Premature senescence, endothelial turnover and accelerated atherosclerosis in SLE: the relationship between circulating endothelial cells, telomere length and lupus factors

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2 Abstract

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Premature senescence, endothelial turnover and accelerated atherosclerosis in SLE: the relationship between circulating endothelial cells, telomere length and lupus factors

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Systemic lupus erythematosus (SLE) is associated with premature onset of coronary heart disease and endothelial dysfunction. To date the mechanisms underlying this remain unclear.

We hypothesise there is premature biological ageing in patients with SLE as evidenced by a reduction in the mean telomere length of PBMC. Premature biological ageing is also evident in the vasculature of patients with SLE and reflected by relatively shortened telomeres of cells involved in vascular repair and regeneration i.e. endothelial progenitor cells (EPC). Furthermore, senescent EPC result in cellular imbalance with a relatively reduced number and/or function of circulating healthy EPC.

We studied 200 SLE patients longitudinally over an average of 5.8 (5.2, 6.3) years and demonstrated progression of carotid plaque burden in 17.5%. Baseline traditional CHD risk factors did not influence plaque progression.

We measured CD34/CD133+ EPC using flow cytometry in 54 SLE patients and 49 controls in cross-sectional study and demonstrated no significant difference between the groups. We further investigated number and function of EPC by enumerating colony-forming unit (CFU) in culture in 39 SLE patients and 27 controls and demonstrated a significant reduction in CFU number in SLE [median (IQR) CFU 5.7 (2.3, 8.0) in SLE *vs.* 10.0 (5.7, 15.0) in controls; p= 0.0016] and this difference was particularly marked in those under the age of 40 years [4 (2, 8) vs. 10.5 (7, 19), p= 0.03].

We measured relative telomere length of PBMC in SLE compared to age–matched controls using real-time qPCR in a cross-sectional study and demonstrated a significant reduction in SLE patients [0.97 (0.47, 1.57) and 1.53 (0.82, 2.29), p = P = 0.0008]. Further, telomere length of DNA extracted from CFU after 7 days in culture was quantified in a preliminary study of 5 SLE patients and 5 controls and demonstrated a trend to telomere length reduction in SLE patients.

In conclusion there was evidence of significant progression of carotid plaque is in this cohort of female SLE patients. Further there is evidence of abnormal endothelial repair and premature senescence in SLE. Results support the hypothesis that there is a premature senescent phenotype in SLE and as such may present a novel therapeutic target to attenuate the risk of CHD in SLE.

3 Declaration

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

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Further information on the conditions under which disclosures and exploitation may take place is available from the Head of Department at the Arthritis Research UK Epidemiology Unit.

A 3 year restriction has been set on the electronic publication of this thesis within the University of Manchester.

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Lastly, I am thankful for the unconditional and constant support of my parents for all my endeavours and Kayes for continual consideration and kindness (and administrative input).

5 Statement about the author

I graduated from the University of Liverpool in 1999 with an MBChB and completed pre-registration and senior house officer posts at the University Hospital Aintree where I attained MRCP in 2002. I commenced rheumatology training in the Northwest deanery in 2003 and completed an MSc in Clinical Rheumatology in 2006 during which I undertook a BILAG-designed multi-centre UK-wide case control study examining risk factors for clinical coronary heart disease in SLE. In 2006, I obtained an ARC Clinical Research Fellowship to fund this research. I will shortly take up a post as a consultant rheumatologist at the East Lancashire Hospitals Trust.

Abstracts related to thesis

- K Williamson, S Haque, IN Bruce, S Stringer, MY Alexander. (2009) The role of heparan sulphate proteoglycans on endothelial progenitor cells in age-related endothelial dysfunction. Human Gene Therapy 20:1417-1545
- S Haque, C Rakieh, F Salway, R Gorodkin, P Ho, LS Teh, P Day, IN Bruce. (2009) SLE Patients Show Evidence of Premature Biological Senescence. Arthritis & Rheumatism 60 (Suppl 10); 920. DOI: 10.1002/art.25999
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- S Haque, J Shelmerdine, Y Ahmad, H Bodill, LS Teh, IN Bruce. (2008) Rate and factors influencing progression of atherosclerosis in SLE. Rheumatology 44 (Suppl 2):i92
- S Haque, Y Ahmad, J Shelmerdine, IN Bruce. (2008) Rate and Factors Influencing Progression and Onset of Atherosclerosis in SLE. Arthritis & Rheumatism 568 (9) Supplement:S324

Publication related to thesis

- Haque, S and Bruce IN. (2009) Cardiovascular outcomes in systemic lupus erythematosus: big studies for big questions. Journal of Rheumatology. Mar;36(3):467-9 (Editorial)
- Haque S, Mirjafari, H, Bruce, IN. (2008) Atherosclerosis in Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE). Current Opinion in Lipidology. 19(4):338-343

Prizes related to thesis

2010 International Lupus Congress 'Cutting Edge of Research' Prize
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6 Introduction

6.1 Systemic Lupus Erythematosus (SLE)

SLE is a complex inflammatory disease characterised by autoimmune dysregulation predominantly affecting women. The first comprehensive descriptions of the disease were documented in the 1800s by Kaposi and Osler [175, 261] but the first classification criteria were recommended by committee consensus in 1971 [72]. Patients with SLE are known to have a wide spectrum of manifestations that evolve over time. An accurate estimation of disease onset can be difficult to determine and for the purpose of clinical studies patients with SLE are usually defined as those that meet the 1997 American College of Rheumatology (ACR) revised classification criteria [155]. These criteria are summarised in **Error! Reference source not found.**. The epidemiology, pathogenesis, major organ involvement and current therapeutic options are summarised below.

6.1.1 Epidemiology of SLE

SLE displays a strong female preponderance, with a female to male ratio of 9 - 13:1. The overall incidence in the UK is approximately 3 – 5 per 100,000 per year although the condition more commonly affects Afro-Caribbean and Asian individuals compared to white individuals [173, 229, 324]. Incidence rates have increased over the past forty years and this might partly be attributed to an increased awareness and recognition of the condition, as well as the introduction and revisions of the classification criteria [155, 336]. Mortality rates in the pre-steroid era were low and the 5-year

survival in the 1950s was estimated at 50% [235]. Recent studies however, have demonstrated improved 5-year survival rates of 95% in Europe and North America [59, 177]. Despite the increased awareness of the disease and improvements in the management of some aspects of the condition, there remains considerable morbidity associated with SLE and the estimated 10 -15 year survival remains significantly reduced at 85% [345].

Table1 Adapted from 1997 Updated Revised Criteria for the Classification of SLE

Clinical Features *	Description
1. Malar Rash	Fixed erythema, flat or raised, over the malar eminences
2. Discoid rash	Erythematous raised patches with adherent keratotic scales
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight
4. Oral Ulcers	Oral or nasopharyngeal, usually painless
5. Arthritis	Non erosive, 2 or more peripheral joints
6. Serositis	Pleuritis or pericarditis
7. Renal disorder	Persistent proteinuria > 0.5 g/d or> 3+ or cellular casts
8.Neurological disorder	Seizures or psychosis
9.Haematological disorder	Haemolytic anaemia or leucopenia or lymphopenia or thrombocytopenia
10.Immunologic disorder	Anti-dsDNA or anti-Sm and/or anti phospholipid antibody
11.Antinuclear antibody	In the absence of drugs

*4 criteria required during the course of the disease [155]

6.1.2 Pathogenesis of SLE

The pathogenesis of SLE is multifactorial involving both genetic and environmental factors ultimately resulting in the formation of antibodies and immune dysfunction.

6.1.2.1 Genetic factors

The heritability of SLE has been recognised for several decades and family clustering of disease in SLE is greater than observed in other autoimmune diseases such as type 1 diabetes or Graves' disease [76]. Twin studies have demonstrated disease concordance between monozygotic twins to be between 24 - 69% compared to 2 to 9 % of dizygotic twins [38, 83]. Recent advances in genotyping, such as candidate gene studies and the genome-wide association studies, have resulted in increased interest in the genetic basis of SLE. Several genetic polymorphisms or DNA alterations of importance to SLE susceptibility have been identified and the expression and function of genes have furthered the understanding of SLE pathogenesis.

As in other autoimmune diseases, HLA class II genes, in particular those coding for T-cell signalling, have been associated with SLE in European and Asian cohorts [118, 130, 141]. HLA class III genes which contain important immune genes e.g. TNF and complement pathway genes have also been found to be associated with SLE [366].

A number of non-HLA genes have been shown to be prevalent in SLE patients. Recent reviews cite more than 25 confirmed susceptibility loci and these include interferon (IFN) regulatory factor 5 (*IRF5*), which has an important role within the interferon pathway, signal transducer and activator of

transcription 4 (*STAT4*), which is involved in cytokine signalling and T cell responses, *PTPN22,* which plays a role in T-cell receptor inhibition, and Fc receptors for immunoglobulin G (*FcγR*) genes which are involved in immune complex clearance and antibody-dependant responses [85, 108]. Interestingly, several polymorphisms have been demonstrated in patients from differing ethnicities and are also common to other autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease, suggesting autoimmune diseases have a similar genetic foundation [149]. Clearly other factors determine the development of differing clinical phenotypes between individual diseases. The genes identified have been shown to have a small effect and there is likely to be significant contribution of gene-environment interactions and epigenetic factors.

6.1.2.2 Environmental factors

The predilection for disease amongst individuals from some ethnicities is well recognised. However it is also recognised that the risk of SLE varies between individuals of the same ethnic background situated in different geographical regions suggesting that environmental factors have an important role in SLE susceptibility e.g. reduced exposure to endemic pathogens such as malaria may lead to loss of protective factors [41]. Conversely some viral infections have been shown to be prevalent in SLE and may trigger immune dysfunction resulting in autoimmunity such as parvovirus B19, cytomegalovirus and Ebstein-Barr virus [282, 342].

Given the striking female preponderance of SLE, several studies have attempted to study the role of hormones on disease susceptibility and activity.

The increased incidence of disease flares in pregnancy and subsequent pregnancy morbidity is well recognised [71, 267]. The role of hormone contraceptives has been studied in several small investigations and a recent systematic review has concluded it is likely that the oral contraceptive pill does not increase the rate of flares in SLE and can safely be used, except in the subgroup of patients with the antiphospholipid syndrome given the increased risk of thrombosis in this group [77].

6.1.2.3 Immune dysfunction

Elements of both the innate and adaptive immune systems have been shown to be defective in SLE. However, the exact nature of these abnormalities remains unclear and it is not known if immune dysfunction observed is the cause or consequence of SLE. Autoimmunity is thought to result from loss of self tolerance and the subsequent formation of antibodies to self-antigens. Autoantibodies have been shown to be present in individuals for many years before the onset of clinical features of SLE [9]. One of the major contributors to the formation of autoantibodies in SLE is thought to be the inadequate recognition and clearance of apoptotic cells resulting in exposure of intracellular antigens to the immune system [129]. Several components of the immune system are likely to contribute to the process of defective clearance of immunogenic material in SLE. Dendritic cells, which primarily have a role in antigen presentation and T cell activation, have been shown to be dysfunctional in SLE [91]. Furthermore abnormalities of T and B cell activation and signalling have been demonstrated in SLE [75]. Toll-like receptors, which have an important role in the activation of both B cells and

dendritic cells, may also play a role in the defective processing of autoantigens in SLE [156]. The role of the complement system in SLE is interesting. C1q is a component of the classical pathway and individuals with homozygous C1q deficiency have a greater than 90% risk of developing SLE [344, 366]. Abnormalities of complement receptors have been demonstrated in SLE and have been implicated in accelerated atherosclerosis in SLE [180, 293].

6.1.2.4 Cytokines profiles in SLE

Abnormalities of cytokine pathways in SLE and their role in pathogenesis have emerged over the past decade. These include cytokines with effects on B cells such as B-cell activating factor (BAFF) also known as B-lymphocyte stimulator (BLyS) protein, interleukin-6 and interleukin-10. Increased levels of BLyS have been demonstrated in SLE and shown to be associated with disease activity [385].

Another important cytokine is interferon (IFN)-α, a member of the type 1 interferon cytokine family. These proteins have a role in immune defence and increased levels are observed in the context of viral infections. They also have a role in physiological growth and differentiation [140]. Increased levels of IFN-α can be detected during a flare in SLE and may have a role in SLE pathogenesis [19, 186]. BLyS and IFN-α are the target of recently developed biologic therapies which may be beneficial in SLE [291].

The pathogenesis of SLE is complex and multifactorial. Pathogenic processes are likely to occur over a long period and ultimately result in clinically apparent manifestations of SLE.

6.1.3 Clinical manifestations of SLE

SLE can result manifest in several organs as illustrated by the classification criteria (Table1). Disease onset is usually insidious and the clinical course is highly variable with a chronic relapsing and remitting pattern. Most patients will have constitutional symptoms, mild skin, musculoskeletal or serological manifestations and slight derangements in the haematological system. However, a proportion of patients will have more severe disease with renal, pulmonary or neuropsychiatric involvement, requiring aggressive immunosuppressant therapy [358].

6.1.4 Antiphospholipid syndrome

A subgroup of patients with SLE will have the antiphospholipid syndrome (APS). APS is characterised by the presence of antibodies to phospholipids in a moderate to high titre, pregnancy morbidity and thrombotic disease [240]. Commonly measured anti-phospholipid antibodies are anticardiolipin antibody (aCL), β_2-glycoprotein I (β_2GPI) or the lupus anticoagulant (LA). Common thrombotic manifestations are deep vein thrombosis or stroke however a minority of patients, estimated at less than 1%, have catastrophic involvement associated with a high mortality [51]. Management strategies include anti-platelet and anticoagulation as well as avoidance of thrombogenic factors e.g. smoking or hormonal contraceptives [100]. Over a third of patients with APS also have SLE i.e. secondary APS [60] and between 23 – 40% of SLE patients have been shown to have antiphospholipid antibodies [214, 308].

6.1.5 Autoantibodies in SLE

Autoantibody formation is a pivotal feature of SLE. A number of immunological factors are likely to contribute to the formation of autoantibodies as briefly discussed in section 6.1.2.3. All patients with SLE are positive for the antinuclear antibody (ANA) in a significant titre, however the ANA is also positive in a number of other autoimmune conditions as well as in a small proportion of healthy individuals [181, 322].

Antibodies directed double-stranded DNA (anti-dsDNA) are very specific for SLE, in a moderate to high titre [321]. Additionally, a number of antibodies directed against extractable nuclear antigens (ENA) are also found in varying proportions of SLE patients depending on the laboratory methodology used and the population of SLE patients studied and these include anti-Ro/SSA, anti-La/SSB, anti-Sm and anti- UI RNP [271]. Antibody detection is a useful diagnostic tool in SLE although the variability of positivity would suggest that these are not directly pathogenic. Anti-dsDNA titres have been shown to correlate with disease activity in some patients with SLE but not all [307]. Anti-dsDNA have been shown to inversely correlate with the more reliable measure of disease activity, C3 and C4 components, which represent activation of the complement system [237].

6.1.6 Overview of management of SLE

Current therapies for SLE include supportive measures such as smoking cessation and avoidance of sunlight, which is known to cause exacerbations of cutaneous SLE [73]. Pharmacological therapies include the use of non-steroidal anti-inflammatories and anti-malarials for mild musculoskeletal

manifestations and immunosuppressive therapies for organ specific disease, although there is little documented evidence for their use. As such there is variation in the therapies used across continents.

Anti-malarial agents such as hydroxychloroquine and chloroquine phosphate have been shown to be effective for cutaneous and musculoskeletal manifestations [363]. Corticosteroid (CS) therapy has been the mainstay of therapy for the last several decades and likely to have had the largest effect on mortality rates in SLE. CS are often used in conjunction with other immunosuppressive, steroid-sparing agents in moderate to severe disease activity. Azathioprine, a purine analogue, is often used as a first line steroid sparing agent and also has effects in the maintenance of severe disease such as lupus nephritis [4]. Mycophenolate mofetil (MMF) is an inhibitor of purine synthesis commonly used in the setting of renal transplants. MMF has shown to be effective for both the induction and maintenance of lupus nephritis as well for non-renal manifestations of SLE [274]. Cyclophosphamide is currently the mainstay of treatment for severe life-threatening disease and is the standard of therapy for lupus nephritis [327]. Therapy for SLE has moved into a new era over the last decade with the development of targeted biological therapies. Improved understanding of the pathogenesis of SLE has lead to the use of B cell depleting agents. The anti-CD20 drug, rituximab has shown much promise in case series and anecdotal reports however randomized placebo-controlled trials have not shown consistent positive results. Belimumab is an inhibitor of BLyS has been shown to be efficacious in two large phase 3 trials [198]. Targets for anti-cytokine therapy of interest currently include IL-10, IL-6, IL-1 and IFN-α [379].

6.2 SLE and Clinical Coronary Heart Disease

The striking incidence of coronary heart disease (CHD) in SLE was first reported over thirty years ago by Urowitz *et al.* [346]. During a 5-year prospective follow-up of 81 patients with SLE, it was observed that the causes of death appeared to follow a distinctive pattern. Those that died early in the course of the disease had evidence of active SLE, whereas all those that died late in the disease had had recent myocardial infarction [346]. This observation has since been confirmed in several studies. Overall, women with SLE have a 5-6 fold increased risk of CHD compared to women in the general population [223]. In addition to the increased overall risk, patients with SLE are susceptible to CHD at a younger age than would be expected. A retrospective study of acute hospital admissions of patients with SLE revealed that in the 18 - 44 years age group, females with SLE were 8.5 times more likely to be admitted due to acute myocardial infarction (MI), compared with non-SLE female patients [369]. A study of 498 females with SLE at the University of Pittsburgh Medical Centre revealed that between the ages of 35 - 44 years, patients were over 50 times more likely to have an MI compared with females of a similar age in the Framingham Offspring study [223] and a case control study of 53 SLE patients with CHD found more than half of the patients developed the coronary event before the age of 55 years [143]. Observational and retrospective studies point to an undeniable relative excess of clinical CHD in SLE, however, because of the low number of actual events, prospective studies to investigate contributing factors would require long periods of follow up and include large numbers of patients. Several

investigators have therefore used surrogate end-points for clinical CHD to investigate factors that may be related to atherosclerotic progression.

6.3 SLE and Subclinical Atherosclerosis

In addition to clinically apparent disease, a significant proportion of patients appear to have evidence of subclinical disease. Several studies have attempted to quantify the burden of subclinical atherosclerosis in patients with SLE.

6.3.1 Circulating Markers of endothelial function

Several previous studies have attempted to measure circulating biological markers of endothelial function using ELISA. E selectin is an endothelial cell-specific surface marker [96] and be detected following endothelial cell activation by cytokines such as TNF-α or IL-1 [356]. Soluble E selectin has been shown to correlate with CHD [329]. The intercellular adhesion molecules ICAM-1 and VCAM 1 are expressed on the endothelium and promote the adhesion inflammatory cells [248, 273]. They have been demonstrated in pathological specimens of atherosclerotic plaque and found to correlate with CHD [137, 278]. These markers however are not specific to the endothelium. Several previous studies have demonstrated increased levels of E selectin, ICAM-1 and VCAM-1 within SLE and these markers are thought to correlate with both SLEDAI and subclinical disease [94, 301, 343, 361]. The non-specific nature of these markers makes the interpretation of increased levels difficult.

6.3.2 Surrogate measures of arthrosclerosis

Uncontrolled cross sectional studies of patients with SLE have found the prevalence of subclinical atherosclerosis to be between 30 – 40% using a variety of markers of subclinical disease including single photon emission computed tomography dual isotope myocardial perfusion imaging [47], coronary artery calcification (CAC) score [222], ankle brachial pressure index [340] and exercise thallium-201 cardiac scintigraphy [311]. Amongst the most widely used measures of subclinical arthrosclerosis in SLE are carotid IMT, carotid plaque, coronary artery calcification scores, pulse wave velocity and flow medicated dilation. The relative attributes of these measures are summarised in Table 2.

CAC scores are measured using electron beam computed tomography (EBCT) and multidetector computed tomography (MDCT). CAC has been shown to occur more frequently and severely in patients with SLE [14]. The main disadvantages are exposure to radiation and expense. Although this technique has good predictive value for coronary events, to date CAC has failed to show sensitivity to change within interventional studies, most notably with statin therapy [231].

Flow mediated dilation (FMD) is measured using ultrasound. This technique assesses brachial artery endothelial response to increased blood flow following shear stress after a period of ischemia in the distal circulatory bed. FMD has been widely used and despite the wide range of variability between individuals, it has been shown to have good sensitivity to intervention at a group level [150, 191]. Subjects may find application of a tourniquet to

induce ischemia difficult, particularly in the context of Raynaud's phenomenon in SLE.

Carotid IMT/carotid plaque and pulse wave velocity will be used in this study owing to local expertise and availability and are discussed in more detail below.

Table 2 Measures of subclinical atherosclerosis

Attribute	Carotid plaque	CIMT	FMD	APWV	CAC
Predictive value of coronary event	Excellent	Good	Fair	Good	Excellent
Technical difficulty	Low	Low	High	Low	High
Reproducibility	Good	Excellent	Fair	Fair	Fair
Cost	Low	Low	Low	Low	High
Safety / tolerability	Good	Good	Fair (tourniquet application)	Fair (patient exposure)	Poor (radiation exposure)

CIMT - Carotid intima media thickness; FMD - Flow mediated dilation; APWV – Aortic pulse wave velocity; CAC – Coronary artery calcification

6.3.2.1 Aortic pulse wave velocity

Aortic pulse wave velocity (APWV) is a non-invasive measure of aortic stiffness used as a research tool. Left ventricular contraction causes ejection of blood into the ascending aorta resulting in acute dilation and generates a pulse wave that propagates along the arterial tree at a finite speed. The propagation velocity can be used as a measure of the aortic distensibility and stiffness, i.e. increased velocity is a reflection of increased aortic stiffness [256]. APWV has been found to correlate with increasing age, CHD- and stroke-associated mortality in hypertensive patients, patients with end-stage

renal disease as well as healthy subjects, independent of cardiovascular risk factors [34, 228, 318]. In addition, cross-sectional study, suggests APWV appears to correlate with burden of carotid plaque and may provide a marker of subclinical atherosclerosis that precedes carotid plaque formation, although prospective studies are lacking [232]. Recent studies have suggested APWV is increased in SLE compared to control, particularly in a pre-menopausal cohort [78, 380].

6.3.2.2 Carotid ultrasonography

High-resolution B-mode carotid wall ultrasonography allows real-time *in vivo* visualisation of the distance between the lumen-intima interface and media-adventitia interface, interpreted as the carotid intima-media thickness (IMT), as well as detection of carotid plaque. Previous large studies have used IMT to measure all stages of atherosclerosis and found IMT to be an independent predictor of CHD [61, 157] as well as cardiovascular risk factors such as hypertension, hyperlipidaemia and family history of CHD [116, 197, 275].

Cross-sectional studies using carotid ultrasonography have demonstrated the prevalence of carotid plaque to be between 29 - 37% in SLE patients compared with 15 - 22% in controls and the excess of carotid plaque in SLE patients has been shown to be particularly marked in those under 55 years [7, 290].

Four recent short-term longitudinal studies have attempted to estimate the rate of progression of subclinical atherosclerosis and elucidate contributing factors. Roman *et al*. [289] followed 158 patients with SLE over a

mean of 2.8 (±0.75) years. This uncontrolled study demonstrated 28% (10% per year) of patients had progressive carotid plaque, defined as an increase in number or more extensive plaque. Factors related to plaque progression in the multivariable analysis were age, disease duration and baseline homocysteine level. Additional factors noted in the univariate analysis that differed significantly between those with no plaque (which represented 49% of patients) and those with progressive plaque, were less aggressive immunosuppression and lower average prednisolone dose. Thompson *et al.* [341] followed 217 SLE patients over 4.19 (±1.97) years and 104 age-matched controls over 4.97 (±0.5) years. Plaque burden was estimated using the plaque index, a summary score of number and size of plaque. SLE patients had a significantly increased rate of plaque progression compared to the controls (27% vs. 10% over study period) and a similar rate of change of intima media thickness (IMT) (0.011mm ± 0.03 vs. 0.008mm ± 0.01 per year). Eleven (5%) SLE patients demonstrated a reduction in plaque. Factors associated with plaque progression in the univariable analysis were age, smoking status and baseline immunosuppressant use. An additional factor identified in the multivariable analysis was a higher baseline C3 level. Rua-Figeuroa *et al.* [293] conducted an uncontrolled study of 101 patients with SLE over a mean of 2.03 years (range 1.49–2.82) and in contrast to Thompson *et al.* demonstrated a significant mean change in the IMT of 0.078 (0.071) mm over the study period. Factors associated with IMT progression in the multivariable analysis were age at diagnosis, postmenopausal status, homocysteine, C3 and C5a levels. This study did not demonstrate any change in the proportion of patients with plaque over 2 years (19%). Lastly de Leeuw

et al. [82] followed 52 SLE patients over 2.7(±0.58) years and demonstrated an IMT progression of 0.012 ± 0.04 mm/year. Of a number of traditional and novel risk factors assessed, only age was found to be associated with IMT progression.

These studies examined patients with established SLE (>10 years of disease duration) of similar age (38-45 years) with relatively quiescent disease activity. Perhaps unsurprisingly age or age at diagnosis was found to be persistently correlated to atherosclerosis progression across the studies, however, not all analyses adjusted for age which may have aided in disentangling age-related variables e.g. age at diagnosis and disease duration. The progression of carotid plaque was greater in the SLE cohort in the only controlled study published. A general population-based study of women aged between 59 – 71 years demonstrated plaque progressed by 18% over 4 years [388] and by this estimate the progression observed in the SLE population in the Roman study is also greater than would be expected. The study conducted by Rua-Figeuroa did not demonstrate a change in the prevalence of plaque over time and this may indicate that a 2 year follow-up is inadequate to detect changes or may be a reflection of the small sample size. IMT progression was less consistent. Differences observed in the change of IMT over time may relate to the relatively short length of follow up or to differences between scanning techniques or perhaps due to inherent gender or ethnicity differences between the cohorts. Correlation of atherosclerosis to traditional risk factors and SLE-related factors were inconsistent.

It should be noted that these measures of subclinical atherosclerosis require specialist equipment or expertise and are not widely available in

routine clinical practice. Further, it is unclear what proportion of patients with progressive subclinical atherosclerosis will develop clinical coronary events.

6.4 Factors Influencing CHD in SLE

Several factors are thought to contribute, at least in part, to clinical and subclinical CHD in SLE including 'classic' Framingham risk factors, as well as SLE-related factors such as systemic inflammation, therapy such as corticosteroids, renal disease and antiphospholipid antibodies. These factors are discussed below.

6.4.1 Classic risk factors

Several studies have attempted to quantify the burden of cardiovascular risk factors in SLE and to estimate the contribution of these factors to either subclinical or clinical CHD.

6.4.1.1 Prevalence of classic CHD risk factors in SLE

It was noted in the Baltimore lupus cohort that 53% of SLE patients had more than 3 risk factors for CHD [269]. Bruce *et al.* demonstrated in a case control study of SLE patients compared with non-SLE controls without clinical CHD, patients with SLE were more likely to have hypertension, diabetes, dyslipidaemia (raised VLDL and triglycerides) and premature menopause [49]. Recent lipid literature would suggest that individuals at high risk of CHD have persistently elevated levels of an altered or pro-inflammatory (pi) HDL. PiHDL not only results in loss of usual atheroprotective actions of HDL, such as cholesterol transport out of vessel walls through the actions of carrier proteins

including Apo-A1, and prevention of LDL oxidation via the actions of the enzyme paraoxonase, but is also thought to have additional deleterious effects such as to enhance LDL oxidation [351]. A higher proportion of SLE patients were found to have piHDL when compared patients with rheumatoid arthritis or healthy controls and piHDL was also found to be associated with CHD in SLE patients in this study [233]. In addition to overt type 2 diabetes, patients with SLE have also been shown to exhibit insulin resistance and the associated clustering of risk factors as part of a metabolic syndrome. The variation of insulin sensitivity in the healthy population is well recognised as an antecedent to type 2 diabetes and probably plays a role in several stages of the development of CHD [20, 158]. Within SLE, compared with age-matched healthy controls, patients have been shown to have an increased incidence of insulin resistance and the metabolic syndrome [69, 95]. The interaction of corticosteroid and traditional risk factors should be noted. In particular, corticosteroid therapy has been shown to be a major factor influencing dyslipidaemia and is discussed below.

6.4.1.2 Classic risk factors and subclinical atherosclerosis in SLE

Cross-sectional studies measuring carotid plaque and IMT have identified the association of classic risk factors with subclinical atherosclerosis in SLE. Manzi *et al.* demonstrated a mean IMT of 0.71 ± 0.14 mm and focal plaque in 40% of SLE females. Hypertension and age were independently associated with plaque and older age was associated with IMT in the multivariable analysis [224]. In contrast to this study Roman *et al.* demonstrated in a similarly aged cohort, that SLE patients with carotid plaque

(37% of the cohort) were more likely to be older, have a higher systolic blood pressure and higher serum cholesterol in the univariable analysis only. However in the multivariable analysis, age at onset and disease duration were the only significant associations with carotid plaque and the authors concluded that SLE was a risk factor for atherosclerosis, independent of classic risk factors [290]. Common carotid IMT was 0.61 ± 0.016mm in this study and did not differ from controls. Ahmad *et al*. demonstrated the median carotid IMT to be 0.51mm (0.44–0.58) and the prevalence of plaque to be 29% in SLE patients. Consistent with the Roman study, Ahmad *et al*. noted a number of classic risk factors associated with carotid plaque in the univariable analysis but that these factors performed less well in multivariable models for SLE. In contrast traditional risk factors performed well in control subjects with regard to carotid plaque [7].

Overall, the contribution of classic CHD risk factors to subclinical atherosclerosis, measured by carotid plaque or IMT, appears to be less important in SLE patients than in the general population.

6.4.1.3 Classic risk factors and clinical CHD in SLE

Few studies have examined clinical CHD as an outcome in SLE. These are summarised in Table 3. In contrast to studies using subclinical atherosclerotic disease as an outcome, recent studies using clinical events as an outcome would suggest a significant contribution of traditional cardiovascular risk factors in SLE [143]. It may be that risk factors vary in their impact at the various stages of atherosclerosis, e.g. systemic inflammation

may play a role in the development of endothelial dysfunction and traditional risk factors may be important factors for plaque rupture.

Table 3 Summary of studies examining risk factors for clinical cardiovascular events in patients with SLE

Author	Mean (range or ±SD) age at time of event	Cardiovascular outcomes	Classic risk factors	Lupus / other factors
Gladman (1987) [123] (N=45)	48 years (25 - 73)	CHD	Hypertension Congestive heart failure Hypercholesterolaemia Hypertriglyceridaemia Hyperglycaemia Diabetes mellitus	Pericarditis Myocarditis
Manzi (1997) [223] § (N=33)	48 years (22 - 72)	CHD	Hypercholesterolaemia Postmenopausal status	Older age at diagnosis* Longer disease duration* Duration of steroid use
Svenungsson (2001) [333] (N= 26)		CHD Stroke or peripheral vascular disease (PVD)	High VLDL, LDL Lipoprotein a Low HDL	ESR, CRP, Orosomucoid α-1-antitrypsin Lupus anticoagulant Homocysteine Osteoporosis Cumulative steroid dose
Petri (1992) [268] (N=19)		CHD	Hypercholesterolaemia Hypertension	Older age at diagnosis Longer disease duration Duration of steroid use
Bessant(2006) [30] (n=29)		‘survivors’ only Stroke PVD	Hypertension High total cholesterol High triglycerides	Lupus anticoagulant Less hydroxychloroquine use
Freire (2006) [110] (N=10)	43 years	CHD Stroke	Older age	Longer disease duration SLE clinical features not assessed

Ho (2005) [154] (N=42)		CHD Stroke PVD Venous thrombosis	Smoking	Mucocutaneous manifestations Serosal manifestations SDI Systemic lupus activity measure Steroid therapy
Urowitz (2007) [348] (N=118)	51 years (± 12.3)	CHD Stroke PVD	Hypertension Smoking Hypercholesterolaemia Number of traditional risk factors	Raynaud's Renal disease Neuropsychiatric disease Vasculitis Elevated prothrombin time Steroid therapy / immunosuppressives Less Anti-malarials
Haque (2010) [143] (N=53)	53 years (± 10)	CHD	Male gender Hypercholesterolaemia Hypertension	SDI Azathioprine therapy Steroid therapy

*Significant variables after controlling for age
§ SLE cases vs. non-SLE controls
N = number of cases with events

Adapted from Haque et al. [143]

6.4.1.4 Risk of CHD associated with classic risk factors

The excess of traditional risk factors in some patients with SLE must, at least in part, explain the clinical picture observed. However the literature also suggests that they do not fully explain the prevalence of CHD observed. Esdaile *at al.* examined 296 patients with SLE in Canada at entry to an observational cohort. They found that over an 8.6 year follow-up period, 34 patients developed a cardiovascular event (defined as CHD, cardiac failure or stroke). When retrospectively estimating the expected risk using the Framingham model, it was demonstrated that the rate of clinical events was greater than expected. After controlling for common risk factors at baseline, patients with SLE were 10 times more likely to have a non-fatal MI and 17 times more likely to die from CHD [99]. Similarly, a case control study of 26 patients with SLE compared with age-matched non-SLE patients with premature CHD found that SLE patients had, on average, one less classic risk factor compared to the population controls [280]. A case control study from the UK-based General Practice Research Database (GPRD) compared 8,688 patients with myocardial infarction with 33,923 controls and demonstrated an odds ratio of MI in the SLE patients, after adjusting for all traditional risk factors, aspirin and non-steroidal anti-inflammatory use, was 2.67 (1.34–5.34), again suggesting that factors other than classic risks are contributing to CHD in SLE [107].

The precise contribution of classic risk factors remains unclear. A randomised placebo controlled trial of atorvastatin (40mg daily) in 200 SLE patients did not show any benefit of the statin using coronary artery calcification as the primary end-point or carotid IMT and plaque as secondary end-points [270]. A number of factors may account for the negative result and include the

relatively short follow up period of 2 years, use of 40mg of atorvastatin rather than 80mg and importantly the use of a primary end-point that is likely to be a relatively fixed, advanced stage of atherosclerosis. Similar results were attained in a small randomised controlled trial using rosuvastatin (10mg daily) that demonstrated no difference in carotid IMT at 2 years between the statin and placebo group [241]. In contrast, a small study has demonstrated an improvement of flow mediated dilation in SLE over a 8 week period with the use of atorvastatin (20mg) [106]. Parallels can be drawn with the use of statins in chronic kidney disease, where studies have shown inconsistent results. Two large clinical trial have suggested there is no reduction in cardiovascular events with lipid lowering agents, however the initial results of the SHARP study, a large randomised placebo controlled trial, has shown a reduction in all-cause cardiovascular events but not in MI [105, 313, 368].

Given the diversity of pathologies observed in SLE i.e. thrombosis, immunologically mediated processes, neoplastic disease and iatrogenic effects related to potent therapies e.g. steroids and cytotoxic drugs, perhaps it is not surprising that traditional risk factors do not fully explain the CHD risk.

6.4.2 SLE-related risk factors and CHD

6.4.2.1 Role of corticosteroids

The adverse effect of corticosteroids (CS) on lipid profiles, hyperglycaemia and hypertension status is well recognised. The correlation of previous steroid use and cardiovascular morbidity in SLE has been documented in several studies [223, 268]. Post-mortem examinations of 36 SLE patients with prior CS therapy were carried out by Bulkley and Roberts and the findings were

compared with the results of autopsies of 20 SLE subjects undertaken in 1941 (pre-steroid era). Age and presence of renal disease (present in approximately 80%) were comparable between the two groups. Sixty-four percent of CS-treated patients had left ventricular hypertrophy (hypertension had been present in 69%) compared with 15% of patients from the pre-steroid era [52]. This study also appears to indicate that the duration of exposure of CS is relevant. Those that had steroids for longer than 12 months were more likely to have coronary artery narrowing. Furthermore, a case control study of 46 female patients with SLE compared with 30 matched healthy controls, found that patients treated with CS had higher triglyceride and total cholesterol levels. SLE patients not taking CS had a lipid profile similar to that of non-SLE controls with the exception of lower levels of HDL in this small study [101]. Bruce *et al.* demonstrated that the cumulative dose of CS correlates with sustained hypercholesterolemia in SLE patients with early disease [48]. A higher average daily dose of CS has been implicated in causing dyslipidaemia in other studies [205], however, steroid treatment may not adversely affect the risk of dyslipidaemia across the whole dose range [217]. Roman *et al.* using carotid plaque measurement as an outcome found that patients with plaque were exposed to a lower average daily dose of CS over the previous 5 years than those without plaque, suggesting that incomplete suppression of inflammation might be a factor and optimal doses of CS might have a beneficial effect on atherosclerosis. It is possible that CS could represent a marker for another confounding factor, such as disease activity (i.e. inflammation), severity or duration. The importance of inflammation in the pathogenesis of atherosclerosis is discussed in section 6.4.2.4. The impact on the risk of CHD of

the various biological effects of CS is difficult to assess. In addition, accurate documentation of the amount of CS exposure is complex, as they are frequently prescribed by different practitioners in both primary and secondary care, as well as being self-administered by patients. Direct comparisons between studies is often not possible as various measures of exposure have been used in different studies e.g. cumulative dose, average daily dose, maximum dose ever or length of exposure. All these factors should be taken into account when considering the effect of CS on the risk of CHD in SLE.

6.4.2.2 Renal disease

The prevalence of renal disease in SLE is estimated at 22 - 41% [59, 165]. The high incidence of cardiovascular morbidity and mortality associated with chronic kidney disease and proteinuria is well documented in the general population [125, 300]. However, within SLE, studies have not shown a consistent association [222, 290]. Notably, nephrotic range proteinuria has been shown to be a major risk factor for carotid atherosclerosis in juvenile-onset SLE and renal disease has also been found to be predictive carotid atherosclerosis in a small prospective observational study [92, 104]. Difficulties arise when making comparisons between studies as measures of renal function vary e.g. serum creatinine level, creatinine clearance and proteinuria. Invariably the therapy profile of patients with renal disease will also differ from non-renal SLE patients, therefore introducing potential confounding variables.

6.4.2.3 Antiphospholipid antibodies

The prevalence of antiphospholipid antibodies in SLE is estimated at 30 - 40% in different studies [145, 214]. The prothrombotic nature of these

antibodies is well documented in the context of APS. Serum lipoproteins contain phospholipids and modified LDL and therefore it is conceivable that they may be a target for antiphospholipid antibodies. Crossreactivity between anticardiolipin antibody (aCL) and antibodies to oxidized LDL has been reported [349]. The possible association between antiphospholipid antibodies and oxidized LDL is interesting, however the exact nature and function of these antibodies is not clear. The key antigenic target for antiphospholipid antibody is β_2-glycoprotein I. *In vitro* studies have shown that β_2-glycoprotein I inhibits uptake of oxidized LDL by macrophage scavenger receptors but that in the presence of antibodies to β_2-glycoprotein I (β_2GPI), uptake of oxidized LDL is increased, suggesting a pro-atherogenic role of antiphospholipid antibodies [146]. Furthermore β_2GPI have been shown to immunolocalise to atherosclerotic plaque and interact with oxLDL [120, 188]. These processes would suggest a role for aCL in both the initiation and instability of atherosclerotic plaque. Autoantibodies directed at the oxLDL/β_2GPI complex have been demonstrated in several groups at high risk of CHD and more recently been demonstrated to predict a poor outcome in patients with the acute coronary syndrome [131]. Recent research has focused on the interaction between aCL and annexin A5. Annexin A5 is a calcium and phospholipid-binding protein that interacts with cellular membranes and is thought to have plaque-stabilising properties. Plasma from SLE patients has been demonstrated to result in reduced binding of annexin A5 to endothelium [58] and a recent *in vitro* study has demonstrated a dose-dependent reduction of annexin A5 binding to human umbilical venous endothelial cells (HUVECS) by monoclonal antibody to anticardiolipin which was abrogated by preincubation of HUVECS

with neutralising pooled intravenous immunoglobulins. The investigators suggest aCL directly interfere with annexin A5 binding to endothelium and may contribute to plaque instability [113].

Animal studies using LDL receptor-deficient mice have demonstrated increased atherosclerotic lesions following immunisation with aCL and β_2GPI (reviewed in [314]). Within human SLE studies, the association of aCL and athrosclerosis is not clear. aCL have been shown to be associated with carotid plaque in patients with SLE [7, 7]. In contrast, another study demonstrated aCL were less likely in SLE patients with plaque compared to SLE patients without plaque [290]. It is unclear if antiphospholipid antibodies may represent a surrogate marker for an underlying affinity for atherosclerosis and further research is needed to establish whether they are directly pathogenic. It is likely that certain subtypes of aCL represent a proatherogenic process, whereas others maybe atheroprotective [253].

6.4.2.4 Atherosclerosis and the immune system

Atherosclerosis was previously considered as a disorder of lipid storage however it has become clear over the last decade that the innate immune system and in particular, inflammation play a key role.

6.4.2.4.1 Innate immunity and atherosclerosis

The innate immune system comprises the non-specific defence processes of the body. These defences include barriers such as the skin and mucosal surfaces, the inflammatory response, pathogen recognition via toll-like receptors (TLR), immune cell recruitment and signalling via cytokines, and complement activation.

The stages of atherosclerosis are now well recognised and schematically described in Figure 1. The initial step in the process is thought to be endothelial cell activation and upregulation of adhesion molecules and chemokines. Recruitment and activation of immune cells such as monocytes and T helper cells are pivotal processes in atherogenesis and likely to be mediated by the local release of chemotactic cytokines such monocyte chemotactic protein-1 (MCP-1), regulated upon activation normal T cell expressed and secreted (RANTES), interferon inducible protein (IP)-10 and interferon-inducible T cell alpha chemoattractant (I-TAC) [42, 136, 218, 354]. Uptake of oxLDL by macrophage scavenger receptors in the subendothelial layer results in the formation of foam cells. The earliest detectable lesion in the formation of atheroma is the fatty streak which is rich in inflammatory cells. This lesion is thought to be dynamic with the potential to regress [212]. The atheromatous lesion later in the developmental stages of CHD consists of a core of foam cells and lipid droplets with a surrounding cap of smooth muscle cells and a collagen-rich matrix. The lesion also contains T cells, macrophages and mast cells which exhibit signs of immune activation and produce pro-inflammatory cytokines. An atheromatous lesion can be relatively stable for many years with the potential for thrombus formation and plaque rupture, causing acute coronary syndromes. Activation of immune cells and release of inflammatory cytokines and proteases are thought to contribute to plaque instability and rupture [142]. Inflammation is therefore thought to contribute to initiation, formation and rupture of atheromatous plaque.

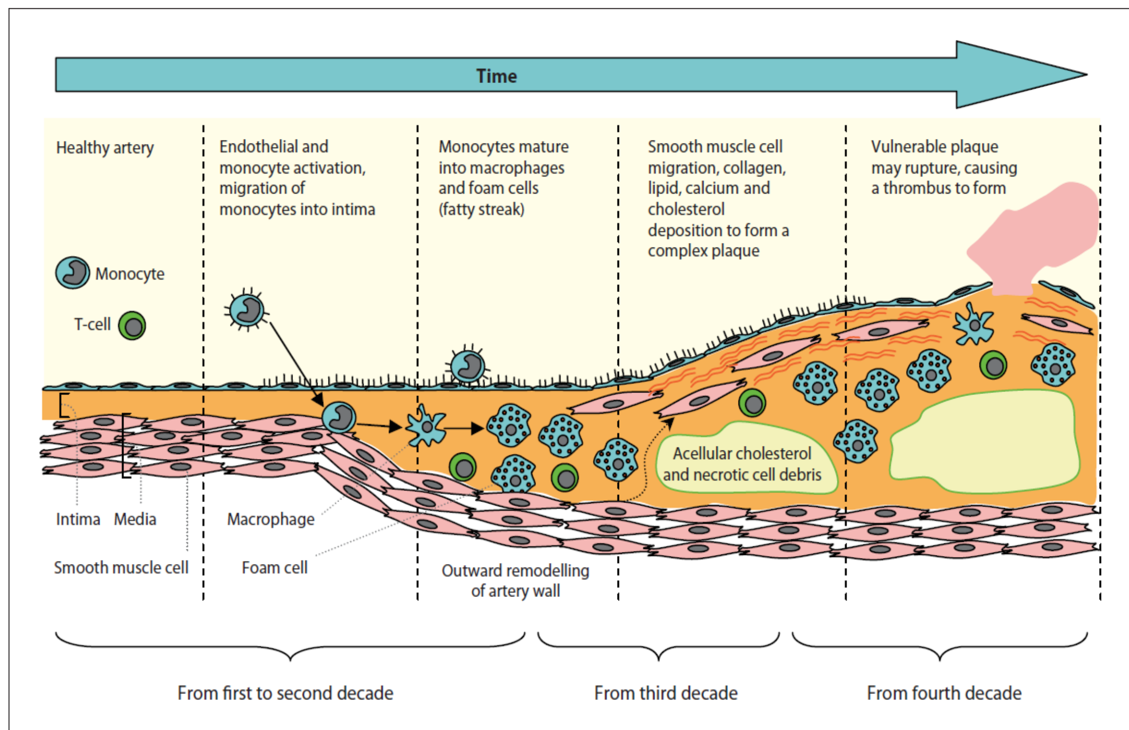


Figure 1 Developmental stages of atherosclerotic plaque. ***Adapted from C Erridge, Journal of Innate Immunity 2009 [97]***

The importance of immune cells in atherosclerosis has become well established, however, more recently another component of the innate immune system, toll-like receptors (TLR), have been shown to have an important role. TLR consist of 10 transmembrane receptors that are involved in recognition and defence against microbial infections. These receptors recognise and engage microbial motifs termed pathogen-associated molecular pathogens (PAMP), such as single-stranded RNA motifs associated with viral infections, resulting in the initiation of inflammatory signalling [182]. Some or all of the 10 subtypes of TLR receptors are expressed on most immune cells and often result in upregulation of expression of inflammatory genes such as endothelial adhesion molecules and chemokines. In particular macrophages express all 10 types. A number of TLR have also been shown to be expressed on arterial endothelial

and smooth muscle cells [98, 378]. Mouse models would suggest a central role for TLR in atherosclerosis. Deletion of specific TLR or their signalling pathways in the atherosclerosis prone ApoE$^{-/-}$ and LDLR$^{-/-}$ mice models have demonstrated a significant reduction in atherosclerotic burden. Furthermore, infusion of TLR ligands has been shown to accelerate atherosclerosis in mice [33, 219, 236, 247]. TLR may also contribute to uptake of oxLDL by macrophages to form foam cells [21, 55]. The exact physiological link between TLR signalling and various stages of atherosclerosis remains a subject of much interest.

The complement system consists of cascade fashion activation of several proteins down 3 separate pathways (classical, mannose binding lectin and alternative) that ultimately collaboratively lead to cleaving of C3 to form C3a and C3b. These fragments subsequently participate in processes involved in the removal of unwanted material from the immune system. The complement system is a key player within the innate immune system and also interacts with the adaptive immune system [364, 365]. Complement components have been shown to be involved in endothelial cell activation and immune cell recruitment via upregulation of MCP-1, IL-6 and VCAM-1 [357]. Complement activation cannot be detected in intact arteries however several *in vitro* studies have been able to demonstrate complement activation in atherosclerotic plaque and in particular within vulnerable plaque [193, 259, 310]. Several mouse studies have also contributed to elucidating the role of complement in atherosclerosis. Controlled studies using atherosclerosis prone LDLr$^{-/-}$ and ApoE$^{-/-}$ knockout mice have demonstrated larger plaque and accelerated atherosclerosis in the context of C3 deficiency or CD59 (complement regulatory protein) deficiency

[53, 266, 377, 384]. C1q has a role in activating the classical pathway and is involved in clearance of apoptotic cells. $C1q^{-/-}$/ $LDLr^{-/-}$ mice have been shown to have accelerated formation of early atherosclerotic lesions containing more apoptotic cells compared to control mice [31, 338]. Of interest, studies have shown high serum levels of C3 to be associated with traditional risk factors and also predictive of MI independent of traditional risk factors [249, 250].

Inadequate clearance of cell debris by the complement system has been implicated in the pathogenesis of SLE and SLE-like syndromes are well recognised in the context of congenital deficiencies of C1q and C4 [272]. The relationship between the complement system and SLE, particularly in the context of atherosclerosis, requires further clarification.

6.4.2.4.2 Inflammatory biomarkers and atherosclerosis

Evidence of immune activation has been demonstrated in the peripheral circulation of patients in the general population with acute coronary syndromes. A small case control study examining patients with stable and unstable angina found an association of clonally expanded T cells with unstable atherosclerotic plaque. Similar T cell receptor sequences were detected in different subjects in this study, implying that chronic antigenic stimulation by a common antigen, possibly triggered by inflammation or infection, contributes to plaque instability and rupture [211]. Vulnerable unstable plaques have been shown to occur more commonly in patients with inflammatory conditions than in controls [16].

Several prospective studies have demonstrated raised baseline levels of inflammatory markers in subjects that have subsequently developed clinical coronary events such as IL-6, CD40L, macrophage inhibitory cytokine-1 and TNF-α [46, 285, 286, 305]. The most widely researched inflammatory biomarker

for CHD to date has been the C-reactive protein (CRP). Several large prospective studies have demonstrated that baseline CRP, assessed using a high sensitivity (hs) assay measuring low levels of inflammation, generally considered to be less than 10mg/l, is predictive of future CHD in both men and women [277, 284, 287]. Moreover, it would appear that hsCRP level may reflect the risk of CHD more accurately than the Framingham risk score [287]. A prospective study of 25,000 healthy women followed over a 10 year period demonstrated that a risk prediction model incorporating the hsCRP and history of premature parental CHD event allowed more accurate prediction of CHD events than the Framingham risk prediction model, in those individuals classified in the 'intermediate risk' category [283]. This observation lead to the JUPITER trial (Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin); a randomised controlled study. The recently reported findings demonstrated that statin therapy significantly reduced coronary events in patients with a normal LDL (<3.4mmol/l) and raised hsCRP (>2mg/l) compared with a placebo drug [288].

Whilst biomarkers such as the CRP are classically not particularly raised in SLE, one recent study has demonstrated a CRP level greater than 9 mg/l is predictive of atherosclerotic damage in SLE defined as the development of angina, MI, heart failure or coronary intervention [276].

6.4.2.4.3 Inflammation, atherosclerosis and SLE

In addition to the wealth of evidence demonstrating subclinical atherosclerosis, autopsy studies have also demonstrated the presence of the 'usual' type of coronary atherosclerotic plaque in SLE patients. Accordingly, the majority of coronary events have been demonstrated to be the result of

atheromatous plaque and not coronary vasculitis [115, 139]. The role of inflammation in atherosclerosis is particularly interesting as systemic inflammation is the hallmark of SLE. Inflammatory processes have been shown to contribute at all stages of the atherosclerosis process, including the initiation with endothelial cell damage. It is increasingly recognised that maintenance of endothelial integrity is crucial to prevent atherosclerosis.

6.5 Endothelial cells

6.5.1 Vascular damage and endothelial dysfunction

The vascular endothelium comprises a single layer of endothelial cells with essential anti thrombotic and barrier functions as well as a role in the regulation of vascular tone. Endothelial dysfunction (ED) can be characterised as a deviation from normal function to a vasoconstrictive, procoagulant, platelet-activating and anti-fibrinolytic state. ED is thought to have a key role in atherosclerosis and related diseases and found to be present in all stages of atherosclerosis [142]. Mechanisms by which vascular damage is repaired are not well understood. Several recent studies have suggested that two groups of endothelial cells (EC) can be detected in the peripheral circulation of individuals with vascular damage. A population of endothelial progenitor cells (EPCs) are thought to be bone marrow-derived and involved in the repair of the endothelium. A population of 'inflammatory' or 'activated' endothelial cells (IECs) are thought to have been shed from the endothelium following an insult [159]. In addition to IEC, endothelial microparticles (EM), thought to be shed from damaged endothelial cells, with a potential role in cell signalling, have also been identified from the serum of individuals with acute coronary syndromes [26, 27, 221]. It is believed that damage to endothelial cells can result in endothelial dysfunction and this is thought to be critical in the formation of atheroma [79, 142]. Understanding endothelial repair mechanism is therefore very important.

6.5.2 Characterisation of Endothelial progenitor cells

Endothelial progenitor cells were first reported by Asahara *et al.* as $CD34^+$ mononuclear cells with the ability to form island-like colonies in culture in the late 1990s [12]. These bone marrow derived cells were shown in a mouse model, under the influence of vascular endothelial growth factor (VEGF), to have the ability to incorporate into blood vessels with experimentally induced ischaemia [13]. These landmark experiments have given an important insight into the biology of vascular repair.

6.5.3 Methods of EPC quantification

Two main methods have been utilised to identify EPC. The first aims to identify cells with specific surface markers for EPC using flow cytometry and the second attempts to identify *in vitro* cell culture characteristics.

6.5.3.1 Flow cytometry identification of EPC

Flow cytometry allows the rapid analysis of multiple characteristics of a population of cells and has been used since the 1950s [74]. Cells labelled with fluorescently-conjugated antibodies are injected in suspension into a fine nozzle, encouraged to flow in single file and illuminated by a laser beam. The direction and intensity of light scattered from an individual cell along with emitted fluorescence is converted into a numerical value and plotted on a histogram. For a given cell, information is recorded relating to light scatter in a forward direction (forward scatter), side scatter and wavelength of fluorescence emitted. Several groups have used fluorescently-conjugated antibodies to specific cell surface markers for analysis with flow cytometry to identify EPC. Flow cytometry has several advantages including good sensitivity, specificity

and reproducibility [183]. The choice of surface markers that correctly identify EPC is the subject of much debate and discussed below.

6.5.3.2 Surface markers to identify EPC

A variety of surface markers to identify EPC have been used by different groups. The majority of studies using flow cytometry have used at least one surface marker to identify a cell of immaturity and at least one marker to identify a cell from the endothelial cell lineage.

The most widely used marker to identify immaturity is CD34 positivity. CD34 is an 110kD surface marker expressed on haematopoietic stem cells, although the exact origin and signals for differentiation of these cells remain unclear. CD34 can be detected on approximately 0.1% of circulating mononuclear cells and thought to act as an adhesion molecule related to endothelial and haematopoietic cells [64]. In addition to CD34, other studies have demonstrated $CD14^+$ (a surface marker found on monocyte populations) cells can also form similar colonies *in vitro*. It is possible both these cell types originate from a common precursor, the 'haemangioblast' [119]. More recently $CD14^+$ cells have been shown not to have an association with CHD or related risk factors [162]. CD133 is a 120kD glycoprotein expressed on progenitor and haematopoietic stem cells [381]. Although the exact function is not clearly understood they are thought to identify an immature population of EPC. Piechev *et al.* demonstrated a subpopulation of $CD34^+$ / $VEGFR2^+$ cells that are also positive for CD133. However CD133 was no longer expressed on mature $CD34^+$ / $VEGFR2^+$ in cells *in vitro* leading the authors to conclude that $CD34^+$/ $CD133^-$/ $VEGFR2^+$ represent a more mature differentiated population of endothelial cells [263]. Furthermore, Friedrich *et al* isolated $CD34^-$/ $CD133^+$/

VEGFR2^{+} and demonstrated that when cultured, these cells upregulate the surface expression of CD34 suggesting that CD34^{-}/ CD133^{+}/ VEGFR2^{+} are precursors of CD34^{+}/ CD133^{+}/VEGFR2^{+} EPC [112]. It is possible that CD34^{+}/VEGFR2^{+} and CD133^{+}/VEGFR2^{+} may represent different subtypes of EPC and CD34^{+}/ CD133^{+}/VEGFR2^{+} may identify a restricted EPC phenotype. One major disadvantage is that these triple labelled cells represent a very small, and therefore difficult to quantify, population of cells. Despite this limitation combinations of these markers have been repeatedly demonstrated to be good biomarkers for disease as summarised in

Table 44.

Table 4 Endothelial progenitor cell surface markers

Experimental model/ tissue type	Cell surface markers	Reference
Circulating PBMCs	CD133^{+}	[119]
Circulating PBMCs	CD34^{+}/ CD133^{+}/ VEGFR2^{+}	[263]
Cord blood	CD34^{+} /CD11b^{+}	[151]
Circulating PBMCs – markers induced in culture from CD133^{-} cells	CD34^{+}/ CD133^{+}/ VEGFR2^{+}	[331]
Circulating PBMCs	CD34^{-}/ CD133^{+} /VEGFR2^{+} (?precursor) & CD34^{+}/ CD133^{+}/ VEGFR2^{+}	[112]
Circulating PBMCs	CD34+KDR+	[303]

It is widely accepted that EPC express vascular endothelial growth factor receptor-2 (VEGFR2 or KDR in humans). The roles of the VEGF receptor family in angiogenesis and vasculogenesis are well recognised [292]. Other endothelial antigens include CD31 and von Willebrand factor. CD31 or platelet endothelial cell adhesion molecule-1(PECAM-1) is known to have roles in

angiogenesis, platelet function, thrombosis and regulation of leukocyte migration through vascular walls [375]. Von Willebrand factor has a role in platelet adhesion to the vascular wall in injury and is a carrier protein of coagulation factor VIII [86]. In addition, the vessel wall itself may be a source of progenitor cells [167].

Another important factor to consider is the units in which cell populations are expressed. Some studies have favoured expression of cells as a count per unit of volume of blood. EPC are estimated to represent between 0.0001% and 0.01% of peripheral cells, making them a ‘rare event’ in flow cytometric analysis. Given the rarity of these events in peripheral blood and the variations in haemodilution between individuals, particularly in patients with cardiovascular disease, this measure can be misleading and likely to be inappropriate. A more suitable measure of cells identified using flow cytometry would be cell number or proportion of total cytometry events.

Identification and quantification of EPC using flow cytometry can be challenging but despite this several studies have reported associations with disease states. Therefore, EPC quantification using surface marker identification remains a relatively widely used technique.

6.5.3.3 Cell culture characteristics of EPC

Flow cytometry allows enumeration of EPC, a population occurring in peripheral blood in low frequency, using a limited number of surface markers. Cell culture has the advantage of allowing expansion of cell numbers and enabling qualitative characteristics to be gathered. In the landmark publication described above, Asahara *et al.* described EPC as cells *in vitro* with the ability to adhere to fibronectin and form colonies (CFU - colony forming units) as early

as following 3 days in culture. Cells in culture were shown to have typical endothelial cell characteristics such as uptake of DiI-labeled acetylated low density lipoprotein (Dil-aCLDL) and expression of surface markers including CD31, KDR, CD34 and Tie-2 [12]. This initial study used CD34+ and KDR+ enriched cells in culture; however several investigators have subsequently utilised unfractionated mononuclear cells and included a pre-plating stage to increase specificity and exclude mature endothelial cells. This modified protocol identifies CFU between 4 – 9 days after culture termed 'early-outgrowth' colonies or CFU-Hill [152] (see Figure 2).

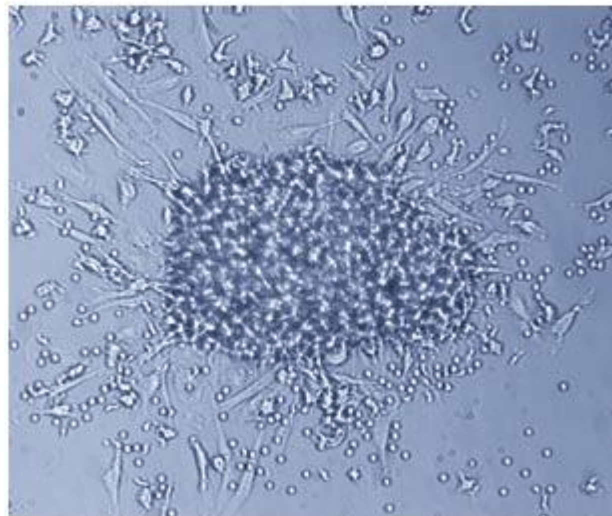


Figure 2 Endothelial cell colony forming unit [152]

Another commonly used protocol identifies 'late outgrowth' colonies derived from adherent mononuclear cells following 7 – 21 days in culture [210] (see Figure 3).

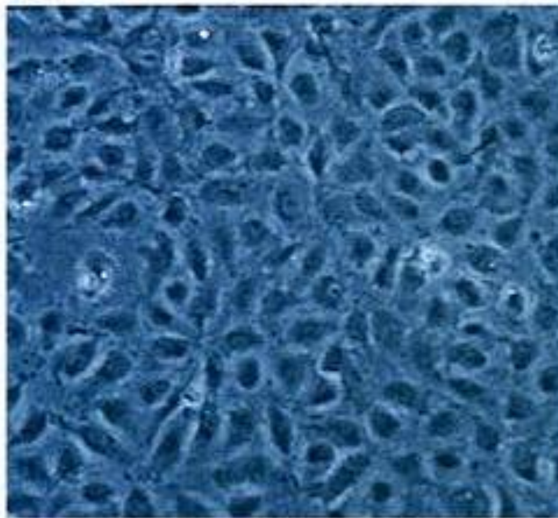


Figure 3 'Late outgrowth' CFU with typical cobbled appearance [210]

Early and late outgrowth cells share a number of features including uptake of Dil-aCLDL and expression of CD31 but differ with regards to surface expression of CD133. They are likely to represent EPC at differing stages of maturation. It should be noted that EPC identified using cell culture are unlikely to represent the same cells identified using flow cytometry and the precise relevance to EPC in the *in* vivo situation remains unclear. Nonetheless, several groups have successfully utilised CFU number as a biomarker in several different disease states including CHD-related diseases and inflammatory rheumatic diseases.

6.5.4 Role of EPC in vascular regeneration

Prior to experiments conducted by Asahara et al in 1997, it was largely believed that 'vasculogenesis' or the *de novo* formation of vessels from uncommitted precursor cells occurred only in the developing foetus. It has since been demonstrated in several animal models that stem cell transfusion and systemic transfusion of expanded *ex vivo* EPC can induce functionally intact endothelial regeneration [174, 190, 316]. EPC induced vasculogenesis is

yet to be demonstrated in human models. However within the setting of CHD, re-endothelialisation of 'EPC-capturing' anti-CD34 coated coronary stents has been shown to have a beneficial effect in terms of stent re-stenosis [8]. In addition, fascinatingly, a recent case report has described treatment of critical limb ischemia with the injection of autologous peripheral blood $CD133^+$ purified stem cells into the gastrocnemius muscle without any side effect resulting in limb salvage and symptomatic relief sustained at 17 months of follow-up [54]. Further examination of the role of EPC in human vasculogenesis and factors influencing this process is required.

6.5.5 EPCs and CHD risk factors

Few studies have quantified EPC using flow cytometry in the context of cardiovascular risk factors. Vasa *et al*. demonstrated a significant reduction of CD34/KDR+ EPC in patients with CHD compared to healthy controls but also found a correlation of these cells to smoking and family history of CHD across all participants [352]. CFU-Hill formation has been the most widely used measure of EPC number and function in association with CHD risk factors. Hill *et al.* measured CFU number in 45 men with a mean (±SE) age of 50 (±2) years and demonstrated a negative correlation with the Framingham risk score (R^2– 0.47, P=0.001). In addition, CFU were reported to be a better predictor of vascular reactivity (measured using flow-mediated brachial-artery reactivity) than traditional CHD risk factors [152]. Increasing age, total cholesterol, triglycerides, low HDL, smoking and diabetes have also been shown to correlate with a reduction in EPC number in culture [63, 134, 189, 264, 339]. A

recent report has demonstrated CD34/KDR+ cells and CFU have a negative correlation with the metabolic syndrome [171]. Importantly there have been reports of enhancement of EPC number with pharmacological agents and these studied are discussed briefly below.

6.5.6 EPCs and CHD disease

Werner e*t al.* recently reported findings of a study examining 519 patients with angiographically documented CHD. Independent of cardiovascular risk factors including age and gender, increased $CD34^+/VEGFR2^+$ EPC level was associated with better outcome over a one year follow-up period. Patients with a higher EPC level had a reduced risk of a major cardiovascular event and death from a cardiovascular event [372]. Schmidt-Lucke *et al.* compared 44 patients with stable CHD (no acute coronary syndrome for at least 3 months) with 33 patients with unstable angina and 43 healthy (not matched for age) controls. They demonstrated a reduction in $CD34^+/VEGFR2^+$ EPC number in patients with CHD compared with healthy controls and a negative correlation, independent of risk factors, between EPC number and cardiovascular events [303]. Shaffer *et al.* reported, in a small study, a reduction in $CD133^+$, $CD34^+$ and $CD133^+$ /$CD34^+$ cells in elderly patients with and without peripheral vascular disease compared to healthy younger adults [312]. Moreover, Fadini *et al.*, demonstrated $CD34^+/VEGFR2^+$ EPC level has a negative correlation with carotid IMT (intima-media thickness) in healthy individuals, independent of risk factors [103]. In addition, EPCs levels have been shown to rise in the first few days following acute myocardial infarction and peak at 7 days post event [226, 315] which may suggest that

EPCs are mobilised as a repair mechanism in response to an acute stress. Therefore any study quantifying EPC in disease states should note the occurrence of recent coronary events to avoid skewing of results.

Overall, several studies to date suggest that EPC number is independently associated with cardiovascular outcome and could be a clinically useful biomarker. Further study is required to establish optimal surface markers for the identification of EPC. Some studies have defined EPC as $CD34^+/VEGFR2^+$ cells, others as $CD34^+/ CD133^+/ VEGFR2^+$and some have described an 'early' population of cells as $CD34^-/ CD133^+/ VEGFR2^+$. CFU number provides a robust and uniform method of detecting EPC and has been shown to be a good biomarker in CHD and related risk factors.

6.5.7 Other factors influencing EPC number and function

Endogenous factors that influence the number and function of EPC remain unclear and of much interest. However, in addition to the pathological processes above, several other exposures have been shown to influence EPC levels.

6.5.7.1 Lifestyle modification

The influence of tobacco smoking on EPC level is interesting. Chronic smoking as discussed above results in a reduction of EPC. However low concentrations of nicotine have been associated with increased EPC levels [367], perhaps representing a physiological response to a low grade insult. Importantly, smoking cessation has been shown to result in a rise in EPC within 4 weeks [189]. In addition, physical activity has been shown to increase EPC in both healthy individuals and patients with CHD [200, 299].

6.5.7.2 Pharmacological agents

HMG-CoA reductase inhibitors have been shown to increase the number of circulating EPC in a mouse model and this might be another mechanism by which statin medications exert a cardioprotective effect [90, 352]. Angiotensin converting enzyme (ACE) inhibiting agents and angiotensin 2 receptor blockers have also been shown to have a positive effect on EPC numbers [166, 238] and this has also been demonstrated within a diabetic population [207]. Chemokines and cytokines such as erythropoietin, GM-CSF and VEGF have also been shown to increase EPC levels [17, 121, 334]. These reports suggest that treatment of CHD risk factors can influence EPC number.

6.5.8 EPCs and SLE

Over the last 4 years since the commencement of this study, other groups have reported on EPC using flow cytometry in SLE. As described above these groups have used a combination of CD34, CD133 and KDR to identify EPC.

Westerweel *et al.* studied 15 females with SLE aged 37 (3) years with inactive disease for at least a year prior to the study and taking low doses of prednisolone (<10mg) compared to 15 age matched healthy females aged 37 (3) years. They demonstrated a reduction of the overall CD34 fraction of cells described as haematopoietic stem cells as well as a reduction of CD34+KDR+ EPCs in SLE patients compared with healthy controls. EPC number correlated positively with ankle brachial pressure index (R^2=0.532, p=0.042) and inversely with cholesterol level (R^2=-0.587, p=0.021). There was no association with disease related factors including disease activity, therapy or serological

findings. CFU-Hill formation was not different between the two groups and the influence of CHD risk factors was not assessed [373].

Denny *et al.* defined EPC as CD34/CD133+ cells in SLE compared to controls. The overall cohort consisted of 135 SLE patients aged 38 (3) years and 60 controls aged 41 (1) years. It should be noted 55% of the controls were females compared with 95% of SLE patients. The proportion of participants included in subgroups of the study is not reported. They found a reduction of EPC in SLE compared to control which remained statistically significant when limiting the analysis to females only. In addition they documented a correlation of EPC with the SLEDAI (R^2=0.42, p = <0.01). Other disease related factors and CHD risk factors were not correlated. Interestingly this study demonstrated SLE EPC have increased Interferon-α (IFN-α) expression in culture compared with controls. Denny *et al.* propose the increased levels of IFN-α in SLE EPC suggest IFN- α may potentiate abnormal vascular repair in SLE [88].

Moonen *et al. I* studied CD34/CD133+ EPC in 44 SLE patients aged 40 ± 12 years and 35 age-matched female controls aged 41 ± 12 years. They demonstrated a reduction of CD34/CD133+ EPC and CFU-Hill in SLE compared to controls. No correlation was noted between EPC or CFU number with regard to disease-related factors. CHD-related factors were not analysed [242].

All these studies consistently noted a reduction of EPC in SLE but varied with regards to clinical correlates. The populations studied were of a similar age. Possible explanations for the discrepancy of the findings between different studies may be related to small sample sizes or due to the differences within the study population such as ethnicity, therapies, disease activity or differences in

disease phenotype. More recently other groups have been unable to demonstrate a difference in EPC as measured by flow cytometry and therefore focussed on CFU formation.

Grisar *et al*. reported on 31 SLE females aged 35 (± 2) years compared 14 healthy female controls aged 39 (± 5) years. They found no difference in CD34/CD133/KDR+ cells between SLE and controls. EPC did appear to correlate with previous renal disease but not with other clinical parameters. They also reported on CFU number in 13 SLE patients and 12 controls and found CFU number was similar between the 2 groups. However, they reported a reduction in migratory and adhesive properties of cells in SLE patients [135].

Ablin *et al*. reported no significant difference in CFU number of 28 SLE patients aged 38 (15) years and 50 controls aged 43 (18) years [3]. SLE patients included males and patients with a prior history of high dose steroid, cyclophosphamide or MMF use were excluded suggesting the group studied were of a 'milder' phenotype. Ablin and colleagues did note an impaired adhesion of cells to fibronectin in SLE patients which was interpreted as a marker of impaired function. CFU number or adhesion scores did not vary with disease activity.

Lee *et al.* enumerated CFU-Hill from 70 SLE patients aged 35 (27–45) and 31 control aged 36 (26.5–44) years. Ninety-two percent of SLE patients were female compared to 84% of the controls. They demonstrated a significantly reduced number of CFU in SLE compared to controls which was independent of leucopoenia. They also quantified CD34/KDR+ cells using flow cytometry and also demonstrated a reduction in SLE compared to controls. Neither CD34/KDR+ cells nor CFU were found to have any significant correlation to

disease-related factors or CHD risk factors. CFU did however have an inverse correlation with CRP. This group measured expression of an interferon inducible gene, *MX1* as a surrogate for serum IFN-I levels. PBMC of SLE patients were found to have significantly increased expression of *MX1 compared* to controls. *MX1* levels were also found to have an association with CFU in SLE. In addition, cell culture with recombinant IFNα2 resulted in a dose-dependent reduction of CFU number. The authors conclude type IFN can be regarded as a novel risk factor for EPC depletion and endothelial dysfunction in SLE [201].

Lastly, Ebner *et al.* measured EPC in 19 SLE females aged 36 (6) years and 19 female controls aged 37 (8) years. They demonstrated reduced CD34//KDR+ cells in SLE but increased level of CD133/KDR+ cells compared to controls. Clinical correlates were not analysed. The authors suggested low levels of endothelial damage may result in increased mobilisation of immature CD133/KDR+ cells in cells [93].

Overall studies of EPC measured using flow cytometry have consistently demonstrated a reduction in CD34/KDR+ cells compared to controls[93, 201, 373]. Two studies have also demonstrated a reduction in CD34/CD133+ cells [88, 242]. Grisar *et al.* al measured the smaller population of CD34/CD133/KDR+ cells and found no difference between SLE and controls. All studies had small sample sizes but the larger of these studies demonstrated a reduction in CFU in SLE compared to controls [201, 242]. The smallest of the studies and that which included patients with a mild phenotype did not find any difference in CFU number in SLE patients suggesting that CFU number may be related to overall inflammatory burden over time. Studies to date have been too

small to detect clinical correlates to EPC or CFU. An interesting link between type I IFN and CFU is emerging within the SLE population and may provide a mechanistic explanation for some observations as well as a potential therapeutic target to attenuate EPC/CFU abnormalities observed.

6.5.9 Inflammatory or damaged endothelial cells in SLE

Somers *et al.* measured apoptotic endothelial cells with CD146/Annexin V surface markers in 75 patients with SLE and reported a correlation with carotid IMT but not with lupus-related factors such as disease activity, damage or duration or with serum markers of vascular damage [323]. In keeping with the findings of Somers *et al.* two other groups have reported increased circulating levels of damaged endothelial cells (EC). Clancy *et al.* reported increased levels of circulating EC positively stained for P1H12 (identifying $CD146^+$ cells) in 38 patients with SLE compared to 16 healthy controls [70] and Rajagopalan *et al.* reported increased CD146/Annexin V positive cells in 43 patients with SLE compared to the same number of age-matched healthy controls and older patients with known CHD. In addition, EC number in this study were found to correlate with brachial artery flow-mediated dilation, a marker for endothelial dysfunction [281].

These preliminary studies lend support to the theory that an imbalance in EC subpopulations with evidence of increased EC apoptosis and reduced numbers of EPC in patients with SLE leads to a reduced vascular regenerative capacity and endothelial dysfunction. Further investigation is required to determine factors associated with endothelial cell abnormalities in SLE. Studies

of clinical and subclinical atherosclerosis in SLE using carotid scanning would suggest age is a significant contributing factor in SLE and that on average atherosclerosis occurs earlier in SLE than controls. Most studies to date examining EPC have matched for age and therefore the contribution of age has not been explored.

6.6 Telomeres and cellular senescence

6.6.1 Telomere structure and function

The unique structures that form the physical ends of linear chromosomes were first reported in the 1930s. McClintock *et al.* noted that, unlike broken ends, the natural ends of maize chromosomes appeared to be protected from forming random attachments to other fragments of chromosomes [230]. In the late 1930s these highly conserved repetitive DNA-protein complexes of hexanucleotide sequences were given the name 'telomeres' originating from the Greek words *'telos'* meaning end and '*meros*' meaning part [245] (Figure 4).

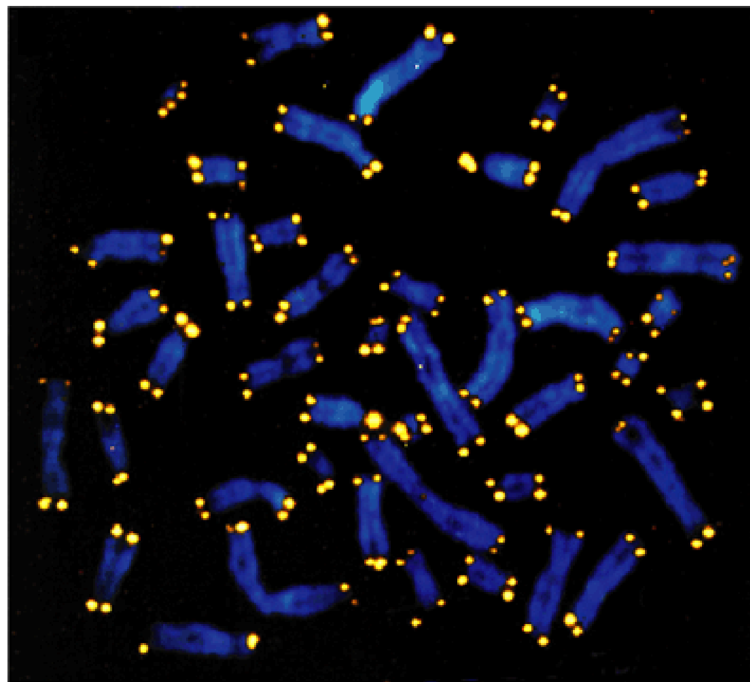


Figure 4 Telomeres at chromosome ends demonstrated using quantitative fluorescence in situ hybridization (Q-FISH). ***Adapted from Oeseburg et al. [257]***

The DNA sequence of human telomeres was identified in the 1980s, as repeats of TTAGGG, highly conserved in evolution [244]. Human telomeres are constituted by tandem DNA repeats ending with a 150 to 200 nucleotide G-rich 3' single-stranded overhang and associated proteins [68, 80, 386]. The G-rich single strand is protected by a complex of proteins. Whilst several associated proteins including those involved in DNA synthesis have been demonstrated in the telomere region, there are thought to be six proteins that are specific to telomeres. These telomere-specific proteins, TRF1, TRF2, hRap, TIN2, POT1 and TPP, form the protective complex termed shelterin [43, 81, 185, 208, 213, 386] (see Figure 5). The proteins contained within shelterin are able to bind double stranded DNA enabling the formation of a loop-like structure termed the telomere- or T-loop. POT1 specifically has been shown to bind single stranded DNA and is thought to bind to the G-rich overhang forming a lesser loop termed the displacement or D-loop, thereby protecting the structure from the enzyme telomerase (see below) involved in telomere elongation [202]. It is likely the protein complex is a dynamic structure that undergoes conformational changes during the different phases of the cell cycle to enable elongation, protection and regulation of the telomere [203, 204].

Figure 5 Schematic of telomere structure and associated proteins of shelterin complex

The length of telomeres in human cells varies between tissue types and is typically between 5 and 15kb in somatic cells [244]. Importantly, DNA polymerases are unable to replicate the DNA lagging single strand of chromosomes ends. As a result of which, telomeres shorten with cell division, a process referred to as the ‘end-replication problem’. With each cell division it is approximated between 50 and 150 base pairs are lost and this loss is cumulative. Therefore, the mean length of telomeres can provide an estimate of the replicative age of a cell [144, 163]. Somatic cells appear to have a finite replicative life span often referred to as the Hayflick limit [147]. Telomere erosion beyond a critical length leads to loss of protection of chromosomes resulting in genomic instability, degradation or end to end fusion. This chromosomal dysfunction will result in proliferative senescence i.e. cessation of

replication or cell apoptosis or less frequently, undergo continued proliferation and develop tumourogenic potential [328]. The mechanisms that trigger these processes remain unclear. For any given cell it might be that a threshold number of short telomeres are required or alternatively perhaps the length of the shortest telomere in the repertoire is the important factor.

6.6.2 Mechanisms of telomere attrition

There is also a great deal of uncertainty regarding the mechanism(s) by which telomere attrition leads to cell cycle arrest or senescence. One of the potential mechanisms is thought to be via activation of the transcription factor p53. Expression of p53 is initiated by cellular assaults such as oxidative stress or DNA damage. Expression of p53 can lead to the activation of several genes and ultimately lead to cellular senescence enabling DNA repair processes or apoptosis [178] [138]. In a mouse model the effects of telomere attrition has been found to be linked to p53 status. In the presence of p53, shortened telomeres and subsequent exposed or damaged DNA do not result in malignant transformation of cells and induce an ‘aged’ phenotype i.e. premature greying or reduced wound healing [132, 294]. However, in the absence of p53, genomic instability induced by telomere attrition leads to accelerated tumourogenesis [66]. This would suggest that telomere attrition and involvement with p53 is an important safeguarding mechanism against tumourogenesis despite the disadvantage of leading to ‘ageing’.

6.6.3 Determinants and maintenance of telomere length

Telomere length between individuals of the same age can be highly variable [260, 335]. The mean length of an individual's telomeres of a given cell/tissue type at any given time will be influenced by many factors. Marked variation of telomere length at birth between individuals is observed and the influence of foetal or stem cell telomere length may be of importance. There is much uncertainty about the factors that influence the determinants of variation of mean length of telomeres between individuals. Interestingly, in the case of peripheral blood mononuclear cell (PBMC) telomere length, there appears to be some genetic influence [128, 254]. Analysis of 115 monozygotic and dizygotic twin pairs between the ages of 2 and 95 years indicated 78% heritability [320]. There are likely to be several environmental factors that influence the rate of replicative senescence of a cell and some of these are discussed below. In addition to these factors that impact on the rate of telomere erosion, telomere length will also be influenced by mechanisms that are present to maintain telomere length.

6.6.4 Telomerase and telomere maintenance

An important mechanism involved in the maintenance of telomeres involves the enzyme telomerase. Components required for the active telomerase complex are telomerase reverse transcriptase (TERT) protein and the telomerase RNA component, often referred to as TERC (Figure 6). The RNA component of this unique enzyme complex provides a template for de novo addition of telomere repeats [133].

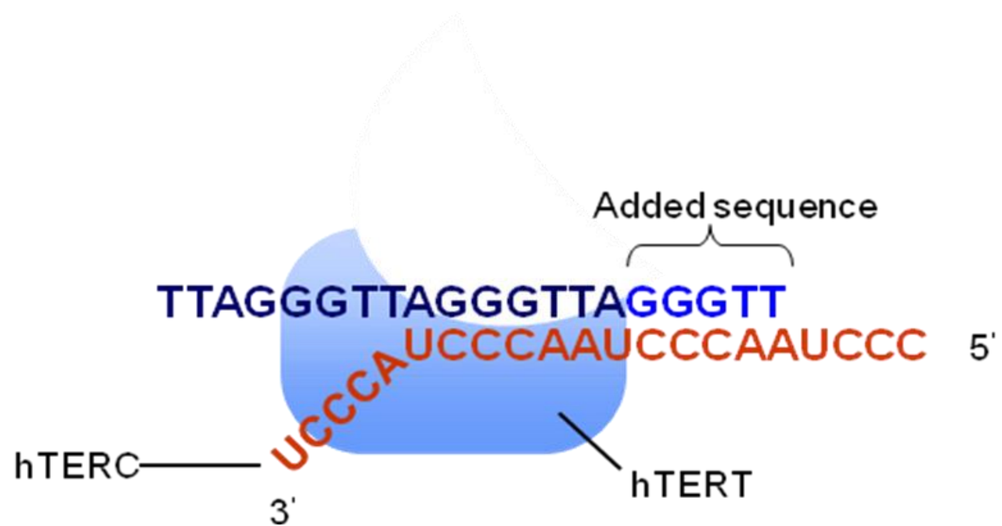


Figure 6 Telomerase structure demonstrating de novo addition of base pairs using the hTERC template

The TERT component of the complex appears to have an essential catalytic role [370]. Although human somatic cells express telomerase RNA, telomerase activity is absent from most of these cells and this leads to the gradual telomere attrition incurred with cell division. The inactivity of telomerase in this context is usually associated with the absence of TERT [39, 279]. Telomerase is known to be expressed in some human germ cell lines [234], epithelial and haematopoietic cell lines [67] and activated lymphocytes [153]. Interestingly, the expression of telomerase appears to cease at some stage during differentiation [10, 109]. In those cells containing telomerase, DNA elongation is thought to occur in the S-phase of the cell cycle and access to the telomere is thought to be regulated by the telomere protein complex [227].

Tumours present an interesting model in which to study telomere elongation mechanisms. The majority of tumours express active telomerase and maintain stable telomeres with proliferation [184] however, this is not the case in all tumour types [50]. Additionally, other conditions exhibit telomere

maintenance in the absence of telomerase. Cells from individuals with Werner Syndrome, a condition characterised by premature ageing, do not exhibit telomerase activity and have been found to have stable telomere lengths. This leads to the notion that there are non-telomerase dependant pathways capable of maintaining telomere length and these have been termed alternative lengthening of telomere (ALT) [199]. Further elucidation of ALT pathways was possible when a telomerase deficient yeast model was developed and it was observed that whilst most telomerase deficient cells died a small subpopulation regained replicative potential by recombination of telomeric tracts. These chromosomal rearrangements were found to be dependant on 2 different genes, RAD50 and RAD51. Strains deficient of these genes lost the survival advantage [215, 216]. To date however, equivalent genetic predispositions in replicating human telomerase-defective cells have not been identified.

6.6.5 Telomere length variation with age

The relationship between telomere shortening and age can be demonstrated in telomerase-deficient mice that develop rapid telomere attrition. *In vitro* studies in mouse models can provide an insight into the consequences of reduced proliferative capacity of cells and the potential regenerative role of telomerase. Mice with telomerase deficiency develop age-related disease such as infertility, heart failure, immunosenescence-related diseases and decreased tissue regeneration (digestive system, skin and haematopoietic system) [36, 37, 148, 206].

Correlative evidence from *in vitro* studies in premature ageing syndromes also provides evidence for variation of telomere length with ageing in humans.

Such models, however, require caution as *in vitro* cells are unlikely to accurately reflect the *in vivo* situation. Dyskeratosis Congenita is a rare genetically heterogeneous syndrome characterised by premature mortality related to bone marrow failure, infections, fatal pulmonary complications or malignancy. Cells from individuals with this condition have been found to have a lower level of telomerase RNA, produce lower levels of telomerase activity and have shorter telomeres than cells from matched controls [239, 360]. Werner Syndrome is a rare autosomal recessive syndrome characterised by development skin atrophy, greying of hair and onset of cataracts, osteoporosis, diabetes and malignancies, typically following puberty and resulting in early mortality. Cells from subjects with this syndrome also exhibit shortened telomeres compared to age-matched controls and interestingly, *in vitro*, the life span of these cells can be extended with the introduction of telomerase [39, 353]. Additionally, cells from older donors do appear to undergo replicative senescence in culture more rapidly than cells from younger donors indicating that telomere length does reflect chronological ageing [144, 304].

Accumulating epidemiological evidence would also suggest that telomere length declines with human ageing. It is thought that telomere length varies greatly between individuals at the extremes of age and can be relatively stable in healthy humans during adult life as demonstrated in Figure 7. Of interest, a study examining the mean length of telomeres of peripheral blood cells of 812 individuals aged between 70 and 100 years of age did not find any association between telomere length and survival [32]. Similar findings are reported in 598 individuals over the age of 85 years [225]. Genomic instability in the ‘very old’

is likely to be multifactorial and the effects of telomere length may be less significant in this context.

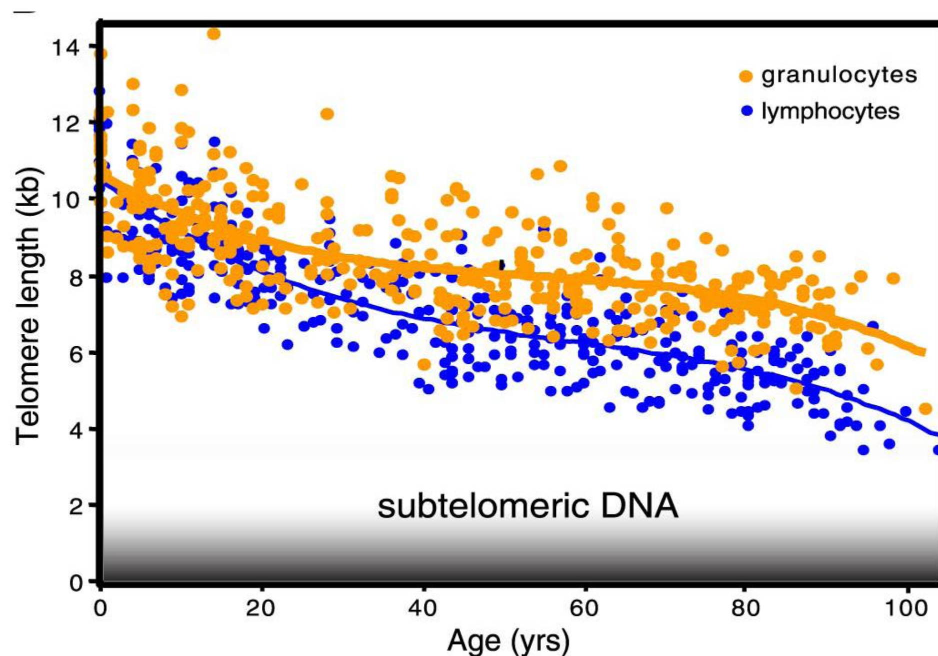


Figure 7 Results of calculated median telomere length in lymphocytes and granulocytes of 400 normal individuals over the entire age range (Baerlocher and Lansdorp, unpublished data) cited by Aubert *et al*.

Additionally some studies have suggested there are gender and ethnicity differences with females and African Americans having longer telomeres [65, 164]. Difficulties arise in study design as telomere length is highly variable between species as well as between individuals within the same species but also between different cells within the same organism [65]. Much of the evidence relating to human ageing and telomere length is based on cross-sectional studies. The results of such studies present the potential issues of cohort effect and censorship with the risk of missing those people with the shortest telomeres due to death prior to sampling. Longitudinal studies can be difficult to conduct as telomere length changes slowly over time and owing to the wide variation, large samples sizes would be required to attain meaningful

results and as such there are no prospective population studies examining age-dependent telomere attrition in 'healthy' individuals.

Many questions remain unanswered. It is not known if premature ageing of cells is caused directly by telomere length reduction or both phenomena are driven by a common process. Also it is unclear if it is possible to modify the mean telomere length of human tissue *in vivo* or in fact whether such an alteration would have an effect on the entire organism. The importance of mean cellular telomere length with regards to whole organism senescence remains an area of much interest.

6.6.6 Measuring telomere length

6.6.6.1 Techniques for measurement of telomere length

Cell population telomere length has been quantified using three main methods namely Southern blotting, quantitative polymerase chain reaction (qPCR) and *in situ* hybridization methods (Q-FISH and flow FISH). Previously the most commonly used of these techniques was Southern blotting to estimate absolute values of telomere repeats. However there are several disadvantages to this method including the requirement of relatively large amounts of DNA, long length of time required to carry out the technique and the potential to underestimate the length of telomeres [18]. Q-FISH uses a peptide nucleic acid probe to visualise telomeres in metaphase spreads. Since cells approaching senescence are less able to enter mitosis, this method can lead to variable results in a mixed population of cells (i.e. senescent and proliferating). In addition, specialist knowledge and equipment is required to perform this technique [295]. Real-time qPCR presents a much faster method of telomere

length measurement. This method uses modified PCR primers to avoid the formation of primer-dimers as far as possible. The final measure is a relative telomere length expressed as a ratio of telomere quantity to a single copy gene quantity (T/S). Advantages of this method are that relatively small amounts of DNA are required and a large number of samples can be measured in a short time [57]. The main disadvantage is that the measure attained is relative telomere length and therefore whilst valid within a population, comparisons between populations cannot easily be made. Measurements of telomere length using qPCR have been shown to have a good correlation to measurements using Southern blotting [45, 56] and therefore this technique is increasingly utilised.

6.6.6.2 Choice of cell population

The techniques available to measure telomere length have the potential to allow measurement of cell populations, single cells or single chromosomes. Peripheral blood mononuclear cell (PBMC) telomere length has been measured extensively to study both ageing in normal populations and in disease states. PBMC telomere length measurement has the advantage of being relatively easy to obtain from study participants as it can be measured from a few millilitres of peripheral blood and can be easily processed. Several studies measuring PBMC telomere length have been shown to have a correlation to ageing and to disease states and therefore PBMC telomere length appears to be a marker for disease.

6.7 Association between telomere length, CHD and risk factors

Cardiovascular disease and CHD risk factors are major contributors to age-related morbidity and mortality in the general population and as such the association of telomere length, a marker of senescence, is of particular interest in this subject. There is a growing body of evidence in both animal and human studies that telomere length is associated not only with clinical CHD but also risk factors associated with CHD.

6.7.1 Telomere length and CHD risk factors

Several studies have explored the association of telomere length with cardiovascular risk factors and some of these studies are summarised in Table 5. There are several small studies indicating an inverse correlation between telomere length and most cardiovascular risk factors. Brouilette *et al.* demonstrated in a case control study of 203 patients with a premature MI (<50 years) and 180 controls, that hypertension, diabetes smoking status and serum cholesterol did not account for reduced telomere length in cases vs. controls [44]. Patients with SLE have been shown to have an excess of traditional cardiovascular risk factors [269] and to what extent these factors account for telomere attrition observed in SLE will be addressed in the current study.

Despite the findings of many correlative studies the question remains whether telomere attrition is a cause or consequence of CVD. Supporting evidence for causation is provided by studying the TERC-deficient mice. Amongst other features of premature ageing these mice develop cardiac dilatation and heart failure [206]. Although, given that telomere maintenance can occur in the absence of telomerase, this mouse model could be considered

to be informative about the effects of telomerase deficiency rather than the effects of telomere ablation *per se*. Nonetheless, several studies suggest that PBMC telomere length appears to correlate with CHD risk factors.

Table 5 CHD risk factors & telomere length

CHD Risk Factor	Cell/Tissue Type	Main Findings	Reference
Gender / oestrogen	Rat kidney, liver, pancreas, lung, brain tissue	Reduced telomere length in males vs. females in all tissue types except brain	Cherif 2003 [65]
	Human WBC*	Age-adjusted telomere length longer in women than in men	Benetos 2001 [24]
Diabetes mellitus	Human WBC*	Reduced telomere length of monocytes from type 2 diabetic patients vs. controls	Sampson 2006 [298]
	Human WBC*	Weight gain and insulin resistance associated with accelerated telomere attrition	Gardner 2004 [117]
	Human WBC*	Age- adjusted telomere shorter in type 1 diabetes vs. non diabetic controls	Jeanclos 1998 [169]
Hypertension	Human WBC*	Inverse correlation between pulse pressure and telomere length	Jeanclos 2000 [170]
	Human WBC*	Shorter telomere length associated with hypertension, increased insulin resistance and oxidative stress	Demissie 2006 [84]
	Telomerase deficient mice	TERC deficient mice exhibit increased plasma endothelin-1 levels and higher systolic and diastolic blood pressure compared with wild type mice	Perez-Rivero 2006 [265]
Obesity	Human WBC*	Inverse correlation of telomere length with BMI. Also shorter telomere length in smokers and ex smokers vs. non smokers	Valdes 2005 [350]
Hypercholesterolemia	Human WBC*	No significant relationship between telomere length and cholesterol	Brouilette 2003 [44]
Smoking			See above – Valdes
	Human WBC*	In COPD§ patients - reduced telomere length of smokers compared to non smoking patients	Morla 2006 [243]

*WBC – white blood cells; §COPD – chronic obstructive airways disease

6.7.2 Telomere length and carotid atherosclerosis

A recent large study within the Framingham cohort of 496 men and 566 women has demonstrated a significant negative association to carotid IMT with PBMC telomere length. Following adjustment for several factors, telomere length remained significantly associated to obesity in men. A further study measured carotid IMT in 762 volunteers and found a significant association that persisted after adjustment for age and CHD risk factors. In the gender stratified analysis, the association remained only in the male cohort [255, 262].

Two recent small studies within a hypertensive cohort and a diabetic cohort have demonstrated significant reduction in PBMC telomere length in those with carotid plaque compared to those without [6, 23].

6.7.3 Telomere length and CHD

Recent studies have examined the role of PBMC telomere length in clinical CHD. Brouilette *et al.* reported significantly reduced telomere length in white blood cell (WBC) DNA from 203 patients with MI under the age of 50 years compared to 180 controls and this was independent of traditional risk factors. In contrast to studies indicating telomere length has a poor correlation with clinical outcome in elderly individuals, Cawthon *et al.* found, in a longitudinal study, mortality in persons aged over 60 years from a cardiac cause was highest in the proportion of individuals with the lowest WBC telomere length [57]. Similarly, a case control study of 20 patients with severe CHD and 20 healthy individuals reported significantly reduced WBC telomeres in cases compared to controls after adjusting for age and gender [297]. A recent

prospective study followed 484 individuals that subsequently developed CHD events and 1058 individuals that remained CHD-free. The risk of CHD was found to be significantly higher in patients with mean telomere length (measured at baseline) within the middle and lowest tertiles compared to the highest tertile. In addition, when patients were stratified for pravastatin therapy this risk appeared to be attenuated despite similar lipid levels between the three groups [45].

The concept that atherosclerosis is an inflammatory process has become widely accepted in recent years [142]. It might be that WBC telomere attrition is a reflection of a systemic inflammatory response or alternatively it might be representative of a more general premature ageing process and increased susceptibility to CHD. Alternatively enhanced telomere attrition may be the result of subclinical atherosclerosis and is therefore apparent prior to the onset of clinically apparent CHD.

6.7.4 Telomere length of endothelial cells

Endothelial dysfunction is recognised as the earliest pathology in the formation of atheroma. There is an emerging body of evidence suggesting that endothelial cells may exhibit a reduction in telomere length. A small comparative study examining coronary endothelial cells obtained during post mortems from 11 patients with CHD found that these cells had shorter telomere length when compared to samples obtained from the coronary arteries of 22 non-CHD patients [258]. Chang *et al.* also examined specimens retrieved at post mortems or after coronary artery bypass surgeries. They reported that increased telomere erosion in vessels correlated with age. In addition, they

reported increased telomere erosion in cells from the iliac artery (a vessel thought to be exposed to high haemodynamic stress) compared to cells from the iliac vein or internal thoracic artery [62].

In conclusion, telomere length reduction has been to be significantly associated with CHD and CHD-related risk factors independently of age. Emerging evidence suggests that telomere attrition and premature cellular senescence might be a potential mechanism by which endothelial dysfunction occurs.

6.8 Telomere length and SLE

To date there have been a few preliminary studies examining the association of telomere length and telomerase in SLE.

Kurosaka *et al.* measured telomere length of PBMC using Southern blotting in 30 SLE patients including 3 males, aged 38 (±11) years and 39 controls including 3 males aged 40 (±11) years. They demonstrated a significantly reduced telomere length in SLE patients compared to controls under the age of 40 years. Telomere length tended to be similar in older SLE patients and age-matched controls suggesting premature ageing of PBMC in the younger SLE age group. The report did not include correlations of telomere length to clinical measures [194]. Kurosaka *et al.* also reported increased peripheral blood mononuclear cell (PBMC) telomerase activity in 55 patients with SLE aged a mean (SD) of 39 (±13) years and 45 healthy controls 42 (±13) years and demonstrated increased telomerase activity in SLE patients compared to controls on all age groups. Telomerase activity within the SLE population was significantly correlated with disease activity measured using a

modified SLEDAI (i.e. SLEDAI excluding complement and anti-dsDNA antibody) with a Spearman's correlation coefficient (R^2) of 0.524 ($p<0.0001$). Other significant correlations to telomerase activity were haemoglobin level (R^2 -0.246, p = 0.038), CRP (R^2 0.325, p = 0.007) and C4 (R^2 -0.477, p = 0.005). No significant correlation was demonstrated with total white cell count or lymphocyte count [195]. The same group further elaborated this work and measured telomerase activity in 37 SLE patients compared with 17 healthy controls and found that B cell telomerase activity was significantly higher in patients with active SLE (SLEDAI > 6) compared with patients with inactive disease or healthy controls. However T cell telomerase activity was increased in SLE patients regardless of disease activity compared with healthy controls. In addition, T lymphocyte telomere length was reduced in SLE patients *vs.* controls but no significant difference was noted with B cell telomere length [196]. These findings might suggest that there is continuous T cell activity in SLE and increased telomerase activity but this is inadequate to maintain telomere length. However in active disease, B cell activation and division occurs and increased telomerase activity is able to maintain telomere length in this population of cells.

Klapper *et al.* measured telomerase activity of CD19+, CD4+, and CD8+ lymphocytes isolated from the peripheral blood of 9 patients with SLE and 9 age-matched healthy controls (details of age not published). Similar to the findings of Kurosaka, Klapper *et al.* found increased telomerase activity in B cells and T cells in the SLE patients [187]. This group attempted to measure telomere length using Southern blotting in these cells but owing to high DNA requirements for this technique, were only able to assess telomere length of

CD4+ cells in 6 SLE patients and 5 healthy controls and did not demonstrate a difference.

A larger study by Honda *et al.* measured telomere length of PBMC using Southern blotting in 58 SLE patients and 51 controls. They found a reduction in telomere length in SLE compared to controls in the overall analysis which was particular marked in those under the age of 45 years and abrogated in those over 55 years. Correlations to potential contributing factors was not analysed in this small study [160].

Wu *et al.* measured PBMC telomere length using Southern blotting in 60 SLE patients with a median (range) age of 34 (16 – 76) years and 26 gender-matched controls aged 30 (29 – 79) years. They demonstrated an overall reduction in telomere length in SLE patients compared to controls which was particularly marked in those under the age of 45 years. The multivariable analysis of selected factors that may influence telomere length demonstrated a significant negative correlation with age and a trend towards a negative correlation with SLEDAI and immunosuppressant use in the previous 6 months [376].

Beier *et al.* used flow-FISH to measure telomere length of PBMC in 22 SLE patients and 20 controls (details of age not reported). Using this technique no difference in telomere length was noted in lymphocyte subpopulations between SLE and control. Neither were there any significant correlations of clinical features to telomere length within the SLE group [22]. The authors noted there was no correlation between telomere length and lymphocyte count.

Overall there would appear to be a reduction in PBMC telomere length in SLE compared to controls [160, 195]. Studies that have analysed telomere

length of lymphocyte subpopulations have not found a significant reduction in SLE compared to controls [22, 187, 196]. The studies reported by Wu *et al.* and Beier *et al.* analysed a limited number clinical factors based on prior knowledge and assumptions of possible associations with PBMC telomere length and did not demonstrate any significant correlations. Importantly, several studies have demonstrated that there is no correlation between lymphocyte count and telomere length in SLE. In addition, no correlation was noted between disease activity and PBMC telomere length suggesting that telomere length is not simply a reflection of immune cell turnover. It should be noted that the majority of patients studied had low disease activity. All of the above reports had relatively small sample sizes and not all controls were gender matched. However, these preliminary results do suggest a reduction in PBMC telomere length in SLE compared to controls that is particularly marked in those under the age of 45 years. None of the studies measured atherosclerosis markers or CHD risk factors in SLE.

6.9 Telomeres and telomerase activity in other inflammatory rheumatic diseases

Lymphocyte activation and proliferation are the hallmark of many inflammatory rheumatic diseases such as rheumatoid arthritis. Telomerase has the ability to initiate indefinite cellular proliferation as in the case of malignancies and telomerase activity has been shown to be increased in activated lymphocytes from healthy individuals [371]. These observations have stimulated some interest in the role of telomerase and telomere length in immune function of patients with inflammatory rheumatic diseases.

6.9.1 Systemic sclerosis

Interestingly a small study comparing patients with connective tissue diseases and 10 patients with Hepatitis C and abnormal liver function tests found that telomerase activity could be detected in 11/17 (64.7%) patients with SLE, 6/11 (54.5%) patients with Sjogren's syndrome, 7/11 (63.6%) of patients with mixed connective tissue disease and 6/11 patients with systemic sclerosis but no telomerase activity could be demonstrated in the Hepatitis C group. This would suggest that the autoimmune nature rather than inflammatory component of connective tissue diseases might have an effect on telomerase activity [179]. Artlett *et al.* found reduced telomere length in 43 patients with systemic sclerosis and in their 182 family members compared to 96 age-matched controls [11]. Interestingly, a proportion of these family members were spouses indicating that in some cases there might be significant influence of environmental factors on telomere length such as tobacco usage.

6.9.2 Rheumatoid arthritis (RA)

Small controlled studies have demonstrated telomere length reduction in the PBMC, osteoblasts, synovial lymphocytes and CD34+ haematopoietic progenitor cells in patients with RA [89, 326, 383]. The findings reported with regard to telomerase activity however are conflicting with increased and reduced activity reported compared to controls [114, 382]. Of particular interest in the rheumatoid arthritis population is the association of telomere length and HLA haplotype. The presence of the HLA-DRB1 shared epitope is believed to convey susceptibility to RA and in particular to disease severity [127, 251, 325]. The two widely hypothesised explanations for this association previously were

the possibility of presentation of an ‘arthritogenic’ peptide or induction of autoreactive T cell responses. In view of the diversity of the structure of these molecules and lack of collaborative evidence, these theories appear to be unlikely. Interestingly, Schonland *et al.* have reported that the *HLA-DRB1*04* haplotype is associated with telomere shortening in a small sample of patients. Both healthy individuals (n=37) and those with RA that were *HLA-DRB1*04*$^{+}$ had reduced telomere length compared to *HLA-DRB1*04* $^{-}$ individuals [306]. More recent data on a larger cohort (176 RA patients vs. 1151 healthy controls) has also reported that short telomeres correlate with shared epitope status in RA but not in controls. Also there was no correlation with disease duration or disease activity estimated using the CRP [326]. These studies might provide a new insight into the role of the shared epitope and susceptibility to RA. Interestingly, Schonland *et al.* also found that reduced telomeres were not isolated to T cells but also found in granulocytes suggesting that senescence is predetermined at the haematopoietic stem cell level. These data may suggest a genetic susceptibility to premature senescence in several cell lines and lend support to the theory that patients with connective tissue diseases might be genetically predestined to develop disease in several different organ systems and environmental exposures might enhance/trigger that risk.

In keeping with the SLE studies of telomere length, studies have demonstrated a reduction in telomere length but not demonstrated an association of disease activity and inflammatory burden with telomere length. The findings would suggest other unmeasured factors may contribute to telomere length. Studies to date have not examined telomere length in the context of atherosclerosis in the rheumatic diseases.

7 Hypothesis

There is premature biological ageing in patients with SLE as evidenced by a reduction in the mean telomere length of PBMC. Premature biological ageing is also evident in the vasculature of patients with SLE and reflected by relatively shortened telomeres of cells involved in vascular repair and regeneration i.e. endothelial progenitor cells (EPC). Furthermore, senescent EPC result in cellular imbalance with a relatively reduced number and/or function of circulating healthy EPC.

8 Aims and objectives

Overall aims

1. Describe demographic and clinical features of study cohort
2. Describe the burden of carotid atherosclerosis in the study cohort
3. Describe progression of carotid atherosclerosis in SLE cohort longitudinally
4. Measure EPC in SLE compared to healthy controls in a cross-sectional study, ascertain potential contributing factors and correlation to subclinical atherosclerosis
5. Measure telomere length of SLE patients compared to healthy in a cross-sectional study, ascertain potential contributing factors and correlation to subclinical atherosclerosis
6. Develop a method and measure telomere length of EPC in SLE compared to healthy controls in a preliminary cross-sectional study

The objectives of individual chapters will be summarised at the beginning of each chapter.

9 General methods

9.1 Study design, venue and funding

This is a cross sectional, secondary care based observational study. All participants were assessed at a single central site (the Wellcome Trust Clinical Research Facility, Manchester Royal Infirmary). All laboratory experiments were conducted at the University of Manchester. The study was funded by a clinical research fellowship from the Arthritis Research Campaign.

9.2 Ethical approval

Ethical approval was secured for the study from the National Research Ethics Service (see Appendix 1). Local approval was obtained from each of the participating centres, namely, South Manchester Hospital Trust, East Lancashire Hospitals NHS Trust, Stepping Hill Hospital, North Manchester General Hospital and Blackpool, Fylde and Wyre Hospitals NHS Trust.

9.3 Patient recruitment

Rheumatology centres across the Northwest region of the UK were invited to suggest patients with SLE for the study. The majority of participants were recruited from one centre (Central Manchester and Manchester Children's University Hospital NHS trust). The study was publicised in local newspapers, the BBC health online pages, a Lupus UK newsletter and at Lupus patient education days to encourage patient and healthy control participation [2] (Appendix 2). Study information was forwarded to all participants and they were then contacted on a further occasion to allocate a clinic date and time

(Appendix 3). General practitioners of all participants were informed (Appendix 4).

9.3.1 SLE inclusion criteria

Patients recruited satisfy all the following criteria

a. Modified 1997 American College of Rheumatology classification criteria for SLE [155]; diagnosis confirmed with direct casenote review or communication with their physician
b. Female gender
c. Age > 18 years old

SLE patients are excluded if any of the following apply

   a. Current pregnancy
   b. Diagnosis or treatment of a malignancy in previous 12 months

9.3.2 Comparison cohort recruitment

Fifty cases were randomly selected to have endothelial cell quantification and these cases were allocated a control subject matched by gender and age (within 10 years) for comparison. The selected cases were asked to suggest a friend to be a matched control and where it was not possible for a case to suggest a control, an individual was allocated from a list of names of healthy subjects that had participated in a similar study previously. Control participants underwent identical clinical and laboratory assessments to the cases with the exception of SLE-specific assessments e.g. SLE disease activity index.

9.4 Clinical assessment

All participants are reviewed by a trained member of the clinical research team (Ian N Bruce, Chadi Rakieh or I) on at least one occasion. Data collected,

using a standardised proforma (see Appendix 5), include demographic data, presence of cardiovascular risk factors (defined below) and cardiovascular events (personal history from patient) i.e. myocardial infarction, angina, cerebrovascular event or coronary intervention.

9.4.1 Definition of cardiovascular risk factors

1. Hypertension: blood pressure of > 140/90 or current treatment with an antihypertensive drug
2. Hypercholesterolemia: TC > 5.2 mmol/l or LDL-c > 3.2 mmol/l or on therapy
3. A family history of cardiovascular disease: MI, angina or sudden death in a first degree relative: male <55 yrs or female <65yrs;
4. Diabetes mellitus: fasting plasma glucose >7.0 mmol/l or current diabetic therapy

Other information collected included the medical history SLE clinical features including details of the SLE diagnosis. ‘Renal disease’ was defined as any patient with persistent proteinuria, otherwise unexplained microscopic haematuria, chronic renal insufficiency, nephrotic syndrome or any grade of lupus nephritis diagnosed on biopsy.

Two composite measures of disease activity were assessed, namely, British Isles Lupus Assessment group (BILAG) index [168] and SLE disease activity index (SLEDAI) [40] (see Appendix 8 and Appendix 9). Cumulative damage was recorded using the Systemic Lupus International Collaborating Clinics (SLICC) damage index [122]) (see Appendix 10). Details of current and previous SLE therapy were recorded as well as all other current medications. Detail of steroid exposure was documented including previous exposure, average daily dose and length of courses. A number of lifestyle factors including

tobacco and alcohol use and prior use of hormonal preparations were noted. A systemic clinical examination was undertaken.

9.4.2 Anthropometry

Height, weight, pulse rate, blood pressure measurement, waist and hip measurement were undertaken by trained nursing staff.

9.4.3 Laboratory assessment

To aid the clinical assessment and cardiovascular risk assessment, a number of laboratory measures were undertaken and are listed below:

a. Full blood count
b. Renal profile, estimated GFR and urine dipstick
c. High sensitivity C-reactive protein to assess low grade inflammatory activity
d. Fasting blood glucose, insulin, HDL cholesterol and triglycerides as well as body mass index to assess insulin resistance and presence of the metabolic syndrome

9.4.4 Estimation of subclinical CHD

9.4.4.1 Carotid ultrasound

Carotid ultrasound examination was undertaken by one of two vascular technicians on the day of the clinical assessment. Comparison of measurements between the two operators showed good correlation (see Appendix 6).

9.4.4.2 Aortic pulse wave velocity (APWV)

All measurements were performed by one of two trained nursing staff at the time of the clinical assessment. The measurement was performed after an overnight fast and 12 to 24 hours abstinence from all anti-hypertensive

medications. Participants were asked to omit caffeinated beverages, smoking, and alcohol for ≥12 hours before the assessment. Comparison of measurements between the two operators showed good correlation (see Appendix 7).

9.5 General statistical methods

All data were collated and entered into an Access database by a trained research assistant. The data were analysed using STATA 9.2 statistical software. Comparisons were made between *cases* and *controls* by means of a two-sample t-test for normally distributed continuous variables (when explored by the Shapiro-Wilk test) and by chi-square analysis for categorical variables. For non-normally distributed variables, non-parametric tests were determined using Kruskal-Wallis rank test for categorical variables and Spearman's correlation coefficients for continuous variables. Two sided p values (P) of less than 0.05 were considered to be significant. Bland-Altman plots were used to test the extent of agreement between pairs of measurements e.g. carotid measurements taken by different operators or consistency of laboratory experiments [35] by plotting the differences between the pairs on the vertical axis and the mean of each pair on the horizontal axis. A description of the relevant statistical methods can be found in each section if tests other than those described above are utilised.

9.6 Laboratory methods

Methods relating to the relevant results chapter will be discussed at the beginning of each chapter.

10 RESULTS

10.1 Description of SLE patients

SLE patients have been reported to have an excess of CHD risk factors and carotid atherosclerotic burden. In this chapter we aim to ascertain these factors in our study population.

The objectives of this chapter are to:

a. Describe the characteristics of 182 female SLE patients and 70 female controls enrolled into the cross-sectional study
b. Describe the burden of carotid atherosclerosis in the cross-sectional assessment
c. Determine the progression of carotid atherosclerosis in a longitudinal study
d. Determine factors contributing to atherosclerosis progression

10.1.1 Demographics of SLE patients

A total of 182 patients were recruited to the study with a median (IQR) age of 53 (46, 61) years and 38% of patients were under the age of 50 years. See below for age frequencies. The majority of patients were of White-Caucasian ethnicity and 9% of patients were of Afro-Caribbean or Asian ethnicity. See Figure 9 for distribution of ethnicities.

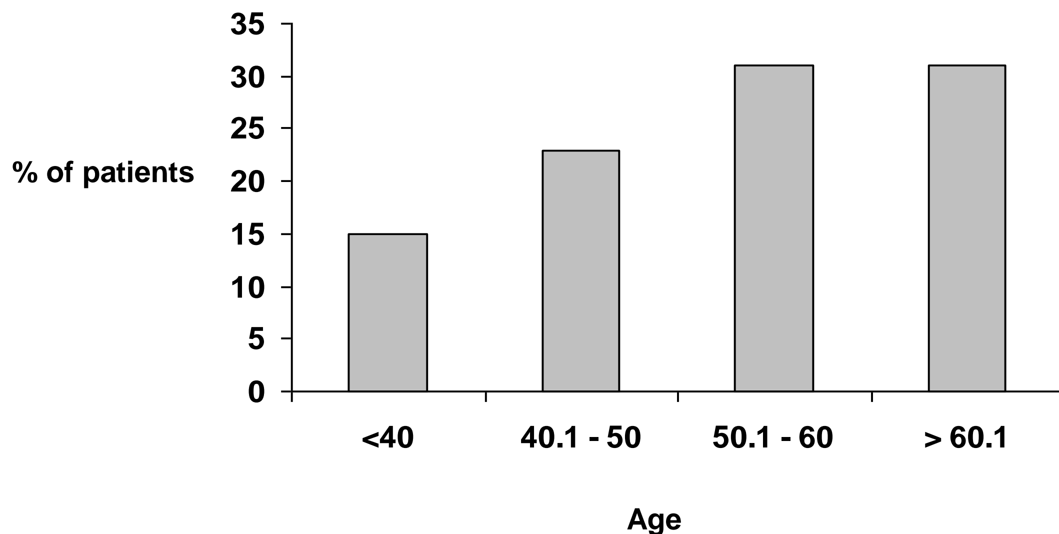


Figure 8 Age distribution of SLE patients

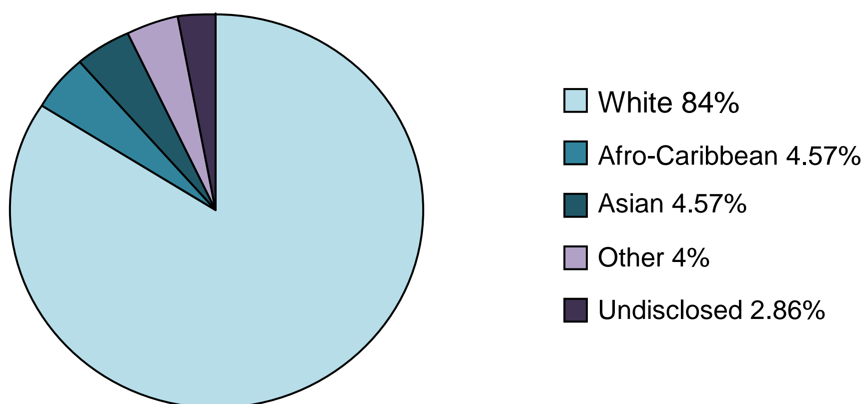


Figure 9 Ethnicity distribution of SLE patients

10.1.2 Clinical and immunological features of SLE patients

The median (IQR) disease duration was 13 (7, 23) years and there was a median (IQR) lag period of 3 (1, 11) years between the onset of the first criteria for diagnosis until four ACR criteria were met. All patients satisfied 4/11 of the 1997 ACR criteria and 101 (55%) satisfied more than four criteria. The most common manifestation was an immunological abnormality although documentation of a positive antinuclear antibody was not available in 18 (10%) of patients. Thirty-eight (21%) patients had biopsy-proven renal disease. See Table 66 for the prevalence of features of the 1997 ACR criteria amongst the SLE patients.

Table 6 Prevalence of ACR criteria features

Clinical Features	n (%) patients
1. Malar Rash	100 (55)
2. Discoid rash	20 (11)
3. Photosensitivity	102 (56)
4. Oral Ulcers	96 (53)
5. Arthritis	124 (68)
6. Serositis	67 (37)
7. Renal disorder	38 (21)
8. Neurological disorder	18 (10)
9. Haematological disorder	87 (48)
10.Immunologic disorder	155 (85)
11. Antinuclear antibody	164 (90)

Immunological profile was repeated at the time of the study assessment and the autoantibody profile is summarised in Table 77. Approximately a

quarter of patients had some immunological evidence of disease activity with a low C4 or high titre of anti-dsDNA antibody.

Table 7 Immunological features of SLE patients

Immunological features at time of assessment	n (%) patients
Anti – Ro antibody	36 (23)
Anti – La antibody	22 (12)
Anti – Sm antibody	2 (1)
Anti – RNP antibody	16 (9)
Anti - cardiolipin antibody [d]	40 (22)
Lupus anticoagulant	53 (29)
Low C3 [a]	5 (3)
Low C4 [b]	46 (25)
High anti - dsDNA antibody [c]	42 (23)

[a] using local laboratory cut-off 0.62 g/L at time of study assessment
[b] using local laboratory cut-off 0.14 g/L at time of study assessment
[c] using local laboratory cut-off 25 IU/ml at time of study assessment
[d] using local laboratory cut-off for IgM anti-cardiolipin antibody 20 IU/ml & IgG anti-cardiolipin antibody 10 IU/ml

10.1.3 Disease activity and damage scores

SLE patients were selected from an outpatient setting and therefore in general had low disease activity. The median SLEDAI score was 2 (0, 5) and in addition to the immunological components of the SLEDAI as highlighted in Table 77, 21% of patients had active arthritis, 15% had evidence of an inflammatory rash, 13% had a leucopoenia and 11% had mouth ulcers at the time of assessment. Eighteen (10%) patients had a SLEDAI score greater than 8. Figure 10 summarises the SLEDAI scores at the time of the assessment. Similar to the SLEDAI scores, the key BILAG items suggest some disease activity within the mucocutaneous, musculoskeletal and haematological systems (Table 8). Fifty-five patients reported previous or current fatigue, an

item not incorporated in the SLEDAI, and therefore contributed to the ‘general’ category of the BILAG grades.

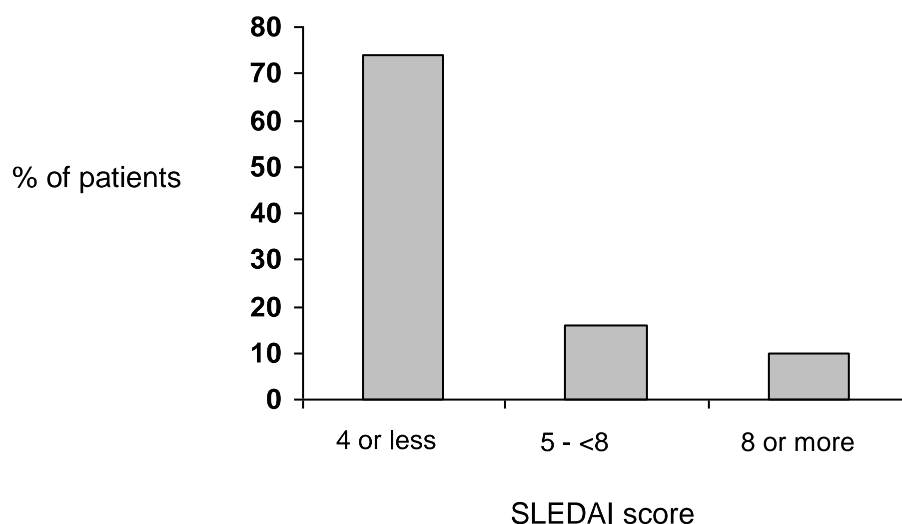


Figure 10 SLEDAI score distribution in SLE

Table 8 Distribution of BILAG scores and components

BILAG item	% of SLE patients with grade				
	A	B	C	D	E
General	2	4	26	-	68
Mucocutaneous	2	13	27	0.5	57.5
Neurological	-	1	6	-	93
Musculoskeletal	-	16	25	-	59
Cardiorespiratory	-	1	9	-	90
Vasculitis	0.5	1	21	-	77.5
Renal	-	5	-	-	95
Haematological	0.5	12	55.5	-	32

The median damage index as measured using the SLICC damage index (SDI) was 1 (0, 2). The most prevalent items of the SDI were scarring alopecia occurring in 31 (17%) of patients, cataract in 27 (15%) and cerebrovascular accident 20 (11%). The prevalence of SDI items amongst this cohort is

summarised in **Error! Reference source not found.**9. Fifteen (9%) of patients gave a past history of

malignancy and 6 of those patients specified carcinoma of the breast.

Table 9 Prevalence of SDI items

SDI item	n (%) patients
Ocular	
Cataract	27 (15)
Retinal damage	9 (5)
Neuropsychiatric	
Cognitive impairment	7 (4)
Seizures	4 (2)
Cerebrovascular accident	20 (11)
Neuropathy	5 (9)
Transverse myelitis	2 (1)
Renal	
GFR< 50%	4 (2)
Proteinuria >3.5g/24/ESRF	4 (2)
Pulmonary	
Pulmonary hypertension	0
Pulmonary fibrosis	4 (2)
Shrinking lung	4 (2)
Pleural fibrosis	2 (1)
Pulmonary infarction	0
Cardiovascular disease	
Angina/CABG	9 (5)
Myocardial infarction	5 (3)
Cardiomyopathy	2 (1)
Valvular heart disease	2 (1)
Pericarditis /pericardectomy	5 (3)
Peripheral vascular disease	
Claudication	2 (1)
Minor tissue loss	0
Major tissue loss	2 (1)
Venous thrombosis/stasis	13 (7)
Gastrointestinal disease	
Organ infarction/resection	9 (5)
Mesenteric insufficiency	0
Chronic peritonitis	0
Stricture/GIT surgery	4 (2)

SDI item continued	% patients
Musculoskeletal	
Muscular atrophy/weakness	2 (1)
Deforming/erosive arthritis	13 (7)
Osteoporotic fracture	4 (2)
Avascular necrosis	2 (1)
Osteomyelitis	2 (1)
Skin	
Chronic scarring alopecia	31 (17)
Extensive scarring	7 (4)
Skin ulceration	2 (1)
Premature gonadal failure	15 (8)
Diabetes	9 (5)
Malignancy	16 (9)

10.1.4 Lifestyle factors of SLE patients

Seventeen patients (9%) of patients were current smokers and the median number of cigarettes smoked per day was 9 (4.5, 20). Seventy-nine (43%) of patients admitted to alcohol consumption and the average number of units consumed was 2 (0, 8) with 16 patients admitting to an alcohol consumption of greater than the recommended 14 units for women. More than half the patients were post menopausal (103 patients) with an average age of menopause of 48 (41, 51) years. Thirty patients had a prior history of hysterectomy at an average age of 39 (35, 43) years. Ninety-three percent (169 individuals) of patients had a previous live birth. Lifestyle factors were ascertained via direct interview and pre-visit questionnaire. Table 10 summarises the lifestyle characteristics of SLE patients.

Table 10 Lifestyle factors of SLE patients

Social factor	n (%) patients
Smoker – ever	78 (43)
Current smoker	16 (9)
Ex-smoker	62 (34)
Alcohol consumption	78 (43)
College or university education	102 (56)
Hormone contraception use	31 (17)
Post menopausal	103 (57)
Hysterectomy	29 (16)
HRT use - ever	60 (33)
Pregnancy	149 (82)
1 or more live birth	139 (93)
1 or more miscarriage	40 (27)
1 or more termination	25 (17)
Marital status	
Single	24 (13)
Married/common law wife	124 (68)
Separated /divorced	24 (13)
Widowed	7 (4)

10.1.5 Treatment factors

Three quarters of patients of patients (76%) had used corticosteroids (CS) during their disease course and 45% were using CS during the study period. Current and previous CS dosages were moderately low. See Table 11 for details of SLE therapy.

Table 11 Details of therapy of SLE patients

Treatment	
Steroid – ever	139 (76)
Current steroid	82 (45)
Current daily steroid dose : median (IQR) mg	8 (5, 10)
Past steroid use	67 (37)
Past daily steroid dose : median (IQR) mg	10 (5, 15)
Anti-malarial current	87 (48)
Anti-malarial (any) – past	25 (14)
Hydroxychloroquine – current	71 (39)
Hydroxychloroquine – past	21 (12)
Azathioprine – current	35 (19)
Azathioprine – past	18 (10)
Mycophenolate – current	17 (9)
Mycophenolate – past	8 (4)
Cyclophosphamide – current	5 (3)
Cyclophosphamide – past	9 (5)
Methotrexate – current	14 (8)
Methotrexate – past	9 (5)
Cyclosporin – current	3 (20)
Cyclosporin – past	2 (1)

All values n (%) unless other stated

10.2 Description of control population

10.2.1 Demographics of controls

A total of 70 control females were recruited with a median (IQR) age of 51 (39, 60) (39, 60) years and 46% were under the age of 50 years. The age distribution of control control participants is described in

Figure 11. Similar to the patient population, the majority of controls were of White-Caucasian ethnicity and 8% were of Afro-Caribbean or Asian ethnicity. See Figure 12 for distribution of ethnicities.

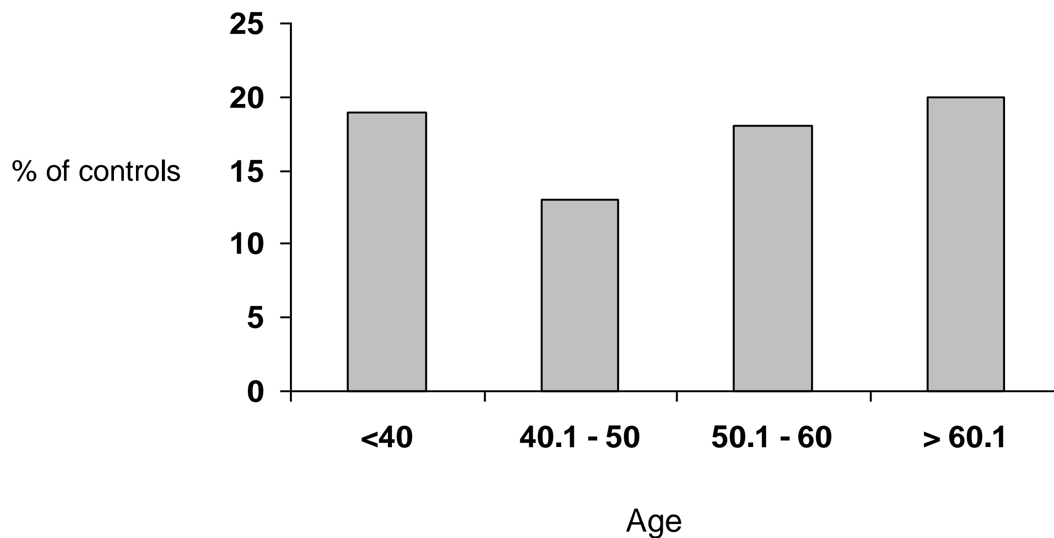


Figure 11 Age distribution of controls

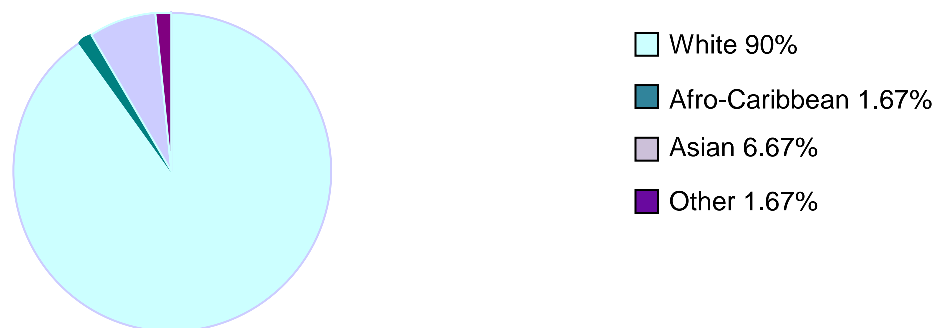


Figure 12 Ethnicity distribution of controls

10.2.2 Lifestyle factors of controls

SLE patients and controls were reasonably well matched with regard to social factors. Overall 50% of controls had ever smoked however a slightly higher percentage of controls remained current smokers (20%) were current smokers and the median number of cigarettes smoked per day was 15 (7, 9). Sixty-two (89%) controls admitted to alcohol consumption and the average number of units consumed was 8 (4, 10) with 10 (14%) of controls admitting to an alcohol consumption of greater than the recommended 14 units for women. Just less than half of the controls were post menopausal (31 individuals) with an average age of menopause of that was very similar to the SLE patients of 47 (40, 51) years. Ten controls had a prior history of hysterectomy at an average age of 40 (38, 43) years. Sixty percent (42 individuals) had a previous live birth. Table 12 summarises the lifestyle characteristics of the control population.

Table 12 Lifestyle factors of controls

Social factor	% controls
Smoker – ever	35 (50)
Current smoker	21 (30)
Ex-smoker	14 (20)
Alcohol consumption	62 (89)
College or university education	44 (63)
Hormone contraception use	46 (66)
Post menopausal	31 (44)
Hysterectomy	10 (14)
HRT use - ever	17 (24)
Pregnancy	46 (66)
1 or more live birth	28 (60)
1 or more miscarriage	17 (36)
1 or more termination	2 (4)

10.2.3 Discussion

SLE patients were recruited from existing databases in the Northwest of the UK and are representative of the ethnicity of that population. The majority of SLE patients (127/182) were recruited from a cohort of patients that participated in a previous study assessing factors contributing to carotid atherosclerosis and therefore recruited ‘older’ patients. As a results, the majority of the SLE patients included in this study were older than 55 years and therefore postmenopausal, in comparison to the control population which had a more even spread of participants across the age-range. This will limit the sample size of analyses of younger patients. Age-adjusted statistical analyses will be taken undertaken when comparisons are made with controls to account for the age differences. In keeping with other studies, all patients satisfied the 1997 ACR classification criteria for SLE. The SLE patients appear to be representative of a European cohort of SLE patients with respect to clinical features and antibody profiles. Cervera *et al*. previously reported the prevalence of clinical features in 1000 patients and observed arthritis in 84%, pleurisy in 36% and skin disease in 58% of patients. The reported prevalence of renal (39%) and neurological disease (27%) was slightly higher and oral ulceration (24%) slightly lower than observed in this cohort [59]. Previous studies have reported the expected frequency of anti-Ro to be 20-60%, anti-La 15-40%, anti-Sm 3-30% and anti-RNP 10-30% [5, 302]. ANA was not re-tested during the study period but historical information was gathered from hospital record review. The platform measuring anti-cardiolipin antibody titres was changed during the study period; as a result different titres were not comparable between patients. Anti-cardiolipin antibody was therefore classified as a positive or negative result according to the

suggested laboratory cut-off rather than defined as medium or high titres as specified in the classification of the APS. Lupus anticoagulant was not measured in all participants owing to prescription of medications that would interfere with the result. Lupus anticoagulant was therefore not used as a variable in the analyses.

The disease activity score is low as would be expected in patients recruited from an outpatient setting. The majority of patients had established disease with a median duration of 13 years and the average SDI of 1 (0, 2) is as would be expected. A mean (SD) SDI of 1.19 (1.59) at 10 years of disease duration and 1.9 (1.99) at 15 years has previously been reported [124]. We observed a higher rate of malignancies in this cohort at 9% compared to the estimates of 4-5% previously reported a Hungarian and an international cohort and this may represent geographical differences [28, 337]. Breast cancer was present in 6 (3%) of patients and this is slightly higher than a prevalence of 1.7% previously reported [29].

Less than half the patients were taking corticosteroids at the time of the study (45%) and the average daily dose in these patients was low at 8 (5, 10) mg. A similar number of patients were taking anti-malarial drugs but as expected in a cohort with low disease activity, less than 1/5 of patients were taking immunosuppressants such as azathioprine, mycophenolate and cyclophosphamide. Our findings are therefore unlikely to be an effect of disease severity or high inflammatory burden and more likely to be representative of a wider SLE population

Control subjects were chosen from a historical database or recruited from amongst friends and family of patients from the same geographical area, in an

attempt to limit demographic and lifestyle factors. Nonetheless, as might be expected, the prevalence of smoking, alcohol intake and hormone contraception use was increased in the control patients. Those factors that are less likely to be related to medications or medical advice such as further or higher education, prevalence of hysterectomies or miscarriages/terminations were remarkably similar between the groups (see Table 10 and Table 12).

The prevalence of cardiovascular risk factors is discussed below.

10.3 Cardiovascular risk factors, subclinical atherosclerosis and cardiovascular disease

10.3.1 Cardiovascular risk factors in SLE patients

A number of cardiovascular risk factors were assessed. Definitions are summarised in the methods chapter (section 9.4.1). Fasting blood glucose and lipid profiles were measured on the day of the visit. The comparison of cardiovascular risk factors between SLE patients and controls is summarised in Table 13. Definitions of high lipid levels are based on The Third Report of the National Cholesterol Education Program (Adult Treatment Panel III) [1]. As none of the measured values of triglycerides were very high i.e. above 4.5 mmol/L, LDL cholesterol was calculated using the Friedewald formula [111]. See Figure 13.

LDLc = total cholesterol – HDLc – (triglycerides/5)

Figure 13 Friedewald formula of LDL calculation

There was a significant difference between SLE patients and healthy controls with regard to age; therefore all further analyses are adjusted for age. The

proportion with hypertension, as defined by a blood pressure greater than 140/90 or current anti-hypertensive medications was significantly higher in the SLE patients. The proportion of individuals taking anti-lipid therapy was greater in the SLE group. Overall measures of total cholesterol and LDL cholesterol were higher in the control group. All these factors remained significantly associated after adjustment for age. Other traditional risk factors did not differ.

Table 13 Comparison of cardiovascular risk factors in SLE patients and controls

Cardiovascular risk factor	SLE patients (n=182)	Controls (n=70)	P value
Age at assessment: med (IQR) years	53 (46, 61)	50 (39, 60)	0.01
Hypertension: n (%)	66 (36%)	7 (10%)	<0.001
Systolic blood pressure: mean (SD)	129 (20)	124 (19)	NS
Diastolic blood pressure: mean (SD)	71 (10)	68 (16%)	NS
Hypercholesterolaemia: n (%)	74 (41%)	15 (21)	0.006
Total cholesterol: mean (SD)	4.7 (0.9)	5.1 (1.0)	0.0013
Anti-lipid therapy: n (%)	68 (37%)	5 (7%)	0.006
Triglycerides: mean (SD)	1.2 (0.6)	1.1 (0.7)	NS
LDL : mean(SD)	2.7 (0.9)	3.2 (1.0)	0.0002
HDL : mean(SD)	1.7 (0 .5)	1.7 (0.4)	NS
VLDL: mean(SD)	0.5 (0.3)	0.4 (0.3)`	NS
High cholesterol (>6.2 mmol/L): n (%)	9 (5%)	11 (16%)	0.004
High LDL (>4.1 mmol/L): n (%)	12 (7%)	14 (21%)	0.001
High triglycerides (>2.3 mmol/L): n (%)	8 (4%)	6 (9%)	NS
Low HDL (<1.03)	4 (2%)	0	NS
Diabetes mellitus: n (%)	10 (5%)	1 (1%)	NS
Fasting blood glucose : med (IQR)	4.5 (4.2, 5.1)	4.7 (4.5, 4.9)	NS
Family history of CHD: n (%)	61 (34%)	21 (30)	NS
BMI: med (IQR)	26 (23, 31)	25 (23, 28)	NS
Waist circumference : med (IQR)	91 (81, 100)	86 (79, 95)	NS
Waist:hip ratio : med (IQR)	0.88 (0.84, 0.93)	0.87 (0.82, 0.92)	NS
CRP	2.1 (0.8, 5.3)	1.9 (0.4, 3.9)	NS

10.3.2 Cross sectional assessment of carotid IMT and plaque

Control subjects had a trend towards a higher IMT compared to SLE patients (0.075 (0.122) vs. 0.062 (0 .014) cm, p=NS) and adjusting for age did not have a significant effect. A higher proportion of SLE patients had the presence of any carotid plaque compared to controls (45% vs. 23%, p=0.002). When adjusting for age controls remained less likely to have carotid plaque (OR 0.34, 95%CI 0.16, 0.77, p=0.009). Further, a higher proportion of SLE patients had medium or large-sized plaque (9.6% vs. 6%) although this was not a statistically significant difference. The age of SLE participants with plaque was higher than those without (59.0 *vs.* 47.6 years, p<0.0001) and therefore all further analysis of plaque has been adjusted for age at assessment. There were 6 controls and 26 SLE patients that were not of white Caucasian ethnicity but ethnicity did not have an association with carotid plaque.

10.3.2.1 Univariable associations of carotid plaque in SLE

The association of cardiovascular risk factors, present at the time of assessment, with plaque within the SLE population is summarised in Table 14. Traditional cardiovascular risk factors significantly associated with carotid plaque within the SLE population were hypercholesterolaemia and smoking. HsCRP was associated with a small increase risk of carotid plaque (with a one-unit increase in the hsCRP translating to a change in the odds for plaque of 1.10).

Table 14 Univariable analysis of traditional CHD risk factors associated with carotid plaque in SLE

Cardiovascular risk factor	Age-adjusted odds ratio	95% confidence interval
Hypertension (>140/90mmHG)/medication	1.57	0.75, 3.26
Hypercholesterolemia (>6.2mmol/L)/statin	2.13	1.01, 4.50*
Smoking – ever	2.41	1.17, 5.00*
Smoker – current	1.15	0.31, 4.23
Family history of CHD	1.13	0.51, 2.52
High BMI (>25 kg/m^2)	1.55	0.74, 3.25
Diabetes	2.20	0.31, 15.50
hsCRP	1.10	1.01, 1.21*
Systolic blood pressure	1.01	0.99, 1.03
Total cholesterol	0.68	0.46, 1.02

*factors significantly associated

A number of SLE-related factors were also considered and are summarised in Table 15. Factors analysed include the presence of any component of the ACR classification criteria and autoantibodies, at any time during the course of disease. Disease activity and damage indices analysed are as measured on the day of assessment. The uses of anti-malarials or immunosuppressants at any time of the disease course were also analysed. The current or past use of azathioprine was analysed separately in view of previous reports of an association with both subclinical and clinical atherosclerosis in SLE [7, 92, 143, 154].

Only two SLE patients had a prior history of vasculitis and therefore logistic regression using this variable was not possible. Similarly only one patient was anti-Sm antibody positive and therefore this variable was not

analysed. SLE- related factors were not significantly associated with carotid plaque in this analysis.

Table 15 Univariable analysis of SLE-related factors associated with carotid plaque in SLE patients

SLE-related risk factor	Age-adjusted odds ratio	95% confidence interval
Clinical features		
Malar Rash	1	0.49, 2.05
Discoid rash	0.36	0.10, 1.31
Photosensitivity	0.41	0.44, 1.85
Oral Ulcers	0.63	0.31, 1.30
Arthritis	0.91	0.43, 1.94
Serositis	0.70	0.34, 1.47
Renal disorder	1.93	0.80, 4.66
Neurological disorder	0.75	0.24, 2.30
Haematological disorder	0.71	0.35, 1.47
Vasculitis	-	-
Serology		
Anti – Ro antibody	0.53	0.23, 1.23
Anti – La antibody	0.58	0.19, 1.76
Anti – Sm antibody	-	-
Anti – RNP antibody	0.78	0.23, 2.63
Anti - cardiolipin antibody [d*]	0.77	0.29, 2.04
Lupus anticoagulant	1.82	0.79, 4.13
Measures of disease activity and damage		
Low C3 [a*]	2.84	0.23, 38.77
Low C4 [b*]	1.02	0.49, 2.09
High anti - dsDNA antibody [c*]	1.89	0.46, 3.08
SLEDAI score	0.97	0.89, 1.07
SDI	1.09	0.87, 1.37
Therapy		
Steroid ever	1.11	0.42, 2.91
Steroid current	1.07	0.51, 2.22
Anti-malarial use	0.65	0.31, 1.35
Any immunosuppressant use	1.74	0.82, 3.68
Azathioprine use	1.00	0.45, 2.26

*a-d as defined in Table 77

10.3.2.2 Multivariable associations of carotid plaque in SLE patients

Multivariable logistic regression was used to identify those factors independently associated with carotid plaque in SLE patients. Factors included in the stepwise regression model were those that were significantly associated in the univariable analysis namely, age at time of assessment, hypercholesterolemia, history of current or previous smoking and hsCRP.

Table 16 Multivariable analysis of factors associated with carotid plaque in SLE patients

Risk factor	Odds ratio	95% confidence interval
Age	1.15	1.09, 1.21*
Hypercholesterolemia	1.77	0.81, 3.88
Smoking – ever	3.34	1.49, 7.45*
hsCRP	1.12	1.02, 1.23*

*factors significantly associated

The factors remaining in the regression model i.e. independently associated with plaque were age, smoking and hsCRP but all except smoking had a small effect on the odds of plaque. See Table 16 for detail.

10.3.2.3 Univariable associations of carotid IMT (CIMT) in SLE

Traditional risk factors associated with CIMT in the cross-sectional study are summarised in Table 17. A standardised coefficient β (SE) is utilised to assess the association of factors with CIMT. Please see section 10.4.1 for further details about the β coefficient and it's interpretation. As would be expected, age and systolic blood pressure were significantly associated with CIMT but after correction for multiple testing, there was no significant association with other CHD risk factors.

Table 17 Univariable analysis of traditional CHD risk factors associated with carotid IMT in SLE

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
Age	.4689399 (.0000876)	<0.0001	-	-
Total cholesterol	.1911229 (.0011539)	0.012	.1169639 (.0010553)	0.096
Hypercholesterolemia	.0346571 (.0022235)	NS	-.0872572 (.0020598)	NS
Statin therapy	.0181806 (.002235)	NS	-.0803001 (.0020536)	NS
Systolic blood pressure	.3200345 (.000051)	<0.0001	.1605941 (.0000542)	0.037
Hypertension	.0448302 (.0022269)	NS	-.0471155 (.0020676)	NS
Anti-hypertensive therapy	.0473421 (.0022085)	NS	-.048331 (.002033)	NS
Fasting glucose	.0891071 (.001599)	NS	-.0110168 (.0001685)	NS
Type 2 diabetes	-.0489072 (.0051871)	NS	-.1180383 (.0046425)	NS
Smoking ever	.1519777 (.0021293)	0.043	.0956243 (.0019392)	NS
Body mass index	.057198 (.0001851)	NS	-.0110168 (.0001685)	NS

Table 18 Univariable analysis of SLE-related factors associated with CIMT in SLE patients

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
Renal disease	.1204848 (.0026112)	NS	-.0602664 (.0023664)	NS
Creatinine	-.0290138 (.0000204)	NS	-.0699325 (.0000265)	NS
SDI	.0519586 (.000656)	NS	-.0184051 (.0006308)	NS
SLEDAI	.0215067 (.0003008	NS	.0198833 (.0002734)	NS
C3	.1208456 (.0038758)	NS	.0511975 (.0036754)	NS
Ro	-.0536456 (.0025316)	NS	-.0479568 (.002284)	NS
La	.0232183 (.0033775)	NS	-.0009089 (.0030917)	NS
Sm	-	NS	-	NS
RNP	-.0873208 (.003616)	NS	-.0530032 (.0034286)	0.07
Anti-cardiolipin antibody	.0119319 (.0030863)	NS	.0163385 (.002772)	NS
Anti-malarial use	.0119319 (.0030863)	NS	.0163385 (.002772)	NS
Immunosuppressant use	-.1289854 (.002149)	NS	-.0090888 (.0020169)	NS
Azathioprine use	-.1082384 (.0023343)	NS	.0107513 (.0021814)	NS
Steroid use current	-.2291141 (.0022072)	0.003	-.1450267 (.0020464)	0.041
Steroid exposure ever	-.0674298 (.003058)	NS	.0033906 (.0028292)	NS
Steroid dose	.3414842 (.0000698)	0.002	.2918351 (.0000618)	0.003
Aspirin use	-.0278989 (.005908)	NS	.023949 (.0053002)	NS

A number of SLE –related factors were analysed for associations with CIMT and are summarised in Table 18. Eighty-two (45%) patients were taking oral steroids at the time of the study. The average dose of steroid used was low at 8 (5, 10) mg/day. Current steroid use had a significant and negative

correlation with CIMT suggesting that the use of low dose steroid may be beneficial with regard to CIMT. However, current steroid dose had a significant and positive correlation suggesting higher doses of steroid may be detrimental to IMT. The influence of baseline factors on CIMT is of much interest and discussed below.

10.4 Longitudinal assessment of carotid IMT and plaque

A number of SLE patients were followed longitudinally. A cohort of 200 white SLE females was recruited to a previous study and had measurement of cardiovascular risk factors, SLE-related factors, carotid plaque and IMT. The clinical and serological features of these patients is summarised in Table 19.

Table 19 Description of 200 SLE patients recruited to a previous study

Age: med (IQR) yrs	48 (41.5–56)
Age at diagnosis: med (IQR) yrs	36.4 (11.8)
Disease duration: med (IQR) yrs	11.7 (9.4)
Previous cerebrovascular event: n (%)	16 (8)
Previous coronary event: n (%)	7 (3.5)
Peripheral vascular disease: n (%)	1 (0.5)
Ever ANA positive: n (%)	188 (94)
Ever dsDNA positive: n (%)	115 (57.5)
Ever aCL or LAC positive: n (%)	73 (37)
Antiphospholipid syndrome: n (%)	21 (10.5)
Current steroid therapy: n (%)	106 (53)
SLEDAI-2K: med (IQR)	1 (0–4)
SLICC damage index: med (IQR)	0 (0–4)
Current anti-malarial therapy: n (%)	105 (52.5)
Current immunosuppressive therapy: n (%)	74 (37)
Postmenopausal: n (%)	94 (47)
Current smoker: n (%)	40 (20)
Ex-smoker: n (%)	57 (28.5)
Family history of premature CHD: n (%)	53 (26.5)
Total cholesterol: med (IQR) mmol/l	5.1 (4.3–6.0)
Fasting plasma glucose: med (IQR) mmol/l	4.6 (4.3–4.9)
Systolic blood pressure: med (IQR) mmHg	126 (116–140)
Previous or current hypertension: n (%)	83 (41.5)
BMI: med (IQR) kg/m^2	26.0 (23.2–30.1)

Adapted from Ahmad et al. [7]

Of those 200 patients, 127 participated in the current study and therefore had a repeated measurement of carotid plaque and IMT by the same two carotid scanning technicians. The 127 patients were followed longitudinally for a median (IQR) duration of 5.8 (5.2, 6.3) years, had a baseline median (IQR)

age of 49 (44, 56) years and disease duration of 11(4, 18) years. Seventy-three of the 200 patients initially recruited did not participate in the follow up study and outcomes of these patients are described in Table 20. One patient from the previous study did not fulfil SLE classification criteria and therefore did not qualify to participate in this study. Ten patients had died during the follow up period. There was one suicide, one patient suffered a ruptured aortic aneurysm, one had a cerebral haemorrhage, one had a gastric bleed and 4 patients had a malignancy (cervical, intracerebral, lung and liver). The cause of death was unknown in 2 patients.

Table 20 Outcome of 200 patients form previous study

Outcome of 200 patients	Number of patients
Change of address / no current contact details	42
Declined participation in follow up study	20
Deceased	10
Ineligible	1
Participated in follow up study	127

Of the patients that declined participation, one had a throat cancer, one had bladder cancer and another had a cerebrovascular accident. A number of patients declined participation due to social reasons (sick relatives, child care and employment). The patients that participated in the follow up study tended to be older but did not differ significantly to those that were not followed up with regards to age, disease duration, CHD risk factors or SLE-related factors.

A comparison of SLE patients followed up and those lost to follow up is summarised in Table 21.

Table 21 Baseline features of SLE patients followed up compared to SLE patients lost to follow up

Baseline factors	SLE patients followed up N=127	SLE patients lost to follow up N=73	P value
Age: median (IQR) yrs	49 (44, 56)	45 (37, 55)	NS
Disease duration : median (IQR) yrs	11 (4, 18)	7 (4, 16)	NS
Total cholesterol : mean (SD) mmol/L	5.1 (1.1)	5.3 (1.4)	NS
Systolic blood pressure: mean (SD) mmHg	132 (20)	125 (19)	0.03
Hypertension (%)	32	31	NS
Fasting glucose: mean (SD) mmol/L	4.5 (0.8)	4.8 (0.9)	NS
Smoking ever (%)	43	56	NS
CHD (%)	4	3	NS
Family history of CHD (%)	27	27	NS
Body mass index (kg/m^2)	27 (6)	27 (6)	NS
Renal disease (%)	13	23	NS
SDI: median (IQR)	0 (0, 1)	0 (0, 2)	NS
SLEDAI: median (IQR)	1 (0, 4)	0 (0, 2)	NS
Anti-cardiolipin antibody positive (%)	32	27	NS

10.4.1 Carotid IMT progression in SLE patients

The average baseline and follow up carotid (C) IMT displayed skewed distributions and therefore the median and IQR are used to describe these outcomes in contrast to the change in CIMT over time and the follow up time which were normally distributed and therefore the mean and standard deviation are used. Table 22 summarises the change in CIMT observed over time in 127 SLE patients. Whilst the majority of patients demonstrated some progression of IMT 8/127 had a regression of IMT. These 8 patients had a median baseline age of 53.5 (45, 60.5) years.

Table 22 Rate of CIMT progression in SLE

Baseline CIMT : median (IQR) cm	0.05 (0.04, 0.06)
Follow up Baseline CIMT : median (IQR) cm	0.06 (0.05, 0.07)
CIMT change : mean (SD) cm	0.012 (0.011)
Follow up time : mean (SD) yrs	5.72 (0.89)
Change in CIMT: mean (SD) cm/yr	0.002 (0.001)

CIMT change increased with age when stratified by age groups (see Table 23).

Table 23 Rate of change of CIMT in SLE stratified by age

Age : years	Change in CIMT : mean (SD) cm
<40 (n=6)	0.009 (0 .005)
40.1 – 50 (n=26)	0.011(0.008)
>50.1 (95)	0.013 (0.008)

Table 24 summarises factors associated with CIMT progression in SLE. Since baseline factors are measured in different units, to allow a more direct comparison between the strength of associations, the coefficients have been standardised. The β coefficient is not measured in units of the baseline factor but rather in standard deviations. This transformation into standard scores prior to running the regression model allows comparison of strength of coefficient/association between the variables. Total cholesterol had a small negative association with CIMT change over time in SLE i.e. a one standard deviation decrease in total cholesterol would yield a 0.19 standard deviation increase in the predicted IMT change.

Table 24 Univariable analysis of traditional CHD risk factors associated with CIMT progression in SLE

Baseline factors	Age-adjusted β (SE)	P value
Age	0.0462949 (0.0001032)	NS
Disease duration	-0.0809026 (0.0000996)	NS
Total cholesterol	-0.1944166 (0.0008191)	0.03
Hypercholesterolemia	0.0748241 (0.0028293)	NS
LDLc	-0.0782959 (2.9×10^{-6})	NS
Triglycerides	-0.062034 (2.0×10^{-6})	NS
Systolic blood pressure	-0.1727444 (0.0000467)	0.06
Hypertension	-0.0423764 (0.0020329)	NS
Fasting glucose	-0.1098229 (0.0012462)	NS
Type 2 diabetes	-0.014464 (0.0075498)	NS
Smoking ever	0.0530166 (0.0019157)	NS
CHD	0.0118195 (0.0015101)	NS
Family history of CHD	-0.0723708 (0.0021466)	NS
Body mass index	-0.1102952 (0.0001665)	NS
Metabolic syndrome	0.1755169 (0.0022396)	0.05
Waist circumference >88cm	-.0553981 (.0019987)	NS
HDL	-.0720337 (0.00002)	NS

Factors other than lower cholesterol associated with IMT progression were lower systolic blood pressure and the presence of the metabolic syndrome (NCEP ATP III panel defined metabolic syndrome in women as the presence of three or more of the following, increased waist circumference (>88 cm); elevated triglycerides (≥150 mg/dl); low HDL cholesterol (<50 mg/dl in women);

hypertension (≥130/≥85 mmHg); and impaired fasting glucose (≥110 mg/dl) [102]. Other traditional risk factors were not associated with IMT progression in this cohort of SLE patients.

Table 25 Univariable analysis of SLE-related factors associated with CIMT progression

Baseline factors	Age-adjusted β (SE)	P value
Renal disease	0.0633667 (0.001564)	NS
Creatinine	0.0032491 (0.0000163)	NS
SDI	-0.1834327 (0.0008044)	0.04
SLEDAI	0.0623867 (0.0004039)	NS
C3	-0.0307353 (0.0035119)	NS
C4	-0.1736865 (0 .0002623)	0.05
Ro	-0.0040715 (0.002221)	NS
La	0.0498219 (0.002762)	NS
Sm	-	-
RNP	0.0553044 (0.0031001)	NS
Anti-cardiolipin antibody	-0.0861927 (0.0020555)	NS
Anti-malarial use	-0.1204946 (0.0027453)	NS
Anti-platelet use	-0.0219427 (0.0020783)	NS
Statin use	0.0511282 (0.0031008)	NS
Steroid exposure ever	-0.0430254 (0.0022728)	NS
Average steroid dose	0.108774 (0.0001689)	NS
Total steroid dose	-0.0017469 (1.1×10^{-6})	NS

Amongst SLE-related factors, a lower total SDI score was associated with IMT progression. Other SLE–related factors were not associated with carotid plaque.

Table 26 Multivariable associations of IMT progression in SLE

Baseline factors	Fully-adjusted β (SE)	P value
Age	0.2025859 (0.0001119)	0.04
Systolic blood pressure	-0.2252302 (0.0000522)	0.03
Total cholesterol	-0.189022 (0.0008232)	0.04
Metabolic syndrome	0.1840898 (0.0021699)	0.04
C4	-0.1513526 (0.0002495)	NS
SDI	-0.181023 (0.000764)	0.03

In the multivariable analysis, after adjusting for age and traditional risk factors at baseline, a SDI remained significantly and negatively associated with IMT progression; suggesting a higher SDI correlates with reduced IMT progression. SeeTable 26. The items within the metabolic syndrome were examined further. IMT progression did not have a significant association with blood pressure criteria (age-adjusted β -.0622875, SE .0027712, p = NS), waist circumference as described above, high triglycerides (age-adjusted β -.0884884, SE .0029132, p = NS) or impaired fasting glucose (age-adjusted β -.0983361, SE .0062526, p = NS). Low HDLc did however have a significant association (age-adjusted β .246591, SE .0043239, p = 0.006) suggesting a low HDL correlates with increased IMT progression and is the major contributor to the metabolic syndrome association.

10.4.2 Carotid plaque progression in SLE

At the baseline assessment 34/127 (27%) of patients demonstrated at least one carotid plaque. At the follow up assessment 63/127 (50%) of patients demonstrated at least one plaque. Change in carotid plaque is summarised in Figure 14. Nearly half of the patients (59/127) had no plaque at either time point, 4 patients had a lower plaque index at the follow up assessment consisting of 2 patients that had one small plaque at the first assessment and no observable plaque at the second assessment and 2 patients that had one medium plaque (30-50 % of vessel diameter) at the first assessment and one small plaque (<30% of vessel diameter) at the second assessment.

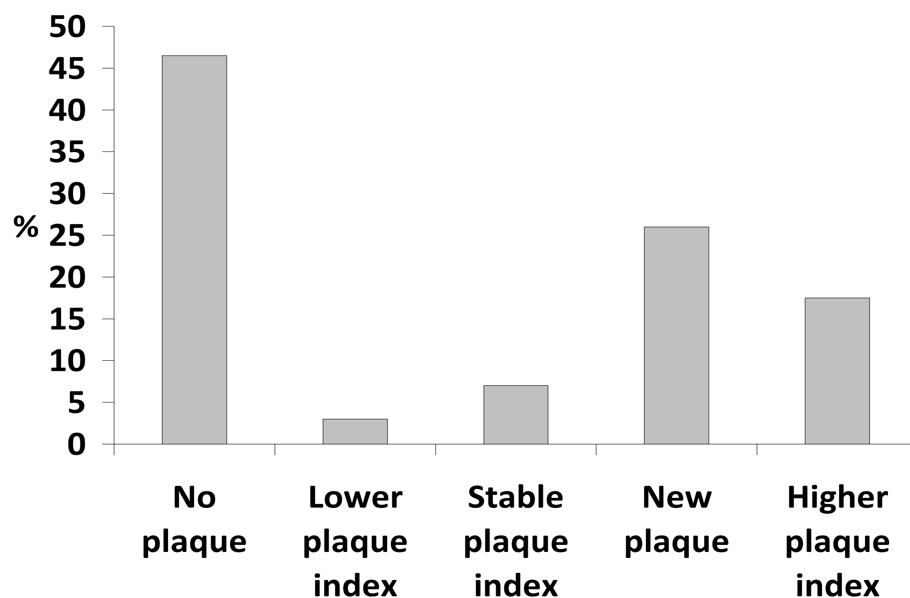


Figure 14 Comparison of baseline and follow up carotid plaque in 127 SLE patients

Thirty-three patients (26%) had no plaque at the baseline and had at least one plaque at the second assessment, 9 (7%) patients had a stable plaque

index and 22 (17.5%) had a higher plaque index at the second assessment. Figure 15 summarises the change of plaque index over time in those 22 patients that had an increased plaque index at the second time point. The majority of patients that demonstrated some plaque progression started with a grade 1 (one small plaque) or grade 2 (one medium plaque or multiple small plaque) and progressed to more extensive plaque.

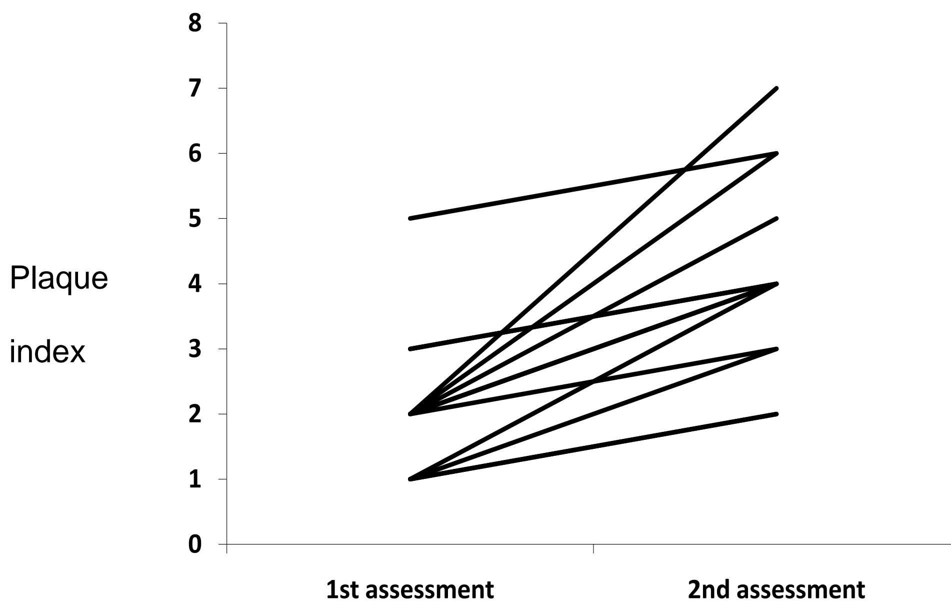


Figure 15 Change of carotid plaque index over time in SLE patients with a higher plaque index at the 2nd assessment

Factors associated with plaque progression, defined as the onset of new plaque or a higher plaque index at the second assessment are summarised in Table 27. Baseline age was significantly associated with plaque progression (OR 1.14, 95% CI 1.07, 1.20) and therefore all further analysis of plaque progression is adjusted for age.

Table 27 Univariable analysis of CHD risk factors: plaque progression (n=55) *vs.* no plaque (n=59) in SLE

Baseline factors	Age-adjusted odds ratio	95% CI
Disease duration	1.02	0.98, 1.06
Total cholesterol	1.07	0.70, 1.46
Hypercholesterolemia	4.87	0.98, 24.12
LDLc	1.07	0.65, 1.78
Triglycerides	1.45	0.71, 2.94
Systolic blood pressure	1.02	0.99, 1.04
Hypertension	0.99	0.38, 2.61
Fasting glucose	0.92	0.40, 2.91
Type 2 diabetes	0.64	0.03, 14.62
Smoking ever	1.95	0.83, 4.56
Family history of CHD	0.45	0.17, 1.18
Body mass index	1.04	0.97, 1.12
Metabolic syndrome	1.47	0.55, 4.02

*p<0.05

Four patients at the baseline assessment had a diagnosis of CHD and three of these patients were found to have a higher plaque index at the second assessment and one had a stable plaque index. Over the 5.8 (5.2, 6.3) years of follow up 4 patients developed a new CHD event. Of those 4 patients, 2 developed a new plaque and 2 had a stable plaque index. After adjustment for age baseline traditional CHD risk factors were not significantly associated with carotid plaque progression.

Table 28 Univariable analysis of SLE-related factors: plaque progression (n=55) *vs.* no plaque (n=59) in SLE

Baseline factors	Age-adjusted odds ratio	95% CI
Renal disease	1.55	1.52, 1.99
Creatinine	0.99	0.99,1.00
SDI	1.26	0.89, 1.80
SLEDAI	0.94	0.77, 1.13
C3	2.13	0.51, 8.95
C4	0.94	0.00, 6.41
Ro	0.27	0.10, 0.75*
La	0.34	0.15, 1.15
Sm	-	-
RNP	0.49	1.16, 2.05
Anti-cardiolipin antibody	3.92	1.41, 10.91
Anti-malarial use	0.36	0.09, 1.35
Anti-platelet use	0.64	0.25, 1.61
Statin use	8.38	0.99, 70.99
Steroid exposure ever	1.14	0.42, 3.06
Average steroid dose	1.01	0.94, 1.06
Total steroid dose	1.00	0.99, 1.00

*p<0.05

SLE–related factors did not have a significant association with carotid plaque with the exception of the anti-Ro antibody which appeared to have a protective effect (p=0.012). The analysis was repeated comparing those with no plaque at both assessments to those with new onset of plaque at the second assessment. Baseline age was associated with a 12% increase in the odds of new plaque with each year increment and therefore all factors were again adjusted for age.

Table 29 Univariable analysis of risk factors: new onset of plaque (n=33) vs. no plaque (n=59) in SLE

Baseline factors	Age-adjusted odds ratio	95% CI
Disease duration	0.99	0.94, 1.04
Total cholesterol	0.90	0.38, 1.39
Hypercholesterolemia	2.83	0.49, 16.49
LDLc	1.01	0.56, 1.81
Triglycerides	0.98	0.42, 2.30
Systolic blood pressure	1.02	0.99, 1.05
Hypertension	0.77	0.25, 2.31
Fasting glucose	1.29	0.51, 3.21
Type 2 diabetes	1.05	0.05, 22.22
Smoking ever	1.13	0.43, 2.96
Family history of CHD	0.45	0.15, 1.34
Body mass index	1.04	0.97, 1.13
Metabolic syndrome	0.90	0.28. 2.96
Renal disease	0.49	0.11, 2.14
Creatinine	0.98	0.94,1.02
SDI	1.15	0.76, 1.75
SLEDAI	0.95	0.78, 1.17
C3	1.93	0.38, 9.77
C4	0.93	0.00, 6.32
Ro	0.27	0.09, 0.86*
La	0.48	0.14, 1.72
Sm	-	-
RNP	0.21	1.02, 1.81
Anti-cardiolipin antibody	3.23	1.06, 9.89*
Anti-malarial use	0.38	0.09, 1.64
Anti-platelet use	0.43	0.14, 1.31
Statin use	3.51	0.33, 36.83
Steroid exposure ever	0.88	0.31, 2.54
Average steroid dose	0.97	0.89, 1.04
Total steroid dose	1.00	0.99, 1.00

*p<0.05

The Ro antibody was associated with a reduced risk of new onset of plaque (p=0.03) and the anti-cardiolipin antibody was associated with an increased risk of new plaque (p=0.04). After adjusting for age and traditional risk factors in a multivariable logistic regression model, these factors were no longer significantly associated with new onset of plaque.

10.4.3 Discussion

One-hundred and eighty-two patients with SLE and 70 healthy controls were recruited to a cross sectional study to examine carotid IMT and plaque. SLE patients have an increased age adjusted prevalence of hypertension compared to controls however, the average systolic blood pressure of SLE patients was within a normal range (129 [20] mmHg). Patients have lower total cholesterol and LDLc compared to controls and this is likely to be related to the use of statin drugs, reported in 41% of patients. The proportion of patients taking statins has increased significantly from 10% reported by Ahmad *et al.* previously and is likely to be due increased awareness of cardiovascular diseases in SLE, the older average age of the cohort and may be in part related to proposed guidelines for the management of risk factors published in 2003 [362]. The prevalence of diabetes in this cohort tended to be higher than in controls and was similar to that reported from the SLICC inception cohort [347].

SLE patients and controls do not differ significantly with regards to carotid IMT in this study. Of the traditional risk factors only systolic blood pressure correlates with CIMT in the cross sectional study. The carotid IMT in SLE patients is 0.075 (0.122) cm and this is higher than previously reported in cross sectional studies. Roman *et al.* reported an IMT of 0.06 (0.012) cm in SLE

patients compared to a significantly lower IMT in controls however, the mean age of patients in the Roman study was 44 (13) years and controls was 44 (12) years [290]. In contrast, the median age of patients in this study is 53 (46, 61) years and controls 50 (39, 60) and the difference in age between these studies may explain the discrepancy in the IMT noted. However, consistent with our findings, several other groups have reported a similar IMT between SLE and controls [172, 332, 374]. The lack of IMT thickness in SLE compared to controls is perhaps surprising in view of the abundance of literature in the general population demonstrating that IMT is an independent predictor of coronary events. However IMT has also been shown to correlate with and predict traditional CHD risk factors in the general population [61, 157] and the lack of correlation within SLE may be yet another reflection of the differences in aetiology of CHD within SLE. Of the disease related factors, only use of steroid was correlated to CIMT. Interestingly the use of any steroid was associated with a lower CIMT, however a higher dose was correlated to increased CIMT. This finding is in keeping with the theory that judicious use of steroids to control inflammation is beneficial but excessive use may lead to detrimental metabolic effects.

Carotid plaque prevalence in the current study is significantly greater than in controls and this is in keeping with previous studies [290]. CHD risk factors associated with carotid plaque are age, high cholesterol, smoking history and hsCRP. SLE related factors did not correlate to the presence of carotid plaque in this study. Multivariable analysis demonstrated an association with age, smoking and hsCRP independent for other factors. The association with hsCRP is particularly interesting since CRP is classically within normal laboratory limits

in SLE patients. Low level rise in hsCRP may reflect ‘grumbling’ subclinical inflammation better than currently used composite clinical scores used to detect disease activity. Previous studies have demonstrated the association of hypertension, diabetes, SDI, prednisolone use and immunosuppressant use [290] with plaque as well as hypertension and C3 levels [220]. The differences in factors associated with carotid plaque may be due to the differential influence of risk factors with increasing age or geographical difference as both previous studies were of North American patients. The differences in results may also be the result of a smaller sample size in the current study.

Two hundred SLE patients were followed longitudinal to assess the rate of progression and correlates of carotid atherosclerosis. Seventy-three of these patients were lost to follow and this may reduce the validity of the results, however as demonstrated in Table 21, that there are no significant differences with regard to age, CHD-related factors or SLE-related factors amongst the patients that were followed up and those that were not. We were not able to ascertain the cause of death in 2/10 patients however, of those where a cause of death was determined; none of the patients had a CHD event therefore reducing the risk of left censorship. Additionally, 20 patients declined participation in a follow up study due to a number of social or health related issues and none of these patients had a CHD event. The 127 patients that were followed up were assessed following a mean of 5.72 (0.89) years. The mean change in IMT was 0.002 (0.001) cm/year and was greatest in patients over the age of 50 years. A large study within the general population has previously estimated common carotid IMT progression at 0.0010 cm/yr and the current study would therefore suggest a greater rate of IMT progression in SLE patients

than would be expected in this age group [161]. IMT progression in this study is similar to previous estimates of IMT progression in SLE (between 0.0011 – 0.0039 cm/yr) [82, 289, 293]. However one recent controlled study has suggested that IMT progression in SLE is less than or similar to the control population [341]. In the current study, univariable factors associated with IMT change were total cholesterol, the metabolic syndrome, SDI and C4 levels. In the multivariable model, metabolic syndrome, and in particular the low HDLc component is associated with IMT progression.

Paradoxically, SDI was negatively correlated with IMT progression suggesting a higher SDI correlates with less IMT progression. One possible explanation is a surviving cohort effect i.e. those with higher SDI have left the cohort or alternatively may be a reflection of the small number of patients included in the multivariable model. Table 30 summarises previous longitudinal studies of carotid IMT and plaque progression. Age at diagnosis and C3 have previously been shown to be associated with carotid IMT progression but these factors could not be verified in the current study. Other factors previously associated with IMT progression were homocysteine levels and C5a levels but these factors were not studied in the current study.

Onset of new carotid plaque was observed in 26% of patients and a higher plaque index at the second assessment was observed in 17.5%. No plaque at either time point was observed in 46.5% and this is similar to previous studies within SLE. A general population-based study of women aged between 59 – 71 years demonstrated plaque progressed in 18% over 4 years [388] and by this estimate the progression observed in the current study is greater than would be expected in the general population. Plaque regression was noted in 3% of

patients and this compares to a previous study that demonstrated regression in 5% [341]. Those patients with plaque regression had a median (IQR) age of 55.5 (42.5, 61) years at the baseline and disease duration of 18 (11, 28) years. Two of the four patients were taking aspirin at the baseline assessment and none were taking a statin drug. In those patients with a higher plaque index at the second assessment, the magnitude of change was at least double over time in the majority of patients. Baseline age was the only factor associated with plaque progression and the anti-Ro antibody was associated with reduced risk of new plaque and may represent patients with a 'mild' phenotype. Interestingly, the point estimate for the anti-malarial use (0.38) was similar to the anti-Ro antibody estimate but did not reach significance. Anti-cardiolipin antibodies, defined as the presence of 2 positive results at any time during the disease duration, are associated with plaque progression with an odds ratio of 3. This association has not been found in other longitudinal studies. Baseline steroid exposure, cumulative steroid dose or immunosuppressant uses were neither predictive nor protective of carotid plaque progression in the current study.

Age has been repeatedly associated with atherosclerosis progression and this may reflect a longer duration of exposure of various risk factors and their cumulative effect on the vasculature. Of interest, of the 4 patients that developed a new CHD event over the follow up period, 2 had plaque at baseline whilst 2 patients did not. The very small number of patients with events restricts any comment about the predictive utility of carotid plaque in SLE.

A number of limitations need to be considered when interpreting these findings. The sample size in the current study of 127 patients is relatively small and whilst the average follow up period of over 5 years is longer than previous

studies, this is still a relatively short period of follow up. Plaque progression was analysed as a dichotomised variable and therefore didn't allow use of all of the data most efficiently. There is some debate about which measure of plaque progression to use in longitudinal analyses. It should be noted that risk factors for disease incidence may be different to those for disease progression or severity. In this regard, the most appropriate comparison groups may be those with no plaque *vs*. those with new plaque over time. To obtain meaningful results from such analysis would require long-term follow up of a large cohort of patients. The differences observed in this study with regard to IMT may reflect the differences in measurement as some previous studies have limited measurement to one part of the carotid artery [82]. Additionally, unlike previous studies, the current study was limited to white Caucasian females. In other studies up to 50% of patients were of none white Caucasian ethnicity and also included male patients.

Table 30 Summary of longitudinal studies of carotid IMT and plaque progression

Author	Baseline age : mean (SD) years	Average follow up period: years	Sample size	IMT progression: mean (SD) cm/yr	Plaque progression over study period	Factors associated with no plaque vs. new / more plaque or IMT* progression
Roman et al.[289]	-	2.8 (±0.75)	158	-	No plaque 48.5% New plaque 15.8% More plaque 12.7%	Age at diagnosis Disease duration Homocysteine level
Thompson et al. [341]	45.1 (10.3)	4.19 (±1.97)	217	0.0011 (0.003)	New / more plaque 27%	Higher serum C3 levels Immunosuppressant use
Rua-Figueroa et al. [293]	41.5 (11.7)	2.03 (range 1.49–2.82)	101	0.0039 (0.004)	New/more plaque 4%	Age at diagnosis * Homocysteine* C3 and C5a*
de Leeuw et al. [82]	37 (14)	2.7(±0.58)	52	0.0012 (0.004)	-	Age *

The current study has highlighted the difficulty in identifying risk factors for carotid atherosclerosis progression and would suggest factors other than traditional CHD risk factors and commonly measured SLE–related factors are contributing to atherosclerosis progression but these factors, as yet, remain to be elucidated. The repeated observation that age contributes to atherosclerosis progression in SLE and that atherosclerosis occurs at a younger age than would be expected in the general population is particularly interesting and lends support to the hypothesis that age-related vasculature changes are particularly marked in SLE patients and may be related to reduced regenerative capacity of the vasculature in these patients.

10.5 Endothelial progenitor cells (EPC)

Endothelial dysfunction has been shown to be an important trigger of atherosclerosis and maintenance of endothelial integrity is thought to be crucial to prevent atherosclerosis. Endothelial progenitor cells are bone marrow derived cells involved in repair and regeneration of the endothelium.

The objectives of this chapter are to:

a. Quantify EPC number in SLE

b. Identify factors associated with EPC in SLE

c. Describe relationship of EPC to atherosclerotic markers in SLE

10.5.1 Endothelial progenitor cell (CD34+CD133+) quantification protocol

10.5.1.1 Preparation of blood sample

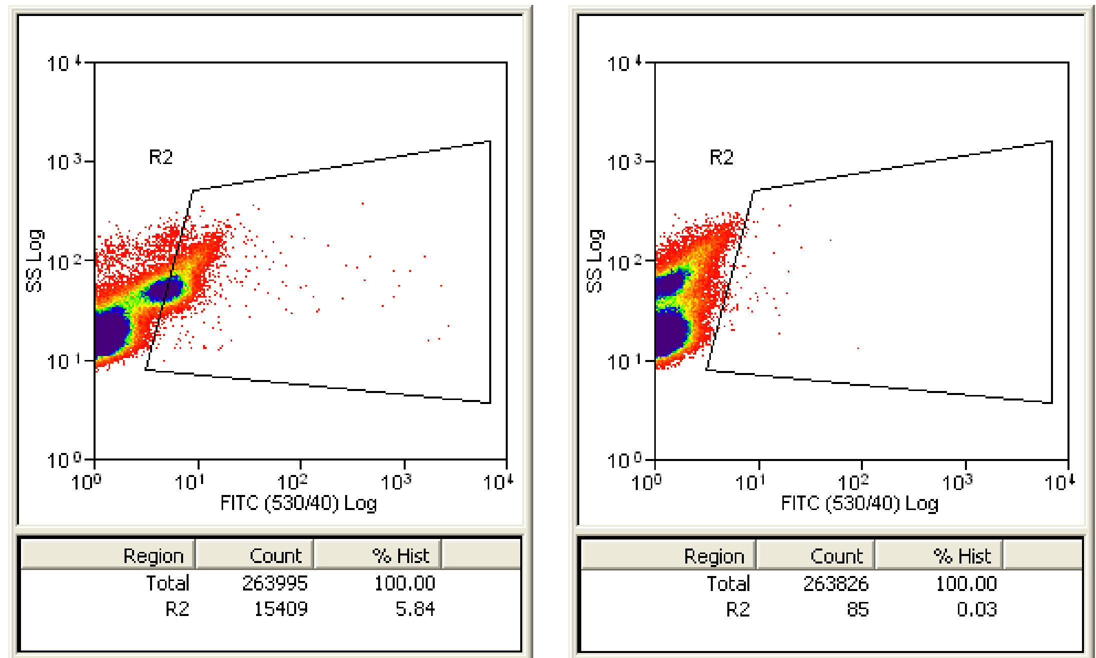
Peripheral venous blood (20 ml into EDTA containers) was drawn from study participants. An EPC isolation and enumeration protocol was adapted from a previously described technique [372] following a period of training at the protocol author's laboratory in Bonn, Germany. Fresh whole blood was diluted in a 1:1 ratio with phosphate buffer solution (PBS without calcium and magnesium x1, Lonza, Wokingham, UK). Diluted blood was then layered onto Lymphoprep (Cambrex Biosciences, Wokingham, UK) in a 2:1 ratio and centrifuged at 2000 rpm for 30 min. The PBMC layer was then harvested using a pastette as this population of cells contains the cells of interest. The cells harvested using this gradient preparation contained several cell types other than mononuclear cells when viewed under a microscope. In addition, a recent

publication would also suggest that cells harvested using Lymphoprep result in isolation of fewer CD34 positive cells compared to an alternative, Ficoll-paque PLUS (GE Healthcare, Little Chalfont, UK) [309]. Optimum results were obtained by layering diluted blood (as described above) onto Ficoll-paque (2:1) and centrifuging at 2400 rpm for 40 min, without breaks. PBMC were then washed in PBS, counted and divided into 10^6 cell aliquots.

10.5.1.2 Enumeration of circulating EPC using flow cytometry

Mononuclear cells were labelled with conjugated antibodies to identify surface antigens specific to EPC and analysed using flow cytometry using conjugated antibodies for CD34, CD133 and KDR. Initially, 10^6 cells were stained consecutively with flourescein isothiocyanate (FITC)-conjugated anti-human CD34 monoclonal antibody (mab) (Miltenyi Biotech, Germany), allophycocyanin (APC)-conjugated anti-human KDR mab (R & D Systems, USA) and phycoerythrin (PE)- conjugated anti-human CD133 (Miltenyi Biotech, Germany) as described previously [372]. A 10^6 cell aliquot was also stained consecutively with isotype control antibodies (FITC-conjugated anti-mouse IgG2a antibody (Miltenyi Biotech, Germany), PE-conjugated anti-mouse IgG2b (Miltenyi Biotech, Germany) and APC-conjugated anti-mouse IgG1antibody (R & D Systems, USA) for comparison. Unlabelled cells were also analysed to assess the autofluorescence of the cells. Initial analysis using this protocol resulted in a high degree of non-specific binding. Density plot 1 of Figure 16 demonstrates the characteristics of unlabelled cells. Each dot represents a single cell or event on this two parameter histogram. The x-axis of this

histogram represents increasing levels of fluorescence detected. The y-axis represents the amount of side-scattered light from each cell.



1. Flow cytometry analysis of unlabelled PBMC - very few events positive (in the boxed region)

2. Flow cytometry analysis of PBMC stained with isotype controls – many cells staining non-specifically

Figure 16. Flow cytometry analysis of PBMC

A series of experiments were carried out altering several factors consecutively in order to eliminate non-specific binding with all three antibodies separately. Samples were incubated for 5, 10, 15 and 20 minutes in separate experiments. Samples were also incubated using differing volumes of antibody namely 5ul, 10ul and 15ul. Optimal results for the CD34 and CD133 antibodies were obtained by making the following changes to the protocol:

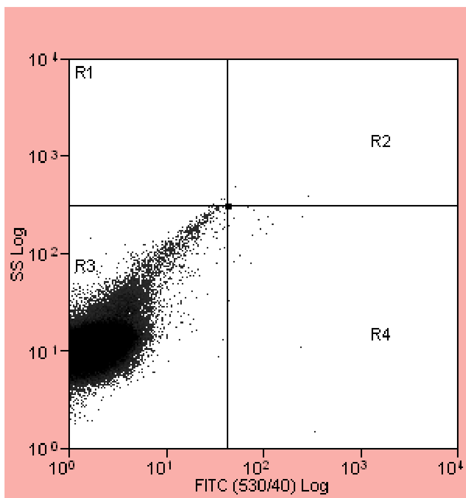
a. Pre-treating the sample with an Fc blocking agent (Miltenyi Biotech, Germany) to saturate non-specific antibody binding sites (10ul for 10 min)

b. Simultaneously incubating with antibodies (for 10 min) rather than consecutively

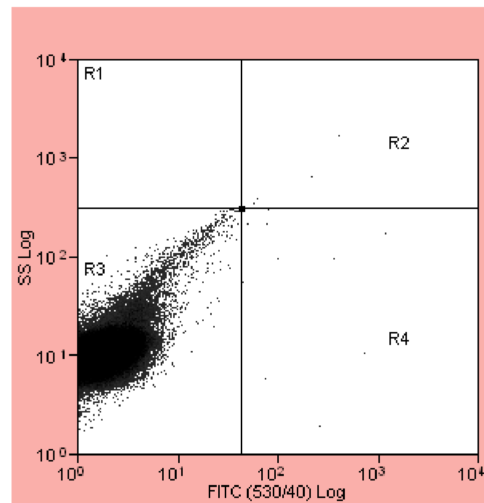
c. Staining with smaller volumes of antibody (5ul rather than manufacturer's recommended volumes)

Simultaneously incubating with CD34 and CD133 antibodies did not change yield of cells enumerated using flow cytometry.

Figure 17 shows a representative result of analysis on cells using an optimised protocol with several altered parameters and demonstrates very little difference between the unlabelled and isotype – control labelled cells, indicating that events detected using the positive conjugated-antibodies represent genuine cells with the surface antigen of interest.



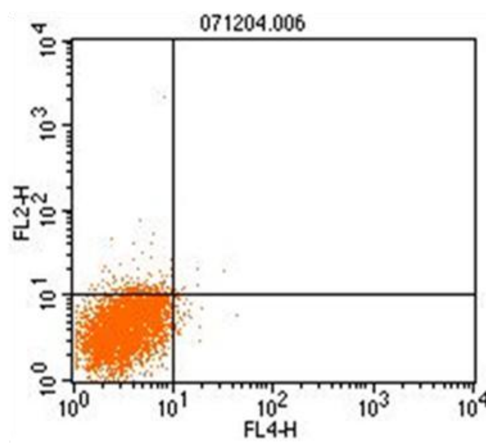
Flow cytometry analysis of unlabelled cells



Flow cytometry analysis of cells labelled with isotype control antibody (FITC and PE)

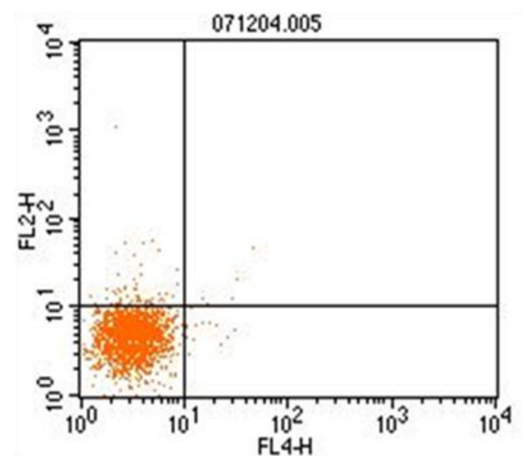
Figure 17 Flow cytometry of PBMC with optimised protocol for CD34 and CD133

A number of protocol modifications were made using the KDR antibody including differing the incubation time and temperature as well as the volume of antibody used. However, as demonstrated in Figure 18, results could not reliably and consistently identify antibody-labelled cells distinct from isotype control-labelled cells. At the time of the study conjugated anti-human KDR mab was not available from any other manufacturer and therefore KDR labelling was not used in further experiments.



Quad	% Gated	X Mean	Y Mean
UL	3.27	3.63	34.30
UR	0.26	27.20	20.75
LL	95.73	3.37	4.86
LR	0.74	15.74	5.63

Flow cytometry analysis of cells labelled with isotype control antibody (APC)

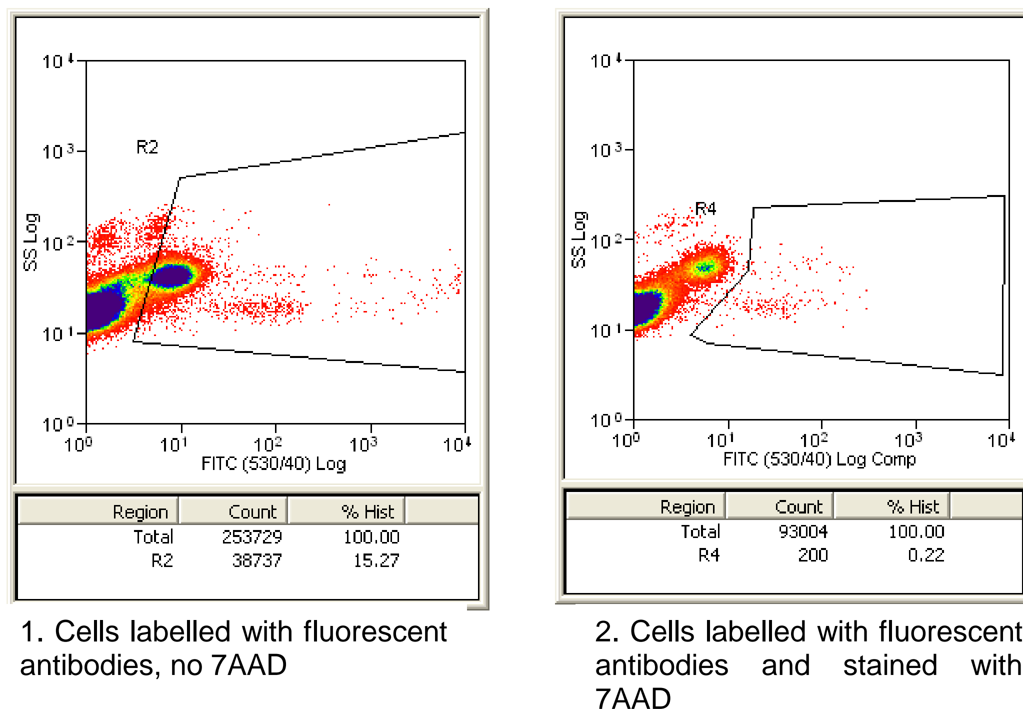


Quad	% Gated	X Mean	Y Mean
UL	4.88	4.83	24.23
UR	0.21	14.74	14.18
LL	93.83	3.54	4.46
LR	1.07	12.63	6.32

Flow cytometry analysis of cells labelled with APC-conjugated KDR antibody

Figure 18 Flow cytometry analysis using KDR antibody after protocol modifications

In addition, a number of events were detected with a very high degree of fluorescence (far right quadrant of density plot 2 of Figure 16). These events are likely to represent clumped debris or dead cells. Incubation of cells with 7 amino-actinomycin D (Sigma Aldrich, UK), a DNA intercalater, which is excluded by viable cells (0.4ul incubated for 20 min), was carried out and enabled these cells to be excluded from the flow cytometry analysis.



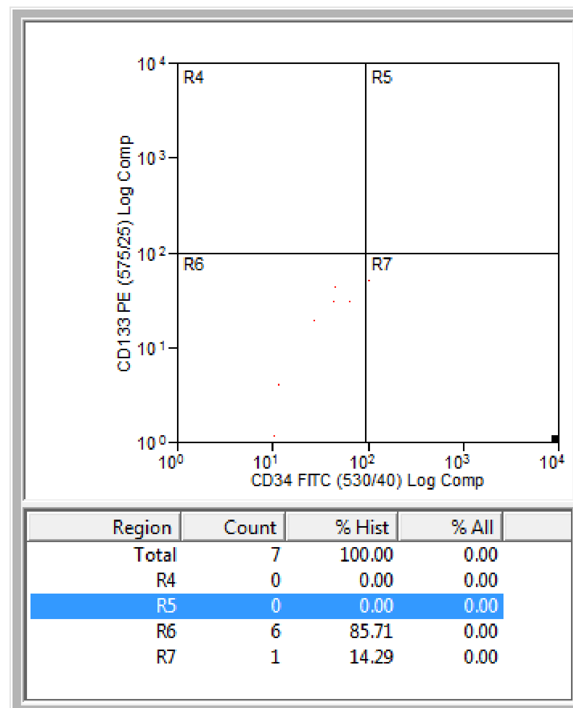
1. Cells labelled with fluorescent antibodies, no 7AAD

2. Cells labelled with fluorescent antibodies and stained with 7AAD

Figure 19 demonstrates density plots of cells labelled with antibodies with and without the addition of 7 AAD.

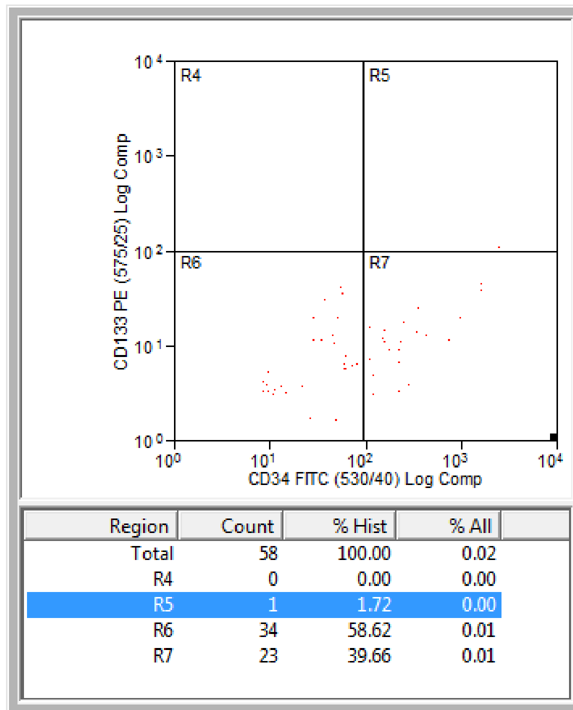
Figure 19 demonstrates density plots of cells labelled with antibodies with and without the addition of 7 AAD. The second density plot in Figure 20 demonstrates fewer events in the extreme of the x-axis when cells that have stained with 7 AAD are accounted for. All participant samples were aliquoted and 10^6 cells were stained with 2 antibodies separately (CD34 & CD133) as well as in dual combination to allow compensation of the wavelength of light detected with flow cytometry as well with isotypes (separately and dual combination) and compared to an unlabelled cell sample prior to enumeration of

CD34 + CD133 + cells. Flow cytometry analysis was undertaken within one hour of cell labelling. All PBMC were analysed including the lymphocyte and monocyte populations as current literature would suggest that EPC have physical characteristics intermediate to these populations [296]. A comparison of numbers of EPC between SLE patients and age (within 10 years) and gender-matched non-SLE controls were undertaken assessing the numbers of CD34+ CD133+ cells. Figure 20 demonstrates analysis of participant (JB). The samples have been gated to exclude 7-AAD positive events and to include those cells identified within the gate of plot 2Plot 1 of Figure 20 demonstrates the autofluorescence of unlabelled cells; plot 2 demonstrates cells labelled with isotype control antibodies and plot 3 demonstrates cells labelled with both antibodies simultaneously.



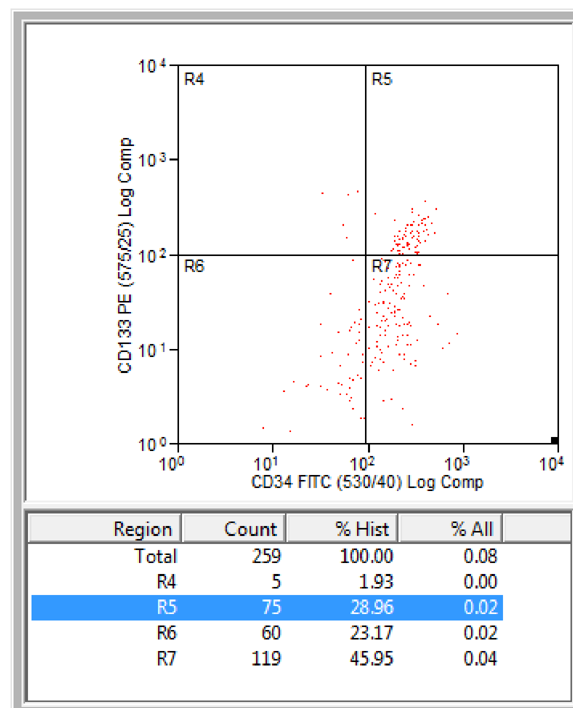
Region	Count	% Hist	% All
Total	7	100.00	0.00
R4	0	0.00	0.00
R5	0	0.00	0.00
R6	6	85.71	0.00
R7	1	14.29	0.00

1. Unlabelled cells



Region	Count	% Hist	% All
Total	58	100.00	0.02
R4	0	0.00	0.00
R5	1	1.72	0.00
R6	34	58.62	0.01
R7	23	39.66	0.01

2. Cells labelled with isotype control antibody (FITC and PE)



Region	Count	% Hist	% All
Total	259	100.00	0.08
R4	5	1.93	0.00
R5	75	28.96	0.02
R6	60	23.17	0.02
R7	119	45.95	0.04

3. Cells labelled with FITC-conjugated CD34 mab and PE-conjugated CD133 mab

Figure 20 Flow cytometry analysis of PBMC gated for 7-AAD negativity. R5 demonstrates CD34+CD133+ cells of interest

The density plots of Figure 20 are able to show that the optimising steps have ensured that only cells positive for the surface markers of interest have been identified and enumerated. CD34+ CD133+ cells are identified in the R5 gate of plot 3. The percentage of CD34+ CD133+ cells in R5 are used in the analysis.

10.5.2 Endothelial cell colony-forming units protocol

Endothelial cell number and function were assessed by quantifying the number of CFU formed in culture. The protocol used was adapted from techniques described by Asahara [12] and Hill [152]. PBMC were separated from 20 ml of venous blood using a ficoll gradient as described above. The recovered cells were washed once in PBS and once in supplemented endothelial media (EGM®-2 Endothelial Cell Growth Medium-2, Lonza). To avoid contamination with mature endothelial cells and other cell types, PBMC were pre-plated on a 12 well fibronectin-coated plate (Becton Dickinson Biosciences, UK) in 1ml of media. After 48 hours, 10^6 non-adherent cells were then washed in endothelial media and re-plated on a 24 well fibronectin-coated well (Becton Dickinson Biosciences, UK) in 500ul of supplemented media. Optimal results were obtained with media change every 3 days. Initial supplementation with 2% foetal bovine serum (FBS) resulted in suboptimal colony formation at day 7 (Figure 21). Supplementation with 20% FBS resulted in enhanced CFU formation (Figure 22). Colonies are counted manually at day 7. A colony is defined as a clump of cells with cells radiating from the peripheries [152].

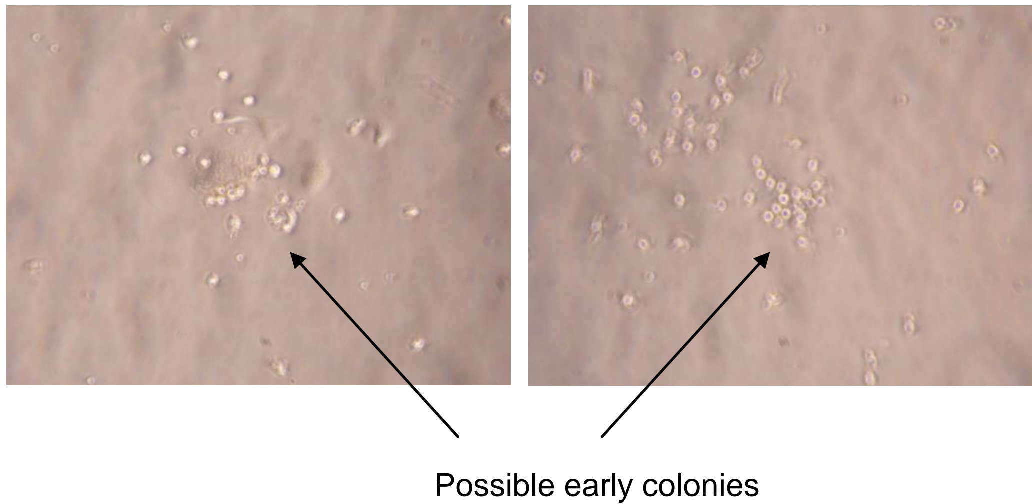


Figure 21 CFU enumeration using 2% FBS supplementation to media

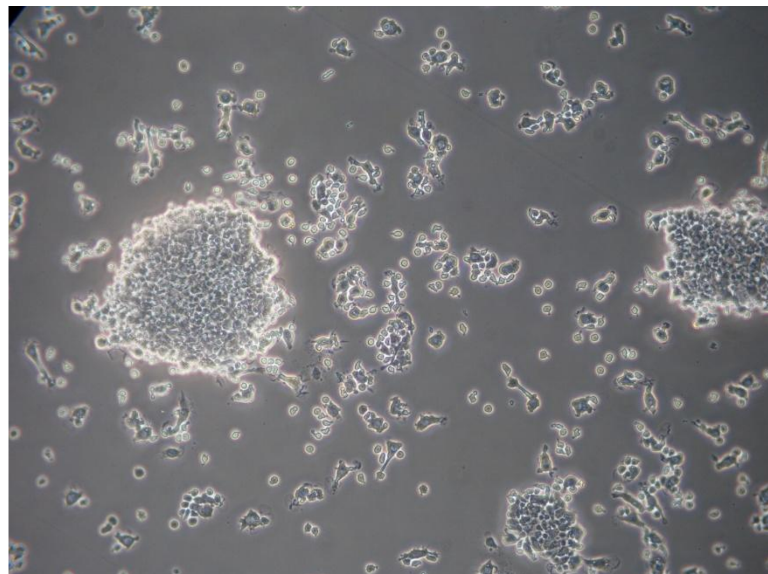


Figure 22 Endothelial colony-forming units (CFU) at day 7

CFU were characterised using immunohistochemistry to demonstrate origin from endothelial cells. Blue fluorescent DAPI (DAPI™ nucleic acid stain, Lonza) was used to stain nuclei as previously described [176]. CFU were stained with PE Mouse Anti-Human CD31 (PECAM-1, BD Biosciences) as

CD31 is highly expressed on endothelial cells as well as platelets and neutrophils. CD31 is thought to play an important role in transendothelial migration of leukocytes in inflammatory responses [246, 252]. CFU were also stained with acetylated low density lipoprotein, labelled with 1,1\'-dioctadecyl –3,3,3\',3\'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL). DiI-Ac-LDL labels vascular endothelial cells and macrophages. Within labelled cells the lipoprotein is degraded by lysosomal enzymes and the DiI (fluorescent probe) accumulates in the intracellular membranes [126, 359]. Figure 23 demonstrates staining of CFU with DiI-Ac-LDL. Figure 24 and Figure 25 demonstrate positive staining with CD31.

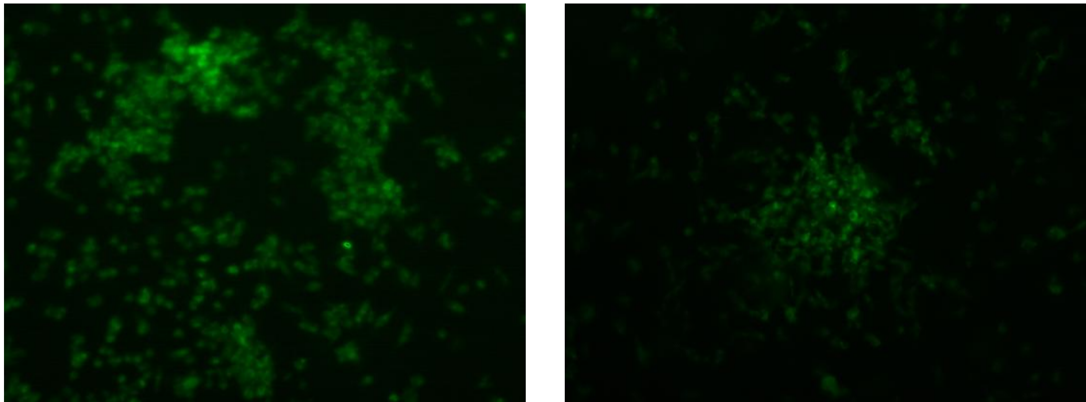


Figure 23 CFU stained positively with DiI-Ac-LDL (green) at day 7 (x20)

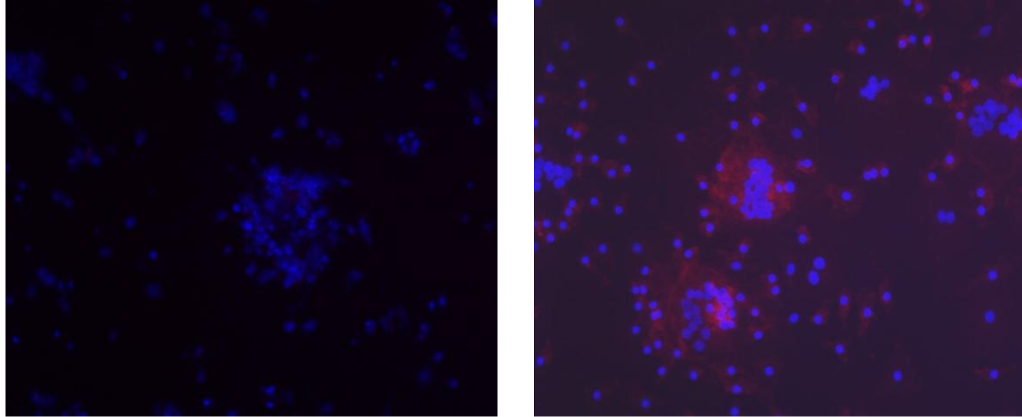


Figure 24 CFU with nuclei stained blue with DAPI (x20)

a. **CFU cells stained with control antibody (negative)**
b. **CFU stained with CD31 antibody (positively stained red)**

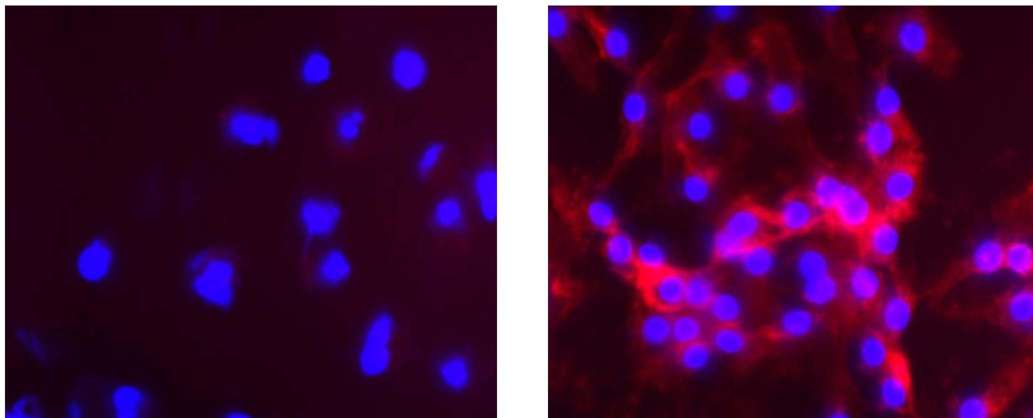


Figure 25 CFU with nuclei stained blue with DAPI (x63)
a. **CFU cells stained with control antibody (negative)**
b. **CFU stained with CD31 antibody (positively stained red)**

The isolation of PBMC prior to plating was used to exclude other cell types prior to culture. Subsequent staining of CFU with both diI-Ac-LDL and CD31 was accepted as evidence of endothelial cells origin.

10.5.3 CD34+ CD133+ EPC number in SLE and controls

EPC were quantified using the above protocol in 54 SLE patients and 49 controls. The characteristics of SLE patients and controls are summarised in Table 31. SLE patients were older than controls and therefore further analysis will be age-adjusted. The overall characteristics reflect those of the entire cohort.

Table 31 Characteristics of SLE patients and controls included in EPC analysis

	SLE patients (n=54)	Controls (n=49)	P value
Age at assessment: med (IQR) years	53 (47, 59)	50 (31, 59)	0.03
Hypertension: n (%)	15 (28)	4 (9)	0.012
Systolic blood pressure: mean (SD)	130 (22)	123 (20)	NS
Hypercholesterolaemia: n (%)	25 (46)	10 (20)	0.006
Total cholesterol: mean (SD)	4.7 (1.1)	5.1 (1.0)	NS
Diabetes mellitus: n (%)	2 (3)	0	NS
Fasting blood glucose : med (IQR)	4.8 (4.4, 5.2)	4.6 (4.5, 4.8)	NS
Family history of CHD: n (%)	18 (36)	14 (33)	NS
BMI: med (IQR)	26 (25, 31)	25 (23, 28)	NS

The number of CD34+ CD133+ cells did not differ between SLE patients and controls (Figure 26). CD34+ CD133+ cells did have a significant correlation to age with a small regression coefficient of 0.0006 (p=0.006). After adjusting for the difference in age at the time of assessment , there was no significant difference between SLE patients and control with regard to number of CD34+CD133+ cells (R^2 = -0.005, p=NS). Additionally, there was no difference in number of CD34+CD133+ cells in those participants under the age of 50 years (Figure 27).

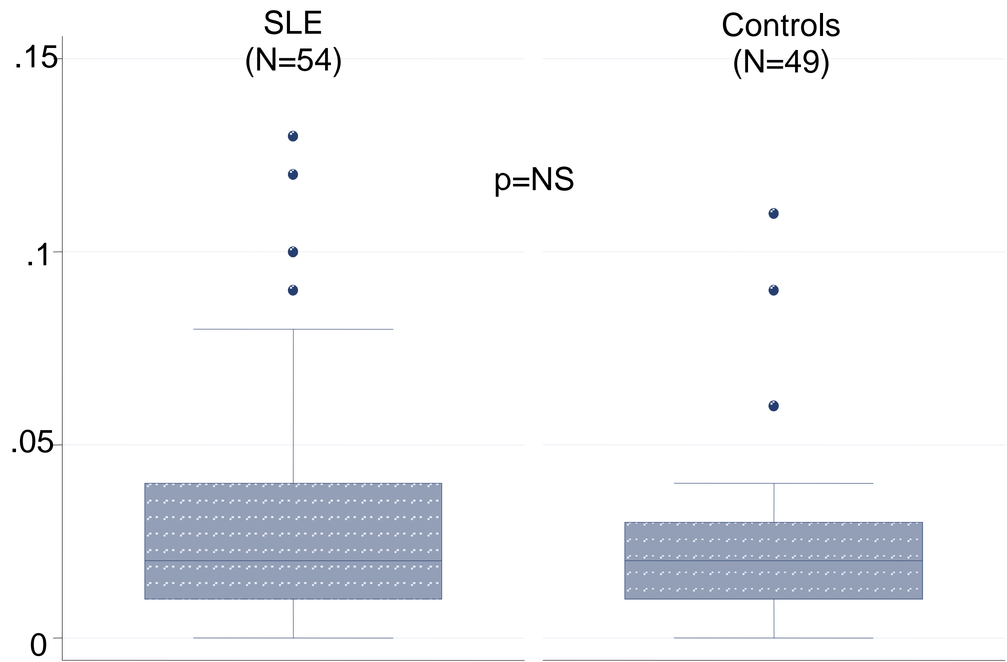


Figure 26 No significant difference of CD34+ CD133+ cell number between SLE patients and controls

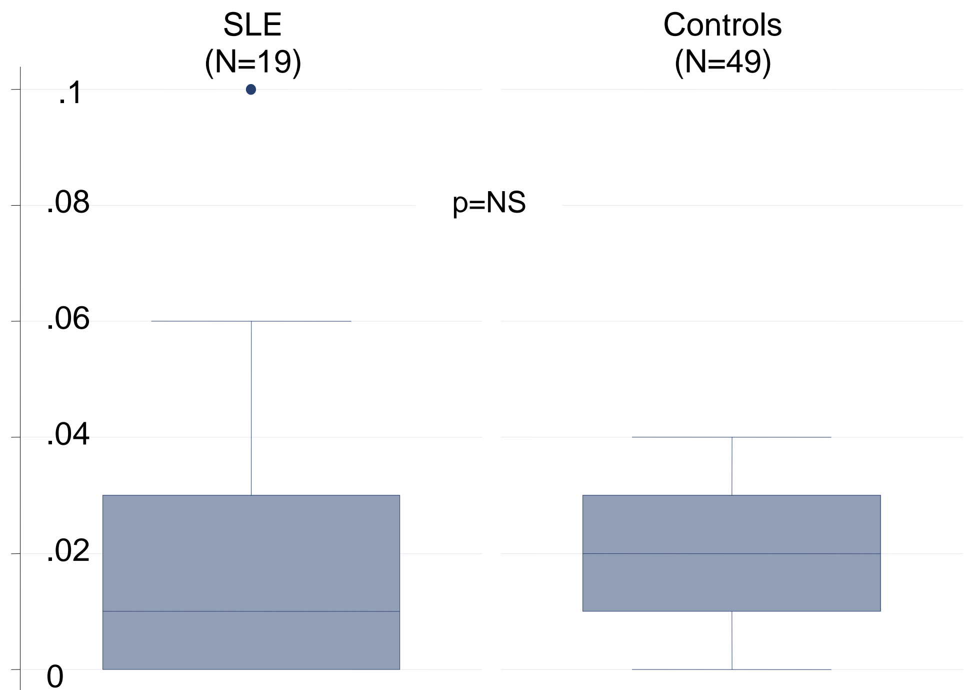


Figure 27 No significant difference of CD34+ CD133+ cell number between SLE patients and controls less than age 50 yrs

10.5.3.1 Factors associated with EPC

Factors associated with EPC in SLE were determined. Of interest, EPC number in controls was significantly correlated to age (β 0.33 (0.0002), p = 0.02) in contrast to SLE patients, where there was no correlation (β 0.22 (0.0004), p = 0.12).

Table 32 Traditional risk factors associated with CD34/CD133+ EPC in SLE

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
Age	.2187042 (.0004348)	NS	-	-
Total cholesterol	.0270632 (.0028849)	NS	-.0390989 (.0029037)	NS
Hypercholesterolemia	.2484947 (.0060021)	0.01	.1664164 (.006553)	NS
Statin therapy	.0828812 (.0100547)	NS	.0564684 (.010117)	NS
Systolic blood pressure	.2138145 (.0001397)	0.033	.1074157 (.0001556)	NS
Hypertension	.1846548 (.0075668)	NS	.1242826 (.0076618)	NS
Anti-hypertensive therapy	.16305 (.0098648)	NS	.1344704 (.0100758)	NS
Fasting glucose	.1358739 (.0042327)	NS	.0591029 (.0043744)	NS
Type 2 diabetes	-.0022219 (.0233291)	NS	.0184777 (.0235775)	NS
Smoking ever	.1689538 (.0058025)	0.08	.1468692 (.0058145)	NS
Body mass index	.0426935 (.0005145)	NS	-.0301414 (.0005203)	NS

Hypertension and systolic blood pressure both correlated with EPC in the unadjusted analysis but were no longer associated when adjusted for age. Other traditional cardiovascular risk factors were not correlated to EPC number in SLE patients (Table 31).

Table 33 Disease-related factors associated with EPC number in SLE

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
Renal disease	.0475128 (.0091376)	NS	.0555139 (.0089372)	NS
Creatinine	.0223855 (.0000931)	NS	.0188541 (.0000914)	NS
SDI	.0116419 (.0024569)	NS	.0422901 (.0025584)	NS
SLEDAI	.1316361 (.0013507)	NS	.1472096 (.0013569)	NS
C3	-.1353044 (.013717)	NS	-.1922454 (.0145901)	NS
Ro	.1239281 (.008498)	NS	.0796516 (.0084726)	NS
La	.1040494 (.0098589)	NS	.0826437 (.0096874)	NS
Sm	-	NS	-	NS
RNP	-.1781443 (.0149481)	NS	-.1772241 (.0145911)	0.07
Anti-cardiolipin antibody	.0920775 (.0099209)	NS	.0773991 (.0096769)	NS
Anti-malarial use	.0641122 (.0065244)	NS	.0038135 (.0066523)	NS
Immunosuppressant use	.2011236 (.0063224)	NS	.1803834 (.0063672)	NS
Azathioprine use	.0635077 (.008045)	NS	.0655233 (.0079106)	NS
Steroid use current	.1684823 (.0073626)	NS	.1417321 (.0072287)	NS
Steroid exposure ever	.1756041 (.0057666)	NS	.1574951 (.0058799)	NS
Aspirin use	-.0595755 (.0150974)	NS	-.0478631 (.01484)	NS

SLE-related factors were not correlated with EPC number. Furthermore, EPC did not correlate with aortic stiffness as measured by aortic pulse wave velocity β (SE) 0.011 (0.006), p=NS), CIMT (β (SE) -0.17 (0.51), p=NS) or plaque (β (SE) 0.09 (0.01), p=NS).

10.5.4 CFU in SLE

CFU were enumerated in 39 SLE patients with a median (IQR) age of 53 (46, 58) years and 27 controls aged 43 (28, 55) years. SLE patients formed significantly fewer CFU compared to healthy controls. The median (IQR) of CFU in SLE was 5.7 (2.3, 8.0) and in controls was 10.0 (5.7, 15.0); p= 0.0016 as demonstrated graphically in Figure 28.

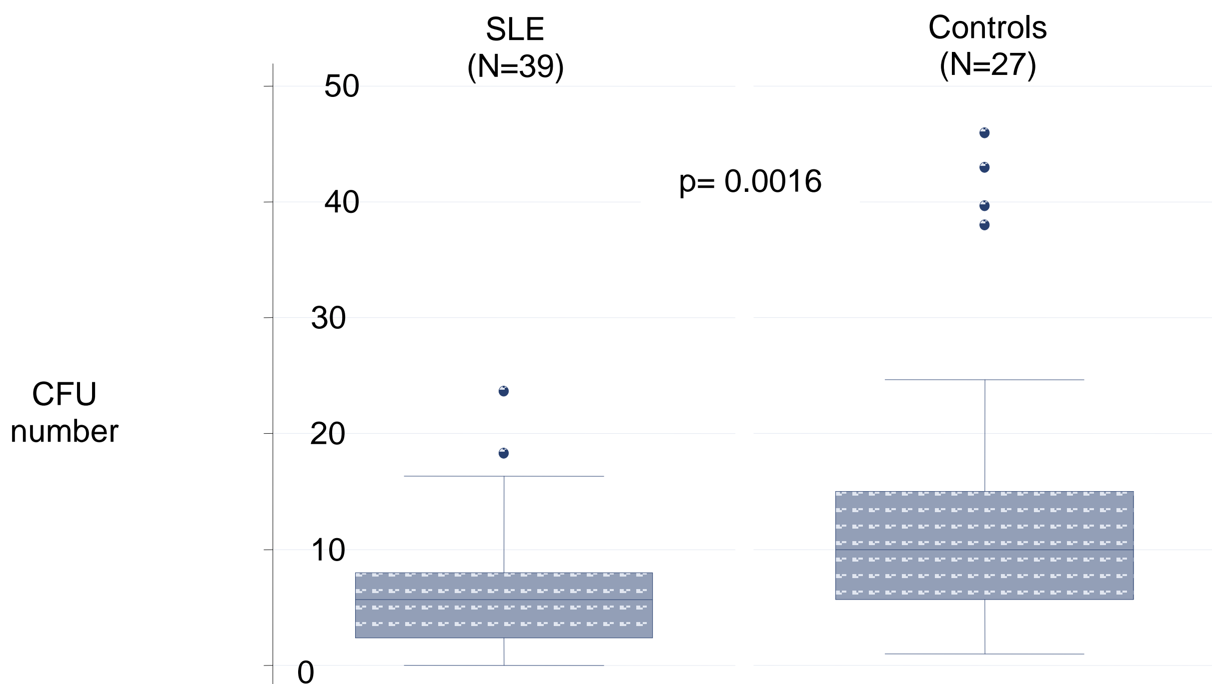


Figure 28 Mean CFU number lower in SLE compared to controls

The difference in number of CFU between SLE patients and controls remained significant after adjusting for age; β (SE) 0.40 (2.49), p=0.002. The difference in the number of CFU was particularly marked amongst the 6 SLE patients and 12 controls under the age of 40 years; median CFU 4 (2, 8) vs. 10.5 (7, 19), p= 0.03 and less marked in those over the age of 50 years

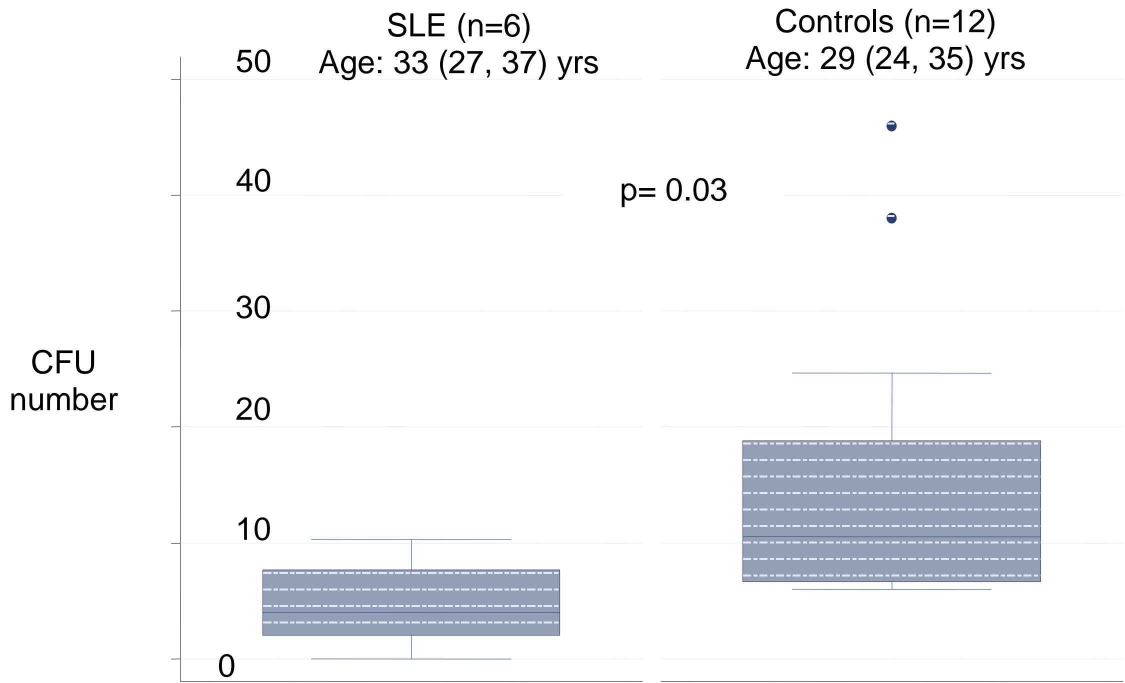


Figure 29 Mean CFU number lower in SLE compared to controls in those less than 40 years old

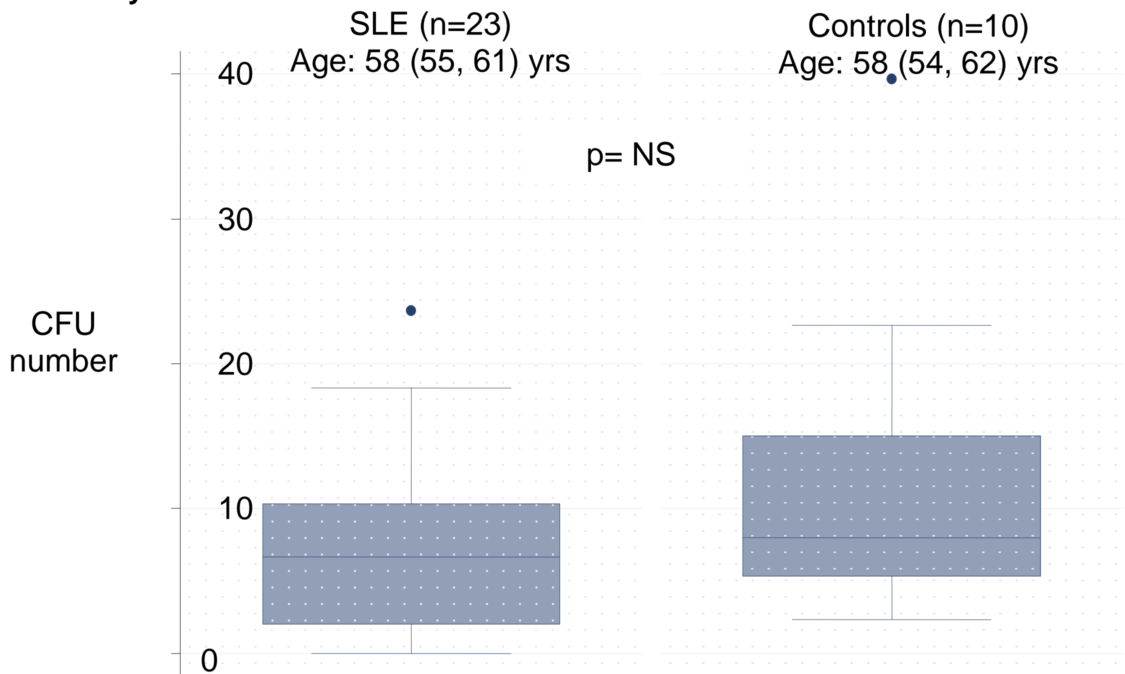


Figure 30 Mean CFU number lower in SLE compared to controls in those more than 40 years old

The numbers of CFU with greater than 50 cells in the central cluster were enumerated. SLE patients formed an average of 0 (0, 2) large CFU compared to 3 (0, 10) formed by controls; $p = 0.004$. Furthermore 11/39 (28%) of SLE patients formed any large CFU compared with 17/27 (63%) of controls; $p=0.02$.

10.5.4.1 Factors associated with CFU number in SLE patients

Factors associated with CFU formation within the SLE population (n=39) were analysed. Traditional cardiovascular risk factors did not correlate with CFU formation. SLE-related factors including renal disease, antibody profile, disease activity, damage index and therapy did not correlate with CFU number (Table 35). However the presence of anti-cardiolipin antibodies (aCL) was positively correlated with CFU number and remained significant when adjusted for age. One possible explanation for this is the use of aspirin however the correlation with aCL also remained positive when adjusted for aspirin therapy (β 0.3580549, $p = 0.023$). Similarly the correlation of aspirin therapy with CFU number remained significant after adjustment for age and aCL (β 0.4139033, $p = 0.011$).

Table 34 Traditional risk factors associated with CFU formation in SLE

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
Age	.0393367 (.0834571)	NS	-	-
Disease duration	-.0615574 (.0826601)	NS	-.0807062 (.0877457)	NS
Total cholesterol	.2240077 (.8563361)	NS	.2186055 (.8779495)	NS
Hypercholesterolemia	.1945315 (1.787787)	NS	.1732577 (2.016818)	NS
LDLc	.1105569 (.9475673)	NS	.1051584 (.9708252)	NS
Triglycerides	-.0872086 (1.356703)	NS	-.0721542 (1.398444)	NS
Systolic blood pressure	.125241 (.044178)	NS	.1046377 (.0467203)	NS
Hypertension	.1142344 (2.029275)	NS	.1031204 (2.081757)	NS
Fasting glucose	.0233524 (1.326692)	NS	.0129338 (1.370776)	NS
Type 2 diabetes	.1442572 (5.163697)	NS	.1418649 (5.267045)	NS
Smoking ever	-.1502769 (1.576456)	NS	-.1535755 (1.601833)	NS
Family history of CHD	.0660778 (1.918217)	NS	.0424189 (1.993597)	NS
Body mass index	-.2637483 (.1581398)	NS	-.263916 (.1602434)	NS

Table 35 Disease-related risk factors associated with CFU formation in SLE

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
Renal disease	-.0812705 (2.142181)	NS	-.0978295 (2.202384)	NS
Creatinine	-.119968 (.0139802)	NS	-.1240429 (.0146683)	NS
SDI	-.2582043 (.4773714)	NS	-.2339211 (.5252232)	NS
SLEDAI	.1016079 (.2458143)	NS	.1458744 (.2585666)	NS
C3	.1497136 (2.97865)	NS	.2821646 (3.505684)	NS
C4	.0861817 (15.09932)	NS	.1421088 (16.1829)	NS
Ro	.1451259 (2.126536)	NS	.1344198 (2.215996)	NS
La	.1040958 (2.703892)	NS	.1029149 (2.773674)	NS
Sm	-		-	
RNP	-.1716301 (3.049402)	NS	-.1792056 (3.107327)	NS
Anti-cardiolipin antibody	.3629715 (2.595709)	0.03	.3859076 (2.658597	0.02
Anti-malarial use	-.0084068 (1.699944)	NS	-.0207861 (1.84168)	NS
Immunosuppressant use	-.1560263 (1.741649)	NS	-.1746781 (1.850143)	NS
Azathioprine use	-.1553937 (1.728435)	NS	-.170063 (1.776749)	NS
Steroid use current	-.0127074 (1.781797)	NS	-.0290727 (1.832436)	NS
Steroid exposure ever	.0016071 (1.742038)	NS	.0295924 (1.801269)	NS
Statin use	.2754097 (1.717979)	NS	.2714922 (1.819499)	NS
Aspirin use	.3225228 (2.163891)	0.045	.3384277 (2.248571)	0.045

The average CFU number was greater in those taking aspirin compared to those not (7 [2, 18]) *vs.* (5 [2, 7]; p =0.045). The median CFU number was similar between those taking statins and those not.

The correlation of CFU number and markers of subclinical atherosclerosis were assessed. Aortic stiffness was measured by the aortic pulse wave velocity (APWV) and the median (IQR) APWV amongst the 39 SLE patients was 5.7(3.8, 8.6) m/s. CIMT was 0.062 (0.053, 0.070) cm and 12 (31%) patients had one or more plaque. CFU number did not correlate with APWV, CIMT or carotid plaque in this group of patients. One patient had a history of myocardial infarction and this patient did not form any CFU in culture.

Table 36 CFU and markers of atherosclerosis in SLE

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
APWV	-.1054126 (.1319949)	NS	-.1098138 (.134661)	NS
CIMT	.1865284 (92.18052)	NS	.2925301 (101.626)	NS
Carotid plaque	-.1694966 (1.788406)	NS	-.1351411 (-.1351411)	NS
Carotid plaque progression	-.2583806 (2.338805)	NS	-.2207638 (2.527775)	NS
CIMT progression	.2144084 (125.3675)	NS	.1811628 (133.1052)	NS

10.5.4.2 Discussion

CD34+CD133+ EPC in SLE

EPC were quantified in 54 SLE patients and 49 healthy controls using flow cytometry. This group of patients were chosen at random and were representative of the overall cohort as demonstrated in Table 311. Whilst a statistically significant higher proportion of SLE patients were hypertensive the systolic blood pressure indicates that these patients had well controlled blood pressure. Similarly, whilst a higher proportion of patients had a diagnosis of hypercholesterolaemia and were therefore more likely to be on statin therapy, there was no significant difference in the overall cholesterol titre.

CD34, CD133 and KDR were chosen as surface markers to identify EPC based on previous literature [12, 112, 263, 331]. However, following several experiments to optimise the use of the KDR antibody and personal communication with other groups, it was decided that this antibody could not be reliably utilised. EPC were therefore defined as CD34+CD133+ cells. CD34 is a marker expressed on bone marrow-derived haematopoietic cells and mature endothelial cells in contrast to CD133 which has been shown to be expressed on haematopoietic progenitor cells but not on mature endothelial cells [119, 263, 317] indicating that CD34+CD133+ cells are identifying an immature subpopulation of EPC.

There was no significant difference in the number of CD34+CD133+ cells between SLE patients and controls. Of interest age was correlated to CD34+CD133+ cells within the control population but not within the SLE group. This may in part be due the narrow age group of SLE patients studied (IQR 47 – 59 years) in comparison to the control group (IQR 31 – 59 years). Traditional

risk factors did not correlate with EPC number in this study. Seven (13%) of patients were taking statin therapy and 16 (30%) were taking an anti-hypertensive medication. Statin or anti-hypertensive therapy use did not correlate with EPC number. EPC number did not correlate to markers of atherosclerosis including APVW, CIMT or carotid plaque. Disease-related factors also did not correlate to EPC number in this study including the clinical phenotype of patients, measures of disease activity or damage, antibody profile or therapies. Since the commencement of this study, 3 other groups have reported on EPC in SLE. One previous study has demonstrated a reduction of CD34+KDR+ EPCs in 15 SLE patients compared with 15 healthy controls [373] and another of 30 patients with SLE compared with 14 healthy controls reported a reduction of $CD34^+/CD133^+/VEGFR2^+$ EPCs in patients with SLE. EPC level in the latter study was correlated with SLEDAI but not age or Framingham score [87]. Lastly Moonen *et al*. reported a reduction in CD34+CD133+ EPC in 20 SLE patients compared to 20 healthy controls [242]. Possible explanations for the discrepancy of the findings between different studies may be related to small sample sizes or due to the differences within the study population such as ethnicity, therapies, disease activity or differences in disease phenotype. Furthermore, previous studies have identified cells with a different combination of surface markers and therefore may have identified a different subpopulation of EPC. The group that measured CD34+CD133+ cells studied a population of patients with a mean age 40 ± 12 years [242]. Our patients were on average a decade older and it is possible that the discrepancy in EPC number between SLE and controls is less marked with advancing age. One other important difference in the methodology used by different groups is

the quantification of cells. Some groups have included absolute counts per volume of blood (e.g. [373] whilst others have measured percentage of the mononuclear cell population [135] as in this study. Lymphopenia amongst patients may possibly result in an underestimation of EPC in our population since a 1/3 (n = 18) of our patients had a lymphocyte count of less than 1.0. However, we did not observe a significant correlation between lymphocyte count and EPC number in our study (β – 0.07 (3.29), p =NS). It is also possible that lymphopenia may result in bone marrow stimulation and in which case relatively more progenitor cells may be detectable. More recent studies of EPC in SLE are in keeping with the findings of the current study and have demonstrated no significant difference of number of EPC measured using flow cytometry in SLE patients compared to controls or even a slight excess of EPC in SLE [93, 135].

The lack of EPC deficiency in SLE is in contrast to other chronic conditions resulting in excess CHD e.g. diabetes or rheumatoid arthritis where EPC number has been shown to be reduced [134, 339]. One possible contributing factor is the relative lack of CRP response in SLE. A previous study using blood from healthy males has demonstrated increased apoptosis of EPC in culture when treated with CRP at concentrations > 15 μg/mL. Similarly, patients with type 2 diabetes with a high hsCRP have been shown to have reduced number of EPC compared to those with low hsCRP [192, 355]. Furthermore, absolute numbers of EPC may be less important in SLE. The EPC present in SLE may be functionally impaired and therefore the total number of ‘effective’ EPC in SLE would therefore be reduced. SLE patients have been shown to have accelerated atherosclerosis and increased endothelial dysfunction [95, 290]

compared to controls and it may be that an EPC number comparable to healthy controls is not able to effectively regenerate and maintain the vasculature. It must be considered that cells identified using these surface markers using flow cytometry may in fact not be EPC but another population of cells also arising from the haematopoietic stem cell lineage. Of interest, whilst CD34+CD133+ cells were not different between patients and controls, they did show a positive correlation with CFU (R^2 0.30, p =0.01) which was not attenuated following adjustment for age.

CFU in SLE

CFU formation was assessed in 39 SLE patients and 27 healthy controls. CFU formation is likely to reflect a composite marker indicating both the number of EPC and also functional ability to migrate to a cluster of cells and adhere to fibronectin. SLE patients were found to form significantly fewer CFU compared to controls and the difference was particularly marked in the younger age groups.

Ablin *et al*. reported no significant difference in CFU number of 28 SLE patients and 50 controls [3]. A number of differences between those patients and the current cohort need to be considered when interpreting their findings. SLE patients were on average 37.7 ± 14.7 years old and controls 43.3 ± 18.2 years old and included males. Patients with a prior history of high dose steroid, cyclophosphamide or MMF use were excluded suggesting the group studied were of a less severe phenotype. Fewer patients had a diagnosis of hypertension (12.9%) or hypercholesterolaemia (6.5%) compared to the current study. Ablin and colleagues did note an impaired adhesion of cells to fibronectin in SLE patients which was interpreted as a marker of impaired function. CFU

number or adhesion scores did not vary with disease activity. Grisar *et al*. also reported on CFU number in 13 SLE patients and 12 controls. The ages of these patients were not reported but the overall population of 31 SLE patients were aged 35 ± 2 years. CFU number was similar between the 2 groups but they too reported a reduction in migratory and adhesive properties in SLE patients. Small sample size and several differences in the study populations as highlighted above may account for the discrepancy in number of CFU noted in these 2 studies compared to current study.

Comparable with our findings, 2 other recent studies have reported reduced CFU number in SLE compared to controls. Moonen *et al*. quantified CFU in 10 SLE patients and 10 healthy controls. The author noted, as we did, that the morphology of the CFU were very different amongst SLE patients. Figure 31 demonstrates the morphological differences of CFU in SLE patients and controls from my study.

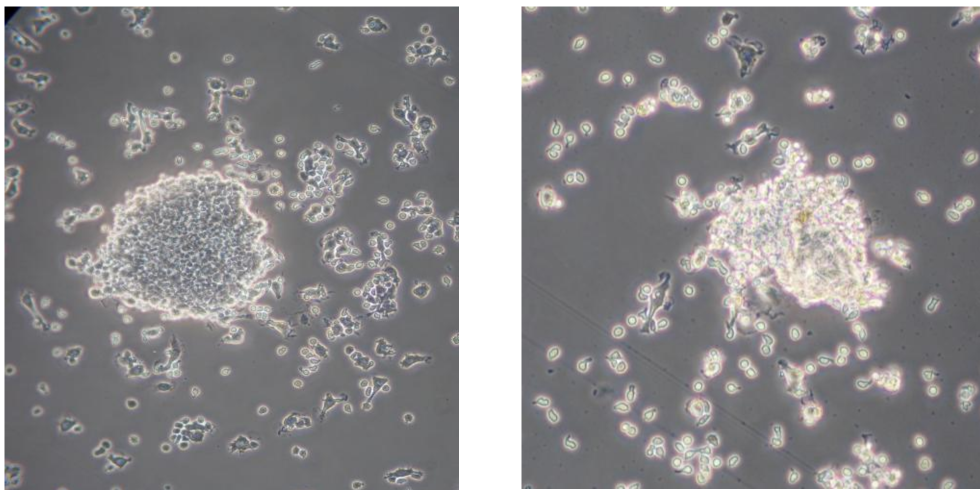


Figure 31 Examples of CFU (x50) a. typical CFU from a control. b. typical CFU from an SLE patient

SLE patients included in the study by Moonen *et al.* had quiescent disease and demonstrated a reduced number of CFU compared to controls but noted adhesion and migratory properties were similar between the two groups. Lee *et al.* reported on 70 SLE patients with an average age of 35 (27-45) years and 31 healthy controls aged 36 (26.5-44) years. The SLE population included males and 2/3 of patients were non-white Caucasian. They found SLE patients had significantly fewer CFU than controls but no correlation of CFU to traditional cardiovascular risk factors or SLE-related factors. Of particular interest, this group found CFU depletion was particularly marked in those patients with high Type I interferon (IFN-I) levels. Further they demonstrated that IFN-I inhibited EPC proliferation in culture and that IFN-I was also associated with endothelial dysfunction on SLE. The authors suggested endothelial dysfunction is mediated via IFN-I actions on EPC. IFN levels have previously been shown to correlate to disease activity and damage in SLE and this proposed contribution to atherosclerosis is of much interest [25].

Studies with a small sample have not commented on clinical factors related to EPC/CFU number. The largest study to date was reported by Lee et al. and included 70 patients with SLE. No significant correlation was detected between CFU and CHD-related or disease-related factors. Age and traditional CHD risk factors were not associated with CFU in our study (Table 34). Aspirin use, however did have a correlation with CFU number ($R2 = 0.10$, $p = 0.045$) which was not attenuated after age-adjustment. This relationship remained unchanged after adjusting for the presence of aCL which itself was correlated with CFU ($R2 = 0.15$, $p = 0.02$). Seven patients (18%) were taking aspirin at the time of the study and many of these patients were taking additional anti-

hypertensive medications which may also be confounding factors. The association of aspirin with CFU number may support the debate for anti-platelet primary prevention of CHD in SLE but requires further investigation. The association of aCL with CFU is interesting as the overall role of aCL in CHD risk is unclear. Previous studies have suggested aCL contribute to the initiation and instability of plaque [120, 188] whilst others have suggested some subtypes of aCL may be atheroprotective [253]. A positive correlation with CFU would support an atheroprotective effect of aCL.

CFU did not correlate with aortic stiffness, CIMT or plaque in this study. APWV and carotid plaque did however indicate a negative association albeit non-significant. A larger sample size may have elucidated a significant relationship. Alternatively CFU abnormalities can potentially be assumed to be an early risk for atherosclerosis in SLE since it was not associated with other markers of atherosclerosis in this study such as carotid plaque.

Studies with larger sample sizes are required to clarify these initial findings. Whilst these small and perhaps preliminary *in vitro* studies provide an interesting potential mechanism for the excess of CHD observed in SLE, further larger studies will be necessary both in patients and animal models to establish a true reflection of *in vivo* actions of endothelial progenitor cells.

10.6 Telomere length in SLE

SLE patients have been shown to have a premature onset of subclinical atherosclerosis and clinical CHD. The early onset of atherosclerosis may be related to premature biological ageing of cells. A premature senescent phenotype may also be contributing to endothelial cell abnormalities observed.

The objectives of this chapter are to

1. Determine if there is evidence of premature biological ageing of peripheral blood cells from SLE patients
2. Determine factors associated with senescent cells
3. Describe association of senescent cells with markers of atherosclerosis

10.6.1 Telomere length measurement

All participants had a 5ml sample of blood taken at the time of the clinical assessment which was frozen at -80 degrees until DNA extraction. Genomic DNA was extracted from whole blood using an 8LX Automated DNA Extractor. DNA was measured using a NanoDrop ND-1000 UV – vis spectrophotometer, normalised to a 10 ng/ µL concentration and stored at -80 degrees. Mean telomere length was measured using a fluorescence-based quantitative real time polymerase chain reaction adapted from the protocol previously described [57] comparing telomere repeat sequence copy number to single-copy gene (RNAseP) copy number in a given sample using his improved primer set (Tel2a and Tel2b) and 36B4 as the single copy control gene as previously described [45]. The following primer sequences (5’- 3’) were used:

Tel1b CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT

Tel2b GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT

36B4F CAGCAAGTGGGAAGGTGTAATCC

36B4R CCCATTCTATCATCAACGGGTACAA

Telomere (T) and 36B4 (S) runs were performed separately. Master mixes for both were made up at the same time to ensure the same SYBR green and qPCR mix was used. The T run was performed for the samples followed immediately by the S run, to keep conditions uniform. Each S sample was plated in the well in the identical corresponding position as the T sample. Each plate included 3 no template control (NTC) samples.

DNA samples were amplified in triplicate in parallel 10.2 µL PCR reactions. Each reaction consisted of:

1. 3µl (30 ng) of DNA sample
2. 5 µl of 2 x mastermix (SensiMix NoRef DNA kit, Quantace, Cat No QT505-20)
3. 0.2 µl SYBR Green (DNA kit, Quantace, Cat No QT505-20)
4. 1µl Tel2a (600nM final concentration) or 36B4F (300nM final concentration)
5. 1µl Tel2b (600nM final concentration) or 36B4R (500nM final concentration)

All PCR were undertaken with the LightCycler 480 real time PCR machine (Roche Diagnostics, UK) with the following cycling conditions:

T 95°C for 10min followed by 30 cycles of 95°C for 15 sec and 58°C for 1min

S 95°C for 10min followed by 30 cycles of 95°C for 15 sec and 58°C for 1min

All reactions were followed by a 10 second cooling period at 40˚C

10.6.2 Telomere length analysis

To determine the telomere length, the amount of product produced in the telomere assay was compared to the amount of product produced from the 36B4 assay. LightCycler 480 software was used to calculate the second derivative of the real-time amplification curve. The peak of the second derivative curve represents the maximum exponential growth of the product. The point 80% back from the peak is defined as the takeoff point, and the amplification efficiency calculated from the section of curve between these two points. The product is calculated for samples in this way. All samples were run in parallel with the same reference sample (from a control participant). Samples were directly compared to the reference sample. The ratio of telomere and 36B4 products is proportional to the average telomere length. The telomere length is therefore expressed as T relative conc / S relative conc (T/S). See Appendix 11 for excel sheet calculation of relative telomere length.

The NTC wells appeared to form some product within the PCR reactions and this is likely to be due to the formation of primer product. Primer products appeared at a much later cycle than the PCR product of interest and therefore were easily distinguished as demonstrated in Figure 32 and Figure 33.

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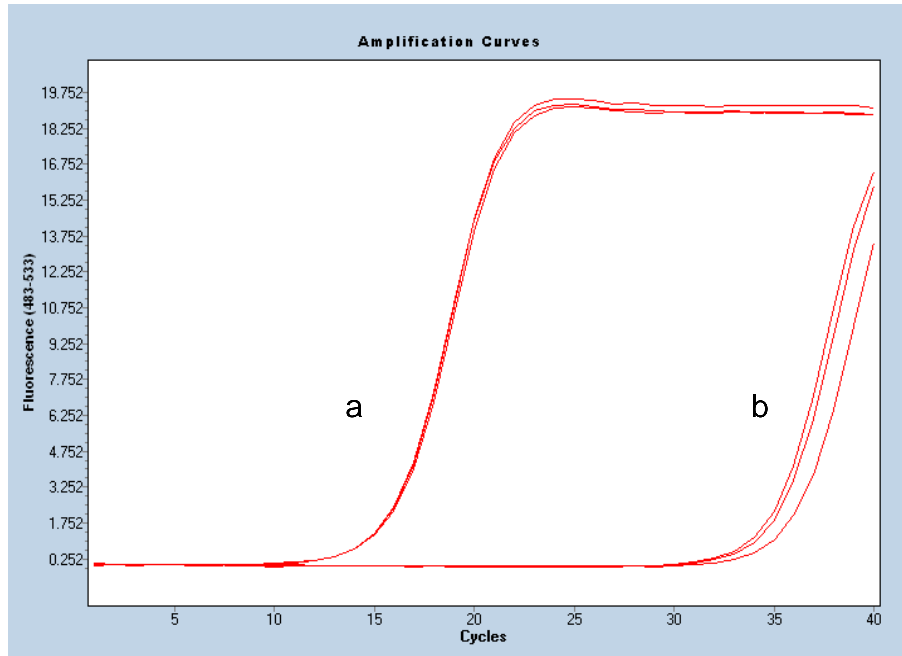


Figure 32 Amplification curve for PCR using telomere primers a. DNA sample in triplicate showing tight replication b. NTC amplifying at much later cycle

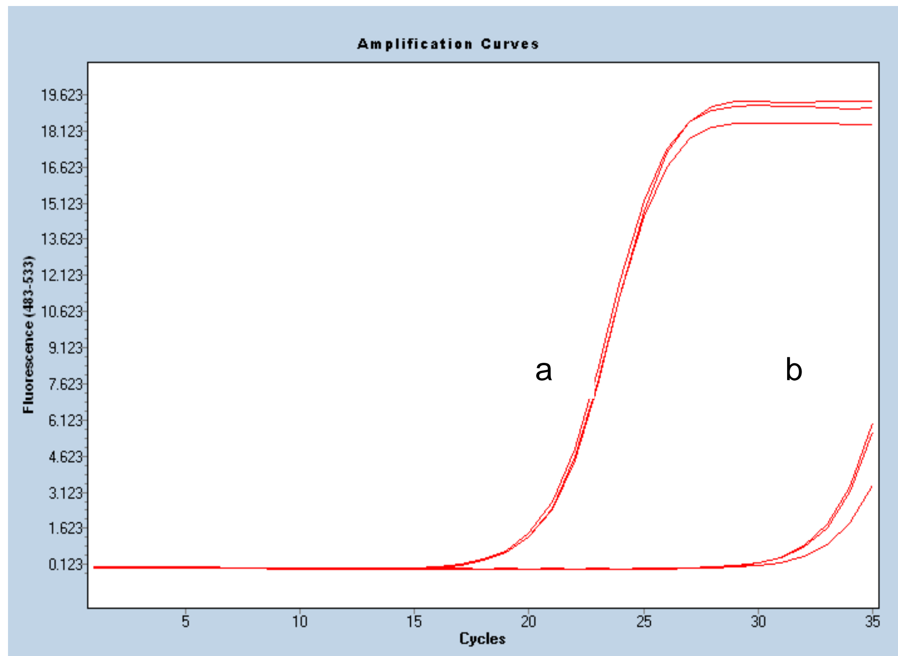


Figure 33 Amplification curve for PCR using 36B4 primers a. DNA sample in triplicate showing tight replication b. NTC amplifying at much later cycle

10.6.3 Telomere length measurement validation

DNA from each individual was plated in triplicate and samples showed very little variance within the triplicates (Figure 34).

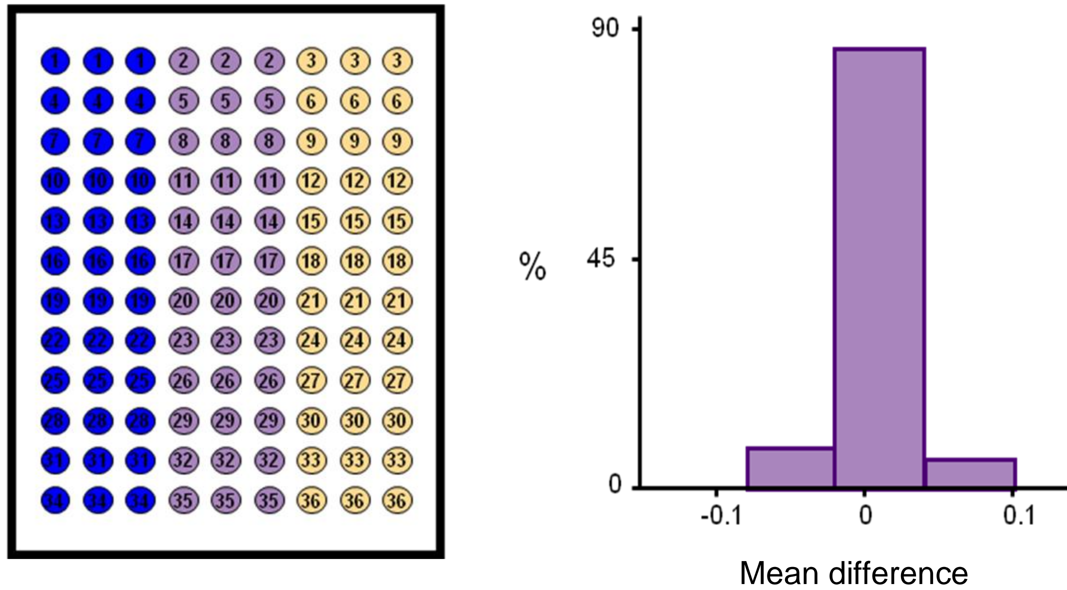


Figure 34 Schematic of PCR plate layout and graph demonstrating little variation of product between triplicate samples

Thirty-five randomly chosen samples were also repeated at a second time-point to ensure repeatability. Figure 36 and Figure 36 demonstrate good repeatability between measurements at different time points.

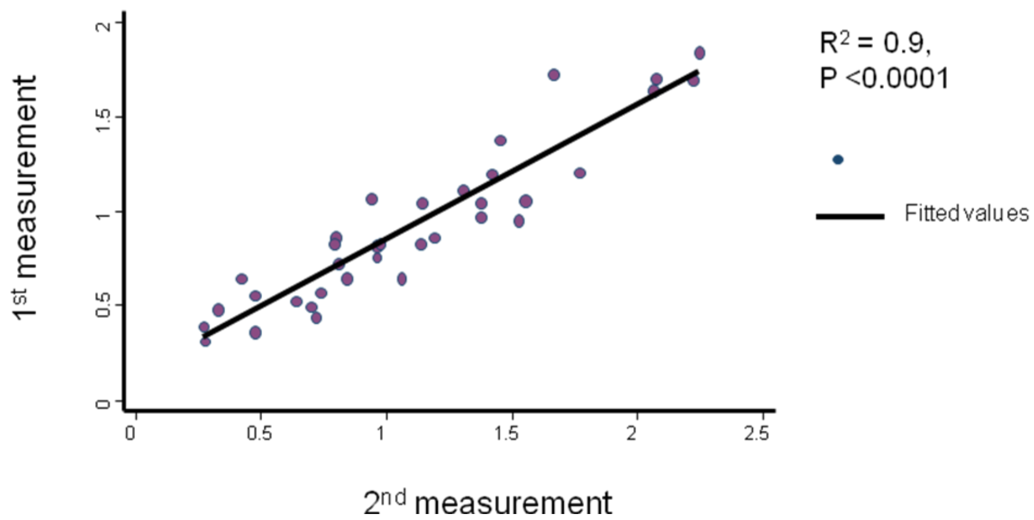


Figure 35 Relative telomere length measured at 2 separate time points show good correlation

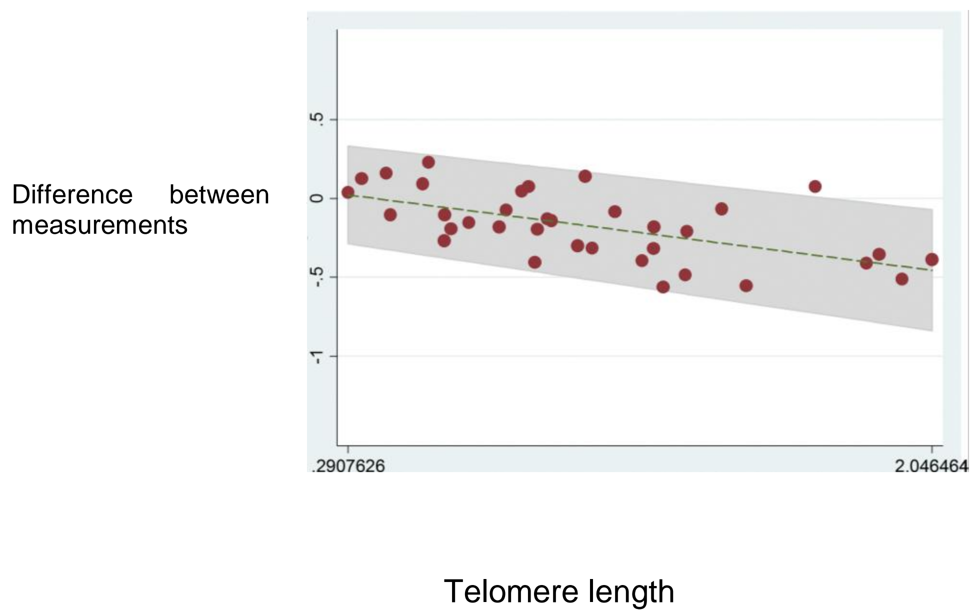


Figure 36 Bland Altman plot of difference between telomere length measurements at 2 time points (measurements outside shaded area excluded from analysis)

10.6.4 Telomere length association with age

Telomere length measurement had an overall negative correlation with age with $R^{2\ \mathrm{of}}$ -0.01, p = 0.09. The correlation was similar between SLE patients and controls (Figure 37).

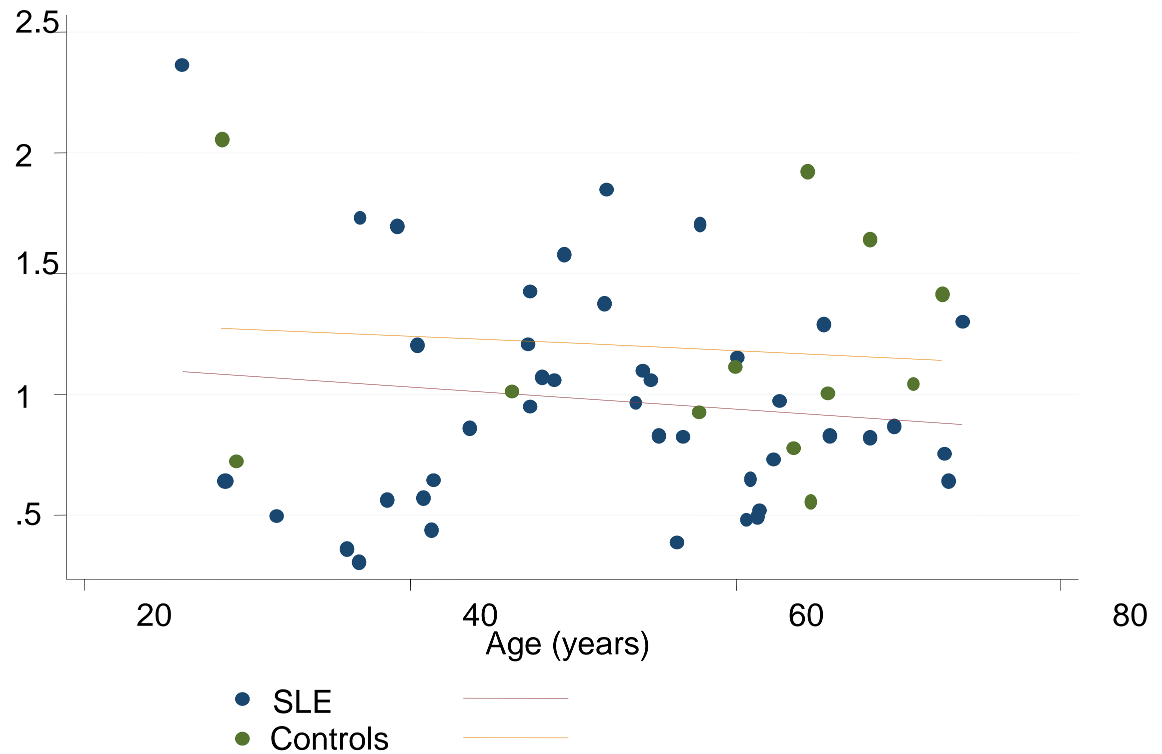


Figure 37 Correlation of telomere length in 63 SLE (R^2 -0.005, p=NS) and 63 controls (R^2 -0.003, p=NS); y-axis represents relative telomere length

10.6.5 Telomere length in SLE and controls

Telomere length was measured in 63 SLE patients and 63 age matched controls. The median (IQR) telomere length amongst SLE patients and controls was 0.97 (0.47, 1.57) and 1.53 (0.82, 2.29) respectively. SLE patients had significantly shorter relative telomere length (Figure 38).

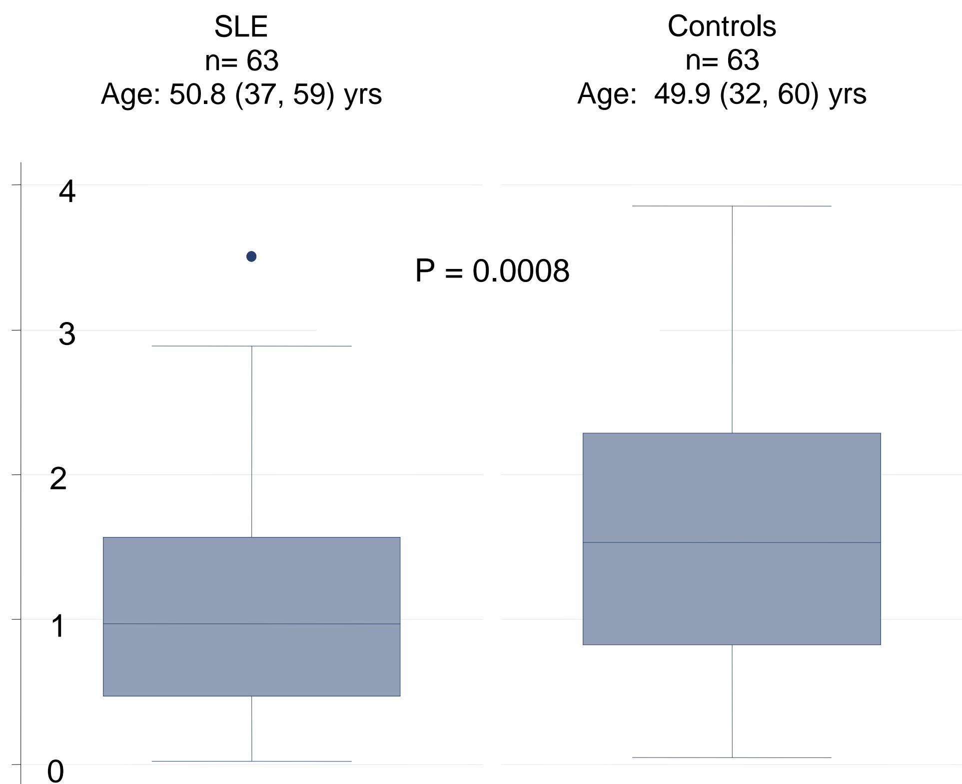


Figure 38 Telomere length in SLE patients and healthy controls; y-axis represents relative telomere length

10.6.6 Factors associated with telomere length in SLE

Telomere length (TL) was measured in a larger cohort of 164 SLE patients with a median (IQR) age 53 (45, 60) years. The median (IQR) telomere length in this group was 1.23 (0.64, 1.86). Traditional CHD risk factors associated with TL is summarised in Table 37. Age did not have a significant correlation with TL in this cohort. Triglycerides had a small but significant positive correlation with TL. Body mass index was also significantly positively correlated to TL with a stronger β coefficient and R^2 of 0.25. Previous history of smoking was not associated with TL; however current smoking had a small significant correlation. Creatinine had a small positive correlation although history of renal disease and eGFR did not (β 0.109, (0. 531), p=NS). Increasing C3 and the presence of anti-Ro antibody was also positively correlated with TL. Current steroid use also had a positive correlation with TL. Eighty (49%) of patients were taking oral corticosteroids at the time of the assessment and average dose was 8 (5, 10) mg. Other immunosuppressants and hydroxychloroquine, which was taken by 102 (62%) of patients was not related to telomere length.

Table 37 CHD risk factors associated with telomere length in SLE

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
Age	.0286394 (.0066339)	NS	-	-
Disease duration	.003198 (.0079396)	NS	-.0059501 (.0083011)	NS
Total cholesterol	.0347914 (.0787449)	NS	.0283767 (.0809326)	NS
Hypercholesterolemia	.127029 (.149541)	NS	.1301897 (.1570589)	NS
LDLc	-.025934 (.0868653)	NS	-.0296583 (.0889616)	NS
Triglycerides	.269633 (.1216939)	0.001	.1216939 (.124337)	0.001
Systolic blood pressure	.1006017 (.0036394)	NS	.0828519 (.0041404)	NS
Hypertension	.1061452 (.1475966)	NS	.0949059 (.1537545)	NS
Fasting glucose	.1165156 (.1065638)	NS	.1065638 (.1114359)	NS
Type 2 diabetes	-.0247151 (.3307528)	NS	-.0273742 (.336669)	NS
Family history of CHD	-.0672614 (.1617464)	NS	-.0769809 (.1651086)	NS
Body mass index	.4999786 (.0109257)	<0.001	.5045071 (.011186)	<0.001
Smoking ever	.0814292 (.1488044)	NS	.075942 (.1540154)	NS
Current smoking	.1656675 (.2411204)	0.04	.1629132 (.2433388)	0.04
Statin use	.039926 (.1567863)	NS	.0376489 (.1621877)	NS
Aspirin use	-.0379615 (.3930576)	NS	-.0369708 (.3968273)	NS
hsCRP	.1505296 (.0152335)	0.054	.1346012 (.0156504)	0.091

Table 38 Disease-related factors associated with telomere length in SLE

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
Renal disease	.1232055 (.1770499)	NS	.1277395 (.1797314)	NS
Creatinine	.1025418 (.0013306)	NS	.1663254 (.0019361)	0.04
SDI	.1306735 (.0428572)	NS	.12404 (.0465613)	NS
SLEDAI	-.055734 (.0192574)	NS	-.0574061 (.0196509)	NS
C3	.3212446 (.2452132)	<0.001	.3148529 (.2590098)	<0.001
Ro	-.2351623 (.1734059)	0.002	-.2389531 (.17638)	0.002
La	-.0575649 (.2418631)	NS	-.0546219 (.2499176)	NS
Sm	-.0646274 (.9466094)	NS	-.0643486 (.9522798)	NS
RNP	-.095034 (.2411683)	NS	-.0636681 (.2573586)	NS
Anti-cardiolipin antibody	-.0444031 (.215287)	NS	-.0496692 (.2172628)	NS
Anti-malarial use	-.0286531 (.1797815)	NS	-.003802 (.1866773)	NS
Immunosuppressant use	.1001962 (.1754039)	NS	.1232227 (.1860104)	NS
Azathioprine use	-.0047449 (.1595307)	NS	.0084572 (.1664905)	NS
Steroid use current	.1455769 (.155517)	0.07	.1603626 (.1587567)	0.054
Steroid exposure ever	.0966537 (.1902879)	NS	.0845827 (.1946587)	NS

The correlation of TL and atherosclerosis was also assessed. Eleven (7%) of patients had a history of CHD (7 patients with MI and 4 patients with angina). The presence of carotid plaque had a small positive correlation with TL suggesting that those patients that had a clinical coronary event had longer telomeres than those did not. Similar results were seen when MI was analysed

separately (adjusted β 0.091 (0.152), p=NS). When dividing the telomere length into 3 equal tertiles and comparing the mean APWV, CIMT and prevalence plaque, similar results are observed.

Table 39 Telomere length in SLE atherosclerosis

Baseline factors	Unadjusted		Age-adjusted	
	β (SE)	P value	β (SE)	P value
APWV	-.0293293 (.0088168)	NS	-.0156208 (.0089319)	NS
CIMT	-.0161027 (5.150632)	NS	-.0711242 (5.797241)	NS
Carotid plaque	.1973369 (.1486059)	0.013	.2031888 (.1772831)	0.032
Carotid plaque progression	.2062594 (.1481292)	0.04	.2195023 (.1723362)	0.062
CIMT progression	-.1178704 (7.453621)	NS	-.1014365 (7.5347)	NS
MI or angina	.1546401 (.2932337)	0.051	.1524812 (.3035895)	0.064

Table 40 Measures of atherosclerosis associated with tertiles of telomeres in SLE

Marker of atherosclerosis	Low	Middle	High	P value
Plaque (%)	33	38	61	0.02*
Intima media thickness: mean (SD)	0.06 (0.02)	0.06 (0.01)	0.06 (0.01)	NS
Pulse wave velocity : mean (SD)	10 (7)	7 (6)	11 (9)	NS

***Comparing low tertile to high tertile**

Relative telomere length had a significant age-adjusted correlation with CFU number in SLE patients (R2 0.1, p = 0.05) but in not in controls (R2 0.01,p=NS).

10.6.7 Discussion

We measured relative telomere length of PBMC from 64 patients with SLE using qPCR. Consistent with our hypothesis, we demonstrate a significant reduction, of approximately 1/3, in telomere length in SLE patients compared to 64 age–matched controls. Further we measured telomere length in a larger study of 164 SLE patients and analysed associated factors. There is no significant correlation of age with TL in our study. This may be a reflection of the small sample size. However it should be noted the IQR of age of our patients is 45 – 60 and previous reports have suggested that telomere length does not show a marked inverse correlation between these ages [15].

CHD risk factors that are associated with telomere length in the age-adjusted analysis are triglycerides, BMI and current smoking. It is possible that these factors present low level oxidative stress and a subsequent response to lengthen telomeres. Telomere length demonstrated an inverse correlation with aortic stiffness which failed to reach statistical significance. Paradoxically, carotid plaque correlates with telomere length suggesting those patients with the longest telomeres are more likely to have plaque. Similarly, there is a correlation with clinical CHD events. When analysing the data by dividing length of telomeres into tertiles, a higher proportion of those with the longest telomeres are found to have carotid plaque. This may be a refection of some censorship i.e. those patients with the shortest telomeres and carotid plaque are deceased and therefore not captured in a cross-sectional study.

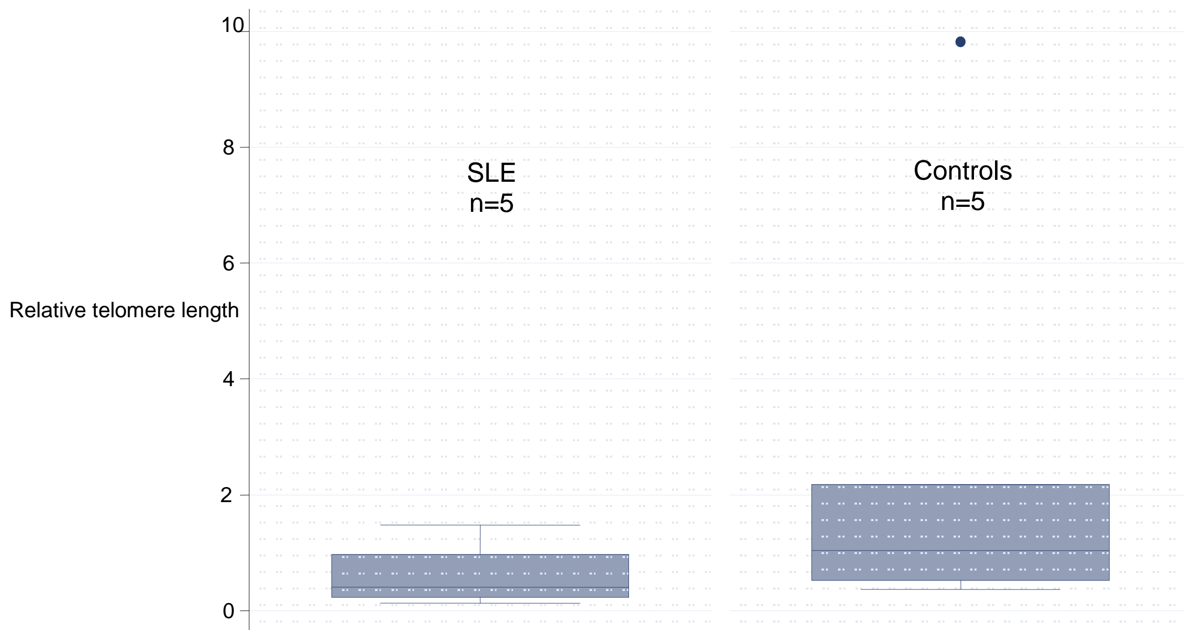
SLE related factors that correlated with telomere length were anti-Ro antibody, C3 complement and current use of steroids. The correlation of telomere length with the anti-Ro antibody is interesting as this antibody was

also associated with reduced carotid atherosclerosis progression. It is possible that this antibody reflects those patients with a less severe disease phenotype or those patients of the Sjogren's disease spectrum. An alternative explanation is that this antibody affords some atheroprotection. The correlation with C3 and current steroid therapy may suggest that reduced inflammatory burden results in a beneficial effect on telomere length. However other measures of inflammation such as CRP or SLEDAI do not demonstrate an inverse correlation. Our study is similar to those previously reported that have shown a reduction in telomere length in SLE compared to controls [271, 274]. Similar to previous studies, we did not find a correlation of telomere length to lymphocyte count (β 0 .01, SE 0.04, p = NS) or disease activity, suggesting that telomere length is not simply a reflection of immune cell turnover. It should be noted in this regard that the majority our patients had low disease activity.

Our study is a cross-sectional analysis and therefore we are unable to determine if our associations with telomere length are the cause or consequence of telomere length reduction. One theory is that disease states lead to accelerated telomere attrition whilst others believe an overall senescent phenotype will result in a propensity for certain diseases. The exact contribution of genetic factors on length of telomere also remains of much interest. Additionally, given the wide variation of telomere length between individuals, a cross-sectional measurement may be less valuable than serial measurements allowing a rate of attrition to be determined. The utility of telomere length as a biomarker has to be questioned in view of the wide variation between individuals and slow rate of change. Rather, it may provide an insight into mechanism underlying disease and present a potential therapeutic target.

We used real-time qPCR to measure telomere length as this method is more time efficient and requires smaller amounts of DNA [18]. Additionally, previous studies have shown a good correlation between measurement with qPCR and Southern blotting [45]. There has been some debate about whether PBMC, a heterogenous group of cells, are the ideal population of cells to study. To date several groups have used PBMC in SLE and in cardiovascular studies and demonstrated PBMC telomere length is a good a marker of disease (please see section 6.7.3 for more details). In addition, small studies have not demonstrated any differences in the telomere length of subpopulations of white cells between SLE and controls. We demonstrate a correlation between PBMC telomere length and mean CFU formation in SLE but not in controls which may suggest that a senescent phenotype contributes to abnormal endothelial repair. However, it is argued that cells from the target organ of interest should be analysed and to this end we have measured telomere length from endothelial cells. In a preliminary study we cultured CFU for 7 days using the protocol described above (section 10.5.2) for 5 SLE patients aged 51 (49, 56) years and 5 controls aged 46 (41, 50) years. We isolated CFU at day 7 of culture and harvested DNA using phenol-chloroform extraction. We then quantified telomere length using qPCR as described in section 10.6.1. Relative telomere length tended to be shorter in SLE CFU compared to control CFU (0.41 [0.23, 0.97] *vs.* 0.93 [0.41, 2.07]; p = NS). See Figure 39.

Figure 39 telomere length of CFU in SLE *vs.* controls



11 Conclusions

We described carotid atherosclerosis progression in a UK cohort of white females with SLE. We designed a study to test the hypothesis that patients with SLE have premature cellular ageing and that this contributes to abnormal endothelial repair and subsequent atherosclerosis.

We have demonstrated carotid plaque progression occurs in 17.5% of SLE patients over an average of 5 years and that baseline traditional CHD risk factors do not contribute to this risk. We demonstrate reduced PBMC telomere length in patients with SLE compared to age-matched controls suggesting premature cellular ageing in our patients. We demonstrate a correlation of PBMC telomere length and CFU number, a measure of endothelial progenitor cell number and function. We also demonstrate an overall reduction of CFU formation in SLE compared to healthy controls suggesting abnormal endothelial repair in SLE. Lastly, we demonstrate a trend to increased cellular ageing of SLE endothelial cells compared to controls. Anti-cardiolipin antibody in our study is associated with carotid plaque progression and with CFU number and this may suggest endothelial cells are stimulated in response to aCL in an attempt to repair the vasculature.

Our study is the largest to date of telomere length in SLE and we have utilised statistical testing to maximise the use of data points e.g. by using variables in a continuous manner where possible. Nonetheless, the sample size remains relatively low and is a weakness of the project. We have conducted cross-sectional studies to examine telomere length and endothelial progenitor

cells and as such are not able to determine if associations are a cause or effect of the variables examined.

11.1 Future directions

Many questions and suggestions are raised by the findings of this study. Firstly, future larger studies are required to verify our results. Studies would need to consist of a large sample size to allow robust conclusions to be drawn, particularly to ascertain the influence of drugs such as statins. Given the low prevalence of SLE; it is likely that multi centre collaborations would be required.

Our group and others have demonstrated reduced endothelial progenitor cells as measured by culture methods in SLE, however precise characterisation of EPC require further clarification and the mechanisms underpinning abnormalities in endothelial cell function e.g. homing signals to sites of damaged endothelium requires further investigation. The observation of a trend to shorter CFU telomeres in SLE is interesting and a larger sample size is required. To date, studies have compared SLE patients to healthy controls. However a more relevant comparison group may be a cohort with another chronic inflammatory condition to elucidate the relative contribution to atherosclerosis in SLE. Prospective studies are required to determine the consequence of low EPC number and to determine whether low EPC number does lead to an excess of CHD.

It is unclear if telomere length is a ‘summary marker’ of replicative and stress induced senescence or is involved in the pathogenesis of atherosclerotic disease. The influence of telomeres on the development of SLE and the

potential effect on clinical phenotype is interesting. Measuring the length of telomeres of patients in a large inception cohort such as the SLICC inception cohort would afford some clarification with regards to this. Ideally, telomere length would be measured in a cohort study to determine if telomere attrition or disease states appear first and initiatives such as The European Prospective Investigation of Cancer (EPIC) – Norfolk, may provide a setting for such a study. Prospective follow up of our cohort is required to determine the consequence of short telomeres.

Appendix 1 Ethics committee approval letter

North West MREC
Greater Manchester Strategic Health Authority
Room 155 - Gateway House
Piccadilly South
Manchester
M60 7LP

Telephone: (0161) 237 2394
Facsimile: (0161) 237 2383

15 November 2005

RECEIVED
17 NOV 2005

Dr Ian Bruce
Senior Lecturer and Consultant Rheumatologist
ARC Epidemiology Unit
The University of Manchester
Stopford Building
Oxford Road
MANCHESTER M13 9PT

Dear Dr Bruce

Full title of study:	**Accelerated atherosclerosis in SLE: Lupus factors, telomere shortening and progression of atherosclerosis**
REC reference number:	**05/MRE08/62**

Thank you for your letter of 10 November 2005, responding to the Committee's request for further information on the above research (and for submitting revised documentation).

The further information has been considered on behalf of the Committee by the Chair (Dr P R Kelsey).

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation (as revised).

Ethical review of research sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the research site(s) taking part in this study. The favourable opinion does not therefore apply to any site at present. I will write to you again as soon as one Local Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at sites requiring SSA.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows: -

The Central Office for Research Ethics Committees is responsible for the operational management of Multi-Centre Research Ethics Committees

Document	*Version*	*Date*
Application	1	11 July 2005
Investigator CV		
Questionnaire	Physical Activity Index	
Questionnaire	Family History Questionnaire	
GP/Consultant Information Sheets	1 (August 2005)	
Participant Information Sheet	2 - Follow-up group (SLE) - November 2005	
Participant Information Sheet	2 - New participants (SLE) - November 2005	
Participant Information Sheet	2 - Healthy Controls – November 2005	
Participant Consent Form	2 - November 2005	
Response to Request for Further Information		10 November 2005

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/MRE08/62	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

C.A.Stokes

p.p. **Dr P R Kelsey**
Chair

E-mail: - northwest.mrec@gmsha.nhs.uk

Enclosures: - Standard approval conditions (SL-AC2)

Copy to: - Dr John Rodgers
Head of the Research Office
University Research Office
Christie Building
The University of Manchester
Oxford Road
MANCHESTER M13 9PL

SF1 list of approved sites

Appendix 2 Study promotion

Arthritis Research Campaign

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RELEASED MAY 2006

Manchester women with lupus in major new study into high risk of heart disease

Women with lupus are to take part in a major study aimed at finding out why they have a hugely higher risk of developing heart disease than the general population.

An Arthritis Research Campaign-funded investigation at the University of Manchester will investigate if lupus patients' blood vessels age at a faster rate than those of healthy people.

Lupus (also known as SLE or systemic lupus erythematosus) is an inflammatory condition that affects mainly younger women. It can affect the joints, the brain, skin, kidneys and other internal organs. Women with lupus have a five to six-fold increased risk of developing coronary heart disease.

Now 250 female patients in the North West and 50 healthy volunteers are being recruited into a study to be performed by Dr Sahena Haque, a rheumatology specialist registrar at the **arc** epidemiology unit in Manchester, who is funded by a three-year clinical research fellowship of £190,000 from **arc**, the UK's fourth largest medical research charity.

Patients will be recruited from clinics at Manchester Royal Infirmary, North Manchester General Hospital in Crumpsall, Wythenshaw and Withington Hospital in south Manchester, and the Royal Blackburn Hospital.

In collaboration with Dr Ian Bruce, Senior Lecturer in Rheumatology at the University of Manchester, Rheumatism Research Centre, Manchester Royal Infirmary, Dr Haque will be investigating why lupus patients develop conditions, which usually affect much older people especially accelerated atherosclerosis (hardening of the arteries).

"Atherosclerosis in lupus develops much earlier, suggesting that the blood vessels in lupus patients may age at a faster rate," explained Dr Haque. "This, coupled with an inability to repair the blood vessels may result in the premature atherosclerosis that we see. The aim of this study is to examine the balance between biological ageing and the ability to repair the blood vessels in lupus patients."

Patients taking part in the study will undergo a full clinical assessment at the

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'Lupus shut down all my organs'

By Jane Elliott
Health reporter, BBC News

For years Chris Anderson had endured a number of baffling and seemingly unrelated and unexplained symptoms.

But it was not until she started to suffer complete organ failure that doctors were finally able to pinpoint her disease as lupus.

Chris first had problems in her 20s

Over the years 57-year-old Chris, from Stockport, Greater Manchester, has needed a heart valve replaced twice, has had three strokes, several mini strokes and a heart attack, which led to a stent being fitted to ease a blocked artery.

She was also diagnosed as an epileptic, had very high-blood pressure and suffered pre-eclampsia during pregnancy.

Diagnosis

"I felt generally terrible and extremely tired," said Chris, who spent three months in hospital after her all her organs began to shut down.

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A RAY OF HOPE IN BATTLE WITH LUPUS

From the Lancashire Telegraph, first published Friday 8th Dec 2006.

A MAJOR health study is being launched in East Lancashire into a rare and debilitating disease.

Lupus is an inflammatory condition that mainly affects younger women.

It attacks the immune system and can affect the joints, the brain, skin, kidneys and other internal organs.

Women living with Lupus have up to five or six times increased risk of developing coronary heart disease.

Two researchers at the Royal Blackburn Hospital will be involved in the study, which is based at the University of Manchester.

They are seeking women from throughout East Lancashire to take part.

More than 100 people in East Lancashire are believed to be living with Lupus, also known as SLE or systemic Lupus erythematosus.

Dr Lee-Suan Teh, consultant rheumatologist said: "It is a valuable and well-thought out study.

"It will hopefully not only help us towards identifying the mechanism and risks which lead to SLE patients developing heart disease but may also point us towards more effective preventative and/or treatment options.

"We have just had ethical approval in this trust to start this study and will be helping to recruit suitable patients soon."

Lupus patient Caroline Morrison-Pinches, 51, of St. George's Avenue, Blackburn, who discovered she had the disease in 1995, welcomed the new study. She said: "It's a good idea. More research needs to be done. However, women should be aware of the condition in the first place. I never knew about Lupus before I found out. Many have problems by the time Lupus is diagnosed, but they don't know they have it.

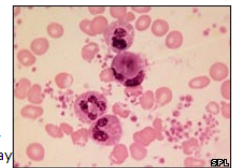
Study

Now Chris is to take part in a major study aimed at finding out why women with lupus have such a high risk of developing heart disease.

The £190,000 Arthritis Research Campaign (ARC)-funded investigation at the University of Manchester will look into whether lupus patients' blood vessels age at a faster rate than others.

Some 250 female patients and 50 healthy volunteers are being recruited for the study by Dr Sahena Haque, a rheumatology specialist registrar at the ARC epidemiology unit in Manchester.

In collaboration with Dr Ian Bruce, senior lecturer in rheumatology at the University of Manchester, Dr Haque will be looking at why lupus patients develop conditions such as artherosclerosis (hardening of the arteries) that usually affect only much older patients.



Lupus cells in a blood smear

"Artherosclerosis in lupus develops much earlier, suggesting that the blood vessels in lupus patients may age at a faster rate," said Dr Haque.

"This, coupled with an inability to repair the blood vessels, may result in the premature atherosclerosis that we see.

"The aim of this study is to examine the balance between biological ageing and the ability to repair the blood vessels in lupus patients."

They hope the study will not only help identify patient with lupus at risk of having angina and heart attacks, but will also try to find the cause and ultimately develop better treatments for the general public.

Appendix 3 Example of Patient information sheet

Central Manchester University Hospitals NHS
NHS Foundation Trust

(Version 3 August 2006)

Patient Information Sheet - new participant
(SLE)

Accelerated atherosclerosis in SLE: Lupus factors, telomere shortening and progression of atherosclerosis

<u>Accelerated atherosclerosis and premature senescence in SLE.</u>

Systemic lupus erythematosus (SLE) is a chronic autoimmune illness that occurs in about 1/2000 women in the UK. It can affect many parts of the body initiated firstly by inflammation and then further complicated by organ damage in some patients. Women with SLE also have approximately a 5-8 fold increased risk of heart disease caused by atherosclerosis (hardening of the arteries). Our research is focused on understanding this process better.

We have found in an initial study that a range of factors are likely to contribute to this increased risk of atherosclerosis and some of these factors included typical risk factors such as smoking and blood pressure as well as the type of lupus a patient has and certain antibodies associated with SLE also seemed to be important. It is also likely that some of the risk is driven by genetic markers associated with SLE and in particular we are interested in measuring telomere length and activity as one such marker. Telomere length is believed to be related to how quickly a cell ages and may be a better marker of cellular age. Since atherosclerosis occurs earlier in SLE we aim to determine whether the cells of SLE patients age at a quicker rate and if this relates to the higher risk of atherosclerosis seen. As we are particularly interested in arteries (blood vessels) we also wish to study how the cells lining blood vessels (endothelial cells) age and how they are replaced naturally in the circulation.

You are being asked to take part in this study because you have SLE and we wish to assess how factors associated with SLE alter the risk of atherosclerosis over time. This will be done by assessing SLE at baseline and also studying the thickness of the lining of the major blood vessel in the neck (carotid artery) to look for signs of early atherosclerosis as well as measurement of blood vessel stiffness. A repeat “stiffness” scan will also be performed every 6 months and the neck scan will be performed 2 years after the initial scan to assess progression of atherosclerosis and how SLE factors including telomere length may affect this.

What will I have to do if I take part?

If you agree to take part you will be asked to visit the Wellcome Trust Clinical Research Facility and to have fasted for 12 hours and avoided alcohol for 48 hours prior to this visit. We will take a medical history and examine you. This will help us to assess the current level of activity of your SLE. A 60ml (approximately 10 tea spoons) blood sample will be taken to measure the level of inflammation in your system as well as

measure your cholesterol, blood glucose and other factors that may influence the risk of heart disease including lupus associated antibodies. A DNA sample will also be taken from this blood sample. A small urine sample (10ml) will also be frozen and stored to measure inflammation markers.

The clinical assessment will be at the Wellcome Trust Clinical Research and this will include a history and examination as well as completing two Quality of Life questionnaires (SF36 and LupusQOL) and a short lifestyle questionnaire. Your treatment will also be recorded including current dose of steroids, immunosuppressive agents and anti malarial drugs.

You will also have two scans performed. The first (carotid scan) will be done using a small ultrasound probe placed on the surface of the neck and does not involve any needles. The scan will examine the main artery in your neck on both sides (carotid arteries) this will assess if there is any evidence of early atherosclerosis. This scan is painless and does not involve any discomfort

In addition to the carotid scan a second scan will also be performed. The second (pulse wave velocity) involves placing a small ultrasound probe at the base of your neck, above the collar bone to measure the wave-form. It does not involve any needles. A second measurement is also taken from the blood vessel at the top of your leg (femoral artery) using the same device. The distance between the 2 probes is then measured. This scan compares the pulsation in your neck with that in the artery at the top of your leg (femoral artery). Again, this is a painless procedure and no needles are involved. We will repeat this scan every 6 months for 2 years.

The genetic sample will be used to measure telomere length. This is the length of the small protein 'caps' at the end of each DNA strand. These are believed to shorten gradually as we age. It will also be used to study other genes. Such genes include genes that alter proteins involved in SLE inflammation, for example, mannose-binding lectin, complement genes and E-selectin, also genes that control cholesterol such as apolipoprotein E. Some of the blood sample will also be used to measure the type ('new' cells involved in repair vs 'old' that have become detached from the blood vessel) and age of endothelial cells. Some of these tests may be done in different laboratories in Europe or the USA. When your sample is sent to these collaborators, it will be coded and they will not be able to identify you.

Overall the visit to the Lupus Research Clinic will take about 1 hour and can be arranged to coincide with your routine clinic appointment if necessary.

We will reimburse you travel expenses by public transport or private car to attend any visits that are in addition to your usual clinic visits.

What are the possible risks of taking part?

Taking the blood sample may cause bruising and scan involves no risk. There is however a chance that we will discover some blood test or scan abnormalities that you were unaware of and are clinically relevant. If we find any unexpected abnormalities in the blood tests or scans a member of the study team will advise you of these and arrange with your agreement any additional tests or referrals that may be appropriate.

This kind of genetic testing does not have any implications for other members of your family and it will not affect your chances of getting life insurance, mortgages etc.

Are there any possible benefits?

As a result of this study we will have detailed information of several important risk factors for heart disease. A member of the research team will discuss all the results

with you when they are available. With your permission they will communicate clinically relevant results to your general practitioner and your own Consultant where appropriate. The results of the genetic tests will remain confidential.

Do I have to take part?

No, taking part is voluntary. If you prefer not to take part you do not have to give a reason. You can also withdraw at any time and again no reason is necessary. Refusal to take part will in no way affect the treatment you receive now or in the future. If at any time you decide that you no longer wish to be included in the genetic part of the study, this sample will be destroyed and not used further in the study.

All information gathered as a result of this study will be treated in the strictest confidence and no publication or reporting of these results will personally identify you in any way.

We would want to inform your GP that you are taking part, with your permission.

What will happen to the blood and genetic samples?

This sample will be a gift to medical research, and will be stored in a secure laboratory. Only authorised personnel associated with this study will have access to the sample. At the end of the study we would like to retain the samples. This may be valuable in future research so we would like to keep it as a coded sample. We will however only use your sample in future research after we have been granted further ethical approval.

What do I do now?

The doctor or study nurse organising this study will contact you to discuss whether you wish to take part and make the necessary arrangement for you.

Thank you for considering taking part in this research. Please do not hesitate to discuss this information with your family, friends or GP if you wish.

For further advice regarding this study you can contact:

Dr Ian Bruce,
Reader and Consultant Rheumatologist
or

Sr Joanna Shelmerdine
University of Manchester
Rheumatism Research Centre,
Central Manchester and Manchester Children's University Hospitals NHS Trust,
Oxford Road,
Manchester, M13 9WL.

Telephone: 0161 276 6841.

MANCHESTER
1824
The University
of Manchester

Appendix 4 GP information letter

Accelerated atherosclerosis in SLE: Lupus factors, telomere shortening and progression of atherosclerosis

GENERAL PRACTITIONER INFORMATION LETTER

5th October 2009

Dear «Title» «GPName»,

Re: *«PatName» «PatSurname» («PatDOB») «PatAddress» «PatArea» «PatTown» «PatPost»*

I am writing to inform you that your patient indicated above is currently taking part in the above study. This is a multi-centre study looking at the progression of atherosclerosis in SLE and whether or not this is associated with telomere shortening (premature ageing) and abnormal endothelial turnover. We aim to compare changes/progression in subclinical atherosclerosis in a well defined population of patients with SLE followed over a 3-5 year period with that seen in a control population.

Your patient has agreed to take part in this study (either as a patient or healthy control) and we therefore plan to review their medical notes, take a medical history and examine them. We will take a 60ml blood sample to look at your patient's level of inflammation as well as to measure their cholesterol, blood glucose and other factors that may influence the risk of heart disease including lupus associated antibodies. A DNA sample will also be taken from this blood sample and a 10ml urine sample will be frozen and stored to measure inflammation markers. Finally, your patient will undergo an ultrasound scan of the carotid artery.

Should the blood tests or scan identify any unexpected clinically relevant abnormalities, your patient will be advised of any additional tests or referrals that may be relevant.

If you require any further information regarding this study please do not hesitate to contact me.

With best wishes
Yours sincerely

Dr I N Bruce
Senior Lecturer and Consultant Rheumatologist

Dr S Haque
Clinical Research Fellow

Appendix 5 Clinic data collection form

CENTRE NAME:____________________ PATIENT INITIALS:______ ______

CENTRE NO:______________________ PATIENT ID NO:________ ______

ASSESSMENT DATE:_____/_____/_____
d m y

For follow – up patients please update any changes since last visit

DEMOGRAPHIC DATA

Date of birth: ______ /_____ /_____ ______ /_____
d m y

Date of diagnosis of SLE: ______ /
d m y

Date of 1st symptom: ______/ ______/______

ACR criteria for diagnosis (tick as appropriate):

- □ Malar rash
- □ Discoid rash
- □ Oral ulcers
- □ Serositis
- □ Arthritis
- □ Photosensitivity
- □ Renal disorder
- □ Neurologic disorder
- □ Haematalogic disorder
- □ Immunologic disorder
- □ ANA

Other features:

□ Raynauds □ Fatigue □ Polymyositis □ Last DXA scan □ Fractures

Marital Status (circle as appropriate):

1 = Single
2 = Married
3 = Widowed
4 = Divorced
5 = Separated
6 = Common law

Education:

Number of years prior to college/university: ______
Number of years at college/ university: ______

*Occupation (specify):*_____________________________

FAMILY HISTORY AND LIFESTYLE

*Alcohol consumption:*______units per week or ______ml per week

Cigarette smoking (delete as appropriate):

Current	Yes / No	If yes, number per day	______
Ex-smoker	Yes / No	If no, number of years smoking	______
		and date stopped	______ / ______ /_____
			d m y

Lifestyle questionnaire completed by patient (delete as appropriate): Yes/No

CLINICAL DATA

*Height:*______cm *Weight (shoes and coat off):*______kg *BMI:* ____________

*Waist/hip ratio:*_____ cm /_____cm *Blood pressure (systolic/diastolic):*_____ / ____________

Antihypertensive therapy (delete as appropriate):

Current Yes / No If yes, specify type (circle as appropriate):

0 = Diuretics	5 = Calcium antagonists
1 = Adrenergic inhibitors	6 = Angiotensin-converting enzyme inhibitors
2 = Central and agonists	
3 = Beta blockers	7 = Other
4 = Direct vasodilators	8 = Combination

In the past Yes / No If yes, specify type (circle as appropriate):

0 = Diuretics	5 = Calcium antagonists
1 = Adrenergic inhibitors	6 = Angiotensin-converting
2 = Central and agonists	enzyme inhibitors
3 = Beta blockers	7 = Other
4 = Direct vasodilators	8 = Combination

Myocardial infarction (circle as appropriate):

In the past Yes / No If yes, specify date(s): ______ / ______ /_____
d m y

______ /_____ / ______

d m
y

Angina (circle as appropriate):

Current Yes / No If yes, specify date of diagnosis: ______ /
______ /_____
d m
y
______ /
______ /_____
d m
y

In the past Yes / No If yes, specify date of diagnosis: ______ /
______ /_____
d m
y
______ /
______ /_____
d m
y

Congestive heart failure (circle as appropriate):

Current Yes / No If yes, specify date(s): ______ /
______ /_____
d m
y
______ /
______ /_____
d m
y

In the past Yes / No If yes, specify date(s): ______ /
______ /_____
d m
y
______ /
______ /_____
d m
y

Angioplasty (circle as appropriate):
Ever Yes / No If yes, specify date(s): ______ /
______ /_____
d m
y
______ /
______ /_____
d m
y

Bypass surgery(circle as appropriate):

Ever Yes / No If yes, specify date: ______ / ______ /_____

d m y

PREVIOUS SLE CARDIAC MANIFESTATIONS

Pericardiditis: Yes / No If yes, specify date(s): ______ / ______ /_____

d m y

______ / ______ /_____

d m y

Myocarditis: Yes / No If yes, specify date(s): ______ / ______ /_____

d m y

______ / ______ /_____

d m y

Endocarditis: Yes / No If yes, specify date(s): ______ / ______ /_____

d m y

______ / ______ /_____

d m y

PERIPHERAL VASCULAR

Intermittent claudication (circle as appropriate):

Current Yes / No If yes, specify date(s): ______ / ______ /_____

d m y

______ / ______ /_____

d m y

In the past Yes / No If yes, specify date(s): ______ / ______ /_____

d m y

______ / ______ /_____

d m y

CEREBROVASCULAR

Transient ischemic attack (circle as appropriate): Yes / No

If yes, specify date(s): ______ /_____ /_____
d m
y
______ /
______ /_____
d m
y

Stroke (circle as appropriate): Yes / No

If yes, specify date(s):_____ /_____ /_____
d m
y
______ /
______ /
d m
y

Type (if known): 1 = Hemorrhagic 2 = Thrombotic

HYPERLIPIDEMIA THERAPY

Current Yes / No If current, specify type (circle as appropriate):
0 = None 4 = Fibrates
1 = Statins 5 = Combinations
2 = Sequestrants 6 = Other
3 = Nicotinic acid

In the past Yes / No If in the past, specify type (circle as appropriate):
0 = None 4 = Fibrates
1 = Statins 5 = Combinations
2 = Sequestrants 6 = Other
3 = Nicotinic acid

HORMONAL FACTORS

Ovarian function (circle all which apply):

1 = Menstruating 5 = Pre-menopausal hysterectomy
2 = Premenarche 6 = Post-menopausal hysterectomy
3 = Postmenopausal 7 = Pre-menopausal hysteroophorectomy
4 = Amenorrhea

Age at menopause ________

Oral contraceptive

Current Yes / No If yes, specify number of years:___ ______

In the past Yes / No If yes, specify number of years:___ ______

Hormone replacement therapy:

Current Yes / No If current, specify type (circle as appropriate):

1 = Estrogen (specify):___________ ______

2 = Estrogen + progesterone

3 = Progesterone only
4 = Other (specify):______________ ______

Current course start date: ______ / ______ / ____
d m y

In the past Yes / No If in the past, specify type (circle as appropriate):

1 = Estrogen (specify):___________ ______

2 = Estrogen + progesterone

3 = Progesterone only
4 = Other (specify):______________ ______

Are you currently pregnant? (circle as appropriate): Yes / No

Gravida / Para: ______ / ____ Miscarriage (No):__________ ______

ENDOCRINE

Hypothyroidism:

Current Yes / No

Ever Yes / No

Diabetes: Yes / No If yes, specify date of diagnosis:___ / ____ / ______

d m y

Specify type (delete as appropriate): Type 1 / Type 2

RENAL
Active nephritis:

Current Yes / No

In the past Yes / No

Nephrotic syndrome:

Current Yes / No

In the past Yes / No

Past medical history:____

____

If follow up patient complete page 9 instead of this page

THERAPY

Steroids:

Current Yes / No If yes, specify course start date: __ / ____ / ______

d m y
and average daily dose:__________ mg

In the past Yes / No If yes, number of previous courses:____________

And average daily dose:____ mg

Number of previous courses of IV steroids (and details):________________________________

____
____

Number of courses in 6 months before event:____________________________
____

Anti-malarials:

Current Yes / No If yes, specify type (circle as appropriate):
1 = Chloroquine 3 = Hydroxychloroquine
2 = Atabrine 4 = Other (specify):_
____

If yes, specify course start date: __ / ____ / ____
d m y
and average daily dose:____ mg

In the past Yes / No If yes, specify type (circle as appropriate):
1 = Chloroquine 3 = Hydroxychloroquine
2 = Atabrine 4 = Other (specify):_
____

Immunsuppressives:

Current Yes / No If yes, specify type (circle as appropriate):
1 = Azathioprine 4 = Cyclosporin
2 = Cyclophosphamide PO 5 = Mycophenolic acid
3 = Cyclophosphamide IV 6 = Methotrexate
7 = Other (specify):_
____

In the past Yes / No If yes, specify type (circle as appropriate):
1 = Azathioprone 4 = Cyclosporin
2 = Cyclophosphomide PO 5 = Mycophenolic acid
3 = Cyclophosphomide IV 6 = Methotrexate

7 = Other (specify):_
____

Current medications:____
____

QUALITY OF LIFE:

Lupus QOL

DOMAIN	Physical health	Pain	Planning	Intimate relationship	Burden to others	Emotional health	Body image	Fatigue
SCORE								

SF36

DOMAIN	Physical function	Role physical	Bodily pain	General health	Vitality	Social function	Role emotional	Mental health
SCORE								

Appendix 6 Carotid ultrasonography protocol

The right and left common carotid artery (CCA), carotid bulb and the first 1.5 cm of the internal and external carotid arteries were examined in longitudinal and cross-sectional planes using the Philips HDI 5000. Intima-medial thickness (IMT) was measured as previously described and validated [319]. Briefly, measurements were made in a longitudinal plane at a point of maximum thickness on the far wall of the CCA along a 1 cm section of the artery proximal to the carotid bulb. Measurements were repeated three times on each side, unfreezing the image on each occasion and relocating the maximal IMT, the average of six measurements were then used to calculate the mean IMT. Carotid plaque was defined if two of the following three conditions were met: (i) a distinct area of protrusion >50% compared with the surrounding area into the vessel lumen, (ii) increased echogenicity than the adjacent boundaries and (iii) IMT >0.15 cm [209].

The degree of plaque was graded using the plaque index outlined below as previously described and validated [330].

Table 41 Plaque index

Grades	Description
0	No observable plaque
1	One small plaque (< 30% of vessel diameter)
2	One medium plaque (30-50 % of vessel diameter) or multiple small plaque
3	One large plaque (>50% of vessel diameter) or multiple plaque with al least one medium sized plaque

Adapted from [330]

Inter-observer variability

Carotid scans were performed by one of two experienced vascular technicians blinded to the all other patients details. Measurements taken by each technician on 10 different subjects has shown a good level of agreement

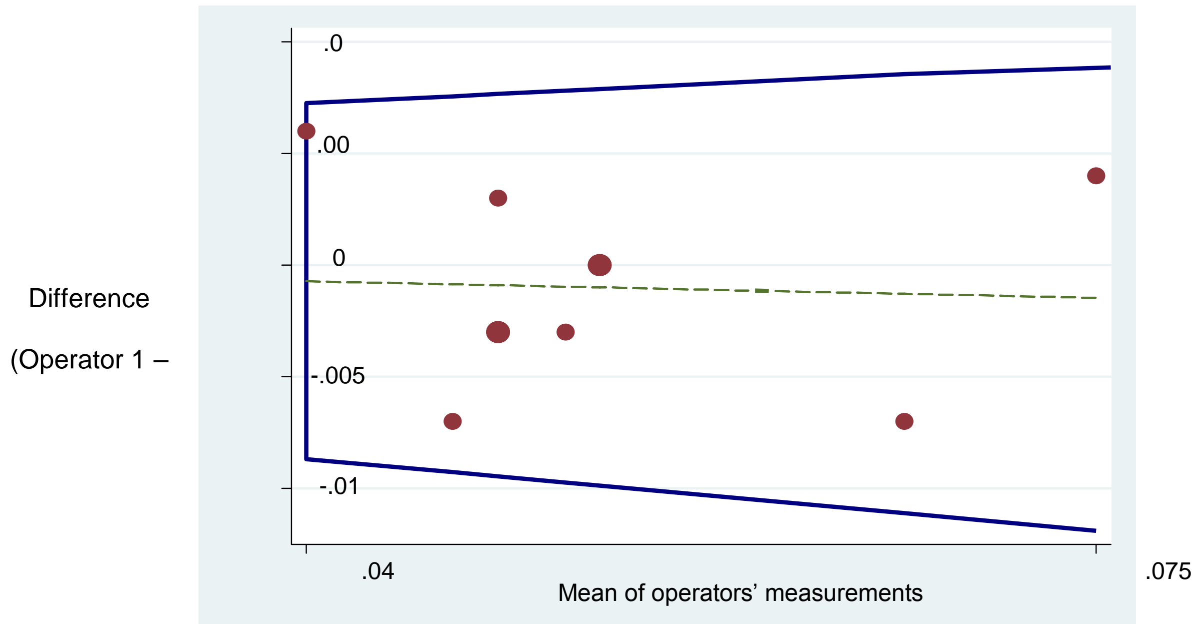


Figure 40 Bland Altman plot of difference between 2 operators (vertical axis) and mean of 2 operators (horizontal axis)

Dashed green line represents the mean difference (- 0.001) and bold blue line represents the 95% limits of agreement (-0.0098, 0.0078) and indicate good agreement between operators.

Appendix 7 Aortic pulse wave velocity measurement

Participants were supine for >5 minutes before recording. All measurements ere undertaken in a temperature-regulated room. Carotid-femoral APWV was determined using the Micro Medical Pulse Wave Velocity machine by sequentially recording ECG-gated carotid and femoral artery waveforms. Distances from sternal notch to the femoral was measured as a straight line between the points on the body surface using a tape measure. A 4Mhz Doppler pencil probe was placed at 45° to the skin surface of the artery and measured with the probe facing towards the flow direction (towards the heart).

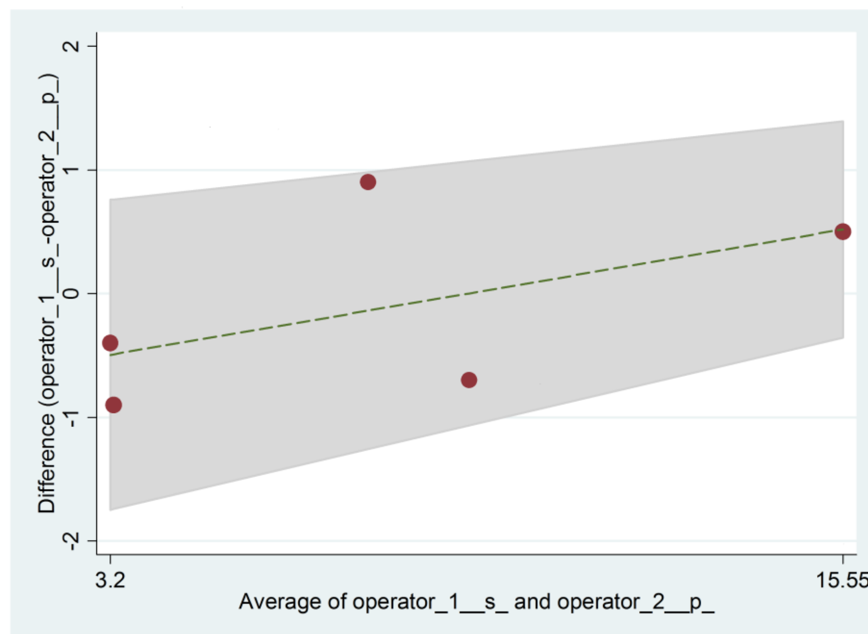


Figure 41 Bland Altman plot of difference between 2 operators (vertical axis) and mean of 2 operators (horizontal axis)

Dashed green line represents the mean difference and bold blue line represents the 95% limits of agreement and indicates little variation between operators.

Appendix 8 BILAG Index

BILAG2004 INDEX **Centre:** **Date:** **Initials/Hosp No:**

Only record items <u>due to SLE Disease Activity</u> & assessment refers to manifestations occurring in the <u>last 4 weeks (compared with the previous 4 weeks)</u>. ♦♦ **TO BE USED WITH THE GLOSSARY** ♦♦

Scoring:
- **ND Not Done**
- **1 Improving**
- **2 Same**
- **3 Worse**
- **4 New**
- **Yes/No OR Value (where indicated)**
- ❑ **indicate if <u>not due to SLE activity</u>**
- **(default is 0 = not present)**

CONSTITUTIONAL

1. Pyrexia - documented > 37.5ºC ( )
2. Weight loss - unintentional > 5% ( )
3. Lymphadenopathy/splenomegaly ( )
4. Anorexia ( )

MUCOCUTANEOUS

5. Skin eruption - severe ( )
6. Skin eruption - mild ( )
7. Angio-oedema - severe ( )
8. Angio-oedema - mild ( )
9. Mucosal ulceration - severe ( )
10. Mucosal ulceration - mild ( )
11. Panniculitis/Bullous lupus - severe ( )
12. Panniculitis/Bullous lupus - mild ( )
13. Major cutaneous vasculitis/thrombosis ( )
14. Digital infarcts or nodular vasculitis ( )
15. Alopecia - severe ( )
16. Alopecia - mild ( )
17. Peri-ungual erythema/chilblains ( )
18. Splinter haemorrhages ( )

NEUROPSYCHIATRIC

19. Aseptic meningitis ( )
20. Cerebral vasculitis ( )
21. Demyelinating syndrome ( )
22. Myelopathy ( )
23. Acute confusional state ( )
24. Psychosis ( )
25. Acute inflammatory demyelinating polyradiculoneuropathy ( )
26. Mononeuropathy (single/multiplex) ( )
27. Cranial neuropathy ( )
28. Plexopathy ( )
29. Polyneuropathy ( )
30. Seizure disorder ( )
31. Status epilepticus ( )
32. Cerebrovascular disease (not due to vasculitis) ( )
33. Cognitive dysfunction ( )
34. Movement disorder ( )
35. Autonomic disorder ( )
36. Cerebellar ataxia (isolated) ( )
37. Lupus headache - severe unremitting ( )
38. Headache from IC hypertension ( )

MUSCULOSKELETAL

39. Myositis - severe ( )
40. Myositis - mild ( )
41. Arthritis (severe) ( )
42. Arthritis (moderate)/Tendonitis/Tenosynovitis ( )
43. Arthritis (mild)/Arthralgia/Myalgia ( )

Weight (kg):	**Serum urea (mmol/l):**
African ancestry: Yes/No	**Serum albumin (g/l):**

CARDIORESPIRATORY

44. Myocarditis - mild ( )
45. Myocarditis/Endocarditis + Cardiac failure ( )
46. Arrhythmia ( )
47. New valvular dysfunction ( )
48. Pleurisy/Pericarditis ( )
49. Cardiac tamponade ( )
50. Pleural effusion with dyspnoea ( )
51. Pulmonary haemorrhage/vasculitis ( )
52. Interstitial alveolitis/pneumonitis ( )
53. Shrinking lung syndrome ( )
54. Aortitis ( )
55. Coronary vasculitis ( )

GASTROINTESTINAL

56. Lupus peritonitis ( )
57. Abdominal serositis or ascites ( )
58. Lupus enteritis/colitis ( )
59. Malabsorption ( )
60. Protein losing enteropathy ( )
61. Intestinal pseudo-obstruction ( )
62. Lupus hepatitis ( )
63. Acute lupus cholecystitis ( )
64. Acute lupus pancreatitis ( )

OPHTHALMIC

65. Orbital inflammation/myositis/proptosis ( )
66. Keratitis - severe ( )
67. Keratitis - mild ( )
68. Anterior uveitis ( )
69. Posterior uveitis/retinal vasculitis - severe ( )
70. Posterior uveitis/retinal vasculitis - mild ( )
71. Episcleritis ( )
72. Scleritis - severe ( )
73. Scleritis - mild ( )
74. Retinal/choroidal vaso-occlusive disease ( )
75. Isolated cotton-wool spots (cytoid bodies) ( )
76. Optic neuritis ( )
77. Anterior ischaemic optic neuropathy ( )

RENAL

78. Systolic blood pressure (mm Hg) value ( ) ❑
79. Diastolic blood pressure (mm Hg) value ( ) ❑
80. Accelerated hypertension Yes/No ( )
81. Urine dipstick protein (+=1, ++=2, +++=3) ( ) ❑
82. Urine albumin-creatinine ratio mg/mmol ( ) ❑
83. Urine protein-creatinine ratio mg/mmol ( ) ❑
84. 24 hour urine protein (g) value ( ) ❑
85. Nephrotic syndrome Yes/No ( )
86. Creatinine (plasma/serum) µmol/l ( ) ❑
87. GFR (calculated) ml/min/1.73 m^2 ( ) ❑
88. Active urinary sediment Yes/No ( )
89. Active nephritis Yes/No ( )

HAEMATOLOGICAL

90. Haemoglobin (g/dl) value ( ) ❑
91. Total white cell count (x 10^9/l) value ( ) ❑
92. Neutrophils (x 10^9/l) value ( ) ❑
93. Lymphocytes (x 10^9/l) value ( ) ❑
94. Platelets (x 10^9/l) value ( ) ❑
95. TTP ( )
96. Evidence of active haemolysis Yes/No ( )
97. Coombs' test positive (isolated) Yes/No ( )

Appendix 9 SLE Disease Activity Index

SYSTEMIC LUPUS ERYTHEMATOSUS DISEASE ACTIVITY INDEX (SLEDAI)

Chart no: ☐ Date of visit: ☐

M.D: ☐ Patient's name: ☐

(Enter weight in SLEDAI Score column if descriptor present at the time of the visit or in the preceding 10 days).

Weight	SLEDAI score	Descriptor	Definition
8	☐	Seizure	Recent onset. Exclude metabolic, infectious, or drug causes.
8	☐	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behavior. Exclude uremia and drug causes.
8	☐	Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function, with rapid onset and fluctuating clinical features. Include clouding of consciousness with reduced capacity to focus, and inability to sustain attention to environment, plus at least 2 of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious, or drug causes.
8	☐	Visual disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serous exudate or hemorrhages in the choroid, or optic neuritis. Exclude hypertension, infection or drug causes.
8	☐	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	☐	Lupus headache	Severe, persistent headache; may be migrainous, but must be nonresponsive to narcotic analgesia.
8	☐	CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	☐	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.
4	☐	Arthritis	More than 2 joints with pain and signs of inflammation (i.e., tenderness, swelling or effusion).
4	☐	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/aldolase or

Weight	SLEDAI score	Descriptor	Definition
4		Urinary casts	Heme-granular or red blood cell casts.
4		Hematuria	>5 red blood cells/high power field. Exclude stone, infection, or other cause.
4		Proteinuria	>0.5 gm/24 hours. New onset of recent increase of more than 0.5 gm/24 hours.
4		Pyuria	>5 white blood cells/high power field. Exclude infection.
2		New rash	New onset or recurrence of inflammatory type rash.
2		Alopecia	New onset or recurrence of abnormal, patchy or diffuse loss of hair.
2		Mucosal ulcers	New onset or recurrence of oral or nasal ulcerations
2		Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2		Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation.
2		Low complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.
2		Increased DNA	>25% binding by Farr assay or above normal range for testing laboratory.
1		Thrombocytopenia	>38 degrees celsius. Exclude infectious cause.
1		Leukopenia	<100,000 platelets/mm^3.
1		Fever	<3,000 white blood cells/mm^3. Exclude drug causes.
		total SLEDAI score	

Systemic Lupus Erythematosus Disease Activity Index (SLEDAI).

Appendix 10 SLICC Damage Index

SLICC/ACR DAMAGE INDEX FOR SYSTEMIC LUPUS ERYTHEMATOSUS

Overview:

The Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) Damage Index for Systemic Lupus Erythematosus (SLE) records damage occurring in patients with SLE irregardless of causation. This can be used to monitor patients over time especially for comparing periods of disease activity and inactivity.

Glossary of Terms

Term	Definition
Damage	Nonreversible change not related to active inflammation occurring since diagnosis of lupus ascertained by clinical assessment and present for at least 6 months unless otherwise stated. **Repeat episodes must occur at least 6 months apart to score 2.** The same lesion cannot be scored twice.
Cataract	A lens opacity (cataract) in either eye ever whether primary or secondary to steroid therapy documented by ophthalmoscopy
Retinal damage	Documented by ophthalmoscopic examination may result in field defect legal blindness
Optic atrophy	Documented by ophthalmoscopic examination.
Cognitive impairment	Memory deficit difficulty with calculation poor concentration difficulty in spoken or written language impaired performance level documented on clinical examination or by formal neurocognitive testing.
Major psychosis	Altered ability to function in normal activity due to psychiatric reasons. Severe disturbance in the perception of reality characterized by the following features: delusions hallucinations (auditory visual) incoherence marked loose associations impoverished thought content marked illogical thinking bizarre disorganized or catatonic behavior.
Seizures	Paroxysmal electrical discharge occurring in the brain and producing characteristic physical changes including tonic and clonic movements and certain behavioral disorders. Only seizures requiring therapy for 6 months are counted as damage.
Cerebrovascular accident (CVA)	Cerebrovascular accident resulting in focal findings such as paresis weakness etc. or surgical resection for causes other than malignancy.
Neuropathy	Damage to either cranial or peripheral nerve excluding optic nerve resulting in either motor or sensory dysfunction.
Transverse myelitis	Lower extremity weakness or sensory loss with loss of rectal and urinary bladder sphincter control
Renal	Estimated or measured glomerular filtration rate $<50\%$ proteinuria $\geq$ 3.5 gm per 24 hours or end-stage renal disease (regardless of dialysis or transplantation)
Pulmonary	Pulmonary hypertension (right ventricular prominence or loud P2); pulmonary fibrosis (physical or radiograph); shrinking lung (radiograph); pleural fibrosis (radiograph); pulmonary infarction (radiograph); resection for cause other than malignancy)
Cardiovascular	Angina or coronary artery bypass; myocardial infarction (documented by electrocardiograph and enzyme studies) ever; cardiomyopathy (ventricular dysfunction documented clinically); valvular disease (diastolic murmur or systolic murmur $>3/6$);

Term	Definition
Peripheral vascular	Claudication persistent for 6 months by history; minor tissue loss such as pulp space ever; significant tissue loss such as loss of digit or limb or resection ever; venous thrombosis with swelling ulceration or clinical evidence of venous stasis
Gastrointestinal	Infarction or resection of bowel below duodenum by history resection of liver spleen or gallbladder ever for whatever cause; mesentric insufficiency with diffuse abdominal pain on clinical examination; chronic peritonitis with persistent abdominal pain and peritoneal irritations on clinical examination; esophageal stricture on endoscopy upper gastrointestinal tract surgery such as correction of stricture ulcer surgery etc. ever by history; pancreatic insufficiency requiring enzyme replacement or with a pseudocyst
Musculoskeletal	Muscle atrophy or weakness demonstrated on clinical examination; deforming or erosive arthritis including reducible deformities (excluding avascular necrosis) on clinical examination; osteoporosis with fracture or vertebral collapse (excluding avascular necrosis) demonstrated radiographically; avascular necrosis demonstrated by any imaging technique; osteomyelitis documented clinically and supported by culture evidence; tendon ruptures
Skin	Scarring chronic alopecia documented clinically; extensive scarring or panniculum other than scalp and pulp space documented clinically; skin ulceration (excluding thrombosis) for more than 6 months
Premature gonadal failure	Secondary amenorrhea prior to age of 40
Diabetes	Diabetes requiring therapy but regardless of treatment.
Malignancy	Documented by pathologic examination excluding dysplasia

Scoring

Organ	Item	Points
Ocular (either eye by clinical assessment)	Any cataract ever	1
	Retinal damage or optic atrophy	1
Neuropsychiatric	Cognitive impairment (e.g. memory deficit difficulty with calculation poor concentration difficulty in spoken or written language impaired performance level) or major psychosis	1
	Seizures requiring therapy for 6 months	1
	Cerebrovascular accident ever (score 2 if more than 1) or surgical resection for causes other than malignancy	1 or 2
	Cranial or peripheral neuropathy (excluding optic)	1
	Transverse myelitis	1
Renal	Estimated or measured glomerular filtration rate $<50\%$ **or**	1
	Proteinuria $\geq$ 3.5 g per 24 hours **or**	1
	End-stage renal disease (regardless of dialysis or transplantation)	3

SLICC/ACR Damage Index for Systemic Lupus Erythematosus continued.

Organ	Item	Points
Pulmonary	Pulmonary hypertension (right ventricular prominence or loud P2)	1
	Pulmonary fibrosis (physical and radiograph)	1
	Shrinking lung (on radiograph)	1
	Pleural fibrosis (on radiograph)	1
	Pulmonary infarction (on radiograph) OR pulmonary resection for cause other than malignancy	1
Cardiovascular	Angina or coronary artery bypass	1
	Myocardial infarction ever (score 2 if more than 1)	1 or 2
	Cardiomyopathy (ventricular dysfunction)	1
	Valvular disease (diastolic murmur or systolic murmur >3/6)	1
	Pericarditis for 6 months or pericardiectomy	1
Peripheral vascular	Claudication for 6 months	1
	Minor tissue loss (pulp space)	1
	Significant tissue loss ever (e.g. loss of digit or limb resection) (score 2 if more than one site)	1 or 2
	Venous thrombosis with swelling ulceration or venous stasis	1
Gastrointestinal	Infarction or resection of bowel below duodenum spleen liver or gallbladder for any cause (score 2 if more than 1 site)	1 or 2
	Mesenteric insufficiency	1
	Chronic peritonitis	1
	Esophageal stricture or upper gastrointestinal tract surgery ever	1
	Pancratic insufficiency requiring enzyme replacement or pseudocyst	1
Musculoskeletal	Muscle atrophy or weakness	1
	Deforming or erosive arthritis (including reducible deformities excluding avascular necrosis)	1
	Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis)	1
	Avascular necrosis (score 2 if more than 1)	1 or 2
	osteomyelitis	1
Skin	Scarring chronic alopecia	1
	Extensive scarring or panniculum other than scalp and pulp space	1
	Skin ulceration (excluding thrombosis) for more than 6 months	1
Other	Premature gonadal failure	1
	Diabetes (regardless of treatment)	1
	Malignancy (exclude dysplasia) (score 2 if more than 1 site)	1 or 2

SLICC/ACR Damage Index for Systemic Lupus Erythematosus continued.

Uncertainties in scoring

- Cerebrovascular accident in glossary but not in original table includes resection for cause other than malignancy as an "or" clause
- Renal damage: assumed that only one of the 3 disease entities mentioned (reduction GFR proteinuria end-stage renal disease) would be scored giving maximum score of 3 rather than 5
- In valvular disease assumed that the 3 limit applied only to systolic murmur; assumed that any diastolic murmur would apply
- Tendon ruptures is listed in the glossary but not in the table
- Primary gonadal failure in the glossary applies only to women; I added testicular failure for males for no substantiated reason.

Interpretation:

- Minimum score: 0
- Maximum score is unclear. According to Stoll (1996) the maximum total is 46 points but I get 47 on adding up his data (he scores a maximum of 2 for skin when it appears to be 3). A breakdown by sections: 47 points (ocular 2 neuropsychiatric 6 renal 3 pulmonary 5 cardiovascular 6 peripheral vascular 5 gastrointestinal 6 musculoskeletal 6 skin 3 other 4). If tendon rupture is added the maximum would be 48.
- The higher the score the more extensive the damage.

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SLICC/ACR Damage Index for Systemic Lupus Erythematosus.

Appendix 11 Example of telomere length calculation

Pos	Name/study_id	TelCp	Average Cp TELOMERE	Pos	Name	36B4 Cp	Average Cp 36B4	ΔCT	ΔΔCT (RELATIVE TO TV4)	RELATIVE TELOMERE LENGTH
A1	15	13.56	13.25666667	A1	15	20.72	20.62666667	-7.37	1.04333	0.485206237
A2	15	13.16		A2	15	20.62				
A3	15	13.05		A3	15	20.54				
A5	41	12.56	12.58333333	A5	41	20.29	20.38333333	-7.8	0.61333	0.653686134
A6	41	12.55		A6	41	20.44				
A7	41	12.64		A7	41	20.42				
A9	65	12.51	12.49666667	A9	65	20.57	20.51666667	-8.02	0.39333	0.761370195
A10	65	12.48		A10	65	20.46				
A11	65	12.5		A11	65	20.52				
A13	94	11.72	11.745	A13	94	20.43	20.54333333	-8.798333333	-0.385003333	1.305862804
A14	94			A14	94	20.54				
A15	94	11.77		A15	94	20.66				
B1	16	12.32	12.2	B1	16	20.25	20.33666667	-8.136666667	0.276663333	0.825498024
B2	16	12.22		B2	16	20.32				
B3	16	12.06		B3	16	20.44				

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