

Examining links between Factor Xa, endothelial dysfunction, adverse pregnancy outcomes and cardiovascular disease in Lupus



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Declaration

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Summary

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune rheumatic disease (ARD) associated with significant morbidity and mortality, which presents mainly in women of childbearing age. Women with SLE have a 5-10-fold increased risk of developing cardiovascular disease (CVD) compared to age and sex-matched controls. Increased CVD risk in SLE is not fully explained by traditional risk factors and other factors such as persistent inflammation, autoantibodies and microparticles (MPs) have been implicated. Women with SLE and previous adverse pregnancy outcomes (APO) are at increased risk of CVD. Other co-morbidities in women with SLE include low bone mineral density (LBMD), which is also linked with CVD. Increasing interest has focused on the involvement of serine proteases (SP) such as anti-Factor Xa (FXa) and anti-Thrombin (Thr) in atherosclerotic plaque formation. These SP are of further interest because they are known to have extended cellular/inflammatory effects beyond coagulation through their activation of protease activated receptors (PARs) and antibodies (IgG) directed against them have been identified in patients with SLE.

To examine the importance of these risk factors in CVD pathogenesis in SLE, I studied a cohort of 100 patients with SLE who had undergone detailed vascular imaging via carotid and femoral ultrasound scans. These patients had no clinical diagnosis of CVD prior to scanning and were subdivided by the presence of subclinical CVD (n=36) and absence of CVD (n=64). I explored associations between atherosclerotic plaque and the presence of anti-FXa and/or anti-Thr IgG by ELISA. Plasma was analysed for presence of endothelial and platelet microparticles (EMPs and PMPs). Samples were stained with Annexin V, CD42a, CD31, CD105 and CD144 and measured using flow cytometry. Furthermore, I carried out retrospective analysis

of the pregnancy experience of 95 women with SLE focusing on APO and CV risk. I then correlated bone density results with CV status. Finally, I carried out a systematic review to determine whether fertility and parity are reduced in patients with SLE.

Of the cohort 95% were female with a mean age of 45.2 (range 20-66; SD 12.4) years. Anti-FXa IgG positivity was found in 33/64 (52%) of patients without plaque and 11/36 (31%) of patients with plaque ($p=0.04$). PMPs were higher in patients with SLE compared to healthy controls ($p=0.025$). Strikingly, almost half the women (45%) with SLE had no children, more than double one would expect in UK women of the same mean age. A total of 61% had APO and rates of miscarriage were higher (31%) than in the general population (15-20%). My systematic review showed an overall reduction in fertility and parity in women with SLE. Of this lupus cohort, 81% had available bone density scans and 65% had LBMD.

This work demonstrates for the first time that anti-FXa IgG may be atheroprotective in patients with SLE. PMPs are higher in patients with lupus, likely due to overall disease burden. APO and miscarriage rates were high which is in keeping with other studies. My study identified much higher rates of nulliparity in women with SLE than in the general population. Reasons underlying this difference are likely to be multifactorial. The systematic review revealed reduced fertility and parity in women with SLE, although this was confounded by differences in design and outcome measures of studies selected for inclusion. Although there was no correlation between lupus and CVD with low BMD, my work has highlighted the importance of screening for osteoporosis in lupus. Overall, the work produced in my thesis has contributed to the identification of risk factors for CVD in patients with SLE. Further research is now required to elucidate the mechanisms involved in SLE CVD progression.

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List of abbreviations

ACR	American College of Rheumatology
ACS	Acute coronary syndrome
ACT	Activated clotting time
AHA	American Heart Association
ANA	Antinuclear antibodies
AnV	Annexin V
Anti-dsDNA	Anti-double stranded DNA antibodies
AMH	Ant-Mullerian hormone
APC	Allophycocyanin
aPL	Antiphospholipid antibodies
APO	Adverse pregnancy outcomes
APS	Antiphospholipid syndrome
ARD	Autoimmune rheumatic disease
ARTs	Assisted Reproduction Techniques
AU	Absorbance units
BAFF	B-cell activating factor
BCA	Bicinchoninic acid assay
BCR	B cell receptor
BD	Becton Dickinson
BILAG	British Isles Lupus Assessment Group
BMI	Body Mass Index
BMD	Bone Mineral Density
BSA	Bovine serum albumin
BV	Brilliant Violet
C	Caucasian origin

CABG	Coronary Artery Bypass Graft
C3	Complement component 3
CAD	Coronary Artery Disease
CD	Cluster of differentiation
CCA	Common carotid artery
CK	Creatinine kinase
CNS	Central nervous system
CRP	C-reactive protein
CT	Computed tomography
CS	Caesarean section
CVD	Cardiovascular Disease
CVE	Cardiovascular Events
CYC	Cyclophosphamide
DDT	Dithiothreitol
DXA	Dual energy x-ray absorptiometry
EBV	Epstein Barr Virus
ECA	External carotid artery
ECM	Extracellular matrix
EGM-2	Endothelial cell growth medium-2
ELISA	Enzyme Linked Immunosorbant Assay
EMP(s)	Endothelial microparticle(s)
ENA	Extractable nuclear antigens
ERK	Extracellular signal-related kinase
ESR	Erythrocyte sedimentation rate
Fab	Fragment antibody binding
FACS	Fluorescence Activated Cell Sorting
FBC	Full blood count
FMD	Flow mediated dilatation

FSC	Forward scatter
FXa	Factor Xa
GIO	Glucocorticoid induced osteoporosis
GnRH	Gonadotrophin Releasing Hormone
GSM	Grey scale median
HC	Healthy control
HCL	Hydrochloric acid
HDL	High-density lipoprotein
HELLP	Haemolysis, elevated liver function tests, low platelets
HCQ	Hydroxychloroquine
HLA	Human lymphocyte antigens
HPF	High powered field
HPV	Human Papillomavirus
HRP	Horse radish peroxidase
HRT	Hormone replacement therapy
HSCT	Hematopoietic stem cell transplant
IC	Immune complex
ICA	Internal carotid artery
ICAM 1	Intercellular adhesion molecule 1
IFN	Interferon alpha
IgG	Immunoglobulin G
IHD	Ischemic heart disease
IL1	Interleukin 1
IMT	Intima-media thickness
iNKT	Invariant natural killer T
ITP	Immune thrombocytopenia
INR	International normalised ratio
IU	International Units

IUD	Intrauterine Device
IUGR	Intrauterine growth restriction
IVF	In vitro fertilization
JNK	c-Jun N-terminal kinase
LA	Lupus anticoagulant
LDL	Low-density lipoprotein
LFT	Liver function test
Lp	Lipoprotein
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MI	Myocardial infarction
miRNA	microRNA
MMF	Mycophenolate mofetil
MMP	Monocytic microparticles
MP(s)	Microparticle(s)
MPS	Maternal placental syndrome
MRI	Magnetic resonance imaging
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NIHR	National Institute of Health Research
NREC	National Research Ethics Committee
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical Density
OPG	Osteoprotegerin
PARs	Proteinase Activated Receptors

PBS	Phosphate buffer solution
PBST	Phosphate buffer saline tween
PC	Positive control
PCI	Percutaneous coronary intervention
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule-1
PIH	Pregnancy Induced Hypertension
PMP(s)	Platelet microparticle(s)
PPI	Patient and public involvement
PPP	Platelet poor plasma
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PVD	Peripheral vascular disease
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RANK	Receptor Activator of Nuclear Factor κ B
RBC	Red blood cell
RCOG	Royal College of Obstetricians and Gynecologists
ROS	Reactive oxygen species
RT	Room temperature
SGA	Small for gestational age
SLE	Systemic Lupus Erythematosus
SLICC	SLE International Collaborating Clinics
SMC	Smooth muscle cells
SP	Serine Protease
SSC	Side scatter

STBM	Syncytiotrophoblast microparticles
TCD	Trinity College Dublin
TF	Tissue Factor
Thr	Thrombin
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor alpha
TReg	Regulatory T cell
TTP	Time To Pregnancy
UCL	University College London
UCLH	University College London Hospital
UPCR	Urinary protein creatinine ratio
US	Ultrasound
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
VWF	Von Willebrand Factor
WBC	White Blood Cell
WHO	World Health Organisation

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1. Chapter One: Introduction

1.1. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune rheumatic disease associated with significant morbidity and mortality. People of all ages can be affected, but it mainly presents in women of childbearing age. It is a complex disease with diverse clinical features. Its clinical manifestations, either subtle or florid, often fluctuate considerably [1]. SLE causes a range of problems, from mild skin and joint symptoms to severe life-threatening renal or cerebral disease. Constitutional symptoms include fever, lymphadenopathy, weight loss and fatigue. Haematological features include haemolytic anaemia, leucopenia and thrombocytopenia. Juvenile SLE is usually more aggressive and has a worse prognosis [2]. Children have been shown to accrue more damage than adults and have higher mortality rates [3]. Women with SLE have an increased risk of adverse pregnancy outcomes (APO). It has been shown that women with SLE and previous APO, such as pre-eclampsia are at increased risk of cardiovascular disease (CVD) [4].

The prevalence of SLE varies according to geographical and racial background. SLE is more frequent in non-Caucasian individuals [5-7]. It is estimated that 3/10,000 in Caucasians to 20/10,000 in Afro-Caribbean's are affected. Figures show an incidence of SLE in the UK 5.8 times higher in females than in males (8.34/100,000 person-years versus 1.44/100,000 person-years) ($p < 0.001$) [8]. There is a 5-10 fold increased risk of CVD in women with SLE compared with age and sex matched controls and this risk is amplified in younger women.

1.1.1. SLE pathogenesis

The precise cause of SLE is unknown and is likely to be multifactorial. Genetic abnormalities, immune dysregulation, hormonal imbalance and the environment all appear to make important contributions see Figure 1.1.

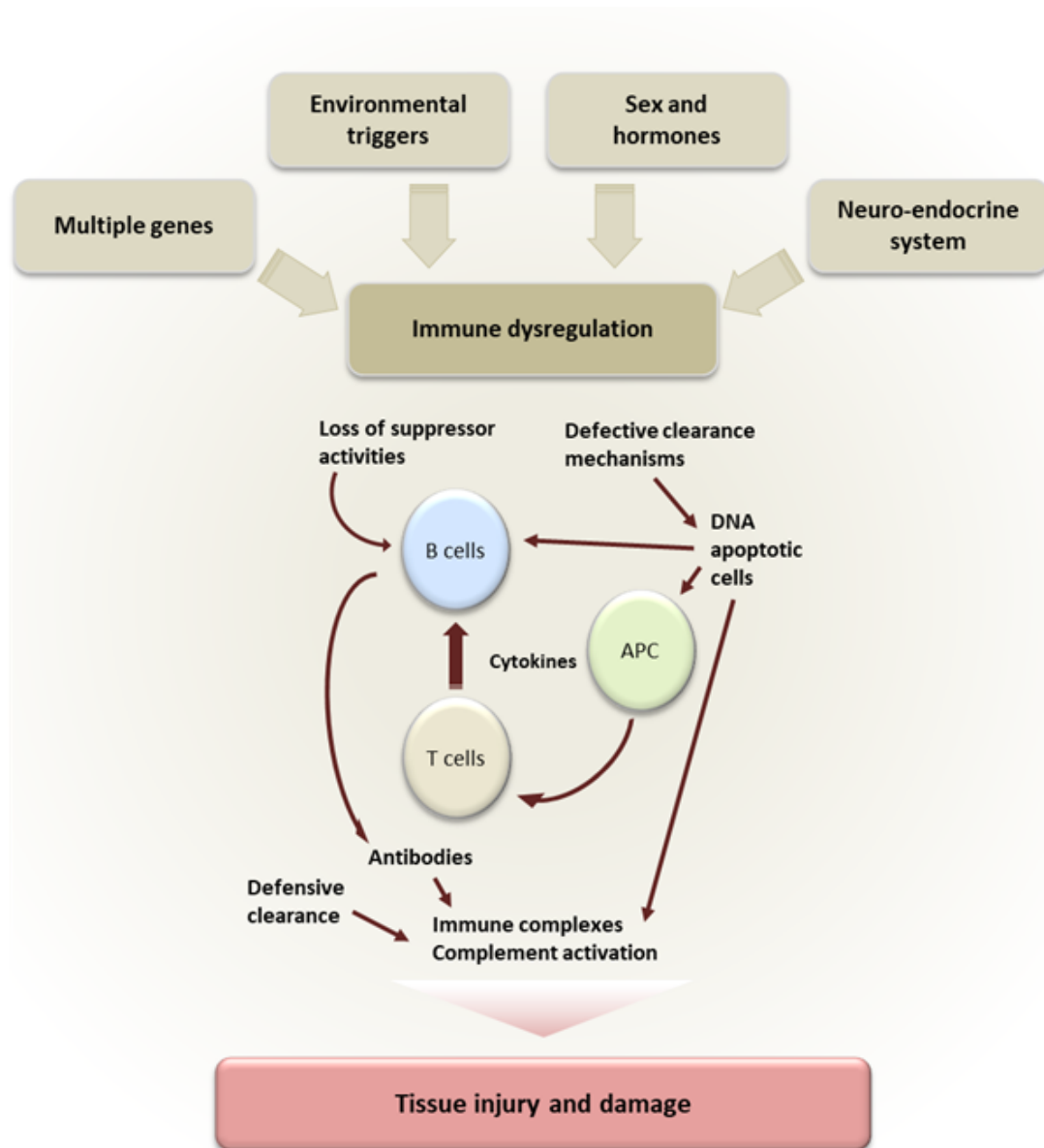


Figure 1.1: Pathogenesis of SLE

The pathogenesis of SLE is summarized as above. Multiple genes, environmental triggers, sex, hormones and the neuroendocrine system contribute to immune dysregulation. Defective clearance of apoptotic cells and immune complexes, modify this susceptibility. The loss of immune tolerance, increased antigen load, excess T-cells and defective B cells lead to cytokine imbalance and the production of pathogenic autoantibodies. Abbreviations: APC; Antigen presenting cell. Adapted from Mok et al. [9]

1.1.2. Genetic abnormalities in SLE

SLE is a disease of polygenic inheritance. Patients with SLE can inherit multiple predisposing genes. The first described genetic link to SLE was the major histocompatibility complex (MHC) on chromosome 6, which contains the human lymphocyte antigens (HLA) [10]. HLA-DRB1*1501 and HLA-DRB1*0301 alleles confer a 2-3 fold increased risk of developing SLE in Caucasians [11]. Genetic mutations can produce a monogenetic form of SLE such as deficiency of complement components C2, C4, and C1q.

Gene Wide Association Studies (GWAS) of up to one million single nucleotide polymorphisms (SNPs) add further to our understanding of SLE with over 50 loci associated with SLE susceptibility [12]. There is 25% concordance amongst monozygotic twins compared to 2% in dizygotic twins in SLE [13]. Genetic abnormalities leading to overproduction of interferon-alpha, complement deficiencies and apoptosis defects can all cause monogenic SLE [14, 15]. The Large Lupus Association Study 2 (LLAS2) has over 13,000 patients with SLE enrolled in the study of about 38,000 SNPs. Over 50 research groups worldwide contributed SLE patients to this consortium.

Polymorphisms in FcγRI, FcγRIIA and FcγRIIIB have been associated with disease severity and increased susceptibility to SLE [16]. Allelic variants of Fc gamma R confer distinct phagocytic capacities providing a mechanism for heritable susceptibility to immune complex disease [17]. HLA-DR3 has been linked to renal disease (and to anti-Ro/La autoantibodies). Polymorphisms such as of APOL1 have been associated with kidney failure in African-Americans [18, 19]. Many other genes have shown associations with SLE, including those involved in lymphocyte signalling (BANK1, CD80, CSK, IL10, SLK, STAT4), innate immune signalling (IRAK1, IRF5, IRF7, IRF8, TLR7, TLR9, TYK2) and immune complex clearance (ATG5, DNASE1, TREX1) [20, 21].

Polymorphisms of IL-10 and TNFS4 have been described and polymorphisms of STAT4 have been correlated with early onset and more severe SLE [19].

1.1.3. MicroRNA

Epigenetic modifications help to explain part of the missing genetic heritability. Chromatin structure and DNA methylation are sensitive to environmental factors. Among post-transcriptional modifications that influence gene expression and DNA methylation, microRNAs (miRNAs) appear to play an important role in SLE pathogenesis. miRNAs are small noncoding molecules that modulate target messenger RNA expression. In patients with SLE, overexpression of miR-126, miR-21, and miR-148a in CD4⁺ T cells has been shown to enhance hypomethylation [22]. In B cells, the overexpression of miR-30a and miR-181b promotes cell proliferation and antibody diversification [23]. miR-21 increases IL-10 levels in CD4⁺ T cells, while miR-31 hyperactivity in T cells reduces IL-2 secretion and negatively regulates Foxp3 expression in regulatory T cells (Tregs) [23]. A reduced expression of miRNA-146 associates with the activation of the Toll-like receptor (TLR)7/9 pathway in plasmacytoid dendritic cells (pDCs), which sustain inflammation through IFN- α production and therefore an immunosuppressive role of this miRNA has been suggested [22].

1.1.4. Cells of the immune system

There is dysregulation of the immune system in SLE with upregulation of B cells, T cells, natural killer cells, monocytes and dendritic cells. The main T cell subsets involved are CD4⁺ T helper (Th) cells and regulatory T cells (Tregs). CD4⁺ T cells are known to regulate B cell autoantibodies via the production of cytokines. Cytokines

including interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 17 (IL-17), type I interferon (IFN), B Lymphocyte Stimulator (BLyS), tumour necrosis factor- α (TNF- α) have all been implicated in SLE pathogenesis.

Neutrophils are also implicated in lupus pathogenesis, characterized by impaired phagocytic ability, increased production of interferon alpha, and increased apoptosis. Neutropenia is common in up to 50% of patients with SLE and is likely to be due to immune-mediated destruction of neutrophils. Neutropenia is associated with the anti-Ro antibody, which can bind and fix complement on the neutrophil surface [24]. The clearance of neutrophils in lupus appears to be impaired [25]. Neutrophils can kill invading microbes by forming neutrophil extracellular traps (NETs). During this process known as NETosis, neutrophil granular proteins and chromatin is released forming extracellular fiber like structures that trap invading bacterial pathogens. This process is dependent on reactive oxygen species (ROS) and requires NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity. Neutrophils in lupus have an increased capacity to undergo netosis and the clearance of NETs appears to be impaired in SLE [26]. Neutrophils release cytokines including BLyS (or BAFF) and APRIL, and can therefore induce B-cell stimulation[27].

B cells play an important role besides the production of autoantibodies. B cells produce cytokines such as IL-6, IL-10, TNF- α , and INF- γ in patients with SLE. Activation of B cells depends on signals transmitted through the B-cell receptor (BCR) and co-receptors, as well as competition for survival factors such as B-cell activating factor (BAFF) [28]. Tregs determine whether a B cell becomes activated or tolerized.

The role of macrophages in lupus nephritis is similar to that of T cells, in that they constitute major infiltrating inflammatory cells in nephritic kidneys. Renal infiltrating macrophages express raised levels of cell markers such as CD11b, CD80, and CD86 and secrete proinflammatory cytokines [29].

Dendritic cells (DCs) are major antigen presenting cells (APCs), which activate antigen-specific T cells. DCs have a crucial role in the control of immunity and tolerance and their uncontrolled activation may be a driver for SLE. The majority of DCs originate from the common myeloid progenitor (MDP) cell, which gives rise to monocytes, plasmacytoid DCs (pDCs) or classical DCs. pDCs are rare in peripheral tissues but they are recruited in to inflammatory sites. They are generally reduced in the blood of patients with SLE due to accelerated migration to inflammatory sites. pDCs preferentially express intracellular Toll-like receptors, including TLR7 and TLR9, that can recognize ssDNA and ssRNA and transduce signals on sensing viral and self-nucleic acids [30]. Immune complexes can activate pDCs to secrete type I IFN through the triggering of TLR7/9, which has proven to be important in SLE pathogenesis [31].

1.1.5. SLE and the environment

Environmental triggers of lupus include ultraviolet (UV) light, drugs and viral infections. UV radiation is the most established environmental factor and the occurrence of a photosensitive rash is included in the American College of Rheumatology (ACR) classification criteria for SLE [32]. Drugs such as hydralazine, procainamide and TNF-antagonists are risk factors for drug-induced lupus [33]. Infections such as EBV have been implicated [34]. Cigarette smoking is also a risk factor for SLE [35]; since smoke increases cellular necrosis. Studies have identified an

association between exposure to petroleum, silica dust, organic solvents, and mineral oils and an increased risk of SLE [36].

1.1.6. SLE and hormones

Approximately 90% of patients with SLE are women, indicating that the female gender is an important factor in lupus development. This contribution may be hormonal or chromosomal. Interestingly, there is an increased incidence of SLE in patients with Klinefelter's syndrome (XXY). Before puberty, the female-to-male 4:1 ratio of SLE prevalence increases to 9:1 after puberty, suggesting a role for female hormones (likely to be due to the presence of oestrogen receptors on immune cells). Studies in animal models of SLE show that oophorectomy delays SLE progression and improves survival in lupus mice [37]. Oestrogens are believed to sustain B-cell autoreactivity [38]. Conversely, testosterone can suppress anti-DNA antibody production [37]. The Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) studies showed that hormone replacement therapy in postmenopausal women with SLE was associated with a small risk of mild-to-moderate flares and that use of combined oral contraceptives in premenopausal women did not significantly increase the risk of flares in women with stable disease. Interestingly, although there is an increased risk of lymphoma in patients with lupus, women with lupus are less likely to develop breast, endometrial and ovarian cancer [39].

1.1.7. Defects in apoptosis and complement regulation

Most clinical manifestations in SLE are a consequence of immune complex deposition. Immune complexes containing IgG autoantibodies can initiate inflammation via activation of immune cells through Fc-gamma receptors as well as complement activation. Defects in complement such as C1q, C2, and C4, are strongly associated with lupus and patients with C1q-deficiency are prone to developing lupus-like disease [40]. The link between SLE and defects in complement is down to their crucial role in the clearance of apoptotic cells. This inefficient clearance of apoptotic cell debris may facilitate inflammation and exposure of autoantigens, resulting in the activation of lymphocytes. Inefficient apoptotic cell removal leads to an increase in nuclear antigens from the superficial surface blebs found on apoptotic cells and thus an increase in anti-nuclear antibodies (ANA).

1.1.8. SLE Criteria

The criteria for SLE classification were initially established in 1971. These were later revised in 1982 [41] and in 1997 [42] by the American College of Rheumatology as shown in Table 1.1. To improve the clinical relevance and to incorporate new knowledge of SLE immunology, the Systemic Lupus Collaborating Clinics (SLICC) revised the ACR criteria in 2012. The SLICC criteria include 11 clinical criteria (e.g. non-scarring alopecia or synovitis) and six immunological criteria (e.g. decreased complement and antiphospholipid antibodies). Photosensitivity is no longer listed in the SLICC criteria. EULAR and ACR have recently updated and presented new 2017 classification criteria for SLE at the annual ACR meeting. These have yet to be confirmed and approved.

Criteria	Definition
Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.
Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging. Atrophic scarring may occur in older lesions.
Photosensitivity	Skin rash as a result of an unusual reaction to sunlight by patient history or physician observation.
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician.
Arthritis	Involving two or more peripheral joints, characterized by tenderness, swelling or effusion.
Serositis	Pleuritis: convincing history of pleuritic chest pain or rubbing heard by a physician or evidence of pleural effusion. <u>OR</u> Pericarditis: documented by electrocardiogram, rub or evidence of pericardial effusion.
Renal disorder	Persistent proteinuria (>0.5g/day or >3+ if quantitation not performed) <u>OR</u> Cellular casts (red cells, haemoglobin, granular, tubular or mixed)
Neurologic disorder	Seizures (in the absence of other precipitating factors) <u>OR</u> Psychosis (in the absence of other precipitating factors)
Hematologic disorder	Haemolytic anaemia with reticulocytosis <u>OR</u> Leukopenia (<4,000/mm ³ on ≥2 occasions) <u>OR</u> Lymphopenia (<1,500/mm ³ on ≥2 occasions) <u>OR</u> Thrombocytopenia (<100,000/mm ³ in the absence of other precipitating factors)
Immunologic disorder	Anti-dsDNA <u>OR</u>

Criteria	Definition
	Anti-Sm <u>OR</u> Anti-Phospholipid antibodies (IgG/ IgM anticardiolipin antibodies) <u>OR</u> positive lupus anticoagulant using a standard method <u>OR</u> False-positive test result for at least six months confirmed by Treponema pallidum immobilization <u>OR</u> Fluorescent treponemal antibody absorption test).
Positive ANA	Abnormal titre of ANA by immunofluorescence <u>OR</u> An equivalent assay at any time point in time and in the absence of precipitating factors (drugs)

Table 1.1: The ACR revised 1997 criteria for the classification of SLE

(Adapted from Hochberg et al. 1997)

2012 SLICC classification criteria for Systemic Lupus Erythematosus

Requirements ≥ 4 criteria (at least 1 clinical and 1 laboratory criteria)

OR Biopsy-proven lupus nephritis with positive ANA or Anti-DNA

Clinical Criteria

1. Acute cutaneous lupus*
2. Chronic cutaneous lupus*
3. Oral or nasal ulcers*
4. Non-scarring alopecia
5. Arthritis*
6. Serositis*
7. Renal*
8. Neurologic*
9. Haemolytic anaemia
10. Leukopenia*
11. Thrombocytopenia ($<100,000/m^3$)

Immunologic Criteria

1. ANA
2. Anti-DNA
3. Anti-Sm
4. Antiphospholipid Ab*
5. Low complement (C3, C4, CH50)
6. Direct Coombs' test

Table 1.2: SLICC 2012 criteria for Systemic Lupus Erythematosus

* See Appendix IV for further details of clinical criteria

1.1.9. Clinical features of SLE

Clinical features include arthralgia, rash, mucosal ulceration, Raynauds phenomenon and photosensitivity. Musculoskeletal features are present in approximately 90% of patients with SLE. Non-specific symptoms also occur including fatigue, in up to 90% of patients [43]. Men with lupus experience rather similar musculoskeletal symptoms compared to women [44]. Raynaud's phenomenon is common (40%) [45]. Approximately 50% of patients with subacute cutaneous lupus and 10% of those with discoid lupus erythematosus meet criteria for SLE [46]. A Hungarian study showed that photosensitivity (20% versus 9%), butterfly rash (61% versus 35.5%) and mucosal ulceration (11.4% versus 4%) were more common in children with SLE than in adults with SLE [47]. Renal involvement occurs in 30-35% of all SLE patients and

diffuse proliferative glomerulonephritis is the commonest histological diagnosis. Renal and cerebral involvement will be discussed further in section 1.1.15.

1.1.10. SLE and Antiphospholipid syndrome

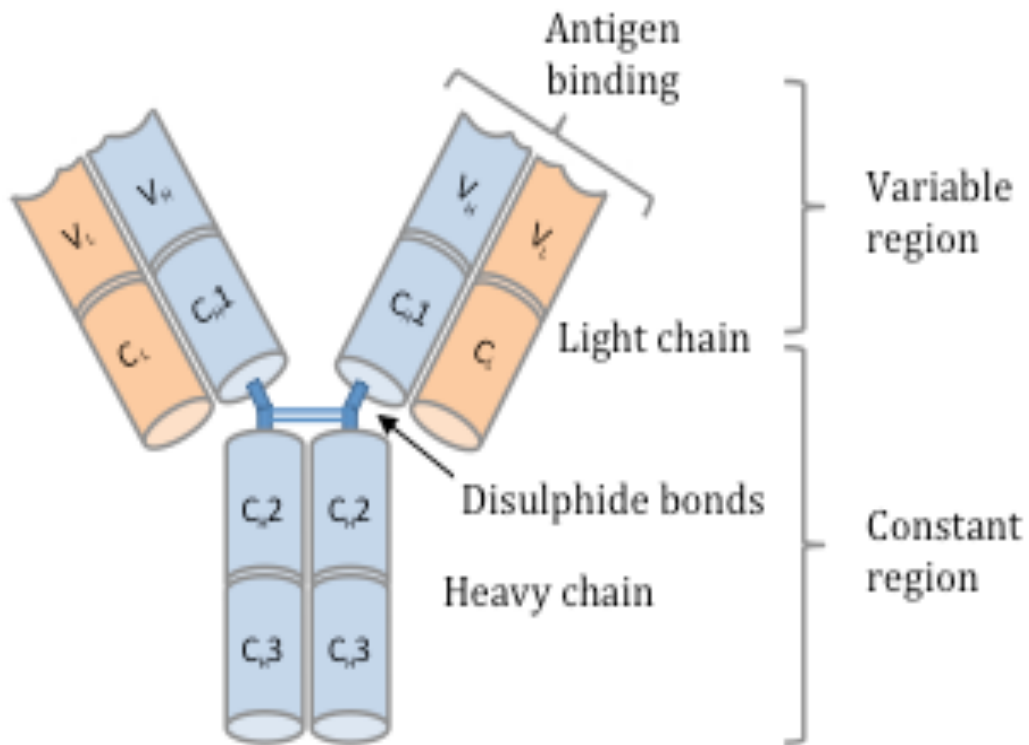
Antiphospholipid antibodies are found in approximately 30-40% of patients with SLE, but only about 10% have APS [48]. Antiphospholipid syndrome (APS) is defined as an association of vascular thrombosis and/or pregnancy complications with presence of antiphospholipid antibodies (aPL), which include lupus anticoagulant (LA), anticardiolipin and anti- β_2 glycoprotein I [49]. It is the most common cause of acquired thrombosis. The pathogenesis of APS is multifactorial. Thrombotic complications in APS can occur in most vessels and numerous pathogenic mechanisms have been implicated. aPL promote thrombosis through inhibition of anticoagulation pathways, impairment of the fibrinolytic system, activation of the complement system, and activation of procoagulant cells [50]. The management of patients with SLE and APS/aPL should include an accurate stratification of vascular risk factors. Low dose aspirin and hydroxychloroquine should be considered as primary prophylaxis. Lopez-Pedrerera and colleagues analyzed the involvement of micro-RNA in atherothrombosis related to patients with APS and SLE. MicroRNA biogenesis was altered in neutrophils and microRNA was associated with atherothrombotic status patients with APS and SLE [51].

1.1.11. SLE and antibodies

Antibodies also known as immunoglobulins (Ig) are large proteins produced by B lymphocytes (or differentiated B cells, known as plasma cells), that neutralize pathogens such as bacteria and viruses and autoantibodies recognize self-proteins. An

antibody recognizes an antigen by its Fab (fragment antibody binding) region. The Fab region is composed of one constant and one variable domain of each of the heavy and light chain, see Figure 1.2. SLE is often associated with hypergammaglobulinemia. IgG antinuclear antibodies (ANAs) have been shown to be elevated in patients with SLE compared with patients with discoid lupus erythematosus (DLE) [52].

ANA are present in over 90% of patients with SLE [9, 53]. Autoantibodies produced against nuclear components of the cell, such anti-double stranded DNA antibodies (anti-dsDNA) are highly specific for SLE. They are present in 70% of patients with lupus and less than 0.5% of healthy controls and their relevance in the pathogenesis of SLE is well established [54]. Anti-extractable nuclear antigens (ENA) are antibodies that target non-DNA antigens. It is thought that ENA are produced by long-lived plasma cells [55]. Subtypes of ENA include anti-Ro/anti-SSA (Sjögren's-syndrome related antigen A), anti-La/anti-SSB, anti-Smith and anti-RNP. Anti-Ro and anti-La antibodies are associated with Sjögren's syndrome that is characterized by dryness in the eyes and mouth, arthralgia and fatigue. Anti-Ro antibodies are associated with an increased risk of congenital heart block in the foetus of pregnant women with lupus, as well as subcutaneous lupus in the newborn [56-58]. Pregnant women with lupus should therefore be screened for these antibodies. Anti-Ro antibodies are correlated with photosensitivity in patients with SLE [59]. Anti-Smith has a prevalence of approximately 10-30% (being much more commonly found in Afro-Caribbean patients than Caucasians), and its presence is considered pathognomonic of SLE [55, 60, 61]. Anti-Smith antibodies are associated with severity of renal involvement. There has been suggestion that EBV has the potential to induce Anti-Smith antibodies [62]. Anti-RNP antibodies are found in 25-47% of patients with SLE and are more prevalent in patients with Raynauds phenomenon and are associated with milder renal involvement [62].

**Key**C_H – Constant heavy chainC_L – Constant light chainV_H – Variable heavy chainV_L – variable light chain**Figure 1.2: Schematic representation of Immunoglobulin G**

This figure illustrates the basic structure of an IgG antibody. The constant region determines the class and subclass of the antibody, and the variable domains, which bind antigen. An antibody recognizes an antigen by its Fab (fragment antibody binding) region. The Fab region is composed of one constant and one variable domain of each of the heavy and light chain.

1.1.12. Laboratory features

Standard laboratory tests that are of diagnostic use in patients with SLE include full blood count (FBC) with differential, urea and electrolytes (U&E), and liver function tests (LFTs). Inflammatory markers including erythrocyte sedimentation rate (ESR) may be raised due to increased disease activity. Measurement of complement is useful as C3 and C4 levels are often low in patients with active disease due to consumption by immune-complex-induced inflammation. LFTs may be mildly elevated in acute SLE or secondary to medications such as non steroidal anti-inflammatory drugs (NSAIDs) or azathioprine. Creatinine kinase (CK) may be elevated in myositis or in overlap conditions. Urine protein creatinine ratio (UPCR) is used to quantify proteinuria. The 2012 ACR guidelines for lupus nephritis suggest that a spot UPCR can substitute for the 24-hour protein measurement and that an active urinary sediment (defined as >5 red blood cells (RBCs) or >5 white blood cells (WBCs) per high powered field (hpf) in the absence of infection, can substitute for cellular casts.

1.1.13. Treatment for SLE

A multidisciplinary approach is essential to ensure quality care for patients with SLE. Patients should ideally be treated in a specialist centre with easy access to other specialists such as nephrologists, neurologists and dermatologists. In addition, involvement of general practitioners, physiotherapists, psychologists and nurse specialists is key to ensuring optimal care. Early and aggressive treatment is crucial in management of SLE in order to prevent irreversible damage of organs. Avoidance of ultraviolet (UVA and UVB) exposure is advised because UV radiation leads to cellular release of DNA, which can trigger lupus [63]. Therefore patients with SLE should avoid the sun and wear high factor (50 plus) sunscreen. In view of this recommendation it is important patients avoid becoming vitamin D deficient.

Treatment of lupus depends on the severity of disease ranging from some patients being treated with low dose hydroxychloroquine (HCQ) only, whilst others require other immunosuppressive or biologic therapies, often in combination, for life-threatening disease. Glucocorticoids are frequently prescribed during a lupus flare with efforts to wean off as soon as possible.

1.1.14. Mild/Moderate SLE

NSAIDs are helpful for musculoskeletal disease, but have significant side effects and should be avoided in those with lupus nephritis. Hydroxychloroquine (HCQ), an anti-malarial, is recommended for all patients with SLE provided there are no contraindications. It is helpful in skin, joint and renal disease and can prevent flares of SLE. HCQ inhibits Toll-like receptors (TLR) and has effects on pro-inflammatory cytokines and therefore reduces disease activity [64]. Binding of HCQ to nucleic acids, masks their TLR-binding epitopes and therefore prevents TLR activation [65]. HCQ also reduces low-density lipoprotein levels in SLE and has been shown to be cardioprotective [66]. A Spanish study of 232 patients with SLE, showed that HCQ might also be protective against thrombosis [67]. HCQ is particularly useful in patients with SLE and secondary APS. Hydroxychloroquine is safe in pregnancy and women with SLE are advised to continue on this medication [68] [69]. Azathioprine is a purine analog that is used as a steroid-sparing agent in mild-moderate SLE, especially in patients who are having recurrent flares while already treated with HCQ. Methotrexate is useful for predominant arthritis in SLE [70].

1.1.15. Renal/cerebral disease

Approximately 30-35% of patients with lupus will have clinically overt renal involvement. Lupus nephritis can be aggressive and the threshold for performing a renal biopsy should be low. Any sign of renal involvement such as proteinuria ≥ 0.5 g/24h, especially with glomerular haematuria and/or cellular casts is an indication for biopsy [71]. Mycophenolate mofetil (MMF) has been shown to be as effective for severe renal lupus (grade III and grade IV) and have less side effects than cyclophosphamide (CYC) [72].

Neuropsychiatric SLE (NPSLE) includes heterogenous manifestations involving both the central nervous system (CNS) and peripheral nervous system (PNS) [73]. It can present in various different ways from mild headaches or cognitive dysfunction to seizures, stroke or coma. Psychosis is uncommon and usually occurs early in the course of the disease. In the UCLH cohort, psychosis was diagnosed in 2.3% of patients [74].

CYC is used only in severe SLE, weighing up the risks of infection, premature menopause, infertility, haemorrhagic cystitis and malignancy. Poor adherence with oral CYC is a major concern and therefore IV CYC is preferable [75]. The National Institute of Health (NIH) protocol is whereby CYC ($0.5-1\text{gm/m}^2$) is given monthly for six months and then quarterly for at least six months. The Euro Lupus Nephritis Trial protocol seems to be as effective and involves six pulses of a fixed dose of 500mg given every fortnightly for a cumulative dose of 3gms, followed by azathioprine as a remission maintenance agent [76]. Cyclophosphamide is the preferred treatment for cerebral lupus.

1.1.16. Biologics

B cell depletion

B cell depleting drugs such as rituximab and ofatumumab, which target anti-CD20 monoclonal antibody have been used in patients with severe refractory SLE [77]. Ofatumumab has also been used in patients who have had infusion reactions to rituximab. Despite the failure of rituximab in the EXPLORER and LUNAR trials [78, 79] rituximab is highly efficacious in many patients and is used regularly to treat lupus [80]. In the EXPLORER study, the safety and efficacy of rituximab in moderate-to-severe nonrenal SLE was evaluated. This study included 257 patients with ≥ 1 BILAG (British Isles Assessment Group) A or ≥ 2 BILAG B scores despite ongoing stable-dose immunosuppressant therapy with either, azathioprine (100 to 250 mg/day), mycophenolate (1 to 4 g/day) or methotrexate (7.5 to 25 mg/week), which was continued during the trial. The BILAG score refers to disease activity in SLE where A is 'highly active' and E is 'never active', and is explained further in section 1.1.19. In this study, patients in both the rituximab and placebo arm received high-dose corticosteroids.

The LUNAR study included 144 patients with class III and IV proliferative nephritis. This investigated the safety and efficacy of 2 x 1,000 mg of rituximab, at 0 and 6 months, compared with placebo in addition to background therapy with MMF 3 g/day and high dose glucocorticoids. The primary endpoint was the proportion of patients with a complete or partial remission of nephritis at twelve months. Confounding factors are likely to have led to the failure of these trials, may include high dose of corticosteroids used, stringent and nonorgan-specific clinical response criteria, too short a follow-up and the sample size. In spite of the problems, it is notable that the ACR and EULAR guidelines for the treatment of lupus nephritis recommend rituximab and it is approved by NHS England. Rituximab has also been used in SLE to treat

concomitant Immune Thrombocytopenia Purpura (ITP) and autoimmune haemolytic anaemia.

Blocking B cell activity factors

Belimumab is a human IgG1 λ recombinant monoclonal antibody to the soluble human B lymphocyte stimulator protein (BLyS) and is the first licensed drug in over sixty years for SLE [81]. Belimumab was approved in 2011 as the first targeted biologic treatment for SLE [82]. Two multicenter phase III trials, BLISS-52 and BLISS-76, evaluated the efficacy and safety of belimumab in patients with SLE.

In BLISS-52, 865 patients were included. Improvement in the SRI (SLE responder index) at week 52 was the primary efficacy endpoint. SRI rates were significantly higher in the belimumab 1 and 10 mg/kg group than with placebo at the end of week 52 (51% and 58% vs. 44%). No significant difference was found between belimumab and placebo with respect to adverse effects [81]. In BLISS-76, 819 patients were recruited for 76 weeks. The patient response rates measured by SRI at week 52 and week 76, were the primary and secondary endpoints, respectively. No significant SRI improvement was seen with belimumab 1 mg/kg compared to placebo (40.6% vs. 33.8%). The improvement was significantly higher in 10 mg/kg belimumab group than placebo at week 52 (43.2% vs. 33.8%) but could not be sustained later and the difference at week 76 was not statistically significant [82].

Atacicept has recently shown encouraging results. Atacicept is another antagonist of BlyS which blocks BlyS from APRIL-mediated B cell activation. The phase IIb ADDRESS II study involved 306 patients with SLE. This was a multicentre, randomised, double-blind placebo-controlled trial. Patients with active SLE were randomised to receive Atacicept (75mg or 150mg) or placebo for 24 weeks. The

primary end point was the SLE responder index 4 (SRI-4) at week 24. Atacicept was shown to be particularly effective in patients who had high disease activity and it had an acceptable safety profile [83].

Anifrolumab [84] is another potentially promising lupus treatment. Anifrolumab is an anti-interferon- α receptor monoclonal antibody has been studied in a phase IIb trial. Patients with moderate to severe SLE (n=305) were randomised to receive IV anifrolumab (300mg or 1gm) or placebo in addition to standard therapy, every 4 weeks for 48 weeks. Anifrolumab reduced disease activity compared with placebo. However, herpes zoster occurred more frequently in the Anifrolumab group.

1.1.17. Other treatment

Intravenous Immunoglobulin (IVIG) and plasma exchange are useful in acute life-threatening disease [85] [86]. IVIG was the first immunosuppressive therapy targeting Fc γ R functions. Purification of Immunoglobulin (Igs) was originally developed and used to treat Ig deficiency and recurrent bacterial infections. Anti-inflammatory immunomodulation also occurs [27].

Hematopoietic stem cell transplantation (HSCT) was first reported in patients with SLE in 1997. However, it has not been widely used due to potential fatal side effects. Burt and colleagues in Northwestern University have conducted stem cell transplants on adults with active treatment resistant lupus [87]. They found that 50% of 48 patients, who underwent HSCT, had disease-free survival at five years with an overall five-year survival rate of 84%. HSCT [88] has also been used for children with severe life-threatening JSLE [89, 90]. A systematic review in 2017, showed that the results of HSCT as a therapeutic option appear promising but because of side effects should only be used in select patients [91].

1.1.18. Biomarkers for SLE

Management of SLE is challenging due to its heterogeneous clinical features, the unpredictable disease course and the difficulty in detecting flares early. Studies have shown that even experienced rheumatologists may differ in their clinical judgment regarding disease activity. For instance, in the study assessing lupus flare in 16 active lupus patients, comparing the BILAG-2004 index and the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) flare instrument, three experienced rheumatologists were at odds with thirteen other rheumatologists in their clinical judgment of disease flare [92]. Severe flare was associated with good agreement between disease activity indices, but mild to moderate flare was much less consistent. Adding to the complexity of measuring disease activity in SLE, some of its features may resemble other conditions. For example, patients often have chronic pain, depression, fever, lymphadenopathy, fatigue or headaches, which may or may not be lupus related. Unfortunately there is no one biomarker that uniformly reflects disease activity in SLE. Clinicians rely on clinical history, examination, bloods, ESR, CRP, C3, C4, dsDNA levels and urinary protein creatinine ratio to monitor disease activity. However, anti-dsDNA antibodies, complement levels and inflammatory markers are not always abnormal in active disease. Novel biomarkers would help in identifying SLE, disease monitoring, and prediction of flares. In particular, biomarkers detecting specific organ involvement, such as risk of development of CVD in lupus would be beneficial.

The National Institute of Health (NIH) Biomarkers Definitions Working Group defines biomarkers as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [93].

SLE biomarkers that have been investigated more recently include MicroRNA, Type 1 Interferon, cell adhesion molecules and complement components. Circulating erythrocyte E-C4d levels appeared higher in patients with lupus nephritis compared to healthy controls [94]. Monocyte chemoattractant protein-1/CCL-2, neutrophil gelatinase-associated lipocalin, urine protein signature and colony-stimulating factor 1 have been researched as biomarkers in lupus nephritis. However, these biomarkers lack validation and need testing in large multicentre studies prior to their use in clinical practice.

1.1.19. Disease activity indices in SLE

Since the initial development of the Classic BILAG index in 1984, major improvements have been made in measurement of disease activity in lupus. The Classic BILAG index focuses on disease activity in eight organs or systems: constitutional, mucocutaneous, neurological, musculoskeletal, cardiovascular/respiratory, vasculitis, renal and haematological [95]. The Classic BILAG index has been recommended for use in clinical trials and has been used worldwide. It has been shown to be reliable, comprehensive and sensitive to change. The optimization of the Classic BILAG index, which evolved into the more sophisticated BILAG-2004 index, has proved to be a sensitive and reproducible tool [96]. The BILAG-2004 index includes an assessment of the ophthalmic and gastrointestinal systems and removal of damage features such as avascular necrosis and calcinosis. The exclusion of fatigue and migraine from the Classic BILAG index, which can be difficult to interpret and are common in those who have active or inactive lupus, further improved the accuracy of the BILAG 2004 index. The updated glossary and terminology made the BILAG-2004 index more user-friendly than the Classic BILAG index. Disease activity is graded between A (highly active) and E (never active) for each system of the body.

A numerical score can be derived from the BILAG providing a global disease activity score. A global BILAG score was initially designed to be calculated from the formula A=9; B=3; C=1; D=0 and E=0 [97]. This scoring system for the Classic BILAG was subsequently optimised to A=12; B=8; C=1; D and E=0 [98]. The BILAG-2004 uses the scoring system A=12; B=8; C=1; D and E=0.

The BILAG-2004 index is one of the only disease indices to undergo such extensive and thorough analysis using robust statistical methods. It is used worldwide in longitudinal studies and major clinical trials. It is based on the physician's intention to treat and has been shown to measure SLE disease activity better than the SLEDAI-2000 [99]. To date, there is no international consensus as to which disease activity index should be used in clinical trials. The complexity of this multi-systemic disease and lack of biomarkers made it exceedingly difficult to develop an accurate disease index. The BILAG-2004 index is the only transitional index that grades disease activity as either being new, the same, worse or improving and incorporates severity into the scoring. The evolution of SLE disease indices is further explained in a paper I published during the course of my MD 'From BILAG to BILAG-based combined lupus assessment-30 years on', see Appendix 1.1, page 260.

1.2. SLE and cardiovascular disease

Patients with SLE are at greater risk for CVD compared to healthy controls. CVD causes 25% of deaths in patients with SLE. This mortality occurs despite management of modifiable risk factors such as smoking, blood pressure and cholesterol. Increased CVD risk in SLE is therefore not fully explained by traditional risk factors [100, 101] and other disease related factors such as persistent inflammation, autoantibodies and microparticles (MPs) [102] have been implicated [103-106]. Women with SLE have a 5-10-fold increased risk of developing CVD compared to age and sex-matched controls [107]. The average age of developing a first CVD event in patients with SLE is 49. CVD has considerable impact upon patients with SLE and is a major contributor to morbidity, fatigue, inability to work and premature mortality. Patients with SLE have an increased risk of arterial and venous [107, 108] thrombotic events. Imaging studies show that patients with SLE have a much higher prevalence of asymptomatic atherosclerotic plaques than age and sex-matched controls [109, 110]. A recent study of 281 patients with lupus and controls showed that subclinical atherosclerosis as measured by mean intima media thickness (IMT) and plaque via carotid ultrasound, was more common in patients with lupus nephritis [111].

Consideration of non-traditional immunological factors and disease activity may hold the key to understanding why CVD risk is increased. It is therefore imperative to gain a better understanding of why accelerated atherosclerosis occurs more frequently in patients with SLE. This knowledge should help develop measures to assess and prevent CVD in patients with SLE. As physicians we cannot alter the non-modifiable risk factors (e.g. age, gender and family history of CVD) but we can improve our management of the modifiable risk factors, for example smoking, hypertension and cholesterol levels. However these factors do not fully explain the reason for CVD in

patients with lupus. My research was focused on examining non-traditional risk factors, which may link CVD and lupus. The areas I chose to study include anti-serine protease antibodies, endothelial and platelet microparticles, adverse pregnancy outcomes and osteoporosis associated with CVD.

1.2.1. Pathogenesis of CVD.

Atherosclerosis is a chronic inflammatory process whereby an arterial wall thickens as a result of invasion and accumulation of macrophages and T-lymphocytes “fatty streaks” and intimal smooth-muscle cells (SMCs) proliferate creating an atheromatous plaque. Raised low-density lipoproteins (LDL) levels and reduced HDL level are well-established risk factors in the general population. Endothelial dysfunction may occur secondary to oxidized LDL or free radicals caused by smoking, hypertension, diabetes, or raised homocysteine levels. Combined with a predisposing genetic background this process may lead to endothelial damage and atherosclerosis [112]. Following endothelial dysfunction, there is increased endothelial permeability mediated by nitric oxide (NO), prostacyclin, platelet-derived growth factor (PDGF) and angiotensin II as well as an increased adhesiveness, mainly mediated by L-selectin, E-selectin, P-selectin and intercellular adhesion molecule 1 (ICAM1).

Atherosclerotic plaque may be stable or unstable. Stable plaques are generally rich in extracellular matrix (ECM) and SMC and are asymptomatic. Unstable plaques are rich in macrophages and foam cells (fat laden macrophages) and the fibrous cap is usually weak and vulnerable to rupture. Ruptures of the fibrous cap expose collagen and other thrombogenic material to the circulation and can lead to thrombus formation in the lumen [113]. These thrombi can occlude arteries or detach and move into the circulation occluding smaller blood vessels. The gradual build-up of atherosclerotic plaque is asymptomatic, but plaque rupture or endothelial erosion can cause

thrombus formation, leading to myocardial infarction (MI) or stroke [114]. The term atherothrombosis, is characterized by atherosclerotic lesion disruption with superimposed thrombus formation, is a leading cause of mortality [115]. Inhibition of tissue factor or Factor Xa (FXa) or thrombin may have the potential to improve outcomes in atherothrombosis [116]. Platelets also play an important role in atherothrombosis. The endothelium releases von Willebrand factor (VWF), which leads to platelet recruitment to the plaque site. Platelets bind to the endothelial cells via an interaction between glycoprotein I and VWF. This interaction leads to platelet activation and production of vasoactive molecules such as thromboxane A₂, adenosine and thrombin as well as other proinflammatory mediators like PDGF, which in turn are involved in the recruitment and activation of inflammatory cells see Figure 1.3.

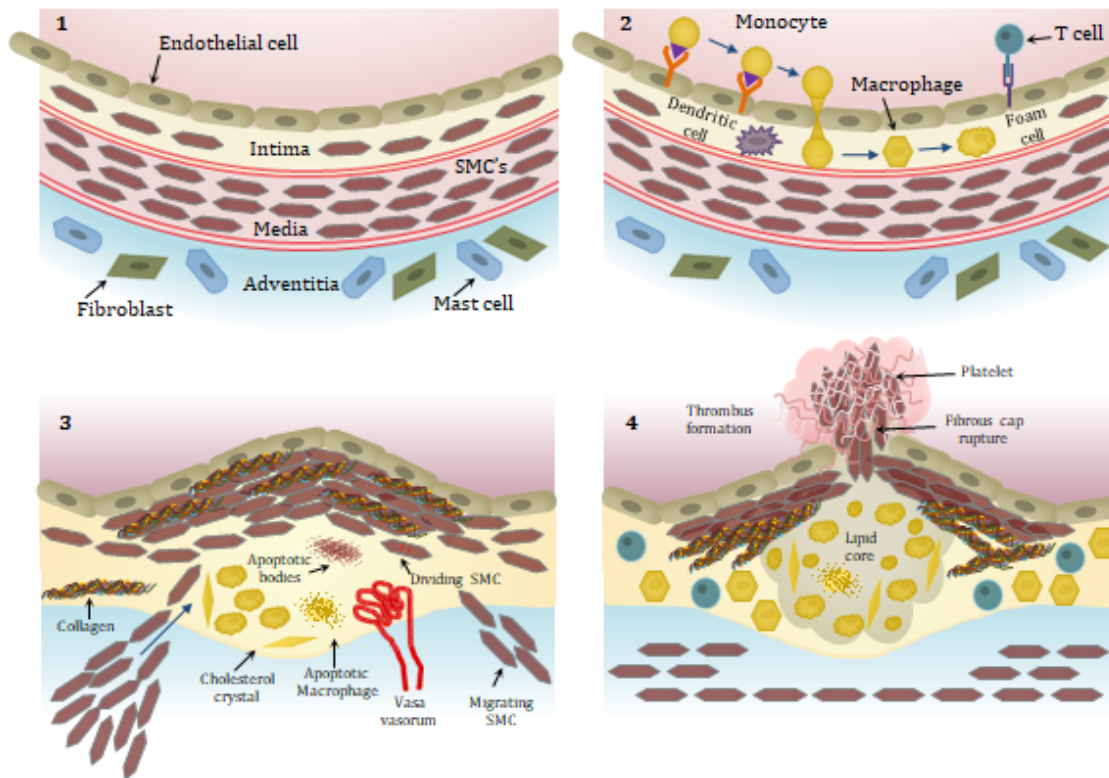


Figure 1.3: Stages of atherothrombosis

Diagram 1: This shows the arterial wall including the endothelium, intima, media with smooth muscles cells (SMC's) and adventitia. Mast cells and fibroblasts are present in the adventitia.

Diagram 2: Monocytes, macrophages invade into the intima. Macrophages transform into foam cells.

Diagram 3: Further invasion of cells into the media occurs. Activation of SMCs, apoptotic bodies, apoptotic macrophages and subsequent build up cholesterol and collagen.

Diagram 4: The formation of the lipid core, fibrous cap rupture and thrombus formation. (Adapted from Libby et al. [117])

1.2.2. Vascular imaging in cardiovascular disease

There are multiple imaging modalities used to assess CVD in patients with SLE [118]. Methods such as computed tomography (CT) are considered unsuitable for assessing asymptomatic patients due to radiation exposure. The sensitivity of cardiac magnetic resonance (MR) for assessing subclinical myocardial dysfunction is undisputed, but its cost prevents more generalised use [119-121].

Carotid ultrasound is a non-invasive and accurate method for assessing atherosclerosis through the measurement of the intima media thickness (IMT) and plaque. An increase in IMT is the result of intimal thickening, smooth muscle hyperplasia and intimal fibrocellular hypertrophy. These features are traditionally seen in arteriosclerosis associated with age-related sclerosis and systemic hypertension [122]. Carotid ultrasound allows measurement of arterial wall thickness and the detection of plaque, its location, size and nature each of which has a strong positive correlation with CVD-related events. For the purposes of this study, 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 >1.2 mm as measured from the media-adventitia interface to the intima-lumen interface” [123, 124]. Grey-scale median (GSM) was the underlying texture analysis used to assess plaque. GSM was calculated as a numerical value, with lower values corresponding to more echolucent plaques i.e. lipid-rich plaques.

1.2.3. The carotid and femoral arteries

The two common carotid arteries are the main arteries that supply the head and neck. The right CCA originates at the bifurcation of the innominate artery whilst the left CCA emerges from the aortic arch. Both CCAs balloon out forming the carotid bulb and

then divide into their main branches, the external carotid artery (ECA) and internal carotid artery (ICA). The ECA supplies the exterior of the head and neck as well the face and eye region. The ICA supplies the brain and intra-orbital cavity and communicates with the Circle of Willis. The common femoral artery (CFA) divides into the superficial and the deep femoral artery at groin level. The superficial femoral artery runs along the inside of the thigh and divides into the calf arteries in the popliteal region, whereas the deep femoral artery divides proximally, eliciting the smaller arteries that supply the muscles of the thigh and buttock [125].

The CCA is relatively long and straight and therefore blood flow is usually laminar, minimizing shear-stress forces on the arterial wall. The bulb region is characterized by turbulent flow due to the anatomical divide. The change in blood flow increases the likelihood of endothelial damage and consequently increased cholesterol deposition and plaque formation. The CFA bifurcation differs from that of the CCA as it does not dilate before dividing.

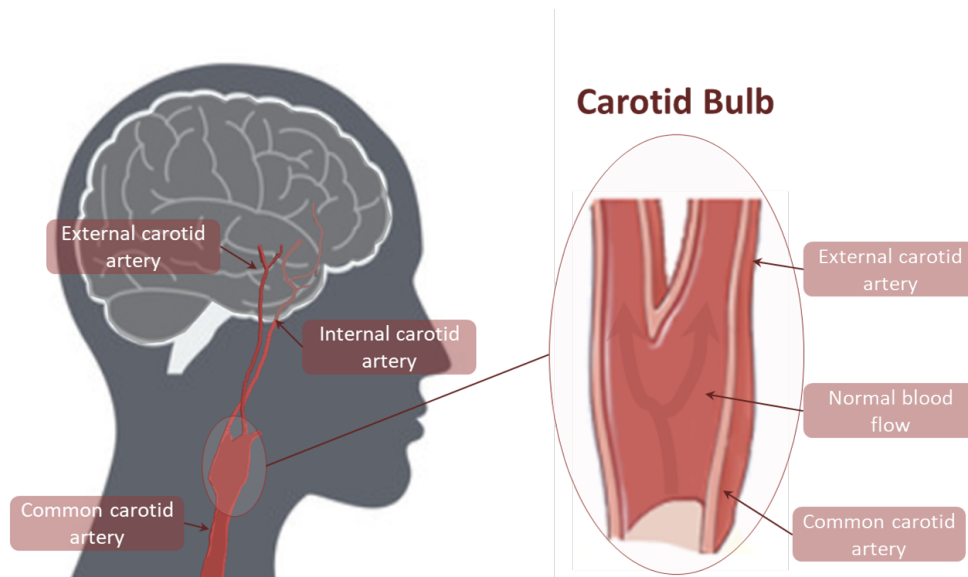


Figure 1.4 Overview of the carotid territories

Common carotid artery (CCA) and its two main branches the internal and external carotid arteries. The carotid bulb is shown in detail.

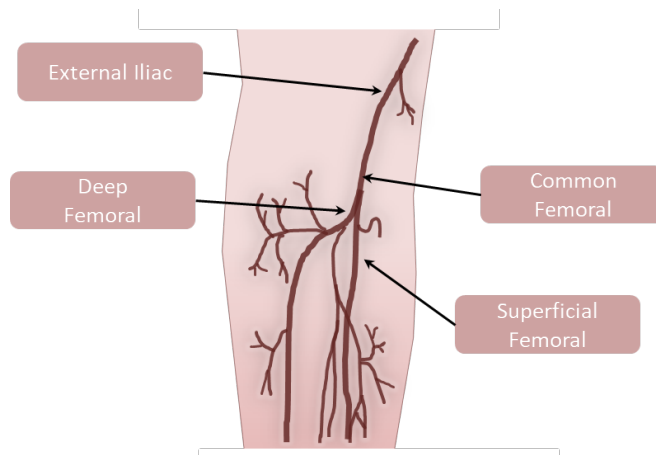


Figure 1.5 Common femoral artery (CFA)

Common femoral artery division into deep and superficial arteries

1.2.4. Antibodies associated with cardiovascular disease and SLE

Antibodies have been implicated in both SLE and CVD pathogenesis. Defective clearance of dying cells in SLE combined with the production and exposure of autoantigens during apoptosis have led to the belief that improperly cleared apoptotic debris constitutes a source of autoantigens capable of triggering autoimmune disease. Antibodies such as anti-high density lipoprotein (anti-HDL) and anti-apolipoprotein A1 (anti-apoA1) [126] and aPL have all been implicated as possible modulators of increased CVD risk in patients with SLE. Interestingly, anti-oxidized LDL (oxLDL) antibodies have been found to be both pathogenic and protective in CVD [127]. Titres of anti-apoA1 are significantly higher in SLE patients with persistent disease activity and correlate inversely with activity of paraoxonase, a key enzyme that gives HDL its anti-oxidant properties [128]. Some studies show a positive association between aPL and carotid atheroma ([106, 129, 130] whilst others have not [131, 132]. In contrast the precise relationship of these autoantibodies with development of CVD in patients with SLE is also uncertain.

1.2.5. Biomarkers in SLE and CVD

There is an unmet need for biomarkers that could identify patients with SLE and subclinical atherosclerosis who may benefit from preventive strategies. Traditional biomarkers often used include cholesterol, triglycerides, LDL and HDL. Non-traditional biomarkers rarely used, include pro-inflammatory HDL, High sensitivity C-reactive protein, apolipoprotein A-1 and soluble TWEAK. Soluble TWEAK is a proinflammatory cytokine belonging to the TNF superfamily. It has been shown to be linked to increased rates of atherosclerosis, inflammation, angiogenesis, and apoptosis [133].

Adipokine cytokines like leptin, adiponectin and ghrelin may be important in the development of atherosclerosis in patients with SLE and in healthy subjects. Low adiponectin and raised leptin concentration have been proposed as biomarkers for patients with renal disease and the metabolic syndrome [134]. Marjon et al. examined adipokines and leptin levels in 35 children as a potential for CV risk in JSLE. One third of patients with JSLE had raised leptin levels. Adiponectin concentrations were associated with prednisolone dose and not associated with raised disease activity scores [135].

Platelet-bound C4d has potential utility in predicting stroke risk and severity. In a cohort of 356 SLE patients, platelet-bound C4d levels were associated with all-cause mortality and ischemic stroke, even after adjustment for age and presence of aPL [136].

A recent multicenter, longitudinal study assessed whether a panel of CV biomarkers (PREDICTS profile) predicted carotid plaque in 210 women with SLE and 100 sex and age-matched healthy controls. The PREDICTS profile included proinflammatory HDL, leptin, soluble TWEAK, homocysteine and traditional cardiac risk factors. A high risk profile defined by presence of ≥ 3 biomarkers (or ≥ 1 biomarker with a diabetic

history) predicted incident carotid plaque with 81% sensitivity and 79% specificity and had a positive predictive value of 40% with a negative predictive value of 95%. A high risk PREDICTS profile increased the odds of carotid plaque by 38-fold in SLE patients and correlated with progression of carotid intima medial thickening [137].

1.3. Factor Xa, thrombin and cardiovascular disease

The importance of the immune system in the pathogenesis of CVD has only relatively recently been appreciated. The term 'atherothrombosis' is now used frequently in the description of CVD. Factor Xa (FXa) and Thrombin (Thr) are serine proteases (SP), which are well known for their role in the coagulation cascade, (see Figure 1.6) and have also been found in atherosclerotic lesions. FXa has been found on various cells including endothelial cells, macrophages, foam cells, intimal smooth muscle cells (SMC), medial SMC and the vasa vasorum [138]. Yusuke et al. showed in 2015 that Factor Xa aggravates atherosclerosis by promoting inflammasome formation through suppressing autophagy. Autophagy is a self-degradative process that leads to proteolytic degradation of cytosolic components at the lysosome [139]. In 2017, Moran et al. demonstrated that parenteral administration of factor Xa/IIa inhibitors limits aortic aneurysm and atherosclerosis in Apolipoprotein E deficient mice [140]. Other components of the coagulation cascade including tissue factor (TF) and FVII are also expressed in macrophages and vascular smooth muscle cells within arterial vessel wall and atherosclerotic lesions. Direct inhibitors of FXa have been shown to reduce pro-inflammatory effects and stabilise atherosclerotic plaque. Fondaparinux, a selective FXa inhibitor, has been shown to promote the stability of atherosclerotic lesions in apolipoprotein E-deficient mice [141]. This effect appears to occur via inhibition of the expression of the inflammatory mediators in plaque and reduced synthesis of MMP-9 and MMP-13 [141].

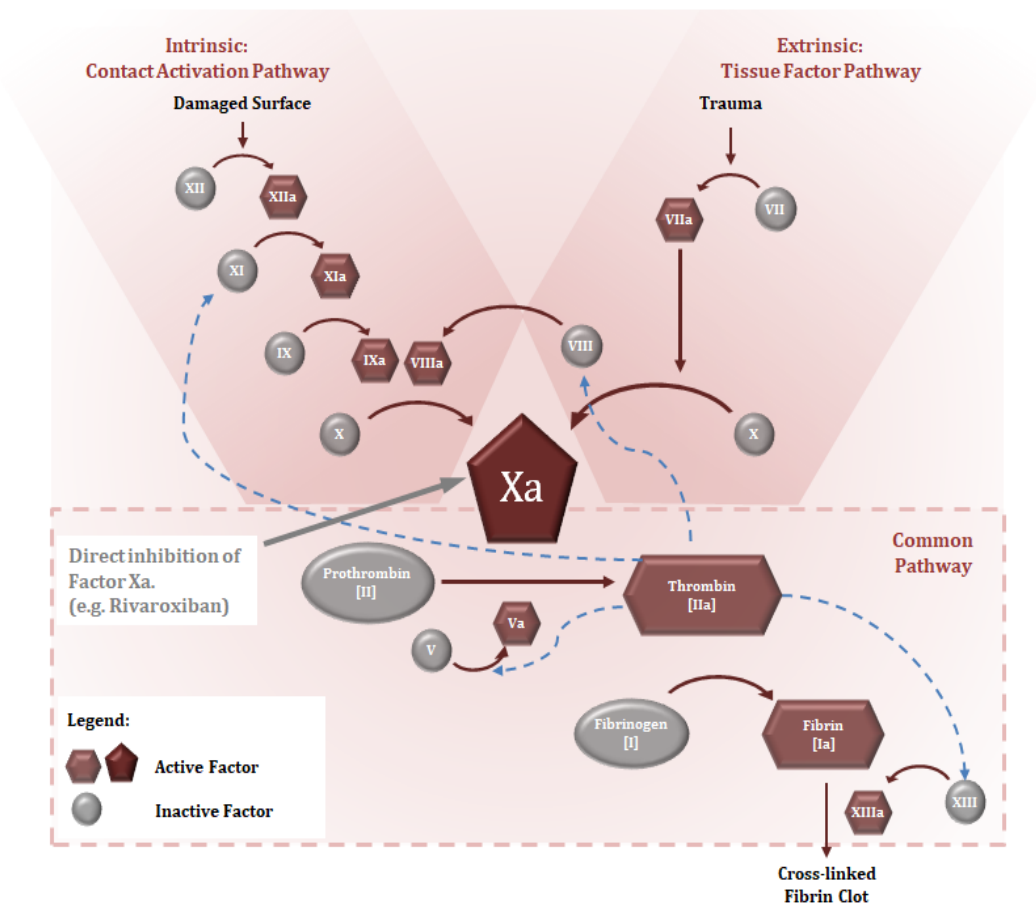


Figure 1.6: Coagulation cascade

The coagulation cascade has been traditionally classified into intrinsic and extrinsic pathways, both of which converge on factor X activation. The main role of the extrinsic, also known as the tissue factor (TF) pathway, is to generate thrombin. Following damage to the blood vessel, FVII leaves the circulation and comes into contact with TF forming an activated complex (TF-FVIIa), which initiates downstream effects. The intrinsic or contact activation pathway begins with the formation of the primary complex on collagen by high-molecular-weight kininogen (HMWK), prekallikrein, and FXII. Pre-kallikrein is converted to kallikrein and FXII becomes FXIIa. Following activation by the extrinsic and intrinsic pathways, the coagulation cascade is maintained in a prothrombotic state by the continued activation of FVIII and FIX to form the tenase complex, until it is down-regulated by the anticoagulant pathways. Activated factor X along with factor V, phospholipids and calcium forms the

prothrombinase complex, which converts prothrombin to thrombin. Thrombin further cleaves fibrinogen to fibrin and activates factor XIII, which crosslinks fibrin polymers incorporated in the platelet plug. This creates a fibrin network, which stabilises the clot.

1.3.1. Factor Xa and inflammation

FXa has pleiotropic effects beyond regulation of haemostasis, see Figure 1.6 (page 53) and vascular injury [142] with important inflammatory properties now being recognized. FXa acts directly on a variety of cells via interactions with a family of receptors known as proteinase activated receptors (PARs) that are described in section 1.3.2. FXa activates PAR1/2 on various cells to modulate inflammation and thrombosis [143]. The FXa-PAR interaction triggers calcium oscillation, along with phosphorylation of intracellular kinases including mitogen-activated protein kinases (MAPKs), extracellular signal-related kinases (ERK) and c-Jun N-terminal kinase (JNK), leading to initiation of different transcriptional programmes. This process promotes cell growth and differentiation, as well as migration, and induction of genes encoding cytokines (such as interleukin-6 (Il-6), interleukin-8 (Il-8) and monocyte chemoattractant protein-1 (MCP-1) as well as receptors such as ICAM (intracellular adhesion molecule) and other proteins.

1.3.2. Proteinase activated receptors

Four PARs (1-4) exist [144] and are activated by SP, including thrombin and FXa. PAR 1 and 2 are ubiquitously expressed on a wide variety of cells; including fibroblasts, mononuclear leucocytes, epithelial cells, neurons, aortic smooth muscle cells and endothelial cells, such as human umbilical vein endothelial cells (HUVEC), see Figure

1.7. Increased levels of anti-SP antibodies have been found in patients with APS and SLE [145-147] and increased expression of PARs have been found in antiphospholipid syndrome (APS) monocytes [148] and a murine model of APS [149]. PAR 1 is the main receptor for thrombin. Previous research at UCL found that increased levels anti-FXa IgG (48.3%) were found in patients with SLE compared with controls (0%) and that purified polyclonal IgG from anti-FXa positive patients had differential avidity to FXa, as well as effects upon the enzymatic, coagulant and PAR mediated cellular activity of FXa compared with control IgG. In contrast, anti-Thr IgG were identified in other autoimmune rheumatic disease [150-152].

PAR activation influences a wide range of physiological responses, such as platelet activation, intimal hyperplasia, maintenance of vascular tone and endothelial barrier protection. Therefore, overactivation of PARs may contribute to inflammation, alterations in vascular tone and disruption of endothelial barrier protection, thus increasing permeability and contributing to the pathogenesis of CVD [153]. Given the central role of FXa in mediating inflammation and thrombosis via PAR activation, I wished to analyse the relationship of polyclonal IgG with anti-FXa positivity and development of subclinical CVD.

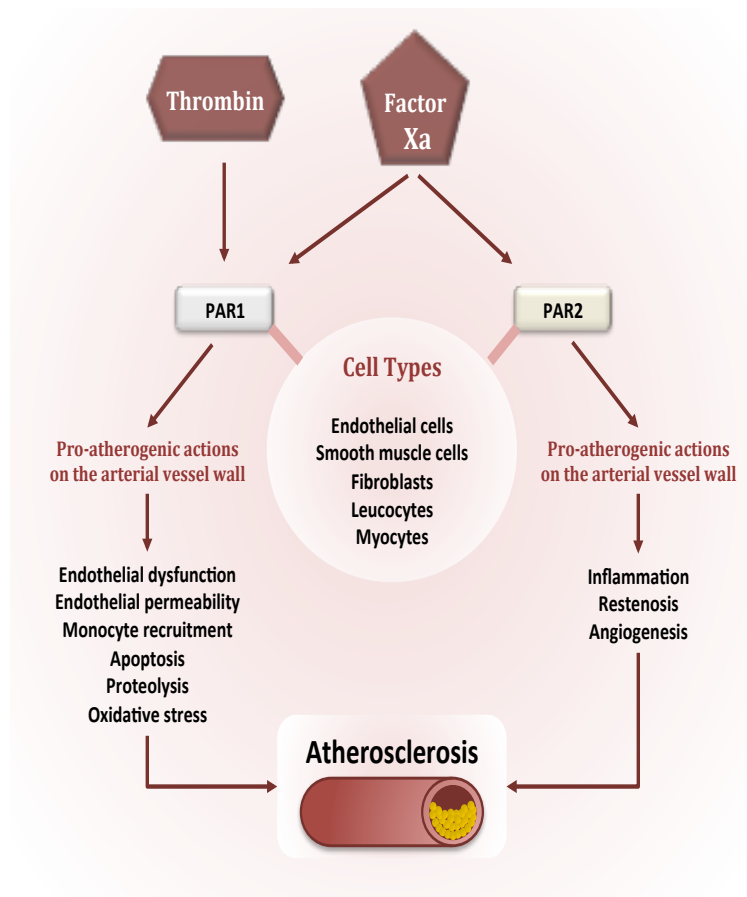


Figure 1.7: The effects of FXa and Thrombin-mediated PAR activation on the arterial wall

This schematic shows the cellular locations of PAR 1 and PAR 2 and how the interaction of FXa and thrombin with PAR1 and PAR 2 can contribute to atherosclerosis.

1.3.3. SLE and thrombosis

Patients with SLE are at an increased risk of arterial [107, 108] and venous [154, 155] thrombotic events which are not fully explained by traditional thrombotic risk factors [100]. This increased thrombotic risk may be partly explained by the presence of autoantibodies in SLE [128], particularly aPL which occur in 20-40% of patients with SLE [58, 156, 157]. APS is an important cause of: stroke and myocardial infarction, especially in people under 50 years old [158, 159]; and recurrent miscarriage [160, 161]. Despite current treatment of long term anticoagulation to reduce vascular thrombosis [162] the incidence of thrombotic complications in APS remains high. Pathogenic aPL bind phospholipid-binding proteins, particularly β 2-glycoprotein I (β 2-GPI) [163, 164], to interact with cells including monocytes, endothelial cells (EC) and trophoblast cells leading to activation of intracellular signalling pathways [165] and the clinical manifestations of APS. Other targets however, such as complement and PARs [166, 167] have been shown to be important in the APS. Increased expression of PARs has been found in monocytes from patients with APS [148] and tissue factor (TF)/FVIIa/PAR2 signalling has been shown to be important in the pathogenesis of aPL mediated pregnancy morbidity in mice [149]. Monoclonal human aPL also cross-react with SP, including thrombin and FXa [147, 168-171] and inhibit their procoagulant effects [146, 147, 168, 172]. Furthermore, 13-54% of patients with APS have anti-SP antibodies and 20-50% of these patients had SLE associated APS [145-147].

1.3.4. Factor Xa Inhibition

Rivaroxaban, a direct FXa inhibitor is now routinely used as primary and secondary thrombo-prophylaxis in several clinical settings. This drug, in contrast to warfarin, has no food/alcohol interactions, few reported drug interactions, and does not require routine monitoring. The RAPS (Rivaroxaban in APS) trial addressed whether rivaroxaban could offer more predictable and efficacious anticoagulation than warfarin in patients with APS in a randomised controlled trial. The outcome of this trial was that rivaroxaban could be an effective and safe alternative in patients with APS and previous venous thromboembolism with a target INR (International Normalised Ratio) of 2-3 [173, 174]. Furthermore, simvastatin and pravastatin decrease TF and PAR2 expression on neutrophils and prevent aPL mediated pregnancy loss in mice [149].

Previous work at UCL showed that anti-thrombin IgG was significantly elevated in patients with APS (35.6%) and patients with SLE who were aPL positive, but lacked APS (SLE/aPL+) (60%) compared to healthy controls (5%). Furthermore, APS-IgG displayed higher avidity for thrombin, and significantly inhibited antithrombin (AT)-III inactivation of thrombin compared with SLE/aPL+ and healthy control IgG [150]. In addition, mutations in the antigen binding sites of a panel of recombinant human monoclonal aPL, which altered their binding to thrombin, predicted pathogenicity in mice [175].

Patients with SLE and APS also had significantly increased anti-FXa IgG levels compared with controls. In addition, anti-FXa IgG isolated from patients with APS had differential avidity to FXa and effects upon the enzymatic and coagulant activity of FXa compared with anti-FXa IgG isolated from patients with SLE who lack APS. Further work demonstrated that FXa mediated Ca^{2+} release from HUVEC was: mediated via PAR1 and 2 dependent signalling mechanisms; enhanced by FXa-

reactive APS IgG as well as SLE/APS- IgG; and blocked by a specific FXa inhibitor, HCQ and fluvastatin [176].

Examination of the importance of anti-FXa IgG-FXa-PAR interactions in patients with SLE and/or APS and exploration of whether anti-FXa IgG positivity may be used as a novel biomarker of sub-clinical CVD would allow targeted treatment with specific FXa inhibitors or statins or HCQ in these patients.

1.4. SLE and microparticles

Endothelial dysfunction is a key player in the development of CVD [177] and may provide the important link between inflammation and atherogenesis. There are a number of different ways to assess endothelial activation and dysfunction, including measurement of adhesion molecule expression, flow-mediated vasodilatation and measuring microparticles (MPs).

There has been increased interest in MPs over the past few years in various conditions including CVD, autoimmune diseases and malignancy. MPs are now believed to be more than cellular debris and serve as circulating biological effectors involved in inflammation, vascular injury, coagulation and angiogenesis. MPs are subcellular membrane-bound vesicles, 0.1 to 1.0 microns in diameter released from cells undergoing cell activation as well as cell death. MPs transmit information from one cell to another and display tissue factor, which promotes thrombosis [178]. MPs are typically identified in terms of size by flow cytometry and exposure of PS, detected by staining with annexin-V. MPs are further characterised by cell surface markers of the potential cells of origin, see Table 1.3. MPs are a rich source of autoantigens, including DNA and have many functions including thrombosis and inflammation.

MPs have been regarded as “liquid biopsies” which can assess the production and clearance of dying and activated cells, which are pivotal in SLE pathogenesis [178]. MPs provide an important source of bioactive molecules that can induce a broad range of immunological and vascular activities. They can provide a source of nuclear and membrane molecules to drive autoantibody and aPL production, eventually leading to immune complex (IC) formation.

MPs are potentially important in the pathogenesis of both autoimmune rheumatic disease (ARD) and CVD. They carry various surface proteins from their cell of origin, such as vascular endothelial cadherin (VE cadherin) (CD144), platelet endothelial cell

adhesion molecule-1 (PECAM-1) (CD31), intercellular cell adhesion molecule (ICAM-1), Endoglin, and E-selectin [179] see Figure 1.8 (page 65). MPs are now regarded as a new surrogate for endothelial health and also have a huge potential to be important biomarkers in early disease detection and treatment.

There are various types of MPs, including endothelial microparticles (EMPs), platelet microparticles (PMPs), monocyte microparticles (MMPs) and syncytiotrophoblast microparticles (STBM). Elevated levels of MPs are associated with many diseases such as congestive heart failure (EMPs), thrombosis (PMPs) and pre-eclampsia (STBM).

Microparticles	Surface antigens
PMP	CD31, CD41, CD42, CD61, CD62P, CD63
EMP	CD31, CD51, CD54, CD62E, CD105, CD106, CD144, CD146, E-selectin, VE-cadherin
MMP	CD14, CD54

Table 1.3: Antigens on the surface of microparticles

This table shows the antigens on the surface of microparticles derived from platelets, endothelium and monocytes. (Adapted by Franca et al. [180] Microparticles as a potential for cardiovascular disease)

MPs may serve as carriers of autoantigens and constituents of immune complexes (ICs) in SLE [181]. MPs generally externalise PS and bind Annexin V in a calcium dependent manner. However, Annexin V negative MPs have been identified in plasma. Changes in levels of MPs in plasma may carry important information in healthy patients and those with CVD [182].

1.4.1. Endothelial microparticles

Approximately 5-15% of circulating MPs arise from endothelial cells and are known as EMPs [183]. They are complex vesicular structures that arise from the activated or apoptotic endothelial cells. EMPs are approximately 100nm to 1 μ m in diameter. They are larger than exosomes (<100nm in diameter) and smaller than apoptotic bodies. EMPs contain ribonucleic acid (RNA) and microRNA [184]. VE cadherin (CD144) has been proposed as one of the most specific markers for EMP detection. EMPs can be generated from HUVEC by stimulation with TNF- α [185] or other inflammatory cytokines such as lipopolysaccharide (LPS). EMPs are strongly linked to endothelial dysfunction and levels are elevated in acute coronary syndromes [186], hypertension [187] and diabetes mellitus [188]. EMPs are increased in patients with SLE, particularly those with circulating aPL [189]. EMPs promote vascular dysfunction and thrombosis by a number of mechanisms, as reviewed by Pericleous et al. [183].

Parker et al. measured EMP levels and vascular function, assessed by flow-mediated dilatation (FMD) of the brachial artery, in 27 patients with active SLE and 22 age-matched controls [102]. They showed that the EMP level was higher and FMD lower, in patients compared to healthy controls and that there was an inverse correlation between EMP and FMD. After treatment of SLE, disease activity and EMP level fell whereas FMD increased. Therefore, they suggested that improved control of inflammatory disease activity, by reducing vascular dysfunction, might reduce CVD risk in patients with SLE.

In patients at high risk of CVD, baseline levels of expressing VE-cadherin (CD144) predicted outcome independent of the Framingham score, (a gender specific algorithm used for estimating an individual's 10 year CV risk)[190]. Therefore EMPs may be used as a biomarker for endothelial dysfunction. EMP release can be

stimulated by passive smoke in healthy subjects [191]. Medications such as nifedipine and vitamin C reduce levels of EMPs. There is some evidence showing that statins have an anti-inflammatory effect on EMP production. PS expression can bind and activate coagulation factors. EMP contain tissue factor (TF), which initiates the extrinsic pathway of the coagulation cascade [185].

1.4.2. Platelet microparticles

Our understanding of PMPs and their mechanisms of formation has grown substantially over the last number of years. From the initial discovery of “platelet dust” by Peter Wolf in 1967 [192], it is now clear that PMPs are not just cellular debris. In fact PMPs appear to play a vital role in intercellular communication and have important functions in many diseases. Studies have shown that PMPs are raised in atherosclerosis, diabetes, cancer, sepsis, and pulmonary hypertension.

PMPs are the most abundant [193], constituting approximately 70-90% of all MP. With regards to cardiovascular disease, PMPs can induce foam cell formation and promote adherence of platelets to endothelial lesions.

The majority of PMPs in healthy individuals derive from megakaryocytes and activated platelets [194]. PMPs are shed from platelet plasma membranes that are undergoing activation or apoptosis. They express the procoagulant PS and function as a transport and delivery system for molecules, participating in haemostasis and thrombosis, inflammation, malignancy, infection, angiogenesis, and immunity. PMPs are distinguishable from other MPs by CD42a. Platelet endothelial cell adhesion molecule-1 is less specific in that it is present on platelets and PMPs, but also on leukocytes. PMPs may be markers or triggers of thrombosis or atherosclerosis. There is paucity however, of information regarding PMPs in patients with SLE and no

research comparing patients with concomitant SLE and atherosclerosis to those without SLE and CVD. Therefore, I felt this was an important area to explore, particularly to determine whether PMP may be potential biomarkers for CVD in SLE, see section 4.2 (page 143).

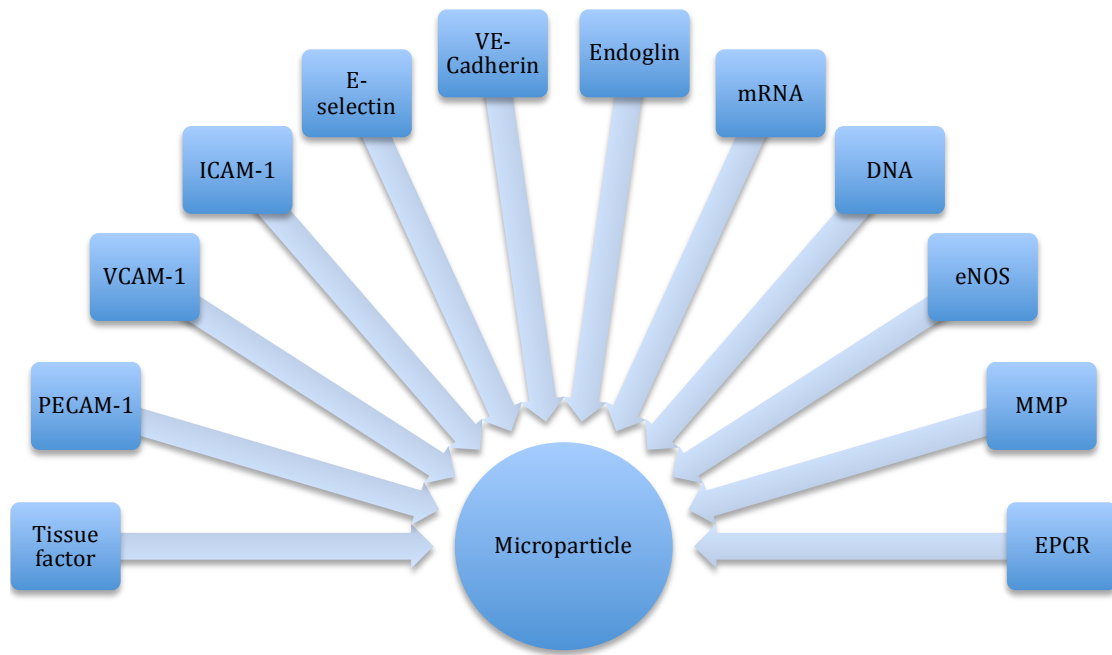


Figure 1.8: Panel of molecules conveyed by microparticles

Adapted from Dignat-George et al. [179] The Many Faces of Endothelial Microparticles

Abbreviations:

EPCR, endothelial protein C receptor;

PECAM-1, platelet endothelial cell adhesion molecule-1; V

CAM-1, vascular cell adhesion molecule-1;

ICAM-1, intercellular cell adhesion molecule-1;

E-selectin, endothelial selectin;

VE-cadherin, vascular endothelial cadherin;

eNOS, endothelial NO synthase;

MMP, matrix metalloproteases;

EPC, endothelial protein C.

1.5. SLE and pregnancy

SLE has a predilection for women of childbearing age and therefore pregnancy is of particular importance. Due to improved management, the survival of patients with lupus has improved over the last few decades. Increasing numbers of women with SLE are now considering pregnancy and having children. Pregnancy in SLE should be considered high risk and these patients require careful preconception planning as well as vigilant medical and obstetric monitoring of their disease to reduce the incidence of potential adverse maternal-foetal outcomes. A recent European League Against Rheumatism (EULAR) paper gives evidence based recommendations regarding family planning, assisted reproduction, pregnancy and menopause in patients with SLE and APS [68].

1.5.1. SLE and adverse pregnancy outcomes

Adverse pregnancy outcomes (APO) in SLE include hypertensive disorders of pregnancy such as pre-eclampsia, intrauterine growth restriction (IUGR) [195, 196], pregnancy loss, premature delivery and caesarean delivery [195-198]. APO are predicted by a number of factors such as active lupus at conception/during pregnancy, lupus nephritis and the presence of maternal anti-Ro/La antibodies, APS as well as hypertension [199]. A US database of 16.7 admissions for childbirth over four years (2000–03) identified 13,555 women with SLE and found that maternal mortality was 20-fold higher among women with SLE. The risks for thrombosis, infection, thrombocytopenia, and transfusion were each 3- to 7-fold higher for women with lupus. These patients had a higher risk for Caesarean sections (odds ratio: 1.7), preterm labour (odds ratio: 2.4), and pre-eclampsia (odds ratio: 3.0) than the general population. Women with SLE were more likely to have co-morbidities, including diabetes, hypertension and thrombophilia associated with APO. Limitations of this

study included that women with SLE were older and more were African American compared with the non-SLE population [196].

1.5.2. Risk factors for APO in women with SLE

As per the EULAR recommendations, assessment of risk factors in pregnant women with SLE for adverse maternal and fetal outcomes is crucial, as part of preconception counseling and implementing preventive strategies pre-pregnancy and during pregnancy. In women with SLE, the frequencies rates of prematurity is 25-30%, pre-eclampsia 10-15% and eclampsia/Hemolysis, Elevated Liver enzyme levels, Low Platelet count (HELLP) (1.0–1.5%). A women with a flare/active SLE during her pregnancy, has an increased risk for pre-eclampsia/eclampsia (OR 12.7), emergency C-section (OR 19.0), early foetal loss (OR 3.0), preterm delivery (OR 5.5). A woman with active lupus nephritis during pregnancy has an increased risk for any adverse maternal outcome (OR 5.3). SLE and hypertension lead to an increased risk of pre-eclampsia; (relative risk 1.8 of preterm birth). High dose corticosteroids at a dose >10-20mg/day increases the risk of preterm birth (OR 3.5) [68]. HCQ reduces the risk of SLE flare during pregnancy and reduces the risk of neonatal lupus and therefore women should continue on HCQ during pregnancy unless contraindicated. Buyon and colleagues showed that the use of HCQ in a mother with anti-Ro antibodies, who had a child with cardiac-neonatal lupus, may reduce the risk of cardiac-neonatal lupus recurrence in a subsequent offspring [200].

1.5.3. Contraindications to pregnancy in women with SLE

Contraindications to pregnancy in patients with SLE include severe pulmonary hypertension, chronic renal insufficiency stage 4-5 (creatinine >220-250 mmol/L;

estimated glomerular filtration rate (GFR) $<30\text{ul/min}$), severe restrictive lung disease, moderate to severe heart failure (left ventricular ejection fraction $<40\%$), and previous severe hypertensive disorders of pregnancy such as pre-eclampsia despite therapy, see [201] Table 1.4. Reasons to defer pregnancy in SLE include severe disease flare within the previous six months; active lupus nephritis and stroke within the last six months see Table 1.5. Of note, complement levels may rise by 15% in normal pregnancy and therefore may remain normal in pregnant women with active SLE.

Contra-indications to pregnancy in SLE
Severe pulmonary hypertension (systolic pulmonary artery pressure > 50mm Hg)
Severe restrictive lung disease (Forced vital capacity < 1 L)
Chronic renal insufficiency (stage 4-5)
Moderate to severe heart failure (Ejection fraction <40%)
Previous severe pre-eclampsia or HELLP despite therapy

Table 1.4: Contraindications to pregnancy in SLE

Reasons to defer pregnancy in SLE
Severe disease flare within last 6 months
Active lupus nephritis
Stroke within the previous 6 months

Table 1.5: Reasons to defer pregnancy in SLE

1.5.4. Disease activity in SLE pregnancy

Active lupus during pregnancy can lead to APO for both mother and foetus. Lupus nephritis, in particular, can be associated with foetal loss, prematurity and growth restriction. The differential diagnosis from pre-eclampsia and lupus nephritis remains a challenge as outlined by Ramires de Jesus et al [202], see Table 1.6.

Assessment of disease activity, renal function and serological markers is important to prevent APO. With regards to the foetus, foetal biometry measurements are essential to screen for placental insufficiency and for small gestational age (SGA). Foetal echocardiography is recommended, especially in patients with positive anti-Ro/ SSA and/or anti-La/SSB antibodies, where there is an increased risk of congenital heart block.

Increased SLE disease activity in pregnancy may be due to increased levels of estrogen, prolactin, and T-helper cell 2 cytokines. Experimental observations suggest that increased activation of complement causes or perpetuates inflammation during pregnancy. Generally, the complement system protects against invading organisms, initiates inflammation and dispose of immune complexes. There now appears to be a link between complement activation and pre-eclampsia. Excessive activation of complement recruits leukocytes and unleashes inflammatory and anti-angiogenic mediators associated with placental insufficiency [203].

Pre-pregnancy counselling, improvements in disease management, and close monitoring have led to a significant improvement in pregnancy outcomes in SLE. The rate of pregnancy loss has decreased from 43% in 1960–65 to 17% in 2000–03 [204]. Ideally an obstetrician and a rheumatologist should be involved in the care of pregnant women with SLE.

Features differentiating pre-eclampsia and lupus nephritis							
Clinical and Laboratory features	Pre-eclampsia				Lupus nephritis		
Hypertension	After	20	weeks	of	Any	time	during
	gestation				pregnancy		
Platelets	Low-normal				Low-normal		
Anti-dsDNA antibodies	Absent or unchanged				Normal to raised		
Creatinine	Normal to raised				Normal to raised		
Serum uric acid	Elevated				Normal		
24-hour urine calcium	<195 mg/dL				>195 mg/dL		
Urinary sediment	Inactive				Active		
Other organs involved	Occasionally	CNS	or	Evidence	of	active	
	HELLP			nonrenal SLE			
Response to steroids	No				Yes		

Table 1.6: Features differentiating pre-eclampsia and lupus nephritis

Adapted from Ramires de Jesus et al [202].

Abbreviations: CNS-central nervous system. The HELLP syndrome stands for- **H**aemolysis, **E**levated **L**iver function tests, **L**ow **P**latelets. This condition is a serious life threatening complication of pregnancy, closely related to pre-eclampsia.

1.5.5. SLE and Maternal Placental Syndrome

Women with SLE are particularly at risk of developing APO, such as maternal placental syndrome (MPS). MPS occurs as a consequence of abnormal placental vessel formation, and refers to hypertensive disorders of pregnancy such as pre-eclampsia, placental infarction and placental abruption [205]. Pre-eclampsia is estimated to complicate 2%–8% of all pregnancies [206]. It is a multisystem disorder characterized by hypertension and proteinuria ≥ 0.3 grams after 20 weeks of gestation, which remains a leading cause of maternal and foetal morbidity and mortality. The World Health Organization (WHO) estimate that pre-eclampsia is responsible for 70,000 maternal deaths [207] and 500,000 infant deaths annually [208]. In severe cases pre-eclampsia may lead to pulmonary oedema, renal/liver dysfunction and eclampsia, (which is the onset of seizures in pre-eclampsia, from the Greek word lightening). Risk factors for pre-eclampsia in the general population include advanced age, twins, previous pre-eclampsia, pre-existing hypertension, diabetes and obesity. Pre-eclampsia is more commonly encountered in the third trimester.

The aetiology of pre-eclampsia is focused on maladaptation of the immune system and defective trophoblast invasion [209]. Pre-eclampsia is associated with the release of anti-angiogenic factors by an ischemic placenta, which may lead to maternal endothelial dysfunction [210]. Angiotensin receptor agonistic autoantibodies have been found in women with pre-eclampsia [211]. Antiangiogenic factors secreted by the placenta to the maternal circulation are considered responsible for systemic endothelial dysfunction, hypertension and multi-organ damage.

MPS has been associated with hypertension, ischemic heart disease (IHD), stroke, venous thromboembolism and CV death. In women with SLE, lupus nephritis, aPL

antibodies, low complement levels and thrombocytopenia are risk factors for MPS [212] and are negative predictors for foetal survival [201].

1.5.6. SLE, APO and CVD

In 1927, Corwin et al reported an association between 'hypertensive toxemia of pregnancy' (now known as pre-eclampsia) and CVD. MPS has been suggested to be a 'failed stress test' identifying women who have an underlying predisposition to CVD and has been shown to double the risk for the development of CVD in later life.

A retrospective analysis [213] using Swedish population registries between 1973 and 2011, found an increased risk of subsequent CVD death in women with SLE whose pregnancies were complicated by MPS. A total of 3,977 women with SLE had 7,410 pregnancies during the study interval. The outcome was death from CV causes, defined as ACS (acute coronary syndrome) or CAD (coronary artery disease), stroke, or PVD (peripheral vascular disease). MPS was defined as any hypertensive disorders in pregnancy, stillbirth, placental abruption, or delivery of a small-for-gestational-age infant. Death from primarily cardiovascular causes occurred in 44 of the 325 women (13.5%). The median age at death from CV causes was 54 years and these women were more likely to have had hypertension and renal disease. MPS was associated with an increased risk of death primarily from CV causes, specifically in a history of placental abruption. Delivery at less than 34 weeks of gestation, particularly when combined with MPS, was also associated with an increased risk of death from primarily cardiovascular causes (adjusted OR 2.49 [95% CI 1.06-5.85]).

Knowledge of APO in patients with SLE who have otherwise favourable modifiable CVD risk factors may prompt more aggressive intervention (e.g. the use of statins even in patients with favourable lipid profiles that do not usually warrant statin use)

than may normally be applied to these patients who would otherwise be considered at low risk for CVD. Therefore, I examined the relationship between APO and the development of carotid and femoral plaque, measured by high sensitivity ultrasound in women with SLE. I also analysed the data to see if there was any association between anti-FXa and anti-thrombin IgG in patients with SLE and concomitant CVD and APO.

1.6. Fertility and parity in SLE

Parity indicates the number of pregnancies reaching viable gestational age. This includes live births and stillbirths as opposed to gravidity, which indicates the number of times a woman has been pregnant, regardless of the pregnancy outcome. E.g. twin pregnancy is counted as one. The reasons for reduced parity in SLE are complex, and potential causes of infertility have been investigated. However, there is no concrete evidence that SLE decreases fertility [214-216]. A higher incidence of SLE (~1%) has been identified among infertile women in the general population than would be expected [217]. The incidence of infertility in SLE is uncertain, and a number of potential factors include renal failure, cyclophosphamide, APS, menstrual disturbances and older age given delays for disease remission and drug washout have been reported [218]. Psychosocial influences are important in fertility also. SLE is associated with depression and fatigue and reduced libido/sexual dysfunction in women. Therefore, a reduction in fertility may actually reflect reduced sexual intercourse. A multicenter study found that 42% of women with SLE (n = 339) diagnosed before 50 years of age had never been pregnant [219]. Stein et al. showed in a study of 119 women with SLE, that they viewed their disease as a barrier to childbearing [220].

Women with SLE, like in the general population should be counseled on fertility issues, such as the negative impact of increasing age and lifestyle exposures e.g. smoking and alcohol consumption. If there are multiple risk factors for impaired fertility, such as the use of cyclophosphamide, ovarian reserve may be assessed in patients with SLE at a younger age than recommended for the general population.

1.6.1. Fertility preservation in SLE

Limited data are available on fertility preservation methods in women with SLE. Cryopreservation of ovarian tissue or oocytes/embryos is poorly investigated. Gonadotropin-releasing hormone (GnRH) analogues can be considered for fertility preservation prior to the use of drugs such as cyclophosphamide. Gonadotropin-releasing hormone analogues (GnRH-a) have been extensively studied and have a good safety and efficacy profile with regards to preservation of fertility [221]. GnRH-a may cause menopause-like symptoms, which reverse upon discontinuation. A study in patients with JSLE aged <21 years suggested that GnRH-a should be administered 22 days before cyclophosphamide is started or continued [222]. If this is not possible, it is recommended to start the GnRH-a during treatment.

1.6.2. Assisted reproduction techniques (ARTs)

Women with SLE who have difficulty conceiving should be reassured that in vitro fertilization (IVF) and other ARTs, such as ovulation induction can be used safely. Evidence on the efficacy and safety of ARTs in women with SLE and/or APS comes from observational studies [223, 224]. Efficacy in terms of pregnancy rate is up to 30%, which is comparable with the general population. ARTs are generally safe if the patient is in remission and is on appropriate antithrombotic treatment if aPL positive.

Patients with SLE and positive aPL should be anticoagulated with low dose aspirin and or anticoagulation, similar to the treatment they would be recommended during pregnancy. The ART induction protocol should be tailored to the individual patient, balancing the safety and effectiveness of the procedure. Ovarian hyperstimulation syndrome can be avoided by using milder hormonal stimulation.

1.6.3. Family planning in SLE

Family planning has become of increased importance for women with SLE. The new EULAR guidelines recognise “an implicit need for change in the mindset of health professionals, shifting from caution against pregnancy towards embracement of pregnancy”. SLE may impact on personal relationships and the decision to have children. Family planning should be discussed as early as possible after diagnosis, ideally at the first clinic appointment. Parents often worry that their children with JSLE may not become future parents and often appreciate reassurance. For patients with JSLE, pregnancy counseling should be addressed in the adolescent transition clinic, particularly when patients have been on long-term teratogenic medications since childhood. It is important to establish early on if patients wish to have children and reassure patients that most women with SLE have successful pregnancies and that measures can be taken to reduce the risks of APO. Pre-conception risk stratification includes assessment of disease activity, autoantibodies, previous pregnancy/CVD morbidity, hypertension and use of drugs.

HCQ, glucocorticoids, azathioprine, ciclosporin A and tacrolimus can be used to prevent or manage SLE flares during pregnancy. MMF, CYC, leflunomide and methotrexate should be avoided in pregnancy. Women with SLE, especially those with lupus nephritis or positive aPL should receive low dose aspirin. In women with SLE-associated APS or primary APS, combination treatment with low dose aspirin and

heparin is recommended to decrease the risk of APO. Calcium, vitamin D and folic acid supplementation should be offered as in the general population.

1.6.4. Contraception in women with SLE

Contraception should be discussed with patients for prevention of unwanted pregnancies especially during disease flares and while taking teratogenic drugs. Oestrogen-containing contraceptives increase the risk of venous thromboembolism and are therefore not recommended in those with aPL, APS or a history of thrombosis. In patients with stable SLE and negative aPL, combined hormonal contraceptives can be considered. In women with positive aPL regardless of APS diagnosis hormonal contraception with progesterone only, must be carefully weighed against the risk of thrombosis. Oral contraceptives, subcutaneous implants, intrauterine device (IUD), can be used in patients with stable SLE and who have a low risk of thrombosis. The copper IUD can be used safely in women with SLE. The levonorgestrel-containing IUD should be used only if the benefits of the released hormone (such as the reduction of excessive menstrual bleeding due to anticoagulation outweigh the risk of thrombosis).

1.6.5. Other female specific issues in SLE

Hormone Replacement Therapy (HRT) can be used for the management of severe vasomotor menopausal manifestations in women with stable lupus and negative aPL. The use of HRT in patients with positive aPL should be carefully weighed against the risk of thrombosis and CVD. Screening for gynaecological malignancies is similar to the general population, with increased vigilance for cervical premalignant lesions if patients are exposed to immunosuppressive drugs. SLE is associated with an increased risk of malignancies, particularly lymphoma. Ovarian, endometrial and

breast cancer appears to be reduced in patients with SLE. Human papillomavirus (HPV) immunisation can be used in women with stable SLE.

1.6.6. SLE, parity and APO

Soh et al. found a higher incidence of cardiovascular events (CVE) among women with lupus who never had children, followed by women with lupus who had pregnancies complicated by APO [225]. CVE was defined as presence of any CV disease (i.e. CAD, stroke or PVD) or death from cardiovascular causes. This study utilized Swedish population registries and identified 3232 women with SLE born between 1951 and 1971. A total of 2317 (72%) were parous and the median age was 49 years. Compared to the parous women, the nulliparous women had more SLE-related morbidity, cardiovascular risk factors and CVE. Amongst the parous women 23.3% (n=539) had a history of MPS. The incidence of CVE was highest amongst the nulliparous group at 3.4 per 1,000 person-years (95% CI 2.91-4.07), followed by those who had pregnancies complicated by MPS (2.8 per 1,000 person-years (95% CI 2.2-3.6)). The probability of a CVE-free survival was lowest in the non-parous group even when compared to those with MPS ($p<0.001$). Associations that link pregnancy complications to later life are not well understood and may include traditional cardiovascular risk factors, inflammation and atherothrombosis.

1.7. SLE and bone mineral density

Survival among patients with SLE has improved over the past number of decades. However comorbidities and complications secondary to prolonged use of glucocorticoids, such as osteopenia and osteoporosis remain important. Decreased physical activity, reduced sun exposure and lupus activity itself, especially active inflammation, play a role. Vertebral fractures occur in up to 10% of patients with lupus and may be secondary to long term steroid use or persistent inflammation. Fracture risk was increased 5-fold in a large cohort of patients with lupus whose mean age was <45 years [226].

Dual-energy x-ray absorptiometry (DXA) is a means of measuring bone mineral density (BMD). The T-score is the bone mineral density (BMD) at the site when compared to the young normal reference mean, which compares a patient's BMD to that of a healthy 30-year-old. Normal BMD is defined by a T-score of -1 or above. Osteopenia is when BMD is below normal and is defined as a T-score between -1 and -2.5. Osteoporosis is defined as a T score below 2.5 and increases the risk of fracture. Low bone mineral density (LBMD) was defined as having either osteopenia or osteoporosis e.g. a T-score below -1. The Z-score compares age-matched normals and is usually used in cases of severe osteoporosis. The Z-score is the number of standard deviations a patient's BMD differs from the average BMD of their age, sex, and ethnicity. This value is used in premenopausal women, men under the age of 50, and in children.

1.7.1. Pathophysiology of bone loss in SLE

The cause of bone loss in SLE is multifactorial. Inflammation is important with pro-inflammatory cytokines and the RANK (Receptor Activator of Nuclear Factor κ B)

/RANKL (RANK ligand) /OPG (osteoprotegerin) pathway implicated as a link between these conditions [227], see Figure 1.9. Bone loss occurs early in the disease, most likely due to cytokines (TNF, Il-1, Il-6) that promote osteoclast function. Proinflammatory cytokines produced in inflamed joints promote release of osteoclast precursors from the bone marrow. Increase in bone resorption occurs to a greater extent than bone formation. During acute and chronic inflammation, activated T cells and synovial-like fibroblasts stimulate osteoclastogenesis through the production of RANKL and TNF. TNF stimulates maturation of osteoclasts and osteoblasts to release RANKL. Osteoclasts are multinucleated cells formed by fusion of mononuclear precursors in the monocyte/macrophage lineage. They are responsible for the degradation of old bone matrix. Osteoblasts are bone-building cells, which differentiate from mesenchymal progenitors.

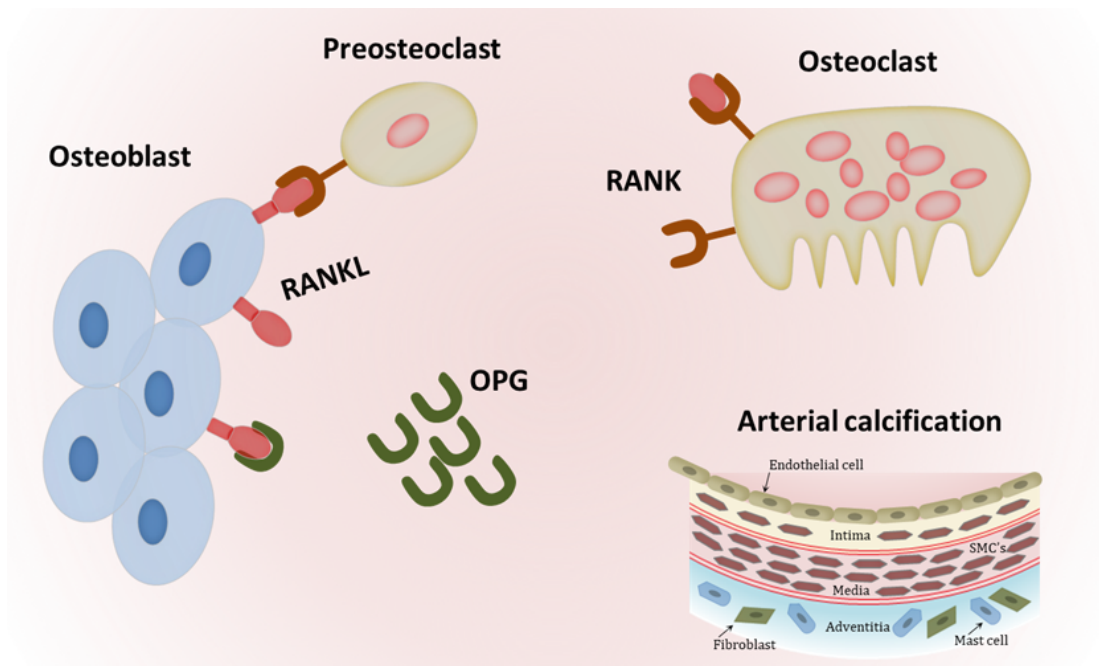


Figure 1.9: RANK/RANKL/OPG system

This figure demonstrates the importance of the RANK/RANKL/OPG system with regards to both bone and arterial calcification.

1.7.2. Osteoporosis screening in women with SLE

Studies have demonstrated suboptimal screening and management of osteoporosis in patients with SLE, despite treatment options that are proven to decrease osteoporosis risk [228]. Screening for osteoporosis should be considered in all patients with lupus. Depending on the severity of lupus, patients may require treatment with steroids, which increases the risk of osteoporosis. The ACR has developed recommendations for the prevention and treatment of glucocorticoid-induced osteoporosis (GIO) [229, 230]. An initial fracture risk assessment should be performed as soon as possible, but at least within 6 months of initiation of long-term glucocorticoid treatment. This assessment should include a history with an evaluation for falls, fractures and other risk factors for fracture e.g. significant weight loss or low body weight, thyroid disease, secondary hyperparathyroidism, family history of hip fracture, history of alcohol use and smoking. The initial absolute fracture risk should be estimated using the WHO FRAX calculator with the adjustment for glucocorticoid dose. The FRAX tool was developed in the University of Sheffield and integrates clinical risk factors and BMD at the femoral neck to calculate the 10-year probability of hip fracture and the 10-year probability of a major osteoporotic fracture. The FRAX tool was derived from studying patient populations in Europe, North America, Latin America, Asia and Australia and has been incorporated into international guidelines.

1.7.3. Management of osteoporosis in women with SLE

Patients with lupus should be advised to limit alcohol use, maintain normal body weight and engage in weight bearing exercise. Early and aggressive treatment for bone preservation should be considered in patients treated with steroids. Replacement of calcium and vitamin D should be recommended if patients are deficient. The ACR recommends 800 to 1000 international units (IU) per day of

vitamin D to achieve therapeutic levels. Supplementation of calcium may have potential risks, as studies have found an association between high calcium intake and elevated risk of cardiovascular death. Bisphosphonates inhibit bone loss, but cross the placenta, have a long half-life and are potentially teratogenic, so ideally should be avoided in women of reproductive age [231]. HRT has been studied for the prevention of bone loss in patients with SLE and has led to an increase in lumbar spine BMD in patients with and ovarian failure. However, hormone therapies often increase the risk of stroke, CVE and thrombosis, so hormone therapy is not typically recommended as a treatment for osteoporosis in patients with SLE.

1.7.4. SLE, bone mineral density and CVD

What is less well established is the link between, SLE, CVD and osteoporosis. Osteoporosis and osteopenia are associated with atherosclerosis in postmenopausal women. This relationship may be secondary to age and other shared traditional risk factors. In SLE however, bone loss occurs in women at a much younger age. Therefore, women with SLE are an ideal population to study to determine the underlying relationship between these conditions.

Atherosclerotic calcification and bone mineralization share common features. Arterial calcification is not merely a passive process of calcium phosphate precipitation. It is a process that is regulated by mechanisms similar to those involved in bone mineralization [232, 233] The mineral in calcium deposits of atherosclerotic plaques has a very similar chemical composition to hydroxyapatite crystals which form the inorganic bone matrix [234].

Calcified plaques have been shown to express bone matrix proteins such as type I collagen, gla (gamma carboxyglutamate)-containing proteins such as osteocalcin and

matrix-gla protein, bone morphogenetic proteins, osteopontin, osteonectin, and bone sialoprotein [234]. Osteogenic cells have been identified in atherosclerotic plaques. These are a subpopulation of vascular smooth muscle cells (VSMC) that is capable of osteoblastic differentiation [235]. Osteoclast-like cells, chondrocyte-like cells, and hematopoietic bone marrow cells were also seen in plaques [236]. Osteoprotegerin in particular has been hypothesized to be the link between bone and CVD. OPG is related to the CV risk in patients suffering from diabetes [237]. Osteoporotic fractures and CV outcomes have been shown to coexist in young women with SLE. Given the close links between SLE, CVD and LBMD, I explored associations between atherosclerotic plaque and LBMD in patients with SLE.

1.8. Unanswered questions:

Increased CVD risk in SLE is not fully explained by traditional risk factors and other disease related factors such as persistent inflammation, autoantibodies and microparticles have been implicated. Women with SLE and previous APO are at increased risk of CVD. The unanswered questions I have identified with regards to these non-traditional risk factors for SLE are:

1. Are patients with SLE and subclinical CVD more likely to have antibodies to Factor Xa and Thrombin, compared to patients without SLE?
2. Do patients with SLE and atherosclerosis have increased EMPs and PMPs than patients without SLE and healthy controls?
3. Are women with SLE and adverse pregnancy outcomes more likely to have subclinical atherosclerosis compared to the general population?
4. Do patients with SLE and atherosclerosis have an increased risk of low bone mineral density?

1.8.1. Hypothesis

The development of subclinical CVD in patients with SLE is associated with the presence of one or more non-traditional cardiac risk factors: anti-FXa and anti-thrombin IgG; EMP and PMP; APO and/or osteoporosis.

1.8.2. Aims/Objectives

The main aim of my research was to study a cohort (n=100) of patients with SLE who had previously undergone detailed vascular imaging. These patients had no clinical diagnosis of CVD prior to scanning and were subdivided by the presence of subclinical CVD in one sub-group (n=36) and absence of CVD on carotid and femoral ultrasound scans in another sub-group (n=64). I compared these two groups for differences in the prevalence of: anti-SP antibodies (FXa and thrombin); EMPs and PMPs; adverse pregnancy outcomes; and reduced bone mineral density.

My objectives for all (n=100) patients were as follows:

1. Test serum samples for antibodies to Factor Xa and Thrombin.
2. Test plasma samples for EMP and PMP levels via flow cytometry.
3. Obtain pregnancy information available on patients with SLE to correlate APO with findings from vascular ultrasound imaging
4. Correlate DXA scan results with cardiovascular status

2. Chapter Two: Materials and methods

2.1. Patient selection

100 patients who attended the SLE outpatient rheumatology clinic at University College London Hospital (UCLH) were included in this research. Serum was available for all patients. None of these patients had a history of CVD-related events or symptoms suggestive of underlying CVD, including angina, intermittent claudication and TIA. These patients had undergone vascular imaging of their femoral and carotid arteries from 2011-2013. Clinical and laboratory data including traditional cardiovascular risk factors were available on these patients. All patients fulfilled the revised classification criteria for SLE (four or more 1997 American College of Rheumatology (ACR) revised criteria) [42]. Healthy controls were recruited from staff at University College London (UCL) and UCLH to provide serum and plasma samples. All subjects gave written informed consent. It was difficult to find exact age and sex-matched controls for practical reasons, as outlined in strengths and limitations, Section 4.3, Page 151. Ethics approval was obtained from the local ethics board (NRES) National Research Ethics Committee- London Hampstead, reference number 12/LO/0373).

In this cohort of 100 patients, 95% were women with a mean age of 45 (SD 12.4; range 20-66) years with disease duration of 16.2 (SD 10.1, range 2 to 46) years. Of this cohort, 56 patients were Caucasian, 25 were Afro-Caribbean, 11 were South Asian and 8 were of a different ethnic background (Chinese or mixed race). A total of 36% had plaque (15 patients with carotid and femoral plaque, 14 with only carotid plaque and 7 with only femoral plaque). 64% had no plaque. Various ultrasound parameters had been previously measured: presence of plaque, number of plaque sites, total plaque area (TPA), intima media thickness (IMT) and grey scale median (GSM), where

lower values implied echolucent inflammatory plaque. Therefore I was able to compare differences between patients with SLE, with and without plaque.

Patients in the SLE cohort had their disease activity recorded using the Classic BILAG (British Isles Lupus Assessment Group) index. This organ-based transitional disease activity index, provides disease activity scores across eight organ systems on an ordinal scale (A to E), based on the physician's intention-to-treat. This index was used to record disease activity for patients at every visit to the lupus clinic. Activity was graded between A (highly active) and E (never active) for each system of the body. Grade A indicates active disease likely necessitating immunosuppressive drugs and/or corticosteroids (or equivalent) dose of more than 20 mg daily or high-dose anticoagulation. Grade B implies moderate disease activity requiring a lower dose of corticosteroids, topical steroids, topical immunosuppressive drugs, anti-malarials, or NSAIDs. Grade C represents mild stable disease, and grade D indicates no disease activity, but suggests the system had previously been affected. Grade E implies no current or previous disease activity.

2.2. Anti-Factor Xa and anti-thrombin assays

Reagents

Coagulation factors were obtained from Haematologic Technologies, Essex Junction, Vermont, USA. Chromogenic substrates for ELISA were from KPL, Gaithersburg, Maryland, USA. Porcine gelatin, bovine serum albumin (BSA) and conjugated antibodies were from Sigma-Aldrich, Suffolk, UK.

2.2.1. Direct ELISA for detection of anti-Factor Xa antibodies

The Factor Xa ELISA to detect anti-Factor Xa antibodies was performed based on a protocol by Yang et al [171]. This assay was previously adapted by Artim-Esen et al [176, 238] at UCL and then further refined by myself. All 100 SLE serum samples were tested in duplicates on 96-well high binding Costar ELISA plates (3590).

The test half of the plates were coated with 50 µl/well of 5 µg/ml of human FXa in Tris-buffered saline (TBS, 0.05 M Tris-HCl and NaCl, PH 7.5). The control half was coated with 50 µl/well of TBS alone. Plates were covered and incubated overnight at 4 °C. To ensure blocking of non-specific binding, 150µl TBS was added containing 0.3 % porcine gelatin (Sigma) to each well. Each plate was then covered and incubated for 1.5 hours at room temperature. Plates were then washed 4-5 times with TBS. Test and control samples were diluted (1:50) in 0.3% gelatin/TBS and loaded in duplicate wells and compared against a calibrator loaded in serial dilutions at 50 µl/well. Horseradish peroxidase (HRP) conjugated to anti-human IgG or IgM was then added at 50 µl/well (diluted 1:2000 in 0.3% porcine gelatine/TBS). Plates were covered and incubated for 1 hour at RT. Plates were then washed 4-5 times with TBS. 100 µl/well

of 3-3'-5-5' TMB (tetramethylbenzidine) substrate was added to each well. ELISA plates were incubated for 15-20 min at room temperature. The reaction was stopped by adding 1% HCL (100µl/well) and read with the Xfluoro 4 plate reader at wavelength absorbance 450nm. Anti-FXa positivity was determined when the test optical density (OD) minus the background OD exceeded the mean OD + 3 SD of healthy controls (HC). Any serum sample with a standard deviation greater than 0.1, from the mean of the HC group was repeated.

2.2.2. Direct ELISA for detection of anti-thrombin antibodies

The Factor Xa ELISA to detect anti-Factor Xa antibodies was performed based on a protocol by Yang et al. [171]. Detection of anti-thrombin antibodies was performed using a MaxiSorp ELISA plate. All 100 SLE serum samples were tested in duplicates.

The test half of the plate was coated with 100 µl/well of human α -thrombin (Haematologic Technologies) at 10 µg/ml in PBS buffer. The control half was coated with 50 µl/well PBS alone. Each plate was covered and incubated overnight at 4°C. These plates were then subsequently washed three times with Phospho-buffered saline tween (PBST) (0.05 %). 200µl of PBS containing 1% BSA was added to each well to block non-specific binding. Plates were covered and incubated for 2 hours at room temperature. MaxiSorp plates were washed three times with PBST (0.05 %). Serum samples were diluted (1:50) in 1% BSA/PBS and loaded in duplicate wells or serial dilutions (50 µl/well). Plates were covered and incubated for 1.5 hours at room temperature, then washed 4-5 times with PBST (0.05 %). Alkaline phosphatase conjugated anti human IgG or IgM was added at 50 µl/well (diluted 1:1000 in 1% BSA/PBS), then incubated for 1 hour at room temperature. Plates were then washed

4-5 times with PBST (0.05 %). PNPP (p-Nitrophenyl Phosphate, Disodium Salt) substrate was prepared according to manufacturers instructions and 100 µl/well was added. Absorbance was read at 405 nm at 15, 30, 45 and 60 min.

2.2.3. Statistical analysis

Statistics were performed using GraphPad Prism, version 5.0a. Results of anti-FXa and anti-Thrombin IgG reactivity were analysed and correlated with ultrasound data. Data were presented as means $\pm$ SEM (standard error of the mean). Multivariable analysis was used to control for confounding risk factors. Non-parametric analyses by one-way ANOVA (Kruskall-Wallis) and Chi-squared calculations were used to determine antibody positivity/negativity and presence/absence of plaque. Differences between means with $p < 0.05$ were considered significant.

2.2.4. Anti-Factor Xa IgG cell experiments

I was interested to measure the effects of polyclonal IgG from anti-FXa positive patients with SLE and subclinical CVD on FXa-PAR mediated signalling pathways. Therefore after the ELISAs, I carried out cell experiments using HUVEC (Human Umbilical Vein Endothelial Cells). Firstly, IgG was purified, concentrated and then quantified using Spectrophotometre protein quantification (Nanodrop).

2.2.5. Whole IgG purification

Reagents used included binding, elution, neutralisation buffer and storage solution.

Serum from patients was used to extract whole IgG for testing in cell culture assays. Purification was carried out using a gravity flow Protein-G column from Pierce. The columns were stored in 0.02% Sodium azide with a screw cap and can be reused up to ten times. After storage, upright at 4°, the columns were allowed to reach room temperature before use. The lid and cap were removed and the storage buffer was removed by gravity from the column. The column was then equilibrated with five column volumes of binding buffer.

Binding Buffer
0.1M Phosphate pH 7.2
13.9 g Monobasic sodium phosphate in 500 mL water (0.2 M stock)
28.4 g Anhydrase sodium phosphate dibasic in 1 L water (0.2 M stock)
117 mL Monobasic + 183 mL dibasic, make up to 600 mL with water (0.1 M phosphate)
Filter Sterilise

Table 2.1: Binding buffer used for IgG purification

Meanwhile the serum was diluted 1:1 in binding buffer with a maximal capacity of 2 mls. Dilutions were greater depending on the viscosity of the patients' serum. The 1:1 mix was then applied to the column and the flow through was collected and stored at 4°C for analysis. The column was washed with 15 columns of binding buffer before elution with 0.1M Glycine (pH 2.7) in two stages.

Elution Buffer
0.1 M glycine pH 2-3
Make 1 M stock (MW in g in 1 L water)
Dilute in water to make 0.1 M glycine in 500 mL
Filter sterilise

Table 2.2: Elution buffer used for IgG purification

Initially 4mls of elution buffer (0.1 M glycine pH 2-3) was applied which contained the IgG. Then another 1ml was added and allowed to wash through to clear the column of miscellaneous debris. Eluted IgG is stable for a short period of time at pH 2.7 but was quickly neutralised using neutralisation buffer (pH 9) at a 1:10 ratio of TRIS-HCL to elute the protein and then stored at 4°C overnight.

Neutralisation buffer
1 M Tris pH 8-9
Made in 1 L

Table 2.3: Neutralisation buffer used for IgG purification

The columns were then washed with 5mls of sodium azide and stored upright at 4°C for further use.

Storage Solution
0.02% Sodium azide in 1% PBS
0.1 g sodium azide in 500 mL 1% PBS

Table 2.4: Storage solution used for IgG purification

Purified Samples were later dialysed through 50kDa concentrator to produce IgG for quantification and endotoxin removal.

2.2.6. IgG concentration

Purified IgG was added to a Millipore ultra centrifugal filter with a cut off of 30 KDa, thus retaining molecules larger than 30KDa such as IgG that is 150KDa. The device was centrifuged at 7,500g for 20 minutes before 2 mL of PBS added and re-centrifuged at 7,500 g for 20 minutes a total of two times. The IgG sample left in the top of the filter was transferred to a clean 1.5ul microcentrifuge tube and made up to 1 mL with PBS. The filter was washed with the extra PBS to ensure all the IgG was collected. The concentration of IgG was determined using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

2.2.7. Spectrophotometre protein quantification of IgG

Spectrophotometry is the measurement of the reflection or transmission properties of a material as a function of wavelength. Protein mass spectrometry allows for the accurate mass determination and characterisation of proteins. 1.5ul microcentrifuge tubes of IgG were removed from the freezer. 5µl was removed and put in new microcentrifuge tube. The nanodrop-1000 Spectrophotometer was initially tested with (Hyclone) PBS to calibrate the machine. Computer settings were set to ND-1000

and Protein A280. The machine probe was washed with 2µl of water in between measurements. Measurements were analysed by calculating the absorbance divided by IgG extinction coefficient.

2.2.8. Endotoxin removal protocol

Endotoxin removal was performed under sterile conditions in the tissue culture hood. Once IgG samples were purified and then concentrated they were put through a Detoxi-Gel endotoxin removing column (Thermo Scientific) to remove endotoxin. The column contains immobilised polymyxin-B beads that binds to the lipid A portion of bacterial lipopolysaccharide and thus removes it from contaminated samples. Columns were equilibrated at room temperature and the storage solution (25% ethanol) was removed by gravity flow. Columns were then washed sequentially with 5mls of 1% sodium deoxycholate/endotoxin-free water (0.22µm filter-sterilised); 4 ml endotoxin-free water; and 4ml sterile PBS. Purified IgG was then added and allowed to enter the column matrix. IgG was eluted by addition of 1.2mls sterile PBS, which was collected in a 1.5ul microcentrifuge tube. The protein concentration was re-tested using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA) to confirm the presence of purified IgG. The concentration of purified IgG was then determined using the IgG ELISA as described further on.

2.2.9. Purified IgG quantification

Following endotoxin removal, purified IgG was determined using an IgG capture ELISA. The test half of a MaxiSorp plate was coated with 400ng/ml of goat anti-human IgG Fc antibody (18885;Sigma, UK) in PBS overnight at 4° whilst the control half of each plate was washed twice with PBS alone to determine the relative backgrounds

for each sample. Coated plates were washed twice with PBS/0.1% Tween-20 and blocked with 100ul of 2% BSA/PBS for one hour at 37°C. After washing three times with PBS/0.1% Tween-20, purified samples were loaded in serial dilutions (50ul/well) onto the plate alongside a standard containing human IgG of known concentration (I2511;Sigma, UK), and were allowed to incubate for 1 hour at room temperature. Plates were then washed three times with PBS/0.1% Tween-20 and a HRP-conjugated anti-human IgG (A6029;Sigma, UK) was added and incubated for a further hour at RT. Plates were then washed three times with PBS/0.1% Tween-20 three times before 100ul/well of HRP substrate was added and incubated for 15mins in the dark at RT before the reaction was stopped by addition of 100ul of stop solution. Absorbance was then read at 450nm using a Tecan GENious Spectra FLUOR plate reader. The concentration of purified IgG samples was determined by comparison with the standard curve generated by the known concentrations of human IgG.

2.2.10. Human umbilical vein endothelial cells (HUVEC)

Human Umbilical Vein Endothelial cells (Lonza Walkersville Inc) (Lot number 0000311953 Product code C2519A) were prepared and manipulated under sterile conditions in a class II biosafety cabinet. Media was made up in 50ml centrifuge tubes using 5mls of FBS (Fetal Bovine Serum) with 45mls of Endothelial cell growth medium 2 (EGM2) (Lonza), containing added ingredients (CC-4176), as listed below.

EGM-2 Growth Medium	Measure
EBM-2 basal medium	500mls
hEGF (human endothelial growth factor)	0.5mls
VEGF (vascular endothelial growth factor)	0.5mls
R3-IGF-1 (Recombinant Insulin like growth factor)	0.5mls
Ascorbic acid	0.5mls
Hydrocortisone	0.2mls
hFGF-β (human fibroblastic growth factor- β)	2.0mls
Heparin	0.5mls

Table 2.5: EGM-2 growth medium

Media was pre-warmed in the 37°C water bath and 15mls was placed in T75 flask (surface area 75cm²). HUVEC of up to passage 4 trypsinized, seeded at a density of 10⁴ cells/well (200 μ L/well) in 96-well flat-bottom plates in air containing 5% CO₂ at 37 °C and were grown to 75% confluency for 48 hours. HUVEC were analysed by light microscopy for confluence. Media was aspirated and 15mls of fresh media pre-warmed to 37°C was added and incubated at 37°C. When the HUVEC had reached close to 100% confluence, cells were split. HUVEC were washed with 5mls of PBS. Pre-warmed (37°C) of Trypsin (3mls) was then added. HUVEC were then returned to the incubator for 2 minutes. EGM (3mls) was then added to inactivate trypsin. HUVEC were then aspirated and placed in 50ml centrifuge tube at 1000g for 5 minutes. The media was then quickly removed leaving the cells in the bottom of the tube. Fresh warm (37°C) media (20mls) was then added and mixed thoroughly by 1 ml pipette.

HUVEC were counted using a haemocytometer and cell counter. 20 μ l of cells were added to 1.5ul microcentrifuge with 20 μ l of tricon blue stain (1:1 dilution factor). All four quadrants were counted (e.g. 16 squares per quadrant).

E.g. 31.5 cells imply 7.875 cells per chamber. This was multiplied by the dilution factor of 2 =15.75

$15.75 \times 10^4 = 157,500$ cells per 1 ml.

e.g. 20 mls of media implies 315,000 cells in falcon tube.

Plates were then filled according to size; 100,000 cells/ml per 12 well plate and 200,000 cells/ml per 24 well plate.

2.2.11. Protein extraction

Cell extracts were prepared as follows. Plates were placed on ice and each well was washed with 1 mL ice cold PBS. Next 100 μ L of lysis (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA 1% NP-40, 0.1% SDS, 0.5% Na-Deoxycholate, 10 mM NaF, 1 mM Na_3VO_4 , and complete mini protease inhibitor cocktail tablets [Roche] was added to each well and left for 10 minutes on ice.

Cells were scraped from the bottom of the wells and transferred to ice cold microcentrifuge tubes, following which cells were lysed by repeated passage through a 26 gauge needle (five times) and then placed on ice for a further 15 minutes. Lysates were spun at 16,060 g, 4°C for 5 minutes to pellet cell debris. Following centrifugation cell lysate supernatants were transferred to microcentrifuge tubes and stored at -20°C for future analysis.

2.2.12. Determination of protein extraction

Protein concentration was determined using the Bicicchoninic acid (BCA) protein Assay (Thermo Scientific, UK). The assay relies on the reduction of copper ions by the

peptide bonds in the lysates and the subsequent chelation of reduced copper ions by BCA, which induces a colorimetric change. Protein standards were made by diluting a 2 mg/mL Albumin Standard to eight concentrations ranging from 25ug/ml to 2mg/mL. A working reagent was then prepared by mixing 50 parts of BCA reagent A (sodium carbonate, sodium bicarbonate, biochonic acid and sodium tartrate in 0.1M sodium hydroxide) with 1 part BCA reagent B (4% cupric acid). 10 μ L of the Albumin Standard was pipetted into wells of a polysorb plate along with 10 μ L of cell lysate supernatants. Next 200 μ L of working reagent was added to each well and the plate was incubated at 37°C for 30 minutes. Following incubation the polysorb plate was read on the TECAN GENios Microplate reader at 560 nm. The protein concentrations of the cell lysate supernatants were determined by comparison to the Albumin Standard.

2.2.13. Protein detection via immunoblot (Western Blot)

Cell lysate sample, loading dye and dithiothreitol (DTT) reducing agent was made up with water to a final volume of 30 μ L. Samples were heated to 95°C for 5 minutes to denature proteins, and then centrifuged at 16,060 g for 3 minutes. Samples were run on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions to separate proteins on the basis of their molecular weight.

MOPS running buffer (50mls running buffer to 950ml water) was added to the running rig, ensuring the gel was completely covered. 500 μ L of BOLT antioxidant was added to the tank to protect the anode and cathode and to prevent the proteins oxidising.

8 μ L of prestained broad range protein marker (7-175 kDa) ladder (New England BioLabs, UK) was added to the first well of the gel and samples containing 30 μ g of

protein were added to the remaining wells. Then 165 volts was applied to the gel for approximately 1 hour or until the dye had run to the bottom of the gel. The resolved proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane as follows. The gel was carefully removed and placed in a transfer cassette next to PVDF membrane and sandwiched between filter paper and sponge pads all pre-soaked in BOLT transfer buffer. Transfer buffer was made up as follows- 50 mls transfer buffer, 100mls methanol and 850mls water). The cassette was placed in an electrophoresis rig containing transfer buffer and at voltage 10 for one hour. PVDF membranes were removed and blocked with 5% BSA (Sigma) diluted in Tris buffered saline 0.1% Tween (TBST) for 1 hour on a shaker, to prevent non-specific binding when the target antibody was added. Membranes were incubated overnight in the cold room at 4°C with different dilutions of rabbit, mouse and goat antibodies, see Table 2.6.

Antibody	Company
Phosphorylated NFkB p65 (Ser ⁵³⁶)	Cell Signaling
Total NFkB p65 (C22B4)	Cell Signaling
Phosphorylated p38MAPK (Thr ¹⁸⁰ /Tyr ¹⁸²)	Cell Signaling
Total p38MAPK	Cell Signaling
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling
Phospho-Akt (Ser473)	Cell Signaling
Total-Akt	Cell Signaling
Phospho-JNK	Cell Signalling
Total JNK	Cell Signalling

Table 2.6: List of antibodies used in HUVEC cell experiments

Following overnight incubation with primary antibodies the membranes were washed three times with TBS-T. Next membranes were incubated for 1 hour in 1:1000/1:5000 dilution anti rabbit, anti goat or anti mouse antibodies in 5% BSA TBS-T. After incubation with the secondary antibody membranes were washed again three times with TBS-T. Protein bands were visualised by chemiluminescence (GE Healthcare) developed onto photographic film and their intensity quantified by densitometric analysis (QuantityOne software, Biorad, USA).

Results were expressed as a ratio of relative expression of phosphorylated protein to total protein. Phosphorylated and total protein for the same signalling protein was analysed on the same membrane. After incubation and detection of the phosphorylated protein membranes were washed for 5 minutes with TBS-T then the phosphorylated protein was removed by washing twice with 0.2 M sodium hydroxide

for 5 minutes. The membrane was washed again with TBS-T before being blocked for 30 minutes in 5% BSA TBS-T. After removal of the phosphorylated antibody membranes were placed overnight with their respective total antibody and the process was repeated.

2.3. Endothelial and platelet microparticles assay

Using 57 available frozen plasma samples (stored at -80°C), I determined EMP and PMP using flow cytometry. The following samples were analysed: patients with SLE and subclinical cardiovascular plaque (n=16); SLE and no subclinical cardiovascular plaque (n=23); and healthy controls (n=18). Plasma samples were available from patients during or around the time of ultrasound scan.

Blood was collected in sodium citrate tubes. Samples were spun in microcentrifuge at 2,800g for 15 minutes. Supernatant was decanted and samples spun at 2,800g as before. Supernatant was removed and known volumes aliquoted into cryovials, and stored at -80°C. Platelet poor plasma was thawed in a 37° degree waterbath. Each sample was centrifuged for five minutes at 5000g and 17000g for 60 minutes. Supernatant was carefully removed leaving 40µl pellet in each tube. Pellets were suspended in Annexin V (AnV) buffer. Annexin Buffer was prepared as per manufacturer instructions by diluting 3 mls of 10x concentration Annexin buffer in 27 mls of sterile water.

2.3.1. Preparation of antibodies and staining of MPs

A 96 well U-bottom plate was used. MPs were stained with 2ug of antibodies in order to determine whether MPs were of platelet or endothelial origin. This was according to the 96 well plate plan. The plate was loaded to stain for EMP or PMP with appropriate controls. MPs were stained for antibodies Cluster of differentiation (CD)31 (PE mouse anti-human BD Pharmingen), CD42a (BV421 Mouse anti-human BD Horizon), CD105 (BD Pharmingen) and CD144 (BV421 mouse anti-human BD biosciences).

Reagent	Company
Annexin V Binding Buffer, 10X concentrate	BD
APC Annexin V	BD

Table 2.7: Materials used to detect microparticles

Cellular source of MPs	Marker	Alternative name	Company
Platelets	CD42a	Glycoprotein (GP) IX	BD
Endothelial cells	CD31	PECAM-1	BD
	CD144	VE-cadherin (Vascular endothelial-cadherin)	BD
	CD105	Endoglin	BD
Isotype control	PE IgG1		BD
	BV421		BD
	IgG1, k		

Table 2.8: Antibodies and isotype controls used to detect microparticles

50µl of resuspended MPs were aliquoted carefully into each well. Brilliant Violet (BV)421 mouse IgG1, k Isotype control BD Horizon, and Phycoerythrin (PE) mouse IgG1K isotope controls, were added.

Samples were incubated at RT in the dark, shaking for 20 minutes. 200 µl of Annexin V buffer was added to each well. Latex beads (Sigma) were prepared by adding 6µl of 3µm latex beads (resuspended well) to 2mls of sterile filtered distilled water. 10 µl of this preparation was then to each well added for counting.

On a 96 well plate up to 12 samples were tested for the presence of EMP and PMP by detection of Annexin V, CD31/CD42a, CD31/CD105 (BV), CD31/CD144, CD105 (PE)/CD42a, Isotype BV, Isotype PE (PE=phycoerythrin, BV Brilliant Violet) and compared with background from an unstained control well.

2.3.2. Flow cytometry

Three-colour flow cytometry was performed using the BD FACSVerse flow cytometer. The size of MPs was determined via forward scatter (FSC) and the granularity of the MPs was detected by side scatter (SSC). Standard beads of different diameters were used for size calibration. A known concentration of sigma latex 3µm beads was used as an internal standard to enable the number of MPs analysed per volume to be calculated. The upper size limit for MPs was taken as 1 µm as per previous studies [179, 239]. The BD FACS Verse flow cytometer settings were adjusted using latex beads, and samples with Annexin V-APC, BV or PE. Samples were acquired at a medium rate for 30-60 seconds. An event is defined as a single particle detected by the instrument and all events were stored. The process of gating involved selecting an area on the scatter plot generated during the flow experiment that decides which events to analyse. Gates determined by forward scatter and side scatter to determine size were set using 0.1 µm, 1.1 µm and 3µm beads.

MPs can be detected by microscopy, enzyme-linked immunoassays and functional assays. Currently flow cytometry is the preferred method because of the ability to quantitate based on beads or flow rate-based method [240]. Flow cytometry previously suffered limitations due to difficulties in sizing and counting in a standardised manner. However, the latest flow cytometers have improved technology, which has helped in detecting MPs as small as 0.1 µm in size. Microbeads calibrated

to specific sizes help with standardised counting. During my experiments, I used the BD Verse Flow Cytometer. The BD Verse flow cytometer is a highly sensitive laser based biophysical technology employed in cell counting, sorting and biomarker detection. Cells/ microparticles are suspended in a stream of fluid and are passed through an electronic detection apparatus. The flow cytometer detects up to thousands of particles per second.

I was interested in determining if there were significant differences in levels of circulating EMP and PMP in patients with SLE compared to healthy controls and if there was a difference between those with or without atherosclerotic plaque, as determined by number of plaques.

2.3.3. Data analysis and calculations

Data was analysed using FlowJo, which is a software package for importing and analysing flow cytometry data. Using FlowJo allowed me to organize and present my samples and view the sample analyses (gates and statistics) e.g. samples could be grouped or sorted by various attributes such as the panel of stained antibodies. Viewing an entire experiment allowed organizing and managing complex cytometry experiments and produces detailed graphical reports.

Number of MPs was calculated using the following equation.

Number of MPs = $(200,000/\text{no of beads counted}) \times \text{no of MPs (AnV+marker+)} \times \text{no of tubes divided into}$

No of mls of plasma

Statistical analysis was carried out using GraphPad Prism. Prior to statistical analysis, data were log transformed, to obtain a normal distribution.

2.4. Pregnancy questionnaire

To examine the relationship between previous APO and presence of sub-clinical CVD in this cohort I acquired pregnancy data on all 95 women with SLE, from the 100 patient cohort (n=95 female, n=5 male), as described in Section 2.1 page 88. I devised a pregnancy questionnaire, shown in Figure 5.1, page 155, to capture clinical information including APO, parity, number of pregnancies, termination of pregnancy (TOP) and number of live births. Information on APO included miscarriage, foetal death, stillbirth pre-eclampsia, hypertension, low birth weight, intrauterine growth restriction (IUGR), abruption placenta, gestational diabetes, Caesarean sections (CS) and preterm labour <37 weeks. This information was collected on all 95 female patients for analysis with aspects of their medical history including renal disease, hypercholesterolemia, hypertension and smoking. BMI was calculated by height and weight of each patient.

The majority of patients were seen during routine consultation at UCLH and the questionnaire was completed via face-to-face interview. Pregnancy questionnaires were collected weekly and data were entered onto a database. Relevant information on remaining patients was collected by subsequent telephone interview. I examined the relationship between APO and presence of subclinical CVD in this cohort. Univariable and multivariable analyses of associations between these variables were then carried out. Interestingly, many patients with SLE had never been pregnant so this issue was then investigated by formulating another questionnaire, described in Chapter 5, see Figure 5.5, page 163.

Prior to devising the questionnaire, a power calculation was performed, assuming that 5 patients who were men were illegible, a plaque prevalence of 33% (as found in the whole female cohort) in patients with normal pregnancies, and an APO prevalence of 43% (i.e. n=39 of the remaining n=90 patients will have an adverse pregnancy) as

was found in previous SLE cohort studies [241]. This calculation led to a 89% power at Type I significance level of 5% to detect a doubling of plaque prevalence (i.e. to 66%), which is compatible with degree of risk found in other studies of APO and CVD in SLE [241, 242]. This sample size calculation was performed based on comparison of two proportions using Stata software (“power” command) by Dr. Colette Smith, Lecturer in Biostatistics in UCL.

2.5. SLE and osteoporosis/CVD

I audited the 100 patients with SLE in this cohort, to see firstly the number of patients who had dual energy absorptiometry (DXA) scans performed. I examined for a correlation between BMD and CVD via presence of plaque, mean number of plaque sites, mean GSM, mean TPA, mean overall IMT, mean common carotid IMT. I also examined for a correlation between LBMD and anti-serine proteases.

3. Chapter Three: Factor Xa and Thrombin:

3.1. Introduction and Aims

As described in the methods section 2.1, page 88, this study involved a cohort of patients with SLE (n=100) in whom subclinical CVD was detected in 36 patients by carotid and femoral ultrasound imaging [243]. The demographics of this cohort are described below and in Table 3.1.

3.2. Demographics of patients with SLE

The majority of the patients were female (95%) and 56% were Caucasian. The only cardiovascular risk factors that differed significantly between the plaque and non-plaque groups in the 100 patients were age, serum triglyceride level and ever having smoked (i.e. ever-smoker versus never-smoker), see Table 3.1 and Table 3.2. Those with plaque were significantly older, significantly more likely to have smoked and had higher triglyceride levels than those without plaque.

The imaging equipment enabled measurement of both carotid and femoral arteries, of not just IMT and presence of plaque but TPA and echolucency as well. Only two patients had thickened IMT in the common carotid artery (CCA) but 36 had plaque, consisting of 14 with carotid plaque only, 7 with femoral plaque only and 15 with both carotid and femoral plaque. Fifteen patients had plaque in at least three sites (each patient had both carotids and femorals scanned). The patients in the plaque group were older (53.8 v's 40.2 years) and had longer disease duration (12 v's 13.1 years) see Table 3.1.

Groups were compared for differences in the prevalence of: anti-serine protease antibodies, anti-Factor Xa and anti-thrombin; EMPs and PMPs and reduced bone mineral density.

Demographics	Plaque (n= 36) (SD, range)	No plaque (n= 64) (SD, range)
Sex (F : M)	33 : 3	62 : 2
Ethnicity		
Asian	2	9
Afro-Caribbean	8	17
Caucasian	23	33
Other	3	5
Mean age at scan *	53.8 (9.2; 27-66)	40.2 (11.4; 20-66)
Mean age at SLE diagnosis	32.7 (11; 16-56)	27.3 (10.4; 8-55)
Mean disease duration at scan	21 (11.7; 3-46)	13.1 (7.5; 2-32)
Global BILAG score at scan	3.9 (6.4; 0-32)	4.5 (4.8; 0-22)

Table 3.1: Demographics and diseases activity of 100 patients with SLE

This table shows the difference in demographics between the plaque and non-plaque groups. The only factor that differed significantly between the two groups was mean age at scan, shown by * (Croca S and Rahman A)

The Classic BILAG was used to determine disease activity clinically. Activity on the date of the sample (current activity) for each organ system was defined as high if the BILAG score was A or B and low if it was C, D or E. Additionally, disease activity was also determined using a numerical overall BILAG score: a score of less than 5 was considered to correspond to low disease activity while of score of 5 or above was deemed to be high.

Traditional Risk Factors for CVD	Plaque (n=36) (SD, range)	No Plaque (n=64) (SD, range)
Smoker (current): Yes vs. No	4 / 32	6 / 58
<u>Ever smoker: Yes vs. No*</u>	<u>15 / 21</u>	<u>12 / 52</u>
Lipid profile (mmol/L)		
Total cholesterol	5.1 (0.9; 2.9-6.5)	4.7 (1.1; 2.5-7.3)
HDL	1.6 (0.5; 0.8-3.0)	1.7 (0.5; 0.8-3.0)
LDL	2.8 (0.8; 1.2-4.2)	2.5 (0.9; 0.8-4.8)
Total cholesterol/ HDL ratio	3.4 (1; 1.8-5.6)	2.9 (0.8; 1.6-5.2)
<u>Triglycerides*</u>	<u>1.3 (0.5; 0.6-2.8)</u>	<u>1.0 (0.4; 0.4-2.3)</u>
Diabetes Yes vs. No	1 / 35	1 / 64
Hypertension Yes vs. No	10 / 26	16 / 48
BP (mmHg)		
Systolic (sBP)	132.3 (15.3; 108-168)	122.9 (15.4; 94-166)
Diastolic (dBP)	75.1 (10.4; 55-96)	76.1 (10.4; 57-109)

Table 3.2: Traditional CVD risk factors in 100 patients with SLE

This table shows the differences in CV risk factors between patients with plaque (n=36) and those with no plaque (n=64)(Croca S, Rahman A)

This table shows the traditional risk factors for both plaque and non-plaque groups.

The only factor that differed significantly between the two groups was ever smoker and serum triglyceride level– shown by *

Current treatment regimen on day of scan	Plaque	No Plaque
Hydroxychloroquine Y vs.N	23 / 13	42 / 22
Immunosuppression Y vs.N	13 / 23	32 / 32
Azathioprine	5	12
MMF	6	14
Others	2	6
Prednisolone dose (mg)	4.7 (8.4; 0-50)	4.5 (4.4; 0-20)
B-cell depletion (ever) Yes vs. No	11 / 25	20 / 44
ACE-inhibitors Yes vs. No	12 / 24	23 / 41
Aspirin Yes vs. No	7 / 29	7 / 57
Statins Yes vs .No	8 / 28	8 / 56

Table 3.3: Treatments used in 100 patients with SLE

This table shows treatments used by both plaque (n=36) and non plaque group (n=64) on the day of the ultrasound scan. There was no significant difference between the two groups. (Croca S, Rahman A)

General Biochemistry and Serology	Plaque	Non plaque
Serum urea (μmol/L)	6.4 (3.5; 2.8-17.5)	5.1 (2.3; 2-11.8)
Serum creatinine (μmol/L)	77.7 (35.2; 40-184)	71.3 (29.7; 42-227)
Serum vitamin D (nmol/L)	55.8 (26.5; 7-114)	63.1 (37.1; 14-228)
Serum albumin (g/L)	43.2 (4.9; 30-50)	43 (5; 19-51)
ESR (mm/h)	23.9 (22.6; 4-86)	19.5 (15.5; 2-71)
CRP (mg/dL)	3.4 (3.5; 0.6-15.5)	2.4 (2.1; 0.6-9)
Serum IgG (g/L)	12.9 (5.4; 5.4-32.3)	13.5 (5.2; 4.6-27.8)
Serum IgM (g/L)	1.0 (1.2; 0.1-6.6)	1.1 (0.9; 0.1-4.9)
Serum IgA (g/L)	2.8 (1.7; 0.1-9.1)	2.9 (1.4; 0.3-5.8)
C3 (g/L)	(0.2; 0.7-1.5)	(0.2; 0.5-1.4)
Anti-dsDNA (IU/L)	109.0 (200.6; <10-712.0)	90.0 (145.5; <10-688)
Anti-C1q Yes vs. No	7 / 21	14 / 34
Anti-cardiolipin (IgG and/ or IgM) Yes vs. No	7 / 26	6 / 56
Anti-β2 GP1 Yes vs. No	3 / 8	6 / 12
Lupus anticoagulant Yes vs. No	7 / 22	5 / 56
Anti-ApoA1 IgG (AU)	138.4 (190.7; 4.5-929.3)	160.5 (247.8; 2.2-1432.9)
Anti-ApoA1 IgM (AU)	367.7 (1043.1; 0.5-3871.8)	281.3 (710.6; 0.5-3871.8)
Anti-HDL (IgG) (AU)	23.0 (27.7; 0-105.5)	15.2 (29.1; 0-194.2)

Table 3.4: Biochemistry and serology of 100 patients with lupus

This table shows the differences in biochemistry between patients with plaque (n=36) and the patients with no plaque (n=64). There was no significant difference between the two groups. (Croca S, Rahman A)

Given that SP have pro-inflammatory effects relevant to atherosclerotic plaque formation and increased levels of serine proteases are found in patients with SLE/APS, I examined whether there was an association between anti-SP and the presence of subclinical CVD in this cohort. This direct ELISA (described in section 2.2, page 90) was optimised and performed as per the protocol described.

3.3. Antibodies against Factor Xa do not associate with CVD

Anti-FXa IgG were found in 44% and anti-Thr IgG in 31%, of all 100 patients with SLE, see Figure 3.1. The percentage of anti-FXa IgG positivity was 33/64 (52%) in patients without plaque and 11/36 (31%) in patients with plaque ($p=0.04$), see Figure 3.2. In contrast, there was no association between plaque and anti-Thr IgG, with the percentage of anti-Thr IgG positivity 22/64 (39%) in patients without plaque being similar to that 9/36 (25%) in patients with plaque ($p=0.3$), see Figure 3.2.

In addition, patients with SLE and plaque who were positive for anti-FXa IgG had fewer plaque sites per patient compared with patients with plaque who were anti-FXa IgG negative ($p=0.02$), see Figure 3.3. There was no association between number of plaque sites and anti-Thrombin IgG status ($p=0.2405$), see Figure 3.4.

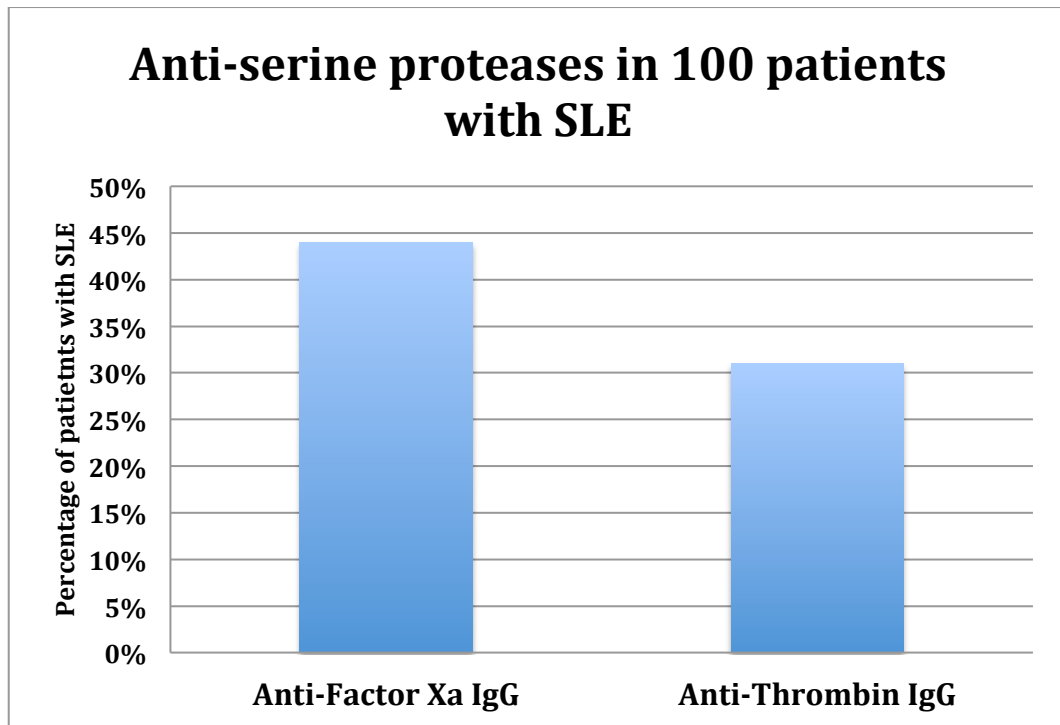


Figure 3.1: Anti-serine proteases in patients with SLE

This figure depicts the percentage of patients with SLE (y-axis) who were anti-FXa IgG and anti-Thr IgG (x-axis).

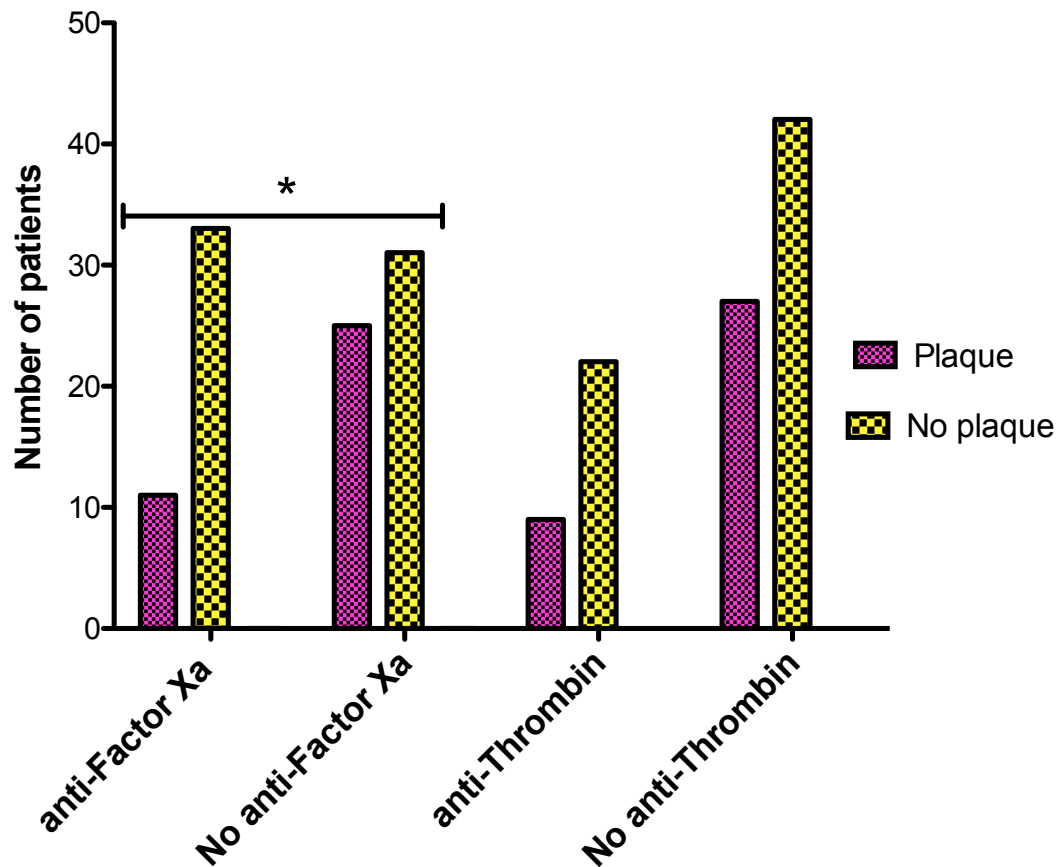


Figure 3.2: Patients with SLE and plaque are less likely to have anti-Factor Xa antibodies than patients with SLE without plaque

This diagram shows the final ELISA results of both anti-FXa and anti-thrombin and their association with plaque. Using Chi² test, GraphPad Prism software v5.0a, there was a statistically significant association between anti-FXa and plaque (* p=0.0422). There was no association between anti-thrombin positivity and atherosclerotic plaque p<0.05.

	Plaque	No plaque
Anti-FXa positive	11	33
Anti-FXa negative	25	31
Ant-Thr positive	9	22
Anti-Thr negative	27	42

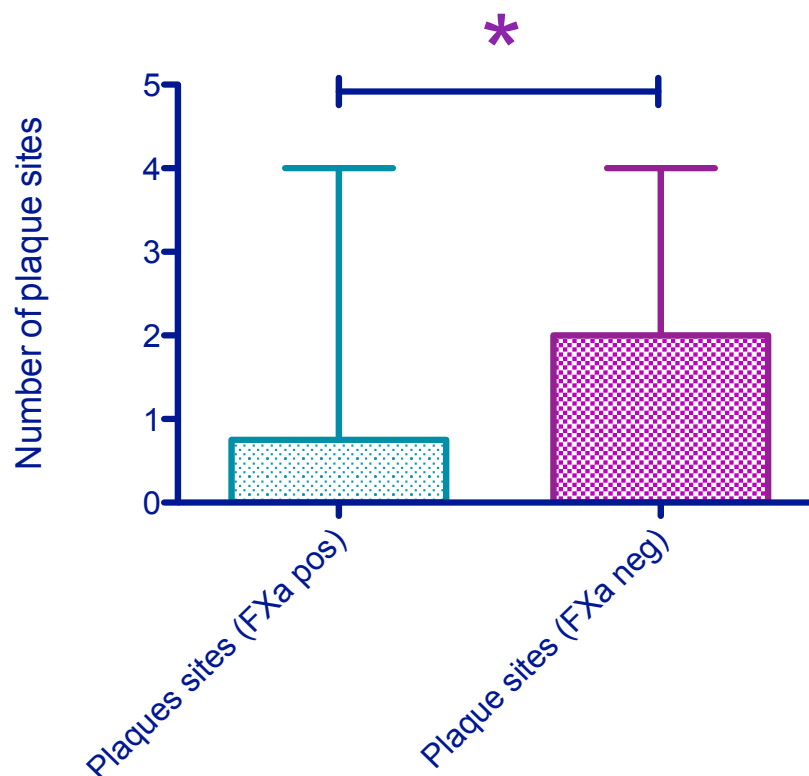


Figure 3.3: Patients with SLE who are anti-Factor Xa positive have less plaque sites than patients with SLE who are anti-Factor Xa negative

This schematic compares the total number of FXa positive and FXa negative patients (x-axis) and the total number of plaque sites (y-axis). Using Mann Whitney U, GraphPad Prism software v5.0a, this was statistically significant * $p=0.0203$.

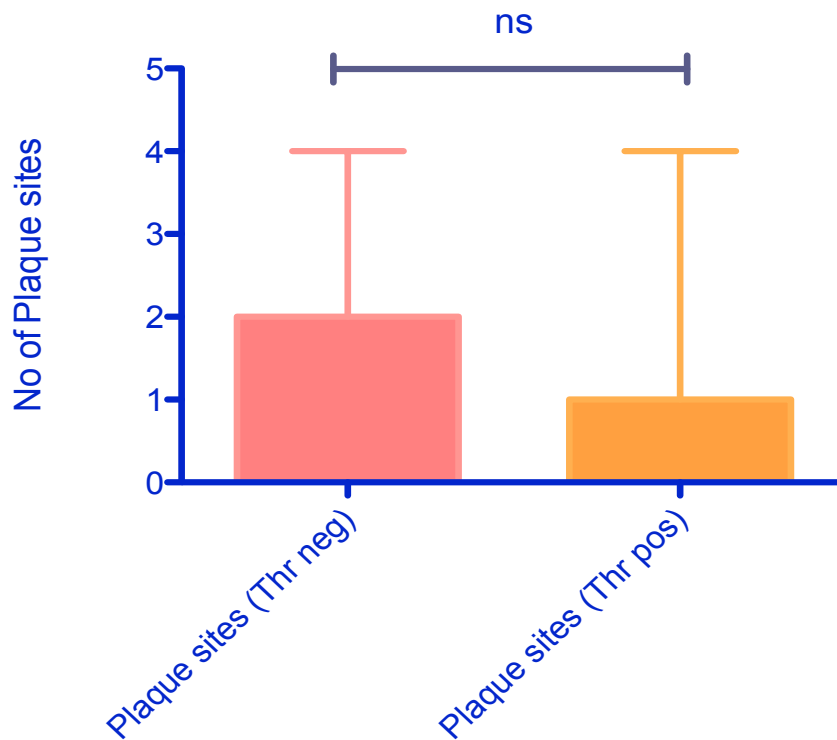


Figure 3.4: Patients with SLE who were anti-Thrombin positive or anti-Thrombin negative had no statistical difference in number of plaque sites

This figure compares the total number of Thr positive and Thr negative patients (x-axis) with the total number of plaque sites (y-axis). Using Mann-Whitney U, GraphPad Prism software v5.0a, this was not statistically significant ($p=0.2405$). Abbreviations: Thr, thrombin; ns, no significance.

3.4. Discussion

Patients with SLE and atherosclerotic plaque were less likely to have Factor Xa antibodies than patients with SLE without atherosclerotic plaque ($p=0.0422$). There was no association between anti-thrombin positivity and presence of atherosclerotic plaque.

FXa and Thrombin have been found in atherosclerotic lesions and may therefore be novel factors in the pathogenesis of CVD. My initial hypothesis was that patients with SLE and subclinical CVD would be more likely to have antibodies to anti-FXa and anti-Thrombin. I was aware that if anti-FXa or anti-thrombin reactive IgG were found to be associated with plaque, then measurement of these antibodies in clinical practice, could add value to CVD risk stratification in patients with SLE. Surprisingly my results showed that patients with SLE and no CVD had the strongest statistical association with anti-FXa antibodies. I also observed that patients who are anti-FXa positive have less plaque sites. One interpretation of these findings is that anti-FXa antibodies have an atheroprotective effect. It is well recognized that the main cause of CVD is an inflammatory disease characterized by immune activation in the artery wall. Perhaps anti-FXa IgG inhibits the inflammatory action of FXa in plaque, or perhaps it is due to other reasons as discussed in the following paragraphs. Other antibodies such as anti-phosphorylcholine antibodies and anti-oxidised LDL antibodies have been reported in the literature to be atheroprotective also [127] [244].

Atheroprotective antibodies in CVD

CVD is virtually unheard of in people living in Kitava, Papua New Guinea [245]. The population appears to be largely uninfluenced by the dietary habits and sedentary lifestyle that are common in developed countries. Frostegart et al. [244] showed that

IgM autoantibodies to phosphorylcholine (anti-PC) measured by ELISA, are significantly higher in the population from Kitava (n=108) who have a traditional lifestyle, as compared to age and sex matched Swedish controls (n=108). They hypothesise that these antibodies are atheroprotective and contribute to the low incidence of CVD reported from Kitava. A further study by this group of a different cohort of 226 hypertensive patients showed that the presence of anti-PC IgM, IgA and IgG1 (but not IgG2) was associated with reduced IMT-progression [246]. They report that anti-PC IgM inhibits cell death and constitutes a strong protection marker for atherosclerosis, particularly in combination with other autoantibodies against modified LDL.

These anti-PC, along with anti-malondialdehyde (MDA) antibodies, were subsequently checked in by a different group from the Karolinska institute in 114 lupus patients and 108 HCs. The presence of both IgM anti-PC and IgG anti-MDA in patients were shown to be associated with a reduced presence of, thus potentially protective against, atherosclerosis in patients with SLE [247].

Oxidised LDL (oxLDL) is another factor that can promote inflammation and immune activation in the artery wall. OxLDL is found in the serum of patients with autoimmune disease including SLE, RA, and APS and has been associated with subclinical atherosclerosis and accumulates in atherosclerotic plaques. OxLDL has been shown to stimulate the production of autoantibodies by B cells. Studies indicate that oxLDL can induce activation of monocytes/macrophages, endothelial cells and T cells. OxLDL promotes inflammation also in immune competent cells from atherosclerotic lesions [248].

There is debate over the role of anti-LDL antibodies. It was originally reported that anti-LDL antibodies were associated with increased risk of atherosclerosis progression and were risk factors for CVD [249]. High levels of anti-oxLDL antibodies

in patients with peripheral vascular disease (PVD) were shown to correlate with more extensive atherosclerotic lesions. Elevated levels of anti-oxLDL antibodies were shown to be related to hypertension, vasculitis, PVD and endothelial dysfunction [250].

In contrast, it has been shown that anti-LDL antibodies are decreased in patients with early signs of CVD-risk such as borderline hypertension and it is suggested that they are atheroprotective. A potentially atheroprotective effect of these antibodies is echoed in other studies [251]. Karvonen et al. showed that IgM autoantibodies to oxLDL have an inverse relation to carotid artery atherosclerosis [252]. The precise cause of these discrepancies is unclear and may in part relate to methodological differences in antibody detection and quantification in these different studies.

Anti-LDL antibodies have been detected in adults, children and patients with CVD. Interestingly anti-LDL levels appear to be higher in children [127]. It is hypothesised that the high levels of anti-LDL in children modulate the antigen and thus protect against the development of atherosclerosis. Shoji et al. showed that oxLDL concentrations in 130 healthy subjects were inversely correlated with the levels of anti-oxLDL antibodies.

B cells contribute to atherosclerosis through subset-specific mechanisms. B-1a cells are atheroprotective because of secretion of atheroprotective IgM antibodies independent of antigen. B-1b cells produce atheroprotective oxidation-specific epitope-reactive IgM antibodies and protect against atherosclerosis in mice and suggest that similar mechanisms may occur in humans [253].

The prevalence/function of anti-FXa and anti-thrombin in SLE in previous studies.

The prevalence and functional effects of anti-FXa and anti-Thr in patients with SLE was studied previously in UCLH, but not in relation to atherosclerosis. Patients with SLE (n=106), APS (n=59), other ARD (n=63) and HC (n=40) had their serum tested for anti-Thrombin, anti-FXa, anti-FVIIa, phosphatidylserine (PS)/FXa and antithrombin (AT)-III by ELISA. Anti-FXa positive IgG were purified to measure their avidity by chaotropic ELISA and their functional effects upon FXa mediated clotting time (FXa-ACT) and enzymatic activity in the presence and absence of antithrombin III (ATIII) a natural inhibitor of FXa.

Anti-FXa IgG were found in patients with SLE (49.1%) and APS (33.9%) ($p < 0.05$), and not in ARD controls and HC. Anti-Thr and anti-PS/FXa IgG were identified in other ARD and anti-FVIIa IgG were low in all groups. The avidity of APS-IgG to FXa was significantly higher than SLE-IgG ($P < 0.05$). The greatest prolongation of FXa-ACT was observed with APS-IgG and the greatest inhibitory effect upon FXa enzymatic activity was found with APS-IgG followed by SLE-IgG compared to HC-IgG. ATIII inhibition of FXa was significantly reduced by APS-IgG compared with HC and SLE ($p < 0.05$) and did not correlate with binding to AT-III. APS anti-FXa IgG was found to have higher avidity to FXa and greater effects upon the enzymatic and coagulant activity of FXa compared with SLE anti-FXa IgG.

The potential role of FXa and anti-FXa antibodies in CVD

To my knowledge this study is the first to identify that anti-FXa antibodies may be potentially protective in patients with SLE and atherosclerotic plaque. The contribution of FXa to plaque formation appears to be either direct via binding of PAR1 and/or PAR2 (which are both present on cardiomyocytes and endothelial cells)

or indirectly through the generation of thrombin. FXa and Thr have significant roles mediating cellular signaling effects associated with the development of atherosclerosis [254]. FXa is produced in atherosclerotic plaque via vascular smooth muscle cells (VSMC), inflammatory cells, and endothelial cells [255].

Parenteral administration of enoxaparin (FXa/IIa inhibitor) and fondaparinux (FXa inhibitor) over 14 days reduced the severity of aortic aneurysm and atherosclerosis in AngII-infused ApoE^{-/-} mice [140]. FXa/FIIa inhibition appear to limit atherosclerosis severity due to down-regulation of vascular PAR-2-mediated Smad2/3 signalling and MMP2 expression. Rivaroxaban, a FXa inhibitor attenuates atherosclerotic plaque progression and destabilisation in ApoE mice, by inhibiting activation of macrophages.

Perhaps anti-FXa IgG has a similar effect as these FXa inhibitors in that perhaps high levels of anti-FXa modulate the antigen and thus protect against the development of atherosclerosis. Alternatively, polyclonal anti-FXa IgG may inhibit uptake of FXa within plaque or inhibit proinflammatory effects mediated by FXa via PARs. Perhaps the low FXa antibody levels in individuals with plaque could be caused by consumption into the atherosclerotic lesions, which are known to contain FXa epitopes, or due to formation of immune complexes.

The potential role of Thrombin and anti-thrombin in CVD

The role of thrombin in generation of plaque has been attributed not only to thrombus formation, but due to its ability to activate platelets [256]. Thrombin-mediated regulation of PDGF influences the migration and proliferation of vascular SMCs leading to plaque formation [257]. It would be interesting to determine a relationship between anti-Thr positivity and PMP levels. Dabigatrin, a direct thrombin

inhibitor was shown to reduce the plaque size in the apoE deficient mouse model. Thrombin has been shown to induce the synthesis of MCP-1 in vascular SM and MCP-1, which is abnormally expressed in the walls of atherosclerotic vessels [258]. Therefore, although I did not find a statistically significant difference between the presence of anti-Thr IgG and atherosclerotic plaque in my cohort, given the evidence, it would be interesting to see if there are significant findings in other lupus cohorts. With over 700 patients with lupus attending UCLH, there is ample opportunity to check these levels in a larger cohort.

iNKT cells in SLE and CVD

In this same SLE cohort of patients in UCLH, a subsequent study by my colleagues [259] showed that the number of invariant natural killer T (*iNKT*) cells in SLE patients with plaque (n=20) was maintained at healthy levels compared to a significant reduction in non-plaque SLE patients (n=30). *iNKT* cells have been shown to contribute to atherosclerosis because of their connection to immune responses and lipids. Interestingly, *iNKT* cells from patients with SLE with asymptomatic plaque produced more interleukin-4 than those with SLE and no plaque. They suggest that *iNKT* cells may have a protective role in patients with SLE who have asymptomatic atherosclerotic plaques [259]. Further larger prospective clinical studies would be required to evaluate these interesting findings.

The advantages of vascular imaging in SLE

Of my cohort, 36% had atherosclerotic plaque as demonstrated on high sensitivity ultrasound. Other studies have shown the prevalence of carotid plaque in SLE ranges from 17% to 40% [106, 131, 260-263] whereas its mean prevalence in HC women is

18% [106, 131, 264]. The Early Vascular Aging (EVA) study assessed the presence of carotid plaque in a cohort of asymptomatic women between the ages of 59 and 71 years and reported a prevalence of plaque of 19%, which is still lower than the average 30% reported for much younger SLE-cohorts [265]. Carotid US in the assessment of atherosclerosis in the context of SLE is important, accurate and safer than other modalities. Patients with SLE who have a serological profile suggesting a higher CVD-risk should have carotid US imaging. The combination of clinical examination, serological profiling and imaging studies could prove to be the key for early diagnosis and primary prevention of CVD and thus allowing for reduced mortality and morbidity among patients with SLE.

3.5. Future work

Further studies are needed to establish the involvement of FXa and Thrombin and antibodies directed against them in the pathogenesis of atherosclerotic plaques, particularly in relation to PAR signalling-mediated cellular events in atherosclerosis. Factor Xa is likely to be the preferred target because of its upstream position in the coagulation cascade and its role in PAR-mediated cellular functions, either through direct activation of PARs or by producing thrombin. Anti-LDL IgG has been demonstrated directly in intimal lesions and as a component of circulating immune complexes, so the presence of anti-FXa IgG and or their immune complexes in atherosclerotic lesions could also be analysed. In addition, it would be interesting to measure circulating Factor Xa levels to examine for an inverse relationship with the presence and titre of anti-FXa IgG.

Future ways to evaluate whether anti-FXa is protective or pathogenic, would involve analysing different epitopes on FXa that determine protectivity or pathogenicity.

Similar to previous studies on atheroprotective antibodies, the mechanism of action anti-FXa needs to be explored. It would be interesting to determine the binding specificities of different anti-FXa isotypes. The effect of anti-FXa on macrophage uptake of apoptotic cells and oxidative stress could be studied by flow cytometry. In my study anti-FXa IgG was tested from serum taken at the time of the scans. Therefore, it would be interesting to test fresh serum samples when these patients are re-scanned to elicit these findings. In addition, it would be important to further analyse anti-FXa IgG-FXa-PAR interactions from fresh samples in patients found to have developed concomitant SLE and CVD on serial imaging.

To my knowledge, anti-FXa levels in SLE have not been tested in children with JSLE and it would be interesting to do so in the future. Children are unlikely to have traditional risk factors, such as smoking that may confound the results and therefore they would be an ideal group to study. It would also be interesting to measure anti-FXa IgG in patients with lupus and established CVD, to determine significance in this group. It would be worth comparing anti-FXa levels in lupus patients in the UK versus a different cohort from another country. E.g. are these antibodies more prevalent in lupus patients living a non-Western lifestyle?

Similar to Factor Xa and thrombin, the proteolytic activities of other serine proteases e.g. TF and anti-FXIIa are increased in early atherosclerotic lesions compared with later atherosclerotic lesions. It would be interesting to determine the role of anti-TF IgG and anti-factor XIIa IgG in lupus patients with plaque versus no plaque and perhaps in those with early versus late atherosclerotic lesions.

The utility of anti-FXa IgG as a future biomarker for CVD in SLE is yet to be determined. Based on my findings the presence of anti-FXa IgG may prove to be a useful marker to stratify the CV outcome of many patients with SLE. Whereby, knowledge of anti-FXa IgG status in patients with SLE may reduce the threshold for

treatment with aspirin or statins in patients without antibodies. By clearly defining the characteristics of anti-FXa IgG, we might further determine whether FXa IgG could be translated from bench to bedside.

4. Chapter Four: Endothelial and platelet microparticles

4.1. Introduction and Aims

Given the importance of endothelial activation in the pathogenesis of CVD and the potential importance of FXa and MPs in this process described in Section 1.3 and Section 1.4, I wished to examine evidence of the involvement of these factors in SLE. My aims were thus to determine whether: anti-FXa positive IgG may have differential effects upon endothelial activation; EMPs and PMPs are increased in patients with SLE; and if there was a difference between those with/without subclinical CVD and healthy controls.

Of the 100 patients described in section 2.1, I tested a total of 57 available samples of which 39 were patient plasma samples. Those with plaque (n=16), no plaque (n=23) and healthy controls (HC) (n=18) were analysed for presence of EMPs and PMPs, as per the protocol described in section 2.3. The 18 HC had an average age of 37 +/- 7 years, 83% were female. It was difficult to find exact age and sex-matched controls for practical reasons, as outlined in strengths and limitations, Section 4.3, Page 151. A total of 88% were Caucasian and 12% other ethnicities and 11% of the HCs were smokers. Samples were stained with Annexin V and platelet and endothelial antibodies, CD42a, CD31, CD105 and CD144. MPs were measured using flow cytometry; see section 2.3.2 (page 106).

Optimisation of experiments to determine IgG interactions with endothelial cells

Based on my interesting findings that anti-FXa levels were higher in those without plaque, my plan was to explore how anti-FXa mediates its effects via endothelial cells. Therefore, I carried out preliminary experiments using HUVEC. There were multiple stages involved in optimisation of these experiments. Firstly, IgG from serum of lupus

patients were purified, concentrated and quantified, see Table 4.1 IgG was also checked to ensure it was endotoxin free and therefore that the effect seen was purely from FXa, see Table 4.2. Samples were diluted to 200ug per ml and tested for endotoxin using the hyglos florescent assay, as per manufacturing instructions. Standard 1-5 is dilution of supplied in kit LPS standard as shown in Figure 4.1. Accuracy was greater than 90% to the level of 0.5.

In parallel with IgG purification, I carried out preliminary HUVEC experiments to examine for the expression of phosphorylated NF κ B, which is well known to be induced downstream of PAR 1 and 2 upon binding of FXa. I also tried to determine the induction of other signalling molecules as described in Section 2.2.13. Six different time points were chosen as shown in Figure 4.2. One hour was the only time where FXa stimulation of NF κ B was higher than media and LPS. Although the one hour results were promising, consistency of signal remained an issue and given my own time constraints as well strong evidence linking microparticles to CV disease, I chose to focus on the study of microparticles instead, described in section 2.3.

SLE samples	IgG (mg/ml)
Sample 1	8.87
Sample 2	18.71
Sample 3	18.01
Sample 4	13.11
Sample 5	27.9
Sample 6	7.06
Sample 7	6.4
Sample 8	23.97
Sample 9	3.91
Sample 10	27.9
Sample 11	6.89
Sample 12	24.95
Sample 13	6.85
Sample 14	15.42

Table 4.1: Results of IgG purification from patients with SLE

This table demonstrated the range of IgG (mg/ml) in 14 patients with SLE.

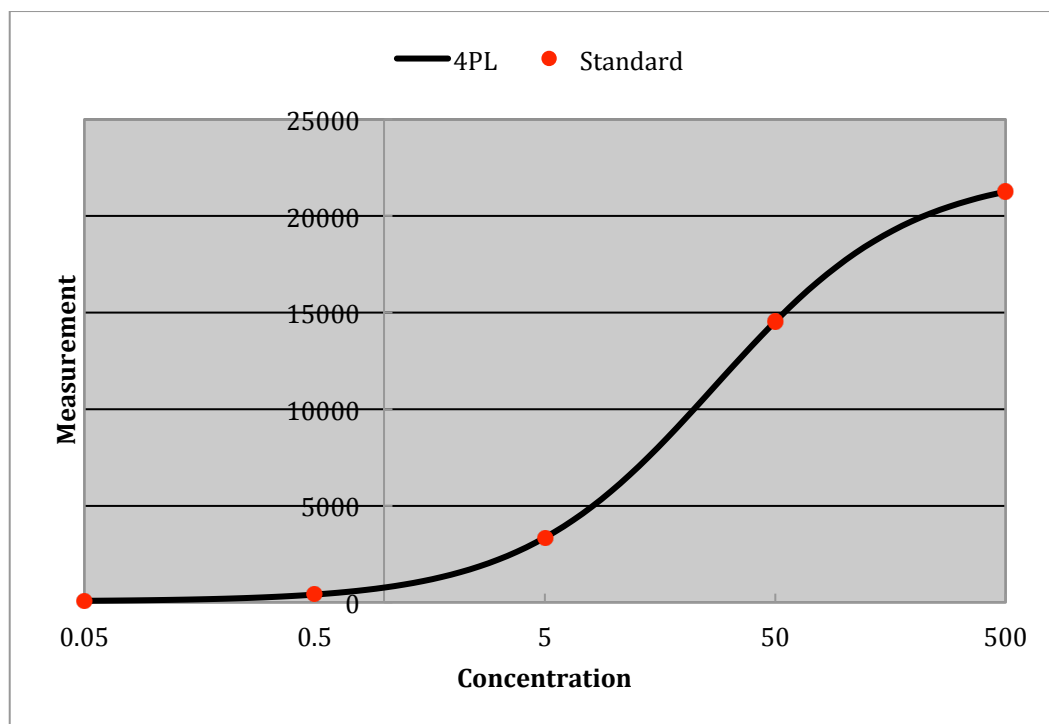


Figure 4.1: Parameter logistic regression standard curve

This figure shows the standard curve used to analyse IgG for endotoxin.

Calibrator	Wells	Concentration	Calculation
Standard 1	A1	500	499.8
Standard 2	B1	50	50.01
Standard 3	C1	5	4.994
Standard 4	D1	0.5	0.5216
Standard 5	E1	0.05	0.03169
Samples 1-14	A2-F3	<0.05	N/A

Table 4.2: IgG Endotoxin levels were undetectable

This table shows that IgG samples (1-14) were below the curve (<0.05) e.g. endotoxin was undetectable as compared to standards (1-5) of known endotoxin concentrations.

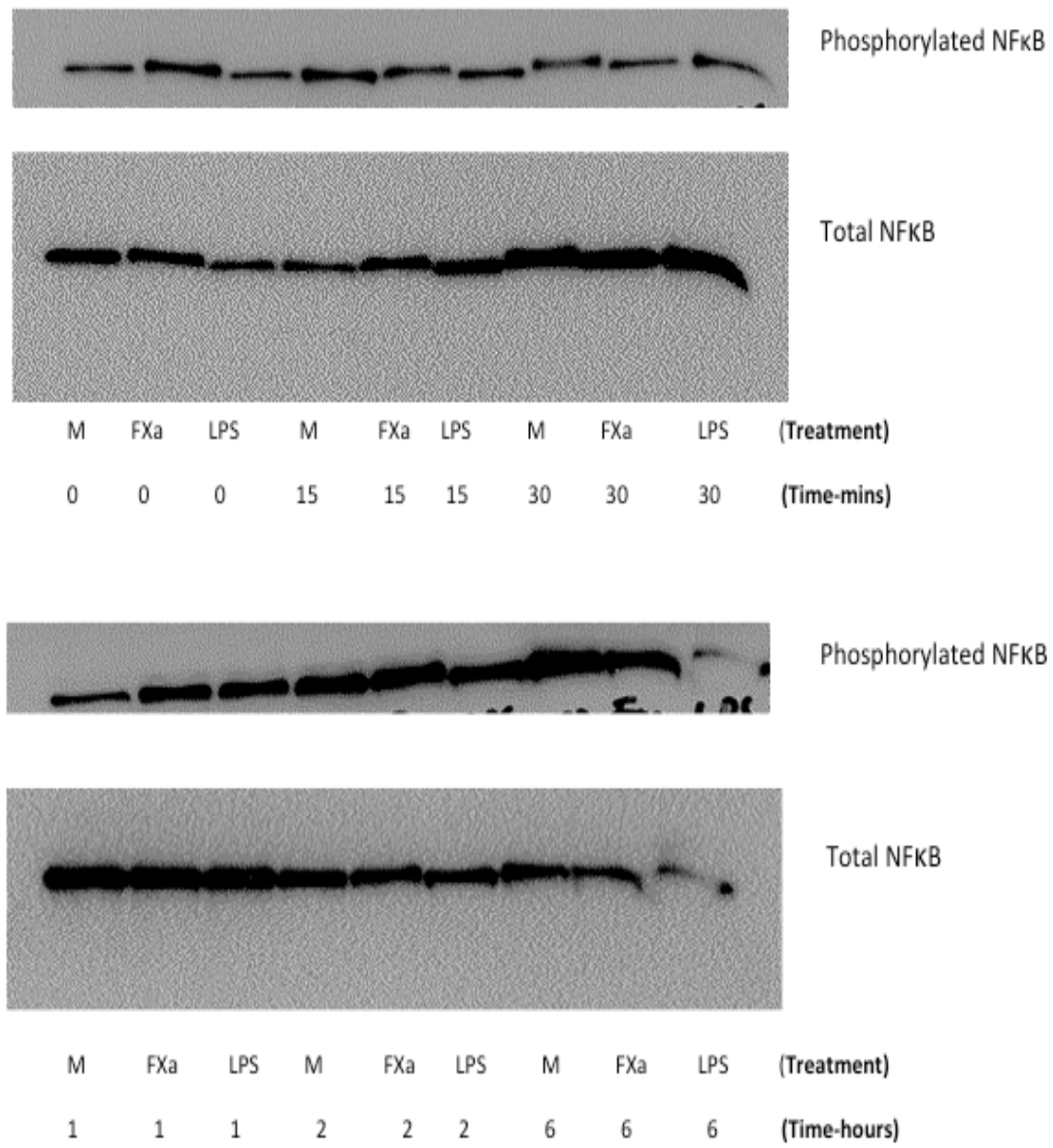


Figure 4.2: Immunoblot demonstrating the effect of FXa on HUVEC

This figure shows the effect of FXa on the expression of phosphorylated NFκB and total NFκB in HUVEC at 0, 15 and 30 minutes and 1, 2 and 6 hours. LPS was used as a control. Abbreviations: M, media; LPS, lipopolysaccharide; FXa, factor Xa.

Optimisation of EMP and PMP experiments

I analysed plasma samples to determine if there was a difference between microparticle (EMP and PMP) levels between patients with SLE, versus healthy controls. My secondary aim was to determine if there was a difference between those with SLE with/without subclinical CVD and healthy controls. Annexin V was used to determine PS positivity. I chose the most specific and sensitive platelet and endothelial antibody markers, CD42a, CD31, CD105 and CD144, as outlined in the figure below. Microparticles were defined as particles that were less than 1.0 μm in diameter, had positive staining for Annexin V. Analyses were performed using Flow Jo v10.1 software.

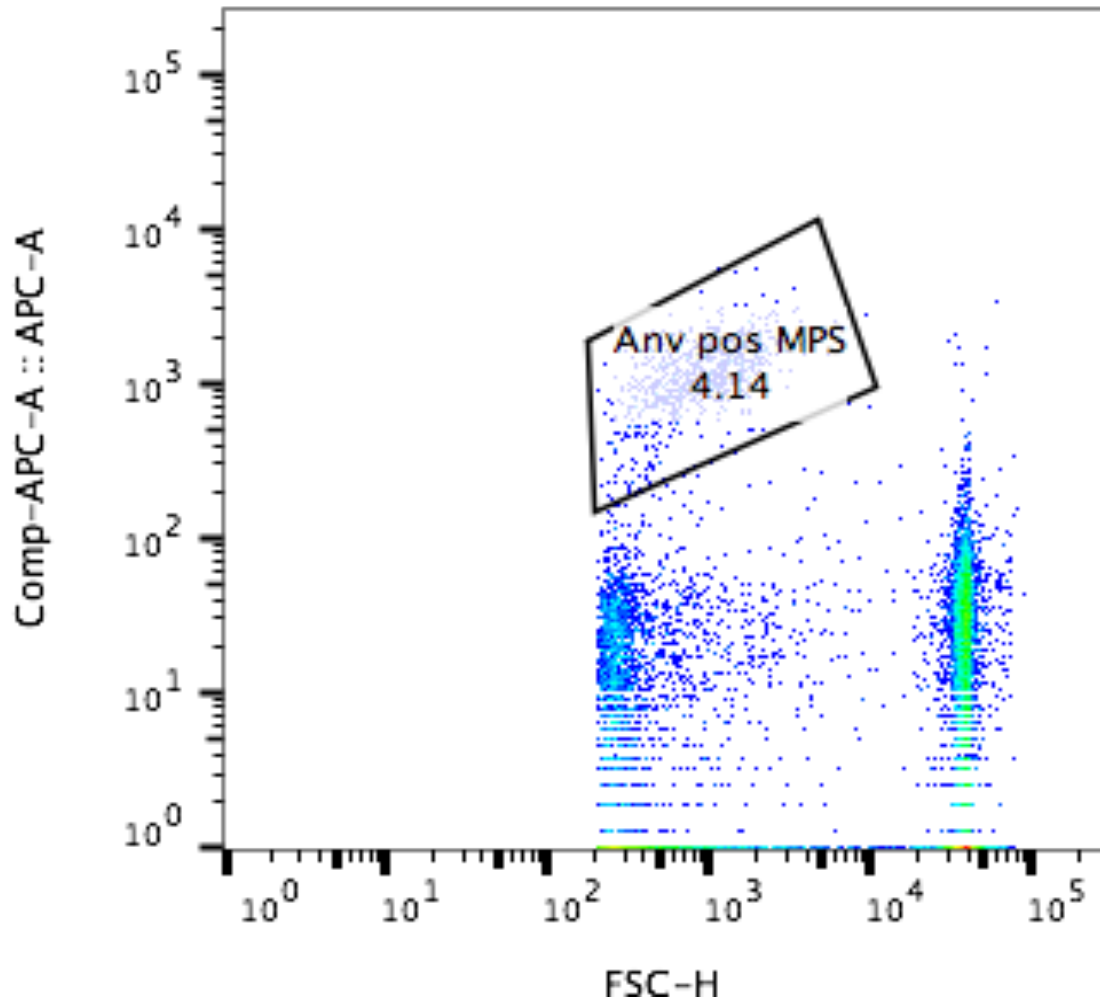


Figure 4.3: Gating of Annexin V positive microparticles

Using Flow Jo v10.1, this figure demonstrates the gating of all microparticles staining positive for Annexin V (Anv pos MPS 4.14) via staining with APC (y-axis) and forward scatter (x-axis). This figure does not differentiate between EMP and PMPs at this stage. Abbreviations: AnV pos MPS, Annexin V positive microparticles; APC, Allophycocyanin; FSC-H, Forward scatter

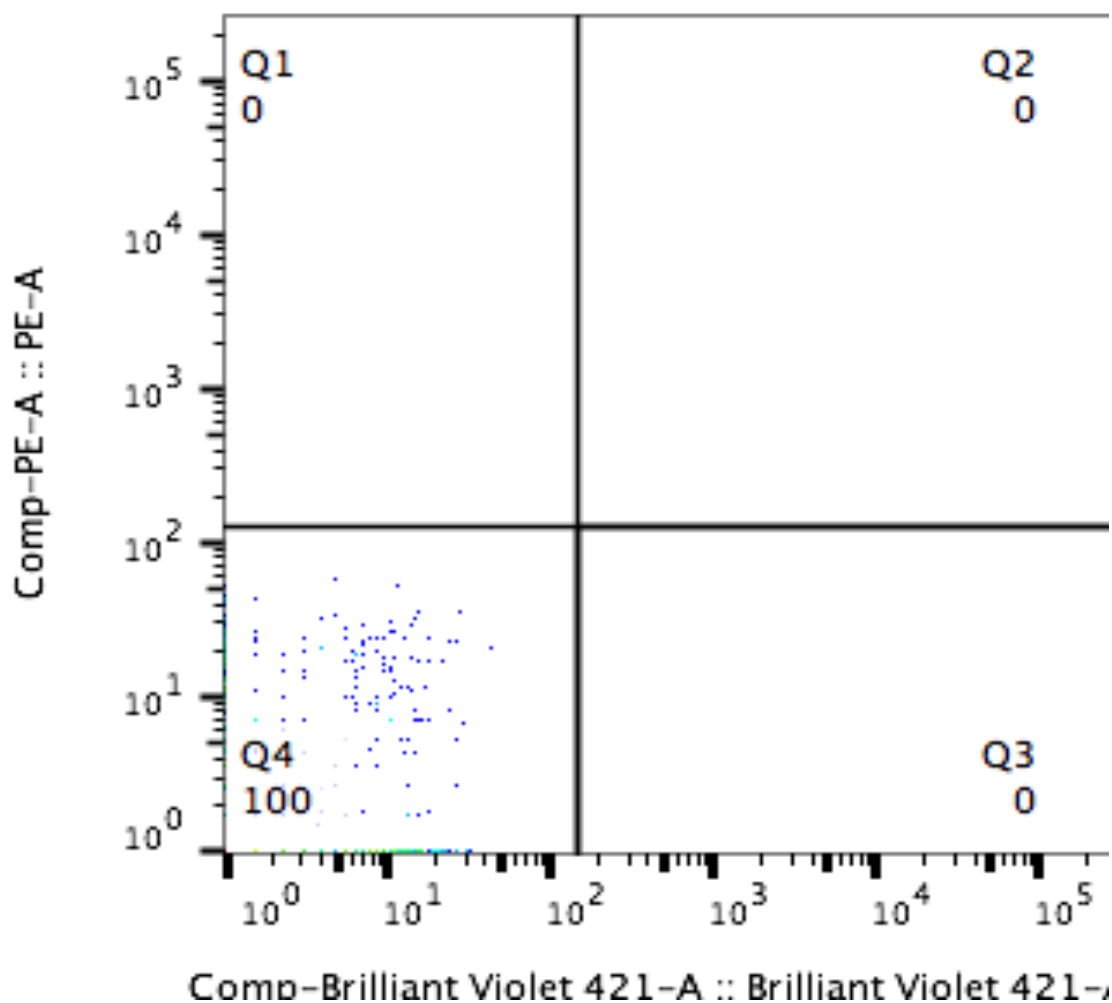


Figure 4.4: Detection of Annexin V positive microparticles

This figure shows Annexin V positive microparticles in the lower left quadrant-Q4, which are unstained for EMP and PMP. There are no microparticles detected in Q1-Q3 because these microparticles do not stain for PE (y-axis) or BV (x-axis). Software Flow Jo v10.1 Abbreviations: PE: Phycoerythrin; BV: Brilliant Violet; Q=Quadrant

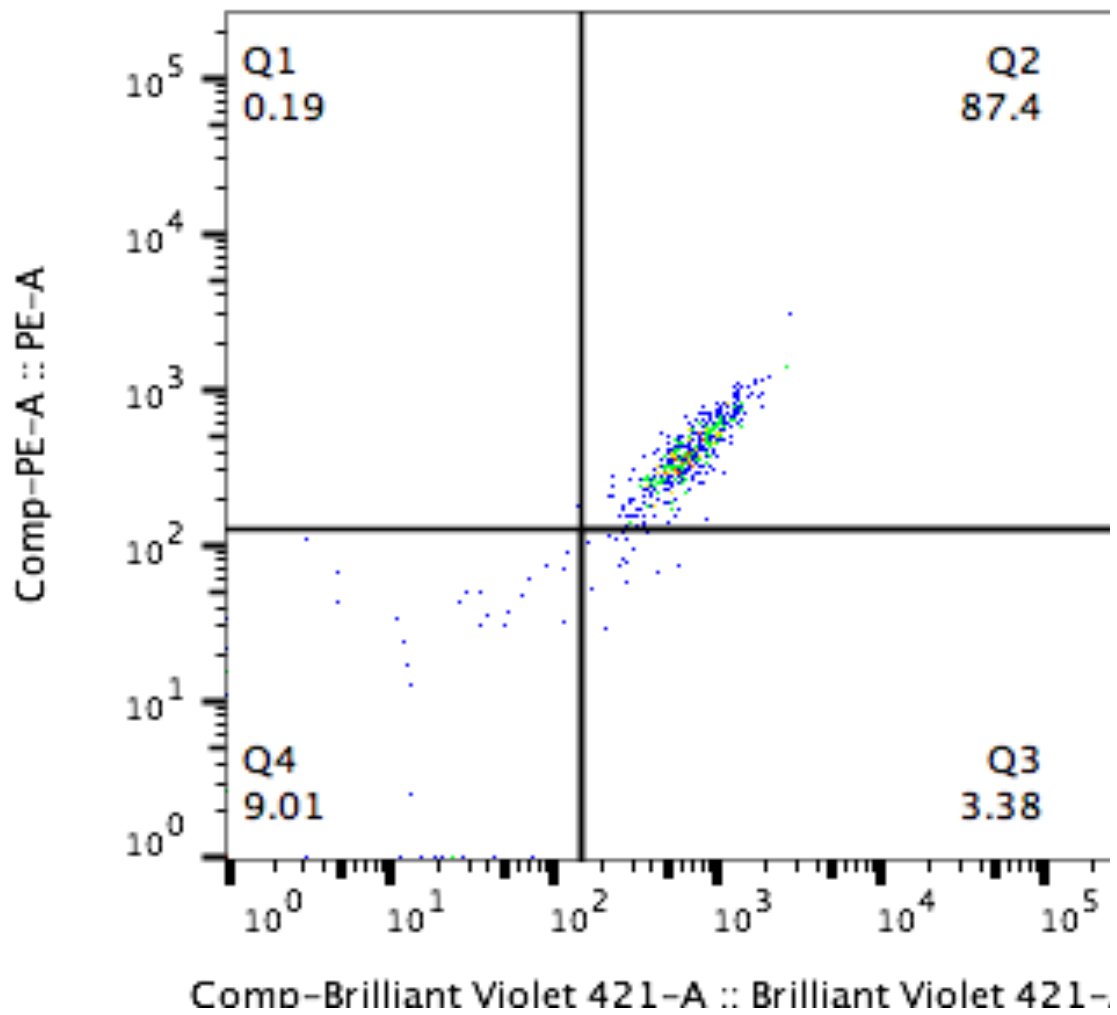


Figure 4.5: Detection of platelet microparticles (PMPs)

Using Flow Jo v10.1, this figure shows the majority of Annexin V positive microparticles predominantly in the upper right quadrant Q2, suggesting double positive staining for CD42a and CD31, used to detect platelet microparticles. PE (y-axis) and BV (x-axis). Abbreviations: PE: Phycoerythrin, BV: Brilliant Violet

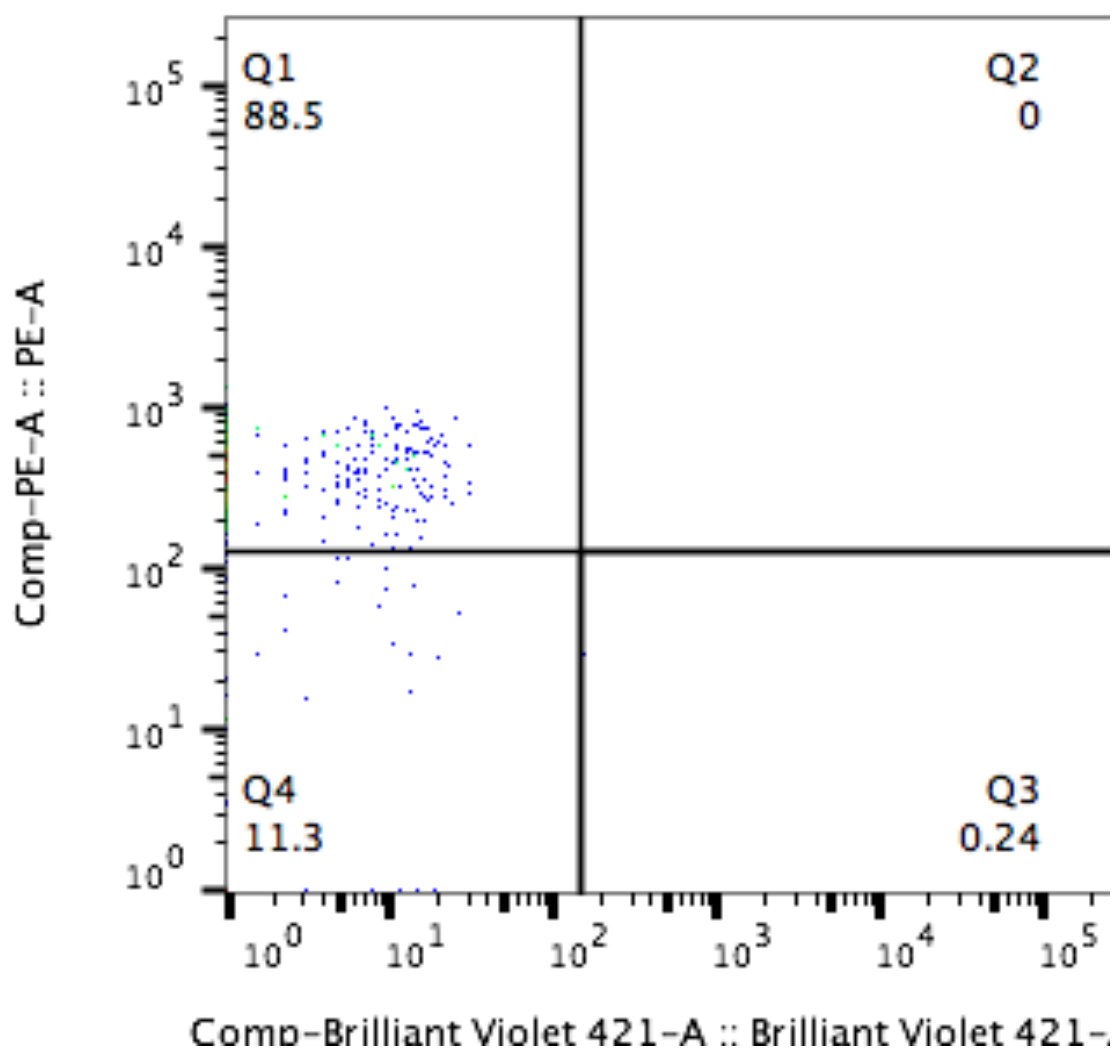


Figure 4.6: Detection of endothelial microparticles (EMPs)

This figure shows Annexin V positive microparticles predominantly in the upper right quadrant Q1, therefore staining positive for CD31 and negative for CD144 used to detect endothelial microparticles. PE (y-axis) and BV (x-axis). Software Flow Jo v10.1

Abbreviations: PE, Phycoerythrin; BV, Brilliant Violet.

4.2. Platelet microparticles are increased in SLE versus HC

Of the cohort described in section 2.1 (page 88), a total of 57 plasma samples (39 patient samples and 18 controls) were tested. The average age of the 39 patients with SLE was 45+/- 12 years. 96% were female. 56% were Caucasian, 18% South Asian, 21% Afrocaribbean, 5% other ethnicities. 18% of the SLE patients were smokers. It was difficult to find exact age and sex-matched controls, as outlined in strengths and limitations, Section 4.3, Page 151. Despite considerable variation in PMP numbers in SLE and HC groups, I found that PMPs were higher in patients with lupus compared to healthy controls ($p=0.0245$); see Figure 4.7. Also, patients with lupus without plaque had more PMPs compared to healthy controls ($p=0.0274$), see Figure 4.8 page 145. There was no statistical significance between those with SLE with plaque and healthy controls or in EMP levels between the three groups. There was no correlation between MPs with disease activity as measured by BILAG.

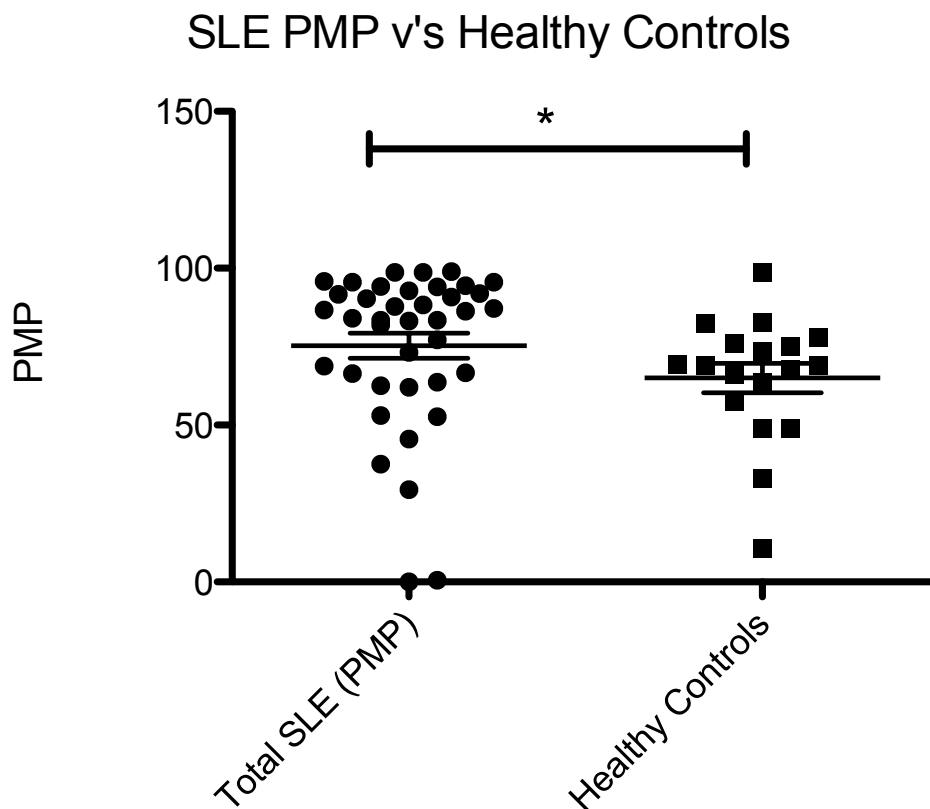


Figure 4.7: Patients with SLE had higher levels of PMPs compared to HCs.

This figure shows the difference in PMPs between all SLE patients e.g. those with plaque and no plaque compared to healthy controls. SLE patients had higher PMP levels than HCs ($p=0.0245$), as demonstrated using GraphPad Prism v5.0a software (Mann Whitney U test) $*=p<0.05$. Abbreviations: HC, healthy controls; PMP, platelet microparticles.

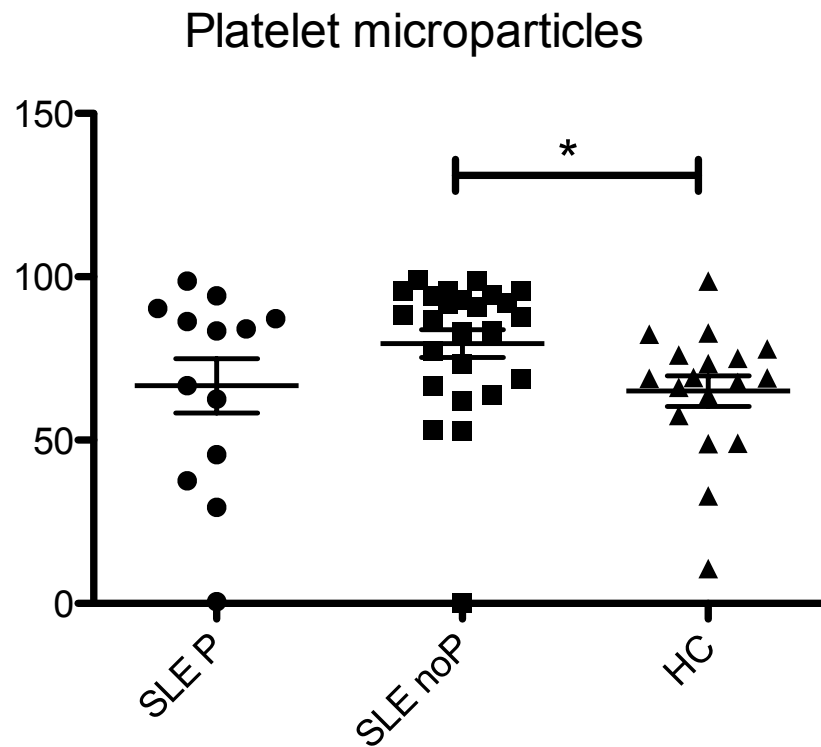


Figure 4.8: Patients with SLE and no subclinical CVD had higher levels of PMPs compared to HCs.

This figure compares three groups. Using one-way ANOVA, GraphPad Prism software v5.0a, there was a statistically significant difference between those with SLE and no plaque and HCs ($p=0.0275$). There was no statistical significance between SLE plaque group and SLE no plaque group ($p=0.1753$) or between SLE plaque and HCs ($p=0.4589$). Abbreviations: SLE P, SLE Plaque; SLE no P, SLE no plaque; and HC, healthy controls, $*=p<0.05$.

4.3. Discussion

In these experiments, I found PMPs but not EMPs to be significantly increased in patients with SLE compared to healthy controls. This relationship however, was only found between patients with SLE and no plaque, since there was no difference between PMPs in patients with SLE and plaque and HC. Therefore, the increase in PMPs was not specific to CVD.

Previous findings of MPs in CVD.

It was an interesting observation that PMPs were higher in patients with SLE with no plaque compared to HCs. The prevailing view is that circulating MPs are harmful; contributing to CVD, and therefore my original hypothesis was that MPs would be more likely to be raised in patients with lupus and subclinical CVD. This view is backed up by other studies. For instance, Morel et al. [266] assessed MP levels within occluded coronary arteries of patients with MI treated with primary angioplasty and compared them with MP levels in peripheral blood. They reported an increase in MPs within arteries, indicating the importance of MPs in the development of coronary atherothrombosis. Williams et al. [267] assessed platelet activation and clinical depression in patients with CAD, because even mild depression, is an independent predictor of increased mortality after MI. They reported that patients with moderate depression and high levels of TNF- α and IL-6 released more PMPs, indicating that a pro-inflammatory component could change platelet function in those patients. A study assessed the effect of clopidogrel with and without rosuvastatin on levels of EMP and PMP in patients with CVD. They showed an increase in the levels of PMP after stopping rosuvastatin and maintenance of only clopidogrel for four weeks and a tendency towards greater EMP levels in those patients. They suggested that an

increase in apoptosis of platelets occurred, and that rosuvastatin might have a protective effect on the endothelium when associated with clopidogrel [268].

However, more recently MPs have been considered to have a beneficial effect on vascular health, which is of interest given my findings. The presumed harmful role of MPs has been challenged because MPs may also be involved in the maintenance and preservation of cellular homeostasis and in promoting defense mechanisms. MPs may also protect against vascular damage [269]. The release of MPs may protect cells against the consequences of external stimuli or stress. It has been shown that endothelial cells escape from complement induce lysis by releasing MPs carrying the lytic complement C5b-9 complex [270]. Interestingly patients with subclinical or less occlusive atherosclerosis have been shown to have more MPs compared with patients with established or symptomatic atherosclerosis [271] suggesting that the ability of the endothelium to release MPs depends on its integrity and viability. A study assessing patients undergoing dobutamine stress echocardiography, reported an elevation in PMPs and EMPs after the echocardiogram, followed by rapid clearance of MPs the following hour in response to cardiac stress [272], suggesting that the release of MPs may be a protective mechanism in those patients. The release of MPs has been shown to protect cells from dangerous or redundant products, compounds, or cellular waste that may accumulate in response to cell stress induced by, for example, CVD risk factors. Therefore, raised PMPs within patients with SLE may be atheroprotective and account for my findings.

Previous findings of MPs in SLE.

Another reason why I found PMPs to be raised in SLE compared to HCs is likely due to lupus itself. In lupus, self-antigens likely derive from the remains of dead and dying cells, as well as from disturbances in clearance. During cell death/activation, MPs can

be released to the circulation. MPs can affect both proinflammatory and anti-inflammatory processes. Previous MP studies in SLE are limited especially in patients with SLE and concomitant CVD. Studies of MPs in lupus are of limited sample size with various different methodological approaches having been used to assess them. It is thus difficult to make direct comparison of the studies. Detecting MPs by flow cytometry is difficult, related to factors such as effects of storage and centrifugation, gating strategy and flow cytometry sensitivity. The range in MP numbers in blood samples is extensive, so a large number of patient samples are needed to assess reliably the number of MPs in the circulation and therefore explore any dysregulation of MPs between disease and controls.

Variation in MP expression in SLE appears to be common and both higher and lower numbers of MPs in the blood of SLE patient compared to those in controls have been reported [273, 274]. Many of the studies of MPs have small numbers of patients e.g. Neilson showed in a study of 29 patients with controls that concentrations of total MPs ($p=0.011$) and AnV-binding (AnV+) MPs ($p<0.0001$) were decreased, compared with controls [181]. Mobarrez et al. had one of the largest cohorts of 280 patients and showed that regardless of MP phenotype, patients with SLE had higher levels of MPs compared to controls [178], however they did not differentiate between those with subclinical CVD as demonstrated by plaque. EMPs have been shown to be increased in patients with active lupus and to be reduced post treatment [33]. There was no statistical significant difference between those with SLE with plaque and healthy controls or in EMP levels between the three groups. Similar to my study, there was no correlation between EMPs or PMPs with disease activity as measured by BILAG.

There are only a few studies looking at PMPs in patients with SLE as listed in a review by Beyer et al. [275]. Raised PMPs in SLE is in keeping with findings from other studies [273]. Pereira et al. showed that there is an association between circulating

PMPs in SLE and an association with thrombin generation and production of a procoagulative state. We know that patients with SLE have an increased risk of thrombosis and CVD, regardless of their APS status. MPs have also been associated with systemic inflammation or risk of thrombosis in rheumatoid arthritis (RA), vasculitis and APS. There is conflicting literature as to whether MPs cause thrombosis, or if they are the result of thrombosis.

Implications of high PMPs in SLE-non CVD.

PMPs are the most abundant MP in plasma and EMPs represent a smaller population, which was in keeping with my results. Kaabi et al. [276] assessed the relationship between MPs and treatment of stable patients with CAD with external counterpulsation (ECP), a therapy which compresses blood vessels to improve blood flow, used for patients with refractory angina pectoris. They found an increase in PMP levels after ECP therapy, and no difference in EMP and MMP levels [276]. My results showed that there were higher levels of PMPs in patients with SLE regardless of CV status. Platelet activation may be a key event in the pathogenesis of SLE. MPs from nucleated cells contain chromatin that can react with anti-DNA and other antibodies to form ICs [277]. These ICs can contribute to lupus pathogenesis by depositing in the tissue, especially in the kidney to induce lupus nephritis [278]. ICs, aPL antibodies and infectious agents are the main activators of platelets in SLE. Platelet activation can be monitored via PMP levels suggesting the potential use of PMP as a biomarker. Platelets promote dendritic cell activation, type I interferon, NETosis, T and B cell activation, which all contribute to the development of SLE [279]. Therefore, PMPs may be a potential biomarker for lupus.

Strengths and weaknesses

My study was unique in that it involved patients with SLE who had detailed CV risk stratification via carotid and femoral ultrasound. Most other studies to date have examined either EMP or PMP in relation to CVD or SLE separately. However, my study examined both EMP and PMP populations in lupus patients with/without CVD. I was fortunate to have access to a modern highly sensitive flow cytometer, which allowed for accurate detection of MPs. It was difficult to find age and sex matched controls and this was a limitation of the study. However, despite the younger age and higher proportion of males in the HC group, there was no significant difference in MP levels between the groups. Also, some of my samples were taken at a different date to the scanned samples, which may have had an impact on the results. My sample size, like most other SLE/MP studies published was small, due to the number of plasma samples available at the time of the scan. In my study I checked Annexin V positive MPs. However, some studies have checked for both Annexin V+ and- MPs that may yield higher results.

4.4. Future work

Further research is required to define the importance of MPs in SLE pathogenesis and to clarify the relationship of subclinical CVD in larger cohorts. Exploring whether therapeutic strategies such as blocking PMP release, or targeting their signalling properties, would be of benefit for patients with SLE, regardless of their CVD status.

It would also be worth exploring MMPs in this cohort also. MMPs are similar to PMPs in that they contain procoagulant substances and are implicated in endothelial dysfunction. Wang et al. showed that MMPs contain IL-1 β and can activate endothelial cells [280]. A previous study measured leukocyte-derived MP (LMP) in 42 patients

with carotid artery stenosis greater than 70%. The patients with unstable plaque had increased levels of the CD11bCD66b+ and CD15+ LMP, suggesting that even less common subpopulations of MPs in plasma, as compared to PMPs, can provide important information regarding clinic atherosclerotic plaque vulnerability in patients with high-grade carotid stenosis [281]. Perhaps PMPs are consumed/deposited in plaque and further research exploring plaque within patients with subclinical SLE versus established CVD, would be of great interest. It would be interesting to test EMP and PMP levels in patients after they have been re-scanned with fresh plasma samples and to check these levels in patients with established CVD.

5. Chapter Five: SLE and pregnancy

5.1. Introduction and Aims

Pregnancy in SLE is associated with higher maternal and foetal morbidity compared to the general population. The main considerations when managing patients with SLE in pregnancy are to control maternal disease and avoid foetal complications. Other challenges however, in SLE pregnancy relate to reduced parity and/or fertility as well as potential associations of APO with future risk of CVD.

Therefore, I carried out a retrospective analysis of the pregnancy experience of women with SLE followed up for a mean of 18 years in the specialist lupus clinic to gather information on pregnancy history, such as APO for correlation with presence of CVD. I collected pregnancy data on 95 women with SLE via questionnaire recording details of parity, gravidity, APO and CVD risk factors as listed in Figure 5.1. Demographic, serological, biochemical data were also available for these patients.

SLE/ Pregnancy Questionnaire (Confidential)		Patient Label																											
1. How many times have you been pregnant? _____																													
2. How many times have you had a: <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">(a) miscarriage? _____</td> <td style="width: 20%;">No of wks _____</td> <td style="width: 40%;">Year _____</td> </tr> <tr> <td>(b) stillbirth? _____</td> <td></td> <td>Year _____</td> </tr> <tr> <td>(c) foetal death? _____</td> <td></td> <td>Year _____</td> </tr> <tr> <td>(d) termination of pregnancy? _____</td> <td colspan="2">If yes, was it for medical reasons? Y/N _____</td> </tr> </table>			(a) miscarriage? _____	No of wks _____	Year _____	(b) stillbirth? _____		Year _____	(c) foetal death? _____		Year _____	(d) termination of pregnancy? _____	If yes, was it for medical reasons? Y/N _____																
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(b) stillbirth? _____		Year _____																											
(c) foetal death? _____		Year _____																											
(d) termination of pregnancy? _____	If yes, was it for medical reasons? Y/N _____																												
3. How many live births? _____ Year _____																													
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(d) Renal disease	Yes _____ No _____																												
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8. Are you currently on: <table style="width: 100%; border: none;"> <tr><td>(a) Aspirin</td><td>Yes _____ No _____</td></tr> <tr><td>(b) Statin</td><td>Yes _____ No _____</td></tr> <tr><td>(c) Warfarin</td><td>Yes _____ No _____</td></tr> <tr><td>(d) Rivaroxaban</td><td>Yes _____ No _____</td></tr> <tr><td>(e) Anti-hypertensive</td><td>Yes _____ No _____</td></tr> <tr><td>(e) Other</td><td>_____</td></tr> </table>			(a) Aspirin	Yes _____ No _____	(b) Statin	Yes _____ No _____	(c) Warfarin	Yes _____ No _____	(d) Rivaroxaban	Yes _____ No _____	(e) Anti-hypertensive	Yes _____ No _____	(e) Other	_____															
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Figure 5.1: Pregnancy questionnaire

5.2. SLE female demographics:

Of the original cohort described in section 2.1, the majority 95% were female which is to be expected given the female to male ratio in lupus. Of these 95 women, the mean age was 45 years and mean follow-up was 18 +/- 11 years. Of this cohort, 7% (7/95) had juvenile onset SLE. A total of 7% were current smokers and 24% ex-smokers. A total of 41% were anti-Ro positive. With regards to aPL antibodies, 14% were LA positive. 14% were aCL positive and 9% were β 2GP positive. A total of 11% (10/95) had concomitant APS. Of this cohort, 64% (61/95) were on HCQ, 18% (17/95) azathiaprine, 20% (19/95) MMF, 32% (31/95) had B-cell depletion therapy, 36% (34/95) ACE-inhibitors, 15% (14/95) aspirin, 17% (16/95) statins, 4% (4/95) tacrolimus and 4% (4/95) methotrexate.

5.3. Women with SLE are more likely to be nulliparous

Of this cohort, 35% (33/95) women had never been pregnant and 45% (43/95) of patients had no children. There were a total of 154 pregnancies, 17% (n=16) had 1 pregnancy; 19% (n=18) had 2 pregnancies; 19% (n=18) had 3 pregnancies; 4.2% (n=4) had 4 pregnancies; 5% (n=5) had 5 pregnancies and 1% (n=1) had 7 pregnancies, see Figure 5.2. A total of 10% of patients had been pregnant but had no children. Women with SLE were more likely to be non-parous compared to the general population (45% versus 20% at age 45). Of the 52/95 (55%) parous women, there were 95 live births. Of the parous patients with SLE, 80% (42/52) had 1-2 children, 15% (8/52) had 3 children and 4% (2/52) had 4 children. No patients with SLE, had more than 4 children, see Figure 5.3. Miscarriage rate was higher 31% versus 15-20% in the general population, as per Table 5.1 (Page 158). In contrast, termination of pregnancy rate appeared slightly lower 30% versus 36% of the general population, as per office of national statistics (ONS).

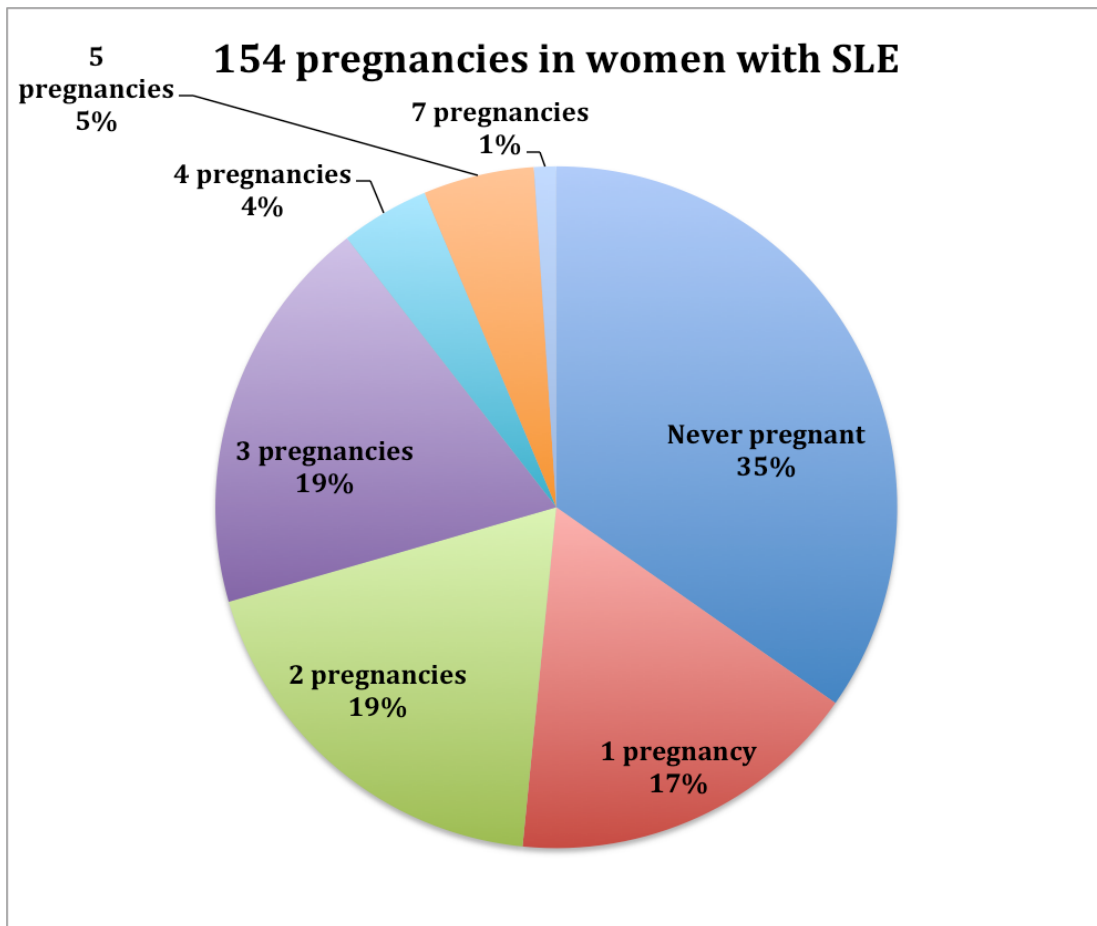


Figure 5.2: Number of pregnancies in women with SLE

A pictorial display of the number of pregnancies per patient as a percentage of the cohort of 95 patients with SLE.

	Women SLE (n=95)	UK population average
Never pregnant	35%	Unknown
No children	45%	20% at age 45*
Miscarriage rate	31%	15-20%
Elective TOP	30%	≈36% by age 45
Average family size (for the 52 who had children)	1.82 children	1.82 children
Number with > 3 children	10.5% (i.e 10/95)	14.3%*

**Office of National Statistics (ONS) UK*

Table 5.1: Pregnancy outcomes in women with SLE compared to the UK population

This table shows comparisons in pregnancy outcomes between women with SLE versus the UK population as per ONS. Abbreviations: TOP, termination of pregnancy.

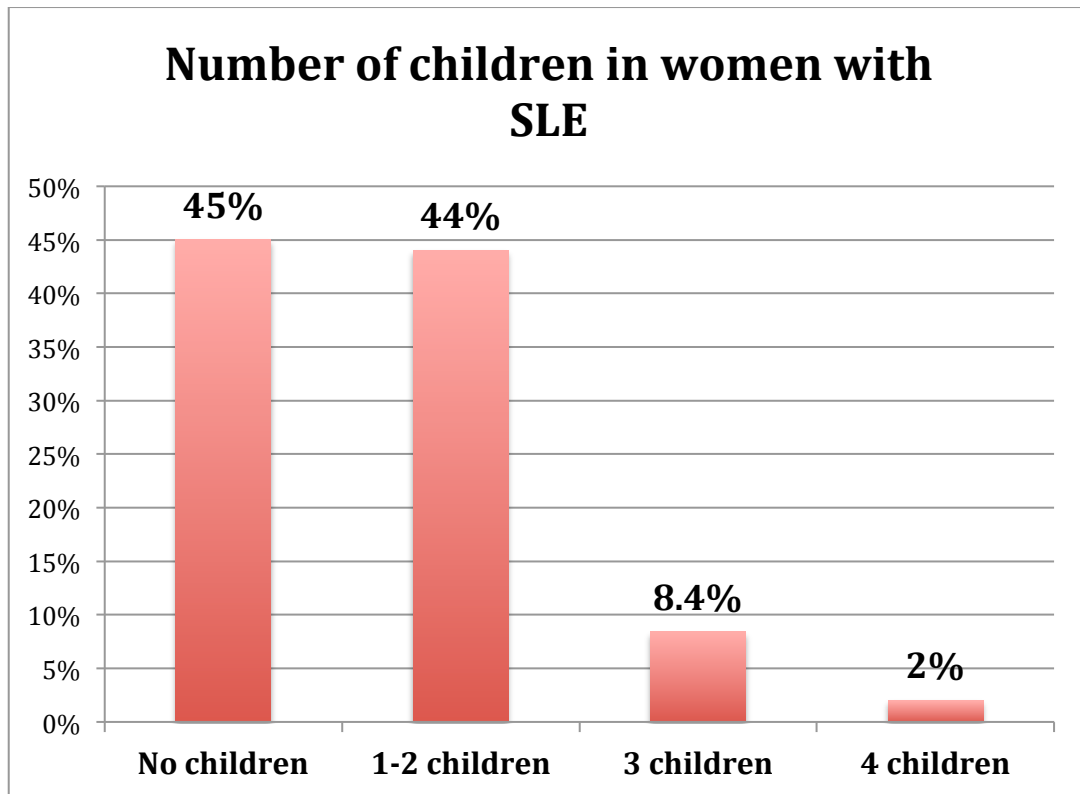


Figure 5.3: Number of children in women with SLE

This figure demonstrates the number of children within this cohort of women with SLE

5.4. Further study of reduced parity in SLE

Given the interesting and unpredicted finding, that 45% of patients had no children, I wished to explore the reasons behind this further. However, I was aware of the sensitive nature of asking women this personal question. Therefore, I decided to engage patients themselves in the process to determine the best way of approaching this topic. I devised an anonymised online survey, using Questionnaire I and II, as outlined below Figure 5.4 and Figure 5.5.

Questionnaire I include the patients' age range and how comfortable patients would feel answering questions in relation to pregnancy as outlined on questionnaire II. Questionnaire I included questions as to whether they felt that research in to pregnancy and lupus was important; whether they would prefer a male or female doctor asking these questions and if they comprehended the medical/non-medical terminology used in the questionnaire. Other questions included preferred method of delivery of the questionnaire, options being online; face-to-face interview; postal; or to fill out the questionnaire while waiting in clinic and drop into a box. I specifically added an area for free text for patient suggestions to gain their insight and ideas regarding changing the proposed questionnaire, or if they had any other comments regarding this research topic.

Questionnaire II explored reasons identified in the literature with regards to reduced fertility/parity in women with SLE. For instance as to whether this was due to personal choice; did not find the right partner; advised not to because of lupus; advised not to because of another disease; medication concerns; concern about disease flare if pregnant; active disease; infertility; fear of passing on lupus to children; fear of passing on another disease to children; fear of not being able to look after children; sexual dysfunction; reduced libido; chronic pain; emotional problems e.g. depression, worsened relationship because of disease/lupus; male factor

problems with sperm; premature ovarian failure; premature menopause or other reasons. Patients were asked if they ever had IVF and if they were an ex-smoker, smoker or never smoker.

I piloted this questionnaire firstly with my clinical and laboratory colleagues. Questions were revised given their sensitive nature. With the help of Lupus UK, a national charity supporting patients with SLE, Questionnaire I and II were posted on the lupus UK website, Facebook and Twitter via survey monkey. Women who were members of Lupus UK were invited to respond.

Questionnaire I

Please read the proposed pregnancy related questionnaire and then answer the following questions

Age: 15-19__ 20-24__ 25-29__ 30-34__ 35-39__ 40-44__ 45-49__ 50+__

(1) How would you feel about answering the following pregnancy related questions?

Comfortable__ Uncomfortable__

(2) Are there any questions in the pregnancy questionnaire that you would feel uncomfortable in answering? If yes, please indicate which ones.

a__ b__ c__ d__ e__ f__ g__ h__ i__ j__ k__ l__ m__ n__ o__ p__ q__ r__ s__ t__ u__

(3) Do you feel that research into pregnancy and lupus is important?

Yes__ No__

4) What do you feel is the best way of doing this pregnancy questionnaire?

Online survey__

Face-to-face interview__

Postal__

Fill out questionnaire while waiting in clinic and drop into box__

(5) Would you prefer a male or female doctor to ask you these questions in person?

Male__

Female__

Either__

Neither__

(6) Do you understand the medical and non-medical terms used below?

a__ b__ c__ d__ e__ f__ g__ h__ i__ j__ k__ l__ m__ n__ o__ p__ q__ r__ s__ t__ u__

(7) Any suggestions on how to change the proposed questionnaire or other comments?

Figure 5.4 Questionnaire I

This questionnaire focused on how comfortable patients would feel answering questions (in Questionnaire II) related to not becoming pregnant.

Pregnancy Questionnaire (Confidential)	Anonymous
Have you ever been pregnant? (If no please proceed)	Yes__ No__
Do/did you want to become pregnant?	Yes__ No__
If never pregnant, was it because of?	
(a) Personal choice	Yes__ No__
(b) Did not find the right partner	Yes__ No__
(c) Advised not to because of lupus	Yes__ No__
(d) Advised not to because of other disease	Yes__ No__
(e) Medication concerns	Yes__ No__
(f) Concern about disease flare if pregnant	Yes__ No__
(g) Active disease	Yes__ No__
(h) Infertility	Yes__ No__
(i) Inability to become pregnant after 12 months	Yes__ No__
(j) Fear of passing lupus to children	Yes__ No__
(k) Fear of passing another disease on to children	Yes__ No__
(l) Fear of not being able to look after children	Yes__ No__
(m) Sexual dysfunction	Yes__ No__
(n) Reduced libido	Yes__ No__
(o) Chronic pain	Yes__ No__
(p) Emotional problems eg depression	Yes__ No__
(q) Worsened relationship because of disease/lupus	Yes__ No__
(r) Male-factor problems with sperm	Yes__ No__
(s) Premature ovarian failure	Yes__ No__
(t) Premature menopause	Yes__ No__
(u) Other	Yes__ No__
Have you ever had IVF?	
Are you an ex-smoker __ smoker __ never smoker __	

Figure 5.5: Proposed questionnaire II

This questionnaire was directed at patients who had never been pregnant and the underlying reasons.

5.5. Results pregnancy questionnaire

Over the course of 33 days, questionnaire I and II were hosted on survey which was posted on the Lupus UK website, Facebook and Twitter. 39 people responded and were included in the analysis. Of the respondents in whom the diagnosis of SLE could not be verified, 97% reported feeling comfortable pregnancy related questions, see

Figure 5.6 and 94% agreed that research into pregnancy and lupus is important, see Figure 5.7.

A lower proportion of patients however, felt uncomfortable answering questions regarding infertility (3%), premature ovarian failure (3%), advice not to become pregnant because of lupus (3%) and sexual dysfunction (5%). Regarding questionnaire delivery; 70% of respondents would prefer an online survey; 21% to fill out a paper questionnaire in clinic and drop into a box; 3% preferred face-to-face interview; and 8% return by post see Figure 5.8. Most (62%) patients had no preference as to whether a male or female doctor delivered the questionnaire, although 31% would prefer a female doctor, Figure 5.9. Some respondents had difficulty understanding certain medical terms e.g. premature ovarian failure (46%).

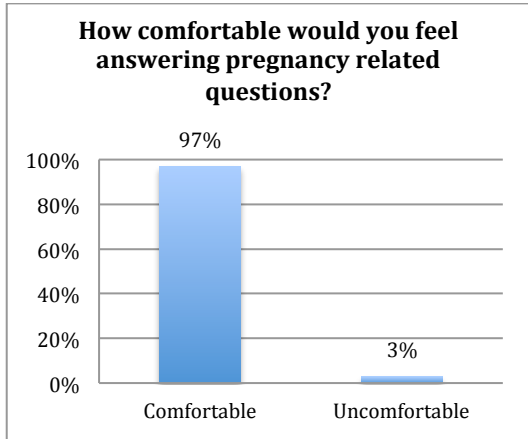


Figure 5.6: Patients feel comfortable answering questions related to pregnancy

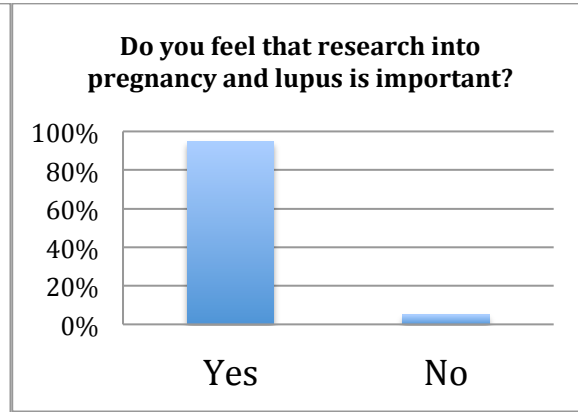


Figure 5.7: Patients feel that research into pregnancy and lupus is important

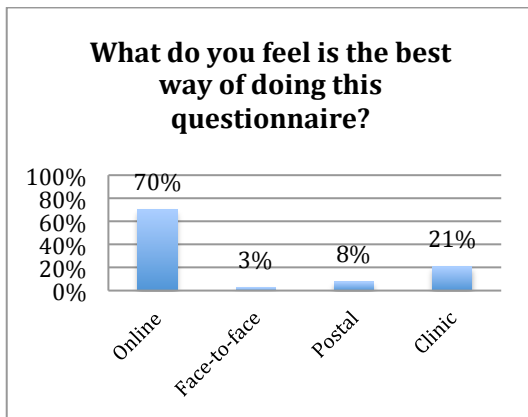


Figure 5.8: Patients prefer online survey as a way of doing this questionnaire

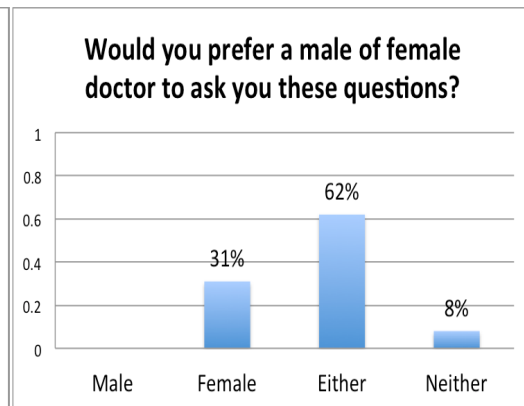


Figure 5.9: Patients preference for gender specific doctor delivering questionnaire

5.6. Pregnancy Questionnaire Feedback

A free text box allowed patients to make other comments and/or suggestions regarding the content of proposed questionnaire II. These responses are shown in Table 5.2. It appeared from the responses that many of these women had been previously pregnant e.g. the general themes identified were from women describing the difficulty in becoming pregnant and regards pregnancy itself. Patients suggested including questions regarding the topics of miscarriages, TOP and difficulties during pregnancy. However, my primary aim of this questionnaire was to focus on women with lupus who had not or could not become pregnant. One of the respondents made a valid point that one would ideally need training in qualitative interviewing for this type of questionnaire.

<u>Suggestion 1:</u> <i>"I would want to be anonymous! I am interested in this topic though, as I had complications with my first baby and almost lost her. My lupus was only diagnosed afterwards, and now I need to find out more about implications for any future pregnancies. I would love to have more children, but am also wary of the heartache I could face if I wasn't so lucky next time."</i>
<u>Suggestion 2:</u> <i>"Pregnancy and miscarriage can be such a sensitive subject that I don't think administration of the questionnaire in a lupus clinic is appropriate. You may find that you get better in depth information via qualitative interviews. You don't need a doctor to ask the questions, you need someone with appropriate training."</i>
<u>Suggestion 3:</u> <i>"You could add to me an obvious question of not continuing in trying for pregnancy due to multiple miscarriages which for me and many can be multiple."</i>
<u>Suggestion 4:</u> <i>"Perhaps include a section for concerns women had before they became pregnant. Could help find some correlation between different situations"</i>
<u>Suggestion 5:</u> <i>"Include questions on miscarriage, difficulty during pregnancy. When were you diagnosed is what age, how are you affected this would have a bearing on your answers is mobility and being able to cope."</i>
<u>Suggestion 6:</u> <i>"Personal choice is quite broad - can encompass no desire for kids or fear that parenting will be difficult due to chronic health issues."</i>
<u>Suggestion 7:</u> <i>"Could add fear of miscarriage/still birth and fear of premature birth. Fear of autism on child."</i>

Table 5.2: Patient feedback and suggestions

This table shows suggestions made by patients in the free text area of the questionnaire.

5.7. Discussion

One of the most striking results from my study was that a large proportion 35% of this cohort had never been pregnant and nearly half the respondents 45%, had no children, more than double what one would expect in UK women of the same mean age (45% versus 20% at the age of 45, as per the office of national statistics. There is no comparative figure for the number of women who have been pregnant in the UK population. The potential reasons for reduced fertility and parity in women with SLE are multifactorial and are outlined in Section 1.6, page 74. I also carried out a systematic review regarding fertility and parity as described in Chapter 7.

There was an increased rate of miscarriage in SLE, which is higher than the estimated 20% found in other studies [282]. This may be due to the fact that 11% of women in this cohort had SLE had APS, which lead to recurrent miscarriages/stillbirths. There was a slightly lower number of TOPs in my cohort (30%) compared to the general UK population (36%) and reasons for this finding are unknown. There is little in the literature regarding TOP and lupus. Foocharoen et al. showed that TOP rate was 27% of their lupus cohort [283]. In my cohort, one patient had a TOP advised due to active lupus. APO including miscarriage and TOP will be discussed separately in this thesis in Chapter 6, page 175. While the precise incidence of infertility in SLE is uncertain, around 1% of infertile women are diagnosed with SLE, which is higher than expected given the incidence of SLE in premenopausal women.

SLE and parity

Despite the high rates of nulliparity within my cohort, there were a number of women of childbearing age with SLE in this cohort (nulliparous women 40.64 +/-13 years), who may potentially go on to have children. For instance, one patient with JSLE was

currently pregnant at the time of the study and another had never been pregnant but had undergone oocyte cryopreservation. Interestingly, similar rates of nulliparity have been found in other studies. Vinet et al. showed that of 339 women diagnosed with SLE before the age of 50, 42% had never been pregnant [284], and factors contributing to lower live birth rate included high disease activity and exposure to cyclophosphamide. In my cohort, a large proportion were on teratogenic drugs including MMF, MTX and ACE-inhibitors, which may have had an adverse impact on women's decisions to become pregnant. Women with SLE are often concerned about the impact of medications on the foetus and the risk of lupus flare if medications are stopped or changed. Women may also be unduly concerned regarding safe medications such as hydroxychloroquine, azathioprine and corticosteroids and may therefore need reassurance that these are safe in pregnancy.

Other reasons for reduced parity include the concern regarding the effect of SLE on pregnancy and the concern regarding the effect of pregnancy on their disease. Personal reasons for apparent reduced parity include a woman's concern regarding their ability to care for the newborn, future disease flares leading to disability and long term effects of maternal SLE upon child health [285]. Reduced parity may also be secondary to psychosocial factors. Depression and anxiety affects up to 40% of patients with SLE [286]. An apparent reduction in fertility may actually reflect reduced frequency of sexual intercourse. For instance, SLE is associated with fatigue, depression and subsequent loss of libido in women [287, 288].

Family Size in SLE

Despite the high percentage of nulliparity within my cohort, and the concerns outlined above regarding becoming pregnant, the average family size appeared similar to the general population, with an average number of 1-2 children. However,

only 10.3% of patients with SLE had three or more children, which falls below the reported 14.3% in the UK population. Clowse et al. reported that 64% of patients with SLE had fewer children than originally planned. Concerns about child health and personal welfare were associated with a lower pregnancy rate. Lee et al. explored family size among 112 Korean women with SLE and compared them to 135 healthy controls. The mean number of live birth was significantly lower in women with SLE ($p < 0.001$). Problems of decreased sexual desire ($p = 0.035$) and menstrual irregularity ($p < 0.0001$) were more common among SLE patients. More women with SLE decided not to have children compared with controls (54.5% vs. 40.7%, $p = 0.031$), and SLE itself was the major cause of the decision. Therefore, these studies echoed my own observation that women with SLE appear to have smaller families than the general population.

Strengths and Limitations

One of the strengths of this study was the ability to have face- to- face consultation with women with SLE regarding their pregnancy history. One of my initial concerns with the pregnancy questionnaire was relying on patients' recollection of their pregnancy history. However, all women had excellent recall, and were able to give specific details including dates, weights and pregnancy complications. Tomeo et al. showed that maternal recall regarding pregnancy details is reliable many years after pregnancy [289]. I also had access to some patients' obstetric notes via the UCLH electronic patient record (EPR). However, a limitation of this study was not having access to the obstetric notes of all the patients.

Patient and public involvement

My decision to get women with SLE involved in the design of the subsequent questionnaire has become a more popular concept. In recent years, patient and public involvement (PPI) has become of increased importance, since it allows patients to have an active and effective role in research and has been proven to improve research [290]. There are benefits in partnership between patients and researchers in designing relevant, participant friendly, ethically sound studies [291]. INVOLVE is a national NHS advisory group that supports PPI in research. INVOLVE suggests that research should be carried out with, or by, patients or members of the public and not just be 'about' or 'for' them. PPI is important for many reasons. From the moral aspect, patients and the public have a right in decisions about research that may affect them. This process includes how research is designed and undertaken and how research findings are disseminated and implemented once a study is completed. Pragmatically, public involvement may ensure that research is relevant and conducted ethically and with sensitivity to involved patients.

There are benefits and challenges of PPI in research. The drive to undertake PPI within research is apparent with many funding bodies requesting that researchers provide evidence of PPI when submitting their proposals including plans for PPI engagement during the research periods. One of the strategic goals for the National Institute of Health Research (NIHR) is that, by 2025, PPI is a 'required part of high quality research'. With regards to my study, I received helpful feedback from patients. However, a limitation of my study was that many of the respondents were previously pregnant and therefore were more interested in the questionnaire being about lupus pregnancy, as opposed to my aim of delineating the reasons as to why women with SLE do not become pregnant.

Mode of administration

The mode of questionnaire administration has been shown to be of upmost importance, as it is likely to have different effects on the quality of the data collected and bias results [292]. The biasing effects of mode of delivery have important implications for research methodology, the validity of the results of research, and for the soundness of public policy developed from evidence using questionnaire-based research.

My study showed that the majority of patients preferred an online portal as opposed to a face-to-face interview. However, this result was likely to be biased given the fact that the questionnaire was presented online. Respondents are also more likely to be interested in their condition and may be more comfortable responding to personal questions regarding pregnancy. My original questionnaire was administered as a face-to-face entity. The benefits of face-to-face interaction are that you administer the questionnaire directly to the patients. However, this mode takes time to complete in a busy clinic and may not allow sufficient time for patients to consider their responses. Postal questionnaires need to ensure correct addresses are available for each patient, and resources available for printing and posting of letters. Online questionnaires require access to a computer and the Internet. Postal and online questionnaires have the advantage that they give patients time to think about their responses, but they may require a contact with the health professional if they have difficulties with questions.

Patient Feedback

The suggestions given by patients were particularly useful to help gain an insight into their perspective and to improve the questionnaire. One patient mentioned fear of

miscarriage, stillbirth and fear of premature birth and fear of a child developing autism. It was interesting to get an insight into patients' views to facilitate the optimizing of future questionnaires. Imagined or actual patient concerns are often different to what healthcare professionals expect, thus PPI is a very helpful and useful exercise. It also enables medical professionals to listen, educate and reassure patients. For instance, reassurance that many women with lupus have normal pregnancies and healthy babies can have a huge positive impact. Following questionnaire feedback, the questionnaire was modified. As an example, there were three responses suggesting that the question regarding sexual dysfunction be removed.

Sexual dysfunction in SLE

It was suggested I remove the question about sexual dysfunction from the questionnaire. Despite the request regarding removal this question, there is much supporting evidence of the existence of sexual dysfunction in SLE, to justify its inclusion. Patients with SLE report lower sexual functioning than other patients with chronic illnesses [293]. Sexual dysfunction in women with SLE has been shown to be associated with vaginal discomfort or pain during intercourse [294]. The risk factors for sexual problems in women with SLE are complicated and multifactorial [295]. Body image, emotional distress, education, disease activity, and depression have been noted to be the most significant causes of impaired sexual function. A recent systematic review reported on the impact of SLE on sexual functioning (n=236 with SLE and 985 female controls). This review showed that there was no significant difference between patients with SLE and healthy controls on desire, arousal, lubrication, orgasm, satisfaction and pain, except for total FSFI (female sexual functional index) [287].

5.8. Future Work

The reasons underlying reduced parity in women with SLE appears to be multifactorial and needs to be explored further. Respondents were interested in getting involved in research from the very early on e.g. at the study design stage. Feedback from patients was of huge benefit to gain an insight into their views. Therefore PPI is a resource that I feel is of benefit in the design of future lupus studies. The online survey appeared to be the most preferable and efficient mode of administration for most patients, although this may not be preferable for older patients. Therefore, perhaps a combination of face-to-face, online and postal administration may be necessary. Further research into the social and/or medical reasons would hopefully shed light on why fewer women with SLE than expected have children. This finding would then help healthcare professionals raise questions such as “have you ever thought of having children?” and explore the reasons sensitively as to why women with SLE cannot or wish not to have children. Only sensitive handling of these conversations may help reassure women with SLE that a healthy pregnancy and healthy baby is a possible, and therefore should not be avoided unnecessarily.

6. Chapter Six: SLE, Adverse Pregnancy Outcomes and CVD

6.1. Introduction and Aims

SLE has strong links to CVD and APO. Women with SLE have a higher risk of APO such as pre-eclampsia and of CVD [241]. I was interested to examine whether women with a past history of APO had subclinical CVD on ultrasound imaging of carotid and/or femoral arteries. Women with SLE and APO developed CV events earlier. Delivery at less than 34 weeks and MPS increased a woman's risk of cardiovascular events 2-fold [296]. It has been suggested that APO should be included when screening for CVD in women with SLE.

My sample size of pregnant women and hence APO was relatively small given the high percentage of nulliparity. A total of 65% (n=62) women with SLE had been pregnant. Of these women, 10% of this cohort had been pregnant, but had no children. There were a total of 154 pregnancies.

6.2. APO in women with SLE

Of these 62 women, 61% (38/62) had APO including miscarriages and C-sections. Therefore, 39% (24/62) of women had no pregnancy complications. Of the entire cohort of 95 women, 55% (52/95) had children. A total of 30% (18/62) of patients had a TOP, for medical and non-medical reasons. TOP was not included in this study as an APO.

The most common APO was miscarriage, which occurred in 31% (19/62), (see Figure 6.1) and in the majority of cases (22%) was a single miscarriage. However, 5% of women had 2 miscarriages, 2% had 3 miscarriages and a further 2% had 5 miscarriages.

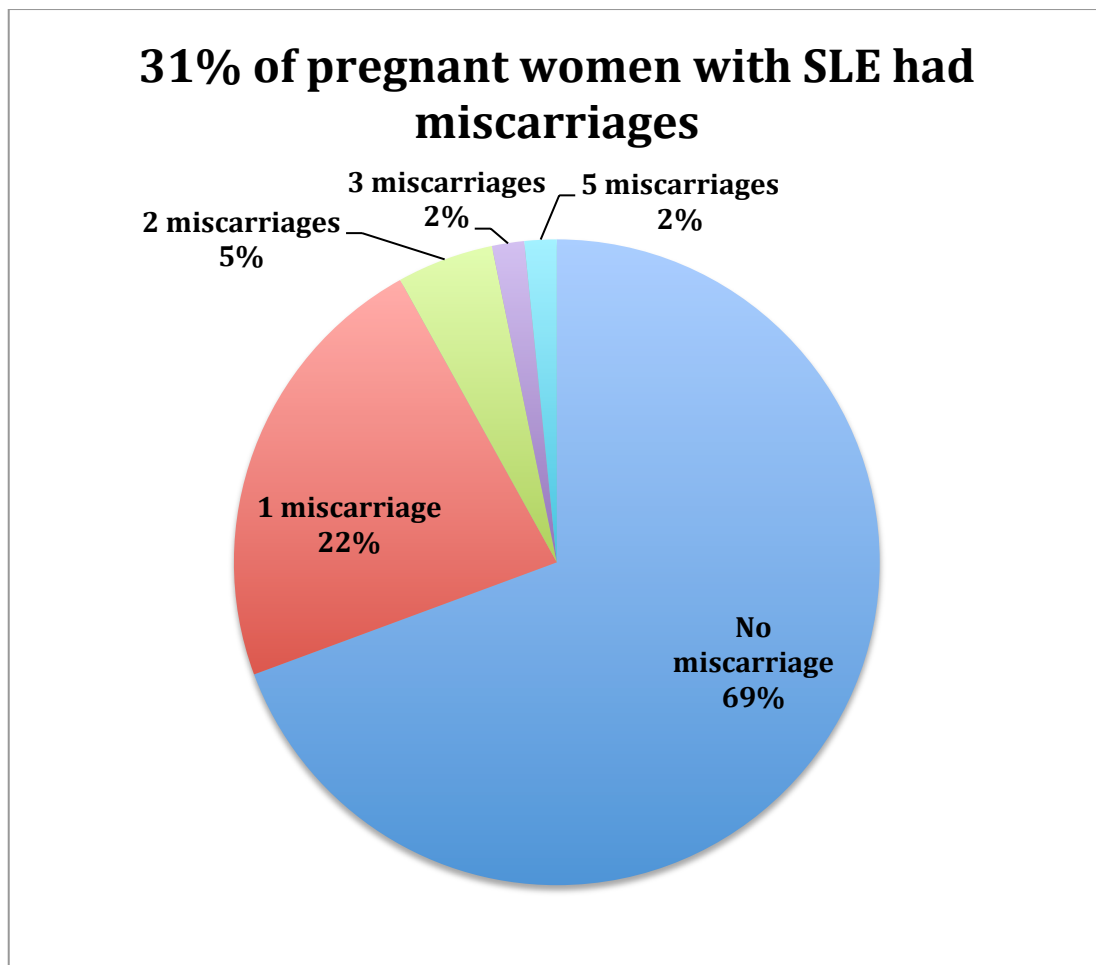


Figure 6.1: Pregnant women with SLE had a higher percentage of miscarriage than the average UK population.

This figure shows the number and percentages of miscarriages in women with SLE.

Of the 62 women who became pregnant, 15% (n=9/62) had preterm labour. A total of 8% (n=5/62) had hypertension during pregnancy of which 2% (n=1/62) developed pre-eclampsia. Caesarean sections were performed in 25% (15/62). Other APO included low birth weight (13%) (n=8/62); placental abruption (3%) (n=2/62); ectopic pregnancy (3% (n=2/62), IUGR (5% n=3/62) and stillbirth (twins) (3%), see Figure 6.2.

Other conditions that women with SLE reported during pregnancy but were not included as APO were Graves's disease during pregnancy, cholestasis in pregnancy and "cervical precancer" in pregnancy.

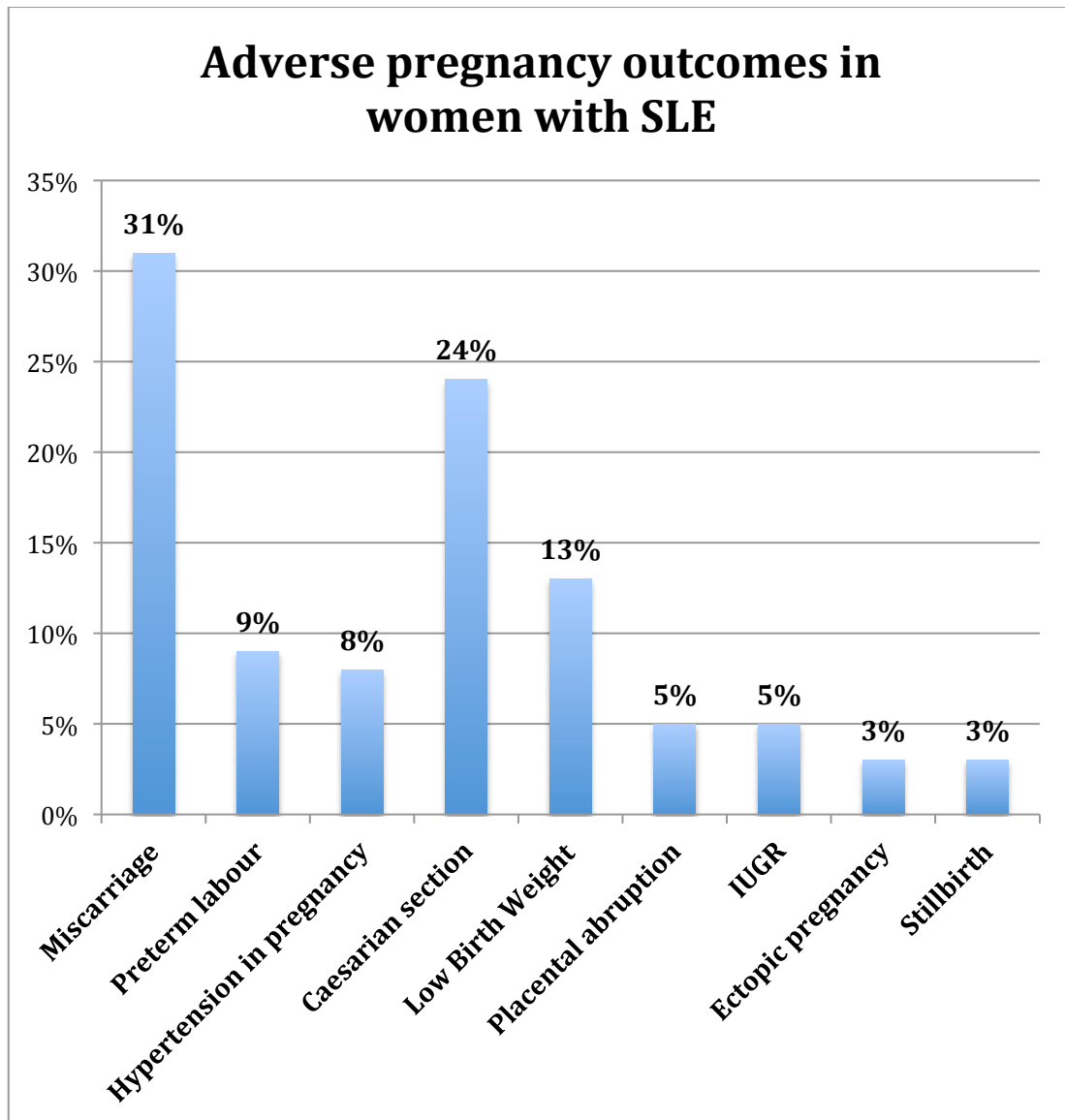


Figure 6.2: Adverse pregnancy outcomes in women with SLE

This figure shows the percentage of APO in women with SLE. Miscarriage was the most common APO, followed by Caesarean section and low birth weight.

Abbreviations: IUGR, Intrauterine growth restriction

6.3. APO in nulliparous women with SLE

Of the 45% (43/95) of women who were nulliparous, 16% (7/43) had TOPs. A total of 9% (4/43) of these nulliparous women had spontaneous miscarriages. One of these nulliparous women with SLE and APS, had 5 miscarriages and no children. She subsequently adopted a child.

6.4. APO in women with SLE and APS

Of the patients with SLE and APS, a total of 4 patients were never pregnant, 1 patient had 5 miscarriages and no children, 1 patient had an early miscarriage, 1 patient had a miscarriage at 15 weeks and 1 patient had preterm labour at 30 weeks, LBW, placental abruption and subsequent TOP, see Figure 6.3.

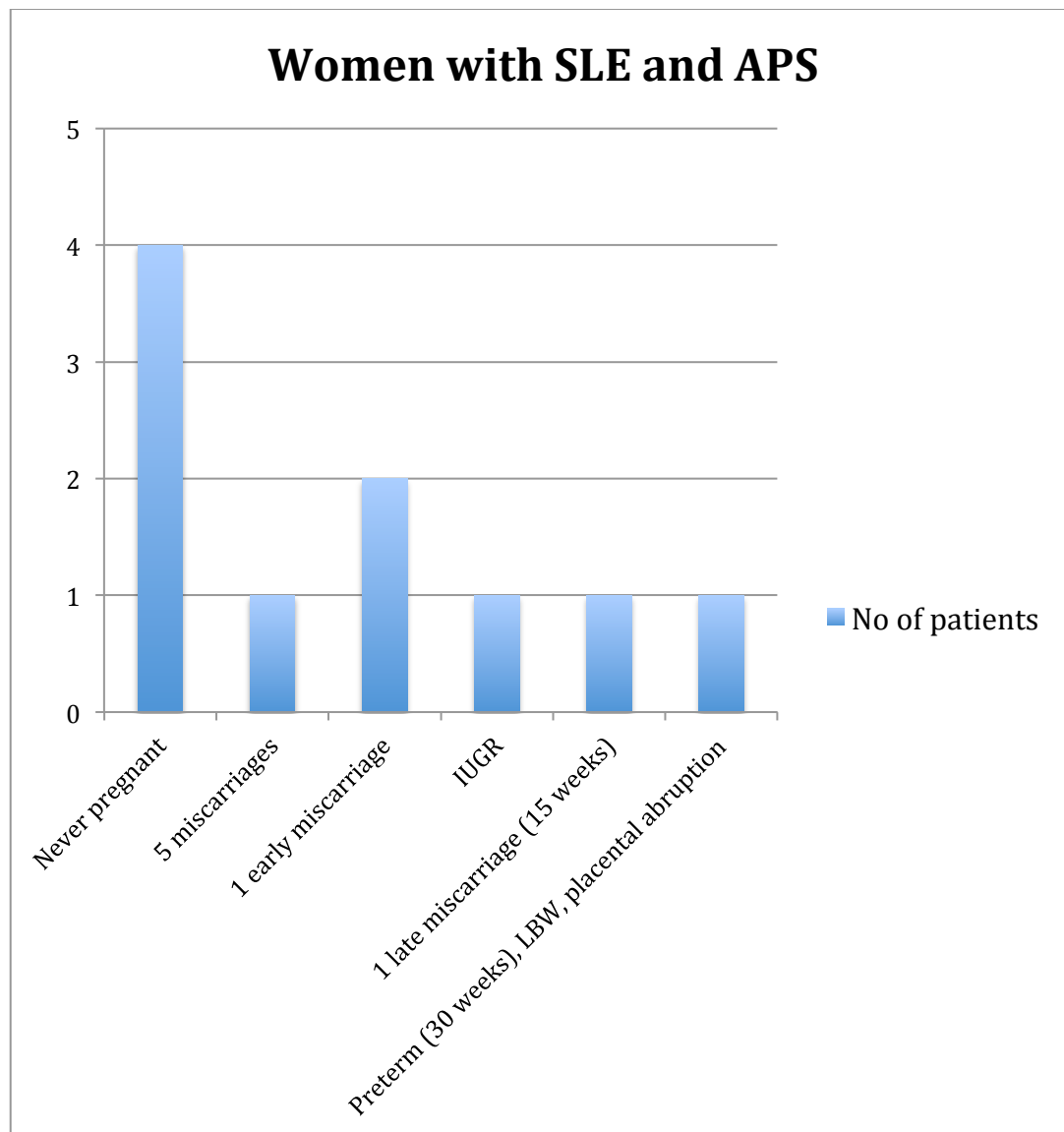


Figure 6.3: Adverse pregnancy outcomes in patients with SLE and concomitant APS

This figure shows the number patients with SLE and APS (x-axis) who had APO including miscarriage, IUGR, LBW, preterm birth and placental abruption (y-axis).

6.5. SLE, APO and Anti-serine proteases

Given the association between APO with CVD, I checked anti-FXa status in these women. The total number of pregnant women (n=62) with APO was (n=38) and no APO (n=24). Of these 38 patients with APO, 14 were anti-factor Xa positive and 24 were anti-factor Xa negative. Of the 24 women with no pregnancy complications, 10 were anti-factor Xa positive and 14 were anti-FXa negative. Of the 38 patients with APO, 12 were anti-thrombin positive and 26 were anti-thrombin negative. Of the 24 women with no APO, 4 were anti-thrombin positive and 20 were anti-thrombin negative.

Women with SLE who had APO were less likely to be anti-Factor Xa IgG positive or anti-thrombin IgG positive, although this difference was not statistically significant (p=0.704, Figure 6.4 and p=0.2421, Figure 6.5).

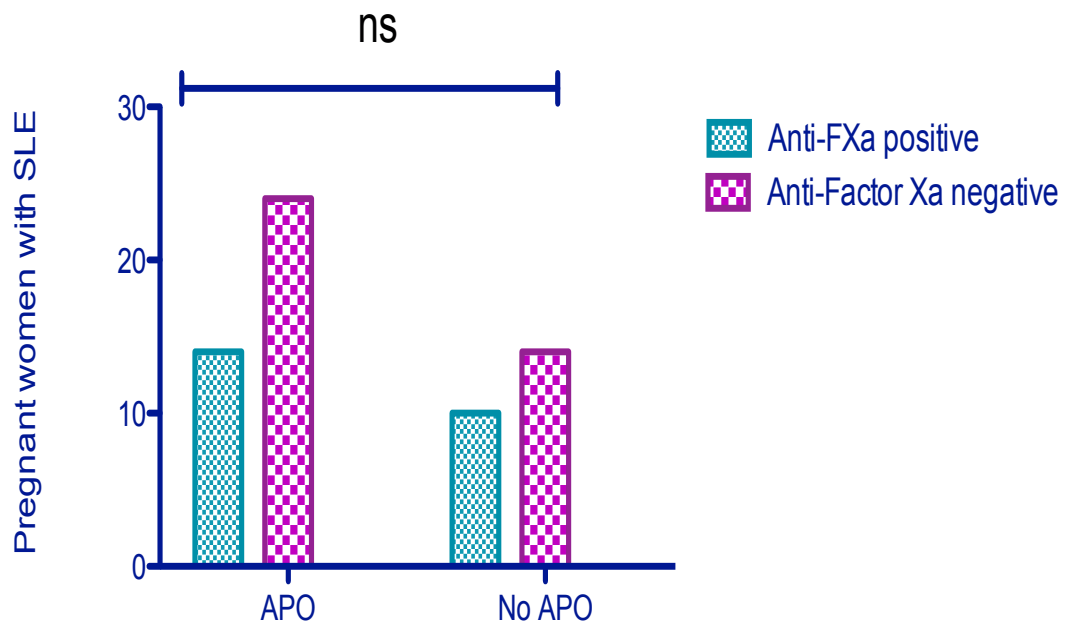


Figure 6.4: Pregnant women with SLE +/- APO and anti-Factor Xa status.

This figure shows all pregnant women (APO and no APO) and the distribution of anti-FXa positivity between APO and no APO subgroups. Using GraphPad Prism v5.0, Chi-squared, there was no statistical significance difference between the two groups ($p=0.704$). Abbreviations: APO, adverse pregnancy outcomes; ns, non-significant.

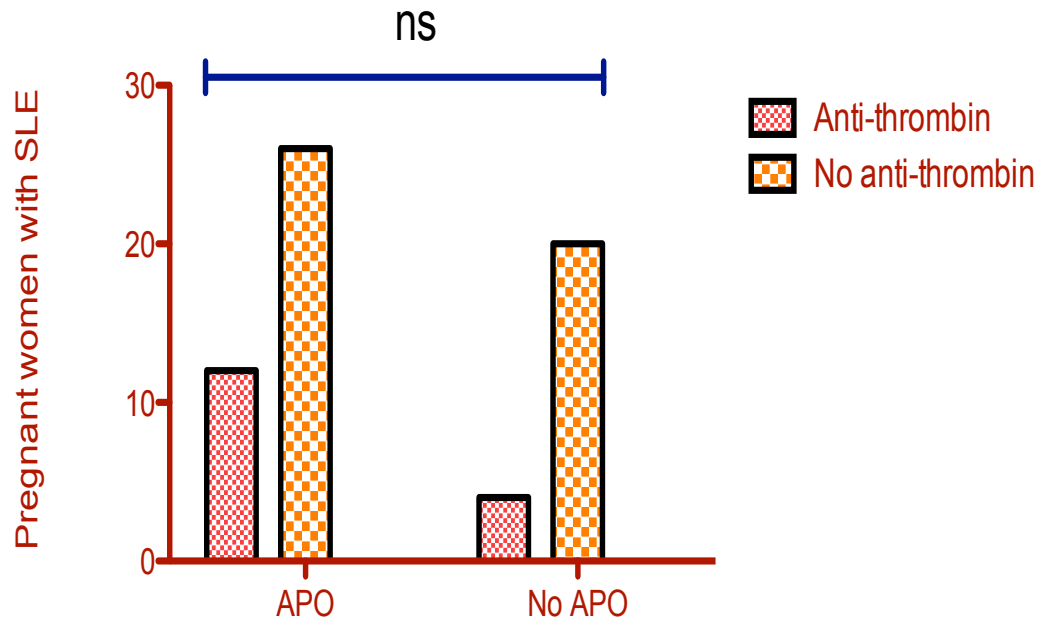


Figure 6.5: Pregnant women with SLE +/-APO and anti-Thrombin status

This figure shows all pregnant women (APO and no APO) and the distribution of anti-Thr positivity between APO and no APO subgroups. Using GraphPad Prism v5.0, Chi-squared, there was no statistical significance difference in anti-Thr in those with APO versus no APO ($p= 0.2421$). Abbreviations; APO, adverse pregnancy outcomes; ns, non-significant.

6.6. SLE and CVD

In this cohort of 95 women with SLE, many had traditional risk factors for CVD. For instance, 40% (n=38/95) had renal disease, 41% (39/95) had hypertension, 18% (17/95) had hypercholesterolemia and 2% (2/95) had diabetes. A total of 7% were current smokers and 24% ex-smokers, see Figure 6.6.

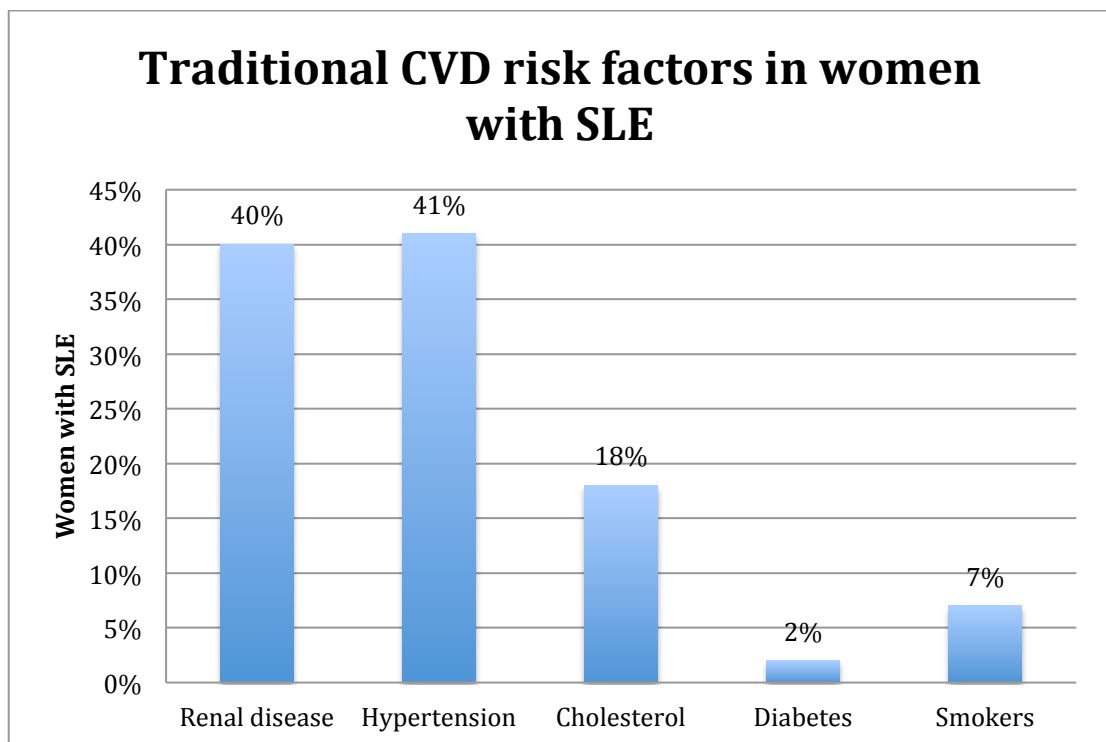


Figure 6.6: Traditional risk factors for CVD in women with SLE

This figure shows the distribution of traditional CVD risk factors within this cohort of women with SLE.

A total of 7.4% (7/95) women subsequently developed established CVD, including MI and stroke. Of these women, 6/95 (6%) had plaque and 1% (1/95) had no plaque, see Table 6.1. The average age of patients having an event was 55 years. Of these patients, only two were anti-FXa positive and one patient was anti-thrombin positive. 5 of the patients were never smokers and 2 patients were previous smokers. Sadly (3%) 3/95 women have passed away since this study started. One woman died from malignant melanoma, one woman died from an MI and another woman died from pulmonary hypertension.

Established CVD in women with SLE	Plaquer	Anti-FXa	Anti-Thr	APO
Microvascular angina	Yes	Neg	Neg	No APO
Myocardial infarction	Yes	Neg	Neg	Miscarriage, abruptio placenta
Myocardial Infarction	Yes	Pos	Neg	No APO
Myocardial Infarction	Yes	Neg	Neg	2 miscarriages, 1 TOP
Stroke	No	Neg	Neg	Never pregnant
IHD	Yes	Pos	Pos	Pre-eclampsia, pre-term, LBW, abruptio placenta
Myocardial infarction	Yes	Neg	Neg	No complications

Table 6.1: Established CVD in patients with SLE and a history of APO

This table shows subsequent established CVD in women with SLE. Two of these patients had no plaque demonstrated on ultrasound. Abbreviations: AVR, aortic valve replacement; CABG, coronary artery bypass graft; IHD, ischemic heart disease.

In the total cohort of women, 35% (33/95) women had plaque and 65% (62/95) of women had no plaque. Of the pregnant women (n=62), 42% (n=26/62) had subclinical cardiovascular plaque. Of the pregnant women with plaque, n=14/26 had APO and n=12/26 had no APO. Of the 36 pregnant women with no plaque, n=24/36 had no APO.

Of these 38 patients with APO, 14 had plaque and 24 had no plaque. Of the 24 women with no pregnancy complications, 12 had plaque and 12 had no plaque, see Figure 6.7.

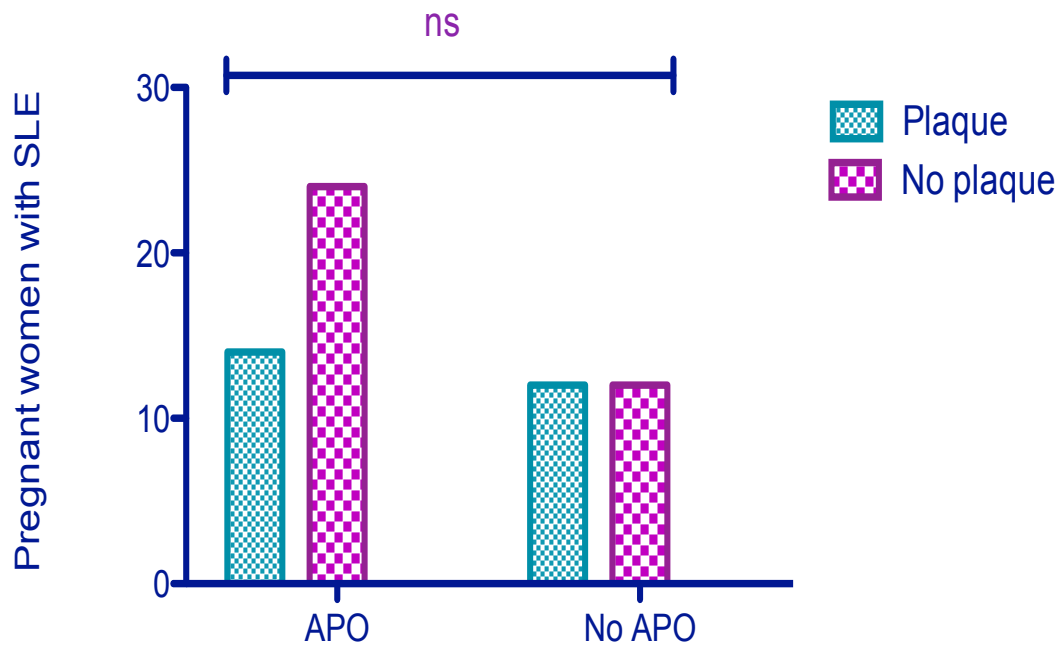


Figure 6.7: Pregnant women with SLE +/- APO and CV plaque status

This figure shows all pregnant women (APO and no APO) and the distribution of plaque between APO and no APO subgroups. Using GraphPad Prism v5.0, chi-squared, there was no statistical significance difference between the two groups ($p=0.3065$).

Abbreviations: ns, non-significant.

	Plaque	No plaque	Total
APO	14	24	38
No APO	12	12	24
Total	26	36	62

6.7. CVD and parity

A total of 21% (7/33) of women with plaque were never pregnant. With regards to those who had no children, 33% (11/33) of nulliparous women had plaque, versus 52% (32/62) nulliparous women with no plaque. The nulliparous women were younger (40.64 +/-13 years) than the parous women (47.19 +/-11 years).

Of the total number of women (n=95), 52 were parous and 43 were nulliparous. Of these 52 parous patients, 22 had plaque and 30 had no plaque. Of the 43 nulliparous women, 11 had plaque and 32 had no plaque. There was no statistically significant difference when comparing the parous and nulliparous groups. Overall there was no relationship between APO, parity and subclinical CVD in this cohort.

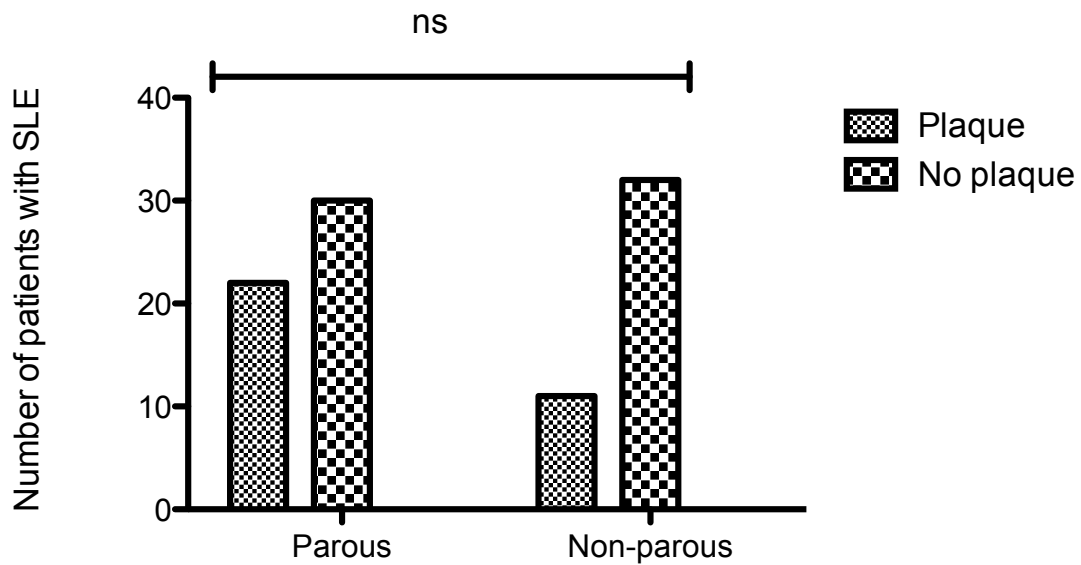


Figure 6.8 Distribution of plaque amongst parous and non-parous women with SLE

This figure shows all women and the distribution of plaque between parous and nulliparous subgroups. Using GraphPad Prism v5.0, Chi-squared, there was no statistical significance difference between the two groups ($p=0.0883$).

	Plaque	No plaque	Total
Parous	22	30	52
Nulliparous	11	32	43
Total	33	62	95

6.8. Discussion

My results showed that the majority of patients (61%) with SLE in this cohort had APO. The rate of miscarriage was much higher (31%) compared to the UK general population (15-20%). Only three of these women who had miscarriages had APS, indicating that SLE itself may confer an increased risk for miscarriage. Retrospective cohort studies show that the miscarriage rate prior to diagnosis of SLE is also higher than among healthy controls [297]. In prospective cohorts, the rate of miscarriage is similar between patients with SLE and HCs, but the rate of stillbirth is 5 to 10-fold higher in women with SLE than in the general population [298-300].

The largest prospective multicentre study of APO in SLE is the Predictors of Pregnancy Outcome: Biomarkers in Antiphospholipid Antibody Syndrome and SLE (PROMISSE) study [301]. APO included foetal death after 12 weeks' gestation (women who had a miscarriage before 12 weeks were excluded), neonatal death prior to hospital discharge; preterm delivery or TOP <36 weeks due to gestational hypertension, pre-eclampsia, or placental insufficiency; small-for-gestational-age (SGA) neonate (birth weight below the fifth percentile). In this cohort, APO occurred in 19% of pregnancies. APO included foetal death after 12 weeks (4%), neonatal death (1%), preterm delivery (9%), and SGA (10%).

In contrast, my results showed that women with SLE had a much higher rate (61%) of APO. However, my study included miscarriages before 12 weeks and C-sections unlike the PROMISSE study. After excluding miscarriages and C-sections and using similar measures as per the PROMISSE study, there were still a larger number of patients with APO (37%) in my cohort. However some of these pregnancies were over thirty years ago and current experience with a specialist obstetric-rheumatology clinic, set up in 2006 in UCLH offers pre-pregnancy counselling and rates of APO are more in line with PROMISSE experience. This clinic identifies risk factors for APO, which are

addressed pre-conception. Risk factors for APO include assessment of SLE disease activity in the last 6-12 months, which can increase the risk of hypertensive complications, foetal morbidity and mortality (OR 5.7 for pregnancy loss, 3.5 for IUGR, and 6.5 for preterm delivery) [68]. Lupus nephritis, previous APO, history of vascular thrombosis, aPL profile, anti-Ro and anti-La antibodies, end stage organ damage also increase the risk of APO.

Lupus anticoagulant and APO

The PROMISSE study reported that lupus anticoagulant (LA) is a primary predictor of APO [302]. My observations were that LA positivity was present in 9 women, 3 of whom were never pregnant. Only one woman who was LA positive, had no pregnancy complications, but otherwise my results reflected the PROMISSE study. Women with SLE who were LA positive had an increased risk of APO; e.g. one patient had a 2nd trimester miscarriage, one stillbirth and 1 preterm infant, another patient had an ectopic and a preterm infant at 5 months; 2 women had miscarriages (1 late at 15 weeks) and 1 patient had a TOP.

Miscarriage rate in SLE

Miscarriage rates are known to be higher in SLE [285]. Reasons for miscarriage in women with SLE include active SLE and APS as well as other general causes such as advanced maternal and paternal age, intercurrent illness, obesity, smoking, alcohol, excess caffeine consumption and genetic abnormalities. A systematic review and meta-analysis of over 517,504 women showed that women who miscarry have a higher incidence of future CVD [303, 304]. Clowse [285] demonstrated an overall rate of miscarriage of 21.7% in the patients with SLE and Hardy showed a rate of 22.1%

[305]. Reasons for the higher percentages of miscarriage in my study may be perhaps due to patients having more active disease prior to pregnancy. However, it is difficult to determine this because my study was done retrospectively. Some women had multiple and recurrent APO. For instance one lady had a preterm baby at 36 weeks due to MPS, IUGR and LBW. The rate of stillbirth was 3% within my cohort as opposed to 0.43% in the UK population. The rate of preterm labour was 9% in my cohort versus the UK figure of 7%. Caesarean section rate was 24%, which is similar to the current population rate of 25% in the UK.

APO and future risk of CVD

The 2011 American Heart Association guidelines identified pregnancy complications as a risk factor for CVD in women. Endothelial dysfunction prior to pregnancy has been suggested to impair the invasion of trophoblasts into the wall of the uterus, which is necessary for pregnancy. Women with a history of recurrent miscarriages have been found to have higher rates of endothelial dysfunction relative to women who experienced uncomplicated pregnancies [306]. In my study, the rate of TOP was lower in those with SLE compared to the general population, and reasons for this finding are unknown and should be explored.

My results showed that 7% of patients went on to develop CVD. The average age of a patient with lupus developing a first CVD event is reported as 49 years and the average age in of women within my cohort was 45 years. Two of the patients with established CVD, e.g. the patient with a coronary artery bypass graft (CABG) and another with IHD and a coronary stent, had never smoked. Their only traditional Framingham risk factor was hypertension. A total of 7% of the cohort was current smokers, which is below the 2015 UK national average for women of 15.3%. It was reassuring that less women with SLE smoke than the national average but concerning

that there are still patients who smoke. As clinicians we need to be advising patients of the major increased risk smokers with SLE have of CVD.

Guidelines for the prevention of CVD in women recommend a referral of women with a history of hypertensive pregnancy to primary care or cardiology in order to facilitate monitoring and control of risk factors. A history of APO such as MPS may also identify women at risk for heart failure, suggesting that increased post-partum vigilance, lifestyle modifications and early detection and treatment of risk factors. In 2000, the SLICC group established a registry for atherosclerosis to determine the incidence, prevalence, nature, and risk factors of atherosclerotic CAD in SLE [307].

Pre-eclampsia and hypertension in SLE

Lin et al. showed that women with SLE (age 44.5 ± 9.7) and prior APO, specifically pre-eclampsia, are more likely to have subsequent hypertension post pregnancy compared to women with SLE and no prior APO [241]. They also showed that women with SLE and pre-eclampsia had an almost four fold increase in the rate of subclinical CVD [241], identified by increased coronary artery calcium on CT [241]. They did not find an association between the presence of carotid atherosclerotic plaque on B mode ultrasound or subclinical CVD. Notably, they did not examine for femoral plaque or report plaque echogenicity or total plaque area (TPA), all of which have been associated with CVD in the general population [308-311]. The patients included in my study had more sensitive ultrasound scanning techniques measuring TPA and echogenicity which have been shown to be a better predictor of CVD than plaque presence alone [308, 310, 311]. It is reported that pre-eclampsia occurs in approximately 2-8% in the general population and in approximately 20% of patients with SLE [312]. My results showed that only 2% (1/62) had definite pre-eclampsia, but a further 6% had hypertension during pregnancy. Notably, the patient with pre-

eclampsia had SLE associated APS and experienced premature delivery at 30 weeks of a LBW (3lbs) infant. She subsequently had a myocardial infarction.

FXa and Thrombin and APO in SLE

Given the known relationship between SLE, APO and CVD, I checked anti-serine proteases in these women. FXa and Thrombin have been found in plaque and APO such as pre-eclampsia is likely related to immunological factors and endothelial dysfunction. I found no association between anti-FXa and anti-Thrombin IgG and APO. To my knowledge, this study is the first to examine for this potential link. I note that only a few patients in my cohort had established CVD and it would be interesting to examine for anti-serine protease antibodies in larger numbers of patients with SLE and established CVD.

SLE, CVD and parity

Soh et al. showed in a study of 3232 women with a median age of 49, that nulliparous women with SLE had a higher incidence of CVD than parous women (n=2317) (72%). Nulliparous women with SLE developed accelerated CVD even when compared to women who had a history of MPS (23.2% n=539) complicating their pregnancies [242]. The probability of a CVE-free survival was lowest in the nulliparous group even when compared to those with MPS ($p<0.001$). Therefore I felt it was worth looking for a difference between these two subgroups within my cohort. Although, it appeared that nulliparous women were in fact less likely to have atherosclerotic plaque, this finding did not meet statistical significance. However, as per the power calculation described in the methods section, I expected a larger proportion of women to have been pregnant and this is a limitation of my study.

Strengths and limitations

One of the strengths of my study was having a well-characterised group of women of whom I had detailed carotid and femoral ultrasound scans. Many pregnancy lupus studies have data regarding the increase in risk of APO in women with SLE, but have not examined this risk in relation to subclinical CVD. A limitation of my study was that it had a smaller sample size than expected given the number of nulliparous women. Also, many of the women had their children in hospitals other than UCLH and therefore I did not have access to their medical notes.

6.9. Future work

The nulliparous women were younger (40.64 ± 13 years) than the parous women (47.19 ± 11.27 years) and therefore the nulliparous women may have children in the future. The WHO definition of reproductive age is 15-49 years and the average age of mothers in the UK in 2016 was 30.4 years, with many women giving birth into their forties. Therefore it would be interesting to follow this cohort and conduct longitudinal analysis, particularly to determine how many women with APO will develop established CVD. Repeat cardiovascular ultrasound scanning of this cohort has already begun in these patients.

7. Chapter Seven: Systematic Review: Are fertility and parity reduced in SLE?

7.1. Introduction and Aims

Many studies report reduced fertility and/or parity [285, 313, 314] in women with SLE. However, this is debatable because other studies report no difference in fertility and/or parity in patients with SLE compared to healthy controls [216, 315, 316]. Therefore, as part of my thesis, I carried out a systematic review to determine if there is a reduction in fertility and parity in patients with SLE. Rheumatoid arthritis was also included in this review as both conditions are characterized by systemic inflammation, affect women during their reproductive years, and often require similar medications. There is also an increased risk of APO in women with RA and there can be complexities in treating these women in pregnancy. However, for the purpose of this thesis, I have focused on the SLE study results. The aim of this systematic review was to determine if fertility and parity are reduced in SLE and to determine the quantity and strength of evidence implicating reductions in fertility and parity these women.

7.2. Methods

MEDLINE and EMBASE databases were searched from their inception for relevant publications through to October 2015, using keywords including SLE and RA combined with fertility and parity, see Table 7.1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines were followed. MeSH terms such as fertility (fertil*) and pregnancy (pregn*) were included. Abstracts were screened to identify eligible articles.

Exclusion criteria included, other rheumatic diseases (including APS); non-rheumatological autoimmune disease; effects of drug treatment on fertility e.g. CYC;

effects of autoantibodies on fertility; assisted reproduction e.g. IVF; effect of fertility on rheumatic disease; non-English papers and conference abstracts, see Figure 7.1.

KEYWORDS
Lupus, SLE, Systemic lupus erythematosus, Lupus nephritis, Autoimmune rheumatic disease, Rheumatoid arthritis, Fertility, Female fertility, Infertility, Subfertility, Sterility, Ovarian reserve, Ovarian dysfunction, Anti-Mullerian hormone, Reproductive function, Gonadal function, Parity, Gravidity, Pregnancy number

Table 7.1 Keywords used in systematic review

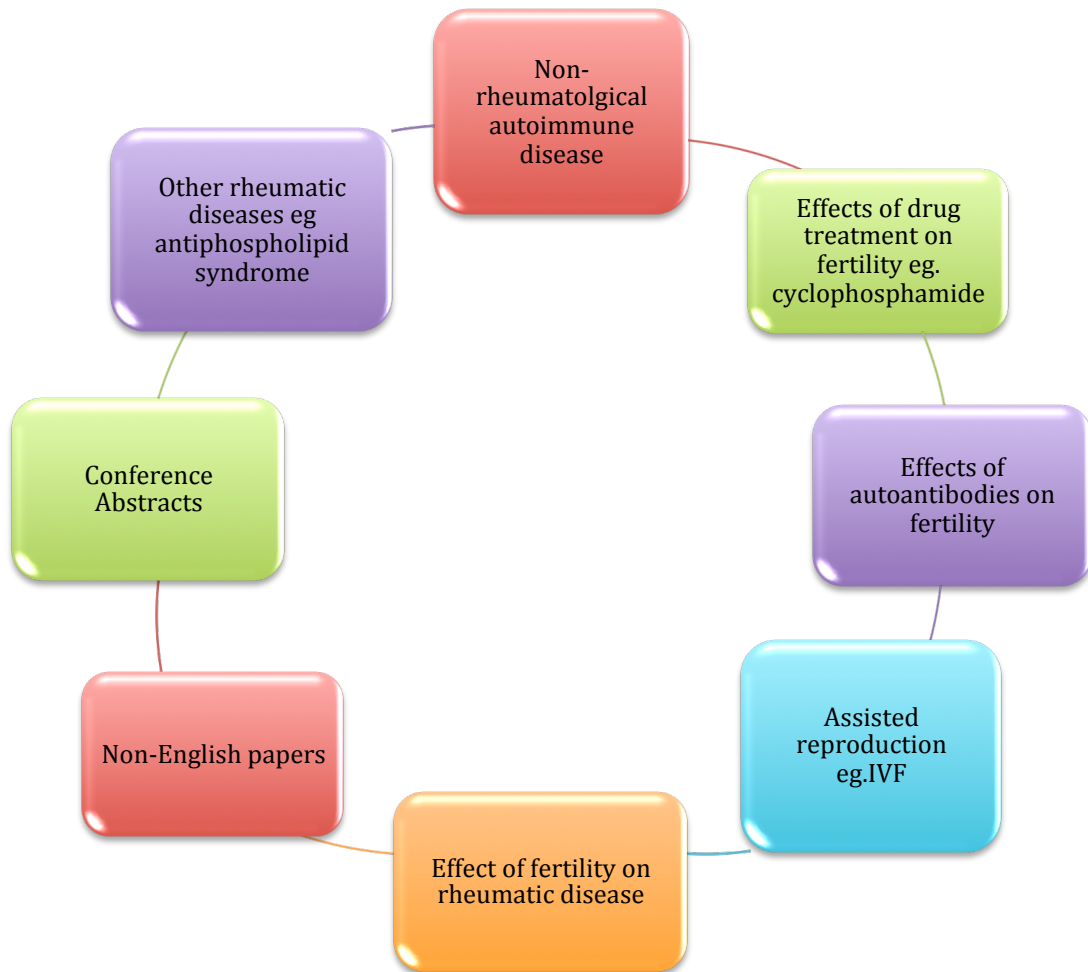


Figure 7.1: Exclusion criteria –systematic review.

Articles were excluded as per reasons outlined above, e.g. other rheumatic disease such as APS, non-rheumatological autoimmune disease, effects of drugs such as cyclophosphamide on fertility, conference abstracts, non-English papers, assisted reproduction, effects of autoantibodies on fertility, effect of fertility on rheumatic disease.

7.3. Results

A total of 2,444 articles were identified, consisting of 1,724 and 720 articles from EMBASE and MEDLINE databases respectively. After removal of 1,840 duplicated articles and exclusion of non-rheumatological conditions, 334 articles were selected for full-length review. 158 papers were excluded because they did not fit the criteria, 35 papers were excluded because of APS, and 63 papers were excluded as they were review articles, 10 papers were excluded because of conference abstracts and 14 papers listed other ARD. Of these, 22 papers related to SLE were identified.

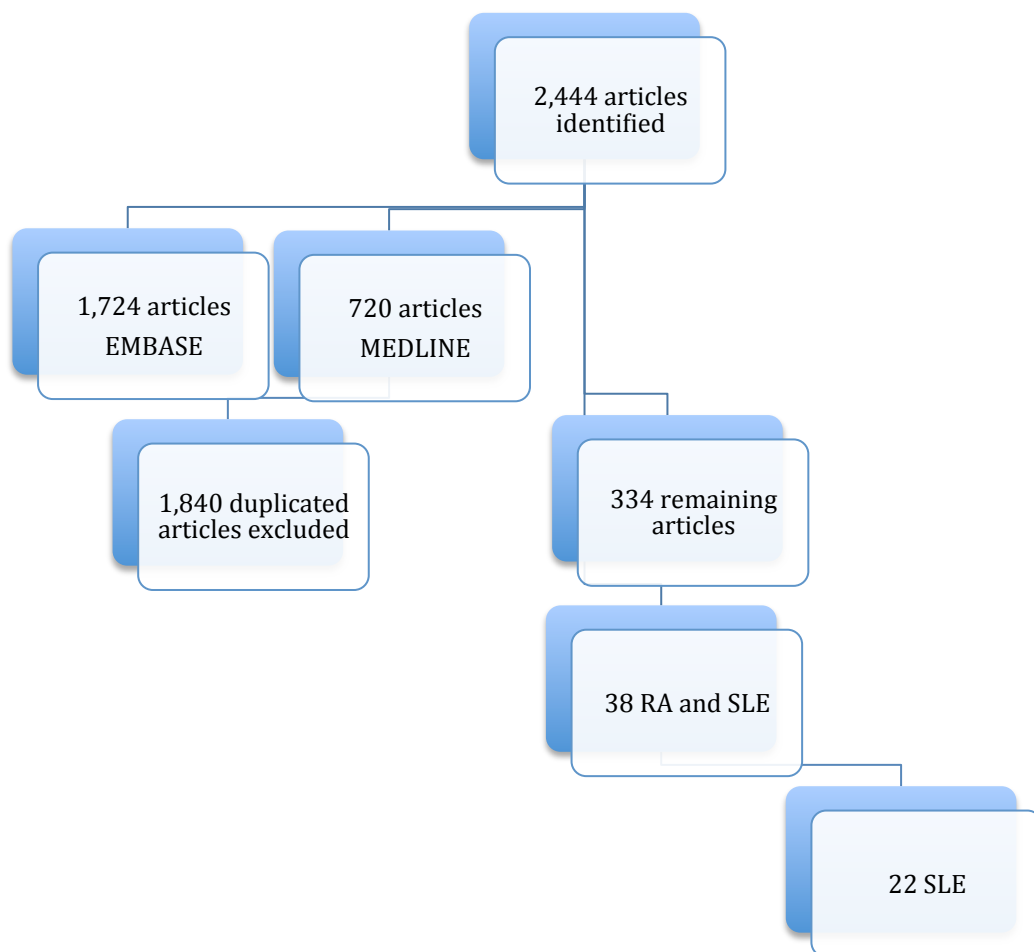


Figure 7.2: Data extraction using PRISMA guidelines

2,444 articles retrospective and prospective papers were identified. After exclusion criteria, 22 papers related to SLE and fertility and parity remained.

A total of 2287 patients with SLE were included. Articles captured retrospective and prospective case control, cohort and cross-sectional studies ranging from years 1974 to 2015. There was variation in SLE diagnostic criteria used. The ACR 1982 criteria and ACR 1997 criteria were predominantly used. Some papers did not report the use of any criteria. There was variation between studies as to how fertility and parity were measured. For instance some papers measured fertility by ovarian reserve as determined by anti-Müllerian hormone (AMH) levels. Other studies reported reduced fertility as time to pregnancy (TTP) greater than twelve months, or by reduced antral follicle count (AFC) as demonstrated on ultrasound. Parity related to increased pregnancy loss, nulliparity, fewer children than desired, lower live birth rates and reduced family size. In the SLE cohort, 5 studies reported no difference in fertility and/or parity. 11 reported reduced fertility/ovarian reserve or menstrual dysfunction affecting fertility and 6 reported reduced parity/pregnancy loss.

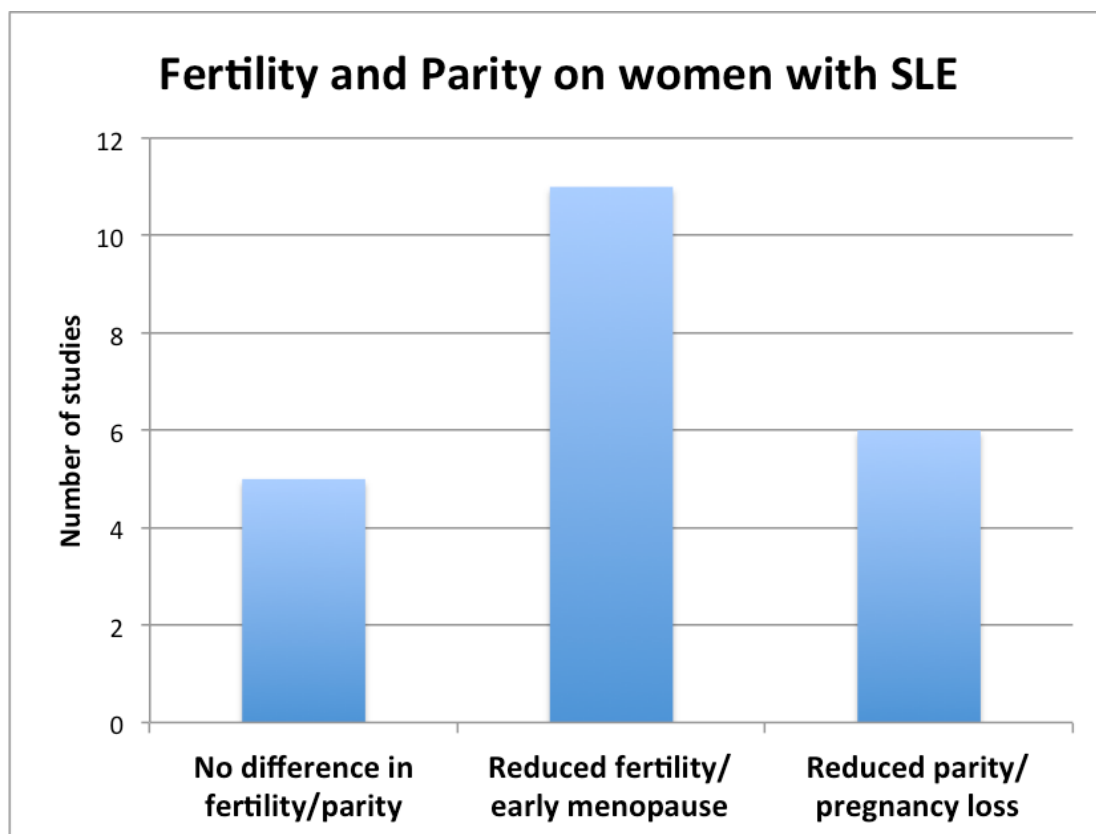


Figure 7.3: Studies demonstrating fertility and parity in patients with SLE

This figure represents SLE studies (n=22) pertaining to reduced fertility and parity in this population.

Studies showing no difference in fertility/parity

Ekbloom et al. [216] demonstrated in 206 women with SLE and 1037 HCs, that the frequency of infertility (16%) in patients with SLE was similar to that of the general population (16%). However, despite menarche being similar in both groups (13 years), menopause occurred earlier (44.9 vs 46.8 years, $p=0.01$). However, the mean number of pregnancies was lower in patients with SLE compared to controls (2.3 vs. 2.5, $p=0.046$). There was no difference in the occurrence of spontaneous and induced abortions compared to controls, but APO was more common in women with SLE [216]. Pasato et al. [316] showed that of 36 patients with SLE (age 18-39), without

current or previous alkylating therapy, that 52% had menstrual disturbances but ovarian function was generally preserved. The mean SLEDAI levels and the frequency ($p=0.02$) and the frequency of patients with a SLEDAI greater or equal to 8 ($p=0.008$) were higher in those with irregular cycles. This suggests that disease activity was a major factor for menstrual disorders in women with SLE [316]. McHugh et al. [315] reported that the rate of pregnancy loss was similar between those with SLE (18%) and the general population (16%) Fraga et al. (1974) showed that women with SLE have a normal fertility rate. Kaufmann et al. (1982) showed that despite normal fertility rates, there was a reduction in parity, from 2.3 live births prior to disease onset to 0.7 live births after disease onset ($p<0.02$). Hardy et al. [305] showed that women with SLE ($n=138$) are at greater risk of pregnancy loss than HC ($n=276$), but the median number of children was the same between the two groups. However non-white women with SLE had more children than white women.

Studies showing a difference in fertility/parity

Ulug et al. [314] measured FSH, estradiol (E2) and LH on cycle day 3 of women with SLE ($n=20$) and compared with HC ($n=20$). All women had an ultrasound on cycle day 3 to determine ovarian volume (OV) and total antral follicle count (AFC). There was a statistically significant reduction in AFC and OV in the SLE group and menstrual irregularity was higher within the SLE group. Malheiro et al. [317] also showed in a small study, SLE ($n=27$), HC ($n=27$) that AFC was significantly reduced in women with SLE ($p=0.029$), despite the fact that FSH levels were similar. Morel et al. [215] reported low AMH levels in patients with SLE who had not been exposed to CYC. Ma et al. [318] showed that AMH ($p=0.000$) values and AFC ($p=0.001$) were significantly lower in the SLE than in HC, despite normal menstruation. Lawrenz et al. [313] echoed these findings, showing that AMH values in patients with SLE ($n=33$) were significantly lower than in HC ($n=33$). There were no significant differences between

the groups regarding number of children and miscarriages and no correlation between the AMH value and the duration of illness or the SLEDAI. Despite mild disease activity, women with SLE had a significantly lower ovarian reserve than age-matched healthy women. Shabanova et al. [319] showed that menstrual cycle disorders were common in women with SLE with oligomenorrhea observed in 54%. A decreased progesterone level (52%), reduced E2 concentration (25%) and increased levels of LH (13%), FSH (9%) and prolactin (10%) were reported in this cohort of lupus patients [319]. Gonzalez et al. [320] reported that lupus disease activity (and Texan-Hispanic ethnicity), were predictors of a shorter time to premature gonadal failure in women with SLE. Nonato et al. [321] showed in a study of 87 patients with SLE, that menstrual alterations occurred in 37.9% and amenorrhoea in 11.5%, which is higher than the general population.

Vinet et al. [219] showed that of a total of 339 women with SLE, the number of live births (n=313) was substantially lower compared to the general population (n=479). Black race and being married or living in common-law was associated with increased live births. There were trends towards fewer live births in those with high disease activity [219]. Clowse et al. [285] showed that of 114 women with SLE, 16% reported infertility and 64% had fewer children than planned. Gupta et al. [322] completed a study in Northern India, via questionnaire and with access to available medical notes. They showed that patients with SLE (n=210) have less living children compared to the national average in India and that patients with SLE have more APO compared to patients with RA [322].

7.4. Discussion

Potential reasons for reduced fertility and parity in SLE

Most of the studies that showed a reduction in fertility/ovarian reserve used hormonal or ultrasound methods to assess and these were relatively recent studies. Reasons for reduced ovarian reserve in SLE are unknown, but increased disease activity appears to have a large influence. Pregnancy loss in SLE is most strongly associated with increased SLE activity prior to and during pregnancy [300]. It has been hypothesised that systemic inflammation from lupus disease activity impairs placentation and promotes cervical ripening or preterm labour, all of which increase the risk for pregnancy loss.

Alkylating agents such as CYC are a well known to cause reduced fertility and therefore these studies were purposefully excluded in this review. NSAIDs may also play a role in infertility [323]. Other potential causes of reduced fertility include autoantibodies. Premature ovarian failure has been shown to be related to a variety of autoantibodies including antibodies to DNA, islet cells and acetylcholine receptors [324]. SLE patients have been shown to have organ-specific antibodies directed against ovaries [325] which have been associated with premature ovarian failure [326]. The presence of aPL in SLE have been associated with reduced ovarian reserve [327] and autoimmune destruction of the ovary could be the primary cause of primary ovarian failure. Interestingly, atherosclerosis in the ovarian artery may cause reduced ovarian volume in SLE patients, which correlates with decreased ovarian reserve [328]. Levels of soluble Fas apoptosis antigen (sFas) are correlated with organ damage, including ovary damage and these levels have been shown to be significantly higher in SLE patients than in HCs [329].

Clowse et al. showed that personal choice had a significant role on parity/family size.

Patients expressed concern for themselves and for potential offspring including inability to care for a child, genetic risks and side-effects of medications [285] which led to a significant reduction in the number of pregnancies, in patients with normal fertility. Other factors contributing to reduced fertility and parity in SLE include increased maternal age, delays to allow for disease remission, menstrual disturbances and renal failure. Other factors such as fatigue, depression and reduced libido may also play a role.

7.5. Conclusion

This systematic review showed that the majority of lupus studies reported reduced ovarian reserve or menstrual dysfunction affecting fertility. Limitations of my analysis included heterogeneity of outcome measures, which included ovarian reserve, anti-müllerian hormone (AMH) levels, TTP (time to pregnancy) >12 months, nulliparity, sterility, premature menopause and pregnancy rates. There were differences between the age of women, disease activity measurement tools and classification criteria used. Additionally, potential confounding factors included the effect of disease activity and differences between older and more recent studies. Therefore, it was difficult to accurately compare across the studies and come to a certain conclusion as to whether fertility and parity is definitely reduced. Adding to this complexity, one study showed that women even with very low ovarian reserve subsequently had children. There was also a variation in the number of participants per study e.g. study groups ranged from 20-339 patients with SLE, which may have skewed the data. Despite these limitations, the majority of studies showed a reduction in fertility and/or parity in patients with SLE. The majority of these studies were small and therefore data from large multicentre cohorts using similar measures for fertility/parity would be extremely valuable.

8. Chapter Eight: SLE and osteoporosis

8.1. Introduction and Aims

As described in section 1.7.4 (page 82), osteoporosis and osteopenia are associated with atherosclerosis. This relationship appears to be independent of age and other shared traditional risk factors. Inflammation has been proposed to be important in the pathogenesis of osteoporosis with pro-inflammatory cytokines and the RANK/RANKL/OPG pathway implicated as a link between these two conditions. An understanding of the biological linkages may set the stage for dual-purpose preventive and therapeutic interventions aimed at reducing bone loss and the progression of atherosclerosis. Therefore I studied the association between atherosclerosis and LBMD in patients with SLE.

8.2. Results SLE and Osteoporosis

Of the cohort of 100 patients described in section 2.1, 81% had a DXA scan performed at UCLH. Although, the remaining 19% had no DXA scan recorded they may have had a DXA done elsewhere. Normal bone mineral density (BMD) was demonstrated in only 32.14% (n=27); osteopenia 53.57% (n=45); osteoporosis 10.71% (n=9), see Figure 8.1.

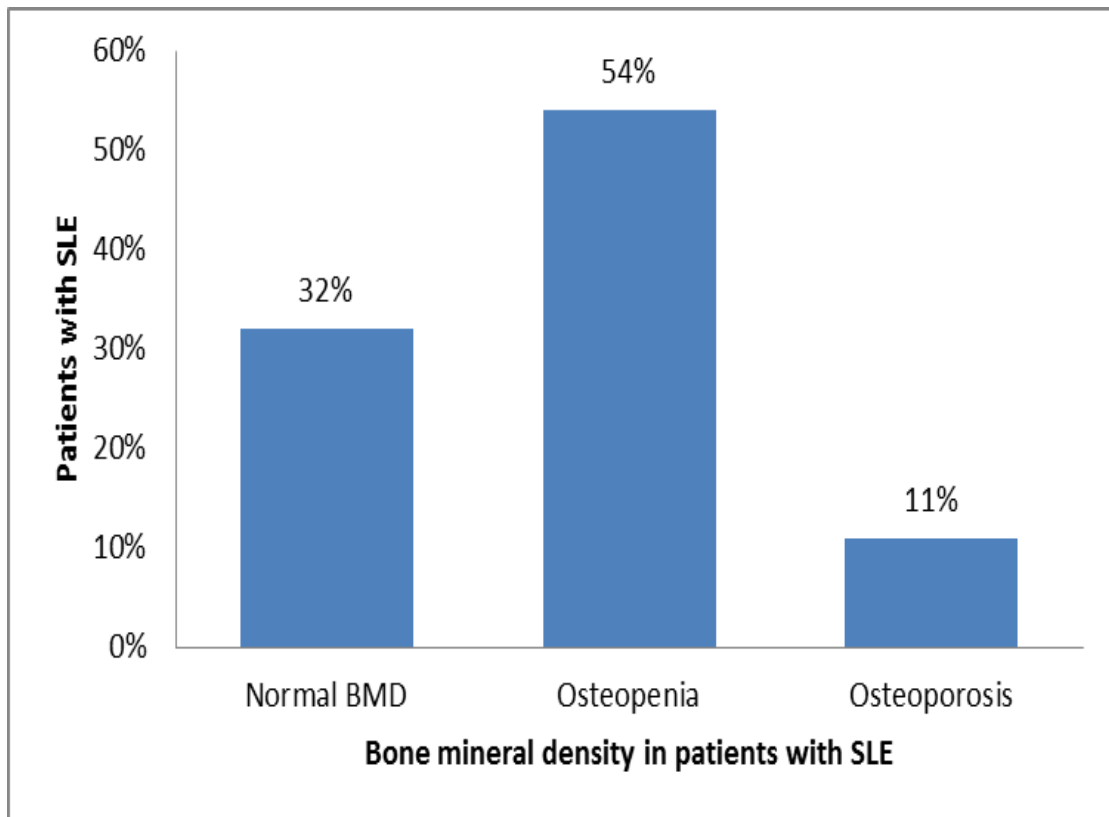


Figure 8.1: Bone mineral density in patients with SLE

The mean age was similar in both the normal BMD (46.18 $\pm$ 13 years), and LBMD groups (45.74 $\pm$ 13 years), see Figure 8.2. All patients in the normal BMD group were female and 93% were female in the low BMD group. The mean corticosteroid dose was 6mg in the patients with normal BMD versus 4.9mg in those with LBMD. Vitamin D levels were slightly higher in those with LBMD; compared to those with normal BMD (mean 59.55 IU versus 50.74 IU respectively), although this difference was not statistically significant ($p=0.0694$), see Figure 8.3. A total of 17.8% of patients with normal BMD were smokers versus 10.4% with LBMD. ESR was similar between the groups (21.52 versus 20.65 respectively). There were a higher percentage of patients on HCQ in those with normal BMD (70.37%) versus 62.96% in those with LBMD. There was no statistical significance between the two groups, see Table 8.1.

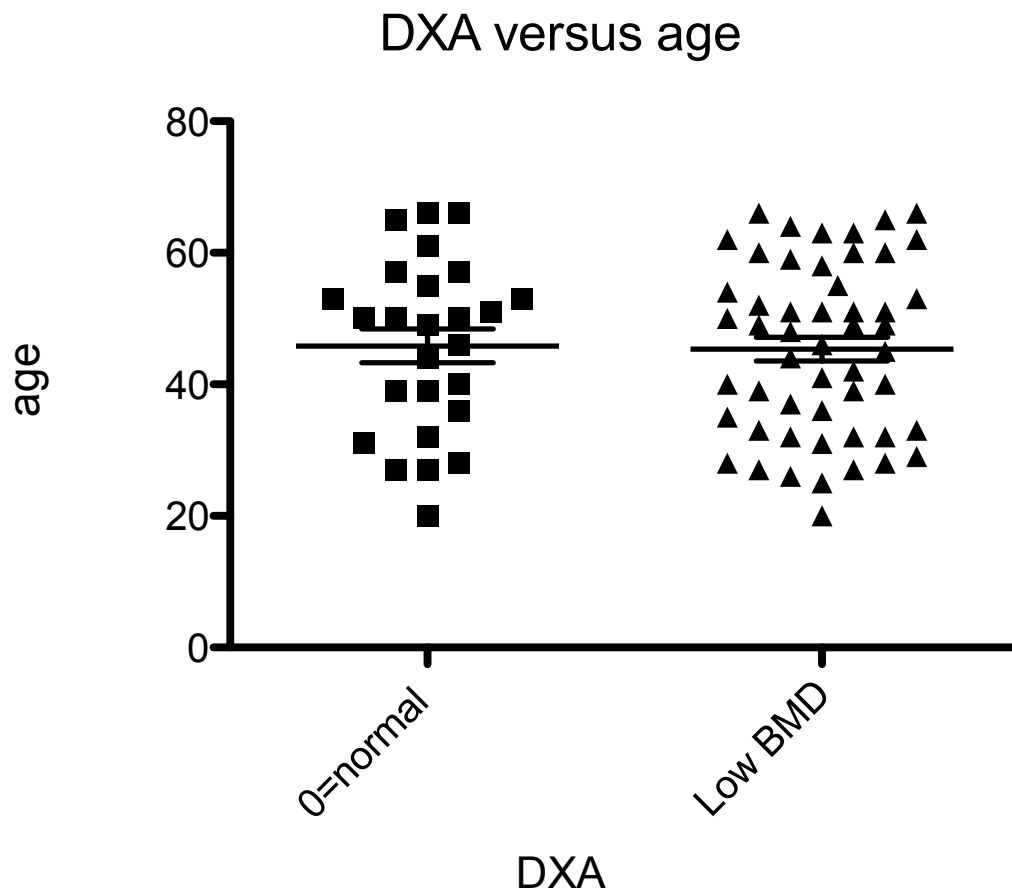


Figure 8.2: Age was similar in those with normal and low bone mineral density

This figure shows the number of patients with normal BMD and LBMD (both x-axis) correlated with patient age (y-axis). Using GraphPad Prism v5.0, Mann-Whitney test, the mean age was similar in both groups ($p=0.8963$)

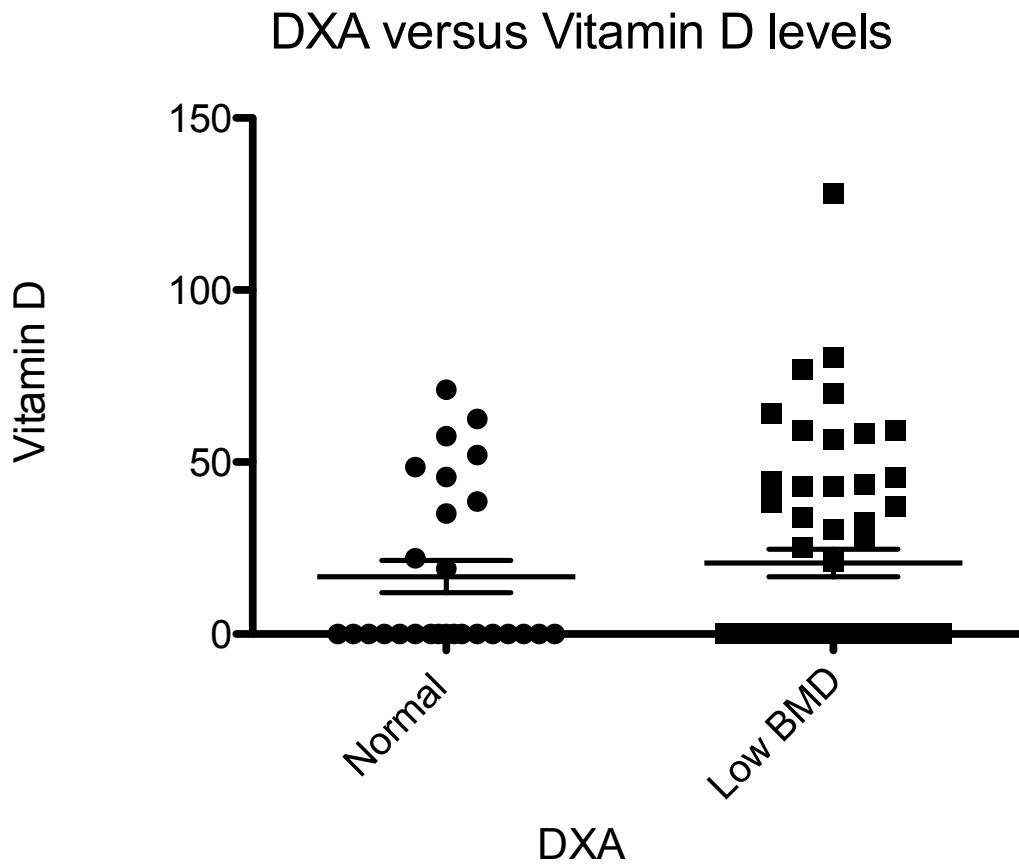


Figure 8.3: Bone mineral density and Vitamin D levels

This figure shows patients with SLE and normal and LBMD (x-axis) correlated with vitamin D levels (y-axis). There was one outlier in the LBMD group, who was on replacement therapy for vitamin D deficiency. Using GraphPad Prism v5.0, Mann Whitney test, there was no statistical significance between the two groups ($p=0.694$).

	Normal BMD	Low BMD	p-value
Age (mean)	46.18+/-13	45.74+/-13	0.8963
Sex (female)	27/27	50/54	0.1469
Corticosteroids (mean daily dose)	6mg	4.9mg	0.9794
Smoker	17.3%	10.4%	0.4179
Vitamin D (mean)	50.74IU	59.55IU	0.0694
ESR	21.52	20.65	0.4138
Hydroxychloroquine	70.37%	62.96%	0.5153

Table 8.1: Confounding variables in patients with normal BMD and low BMD

This table shows the differences between those with normal BMD and LBMD. Using GraphPad Prism v5.0, Mann Whitney test and Chi square (age), there was no statistical significance between the two groups.

Of the 81 patients for whom I had available DXA scans, 40% (32/81) patients had atherosclerotic plaque. Plaque was more commonly found in patients with LBMD compared to those with normal BMD (40.74% versus 37.03% respectively), but this difference did not reach statistical significance (chi squared test $p=0.7479$), see Figure 8.4.

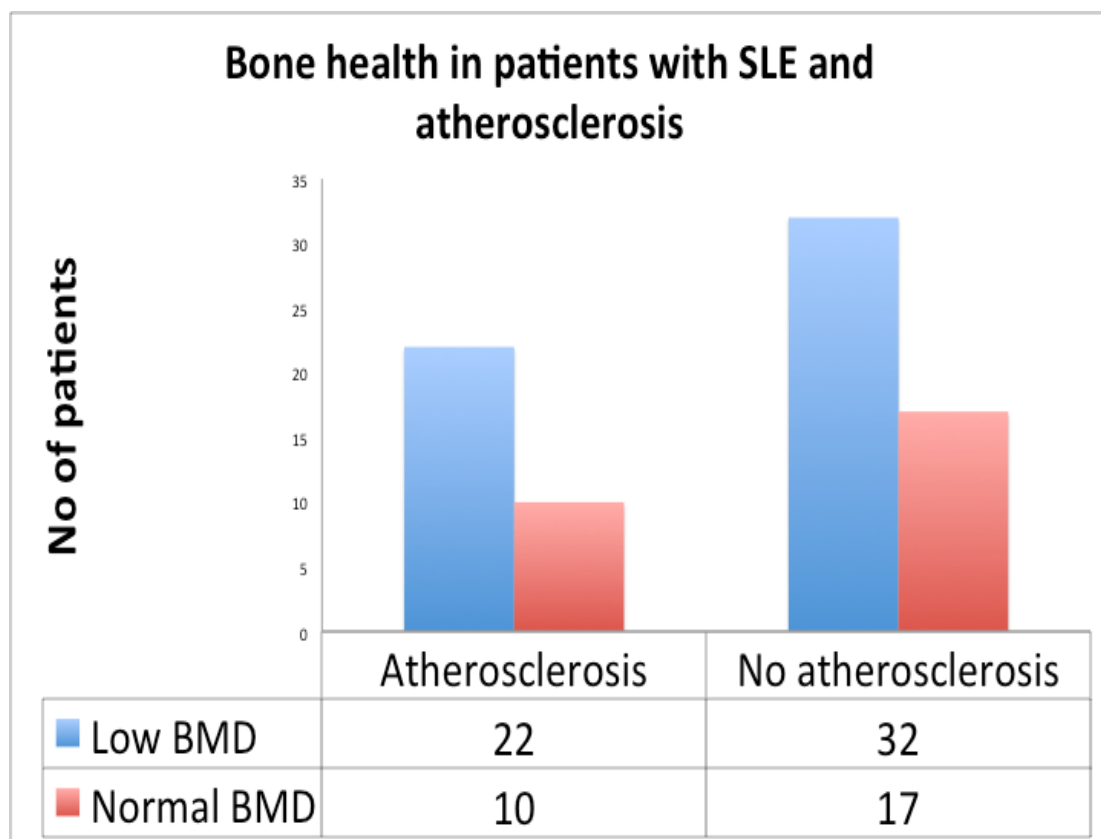


Figure 8.4: Bone health in patients with SLE and atherosclerosis

This figure shows patients with and without atherosclerosis and normal/LBMD. There was no statistically significant difference between atherosclerosis and LBMD (chi-squared test, $p=0.7479$).

The differences between those with SLE and normal BMD, compared to those with SLE and LBMD are shown in Table 8.2. There were differences between the two groups in relation to mean number of plaque sites (0.667, 1.056), $p=0.4059$; mean GSM (16.72, 20.689) $p=0.7121$; mean TPA (50.17, 82.7), $p=0.485$; mean overall IMT (0.0861, 0.0936) $p=0.8138$ and mean common carotid IMT (0.0554, 0.0552) $p=0.6603$. None of these differences were statistically significant (Mann-Whitney t-test).

	SLE and normal BMD	SLE and low BMD	P value
Number of patients	33.3% (n=27/81)	66.6% (n=54/81)	
Presence of Plaque	37.03% (n=10/27)	40.74% (n=22/54)	p=0.7479
Mean no. of plaque sites	0.667	1.056	p=0.4059
Mean GSM	16.72	20.689	p=0.7121
Mean TPA	50.16	82.7	p=0.4850
Mean overall IMT	0.0861	0.0936	p=0.8138
Mean common carotid IMT	0.0554	0.0552	p=0.6603

Table 8.2: The association of BMD to cardiovascular status in patients with SLE

This table shows the results of patients with SLE and low/normal BMD correlated with presence and number of plaque sites along with plaque variables. Using Mann-Whitney t-test, GraphPad Prism v5.0, there were no statistically significant findings. Abbreviations: BMD, bone mineral density; GSM, Grey scale media; TPA-Total plaque area; IMT, intima media thickness.

Given that anti-FXa has cellular/inflammatory effects via PARs and FXa positivity was more prevalent in those without CVD, I assessed whether there was a correlation between anti-serine proteases (anti-FXa and anti-Thr) and BMD. There was no statistical significance between the groups, see Figure 8.5 and Figure 8.6.

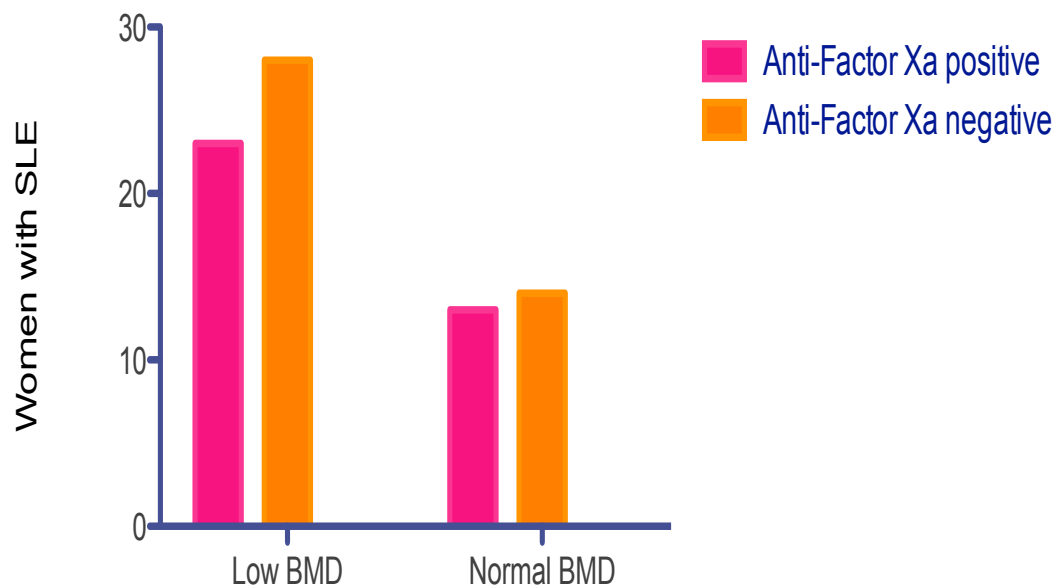


Figure 8.5: BMD and anti-Factor Xa in women with SLE

This figure shows low and normal BMD (x-axis) in relation to anti-FXa IgG status and numbers of women with SLE (y-axis). Using GraphPad Prism v5.0, chi-squared test, there was no statistical significance between the two groups ($p=0.7971$).

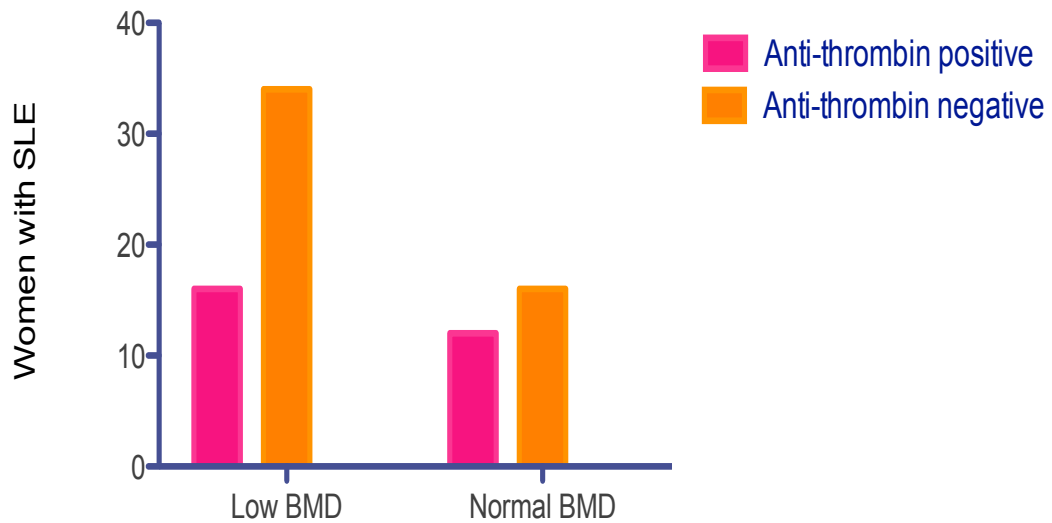


Figure 8.6: BMD and anti-Thrombin in women with SLE

This figure shows low and normal BMD in relation to anti-thrombin IgG status (x-axis) and the number of women with SLE (y-axis). Using GraphPad Prism v5.0, chi-squared test, there was no statistical significance between the two groups ($p=0.3376$).

8.3. Discussion

In this chapter, I explored whether there was an association between SLE and subclinical CVD with LBMD. Although there was no statistical significance between these variables, my study revealed interesting findings. My initial finding in this lupus cohort was that nearly one fifth had no BMD measurement recorded at UCLH. However, I cannot exclude the possibility that a FRAX risk assessment was carried out or that a DXA may have been performed elsewhere. It is recommended that patients with SLE should have osteoporotic assessment, especially if they have risk factors such as being post-menopausal, a history of corticosteroid use, a family history of fracture or other osteoporotic risk factors such as low weight, long term use of proton pump inhibitors, smoking, alcohol intake, and limited weight bearing exercise.

Osteoporosis in SLE appears early in the course of disease and approximately 25% develop organ damage attributed to the musculoskeletal system (including osteoporosis and fragility fractures), within the first 10 years of the disease [226, 330-332]. A large proportion of patients in these studies were on long-term steroids. Therefore osteoporosis assessment should be considered early in patients' disease course.

Secondly, despite the relatively young age of my cohort, a large percentage (65%) had LBMD, which is echoed in other SLE/osteoporosis studies in patients of a similar age [332]. Of my cohort, 11% had osteoporosis, and other studies report figures of 4-24% and 10- 20%, when postmenopausal patients are assessed. The majority of my cohort had long-standing disease (greater than sixteen years), and they were on a mean daily dose of corticosteroids (5mg), which likely influenced these results, as has been shown in other SLE/osteoporosis studies.

SLE and corticosteroids

Carli et al. studied a lupus cohort of similar age and disease duration to my study. They showed that of 186 patients with SLE (mean age 46.4 +/- 13 years, mean disease duration 14.9 +/-9 years), that 52.2% had reduced BMD and 27.9% had osteoporosis. These patients had been on long-term corticosteroids (greater than six months, mean daily dose of 5.4 ± 2.3 mg) [332]. BMD rapidly declines within the first three months of steroid use and peaks at six months, followed by a steady loss with continuous use [333]. The risk of fracture escalates by as much as 75% within the first 3 months after the initiation of glucocorticoid therapy. However, SLE causes significant trabecular bone loss, regardless of whether a patient is on corticosteroid therapy, as shown by Kalla et al. [334]. Low doses of corticosteroids (<6 mg/day) were previously considered 'safe' [335] but there appears to be no safe dose. There is an increased risk of vertebral and nonvertebral fractures with a dose equivalent to 2.5-7.5mg daily [336]. Therefore, withdrawal of steroids should be attempted in all patients with SLE, as recommended by GIO (Glucocorticoid Induced Osteoporosis) prevention and treatment guidelines [337].

SLE and vitamin D

Other factors that should be taken into consideration include reduced vitamin D levels and avoidance of sun exposure [338]. Patients with SLE are advised to avoid the sun and wear factor 50, to prevent lupus flares so are more likely to be vitamin D deficient and thus more prone to LBMD. Also, genetic polymorphisms in patients with SLE may interfere with levels of 25 hydroxy-vitamin D (25-OH-D). Monticielo et al. [339] showed that Fok I vitamin D receptor polymorphism appears to interfere with serum concentrations of 25OHD in patients with SLE. Within my cohort, mean vitamin D levels were within the normal range but were slightly lower in patients with normal

BMD, which was not statistically significant. This finding is likely because patients with LBMD are routinely prescribed vitamin D replacement therapy.

Autoimmunity and bone health

The role of autoimmunity in bone resorption in SLE is very limited. In a cross-sectional study on 34 patients with lupus, an association between the presence of anti-Sm and higher hip bone mass was found, while the presence of anti-Ro was negatively associated with hip BMD [340]. The latter finding may be explained by the fact that patients who are anti-Ro positive are advised to avoid the sun. In the same study there was no relationship between anti-dsDNA and BMD. The potential importance of other antibodies in relation to BMD and SLE, are currently unknown. In RA, patients who were ACPA (antibodies against citrullinated protein) positive, had reduced BMD, in comparison to those who were ACPA negative. ACPA directly induces bone loss by stimulation of osteoclast differentiation. Therefore although I did not find a relationship between anti-FXa and anti-Thr antibodies in my study, perhaps other antibodies in lupus play a role in bone health and would be worth investigating in the future.

SLE, osteoporosis and CVD

A number of studies have reported the association between low BMD and cardiovascular morbidity, but few have looked at this association with SLE. An inverse relationship between bone mass and subclinical CVD, especially in women, has been reported in many studies and reasons are yet to be fully established. Inflammation and immunological factors appear to be key factors in these conditions.

Likewise, many studies have reported an association between low BMD and SLE, but few have reported a link between SLE and CVD with LBMD.

Ramsey-Goldman et al. [341] performed a pilot study of 65 women with SLE who underwent carotid B-mode ultrasound to measure carotid plaque index and IMT and DXA to measure BMD at the lumbar spine and hip. A total of 13 patients also had electron-beam CT to assess coronary artery calcification (CAC). They demonstrated an association between LBMD and an increased carotid plaque index and presence of CAC in this small cohort [341]. The carotid plaque index was higher in patients in the lowest and middle tertiles of hip BMD when compared to patients in the highest tertile of hip BMD. The patients included in Ramsy-Goldmans study had a similar average age of 44.6 years, as was the case in my study. However they did not analyse other variables such as GSM, TPA and mean number of plaque sites, as were evaluated in my cohort. Although there was a trend in my cohort towards LBMD and atherosclerosis in my study, this did not meet statistical significance.

However, another study by Mendoza-Pinto et al. [342] echoed my findings. They studied 122 women with SLE. All patients had BMD, carotid IMT, and plaque assessed by ultrasound. Mean age was 44 years and the mean disease duration was 11 years. Carotid plaque was found in 11% of patients. They found no association between BMD and IMT. However, there was a trend towards higher incidence of vertebral fracture in patients with carotid plaque compared with those without carotid plaque (33% vs 21%, $p=0.2$). They concluded that carotid atherosclerosis is not associated with LBMD and vertebral fractures in patients with lupus. Therefore, this study is more in keeping with my results and reflects the need for further research in this area.

Anti Factor Xa/ Anti Thrombin IgG and BMD

There was no significant difference between the LBMD and BMD groups with atherosclerotic plaque but there appeared to be a trend in this direction. It is well known that there is a common link between osteoporosis and CVD, which occurs regardless of the aging process and is hypothesised to be due to inflammatory or immunological factors. Inflammation and immunologic factors play important roles in osteoporosis, atherosclerosis and SLE. To my knowledge, this study is the first to try to elucidate an immunological link between either anti-FXa IgG and anti-Thrombin IgG and low BMD. Interestingly, ox-LDL has been studied in this area. Ox-LDL has been shown to promote atherogenesis and ox-LDL is also known to inhibit bone cell differentiation [343]. Given the similarities between anti-FXa and anti-oxLDL as being potentially atheroprotective, I felt it was worthwhile exploring whether anti-FXa had a role in bone health also.

Heparin, which inactivates FXa and thrombin via an antithrombin (AT)-dependent mechanism, is a known risk factor for osteoporosis. A recent study showed that by switching to rivaroxaban from warfarin, in patients with atrial fibrillation, there was an associated increase in bone formation markers and a decrease of bone resorption markers. There were also improvements of PWV (pulse wave velocity) and AI (augmentation index), which are measurements of arterial stiffness [344]. Therefore, I explored whether anti-serine protease IgG (anti-FXa and anti-Thr) could be the missing link between LBMD and CVD in patients with SLE. Given that FXa exerts cellular/inflammatory effects via PARS, I hypothesised that anti-FXa may have an anti-inflammatory mechanism of action. This SLE population had a high incidence of low BMD, despite being relatively young (mean age 45 years old) and therefore offered the ideal cohort to study. Anti-FXa and anti-Thr did not prove to be of

particular significance in my study, but perhaps these antibodies could be significant when checked within the complete cohort.

Strengths and limitations

A strength of my study was being able to assess BMD against a wide variety of CV variables including, not only just the presence of plaque, but also the number of plaque sites, mean GSM, mean TPA, mean overall IMT and mean common carotid IMT. Also, I explored if there was a common immunological process implicated in the pathogenesis of CVD and osteoporosis in SLE. A limitation of my study was that DXA results were unavailable on 19% of patients. Perhaps if DXA results were available for the entire cohort it would have influenced my results.

8.4. Future Work

It is not uncommon in a busy lupus clinic with complex patients to forget to screen for osteoporosis. My suggestion is to place a reminder in the electronic patient record (EPR)/ medical notes of all patients with SLE, so that screening is done routinely. I would be interested to re-audit this patient group in the future, once this measure has been put in place for a year. Future aims would be to re-analyse the data once all DXA scans are complete to see if there is a relationship between those with LBMD and subclinical CVD. It would also be worth checking for a relationship between LBMD and those with established CVD once patients have undergone repeat vascular imaging by ultrasound. I would also like to analyse the relationship between other antibodies in SLE to examine for any correlation with BMD. In the future, new paradigms for treatment and prevention of CVD and osteoporosis may emerge from investigating the link between these conditions.

9. Chapter Nine: Discussion and future directions

9.1. Overall findings

The research presented in my thesis focused on several non-traditional factors that were hypothesised to be important in CVD pathogenesis in SLE; anti-serine-protease antibodies, endothelial dysfunction via EMP and PMP production, adverse pregnancy outcomes and LBMD.

My study is the first to demonstrate that patients with SLE and atherosclerotic plaque are less likely to have Factor Xa antibodies than patients with SLE without plaque. My original hypothesis was that anti-FXa may be pathogenic in SLE, and therefore I hypothesised that anti-FXa may play a role as a biomarker for CVD in patients with lupus. However, my results suggest that Factor Xa antibodies may actually be cardioprotective in patients with SLE, similar to anti-LDL antibodies in the general population. Perhaps anti-FXa, like rivaroxaban, inhibits the pro-inflammatory action of FXa in plaque. On the contrary, it could be that anti-FXa is in fact pathogenic and is being consumed/deposited in plaques, analogous to C3 levels in active disease. It must be noted that anti-FXa IgG was checked from serum taken at the time of the scans and not the time of any clinical event. Therefore it is important to test fresh serum samples when these patients are re-imaged and/or have CVE to examine this relationship.

My data shows that PMPs were overall higher in patients with SLE compared to healthy controls and patients with SLE without plaque had more PMPs compared to healthy controls. My observation is consistent with other published reports and builds upon current work. PMPs are likely to be higher due to the overall disease burden in SLE. On the contrary, PMPs may have beneficial role in patients with CVD, as suggested in other studies. Perhaps PMPs are consumed or deposited in plaque and further research exploring plaque within patients with SLE and subclinical CVD versus established CVD would be of great interest.

Strikingly, my study showed that nearly half the cohort of women with SLE was nulliparous. Reasons for this finding are likely multifactorial and could be explored using a new updated pregnancy questionnaire, utilising PPI. A large proportion of women with SLE had APO, which is echoed in the lupus pregnancy literature. My systematic review showed that the majority of studies showed reduction in fertility and/or parity in women with SLE. However, there was heterogeneity between the types of studies, outcome measures, classification criteria, disease activity tools, study size and older versus more recent studies. Therefore, the evidence is confounded by various factors making it difficult to be wholly convinced by this conclusion. Data from large multicentre cohorts using similar measures for fertility/parity would be extremely valuable to try and resolve this issue.

My work showed that patients with SLE and LBMD do not have a significantly increased burden of subclinical atherosclerosis, compared to patients with SLE and normal BMD. However, nearly one fifth of this cohort had missing data and therefore analysis of the entire cohort, may yield more interesting results.

9.2. Future work

There is now a wealth of information available on this cohort of 100 patients with SLE, which provides an ideal opportunity for future work to be done. These patients have had their subclinical cardiovascular status assessed in detail with high sensitive ultrasound scans. These patients have full demographic and SLE-related characterisation, such as disease course, main organ systems involved, immunological profile, disease activity over time and treatment as well as CVD-specific risk factors, such as lipid profile, smoking history and BMI. I have added anti-Factor Xa/anti-thrombin status, EMP, PMP, APO and BMD results to this data set. This extensive data

set may be used to correlate further variables that may potentially influence atherosclerotic burden of patients with SLE.

In order to take my findings forward there are several points to consider. Future work has already begun with regards to repeating the femoral and carotid ultrasound scans to identify which factors influence progression or regression of plaque as well as noting whether any further patients develop asymptomatic/symptomatic CVD. This re-imaging began in 2017 and 24 patients have been re-assessed. To date, of the 36 patients with plaque, seven patients have had a CVD event since their original scan. Only one non-plaque patient has had a CVD event. Of the 24 patients re-scanned, three patients without plaque have subsequently developed plaque. Three patients previously with plaque have developed bigger or more plaque (although not significant to warrant urgent investigations). Six patients are being assessed in clinic to see whether there is need to optimise their CVD risk factors, such as blood pressure and cholesterol.

Having found that anti-FXa IgG levels are higher in patients with SLE and no plaque, future work could explore if polyclonal IgG antibodies purified from patients with SLE have direct effects on cultured HUVEC e.g. to see if this alters intracellular signalling and expression of adhesion molecules. Although I will not have the opportunity to continue this work myself, the laboratory group at UCL has extensive experience of testing the effects of purified antibodies on multiple different cell types in culture [345-347]. In fact, recent work has studied the effects of anti-FXa positive whole IgG fractions and affinity purified anti-FXa specific IgG sub-fractions upon endothelial cells [176]. Artim-Esen et al. demonstrated that FXa stimulation of HUVEC is mediated by PAR-1 and PAR-2 dependent signaling and this response is enhanced by IgG from FXa reactive antibody positive patients with APS, as well as SLE. A specific FXa

proteolytic inhibitor, antistasin, HCQ and fluvastatin blocked this response. Therefore, there is potential that in the future anti-FXa IgG may be used as a biomarker, to assess cardiovascular risk in SLE and thus allow targeted therapy with FXa inhibitors and/or other therapies in these patients. Further research may also involve examining atherosclerotic plaque itself for anti-FXa IgG patients with established CVD, as this has not been done previously.

Preliminary work in this thesis supports published reports, which found that PMPs are higher in patients with SLE than in HCs. It would be interesting to define the importance of PMPs in SLE pathogenesis. Additional functional experiments aimed at addressing the exact role of MPs derived from patients with SLE, as well as the contribution of MPs derived from different vascular bed territories, are required to determine the significance of my clinical observations. Further research may also define whether therapeutic strategies might include blocking PMP release or their signaling properties.

Given the high percentage of nulliparous women identified in this cohort, it is important for health professionals to raise the topic of childbirth with women with lupus. Misconceptions amongst women with regards to having children need to be addressed e.g. informing patients of the low likelihood of passing on lupus to their children. It is essential to educate women regarding their medication options, so that together we can make optimal decisions. Women with SLE who have difficulty conceiving should be reassured that IVF and other ARTs, such as ovulation induction can be used safely. Future work should focus on exploring patients understanding of their condition and its relationship to pregnancy. It would be worth exploring the underlying social and/or medical reasons for reduced parity in women with lupus.

The pathogenesis of APO in lupus is most likely multifactorial including immune dysfunction, abnormal placental development, endothelial dysfunction and an

imbalance between pro-angiogenic and anti-angiogenic factors. It would be interesting to check MP levels, including STBM levels in pregnant women with SLE and to check AMH levels within a young lupus cohort and compare these levels in age and sex matched controls. A better understanding of the pathophysiological mechanisms in lupus pregnancies may help develop tools to better stratify women pre-conceptually and in early pregnancy.

There was no correlation between low BMD in lupus, with CVD and anti-serine proteases. However, there was a trend towards a correlation between LBMD and subclinical CVD in these patients. Perhaps if the whole cohort had had DXA scans it would have influenced my findings. My work has highlighted the importance of BMD screening in patients with lupus and in the future, this group should be re-audited after a reminder has been placed in their medical charts.

Although I focussed on non-traditional causes for the increased CV risk in this cohort, we should not lose sight of the traditional risk factors. In this cohort, a large number of patients were smokers and/or had hypertension and hypercholesterolemia and seven patients had developed a subsequent CV event. Patients with SLE should have their CV risk factors assessed as stringently as a one would expect for a patient with diabetes or a patient with a history of previous MI or stroke. Primary prevention strategies should be undertaken in a timely manner in order to minimize the occurrence of CVD-related events. My work produced in this thesis has contributed to the study of CVD in patients with SLE and further research will hopefully elucidate the mechanisms involved in SLE CVD progression.

10. References

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11. Appendix

1.1. Appendix I

Posters and presentations

1. Murphy CL, Sharp H, Hguyen H, Rahman A, Giles I. Do Patients with Systemic Lupus Erythematosus and Rheumatoid Arthritis Have Reduced Fertility and Parity? A Systematic Review.

American College of Rheumatology conference, Washington 2016

2. Murphy CL, Croca S, Rahman A, Giles I. The pregnancy experience of 85 women with SLE followed in a specialist clinic compared to the general population.

European League Against Rheumatic Disease conference (EULAR), London 2016.

3. Murphy CL, Croca S, Pericleous C, Isenberg D, Rahman A, Giles I. Patients with Systemic Lupus Erythematosus (SLE) and low bone mineral density have no significant increased subclinical atherosclerosis compared to those with SLE and normal bone density

British Society of Rheumatology conference, Glasgow 2016.

4. Murphy CL, Artim-Esen B, Croca S, Isenberg DA, Rahman A, Giles I. Patients with SLE who are Factor Xa positive are less likely to have atherosclerotic plaque Factor Xa

American College of Rheumatology conference, San Francisco 2015

1.2. Appendix II

Publications

1. Murphy CL, C. S. Yee, C. Gordon, Isenberg D. From BILAG to BILAG based combined lupus assessment-30 years on. *Rheumatology (Oxford)*. 2016 Aug;55(8):1357-63.
2. Murphy CL, Ambrose N: Biologics in Rheumatology: New Developments, Clinical Uses and Health Implications, *Biologics in Juvenile SLE Nova Science Publishers 2016* chapter 2 pp. 33-44.
3. Murphy CL, Ioannou Y Ambrose N. Systemic Lupus Erythematosus. *Juvenile Systemic Lupus Erythematosus, Oxford University Press 2016 chapter 8*

1.3. Appendix III

**From BILAG to BILAG based combined lupus assessment-30
years on.**

Review

From BILAG to BILAG-based combined lupus assessment—30 years on

Claire-Louise Murphy¹, Chee-Seng Yee², Caroline Gordon³ and David Isenberg¹

Abstract

Disease activity in SLE can be difficult to measure and there is no biomarker that uniformly reflects disease activity. There are various disease activity scores, but there is no gold standard assessment tool. This is a review of the development of the BILAG index from the classic BILAG disease activity index to the BILAG-2004 disease activity index and composite response criteria. The original classic BILAG index was revised and distinguished nine organs/systems. Features that indicated damage, such as avascular necrosis, were excluded. There was improvement in the glossary, scoring system and software. The BILAG-2004 index has been shown to be reliable, valid and sensitive to change. The BILAG-2004 index has been modified for pregnancy and has also been used in paediatrics. The SLE Responder Index (SRI) and the BILAG-based combined lupus assessment (BICLA) are composite responder indices incorporating the BILAG index. Since the initial development of the BILAG index in 1984, major improvements have been made in the measurement of disease activity in lupus. However, the BILAG-2004 index is the only transitional index that grades clinical features as being new, the same, worse or improving and incorporates severity in the scoring.

Key words: SLE, lupus, lupus activity, BILAG, disease activity indices, clinical trials, autoimmunity, rheumatology, measurement, scoring, BILAG-2004

Rheumatology key messages

- The BILAG-2004 disease activity index is an improved version of the older classic BILAG disease activity index.
- The BILAG-2004 disease index provides a comprehensive way to capture disease activity in patients with SLE.
- The BILAG-2004 disease index is a valid, reliable and sensitive tool for measuring SLE disease activity.

Introduction

SLE is a complex autoimmune rheumatic disease with diverse clinical features. Its clinical manifestations, either subtle or florid, often fluctuate considerably. SLE causes a range of problems, from mild skin and joint symptoms to severe life-threatening disease. Adding to the complexity of measuring disease activity in SLE, some of its features may resemble other conditions. For example, patients often have chronic pain, depression, fever, lymphadenopathy,

fatigue or headaches, which may or may not be lupus related.

There is no biomarker that uniformly reflects disease activity. Anti-dsDNA antibodies, complement levels and inflammatory markers such as ESR are not always abnormal in active disease. Therefore assessment is largely based on clinical history, examination and measurement of organ function, ideally by physicians experienced in managing SLE. However, even experienced rheumatologists may differ in their clinical judgment regarding disease activity. This was seen in the study assessing lupus flare comparing the BILAG-2004 index and the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) flare instrument, where three experienced rheumatologists were at odds with 13 other rheumatologists in their clinical judgment of disease flare [1].

Due to the heterogeneity and complexity of lupus, and taking into consideration inter-assessor variability, the difficulty in measuring disease activity is not surprising. Consequently it is difficult to establish a uniform scoring system.

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Measurement of disease activity is important because it has been shown to predict the accumulation of damage and risk of mortality [2]. Measurement is also necessary in order to assess response to therapy and demonstrate the effects of new treatments. An appropriate choice of endpoints to assess the response to treatment is also vital. Currently there are global and responder indices, but there is no single gold standard assessment tool. This article focuses on the development of one of the most widely used indices, the revised version of the BILAG index known as the BILAG-2004 index [3].

Background

The ideal SLE disease activity index (DAI) should capture changes in disease activity including improvement and worsening in organs and systems. Between the 1950s and 1980s, ~60 different DAIs were published [4]. These were global indices, but none were reliable or validated. It was not until the 1980s that rheumatologists made successful attempts at defining disease activity.

In Toronto, Murray Urowitz, Dafna Gladman and their colleagues produced the SLEDAI. Although this is a global score, it was shown to be reliable, valid and sensitive to change [5]. Meanwhile, in Boston, Liang et al. [4] developed the Systemic Lupus Activity Measure (SLAM). Bombardieri proposed the European Consensus Lupus Activity Measurement and Smolen introduced the National Institutes of Health SLE Index Score [6].

BILAG begins

In 1984 in the UK, the newly formed BILAG group agreed that a global score was not suitable to capture the full range of real-life lupus activity. Using a global score, a patient hospitalized with life-threatening renal lupus may score the same as an outpatient with relatively low disease activity in several other systems. It was felt that it was important to derive an index that captured change in disease activity over time and distinguished partial or complete improvement as well as deterioration. It was based on the principle of the physician's intention to treat [7].

Classic BILAG

Classic BILAG characteristics

The BILAG index focused on disease activity in eight organs or systems: constitutional, mucocutaneous, neurological, musculoskeletal, cardiovascular/respiratory, vasculitis, renal and haematological. When the BILAG index was updated in 2004, the original BILAG index became known as the classic BILAG index to differentiate it from the revised BILAG-2004 index.

The classic BILAG index is a categorical scale with up to 18 questions in each category [8, 9]. The form can be completed on most patients in less than 5 min, either on paper or computer. The urine and blood test results are filled in later. Training is of vital importance. The key to accurate BILAG assessment is only scoring a clinical

feature if the physician is sure it is due to lupus activity. For example, a patient with proteinuria from a urinary tract infection should not be scored for proteinuria in the renal system. For each symptom or sign due to lupus activity, it is determined whether the feature is the same, better, worse or new over the preceding 4 weeks as compared with the previous 4 weeks. The recorded items are then converted into A–E scores for each organ/system. Scoring for the BILAG DAI is based on the concept of intention to treat. An A (action) score implies that the patient requires treatment with at least prednisolone 20 mg daily or immunosuppressive therapy. A B (beware) grade is given to patients requiring a lower level of immunosuppression. A C grade implies contentment, meaning low disease activity requiring little treatment. A D (discount) grade means that the patient had been active in the past but is no longer active. An E (never ever active) grade means that the disease has never been active.

Classic BILAG reliability and validity

With support from the Arthritis Research Campaign (ARC), now Arthritis Research UK (ARUK), the classic BILAG index was shown to be a reliable and valid tool. A study involving five UK centres showed strong support for the intention-to-treat concept in all systems apart from the neurological system [8].

Classic BILAG compared with other DAIs

The classic BILAG index is an ordinal scale based on intention to treat and ostensibly should not be compared with global score tools. However, an ad hoc scoring system was agreed upon so it could be converted into a global score. Using this scoring scale (A=9, B=3, C=1, D/E=0), there appeared to be strong correlation between the classic BILAG index, SLEDAI and SLAM [10]. Subsequently, detailed analysis indicated that a more optimal scoring system was A=12, B=5, C=1, D/E=0 [11].

The classic BILAG index is a transitional index, with categorical scoring for each system, which has advantages over a global score. For example, distinguishing 5000 vs 145 000 $\times 10^6/l$ platelets would not be captured with the SLEDAI. The SLEDAI group subsequently realized the importance of detecting change rather than assigning dichotomous values and developed the SLEDAI-2K Responder Index 50 (SRI-50), which measures $\geq 50\%$ improvement in disease activity [12]. However, this fails to distinguish a persistently active clinical feature, which is unchanged from a feature whose activity is getting worse. In 1991, the SLICC group compared the SLEDAI, SLAM and classic BILAG indices, demonstrating that they are reliable and valid [13].

Classic BILAG clinical trials

In the past 15 years, pharmaceutical companies have become more interested in doing clinical trials in patients with lupus. The US Food and Drug Administration (FDA) reviewed the various DAIs; the classic BILAG index was recommended and has been used worldwide. It has been shown to be reliable, comprehensive and sensitive to

change [14]. It has also been used in real and paper patient exercises performed by the SLICC group [10].

The classic BILAG index has proved very useful in long-term observational studies and in double-blind controlled trials (e.g. azathioprine vs ciclosporin in lupus) [15]. Although, the classic BILAG index was designed for use in adults, it has been used in JSLE [16] and to determine the occurrence and rate of flare [17].

Problems with the classic BILAG index

Problems with the classic BILAG index emerged over time. If there was improvement in an item that previously scored grade A, it would become a C on the next assessment 4 weeks later. If the item remained the same, it would score a B at the third assessment. This reflects poor response to treatment and is consistent with the intention-to-treat premise on which the BILAG index is based, that more treatment is needed to gain further improvement, but it gives the false impression of a deterioration, because of the increase in score. In order to assess flare, it is necessary to identify items that are recorded new or worse. Some of the items were capturing damage (e.g. avascular necrosis) rather than activity. In the renal assessment, it did not capture stable proteinuria, only worsening proteinuria, and it was not validated using methods other than 24 h protein values, such as the urine protein:creatinine ratio. Also, it did not capture most gastrointestinal and ophthalmic manifestations, the neuropsychiatric terminology was out of date and the glossary was inadequate.

BILAG-2004 index

BILAG-2004 characteristics

The classic BILAG index was revised and replaced by the BILAG-2004 index. This new index distinguished nine organs/systems instead of eight. Ophthalmic and gastrointestinal systems were added and the vasculitis section was removed and the items were placed in the appropriate system. Mucocutaneous and neurological systems were arranged more logically and renal disease was better captured. Features that indicated damage, such as sclerodactyly, avascular necrosis, calcinosis and tendon contractures, were removed. Fatigue and migraine were excluded.

The new glossary was more standardized and comprehensive. Terminology was updated and better definitions of various terms were provided.

The BILAG-2004 index improved its scoring system. The A score items now improve to score B and not C. Grade A is defined as patients that would require treatment with medium/high doses of steroids or immunosuppressive drugs and/or high-dose anticoagulation (international normalized ratio >3) [18]. Numerical scoring analysis suggested that A=12, B=8, C=1 and D/E=0 was most appropriate [19].

BILAG-2004 software

The BILAG group worked closely with Gordon Hamilton from ADS-Limathon (Sheffield, UK), who devised BILAG-2004 software (BLIPS) to record activity items and determine the activity index. BLIPS includes demographic variables, SLAM, SLEDAI, SLICC/ACR Damage Index and the 36-Item Short Form Health Survey [9]. This was subsequently made a web-based programme known as iBLIPS, and later Silver-BLIPS. This program has been continually improved, and the eighth version is currently being developed. BLIPS is used in many research studies and clinical trials.

BILAG-2004 research

With the support of ARC/ARUK, the BILAG group performed various validation studies.

Reliability

The BILAG-2004 index was tested for interrater reliability in routine clinical practice. One local physician at each centre and a single study physician separately assessed 97 patients at 11 centres [20]. In addition, two reliability exercises were performed involving 12 and 14 raters respectively with prior training in the use of the BILAG-2004 [18]. Fatigue, migraine/cluster/tension headache, mood disorders and anxiety disorders showed poor agreement between raters and resulted in their removal. Interrater reliability was assessed using kappa statistics and intra-class correlation coefficients (ICCs). Many users gave scores to items even if they were not related to lupus (e.g. rash secondary to eczema), therefore formal training in the use of the BILAG-2004 index is essential to ensure its optimal performance. Having removed certain items, such as fatigue and mood disorders (where attribution to active lupus is often most uncertain), the BILAG-2004 index was shown to be reliable [20].

Validity

A large multicentre cross-sectional study with 369 patients assessed validity. Construct validity (the degree to which a test measures what it claims to be measuring) was determined by assessing disease activity using the classic BILAG, BILAG-2004 and SLEDAI-2000 indices and determining if they correlated with ESR, C3, C4 and anti-dsDNA levels. The BILAG-2004 index, which does not include serology, showed significant association between increasing ESR, decreasing C3 and C4 levels, elevated anti-dsDNA levels and increasing SLEDAI-2K scores.

Because there is no gold standard for disease activity in SLE, change in treatment was used for criterion validity. Criterion validity was confirmed by the increasing strength of association between BILAG-2004 disease activity scores and an increase in therapy. The BILAG-2004 index is a valid measure of disease activity and was recommended for use in clinical trials and outcome studies [21].

Sensitivity

The BILAG-2004 index needed to show sensitivity to change in order to be used in longitudinal studies. Sensitivity to change or responsiveness implies an ability to change with time. A prospective multicentre longitudinal study of 347 patients was performed. Statistical analyses with maximum likelihood multinomial logistic regression were used with change in therapy as the outcome variable. An increase in the overall BILAG-2004 index score was associated with an increase in therapy and inversely associated with a decrease in therapy [3].

There were some concerns regarding renal scoring, as there was a non-significant association between renal score and treatment change (e.g. some patients with A or B scores in the renal system did not have their treatment increased). This discrepancy may have been because of persistent renal disease, intermittent active urinary sediments or pre-existing renal damage. However, the BILAG-2004 index is sensitive to detection of new-onset LN and significant improvement of disease activity in the renal system. Also, it is possible that the time to expected response is a factor here. For example, the BILAG-specified parameters for nephritis are responsive, but they may need to be assessed over a longer time interval than the other domains.

BILAG-2004 systems tally

Analysis of nine systems using the BILAG-2004 index may pose a challenge in clinical studies. Therefore a new method was devised to make it more clinically meaningful and simpler for analysis. The aim was to record the number of systems in which disease activity was increased, decreased or remained the same between two consecutive visits. This was expressed as a tally (BILAG-2004 systems tally) and became a novel way of representing BILAG-2004 index scores longitudinally in clinical trials [22].

This was further simplified into three parts [simplified BILAG-2004 systems tally (sBST)] by grouping major and minor deterioration and persistent activity into a single group and major and minor improvement into another group. Using multinomial logistic regression analysis and receiver operating characteristics (ROC) curve analysis, the sBST was shown to be appropriate for use, as the number of systems with active or worsening disease were independently associated with an increase in therapy.

Comparison of BILAG-2004 with SLEDAI-2000

The BILAG-2004 index captures SLE disease activity better than the SLEDAI-2000 [23]. Rheumatologists trained in the use of both assessed 93 patients with SLE. Disease activity was determined in patients with high activity on the BILAG-2004 (overall score A or B) but a low SLEDAI-2000 score (<6) and patients with low activity on the BILAG-2004 index (overall score C, D or E) but a high SLEDAI score (≥6). Thirty-five patients had

high activity on the BILAG-2004 index but a low SLEDAI-2000 score, of which 48.6% had treatment increased. There were only five patients with low activity on the BILAG-2004 index but a high SLEDAI-2000 score. The SLEDAI failed to capture disease manifestations such as peripheral neuropathy, myelopathy and haemolytic anaemia. Therefore SLEDAI-2000, although shown to be a reliable index to assess overall disease activity, is less able than the BILAG-2004 index to detect active disease requiring increased treatment. This is probably because the SLEDAI is a global index rather than a transitional index.

A study assessed 159 children with LN using the BILAG-2004 index and the SLEDAI-2000. Correlations between 24 h urinary protein and renal pathology index were analysed. The BILAG-2004 index score showed the best correlation with the pathology activity index with type II LN, followed by those with type IV LN. The SLEDAI-2000 showed the best correlation with the pathology activity index with type II LN, followed by those with type I LN. The BILAG-2004 index had a higher ROC curve (area under the curve) (0.93 vs 0.88). The authors showed that the BILAG-2004 index scoring system is more reliable and more comprehensive [24].

BILAG-2004 index and flare

Assessment of disease flare in SLE is challenging. The SLICC group performed an outpatient clinic study with 16 patients to assess flare, comparing the BILAG-2004 index with the flare version of SELENA and physician's global assessment (PGA). The revised SELENA system is organ based and thus is similar to the BILAG-2004 index. Mild, moderate and severe flares were determined depending on the physician's intention to treat. The primary objective was to see if these instruments can be used for identifying flare, as well as if they can distinguish between mild, moderate or severe flare [1].

Each patient was assessed independently by four rheumatologists who were previously trained in using the BILAG-2004 index, SELENA flare index (SFI) and PGA. The BILAG-2004 index flare definition was that a severe flare was defined as an A score for items recorded as worse or new, a moderate flare if a patient had two or more B scores for items recorded as worse or new and a mild flare if a patient had a single B score or if there were three or more C scores. D or E scores implied no lupus activity and no flare.

The SFI distinguished mild flare if no treatment was necessary or if hydroxychloroquine, prednisolone 7.5 mg/day or a non-immunosuppressive therapy is commenced. A moderate flare implied >7.5 mg of prednisolone per day but <0.5 mg/kg/day or immunosuppressive therapy other than cyclophosphamide. Severe flare was defined as prednisolone or equivalent ≥0.5 mg/kg/day or cyclophosphamide or biologic treatment or admission to hospital.

There was agreement of a flare in at least 75% of patients seen and ~80% between BILAG-2004 index flare and SFI. Severe and no flare were easily distinguishable, but there was less differentiation between mild and

moderate flare. ICC was used to assess the reliability of flare by the BILAG-2004 index and SFI compared with PGA. The BILAG-2004 flare had the highest ICC at 0.54, compared with the SFI at 0.21 and the PGA at 0.18, suggesting that it is better at capturing flare than the SFI.

BILAG-2004 pregnancy index

Physiological changes in pregnancy can mimic active lupus. Differentiating proteinuria of pre-eclampsia from LN can be difficult and therefore the major change in the BILAG-2004 index was in the renal system. Hypertension was excluded. Complements (C3 and C4) and anti-dsDNA antibodies were included to help differentiate proteinuria due to LN from proteinuria due to pre-eclampsia in the absence of urinary sediment. Changes in the glossary were emphasized to ensure rheumatologists were aware of the pathophysiological changes in pregnancy. The BILAG-2004 pregnancy index was shown to be a reliable tool for assessment of disease activity in pregnant patients with lupus [25].

Paediatrics

JSLE is similar to adult-onset SLE but there are well-documented differences. JSLE is more aggressive, with more frequent renal, neuropsychiatric and haematological manifestations, and mortality rates are higher. Marks *et al.* [16] showed in a prospective observational 12 month study of 21 patients that the renal BILAG score was able to differentiate between patients with and without nephritis. The classic BILAG index was shown to be a useful tool in monitoring disease activity in JSLE. The excellent responsiveness to change seen in JSLE studies supported the use of the BILAG-2004 index as a response measure in clinical trials in children and adolescents with lupus.

Responder indices

In recent years, composite responder indices have been developed for use in clinical trials, combining individual organ and system assessment indices such as BILAG-2004 with global scores [26]. This development was thought to make the composite indices more responsive than the individual component DAs. However, the superiority of these composite indices has yet to be demonstrated.

SRI

The SRI was the first composite index to be used in lupus randomized control trials. The original SRI combined criteria from the classic BILAG index, SELENA-SLEDAI and PGA. Belimumab, the first drug in 60 years to be approved for SLE, used the SRI as its primary endpoint. A response by SRI was defined as having an at least 4 point reduction from baseline in the SELENA-SLEDAI score without one or more new classic BILAG index A scores or more than one new classic BILAG index B scores compared with

baseline, and no worsening in PGA (no more than a 0.3 point increase from baseline) [27, 28].

BILAG-based combined lupus assessment

The BILAG-based combined lupus assessment (BICLA) is another composite index used in clinical trials [29]. This differs from the SRI in that the BILAG-2004 index is the driver of efficacy. BICLA requires patients to meet response criteria across the BILAG-2004 index, SLEDAI-2000 and PGA. This primary endpoint was used in the phase IIb randomized control trial assessing the efficacy and safety of epratuzumab in patients with moderate to severe SLE. It is being used as the primary endpoint for the ongoing phase 3 study of this drug. To be classified as a BICLA responder, patients must meet the following criteria: BILAG-2004 index improvement in all A and B scores, no more than one new BILAG B score, no worsening of the total SLEDAI-2K score from baseline, no significant deterioration (not >10% worsening) in the 100 mm visual analogue PGA and no treatment failure.

Discussion

Since the initial development of the classic BILAG index in 1984, major improvements have been made in the measurement of disease activity in lupus. Optimization of the classic BILAG index, which evolved into the sophisticated BILAG-2004 index, has proved to be a sensitive and reproducible tool. The BILAG-2004 index is currently used worldwide in longitudinal studies and major clinical trials.

The BILAG-2004 index, vs the classic BILAG index, includes an assessment of the ophthalmic and gastrointestinal systems and removal of damage features such as avascular necrosis and calcinosis. The exclusion of fatigue and migraine from the classic BILAG index, often difficult to interpret and common in those with both active and inactive lupus, further improved accuracy. The updated glossary and terminology made the BILAG-2004 index more user friendly than the classic BILAG index.

The BILAG-2004 index is one of the only disease indices to undergo extensive and thorough analysis using robust statistical methods. The largest multicentre validation study to date was performed on the BILAG-2004 index, showing that it is ideal for use in clinical trials.

The validation and development process emphasized the importance of training of physicians in the use of the BILAG-2004 index. This has been likened to standardization of equipment prior to use. If the BILAG-2004 index is incorrectly used it could adversely affect the results reported in clinical trials.

The new scoring system was another advancement in the BILAG-2004 index. The BST and sBST provide new ways of representing BILAG-2004 index scores longitudinally in clinical trials. Another key development of the BILAG-2004 index was the adaptation of this score for pregnant patients with SLE.

The BILAG-2004 index is better at capturing disease activity requiring an increase in treatment than the

SLEDAI-2000. New composite indices such as the BICLA allow further accuracy in the detection of clinical response across all systems. The BICLA also identifies deterioration in clinical features and excludes treatment failures. However, none of the composite indices have been accepted as the gold standard. In fact, it has been suggested that outcome assessment for flares in lupus trials have been overly sensitive and non-specific [30]. The selection of the most appropriate disease activity tool will depend on the context in which it is used and the questions to be answered.

The frustrating discrepancy between clinical practice (e.g. patients responding to rituximab [31]) and the outcomes of clinical trials remains an issue [32]. Many studies using the classic BILAG have shown rituximab to be an effective treatment in patients with refractory SLE [33–36]. Failure of rituximab in clinical trials appears to be multifactorial [37]. In the Exploratory Phase II/III SLE Evaluation of Rituximab (EXPLORER) and Lupus Nephritis Assessment with Rituximab (LUNAR) trials, patients were on large doses of corticosteroids and immunosuppressive drugs, which affected the results. However, there may be other issues with clinical trial design.

Conclusion

Many disease indices have been developed to assess SLE activity. Those described before the mid-1980s however were clearly inadequate. Even the optimized global score systems continue to have problems in terms of not capturing various clinical features and being limited in detecting sensitivity to change in clinical status.

The BILAG-2004 index is the most comprehensive of the SLE disease activity measures [38]. It is the only transitional index that grades clinical features as being new, the same, worse or improving and incorporates severity in the scoring. Global score indices distinguish whether a feature is present or absent, but do not express the severity of the symptom, and therefore can be misleading.

To date, there is no international consensus as to which DAI should be used in clinical trials. The complexity of this multisystemic disease and lack of biomarkers made it exceedingly difficult to develop an accurate disease index. However, it is remarkable how far we have come in the past 30 years in developing the BILAG-2004 index into a valid, reliable and sensitive tool.

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1.4. Appendix IV:

New Developments, Clinical Uses and Health Implications, Biologics in Juvenile SLE.

BIOLOGICS IN JUVENILE SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT

Juvenile Systemic Lupus Erythematosus (JSLE) is a chronic autoimmune multisystemic disease. The prognosis of JSLE has greatly improved over the past number of decades likely due to a combination of better recognition, early diagnosis as well as aggressive treatment in specialist units. Adult SLE and juvenile SLE have similarities but also important differences. Patients with JSLE have more aggressive disease. They have frequent haematological and renal involvement. The female to male ratio is less pronounced in JSLE. Few clinical trials have been undertaken in paediatric rheumatology, and the use of biologics in JSLE is largely based on their use in the adult population. There is a resultant paucity of efficacy and safety data in JSLE. Reassuringly, clinical experience suggests that biologic drugs may be as efficacious in paediatric settings as they are in adult. In this chapter we review current biologic agents being used and those with potential for use in children and adolescents with JSLE.

Keywords: biologic agents, systemic lupus erythematosus, juvenile SLE, B cells, autoimmunity.

INTRODUCTION

Juvenile SLE is an autoimmune disease with multisystemic involvement. Twenty percent of all SLE patients present before the age of 18 [1]. Median age of onset is around the onset of puberty. The cause of JSLE is unknown. It is likely multifactorial with the immune system, genetics, hormones and the environment all playing a role [2, 3]. In fact all components of the immune system appear to be involved with dysregulation of both the innate and adaptive systems. Therefore the immune system is a prime target for biologic treatment.

Patients with JSLE tend to have more severe disease than adult SLE [4]. Children and adolescents have been shown to accrue more damage than adults and have higher standardized mortality risks

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[5]. Both the disease itself and disease modifying drugs may have detrimental effects on children and adolescents psychological and physical development. The aim of treatment is to maintain remission and to improve overall quality of life and long-term survival.

Constitutional symptoms such as fever, lymphadenopathy, rashes and oral ulceration are more frequently seen. Diffuse proliferative glomerulonephritis is more severe. Haematological features such as haemolytic anemia, leucopenia and thrombocytopenia are more prevalent in JSLE [6]. Autoimmune hepatitis [7] and arthritis is also more frequently seen in JSLE. Interestingly cardiopulmonary features are rare in children and adolescence with JSLE, but they have been shown to be at increased risk of cardiovascular disease [8].

There are also laboratory differences between both diseases. Anti-double stranded DNA, anti-ribosomal P antibodies and anti-histone are all more prevalent in JSLE than in adult onset-SLE [9]. Macrophage activation syndrome is more commonly seen in children and can be life-threatening [10].

Pharmacodynamic and pharmacokinetic differences between adolescents and children need to be taken into consideration [4]. Young children and adults have evolving immune systems. The use of biologics and their potential long-term adverse effects including infertility and infection are unknown. Beukelman et al. showed that patients with JSLE are likely to have an increased risk of malignancy regardless of biologics or medication use [11].

Management requires a holistic multidisciplinary approach focusing on controlling disease rapidly to prevent damage and also providing support for patients and their families. Transfer from paediatric to adult care can be a difficult milestone and should be individualized for each patient. This chapter focuses on biologics currently in use and biologic agents that show promise in the future for children and adolescents with JSLE.

Background:

To date there are no large randomized controlled trials performed on the use of biologic agents in JSLE. These drugs have shown efficacy in adults with SLE and therefore use in children and adolescents is mainly in an ad hoc unlicensed manner.

Biologics are expensive and are generally reserved for patients who have difficult to control disease and who have not responded to traditional DMARD (Disease Modifying Anti Rheumatic Agents) therapy. To date there is only one licensed biologic for use in adult onset SLE and none available in JSLE. Therefore we rely mainly on adult population studies, clinical experience and

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small cohort studies to guide treatment. National and international collaborative studies should help guide better treatment strategies.

Biosimilars appear to have excellent safety and efficacy data comparable to biologics and should help reduce the cost of these agents [12]. They have been introduced into the UK for use in rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis in February 2015. However, the estimated cost saving is approximately 15-30% so they remain an expensive therapy [12].

Biologics in JSLE

Biologics target components of the immune system and therefore better knowledge of immunity is essential in order to develop treatments for JSLE. The immune system appears to be over active with upregulation of B-cells, T-cells, natural killer cells, monocytes and dendritic cells. ANA (antinuclear antibodies) are seen in over 90% of patients with JSLE and adult onset SLE [13]. However, anti-double stranded DNA and low C3 are more common in JSLE.

Cytokines including Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin 17 (IL-17), Type I Interferon (IFN), B Lymphocyte Stimulator (BLyS), Tumour Necrosis Factor- α (TNF- α) have all been implicated in SLE pathogenesis. Anti-TNF drugs have revolutionised the treatment of autoimmune diseases such as juvenile inflammatory arthritis (JIA) but can rarely induce a lupus like syndrome in children and adolescents.

Neutrophil extracellular traps (NETs) are fibrous networks of chromatin and antimicrobial factors that are released by neutrophils to trap and kill pathogens. Increased NET formation (NETosis) or insufficient degradation of NETs can promote autoimmunity due to the contents being exposed to the immune system [15].

Knowledge of the immune system and in particular the **inflammasome** is important. This cytoplasmic complex leads to caspase-1 activation and subsequent production of inflammatory cytokines such as IL-18 and IL-1 β .

Pattern recognition receptors, such as NLRP1 can assemble an inflammasome in response to danger signals or pathogens [16].

Genetic overproduction of interferon- α and complement deficiencies has been shown to lead to monogenic SLE [17].

T cells appear to play a role in SLE disease progression. The main T cell subsets involved in SLE are CD4+ T helper (Th) cells and regulatory T cells (Tregs). CD4+ T cells are known to regulate
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B cell autoantibodies via the production of cytokines. Beresford et al. measured IL-17A and Th1/Th2-related cytokine concentrations from patients with JSLE. IL-17A levels were higher compared to healthy controls and JSLE T cells once activated had a better ability to secrete Th17 associated cytokines [18].

Midgley et al. showed that patients with JSLE have increased expression in low density granulocytes, which are a subset of neutrophils. These appear to correlate with BILAG disease activity scores and dsDNA levels and may have a role in pathogenesis [19].

B cells play a pivotal role in the pathophysiology of JSLE and therefore have been targeted. B lymphocytes have numerous roles including antibody production, act as antigen presenting cells, interact with T-cells, secrete cytokines and modulate dendritic cells. CD20 is a B-cell specific antigen expressed on both immature and mature B cells [20]. There is evidence for the use of two B cell targeted biologics, Rituximab and Belimumab in JSLE [21, 22]. However, to date there are no biologic agents licensed for use in JSLE. Belimumab is the only licensed biologic drug for use in adult onset SLE. Currently there is a randomized control trial investigating the efficacy and safety of belimumab in children and adolescents with JSLE.

Rituximab is a chimeric monoclonal antibody (MAB) targeted against CD20 on immature B cells causing apoptosis of these cells. Rituximab was initially used as treatment for patients with non-Hodgkins lymphoma and subsequently in rheumatology for rheumatoid arthritis. It was observed that patients with both lymphoma and rheumatoid arthritis, who were treated for lymphoma experienced improvement in their rheumatoid arthritis. The success of rituximab in the treatment of rheumatoid arthritis led to a study treating fifty adults with SLE who had failed conventional treatment. Using the BILAG disease activity index, 42% of patients with SLE achieved full remission post rituximab. Double stranded DNA levels dropped and C3 levels improved [23].

Rituximab is beneficial in the treatment of adults with renal disease [24] Its use in JSLE has increased over the past number of years especially for children with refractory lupus nephritis. The optimal dose and timing remains uncertain [25].

Rituximab has a good safety profile and its effect generally lasts between 6 to 12 months. Rituximab has been used to treat children with Immune Thrombocytopenia Purpura (ITP) and autoimmune haemolytic anaemia. It appears effective in the treatment of renal and neuropsychiatric manifestations of JSLE. The two major SLE randomized controlled trials, the EXPLORER (Exploratory Phase II/III SLE evaluation of Rituximab) and the LUNAR trial (Lupus Nephritis Assessment with Rituximab Study), which both enrolled patients age 16-75,

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unfortunately failed to meet their primary end-points in adults [26, 27]. Despite this Rituximab continues to be widely used because patients with SLE improve on this treatment.

The EXPLORER trial assessed rituximab use in 257 patients with moderate to severe systemic lupus erythematosus. Using the Classic BILAG disease activity index, the primary endpoint was to achieve remission at week 24 and to avoid flare at week 52. The trial failed to meet its primary end-point but beneficial effects were noted in African-American and Hispanic patients. Post hoc analysis showed that patients had lower dsDNA and higher C3 levels.

The LUNAR trial involved 144 patients with Class III/IV lupus nephritis. These patients were also given mycophenolate mofetil and glucocorticoids, which may have had an impact on why the study did not reach its primary end-point. Other potential reasons for why these studies failed include trial design or sample size. Despite this frustrating discrepancy, there is plenty of clinical evidence including numerous case reports, which show the benefit of rituximab in SLE[20, 28].

The largest cohort study to date involving children and adolescents with SLE treated with rituximab, was conducted in the United Kingdom. Using the JSLE cohort study database, those with JSLE who had prior rituximab treatment were identified. Retrospective analysis of 63 patients case notes from 2003-2013 were reviewed. The mean age was 12.2 years at diagnosis and 79% were female. All patients had been treated with previous immunosuppressives. They received on average 104 courses of IV rituximab at a dose of 750mg/m² given on two different occasions approximately two weeks apart. Most patients were on glucocorticoids. Patients on rituximab had a reduced BILAG disease activity index score from 4.5 to 3, although this did not reach statistical significance. Patients required less corticosteroids and their lab markers improved. Adverse events including neutropenia, fever, infection and infusion reactions were experienced in 18% [29]. Long-term studies are necessary to assess the safety of rituximab in the future.

A further study by Lightstone et. al. assessed the treatment of patients with rituximab without corticosteroids compared with those treated with conventional treatment [30, 31]. This study suggested that early treatment of patients with rituximab appears to be safe and effective. The Rituxilup Trial is a multicenter randomized control trial that aims to demonstrate whether addition of Rituximab to MMF is helpful in treating a new flare of lupus nephritis and whether it has long lasting steroid-sparing effects with equal efficacy and greater safety than MMF and oral prednisolone. Eligibility criteria include adults and children aged 12-17 years old with active lupus nephritis. If successful, this trial has the potential to dramatically change how lupus nephritis in adults and children with JSLE are managed.

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Children who have had rituximab may develop long-lasting B cell depletion and hypogammaglobulinemia, sometimes requiring immunoglobulin replacement. There is conflicting data regarding immunoglobulin levels post rituximab treatment in children and adolescents. Immunoglobulin levels may be mildly depleted, remain normal or be severely depleted [32].

One of the largest and most detailed analyses of children treated with rituximab reviewed 91 children with immune thrombocytopenia. In total 108 adverse events were noted of which 84% were noted to be mild to moderate. However, one patient developed prolonged hypogammaglobulinemia [33].

Combined Rituximab and cyclophosphamide

A pilot study of twelve children/adolescents with either lupus nephritis or treatment resistant lupus were treated with combination of rituximab 750mg/m² to 1 gram, and cyclophosphamide 750mg/m² over eighteen months. Combined treatment significantly reduced the need for steroids and response was maintained over a 5 year period [34].

BLyS/BAFF

B lymphocyte stimulator (BLyS) also known as BAFF (B lymphocyte activating factor) or TNF superfamily member 13B, is a cytokine that promotes B-cell proliferation and survival [35]. It therefore promotes the secretion of immunoglobulin. BLyS is produced as a 285-amino acid transmembrane protein. It is cleaved at a furin protease site and released in its soluble form. BLyS is produced from myeloid cells and binds to three receptors on the B cell (BAFF-receptor, BCMA (B-cell maturation antigen) and TACI (transmembrane activator and cyclophilin ligand interactor) [36] BLyS is upregulated in response to IL-10 and IFN-gamma [37]. Therefore, blocking BLyS makes therapeutic sense as a treatment option in JSLE.

Hong et al. tested 56 blood samples from patients with JSLE and showed that plasma BLyS protein and blood leucocyte BLyS mRNA levels were significantly elevated in these children. There was correlation between plasma BLyS protein levels with disease activity which adds strength to the fact that BLyS may be a therapeutic target in JSLE [38]. An identical BLyS expression profile was observed in both children and adolescents with JSLE as was found in adult SLE. Interestingly, BLyS expression in patients with JSLE was independent of corticosteroid treatment.

Belimumab is a fully humanized monoclonal antibody that binds to soluble human BLyS. It inhibits the activity of BLyS and has been shown to reduce dsDNA levels in patients with lupus. The FDA first approved Belimumab for use in patients with active SLE in 2011, which is the first drug in sixty years to be approved for SLE [39].

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Belimumab is given intravenously at a dose of 10 mg/kg every fortnight for the first month and then every 28 days. Belimumab has no known drug interactions and dose adjustments are not required for renal or hepatic dysfunction [40]. The SLE Responder Index (SRI) was the composite index used as the primary outcome measure. BLISS 52 and BLISS 72 were the two large randomised controlled multicenter trials that compared belimumab to placebo in patients with SLE who on standard treatment. The patients enrolled had to meet the American College of Rheumatology criteria for the diagnosis of SLE, to have active disease, and to be seropositive (ANA titer $\geq 1:80$ or anti-dsDNA antibody titer ≥ 30 IU per milliliter) at screening.

Both studies showed significant improvement in the SRI with 10 mg/kg being the most effective dose. In the BLISS-76 trial, differences in SRI between belimumab at 10 mg per kilogram and placebo were no longer significant at 76 weeks [40].

Pediatric Lupus Trial of Belimumab Plus Background Standard Therapy (PLUTO) is a multicenter, randomized study to evaluate the safety, pharmacokinetics, and efficacy of belimumab in children and adolescents aged 5 to 17 years old with active systemic lupus erythematosus (SELENA SLEDAI score ≥ 6). The study will consist of three phases: a 52-week randomized, placebo-controlled, double-blind phase; a long term open label continuation phase; and a long term safety follow up phase. The long term open label continuation and safety follow up periods will continue for at least 5 years and possibly up to 10 years from a subject's initial treatment with belimumab.

Atacicept is a human fusion protein that inhibits BlyS and APRIL [41]. APRIL is one of the TNF family cytokine with a stronger affinity to B cell maturation antigen (BCMA) receptor than B cell-activating factor receptor (BAFF-R). BCMA is important in B cell survival. Unfortunately the clinical trial of atacicept with corticosteroid and mycophenolate had to be terminated due to a significant hypogammaglobulinemia among some of the patients [42]. Elolimy et al showed in a study of 29 patients with JSLE that serum BlyS and APRIL were elevated in JSLE compared to controls. Although BlyS correlated with disease activity as measured by SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) ($p=0.042$), serum APRIL levels were inversely related ($p=0.02$). However there was a statistically significant association between elevated serum APRIL levels and negative anti-dsDNA in JSLE patients ($p=0.017$) suggesting the possibility of it being a down-regulator in JSLE.

Epratuzumab is a humanized monoclonal antibody that targets CD22 antigen on B cells [43]. Epratuzumab and rituximab have different effects on B cells. Epratuzumab has distinct effects on cell growth. 786 patients with SLE participated in the EMBODY 1 and 788 patients in EMBODY

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2 trials. Patients included were 18 years of age or older and those with JSLE were not included. In addition to standard treatment with glucocorticoids and other treatments including immunosuppressants or hydroxychloroquine, patients received 600 mg of epratuzumab every week for a period of 4 weeks, 1200 mg every 2 weeks for a period of 4 weeks, or a placebo. Disappointingly, the phase III clinical trial showed that epratuzumab failed to meet its primary end-point.

Patients who are intolerant to Rituximab may benefit from novel anti-CD20 monoclonal antibodies. Ocrelizumab is humanized anti-CD20 IgG1 monoclonal antibody with modifications in the Fc region. A phase III trial of ocrelizumab in adult onset SLE for the treatment of lupus nephritis was ended because of high rates of serious infections. Of note, these patients had also been on concomitant cyclophosphamide, prednisolone and azathioprine or mycophenolate [44].

Ofatumumab is a human IgG1κ MAB that binds CD20 on B cells but at a unique epitope. It has been shown to be effective in the treatment of rheumatoid arthritis but there is limited data in JSLE to date. However, it had beneficial effects in the treatment of a 22 year old lady with severe JSLE from the age of 11, who became intolerant of rituximab [45].

Eculizumab which inhibits complement 5 (C5) was successful in treatment of a 4 year old girl with lupus and diffuse proliferative lupus nephritis. She was treated previously with prednisolone, plasma exchange and cyclosporine and despite this aggressive therapy developed atypical haemolytic uremic syndrome. Eculizumab led to remission of vasculitis, proteinuria and haematuria with normalisation of renal function. This biologic treatment be of benefit for patients with JSLE in the future [46].

Low levels of C1q protein and high titres of C1q antibodies have been shown to be involved in the pathogenesis of JSLE, especially lupus nephritis and therefore may be another therapeutic target [47].

A study analysed serum levels of HMGB1 and IFN-α and LAIR-1 expression on plasmotoid dendritic cells (pDCs) of patients with JSLE. 26 patients with JSLE aged between 8-16 years were tested. It was found that serum levels of HMGB1 and IFN-α in patients with JSLE were significantly increased compared to healthy controls and also in those with active JSLE compared to those with inactive JSLE. LAIR-1 was lower than in healthy controls. Blocking of HMGB1 and its receptors or increasing expression of LAIR-1 on dendritic cells may be a potential therapeutic target. [48].

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Polymorphisms in the inflammasome receptor NLRP1 and adult-onset SLE have been reported. Selected polymorphisms in inflammasome genes were analysed in 90 children and adolescents with JSLE and 144 healthy controls. A single polymorphism in the IL1B gene was associated with JSLE, suggesting that IL-1B is involved in the pathogenesis of SLE [16].

Discussion

Clinical trials have been curtailed in JSLE due to the small number of eligible patients and due to the heterogeneity of disease. International collaboration is imperative in order to discover effective biologic agents to treat JSLE. For this to occur, more children need to be afforded the opportunity to be involved in research. In 2007, the European medicines regulations were updated and children and adolescents are now allowed to enroll in clinical trials, which should facilitate our understanding and management of JSLE in the future.

PRINTO (Paediatric Rheumatology International Trials Organisation) is a non-governmental international research network, which includes 59 countries with the goal to conduct international clinical trials and outcome studies in children with rheumatic diseases [51].

Measurement of disease activity is crucial. The BILAG (British Isles Lupus Assessment Group) index, SLEDAI and SLAM (Systemic Lupus Activity Measure) indices are validated for use in JSLE. The BILAG-2004 index is based on the physician's intention to treat and has been shown to measure SLE disease activity better than the SLEDAI-2000 [52].

The BILAG index was adapted for use in JSLE and subsequently used in the UK Juvenile SLE cohort study and was named the paediatric BILAG (pBILAG). The pBILAG index collects more detailed information about organ-related disease activity than that incorporated within the American College of Rheumatology (ACR) criteria [53]. However, this index was designed for adults with lupus so may not capture the full spectrum of disease.

CONCLUSION

Biologic agents show promise in the treatment of patients with JSLE in the future. Children and adolescents with JSLE have higher mortality rates than adults with SLE and therefore it is essential that we can use biologics in these difficult to treat patients. Children and adolescents with JSLE have evolving immune systems so we should not be depending on adult studies. To date we have no licensed biologic agents for use in JSLE but there is evidence that rituximab is of great benefit.

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Growth delay, obesity and psychological effects of both the disease and treatments can pose major problems in management of children and adolescents. Patients need support so they can live a full life despite having a chronic debilitating disease. Further national and international collaboration is required. Now that children and adolescents are allowed to enrol in studies we should be able to discover new biologic agents should optimise management of children and adolescents in the future.

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1.5. Appendix V:

Systemic Lupus Erythematosus. Juvenile Systemic Lupus Erythematosus.

Chapter 8

Juvenile systemic lupus erythematosus

Claire Louise Murphy, Yiannis Ioannou, and Nicola Ambrose

Key points

- Juvenile SLE is a more aggressive disease than adult-onset SLE and has a higher mortality rate.
- Macrophage activation syndrome is a potentially life-threatening complication of JSLE, which may mimic the underlying disease or be confused with sepsis.
- Transferring care from paediatric to adult care can be a difficult milestone and should be tailored to the individual patient.

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Introduction

Juvenile-onset systemic lupus erythematosus (JSLE) is a chronic complex multisystem autoimmune disease, which represents 15–20% of all SLE cases.¹ Clinical presentation of JSLE is similar to adult-onset SLE, but there are some well-documented differences. JSLE is more aggressive, with more frequent renal and haematological manifestations. Mortality rates when corrected for age are higher than that of adult-onset SLE.² Infantile-onset SLE has a particularly high mortality rate.³ The female to male ratio is approximately 5:1, lower than the 9:1 ratio in adult SLE.⁴ Management of JSLE requires a multisystemic, holistic approach with recognition of psychosocial factors that occur during normal childhood and adolescence.

Epidemiology

JSLE prevalence ranges from approximately 40 cases per 100,000 in Caucasians to more than 200 cases per 100,000 among black people.⁵ SLE is uncommon before puberty. However, the upper cut off age in JSLE studies has varied from 14 and 20 years of age and therefore comparison between juvenile data sets is difficult. Median age of onset is between 12–14 years and it is rare before 5 years of age.⁶

Pathophysiology

As with adult-onset SLE, the cause of JSLE is unknown. It appears to be multifactorial with genetic, immunological, hormonal, and environmental influences. All components

of the immune system seem to be involved with dysregulation of the innate and adaptive systems.

Genetic susceptibility

Congenital complement deficiencies are present in about 1% of patients with JSLE;⁷ the best characterized being C1q deficiency. The disease linked to homozygous C1q deficiency is best described as lupus-like with lower levels of antinuclear antibody (ANA) and double-stranded DNA (dsDNA) antibody positivity, and less renal and cerebral involvement.

Genetics play an important role in the pathogenesis of adult-onset SLE and JSLE. There is an approximate 25% concordance amongst monozygotic twins compared with 2% concordance in dizygotic twins in SLE.⁸ The UK juvenile cohort study showed that 38% of patients with JSLE had a family history of at least one autoimmune disease.⁹

Genome-wide association studies (GWAS) are beginning to add further to our understanding of SLE with over 40 genes being implicated to date. Genes relating to pathways involved in the removal of anti-DNA-nucleosome complexes, such as complement, are among the strongest genetic risk factors.¹⁰ Increased nuclear factor-kappaB (NF- κ B) sensitivity has been identified, via a SLE susceptibility gene UBE2L3 (ubiquitination gene).¹¹

Genetic overproduction of interferon- α , complement deficiencies, and apoptosis defects can lead to monogenic SLE.¹² New genetic techniques should lead to the discovery of new genes and help our understanding of the pathogenesis.

The immature immune system

Over-activity of the immune system appears to be a major factor in SLE with up-regulation of B cells, T cells, natural killer cells, monocytes, and dendritic cells. There are abnormalities in complement, in cytokine pathways, and in apoptosis.¹³ Cytokines including interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin 17 (IL-17), type I interferon (IFN), B lymphocyte stimulator (BLyS), and tumour necrosis factor- α (TNF- α) have all been implicated in SLE pathogenesis. Neutrophil extracellular traps (NETs) are fibrous networks of chromatin and antimicrobial factors that are released by neutrophils to trap and kill pathogens. Increased NET formation (NETosis) or insufficient degradation of NETs can promote autoimmunity due to the contents being exposed to the immune system.¹⁴

There are well described differences between the immune system in children and adults. Young children and infants appear to be more susceptible to infection. They also may have suboptimal responses to vaccines.¹⁵ The total number of immune cells differs depending on age. In the first few months of life, helper T cells peak up to four times higher than in adults and then slowly decline reaching adult levels at around six years of age.¹⁶ IgA levels are lower in infants than in adults. IgG1 and IgG3 reach 60% of adult levels at one year of age and IgG2 and IgG4 at 2–5 years of age.¹⁵ Although some lymphocyte phenotypic differences have been described between young children and adults, very little is known about what functional changes occur at puberty across the innate and adaptive immune system that clearly lower the threshold for the development of JSLE.

Hormonal influences

Although adult-onset SLE affects a higher proportion of females, this is not as high in childhood SLE. One explanation for this is differences in oestrogen profiles. Oestrogen is known to affect the immune system via its oestrogen receptors (ER α and ER β); and *ESR1* and *ESR2* genes encode these receptors. Oestrogens acting via receptors are important in the pathogenesis of SLE. The C variant of rs2234693, a single nucleotide polymorphism (SNP), increases transcription of *ESR1*. The T allele of this SNP was associated with early-onset SLE. A higher prevalence of the *ESR1* C allele among children with JSLE compared with controls suggests that increased activity of the oestrogen receptor is pathogenic. Allele A of the *ESR2* SNP (rs4986938) was associated with Adult SLE.¹⁷ However, how puberty may affect oestrogen receptor expression, and how activation of oestrogen receptors may lower the threshold for lupus again remains unknown. However, in the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) trial, the combined oral contraceptive pill did not increase the risk of SLE flare.¹⁸

The UK Juvenile-Onset SLE study group showed that male patients with JSLE were younger at presentation and were more likely to have a discoid rash compared with females. Males were less likely to have arthritis than females.⁹ However, a recent review showed that there was no clear association between gender and mortality or disease activity in SLE.¹⁹

Environmental influences

UV radiation appears to play a role in the pathogenesis of SLE. There may be an association with low vitamin D levels and SLE. Drugs such as hydralazine, procainamide, and TNF-antagonists are risk factors for SLE. Infections have been implicated. Epstein-Barr virus antibodies have been found to be more prevalent in children and adolescents with JSLE compared with healthy controls (99% vs 70%).²⁰

Clinical features with emphasis on differences between JSLE and adult SLE

Patients with JSLE display a wide range of clinical manifestations, which can fluctuate over time. Some may have mild lupus and others have life-threatening disease.

Systemic features

Fever and lymphadenopathy are more frequently observed in JSLE than in adult-onset SLE. Although Raynaud's is commonly seen in JSLE, it is more commonly seen in adult-onset SLE. Sicca symptoms are more common in adult-onset SLE.²¹ It may be that younger patients have a greater reserve of salivary ducts and remain asymptomatic for longer. JSLE is more severe and has a worse prognosis than adult-onset SLE.²² Please see Table 8.1.

Skin manifestations

A malar rash, and oral ulceration is more common in JSLE than in adult-onset SLE.²³ Alopecia is usually non-scarring and is more common in adults.²⁴ Discoid lesions and

Table 8.1 Key clinical differences between JSLE and adult-onset SLE

Clinical features	Juvenile SLE	Adult-onset SLE
Fever	++	+
Lymphadenopathy	++	+
Raynaud's	+	++
Dry eyes	+	++
Butterfly rash	++	+
Alopecia	++	+
Oral ulceration	++	+
Arthralgia	+	++
Lupus nephritis	++	+
Neuropsychiatric	++	+
Livedo reticularis	+	++

subacute lupus are more frequently seen in adult-onset SLE.²³ Livedo reticularis is less common in JSLE.²⁴

Musculoskeletal manifestations

The reporting of joint pains increases with advancing age. True arthritis may be more common in JSLE, whilst arthralgia and myalgia may be more frequent in adult-onset SLE.^{25,26} Avascular necrosis is more common in children than in adults with lupus.²⁷

Renal disease

Lupus nephritis is more common and severe in JSLE and is more often a presenting feature. As with adults, diffuse proliferative glomerulonephritis is the commonest histological diagnosis. Children may present with severe nephritic syndrome, hypertension, nephrotic range proteinuria, and oedema, but are more commonly asymptomatic.²⁶ One study showed that 47% of JSLE patients had evidence of renal involvement at presentation and 80% at the time of their last review.⁹ Patients with JSLE are more likely to have dialysis compared with those with adult-onset SLE (19% vs 5.7%; $p < 0.001$).²⁸

Neurological manifestations

Neuropsychiatric manifestations may be more common in JSLE (20–45%) compared with adult-onset SLE (10–25%).⁵ However, it is difficult to quantify manifestations, as there is such a wide array, ranging from 15–90%, depending on diagnostic criteria and patient selection. For instance, headaches are frequent but the prevalence of true 'lupus headache' is unknown. It is imperative that all other aetiologies of neuropsychiatric disease are excluded prior to diagnosis. Psychosis with visual hallucinations is the commonest manifestation of neuropsychiatric disease associated with JSLE. Neuropsychiatric manifestations often occur within the first year of disease onset. Seizures are more prevalent in JSLE than in adult-onset SLE.^{21,29} Mood disorders and headaches are frequent. Diagnosing true neuropsychiatric JSLE may be challenging in the adolescent patient, where rebellion and testing boundaries is part of normal development.

Haematological manifestations

Haemolytic anaemia and thrombocytopenia are more common in JSLE than in adult-onset SLE. Patients with a positive ANA and immune thrombocytopenic purpura (ITP) are at higher risk of developing autoimmune diseases such as SLE.³⁰

Gastrointestinal manifestations

Abdominal pain is common in JSLE, and occasionally may be secondary to ascites, pancreatitis, autoimmune hepatitis, or intestinal vasculitis.³¹ There is a greater prevalence of autoimmune liver disease (smooth muscle antibody-positive, biopsy proven) in JSLE compared with adult-onset SLE (9.8% vs 1.3%; $p < 0.001$).³² Also liver disease preceded the diagnosis in many of those with JSLE but in none of the adults.³²

Cardiopulmonary manifestations

Cardiopulmonary involvement is more common in adult-onset SLE than in JSLE.³³ Pleuritis is the most common feature in both groups. Pericarditis appears to be as common in JSLE and adult-onset SLE.²¹ Pulmonary hypertension is rare in SLE.³⁴ Adult-onset SLE is associated with a 10-fold risk of coronary artery disease which is not explained by conventional risk factors.³⁵ One study of 157 patients with JSLE showed pericarditis in 28.7%, cardiomegaly in 33.8%, and arrhythmia/conduction defects in 12.7%.³⁴ Long-term follow-up studies are underway which will help evaluate the true cardiovascular risk in JSLE.

Macrophage activation syndrome and its treatment

Macrophage activation syndrome (MAS) is a potentially life-threatening condition which can complicate JSLE. It is characterized by the infiltration of macrophages in bone marrow and in organs such as the liver, spleen, and lymph nodes. The pathogenesis is not fully understood, but it appears to be due to a defect in natural killer cells and cytokine dysregulation. Activation and uncontrolled proliferation of T lymphocytes and macrophages can lead to cytokine release and widespread haemophagocytosis. Infections (such as Epstein-Barr virus) and drugs may be a trigger. Patients may develop a high fever, headaches, fatigue, disorientation, seizures, and coma, and multisystem involvement may occur. Hyperferritinaemia is an important feature. Haematological features include pancytopenia, elevated liver enzymes, high triglycerides, high lactate dehydrogenase, and elevated D-dimers. Hyponatremia is frequently seen. The coagulation profile may show low fibrinogen, with prolongation of the prothrombin and partial thromboplastin time. Patients may develop purpura and bleed easily. Recognition of MAS in JSLE can be particularly challenging as it may mimic features of the underlying disease (eg, fever and cytopenias) or be confused with sepsis. Based on the HLH-94 protocol, treatment with high dose corticosteroids is necessary. Cyclosporin and etoposide are sometimes needed.³⁷

Antiphospholipid syndrome

There are limited data on antiphospholipid syndrome (APS) in JSLE. The prevalence of antiphospholipid antibodies ranges from 27–66% for anticardiolipin antibodies and 24–62% for lupus anticoagulant.^{38,39} Part of the problem is a lack of clarity with regards to classification of childhood onset APS, as the current classification criteria for APS include recurrent pregnancy loss as one of the clinical features that define disease.⁴⁰ Catastrophic antiphospholipid syndrome (CAPS) in paediatric patients is rare though very serious. One study showed that 10.3% of the 446 patients from the CAPS registry

Table 8.2 Key laboratory differences between JSLE and adult onset SLE

Haematology	Juvenile SLE	Adult-onset SLE
ANA	++	++
Anti-dsDNA	++	+
Anti-Ro	++	++
Anti-La	++	++
Anti-Smith	++	+
Anti-RNP	++	++
Rheumatoid factor	+	++
Anti-cardiolipin (IgG and IgM)	++	+
Lupus anticoagulant	++	+
Anti-ribosomal P	++	+
Low C3	++	+
Haemolytic anaemia	++	+
Thrombocytopenia	++	+
Urine		
Urinary cellular casts	++	+

ANA: antinuclear antibodies; dsDNA: double-stranded DNA.

were below 18 years of age. Of the paediatric patients with CAPS, 68.9% suffered from primary APS and 28.9% from SLE. There appeared to be a higher prevalence of infection as a precipitating factor for in the paediatric population (60.9% vs 26.8% in the adult population; $p < 0.001$) and of peripheral vessel thrombosis (52.2% vs 34.3%; $p = 0.017$).⁴¹ A study of 121 patients with paediatric APS showed that those with primary APS were younger and had a higher frequency of arterial events, whereas patients with APS associated with autoimmune disease were older and had a higher frequency of venous events.⁴²

Immunological manifestations

Antinuclear antibodies are present in over 90% of patients with adult-onset SLE and JSLE.³³ There is a similar occurrence of anti-Ro and anti-La. It is more common to have high anti-dsDNA and low C3 in JSLE. Anti-histone and anti-ribosomal P antibodies are more common in JSLE.⁴³ Please see Table 8.2.

Treatment

A multidisciplinary approach is essential to ensure quality care for patients with JSLE. Patients should be treated in a specialist centre where related specialists are also available (eg, haematologists, nephrologists, neurologists). Involvement of the general practitioner, physiotherapist, occupational therapists, psychologists, play therapists, nurse specialists, social workers, and school teachers is key to ensuring optimal care.

Avoidance of UVA and UVB exposure is advised. Therefore patients with JSLE should avoid excessive sun and wear high factor sunscreen.

Early recognition and aggressive treatment is crucial in the management of JSLE in order to prevent irreversible damage. Due to the higher prevalence of renal and cerebral involvement, more intensive immunosuppression is usually required in JSLE.⁴⁴ Glucocorticoids are prescribed frequently with efforts to wean off as soon as possible. Children tend to have higher levels of glucocorticoids than adults, which contribute to damage, and may be a reason why children have an increased incidence of avascular necrosis compared with adults. Non-steroidal anti-inflammatory drugs (NSAIDs) are helpful for musculoskeletal disease but have significant side effects.

Mild/moderate JSLE

Hydroxychloroquine is helpful in skin and joint disease and can prevent flares of SLE. It inhibits toll-like receptors 7 and 9 and has effects on pro-inflammatory cytokines and reduces disease activity.⁴⁵ It also reduces low-density lipoprotein levels in SLE and appears to be cardioprotective.⁴⁶ There is also evidence to show that hydroxychloroquine is protective against thrombosis.⁴⁷ It is recommended for all patients with JSLE provided there are no contraindications.

Azathioprine (AZA) is a purine analogue that is used as a steroid-sparing agent in mild-moderate SLE, especially in patients who are having recurrent flares.

Methotrexate is useful in patients with lupus for musculoskeletal disease.

Renal/cerebral disease

Mycophenolate mofetil (MMF) is useful in lupus nephritis in JSLE like in adult-onset SLE.⁴⁸ MMF has been shown to be as effective for severe renal lupus (grades III and IV) as cyclophosphamide, and has fewer side effects.⁴⁹ The MAINTAIN nephritis trial compared MMF with azathioprine for long-term immunosuppression and reported that although there were fewer renal flares in the MMF group, this did not reach statistical significance. Therefore, both MMF and azathioprine are reasonably effective in maintaining remission;⁵⁰ however, data for JSLE is lacking.

Cyclophosphamide (500–750 mg/m² per month for six months, or the Euro-Lupus protocol of 500 mg fortnightly for 6 doses) is used only in severe JSLE, weighing up the future risks of infertility and malignancy. Cyclophosphamide is the preferred treatment in neuropsychiatric JSLE. Prepubertal girls seem to be protected from the gonadotoxic effects of cyclophosphamide.⁵¹ The gonadotrophin releasing hormone (GnRH) analogue, triptorelin, is used for ovary protection in JSLE, and a recent randomized control trial has shown it to be effective in patients.⁵² Gonadal functioning and preservation of reproductive function in JSLE has been explored but remains uncertain.^{51,53,54}

B cell depletion

Rituximab is a chimeric anti-CD20 monoclonal antibody (MAB) and has been used successfully in patients with refractory JSLE.⁵⁵ Rituximab has been used in JSLE to treat Immune Thrombocytopenia Purpura (ITP), and autoimmune haemolytic anaemia, renal and neuropsychiatric JSLE and appears to be safe and efficacious. Despite failure of rituximab in the EXPLORER and LUNAR trials,^{56,57} rituximab does appear to be efficacious and is widely used.⁵⁸ Novel anti-CD20 MABs may present novel options for patients allergic to Rituximab. **Ocrelizumab** is a humanized MAB and **ofatumumab** is a human IgG1κ MAB that binds CD20 on B cells but at a unique epitope. Although unlicensed, ofatumumab may be an effective alternative therapy for patients with SLE intolerant to rituximab.⁵⁹

Belimumab is a MAB to the soluble human B lymphocyte stimulator protein and has been shown to be effective in adult-onset SLE. Clinical trials with belimumab in children with SLE are currently in progress.⁶⁰

Epratuzumab, an anti-CD22 human MAB currently in phase III clinical trials and may be promising in JSLE.⁶¹

Other available treatments

Intravenous immune globulin (IVIG) and plasma exchange may be useful in acute life-threatening disease.

Stem cell transplantation may be used in patients with severe disease, unresponsive to other therapy.^{62–64} One study showed that three of six severely ill patients (all female, age 15–29 years) with severe refractory SLE died post stem cell transplantation and three survived and remained in remission.⁶³ Stem cell transplantation is not widely used due to the potentially fatal side effects.

Specific adolescent concerns

Long-term prognosis has improved for patients with SLE with the 10-year survival rate now over 90%.^{65,66} The emphasis is on maintaining health, preventing damage, achieving growth, and successfully transitioning from paediatric to adult care.

Access to care and delays in initial diagnosis are well reported in JSLE.⁶⁷ Patients treated early and aggressively have a better prognosis than those who have their treatment delayed. The UK JSLE cohort study showed that there is a wide variation in time to JSLE diagnosis. However, overall prognosis is improving and life expectancy is now much longer.

Transferring care from paediatric to adult clinics

Moving from the paediatric JSLE clinic to the adult SLE clinic can be a difficult milestone for children and parents. The transition is not a single event and should occur over several years. Research has shown that adolescents with rheumatic conditions rate their mental health, use of health services, and activities more negatively than those without chronic disease or with other chronic diseases.⁶⁸ Transition to adult care should be tailored to each individual patient.

School attendance, vocational planning

Adolescents with JSLE are at higher risk for delayed psychosocial and cognitive development than their peers. Additionally clinic appointments, medication monitoring blood tests, side effects, fatigue, and disease flare ups lead to reduced school attendance. A holistic approach needs to be taken in managing their individual needs, taking vocational planning and school attendance into consideration.

Self-management

As children grow up, they need to learn about their condition, medications, self-administration, and monitoring requirements. Adolescents may respond to a problem-solving approach rather than a paternalistic approach.

Bone health

Osteopenia affects approximately 40% of those with JSLE.⁶⁹ Vertebral fractures occur in up to 10% of patients. Patients should ensure sufficient intake of vitamin D and

calcium to prevent osteopenia or osteoporosis. Weight-bearing exercise is crucial in maintaining bone strength. Bisphosphonates have a long half-life and are potentially teratogenic so ideally should be avoided in adolescents. Dual-energy X-ray absorptiometry (DXA) use in children is controversial but it is generally recommended at the time of diagnosis and every two years thereafter.

Growth

Growth failure occurs in approximately 15% of patients with JSLE and therefore regular measurement of height and weight is essential. Active disease, corticosteroids, and concomitant autoimmune disease such as thyroid disease may result in short stature. Growth hormone in JSLE may result in increased disease flares.⁷⁰ Early referral to endocrinology is recommended as soon as growth failure is apparent.

Cardiovascular health

Like adults with SLE, patients with JSLE have an increased cardiovascular risk. Atherosclerosis begins early in life even in healthy children. Risk factors such as dyslipidaemia, high blood pressure and glucose, smoking, and lack of exercise should be addressed. Aspirin and hydroxychloroquine should be considered in JSLE especially if antiphospholipid antibody positive. Schanberg et al. studied the use of atorvastatin over three years in JSLE and there was no statistically significant benefit.⁷¹ The APPLE (Atherosclerosis Prevention in Pediatric Lupus Erythematosus) study suggests atorvastatin may reduce atherosclerosis progression in pubertal patients with SLE with higher C-reactive protein. Further research in this area is needed.⁷²

Malignancy

Little is known regarding the risk of malignancy in JSLE. The risk of non-Hodgkin's lymphoma appears to be increased in adult-onset SLE.⁷³ A cohort study of 1020 juvenile patients aged under 18 years, were observed for an average of 7.8 patient years. Two patients developed non-Hodgkin's lymphoma and one patient had leukaemia. The non-haematological cancers included one bladder, one breast, one thyroid and one brain, three head and neck and four non-specified cancers. Although this study showed an increased malignancy risk in patients with JSLE compared with the general population, this translated to a relatively low absolute risk (1.75 cancers per 1000 patient years). The risk may be higher after patients have transferred to adult care.⁷⁴ Longer term studies are required.

Vaccinations

Patients with JSLE are at increased risk of infection. Annual influenza vaccine and 5-yearly pneumococcal vaccines are recommended. There is an increased risk of cervical intraepithelial neoplasia (CIN) in SLE.⁷⁵ Adolescent girls should receive human papilloma vaccine and have regular cervical smears. Live vaccinations are contraindicated while on immunosuppressant therapy and for three months after discontinuation.

Fertility and contraception

Active disease is associated with delayed menarche, amenorrhoea, or oligomenorrhoea. Oestrogen-based contraception is contraindicated in patients with APS associated with JSLE, because of a risk of thrombosis. However, in those without antiphospholipid antibodies, the combined oral contraceptive pill may be prescribed.⁷⁶

Medication toxicity

High-dose steroids may lead to premature atherosclerosis, secondary osteoporosis, and glucose intolerance. In adolescence, obesity, growth delay, hirsutism, and striae may have a significant impact for body image and lead to poor adherence.

Research

Performing clinical trials is curtailed in JSLE due to the small number of eligible patients and due to the heterogeneity of the disease. To date, treatments have been based on those used in adult-onset SLE. Patients with rheumatic disease, regardless of their age, should have the opportunity to participate in research. PRINTO (Paediatric Rheumatology International Trials Organisation) is a non-governmental international research network, which includes 59 countries with the goal to conduct international clinical trials and outcome studies in children with rheumatic diseases.⁷⁷

Measurement of disease activity is crucial, especially now that various biologics are undergoing clinical trials. The BILAG (British Isles lupus assessment group) index, SLEDAI (systemic lupus erythematosus disease activity index) and SLAM (systemic lupus activity measure) indices are validated for use in JSLE.⁷⁸ The BILAG-2004 index is based on the physician's intention to treat and has been shown to measure SLE disease activity better than the SLEDAI-2000.⁷⁹

The BILAG index was adapted for use in JSLE,⁸⁰ and subsequently used in the UK juvenile SLE cohort study and was named the paediatric BILAG (pBILAG). The pBILAG index collects more detailed information about organ-related disease activity than that incorporated within the American College of Rheumatology (ACR) criteria.⁸¹ However, this index was designed for adults with lupus so may not capture the full spectrum of disease.

New biomarkers

Novel biomarkers would help in identifying JSLE, disease monitoring, and prediction of flares.⁸² There has been much interest in looking for novel biomarkers for SLE and JSLE. Those published include monocyte chemoattractant protein-1/CCL-2, neutrophil gelatinase-associated lipocalin, urine protein signature, and colony-stimulating factor 1.

Other biomarkers being investigated in JSLE include microRNA, type 1 interferon, cell adhesion molecules, complement components, ~~and others~~. Recent studies show that complement split products may be better than the traditional C3 and C4. Circulating erythrocyte E-C4d levels appeared higher in patients with lupus nephritis compared with healthy controls.⁸³

Conclusion

JSLE is similar to adult-onset SLE, but there are distinct differences in clinical features, serology, and management requirements. It is more aggressive, with a higher prevalence of renal manifestations requiring high-dose immunosuppression. Obesity, growth delay, and psychological effects of both the disease and treatments can pose major problems in the management of children and adolescents. A holistic, multidisciplinary approach is necessary. Patients need support so that they can live a full life despite having a chronic debilitating disease. International collaboration and further research is needed to optimize care for these patients.

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1.6. Appendix VI

2012 SLICC criteria

CLINICAL CRITERIA

(1) Acute Cutaneous Lupus OR Subacute Cutaneous Lupus

- **Acute cutaneous lupus:** lupus malar rash (do not count if malar discoid), bullous lupus, toxic epidermal necrolysis variant of SLE, maculopapular lupus rash, photosensitive lupus rash (in the absence of dermatomyositis)
- **Subacute cutaneous lupus:** nonindurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with post inflammatory dyspigmentation or telangiectasias)

(2) Chronic Cutaneous Lupus

- Classic discoid rash localized (above the neck) or generalized (above and below the neck), hypertrophic (verruccous) lupus, lupus panniculitis (profundus), mucosal lupus, lupus erythematosus tumidus, chilblains lupus, discoid lupus/lichen planus overlap
-

(3) Oral Ulcers OR Nasal Ulcers

- Oral: palate, buccal, tongue
- Nasal ulcers
- In the absence of other causes, such as vasculitis, Behcet's disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis, and acidic foods

(4) Nonscarring alopecia

- Diffuse thinning or hair fragility with visible broken hairs, in the absence of other causes such as alopecia areata, drugs, iron deficiency, and androgenic alopecia

(5) Synovitis involving 2 or more joints

- Characterized by swelling or effusion
- OR tenderness in 2 or more joints and at least 30 minutes of morning stiffness

(6) Serositis

- Typical pleurisy for more than 1 day OR pleural effusions OR pleural rub
- Typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day OR pericardial effusion OR pericardial rub OR pericarditis by electrocardiography

- In the absence of other causes, such as infection, uremia, and Dressler's pericarditis

(7) Renal

- Urine protein-to-creatinine ratio (or 24-hour urine protein) representing 500 mg protein/24 hours OR red blood cell casts

(8) Neurologic

- Seizures, psychosis, mononeuritis multiplex (in the absence of other known causes such as primary vasculitis), myelitis, peripheral or cranial neuropathy (in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus), acute confusional state (in the absence of other causes, including toxic/metabolic, uremia, drugs)

(9) Hemolytic anemia

(10) Leukopenia (<4000/mm³) OR Lymphopenia (<1000/mm³)

- Leucopenia at least once: In the absence of other known causes such as Felty's syndrome, drugs, and portal hypertension.
- Lymphopenia at least once: in the absence of other known causes such as corticosteroids, drugs, and infection

(11) Thrombocytopenia (<100,000/mm³)

- At least once in the absence of other known causes such as drugs, portal hypertension, and thrombotic thrombocytopenic purpura

IMMUNOLOGIC CRITERIA

(1) ANA level above laboratory reference range

(2) Anti-dsDNA antibody level above laboratory reference range (or 2-fold the reference range if tested by ELISA)

(3) Anti-Sm: presence of antibody to Sm nuclear antigen

(4) Antiphospholipid antibody positivity, as determined by

- Positive test for lupus anticoagulant
- False-positive test result for rapid plasma reagin
- Medium- or high-titer anticardiolipin antibody level (IgA, IgG, or IgM)
- Positive test result for anti-2-glycoprotein I (IgA, IgG, or IgM)

(5) Low complement (C3, C4, or CH50)

(6) Direct Coombs' test (in the absence of hemolytic anemia)