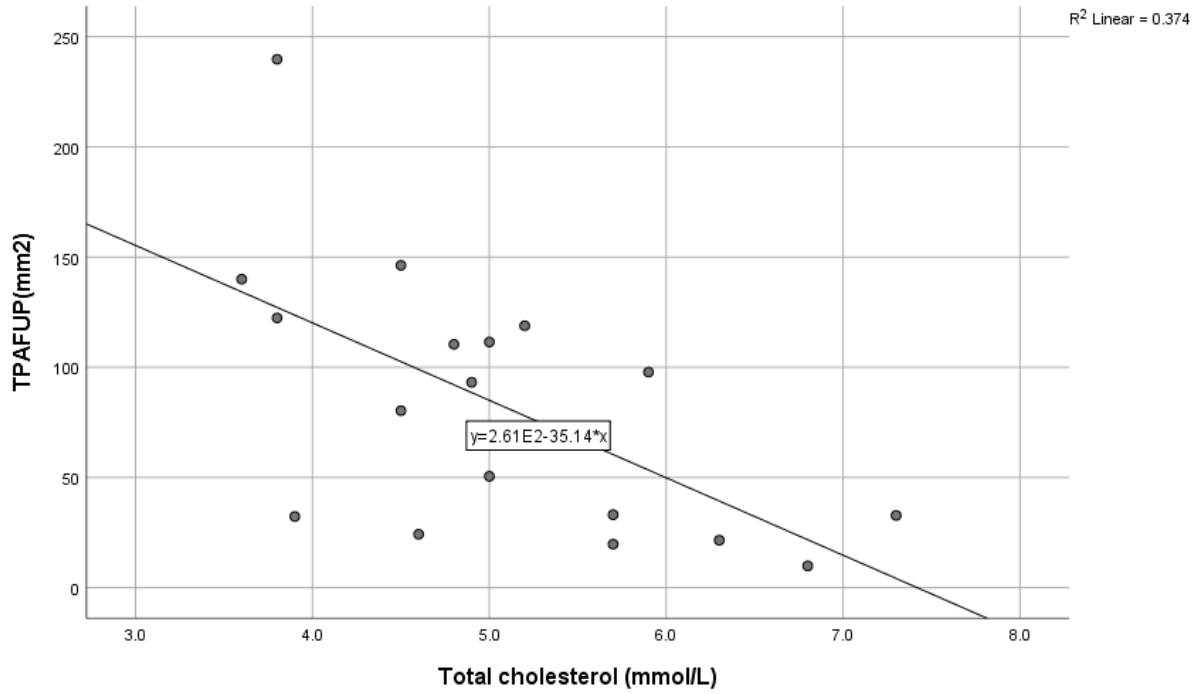


Figure 26- TPA plotted against total cholesterol.

Six outliers with a TC of >5.5 mmol/L.

3.5.8 TPT (Total Plaque thickness) and TPL (Total Plaque Length)

The mean TPT was 0.63 cm (SD 0.36; range 0.14-1.27) and TPL was 3.78 cm (SD 2.14; range 0.9-9). The mean increase in TPT per year was 0.038 cm (SD 0.02; range -0.006-0.09) and for TPL 0.071cm (SD 0.11; range -0.07-0.52).

The univariable linear regression analysis of the determinants of TPT are shown in table 31. Total Cholesterol, triglycerides and HDL were significant predictors of TPT in the univariable analysis. HDL and Total Cholesterol were not independent predictors in the multi-variable linear analysis in table 32.

Univariable linear regression	**Beta p value**
Disease duration	-0.085 0.648
Age at scan	0.113 0.544
Systolic BP at scan	-0.166 0.373
Diastolic BP at scan	0.067 0.722
Ever anti-La positive	0.257 0.164
Ever anti-RO positive	-0.202 0.276
Ever anti-Sm positive	0.155 0.405
Ever anti-RNP positive	0.426 0.017
Raised dsDNA antibodies	-0.1 0.607
Low C3	0.108 0.576
ESR	0.064 0.734
CRP	-0.122 0.522
Ever anti-cardiolipin positive	0.074 0.691
Ever anti-β2 GP1 positive	-0.120 0.519
Ever Lupus Anticoagulant positive	-0.149 0.423
Ever anti-C1q positive	-0.371 0.118
Anti-ApoA1 IgG	0.023 0.938
Homocysteine	0.031 0.951

Total Cholesterol	**0.059**
	0.033
Triglycerides	**0.464**
	0.026
LDL	-0.261
	0.299
HDL	**-0.499**
	0.015
Cholesterol/HDL ratio	0.230
	0.290
Use of Statins	0.073
	0.698
Use of Aspirin	-0.251
	0.181
Treatment with	-0.251
immunosupressants	0.181
B-cell depletion ever	0.353
	0.052
Treatment with	0.135
hydroxychloroquine	0.469
History of Lupus Nephritis	0.021
	0.909
Diabetes	0.167
	0.368
Prednisolone use	0.252
	0.172
Prednisolone >5mg	0.165
	0.542
Ever smoker	0.066
	0.722
Activity within the last two	0.165
years	0.542
Median Global BILAG	0.018
score at time of scan	0.927

TABLE 31- DETERMINANTS OF PLAQUE TPT IN CM (SUBGROUP OF PATIENTS WITH PLAQUE ONLY)

Blood results are from within 3 months of scan and lipids from with a year of scan.

Treatments or use are defined at the time of scan, excluding B-cell depletion.

Activity within the last two years defined as presence of any BILAG A or B score in the previous 2 years before scan. BILAG Score calculated using the formula A=12, B=8, C=1, D=0, E=0.

Multiple linear regression	Beta	P	
HDL	-.361	0.138	r^2 (adjusted) = 0.213
Total Cholesterol	-.231	0.329	F = 3.976
			P = 0.035

TABLE 32 - MULTI-VARIABLE ANALYSIS OF THE DETERMINANT OF TPT (CM)

HDL and Total Cholesterol are not independent predictors in the multi-variable analysis. This final model with two fits better (with the highest r^2 and F values) than a model including more independent variables.

For TPL, the univariable linear regression analysis is shown in table 33. Total cholesterol, HDL, triglycerides, B-cell depletion ever, and Diabetes were significantly associated with TPL. Table 34 shows the best model for multivariable analysis of the determinants of TPL, where HDL was independently predictive.

Univariable linear regression	**Beta p value**
Disease duration	-0.085
	0.648
Age at scan	0.174
	0.358
Systolic BP	-0.157
	0.407
Diastolic BP	0.033
	0.863
Ever anti-La positive	0.143
	0.444
Ever anti-RO positive	-0.073
	0.694
Ever anti-Sm positive	-0.146
	0.434
Ever anti-RNP positive	0.393
	0.300
ESR	-0.086
	0.646
CRP	-0.112
	0.554
Raised dsDNA antibodies	-0.118
	0.544
Low C3	-0.052
	0.788
Ever anti-cardiolipin antibodies positive	0.083 0.658
Ever Lupus Anticoagulant positive	-0.122 0.515
Ever anti-β2 GP1 positive	-0.009
	0.963
Ever anti-C1q positive	-0.282
	0.229
Anti-ApoA1 IgG	0.030
	0.907
Homocysteine	-0.041
	0.894
Total Cholesterol	**-0.581**

	0.005
Triglycerides	**0.552**
	0.013
LDL	-0.354
	0.106
HDL	**-0.578**
	0.005
Cholesterol/HDL ratio	0.197
	0.380
Treatment with	0.148
hydroxychloroquine	0.426
Treatment with	0.033
immunosupressants	0.860
B-cell depletion ever	**0.404**
	0.024
Use of Statins	0.230
	0.214
Use of Aspirin	-0.110
	0.564
History of Lupus Nephritis	0.012
	0.984
Diabetes	**0.361**
	0.046
Prednisolone use	0.251
	0.174
Prednisolone dose>5mg	0.386
	0.155
Ever smoker	0.116
	0.536
Activity within the last two	0.215
years	0.254
Median BILAG Score at	0.172
time of scan	0.372

TABLE 33- DETERMINANTS OF TPL (SUBGROUP OF PATIENTS WITH PLAQUE ONLY) IN A LINEAR REGRESSION

Blood results are from within 3 months and lipids from within 12 months of scan.

Treatments or use are defined at the time of scan, excluding B-cell depletion

Activity within the last two years defined as presence of any BILAG A or B score in the previous 2 years before scan. BILAG Score calculated using the formula A=12, B=8, C=1, D=0, E=0.

Multiple Linear regression	**Beta** **p value**		
Linear regression	Beta	P	
B-cell depletion	0.269	0.143	**r^2 (adjusted) = 0.527**
Diabetes	0.335	0.063	F = 8.809
HDL	**-.505**	**0.005**	P = 0.001

TABLE 34- MULTIVARIABLE ANALYSIS OF DETERMINANTS OF TPL

HDL remains an independent predictor. This final model fits better (with the highest r^2 and F values) than a model including more independent variables.

3.5.9 Grey Scale Media (GSM)

The median GSM of patients with 36 baseline plaque patients was 47 (SD 21, range 14-112). For carotid plaque only it was 47 (SD 14, range16-77) and femoral plaques 51 (SD 25, range 14-112). Over 50% of plaques analyzed had GSM < 60. The majority of plaques are predominantly echolucent.

The the median baseline GSM was 54 (SD 23, range 45-112) in the six plaque patients that had events. The follow up cohort's median GSM value was 64 (range 4-101). There were no correlations with follow-up GSM with demographic and clinical factors (table 35).

Univariable linear regression	**Beta** **p value**
Disease duration	0.212 0.279
Age at scan	0.222 0.256
Systolic BP at scan	0.161 0.415
Diastolic BP at scan	-0.020 0.919
Ever anti-La positive	-0.126 0.521
Raised dsDNA antibodies	-0.014 0.944
Low C3	-0.050 0.806
ESR	0.169 0.391
CRP	0.035 0.862
Ever anti-LA positive	0.126 0.521
Ever anti-RO positive	-0.124 0.531
Ever anti-Sm positive	0.096 0.632
Ever anti-RNP positive	0.182 0.355
Ever anti-C1q positive	-0.292 0.273
Ever anti-Cardiolipin positive	-0.102 0.606
Ever anti-β2 GP1 positive	0.033 0.866
Ever Lupus Anticoagulant positive	-0.104 0.599
Homocysteine	0.375 0.229
Anti-ApoA1 IgG	-0.326 0.217

Total Cholesterol	-0.120
	0.605
Triglycerides	0.048
	0.837
LDL	-0.054
	0.816
HDL	-0.179
	0.437
Cholesterol/HDL ratio	0.198
	0.390
Treatment with	0.134
hydroxychloroquine	0.498
Treatment with	0.033
immunosupressants	0.866
B-cell depletion ever	0.182
	0.354
Use of Statins	0.166
	0.398
Use of Aspirin	0.233
	0.233
Diabetes	0.094
	0.633
History of Lupus Nephritis	-0.024
	0.905
Prednisolone use	0.077
	0.696
Prednisolone dose>5mg	-0.104
	0.701
Ever smoker	0.321
	0.096
Activity within the last two	0.217
years	0.268
Median Global BILAG	0.316
score	0.109

TABLE 35- DETERMINANTS OF GSM (SUBGROUP OF PATIENTS WITH PLAQUE ONLY) IN A LINEAR REGRESSION

Blood results are from within 3 months of scan and lipid results are from within a year of scan.

Treatments are at the time of scan, excluding B-cell depletion.

Activity within the last two years defined as presence of any BILAG A or B score in the previous 2 years before scan.

BILAG Score calculated using the formula A=12, B=8, C=1, D=0, E=0.

3.5.10 CVD Outcomes

Seven events were recorded in the original cohort of 100 patients, among which three were in the cohort that were rescanned. Table 36 describes the demographics and types of events in the seven patients. The average age of the patients who had an event was 55 years. CVD occurred in 6/36 patients with plaque compared with 1/64 without plaque (p=0.002). The average number of plaque sites was 2.4 (CVD patients) vs 0.7 (non-CVD (p=0.02)). The development of CVD was significantly associated with age (p=0.04), disease duration (p=0.02), and plaque thickness (p=0.03). The mean GSM at baseline for patients with events was 60 (SD 26, range 39-112).

Patient	Gender	Age at event	Ethnicity	Event	Smoker	CVS risk factors
1	F	51	Caucasian	Angina	Previous	
2	F	61	Caucasian	NSTEMI	Never	Hypertension
3	**F**	**60**	**Caucasian**	**CABG**	**Never**	**Hypercholesterolemia, Hypertension**
4	F	54	Asian	CABG	Never	PVD
5	F	66	Caucasian	IHD	Never	Hypertension
6	**F**	**53**	**Caucasian**	**NSTEMI**	**Previous**	
***7**	***F***	***42***	***Asian***	**CVA**	***Never***	***Hypertension and Hypercholesterolemia,***

TABLE 36- **DESCRIPTION OF THE SEVEN PATIENTS FROM THE ORIGINAL COHORT OF 100 PATIENTS WHO HAD CVD EVENTS OVER A 5-YEAR FOLLOW UP PERIOD**

*This is the non-plaque patient. The three patients highlighted in bold are the patients who were re-scanned.

3.5.11 Discussion

Various modalities for detecting subclinical atherosclerosis have reported the presence of plaque in SLE patients as being between 30-40% (Manzi, Selzer et al. 1999, Roman, Shanker et al. 2003, Selzer, Sutton-Tyrrell et al. 2004, Farzaneh-Far, Roman et al. 2006). In this study, I investigated the rates and determinants of atherosclerosis longitudinally in a SLE cohort, which had been scanned at baseline. Sixty-nine of the original 100 patients had a second follow up scan.

Over a median of 5 years of follow up, 9% (6/69) of patients developed new plaque and 26% (18/69) had plaque progression (increase in plaque number). In a recent study from a UK cohort study (Haque, Skeoch et al. 2018), new plaque developed in 27% of patients and progression was observed in 41% (which included increase in plaque number and plaque index). Similar findings were reported in the Hopkins Cohort study (Kiani, Vogel-Claussen et al. 2012). A possible reason differences in rates in my study may be due the median age of the patients in my study who were re-scanned were younger, 46 years vs 55 years (Haque, Skeoch et al. 2018).

Predictors of plaque

Comparing patients with and without plaque in my study, patients with plaque unsurprisingly were significantly older (mean age of 57 vs 46 years, $p<0.01$) and had a longer disease duration (20 vs 14 years, $p<0.01$). Roman et al also reported these findings in their study of 204 SLE patients from a New York cohort, showing that the presence of plaque was significantly associated with older age (52 vs 39, $p<0.01$) and longer disease duration (173 vs 129 months $p<0.01$) on univariate analysis.

These findings have also been reported in other SLE cohort (Manzi, Selzer et al. 1999, Selzer, Sutton-Tyrrell et al. 2004, Urowitz, Ibanez et al. 2007).

Plaque patients had a higher systolic BP than non-plaque patients (139 vs 123 mmHg, $p<0.01$). Manzi et al reported higher systolic BP as a being a significant risk factor for plaque (OR 1.03, CI (1.01-1.06, $P<0.007$) (Manzi, Selzer et al. 1999). Roman et al also found significantly higher systolic blood pressure in plaque vs non plaque patients (116 vs 107, $p<0.001$) and greater total cholesterol levels as the only conventional risk factor to independently predict the presence of carotid plaque (Roman, Moeller et al. 2006).

I did not find significant differences between cholesterol levels between the two groups. Total cholesterol was not significantly raised in plaque compared with non-plaque patients (5.1 mmol/L in plaque vs 4.6 in non-plaque patients, $p=0.69$). LDL cholesterol was also not significantly raised in plaque vs non-plaque patients (2.7 vs 2.5 mmol/L, $p=0.68$). Treating SLE as a coronary heart disease equivalent, some would argue that LDL levels of greater than 2.7mmol/L should be treated to reduce CVD risk in line guidelines with the guidelines for other diseases such as diabetes (Wajed, Ahmad et al. 2004). In addition, no significant differences between groups with respect to HDL, triglycerides and total-cholesterol: HDL ratio were found. In another study, SLE patients with chronically elevated total cholesterol (>5.2 mmol/L for 3 years) were shown to have more CV events than those with normal TC level (27.8% vs. 3%) (Bruce, Urowitz et al. 1999). Furthermore, as shown in my metabolomics chapter, it may well be that other lipid markers such as VLDL are more important for predicting CVD risk in SLE individuals.

When comparing treatment regimens between plaque and non-plaque patients, there were no significant differences between use of hydroxychloroquine, immunosuppressants, prednisolone, ACE-inhibitors, B-cell depletion agents and statins. Aspirin use was significantly different, being used in more plaque patients (23%) compared to non-plaque patients (5%), p=0.029. Aspirin was not started on the basis that these patients were known to have plaque.

Mean complement levels were significantly raised in plaque patients (1.14mg/dl) vs non-plaque (0.97mg/dl), p=0.025, but these values are within normal range (≥0.9g/L). Median anti-dsDNA antibody levels were significantly raised in non-plaque patients (34 vs 7 IU/L, p=0.029). However, as with complement levels these levels were within normal range (<50 IU/L).

Historical positivity of anti-La antibodies appeared to be protective for the development of plaque with a significantly greater number of non-plaque patients testing positive (26%) compared to those with plaque (3%), p=0.009. There were no significant differences in the presence of anti-phospholipid antibodies between groups. This as far as I am aware, has not been reported previously. Haque et al, conversely reported that the presence of anti-Ro antibodies were associated with plaque progression (OR 0.31; 0.11 to 0.86) (Haque, Skeoch et al. 2018).

In this study, the multi-variable analysis for the predictors of plaque showed that age at scan>52 years (OR 10.41, CI 2.66-40.80) and systolic BP>133 (OR 5.26, CI 1.396 – 19.862) remained independent predictors of plaque.

CVD outcomes and presence of plaque

This study revealed that 6 out of 7 patients who developed CVD events had plaque, with a low median GSM of 54 at baseline, showing a strong association between the presence of plaque and CVD events. This has been shown in previous studies where the presence of plaque at baseline was predictive of future CVD events in SLE (Roman, Shanker et al. 2003, Kao, Lertratanakul et al. 2013, Haque, Skeoch et al. 2018). There were no patients who had plaque regression in the follow up scan compared to baseline and may be due to the absence of targeted treatments.

Scanning outcomes

The mean IMT (0.130 vs 0.08, p<0.01) and CIMT (0.07 vs 0.06, p<0.01) were significantly raised in plaque vs non-plaque patients. In a large systematic review and meta-analysis of SLE patients (4814 cases and 37773 controls) a higher CIMT (weighted mean difference 0.07mm; 95% CI:0.06,0.09;P<0.001) and an increased prevalence of carotid plaque (OR;2.45;95%CI:2.02,2.97; p<0.001) was reported in patients with SLE. In this study, 71 studies of CIMT were analysed (Wu, Liu et al. 2016). A limitation of this meta-analysis was the heterogeneity in ultrasound protocols in measuring CIMT and most studies were based on cross-sectional design; therefore do not prove causation (Wu, Liu et al. 2016). Manzi and colleagues followed 217 female patients for ten years and showed that progression of carotid plaque occurred in 27% while carotid intima-media thickness (IMT) increased by a mean of 0.011mm/year (Thompson, Sutton-Tyrrell et al. 2008)**.** Kao et al evaluated the association between baseline CIMT and plaque with future CV events in SLE in a population of 392 SLE patients free of CVD at baseline, with a mean follow up of 8

years. For every 0.05 mm increase in baseline CIMT the risk of CVD increased by a factor of 1.35 (95% CI 1.12-1.64) and the presence of plaque at baseline increased this risk by a factor of 4.26 (95%CI 1.23 to 14.83) (Kao, Lertratanakul et al. 2013).

My multi -variable analysis showed CIMT was independently associated with age (beta 0.379, p<0.001) and systolic blood pressure (beta 0.421, p<0.001). Increasing age was also associated with IMT (beta 0.396, p=0.001) in my study. In Wu et al's meta-analysis, CIMT was found to correlate with traditional risk factors (age, TGs, and HDL) and disease related factors (disease duration, steroids, ESR and SLEDAI) (Wu, Liu et al. 2016). In my cohort, age at scan, systolic and diastolic BP were significantly associated with CIMT in the univariate analysis, but only age and systolic BP were independently associated in the multi-variable analysis.

However, there are limitations to measuring IMT. Reduced mean IMT were found in patients with lupus compared to controls, despite prevalence of plaques being much higher in the lupus patients (Roman, Shanker et al. 2003). A better predictor of CVD over IMT is TPA as reported in the Tromso study and particularly for women (Johnsen, Mathiesen et al. 2007). In my study, factors negatively associated with TPA were TC and HDL on univariate analysis. The biological effect of higher TC decreasing TPA may be explained by the effect of HDL. The subgroup of six outlying patients with high TC (>5.5mmol/L) had median levels of HDL which were higher at 2.2 compared to 1.7 mmol/L in the non-outlier groups (NR>1.5 mmol/L). The inverse association of HDL with CVD outcomes and CVD prediction is reported in the general population (Toth, Barter et al. 2013, Goff, Lloyd-Jones et al. 2014) and may

therefore be of significance in SLE patients who traditionally have low HDL (Borba and Bonfa 1997).

Measurement of TPA in SLE is not widely reported. The only study, which looked at TPA in SLE, as far as I am aware, is the baseline scans study of my cohort, (Croca, Griffin et al. 2020). In this paper, TPA was associated with prednisolone use (beta 0.435, p=0.011). A possible reason why my results may not have reflected this may be due to better disease control during the interval from the first scan. In my study, there were fewer patients on steroids at the time of the second scan (61% of plaque patients were taking steroids at the time of the first scan compared to 52% of plaque patients at the second scan.

The potential use of TPA in identifying higher risk individuals was shown in a paper by Perez et al (Perez, Garcia et al. 2016). They studied a population of 2035 Argentines with no history of CVD, and added TPA to a Framingham risk score calculation to risk stratify and compared the reclassification. They reported a change in the risk category for 768 patients (491 increased risk (24.1%) and 277 decreased risk (13.6%) (Perez, Garcia et al. 2016). Cardioprotective treatment was intensified in the patients with increased risk. The population studied in this paper was different to my study as it had a more males (57%), a higher mean age of 59 years (compared to 46 in my study) and a higher prevalence of traditional cardiovascular risk factors (35% hypertensive, 27% hypercholesterolaemic and 14% diabetic).

For the other scanning outcomes looked at in the univariable analysis, for both TPL and TPT, HDL was significant in the univariable analysis and remained an independent predictor for TPL in the multi-variable analysis.

As described in the background, besides the size (area and or volume), the composition of the plaque has a substantial influence on the risk of CVD related events. The GSM score has a positive relationship with plaque echogenicity (i.e. echogenic plaques have a high GSM, echolucent plaques have a low GSM) (Sztajzel, Momjian et al. 2005). The median baseline GSM value for the SLE cohort was on the lower end (47).

The consensus in the literature is that the presence of echolucent carotid plaques is associated with a significant increase of cerebral events compared with echogenic plaques (Mathiesen, Bonaa et al. 2001, Biasi, Froio et al. 2004, Johnsen, Mathiesen et al. 2007, Kakkos, Stevens et al. 2007, Kakkos, Sabetai et al. 2009, Singh, Atam et al. 2013, Ariyoshi, Okuya et al. 2015). Increased plaque echolucency is associated with increased risk of subsequent ipsilateral ischaemic stroke in both symptomatic (Gronholdt, Nordestgaard et al. 2001) and asymptomatic (Huibers, de Borst et al. 2016) patients with plaques causing >50% stenosis of the carotid artery. Carotid plaque echolucency was associated with increased risk of coronary events in 357 Japanese patients with chronic coronary artery disease who were being treated with statins (Uematsu, Nakamura et al. 2014). A proposed mechanism for these findings is that lipid-rich plaques (plaques with low echogenicity) are more unstable and prone to rupture increasing the risk of CVD (Yamagami, Kitagawa et al. 2004, Halvorsen, Johnsen et al. 2009, Huibers, de Borst et al. 2016). It is important to note however

that these studies have been done in the general population in specific risk groups (e.g. diabetic patients) and usually in populations with a greater proportion of male subjects. Therefore, it may be difficult to transpose these findings to the setting of SLE, were the typical patients are relatively young women with few traditional CVD-risk factors.

The Tromso study (Johnsen, Mathiesen et al. 2007) showed that women with the most echolucent plaques had 2.87-fold higher risk of developing MI compared to those with no plaque and is better extrapolated to SLE patients who are predominately women. However, it is also important to bear in mind that the women in this study were older (mean age 65.6 years) than typical patients seen in an SLE clinic.

I did not find any factors that were predictive of GSM in my study. In the study by Croca et al (Croca, Griffin et al. 2020), GSM was positively associated with persistent disease activity. Persistently active disease was associated with more echogenic plaques (beta 0.43, p=0.004). Whether there is any relationship between GSM and disease activity is yet to be elucidated in SLE. In a study of 246 non-SLE patients (including 80 with clinical history of stroke or TIA) the authors found that low echogenicity was associated with raised serum interleukin-6 levels but there was only a borderline association with C-reactive protein level (Yamagami, Kitagawa et al. 2004). Subsequently, in a much larger report of 5434 subjects from the Tromso study, Halvorsen et al found no association between plaque echogenicity and either fibrinogen or C-reactive protein in either sex whereas raised white cell count was associated with echogenicity in women only (Halvorsen, Johnsen et al. 2009).

Several groups have reported the benefits of treatment with statins in regards to increasing plaque echogenicity even in non-hypercholesterolemic individuals, regardless of changes in plaque size or IMT thickness (Watanabe, Sugiyama et al. 2005, Kadoglou, Gerasimidis et al. 2008, Yamagishi, Kato et al. 2009). However, such a strategy may not be so feasible in SLE patients who are younger, predominantly female and of childbearing age.

Strengths and Limitations

A strength of this component of my thesis include the detailed scanning analysis being carried out by a single experienced ultrasonographer for all patients, with the use of the same protocol as the baseline study. In addition, comprehensive data on clinical, serological and treatment variables are available for all patients in this multi-ethnic cohort.

The femoral arteries were included in the US assessment, which has increased the sensitivity of the method as 13% (9/69) only had plaque in the femorals, and therefore increases the chances of identifying plaque. Most studies in SLE CVD have only assessed the carotids. Atherosclerosis involves the arterial bed globally and involvement of multiple territories increases the chances of identifying plaque. Scanning the femoral arteries may be important for risk stratification. Brevetti et al showed that compared with patients who only had coronary artery disease patients with peripheral artery disease had a greater atherosclerotic burden and echolucent plaques (Brevetti, Sirico et al. 2008). The presence of femoral plaques (particularly with a reduced GSM) had an increased risk of cerebrovascular disease.

My study not only assessed IMT at the carotids, but also measured overall IMT (which included the at the right and left bulb region). In most studies, IMT is measured at the CCA because high measurement precision is easily obtained from the artery. Plaques usually occur at sites of non-laminar flow such as the bulb regions and the proximal internal carotid segment. Therefore, the pathological process leading to plaque formation by measuring CIMT alone may reflect different aspects of atherogenesis (Johnsen, Mathiesen et al. 2007).

A limitation of this study that need to be considered when interpreting results is the modest sample size of 69 in this study. However, the average follow up period of 5 years is longer than previous reported studies, although this is still a relative short follow up. Both of these factors may have influenced the ability to detect correlations between subclinical and clinical events.

The full complement of traditional cardiovascular risk factors were not assessed; for example factors such as BMI and family history were not recorded in this study. I did not have data on cumulative corticosteroid doses, which could be more relevant to build-up of atherosclerosis over time.

The population studied were predominantly women, with few diabetics or current smokers and most had low disease activity. Therefore, the results may not be generalisable to all SLE populations and I may have missed potential effects of these variables on ultrasound outcomes.

To conclude, amongst the 69 patients who were re-scanned in this study, 26% had progression and none had decreased plaque over a median of five years follow-up. Seven percent of patients from the original cohort developed an event within 5 years from the date of scanning. Patients with plaque were older and had a longer disease duration. Age >52 years and a systolic BP>133 mmol/ mg were independent predictors of plaque progression. HDL was predictive of TPA on univariable but not multivariable analysis.

This study shows that modifiable risk factors may need to be monitored and treated more stringently to improve CVD outcomes in the SLE population. Measurement of novel ultrasound variables such as TPA and echolucency may identify more modifiable risk factors that can be used to improve CVD outcomes in patients with SLE. Metabolomic studies to identify markers that are more strongly associated with subclinical atherosclerosis and further longitudinal studies in large multi-centres will be helpful to develop a better understanding.

4 Final Discussion

It has been long recognized that SLE patients have an increased risk of CVD, but many questions remain unanswered about its precise pathophysiology, the factors that drive the development and progression of atherosclerosis and how patients can be risk stratified and managed. In my thesis, I have explored possible immunological markers such as anti-ApoA1, serum metabolomics and surrogate markers such vascular ultrasound to understand if these factors could help address some of these unanswered questions. RA patients also have an increased risk of CVD disease compared to the general population. In a small group of RA patients, I explored whether there were differences in the role of *i*NKT cells and serum metabolomics compared to SLE patients.

I have found that serum metabolomics and ultrasound may have a role in risk stratification of CVD patients with SLE.

Role of immunological markers

I did not find a role for anti-ApoA1 antibodies in predicting CVD risk in SLE and this is in keeping with previous results from our group (O'Neill, Giles et al. 2010, Croca, Bassett et al. 2015), despite there being a reported association of anti-apoA1 antibodies with atherosclerosis in RA (Vuilleumier, Bas et al. 2010). It may be that there are other forms of HDL that are more relevant to SLE in predicting risk of CVD such as pro-inflammatory HDL (McMahon, Grossman et al. 2009, McMahon, Skaggs et al. 2014). In SLE, changes in the composition and function of HDL have been

reported leading to the impairment of the function of HDL. For example, multiple studies have shown a significant reduction in PON- 1 and PON- 3 activity in SLE patients (Delgado Alves, Ames et al. 2002, Batuca, Ames et al. 2009, Gaal, Tarr et al. 2016), reducing the anti-oxidant abilities of HDL. Pro-inflammatory HDL has reduced cholesterol efflux capacity impairing its capacity to perform its normal anti-inflammatory and anti-oxidative functions (Kim, Yu et al. 2020). Forty-four percent of SLE patients were reported to have increased pro-inflammatory HDL in comparison to only 4.1% of controls and 20.1% of rheumatoid arthritis patients (McMahon, Grossman et al. 2006). Pro-inflammatory HDL was also shown to be associated with increased carotid intima- media thickness and plaque by carotid ultrasound, suggesting that dysfunctional pro-inflammatory HDL significantly contributes to the development of subclinical atherosclerosis in SLE. McMahon et assessed pro-inflammatory HDL by assessing its ability to oxidise LDL through functional assays and measurement of oxidised LDL. Normal HDL prevents prevents oxidation of LDL whereas pro-inflammatory HDL does not (McMahon, Grossman et al. 2009). Further longitudinal studies are required to see if pro-inflammatory HDL is associated with the development of CVD in SLE. I did not investigate pro-inflammatory HDL in my thesis as I decided to focus on metabolomic assays, which are more likely to be applicable to clinical practice.

My preliminary data on subclinical RA serum being able to induce an *i*NKT phenotype in healthy PBMC's, did not show any differences in responses between RA-P and RA-NP phenotype. This is different to what was found in SLE patients, where patients with pre-clinical plaque were described as having a distinct anti-inflammatory *i*NKT cell phenotype. Possible explanations for this may be that *i*NKT

cell frequency was not directly measured from patient's blood unlike in Smith *et al's* study, and the RA cohort I tested were also a small sample and were all Caucasian. In addition, compared to the SLE cohort examined by Smith *et al,* my RA patients were older and had more males and perhaps other factors could dominate over the role of *i*NKT cells in RA. Therefore, the role of *i*NKT cell in subclinical disease in RA is yet to be determined and larger multi-centre studies are required (Smith, Croca et al. 2016).

Role of metabolomics factors

My study looking at SLE patients with plaque and with no plaque has shown that that VLDL – particularly the small particles are associated with the development of subclinical atherosclerosis.

The top VLDL particles with the highest AUC values for VLDL metabolites were XXL-VLDL-PL (0.68), L-VLDL-FC (0.67), XS-VLDL-TG (0.67), XL-VLDL-PL (0.66) and M-VLDL-FC (0.65) differed significantly between the SLE-NP and SLE-P groups and showed high areas under the curve (AUC) and sensitivities and specificities of >67% for ability to distinguish the P and NP groups. By combining the metabolites with the highest sensitivity and highest specificity, I generated a composite test such that a patient is positive if XXL-VLDL-PL > 0.0030 mmol/l and M-VLDL-FC > 0.04 mmol/l. This composite test distinguished SLE-P from SLE-NP with sensitivity 64%, specificity 79%, positive predictive value 65% and negative predictive value 76%.

I also found significant differences in various sizes of VLDL and in one size of HDL between patients with SLE (86 serum samples) and HC.

My findings suggesting that measurements of VLDL metabolites may be useful in stratifying risk of CVD in patients with SLE. High levels of VLDL particles measured by NMR spectroscopy were associated with an increased risk of cardiovascular disease in the general population and with diabetes. (Freedman, Otvos et al. 1998, Colhoun, Otvos et al. 2002, Mahajan, Zaid et al. 2019). VLDL is the main carrier of triglycerides and triglycerides are known to be an independent factor for CVD (Prenner, Mulvey et al. 2014).

Standard lipid panels in clinical practice are limited as they do not report levels of VLDL-cholesterol at all and report single values for LDL-Cholesterol and HDL cholesterol, making an assumption that these are single entities. However, lipoprotein particles span a continuum of size, density, cholesterol content and triglyceride content. These standard lipid profiles fail to identify many lipoprotein abnormalities, which may contribute to elevated CVD risk. Therefore, my combined test with VLDL metabolites that that differed significantly between the SLE-NP and SLE-P groups could be applied to make a putative test to distinguish these groups.

My findings for RA patients showed that sixty percent of the top 15 significant metabolites were in the HDL subclasses, and the mean levels of these metabolites were significantly higher in HC vs RA patients in keeping with the literature (Dursunoğlu, Evrengül et al. 2005, Georgiadis, Papavasiliou et al. 2006). I was unable to find differences in RA-P vs RA-NP as my dataset was small (only 13) and it may be a limitation that all were Caucasian.

Vascular ultrasound

The results of my vascular US study of 69 SLE patients who were re-scanned a median of 5 years after baseline scans, revealed that 9% (7/69) of patients developed new plaque and 26% (18/69) had plaque progression (increase in plaque number) and almost all patients who developed CVD events had plaque at baseline (6/7). No patients had plaque regression. Plaque at baseline has been shown to be a predictor of future CVD events in patients with SLE (Roman, Shanker et al. 2003, Kao, Lertratanakul et al. 2013, Haque, Skeoch et al. 2018) which is clearly important given that over a quarter of my cohort had plaque progression.

My study confirmed, in line with previous studies that age and disease duration were predictors of plaque (Manzi, Selzer et al. 1999, Selzer, Sutton-Tyrrell et al. 2004, Urowitz, Ibanez et al. 2007). Increasing age was also associated with increased IMT and CIMT, which have been shown to be associated with plaque by other groups (Manzi, Selzer et al. 1999, Roman, Shanker et al. 2003, Farzaneh-Far, Roman et al. 2006, Thompson, Sutton-Tyrrell et al. 2008, Gustafsson, Herlitz Lindberg et al. 2017, Tektonidou, Kravvariti et al. 2017, Theodorou, Nezos et al. 2018). In my study, age >52 years and a systolic BP>133 mmol/ mg were independent predictors of plaque progression. Increased systolic BP as a risk factor for plaque in SLE has been reported previously (Manzi, Selzer et al. 1999, Roman, Shanker et al. 2003).
The mean overall IMT (0.13 vs 0.08 mm, $p<0.01$) and CIMT (0.07 vs 0.06 mm, $p<0.01$) was significantly raised in plaque vs non-plaque patients. Increases in baseline CIMT have been reported to increase CVD risk in SLE patients (Kao,

Lertratanakul et al. 2013). I found CIMT was independently associated with systolic blood pressure, which reinforces the importance of tight BP control in these patients. I looked at novel US outcomes, such as TPA and plaque echogenicity. HDL was associated with TPA on univariate but not multivariate. The Tromso study showed that TPA was a better predictor of CVD in women than IMT (Johnsen, Mathiesen et al. 2007) and women with echolucent plaques had a higher risk of developing MI (and both of these findings are relevant to the population of patients with SLE who are primarily women)**.** The median GSM of the cohort at baseline was 47, reflecting echolucent plaques**.** Echolucent plaques have been associated with an increased risk of cerebral vascular and cardiac events (Uematsu, Nakamura et al. 2014, Ariyoshi, Okuya et al. 2015). In my study, factors negatively associated with TPA were TC and HDL on univariate analysis. One other study looking at TPA in SLE suggests that it may be associated with prednisolone dose (Croca, Griffin et al. 2020). Therefore TPA may not only be of value in identifying those patients at high risk, but also target patients in whom disease control should be managed with immunosuppressive drugs (rather than steroids) and more stringent targeting of cholesterol profiles.

In my audit of traditional risk factor management in SLE, I showed that it was possible to manage conventional risk factors such as BP well, as only 2% of the large Lupus cohort had undiagnosed hypertension. However, I defined hypertension as a systolic ≥140mm/mg, but from my findings from the vascular ultra-sound study it may be that, we need to define more stringent parameters. I found that management of lipid levels according to pre-set parameters was more difficult to achieve, and this

is a fact worth considering if planning to use metabolomic data in management of CVD risk in patients with SLE.

Future work and summary

Based on my findings from my thesis, I propose figure 27 as a pathway for identifying SLE patients with increased CVD in SLE, to help identify those at highest risk and target risk factor management. Future work will help identify immunological markers that could readily identify patients who are at moderate risk. More detailed measures of multiple metabolomics biomarkers using techniques such as nuclear magnetic resonance (Soininen, Kangas et al. 2015) could identify serological lipid profiles associated more tightly with subclinical atherosclerosis in these patients.

A strength of my thesis is that I have constructed a large database of re-scanned SLE patients with extensive data that provides a useful data set to build upon the work I have presented here. The database may be used to explore further the potential implications of other variables, which can potentially influence the increased burden and progression of CVD in patients. Further longitudinal studies from multiple centres using ultrasound markers such as TPA and echolucency will help us improve risk stratification and identify potentially modifiable risk factors.

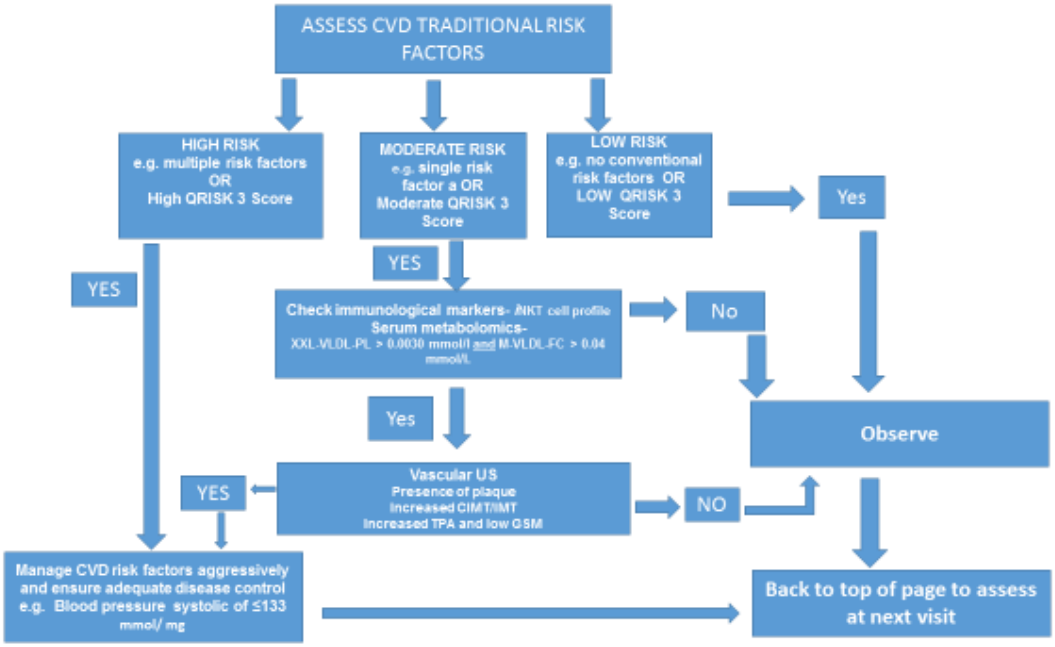


FIGURE 27- RISK STRATIFICATION AND MANAGEMENT FLOW CHART OF CVD RISK IN SLE

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