Vitamin D and endothelial function and repair in Systemic Lupus Erythematosus

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Abstract

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Vitamin D and endothelial function and repair in Systemic Lupus Erythematosus
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Introduction
Patients with Systemic Lupus Erythematosus (SLE) have increased cardiovascular risk, endothelial dysfunction, and abnormal endothelial repair mechanisms. Vitamin D deficiency is common in SLE and has been associated with active disease and increased vascular stiffness. Myeloid angiogenic cells (MACs) repair damaged vessels by secretion of angiogenic factors and may be a target for vitamin D. Vitamin D may therefore be a novel therapy to improve cardiovascular risk in SLE patients. This study aimed to determine the effects of vitamin D on endothelial function and repair in patients with SLE.

Methods
The effects of the active form of vitamin D (1,25(OH)2D3) were studied on MACs from vitamin D deficient SLE patients ex vivo. Functional models were developed to study MAC migration, adhesion and interaction with endothelial cells. Additional experiments used a model of healthy MACs treated with IFN-alpha. An observational study of clinically stable vitamin D deficient SLE patients being treated with high dose vitamin D over 3 months was used to investigate the effects of vitamin D on endothelial function as measured by flow-mediated dilatation (FMD).

Results
MACs expressed markers consistent with an M2 macrophage phenotype and they enhanced endothelial network formation in vitro. SLE patients had an increased number of MACs; however, these were dysfunctional compared to healthy controls. Vitamin D increased the number, changed the phenotype and improved the functional capacity of SLE MACs ex vivo. In addition, the angiogenic capacity of SLE MACs was restored toward that of healthy controls via a reduction in the anti-angiogenic cytokine IP10. Vitamin D-treated MACs were also more able to protect endothelial cells against TNF-mediated down-regulation of endothelial nitric oxide synthase (eNOS). In SLE patients treated with vitamin D, there was a strong correlation between the change in serum 25(OH)D and the change in the ratio of endothelium-dependent:independent dilatation (r=-0.650, p=0.006). This was accompanied by an increase in the number of MACs at 3 months (p=0.015). These observations were independent of changes in serum PTH, calcium or lupus disease activity.

Conclusions
Vitamin D can target MACs and therefore offers a novel approach to improve endothelial repair in patients with SLE. In addition, vitamin D treatment in lupus patients resulted in an improvement in endothelial function, related to the change in vitamin D status. These results suggest that vitamin D could improve surrogate markers of cardiovascular disease and thus reduce cardiovascular risk in this patient group.
Declaration
No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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I would like to begin by thanking Ian Bruce for his continual support, advice and guidance from the early days of planning the fellowship right through to its completion.

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Finally, I would like to thank my family, especially my parents, who offered unrivalled support encouragement and patience not just over the last 4 years but throughout.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)$_2$D</td>
<td>1,25-dihydroxyvitamin D</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-hydroxyvitamin D</td>
</tr>
<tr>
<td>ACEA</td>
<td>Anti-endothelial cell antibody</td>
</tr>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ANA</td>
<td>Antinuclear antibody</td>
</tr>
<tr>
<td>aPWV</td>
<td>Aortic pulse-wave velocity</td>
</tr>
<tr>
<td>BILAG</td>
<td>British Isles Lupus Assessment Group</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplant</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAC</td>
<td>Coronary artery calcification</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>cIMT</td>
<td>Carotid intima-media thickness</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBP</td>
<td>Vitamin D binding protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRA</td>
<td>Diastolic reflection area</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ED/EI</td>
<td>Endothelium-dependent / endothelium-independent</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGM</td>
<td>Endothelial growth media</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>EMP / MP</td>
<td>Endothelial microparticle / microparticle</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FMD</td>
<td>Flow-mediated dilatation</td>
</tr>
<tr>
<td>GTN</td>
<td>Glyceryl trinitrate</td>
</tr>
<tr>
<td>HAoEC</td>
<td>Human aortic endothelial cell</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy control</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
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<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human venous endothelial cell</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>Interferon alpha receptor</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMT</td>
<td>Intima-media thickness</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon-sensitive gene</td>
</tr>
<tr>
<td>IU</td>
<td>International uni</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JSD</td>
<td>Jugulum-symphysis distance</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase insert domain receptor</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LDG</td>
<td>Low density granulocyte</td>
</tr>
<tr>
<td>LDL / VLDL</td>
<td>Low-density lipoprotein / very low-density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>Myeloid angiogenic cell</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl 96 tetrazolium bromide</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OEC</td>
<td>Outgrowth endothelial cell</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegrin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAT</td>
<td>Peripheral artery tonometry</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTIO</td>
<td>2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide</td>
</tr>
<tr>
<td>PVD</td>
<td>Peripheral vascular disease</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-immunoprecipitation assay</td>
</tr>
<tr>
<td>RNA / mRNA / miRNA</td>
<td>Ribonucleic acid / messenger ribonucleic acid / micro ribonucleic acid</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SACQ</td>
<td>Serologically active, clinically quiescent</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal cell-derived factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SLAM(-R)</td>
<td>Systemic Lupus Activity Measure (-Revised)</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>Systemic Lupus Erythematosus Disease Activity Index</td>
</tr>
<tr>
<td>SLICC</td>
<td>Systemic Lupus International Collaborating Clinics</td>
</tr>
<tr>
<td>SMR</td>
<td>Standardised mortality ratio</td>
</tr>
<tr>
<td>SR-A</td>
<td>Scavenger receptor class A</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Full Form</td>
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<td>-------</td>
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</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIA</td>
<td>Transient ischaemic attack</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>UCTD</td>
<td>Undifferentiated connective tissue disease</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D response element</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Vascular endothelial growth factor receptor-2</td>
</tr>
<tr>
<td>vWF</td>
<td>Von-Willebrand factor</td>
</tr>
<tr>
<td>WHI</td>
<td>Women’s Health Initiative</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Preface

I graduated from the University of Birmingham with an MBChB (with honours) in 2005. Whilst an undergraduate I undertook an intercalated degrees and was awarded a BMedSc(Hons) in Neuroscience (first class). I completed my Foundation Year 1 post at City Hospital, Birmingham and then an Academic Foundation Year 2 in the same trust. During this time I had the opportunity to undertake a period of research with Caroline Gordon focussing on the clinical response to rituximab in patients with refractory SLE. Following this I pursued my interest in rheumatology and moved to the North West to undertake an Academic Clinical Fellowship in rheumatology supervised by Ian Bruce and Yvonne Alexander. During this time I gained some basic laboratory skills including human cell culture. I obtained my MRCP in 2008 and began specialist rheumatology training in 2009. In 2010 I was awarded an NIHR Manchester Biomedical Research Centre (BRU) Clinical Research Fellowship. This was a 1-year pump-priming award which allowed me to begin the clinical study and obtain sufficient data to secure a longer fellowship. In 2011 I was awarded a North West England MRC Clinical Pharmacology and Therapeutics Clinical Research Training Fellowship. This fellowship was funded by collaboration between the MRC, AstraZeneca, GlaxoSmithKline, ICON and the Medicines Evaluation Unit.

Publications arising from this thesis


Reynolds JA, Roberston, AC, Bruce IN, Alexander MY. Improving cardiovascular outcomes in rheumatic diseases: Therapeutic potential of circulating endothelial progenitor cells. *Pharmacology & Therapeutics.* 2014;142(2):231-43

Prizes

North West Deanery SpR Research Afternoon – Best Oral Presentation, June 2011

Institute of Inflammation and Repair – Best poster prize 2012 (University of Manchester)

North West Deanery SpR Research Afternoon – Best Oral Presentation, June 2013

Centre for Musculoskeletal Research PhD Showcase – Best Presentation (3rd Year), March 2011
Chapter 1: Introduction

This chapter will introduce the clinical features and pathogenesis of Systemic Lupus Erythematosus and the prevalence of cardiovascular disease in this patient population. The pathophysiology of cardiovascular disease in SLE will be described with an emphasis on endothelial repair mechanisms and the potential role of vitamin D deficiency.
1 Introduction

1.1. The epidemiology and aetiology of SLE

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterised by systemic inflammation and the development of antibodies against nuclear antigens. The clinical presentation of lupus is varied and extends from constitutional symptoms, mucocutaneous disease and inflammatory arthritis through to potentially life-threatening deep organ involvement (1). The disease course of SLE is often unpredictable with periods of disease flare followed by lower disease activity or remission. In the UK and USA, the prevalence of lupus is estimated at 12.5-78.5/100,000 population with a female: male ratio of around 9:1 (2;3). The wide variation in the estimated prevalence may be due to differences in the geographical location of the studies, the ethnicity distribution within the study population or the age/gender of subjects included. The incidence of lupus is much more difficult to determine due to the rarity of the condition within the general population. In the USA and Europe the incidence has been estimated at 2-7.6/100,000 people/year (2).

There is significant ethnic variation both in the prevalence of SLE and in the relative distribution of organ involvement. SLE is around 2-4 times more frequent in patients from a non-white background (4). In an international inception cohort of early disease (within 15 months of diagnosis), non-white patients had more ACR classification criteria than white patients at baseline (5). In other studies, when compared to white patients, Black African, Hispanic and Asian patients had more severe disease, more frequent flares, and a predisposition to developing renal disease (4;6-8).

1.1.1. The immunopathology of SLE

The cellular and molecular basis of SLE is complex and not fully understood. The principle defects are impaired clearance of apoptotic cellular debris, resulting in the persistence of nuclear antigens, and the loss cellular of tolerance leading to the development of autoreactive lymphocytes toward these self-antigens. The production of autoantibodies and the subsequent deposition of immune complexes in tissues results in systemic inflammation.

The failure of both central and peripheral B cell selection processes results in the persistence of auto-reactive cells, primed by the presence of autoantigen (9). Reduced clearance of apoptotic bodies in SLE may arise due to both increased apoptosis (which could overwhelm the system) or impaired phagocytosis. Lymphocytes from lupus patients show an increased
proportion of cells undergoing apoptosis and serum from lupus patients can induce monocyte and lymphocyte apoptosis in vitro (10;11). The increased autoantigen load in SLE is further exacerbated by a reduction in the phagocytic capacity of macrophages (12).

Tolerogenic dendritic cells (DCs) are important for the development of regulatory T cells (Tregs). Patients with SLE have fewer tolerogenic dendritic cells than healthy subjects (13). In addition, dendritic cells from lupus patients also show reduced capacity to generate regulatory T cells (14). Tregs have a pivotal role in immune tolerance and both fewer cells, and defects in their function, are implicated in autoimmune diseases (15). Most studies have found a reduction in Tregs in lupus patients, which correlates with both active disease and increases in steroid therapy (16).

Changes in the T cell compartment are also evident in SLE with an increase in the ratio of inflammatory (Th1) to immunomodulatory (Th2). An increased Th1:Th2 ratio is associated with disease activity (17). In addition, lupus patients have an increased Th17:Th1 ratio and a corresponding increase in serum levels of IL-17 (18;19).

B cells are hyperactive in SLE with an increase in the ratio of memory:naïve B cells and reduced expression of the B cell inhibitory receptor FcγIIB (20). Plasma cells, which are the source of autoantibodies, are increased during SLE flares. The source of these cells (derived from either memory B cells or the activation of naïve B cells) is uncertain. An increased proportion of immature naïve B cells is also seen in SLE which may be due, at least in part, to over-expression of B cell activating factor (BAFF), also termed B lymphocyte stimulator (BLys) (21).

1.1.2. The role of type 1 interferon in SLE pathogenesis
The dominant inflammatory cytokine in SLE is type-1 interferon (IFN) (22). This family of cytokines consist of 7 classes (α,β,δ,ε,κ,τ,ω) which signal through a dimeric membrane receptor complex. Binding of the ligand to the IFNAR2 subunit activates Janus Kinase 1 (JAK1) within the intra-cellular compartment, leading to phosphorylation of tyrosine residues within Signal Transducer and Activator of Transcription (STAT) proteins. These STAT complexes bind interferon stimulated response elements (ISREs) within the promoter regions of interferon stimulated genes (ISGs).

Interest in the role of type 1 IFN in SLE followed observations that treatment with IFNα2b induced an SLE-like syndrome in some patients with chronic hepatitis C (23). Furthermore, the
expression of IFNα in lupus-prone NZB/WF₁ mice results in a persistent production of antibody-producing plasma cells, and in the rapid development of glomerulonephritis and premature death (24). Healthy plasmacytoid dendritic cells (pDCs) produce IFNα in response to pathogenic nucleic acids (either viral or microbial) via interactions with the toll-like receptors 7-9 (TLR7-9). These responses usually terminate within a few hours, but persist in autoimmune states such as SLE (25). It is proposed that in SLE the increase in IFNα production is due to stimulation by i) immune complexes, or ii) native nucleic acids which persist due to a failure in apoptotic mechanisms as described above (26;27). It is noteworthy that a number of rare genetic diseases which are associated with high levels of type 1 interferon (termed “interferonopathies”) often have clinical features in common with adult lupus (28).

The increased expression of ISGs in lupus (often termed the “interferon signature”) has been associated with some clinical features and overall disease severity. Feng et. al. (2006) demonstrated that ISG expression within peripheral blood mononuclear cells (PBMCs) was increased in patients with SLE compared to both healthy controls and patients with other inflammatory disease (29). Furthermore, increased ISG expression was associated with lupus disease activity, low serum complement, higher ds-DNA and the presence of lupus nephritis. SLE patients with a high IFNα score may also have a different clinical and serological phenotype with increased expression of anti-Ro, U1 RNP, Smith and ds-DNA antibodies and more prevalent renal disease, lupus damage and lower serum complement (30). The association of the interferon signature with disease activity has been replicated both in patient whole blood samples, and in gene expression studies of epithelial cell lines treated with lupus serum (31;32). Blockade of IFN in clinically active lupus patients using a monoclonal antibody (sifalimumab) suppresses both the interferon signature and expression of other inflammatory molecules (e.g. TNFα and IL-10) (33). The mechanism by which increased IFN production potentiates disease activity and organ damage in SLE is the subject of further investigation.

1.2. The clinical features of SLE

1.2.1. Classification of SLE
The difficulty in determining the incidence and prevalence of SLE is due, at least in part, to the heterogeneous nature of the condition. The diagnosis of SLE is based primarily on its clinical features and is supported by laboratory investigations. For the purpose of clinical research studies, the 1992 Updated American College of Rheumatology (ACR) Classification Criteria
remain the most widely used (34;35). A patient can be classified as having SLE if they fulfil any 4 of the 11 criteria listed in table 1-1.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Brief definition notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Malar rash</td>
<td>Fixed erythema tending to spare the nasolabial folds</td>
</tr>
<tr>
<td>2 Discoid rash</td>
<td>Erythematous, raised with keratotic scaling and follicular plugging</td>
</tr>
<tr>
<td>3 Photosensitivity</td>
<td>Patient history or physician observation</td>
</tr>
<tr>
<td>4 Oral ulcers</td>
<td>Oral or nasopharyngeal</td>
</tr>
<tr>
<td>5 Arthritis</td>
<td>Non-erosive with tenderness, swelling or effusion in ≥2 peripheral joints</td>
</tr>
<tr>
<td>6 Serositis</td>
<td>Pleurisy: Convincing history of pleuritic pain, pleural rub, or effusion or Pericarditis: ECG evidence, rub or effusion</td>
</tr>
<tr>
<td>7 Renal disorder</td>
<td>Persistent proteinuria (&gt;0.5 g/day or &gt;3+ by dipstick) or cellular casts on microscopy (red, granular, tubular or mixed)</td>
</tr>
<tr>
<td>8 Neurological disorder</td>
<td>Seizures or psychosis in the absence of drugs or metabolic derangements</td>
</tr>
<tr>
<td>9 Haematologic disorder</td>
<td>At least 1 of:</td>
</tr>
<tr>
<td></td>
<td>• Haemolytic anaemia with reticulocytosis</td>
</tr>
<tr>
<td></td>
<td>• Leucopenia (&lt;4000/mm³) on ≥2 occasions</td>
</tr>
<tr>
<td></td>
<td>• Lymphopenia (&lt;1500/mm³) on ≥2 occasions</td>
</tr>
<tr>
<td></td>
<td>• Thrombocytopenia (&lt;100,000/mm³) without drug cause</td>
</tr>
<tr>
<td>10 Immunologic disorder</td>
<td>At least 1 of:</td>
</tr>
<tr>
<td></td>
<td>• Anti-DNA antibody</td>
</tr>
<tr>
<td></td>
<td>• Anti-Smith antibody</td>
</tr>
<tr>
<td></td>
<td>• Positive antiphospholipid antibodies identified by:</td>
</tr>
<tr>
<td></td>
<td>o abnormal serum level of IgM or IgG anticardiolipin antibodies</td>
</tr>
<tr>
<td></td>
<td>o positive lupus anticoagulant</td>
</tr>
<tr>
<td>11 Antinuclear antibody</td>
<td>Immunofluorescence or other assay in the absence of drugs known to be associated with “drug-induced” lupus</td>
</tr>
</tbody>
</table>

Table 1-1: The 1997 Updated ACR Classification Criteria for SLE
In order to be classified as having SLE patients must fulfil ≥4 of the 11 criteria.

The Systemic Lupus International Collaborating Clinics (SLICC) classification criteria were published in 2012 to address some of the criticisms of the ACR criteria (36). The principle difference from the ACR criteria is the separation of clinical and immunological criteria. In order be classed as having lupus, a patient must have 4 criteria of which at least 1 must be clinical and 1 immunological. In addition, the presence of biopsy proven nephritis (which must be compatible with lupus) and either ANA or ds-DNA antibodies is sufficient for the classification of SLE. Other changes include an expansion of skin criteria and immunological
criteria. Whilst the SLICC criteria are more comprehensive, they have not yet been widely adopted and have received some criticism (37).

### 1.2.2. Disease activity, organ damage and quality of life

The assessment of lupus disease activity relies on attributing numerical scores to clinical features which are due solely to active SLE. This can have considerable challenges given the prevalence of comorbidities and use of immunosuppressant and other drugs (which may cause leukopenia for example) in this patient group. The Systemic Lupus Activity Measure, Revised (SLAM-R) and European Consensus Lupus Activity Measurement (ECLAM) are now less favoured than other measures. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was developed in the USA to offer a composite global score of disease activity. This score uses a weighted system such that more severe features e.g. neurological involvement and vasculitis attract a higher score (38). The SLEDAI score has undergone multiple modifications including the SLEDAI-2K which captures both new and ongoing disease activity (39).

Instead of using a global score, The British Isles Lupus Assessment Group 2004 (BILAG-2004) index documents activity in each of 9 organ systems over a 4 week period compared to the 4 weeks prior (40). This has the advantage of allowing changes in individual organ systems to be tracked over time. Furthermore, the definitions of disease activity are considered in terms of whether they would prompt the physician to modify or change therapy.

The principle measurement of lupus-related organ damage is the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SLICC/ACR-DI) (41). This offers a numerical score of up to 41 across 38 items. All damage is recorded irrespectively of whether it can be clearly attributed to SLE, providing it is present for at least 6 months and not expected to reverse.

Closely related to organ damage is the concept of health-related quality of life (QoL). This can be measured by both general scoring systems (e.g. the Medical Outcomes Study Short-Form 36, SF-36) (42) or SLE-specific indices (e.g. LupusQoL) (43). Patient-reported QoL is invariably lower in SLE compared to matched controls both in terms of physical functioning and also psychological and emotional status (44).
1.2.3. Mortality in SLE
Whilst over the last 50-60 years there has been a significant reduction in mortality due to SLE, the 5-year survival remains at approximately 95%. Given that lupus is predominantly a disease of women of child-bearing age, a 5 year mortality rate of 1:20 remains undesirably high. A seminal study by Urowitz et al. (1976) described a bimodal pattern of mortality in SLE (45). In early disease (within 1 year of diagnosis) deaths were due to active lupus, most notably lupus nephritis. In later disease (mean 8.6 years from diagnosis, range 2.5-19.5 years) deaths were predominantly due to cardiovascular disease. A subsequent study confirmed that moderate to severe atherosclerosis was commonly seen in patients later in their disease course and often contributed to their death (46).

1.3. The association between SLE and premature cardiovascular disease

1.3.1. Cardiovascular outcomes
Patients with SLE have a markedly increased risk of cardiovascular disease (CVD) compared to the general population. The estimates of this increased risk vary between different cohorts. The risk is considered to be around 2-2.5 fold across all lupus patients but is significantly higher in young patients. In the study by Manzi et al. (1997), the Relative Risk (RR) of myocardial infarction (MI) was 2.47 (0.8, 6) in patients aged 45-54 years but 52.4 (21.6, 98.5) in those aged 35-44 years (47). The risk to younger patients is exemplified in a retrospective study of postpartum women between 1999 and 2003 followed until the end of December 2014. In this study patients with SLE had an adjusted hazard ratio (HR) of 54.43 (16.0, 185) for MI, of 228 (43.3, 1203) for percutaneous coronary intervention (PCI) and 11.7 (7.97, 17.1) for all-cause mortality (48). The risk of a CVD event following hospitalisation of lupus patients is greater than for any other autoimmune disease. In a large study of 336,479 Swedish patients with autoimmune disease the standardised incidence ratio (SIR) for CVD within a year after hospitalisation in patients with SLE was 4.94 (4.15, 5.83) (49). These data point towards an association between active SLE and CVD risk.

The increase in CVD is seen not only in predominantly Caucasian cohorts but also those containing large numbers of South East Asian, Hispanic and Afro-Caribbean patients (50;51). Furthermore, consistent with variations seen in disease activity, premature CVD-related events are more common in Black or Hispanic SLE patients (52).
### 1.3.2. Carotid intima-media thickness

Carotid intima-media thickness (cIMT) is an independent predictor of future CVD events in the general population. In a meta-analysis of 8 studies (37,197 subjects) by Lorenz et. al. (2007) the RR of MI was 1.15 (1.12, 1.17) for each 0.1mm increase in cIMT (53). Furthermore, the addition of cIMT to existing risk prediction models significantly improved reclassification of patients (54). Numerous studies have identified increased cIMT in patients with SLE. For example in a cross-sectional study by Colombo et. al. (2007) of 30 SLE patients and 30 age- and sex-matched controls there was a significant increase in common carotid IMT in the lupus population (0.73 [0.12] vs. 0.66 [0.11] mm, p<0.05) (55). Interestingly, Leonard et. al. (2011) demonstrated that whilst total cIMT was increased in lupus patients, this was due to an increase in the diameter of the intimal layer and a decrease in the medial layer (56). Measurement of both parameters has been found to better discriminate between CVD and patients without CVD in the general population (57).

Both traditional cardiovascular risk factors (age (58-63), diabetes mellitus (58), smoking (64), waist circumference/BMI (58;59;65), previous CVD event (63), oxidised LDL (66)) and lupus-specific factors (lower C4 (58), higher CRP (58), lupus damage (63;67), disease duration (60), cumulative steroid dose (59;62), and African American background (59;64)) have been associated with cIMT in SLE patients.

In the general population, progression of cIMT is associated with risk of vascular events (68). Progression of cIMT has been found to be accelerated in SLE compared to controls (61) in some studies but not others (69). The progression of cIMT is associated with age at diagnosis, serum homocysteine and C3 and CSa levels (70).

### 1.3.3. Carotid plaque

The presence of carotid plaque is an important predictor of future cardiovascular events in the general population. In a study by Plichart et. al. (2011) it was the presence of plaque, but not increased cIMT without plaque, that predicted CVD risk in subjects without existing cardiovascular disease (71). This observation has been confirmed by others who found that whilst cIMT (especially internal carotid IMT) predicted future CVD events, carotid plaque was a better predictor (72).

In a study by Ahmad et. al. (2007) of 200 lupus patients and 100 controls there was a significantly greater prevalence of plaque in younger patients (<55 years old). This was found to be both at the carotid bulb and internal carotid artery (66). There were a number of lupus-
related factors associated with carotid plaque in this study including age, disease duration, lupus damage, steroid therapy and anticardiolipin antibodies.

In patients with SLE, atherosclerotic plaque in the carotid and femoral arteries can occur without underlying increases in IMT. In a study by Frerix et. al. (2014) of 100 SLE patients the presence of either carotid or femoral plaque was associated with an increased risk of cardiovascular events (HR 5.92 [1.55, 22.67]) over a mean follow-up of 60.5 months (73).

The progression of atherosclerotic plaque in SLE patients is associated with age, male gender, systolic blood pressure, diabetes mellitus, disease duration, LDL cholesterol, steroid therapy, previous cardiac history and nephrotic range proteinuria (63;74-77). Rhew et. al. (2009) found ethnic differences in the prevalence of subclinical CVD in lupus patients. In this study African American women with lupus had increased cIMT and carotid plaque compared to other ethnic groups (78). This difference was, however, most likely due to differences in blood pressure, steroid use, SLE disease activity and SLE damage, which were all increased in the African American patients.

1.3.4. Coronary artery calcification
Patients with SLE have an increased prevalence of calcification across all vascular beds but most notably in the coronary arteries (coronary artery calcification (CAC)) (79). In a study by Asanuma et. al. (2003), SLE patients had significantly increased CAC scores compared to healthy controls (68.9±244.2 vs. 8.8±41.8, p<0.001) (80). Similarly to cIMT and plaque, Yiu et. al. demonstrated that CAC occurs at a younger age in SLE being detectable in 40% patients <40 years old (79). In addition, CAC also shares common risk factors with cIMT/plaque including age, hypertension, disease duration, lupus damage, cumulative lupus activity and cumulative steroid dose (81-83).

1.3.5. Flow-mediated dilatation (FMD)
Flow-mediated dilatation (FMD) is a non-invasive measurement of peripheral endothelial function. FMD quantifies changes in the brachial artery diameter, in response to shear stress and reactive hyperaemia, induced by prolonged vessel occlusion with a blood pressure cuff on the forearm inflated to supra-systolic pressures. On cuff release, it is postulated that changes in shear stress are detected by endothelial cells, leading to endothelial nitric oxide synthase (eNOS) activation (84). The generation of nitric oxide (NO) by this process leads to smooth muscle relaxation and thus dilatation of the artery. This dilatation is therefore a surrogate
marker for downstream NO release and thus endothelial health (85). Whilst FMD is considered to be primarily driven by NO, some groups have proposed that other vasodilators (prostacyclin and endothelium-derived hyperpolarising factor) may also have a role in FMD (86). Given that NOS inhibition abolishes FMD when the cuff is placed on the distal forearm (but perhaps not at other sites) it could be suggested that using this technique at least, NO is the principle mediator of FMD (87). Commonly after measurement of FMD the response of the vessel to glyceryl trinitrate (GTN) is measured. This vasodilator acts directly on the vascular smooth muscle and as such, allows calculation of endothelium-dependent: endothelium independent dilation (ED/EI).

The reactivity of the brachial artery correlates strongly with the reactivity of the coronary endothelium ($r=0.79$, $p<0.001$) and thus offers a non-invasive estimate of coronary vessel function (88). Reduced FMD predicts an increased risk of cardiovascular events in the general population (89;90). A recent meta-analysis of 14 studies by Inaba et. al. (2010) demonstrated that for a 1% increase in FMD, the relative risk (RR) (95% CI) of a cardiovascular event was 0.87 (0.83-0.91) (91).

A significant reduction of FMD in lupus patients compared to matched controls has been reported in 12/15 (80%) of published studies (between 2002 and 2014) and may therefore be an important prognostic marker of future CVD. The results of studies investigating FMD in SLE compared to a control population are summarised in table 1-2. Mak et. al. (2011) conducted a meta-analysis of the studies published between 2002 and 2009. Across 13 studies there were data from 580 SLE patients and 381 controls. Endothelium-dependent (FMD) but not endothelium-independent (GTN-mediated) dilatation was significantly lower in SLE compared to controls ($p<0.001$, $p=0.167$ respectively). Impaired FMD was independently associated with age and lupus disease duration but not traditional cardiovascular risk factors or prednisolone use (92).
<table>
<thead>
<tr>
<th>Author</th>
<th>Population Studied</th>
<th>Lower FMD in SLE</th>
<th>Details of FMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lima <em>et. al.</em>, 2002 (93)</td>
<td>69 pre-menopausal female SLE patients 35 age-matched female controls</td>
<td>Yes</td>
<td>5.0 (±5.0)% in SLE vs. 12.0 (±6.0)% in controls, p&lt;0.001</td>
</tr>
<tr>
<td>El-Magadami <em>et. al.</em>, 2004 (94)</td>
<td>64 female SLE patients 38 controls</td>
<td>Yes</td>
<td>Median (range) 3.6 (-6.3, 13.7)% in SLE vs. 6.9 (-6.6, 17.8)% in controls , p&lt;0.01</td>
</tr>
<tr>
<td>Johnson <em>et. al.</em>, 2004 (95)</td>
<td>5 SLE patients with CVD 5 SLE patients with subclinical CVD 5 SLE patients without CVD 5 control patients</td>
<td>Yes (only in SLE patients with existing CVD)</td>
<td>FMD 0.11 (±3.36)% in CVD patients compared to 11.1 (±2.63)% in controls (p=0.018) Impaired ED/EI* ratio (0.12 [± 0.43] vs. 1.15 [± 0.64], p=0.05) Similar FMD and ED/EI in subclinical CVD and CVD free patients</td>
</tr>
<tr>
<td>Rajagopalan <em>et. al.</em>, 2004 (96)</td>
<td>43 female SLE patients 43 non-lupus patients with CVD (29% female) 43 healthy controls (50% female)</td>
<td>Yes</td>
<td>FMD lower in SLE (3.7 [±3.5]% and CVD (1.4 [±5.7])% compared to healthy controls (6.5 [±3.5]%), p&lt;0.05 for controls vs. SLE or CVD (no significant difference between SLE and CVD)</td>
</tr>
<tr>
<td>Kiss <em>et. al.</em>, 2006 (97)</td>
<td>61 SLE patients 26 age/sex-matched controls</td>
<td>Yes</td>
<td>FMD impaired in SLE patients (7.3 [±5.2]% compared to controls (9.7 [±3.87]%) (p=0.013 and significant difference between SLE patients with and without CVD (5.5 [±4.4]% vs. 8.8[±5.3]%) (p=0.01)</td>
</tr>
<tr>
<td>Wright <em>et. al.</em>, 2006 (98)</td>
<td>32 SLE patients and 19 controls</td>
<td>Yes</td>
<td>FMD significantly lower in patients (2.4 [-2.1, 10.7]%) compared to controls (5.8 [1.9, 14.0]% (p=0.037)</td>
</tr>
<tr>
<td>Karadag <em>et. al.</em>, 2007 (99)</td>
<td>25 SLE patients (inactive disease) 22 controls</td>
<td>Yes</td>
<td>7.1 (±2.1)% in SLE vs. 11.4 (±1.2)% in controls, p&lt;0.001</td>
</tr>
<tr>
<td>Piper <em>et. al.</em>, 2007 (100)</td>
<td>22 SLE patients 22 age/sex- matched controls</td>
<td>Yes</td>
<td>Lower median (IQR) in SLE (5.6 [3.1, 7.2]% compared to controls (8.0 [6.3, 9.3]) (p=0.001). No relationship to future CVD events or damage</td>
</tr>
<tr>
<td>Valdivielso <em>et. al.</em>, 2008 (101)</td>
<td>26 SLE patients and 21 age/sex-matched controls</td>
<td>Yes</td>
<td>12.4 (±4.4)% in SLE vs. 16.9 (±5.6)% in controls, p&lt;0.05</td>
</tr>
<tr>
<td>Svenungsson <em>et. al.</em>, 2008 (102)</td>
<td>26 SLE patients without CVD 26 age/sex- matched controls.</td>
<td>No</td>
<td>6.4 (±4.2)% in SLE vs. 5.1 (±5.0)% in controls (not significant) and number of patients with FMD&gt;4.85% (population mean) greater in patients than controls (p=0.05)</td>
</tr>
<tr>
<td>Ghosh <em>et. al.</em>, 2009 (103)</td>
<td>60 patients (54 female) 38 controls All South Asian</td>
<td>Yes</td>
<td>10.0 (±5.5)% in SLE vs. 19.0 (±6.4)% in controls, p&lt;0.001</td>
</tr>
<tr>
<td>Cyriene <em>et. al.</em>,</td>
<td>30 SLE patients and</td>
<td>No</td>
<td>No difference in median (IQR) FMD</td>
</tr>
</tbody>
</table>

**Notes:**
- FMD: Flow-mediated dilation
- ED: Endothelial dysfunction
- EI: Endothelial integrity
- CVD: Cardiovascular disease
- IQR: Interquartile range
### Table 1-2: Summary of studies of FMD in SLE patients in which a control population was included

This table shows details of studies of FMD in patients with SLE compared to a control population. The principle findings of these studies are shown. Results presented as mean (±S.D) unless otherwise stated.

*ED/EI = endothelium dependent/endothelium independent dilatation
**PFG = post-GTN-FMD gap (i.e. difference between GTN-dependent dilatation and FMD)

<table>
<thead>
<tr>
<th>Year (Ref)</th>
<th>Patient Description</th>
<th>Control</th>
<th>Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009 (104)</td>
<td>66 healthy controls</td>
<td>between SLE (9.25 [5.2]%) and controls (9.69 [3.3]%)</td>
<td></td>
</tr>
<tr>
<td>Aizer et al., 2009 (105)</td>
<td>30 SLE patients and 31 controls</td>
<td>No</td>
<td>No difference in FMD between SLE (12.5 [5.1]%) and controls (12.5 [4.5]%)</td>
</tr>
<tr>
<td>Zhang et al., 2009 (106)</td>
<td>111 SLE patients and 40 controls</td>
<td>Yes</td>
<td>10.9 (±5.4)% in SLE vs. 14.2 (±4.1)% in control, p&lt;0.001</td>
</tr>
<tr>
<td>Ahmadi et al., 2011 (107)</td>
<td>84 SLE patients and 18 controls</td>
<td>Yes</td>
<td>Impaired in 48.8% patients compared to 5.5% controls in terms of PFG** (statistical test not applied)</td>
</tr>
<tr>
<td>Stalc et al., 2011 (108)</td>
<td>42 SLE patients (21 with secondary APS) and 22 controls</td>
<td>Yes</td>
<td>Median (IQR) 7.7(1.9, 12.1)% in SLE vs. 14.6 (11.2, 21.1)% in controls (p&lt;0.001 across the 3 groups). No difference between patients with and without APS (7.8% vs. 7.7%)</td>
</tr>
<tr>
<td>Somers et al., 2012 (109)</td>
<td>95 SLE patients and 38 controls</td>
<td>Yes</td>
<td>4.0 (±4.7)% in SLE vs. 5.7(±4.1)% in controls (p=0.05). FMD correlates with IFN-score (expression of interferon-sensitive genes)</td>
</tr>
<tr>
<td>Parker et al., 2013 (110)</td>
<td>27 SLE (active disease) 22 age-matched controls</td>
<td>Yes</td>
<td>Median (IQR) 1.63 (-1.2, 5.3)% in SLE vs. 3.5 (3.0, 8.6)% in controls (p=0.04). Improvement in FMD as disease activity reduced 1.63% to 4.56% (p=0.1)</td>
</tr>
<tr>
<td>Conti et al., 2014 (111)</td>
<td>50 SLE patients 25 controls 18 Primary APS</td>
<td>Yes</td>
<td>6.5 (±6.6)% in SLE vs. 14.4 (±9.2)% in controls (p&lt;0.001). 1. Also lower in SLE compared to APS (6.5% vs. 13.4 (±12.3)%, p=0.03)</td>
</tr>
<tr>
<td>Barnes et al., 2014 (112)</td>
<td>12 sedentary SLE 14 physically active SLE 15 healthy controls</td>
<td>Yes</td>
<td>3.6 (±1.3)% in SLE vs. 8.1 (±1.2)% in controls (p&lt;0.05) but no different between physically active SLE and controls (p=0.73, raw values not given)</td>
</tr>
</tbody>
</table>

Abnormal FMD in SLE also shows evidence of reversibility, and may be modulated by control of disease activity and other interventions. Parker et al. (2013) observed changes in FMD in 27 patients with active SLE treated with either traditional immunosuppression or rituximab. FMD was significantly impaired in SLE patients compared to controls (2.86 [0.60, 5.32]% vs. 6.81 [3.46, 8.57]%, p=0.03) at baseline and showed a trend towards improvement over time following better control of disease activity (1.63 [0.54, 4.47]% to 4.56 [1.71, 5.87]%, p=0.10) (110).

Drugs which are known to have cardiovascular benefit in the general population can also influence FMD in lupus patients. In an open-label study of atorvastatin (20mg/day for 8...
weeks) there was a significant improvement in FMD in the treatment group (1.9[±3.9]% vs. -0.3[±1.8]%, p=0.009) (113). Interestingly, this improvement was associated with a significant improvement in disease activity in the intervention group (SLEDAI score 4.47 to 3.08, p<0.001) whilst the untreated group had a worsening of disease (SLEDAI 3.33 to 4.33, p=0.02). Similarly a 24-week randomised controlled trial of omega-3-polyunsaturated fatty acids showed a significant improvement in FMD from 3 (0.5, 8.2)% to 8.9 (1.3, 16.9)%, p<0.001. Again this was associated with significant improvement in disease activity by both the SLAM-R and BILAG indices (114).

These observations support that idea that impaired FMD may be driven, at least in part, by disease activity. However, factors other than disease activity are also likely to be important. Torres dos Reis-Neto et al. (2013) studied SLE patients with stable disease (mean SLEDAI=2) and found a significant improvement in FMD following a 16-week supervised exercise programme (6.3 [6.7]% to 14.1 [9.1]%, p=0.006) (115).

1.3.6. Aortic pulse wave velocity
Degenerative stiffness of the arterial tree (arteriosclerosis) represents an early pathological change in vessel function. Aortic pulse-wave velocity (aPWV) is widely considered to be the gold standard for the non-invasive measurement of aortic stiffness (116). The general principle is that the speed of the pulse wave can be determined by measuring the time for a pulse to travel between 2 points along an artery of known length. Whilst measurements of aPWV can be made using magnetic resonance imaging or echocardiography, the most commonly used techniques are currently applanation tonometry or oscillometry. In the former, the pulse transit time is measured between 2 sites (commonly the carotid and femoral arteries) and expressed in terms of the estimated distance between the 2 points. The oscillometric method is based on pulse-wave analysis and measures the time between the pulse wave and the reflected wave (which occurs after the pulse wave reaches the resistance vessels) (117). This methodology is described in more detail in Chapter 2. These methods are not without their difficulties and a consensus document was published on 2006 on behalf of the European Network for Non-invasive Investigation of Large Arteries. This paper suggested that aortic pulse wave velocity and pulse wave analysis should be combined in future studies (118). Furthermore even the use of automated devices can be technically challenging as aPWV is influenced by a variety of factors including blood pressure, caffeine, alcohol and smoking (119).
In a large study of 1678 subjects aged 40-70 years from the general population by Willum-Hansen et. al. (2006) aPWV was an independent predictor of future cardiovascular events over a mean follow-up of 9.4 years. This effect remained significant after adjustment for age, gender, BMI, mean arterial blood pressure (BP), heart rate (HR), lipids and diabetes mellitus (120). In 2010, Valchopoulos et. al. conducted a meta-analysis of 17 longitudinal studies including subjects from the general population and patients renal disease, diabetes mellitus and hypertension. This analysis contained a total of 15,877 individuals followed up for a mean of 7.7 years. An increase of aPWV by 1m/s (or 1 standard deviation) resulted in an increase in total CVD events of 14% (or 46%) respectively (121). Most recently a further meta-analysis by Ben-Shlomo et. al. (2014) showed that aPWV was an independent predictor of CVD (HR 1.3 [1.18, 1.43]) in 17,635 subjects (122).

A number of studies have shown increased arterial stiffness in patients with SLE compared to controls (123-127). In premenopausal women with established lupus Selzer et. al. (2001) identified a number of factors associated with aPWV, most notably in premenopausal women. These included increased C3 levels, ds-DNA antibody positivity, lower leukocyte count, increased mean arterial BP and the presence of carotid plaque (128). The use of hydroxychloroquine appeared to be “protective” against vascular stiffening. In a small study of pre-menopausal lupus patients only traditional risk factors were associated with aPWV: principally age, BMI, and blood pressure (125). Associations between vascular stiffness and the SLICC/ACR-DI (126;127), systolic BP (129;130), fasting insulin (131) and the metabolic syndrome (132) have also been demonstrated by other groups. In support of the observation that a mixture of traditional CVD- and lupus-related factors which are associated with aPWV, Valero-Gonzalez et. al. (2014) found that in a multivariable analysis both the metabolic syndrome (OR [95% CI] 1.5 [1, 2.32]) and lupus damage (6.6 [1.2, 38]) were independently associated with aPWV (133). Furthermore the presence of concomitant anti-phospholipid syndrome may further increase vascular stiffness in lupus patients (134).

1.3.7. Vascular damage

The plasma compartment contains a large number of circulating subcellular particles. These can be classified, from smallest to largest, into 3 principle groups: exosomes (typical diameter 30-100nm), microparticles/microvesicles (100nm-1µm), and apoptotic bodies (1-5µm) (135). Current opinion is that cellular activation is associated with an increased rate of membrane remodelling in activated cells. Phosphatidyl serine (PS) which is usually present on the cytoplasmic side of the membrane becomes increasingly found on the outer surface of the
membrane (termed “flopping”). This results in changes in surface tension, membrane budding/blebbing and finally the shedding of microparticles (MPs) (136). There has been suggestion that MP release is a protective mechanism for the cell in response to external stressors. A study by Hussein et. al. (2008) demonstrated that blocking MP release by HUVECs which were treated with an apoptotic agent (staurosporin) resulted in accumulation of caspase 3 within the cells and rapid cell detachment (137). Consistent with the idea that MPs are a cellular response in vivo, patients with acute coronary syndrome (but not stable angina) show a rapid increase in MPs following onset of the acute event (138). Microparticles have been detected in the circulation from a variety of cell types including endothelial cells, leukocytes, platelets and erythrocytes (139). The stages of MP generation are shown in figure 1-1.

![Figure 1-1: The formation of endothelial microparticles](image)

**Figure 1-1: The formation of endothelial microparticles**
The formation of EMPs in response to an external stimulus involves the loss of membrane asymmetry. This occurs via the activation of floppase (which moves proteins from the inner membrane surface to the outer surface) and scramblase, and the inhibition of flippase (which opposes floppase). Activation of these enzymes and the subsequent rearrangement of cytosolic proteins are calcium-dependent.

LPS = lipopolysaccharide, TNFα = tumour necrosis factor alpha, IL-1 = interleukin 1, LDL = low density lipoprotein, ROS = reactive oxygen species.

Figure adapted from Boulanger et. al. (2006) (140)

### 1.3.7.1. Detection of endothelial microparticles (EMPs)
Typically MPs are detected in plasma samples by flow cytometry. Repeated centrifugation removes unwanted cell debris and platelets resulting in platelet-poor plasma (PPP). After
labelling MPs with annexin-V (which binds to PS) and fluorescent antibodies directed against cell surface markers of interest, 10µm beads can be added to enable quantification of MPs (141). The ideal combination of surface markers to correctly identify EMPs is not clear. For example, CD144 is highly specific for endothelial cells but has poor sensitivity. Instead, combinations of markers to identify MPs of endothelial origin whilst actively excluding MPs from other sources have been used (e.g. CD31+/CD41, CD31+/CD42b, CD105+/CD45) (142). Of these, CD31+/CD42b is a commonly used combination (143-145). It is important to recognise that CD31 is not specific to endothelial cells and is present on both platelets and some leukocytes. The use of CD42b excludes platelet-derived MPs but does not differentiate between leukocyte MPs and EMPs. In addition to the difficulties in identifying the source cell type of the MPs, there is evidence that surface marker expression may differ within a single cell type, depending upon the stimulus for MP generation. The expression of surface markers (notably CD31) and annexin V-binding has been shown to be increased in EMPs produced by EC apoptosis compared to activation (146).

Recently, a study using sophisticated electron microscopy techniques by Arraund et al. (2014) demonstrated that only a small number of circulating extracellular vesicles (comprising both exosomes and microparticles by size criteria) express PS. Furthermore, flow cytometry was only able to detect a small fraction of the particles (147).

### 1.3.7.2. EMPs as a biomarker

The number of circulating EMPs has been associated with many vascular diseases including acute coronary syndrome, hypertension, type II diabetes, peripheral artery disease and end-stage renal failure (reviewed by Boulanger et al. (2006) (140)). EMPs may also offer some prognostic information. Recently a study has shown that in patients with heart failure, increased CD144+ EMPs was associated with an increased risk of future cardiovascular events (HR [95% CI] 2.43 [1.03,5.69]) but not all-cause mortality (HR 2.10 [0.83,5.32]) (148).

### 1.3.7.3. EMPs in SLE

Early evidence for an association between EMPs and inflammatory disease was reported by Combes et al. (1999). This group demonstrated that in response to TNFα, endothelial cells in vitro produced surface blebs and detached vesicles less than 1.5µm in diameter. These EMPs expressed CD31 and αvβ3 (CD51-CD61, vitronectin receptor), could be labelled with annexin-V and were detectable as CD31+/αvβ3+ events in the peripheral blood (149).

Increased concentrations of microparticles have been noted in SLE in patients with both active disease and clinically stable disease. In a study by Duval et al. (2010) of 27 stable SLE patients
and 23 healthy controls, EMPs were defined as flow cytometry events which were CD105+/CD146+/CD45-. The number of EMPs was significantly higher in SLE compared to controls (2755 EMPs/µl vs. 130 EMPs/µl, p<0.001) (150). There was also a trend towards increased numbers of EMPs in patients with secondary antiphospholipid syndrome. CD146 (melanoma cell adhesion molecule, MCAM) is relatively specific for endothelial cells and their progenitors. Interestingly, an apoptotic marker was not used in this study which may have altered the subpopulation of MPs identified. In contrast, Crookston et. al (2013) found no difference in EMPs between SLE patients and controls (defined as CD144+ particles on size criteria) (151).

Similar to changes seen in FMD, the number of circulating EMPs has been shown to be both elevated in patients with active disease and to fall dramatically as this disease activity improves with treatment. In the study by Parker et. al. (2013) EMPs (defined as CD31+/AnnV+/CD42b-), were increased in patients compared to controls, reduced over time as disease activity improved and correlated with endothelial dysfunction. After a mean duration of 20 weeks after changing therapy, the number of EMPs fell from 166,982/ml to 55,655/ml (p=0.02) (110). The correlation between AnnV+/CD31+ EMPs and endothelial dysfunction has also been reported in patients with CVD (152).

The work of Neilsen et. al. (2011) has focussed on non-cell-specific microparticles (i.e. including those from leukocyte and platelet sources in addition to EMPs). In a study of 70 patients with moderately active SLE (mean SLEDAI=5) and 29 controls they reported no difference in the number of EMPs (CD146+/AnnV-), and lower numbers of platelet (CD42b+/AnnV-) and leukocyte microparticles (CD45+/AnnV-) (153). Of interest, this group also reported lower total numbers of AnnV+ microparticles and an increase in AnnV- microparticles. The source and relevance of AnnV- particles is unclear. The authors suggest that these could be intact chylomicrons, but they could also represent other entities. Gyorgy et. al. (2011) demonstrated that immune complexes and other protein structures share similar physical properties making it difficult to distinguish them from microparticles by flow cytometry (154). Annexin V is likely to be an important marker as serum levels have been shown to be independently associated with both cIMT and FMD in SLE patients (155).

More recently the content of EMPs has been demonstrated by proteomic analysis. Microparticles (non-endothelial-specific) from lupus patients contained higher levels of immunoglobulins and complement, and these MPs correlated with disease activity (156).
1.3.7.4. Interaction of EMPs with the vasculature

In the disease state, EMPs may be more than simply markers of inflammation and may have a role in the progression of the inflammatory process. In healthy controls there is a correlation between EMPs and both IL-6 and C-reactive protein (CRP) (157). There is currently much interest in the role of MPs as circulating transporters for microRNAs. A study by Diehl et. al. (2012) used Next Generation Sequencing to investigate the miRNA profile of MPs generated by LPS-stimulation of HUVECs, and from human subjects with, and without CVD. In both cases, the miRNA profile was different between stimulated and unstimulated cells, and in the presence or absence of CVD (158). Similarly, LPS-induced monocyte MPs contain increased TNFα, IL-6 and IL-8 transcripts compared to MPs generated by non-stimulated monocytes (159). To date, both positive and negative effects of MP-mediated transfer of specific miRNAs have been observed in terms of increasing angiogenesis (miRNA-150) (160), increasing endothelial migration/proliferation (miRNA-126) (161) or stimulating apoptosis (miRNA-223) (162).

With respect to SLE, further studies are needed to determine the factors which drive EMP production in lupus, whether EMPs have prognostic value and how they may contribute to vascular damage or repair. It would also be interesting to determine whether all immunosuppressant therapies reduce EMPs by the same amount, or if some agents offer additional vasculoprotective effects.

1.4. Mechanisms of CVD in SLE

The pathogenesis of CVD in patients with SLE is complex and not fully understood. It is likely that a combination of traditional risk factors and SLE-related factors both contribute to the increased risk. In addition, some lupus-related cytokines (notably type I interferon) may play a role in development of CVD. It has been proposed that the increased risk is due, at least in part, to failed endothelial repair mechanisms; a process in which type I interferon may have a key role.

1.4.1. Development of atherosclerosis is an inflammatory process

The development of atherosclerosis is a complex inflammatory process which begins with endothelial dysfunction and progresses towards the formation of a lipid-laden plaque with smooth muscle hypertrophy. Endothelial dysfunction is characterised by reduced nitric oxide (NO) availability, up-regulation of adhesion molecules and chemokines and increased platelet aggregation (163). In response to these changes an oxidative environment develops leading to
lipid modification (which can further inhibit NO production) and monocyte/macrophage recruitment, both of which lead to the development of oxidised-lipid-laden macrophages (foam cells). These macrophages release pro-inflammatory mediators (notably IL-1β) which stimulate endothelial up-regulation of adhesion molecules, recruitment of more leukocytes to the area and vascular smooth muscle cell proliferation (164). This innate immune response is then followed by an adaptive response where T cells, which recognise oxidised LDL and other modified antigens, further propagate the inflammatory response (165).

Reduced NO availability is often accompanied by production of superoxide free radicals. Uncoupling eNOS results in the imbalance between NO and superoxide ($O_2^-$) production. One mechanism by which this occurs is deficiency of the eNOS cofactor tetrahydrobiopterin (BH$_4$) which can occur when endothelial cells are exposed to lipids (166).

1.4.2. Traditional cardiovascular risk factors in SLE
The prevalence of traditional cardiovascular risk factors is increased in patients with SLE. In 1992 Petri et al. described the prevalence of traditional risk factors in the Hopkins Lupus Cohort. In this cross-sectional study approximately half of the patients had ≥3 traditional CVD risk factors. Of particular note was the prevalence of obesity, hypercholesterolaemia and a sedentary lifestyle (167). In the Toronto Risk Factor Study, 250 SLE patients were compared to 250 healthy primary care controls. The lupus population had an increased prevalence of hypertension, diabetes mellitus, serum creatinine, waist:hip ratio and dyslipidaemia (increased VLDL and triglycerides) (168). In a retrospective case-control study comparing SLE patients with and without CVD, those with a verified CVD event were more likely to be hypertensive and have a positive family history of CVD. This study also found a trend towards increased hyperlipidaemia, a higher rate of smoking and a higher BMI prior to the event (169). The number of traditional CVD risk factors has been shown to accumulate over a short period of time following diagnosis of SLE. In the SLICC inception cohort the number of traditional cardiovascular risk factors (including smoking) increased over the 1st 3 years following enrolment (170).

Importantly, in the Toronto Risk Factor Study, the estimated 10-year CVD risk (using the Framingham multiple risk factor assessment) was the same in the lupus group and the control group. Given the increase in CVD described above, traditional risk factors do not fully explain the increased risk. Similarly, in a retrospective analysis the relative risk for CVD remained elevated in SLE (RR 7.5 [5.1, 10.4]) after adjustment for traditional risk factors (171).
Smoking is an important risk factor for CVD in the general population. In the Toronto Risk Factor study, smoking was no more common in SLE than controls (168). Of note, however, the study by Urowitz et. al. (2008) mentioned above showed an increase in smoking in the first 3 years following diagnosis.

The metabolic syndrome (MetS) is also increasingly recognised as a predictor of cardiovascular risk in the general population. Subjects with MetS have an increased risk of developing CVD and of death after adjustment for traditional risk factors (RR 1.54 [1.32, 1.97]) (172). MetS also appears to be common in patients with SLE, varying from 18%-30% depending on the definition used and the population studied (173). The metabolic syndrome is also prevalent early in the disease course. In an international inception cohort of lupus patients, the prevalence of MetS was 16% at baseline and associated with age, ethnicity, steroid use, immunosuppressant use and renal disease (174). Furthermore, the MetS phenotype may be different in SLE with a greater contribution from hypertension and low HDL rather than obesity (175).

Recently the distinction between “traditional” and “lupus-related” CVD risk factors has become less clear. A study by Lozovoy et. al. (2014) found that hypertension in SLE is associated with disease activity and expression of pro-inflammatory cytokines including IL-17 (176).

Dyslipidaemia is also common amongst patients with SLE compared to controls. Patients with SLE have been shown to have increased total cholesterol, triglycerides (TG), very low density lipoprotein (VLDL) and small, dense sub-fractions of low density lipoprotein (LDL) (177;178). Differences in the lipid profile can also be seen between lupus patients with and without CVD. In a study by Hua et. al. (2009), lupus patients with CVD had increased VLDL and decreased small HDL (sHDL) compared to SLE patients without CVD (179). Furthermore, oxidised LDL (oxLDL) is increased in patients with SLE and correlates with cIMT (180).

1.4.3. Lupus-specific CVD risk factors

1.4.3.1. Antiphospholipid antibodies

The presence of antiphospholipid antibodies has been identified as an independent predictor of vascular events in patients with SLE (OR 4.72 [1.68, 13.2]) (181). This is perhaps unsurprising given that the final stage in the pathogenesis of a cardiovascular event is the formation of thrombus. One of the mechanisms by which CVD events are directly increased by
anti-cardiolipin antibodies is by reduced binding of the anti-coagulant protein annexin V to endothelial cells in the presence of anti-cardiolipin antibodies (182).

There is less evidence that antiphospholipid antibodies are associated with the development of atherosclerosis. In a study by Djokovic et al. (2013) increased cIMT was more prevalent in lupus patients with secondary APS (48.3% vs. 16.1%, \( p=0.008 \)) and independently predicted cIMT progression in a multivariable model (OR 6.43 [1.26-32.0]) (183). Whilst APS may logically result in increased thrombosis (and thus cardiovascular events) the cause of increased cIMT in APS is not known. In terms of plaque, in a case-control study by Ahmad et al. (2007), there was an increased prevalence of plaque in patients with antiphospholipid syndrome (3.87 [1.53, 9.74]) or IgG anti-cardiolipin antibodies (2.26 [1.17, 4.36]). Conversely, Roman et al. (2003) found that the presence of anti-phospholipid antibodies was not associated with the presence of carotid plaque (184). Interestingly in this study, anti-cardiolipin antibodies were more common in patients without plaque [11.5% vs. 2.8%, \( p=0.04 \)] although the significance of this is currently unclear. Other groups have also not found an association between anti-phospholipid antibodies and CVD in SLE (75;185).

1.4.3.2. Anti-endothelial antibodies
The role of anti-endothelial cell antibodies (AECAs) in the development of CVD in SLE remains controversial. In a small study of 27 SLE patients, ACEAs were identified in 24/27 (89%) of subjects but were not associated with markers of endothelial damage (150). In vitro studies have identified that AECAs can cause endothelial cell activation (increased adhesion molecule expression) and/or apoptosis (186).

1.4.3.3. Adhesion molecules
In a cross-sectional study by Rho et al. (2008) coronary atherosclerosis in SLE (measured by coronary artery calcium) was significantly associated with circulating E-selectin, Vascular cell adhesion molecule-1 (VCAM), Intercellular adhesion molecule (ICAM) and Tumour necrosis factor-α TNFα (187). Recently, serum E-selectin has been associated with carotid plaque in lupus patients (188). Circulating soluble markers have also been shown to predict CVD events in patients with SLE over time. In a prospective study (mean follow-up 8.3 years), increased levels of von Willebrand factor (vWF) and VCAM-1 were associated with future cardiovascular events (HR 2.05 [1.23, 3.42] and 1.78 [1.20, 2.65] respectively) (189).

1.4.3.4. Lupus disease activity and immunosuppressant therapy
Prospective studies have identified a positive association between SLE disease activity and damage (which suggests prolonged or previously severe activity) and risk of cardiovascular disease (51;167;190). In a prospective study by Lertrantanakul et al. (2013) lupus damage was
associated with progression of coronary artery calcium whilst disease activity (defined by hypocomplementaemia) was associated with progression of aortic calcification. Interestingly, corticosteroid use was associated with progression of calcification at both sites (191). Steroid use (previous or cumulative) has been associated with CVD in some studies (47;167;192) but not others (193). This may be because although corticosteroids per se increase CVD risk, possibly via development of MetS, suppression of inflammation has a more beneficial effect on cardiovascular risk. Magder et. al. (2012) noted that although previous steroid use was not associated with CVD risk, a current dose of 20mg/day was associated with increased risk after adjustment for disease activity (193). This suggests that steroids may also increase cardiovascular risk acutely.

1.4.3.5. Type 1 interferon and cardiovascular disease
There is currently much interest in the role of type 1 interferon in the development of vascular damage and atherosclerosis in SLE. Early evidence came from an observational study by Takase et. al. (2001) of patients with hepatitis C undergoing recombinant interferon-α2b (IFNα2b) therapy. In this study of only 10 patients, IFNα2b resulted in significantly reduced FMD (but not endothelium independent dilatation) and reduced exercise tolerance after 4 weeks of treatment (194). In patients with SLE, those with increased expression of the ISG MX1 in PBMCs have significantly impaired endothelial function as measured by peripheral arterial plethysmography (195). Expression of this signature is not confined to leukocytes and the expression of 3 ISGs (PRKRA, IFITM1 and CD69) has been shown to be significantly up-regulated in platelets from lupus patients compared to controls. In the lupus population, expression of these proteins in platelets was increased in those with a history of cardiovascular disease (196).

Recently, Somers et. al. (2012) adopted the approach of using Principal Component Analysis to identify whether predetermined ISGs (PRKR, IFI44, IFI44L, MX1 and IFIT1) were associated with subclinical CVD in patients with SLE. In this study of 95 SLE patients and 38 HCs, different ISG principal component scores were associated with different forms of subclinical CVD (for example the sum of IFIT1, IFI44, MX1 and IFI44L was associated with cIMT whilst FMD was more closely associated with PRKR) (109). The mechanisms by which IFNα causes premature atherosclerosis are not fully understood. A direct effect is suggested as the endothelial cells from SLE patients express an interferon signature (197). Indirectly, IFN may have important effects on macrophages within developing atherosclerotic lesions. IFNα can increase oxLDL uptake by macrophages, via up-regulation of scavenger receptor class A (SR-A), thus increasing
formation of foam cells (198). IFNα may also have a deleterious effect on endothelial repair mechanisms as described below.

1.4.3.6. **Neutrophils and Neutrophil Extracellular Traps (NETs)**
In response to stimuli (including IL-8 and LPS), neutrophils produce large extracellular matrices comprising DNA, histones and proteins from primary and secondary granules (including neutrophil elastase and myeloperoxidase); formation of these structures is termed NETosis (199). Both LDGs and lupus neutrophils induce endothelial cell apoptosis as demonstrated by co-culture with HUVECs (200). In further studies, inhibition of NET formation significantly attenuated endothelial apoptosis induced by lupus neutrophils/LDGs (201). Endothelium-dependent vasorelaxation is significantly impaired by NETs. In a study by Carmona-Rivera et al. (2014) matrix-metalloprotease-9 (MMP-9) present in NETs activated MMP-2 and induced endothelial dysfunction. Furthermore, immune complexes containing MMP-9 and anti-MMP-9 in SLE patients induced NETosis and enhanced MMP-9 activity which can lead to further vascular damage (202). Figure 1-2 summarises the roles of traditional and lupus-specific cardiovascular risk factors in the development of atherosclerotic plaque in SLE.
Both traditional and lupus-specific cardiovascular risk factors are proposed to increase the CVD risk in SLE patients. LDGs produce NETs which both directly damage the vasculature and stimulate IFNα release from pDCs. The effect of IFNα on the vessel is presumed deleterious. Other important factors are pro-inflammatory lipids and circulating immune complexes. LDG = low-density granulocyte, pDC = plasmacytoid dendritic cell, IFN = interferon, NETs = neutrophil extracellular traps. Adapted from Kahlenberg et. al. (2013) (203)

1.5. Endothelial repair in SLE

1.5.1. Endothelial repair mechanisms
The maintenance of endothelial integrity is important for the health of the vasculature. Similar to other tissues, the endothelium is in a continual state of “wear and repair”. This can occur principally via 2 mechanisms: the proliferation of mature endothelial cells (angiogenesis) and the differentiation of endothelial precursors or progenitors to form new vessels (neovasculogenesis). This complex process appears to be regulated by progenitor cells both in the circulation and resident in the vasculature (204). The first report of a population of circulating pro-angiogenic cells was by Asahara et. al. (1997). In this work CD34+ peripheral blood mononuclear cells (PBMCs) were cultured in vitro and found to express endothelial cell markers CD31, Kinase insert domain receptor (KDR), and E-selectin. Furthermore there cells were able to migrate towards and contribute to new vascular structures in a murine hind-limb ischaemia model (205). These observations led to the term “endothelial progenitor cell” (EPC).
Deficiency of circulating EPCs leading to reduced vascular repair is now proposed as an important contributor to endothelial dysfunction (206).

In patients with CVD there is a release of EPCs into the circulation following MI (207). A similar increase in EPC numbers is seen following a stroke and a greater number of circulating EPCs is associated with an improved neurological outcome (208;209). The stimulus for EPC release is not yet known but may be specific for vascular damage as a systemic inflammatory response induced by bacterial antigens does not mobilise EPCs from the bone marrow (210). Once in the circulation, the migration of EPCs towards sites of vascular damage appears to be stromal cell-derived factor 1 (SDF-1)-dependent (211).

### 1.5.2. The nomenclature of cells involved in endothelial repair

Since the seminal work of Asahara et. al. it has become clear that “EPCs” are not a single cell population. Two distinct populations of cells are involved in endothelial repair and were identified primarily on the basis of the time to emergence in ex vivo culture systems (212). Further work has been able to distinguish between these cell types both morphologically and functionally, and also on the basis of gene expression profiles (213;214).

The first is a population of myeloid cells which appear early in culture (usually <14 days) and were initially termed “early-EPCs”. The principle features of these cells are i) expression of myeloid cell markers (CD14, CD45, CD115) but also some endothelial markers (CD31, CD105, vWF), ii) poor proliferative capacity in vitro, iii) inability to incorporate into developing vessels in vivo or form networks in vitro, iv) capacity for phagocytosis, and v) secretion of angiogenic factors (213). These cells have been identified by many groups and have acquired different names including “early outgrowth-EPCS, circulating angiogenic cells and myeloid angiogenic cells”. In this thesis those cells which are of myeloid origin, occur early in culture and augment endothelial repair by paracrine secretion of angiogenic factors are termed myeloid angiogenic cells (MACs).

Medina et. al. (2011) proposed that MACs express markers of M2 (alternatively-activated) macrophages (215). The principle differences between the macrophage subtypes in terms of surface marker expression have been extensively reviewed by Tonioka et. al. (2012) (216). Whilst the precise nature of macrophage 2 subtypes requires further investigation, the current consensus opinion is that M1 (classically-activated) macrophages have pro-inflammatory activity, whilst M2 macrophages have immunosuppressive and tissue restorative functions.
The situation is complicated further by the observation that these cells can form colonies in vitro if those cells which do not adhere to the fibronectin matrix are re-plated after 48 hours. These colony forming units (CFUs) are sometimes termed CFU-Hill after the work of Hill et. al. (2003) which identified an association between the number of CFUs and the Framingham Risk Score (217). The colonies have a distinct phenotype with central rounded cells surrounded by a periphery of spindle-shaped cells (as described by Asahara et. al.). Importantly, despite these morphological differences, these cells share gene expression profiles with MACs and monocytes (218).

In contrast, the second population of cells occur much later in culture with colonies forming between days 14 and 21 (213). These cells i) express endothelial markers but not myeloid markers, ii) have high proliferative capacity in vitro, iii) display a characteristic cobblestone morphology and iv) form networks in vitro and true vessels in vivo. These cells also have many names including “late-EPCs, late outgrowth-EPCs, endothelial outgrowth cells, outgrowth endothelial cells, and endothelial colony forming cells”. In this thesis these cells will be termed outgrowth endothelial cells (OECs). The principle differences between MACs and OECs are shown in figure 1-3.
Both MACs and OECs are derived from the bone marrow. MACs express the myeloid markers CD14 and CD45, whilst the precursors of OECs express CD34 and CD133. Both of these OECs markers are lost as the cells differentiate towards mature endothelial cells. Whilst only OECs can contribute to vasculogenesis, MACs may augment this process by secretion of pro-angiogenic factors in addition to promoting angiogenesis. The cells have different morphologies and resemble either macrophages (MACs) or mature endothelial cells (OECs). Whilst OECs form clear networks in vitro, MACs are unable to do so. Adapted from Reynolds et. al. (2014) (219).

1.5.3. The Measurement of circulating progenitor cells
Circulating progenitor cells have also been quantified in the blood using flow cytometry. At present, no consensus exists regarding the optimum panel of markers to identify these cells. The most widely used panel is CD34, CD133, and Vascular endothelial growth factor receptor-2 (VEGFR-2) although some groups use only 2 markers at any time. Some groups also favour the use of negative markers and exclude cells on the basis of CD45, CD3, and CD56 expression. To try to improve consistency the European League Against Rheumatism (EULAR) Scleroderma Trials and Research Group proposed that circulating cells could be identified as CD34+/CD133+/VEGFR-2+ (220). This approach has been independently validated in scleroderma but not yet in lupus (221). Other groups have, however, proposed that these cells should be CD34+/VEGFR-2+/CD45dim (222). Critically it has not yet been demonstrated which subpopulation of circulating cells identified in this manner differentiate into OECs. These cells are therefore most appropriately referred to as “circulating putative OEC precursors”.

Figure 1-3: Overview of MACs and OECs
Both MACs and OECs are derived from the bone marrow. MACs express the myeloid markers CD14 and CD45, whilst the precursors of OECs express CD34 and CD133. Both of these OECs markers are lost as the cells differentiate towards mature endothelial cells. Whilst only OECs can contribute to vasculogenesis, MACs may augment this process by secretion of pro-angiogenic factors in addition to promoting angiogenesis. The cells have different morphologies and resemble either macrophages (MACs) or mature endothelial cells (OECs). Whilst OECs form clear networks in vitro, MACs are unable to do so. Adapted from Reynolds et. al. (2014) (219).
1.5.4. Cells involved in endothelial repair in SLE

1.5.4.1. Myeloid Angiogenic Cells (MACs)

Only a small number of studies have quantified the number of MACs in patients with SLE. In the study by Denny et al. (2007) lupus patients had fewer MACs compared to controls with significantly reduced monolayer formation. These cells were cultured for 21 days and may therefore represent a mixed OEC/MAC population (223). A small study by Westerweel et al. (2007) found no difference in MAC number after 7 days, although the control population in this study had higher serum cholesterol which may be important given the relationships between myeloid cells and lipids (224). MACs are also reduced in the NZB/W F1 murine model of SLE. A study by Thacker et al. (2010) found fewer MACs in NZB/W mice compared to the BALB/c background strain both in early disease and once active lupus was apparent (200).

The function of MACs in vitro is also impaired in SLE in terms of colony formation, migratory capacity and expression of angiogenic factors. The CFU assay shows fewer colonies in patients with SLE in most (195;225;226) but not all studies (227). In addition to CFU number, MACs from SLE patients also appear to form smaller colonies than those from healthy controls, with notably fewer spindle-shaped cells (228). Migration of MACs towards both VEGF and TNFα is impaired in SLE (228;229). The expression of VEGF and Hepatocyte growth factor (HGF), which are important pro-angiogenic factors, is also lower in SLE OEC/MACs at both the transcript and protein level (223).

1.5.4.2. Outgrowth Endothelial Cells (OECs)

Much less is known about the function of OECs in SLE. A study by Deng et al. (2010) found that OECs in active SLE patients were impaired in terms of adhesion to fibronectin, migration towards VEGF and tubule-formation on Matrigel compared to healthy controls. Lupus OECs also expressed around 4-fold higher levels of IL-6 (230). Whilst the cause of this dysfunction is unknown, factors which influence MAC function such as IFNα may be implicated.

1.5.4.3 Circulating OEC precursors

Numerous studies have compared the number of circulating putative OECs in patients with lupus with healthy controls. As described above, direct comparison between studies is difficult due to differences in markers used. Of 10 published studies, 5 found fewer cells in SLE (195;223;224;228;231), 2 found an increased number (232;233) and 2 no difference. The study by Ebner et al. (2010) found an increase in VEGFR-2+/CD133+ but a decrease in VEGFR-
$2^+$/CD34$^+$ cells in SLE (229). The differences may be due to the markers used but other factors need to be considered including whether cells are from PBMCs or from whole blood, delays in sample processing and whether necrotic/apoptotic cells are excluded. Furthermore, patient-related factors including lymphopenia, disease activity/duration, concurrent therapies, and coexisting CVD may also influence cell number (234).

Most recently, Castejon et. al. (2014) identified an association between circulating EPC precursors and aPWV in lupus patients. Patients with abnormal PWV (after consideration of age and BP) had lower numbers of CD34$^+$/VEGFR-2$^-$ cells (1.00 v.s. 1.33, p=0.01) regardless of coexistent cardiovascular disease. There was no association between cIMT or carotid plaque and CD43$^+$/VEGFR-2$^-$ cells although cIMT was associated with an increased number of possible apoptotic endothelial cells (CD146$^+$/AnnV$^+$) (235). The association between EPCs and PWV may however be mediated by those traditional cardiovascular risk factors which were associated with EPC numbers (BP, smoking, hyperglycaemia, metabolic syndrome). It should also be noted that the origin of circulating CD146$^+$ cells is uncertain, although this is considered a marker for mature endothelial cells, these cells do not express other endothelial markers (CD31 or CD51/61) (236).

1.5.5 Mechanisms of impaired endothelial repair in SLE

1.5.4.3 Type 1 interferon

Interferon alpha is associated with impaired MAC function in patients with SLE. The number of CFUs is lower in patients with higher PMBC expression of the ISG MX1. This can be replicated in vitro as the addition of IFNa2b to MAC cultures decreases CFUs in a dose-dependent manner (and is restored by IFNα blockade) (195). Similarly in the study by Denny et. al. (2007) OEC/MAC populations treated with IFNα showed increased apoptosis (reduction in CD11c$^+$, increased Annexin-V$^+$) whilst lower concentrations skewed the OEC/MACs towards a non-angiogenic phenotype (reduced CD14$^+$ and increased CD86$^+$) (223). This study also suggested that this effect may occur in vivo as IFNα present in SLE serum significantly reduced OEC/MAC number. Similarly, the reduction in numbers of MACs seen in NZB/W mice is replicated when IFNα is added to cells from the control mouse (BALB/c) in vitro (237). In further experiments the lupus-prone New Zealand mixed 2328 (NZM) model was crossed with an IFNα-receptor (IFNAR) knockout mouse. This NZM IFNAR$^{-/-}$ model (INZM) had significantly increased MACs in both bone marrow and spleen compared to NZM. Furthermore, in vivo angiogenesis as measured by subcutaneous Matrigel plugs was also enhanced. The opposite effect was seen when IFNα was over-expressed in NZM mice. These animals had fewer MACs and impaired
endothelial function as measured by aortic ring myography compared to the control group (238).

Gene expression studies have identified that IFNα treatment results in down-regulation of VEGF-A and IL-1 pathways in OEC/MACs (239). The source of IFNα has been identified as low-density granulocytes (LDGs) which are present in the PBMC fraction and secrete IFNα (200). Consistent with this, ISGs (IFI44, MX1, IFIT1) are significantly up-regulated in SLE OEC/MAC cultures (223).

1.5.4 Rationale for the investigation of vitamin D and MAC function
There is evidence that vitamin D modulates macrophage function in a manner that is both anti-inflammatory and increases bacterial killing. Given that MACs are myeloid cells with some features of M2 macrophages (215) it is proposed that vitamin D will also influence MAC function. The first observations of calcitriol in macrophage function came from studies of M. tuberculosis infection. Toll-like receptor (TLR2/1) activation resulted in up-regulation of both the VDR and 1α-hydroxylase. This in turn induced cathelicidin (LL-37) and augmented intracellular killing of the mycobacteria (240). Indeed expression of cathelicidin, an important anti-microbial peptide, is directly regulated by 1,25(OH)2D with a vitamin D response element (VDRE) present in the promoter region of the gene (241). Calcitriol also reduced macrophage expression of pro-inflammatory cytokines in vitro including IL-1β, IL-6 and TNFα (242). This effect was even greater in primary macrophages obtained from patients with rheumatoid arthritis (243). In addition to cytokine expression, vitamin D can modulate cellular function in terms of adhesion and migration (244). The direct regulation of macrophage gene expression and changes in cell function suggests that MACs may be a target cell for vitamin D, and that vitamin D may be able augment endothelial repair mechanisms.

1.6 Physiology of vitamin D
Vitamin D is a steroid hormone which has a central role in calcium homeostasis. The discovery of vitamin D followed the observation that a fat-soluble factor present in cod-liver oil (vitamin A) could cure xerophthalmia in young rats. It then followed that a canine model of rickets was also cured by cod-liver oil. This effect was initially attributed to vitamin A until it was discovered that vitamin A was destroyed by heating, but that anti-rickets effect (obtained from vitamin D) was not (245). The first observation of a protective effect of cod liver oil against rickets in children was published in the Lancet in 1922 (246). Subsequent observations
that sunlight could also cure rickets led investigators to identify that vitamin D synthesis could occur in vivo. Subsequently, Holick et. al. (1980) conclusively demonstrated that previtamin D₃ is derived from the photoconversion of 7-dehydrocholesterol (7-DHC) by UV radiation at a wavelength of 280-320nm (247). Only 10-15% of 7-DHC is converted to previtamin D₃, due to the formation of inert compounds (lumisterol, pyrocalciferol, isopyrocalciferol and tachysterol) (248). Pre-vitamin D₃ then undergoes non-enzymatic transformation into vitamin D₃ (cholecalciferol) which enters into the circulation, bound to vitamin D binding protein (DBP), with serum concentrations peaking at 12-24 hours following UV exposure (figure 1-4) (249).

The efficiency of cutaneous vitamin D synthesis is limited by many different factors (table 1-3). Unlike cholecalciferol, vitamin D₂ (ergocalciferol) is an exogenous compound in humans and obtained from the diet. In a standard Western diet most of the ergocalciferol is obtained from oily fish (notably cod liver oil) and fortified dairy products (250).

**Figure 1-4: Synthesis of vitamin D in the skin under the influence of UV radiation**

The conversion of pro-vitamin D₃ to pre-vitamin D₃ occurs in the skin under the action of UVB radiation. Non-enzymatic conversion to vitamin D₃ is strongly temperature dependent. Much of the pre-vitamin D₃ is metabolised to inert compounds (principally lumisterol and tachysterol). 7-DHC = 7-dehydrocholesterol, DHCR7 = 7-dehydrocholesterol reductase.
Factors Influencing Vitamin D Synthesis

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<td>7-dehydrocholesterol availability in the skin (7-DHC concentrations decrease with age)</td>
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<td>Activity of 7-dehydrocholesterol reductase</td>
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<td>Wavelength of UVB radiation</td>
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<td>Exposure to radiation (high doses increase formation of inactive isomers)</td>
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<tr>
<td>Time of day</td>
<td></td>
</tr>
<tr>
<td>Skin pigmentation (reduced synthesis in pigmented skin)</td>
<td></td>
</tr>
<tr>
<td>Use of sunscreen/sunblock/protective clothing</td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1-3: Factors influencing cutaneous vitamin D synthesis**

(Adapted from Lehmann and Meurer, 2010) (249)

However, both vitamin D₂ and D₃ are relatively metabolically inactive and require 25- and 1-alpha hydroxylation. Blunt et al. (1968) were the first group to identify 25-hydroxyvitamin D (25(OH)D) as a metabolite of vitamin D with greatly increased bioactivity (251). Isolation of the hepatic circulation in rats demonstrated that 25-hydroxylation occurs uniquely within the liver (252). Only 3 years later the fully active metabolite, 1α,25-dihydroxyvitamin D (1,25(OH)₂D₃) was identified, and its ability to regulate intestinal calcium absorption demonstrated (253). The metabolism of 25(OH)D to 1,25(OH)₂D occurs predominantly in the mitochondria of the kidney by the enzyme 1-alpha-hydroxylase (CYP27B1) (254). Unlike the 25-hydroxylation, which is only dependent on the availability of vitamin D, 1-alpha-hydroxylase is subject to exquisite regulation by parathyroid hormone (PTH), calcitonin and 1,25(OH)₂D itself. There has also been recent interest in the extra-renal expression of CYP27B1. Whilst CYP27B1 is expressed in a wide range of tissues including skin, exocrine glands and the vasculature, the physiological importance of peripheral 1α-hydroxylation is not clear. In both the placenta and activated macrophages (in granulomatous disorders such as sarcoidosis) the activity of CYP27B1 is such that detectable levels of 1,25(OH)₂D from these sources can be found in the serum (255).

The serum concentrations of 1,25(OH)₂D and to a lesser extent, 25(OH)D are regulated by the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24A1). This was demonstrated by a failure of clearance of the active hormone in 24-hydroxylase deficient mice (256). This has clinical relevance as loss-of-function mutations within CYP24A1 result in prolonged hypercalcaemia and nephrocalcinosis and nephrolithiasis (257;258). The principle regulator of CYP24A1 expression is 1,25(OH)₂D₃ which initiates transcription via a VDRE located proximal to the transcription start site of the gene (259).
1.6.1 Vitamin D transport
Vitamin D is a hydrophobic molecule and in the plasma is strongly bound to vitamin D binding protein (DBP). DBP is a 58 kDa glycoprotein and is also known as group-specific component of serum (Gc-globulin). The concentration of DBP in the serum is around 100-fold that of 25(OH)D suggesting that it is usually <1% saturated (260). Approximately 88% of 25(OH)D and 85% of 1,25(OH)₂D in the serum is bound to DBP although the affinity for 25(OH)D is 10x greater than for 1,25(OH)₂D (261).

1.6.2 The vitamin D receptor
The vitamin D receptor (VDR) is one of 48 members of the superfamily of nuclear hormone receptors which regulate gene expression by either inducing or repressing transcription. Baker et. al. (1988) successfully cloned the human VDR, demonstrating that it was a protein of ~50kDa, with 427 amino acids and structural homology to the thyroid hormone receptor (262). The receptor consists of three distinct domains; i) the zinc-finger DNA binding domain at the N-terminus, ii) the hinge region, and iii) the C-terminal ligand-binding domain (figure 1-5) (263). The hydrophilic DNA binding domain has nine cysteine residues which are strongly conserved between nuclear hormone receptors, and which confer zinc finger formation and thus binding to VDREs (264). The domains of the VDR are not, however, as clearly defined as presented above and amino acid residues within the C-terminal region may also be important for VDRE binding (265).

![Diagram of VDR structure]

**Figure 1-5: The structure of the VDR**
Numbers represent the amino acid residues from the N-terminus. The sites of transactivation (marked) are where co-stimulators and co-repressors bind.

The C-terminal region is perhaps of greatest interest as is it the site of ligand binding, and contains 12 α-helices critical for ligand-induced heterodimerisation of the VDR with the
retinoid X receptor (RXR) (266). It is this heterodimerisation of the VDR with the RXR that transforms the inactive receptor into a receptor complex capable of binding VDREs.

The distribution of VDREs within vitamin D-related genes is variable. Some genes (e.g. osteocalcin) contain a VDRE proximally within the promoter region, whilst other genes contain multiple VDREs, all of which need occupation by the VDR-RXR complex for maximal activation (267). The VDR-RXR complex has been implicated as responsible for induction or repression of a vast number of different genes. It is important to recognise that the regulation of gene expression is both time- and cell-specific (e.g. in THP-1 cells 638 genes were regulated with 4 hour treatment, whilst only 229 were regulated by 36 hour-treatment of human lymphoblastoid cells, with only 5.6% overlap) (268;269).

The complexity of VDR-RXR signalling is increased further by the existence of numerous co-activators and co-repressors. Important co-activators include the Vitamin D-receptor Interacting Protein (DRIP) and steroid receptor co-activator (SRC) complexes, the latter of which has histone acetyl transferase (HAT) activity (270). Interestingly, different dominant co-regulators are seen bound to the VDR depending upon the physiological state of the cell (e.g proliferating, differentiated) and these result in differential gene transcription (figure 1-6).

Figure 1-6: Simplified overview of VDR signalling
The VDR and RXR are localised and bound to co-repressors (A). Ligand binding leads to release of the now tightly formed VDR-RXR complex from the co-repressor. The VDR is phosphorylated (P) and binds to co-activators (B). The co-activator dissociates from the complex as vitamin D receptor interacting proteins (DRIPs) bind and transcription begins (C). The VDR becomes degraded or recycled for further signalling. The active ligand is dissociated and degraded (D). Figure adapted from Jones et. al. (1998) (263)
The nuclear VDR is not, however, the sole receptor for 1,25(OH)\(_2\)D. In 1994, Nemere et. al., characterised a membrane bound receptor which could rapidly increase calcium absorption in chick intestinal epithelia (271). The exact nature of the membrane-bound VDR is unclear and Norman et. al. (2001) have postulated potential signalling mechanisms including intrinsic kinase activity and G-protein coupling (272). Interestingly, the nuclear and membrane VDRs selectively recognise different shapes of 1,25(OH)\(_2\)D. This is particularly relevant as the activated form of vitamin D has conformational flexibility which may be influenced by the surrounding environment. Recently using CYP27B1\(^{-/-}\) cells it has been demonstrated that 25(OH)D can also directly regulate gene expression. Expression of the vitamin D-regulated gene CYP24A1 was saturated with 500nM 25(OH)D compared to only 10nM 1,25(OH)\(_2\)D (273). This has important implications given that 25(OH)D is present at around 10\(^3\)-fold greater concentrations and is much less tightly regulated than 1,25(OH)\(_2\)D.

Finally, vitamin D also exists as C-3 epimers: principally 3-epi-25(OH)D\(_3\), 3-epi-1,25(OH)\(_2\)D\(_3\) and 3-epi-24,25(OH)\(_2\)D\(_3\). These epimers exist due to changes in the orientation of the OH group at position C-3 of the A-ring. The process of C-3 epimerisation is cell specific and does not appear to effect the action of either CYP21B1 or CYP24A1. The C-3 epimers show significantly lower binding to both DBP and the VDR (274). Although the epimers were initially only seen in neonates they have been recently identified in the serum of a small number of adults (275). The physiological relevance of these compounds is not yet known.

1.6.3 Vitamin D and bone health
The principal role of vitamin D is regulation of serum calcium and phosphate promoting bone mineralisation. Stimulation of calcium absorption (transcaltachia) occurs via up-regulation of calcium channels/transporters within the epithelium of the small intestine (276). Vitamin D raises serum calcium and phosphate in order to optimise the blood calcium-phosphate product, resulting in the precipitation of hydroxyapatite upon the type I collagen matrix of bone. Deficiency in vitamin D therefore results in hypomineralisation and the development of rickets or osteomalacia. The association between vitamin D deficiency and osteoporosis is more controversial. Some studies have identified an association between vitamin D deficiency and osteoporosis or low BMD (277-279) whilst others have not found such an association (280-282). Similarly, in a recent large meta-analysis, vitamin D supplementation did not appear to significantly reduce the risk of fractures (283).
Vitamin D also suppresses parathyroid hormone (PTH) release both directly (via a VDRE within the parathyroid gland), and indirectly by up-regulation of the calcium-sensing receptor within the parathyroid (284). PTH stimulates renal 1α hydroxylation of 25(OH)D, thus forming a feedback loop. In addition to effects on calcium absorption and PTH secretion, 1,25(OH)₂D₃ can directly signal in both osteoblasts and osteoclasts. Within osteoblasts, the regulation of gene expression appears to be strongly influenced by the stage of osteoblast maturation (285). Similarly, whilst vitamin D may increase osteoclast formation, there may be a contrasting reduction in bone resorption (286).

Recently there has been much interest in the extra-skeletal effects of vitamin D. These range from immunomodulation, cancer prevention, insulin sensitivity and vascular function. VDRs have been identified in a wide range of tissues including the skin, colon, pituitary gland, heart, liver, ovary and skeletal muscle (287). Consequently, vitamin D deficiency has been associated with a broad range of autoimmune conditions (including type I diabetes, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis), degenerative brain diseases and malignancy (288). The effects of vitamin D on the function of the cardiovascular and immune systems relevant to SLE are discussed below.

### 1.7 Vitamin D deficiency

#### 1.6.1 Measuring serum vitamin D levels

The accurate and reliable measurement of circulating 25(OH)D and its metabolites is essential for both clinical studies and routine clinical practice. Serum 25(OH)D is considered to provide a more accurate reflection of body vitamin D stores than its active metabolite 1,25(OH)₂D due to a half-life of 2-3 weeks as opposed to approximately 6 hours. However, measurement of vitamin D is technically challenging as 25(OH)D is a remarkably hydrophobic molecule which strongly binds to DBP. Presently, five different methodologies have been described; competitive protein binding assay (CPBA), radioimmunoassay (RIA), enzyme-linked immunosorbant assay (ELISA), high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS). Of these, only the direct methods (HPLC and LC-MS) are able to differentiate 25(OH)D₂ (ergocalciferol) from 25(OH)D₃ (cholecalciferol). The CPBA is now rarely used due to its complex methodology and poor reproducibility (289). The RIAs and ELISAs offer more rapid and relatively robust measurements of total 25(OH)D. Some variability still occurs which may reflect the detection of other vitamin D metabolites notably 24,25(OH)₂D which may contribute to 10-15% of the estimate of 25(OH)D (290). Furthermore,
it is important to recognise that marked differences exist between commercially available assays (291). This has implications when comparing serum vitamin D levels derived via different assay kits.

The direct methods for quantifying vitamin D are more costly, and in the case of HPLC more time consuming, but have clear advantages over the indirect assays. LC-MS is a reliable method with intra- and inter-day variance of <6%, and correlates well with the results of the radioimmunoassay (292). Indeed, Roth et al. (2008) demonstrated that when compared to LC-MS, HPLC was the only method which did not result in significant variability in the obtained values for 25(OH)D (293).

1.6.1 Definition of vitamin D deficiency
The definition of vitamin D deficiency remains at present, one of considerable controversy. In the UK, serum 25(OH)D is often quoted in ng/ml, whilst in the US nmol/l is favoured. The conversion factor for nmol/l from ng/ml is approximately 2.5 (i.e. 20ng/ml ~ 50nmol/l). The current working definition of deficiency is based on the effects of 25(OH)D on bone health, given that there is no definitive evidence for the extra-skeletal effects of 25(OH)D. The principal observation which set the initial threshold was, that at levels below 20ng/ml, serum PTH begins to rise (294). However, this value is much lower than that at which bone demineralisation is evident. In a study by Priemel et al. (2010) the histology of iliac crest biopsies of 675 subjects was analysed. In this group the pathological accumulation of osteoid (based on the conservative osteoid volume/bone volume ratio of 2%) was only absent in those with 25(OH)D >30ng/ml (75nmol/l) (295).

A consensus opinion of authors in 2005 suggested a target of 50-80nmol/l for fracture prevention, but an optimum of 70-80nmol/l for general health (296). Similarly, Bischoff-Ferrari et al. (2006) proposed a serum 25(OH)D level of >30ng/ml (75nmol/l), and a target of 36-40ng/ml (90-100nmol/l). Most recently, an international committee of experts agreed that a target of 30-40ng/ml (75-90nmol/l) should be set by considering the role of vitamin D in areas such as cardiovascular disease, autoimmunity and cancer, rather than focusing of musculoskeletal health (297). Many authors presently accept serum 25(OH)D <20ng/ml as representing deficiency, 20≤30ng/ml as insufficient and >30ng/ml as vitamin D replete.
1.6.1 Environmental determinants of vitamin D status

Vitamin D exhibits both geographical and seasonal variation within the general population. A large cross sectional study of over 3000 women in Aberdeen demonstrated a peak in serum 25(OH)D in autumn (mean [sd] 23.7 [9.9] ng/ml) and a nadir in spring (19.7 [7.6] ng/ml) (298). The seasonal difference reported in this study was relatively modest, and differences of 10ng/ml between the ends of summer and winter (28.4ng/ml to 18.3ng/ml) have been reported in Manchester, UK (299). It is possible that the effect of seasonal changes in UV intensity is attenuated at more northern latitudes, leading to more widespread vitamin D deficiency. Indeed, the seasonal differences in 25(OH)D at northern latitudes of the UK appear to be less marked than those seen at more southern latitudes (300).

There is a strong association between 25(OH)D and BMI which may be due to adipose tissue acting as a “sink” for 25(OH)D. Weight loss has been found to increase serum 25(OH)D levels (301). In addition baseline BMI predicts serum 25(OH)D changes following oral supplementation (302). This suggests that the normal range for vitamin D may be dependent on BMI and/or adiposity.

1.6.2 The genetic contribution to vitamin D status

In the general population, both genome-wide association studies (GWAS) and targeted genotyping have identified associations between serum 25(OH)D and SNPs within or near VDR, DBP, DHCR7 (which converts 7-dehydrocholesterol to cholesterol) and CYP2R1 (responsible for 25-hydroxylation of cholecalciferol) (303-308). In the study by Wang et. al. (2010) of 33,996 subjects, those with a genotype score (comprising 3 alleles in DNP, near DHCR7 and CYP2R1) in the highest quartile had an OR of 1.92 (1.70-2.16, p=1x10^{-26}) of having 25(OH)D<50ng/ml compared to the lowest quartile (307). Genetic factors may also be important in autoimmune diseases as preliminary studies found that polymorphisms within DBP were associated with lower levels of 25(OH)D and 1,25(OH)_{2}D in patients with type 1 DM (309). Environmental factors including the season of blood sample collection and dietary vitamin D intake both modified the effects of genetic variation in DBP and CYP2R1 indicating that genotype alone does not determine serum 25(OH)D (306). Furthermore in a study of African Americans a genotype score generated from risk alleles across 3 SNPs only accounted for 4.6% of the variation in serum 25(OH)D (310).
1.6.3 Vitamin D toxicity

Vitamin D toxicity, as manifest by hypercalcaemia and hyperphosphataemia, has been reported when serum 25(OH)D increases to 150ng/ml (or lower in patients with chronic granulomatous disorders) (250). Doses of up to 10,000IU/day, with corresponding serum 25(OH)D levels of 88ng/ml are reported as safe (311). The North American Institute of Medicine (IOM) has adopted a more conservative approach with a safe serum level of 50ng/ml (312). Interestingly, studies in 1α–hydroxylase-deficient mice suggest that 25(OH)D rather than its active metabolite may be responsible for hypercalcaemic toxicity (313). This observation warrants further investigation.

1.7 Vitamin D and CVD risk in the general population

There have been a large number of studies investigating the association between vitamin D deficiency and cardiovascular disease in both the general population and high risk populations (e.g. type II diabetes mellitus). These will be discussed below in terms of observational studies, interventional trials and experimental studies (comprising smaller studies with surrogate markers of CVD, animal models and in vitro studies).

1.7.1 Observational studies of vitamin D and CVD

1.7.1.1 Cardiovascular outcomes

A large number of observational cohort studies within the general population have reported independent associations between lower levels of vitamin D and risk of CVD and CVD-related mortality. In a meta-analysis of 73 cohort studies (849, 412 subjects) those in the bottom tertile of 25(OH)D had a RR of death from CVD of 1.14 (1.01, 1.29) (314). In the largest single study of 18,225 men (Health Professionals Follow-up Study) subjects with 25(OH)D ≤15ng/ml had an increased risk of MI over 10 year follow-up compared to those with 25(OH)D ≥30ng/ml. In an unadjusted analysis the RR of MI was 2.42 (1.53, 3.84) which remained significant after adjustment for traditional CVD risk factors (2.09 [1.24, 3.54]) (315). Similar risk was found in the Framingham Offspring Cohort Study (1739 subjects) which found that those with 25(OH)D <15ng/ml had an adjusted HR for a cardiovascular event of 1.62 (1.11, 2.36), most notably in those with hypertension. Importantly, this study suggested that the relationship between 25(OH)D and risk of a CVD event is non-linear. The relationship has a U-shaped distribution with increased risk of CVD at low levels of vitamin D, and a smaller but clear increased risk with high levels of vitamin D (in this case >30ng/ml). This same distribution was found by Perna et al. (2013) although the increase in risk with 25(OH)D >30ng/ml was much
less marked (316). Furthermore, the effects of low vitamin D on CVD risk seem to be exaggerated in higher risk populations. In a study by Dobnig et al. (2008) patients referred to a tertiary centre for investigative angiography the adjusted hazard ratio cardiovascular mortality was 2.22 (1.57, 3.13) (317). Similarly, in Argentinian patients with suspected acute coronary syndrome (ACS), comparing those in the highest quartiles of 25(OH)D with the lowest gave a HR of 0.37 (0.11, 0.94) for sudden cardiac death over 2 years (318).

Not all studies have found an association between 25(OH)D and cardiovascular events. In the 3rd National Health and Nutrition Examination Survey (NHANES III) study of 13,311 adults, those in the lowest quartile had an increased risk of mortality (adjusted rate ratio 1.26 [1.08, 1.46]) but only a trend towards increased CVD-related mortality (rate ratio 1.20 [0.87-1.64]) (319). Similarly, others have found associations between vitamin D levels and other adverse events (all-cause mortality, stroke) but not coronary CVD (320;321).

In subjects with established CVD the association between vitamin D and CVD events is less clear. In a study of patients undergoing rehabilitation following an MI by Grandi et al. (2010) there was no association between low vitamin D (in terms of either <15ng/ml or lowest quartile) and future CVD events (322). More recently the Heart and Soul study of patients with stable CVD found an association between 25(OH)D and future CVD events which did not remain statistically significant in a complete model containing traditional CVD risk factors and PTH (323). Again, the relationship between vitamin D and CVD risk was non-linear.

Whether or not serum 25(OH)D has less of an effect on CVD risk in the context of established CVD remains unclear. In the study by Dobnig et al. (described above) 65-75% of patients had coronary artery disease and 25(OH)D remained independently associated with future CVD events (317).

1.7.1.2 Surrogate markers of CVD
Studies have identified associations between 25(OH)D and subclinical CVD including cIMT, coronary calcification, vascular stiffness and endothelial dysfunction. In an older population of 654 adults (mean age 75.5 years) without known CVD, lower 25(OH)D was associated with increased internal carotid intima-medial thickness (IMT) but not common carotid IMT (324). This may be of particular relevance to SLE as lupus patients preferentially have increased internal carotid artery disease (66). An association has also been seen in patients with symptomatic peripheral vascular disease, those undergoing angiography for suspected CVD.
and an inner city asymptomatic population (325-327). However other groups have not seen this association (328-332).

Within the general population low 1,25(OH)₂D has been associated with coronary artery calcification (CAC), assessed using electron-beam CT, in some (333;334) but not all studies (335). As 1,25(OH)₂D₃ levels are a poor marker of vitamin D status, it is more useful to examine associations between CAC and 25(OH)D. More recently, 25(OH)D deficiency has been shown to have importance in the development of new CAC but not in either the prevalence of CAC at baseline, or lesion progression in patients with pre-existing CAC (336). In this study a 10ng/ml reduction in 25(OH)D was associated with a 23% increase of CAC. There have been similar findings in patients with type 1 and type 2 DM, (337;338).

Three studies have demonstrated an association between impaired flow-mediated dilatation (FMD) and serum 25(OH)D in the general population. Tarcin et. al. (2009) selected those with significant vitamin D deficiency (<10ng/ml) and showed a mean FMD of 7.0 ±3.2% compared to 11.2±5.2% in the control group (P<0.001) (339). Similarly, Jablonski et. al. (2011) grouped subjects into deficient (<20ng/ml), sufficient (20-29ng/ml) and replete (≥30ng/ml) and showed a significant stepwise increase in FMD of 3.2±0.3%, 3.7±0.2% and 4.6±0.4% (340). This trend between increasing concentrations of vitamin D and increased FMD has also been replicated by Syal et. al. (2012) (341). Most recently in high risk patients, low vitamin D was associated with both impaired endothelial function (as measured by FMD) and reduced epicardial flow velocity (327).

Separating the effects of 25(OH)D from those of PTH is difficult given the strong reciprocal relationship between the two measurements. In some studies, 25(OH)D remains associated with aPWV after adjustment for serum PTH (342), whilst in others it does not (343).

1.7.1.3 Cardiovascular risk factors
When considering the observational studies described above it is important to recognise that vitamin D deficiency is associated with traditional cardiovascular risk factors including; age (317;344-346), hypertension (307;317;345;346), obesity (307;315;317;340;345-347), smoking (315;317), type 2 DM (317;345-348) and low HDL (315;317). Additionally, in some studies vitamin D deficient patients had a more sedentary lifestyle (315;317;345). Of all of the cardiovascular risk factors, special attention has been given to the metabolic syndrome. Numerous studies have identified a significant relationship between vitamin D deficiency and the metabolic syndrome (reviewed by Gulseth et. al. 2013 (349)). A recent meta-analysis of 16
cross-sectional studies found a linear inverse relationship between vitamin D and metabolic syndrome (OR 0.87 [0.83, 0.92] for each 10ng/ml increase in serum 25(OH)D) (350). This is of particular interest given the association between SLE and MetS described above.

1.7.2 Interventional studies
Fewer studies have investigated the effect of vitamin D supplementation of CVD risk. In the meta-analysis by Chowdhury et al. (2014) of 22 randomised controlled trials, the risk of all-cause mortality was lower in subjects receiving cholecalciferol (RR 0.89 [0.80, 0.99]) but not in those receiving ergocalciferol (RR 1.04 [0.97, 1.11]) (351). The largest single intervention study (the Women’s Health Initiative, WHI) investigated the effects of combined low-dose calcium plus vitamin D or placebo in 36,282 postmenopausal women. The most recent report in 29,862 of these women after 7 years intervention and 4.9 years additional follow-up showed no change in CVD risk with vitamin D supplementation (352). Interestingly this study allowed personal vitamin D supplementation of up to 600 IU/day (later increased to 1000IU/day) whilst the intervention dose was only 400IU/day.

The low-dose supplementation in the WHI study also had no effect on traditional cardiovascular risk factors. There was a small statistically (but not clinically) significant difference in apolipoprotein B100 but not in any other parameters measured (353). Similarly, in an Irish study supplementation of up to 600IU/day during winter months (October-March) had no effect on CVD risk factors. Interestingly, however, in younger adults aged 20-40 years the highest dose of 600IU/day was needed to prevent the seasonal decline in 25(OH)D concentrations (354). There have, however, been a small number of trials which have shown some benefit of vitamin D on cardiovascular risk factors. Salehpour et al. (2012) found that over a period of 12 weeks cholecalciferol (25ug/day, equivalent to around 1000IU) increased HDL and apoA-1 (355). In contrast, in women with polycystic ovary syndrome, vitamin D treatment resulted in decreased total cholesterol, triglycerides and VLDL but had no effect on HDL, LDL or apoA-1 (356). In a study of patients with type II DM, oral calcitriol resulted in significant beneficial changes in lipids (LDL and HDL), HbA1C and diastolic BP in only 8 weeks (357). The effect of vitamin D on lipids is therefore currently uncertain. In contrast to the above study, Ponda et al. (2012) found a trend towards increased LDL (but no change in other lipids) in vitamin D deficient adults treated with 50,000 IU/week for 8 weeks (358).
1.7.3 Experimental and translational studies

1.7.3.1 Vitamin D and flow-mediated dilatation and vascular stiffness
There have been numerous studies which have investigated the effects of vitamin D on endothelial function as measured by flow-mediated dilatation. These include both open-label studies and randomised controlled trials conducted both in the general population and in chronic disease states. The studies of vitamin D supplementation/therapy on FMD are summarised in table 1-4.
<table>
<thead>
<tr>
<th>Author</th>
<th>Population Studied</th>
<th>Baseline 25(OH)D</th>
<th>Vitamin D Dose</th>
<th>Study Duration</th>
<th>Improvement in FMD?</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitalia et al. (2014) (359)</td>
<td>Chronic kidney disease (n=26)</td>
<td>Deficient Mean = 17.2ng/ml</td>
<td>300,000 IU twice (baseline and 8 weeks)</td>
<td>16 Weeks</td>
<td>Yes</td>
<td>Increase in FMD from 3.1±3.3% to 6.1±3.7% (p=0.001)</td>
</tr>
<tr>
<td>Witham et al. (2013) (360)</td>
<td>Healthy South Asian women</td>
<td>Deficient Mean = 10.8ng/ml</td>
<td>100,000IU (n=25) or placebo (n=25)</td>
<td>4 weeks and 8 weeks</td>
<td>No</td>
<td>Mean change in FMD of 0.0% compared to placebo (p=0.98)</td>
</tr>
<tr>
<td>Yiu et al. (2013) (361)</td>
<td>Type II diabetes (n=100)</td>
<td>Insufficient Mean = 21.1ng/ml</td>
<td>5000IU/day (n=50) or placebo (n=50)</td>
<td>12 weeks</td>
<td>No (positive trend)</td>
<td>Change from mean 3.39 (±2.7)% to 4.9% (p=0.11) in treated and 3.4 (±2.0)% to 3.37% (p=0.95) in placebo</td>
</tr>
<tr>
<td>Can et al. (2012) (362)</td>
<td>Behcet’s disease (n=36), RA (n=33), HC (n=51)</td>
<td>Deficient Mean = 17.8ng/ml</td>
<td>1000IU/day</td>
<td>12 weeks</td>
<td>No (positive trend)</td>
<td>No significant change in 25(OH)D with replacement (44.5nmol/l to 47.5nmol/l) Change in Behcet’s group of 6.3% to 7.8% (p=0.4)</td>
</tr>
<tr>
<td>Gepner et al. (2012) (363)</td>
<td>Postmenopausal women (n=114)</td>
<td>Replete Mean 30.3ng/ml</td>
<td>2500IU/day (n=57) or placebo (n=57)</td>
<td>16 weeks</td>
<td>No</td>
<td>Change in FMD of 0.3 (3.4)% in treated group and 0.3 (2.6)% in placebo (p=0.77)</td>
</tr>
<tr>
<td>Longenecker et al. (2012) (364)</td>
<td>HIV-positive patients on stable therapy</td>
<td>Deficient All &lt;20ng/ml</td>
<td>4000IU/day (n=30) or placebo (n=15)</td>
<td>12 weeks</td>
<td>No</td>
<td>Median (IQR) change in 25(OH)D of 5 (-0.9, 7.4)ng/ml in treated group. Median change in FMD of 0.55(-1.1, 2.1)% in treated and 0.29% (-1.6, 1.8) in control group</td>
</tr>
<tr>
<td>Witham et al. (2012) (365)</td>
<td>Subjects with history of stroke</td>
<td>Deficient Mean = 15.5ng/ml</td>
<td>100,000IU (n=30) or placebo (n=28)</td>
<td>8 and 16 weeks</td>
<td>Yes (8 weeks)</td>
<td>Greater change in FMD in treated group at 8 weeks (6.9[3.5]% vs. 3.7[3.1]%, p=0.007) – due to reduction in FMD in placebo group</td>
</tr>
<tr>
<td>Study</td>
<td>Participants</td>
<td>Deficiency Status</td>
<td>Intervention</td>
<td>Duration</td>
<td>Outcome</td>
<td>Additional Findings</td>
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<tr>
<td>Harris et al. (2011) (366)</td>
<td>Overweight African-American Adults</td>
<td>Deficient</td>
<td>Mean = 13.7ng/ml</td>
<td>60,000IU/month (n=22) or placebo (n=23)</td>
<td>16 weeks</td>
<td>Yes</td>
</tr>
<tr>
<td>Witham et al. (2010) (367)</td>
<td>Type II diabetes</td>
<td>Deficient</td>
<td>Mean = 16.4ng/ml</td>
<td>200,000 IU (n=20) 100,000 IU (n=19) Placebo (n=22)</td>
<td>16 weeks</td>
<td>No</td>
</tr>
<tr>
<td>Tarcin et al. (2009) (339)</td>
<td>Healthy subjects (n=46)</td>
<td>Deficient</td>
<td>Mean = 8.2ng/ml</td>
<td>300,000IU intramuscularly monthly (n=23)</td>
<td>12 weeks</td>
<td>Yes</td>
</tr>
<tr>
<td>Sugden et al. 2008 (368)</td>
<td>Type II diabetes (n=34)</td>
<td>Deficient</td>
<td>Mean = 16.1ng/ml</td>
<td>100,000 IU single dose (n=17) or placebo (n=17)</td>
<td>8 weeks</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1-4: A summary of studies investigating the effect of vitamin D on endothelial function

This table summarises interventional studies of vitamin D where the primary or secondary outcome was a change in FMD. Both open-label and placebo-controlled trials are included. FMD = flow mediated dilatation, RA = rheumatoid arthritis, HC = healthy control.
These studies showed significant improvement in endothelial function in 5/11, a positive trend in 2/11 and no effect in 4/11. It is difficult to identify a clear trend, although with the exception of the study by Witham et. al. (2013), positive studies tended to have participants with lower baseline 25(OH)D values (360). There does not appear to be any difference in the outcome of the study between those which were open-label and those which were placebo-controlled.

In addition, a small number of studies have investigated the effect of vitamin D using other measures of endothelial function. Sokol et. al. (2012) conducted a placebo controlled trial of 50,000IU ergocalciferol weekly in vitamin D deficient patients with coronary artery disease found no difference in reactive hyperaemia peripheral artery tonometry (PAT) after 12 weeks (369). In contrast, in healthy subjects normalisation of 25(OH)D levels to >30ng/ml has been associated with increases in the reactive hyperaemic index (as measured by PAT) (370). Similarly, a single supplement of 100,000IU cholecalciferol in patients with peripheral artery disease caused no change in augmentation index after 1 month, but a trend towards an increase in reactive hyperaemia as measured by laser Doppler flux (371).

Studies of the effect of vitamin D on arterial stiffness have also shown variable results. In a study of adolescents, 2000IU cholecalciferol daily resulted in a significant decrease in carotid-femoral PWV in the treated group (5.41 to 5.33 m/s) whilst there was in increase in the control group (5.38 to 5.71 m/s), p=0.031 and p=0.016 respectively (372). In contrast Whitman et. al. (2013) found no change in either augmentation index or PWV after a single dose of 100,000IU in South Asian women living in the UK (360). Duration of treatment may be important as the augmentation index significantly improved (32.9% to 25.9%, p=0.0001) in patients with type II diabetes given 1000IU/day for 12 months (373).

1.7.3.2 Genetic associations of vitamin D metabolism in SLE
There have been reported associations between 4 polymorphisms within the VDR and the presence of SLE: FokI (rs2228570), BsmI (rs1544410), Apal (rs7975232) and TaqI (rs731236). Meta-analyses of 11 studies in SLE demonstrated an association between the both BsmI and FokI polymorphisms and SLE which was strongest in the Asian populations (374;375). The association was seen for both the presence of the BsmI B allele and the BB genotype. In the analysis by Mao et. al. (2014), the FokI polymorphism (FF genotype) was only associated with lupus in Asian subjects (375). Whilst it was not associated with lupus, the TaqI polymorphism was associated with primary biliary cirrhosis in a
meta-analysis of around 3500 subjects (376). VDR polymorphisms have also been associated with autoimmune thyroiditis and Addison’s disease (377;378).

Interestingly, serum 25(OH)D level may be influenced by the VDR genotype. Lupus patients with the FokI ff genotype have significantly higher levels of 25(OH)D compared to those with the FF genotype (27.9nmol/l vs 19.1nmol/l) (379). This observation suggests that as vitamin D levels are determined in part by VDR genotype, the definition of a normal range for 25(OH)D may need to be adjusted for these patients.

Polymorphisms in other components of the vitamin D pathway e.g. 1α-hydroxylase (CYP27B1) and DBP have not been investigated in SLE. In other autoimmune diseases (including type 1 DM, multiple sclerosis and inflammatory bowel disease) SNPs within the vitamin D metabolism pathway have been identified with disease risk, progression and severity (380-383).

Vitamin D may directly regulate the transcription of genes which are relevant to autoimmune disease. A ChIP-seq study by Ramagopalan et. al. (2010) identified vitamin D binding in a region containing a SNP previously associated with SLE (rs 13385731) (268). This SNP lies within the RASGRP3 gene and is associated with increased prevalence of malar rash in Chinese lupus patients (but interestingly reduced discoid rash and reduced ANA positivity) (384). The importance of this gene in lupus pathogenesis is thus currently unclear.

**1.7.3.3 Vitamin D in animal models of CVD**

A small number of experimental studies have investigated the effects of vitamin D supplementation or deprivation in mouse models of CVD. These have utilised both the apolipoprotein E knock-out (ApoE⁻/⁻) or LDL-receptor knockout (LDLR⁻/⁻) models. The interventions variable considerably between studies and include vitamin D deficient chow, oral or parenteral vitamin D administration, or combination with the VDR⁻/⁻ model (table 1-5).
<table>
<thead>
<tr>
<th>Author</th>
<th>Model</th>
<th>Intervention</th>
<th>Duration</th>
<th>Outcome</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schmidt et. al. (2014)</td>
<td>LDLR&lt;sup&gt;-/-&lt;/sup&gt;, WT</td>
<td>Western diet with low (50IU/kg) or adequate vitamin D (1000 IU/kg)</td>
<td>32 weeks</td>
<td>Aortic valve calcification</td>
<td>Significantly increased calcification in D-deficient mice Increased plaque lipids and TNFα</td>
</tr>
<tr>
<td>Weng et. al. (2013)</td>
<td>LDLR&lt;sup&gt;-/-&lt;/sup&gt;, ApoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Vitamin D deficient or standard chow, then high fat vitamin D deficient or standard</td>
<td>6 weeks then 8-10 weeks</td>
<td>Blood pressure Atherosclerosis</td>
<td>Deficient mice had higher BP and increased plasma renin Increased thoracic and abdominal atherosclerosis</td>
</tr>
<tr>
<td>Ish-Shalom et. al. (2012)</td>
<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>IP 0.25ng/g body weight calcitriol or vehicle alternate days</td>
<td>8 weeks</td>
<td>Blood pressure Atherosclerosis</td>
<td>Treated mice had reduced atherosclerosis (aortic sinus), lower BP and reduced renin</td>
</tr>
<tr>
<td>Szeto et. al. (2012)</td>
<td>LDLR&lt;sup&gt;-/-&lt;/sup&gt;/VDR&lt;sup&gt;-/-&lt;/sup&gt; mice LDLR&lt;sup&gt;-/-&lt;/sup&gt; with Rag-1&lt;sup&gt;+/+&lt;/sup&gt;/VDR&lt;sup&gt;-/-&lt;/sup&gt; BMT (no mature B/T lymphocytes)</td>
<td>High fat diet</td>
<td>8 or 12 weeks</td>
<td>Atherosclerosis</td>
<td>Increased atherosclerosis in LDLR&lt;sup&gt;-/-&lt;/sup&gt;/VDR&lt;sup&gt;-/-&lt;/sup&gt; despite lower cholesterol Further increased in Rag-1&lt;sup&gt;+/+&lt;/sup&gt;/VDR&lt;sup&gt;-/-&lt;/sup&gt; BMT mice Increased renin and renin receptor</td>
</tr>
<tr>
<td>Schmidt et. al. (2012)</td>
<td>VDR&lt;sup&gt;-/-&lt;/sup&gt; LDLR&lt;sup&gt;-/-&lt;/sup&gt; (diet treated)</td>
<td>Low (50IU/kg), recommended (1000IU/kg) or high (10000IU/kg)</td>
<td>16 weeks</td>
<td>Aortic calcification</td>
<td>Increased in VDR&lt;sup&gt;-/-&lt;/sup&gt; and LDLR&lt;sup&gt;-/-&lt;/sup&gt; fed low vitamin D diet</td>
</tr>
<tr>
<td>Takeda et. al. (2010)</td>
<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>20ng or 200ng calcitriol by gastric intubation twice weekly</td>
<td>12 weeks</td>
<td>Atherosclerosis</td>
<td>Reduced plaque with high vitamin D diet Increased Tregs</td>
</tr>
<tr>
<td>Husain et. al. (2010)</td>
<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>IP paricalcitol 200ng 3 times/week</td>
<td>16 weeks</td>
<td>Atherosclerosis</td>
<td>Reduced in paricalcitol Increased eNOS expression</td>
</tr>
</tbody>
</table>

Table 1-5: A summary of studies of the effects of vitamin D in animal models of CVD

ApoE = Apolipoprotein E, VDR = vitamin D receptor, LDLR = low density lipoprotein receptor, WT = wild type, Treg = regulatory T cell, BMT = bone marrow transplant, eNOS = endothelial nitric oxide synthase
In all of these studies, vitamin D therapy resulted in reduced atherosclerosis, blood pressure and calcification (whilst dietary vitamin D restriction or the VDR−/− has resulted in worsening of these parameters). The changes in blood pressure appear to be mediated by the renin-angiotensin system (387). The mechanism for reduced plaque formation is less clear. Takeda et al. (2010) suggested that the effects of vitamin D were mediated via the immune system. In ApoE−/− mice, 1,25(OH)_{2}D_{3} reduced macrophage and T cell infiltration of the arterial wall, increased Treg numbers in the spleen and reduced dendritic cell maturation (389). Interestingly, the changes in Treg cells may be secondary to the effects of vitamin D on innate immunity. In the study by Szeto et al. (2012), increased plaque was still seen in a complex model of LDLR−/− receiving bone marrow transplant from VDR−/−Rag-1−/− mice. The Rag-1−/− animals are unable to produce functional lymphocytes. The persistence of increased plaque in these experiments suggests the effects are not due to changes in T cell activity (388). Interestingly, in these models the increase in plaque was seen despite decreases in circulating lipids. Others have shown that vitamin D deficiency increases the presence of lipids in plaque but did not report serum levels (385).

Most recently, Andrukhova et al. (2014) suggested that VDR−/− mice have defects in the nitric oxide pathway. In these animals (fed a “rescue diet” containing increased concentrations of calcium and phosphate) there were significantly lower concentrations of nitrate and nitrite in the serum and urine. Furthermore, eNOS expression was down-regulated in aortic tissue at both the mRNA and protein level. Transcriptional regulation of eNOS by vitamin D was suggested by increased expression in aortic rings incubated with both 25(OH)D or 1,25(OH)_{2}D_{3} in the WT mice but not the VDR−/− mice (391). A small number of in vitro studies support the concept that vitamin D can regulate NO production. Calcitriol increases NO production in both porcine aortic endothelial cells and HUVEC under basal conditions (392;393). In human endothelial cells treated with advanced glycation end products (AGEs), calcitriol attenuated eNOS downregulation, increased NO bioavailability and reduced IL-6 production (394). Of note, AGEs have recently been shown to be increased in lupus patients, as associated with disease duration (395).

**1.7.3.4 In vitro studies of vitamin D and the endothelium**

The vitamin D pathway has been shown to be active within the vasculature. Endothelial cells express both the VDR and 1α-hydroxylase (396). Interestingly, 1α-hydroxylase in HUVEC cells is up-regulated by pro-inflammatory cytokines (TNFα) (397). Despite the identification of the VDR in the endothelium occurring in 1989, few studies have investigated the effects of vitamin D on the endothelium.
Vitamin D may directly protect the endothelium against environmental stressors. The expression of apoptosis-related genes in HUVECs treated with hydrogen peroxide is reduced by vitamin D in vitro (398). In addition, Kudo et al. (2012) demonstrated a protective effect of vitamin D on an in vitro model of Kawasaki disease. In these studies, TNFα significantly increased expression of adhesion molecules by human coronary endothelial cells. The addition of 1,25(OH)₂D₃ significantly reduced expression of VCAM-1 and IL-8 by these cells (399).

The effect of vitamin D on endothelial cell proliferation is controversial. Whilst some studies have shown increased proliferation (392), others have found that vitamin D has anti-proliferative effects (397;400;401). Notably, however, inhibition of angiogenesis appears to be most commonly seen in models of malignancy (tumour-derived cells or mouse models). These differences may also relate to differences in the source of the endothelial cells tested as there is marked heterogeneity in the physiology of cells derived from different parts of the vascular tree.

1.7.4 Vitamin D and endothelial repair
There have been a small number of studies investigating the relationship between vitamin D status and circulating putative OEC precursors. In healthy controls Mikirova et al. (2010) found significantly fewer CD34⁺/KDR⁺ EPCs in subjects with 25(OH)D <30ng/ml compared to >30ng/ml (402). In this study, an independent effect of vitamin D on EPC number could not be determined as EPC levels were also significantly lower in those with high glucose, VLDL and blood pressure. In a study by Yiu et al. (2011) patients with type II DM had fewer circulating CD133⁺/KDR⁺ EPCs (but not CD34⁺/KDR⁺) compared to age- and sex-matched controls. The number of these cells also increased significantly between groups with vitamin D deficiency (<20ng/ml), insufficiency (20-30ng/ml) and sufficiency (>30ng/ml). Vitamin D deficiency (not insufficiency) remained independently associated with CD133⁺/KDR⁺ EPC counts after adjustment for age, gender and traditional cardiovascular risk factors. Whist both impaired FMD and lower CD133⁺/KDR⁺ EPCs were associated with vitamin D deficiency, there was no relationship between FMD and EPC number (403). The same authors then investigated the effect of vitamin D supplementation on EPC number in a double-blind, randomised controlled trial of 5000IU/day or placebo. There was no change in EPC number in the treated group, but a statistically significant reduction in the placebo group (361). The overall treatment effect was not significant. Interestingly, the mean baseline 25(OH)D level in the treatment group was 21.1ng/ml (i.e. insufficient) and 21.9ng/ml in the control group. The lack of effect observed in
patients with vitamin D insufficiency (rather than deficiency) is similar to the FMD study by Yiu et. al. described above.

In terms of MACs, Mikirova et. al. (2010) investigated surface marker expression in HC MACs after 5 days culture. The study showed that significantly fewer MACs from low vitamin D subjects expressed KDR (21% vs. 34%), CD105 (72% vs. 89%) and CD62E (77% vs. 95%) by flow-cytometry than MACs from high vitamin D subjects (using a high 25(OH)D threshold of 40ng/ml) (402). Whilst CD105 (endoglin) is expressed on both endothelial cells and macrophages, KDR and CD62E (e-selectin) are specific for endothelial cells. The proportion of cells expressing KDR is higher than expected for MACs at day 5 (404). The high number of cells expressing CD62E is interesting as MACs are typically CD62E-negative (405), whilst OECs are CD62E-positive but typically do not arise so early in culture (406).

There are no studies of the effects of vitamin D on MAC function and only a single study has reported the in vitro effects of vitamin D on OECs. Grundmann et. al. (2012) demonstrated that OECs from umbilical cord blood had increased proliferative capacity and greater network formation on Matrigel in response to 10nM 1,25(OH)₂D. These functional changes were mediated via the VDR and associated with increased VEGF expression and pro-MMP-2 activity (407). The effect of vitamin D on OEC function in vivo has not yet been reported.

1.8 The roles of vitamin D in SLE
There is currently much interest in the potential roles of vitamin D in the aetiology, pathogenesis and clinical course of SLE. This section will discuss the prevalence of vitamin D deficiency in the SLE population and some of the factors which predispose these patients to low vitamin D. The potential roles of vitamin D in immune system function will be described before discussing the associations between vitamin D deficiency and CVD in patients with SLE.

1.8.1 Prevalence of vitamin D deficiency in SLE
Numerous studies have reported that patients with SLE have significantly lower vitamin D levels compared to controls. Müller et. al. (1995) were the first group to demonstrate reduced serum 25(OH)D in patients with SLE compared to a control group (median [IQR] 13 [10-27]ng/ml vs. 27 [18-32]ng/ml, p=0.0008) (408). Of 15 studies comparing SLE to a HC population, 12 (80%) have found significantly lower 25(OH)D in SLE and a further 2 (13.3%) showing a trend towards lower levels. Two studies have compared vitamin D levels in SLE to other disease groups. In a small study by
Huisman et. al. (2001) there was an increased prevalence of deficiency in patients with SLE compared to a control population with fibromyalgia (409). A much larger study by Broder et. al. (2010) found increased 25(OH)D in patients with RA compared to either SLE or type II diabetes (410).

Those studies which found a difference in 25(OH)D between HCs and SLE included groups from North and South America, Europe, North Africa, The Middle East and South East Asia. Only a single study, conducted in India found no difference between SLE patients and healthy controls. In this study, 50/129 (38.8%) of patients were being treated with corticosteroids, calcium and vitamin D. In this group the 25(OH)D was, perhaps unsurprisingly, higher than controls. Those not requiring treatment had similar levels of 25(OH)D to controls, and may have had less severe disease (as treatment was not required). Furthermore, in this study there was marked vitamin D deficiency amongst the control group (mean 13.4ng/ml, 94% had 25(OH)D<30ng/ml) which may have masked any difference between HCs and SE patients (411). Studies comparing the prevalence of vitamin D deficiency in SLE to a control group are summarised in table 1-6.
<table>
<thead>
<tr>
<th>Group</th>
<th>Location</th>
<th>Population</th>
<th>Method and Definition</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mandal et. al. (2014)</td>
<td>India</td>
<td>129 SLE patients 100 healthy controls</td>
<td>ELISA Deficiency &lt;10ng/ml Insufficiency &lt;30ng/ml</td>
<td>Higher 25(OH)D in SLE patients on replacement therapy, no different between untreated and HCs (exact values not given). Low 25(OH)D associated with increased disease activity, ds-DNA, IFNα.</td>
</tr>
<tr>
<td>Emerah et. al. (2013)</td>
<td>Egypt</td>
<td>107 SLE patients 129 healthy controls</td>
<td>ELISA Deficiency &lt;20ng/ml Insufficiency &lt;30ng/ml</td>
<td>Lower in SLE compared to HCs (27.4±15.4)nmol/l vs. 38.1±15.9, p&lt;0.001. Low 25(OH)D associated with disease activity, ds-DNA, leukopenia.</td>
</tr>
<tr>
<td>Abou-Raya et. al. (2013)</td>
<td>Egypt</td>
<td>267 SLE patients 175 controls (age, gender and BMI-matched)</td>
<td>Immunoassay Deficient &lt;10ng/ml Insufficient &lt;30ng/ml</td>
<td>Lower in SLE compared to HC (mean 19.8ng/ml vs. 28.7ng/ml, standard deviation not given).</td>
</tr>
<tr>
<td>Fragoso et. al. (2012)</td>
<td>Brazil</td>
<td>78 SLE patients 64 healthy controls</td>
<td>Direct chemiluminescent immunoassay Insufficiency and deficiency &lt;30ng/ml</td>
<td>Lower in SLE compared to controls (mean[range] 29.3[6.1-55.2]ng/ml vs. 33.1[15.9-63.8]ng/ml, p=0.041).</td>
</tr>
<tr>
<td>Bogaczewicz et. al. (2012)</td>
<td>Poland</td>
<td>49 SLE patients 49 age/sex-matched controls</td>
<td>Direct chemiluminescent immunoassay Deficiency &lt;20ng/ml Insufficiency 21-29ng/ml</td>
<td>Lower in SLE compared to controls in summer months (18.4±9.1)ng/ml vs. 31.3±12.7, p=0.0005). Trend towards lower 25(OH)D in winter.</td>
</tr>
<tr>
<td>Stockton et. al. (2012)</td>
<td>Australia</td>
<td>26 SLE patients 21 healthy controls Low disease activity (mean SLEDAI 4.3)</td>
<td>Direct chemiluminescent immunoassay</td>
<td>No difference in mean 25(OH)D between SLE and controls (73.9±27.9)nmol/l vs. 62.6±13.1)nmol/l.</td>
</tr>
<tr>
<td>Ritterhouse et. al. (2011)</td>
<td>USA</td>
<td>32 SLE patients 32 matched controls</td>
<td>ELISA Deficiency ≤20ng/ml</td>
<td>Lower in SLE compared to HC (median[IQR] 17.3 [11.9, 21.2]ng/ml vs. 29.4 [19.0, 36.3]ng/ml, p=0.003) but also low in ANA-positive HC (17.4 [14.5, 25.8]ng/ml). No association with disease activity.</td>
</tr>
<tr>
<td>Hamza et. al. (2011)</td>
<td>Egypt</td>
<td>60 SLE patients 60 healthy controls</td>
<td>ELISA Deficiency &lt;10ng/ml</td>
<td>Lower in SLE compared to HC (26.3 [12.1]ng/ml vs. 42.7 [9.2]ng/ml, p&lt;0.001).</td>
</tr>
<tr>
<td>Reference</td>
<td>Location</td>
<td>Study Design</td>
<td>Method</td>
<td>25(OH)D Levels</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>----------</td>
<td>--------------</td>
<td>-------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Broder et al. (2010) (410)</td>
<td>USA</td>
<td>123 patients with SLE, 100 with RA, 3,691 with type 2 DM.</td>
<td>Direct chemiluminescent immunoassay</td>
<td>Median (IQR) 25OHD levels significantly higher in the RA group, 20.9 (16.1, 29.9)ng/ml, compared with SLE 18.0 (11.3, 25.4) ng/ml, and T2DM group, 16.8 (11.1, 24.2)ng/ml (p &lt; 0.0001).</td>
</tr>
<tr>
<td>Kim et al. (2010) (418)</td>
<td>Korea</td>
<td>104 patients with SLE 49 controls</td>
<td>Radioimmunoassay</td>
<td>Lower 25(OH)D in SLE (42.5 [±15.1]ng/ml vs. 52.72 [±15.19]ng/ml, P&lt;0.001). No association with SLEDAI score but low 25(OH)D associated with low C3</td>
</tr>
<tr>
<td>Damanhouri et al. (2009) (419)</td>
<td>Saudi Arabia</td>
<td>165 SLE patients (148 female) 214 controls</td>
<td>Direct ELISA</td>
<td>Mean 25(OH)D lower in SLE (23.5 [±13.7]nmol/l vs. 64.5 [± 23.9], p&lt;0.001) Insufficient = 98.8% vs 55% Deficient = 89.7% vs 20%</td>
</tr>
<tr>
<td>Borba et al. (2009) (420)</td>
<td>Brazil</td>
<td>36 female patients with SLE 12 with “high” activity (mean SLEDAI = 22) 24 with “low” activity (mean SLEDAI = 1.7) 26 controls 50% Caucasian</td>
<td>Radioimmunoassay</td>
<td>Lower 25(OH)D in active group (17.4 [±12.5]ng/ml) vs. inactive (44.6 ± 14.5ng/ml) and controls (37.8 [±13.7]ng/ml) p&lt;0.001</td>
</tr>
<tr>
<td>Orbach et al. (2007) (421)</td>
<td>Israel</td>
<td>138 patients with SLE Controls from parallel study</td>
<td>Two-site immunoluminometric assay</td>
<td>Mean 25(OH)D 11.9 (±11.1)ng/ml vs. 21.6 ng/ml in parallel study. Standard deviation not given for controls. No correlation with disease activity (ECLAM)</td>
</tr>
<tr>
<td>Kamen et al. (2006) (422)</td>
<td>USA</td>
<td>123 patients with SLE 240 controls</td>
<td>Method not reported</td>
<td>Lower 25(OH)D in cases compared to control (21.6 [±12.9]ng/ml vs. 27.4 [±15.7]ng/ml). Significant in Caucasians but not African Americans.</td>
</tr>
<tr>
<td>Muller et. al. (1995)</td>
<td>fibromyalgia</td>
<td>Description</td>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>21 patients with SLE (29 patients with RA, 12 patients with OA) 72 healthy controls</td>
<td>Radioimmunoassay</td>
<td>Median [IQR] 25(OH)D in SLE was lower than controls or OA; 13(10-27) ng/ml vs. 27(18-32) ng/ml vs. 32(21-34) ng/ml respectively (p=0.0008, p=0.0168)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1-6: Summary of studies comparing serum vitamin D levels in SLE with a control group**

This table summarizes studies of patients with SLE in which the 25(OH)D was measured and compared to that in a control group. 25(OH)D is presented as mean (sd) unless otherwise stated.

RA = Rheumatoid Arthritis, ECLAM = European Consensus Lupus Activity Measurement, SLEDAI = Systemic Lupus Erythematosus Disease Activity Index, DM = Diabetes Mellitus, HC = healthy control, ELISA = enzyme-linked immunosorbent assay, IFN = interferon
1.6.1 Factors associated with vitamin D deficiency in SLE

1.8.1.1 Ethnicity
As described above, SLE is recognised to be more prevalent (and more severe) in patients of Afro-Caribbean or South Asian origin (423). The majority of the studies reporting vitamin D deficiency in SLE have focussed on defined populations, within discrete geographical areas and of a single ethnic origin. Only the study by Kamen et. al. (2006) which enrolled 138 (38%) African-American subjects has demonstrated clear ethnic differences in serum 25(OH)D within the lupus population (422). Other studies have included an 80% Caucasian group (424), and a 39% African-American/33% Hispanic group (410), both of which have failed to demonstrate any significant variation.

1.8.1.2 Photosensitivity
Photosensitivity is a common feature of SLE and can result in the development of rashes or in a systemic flare of the disease (425). It is therefore unsurprising, given the importance of UV light in vitamin D synthesis, that sunlight avoidance or use of sunscreen increases the prevalence of vitamin D deficiency (426-429) and can attenuate seasonal variation in 25(OH)D levels (430). Reports of seasonal variation in 25(OH)D amongst patients with generalised SLE are often inconsistent but in many cases the season in which the sample was obtained is not reported. In those studies which describe seasonal variation, vitamin D shows a trend towards being lowest in the winter months (422) but this is only statistically significant in some reports (410;424;431). When compared to patients with allergic skin diseases or healthy controls, patients with cutaneous lupus have an increased prevalence of vitamin D deficiency in both the summer and winter months (432). Interestingly, a small open-label study by Cutillas-Marco et. al. (2014) found that treatment of vitamin D deficient patients with cutaneous lupus resulted in a significant improvement in skin disease after 1 year (433).

1.8.1.3 Renal disease
Renal involvement in SLE is common and has a prevalence of approximately 12-14% in Caucasian SLE patients (434;435). The kidneys are the source of circulating active vitamin D metabolites and as such the presence of lupus renal disease (as inferred by elevated serum creatinine) is associated with lower serum 1,25(OH)_2D (424). Intriguingly, however, markers of renal disease (reduced glomerular filtration rate, increased serum creatinine, proteinuria) are also associated with lower 25(OH)D (410;422;424;436). This observation does not have a clear explanation as reduction of CYP27B1 activity as occurs in renal disease would be expected to inhibit 25(OH)D metabolism and therefore possibly increase serum 25(OH)D.
1.8.1.4 Corticosteroid and immunosuppressant therapy
Therapeutic interventions have an impact upon vitamin D metabolism. In the treatment of SLE, both corticosteroids and anti-malarial therapy (predominantly hydroxychloroquine) have potential consequences for the vitamin D endocrine system.

Corticosteroids are an important and frequently prescribed treatment for active SLE. The effect of exogenous corticosteroids upon vitamin D metabolism remains controversial. Klein et. al. (1977) demonstrated that patients receiving high doses of prednisolone (15-100mg/day) had impaired intestinal calcium absorption, secondary to reduced serum 25(OH)D (437). This finding has not, however, been demonstrated by other groups (438). Animal studies have suggested that in the context of steroid use, reduced calcium transport occurs due to the increased metabolism of 1,25(OH)D to an inactive metabolite, rather than impaired activation of 25(OH)D (439). This reflects the observation that in children, treatment with glucocorticoids reduces serum 1,25(OH)D (440) and recently, steroids have been shown to increase transcription of vitamin D 24-hydroxylase (441).

In SLE the association between steroid treatment and serum 25(OH)D remains controversial. Of those studies reporting cumulative or mean steroid dose, only Toloza et. al (2010) show an association (424) whilst other groups do not (418;442). Interpreting the effect of steroid dose in isolation of disease activity is difficult as patients with more active disease are more likely to be receiving higher steroid doses. The studies discussed above have too few subjects to demonstrate conclusively an independent effect of glucocorticoid therapy.

The use of hydroxychloroquine is well established in the management of hypercalcaemic sarcoidosis. In this setting, hypercalcaemia is a consequence of increased serum 1,25(OH)D, generated by the unregulated expression of 1-α-hydroxylase within activated macrophages. Hydroxychloroquine (and chloroquine) inhibits the conversion of 25(OH)D to 1,25(OH)D leading to reduced calcium absorption and improvement in hypercalcaemia, whilst having no effect on serum 25(OH)D (443). Indeed in patients with SLE, hydroxychloroquine treatment has no effect on serum 25(OH)D (418) but may result in lower serum 1,25(OH)D (409). Similar to the studies reporting associations between vitamin D and glucocorticoids, these findings have not been universally replicated (424).

1.8.1.5 Anti-calcitriol antibodies
In a study of 171 patients with SLE, anti-1,25(OH)D antibodies were identified in 4% patients (444). Binding of 1,25(OH)D to antibody may result in increased conversion of 25(OH)D to its active metabolite, and hence reduce serum 25(OH)D. Anti-vitamin D antibodies are, however,
present in relatively low numbers in SLE and are unlikely to have a significant role in reducing serum 25(OH)D given the marked prevalence of vitamin D deficiency.

### 1.8.2 Vitamin D and disease activity in SLE

#### 1.8.2.1 Vitamin D and immunomodulation

Vitamin D has broad immunomodulatory effects on both the innate and adaptive immune systems which may be relevant to disease activity and progression in SLE. As describe above, a hallmark of SLE is the production of autoantibodies due to B cell hyperactivity and the failure of removal of autoreactive cells. Lupus PBMCs spontaneously produce immunoglobulin ex vivo. The addition of 1,25(OH)$_2$D$_3$ has been shown to reduce both polyclonal IgG and anti-dsDNA antibody production by PMBCs derived from SLE patients (445). This effect has also been demonstrated in SLE patients treated with high-dose (100,000IU weekly for 4 weeks) cholecalciferol. In the study by Terrier et. al. (2012) patients had reduced ds-DNA titres and fewer memory (IgD-CD27) B cells (446).

Dendritic cells (DCs) have an important role in the activation of T cells. Both DCs and monocytes express 1α-hydroxylase and the VDR (447). Calcitriol inhibits the differentiation of monocytes to DCs and the maturation of dendritic cells in response to LPS or TLR agonists in vitro. This is associated with reduced expression of MHC class II and co-stimulatory molecules (CD80, CD86) and subsequent reduced T cell activation (448). A similar effect of vitamin D has been demonstrated in monocyte-derived dendritic cells (MDDCs) from patients with SLE (449). Furthermore, expression of ISGs by these MDDCs in response to lupus serum is attenuated by 1,25(OH)$_2$D.

B cells express 1α-hydroxylase, 24-hydroxylase and the VDR. Exposure to 1,25(OH)$_2$D$_3$ in vitro results in inhibition of B cell proliferation, induction of apoptosis and reduced differentiation into plasma cells (450). All of these processes could potentially counter the B cell hyperactivity seen in SLE. Interestingly Linker-Israeli et. al. (2001) also found increased numbers of T, B and natural killer (NK) cells expressing the VDR in lupus patients compared to controls. This suggests that in SLE the immune system may have increased sensitivity to vitamin D and its analogues.

T lymphocytes are also sensitive to vitamin D and vitamin D therapy results in an increase in naïve T cells, an increase in regulatory T cells and a reduction in Th1 and Th17 cells in a time-dependent manner (446). Expression of the VDR is seen in CD4$^+$ T cells and is increased by
around 5-fold when T cells are activated in vitro (451). Vitamin D can both inhibit T cell proliferation and favour polarization of Th1 cells towards the Th2 phenotype. This switch is associated with increased production of IL-4, IL-5 and IL-10 and may restore the abnormal Th1/Th2 balance observed in SLE (452).

1.8.2.2 Animal models of lupus
Two studies have investigated the effects of vitamin D in murine models of lupus. In the study by Lemire et al. (1992), intraperitoneal (IP) 1,25(OH)2D3 reduced proteinuria and skin lesions in the MRL/lpr model. Furthermore there was a trend towards reduced levels of anti-ssDNA antibodies (453). This model also develops severe lymphadenopathy occurring from around 12 weeks of age. Administration of vitamin D had no effect on lymphoid hyperplasia. The NZB/W F1 model develops a lupus phenotype at a much later age than the MRL/lpr model. In a study by Vaisberg et al. (2000) IP cholecalciferol at a low dose (3µg) but not high dose (10µg) resulted in worsening renal disease in female mice (but not male mice) (454).

1.8.2.3 Vitamin D and clinical disease activity in SLE
Vitamin D deficiency has been associated with both clinical disease activity and serological markers of disease in SLE patients. The largest interventional study was carried out in the Hopkins’ Lupus Cohort and included 1,006 patients. Lupus patients with 25(OH)D <40ng/ml were treated with 50,000 IU ergocalciferol weekly and 400IU cholecalciferol daily in an open-label design. In this study, an increase in 25(OH)D was associated with a small decrease in disease activity. A 20ng/ml increase in 25(OH)D was associated with a change in SLEDAI of -0.22 (-0.41, -0.02) (p=0.032). Interestingly, this association was only significant in those with 25(OH)D <40ng/ml after treatment (455). The relationship between vitamin D levels and disease activity remains unclear. Whilst some studies have found an association between 25(OH)D and disease activity (379;411;431;456-458) but others have not (416;418;421;459;460). Similarly, some studies have found an association between low 25(OH)D and low serum complement (418;458;461), and/or increased ds-DNA titres (379;411;431;456-458). In the Hopkins’ cohort described above, there was a complex relationship between 25(OH)D and complement. At baseline, higher 25(OH) was associated with lower C3 and C4 complement although after receiving vitamin D, both C3 and C4 levels increased (455).

There is some evidence that 25(OH)D may modulate flare in non-African American lupus patients. In the Ohio SLE study, 25(OH)D levels were lower during flares compared to the months prior to flare and there was a trend towards more flare during low-daylight months (462). It is currently less clear whether vitamin D therapy can reduce disease activity in SLE.
patients. A randomised, double-blind placebo controlled multicentre trial of 57 vitamin D deficient SLE patients examined the effect of a lower dose (2000IU/day) or higher dose (4000IU/day) on the expression of the IFN-signature. At 12 weeks there was no difference in the expression of the IFN-signature between the groups. However it should be noted that only 11/18 patients in the higher dose group, and 5/15 patients in the lower dose group, achieved serum 25(OH)D>30ng/ml (463). In contrast, a randomised, double-blind placebo controlled trial of 267 lupus patients receiving 2000 IU/day cholecalciferol for 12 months of therapy found a significant reduction in SLEDAI score both in patients who were vitamin D deficient, and vitamin D insufficient at baseline (4.9 [3.5] to 3.0 [2.5], p=0.01 and 4.9[3.6] to 3.2 [2.8], p=0.05 respectively). No change in disease activity was seen in the placebo group. Additionally after 12 months patients receiving vitamin D had significantly lower autoantibodies (anti-dsDNA, anti-Sm), lower pro-inflammatory cytokines (IL-1, IL-6, IL-18, TNF-α) and increased C4 complement (412).

There is currently no clear evidence that vitamin D can modulate the development or progression of SLE. An observational study of patients with undifferentiated connective tissue disease (UCTD) found significantly lower serum vitamin D levels than controls (33.0 ± 13.4ng/ml vs. 39.9 ± 11.7ng/ml, p=0.01) (427). Interestingly, the 35/161 (21.7%) of these patients who developed a formal connective tissue disease during the follow-up period had much lower vitamin D than those who retained an undifferentiated phenotype (14.7ng/ml vs. 33.0 ng/ml, p=0.0001).

Some groups report that vitamin D may act as a negative acute phase reactant although this has only been found in some studies. A significant reduction in serum 25(OH)D has been reported following elective orthopaedic surgery (464), but not following MI (465) or during infection with malaria (466). The mechanism for this change in serum 25(OH)D is not understood although Waldron et. al. (2013) report a fall in serum DBP which may be responsible, at least in part, for the fall in 25(OH)D (464).

1.8.3 Vitamin D and CVD in SLE
Early suggestions that vitamin D may be associated with CVD in SLE came from a study by Ramsey-Goldman and Manzi (2001). In this pilot study, low bone mineral density (which could be due in part to low vitamin D) was associated with carotid plaque and coronary artery calcium (467). In a larger cross-sectional study of 181 female lupus patients 25(OH)D was significantly associated with presence of diabetes mellitus, LDL cholesterol, serum fibrinogen
and SLEDAI score after adjustment for age, season and ethnicity. In a more comprehensive regression model (adjusting for age, season, BMI, SLEDAI and SDI) this association was no longer seen (468). In this study there was no association between 25(OH)D and subclinical CVD in terms of cIMT, CAC or plaque. In support of these observations, Mok et al. (2012) also found an association between low 25(OH)D and cardiovascular risk factors in patients with SLE. When compared to patients with 25(OH)D>15ng/ml, deficient patients had significantly higher LDL cholesterol and TGs and lower HDL (456). In addition there was a significant association between 25(OH)D and disease activity (SLEDAI score) after adjustment for age, gender, disease duration, sun exposure and drug exposure. Similarly to Wu et al. there was no clear association between 25(OH)D and atherosclerosis.

In contrast to the above, some studies in SLE have found an association between 25(OH)D and subclinical CVD in patients with lupus. A small cross-sectional study of 51 African American lupus patients found that those with carotid plaque had lower 25(OH)D and longer disease duration (no difference in age) but also lower SLEDAI scores and anti-dsDNA titres (469). The lower SLEDAI scores and antibody titres may reflect differences in immunosuppressant therapy between the groups although these are not reported. Previously we have demonstrated an association between 25(OH)D and aPWV (but not cIMT or carotid plaque) in female lupus patients with low disease activity. Patients with 25(OH)D ≤ 20ng/ml had significantly increased aPWV (8.2 [4.7, 11.6] m/s vs. 5.4 [3.3, 8.9] m/s, p=0.03). In regression models, 25(OH)D remained significantly associated with aPWV after adjustment for traditional cardiovascular risk factors. This association no longer remained significant after adjustment for disease activity (470). This suggests that the association between 25(OH)D and increased arterial stiffness is mediated through disease activity. However, this cross sectional study did not address whether a change in serum 25(OH)D is able to modify either vascular function or disease activity.

A prospective study of patients enrolled in the Hopkins Lupus Cohort did not find any association between baseline 25(OH)D (measured at a single time point) and progression of CAC or cIMT over 2 years. At baseline, however, there was a trend towards increased CAC scores in those patients who were deficient (<21ng/ml) compared to replete (≥32ng/ml) (11% vs. 3%). Lower baseline 25(OH)D was also associated with high-sensitivity CRP (hsCRP) (1.33mg/l vs. 0.75mg/l) but did not predict changes in hsCRP over time (471). The largest prospective study in SLE (890 patients) was recently reported by the SLICC group. At baseline, patients in the highest quartile of 25(OH)D were significantly less likely to have hyperlipidaemia and hypertension and also had lower disease activity. Over time, there was a...
trend towards fewer cardiovascular events in patients with higher vitamin D levels but this was not statistically significant (472).

1.9 Summary
SLE is associated with an increased risk of cardiovascular disease. Endothelial dysfunction is also increased in the lupus population and represents the first stage in the development of atherosclerotic disease. Both traditional and lupus-specific risk factors contribute to the accelerated CVD. In SLE, the generation of neutrophil extracellular traps and type 1 interferon signalling appear to be important, although it is not clear how IFNα may directly cause vascular damage. There is good evidence that IFNα impairs endothelial repair mechanisms in SLE patients which may contribute to vascular damage and endothelial dysfunction. Two populations of cells have been identified as important in endothelial repair. Of these, myeloid angiogenic cells (MACs) are particularly sensitive to IFNα. These myeloid cells may therefore be a novel target to improve vascular repair in lupus patients.

Vitamin D deficiency is common in SLE and associated with both active disease and subclinical CVD. In the general population, vitamin D deficiency is an independent risk factor for CVD. Furthermore, high dose treatment with vitamin D improved endothelial function in some studies, and there appears to be a modest effect on SLE disease activity. Given that myeloid cells are known express the VDR and response to vitamin D, there is rational to investigate the effects of vitamin D on MAC function.

This thesis will therefore describe the development of in vitro models of endothelial function and repair, the effect of vitamin D on these models and finally the effects of high dose vitamin D on endothelial function in patients with SLE.
Chapter 2: Hypothesis and aims
2 Hypothesis and aims

2.1 Hypothesis
Patients with SLE have significant endothelial dysfunction which leads to an increased risk of clinical cardiovascular disease. This dysfunction is likely due, at least in part, to failure of endothelial repair mechanisms. This thesis will investigate whether vitamin D can target endothelial repair in this patient group and result in changes in endothelial function. The two hypotheses for this work are:

1) Vitamin D can target myeloid angiogenic cells (MACs) and thus improve the potential for endothelial repair in patients with SLE.

2) In vitamin D deficient SLE patients, high-dose vitamin D therapy has a beneficial effect on endothelial function.

2.2 Aims
The aims of the study with respect to the first hypothesis are to:

1) Develop suitable *in vitro* models of endothelial cell function and repair using cells from patients with SLE and to reproduce the lupus phenotype *in vitro* using cells from healthy subjects cultured in the presence of interferon.

2) Determine the effects of vitamin D on these models *in vitro* with specific focus on MAC function.

The aim of the study with respect to the second hypothesis is to:

1) Determine the effects of vitamin D therapy on non-invasive measures of endothelial function, vascular stiffness and endothelial damage in vitamin D deficient patients with SLE compared to a control group of vitamin D replete patients.
Chapter 3: Methods

This chapter will describe the observational study and the laboratory methods used.
3 Methods

3.1 Study setting and funding
The research was undertaken in the Arthritis Research UK Centre for Epidemiology in the Institute of Inflammation and Repair, and in the Institute of Cardiovascular Sciences. The sponsor for this study was The University of Manchester. The research was funded by a one-year Manchester Biomedical Research Centre (BRC) Fellowship and a three-year North West England MRC Clinical Pharmacology and Therapeutics Clinical Research Training Fellowship.

3.2 Ethical approval
The study was approved by the North West 1 Research Ethics Committee (11/NW/0008). Further approvals were obtained from the Research and Development Department at Central Manchester University Hospitals NHS Foundation Trust (CMFT) and from the Scientific Advisory Board at the NIHR/Wellcome Trust Manchester Clinical Research Facility (MCRF).

3.3 Study design
This was an experimental medicine study to investigate the effect of vitamin D on cardiovascular function and disease activity in patients with SLE. This involved an observational cohort study utilising non-invasive markers of vascular function and detailed studies of endothelial repair mechanisms ex vivo.

Patients were recruited from a single centre (CMFT). Eligible patients were given a patient information sheet at their routine clinic appointment and then contacted 2-7 days later to arrange the screening visit. Participant Identification Centres (PICs) were also established during the course of the study at three peripheral sites; Salford Royal NHS Foundation Trust, Pennine Acute NHS Trust and East Lancashire NHS Trust, although no participants were recruited from these sites during the study period. Patients were screened for eligibility on the CMFT site and the main study visits were carried out at the WTCRF. All patients provided informed written consent.

At screening, vitamin D deficient (<20ng/ml) SLE patients were recruited into the treatment group and vitamin D replete (>30ng/ml) patients recruited into the control group. Deficient patients were assessed at baseline (before treatment) and after 3 months. The control group underwent identical assessments and measurements 3 months apart. This time point reflects...
that used by others to study the effects of vitamin D on flow-mediated dilatation in healthy subjects (339). The study design is shown in figure 3-1.

**Figure 3-1: Design of the observational study**

Patients with stable SLE were stratified on the basis of their 25(OH)D status at screening into deficient (<20ng/ml) and sufficient (>30ng/ml). Both groups underwent an assessment of vascular function and disease activity at baseline and 12 weeks later. Those patients with 25(OH)D deficiency received cholecalciferol from their general practitioner in accordance with local clinical guidelines.

### 3.4 Subject recruitment

Female patients aged 18-70 with a physician diagnosis of SLE who fulfilled ≥4 modified 1997 ACR criteria for the Classification of SLE were enrolled into the study (34;35). Furthermore all patients needed to have stable SLE disease defined as no planned changes in therapy (except small changes in oral steroid dose). All patients needed to be able to provide informed written consent.

The exclusion criteria were:

- Pregnancy or planned pregnancy in the next 6 months
- Chronic kidney disease (CKD) of stage III or greater
- Treatment with anti-epileptic therapy
- Hypercalcaemia, primary hyperparathyroidism or inherited disorders of calcium or vitamin D metabolism.
- Planned initiation of a HMG-CoA reductase inhibitor during the course of the study
A small number of healthy female control subjects were recruited opportunistically from the University of Manchester. All healthy subjects were screened for vitamin D deficiency and those with 25(OH)D <20ng/ml at screening were excluded from the remainder of the study.

3.5 The screening visit
The primary purpose of the screening visit was to obtain written consent and to determine the participants' serum 25(OH)D level. A clotted blood sample was taken and 25(OH)D was measured by liquid chromatography-mass spectrometry in the Specialist Assay laboratory at CMFT. In addition to measuring 25(OH)D, lupus disease activity was recorded and aortic pulse-wave velocity (aPWV) measured using the TensioMed Arteriograph™ as described below. Table 3-1 summarises the data collected at the screening visit.

<table>
<thead>
<tr>
<th>Data Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic demographic details (age, gender)</td>
</tr>
<tr>
<td>Disease activity assessments (SLEDAI-2K, BILAG)</td>
</tr>
<tr>
<td>Pulse-wave velocity (TensioMed Arteriograph™)</td>
</tr>
<tr>
<td>Serum 25(OH)D</td>
</tr>
<tr>
<td>Current vitamin D supplement use</td>
</tr>
</tbody>
</table>

Table 3-1: Summary of data collected during the screening visit

Subjects were stratified on the basis of the serum 25(OH)D into: <20ng/ml (treatment group) and >30ng/ml (control group). SLE patients with 25(OH)D 20-30ng/ml were excluded from the longitudinal study. The General Practitioner of all deficient subjects (SLE patients or healthy controls) was informed of the 25(OH)D level and, as per our usual practice, they were asked to prescribe vitamin D replacement therapy in accordance with CMFT guidance. These guidelines state that patients with 25(OH)D <10ng/ml should receive replacement therapy with 40,000IU cholecalciferol daily for 10 days (or 300,000IU as a single dose). After 1 month, patients should then receive maintenance therapy of 20,000IU per week. Patients with 25(OH)D 10-20ng/ml are recommended to receive the maintenance dose only (i.e. 20,000IU per week). The decision to treat and the actual protocol used was left to the discretion of the GP and patient.

Patients were allowed to be considered for screening if they were already prescribed vitamin D supplements. These were defined as low dose (the equivalent of Calcichew D3 Forte twice daily i.e. <25,000 IU per month) or high dose (≥25,000 IU per month).
3.6 Assessment visits
All assessment visits were undertaken at the MCRF. At the first visit a comprehensive medical history was taken to include details of previous SLE features, drug history, menstrual history and a comprehensive cardiovascular history. Specific attention was given to cardiovascular risk factors including; previous cardiovascular events, family history, smoking status and medication use. In addition, patients were asked about risk factors for vitamin D deficiency (sunlight avoidance, photosensitivity and malabsorption syndromes). A full clinical examination was not undertaken unless to verify/exclude features of ongoing disease activity or SLE-associated damage as required by the disease activity and damage indices (see below). Anthropomorphic measurements (height, weight, hip circumference) were undertaken by a trained nurse. Dipstick urinalysis was also undertaken.

As part of the clinical assessment the following blood tests were conducted by the clinical pathology services at CMFT.

- Full blood count
- Renal profile
- Serum calcium and albumin (to calculate corrected calcium)
- Erythrocyte sedimentation rate (ESR)
- Fasting glucose
- Lipid profile (total cholesterol, LDL, HDL, triglycerides)
- Immunology profile (ANA, ds-DNA, serum complement)
- Anti-cardiolipin antibodies (IgM and IgG)

3.6.1 Measurement of vascular function
3.6.1.1 General considerations
All assessments at the MCRF were conducted in a temperature-controlled room maintained at 20-24°C. Participants were fasted from midnight and allowed water only. Subjects were also asked not to consume alcohol for 48 hours prior to the visit and to avoid caffeine or smoking on the morning of the visit. All measurements were made between 9am and 11am. Patients were also asked not to take any medication (to include vasoactive medication) on the morning of the visit. Measurements were made with the participant in a supine position (head raised to approximately 10°C) following a period of rest as described below.
3.6.1.2 **Aortic pulse-wave velocity (aPWV)**

The TensioMed Arteriograph™ provides an automated measurement of aortic pulse-wave velocity. This device has been validated against measures of aPWV obtained invasively during cardiac catheterisation. In this study the Pearson correlation coefficient between the Arteriograph™ and invasive measurements was \( r = 0.91 \) (\( p < 0.0001 \)) (473). A blood pressure cuff inflated to 35mmHg above systolic pressure is used to transmit the oscillations in pressure from the underlying brachial artery. This is therefore an indirect measurement of the pressure changes within the vessel (474). The cuff detects the time period between, and the amplitude of, the first wave and the reflected wave. The time between the start of the first and reflected wave is related to the jugulum-symphysis distance (JSD). In addition, the Arteriograph provides measurement of the brachial augmentation index (and an estimation of the aortic augmentation index) by calculating the difference between the pressures of the 2 waves in relation to pulse pressure. A summary of the measurements obtained is shown in figure 3-1 (adapted from Baulmann et al. 2008 (474))

![Diagram of aortic pulse-wave velocity](image)

**Figure 3-2: Principles of measurement of pulse-wave velocity and brachial augmentation index**

The Arteriograph™ provides automated calculation of the aPWV based on the time difference between primary and reflated waves (top panel) and the difference in amplitude between the 2 waves (bottom panel).

Arteriograph™ measurements were made by JR at the screening visit and by trained nursing staff at the MCRF according to the manufacturer’s instructions. Subjects were placed in a supine position and allowed to rest for 10 minutes. The measurement of the jugulum-symphysis distance (JSD) is then performed by placing the cuff at the level of the brachial artery and recording the time delay between the first and reflected waves.
symphysis distance (JSD) was undertaken once only at the screening visit and then used for each subsequent aPWV measurement. The JSD was measured using custom-made callipers to measure the direct longitudinal distance between the 2 points (jugulum/sternal notch and symphysis pubis) and thus remove errors due to body habitus. A blood pressure cuff was placed in the standard position (overlying the brachial artery) of the dominant arm. The Arteriograph then automatically performs the measurement algorithm over a period of 2-3 minutes.

3.6.1.3 Flow-mediated dilatation (FMD)
Assessment of flow-mediated dilatation was undertaken by a single operator (JR). Guidelines have been published in an attempt to standardise FMD measurement between studies and to reduce measurement error (85;475). Subjects were positioned with the right arm abducted to 90° onto a table and a blood pressure cuff was placed around the centre of the forearm. Subjects were then allowed to rest for 10 minutes. The brachial artery was identified 5-10cm above the antecubital fossa using a 17-5mHz probe held at approximately 60° to the skin. All FMD measurements were made using a Philips iU-22 ultrasound machine. The probe was fixed in position using a custom-made stereotactic probe holder, to minimise probe movement, and the depth and contrast of the image adjusted to provide a clear border between vessel lumen and wall. The pulse-trace was simultaneously visualised on the screen to ensure a that change in flow occurred at cuff deflation. All images were recorded as a loop of 15 seconds duration to ensure that the file size did not exceed the maximum capacity of the US machine. A baseline recording of 30 seconds was made. The cuff was then inflated to 80mmHg above systolic blood pressure or to 200mmHg whichever was the greater. The cuff remained inflated for 5 minutes. Recording of the deflation sequence was started 5 seconds prior to cuff deflation and continued for a total of 2 minutes (8x15 seconds). After a further 5 minutes rest, a repeat baseline measurement was made to allow for minor movement in the probe position. A glyceryl trinitrate (GTN) tablet (300 micrograms) was administered sublingually and after a period of 1 minute, a 2 minute recording was made (8x15 seconds).

Image analysis was undertaken “off-line” by importing loop recordings into the Vascular Research Tools:Brachial Analyzer for Research v.5.10.9 software (Medical Imaging Applications, USA). The region of interest (ROI) was identified based on a) the position of maximum clarity between vessel and lumen and b) the observation of pulsatile changes in artery diameter. The software determines the arterial diameter for each frame of the sequence. A quality control measure was adopted such that any frame where the reported confidence in accuracy of the measurement was <70% was dropped (as per the manufacturer’s
recommendation). The same ROI was used for both the baseline and deflation sequences. Where the position of the artery changed following deflation, a new ROI was identified for the pre- and post-GTN sequences. The arithmetic mean of the diameter was calculated for the baseline sequences. For the deflation and GTN sequences the 15 second sequences were joined together and a cubic spline fitted (using STATA SE v.11.2). The maximal diameter for each sequence was then determined using this derived line. Figure 3-3 shows the measurement of artery diameter using Brachial Analyzer software.

Figure 3-3: Analysis of flow-mediated dilatation
Recorded video loops of the brachial artery were imported into Brachial Analyzer for Research software. Automated edge-tracking (purple) was used to measure the diameter in each frame (a). The read-out provides detailed diameter recording and a suitable position for the ROI is determined by the presence of a pulsatile trace (b). Representative deflation sequence with cubic spline fitted (red). The %FMD is based on the % change in diameter following cuff deflation (c). *baseline diameter shown for illustration only; the diameter was obtained from separate video sequences.

FMD and GTN-mediated dilatation were calculated according formula:

\[
\text{% FMD (or GTN-mediated) = \left( \frac{\text{mean peak diameter} - \text{mean baseline diameter}}{\text{mean baseline diameter}} \right) \times 100}
\]
Given that the health and function of the smooth muscle layer within the vessel has an influence on dilatation, the ratio of endothelium-dependent:endothelium-independent dilatation was also calculated using the following formula:

\[
\text{ED:ID} = \left( \frac{\% \text{FMD}}{\% \text{GTN-mediated dilatation}} \right) \times 100
\]

### 3.6.1.4 Circulating endothelial microparticles (EMPs)

Endothelial microparticles (EMPs) are small circulating fragments of endothelial cell membrane released from activated and apoptotic cells (476). The number of EMPs has been show to correlate with both endothelial dysfunction and disease activity in SLE (110). EMPs were quantified from whole blood as using a method validated by Parker et. al. (110;477).

Whole blood was collected in a citrate tube (4ml total) and processed within 1 hour of collection. The sample was centrifuged at 1700 x g for 10 minutes at 4°C and the plasma removed. Plasma samples were centrifuged at 20,000 x g for a further 10 minutes at 4°C. The platelet-poor plasma (PPP) was carefully removed from the platelet pellet and stored at -80°C for analysis in batches.

EMPs were labelled with 3 fluorescent markers with minimal overlap on the fluorescent spectrum: Annexin-V (eFluor 450-conjugated, eBioscience, USA), anti-CD31 (PE-conjugated, BD Bioscience, UK) and anti-CD42b (APC-conjugated BD Bioscience, UK) (table 3-2). PPP samples were thawed on ice and 50μl added to 900μl Annexin V buffer (eBioscience, USA) and 50μl of 10μm diameter Flow-Count™ Fluorospheres (Beckman Coulter, UK) of known concentration. To each sample 10μl Annexin V was added and 5μl each of anti-CD31 and anti-CD42b antibodies. Samples were well mixed and incubated on ice for 10 minutes in the dark prior to analysis. The process of EMP isolation is shown in figure 3-4.
Figure 3-4: Isolation of endothelial microparticles from whole blood
Schematic representation of the isolation of EMPs. Platelet-poor plasma (PPP) is derived from whole blood. EMPs are fluorescently labelled with anti CD31 and anti CD42b antibodies and Annexin V. Samples are then combined with counting beads and quantified using flow cytometry.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Conjugate</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin-V</td>
<td>eFluor 450</td>
<td>405</td>
<td>450/50</td>
</tr>
<tr>
<td>CD31</td>
<td>Phycoerythrin (PE)</td>
<td>488</td>
<td>575/25</td>
</tr>
<tr>
<td>CD42b</td>
<td>Allophycocyanin (APC)</td>
<td>633</td>
<td>665/20</td>
</tr>
</tbody>
</table>

Table 3-2: Fluorescent markers used in detection of EMPs

All samples were run on a Cyan ADP flow cytometer (Beckman Coulter, UK) by an experienced technician in the Faculty of Life Sciences. The threshold was set on the forward scatter at 0.05%. This allowed maximum detection of small particles with minimal background. The sample was run until 1000 events had been counted in the bead region. Numbers of EMPs were calculated using Summit v4.3 (DAKO Inc, USA). EMPs were defined as CD31+/AnnexinV+/CD42b- events. After exclusion of the bead region, samples were gated to only include CD42b negative events. Within this population, CD31+/AnnexinV+ events were then identified (figure 3-5).
Figure 3-5: Identification of EMPs by flow cytometry

Distribution of events by log forward and log side scatter shows a spread of events with a clear bead population in the boxed region (R1) (a). Events were gated to include only CD42b− events (APC labelled antibody) (b). Plotting AnnV− events against CD31− events reveals a discrete population of EMPs (circled) (c). Re-gating plot (a) to include only the EMPs from plot (c) shows that all of the events are similar in size, and smaller that the counting beads (d).

The number of EMPs/ml plasma was calculated using the following formulae:

1) Concentration of beads in sample = (volume of beads used [50μl] x stock bead concentration beads)/total sample volume [1020μl]
2) Volume of sample analysed = number beads counted / concentration of beads in sample
3) Number EMPs per ml = (Number events in gate / volume of sample analysed) x 1000

3.6.2 Assessment of Lupus Disease Activity and Damage

All Lupus patients underwent a clinical assessment of both SLE disease activity and lupus-related damage. To measure damage the SLE Disease Activity Index 2000 (SLEDAI-2K) (39) and the updated British Isles Lupus Assessment Group Disease Activity Index (BILAG-2004) (40) were used. Whilst SLEDAI-2K gives a numerical score indicating total lupus disease activity, BILAG scores 9 individual organ systems from A-E, with A=high activity, B=moderate activity, C=mild activity and D/E=no activity. The global BILAG score was also calculated as per Yee et.
al. (2010) (478). This weights scores (A=12, B=8, C=1, D/E=0) and provides a total numerical score across the 9 organ systems. The Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SLICC/ACR-DI) was used to determine cumulative damage. Quality of Life was determined using the Short Form 36 (SF-36) (42), LupusQoL (43) and Fatigue Severity Scale (FSS) (479). The Digit Symbol Substitution Test (DSST) was used to provide a rapid assessment of cognitive function (adapted from (480)).

The data collected at the assessment visits (at baseline and at 3 months) are summarised in table 3-3:

<table>
<thead>
<tr>
<th>Data Collected</th>
<th>Brief Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical blood parameters</td>
<td>Including 25(OH)D, PTH, immunology profile, fasting lipids and glucose</td>
</tr>
<tr>
<td>Vitamin D deficiency risk factors</td>
<td>Sunlight avoidance, photosensitivity, malabsorption syndrome</td>
</tr>
<tr>
<td>Vascular stiffness</td>
<td>aPWV (TensioMed Arteriograph™)</td>
</tr>
<tr>
<td>Endothelial function</td>
<td>FMD</td>
</tr>
<tr>
<td>Endothelial damage</td>
<td>EMPs</td>
</tr>
<tr>
<td>Disease activity</td>
<td>SLEDAI-2K, BILAG-2004</td>
</tr>
<tr>
<td>Lupus-related damage</td>
<td>SLICC/ACR-DI</td>
</tr>
<tr>
<td>Quality of life and cognitive function</td>
<td>SF-36, LupusQoL, FSS, DSST</td>
</tr>
</tbody>
</table>

Table 3-3: Summary of data collected at the assessment visits

3.7 Statistical analysis of the observational study

3.7.1 Primary outcome
The primary outcome for the study was the change in endothelial function as measured by FMD after vitamin D therapy.

3.7.2 Secondary outcomes
The pre-defined secondary outcomes were:

- Correlation between change in vitamin D and change in FMD
- Changes in vascular stiffness, blood pressure, lipid profile and markers of vascular damage
- Changes in clinical and laboratory measurements of disease activity
In this experimental medicine study the effects of vitamin D on endothelial repair mechanisms \textit{ex vivo} were also investigated.

Power calculations were conducted to identify the number of vitamin D deficient SLE patients to be recruited. As no studies have yet investigated the effects of vitamin D on endothelial function in SLE, effect sizes have been estimated from studies in type II diabetes mellitus and healthy subjects.

The primary power calculation was based on the reported association of $r=0.45$ between serum 25(OH)D and FMD (339). In patients with type II diabetes, a single dose of 100,000IU cholecalciferol increased 25(OH)D by 6.12ng/ml at 8 weeks. Corretti \textit{et. al.} (2002) proposed that the minimum statistically significant difference in FMD that can be obtained in intervention trials is 1.5-2% (475). In the studies by Sugden \textit{et. al.} (2008) and Tarcin \textit{et. al.} (2009) the improvement in FMD was 2.35% and 3.4% respectively (339;368) after vitamin D supplementation. Therefore, assuming a minimum increase in serum 25(OH)D of 6.12ng/ml and $r=0.45$ between 25(OH)D and FMD then 42 patients would be needed to detect a 2% absolute change in FMD with 80% power.

3.8 Study of endothelial and myeloid angiogenic cell function

Details of the laboratory materials used are given in Appendix 1. The cell culture media used and recipes for buffers and other solutions are described in Appendix 2.

3.1.1 Culture of human endothelial cells
All cell culture was performed under sterile conditions in a class II laminar flow safety cabinet. Incubation was carried out in a humidified atmosphere at 37°C and 5% CO$_2$ unless otherwise stated.

3.1.1.1 Maintenance of endothelial cell culture
Human aortic endothelial cells (HAoEC) (Promocell, Germany) were cultured in Endothelial Cell Growth Medium MV2 (EGM-MV2, Promocell, Germany) supplemented with 5% (v/v) foetal bovine serum (FBS, Sigma-Aldrich, UK), 100 U/ml penicillin and 100μg/ml streptomycin (Sigma-Aldrich, UK) and EGM-MV2 supplement pack comprising of; epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), ascorbic acid and hydrocortisone. Media was removed and replaced with fresh warm media every 48 hours unless stated otherwise.
Human venous endothelial cells (HUVEC) (Promocell, Germany) were cultured in Endothelial Cell Growth Medium (ECGM, Promocell, Germany) supplemented with 5% (v/v) foetal bovine serum (FBS, Sigma-Aldrich, UK), 100 U/ml penicillin and 100μg/ml streptomycin (Sigma-Aldrich, UK) and EGM supplement pack comprising of; endothelial cells growth supplement (ECGS, bovine hypothalamic extract), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), heparin and hydrocortisone.

At 80-90% confluence cells were washed twice with warm Hank’s Balanced Salt Solution (HBSS) and then incubated with 100μl/cm² warmed 1% trypsin-EDTA (Sigma-Aldrich, UK) until detachment was observed. The trypsin was neutralised by the addition of an equal volume of ECGM/EGM-MV2 and the cells were centrifuged to a pellet at 220x g at 22°C for 3 minutes. The supernatant was decanted, cells were then re-suspended in warm media and counted using a Neubauer haemocytometer under an Olympus CKX41 microscope. Dead cells were excluded from enumeration by counting cells in 0.2% trypan blue (Life Technologies, UK). For propagation, cells were typically seeded into tissue culture flasks containing pre-warmed medium at a density of 20,000 cells/cm². All cells were used at passages 3-10.

3.1.1.2 Freezing/thawing of endothelial cells
Confluent cells were detached using 1% trypsin-EDTA as above and centrifuged at 220x g for 3 minutes. The supernatant was removed and cells re-suspended in room temperature cryo-SFM (Promocell, Germany). Cell vials were cooled at a rate of 1°C/minute in a Nalgene 5100 Cryo freezing container at -80°C. For longer term storage, cells were transferred to the vapour phase of liquid nitrogen. Frozen cells were rapidly warmed in 37°C in a water bath until almost defrosted and then immediately transferred into a tissue culture flask containing warm medium. Cells were allowed to adhere overnight. Media was replaced the following day to remove traces of the Cryo-SFM media and cells were cultured as described above.

3.1 Effect of IFN on gene expression in endothelial cells

3.1.1 Isolation of RNA
RNA was isolated from HAoECs to examine the effect of IFNα2b on gene expression. Total RNA was extracted using a modified phenol-chloroform method. After disruption of the cellular monolayer, RNA is separated from DNA and protein then precipitated from the aqueous solution using isopropanol. Guanidine thiocyanate denatures RNases and RNA-binding proteins. All reagents and plastic-ware used in the isolation of RNA were molecular grade and RNase-free.
HAoECs were grown to 90% confluence in T25 tissue culture flasks. After serum starvation overnight (1% FBS EGM-MV2) cells were stimulated with 5% FBS EGM-MV2 with 10ng/ml IFNα2b or vehicle control. After 6 hours, the media was removed, cells washed in PBS and lysed with 1ml TRReagent® (Ambion, UK) per 25cm². The solution was homogenised by pipetting and stored at -80°C for up to 6 weeks.

The frozen samples were thawed on ice and centrifuged at 12,000 x g at 4°C to remove debris. The samples were transferred to clean tubes and 100µl 1-bromo-3-chloropropane (BCP, Sigma-Aldrich UK) added. Samples were mixed thoroughly and incubated at room temperature for 3 minutes. The aqueous and organic phases were separated by centrifugation at 12,000 x g at 4°C for 15 minutes. The aqueous phase (approximately 500µl) was transferred to a clean tube. The RNA was precipitated by the addition of 250µl of isopropl alcohol (Sigma-Aldrich, UK) and 2µl Glycobluem™, (Ambion, UK), incubation at room temperature for 10 minutes and centrifugation at 12,000 x g at 4°C for 20 minutes. The blue RNA pellet was washed in 1ml 75% (v/v) ice-cold ethanol (Sigma-Aldrich, UK) and centrifuged for 12,000 x g at 4°C for 20 minutes. The ethanol was carefully removed and the pellet was air dried. Once all traces of ethanol had been removed, the pellet was re-suspended in 21.2µl Tris-EDTA (TE) buffer (Ambion, UK). The concentration of RNA was measured using a ND-1000 Nanodrop photospectrometer (ThermoScientific, UK).

3.1.2 DNase treatment
Genomic DNA (gDNA) may be amplified in PCR reactions resulting in a false signal. To reduce gDNA contamination, RNA samples were treated with DNase 1 enzyme (Ambion, UK) which hydrolysises single- and double-stranded DNA according to the manufacturer’s instructions. Up to 10µg RNA was treated with 5µl of 10x DNase 1 buffer and 2µl DNase 1 (both Ambion, UK) and diluted to 50µl total volume. The samples were incubated at 37°C for 30 minutes and transferred to ice. Samples were then diluted to 100µl total volume with RNase-free water (Sigma, UK). The RNA was extracted by adding 100µl of phenol:chloroform:1AA (25:24:1, Ambion, UK), mixing the samples to an emulsion and centrifuging at 12,000 x g for 5 minutes at 4°C. The aqueous layer (containing the RNA) was then carefully removed to a clean tube and the RNA precipitated by adding 10µl ammonium acetate (Ambion, UK), 330µl ethanol, 1µl Glycobluem™ and incubated for 30 minutes on ice. The tubes were centrifuged at 12,000 x g for 20 minutes at 4°C and the resulting RNA pellet was washed with 1ml 75% ethanol and
centrifuged again for 5 minutes at 12,000 x g at 4°C. The ethanol was removed and the pellet air-dried and re-suspended in TE buffer as above.

3.1.3 Reverse transcription
Complementary DNA (cDNA) was produced from RNA by reverse transcription using the Precision nanoScript™ Reverse transcription kit (PrimerDesign Ltd, UK) as per the manufacturer’s protocol. A total of 1µg of RNA was reverse transcribed in each reaction. For the annealing step, each sample was incubated with 1µl random nonamer primers and 1µl oligo-dT primers in a total volume of 10µl at 65°C for 5 minutes. Samples were then immediately placed on ice. To each tube, 2µl dithiothreitol (DTT) (100mM), 1µl dNTP mix (10mM), 2µl 10x buffer, 4µl water, and 1µl nanoScript enzyme was added. The extension step was then carried out at 25°C for 5 minutes, 55°C for 20 minutes and 75°C for 15 minutes. The cDNA was stored at -20°C until required.

3.1.4 Reverse-transcription PCR (RT-PCR)
Prior to undertaking the exon gene array, the expression of an IFN-sensitive gene (IFI44) was determined using conventional RT-PCR. Each 10µl reaction contained 50ng cDNA, 0.2µl dNTP, 0.2µl Taq DNA polymerase, 1µl 10x DNA polymerase buffer (all from Roche Diagnostics, UK) and the appropriate forward and reverse primers (40nM) (Eurofins MWG Operon, Germany). The primers used are given in table 3-4. PCR was carried out according to the following protocol: Initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 20 seconds, annealing for 40 seconds and extension at 72°C for 40 seconds. A final extension step at 7 minutes at 72°C was carried out before PCR products were separated on a 1% (w/v) agarose gel (in TBE buffer) containing 0.005% (v/v) ethidium bromide. PCR products were visualised under a UV light source.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: CCACCCATGGCAAATTCCATG</td>
<td>64°C</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCTAGACGGCAGGTCCAGGCCACC</td>
<td></td>
</tr>
<tr>
<td>Interferon-induced protein (IFI44)</td>
<td>Forward: TTGGAGGGAAGCGGCTTAGCCT</td>
<td>62°C</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGGACCCAGCAGCAGAAATTCGT</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-4: Primer sequences used in RT-PCR in HAoECs

3.1.5 Exon Gene Array
To investigate the effect of IFNα2b on gene expression in human endothelial cells a gene-level analysis was performed. HAoECs were treated with 10ng/ml IFNα2b or vehicle control in 2
individual experiments for 6 hours. The RNA was extracted as described above. The RNA integrity was measured using using an Agilent 2100 Bioanalyzer, running the RNA 6000 NanoAssay (Agilent Technologies, USA). A RNA Integrity Number (RIN) of >6 was considered suitable for gene array analysis. All samples used had an RIN of ≥9.90. Samples were run on an Affymetrix Human Gene 1.0 ST Array (version 2.0) (481). This array contains $1.4 \times 10^6$ probe sets with approximately 40 probe sets per gene. The data were normalised and expression analysis conducted using Affymetrix GCOS software, dChip and RMA (482). Components Analysis and t-tests, were performed using maxdView (available from http://bioinf.man.ac.uk/microarray/maxd/).

Differentially expressed genes were defined as those with a q-value ≤0.2 (Bonferroni correction to adjust for multiple testing) and a fold change ≥2. Gene ontology analysis was performed using the DAVID functional Annotation tool (483;484) and displayed using Cytoscape (v.2.8.3) (485) or using Ingenuity IPA (Ingenuity Systems Inc, USA). The normalised data files are published on the ArrayExpress website under the reference E-MEXP-3746 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-3746/).

### 3.2 Investigation of the effects of interferon-alpha on human endothelial cell function

#### 3.2.1 Proliferation: cell counting

HAoECs were seeded onto 9mm glass coverslips (Scientific Laboratory Supplies, UK) in the bottom of 24 well culture plates at a density of $2 \times 10^4$ cells/well. Cells were allowed to adhere overnight and then serum starved (EGM-MV2 without FBS) for 4 hours. At time=0 hours the media was replaced with EGM-MV2 5%FBS ± 10ng/ml interferon alpha 2b (IFNα, Promokine, Germany). At each time point (0, 24, 48 and 72 hours) the coverslips were removed, washed in PBS+ (containing calcium/magnesium) and fixed with 5% (w/v) trichloroacetic acid (TCA) for 30 minutes. The coverslips were then washed twice in PBS and stored wet at 4°C until staining. Cell nuclei were stained using filtered Mayer’s Haemalum (Sigma-Aldrich, UK) for 5 minutes at room temperature and then washed in running tap water. The coverslips were inverted and mounted onto glass slides with Ultramount™ aqueous permanent mounting medium (DAKO UK Ltd, UK) and dried at 70°C for 20 minutes. Treatment groups were masked and images from 3 random fields per coverslip were obtained using a Leica DM5000B microscope and a DFC320 colour camera. The number of nuclei were manually counted using ImageJ (http://rsb.info.nih.gov/ij) software.
3.2.2 Proliferation: MTT assay

The Roche Cell Proliferation Kit 1 (Roche, UK) uses mitochondrial activity as a surrogate marker of cellular proliferation. The soluble yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl 96 tetrazolium bromide, MTT) is cleaved by mitochondrial succinate dehydrogenase into insoluble formazan crystals. These are then dissolved to produce a coloured solution, the intensity of which can be measured using an optical plate reader. The amount of formazan produced is proportional to the number of metabolically active cells.

HAoECs and HUVECs were seeded into a 96 well plate at 1x10^4 cells/cm^2 in a total volume of 100µl of medium and allowed to adhere overnight. Cells were treated with IFNα2b (0.1-100ng/ml) or vehicle (sterile water) in triplicate. Positive and negative controls contained 10ng/ml vascular endothelial growth factor-a (VEGF-A, Promokine, Germany) or 10µM of the nitric oxide scavenger carboxy-PTIO (Sigma-Aldrich, UK) respectively. At each time point (0, 24, 48 and 72 hours) 10µl MTT (final concentration 0.5mg/ml) was added and the cells incubated for 4 hours. To each well 100µl of solubilisation solution (10% w/v SDS in 0.1M HCl) was added and the plate incubated overnight at 37°C. The absorbance (570nm, A_570) and background absorbance (750nm, A_750) were read using a BioTek ELx800 optical microplate reader running Gen5 software (BioTek Instruments, USA). Values were calculated by subtracting background absorbance from the readings (A_570-A_750).

3.2.3 Nitric oxide production

3.2.3.1 Rationale for measurement of NO

The generation of NO is an important function of endothelial cells. Reduced NO bioavailability is associated with dysregulation of vascular tone, increased smooth muscle cell proliferation, leucocyte adhesion to the endothelium and platelet aggregation (486). Nitric oxide can be measured using fluorescent probes which react directly with NO (487), electrochemical probes (488) or by the measurement of the principle metabolites of NO (nitrate and nitrite) (489). The Griess reaction measures the production of a violet-coloured azo dye which occurs when nitrite reacts with sulfanilic acid. The inclusion of nitrate reductase converts all nitrate in the sample into nitrite which can then be measured by the above reaction.

The production of nitric oxide (NO) was measured using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, USA) according to the manufacturer’s instructions. This kit utilises Griess reagents to convert nitrite into a purple azo compound which can be measured
using an optical plate reader. Nitrate reductase converts all available nitrate into nitrite, thus giving a measurements of total nitrate and nitrite.

HAoECs were seeded into 24 well plates (1x10^4 cells/cm^2) in EGM-MV2 (0.5ml/well) and grown to confluence. After treatment with IFNα2b (0.1-10ng/ml) for 6 and 24 hours, the conditioned media was stored at -20°C. A standard curve of nitrite (0-35µM) was prepared in EGM-MV2. Each sample (80µl) was measured in duplicate and incubated with nitrate reductase and enzyme cofactors at room temperature for 2 hours. Following this, 50µl of Griess reagents R1 and R2 were added. After 10 minutes the absorbance was read at 550nM. A standard curve was constructed and the concentration of total nitrate and nitrite measured using the following formula:

\[
[nitrate + nitrite] (\mu M) = \left( \frac{A_{540} - y - \text{intercept}}{\text{slope}} \right) \times \left( \frac{\text{total volume}}{\text{sample volume}} \right)
\]

The lower limit of detection of the assay is 2.5µM.

### 3.2.4 Tubule formation assays

#### 3.2.4.1 2D network formation

Endothelial cells have the capacity to form networks when cultured on complex matrices in vitro. This forms the basis of in vitro angiogenesis assays (490). The term “networks” is preferred as although the structures resemble capillary networks, these cords may not have a functional lumen (491). This is in contrast to in vivo angiogenesis assays in which true vessels form and haemoglobin can be identified within the lumen (492). Matrigel™ is a commonly used complex matrix and is obtained from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. The exact composition of Matrigel is not known but it contains structural proteins including collagen and laminin. Numerous growth factors which may interfere with angiogenesis assays are also found in Matrigel but their effect is attenuated by the use of a growth factor-reduced (GFR) matrix.

GFR Matrigel was thawed at 4°C on ice overnight and then 10µl placed in the lower chamber of µ-slide angiogenesis (IBIDI, Germany). The gel was allowed to polymerise at 37°C for 30 minutes. HAoEC and HUVEC (3.5x10^3 cells and 5x10^6 cells respectively) were added to the upper chamber in a total volume of 50µl of medium. IFNα2b (10ng/ml) or vehicle (with VEGF and PTIO positive and negative controls as above) was added to the cell suspension at this stage. The slides were incubated for 14 hours at 37°C in a humidified chamber. Tubules were visualised using an inverted phase-contrast microscope (Olympus CKX41) with 3 replicates for
each condition. The number of branch points (junctions) in 3 random fields/well was enumerated using ImageJ software.

3.2.4.2 3D network formation
Endothelial cells also form 3D tubule networks in a collagen matrix. This method, adapted from Abici et al. (2011) (493) was used to support the observations seen in the 2D matrix. Each well in a 96 well plate contained 53.8 µl of collagen matrix containing endothelial cells. This matrix comprised of a gel solution and a HAoEC suspension in a 5:1 ratio (with a final number 6x10^4 cells/well). The gel solution was made by mixing an alkaline cell media solution (solution 1) and an acidic type 1 collagen mixture (solution 2) on ice (see appendix 2). The gel mixture was polymerised at 37°C for 30 minutes before 200µl EGM-MV2 was added. Cultures were continued for 72 hours and the media changed every 24 hours. IFNα2b was added to either the media, or the gel mixture to give final concentrations of 10ng/ml or 50ng/ml respectively. Images were captured in 3 random fields per well as described above.

3.3 Isolation of myeloid angiogenic cells

Human peripheral blood (50mls) was obtained from healthy control subjects and SLE patients using standard venepuncture. Samples were collected into 5 x 10ml tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent blood coagulation. Myeloid angiogenic cells (MACs) were isolated using previously described protocols (494-496) with some modifications.

Whole blood was diluted 1:1 with warm phosphate buffered saline (PBS) without calcium or magnesium (Sigma-Aldrich, UK) and then 25ml was carefully layered onto 20ml Ficoll-paque PLUS (GE Healthcare Life Sciences, UK) in 50ml tubes. Samples were then centrifuged at 740 x g at 18°C for 40 minutes. The superficial layer was carefully removed and peripheral blood mononuclear cells (PBMCs) were removed from the buffy coat layer. Cells were washed in warm PBS and centrifuged at 510 x g for 10 minutes. The cell pellet was re-suspended into 4ml of warm EGM (Lonza, Switzerland). The number of viable cells were counted (with trypan blue exclusion as above) and plated at a density of 1x10^6 cells/cm^2 into plates coated with 1µg/cm^2 human fibronectin (R&D Systems, UK). To ensure adherence, those cells plated into glass chamber slides (BD Bios) were coated with 1µg/cm^2 fibronectin in 0.1% bovine gelatin solution in PBS. The day on which cells were plated was considered day 1.
After 4 days in culture (day 5) cells were washed with warm PBS and fresh media replaced. The cell culture media used for MACs was EGM supplemented with 20% (v/v) FBS, 100 U/ml penicillin and 100μg/ml streptomycin and the EGM BulletKit (Lonza) comprising of; Bovine brain extract (BBE) with heparin, hydrocortisone, epidermal growth factor (EGF), ascorbic acid and gentamicin and amphotericin B (GA-1000) as previously described (223). The volume of media was maintained at 0.5ml/10x6 cells. All cells were used after 7 days of culture (day 8) as recommended (406).

3.4 Phenotypic characterisation of MACs

3.4.1 Confirmation of MAC phenotype
Cellular uptake of 1,1'-dioctadecyl-3,3',3',3'-tetramethyl-indocarbocyanine perchlorate (DiI)-labelled acetylated low-density lipoprotein (DiI-Ac-LDL, Biomedical Technologies, USA) and binding of fluorescein isothiocyanate (FITC)- conjugated Ulex euopaeus agglutinin 1 (UEA-1, Vector Laboratories, UK) was used to confirm the presence of a MAC phenotype as described (495) with minor modifications. MACs at day 8 were incubated with dil-Ac-LDL (diluted 1:80) in EGM for 3 hours at 5% CO2 at 37°C. Cells were fixed by replacing 50% of the media with 4% (w/v) paraformaldehyde (PFA, Sigma-Aldrich UK) for 20 minutes at room temperature. This was then removed and fresh 4% PFA added for a further 20 minutes. Cells were then washed twice in PBS and incubated with UEA-1 (1:50) in PBS for 1 hour in the dark. MACs were washed a further 2 times in PBS, then mounted using VECTASHIELD® with 4’, 6-diamidino-2-phenylindole (DAPI) counterstain (Vector Laboratoies, UK) and glass coverslips. Slides were stored at 4°C and visualised using a Leica DM5000B microscope with Leica DFC320 camera and the Leica Application Suite software (version 3.7, Leica, UK).

3.4.2 Phagocytosis by MACs
The phagocytosis and clearance of cellular debris is an essential function of macrophages (497). The phagocytic capacity of MACs was determined using a Phagocytosis Assay Kit (FITC IgG) according to the manufacturer’s instructions (Cayman Chemicals, USA). Briefly, MACs at day 8 were incubated for 24 hours with a 1:10 solution of latex beads-rabbit IgG-FITC complex in EGM. The media was then replaced and cells visualised using a Leica AS MDW inverted fluorescent microscope (Leica, UK) with a CoolSNap HQ CCD camera (Photometrics, UK). The bright field and fluorescent images were merged using ImageJ software (NIH, USA).
3.4.3 Immunocytochemistry
PBMCs obtained as described above were plated at 1x10^6 cells/cm^2 into 8 well chamber slides (Thermo Scientific) coated with fibronectin-gelatin. Prior to immunostaining, cells were fixed in 4% PFA as described above. The fixed cells were washed twice in PBS and then permeabilised with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 5 minutes. Non-specific antigens were blocked using a goat blocking solution containing 10% (v/v) normal goat serum (Vector Laboratories, UK) and 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, UK) in PBS for 1 hour. The blocking solution was removed and cells were incubated for 1 hour with the primary antibodies diluted in goat blocking solution (see table 3-5). Negative controls comprised either omission of primary antibody or normal mouse immunoglobulin (IgG1, DAKO UK Ltd, UK) at the same concentration as the primary antibody. Cells were washed for 3 x 5 minutes in PBS and then incubated with the appropriate secondary antibody for 45 minutes: Alexa Fluor® 488-conjugated goat anti-rabbit (Molecular Probes, Invitrogen, UK) or Dy-Light™ 488-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, USA) both diluted 1:200 in goat blocking solution ± 1μg/ml Phalloidin–Tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC, Sigma-Aldrich, UK) to stain F-actin. Slides were washed again in PBS x 3 and mounted in VECTASHIELD® with DAPI. Fluorescent images were obtained as above.

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Product Code</th>
<th>Source</th>
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<td>DAKO UK Ltd</td>
</tr>
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<tr>
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<td>Mouse</td>
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<td>Sc-13133</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

Table 3-5: Antibodies used in the immunocytochemical analysis of MACs

3.4.4 Investigation of surface marker expression by RT-PCR
The expression of cell surface markers in MACs was determined using conventional two-step RT-PCR. RNA was isolated from MACs by adding 1ml TRI-Reagent to 3 wells (total area approximately 6cm^2), and from 5x10^6 PBMCs immediately after density separation. The RNA was extracted and reverse transcribed to cDNA as described above. PCR was carried out with a denaturing step (95°C for 1 minute), 30 cycles of; 95°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds, and 72°C for 7 minutes. All primers were
obtained from Eurofins MWG Operon (Germany) and are listed in Table 3-6. PCR products were resolved on a 1.5% agarose gel as described above.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Actin</td>
<td>Forward: GACCTGACTGACTACCTCATGA)&lt;br&gt;Reverse : AGCATTTTCGGTGGACGATGGAG</td>
<td>59°C</td>
</tr>
<tr>
<td>CD31</td>
<td>Forward: CAACAGACATGGAACAAGG  &lt;br&gt;Reverse : TCCTGGATGTGAAGTTGGGC</td>
<td>53°C</td>
</tr>
<tr>
<td>CD45</td>
<td>Forward: ACAGCCAGACCTTTTCTAC  &lt;br&gt;Reverse : GTGACAGTGAAGDGAGCACGA</td>
<td>54°C</td>
</tr>
<tr>
<td>CD163</td>
<td>Forward: TTGCCAGCAGCTTAATGTG  &lt;br&gt;Reverse : AGGACAGTGTTGGGACTGG</td>
<td>55°C</td>
</tr>
<tr>
<td>vWF</td>
<td>Forward: ACATCAGCCAGGCTGCAAGTA  &lt;br&gt;Reverse : CACAGAGCAGAAGCATGCAAG</td>
<td>56°C</td>
</tr>
<tr>
<td>CD68</td>
<td>Forward: CAACTGGTGACAGACAGCTTA  &lt;br&gt;Reverse : GTGTTTGTGGCTCTTGGT</td>
<td>57°C</td>
</tr>
<tr>
<td>CD14</td>
<td>Forward: CGGCGGTGACACCTAGAG  &lt;br&gt;Reverse : GCCTACCAGTAGCTGACAG</td>
<td>57°C</td>
</tr>
<tr>
<td>CD206</td>
<td>Forward: GGCGGTGACCTCACAAGTAT  &lt;br&gt;Reverse : GTACCAGTCCCTGCCTTCA</td>
<td>57°C</td>
</tr>
<tr>
<td>CD83</td>
<td>Forward: GGATGAGAGGGTGCTATCCA  &lt;br&gt;Reverse : CTTCGTGAAAGTCCCTTCTGC</td>
<td>59°C</td>
</tr>
</tbody>
</table>

Table 3-6: Primer sequences for markers of endothelial cells and macrophages

3.4.5 Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)
Cell surface marker expression was investigated further using reverse transcription quantitative real time PCR (RT-qPCR) using SYBR green 1 fluorescent dye. This technique utilises the DNA-binding properties of SYBR green which has minimal fluorescence in the presence of single-stranded DNA. As the PCR progresses, SYBR green intercalates with the double-stranded DNA and fluoresces. This fluorescence is measured relative to a passive reference dye (ROX). The threshold cycle (Ct) value is the cycle number at which the fluorescence passes the set threshold. The Ct value is therefore inversely related to the amount of template present in the initial sample and the difference between Ct values is 2-fold. The principle of RT-qPCR using SYBR green is shown in figure 3-6:
Figure 3-6: Schematic diagram to show the principle of RT-qPCR using the SYBR green detection method

During the annealing step SYBR green intercalates with the double stranded DNA. As DNA synthesis continues during the extension steps, further dye is incorporated. The fluorescence is measured each cycle and accumulates as the reaction progresses. The amount of fluorescence is therefore proportional to the amount of newly synthesised DNA. The threshold cycle (Ct) value gives the number of cycles after which the fluorescent signal passes a pre-determined threshold.

RNA was extracted from MACS and 1μg reverse transcribed to cDNA as described above. cDNA was diluted to 5ng/μl with RNAse free water. Each 10μl PCR reaction was carried out in duplicate and contained: 2.5μl (12.5ng) cDNA, 0.5μl primer mix (PrimerDesign Ltd, UK), 2μl water and 5μl Precision™ Mastermix with low ROX (PrimerDesign Ltd, UK). Primers were all designed by PrimerDesign and pre-validated (table 3-7). The appropriate reference genes for endogenous controls were determined using the geNorm reference gene assay (PrimerDesign Ltd, UK). The primer sequences for the selected controls (ATP5B and CYC1) are not available. Samples were run on a 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The cycle conditions used were: 95°C for 10 minutes then 40 cycles of denaturation (95°C for 15 seconds), annealing (60°C for 60 seconds) and extension (60°C for 60 seconds). At the end of 40 cycles a melt-curve was produced using: 95°C for 15 seconds, 60°C for 1 minute then 1% ramp up to 95°C finishing at 60°C for 15 seconds.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
</table>
| CD14 | Forward: CCTAAGATCCAAGACAGAATAATGAA  
Reverse : TTTTAATAAAGGTGGGGAAAGG |
| CD68 | Forward: ACCACCACCTCAGGACAA |

Table 3-7: Primer Sequences for selected controls.
Reverse : GTGCTATTGGTTGGATGAA
CD86 Forward: TGGATAATGGGATGAATGGAAGGA
Reverse : CGTAGGACATCTGGAGCTAAG
CD206 Forward: TGGGTTCCCTCTGTAGGCTAAG
CCR7 Forward: AAGCCCTTTCTCTCCCTATC
Reverse : ATGGTTCTTGCCTTTGAAATA
ATP5B Sequence not available
CYC1 Sequence not available

Table 3-7: RT-pPCR primer sequences for macrophage markers

Gene expression was calculated as the fold expression of the gene of interest (GOI) relative to that of the endogenous control (ΔCt) or to the expression in the untreated control sample (ΔΔCt) according to the following formulae:

ΔCt: ratio = 2^{Ct (GOI) – Ct (Ref gene)}
ΔΔCt: ratio = 2^{ΔCt (sample of interest) – ΔCt (control sample)}

The Ct value for the reference genes was obtained by calculating the geometric mean of the Ct values for the 2 reference genes used.

3.5 Expression of the vitamin D receptor

The presence of the vitamin D receptor (VDR) in MACs was determined western blotting. All Western blots were carried out using the mini-PROTEAN electrophoresis tank and mini-Trans blot for wet transfer (Bio-Rad, UK).

3.5.1 Cell lysate preparation
MACs were isolated and cultured as described in 6-well culture plates at a density of 1x10^6 cells/cm^2. On days 2, 4 and 8, cells were lysed by washing twice in PBS then scraping cells into cold RIPA buffer (200μl/well) which had been supplemented with 1% (v/v) Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, UK). Samples were centrifuged at full speed (16,660x g) for 5 minutes to remove cell debris. Cell lysates were also obtained from 5x10^6
PBMCs, centrifuged at 500xg for 5 minutes to form a pellet and then re-suspended in 200µl cold RIPA buffer with sonication for 5 minutes.

The concentration of protein was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific, UK) according to the manufacturer’s instructions. The BCA method relies on the reduction of Cu$^{2+}$ to Cu$^{1+}$ by proteins in alkaline conditions. A purple colour is then produced which can be detected at 540-590nm. Pre-prepared albumin standard (BSA) was used to produce a standard curve of concentration 0-2000µg/ml. The working reagent (Reagent A: Reagent B at a ratio of 1:50) Samples were analysed in triplicate (10µl/well) by the addition of 200µl working reagent (50 parts Reagent A and 1 part Reagent B) and incubation at 37°C for 30 minutes. The plate was read using a BioTek ELx800 optical microplate reader at 570nm. The protein concentration was then extrapolated from the standard curve. Protein concentration was adjusted to 1.25mg/ml using dH$_2$O and then 5x Laemmli buffer added at a ratio of 4:1 (final protein concentration 1mg/ml). If the starting concentration was <1.25mg/ml, the Laemmli buffer was added directly and the volume of sample was adjusted on loading the gel to ensure even loading of protein. Prior to loading all samples were heated to 99°C for 5 minutes to denature the protein, cooled on ice and briefly centrifuged.

3.5.2 Gel electrophoresis and protein transfer

The protein samples were resolved on an 8% SDS-Polyacrylamide resolving gel adapted from Smith and Titheradge (1998) (498). The composition of the gel is shown in table 3-8. The ammonium persulphate (APS) initiates polymerisation whilst the TEMED (Tetramethylethlenediamine) acts as a polymerisation catalyst. The stacking gel solution was carefully pipetted into the mini-gel sandwich to a level approximately 1cm below the teeth of the comb. The gel was covered with a layer of water and allowed to polymerise. The water was removed and the stacking gel and comb added. The gel was placed in the tank and surrounded by running buffer comprising: 25mM Tris, 192mM glycine and 1% (w/v) SDS. A total of 20µg of each denatured samples were loaded into the wells along with 5µl of ColorPlus pre-stained protein ladder (New England Biolabs UK Limited).

<table>
<thead>
<tr>
<th>Resolving Gel (10%)</th>
<th>Stacking Gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ml 30% acrylamide solution</td>
<td>1ml 30% acrylamide solution</td>
</tr>
<tr>
<td>4.5ml 1.5M Tris (pH 8.7)</td>
<td>2ml 1M Tris-HCl (pH 6.8)</td>
</tr>
<tr>
<td>200µl 10% SDS (w/v)</td>
<td>83µl 10% SDS</td>
</tr>
<tr>
<td>5.3ml dH$_2$O</td>
<td>5ml dH$_2$O</td>
</tr>
</tbody>
</table>
Table 3-8: Composition of SDS-PAGE gels

<table>
<thead>
<tr>
<th></th>
<th>Host Species</th>
<th>Dilution</th>
<th>Diluent</th>
<th>Product Code</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDR</td>
<td>Mouse</td>
<td>1:200</td>
<td>1% milk in TBS-T</td>
<td>sc-13133</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rabbit</td>
<td>1:2500</td>
<td>0.1% milk in TBS-T</td>
<td>5174</td>
<td>Cell Signalling, USA</td>
</tr>
</tbody>
</table>

Samples were run at 50v until the samples reached the top of the resolving gel. The voltage was then increased to 120v for approximately 40 minutes.

The gel was carefully removed from the tank, rinsed in distilled water and the stacking gel removed. The gel was placed into the transfer cassette on top of 0.45µm nitrocellulose membrane (Bio-Rad, UK) and sandwiched between wet filter paper and sponges. The cassette was immersed in fresh transfer buffer (comprising 25mM Tris, 190mM glycine and 20% (v/v) methanol) in the blotting tank. Transfer of protein was carried out at 40v for 90 minutes with constant stirring. On completion, the membrane was rinsed in TBS-T (comprising 137mM NaCl, 2.7mM KCl, 19mM Tris Base, 0.1% Tween 20) and the protein visualised using Ponceau S solution (Sigma-Aldrick, UK). The Ponceau was completed removed by successive washing with TBS-T prior to proceeding with protein detection.

3.5.3 Detection of proteins

Blocking of the membrane was carried out in order to reduce non-specific binding of the primary antibody. The membrane was blocked using 5% (w/v) non-fat milk in TBS-T for 1 hour with constant agitation. The membrane was then washed for 3x10 minutes in TBS-T. To avoid the need for stripping the membrane, the membrane was cut to allow probing for both the protein of interest and the loading control protein. The antibodies used are described in table 3-9. The VDR was probed with 1:200 mouse monoclonal anti-VDR antibody (Santa Cruz, USA) in 1% (w/v) milk in TBS-T and GAPDH probed with 1:2500 rabbit polyclonal anti-GAPDH antibody (Cell Signalling, USA) in 0.1% (w/v) milk in TBS-T at 4°C overnight (table 3-9). GAPDH was selected as the loading control as both β-actin and α-tubulin had variable expression between PBMCs, MACS and HAoECs.
The next day the membrane was washed 3x10 minutes in TBST-T at room temperature to remove unbound primary antibody. Appropriate HRP-conjugated secondary antibodies were used to detect the primary antibody for 1 hour at room temperature. Unbound secondary antibody was removed by 3x10 minute washes in TBS-T. Chemiluminescence was used to detect the antibodies by 1 minute incubation with fresh reagent from the SuperSignal West PicoScript Kit (Thermo Scientific, USA). Exposure of KODAK X-OMAT AR (XAR-5) film (KODAK, UK) to the membrane was carried out in the dark and immediately developed. For quantification of band density, the films were scanned using standard image acquisition software and densitometry was then performed using ImageJ software.

### Table 3-9: Antibodies used in Western blotting

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Blocking</th>
<th>Catalogue Number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse HRP</td>
<td>Goat</td>
<td>1:10,000</td>
<td>0.1% gelatin in TBS-T</td>
<td>111-035-068</td>
<td>Jackson Immunoresearch, USA</td>
</tr>
<tr>
<td>Anti-rabbit HRP</td>
<td>Goat</td>
<td>1:10,000</td>
<td>0.1% gelatin in TBS-T</td>
<td>111-035-003</td>
<td>Jackson Immunoresearch, USA</td>
</tr>
</tbody>
</table>

3.6 Functional assessment of MACs

#### 3.6.1 Cell counting
MACs were isolated as above and cultured in 8-well chamber slides for 7 days with 1,25(OH)$_2$D$_3$ (Sigma-Aldrich, UK) or vehicle control. On day 8, MACs were labelled with dil-AL-LDL as above, fixed and mounted with fluorescent mounting medium. Three random fields were imaged per well in triplicate wells (9 images per experimental condition) using fluorescent microscopy (Leica DM5000B). The number of cells per field was counted using ImageJ software. Images were processed to convert the cells to a binary image, adjust for cells overlying each other, and then cells of sufficient size automatically enumerated.

#### 3.6.2 Migration assay
The migratory capacity of MACs was determined using a Transwell® migration assay. MACs at day 8 (cultured ± 10μM 1,25(OH)$_2$D$_3$) were incubated in basal EBM with 1% (w/v) bovine serum albumin for 3 hours. The cells were carefully washed twice in warm PBS and detached using enzyme-free cell dissociation buffer (Gibco, UK). Cells were centrifuged at 500x g for 5 minutes and then re-suspended in a small volume of EBM. Viable MACs were counted (with Trypan Blue exclusion) and diluted to a concentration of 1x10$^6$ cells/ml in basal EBM. The top
and bottom surface of each insert (8μm pore diameter) was coated with human fibronectin as described above. MACs (1x10^5 cells) were placed into the top chamber. The bottom chamber contained 600μl basal EBM ± 100ng/ml Stromal cell-derived factor-1-alpha (SDF-1α, Promokine, Germany) in duplicate. The plates were incubated at 37° and 5% CO₂ for 8 hours. The inserts were then removed, and washed in PBS. The upper surface of the membrane was scrubbed with a cotton-tip to remove cells which had not migrated. The migrated cells were then fixed in 100% ice-cold methanol overnight. The inserts were washed again in PBS and stained with Mayer’s Heamalum for 5 minutes and rinsed in tap water for a further 5 minutes. The polyester membrane was then carefully removed, and mounted onto glass microscope slides. The number of migrated cells was counted in 3 random fields per membrane using a Leica DM5000B microscope and DFC320 colour camera and ImageJ software. The cells were enumerated automatically using an ImageJ algorithm:

3.6.3 Cytokine production
The production of IL-6 and IP-10 by MACs was determined by “sandwich” Enzyme-Linked Immunosorbant Assay (ELISA). Each cytokine was measured independently using the IL-6 Human ELISA kit (Abcam, UK) and Human IP10 ELISA kit (Invitrogen, USA) respectively according to the manufacturer’s instructions. The principle of a sandwich ELISA is that plates are coated with a capture antibody which binds the antigen of interest in the sample (MAC supernatant). A biotinylated detection antibody is then added followed by a streptavidin-horseradish peroxidise complex. A chromogenic substrate is then added. Once sufficient colour has developed the reaction is terminated and the absorbance measured on a plate reader. The cytokine concentrations are determined by reading values from a standard curve of known concentration. In both cases absorbance was measured at 450nm using a BioTek ELx800 optical microplate reader running Gen5 software (BioTek Intruments, USA). The principles of the sandwich ELISA are show in figure 3-7.
Figure 3-7: Principle of the sandwich ELISA
The sandwich ELISA plate contains antibody bound to its surface. The antigen binds to this antibody and is then “sandwiched” by addition of a biotinylated antibody. The signal is amplified by the addition of an avidin-HRP complex which binds to the biotin. A coloured substrate then develops which is proportional to the amount of biotinylated antibody and compared to a standard curve.

3.6.4 Angiogenic factor production
The secretion of angiogenic factors by MACs was measured using a Bio-Plex Pro™ Human Angiogenesis 9-Plex Panel suspension array according to the manufacturer’s instructions. This assay utilises magnetic beads each of which has a unique fluorescent signature. Each bead is coated with antibodies to recognise a specific angiogenic factor. A tagged reporter is then added and the beads analysed using a Bio-Plex® 200 multiplex array reader. The combination of lasers gives information on both the quantity of reporter (and thus target antigen concentration) and the fluorescent signature of the bead (identifying which angiogenic factor is being measured). This allows the simultaneous measurement of 9 common growth factors within a single sample of MAC conditioned media. The assay was carried out in accordance with the manufacturer’s instructions. Briefly, samples were thawed on ice and standards diluted in EGM 20%. The diluted magnetic beads were added to each well of the assay plate, incubated and washed in assay buffer using vacuum filtration. To each well 50µl of standard, sample or EGM 20% control was added and the plate incubated with shaking for 1 hour at room temperature. After further washes, the plate was incubated with biotinylated detection
antibodies and finally the streptavidin-PE reporter. After washing the beads were re-suspended in assay buffer and the plate read using a target of 50 beads/well. The concentration of angiogenic factor was then extrapolated from the standard curve and reported in pg/ml.

3.7 Interaction of MACs and endothelial cells

3.7.1 Endothelial adhesion assay

The ability of MACs to adhere to endothelium was measured using an endothelial adhesion assay. HAoECs (2x10⁴/well) were cultured in 8-well chamber slides in EGM-MV2 5% FBS until confluent. HAoECs where then activated by the addition of 10ng/ml TNFα for 6 hours. MACs were labelled using Cell Tracker™ Green 5-Chloromethylfluorescein Diacetate (CMFDA) tracker (Molecular Probes, USA) (diluted to 10µM in serum-free EGM) for 30 minutes. The media was then replaced with EGM 20% FCS for a further 30 minutes. MACs were detached using enzyme-free dissociation buffer as described above and the cells centrifuged at 510x g for 5 minutes. The cell pellet was re-suspended in EGM and MACs counted by trypan blue exclusion. The EGM-MV2 was removed from the HAoECs and MACs (30,000/well in 500µl EGM) was added to the chamber slide for 1 hour in triplicate. Any non-adherent MACs were removed by washing the slides three times in cold PBS. The slides were then fixed in 100% ice-cold methanol for 20 minutes, rinsed with PBS and mounted in aqueous mounting medium with DAPI. Fluorescent images were acquired as above in 3 random fields/well and the number of attached green MACs was enumerated.

3.7.2 Tubule formation assay

An important function of MACs is the ability to augment angiogenesis in vitro (and in vivo). This angiogenic capacity was determined by measuring the effect of MAC supernatant on endothelial tubule formation. As described above, endothelial cells rapidly form tubule-like networks in complex matrices. Matrigel (10µl/well) was placed into well of µ-slide angiogenesis slides and allowed to polymerise for 30 minutes. The supernatant from healthy (±IFNα2b) and lupus (±1,25(OH)₂D₃) MACS was carefully removed and centrifuged at 500 x g for 5 minutes to remove cell debris. HAoECs were detached, centrifuged and re-suspended in EGM 20% FCS. The HAoECs were then counted and suspended in the MAC supernatant (the cell suspension accounted for <10% of the total volume to prevent dilution of the MAC supernatant) or EGM 20% as control. HAoECs were placed on Matrigel at 3,500 cells/well (5 replicates per condition) and incubated in a humidified environment for 14 hours. A single
image was taken of each well at x4 magnification using an inverted phase-contrast microscope (Olympus CKX41).

An algorithm was developed to allow the quantification of HAoEC tubule network parameters modified from that described by Guidolin et. al. 2010 (499). Images of the networks were imported into ImageJ software, changed into black and white images, and the network reduced to a single pixel thickness. The “Analyse Skeleton” plug-in for ImageJ was then used to quantify parameters of the network in terms of the number of branches, number of junctions and total pixel area (500). The number of closed loops/polygons was enumerated manually.

3.7.3 Expression of nitric oxide synthase
The effect of MAC supernatant on HAoEC eNOS expression was determined in the presence and absence of 10ng/ml TNFα. To allow for sufficient replicates, HAoECs were seeded into 24-well culture plates (2x10⁴ cells/well) and allowed to grow to confluence. Cells were treated with MAC conditioned media (500μl) or EGM 20% FCS alone ± 10ng/ml TNFα. RNA was extracted using the RNeasy Plus Micro Kit (Qiagen, Germany) according to the manufacturer’s instructions. This procedure uses spin columns to isolate RNA from a small number of cells. After cell lysis and homogenisation of the sample, genomic DNA is removed using a spin column. The sample is then mixed with ethanol which favours binding of mRNA (but not small RNA fragments e.g. tRNA) to the silica membrane of the column. After multiple washing steps, RNase-free water is used to elute the RNA from the column. The RNA was then quantified and reverse-transcribed as above.

Expression of eNOS was determined in triplicate samples using RT-qPCR as described above. The primer sequence is shown in table 3-10. The delta-delta Ct method was used to calculate fold changes in eNOS expression relative to the untreated sample.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>Forward: ACAAGAGTTATAAGATCCGCTTCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCTGCACTGTCTGTGTTACTG</td>
</tr>
<tr>
<td>ACTB (B-actin)</td>
<td>Primer sequence unavailable</td>
</tr>
<tr>
<td>ATP5B</td>
<td>Primer sequence unavailable</td>
</tr>
</tbody>
</table>

Table 3-9: Primer sequences for eNOS expression in HAoECs
The presence of eNOS was also determined using Western blotting. HAoECs were cultured in T25 tissue culture flasks until confluent. Cells were then treated with 3ml of MAC conditioned media or EGM 20% FCS ± 10ng/ml TNFα. After 6 hours the cells were washed and protein extracted as described above. Samples were resolved on a 7% SDS-PAGE gel and transferred to nitrocellulose membrane. After blocking of the membrane in 5% (w/v) milk in TBS-T (5% (w/v) BSA in TBS-T for eNOS) the membrane was probed using 1:1000 rabbit polyclonal anti-eNOS antibody (Cell Signalling, USA) in 5% BSA and 1:10000 mouse monoclonal anti-tubulin antibody (Cell Signalling, USA) in 5% milk. After overnight incubation and washing, both HRP-conjugated secondary antibodies were added at 1:10,000 dilution in 0.1% gelatin in TBS-T. Chemiluminescent detection was used as described above.

3.8 Contribution of the Candidate
The candidate conceived the project idea and design in conjunction with his supervisory team. The measurement of Arteriograph was performed by the candidate at the screening visit and at other study visits by trained nursing colleagues and supervised by the candidate. In addition, the candidate consented all participants into the study, performed all clinical assessments and measurement of FMD. Clinical biochemical, haematological and immunological parameters were measured in routine clinical pathology labs at CMFT. Measurement of 25(OH)D and cytokines in human sera was performed by Dr J Berry and Mr P Pemberton respectively. Flow cytometry was performed by Mr M Jackson in the Faculty of Life Sciences. The exon gene array was undertaken in the Bioinformatics Core Facility within the Faculty of Life Sciences. The candidate undertook the analysis of the normalised dataset. All laboratory work (cell culture, functional cell assays, and molecular biology techniques) was undertaken solely by the candidate. The candidate is also responsible for all data analysis, data presentation and interpretation of the results.
Chapter 4: Results

Modelling endothelial cell function in SLE: effect of IFN-alpha on human aortic endothelial cells \textit{in vitro}

\textit{Interferon-alpha has been implicated in the development of vascular damage in patients with SLE. This chapter will describe the effects of IFN\alpha on human endothelial cells in vitro.}
4 Modelling endothelial cell function in SLE: effect of IFN-alpha on human aortic endothelial cells in vitro

4.1 Introduction
Type 1 interferon has been implicated in the development of vascular damage and premature atherosclerosis in patients with SLE. Both an interferon signature and individual interferon sensitive genes (ISGs) have been associated with endothelial dysfunction and subclinical CVD in lupus patients (109;195). In patients with hepatitis C, the induction of endothelial dysfunction by IFNα2b points towards a direct effect of type 1 IFN on vascular function (194). This is supported by the observation that the endothelium in SLE patients expresses an interferon signature (197).

If IFNα were to directly damage endothelial cells in vitro, this would offer an attractive model to study the mechanisms of endothelial dysfunction in lupus and to screen novel vasculoprotective agents. To date only a small number of studies have investigated the effects of type I interferons on endothelial cells in culture. Only a single study has investigated the effects of IFNα on human arterial endothelial cells in vitro. Gomez et. al. (2003) found an increase in proliferation but did not investigate any other endothelial cell functions (501).

This chapter describes experiments conducted whilst trying to develop a model of endothelial cell dysfunction relevant to patients with SLE. Given the association between type I IFN and vascular damage in SLE, it was hypothesised that this may be mediated by IFNα. Therefore, the aim was to develop a relevant model of endothelial cell dysfunction in which to study the effects of vitamin D. The results contained within this chapter have been published in the Journal of Interferon and Cytokine Research (502).

4.2 Aims of this chapter
The specific aims of this chapter are to:

- Describe the effects of IFNα on human aortic endothelial cell function in vitro
- Identify whether an IFNα model might be suitable to test the effects of vitamin D

4.3 Methodological approach relevant to this chapter
In terms of investigating the effect of IFNα on endothelial cells two approaches were taken. Firstly, cellular assays were performed in order to identify the effects of IFNα on pre-defined cell functions. There are multiple functions of endothelial cells which can be modelled in vitro
with quantitative outputs. Whilst some of these serve as markers of endothelial health (e.g. proliferation, NO production), others are markers of endothelial damage (e.g. apoptosis, adhesion molecule expression) (figure 4-1).

Figure 4-1: Functions of endothelial cells suitable for development into in vitro models
Endothelial cells have many functions which can be measured using in vitro culture systems. Some of these, such as proliferation, can be readily quantified and thus developed into a reliable and reproducible assay. NO=nitric oxide, EMP=endothelial microparticle

In this chapter, the effect of IFNα on human aortic endothelial cell (HAoEC) function was studied using in vitro models of proliferation, network formation and NO bioavailability. Secondly, the effect of IFN on the HAoEC transcriptome was investigated. This hypothesis-generating approach was used to identify novel pathways by which endothelial cell function may be modified beyond the functional assays described above. In both functional and transcriptome experiments cells were used at passages 4-10 as recommended (503).

4.4 Effect of IFN on endothelial cell function

Prior to functional studies, the change in the expression of a single ISG, interferon-induced protein 44 (IFI44), was determined by RT-PCR to ensure that HAoECs were able to respond to IFNα2b in vitro. GAPDH was used as a reference gene to enable relative quantification by densitometry. Compared to either vehicle control or TNFα (10ng/ml), IFNα2b at concentrations of 0.1-100ng/ml resulted in up-regulation of IFI44 after 6 hours (figure 4-2). Little difference in IFI44 expression was seen between the concentrations, and no morphological changes in the cells were observed. A concentration of 10ng/ml was selected for further studies.
The expression of IFI44 in HAoECs is increased after 6 hours treatment with IFNa2b. The agarose gel showing increased expression of IFI44 in response to IFNa2b, and lower expression in untreated cells or cells treated with TNFα is shown in (a). A single PCR product was observed with a 527 base pair size corresponding to IFI44. The relative expression of IFI44 compared to the reference gene GAPDH shows an increase of around 3.5-fold in cells treated with IFNa. Densitometry was performed using ImageJ software and is shown in (b).

### 4.4.1 Proliferation

Endothelial cell proliferation in response to IFNa2b was initially assessed by counting the number of cell nuclei at 24, 48 and 72 hours in HAoECs cultured in the presence or absence of 10ng/ml IFNa2b. HAoECs showed a linear increase in number over 72 hours. No difference was seen in the number of cells at any of the time points (p=0.56, p=0.20, p=0.72 respectively) in n=3 independent experiments (figure 4-3).
Figure 4-3: Effect of IFNα on HAoEC proliferation measured by cell counting

The number of cells at defined time points was enumerated in n=3 random fields in triplicate. Representative images are shown in (a) from n=3 independent experiments. Cell nuclei were stained blue with haematoxylin. Magnification x20, scale bar=100µm. No difference was seen in the number of HAoECs between untreated and treated (10ng/ml IFNα2b) at any time point. The columns represent mean number of cells per random field and error bars show standard error. Means were compared by paired t tests (b).

In addition, proliferation was measured using an MTT assay in which mitochondrial activity acts as a surrogate marker for the number of viable cells. To provide further validation of the results obtained above, HUVEC cells were also used to investigate whether any effect was specific to HAoECs. In these experiments HAoECs from 2 donors were used and HUVECs were from pooled donors. Furthermore, VEGF-A and the NO scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (PTIO) were used to stimulate and inhibit cell proliferation respectively.

In both HAoECs and HUVECs, VEGF-A (20ng/ml) resulted in a modest but significant increase in the number of metabolically active cells at 24 hours (p=0.022 for both cell types) (figure 4-4). Similarly, PTIO (10µM) resulted in significantly fewer cells after 24 hours (p=0.002 for HAoEC and p=0.007 for HUVEC). In HAoEC, 10ng/ml IFNα2b had no effect on proliferation at 24, 48 or 72 hours (p=0.22, p=0.24, p=0.12 respectively) (figure 4-5). In contrast to HAoEC, in HUVEC there was a small but statistically significant increase in proliferation at 24 hours with IFNα2b (fold change 1.07, p=0.047). This effect persisted at 48 hours but was no longer statistically significant at this time point (fold change 1.173, p=0.13).
Figure 4-4: Effect of IFNα2b on HAoEC and HUVEC proliferation measured by MTT assay at 24 hours
The MTT assay was validated in both HAoEC and HUVEC using both a stimulator (VEGF-A, 20ng/ml), and an inhibitor (PTIO, 10µM), of endothelial proliferation. In both cell types, proliferation, measured as the difference in absorbance in treated compared to untreated cells, was significantly increased and reduced by VEGF-A and PTIO respectively after 24 hours. Columns show mean relative difference in proliferation (normalised to vehicle control=1) and the error bars show standard error. Comparisons made by paired t tests *p<0.05, **p<0.01. For HAoEC the columns show pooled results from n=8 and n=5 independent experiments for IFN and VEGF/PTIO respectively, in HUVEC n=3 independent experiments were conducted for all treatments.

Figure 4-5: Effect of IFNα2b on HAoEC and HUVEC proliferation over 72 Hours measured by MTT assay
The effect of IFNα2b on HAoEC and HUVEC proliferation was measured over 72 hours (a, c). There was no difference in proliferation with IFNα2b in HAoECs at any time point (a) but a small increase at 24 hours in HUVEC with a trend towards an increase at 48 hours (b). Columns show mean proliferation (normalised to vehicle control=1), and error bars show standard error, for combined results of triplicate wells in n=3 independent experiments. Comparisons were made by paired t tests *p<0.05, **p<0.01.
4.4.2 Endothelial cell network formation

The Matrigel model was used to determine the effects of IFNα2b on in vitro HAoEC tubule formation by plating endothelial cells on the matrix in the presence of IFNα2b or vehicle control (sterile water). The positive and negative regulators VEGF-A and PTIO were used as described for the proliferation assay above. Initial experiments were undertaken to identify the optimum density and time-point for tubule development (data not shown). IFNα2b had no effect on tubule formation in this model (mean number of junctions 23.1 vs. 23.5, p=0.371) whilst VEGF-A increased the number of junctions (28.8 vs. 23.5, p=0.026) and PTIO decreased the number (9.48 vs. 23.5, p=0.0012) (figure 4-6).

Figure 4-6: Effect of IFNα2b on HAoEC network formation in Matrigel
HAoECs formed networks when plated onto Matrigel which were clearly visible at 14 hours. The junctions (arrowheads) of these networks were quantified in random fields to determine the relative density of the network formed. No clear difference was seen between HAoECs incubated with 10ng/ml IFNα2b or vehicle control. Representative images are shown in panel (a). Magnification x10, Scale bar=500µm. IFNα2b did not affect HAoEC network density at 14 hours (p=0.371). The density was significantly impaired by the NO scavenger PTIO and increased by VEGF-A (b). The graph shows combined results for 3 random fields per well, in triplicate over n=6 independent experiments (n=3 for VEGF and PTIO). Columns show the mean and error bars show standard error. Comparisons made using paired t-tests between each condition and the control, *p<0.05, **p<0.01.

Similar to the proliferation studies above, HUVECs were used as an additional endothelial cell type to determine whether any effect was specific to aortic endothelial cells. No difference was seen in network density with HUVEC (mean [sd] number of junctions 20.0 [3.1] vs. 22.0 [0.9], p=0.339).

The testing of agents on more than 1 matrix is recommended as the mechanisms involved in network formation are partly dependent on the matrix used (504). Therefore, in order to
validate the results obtained above HAoECs were also allowed to form networks in a threedimensional collagen matrix. This methodology was based on that described by Aimes et al. (2003) (505) and is described in detail in Chapter 2. Quantification of the network density is more difficult as the network forms in 3 dimensions, and as such a comparison of overall morphology in the presence/absence of IFNa2b was made. No differences were apparent in the morphology of networks formed by HAoECs in a collagen matrix containing 50ng/ml IFNa2b and those without (figure 4-7).

![Image]

**Figure 4-7: HAoEC network formation in a 3D collagen matrix**

HAoECs form a complex network in collagen with clear branches and junctions. A phasecontrast image of the network in collagen reveals both HAoEC branches (arrowhead) and junctions (*) (a). No differences were seen between HAoECs in collagen gel without (i) and with IFNa2b (ii) (b). The final concentration of IFN within the gel was 50ng/ml. The networks show branches (arrowhead), junctions (*) and cells which have not formed part of the network (white arrow). Representative images from n=3 independent experiments. Magnification x20, scale bar = 125µm

**4.4.3 Nitric oxide production**

The effect of IFNa2b on nitric oxide bioavailability was measured over a dose-range of 0.1-10ng/ml at both 6 and 24 hours. The presence of total nitrate and nitrite was measured using a colorimetric assay as described above. No significant difference was seen between vehicle-treated cells and IFNa2b-treated at any of the concentrations (0.1, 1 and 10ng/ml) at either time point (figure 4-8). The detection limit for this assay is 2.5µM.
Co-culture of HAoECs with IFNα2b had no effect on the concentration of total nitrate and nitrite in the culture media after either 6 or 24 hours. Results represent mean concentrations from n=3 experiments in duplicate. NO production was also measured at 2 hours in n=2 experiments but again showed no difference (data not shown). Columns represent the mean and the error bars show standard error. A 2-way ANOVA was used with comparisons between each IFN concentration and the untreated group (Tukey's correction for multiple testing). The p-value represents the differences between concentrations of IFN. No condition was statistically different from the control in multiple comparisons at either time point.

4.5 Effect of IFN on gene expression in HAoEC

The effect of IFNα2b on gene expression in HAoECs was assessed using an exon microarray. HAoECs at 90% confluence were serum starved for 4 hours then incubated with 10ng/ml IFNα2b or vehicle control for 6 hours in n=3 independent experiments. The integrity of the RNA was measured using an Agilent 2100 Bioanalyser as described above. One sample pair failed the assessment of RNA quality and was thus rejected. The remaining samples had a RNA integrity number (RIN) of ≥9.90. A RIN of >6 is considered adequate for microarray studies. Representative electropherograms of the RNA integrity are shown in figure 4-9.
A primary analysis was undertaken to determine the number of genes significantly regulated in HAoECs by IFNα2b. A differentially expressed gene (between treated cells and the control) was defined as being up- or down-regulated by a factor of ≥2-fold with statistical significance. In order to allow for multiple comparisons, a q-value of ≤0.2 was used to correct for the false discovery rate. The expression of 198 genes was regulated by IFNα2b although only 5/198 (2.5%) were down-regulated. Notably, some genes were markedly up-regulated by IFN including IFIT1 (112.4-fold) and IFI44L (93.1-fold). Those genes regulated by >20-fold are shown in table 4-1. Many of these genes have a defined role in the control of viral infections. IFIT4 was the most strongly up-regulated gene and encodes a sensor of single-stranded RNA which binds viral mRNA. Similarly IFI44L has antiviral activity against hepatitis C although the mechanism of action is less clear.

This table lists all of the genes differentially regulated by IFNα by more than 20-fold. The gene ID is given along with the name of the gene.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>Fold change (IFN vs. Control)</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFIT1</td>
<td>Interferon-induced protein with tetratricopeptide repeats</td>
<td>112.4</td>
<td>8.36x10^-5</td>
</tr>
<tr>
<td>IFI44L</td>
<td>Interferon-induced protein 44-like</td>
<td>93.10</td>
<td>0.000282</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Chemokine (C-X-C motif) ligand 10</td>
<td>92.09</td>
<td>0.000606</td>
</tr>
<tr>
<td>CXCL11</td>
<td>Chemokine (C-X-C motif) ligand 11</td>
<td>88.24</td>
<td>0.000282</td>
</tr>
<tr>
<td>IFIT3</td>
<td>Interferon-induced protein with tetratricopeptide repeat</td>
<td>82.37</td>
<td>0.000132</td>
</tr>
<tr>
<td>OAS2</td>
<td>2'-5'-oligoadenylate synthetase 2</td>
<td>57.80</td>
<td>8.36x10^-5</td>
</tr>
<tr>
<td>RSAD2</td>
<td>Radical S-adenosyl methionine domain containing 2</td>
<td>53.84</td>
<td>8.36x10^-5</td>
</tr>
<tr>
<td>MX2</td>
<td>Myxovirus [influenza virus] resistance 2 (mouse)</td>
<td>49.25</td>
<td>8.36x10^-5</td>
</tr>
<tr>
<td>IFIT2</td>
<td>Interferon-induced protein with tetratricopeptide repeats</td>
<td>35.83</td>
<td>0.000299</td>
</tr>
<tr>
<td>OAS1</td>
<td>N2',5'-oligoadenylate synthetase 1</td>
<td>32.85</td>
<td>0.000114</td>
</tr>
<tr>
<td>MX1</td>
<td>Myxovirus [influenza virus] resistance 1,</td>
<td>31.83</td>
<td>8.36x10^-5</td>
</tr>
<tr>
<td>GBP4</td>
<td>Guanylate binding protein 4</td>
<td>30.59</td>
<td>0.000299</td>
</tr>
<tr>
<td>IFI44</td>
<td>Interferon-induced protein 44</td>
<td>27.61</td>
<td>0.002803</td>
</tr>
<tr>
<td>EPSTI1</td>
<td>Epithelial stromal interaction 1 [breast]</td>
<td>25.68</td>
<td>0.000299</td>
</tr>
<tr>
<td>HERC6</td>
<td>Hect domain and RLD 6</td>
<td>25.64</td>
<td>0.000225</td>
</tr>
</tbody>
</table>

Table 4-1: Genes regulated in HAoECs by IFNα2b by >20-fold

Gene-level expression analysis was undertaken and comparisons were made with a dataset generated by Heathfield et. al. (2012) (506). In this dataset, HAoECs were incubated with 10ng/ml TNFα or vehicle for 6 hours. The experimental design was therefore sufficiently similar to allow direct comparison.

The number of significantly regulated genes was similar between HAoECs treated with IFNα2b and TNFα (207 and 198 respectively), although only 20 genes were common to both groups. In addition, the ratio of genes up-regulated:down-regulated was much greater in IFN-treated cells compared to those treated with TNF. Figure 4-10 shows volcano plots of gene expression and the overlap of genes regulated by both cytokines. Of the 20 genes which were up-
regulated by both cytokines there were a number of recognized interferon-sensitive genes including MX1, CXCL10 and IFIH1. No gene was down-regulated by both cytokines.

![Figure 4-10: Comparison of differentially regulated genes in HAoECs by IFNα2b and TNFα](image)

Volcano plots to show the distribution of genes significantly up- and down-regulated by IFNα2b (a) and TNFα (b). The vertical dotted lines show a fold change of 2 (0.301 on log scale). Transcripts above the horizontal dotted line were regulated with a p value of <0.05. The transcripts in blue were differentially expressed by a factor of 2 fold which was statistically significant. Compared to TNFα, IFNα2b resulted in a marked skewing of the plot towards the right, with very small numbers of genes being significantly down-regulated. The Venn diagram shows a small overlap in the expression profiles of genes differentially regulated by TNF and IFN (n=20). The diagram includes both significantly up- and down-regulated genes (fold change ≥2, q≤0.2) although no gene was regulated in different directions by the two cytokines.

### 4.5.1 Gene ontology analysis
Gene Ontology (GO) analysis was used to classify over-represented transcripts according to biological processes. Significantly regulated genes were identified and imported into the DAVID bioinformatics database v6.7 (NIH, Bethesda, USA) (483;484). The number of genes in each biological process was enumerated, and categories of biological processes were combined where appropriate. The most commonly represented process was “immune system” (63 genes) followed by “transcription/translocation” (25 genes) and “cell signalling” (22 genes). Within the immune system category, the genes were principally involved in “viral replication” (14 genes), “cell signalling” (14 genes) “antigen presentation” (11 genes) (figure 4-11).
198 transcripts were differentially regulated by IFNα2b. Of these, the most over represented category was the immune system (a). Within the immune system category of 63 genes most of the genes fall into groups with functions of modulating viral replication, intracellular signalling and antigen presentation (b). Bars represent the number of genes – bars to the left of the vertical axis show the number of down-regulated genes and bars to the right show the number of up-regulated genes.

Further functional analysis was undertaken by importing the DAVID dataset into Cytoscape (NIH, Bethesda, USA) which allows visualization of complex networks (485). Groups of genes of known biological function formed 5 major groups: i) immune and viral response, ii) cytokine and chemokine signalling, iii) nucleotide binding, iv) zinc finger and v) SPRY and SPRY-like (which are involved in regulating tyrosine kinase signalling) (figure 4-12). Of note, principal groups including cell adhesion molecules (including e-selectin, VCAM-1 and ICAM-1) which were over-represented following treatment of HAoEC with TNFα were not seen with IFNα (506).
Significantly regulated genes cluster into 5 principle super-groups. Within these, 2 are associated with cytokine signalling (yellow and green). The zinc finger and nucleotide binding groups (red and pink) reflect the role of IFNα in inhibition of viral replication and transcription of anti-viral genes. The central node defines the role of IFNα in regulation of the immune system and the anti-viral response. The genes are grouped and colour coded according to the functional group defined by DAVID and the lines connect genes with known interactions. Each box represents a functional group of genes. The white boxes represent groups of genes which do not fall into any of the 5 categories.

SRPY = sprout genes (inhibitors of growth factor-induced tyrosine kinase signalling).

As described above, a small number of genes were regulated in HAoEC by both IFN and TNF. In terms of function there was no clear association between these 20 genes. Table 4-2 lists the 20 genes and their GO functional classification as identified by DAVID. These genes are spread across the functional groups shown in figure 4-12 above.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Functional Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAP1</td>
<td>Immune / Antigen Presentation</td>
</tr>
<tr>
<td>IFIT3</td>
<td>Immune / Cell Signalling</td>
</tr>
<tr>
<td>MX1</td>
<td>Immune / Transporter/Channel</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Immune / Chemotaxis</td>
</tr>
<tr>
<td>GBP1</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>IRF1</td>
<td>Transcription/Translation</td>
</tr>
<tr>
<td>CX3CL3</td>
<td>Immune / Chemotaxis</td>
</tr>
<tr>
<td>TNFSF10</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>CTSS</td>
<td>Proteolysis</td>
</tr>
<tr>
<td>CXCL11</td>
<td>Immune / Chemotaxis</td>
</tr>
<tr>
<td>IFI30</td>
<td>Proteolysis</td>
</tr>
<tr>
<td>PARP14</td>
<td>DNA Protection</td>
</tr>
<tr>
<td>GBP3</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>SQRDL</td>
<td>Transcription/Translation</td>
</tr>
<tr>
<td>IFIH1</td>
<td>Immune / Viral Replication</td>
</tr>
<tr>
<td>APOL6</td>
<td>Lipids</td>
</tr>
<tr>
<td>GBP4</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>DTX3L</td>
<td>DNA Protection</td>
</tr>
<tr>
<td>SAMD9L</td>
<td>Unknown</td>
</tr>
<tr>
<td>RBM43</td>
<td>Transcription/Translation</td>
</tr>
</tbody>
</table>

Table 4-2: Genes up-regulated in HAoECs by both IFNα2b and TNFα

4.6 Discussion

Primary HAoECs responded to IFNα2b in vitro. Analysis of the transcriptome shows a marked induction of interferon sensitive genes (ISGs) after 6 hours. Many of these genes were up-regulated by over 20-fold. The number of genes regulated was similar to that seen in a comparable study using TNFα (506). GO analysis showed that the majority of genes up-regulated were involved in the immune response, particularly the host response to virus, leukocyte chemotaxis, and cytokine and intracellular signalling.

In addition, a clear IFN-signature was seen in HAoECs. Of the 6 ISGs included in the IFNα bioassay described by Hua et. al. (2006) (PTKR, IFIT1, IFI44, MX1, Clorf29, CXCL9) 4 were up-regulated in HAoECs (IFIT1, IFI44, MX1, CXCL9) (31). The results obtained in our studies above are more similar to the expression of ISGs seen in venous endothelial cells from patients with SLE. The 7 ISGs identified in the study by Goldenberg et. al. (2011) (IFI3, IFI44L, IFI6, MX2, IFITM1, OAS1, OAS2) were all up-regulated in HAoECs. Notably, other transcripts which were down-regulated in the venous cells (including TGFβ receptor genes) were not altered in HAoECs in vitro (197). Interestingly, some ISGs were also up-regulated by TNFα although the fold change was markedly lower. This suggests that the IFN-signature may be less specific to IFNα than suggested. This may therefore have implications for the interpretation of bioassays in which patient serum is used.
Although TNFα up-regulated cell adhesion molecules (e.g. VCAM-1, ICAM), no such changes were observed with IFNα2b. In addition, gene ontology and functional cluster analysis did not show over-representation in pathways implicated in endothelial dysfunction (e.g. NO pathways, thrombosis/coagulation or reduced cell proliferation). This strongly suggests that in this model, IFNα does not have a major effect on HAoECs beyond its role as an anti-viral cytokine.

Endothelial cell function in response to IFNα2b was assessed in terms of NO production, network formation and proliferation; tubule formation and proliferation were investigated using 2 different methodologies. IFNα2b had no effect on NO production, which supports the observation that the NO pathway was not over-represented in the gene array analysis.

Similarly, IFNα2b had no effect on HAoEC network formation either on Matrigel or in collagen, or on HUVEC network formation on Matrigel. The use of both an angiogenesis stimulator (VEGF-A) and inhibitor (PTIO) gives confidence regarding the validity of this assay. Whilst the Matrigel assay is a well-recognised in vitro model of angiogenesis, there are some important differences from in vivo angiogenesis. Firstly, whilst Connolly et. al. (2002) identified a lumen in the network structures (507), others have not (491). As discussed above, the term “cord” is preferable to “tubule” unless a lumen has been conclusively demonstrated. Secondly, the networks form on the surface of the matrix, whilst in vivo the network forms in a three dimensions. The 3D assays (using Matrigel, fibrin or collagen as above) may provide a more physiological environment. However, these assays have additional difficulties as they take longer to perform, the thickness of the gel is critical to ensure sufficient oxygen diffusion, and the analysis of a 3D network is more complex (504). A Matrigel plug model is also commonly used to examine in vivo angiogenesis. In this model 500-1000µl of Matrigel is injected subcutaneously into the mouse and harvested 7-14 days later. Angiogenesis can then be quantified either by histology, estimation of plasma volume or haemoglobin concentration within the plug (508;509). Importantly some angiogenic factors may affect network formation in vivo, but have no effect in vitro (reviewed by Liekens et. al. (2001) (510)). Although IFNα had no effect on network formation in the model above it does not conclusively demonstrate that IFNα has no effect on angiogenesis. In a study by Thacker et. al. (2012) angiogenesis was impaired in the New Zealand Mixed 2328 (NZM) mouse (using a Matrigel plug assay and haemoglobin quantification) but significantly improved in a NZM IFNαR⁻² mouse (238).

IFNα2b had no effect on HAoEC proliferation at up to 72 hours when measured either by cell counting or MTT assay. Similarly to the Matrigel assays, the use of VEGF-A and PTIO assists with the interpretation of the results. As described previously, only Gomez et. al. (2003) have
investigated the effect of IFNα on HAoEC proliferation (501). In this study, IFNαA (500IU/ml) increased [³H]-thymidine incorporation by around 2.5-fold after 72 hours. The principle differences between this experiment and the ones described above are: i) high concentrations of FBS used (20% compared to 5%) which may interact with IFNα, and ii) measurement of proliferation by DNA synthesis compared to cell number/mitochondrial activity. High concentrations of FBS have been shown to interfere with test agents in cell culture (511). The choice of assay is also important and the correlation between different measures of cellular proliferation can be poor, especially between MTT-like methods and measures of DNA synthesis (e.g. thymidine incorporation) (512).

In HUVECs, IFNα2b induced a subtle but statistically significant increase in proliferation at 24 hours. The magnitude of the increase was small, and notably less than that seen with VEGF-A. The results of other groups are contradictory, with studies showing an increase (501), decrease (513) or no change (514) in proliferation. The relevance of these findings to endothelial dysfunction and atherosclerosis is not clear as HUVEC originate from the venous side of the circulation which does not develop plaque. It is well recognised that endothelium from different vascular beds are heterogeneous in terms of function, gene expression and response to the extracellular environment (reviewed by Aird (2007)) (515). In the context of in vitro culture, endothelial cells from different vascular beds can have notable differences in terms of morphology, growth rate and total proliferative capacity/survival (516). Similarly, HUVEC are obtained from a foetal source whilst HAoECs are typically from adult sources. Even within the same vascular bed, cells undergo transcriptional changes as they mature. In corneal endothelial cells, for example, 8 genes within the TGF-β signalling pathway are significantly over-represented in foetal cells compared to mature adult cells (517). The effect if IFNα on endothelial cell function may therefore be strongly related to the source of the cells used both in terms of their origin and stage of maturity.

The type 1 IFN family consists of 7 classes (α, β, δ, ε, κ, τ and ω) all of which signal via the transmembrane IFNα-Receptor (IFNαR). The IFRNαR comprises 2 chains, IFN-αR1 and IFNα-R2 and the binding of IFNα to its receptor results in the activation of the JAK-STAT signalling pathway (although there are reports that other signalling pathways may be activated including PI3K, Akt and NFκB) (518). Although IFNα and IFNβ signal via the same receptor, they have different physiological effects. Of relevance, endothelial cell proliferation has been shown to be inhibited by IFNβ but not IFNα in both human microvascular endothelial cells and HUVEC (514). This may be due to an increased binding affinity of IFNβ for the IFNαR (519).

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¹ Equivalent to approximately 1.5-2.5ng/ml (estimated conversion is 3-5pg per IU)
In addition, 12 different IFNα proteins have been identified. Across these subtypes, a 10-fold difference in receptor binding has been reported (520). This study used only IFNα2b (a product of the IFNA2 gene). The similarities in the structures of the IFNα subtypes make it difficult to distinguish between them by ELISA, and thus the relative expression of the different IFNα subtypes in SLE is therefore not known. IFNα2b has been reported to induce a lupus-like syndrome in patients undergoing treatment for chronic hepatitis C and may therefore be an important subtype in patients with idiopathic lupus (23). As the aim of these experiments was to develop a model with relevance to SLE, this subtype was selected. It cannot be assumed, however, that the other subtypes of type 1 IFN do not impair endothelial cell function.

If IFNα induces the expression of an interferon signature but doesn’t otherwise negatively affect HAoEC function then it is possible that IFNα could mediate vascular damage and increase CVD risk by targeting other cells. Macrophages exposed to IFNα up-regulate the expression of the scavenger receptors class A (SR-A). SR-A leads to increased LDL-uptake and the formation of foam cells. Importantly, SR-A mRNA expression is increased in the PBMCs of lupus patients (198). In addition, IFNα results in up-regulation of Toll-like receptor 4 (TLR4) expression in myeloid cells and increased sensitivity to TLR4 ligands (including IL-12, IL-23 and MMP-9) (521). Furthermore, IFNα in atherosclerotic plaque is able to regulate T cell function and increase smooth muscle apoptosis by increasing T cell expression of TNF-related apoptosis-inducing ligand (TRAIL) (522). Platelets may also be a target for IFNα as an interferon signature is found within the platelets of SLE patients, more so in patients with vascular disease (196). As demonstrated by the studies in HAoECs, expression of an interferon signature does not always correlate with impaired function and so experiment studies of platelet function are needed. Finally, IFNα can directly impair endothelial repair mechanisms. MACs and mixed MAC/OEC populations are sensitive to IFNα resulting in increased apoptosis and impaired angiogenesis. The effect of IFNα on vascular repair is discussed in more detail in the next chapter. Figure 4-13 summarises some of the cell types which may be a target in IFN-mediated vascular damage in SLE.
Type 1 IFNs have effects on multiple cell types within the vasculature. As described above, IFNα induces an interferon signature in endothelial cells but has little effect on cell function. In contrast, however, IFN increases LDL-uptake by macrophages promoting foam cell formation, and up-regulation of TLR4 which promotes release of pro-inflammatory cytokines (TNFα). Exposure of T cells to IFNα increases pro-apoptotic mechanisms and smooth muscle cell apoptosis. In addition, IFNα also has detrimental effects on the 2 principle cell types involved in endothelial repair.

**TLR=toll-like receptor, MAC=myeloid angiogenic cell, OEC=outgrowth endothelial cell**

*IFNα inhibits pulmonary artery smooth muscle cell proliferation but the pulmonary system differs markedly from the arterial system and so it may not be appropriate to extrapolate this observation (523). The effect on smooth muscle cells from other vascular beds is not known.*

### 4.7 Summary of results:

- Human aortic endothelial cells express an interferon signature when exposed to IFNα2b
- Gene-level expression analysis shows up-regulation of genes with anti-viral function and roles in cytokine signalling in HAoECs treated with IFNα2b for 6 hours
- IFNα2b does not affect HAoEC proliferation, network formation in matrix or nitric oxide production
- IFNα2b alone does not significantly impair HAoEC function *in vitro* and cannot be used to model vascular damage in patients with SLE
Patients with SLE have impaired endothelial repair mechanisms which may contribute to the development of cardiovascular disease. This chapter describes the development of in vitro models of myeloid angiogenic cell function using cells from SLE patients and from healthy controls cultured with IFNα.
5 Modelling endothelial repair in SLE

5.1 Introduction
Endothelial repair is an essential process in order to maintain the integrity of the endothelium. Whilst both OECs and MACs are likely to contribute to endothelial repair in vivo, MACs in particular have been reported to be both reduced in number and dysfunctional in patients with SLE.

SLE patients appear to have significantly fewer MACs compared to HCs, an effect due at least in part, to IFNα (223). The addition of SLE serum or IFNα to HC MACs resulted in significant MAC apoptosis. Of note, the cells used in this study may not represent a pure MAC population and are likely to contain a mixture of MACs and true EPCs due to the prolonged culture time (7-21 days). A similar picture is seen in the NZB/W F1 murine model of SLE which shows reduced number of MACs (cultured for 7 days only) in both early and late disease (237).

In terms of MAC function, the colony forming capacity (originally described for “early-EPCs”) offers a simple global measure of function. The number of such colony forming units (CFUs) is lower in SLE compared to controls in most (195;225;524-526) but not all studies (227). The negative study by Ablin et. al. (2011) also contained a healthy control group with increased prevalence of CVD risk factors. These colonies are also smaller in SLE and display abnormal morphology (228;524). Although the relationship between CFUs and endothelial repair is still unclear, there is an association between the number of CFUs, interferon-alpha and endothelial function measured by peripheral artery tonometry (195).

The ability of MACs to migrate towards chemotactic factors is impaired in SLE. Chemotaxis assays show that compared to MACs from HCs, SLE MACs migrate less well towards higher (100ng/ml) but not lower (50ng/ml) concentrations of VEGF-A (228;229). A trend towards reduced migration towards TNFα has also been shown, although the relevance of TNFα in MAC mobilisation and endothelial repair is unclear (228).

The ability of MACs to adhere to inactivated endothelial cells (HUVEC) appears to be reduced in SLE although other endothelial cell types have not been investigated (225). Adhesion to basal laminae has shown conflicting results with increased adhesion (227) or no difference (228).

Lupus MAC/EPCs express significantly lower levels of VEGF and HGF at both the transcript and protein level (223). Furthermore, IFNα results in transcriptional down-regulation of VEGF-A in
both SLE and HC MACs (200). The relevance of changes in expression of these factors is yet to be determined.

IFNα appears to negatively modify MACs in patients with SLE. MACs/EPCs in lupus express increased levels of ISGs compared to HCs. IFNα is toxic to MAC/EPC, reducing the formation of monolayers in vitro and inducing MAC/EPC apoptosis (223). These effects are ameliorated by blockade of either circulating IFNα or the type 1 IFN receptor. Furthermore, IFNα results in transcriptional down-regulation of VEGF-A in both SLE and HC MACs (200). This effect is mediated by repression of IL-1β pathways (down-regulation of IL-1α/β and IL-1R1 and up-regulation of IL-1R antagonist and the decoy receptor IL1-R2) (239). Evidence for the effect of IFNα in vascular function and repair has been shown in animal models. Compared to the lupus-prone NZM2328 mouse, the NZM2328 IFNαβR−/− model shows increased numbers of MACs, increased in vivo angiogenesis (using a Matrigel plug model) and improved endothelial function (238). The detrimental effects of IFNα in vascular function are not confined to lupus models of SLE, as knock-out of the IFNαR increases MACs, improves angiogenesis, and importantly reduces atherosclerotic plaque in ApoE−/− mice.

This chapter describes the development of models to investigate the ex vivo function of MACs derived from patients with SLE. These functional assays were developed in order to investigate the effects of vitamin D on endothelial repair mechanisms in vitro. In addition, the effect of IFNα on HC MACs was studied to try to replicate a lupus phenotype in vitro.

5.2 Aims of this chapter
The specific aims of this chapter are to:

- Characterise MACs derived from healthy subjects in terms of myeloid cell subtype
- Demonstrate that MACs interact with endothelial cells and have angiogenic capacity in vitro
- Compare the function of MACs in terms of migration, adhesion and angiogenesis between healthy controls and vitamin D deficient SLE patients
- Identify whether IFNα treatment of healthy MACs can replicate a lupus phenotype
5.3 Isolation and characterisation of MACs

5.3.1 Optimisation of the isolation of MACs from peripheral blood
MACs were isolated from the peripheral blood of vitamin D deficient (25(OH)D<20ng/ml) and replete (>30ng/ml) lupus patients and from healthy controls (HC). These subject populations are described in detail in Chapter 7. Cells were initially plated at 2x10⁶/cm² as described by Denny et al. (2007) (223), but this resulted in the spontaneous formation of colonies of cells similar to those described as “early-EPCs” (see figure 5-1). Furthermore the high density of cells resulted in difficulty in cell detachment. A lower density of 1x10⁶/cm² (as described by Mangialardi et al. (2011) (496)) resulted in a more evenly distributed cell population. MACs were cultured in EGM as per Denny et al. rather than EGM-2 as favoured by some groups (215;496) as EGM does not contain recombinant growth factors (particularly VEGF-A) which would interfere with angiogenesis assays and analysis of the growth factor profile of MACs (see Chapter 6).

Cells at day 8 were confirmed as having a MAC phenotype by the uptake of Dil-Ac-LDL and the binding of UEA-1 lectin as described by Denny et al. (2007) (figure 5-1) (223). All cells which took up Dil-Ac-LDL were also able to bind lectin and so Ac-LDL-uptake positivity alone was used for enumeration of MACs in order to reduce the number of steps involved and thus minimise cell detachment (see below).
Figure 5-1: Assessment of Ac-LDL uptake and UEA-1 by MACs
Phase-contrast micrographs of MACs plated at high and low density (a). Colony formation is seen by day 8 in MACs plated at 2x10^6/cm^2 (i,ii) whilst a lower density of 1x10^6/cm^2 results in a more even cell layer (iii). Magnification x4, scale bar=250µm (i) and magnification x10, scale bar=100µm (ii, iii).
Fluorescent images of MACs at day 8 to confirm the MAC phenotype (b). MACs are able to take up labelled Ac-LDL (red, i) and bind UEA-1 (green, ii). All cells are dual positive (yellow/orange denotes overlap) (iii). Magnification x40, scale bar=25µm.

Although Dil-Ac-LDL-uptake and lectin binding is considered characteristic of MACs (223;496), further more detailed characterisation was undertaken in terms of surface marker expression and phagocytic and migratory capacities.

5.3.2 Phenotypic characterisation of MACs by immunocytochemistry
MACs were characterised by immunocytochemistry using a combination of myeloid (CD45, CD68) and endothelial markers (CD31, vWF). The marker CD3 was also used to ensure that the myeloid culture was not contaminated with any lymphocytes. CD3 is part of the T cell receptor complex and is present on T cells at all stages of development (527).

CD45 (protein tyrosine phosphatase receptor, type C) is a transmembrane protein expressed on the surface of all haematopoietic cells except mature red cells (528). CD68 is a glycoprotein expressed within the lysosomal membranes of monocytes and macrophages (529).

The endothelial marker CD31 (platelet endothelial cell adhesion molecule, PECAM-1) forms part of endothelial cell junctions and so is expressed predominantly at the cell surface (530). In addition to endothelial cells and platelets, CD31 is also expressed on
monocytes/macrophages, neutrophils and lymphocytes (531). Von-Willebrand factor (vWF) by contrast is expressed predominantly on endothelial cells and platelets but not on leukocytes (530). On microscopy, vWF shows a characteristic granular pattern of staining. vWF was used to ensure that no true EPCs (OECs) had appeared in the culture after 8 days. Unlike MACs, OECs strongly express both CD31 and vWF (532).

MACs strongly expressed both CD45 and CD68 confirming a monocyte/macrophage cell type (figure 5-2). As expected, the CD45 staining was predominantly at the cell membrane whilst CD68 was localised within the cytoplasm. MACs weakly expressed CD31 relative to HAoECs, with most staining at the cell membrane. Unlike HAoECs, MACS did not express any detectable vWF. There was also no detectable CD3 staining in the MAC culture.

To support the strong expression of M2 macrophage markers seen using RT-PCR (see below), further staining was used to identify CD14 and CD206. CD14 is a co-receptor for the detection of bacterial lipopolysaccharide. It is expressed predominantly by monocytes and macrophages, and to a lesser extent by dendritic cells and neutrophils (533).
Immunocytochemical analysis of common myeloid and endothelial markers in MACS (a). Positive staining is detected in MACs probed for CD45, CD68, CD31, CD14 and CD206 (ii, iii, v, vii, viii) but not mouse IgG control, CD3 or vWF (i, iv, vi). As a comparator, HAoECs strongly express CD31 (ii) and vWF (iii) (b). Magnification 40x, scale bar=20µm.

5.3.3 Characterisation of MACs by RT-PCR and RT-qPCR

The expression of the markers used above was validated and extended further by RT-PCR. Expression in MACs (day 8) was compared to PBMCs from the same subject, and HAoECs. All 3 cell types expressed β-actin which was used as the reference gene (figure 5-3). Confirming the results of the ICC above, MACs expressed both CD31 and the myeloid markers CD45 and CD68. In addition, MACs expressed CD14 and lower levels of CD83 (a marker of dendritic cells). MACS very strongly expressed CD206 and to a lesser extent CD163 (both markers of M2 macrophages) compared to PBMCs. CD206 is more strongly expressed on the M2 macrophage subtype than M1 (216). HAoECs expressed CD31 and vWF but none of the myeloid markers.
The relative expression of endothelial and myeloid markers between PBMCs, MACs and HAoECs was measured by RT-PCR. MACs express all myeloid markers, but most strongly express CD68 and CD206. HAoECs were used as a negative control and express endothelial markers only.

Quantitative real-time PCR (RT-qPCR) was used to quantify differences in the expression of myeloid markers between MACs and the PBMCs from which the MACs were derived. RT-qPCR uses reference genes in order to reliably quantify expression of the gene of interest adjusting for the total amount of cDNA used. Therefore, it is important to consider reference gene stability when using this method. The geNorm reference gene assay (Primer Design, UK) was used to identify which reference genes were most stable within MACs. In addition, PBMC and THP-1 cells were used to provide a broad spectrum of myeloid cells. The analysis showed that CYC1 and ATP5B in combination was the most stable reference gene pairing across MACs derived from different subjects (figure 5-4). These were also the most stable across samples from the 3 cells types (THP-1 cells, PBMCs and MACs) although there was greater variability than within MACs alone.
Figure 5-4: Stability of the expression of potential reference genes in myeloid cells
The geNORM software package was used to identify the expression stability value (M-value) of candidate reference genes (18s, B2M, β-actin, GAPDH, CYC1, ATP5B). The M-value represents the average stability of a gene compared with the other reference genes. The optimum genes are then identified by stepwise exclusion of the least stable gene. In MACs from different subjects (total n=9 samples) CYC1 and ATP5B were the most stably expressed genes (a). In a population of myeloid cells from different sources (total n=15 samples including MACs, PBMCs and THP-1 cells) the same genes are the most stable although the M-value is much greater (b).

For RT-qPCR more specific markers of macrophages were chosen based on a review paper by Tomioka et. al. (2012) (216). CD14 and CD68 were chosen to identify macrophages as both of these were more strongly expressed in MACs compared to PBMCs in the experiment above.

As described in the introduction MACs have been reported to express markers of M2 (alternatively-activated) macrophages. To identify M1 macrophages, CCR7 and CD86 were selected. CCR7 is only expressed on M1 macrophages whilst CD86 is expressed on M1 and the M2b-subtype. CD206 is expressed on M2 and to a lesser extent on M1 and is considered to be relatively specific for M2 cells (215).

When compared to healthy PBMCs, HC MACs expressed greater levels of CD14 and CD68 (mean increase 2.6-2.8-fold) and significantly greater levels of CD206 (mean increase 353-fold, p=0.004 after correction for multiple comparisons, n=4 in each group). MACs also expressed around 5-fold lower levels of CCR7 although this was not statistically significant (figure 5-5).
5.3.4 Phagocytosis by MACs

The ability to remove cell debris and pathogens by phagocytosis is an important function of macrophages. MACs at day 8 were incubated with fluorescent-labelled Latex beads to demonstrate phagocytosis. The MAC cell population contained cells which were able to ingest the beads (with morphology identical to MACs after incubation with dil-Ac-LDL) and smaller rounder cells which did not (figure 5-6). The distribution of the beads was relatively uniform amongst the MACs.

Figure 5-5: Expression of M1 and M2 macrophage markers by MACs

RT-qPCR analysis showed that when compared to healthy PBMCs, MACs expressed significantly higher levels of the M2 macrophage marker CD206 at the transcript level. CD14, CD68 and CD86 were modestly increased but CCR7 was lower. Columns represent the mean (SE) expression normalised to that of CYC1 and ATP5B and is shown on a log scale. Comparisons between PBMCs and MACs were made using an unpaired t-test (n=4 subjects in each group). Initially all genes except CD14 showed a statistically significant difference between PBMCs and MACs but after correction for multiple comparisons only CD206 remain significant (Holm-Sidak multiple comparison test, α=0.05).
Figure 5-6: Phagocytosis by MACs
Light microscopy shows that after 7 days in culture MACs remain a heterogeneous population with different cell morphologies visible (a). After incubation with IgG-FITC-labelled Latex beads (green) there is considerable uptake by MACS (b). An enlargement of the boxed area in (b) shows cells which clearly take up the beads (black arrow) compared to smaller cells which do not (white arrow) (c). Magnification x10 in (a) and (b), error bar=200µm.

5.4 Development of models of MAC function ex vivo

In order to study the effects of vitamin D on MACs ex vivo it was necessary to develop in vitro models of MAC function. Models were developed based on the principle that for successful endothelial repair MACs needed to be able to i) migrate towards the endothelial layer, ii) adhere to endothelial cells and iii) have a pro-angiogenic capacity in order to stimulate endothelial repair. On this basis, cell models were developed to study MAC number ex vivo, migration, adhesion to endothelial cells and angiogenesis in terms of a model of endothelial network formation. These models are summarised in figure 5-7.
Cell-based assays were developed to study each of the main functions of MACs detailed above. Each assay was conducted independently and was developed in order to investigate the effects of vitamin D on MAC function.

### 5.4.1 Cell number

The number of MACs was measured by Dil-Ac-LDL uptake at days 2 and 8 as described above. There was a significant increase in the percentage of cells with the MAC phenotype during the culture period (figure 5-8). Comparisons were also made between the number of MACs at day 8 between SLE patients and HCs. There were significantly more MACs obtained from SLE patients compared to HCs (mean [sd] 105 [52.4] vs. 167 [72.4], p=0.038).
The proportion of MACs within the cell population increased from day 2 to day 8 (n=3 healthy controls) (a). The columns represent mean % of cells, error bars represent standard error. Analysis was by paired t-test, *p<0.05. The mean number of MACs per random field at day 8 was significantly higher in SLE patients compared to healthy controls (b). There were n=10 HCs and n=30 SLE patients with MAC data. In the SLE population, n=17 were vitamin D deficient and n=13 replete. Each subject contributed triplicate wells, with 3 random fields imaged per well (i.e. 9 fields per patient). The horizontal bar represents the mean. Comparison between groups was made using a two-tailed t-test, **p<0.01.

5.4.2 Migration of MACs
If MACs are to augment endothelial repair by the paracrine secretion of pro-angiogenic factors then it is essential that MACs are able to migrate towards endothelial cells. Furthermore, changes in this migratory capacity have the potential to affect endothelial repair. The interaction between MACs and endothelial cells was initially investigated by observation of MAC co-localisation with an endothelial cell network.

Endothelial cells form complex networks when plated onto appropriate basal laminas (e.g. Matrigel™) as described in detail below. When MACs were labelled with a green cell tracker (as described for the adhesion assays), and co-cultured with HAoECs for 14 hours there was a clear association between the two cell types. Whilst MACs lined up against an endothelial network, they were not able to form similar networks when cultured alone (figure 5-9).
Figure 5-9: Co-localisation of MACs to aortic endothelial networks in Matrigel
HAoECs form branching networks on Matrigel. MACs (green) clearly co-localised with these networks (a). In the absence of HAoECs, MACs plated on Matrigel remained in a monolayer and did not organise into a network (b). The images shown are (i) bright field, (ii) fluorescent microscopy, and (iii) merged image. Magnification 20x, scale bar=100µm.

Time-lapse microscopy was then used to investigate further the relationship between MACs and endothelial cell networks. HAoECs and fluorescent-labelled MACs were plated together onto Matrigel. Over 14 hours the endothelial cells formed networks and the MACs co-localised to the cord structures (figure 5-10). Although the co-localisation experiment demonstrated that MACs could adhere to endothelial cells, it did not demonstrate true migration towards HAoECs. To show this, HAoECs were allowed to establish networks in Matrigel-coated 96 well plates for 14 hours prior to the addition of fluorescent-labelled MACs. Over the time course of 7 hours, MACs clearly migrated both towards each other and the HAoECs forming clusters on the surface of the network (figure 5-10).
Figure 5-10: Migration of MACs towards endothelial cells
As HAoECs (unlabelled) form networks in Matrigel, MACs (green) move along with the endothelial cells and line-up with the cords and nodes (a). The networks were allowed to develop over 14 hours and imaged every 15 minutes using time-lapse microscopy. Magnification x5, scale bar=250µm. To demonstrate true migration, MACs were added to pre-formed HAoEC networks and were observed to migrate towards these structures (b). There is clear migration by 2 hours and this process is almost complete by 6 hours. Magnification x20, scale bar=50µm.

5.4.2.1 Development and optimisation of the migration assay
In order to quantify MAC migration a Transwell® migration assay was established. This is based on the Modified Boyden chamber and measures the movement of adherent cells through pores within a polycarbonate membrane. This assay therefore measures cell movement towards a chemotactic agent (chemotaxis) rather than just random cell motility (chemokinesis). SDF-1α was selected as the chemotactic agent as this has been identified as an important factor in the recruitment and retention of pro-angiogenic cells around developing vessels (534). Furthermore, chemotactic assays show that SDF-1 induces MAC migration in a dose-dependent manner (211). The assay was optimised in terms of the concentration of SDF-1α used, the size of pore in the chamber membrane and the presence/absence of a fibronectin coating on the membrane (figure 5-11). In all assays basal cell media (no growth factors) with 1% bovine serum albumin (BSA) was used. This eliminated
any effect of chemotactic factors which may be present in FBS.

Figure 5-11: Optimisation of the MAC migration assay
MACs migrate towards SDF-1 in a dose-dependent manner. Representative images of MACs (blue) on the underside of the membrane after 8 hours in the absence of SDF-1 (i) or with 100ng/ml SDF-1 in the lower chamber (ii) (a). Magnification 10x, scale bar=200µm. The number of migrated MACs is dependent on the concentration of chemoattractant (b). At a concentration of 100ng/ml, the increase in migration was approximately 4-fold. The greatest difference between control wells and wells with SDF-1 was seen with the larger pore size (8µm) and in the presence of fibronectin coating (c). Columns represent the mean number of migrated cells, error bars show standard error. Analysis by two-way ANOVA and Sidak’s multiple comparisons test. *p<0.05, ****p<0.0001, n.s.=non-significant.

Based on optimisation experiments above, the chemotaxis assay was performed using fibronectin-coated membranes with 8µm pores and 100ng/ml SDF-1α in the bottom chamber. This concentration of SDF-1α has successfully been used by other groups (211).

5.4.2.2 Migratory capacity of MACs from healthy controls and SLE patients
MACs from SLE (n=6 vitamin D deficient, n=4 vitamin D replete) patients showed significantly impaired migration compared to healthy controls (figure 5-12). Migration was standardised to that seen in chambers without SDF such that a relative migration of 1 indicates no detectable chemotaxis. A comparison of migratory capacity between vitamin D-deficient and replete patients is described in Chapter 6.
The chemotactic response to SDF-1α (100ng/ml) is significantly impaired in cells from vitamin D deficient and replete SLE patients (n=10) compared to healthy controls (n=6). Each condition was carried out in duplicate with 5 random fields visualised per well. Columns represent mean relative migration and error bars show standard error. Comparison was made using a two-tailed t test, ***p<0.001.

5.4.3 Adhesion of MACs to endothelial cells

5.4.3.1 Development of the adhesion assay

It is recognised that in order to promote angiogenesis, MACs and other progenitor cells must be able to adhere to the endothelium (535). The mechanism is not fully understood but bone marrow-derived mononuclear cells have been shown to adhere to endothelial cells via β-integrins, a process augmented by monocyte chemoattractant protein-1 (MCP-1) (536). MACs have the capacity to adhere directly to a variety of basal laminae (including fibronectin and fibrinogen) but also to endothelial cells via adhesion molecules (intracellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1]) (227;228;537). A model of adhesion was developed using HAoECs and 10ng/ml TNFα for 6 hours. This dose and duration was used as it produced significant up-regulation of the adhesion molecules ICAM-1, VCAM-1 and E-selectin in HAoECs (506). In order to compensate for variation in HAoEC density or MAC concentration, each experiment was then adjusted to determine the relative increase in adhesion to TNF-activated HAoEC compared to un-activated cells.

5.4.3.2 Adhesion assay results for healthy controls and SLE patients

The adhesion of both healthy (n=5) and vitamin D-deficient lupus (n=4) MACs to the endothelium was significantly increased in the presence of TNFα (figure 5-13). There was no
difference in adhesion between healthy control and SLE MACs (mean [sd] relative adhesion 2.1 [0.4] vs. 2.2 [0.4], p=0.26).

**Figure 5-13: Adhesion of MACs to TNFα-activated endothelium**

The mean number of adherent healthy (n=5) or lupus (n=4) MACs was significantly increased by activating the endothelium with 10ng/ml TNFα (a,b). Panel (a) shows healthy MACs and panel (b) shows SLE MACs. The columns represent the mean number of adherent cells from 3 random fields per well carried out in triplicate for each subject, error bars show standard error. Representative images show labelled MACs (green) adherent to a monolayer of HAoECs (c). The large nuclei of HAoECs were stained with DAPI (blue). Magnification x20 Scale bar=100µm. There was no difference in relative adhesion between MACs from healthy controls or lupus patients (d). Comparisons using a ratio paired t-test for (a) and (b) and two-tailed t-test for (d), **p<0.01, n.s.=non-significant.

### 5.4.4 Angiogenic capacity of MACs

#### 5.4.4.1 Development and optimisation of the angiogenesis assay

The final component of the MAC model was the quantification of the angiogenic capacity of MACs. The Matrigel assay is a widely used model of angiogenesis (538). Endothelial cells spontaneously form networks on this matrix, the density of which can be quantified. Growth factor reduced (GFR) Matrigel was used for all experiments as a high concentration of these factors within the Matrigel itself could mask any effects of MACs. HAoECs were plated onto Matrigel in either the conditioned media from MACs or EGM-20% (the same media in which MACs were cultured).
Images of the resultant capillary network were captured and subjected to image analysis in ImageJ software (http://rsb.info.nih.gov/ij). An algorithm was developed based on a description by Guidolin et al. (2010) (499). This allowed quantification of the morphological features of the network including the number of branches, number of branch points/junctions, and an approximation of the network area (figure 5-14). The number of closed loops (polygons) was enumerated manually. To reduce background noise all fragments of the networks with a mean branch length <10 pixels were excluded. For each experimental condition n=5 replicates were used.

Figure 5-14: Quantification of endothelial network formation in Matrigel
A diagram of the Matrigel angiogenesis model (a). HAoECs were plated onto 10µl GFR Matrigel in a µ-angiogenesis slide with a “well within a well” structure in a small volume (50µl) of EGM-20% or MAC-conditioned media. The HAoECs formed networks on the Matrigel (m) but retain a cobblestone morphology on the plastic section of the well (*) (b). Quantification of network parameters required a multi-stage image development process (c). The original image (i) underwent background subtraction and was transformed into a binary image (ii). The gaps between cells were closed (iii) and the image converted to a skeleton image with branches a single pixel in diameter (iv). A magnified image of the skeletonised network (v) shows the parameters used: number of junctions (blue circles), branches and closed loops (dotted circle). Magnification x4, scale bar=250µm.
5.4.4.2 The angiogenic capacity of MACs from healthy controls and SLE patients

The conditioned media from HC MACs significantly increased the density of the endothelial cell network in all 4 of the measured parameters: mean number of closed loops (25.5 vs 41.0), branches (396 vs 561), junctions (216 vs 264) and total pixels (31,332 vs 36,923) (figure 5-15). Compared to EGM-20% the greatest difference was seen in closed loops (1.61-fold) and the smallest in total pixels (1.18 fold). To compare the effects of HC and SLE MAC-conditioned media, the parameters were standardised to growth media for each experiment. There was a trend towards reduced network formation by SLE MACs although this did not reach statistical significance in any of the 4 parameters. The largest differences between HC and SLE MACs were seen in terms of closed loops and number of branches.
Figure 5-15: Angiogenic capacity of MACs from healthy subjects and patients with SLE

The MAC-conditioned culture media significantly increased the density and complexity of HAoEC network formation on Matrigel (a). There was an increase in the number of closed loops (i), branches (ii), junctions (iii) and the total number of pixels in the skeletonised network (iv). The graph shows the pooled results from n=9 healthy controls. The SLE MACs showed a trend towards impaired network formation in all 4 parameters in this model (b) although this was not statistically significant (i-iv). Panel (b) shows combined results from (n=9 HC and n=10 vitamin D-deficient SLE patients). Each experiment was normalised to network formation in growth medium alone to allow comparison between SLE and HC. In (a) and (b) the columns represent mean and the error bars show standard error. Comparison was made using ratio paired t tests (a) and unpaired two-tailed t tests (b). **p<0.01, ***p<0.001, ****p<0.0001.
5.5 The effect of interferon-alpha on MAC function in vitro

There is mounting evidence that IFNα is detrimental to vascular health. As demonstrated in Chapter 4, this effect is likely not due to direct effects of type-1 IFNs on the endothelium. The following series of experiments aimed to investigate the effects of IFNα on healthy MACs with the aim of developing a model of failed endothelial repair relevant to SLE.

5.5.1 IFNα and MAC survival, migration and adhesion
HC MACs were treated with IFNα2b or vehicle (sterile water) from plating onto the fibronectin through until day 8. IFN was added again when the media was changed at day 4. Incubation of MACs with IFNα2b (0.01-50ng/ml) significantly reduced the number of MACs at day 8. The lowest concentration reduced the number of HC MACs by 50% (figure 5-16). A higher dose of 0.1ng/ml reduced MACs by around 70% leaving sufficient cells for the functional studies. There was a trend towards increased adhesion and reduced migration by HC MACs incubated with 0.1ng/ml IFNα2b but this was not statistically significant (figure 5-16).
IFNα2b decreased the number of MACs surviving to day 8 in a dose-dependent manner (a). The number of MACs was decreased by approximately 50% with 0.01ng/ml IFNα2b. Pooled results from n=5 healthy controls. Representative images showing the number of MACs (identified by Dil-Ac-LDL uptake, red) with increasing concentrations of IFNα2b (b). Magnification x10, scale bar = 100µm. Although incubation with IFNα2b for 8 days produced a trend towards increased adhesion and reduced migration this was not statistically significant (c,d). Experiments of adhesion and migration used n=4 healthy controls in each. Columns show mean and error bars represent standard error. Comparison was made using a two-tailed t-test.

The effect of IFNα2b on angiogenesis was assessed at a low dose (0.1ng/ml) and at a higher, toxic dose (10ng/ml). Incubation with 0.1ng/ml IFN-alpha had no significant effect on any of the 4 parameters measured, although there was a trend towards a small reduction in the number of closed loops and total pixel area (figure 5-17). The higher dose significantly reduced the number of closed loops, but increased the number of junctions and showed a trend towards an increased number of branches.
Incubation of MACs with IFNα2b at a high concentration (10ng/ml) significantly reduced the number of closed loops in the HAoEC networks (a). A lower concentration (0.1ng/ml) produced a smaller, non-significant effect on the same parameter. There was no effect of either concentration on the number of branches or total number of pixels (b, d). IFNα2b 10ng/ml resulted in a statistically significant, but small increase in the number of junctions (c). Columns represent mean and error bars show standard error. Results from n=3 healthy controls for each concentration of IFN, normalised such that growth media alone=1. Comparisons between untreated and IFN-treated made using ratio paired t tests, *p<0.05.

5.6 Discussion

This chapter describes the development of in vitro models of MAC function and the abnormalities in endothelial repair mechanisms in patients with SLE. We found that MACs from SLE patients are increased in number but dysfunctional in terms of migration and angiogenesis. IFNα alone does not fully replicate the lupus phenotype in HC MACs.

MACs were extensively characterised in terms of surface marker expression using both RT-PCR and immunocytochemistry. This demonstrated an increased expression of M2 macrophage markers compared to M1, most notable when comparing MAC expression to that of PBMCs. Since M2 macrophages are deemed to have an anti-inflammatory phenotype, these cells may also have a role in controlling inflammation in SLE. Whilst examination of surface markers at the transcript level is less preferable than analysis by flow cytometry, the cells adhered
strongly to the fibronectin which resulted in undesirable background staining (data not shown). Importantly there was concordance between the data obtained from immunocytochemistry, RT-PCR and RT-qPCR. No panel of markers has yet been identified to clearly differentiate between macrophage subtypes. Indeed, many of the markers used above are also expressed on the surface of dendritic cells (CD31, CD14, CD45, CD206). Although it is possible that some dendritic cells exist within the MACs, CD68 and CD163 were increased in MACs compared to PBMCs and are only expressed on macrophages (539). Furthermore, CD83, which is an important marker for dendritic cells (540) was expressed at a lower level in MACs compared to PBMCs.

Contamination of the MAC population with true OECs is of concern as both cell types are isolated by the culture of PBMCs in endothelial growth media. Although OEC enrichment is more successful using a type 1 collagen matrix (532) they have also been isolated using a fibronectin matrix (406). In order to reduce this, cells were cultured for a total of 7 days similar to Mangialardi et al. (2011) (496). Longer periods of culture (up to 21 days) result in a population of cells which express some characteristics of OECs (e.g strong expression of vWF) (223). In this study, there was minimal expression of vWF at the transcript level and vWF was not detected by immunocytochemistry. However low levels of vWF does not prove that OECs are present. Propki et al. (2009) has demonstrated that MACs are able to ingest markers of platelets (e.g. CD31) which are present in the initial PBMC cell preparation (541). Further evidence that the population are not OECs comes from the absence of cells with endothelial morphology, the inability to form networks in Matrigel and the phagocytic capacity of the cells.

The increased number of MACs observed conflicts directly with the work of others who demonstrated fewer MAC/EPCs and CFUs in SLE compared to healthy controls (195;223;225;524-526). These differences may be due to our lupus patients having inactive disease (mean SLEDAI score=0) or the effect of concomitant corticosteroids or immunosuppressants. In addition, these studies used different culture methods which limit direct comparison. In the study by Denny et al. (2007) the mean SLEDAI score was 4.8, consistent with moderate disease activity and culture was prolonged as discussed previously. In this study the population was mostly Caucasian, whilst in the study by Lee et al. (2007) only 36% of the subjects were Caucasian. The effects of ethnicity on MAC number are not known. Furthermore, the relationship between the number of MACs and the number of CFUs is not known as the colony-forming capacity is a better representation of MAC function rather than number.
The co-localisation of MACs with endothelial cells is important for their paracrine function. The results above clearly demonstrate that MACs can migrate towards and adhere to endothelium \textit{in vitro}, with a preference for activated endothelium. This is in agreement with Sieveking \textit{et al.} (2008) who demonstrated that whilst MACs could be found adjacent to developing vessels, they were not incorporated within the vessel itself. The chemotactic factor SDF-1 is important in MAC migration \textit{in vivo}, and the results above confirm that this can be developed into an \textit{in vitro} model (211;525;542). The Transwell® assay demonstrated marked impaired migratory capacity toward SDF-1 by SLE MACs compared to those from HCs. The mechanism of this is unclear, and may simply be a reflection of age as the SLE population was significantly older than the HCs. It has been observed previously that migration of true EPCs declines with age, due in part to lower CXCR4 expression (532). In the study by Ebner \textit{et al.} (2010) however, impaired migration to VEGF-A was seen in SLE compared to age-matched HCs suggesting that this observation may be due to SLE rather than aging (229).

A negative correlation between serum SDF-1 and MAC number has been reported in healthy subjects which may be due to a single nucleotide polymorphism (SNP) in the SDF1 gene (rs2297630) (543). This finding is in contradiction to other studies above which show that SDF-1 mobilises MACs cells from the bone marrow and increases CFU formation (544). Notably, a second SNP (rs1801157), whilst not differentially prevalent between SLE patients and HCs, has been associated with the presence of nephritis in lupus patients (545).

The conditioned media from MACs demonstrated clear angiogenic capacity in the Matrigel endothelial network model. Whilst the Matrigel assay is a well-recognised model of angiogenesis (546), some argue that the networks formed are not true capillary networks and as such, the term “angiogenesis” should be avoided (546). Regardless of nomenclature, the increased network formation by MACs observed in this model, mirrors that detected both using a Matrigel plug \textit{in vivo} and in the mouse hind-limb ischaemia model (205;211;525;547). On this basis, it is therefore reasonable to interpret this model as a measure of angiogenic capacity, even if the networks formed are not true capillary tubules. The potential factors responsible for this angiogenic effect are discussed in Chapter 6. Lupus MACs from vitamin D deficient patients showed a trend towards reduced angiogenic capacity although this did not reach statistical significance. The lupus patients had inactive disease and were taking combinations of corticosteroids/immunosuppressants (see Chapter 7) which could alter MAC function. It would be interesting to examine the angiogenic capacity of MACs from active SLE patients.
Incubation of MACs with IFNα2b results in significantly reduced numbers after 7 days culture. This was a dose-dependent effect although the smallest dose of IFN used (0.01ng/ml) resulted in a 50% reduction in the number of MACs. Other studies have shown that SLE patients with higher levels of IFN (as measured by expression of the ISG MX1) have fewer CFUs and that IFNα added to MACs in vitro significantly reduces both total MAC and CFU number (195;223). This appears to be due to MAC apoptosis with an increase in annexin-V positive cells. The source of IFNα within MACs has been identified as a population of low-density granulocytes (LDGs) which synthesise IFN and induce endothelial toxicity (200). Interestingly, low dose IFN did not significantly affect the migratory or adhesive capacity of MACs. This is in marked contrast to the reduced SDF-1 mediated migration seen in SLE MACs.

The effects of MAC conditioned media in the Matrigel model trended towards being reduced with low dose (0.1ng/ml) IFNα but were not statistically significant. The higher dose (10ng/ml) reduced the network density in terms of closed loops towards that of growth media alone. This is consistent with this dose causing around a 90% reduction in MAC number. Interestingly, this dose was associated with a small but significant increase in the number of junctions and a trend towards an increase number of branches. This could be explained by a more fragmented network which would have fewer numbers of closed loops overall but areas with branching cells not communicating with each other. Although a threshold was set to exclude small fragments, it is possible that this was too low to exclude background noise. It could be hypothesised, that given 10ng/ml IFNα2b markedly induced MAC apoptosis the conditioned media could have resulted in some endothelial cell damage.

Whilst IFNα has a role in the failure of endothelial repair, the addition of IFNα2b alone to HC MACs does not fully replicate the lupus phenotype. As discussed in Chapter 4, this may be due to different effects amongst the type 1 interferons, or interactions with other cytokines, as it is most likely that IFNα2b does not act alone in SLE patients. The utility of an IFN-model using MACs may therefore be limited. IFNα clearly reduces MAC number, but in our patient population MACs were increased. Further work is needed to fully understand the effects of IFNα on endothelial repair mechanisms.

5.7 Summary of Results

- MACs can be readily isolated from whole blood in patients with SLE and used in ex vivo assays of migration, adhesion and angiogenesis.
- Surface marker expression is consistent with an M2 macrophage phenotype.
- The number of MACs is increased in SLE, but function is impaired in terms of migration and angiogenesis in *in vitro* models (but no difference in terms of adhesion to endothelial cells).
- IFN-alpha significantly reduces MAC numbers but has variable effects on angiogenic capacity and no significant effect of migration - it does not fully replicate the lupus phenotype.
Chapter 6: Results

Vitamin D and myeloid angiogenic cell function

This chapter describes the effect of vitamin D on the function of the myeloid angiogenic cell models. The interaction between myeloid angiogenic cells and endothelial cells is also considered.
6 Vitamin D and myeloid angiogenic cell function

6.1 Introduction

Myeloid angiogenic cells (MACs) are a potential target cell for vitamin D. Macrophages express functional vitamin D receptors (VDRs), the activation of which can modulate gene expression, intracellular bacterial killing and cytokine production by macrophages (240;548). A small study by Mikirova et al. (2010) reported that MACs from vitamin D deficient (<40ng/ml) and replete (≥40ng/ml) healthy subjects showed different expression of endothelial surface markers (402). This suggests that the phenotype of these cells may be influenced by vitamin D status. In addition to inducing phenotypic changes vitamin D may also modulate MAC function. In response to vitamin D, human macrophages from patients with type 2 diabetes have shown reduced migratory capacity (549).

It is not known, however, whether MACs from patients with SLE will respond to vitamin D. Positive effects on MAC function in response to vitamin D would support the hypothesis that vitamin D can improve endothelial repair. Using the models of MAC function described in Chapter 5, it is possible to study the effects on vitamin D on MACs in vitro.

6.2 Aims of this chapter

The specific aims of this chapter are to:

- Identify the effects of vitamin D on the number and phenotype of MACs
- Determine whether vitamin D can modulate SLE MAC function in terms of migration, adhesion and angiogenic capacity
- Investigate the mechanism by which vitamin D might increase angiogenic capacity of MACs and whether MACs are able to regulate endothelial cell gene expression

6.3 Methodology relevant to this chapter

Firstly, the direct effects of 1,25(OH)2D on MACs were investigated followed by studies of using models of MAC-endothelial cell interaction. These latter models utilised MAC-conditioned media to determine the effects of soluble factors secreted by MACs on HAoEC function. The
final section of this chapter describes studies designed to identify vitamin D-regulated molecules secreted by MACs, which may regulate *in vitro* angiogenesis.

The first models utilised HC and SLE MACs at day 8 (differentiated in the presence or absence of IFNα or vitamin D as appropriate) and the second used the conditioned media from MACs at day 8. Figure 6-1 summarises the experimental design and derivation of both MACS and conditioned media.

The focus of this work is the effect of 1,25(OH)$_2$D$_3$ on MACs obtained from vitamin D deficient SLE patients. All MACs used were from patients with 25(OH)D<20ng/ml, recruited as part of the observational study, unless otherwise stated. Control MACs were obtained from HCs with 25(OH)D >20ng/ml.

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**Figure 6-1: Conditions used to derive and treat SLE and healthy control MACs**

All MACs were isolated from PBMCs and cultured for a total of 7 days on human fibronectin. The day on which the cells were plated onto fibronectin was termed Day 1. SLE MACs were cultured in the presence or absence of 10nM 1,25(OH)$_2$D$_3$ and healthy control MACs in the presence of absence of 0.1ng/ml IFNα2b (and 1,25(OH)$_2$D$_3$ for some experiments). Cell culture media was changed after 4 days and any treatments were replaced. The conditioned media was generated over 3 days and removed at day 8 (represented by *).

HC = healthy control, D1,D5,D8 = day 1, day 5, day 8
6.4 MACs express the vitamin D receptor and undergo morphological change in response to vitamin D

Expression of the VDR was determined in MACs by Western blotting. Protein was obtained from PBMCs prior to plating onto fibronectin and then from MACs after 2, 4 and 8 days of culture. This provided samples at different stages of MAC differentiation. Samples were compared within individual donors. HAoECs were also included as a comparison cell type. The expression of the VDR was compared to that of a control protein (GAPDH) and densitometry was used to determine the relative expression. Expression of the VDR was notably increased after 24 hours culture (day 2) but had decreased by day 4 (figure 6-2). After 7 days in culture VDR expression was similar to that in HAoECs and PBMCs.

Figure 6-2: Expression of the VDR in MACS
The expression of the VDR in MACs was investigated by Western blot. MACs at day 2 show a marked increase in VDR expression compared to the PBMCs from which they are derived. Expression was lower at day 4, and reduced further still by day 8. A representative blot to show the VDR (approximately 50kDa) and GAPDH (37kDa) (a). Densitometry of the VDR bands relative to GAPDH shows that expression at day 2 is around 15-fold higher than in PBMCs (b). Expression at day 8 and in untreated HAoEC is negligible. The columns show the mean expression relative to GAPDH, combined results from n=2 experiments (n=1 for D4).

MACs from patients with SLE underwent morphological changes when cultured with $1,25(OH)_2D$ for 7 days. Lupus MACs typically exhibit irregular “fried egg” morphology with a central nucleus. Incubation with vitamin D resulted in more spherical cells. The difference in morphology was statistically significant when quantified in n=5 subjects (p=0.002) (figure 6-3).
Fluorescent microscopy was used to identify changes in MAC morphology after incubation with vitamin D. MACs at day 8 were labelled with dil-AC-LDL (red) and imaged in 3 random fields per well. Representative images are shown in (a), (i) and (ii). ImageJ software was used to produce false-colour binary images of the cells (ii and iv). The cells incubated with 10nM 1,25(OH)$_2$D$_3$ appeared more spherical (iii, iv) than those incubated with vehicle (i, ii). ImageJ was also used to measure the “roundness” of the cells using a standard algorithm (b). The columns show the mean roundness of MACs from n=5 vitamin D deficient lupus patients and the error bars show the standard error. The graph shows pooled results from 3 random fields per well in triplicate for each condition. Comparison was made by paired t test, **p<0.01.

The changes in morphology suggest that vitamin D may alter the phenotype of the MACs. As described in chapter 5, MACs express surface markers consistent with an M2 (alternatively-activated) phenotype. RT-qPCR as described previously was used to compare expression of surface markers between healthy control MACs and SLE MACs in the presence/absence of 10nM 1,25(OH)$_2$D$_3$. CD68 was selected as a global macrophage marker. CD86 and CCR7 were used to identify cells polarised towards the M1 phenotype and CD206 to identify M2 cells. Of the 4 markers used, there were significant changes in the expression of CD206 and CD68. Both of these markers were expressed at significantly higher levels in lupus MACs compared to HC MACs. The incubation of lupus MACs with vitamin D reduced expression of CD206 and CD68 such that there was no difference between HC and vitamin D-treated lupus MACs (figure 6-4). CCR7 levels were low, making comparison difficult however the same trend was seen.
MAC surface marker expression was measured by RT-qPCR. The columns show the relative mean expression normalised to 2 reference genes (ATP5B and CYC1) and the error bars show standard error. The results are combined from n=4 HC and n=5 vitamin D deficient SLE subjects. Analysis was by 2-way ANOVA with Dunnet’s test for multiple comparisons. Expression was compared against the HC sample for each surface marker. Vitamin D deficient SLE MACs expressed significantly higher CD206 and CD68, and a trend toward lower CD86. Incubation of MACs with 10nM 1,25(OH)$_2$D$_3$ changed the expression profile toward that of HC MACs, ***p<0.001, ****p<0.0001.

The change in morphology and reduced expression of CD206 and CD68 suggests that vitamin D may inhibit PBMC differentiation into macrophages. If the effect of vitamin D was to drive polarisation from a M2 to M1 phenotype then a compensatory increase in CCR7 would be expected. All markers measured were reduced by 1,25(OH)$_2$D$_3$ which suggests a global reduction in macrophage differentiation.

A systems approach was then used to investigate whether vitamin D may have effects on macrophage development, activation or polarisation. Kupfer et. al. (2013) performed transcriptional profiling on PBMCs from 12 healthy subjects in the presence or absence of 10nM 1,25(OH)$_2$D$_3$ for 24 hours (550). An Illumina HumanHT-12 v3 Expression Array was used and the normalised dataset is freely available in the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo, accession number GSE50012). Samples were selected and analysed using GEO2R software to identify transcripts which were differentially expressed between PBMCs treated with 1,25(OH)$_2$D$_3$ and vehicle (ethanol). The resultant dataset was explored using Ingenuity IPA Software (Qiagen, Redwood City, USA). Differentially regulated genes were defined as those with a log-fold change of 0.39 with p≤0.01.
Analysis of the genes by biological function showed that the group containing the largest number of regulated genes was “haematological system development and function”. Other large groups included “cellular movement”, “inflammatory response” and “immune cell trafficking” (figure 6-5).

Figure 6-5: Heatmap of gene expression in PBMCs in response to vitamin D
A heatmap generated using Ingenuity IPA software to show the major clusters of genes regulated by 1,25(OH)$_2$D$_3$ in PBMCs. The families are generated from a database of biological interactions. The largest group regulated by vitamin D is “haematological system development and function” (highlighted in yellow). The colours represent the z-scores. Darker blues identify clusters of genes which are mostly down-regulated in the dataset, whilst darker reds show clusters with more up-regulated genes.

Within the largest family, there was an over-representation of genes in the group “differentiation of macrophages” (z-score -2.212$^2$, -log p =6.914). Network analysis of genes involved in the “differentiation of macrophages” group allowed the identification of individual genes. Figure 6-6 shows those genes which belong to the group and the effect of their regulation on the biological process of macrophage differentiation. The majority of genes in this group were down-regulated by 1,25(OH)$_2$D$_3$ and the overall effect was towards inhibition of differentiation. The genes CAMP and PPARG are both inhibitors of differentiation and so up-regulation of these still results in an overall suppression of the biological process. The genes in this group are described briefly in table 6-1.

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$^2$ The z-score represents the difference between observed and predicted values where < -2 or >2 is significant. The z score is, however, only a measure of the direction of change and not the magnitude of the change.
Analysis of microarray data from healthy subject PBMCs treated with 10nM 1,25(OH)$_2$D$_3$ for 24 hours (550). Comparison between treated and untreated PBMCs and subsequent network analysis using Ingenuity IPA software (Qiagen, Redwood City, USA) showed a significant reduction in expression of genes involved in macrophage activation. Genes in green were down-regulated and those in red genes were up-regulated by more than 2-fold. The intensity of the red/green colour shows the magnitude of the change. Blue lines show that the state of the gene leads to inhibition of macrophage differentiation. Yellow lines show that the effects are inconsistent with the state of the downstream molecule. Grey lines identify effects that could not be predicted.*genes with multiple identifiers within the dataset.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Name</th>
<th>Fold Change (log ratio)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>Cluster of differentiation 40</td>
<td>-0.937</td>
<td>Receptor for CD40L which induces macrophage activation</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Colony stimulating factor 1 receptor (CD115)</td>
<td>-0.770</td>
<td>Tyrosine kinase transmembrane receptor which controls differentiation and function of macrophages</td>
</tr>
<tr>
<td>CSF2RA</td>
<td>Colony Stimulating Factor 2 Receptor, Alpha</td>
<td>-1.234</td>
<td>Ligand-binding subunit of GM-CSF receptor. Activation of precursors leads to granulocyte production</td>
</tr>
<tr>
<td>FES</td>
<td>Feline sarcoma oncogene</td>
<td>-0.607</td>
<td>Regulation of actin cytoskeleton, microtubules and cell attachment</td>
</tr>
<tr>
<td>ID2</td>
<td>Inhibitor of DNA binding 2</td>
<td>-0.440</td>
<td>May be an inhibitor of tissue-specific gene expression. Positively regulates macrophage differentiation</td>
</tr>
<tr>
<td>IL-15</td>
<td>Interleukin 15</td>
<td>-0.508</td>
<td>Cellular proliferation and maturation</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
<td>-2.353</td>
<td>Differentiation of monocytes to macrophages</td>
</tr>
<tr>
<td>IRF7</td>
<td>Interferon regulatory</td>
<td>-0.487</td>
<td>Monocyte to macrophage differentiation</td>
</tr>
</tbody>
</table>

*Figure 6-6: Vitamin D regulated genes in PBMCs associated with reduced macrophage differentiation*
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Effect</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP9</td>
<td>Matrix metalloproteinase 9</td>
<td>-1.596</td>
<td>Expressed by macrophages on activation</td>
</tr>
<tr>
<td>PRDM1</td>
<td>PR domain containing 1, with ZNF domain</td>
<td>-0.760</td>
<td>Transcriptional repressor – binds to DNA through zinc fingers, promotes macrophage differentiation</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma 1</td>
<td>-0.0473</td>
<td>Tumour repressor gene, regulated macrophage differentiation by unknown mechanism</td>
</tr>
<tr>
<td>TLR1</td>
<td>Toll-like receptor 1 (CD281)</td>
<td>-0.575</td>
<td>Macrophage activation by Gram-positive bacteria. Up-regulated during macrophage differentiation</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokine signalling 1</td>
<td>-0.577</td>
<td>Negative regulator of macrophage differentiation. Role in M2 polarisation of macrophages</td>
</tr>
<tr>
<td>TLR6</td>
<td>Toll-like receptor 6</td>
<td>-0.651</td>
<td>Macrophage activation by Gram-positive bacteria</td>
</tr>
<tr>
<td>TNFSF10</td>
<td>Tumour necrosis factor superfamily, member 10, CD253, TNF-related apoptosis-inducing ligand (TRAIL)</td>
<td>-1.092</td>
<td>Induces cellular apoptosis. Macrophage lipid uptake. Role in macrophage differentiation not clear</td>
</tr>
</tbody>
</table>

**Up-regulated**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Effect</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARG</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
<td>1.462</td>
<td>Primes macrophages towards M2 differentiation</td>
</tr>
<tr>
<td>CAMP</td>
<td>Cathelicidin antimicrobial peptide</td>
<td>2.624</td>
<td>Anti-microbial peptide. Uncertain role in macrophage differentiation</td>
</tr>
</tbody>
</table>

**Table 6-1: Genes regulated by vitamin D which suppress macrophage activation**

The table includes only those genes which inhibit of macrophage differentiation or in which the effect could not be predicted from the dataset.

### 6.5 Effect of vitamin D on MAC survival *in vitro*

The effects of 1,25(OH)\(_2\)D\(_3\) on MAC survival *in vitro* were investigated i) using MACs from 25(OH)D-deficient SLE patients and ii) using an IFN\(\alpha\) model of HC MACs.

The number of MACs was not significantly different between vitamin D deficient (<20ng/ml) and vitamin D replete (>30ng/ml) SLE patients, although there was a trend toward a higher number of MACs in the replete population. There was however marked variability in MAC number, particularly within the deficient patients, with a range of 34.2 to 257.3 cells per random field. The mean (sd) number was 162.2 (75.3) in deficient patients and 194.4 (66.1) in replete patients (p=0.223). MACs from deficient patients were cultured in increasing concentrations of 1,25(OH)\(_2\)D\(_3\) (from 0.1nM to 100nM) to determine whether there was a dose-dependent effect on MAC number. The effect on MAC number was variable and was strongly influenced by the number of cells as baseline. Splitting the group by those with MACs<150 per field and >150 per field at baseline showed a significant increase in those with lower numbers (p=0.04) and a trend towards a smaller decrease (p=0.09) in those with higher numbers at baseline. Further analysis showed a strong negative correlation between the
number of cells at baseline and the increase in number in response to 10nM 1,25(OH)₂D₃ (r=-0.754, p=0.0098).

**Figure 6-7: Effect of incubation with vitamin D on number of MACs from SLE patients**

The mean number of MACs at baseline was not different in vitamin D deficient and vitamin D replete SLE patients (a). In vitamin D deficient patients there was a variable response to 1,25(OH)₂D₃ at concentrations of 0.1-100nM (b). Subjects with lower numbers of MACs at baseline tended to have higher numbers after incubation with vitamin D, whilst those with higher numbers showed little change. Stratifying the group by baseline number of MACS greater or less than 150/field shows a significant increase in number of MACs in those <150 at baseline, and small non-significant downward trend in those >150. The increase in number of MACs in response to 10nM vitamin D is strongly negatively correlated with the number of MACs at baseline. Panel (d) shows a scatter plot of the change in number in response to vitamin D against the number at baseline. In panel (a) the columns show mean number of MACs and the points show the values for individual patients. Each point represents the mean number of 3 random fields per well with 3 wells per condition. Comparisons were made by unpaired t tests in (a), *p<0.05. In (b) and (c) the points show the mean number of cells from n=11 SLE patients and the error bars show standard error. In (c) the lines show linear regression models and (d) shows the Spearman r correlation coefficient.

An increase in MAC number in response to 1,25(OH)₂D₃ was detected in SLE patients who were vitamin D deficient at baseline, but not in patients with 25(OH)D>30ng/ml. The mean (sd) number of MACs (per random field) in deficient patients increased from 159.1 (75.3) to 194.5 (44.6) (p=0.044), but slightly decreased in replete patients: 193.0 (69.2) to 179.5 (67.4) (p=0.116) (figure 6-8). There was no association between the serum level of 25(OH)D and the response to 1,25(OH)₂D₃ in vitro (data not shown).
Figure 6-8: Effect of vitamin D in vitro on MAC number in deficient and replete SLE patients

The number of MACs from vitamin D deficient SLE patients is significantly increased by incubation with 10nM 1,25(OH)\textsubscript{2}D\textsubscript{3}, whilst no difference is observed in the number of MACs from replete patients. The columns show the mean number of MACs per random field and the points show the values for individual subjects. Each point shows the mean from 3 random fields per well in triplicate for each condition. The mean numbers with and without vitamin D were compared using a paired t-test, *p<0.05.

To validate the above results and to try to minimise some of the variability seen in MAC number, 1,25(OH)\textsubscript{2}D\textsubscript{3} was also tested in an IFNα model. HC MACs were isolated and cultured with 0.1ng/ml IFNα2b ± 0.01-100nM 1,25(OH)\textsubscript{2}D\textsubscript{3} for 7 days. In contrast to the variable effect found in SLE, the number of MACs increased in a dose-dependent manner (figure 6-9). When compared to cells with IFNα alone, vitamin D significantly increased MAC numbers at concentrations from 0.1nM to 100nM. A three parameter dose-response curve was fitted with $r^2=0.508$. The EC\textsubscript{50} of the curve was 4.821nM.
Figure 6-9: Dose-dependent effect of vitamin D on MAC survival in an IFNα-model
Representative images of HC MACs at day 8 after incubation with IFNα2b (0.1ng/ml) for 7 days ± 1,25(OH)₂D₃ (a). The number of MACs was increased in the presence of increasing concentrations of 1,25(OH)₂D₃. MACs were quantified on the basis of dil-Ac-LDL uptake (red) and cell nuclei were stained with DAPI (blue). Images at 10x magnification, scale bar = 50µm. A dose response-effect on MAC number was demonstrated with increasing concentrations of 1,25(OH)₂D₃ (b). The number of MACs is expressed relative to the number enumerated in untreated wells (untreated = 1). The fitted curve has an EC₅₀ of 4.82nM. The points show the mean number of MACs and the bars show standard error. Combined results of n=5 independent experiments. In each experiment 3 random fields per well were enumerated in triplicate. Paired t-tests were used to compared mean number between each concentration of vitamin D and MACs cultured without vitamin D, ***p<0.001, ****p<0.0001.

6.6 Vitamin D MAC migration and adhesion

MAC function was also measured in terms of migration and adhesion using both SLE MACs and an IFN-model (as described in Chapter 5). All cells were incubated with and without 10nM 1,25(OH)₂D₃ for 7 days before the functional assay was performed.

To assess migration, cells were placed in FBS-free EGM (containing 1% BSA) and were placed in the top section of a fibronectin-coated Transwell chamber. Migration was measured towards 100ng/ml SDF-1α (chemotaxis) or basal media alone (chemokinesis) over 8 hours. For each condition, the relative migration was determined by the ratio of chemotaxis:chemokinesis. The effects of IFNα on HC MAC migration/adhesion have been described above. There was no
difference in the migratory capacity of MACs from vitamin D deficient and replete patients (data not shown). Vitamin D had no significant effect on MAC migration in either the IFN model or on MACs from vitamin D-deficient patients (p=0.535, p=0.375 respectively). There was a trend towards increased migration in lupus MACs in response to vitamin D but this did not reach statistical significance over n=6 independent experiments (1.20 vs. 1.57, p=0.375).

Adhesion was determined using the TNF-activated HAoEC model described previously. Briefly, fluorescent-labelled MACs were allowed to adhere to HAoECs which had been previously incubated with and without 10ng/ml TNFα. The number of adherent MACs was enumerated in random fields and normalised to the number adherent to non-activated HAoEC (to obtain the relative number of adherent cells). Similarly to the results for migration, incubation with 1,25(OH)₂D₃ had no effect in either model (p=0.452 for the p=0.236 for lupus MACs) (figure 6-10).

**Figure 6-10: Vitamin D and MAC migration and adhesion in SLE MACs and an IFN Model**

In the IFN model of HC MACs, the addition of 10nM 1,25(OH)₂D₃ had no effect on MAC migration towards SDF-1α or adhesion to TNFα-activated endothelium (a,b). Similarly, MACs from vitamin D deficient SLE patients did not significantly change in terms of either migratory or adhesive capacity after incubation for 7 days with 10nM 1,25(OH)₂D₃ (c,d). In all graphs the columns represent the mean number of cells and the error bars show standard error. Graphs show combined results from n=5 and n=6 independent experiments of migration (for IFN model and SLE respectively) and n=4 experiments for adhesion. Comparisons were made by paired t-tests.
6.7 Vitamin D and MAC-endothelial interaction

Following the investigation of MAC function in response to vitamin D, the effects on MAC-endothelial cell interactions were studied. As described above, MACs can migrate towards endothelial cells, but do not differentiate into mature endothelial cells, or become fully incorporated into developing endothelial networks. Modulation of endothelial function therefore requires secretion of factors by MACs in a paracrine manner. To assess this, the conditioned media from MACs at day 8 was used. As the cell culture media is changed after 4 days, the media on day 8 therefore contains the factors secreted by MACs over a period of approximately 72 hours. The effect of this media was studied in terms of regulation of endothelial nitric oxide synthase (eNOS) and in the in vitro model of angiogenesis.

6.7.1 MACs and nitric oxide synthase expression

A model was developed to determine the effects of day 8 MAC-conditioned media from SLE MACS isolated with and without 1,25(OH)₂D₃ on eNOS expression in HAoECs. All HAoECs were serum-starved in EGM-1% FBS for 3 hours prior to incubation with the conditioned media for 6 hours. This approach is summarised in figure 6-11. In order to replicate a disease state and look for a potential protective effect, TNFα (10ng/ml) was used in some experiments to down-regulate eNOS expression.

Figure 6-11: Investigation of MAC conditioned media on the expression of eNOS in HAoECs

MACS were derived from vitamin D deficient SLE patients after culture for 7 days in the presence or absence of 10nM 1,25(OH)₂D₃. After this time, the conditioned media was added to HAoECs for 6 hours with or without 10ng/ml TNFα. Controls were generated by using EGM-20% FCS with and without 10ng/ml TNFα or 10nM 1,25(OH)₂D₃. After 6 hours RNA was extracted from the cells or protein lysates generated as described in Chapter 3. Gene expression was analysed by RT-qPCR and protein expression by Western Blot.
Similar to the studies of gene expression in MACs, a reference gene assay was used to identify suitably stable genes in HAoECs. Samples of HAoECs treated with TNFα (n=3), MAC-media alone (n=3), TNF and MAC media (n=3), or unstimulated (n=3) were used to compare the expression of 6 candidate reference genes. The genes ATP5B and CYC1 were both stably expressed in HAoECs (figure 6-12). No additional benefit was gained from using 3 reference genes instead of 2 (data not shown).

**Figure 6-12: Identification of stable reference genes in HAoECs**
A GeNorm reference assay was undertaken to identify which reference genes were most stable in HAoECs treated with TNFα and MAC conditioned media. The graph shows the M-value (average gene stability compared to the other genes) across the total of n=12 samples, ATP5B and CYC1 were the most stably expressed genes. The M-values were low suggesting that the genes were highly stable.

In unstimulated HAoECs, 10nM 1,25(OH)₂D₃ alone had no effect on eNOS expression after 6 hours (p=0.440). Both SLE MAC- and vitamin D-treated SLE MAC-conditioned media resulted in a slight increase in eNOS expression which was not statistically significant (p=0.440 and p=0.910 respectively). TNFα (10ng/ml) significantly reduced eNOS expression in HAoECs with a mean (sd) relative expression of 0.324 (0.141) (p=0.014) compared to control HAoECs. In this model, both SLE MAC- and vitamin D-treated SLE MAC-conditioned media significantly attenuated TNF-induced eNOS down-regulation (p=0.0485 and p=0.0249 respectively). In addition, there was a significant difference in eNOS expression between HAoECs incubated
with the conditioned media of untreated and vitamin D-treated MACs (relative eNOS expression 0.393 [0.178] vs. 0.465 [0.219], p=0.0113) (figure 6-13).

![Figure 6-13: Changes in HAoEC eNOS expression in response to MAC-conditioned media](image)

In the first experiments, the expression of eNOS in unstimulated HAoECs was determined using RT-qPCR and the ddCT method adjusting for the reference genes CYC1 and ATP5B. Vitamin D alone (in growth media) had no effect on eNOS expression. Although there was a trend towards increased expression with MAC-conditioned media (both with and without vitamin D) this was not statistically significant (a). TNFα down-regulated eNOS expression in HAoECs. Vitamin D alone did not significantly change eNOS expression. eNOS expression was increased in HAoECs incubated with MAC-conditioned media, with significantly higher levels with vitamin D-treated MAC media (b). In both (a) and (b) the columns show mean expression and the bars show standard error for n=3 independent experiments using conditioned media from n=3 vitamin D deficient SLE patients. Comparisons were made by ratio-paired t-tests as shown and conditions to be compared were identified a priori, *p<0.05, **p<0.01.

To support the changes in eNOS at the transcript level, western blotting was undertaken to investigate the correlative changes in protein expression. The same experimental conditions as described above were used (with TNFα-induced down-regulation of eNOS). There was a trend towards down-regulation of eNOS in TNF-treated compared to control HAoECs (relative expression=0.597, p=0.0507). Similarly, conditioned media from vitamin D-treated and untreated lupus MACs resulted in a trend towards increased eNOS expression which was not statistically significant from TNFα alone (p=0.141 and p=0.525 respectively). There was
however a significant difference between paired samples of vitamin D treated and untreated MACs (p=0.0098) (figure 6.14).

Figure 6-14: Changes in eNOS protein expression in HAoECs by MAC-conditioned media
Changes in the expression of eNOS at the protein level were determined by Western Blotting. The experimental design as described in figure 6-16 was used. A representative image is shown in panel (a). The lanes contained the following samples and correspond to the graph in panel (b): 1) Untreated, 2) TNF alone, 3) TNF and vitamin D, 4) TNF and SLE MAC conditioned media and 5) TNF and vitamin D-treated SLE MAC conditioned media. The band densities were quantified using ImageJ software and normalised such that untreated=1. The SLE MAC-conditioned media trended towards increased eNOS expression compared to TNFα alone. eNOS expression was significantly higher in HAoECS treated with vitamin D-treated MAC conditioned media compared to MACs not treated with vitamin D. Vitamin D alone did not change eNOS expression. The columns show the mean relative eNOS expression from n=4 independent experiments and the error bars show standard error. Comparisons were made by ratio paired t-tests as above, **p<0.01.

6.7.2 Vitamin D and MAC-mediated angiogenesis
The Matrigel model of in vitro angiogenesis described in Chapter 5 was used to identify whether incubation with 1,25(OH)2D3 modified the angiogenic capacity of MACs. The conditioned media of MACs (cultured with and without 10nM 1,25(OH)2D3) was compared to EGM-20% FCS alone (with a control comprising EGM plus vitamin D). The network density was quantified in terms of total number of pixels and closed loops as these parameters showed the greatest differences between SLE and HC MACS.

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The differences between the treatment groups were more marked when the network was quantified in terms of closed loops than total pixel area. Vitamin D alone had no effect on the number of closed loops and a very small (but statistically significant effect) on the number of pixels (p=0.0103). SLE-MAC conditioned media significantly increased the network density by both parameters (p=0.0138 for total pixels and p=0.0007 for closed loops). A comparison of MAC-conditioned media showed that vitamin D significantly increased the angiogenic capacity of lupus MACS in terms of closed loops (p=0.023) but not total pixels (p=0.661) (figure 6-15).

**Figure 6-15: Vitamin D and MAC-mediated HAoEC angiogenesis**

HAoECs were placed on Matrigel in conditioned media from MACs cultured with and without 10nM 1,25(OH)$_2$D$_3$ for 14 hours. The controls were growth media ± 1,25(OH)$_2$D$_3$. Network density was quantified as described in Chapter 5. Vitamin D alone had a very small but statistically significant effect on total network area (in terms of pixels) (a). MAC conditioned media also significantly increased the number of pixels but media from MACs cultured with vitamin D did not result in a larger network. The number of pixels or closed loops were normalised to growth media=1. In terms of closed loops, MAC-conditioned media significantly increased network density with a further significant increase when MACS were incubated with vitamin D. Vitamin D alone had no effect on the number of closed loops. In both graphs, the columns show mean of n=8 independent experiments (conditioned media) and n=3 experiments (vitamin D alone) and the error bars show standard error. Comparisons were made between predetermined groups using paired t tests, *p<0.05, ***<p<0.001.
As described above, 1,25(OH)₂D₃ had a significant effect on the number of MACs at day 8 in a manner that was dependent upon the baseline number of MACs. It was postulated that the increase in the angiogenic capacity in the Matrigel model may be due to an increase in MAC numbers.

There was no correlation between the number of MACs and the relative network density (figure 6-16). The effect of 1,25(OH)₂D₃ was therefore unlikely to be due to changes in cell number but may reflect changes in the expression of pro-angiogenic factors within the MAC population.

**Figure 6-16: Correlation between number of MACs and *in vitro* angiogenesis**

No correlation was seen between the number of MACs and the extent of angiogenesis in the Matrigel model either in terms of the raw number of closed loops (a) or relative angiogenesis expressed as closed loops normalised to growth media alone=1 (b). The Spearman \( r \) correlation coefficient was used in both (a) and (b).

### 6.7.3 Vitamin D and the angiogenic profile of MACs

The angiogenic profile of both HC and SLE MACs was studied using a multiplex assay. The Bio-Plex Pro Human Angiogenesis Assay is a 9-plex panel of common angiogenic factors: Vascular endothelial growth factor-A (VEGF-A), platelet-derived growth factor-BB (PDGF-BB), platelet endothelial cell adhesion molecule-1 (PECAM-1), interleukin-8 (IL-8), hepatocyte growth factor (HGF), leptin, follistatin, angiopoietin 2 and granulocyte-colony stimulating factor (G-CSF).

These factors were measured in both HC MAC-conditioned media and in EGM-20% FCS alone (to exclude contribution of factors in the FBS or in the bovine brain extract added to the media). None of the 9 factors in the assay were added to EGM as a recombinant growth factor. Of the 9 factors measured, all were detected in the conditioned media with the exception of angiopoietin-2. The conditioned media contained significantly higher concentrations of VEGF-A, PDGF-BB, PECAM-1 and HGF with a non-significant trend towards higher concentration of IL-8, leptin and follistatin (figure 6-17). There were only very low
concentrations of G-CSF in conditioned media which was not significantly different from EGM alone.

**Figure 6-17**: Expression profile of pro-angiogenic factors by healthy MACs

The expression of pro-angiogenic factors was measured in the media of MACs and compared to EGM-20% FCS. Angiopoietin-2 was not detectable in any sample (data not shown). MACs secreted significantly higher levels of VEGF-A, PDGF-B, PECAM-1 and HGF, with a trend towards increased IL-8, leptin and follistatin. The columns show the mean concentration in pg/ml on a logarithmic scale and the bars show standard error. A total of n=3 growth media samples and n=8 healthy MAC samples were analysed. Comparisons were made by t test, *p<0.05, **p<0.01.

The expression profiles were then compared using conditioned media (at day 8) from untreated and vitamin D-treated lupus MACs and untreated and IFNα-treated HC MACs. A low concentration (0.1ng/ml) IFNα2b was used to module the MAC response without causing significant reduction in cell number. There was no significant difference in the expression of any of these factors between HC and SLE MACs (figure 6-18). In lupus MACs there was little change in the secretion of pro-angiogenic factors in response to 10nM 1,25(OH)2D3. The exception was a significant reduction in follistatin from a mean (sd) 42.9 (25.7) to 21.7 (12.9) pg/ml (p=0.044). In contrast, there was no difference in follistatin concentration between HC and IFN-treated HC conditioned media (29.6 [16.0] vs. [28.8 [14.4] pg/ml, p=0.905).

Whilst 1,25(OH)2D3 had only a modest effect on lupus MACs, IFNα resulted in significant changes in the expression of 4 angiogenic factors by HC MACs. There was a significant reduction in the concentration of HGF when MACs were cultured with IFN (1584 [664] vs. 646.6 [269.4] pg/ml, p=0.0017). The other factors which were changed by IFN were all increased: VEGF-A (p=0.016), leptin (p=0.049) and IL-8 (p=0.021).
Figure 6-18: Secretion of pro-angiogenic factors in response to vitamin D and interferon-alpha.

The concentration of pro-angiogenic factors in the media of SLE and HC MACs cultured in the presence/absence of 10nM 1,25(OH)2D3 or 0.1ng/ml IFNα2b was measured using a multiplex array. Paired samples were measured from MACs derived from n=10 SLE patients and n=8 healthy controls. HC MACs treated with IFNα expressed significantly lower levels of HGF and increased levels of IL-8, leptin and VEGF-A. Vitamin D resulted in a statistically significant reduction in the expression of follistatin. No differences were seen between any of the conditions and levels of PECAM-1 or GCSF (data not shown). The graph shows the mean values for each cytokine. Comparisons were made by paired (SLE MAC ± vitamin D, and HC ± IFNα) or unpaired (SLE MAC vs. HC MAC) t tests, *p<0.05, **p<0.01.
6.7.4 Identification of other factors regulated by 1,25(OH)$_2$D$_3$ in SLE MACs

A bioinformatics approach was taken to identify potential angiogenic factors which could be regulated by vitamin D in SLE MACs. The dataset from Kupfer et. al. (2013) described above was used to identify which secrete pro-angiogenic factors were up-regulated by 1,25(OH)$_2$D$_3$ and which anti-angiogenic factors were down-regulated in PBMCs after 24 hours. The factors up- and down-regulated are shown in table 6-2.

| Anti-angiogenic Factors Down-Regulated in Healthy PBMCs                        |
|-------------------------------|--------------------------|----------------|-----------------|----------------|
| Gene Symbol | Name                          | Log Fold Change | p value         | Effect                                      |
| APOE         | Apolipoprotein E              | -0.7           | 4.72 x 10$^{-4}$ | Binds to HSPGs on cell surface, blocking binding of growth factors e.g. VEGF |
| THBS1        | Thrombospondin 1             | -2.62          | 2x10$^{-4}$    | Inhibits endothelial cells adhesion, motility and growth. It also interacts with numerous proteases involved in angiogenesis, including plasminogen, urokinase, matrix metalloproteinase, thrombin, cathepsin, and elastase |
| CXCL9        | Chemokine (C-X-C motif) ligand 9 | -2.9         | 7.7310$^{-6}$  | Reduced VEGFR2 (KDR), phospholipase Cy (PLCγ), and extracellular signal-regulated kinase (ERK) phosphorylation - a direct counter-regulatory molecule of VEGF signalling |
| GSN          | Gelsolin                     | -1.56          | 1.53x10$^{-10}$ | Regulator of actin cross linkage – role in angiogenesis not clear |
| MMP12        | Matrix metallopeptidase 12 (macrophage elastase) | -0.54 | 0.00732 | MMP-12 is a key regulator of macrophage infiltration and inflammation, contributing to retinal vascular dysfunction and pathological angiogenesis MMP-12 may block angiogenesis by converting plasminogen to angiostatin, which is one of the most potent angiogenesis antagonists. |
| VASH1        | Vasohibin 1                  | -0.715         | 1.11x10$^{-7}$ | Inhibits migration, proliferation and network formation by endothelial cells as well as angiogenesis. This inhibitory effect is selective to endothelial cells as it does not affect the migration of smooth muscle cells or fibroblasts. |
| CXCL10       | Chemokine (C-X-C motif) ligand 10, IP10 | -2.88         | 3.85x10$^{-8}$ | Inhibits endothelial cell proliferation. Inhibits basic fibroblast growth factor-induced neovascularization of Matrigel |
injected subcutaneously into athymic mice. In addition suppresses endothelial cell differentiation into tubular capillary structures in vitro. No effect on endothelial cell growth, attachment, and migration as assayed in vitro.

CCL13 Chemokine (C-C motif) ligand 13 -3.71 2.86x10^{-13} Important for leucocyte recruitment – role in angiogenesis not clear

### Pro-angiogenic Factors Up-Regulated in Healthy PBMCs

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Name</th>
<th>Log Fold Change</th>
<th>P value</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMC1</td>
<td>Laminin, gamma 1</td>
<td>1.20</td>
<td>2.49x10^{-7}</td>
<td>Increases tubule formation by endothelial cells – important component of Matrigel</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
<td>1.04</td>
<td>0.0019</td>
<td>Recruit HUVECs and stimulate angiogenesis in tumour cells</td>
</tr>
<tr>
<td>HTRA1</td>
<td>HtrA serine peptidase 1</td>
<td>2.27</td>
<td>1.29x10^{-4}</td>
<td>Regulation of retinal angiogenesis – cleaves IGF-binding proteins</td>
</tr>
<tr>
<td>ORM1</td>
<td>Orosomucoid 1</td>
<td>2.76</td>
<td>1.01x10^{-6}</td>
<td>Stimulates angiogenesis in the CAM assay</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
<td>2.72</td>
<td>7.88x10^{-12}</td>
<td>Induces angiogenesis in vitro and in vivo – increased endothelial proliferation, increased migration, increased tubule formation of dermal microvascular cells but not HUVEC.</td>
</tr>
<tr>
<td>GREM1</td>
<td>Gremlin 1, DAN family BMP antagonist</td>
<td>0.65</td>
<td>2.41x10^{-4}</td>
<td>Novel agonist at VEGFR2</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Chemokine (C-X-C motif) ligand 12 (SDF-1)</td>
<td>0.59</td>
<td>1.1x10^{-6}</td>
<td>Stimulates cell proliferation and capillary tube formation, attenuates HMEC apoptosis.</td>
</tr>
<tr>
<td>PLAU</td>
<td>Plasminogen activator, urokinase (uPA)</td>
<td>0.739</td>
<td>4.12x10^{-4}</td>
<td>Higher endothelial levels of uPA correlate with increased angiogenic capacity.</td>
</tr>
</tbody>
</table>

Table 6-2: Up-regulated pro-angiogenic factors and down-regulated anti-angiogenic factors in PBMCs in response to vitamin D.

Of the above molecules, IP10 was selected for further investigation because its expression has recently been associated with both IFNα expression and disease activity in patients with SLE (551). Furthermore, IP10 has been shown to inhibit angiogenesis both of HUVECs in vitro and in an in vivo Matrigel plug model (552).

Confirmation that IP10 was regulated by 1,25(OH)$_2$D$_3$ was sought by measuring the concentration of IP10 in the conditioned media of MACs. An ELISA was performed using media
from day 8 (thus containing factors secreted over a 72 hour period) from SLE MACs (± vitamin D) and HC MACs. The ELISA has a detection limit of 2pg/ml, any samples below this limit were arbitrarily assigned a value of 1pg/ml (0.5x the lower limit of detection). There was a trend towards lower IP10 from HC MACs compared to SLE (15.1 [32.5] vs. 36.0 [91.8] pg/ml, p=0.127). Culture of SLE MACs with 10nM 1,25(OH)₂D₃ resulted in a significant reduction in IP10 (36.0 [91.8] vs. 12.9 [36.0] pg/ml, p=0.0006) (figure 6-19).

The final stage was to determine whether blockade of IP10 could increase the angiogenic capacity of SLE MACs. The same in vitro Matrigel model as described above was used. Conditioned media from n=4 deficient SLE patients cultured in the presence/absence of 1,25(OH)₂D₃ was added to the assay along with 2.5µg/ml neutralising anti-IP10 antibody. Network formation was assessed at 14 hours as described above. As demonstrated previously, both lupus MAC and vitamin D-treated MAC media significantly increased the number of closed loops (p<0.0001 for both). The effect of MAC media was significantly increased by IP10 blockade in untreated MACs (p=0.015) but not those incubated with vitamin D (p=0.238) (figure 6-20). Furthermore, the difference between vitamin D-treated MACs and untreated MACs was no longer detected in the presence of anti-IP10 antibody.

Figure 6-19: Secretion of IP10 by SLE MACs in response to vitamin D
Levels of IP10 were measured by ELISA in the media from vitamin D-deficient SLE MACs at day 8. There was a trend towards higher levels of IP10 in the media from vitamin D deficient SLE patients compared to healthy controls (p=0.127) (a). Incubation with 10nm 1,25(OH)₂D₃ significantly reduced the concentration of IP10 (b). The columns show the mean concentration of IP10 and the points show results for individual subjects. Healthy and SLE values were compared using a t test and SLE ± vitamin D samples compared using a ratio paired t test, ***p<0.001.
**Figure 6-20**: Effect of IP10 blockade on SLE MAC function in an *in vitro* model of angiogenesis
IP10 activity was blocked by addition of anti-IP10 neutralising antibody (2.5µg/ml) in the angiogenesis model described above. IP10 blockade significantly increased the angiogenic capacity of SLE MAC-conditioned media, but had no significant effect on vitamin D-treated MAC media. Columns show the mean number of closed loops normalised to growth media alone of combined results of n=4 experiments, and the error bars show standard error. Comparison by ratio paired t-tests, *p<0.05.

### 6.8 Discussion

This chapter describes the effect of 1,25(OH)$_2$D$_3$ on the phenotype and function of SLE MACs using different *in vitro* models. We found that vitamin D increased the number of SLE MACs and reduced expression of macrophage surface markers. Whilst having no effect on migration or adhesion, vitamin D increased the angiogenic capacity of MACs via the down-regulation of IP10. In addition, vitamin D augmented the ability of MACs to protect endothelial cells against TNF-mediated eNOS down-regulation.

In some experiments, the effects were also investigated using the IFNα model described in Chapter 5. The effects of 1,25(OH)$_2$D$_3$ are summarised in figure 6-21.
Figure 6-21: Summary of the effects of vitamin D on lupus MAC function
The diagram shows some of the functions of MACs modulated by 1,25(OH)_{2}D_{3} in vitro.

The experiments described all utilised 1,25(OH)_{2}D_{3} rather than 25(OH)D. Although myeloid cells express 1α-hydroxylase (CYP27B1) it is not constitutively active in vitro (553;554). Although others have reported that TNFα is sufficient to activate the enzyme in MACs from healthy subjects, 1,25(OH)_{2}D_{3} could not be generated even after the addition of TNFα (see Appendix 3). The active form of vitamin D was therefore used for this study.

The most notable change was that 1,25(OH)_{2}D_{3} altered the morphology of MACs with an increase in the roundness of cells. In PBMCs, 1,25(OH)_{2}D_{3} reduced the expression of genes important for macrophage differentiation which may explain some of the changes seen. The population of MACs resembled M2 macrophages under light microscopy as described by Zajac et. al. (2013). These cells have rounded, fried egg morphology in direct contrast to the spindle-shaped M1 macrophages (555). On incubation with 1,25(OH)_{2}D_{3} the MACs became significantly more rounded. This may represent further M2 polarisation but this would not be consistent with the dramatic reduction in CD206 expression. It is therefore possible that this increased roundness was the result of a global reduction in macrophage differentiation and increased number undifferentiated macrophages. This would be consistent with the reduced expression of CD68, CD206 and CCR7 in response to vitamin D. Importantly, the surface marker expression in vitamin D-treated SLE MACs approached that of healthy controls. It could be proposed, therefore, that vitamin D restores a state of increased activation in SLE back towards a normal state of activation. Exploration of the dataset generated by Kupfer et. al. (2013) also supports this observation, as genes important in macrophage differentiation were significantly down-regulated by 1,25(OH)_{2}D_{3} after 12 hours (550). Of interest, the feline
sarcoma oncogene (FES) has a role in regulation of the cellular cytoskeleton which may explain the morphological differences observed in MACs described in figure 6-3. This gene also induces the differentiation of myeloid progenitors into macrophages (556).

Interestingly, a contradictory effect on macrophage differentiation was reported by Kreutz et al. (1990) who found that 1,25(OH)₂D₃ induced monocyte differentiation into macrophages in the absence of serum (557). In the study described above, however, the effects of 1,25(OH)₂D₃ were being investigated on a population of cells derived from PBMCs (rather than pure monocytes) in the presence of 2 strong stimulators for differentiation (fibronectin and a high concentration of serum).

In both SLE and the IFNα model, 1,25(OH)₂D₃ increased the number of MACs at day 8. The increase in the number of MACs in response to vitamin D was strongly dependent on the number of MACs at baseline. SLE patients with higher numbers at baseline showed a negligible change in MAC numbers demonstrating a ceiling effect. Those patients with higher MAC number may therefore not have the capacity to generate more MACs in response to vitamin D. By contrast, in the IFN model the basal number of MACs was very low. This model generated a clear dose-response curve with an EC₅₀ of 4.82nM. Polly et al. (2000) reported that the EC₅₀ for the VDR transfected into Cos-7 cells was 4nM (558). This suggests that the effect of 1,25(OH)₂D₃ on MAC number is mediated via the VDR and not due to off-target effects as a result of supraphysiological concentrations of the ligand. In this study, dil-Ac-LDL was used as a marker of MACs. Although it is possible that the observed increase in MACs is due to increases in LDL-uptake this is less likely as Riek et al. (2013) demonstrated that the treatment of differentiated macrophages with 1,25(OH)₂D₃ actually reduced cholesterol uptake (549). The two observations above therefore suggest that whilst vitamin D increases the number of MACs in SLE patients, the activation status and polarisation of these cells is modulated to more closely resemble that observed in healthy control subjects.

Vitamin D did not modulate either MAC migration or adhesion in MACs from SLE patients of the IFN model. In terms of the IFN models, these may not be the most appropriate models to use, as IFNα2b alone did not significantly impair or enhance either of these functions. Given that there was no effect in the SLE model either, it is likely that these observations represent a true lack of effect. The effect on migration may be cell type specific, as a positive effect on migration has been observed in dendritic cells (559) and vascular smooth muscle cells (560), with an inhibitory effect in T cells (561).
In terms of adhesion, the endothelium, rather than myeloid cells, may be the target cell for vitamin D. In the study above, treatment of MACs with 1,25(OH)$_2$D$_3$ had no effect on adhesion to endothelium. In contrast, however, the coronary endothelium down-regulates VCAM-1 in response to 1,25(OH)$_2$D$_3$ (399). This observation may be limited to specific vascular beds as others have shown that vitamin D increases monocyte adhesion to HUVEC via the up-regulation of adhesion molecules (397). It is therefore difficult to be certain of the role of vitamin D in leukocyte adhesion, but any effects, positive or negative, do not appear to apply to MAC adhesion to aortic endothelium.

Endothelial dysfunction in the context of inflammation is associated with a reduction in NO bioavailability (562). The regulation of eNOS expression is an important determinant of NO bioavailability within the vasculature and reduced eNOS expression occurs in endothelial dysfunction (563). Talmor et. al. (2008) previously demonstrated that 1,25(OH)$_2$D$_3$ can directly protect HUVECs against advanced glycation end product (AGE)-mediated eNOS down-regulation (394). Circulating concentrations of AGES are increased in patients with SLE and as such, this mechanism may be important in these patients (395). In the studies described above, there was no significant effect of 1,25(OH)$_2$D$_3$ alone on TNF-mediated eNOS down-regulation in HAoECs. Instead, there was an augmentation of the protective effects of MACs on eNOS expression. As the mechanisms by which reduced NO bioavailability (and subsequent endothelial dysfunction) occurs in SLE are not known, it is difficult to directly model this in vitro. Therefore, TNFα was selected as down-regulation of eNOS at the transcript level has been well documented. Lai et. al. (2003) identified that TNF-α destabilises eNOS mRNA via the expression of ribonucleoprotein (RNP) complexes (564). The lupus MACS secreted a factor (or factors) which increased eNOS expression in this model. It is unclear whether this is due to a direct effect on eNOS expression, as there was a small increase in eNOS even in the absence of TNF, although this did not reach statistical significance. A significant effect was seen only when TNF was used to down-regulate eNOS. MACs may therefore secrete factors which interfere with TNF signalling or the RNP complexes. Further experiments would be needed to identify the mechanism by which this occurs. Importantly, the incubation of MACs with 1,25(OH)$_2$D$_3$ augmented this process resulting in significantly increased eNOS at both the transcript and protein level. It is unclear whether this is due to up-regulation of beneficial soluble factors or whether this is due to an increased total number of MACs.

Vitamin D alone did not modulate angiogenesis in the HAoEC Matrigel model. There was a very small (but statistically) significant increase in the number of pixels, but no change in the
number of closed loops. The closed loop parameter is likely to be more representative of the network density whilst pixel number may be influenced by other effects including endothelial cell proliferation or elongation (499). Other groups have demonstrated that vitamin D alone has anti-angiogenic effects in vivo using the chick embryo chorioallantoic membrane model (565) and a murine model of breast neoplasia (401). Furthermore, 1,25(OH)₂D₃ inhibited bovine aortic endothelial cell proliferation and network formation in collagen. Our results do not support vitamin D-mediated inhibition of angiogenesis and this may represent differences in the models used in terms of species (bovine vs. human) or cell matrix (collagen vs. Matrigel).

In contrast, the conditioned media from MACs significantly increased angiogenesis in this model, which was increased further when the MACs were cultured in the presence of 1,25(OH)₂D₃. The pro-angiogenic factors which mediate this effect are not clear. Medina et al. (2011) proposed that IL-8 secreted by MACs increases angiogenesis through transactivation of VEGFR2 (215). The multiplex array showed that MACs express very high levels of IL-8 albeit at highly variable concentrations. Whilst this may explain much of the angiogenic capacity of the MACs, IL-8 expression was not changed by vitamin D and so would not explain the significant increase in angiogenesis seen with vitamin D-treated MACs. Of the 8 traditional pro-angiogenic factors detectable in MACs, the only one significantly regulated was follistatin, which was down-regulated by 1,25(OH)₂D₃. Follistatin is a glycoprotein which primarily functions as a neutraliser of members of the TGF-β superfamily. In vitro and in vivo models have demonstrated a pro-angiogenic effect of follistatin (566) and can also be regulated via the nuclear glucocorticoid receptor. In the context of dexamethasone-mediated regulation, this is biphasic with up-regulation at lower concentrations and down-regulation at higher concentrations (567). It is not known whether the change in expression in response to vitamin D shows a similar pattern. It is clear, however, that a reduction in follistatin does not explain the increased angiogenesis in the Matrigel model. Interestingly, follistatin also up-regulates the macrophage scavenger receptor (MSR) in macrophages then promotes LDL uptake and foam cell formation (reviewed by Kozaki et al. (1998) (568)). In this context, vitamin D may have opposing effects to IFNα on foam cell formation in SLE patients.

It is more likely, therefore, that the effects of vitamin D on MAC angiogenic capacity are mediated via the down-regulation of IP10 (CXCL-10). As previously described, this IFN-regulated protein is expressed in active SLE and inhibits angiogenesis in vitro. The isolation of MACs in the presence of 1,25(OH)₂D₃ reduced IP 10 levels significantly such that <50% were below the threshold of detection for the assay. Neutralisation of IP10 increases angiogenesis by untreated, but not vitamin D-treated MACs. Others have shown that IP10, signalling
through its receptor CXCR3, inhibits expression of m-calpain (caplain-2), in direct opposition to VEGF (569;570)

Consideration should also be given to the effect of 1,25(OH)₂D₃ on MAC polarisation and how that may influence angiogenesis. It is recognised that the M2 subtype of macrophages have pro-angiogenic activities (571) however the results above suggest that vitamin D increases angiogenesis but also reduces macrophage differentiation and the expression of the M2 marker CD206. The M2 subset of macrophages comprises M2a, M2b and M2c subtypes all of which have different roles. It is not known whether vitamin D suppressed the development of all 3 of these subtypes equally or preferentially targets a particular subset. This may have clinical relevance as the M2b subtype is implicated in lupus nephritis (572). The results suggest that despite a reduction in M2 macrophages there is still sufficient expression of pro-angiogenic factors and that the suppression of IP10 may be more important in this model.

All of the experiments conducted above were confined to MACs derived from vitamin D deficient SLE patients. It is not clear, therefore, whether the beneficial effects seen in vitro are limited to patients with clinical vitamin D deficiency. The gene expression analysis of Kupfer et al. (2013) was conducted in 18 subjects of unknown vitamin D status. The changes in gene expression, including IP10, may therefore not be dependent on serum vitamin D levels. Similarly, the expression of pro-inflammatory cytokines by macrophages from patients with RA was modulated by vitamin D in vitro without limiting the study to vitamin D deficient patients (243). Further studies are needed to determine if the effects of 1,25(OH)₂D₃ can be replicated in vitamin D replete patients and in otherwise healthy subjects.

6.9 Summary of results

- MACs in vitro express the VDR transiently and change morphology in response to calcitriol in a manner suggesting reduced differentiation.
- Vitamin D increases the number of MACs both in SLE patients and in an IFN-model
- Migratory capacity and adhesion to activated endothelium are not modulated by vitamin D
- In a TNFα model, vitamin D augments the ability of SLE MACs to attenuate eNOS down-regulation in HAoECs at the transcript and protein level
- Vitamin D further increases the angiogenic capacity of MACs in an in vitro model which may be mediated by reduced expression of the anti-angiogenic factor IP10
Chapter 7: Results
The effect of vitamin D on endothelial function in patients with SLE

An observational study was conducted to determine the effects of vitamin D on non-invasive measures of endothelial function in vitamin D deficient patients. This chapter describes the relationship between vitamin D and markers of endothelial function and damage and disease activity.
7 The effect of vitamin D on endothelial function in patients with SLE

7.1 Introduction

The prevalence of endothelial dysfunction is increased in patients with SLE and represents an early stage in the development of clinical CVD. In other patient groups, vitamin D has been shown to improve endothelial function over 8-12 weeks. Given the effects of vitamin D on endothelial repair mechanisms \textit{in vitro}, it is hypothesised that this may translate into an improvement in vessel function in the patient population.

This chapter describes the effect of vitamin D on endothelial function in patients with stable SLE in an experimental medicine observational study. As secondary aims the effects of vitamin D on vascular stiffness, vascular damage and markers of disease activity were determined. A healthy control population was recruited in order to perform validation studies of FMD and aPWV (contained within Appendix 4). Some limited additional data was collected from healthy control subjects during the validation studies. The differences between the SLE population and HC population are described in more detail in Appendix 5.

7.2 Aims of this chapter

The specific aims of this chapter are to:

- Identify factors associated with vitamin D status and with aPWV in patients attending the screening study
- Compare indices of vascular function (FMD and aPWV), endothelial damage (EMPs), and disease activity between vitamin D deficient and vitamin D replete SLE patients
- Determine the changes in vascular function and disease activity over time in vitamin D deficient patients receiving supplementation compared to the vitamin D replete group
The analysis is therefore considered in 3 sections:

- **Screening Study**
  A cross-sectional analysis of factors associated with vitamin D status and aPWV in SLE patients attending the screening study visit. Differences in control subjects and lupus patients are described briefly here and in more detail in Appendix 5.

- **Cross-sectional Analysis of SLE Patients at Baseline**
  This cross-sectional analysis compares vitamin D deficient and vitamin D replete lupus patients in terms of FMD, aPWV, EMPs and disease activity.

- **Longitudinal Analysis of SLE Patients Receiving Vitamin D Supplementation**
  A longitudinal analysis of changes in vascular function and disease activity relative to the changes in serum 25(OH)D in SLE patients over a 3 month period.

### 7.3 Statistical methods relevant to this chapter

This chapter primarily uses descriptive statistics to investigate differences between vitamin D deficient and replete SLE patients and the effects of vitamin D therapy on the deficient population (using the replete population as a reference). Non-parametric testing (Mann-Whitney U test and Chi-squared test) was used principally as the majority of variables were not normally distributed within the population. Where appropriate and if the data was normally distributed parametric tests (t-test) were used. Similarly, correlation between variables is reported as the Spearman r correlation coefficient (non-parametric). To investigate the relationship between some variables further, and to allow for adjustment of confounding factors including age, regression models were used. Linear regression was used if the variable had a normal distribution or could be transformed into a normal distribution. If this was not possible then the variable was divided into quantiles and ordered logistic regression used. In stepwise regression models the significance threshold was set to p=0.1. The number of variables which could be included in the regression models was limited by the number of participants in the study.
7.4 Screening study

A total of 57 patients with SLE and 19 healthy control subjects were screened for inclusion in the main study. Limited data was collected in the screening study as described in the methods.

Although attempts were made to ensure that the populations were as closely related as possible, the control subjects differed from the lupus patient group. The healthy controls were significantly younger than the SLE group with a median (IQR) age of 30.7 (25.0, 32.9) years vs. 50.2 (41.7, 59.1) years (p<0.001). Whilst the median serum 25(OH)D did not differ between the two groups, a significant number of SLE patients (29/57) were taking low or high dose vitamin D supplements. Exclusion of patients taking vitamin D still showed similar 25(OH)D concentrations between patients and healthy controls (median [IQR] 18.3 [14.2, 29.1] vs. 20.4 [13.5, 28.9]ng/ml, p=0.827). There were also seasonal differences in the timing of the screening visit: more controls were seen in spring and summer and more SLE patients seen in autumn and winter (p=0.048). The demographic characteristics are shown in table 7-1 and figure 7-1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy (n=19)</th>
<th>SLE (n=57)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30.7 (25.0, 32.9)</td>
<td>50.2 (41.7, 59.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>20.4 (13.5, 28.9)</td>
<td>22.7 (16.0, 34.1)</td>
<td>0.157</td>
</tr>
<tr>
<td>Patients taking vitamin D treatment (n, %)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>None</td>
<td>19 (100)</td>
<td>28 (49.1)</td>
<td></td>
</tr>
<tr>
<td>Low dose*</td>
<td>0</td>
<td>19 (33.3)</td>
<td></td>
</tr>
<tr>
<td>High dose**</td>
<td>0</td>
<td>10 (17.5)</td>
<td></td>
</tr>
<tr>
<td>Season (n, %)</td>
<td></td>
<td></td>
<td>0.048</td>
</tr>
<tr>
<td>Winter</td>
<td>3</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>9</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>5</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>2</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Table 7-1: Characteristics of healthy controls and SLE patients at screening

Date is expressed as n (%) or median (IQR). Statistical comparison by Chi-squared (categorical data) or Mann-Whitney U test (continuous data). Seasons were defined as follows: winter (Dec, Jan, Feb) spring (Mar, Apr, May), summer (Jun, July, Aug) and autumn (Sept, Oct, Nov).
Patients and healthy controls were recruited throughout the year. Almost half of the healthy controls were recruited in spring, whilst SLE patients were more likely to be recruited in autumn and winter. The columns show the % number of subjects in each group recruited in each season.

The screening population had inactive SLE disease with a median (IQR) SLEDAI and global BILAG scores of 0 (0, 2) and 0 (0, 1) respectively. There was no difference in aPWV between the healthy controls and SLE patients in a model adjusting for age OR (95%CI), 0.90 (0.12, 3.8), p=0.886. Similarly, there were no differences in blood pressure or brachial artery augmentation index. A comparison of vascular function between the healthy controls and SLE patients is described in more detail in Appendix 5.

7.4.1 Factors associated with serum vitamin D in SLE patients and healthy controls
In the screening population there was no correlation between vitamin D level and age in either SLE patients or healthy controls (figure 7-2). In the lupus patients there was a trend towards increasing 25(OH)D levels with increasing age (Spearman’s r=0.251, p=0.059). This correlation was no longer apparent when only patients who were not taking vitamin D supplements were considered (r=0.162, p=0.420). Patients taking low and high dose vitamin D supplements tended to be older (median [IQR] age 52.9 [43.8, 62.3] and 51.6 [49.1, 58.0] years respectively) compared to those that did not (46.9 [30.1, 58.2]) (p=0.182).
There was no correlation between age and 25(OH)D concentration in patients with SLE either in the group as a whole (Spearman’s r=0.251, p=0.059) or in those not taking vitamin D supplements (r=0.162, p=0.420) (a). Similarly, in the healthy controls (none of whom were taking supplements) there was no correlation between age and vitamin D level (r=0.086, p=0.735) (b).

Vitamin D levels in patients and controls were the highest in autumn, and lowest in winter (30.6 [19.9, 34.2] vs. 17.8 [13.0, 22.3] ng/ml). The difference in 25(OH)D across seasons was statistically significant (Kruskal-Wallis, p=0.014) for the whole population and just failed to reach significance in SLE patients alone (p=0.059) (figure 7-3).

Seasonal variation was seen in serum 25(OH)D levels in the screening population (both healthy controls and patients with SLE) (a). In SLE patients alone, the highest 25(OH)D level in any given season tended to be in patients receiving high dose vitamin D supplementation (b). Patients taking low dose supplements had 25(OH)D levels similar to those on no treatment. In both graphs the points represent individual subjects and the bars show the median value for the group.
Vitamin D levels were normally distributed within the lupus population, and so linear regression was used to identify factors associated with serum 25(OH)D in SLE using the independent variables listed in table (7-2). In an unadjusted analysis, only season (summer and autumn) and high-dose vitamin D therapy were associated with 25(OH)D. After adjustment for age, only the sample being taken in summer and high-dose vitamin D remained associated. In a backwards stepwise regression model containing age, season and vitamin D treatment, only prior vitamin D treatment was an independent predictor of serum 25(OH)D levels (p<0.001).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted</th>
<th>Age Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age</td>
<td>0.0228</td>
<td>-0.002, 0.047</td>
</tr>
<tr>
<td>Season*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>0.570</td>
<td>-0.330, 1.47</td>
</tr>
<tr>
<td>Summer</td>
<td>1.17</td>
<td>0.288, 2.05</td>
</tr>
<tr>
<td>Autumn</td>
<td>0.818</td>
<td>0.039, 1.60</td>
</tr>
<tr>
<td>Prior vitamin D treatment#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>0.186</td>
<td>-0.394, 0.767</td>
</tr>
<tr>
<td>High dose</td>
<td>1.78</td>
<td>1.06, 2.50</td>
</tr>
<tr>
<td>SLEDAI score</td>
<td>-0.033</td>
<td>-0.285, 0.218</td>
</tr>
<tr>
<td>Global BILAG</td>
<td>0.027</td>
<td>-0.109, 0.162</td>
</tr>
</tbody>
</table>

Table 7-2: Predictors of 25(OH)D in SLE patients at screening
Linear regression analysis shows that only high-dose vitamin D therapy (>25,000 IU/month) and samples taken in the summer are associated with 25(OH)D in the screening population in an age-adjusted model.

*season when compared to winter # vitamin D therapy when compared to no therapy

7.4.2 Vitamin D and arterial stiffness in patients with SLE
In this screening cohort, the median (IQR) aPWV was 9.1 (7.0, 11.8) m/s. In an exploratory analysis there was a strong correlation between aPWV and both age (r=0.657, p<0.0001), and mean arterial blood pressure (r=0.5088, p<0.0001) but not 25(OH)D (r=0.125, p=0.367) (figure 7-4).
There was a strong correlation between aPWV and age (a) and mean arterial blood pressure (MAP) (b) but not 25(OH)D (c) in patients with SLE. Figures show scatter plots and the spearman r coefficient.

In the SLE patients, aPWV could not be readily transformed into a normal distribution and was thus analysed in tertiles. There was a significant increase in age and blood pressure across tertiles (p<0.001 for age and p=0.006 for systolic BP) (table 7-3). There was no association between aPWV and 25(OH)D and SLEDAI score, but a trend towards an association between tertiles of aPWV and BILAG score (p=0.065).
Table 7-3: Descriptive analysis of SLE-related and cardiovascular parameters and aortic pulse-wave velocity.

Data expressed as n (%) or median (IQR) and analysed by Chi-squared and Mann-Whitney U tests respectively. Age increased with tertiles of aPWV as did all 4 measures of blood pressure. *after exclusion of patients on high dose vitamin D supplements

BP = blood pressure, PP = pulse pressure, MAP = mean arterial blood pressure, HR = heart rate

The diastolic reflection area (DRA) is a parameter generated by the Arteriograph representing the quality of diastolic filling of the coronary arteries (taking into account the duration of diastole and the expected and measured diastolic curves). A higher DRA is associated with more optimal arterial filling. The equation used to calculate this parameter is not available from the manufacturer.

The association between aPWV and 25(OH)D, disease activity, and other cardiovascular parameters was determined using univariate ordered logistic regression in an unadjusted model as well as in models adjusting for age, and for age and mean arterial blood pressure (table 7-4). The age-adjusted analysis showed a significant association of aPWV with blood pressure (systolic, diastolic and mean arterial) but not with vitamin D.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted</th>
<th>Age-adjusted</th>
<th>Adjusted (age, mean arterial BP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>p</td>
</tr>
<tr>
<td>Age</td>
<td>1.16</td>
<td>1.09-1.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>1.03</td>
<td>0.986-1.08</td>
<td>0.182</td>
</tr>
<tr>
<td>Prior Vitamin D (low or high dose)</td>
<td>1.592</td>
<td>1.30-4.69</td>
<td>0.183</td>
</tr>
<tr>
<td>Season</td>
<td>Ref</td>
<td>0.812</td>
<td>1.27</td>
</tr>
<tr>
<td>Winter</td>
<td>Winter</td>
<td>0.186-3.53</td>
<td>0.762</td>
</tr>
<tr>
<td>Spring</td>
<td>Spring</td>
<td>0.276-5.80</td>
<td>0.762</td>
</tr>
<tr>
<td>Summer</td>
<td>Summer</td>
<td>0.255-3.80</td>
<td>0.762</td>
</tr>
<tr>
<td>Autumn</td>
<td>Autumn</td>
<td>Ref</td>
<td>0.781</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>0.607</td>
<td>0.388-0.948</td>
<td>0.028</td>
</tr>
<tr>
<td>Global BILAG</td>
<td>1.04</td>
<td>0.824-1.32</td>
<td>0.734</td>
</tr>
</tbody>
</table>

Table 7-4: Association between aPWV and demographic and cardiovascular parameters in patients with SLE.

In an unadjusted univariate analysis, aPWV was significantly associated with age, SLEDAI score, blood pressure and DRA. After adjustment for age, only blood pressure parameters remained significantly associated.

A backwards stepwise ordered logistic regression model using the variables age, 25(OH)D, SLEDAI score, MAP and DRA was used to identify which of these variables were independent predictors of aPWV in SLE patients. In this model there was a positive association between aPWV and age (p=<0.001) and MAP (p=0.012) but a negative association with SLEDAI score (p=0.042).

7.5 Cross-sectional analysis of SLE patients at baseline

A total of 40 patients proceeded from the screening study into the main study. The patient flow is show in figure 7-5. Patients were split in to 2 groups: vitamin D deficient (serum 25(OH)D <20ng/ml) and replete (serum 25(OH)D>30ng/ml). The deficient group contained 22 patients and the replete group 18 patients. Patients in the replete group were significantly
older than those in the deficient group (median [IQR] age 46.9 [44.5, 57.6] vs. 57.8 [52.7, 64.6] years, \( p=0.007 \)) and had a trend towards a longer disease duration (table 7-5). There were more patients from non-Caucasian ethnic groups in the vitamin D deficient group although this was not statistically significant (\( p=0.072 \)).

**Figure 7-5: Patient flow during the study**

Of the 58 patients screened a total of \( n=40 \) were eligible for inclusion in the main study. Of these, \( n=22 \) were vitamin D deficient and \( n=18 \) vitamin D replete. In the deficient group, all 22 patients attended follow-up. In the replete study, \( n=16 \) attended the follow up visit.
Table 7-5: Demographic characteristics of the vitamin D deficient and replete cohorts. BMI=body mass index, ANA=anti-nuclear antibody, PTH=parathyroid hormone, ACR=American College of Rheumatology. Comparisons were made using the Mann-Whitney U test (continuous variables) or Chi-squared test (categorical variables).

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Deficient (n=22)</th>
<th>Replete (n=18)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>46.9 (44.5, 57.6)</td>
<td>57.8 (52.7, 64.6)</td>
<td>0.0066</td>
</tr>
<tr>
<td><strong>Disease duration (years)</strong></td>
<td>12.7 (6.94, 18.7)</td>
<td>25.1 (10.6, 31.4)</td>
<td>0.116</td>
</tr>
<tr>
<td><strong>Caucasian</strong></td>
<td>16 (72.7%)</td>
<td>17 (94.4%)</td>
<td>0.072</td>
</tr>
<tr>
<td><strong>Ethnic Origin</strong></td>
<td>16</td>
<td>17</td>
<td>0.406</td>
</tr>
<tr>
<td>Caucasian</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Black Caribbean</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pakistani</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Waist circumference (cm)</strong></td>
<td>83.5 (71.0, 91.0)</td>
<td>85.5 (76.0, 95.0)</td>
<td>0.596</td>
</tr>
<tr>
<td><strong>Hip circumference (cm)</strong></td>
<td>98.5 (88.0, 105)</td>
<td>93.0 (89.5, 104)</td>
<td>0.913</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>25.4 (22.0, 29.1)</td>
<td>25.0 (21.3, 28.8)</td>
<td>0.568</td>
</tr>
<tr>
<td><strong>25(OH)D (ng/ml)</strong></td>
<td>13.1 (10.3, 16.5)</td>
<td>34.5 (30.8, 40.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>PTH (pg/ml)</strong></td>
<td>47.0 (38.0, 66.0)</td>
<td>37.0 (29.0, 50.0)</td>
<td>0.096</td>
</tr>
<tr>
<td><strong>Calcium (mmol/l)</strong></td>
<td>2.34 (2.24, 2.38)</td>
<td>2.36 (2.32, 2.42)</td>
<td>0.634</td>
</tr>
<tr>
<td><strong>Vitamin D supplements</strong></td>
<td>14</td>
<td>6</td>
<td>0.005</td>
</tr>
<tr>
<td>None</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Risk Factors for Vitamin D Deficiency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self-reported photosensitivity</td>
<td>15 (68.2%)</td>
<td>7 (38.9%)</td>
<td>0.064</td>
</tr>
<tr>
<td>Active sun avoidance</td>
<td>14 (63.6%)</td>
<td>10 (55.6%)</td>
<td>0.604</td>
</tr>
<tr>
<td>High-factor sunblock use</td>
<td>12 (54.5%)</td>
<td>8 (44.4%)</td>
<td>0.525</td>
</tr>
<tr>
<td>Covered arms/legs</td>
<td>9 (40.9%)</td>
<td>1 (5.60%)</td>
<td>0.010</td>
</tr>
<tr>
<td>Covered face</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Malabsorption syndrome</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><strong>SLE Disease Features</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. ACR criteria</td>
<td>5 (4, 6)</td>
<td>5 (4, 6)</td>
<td>0.748</td>
</tr>
<tr>
<td>Renal disease (ever)</td>
<td>7 (31.8%)</td>
<td>1 (5.60%)</td>
<td>0.039</td>
</tr>
<tr>
<td>ANA positive (ever)</td>
<td>21 (95.4%)</td>
<td>16 (88.9%)</td>
<td>0.433</td>
</tr>
</tbody>
</table>

The plasma PTH level tended to be lower in the vitamin D replete group (p=0.096) but there were no differences in serum calcium.

The median number of ACR criteria which was met was 5 (4, 6) in both groups. The distribution of these criteria was similar, although vitamin D deficient patients were significantly more likely to have a history of renal disease (7 patients vs. 1 patient, p=0.039) (figure 7-6). The clinical assessment included questions to identify risk factors for vitamin D in the study population. Similar to the pattern seen in the ACR criteria above, there was a trend towards self-reported photosensitivity being more common in the vitamin D deficient group.
(p=0.064). Both groups reported using high factor sun block, but the vitamin D deficient group were significantly more likely to ensure that arms and legs were covered when outdoors (table 7-6).

![Distribution of ACR criteria between SLE patients](image)

**Figure 7-6: Distribution of ACR criteria between SLE patients**
The presence of individual ACR criteria was similar between the 2 groups. The only criterion to show statistical significance (Chi-squared test) was renal disease which was much higher in the vitamin D deficient group compared to the control group, *p<0.05.

### 7.5.1 Comparison of disease activity, therapy and traditional cardiovascular risk factors between vitamin D deficient and replete SLE patients

#### 7.5.1.1 Disease activity and therapy
There was no difference in clinical or laboratory markers of disease activity between the 2 groups (table 7-6). It should be noted that the entry criteria for the study required stable patients (i.e. those with no planned change in SLE therapy). No differences were seen between deficient and replete patients in terms of serology (including ds-DNA titre and serum complement), inflammatory markers (ESR and high-sensitivity CRP) or cytokine expression.
Clinical Assessment of Disease | Deficient (n=22) | Replete (n=18) | p
---|---|---|---
SLEDAI | 0 (2) | 0 (2) | 0.738
SACQ (from SLEDAI) | 7 (31.8%) | 6 (33.3%) | 0.919
BILAG (at least 1 A or B) | 1 (4.54%) | 2 (11.1%) | 0.433
Global BILAG* | 1 (1) | 0 (1) | 0.250

Laboratory
ANA +ve | 17 (77.2%) | 11 (61.1%) | 0.267
Ds-DNA (iu/ml) | 4.0 (2.0, 11.5) | 2.0 (1.0, 8.0) | 0.169
Ds-DNA +ve (>13.9iu/ml) | 6 (27.2%) | 3 (16.7%) | 0.424
C3 (g/l) | 1.065 (0.81, 1.29) | 0.985 (0.81. 1.22) | 0.568
C4 (g/l) | 0.175 (0.11, 0.23) | 0.170 (0.12, 0.21) | 0.957
ESR (mm/hr) | 11 (5.0, 25.5) | 12 (8.0, 20.0) | 0.570
hs-CRP (mg/l) | 1.44 (0.44, 4.55) | 1.28 (0.85, 1.85) | 0.630
IL-6 (pg/ml) | 0.245 (0.25, 1.99) | 0.530 (0.25, 1.15) | 0.855
Detectable IL6# | 5 (22.7%) | 3 (16.7%) | 0.634
IP-10 (pg/ml) | 122 (80.0, 171) | 120 (79.0, 169) | 0.910
TNF-alpha (pg/ml) | 1.00 (1.00, 41.0) | 1.00 (1.00, 151) | 0.558
Detectable TNF-alpha# | 10 (45.5%) | 9 (50.0%) | 0.775
BAFF (pg/ml) | 106 (80.0, 134) | 117 (83.4, 153) | 0.887

Table 7-6: Clinical and laboratory assessment of disease activity in SLE patients

The values in the table show the median (IQR) or n (%) and differences were investigated using Mann-Whitney U tests and Chi-squared tests respectively.

*the global BILAG score was calculated by attributing a numerical value to each score such that A=12, B=8, C=1, D/E=0 as per Yee et. al. (2010) (478).

# the number of subjects with detectable levels of IL6 and TNFα is presented as many patients had values below the limit of detection for the assay

SACQ=serologically active, clinically quiescent, hs-CRP = high sensitivity CRP, BAFF = B cell activating factor

In contrast, there were marked differences in drug treatments between the 2 groups.

Prednisolone was prescribed to a total of 11/40 (27.5%) patients but this was much more common in the vitamin D deficient group (45.5% vs. 5.6%, p=0.005) (table 7-7). Similarly, significantly more vitamin D deficient patients were taking an immunosuppressant than those in the replete group. Of the 14 patients prescribed an immunosuppressant these were principally methotrexate, mycophenolate mofetil and azathioprine (figure 7-7). There was not, however, any difference in anti-malarial usage between the 2 groups.
Table 7-7: Drug therapy at baseline in SLE patients
The majority of SLE patients were taking either prednisolone or an antimalarial agent. There were significant differences between the deficient and replete patients in terms of steroid and immunosuppressant use. Deficient patients were more likely to be taking prednisolone and immunosuppressant therapy than replete patients. Comparisons were made using the Chi-squared test (or Mann-Whitney U test for median steroid dose). *mean dose for information in patients taking prednisolone.

Figure 7-7: Distribution of drug therapy at baseline in SLE patients
The columns show the number of SLE patients prescribed each drug according to their vitamin D status at baseline. MMF = mycophenolate mofetil.

7.5.1.2 Traditional cardiovascular risk factors
There was little difference in the majority of CVD risk factors between the 2 groups (table 7-8). Interestingly, however, the patients in the vitamin D deficient group had significantly lower systolic blood pressure and a trend towards lower diastolic blood pressure although in an age adjusted linear regression model this association was no longer significant (β 0.748, 95% CI (0.219, 1.28), p=0.144).
<table>
<thead>
<tr>
<th>Cardiovascular</th>
<th>Deficient (n=22)</th>
<th>Replete (n=18)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mmHg)</td>
<td>124 (120, 135)</td>
<td>139 (133, 150)</td>
<td>0.023</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>78.0 (75, 84)</td>
<td>85.0 (77, 88)</td>
<td>0.130</td>
</tr>
<tr>
<td>History of hypertension (current or prior therapy)</td>
<td>9 (40.9%)</td>
<td>10 (55.6%)</td>
<td>0.356</td>
</tr>
<tr>
<td>Antihypertensive treatment</td>
<td>8 (36.4%)</td>
<td>7 (38.9%)</td>
<td>0.870</td>
</tr>
<tr>
<td>Smoker – current</td>
<td>3 (13.6%)</td>
<td>2 (11.1%)</td>
<td>0.810</td>
</tr>
<tr>
<td>Smoker – current or ex</td>
<td>7 (31.2%)</td>
<td>10 (55.6%)</td>
<td>0.131</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.5 (0.90)</td>
<td>4.6 (1.0)</td>
<td>0.681</td>
</tr>
<tr>
<td>Diagnosis of diabetes</td>
<td>1 (4.54%)</td>
<td>0</td>
<td>0.360</td>
</tr>
<tr>
<td>Previous history of CVD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>2 (9.1%)</td>
<td>4 (22.2%)</td>
<td>0.247</td>
</tr>
<tr>
<td>Angina/MI</td>
<td>0</td>
<td>2 (11.1%)</td>
<td>0.109</td>
</tr>
<tr>
<td>TIA/stroke</td>
<td>2 (9.1%)</td>
<td>2 (11.1%)</td>
<td>0.832</td>
</tr>
<tr>
<td>PVD</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Heart Failure</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>4 (18.2%)</td>
<td>7 (38.9%)</td>
<td>0.145</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.60 (4.2, 2.9)</td>
<td>4.75 (4.2, 5.6)</td>
<td>0.413</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.69 (1.53, 1.98)</td>
<td>1.73 (1.48, 1.96)</td>
<td>0.778</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>2.25 (2.00, 2.84)</td>
<td>2.70 (2.32, 3.24)</td>
<td>0.242</td>
</tr>
<tr>
<td>TGs (mmol/l)</td>
<td>0.80 (0.55, 1.15)</td>
<td>0.90 (0.60, 1.30)</td>
<td>0.177</td>
</tr>
<tr>
<td>Cholesterol:HDL ratio</td>
<td>2.60 (2.28, 3.02)</td>
<td>2.57 (2.34, 3.28)</td>
<td>0.844</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>68.0 (56, 75)</td>
<td>64.5 (56, 78)</td>
<td>0.946</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>8 (36.3%)</td>
<td>13 (72.2%)</td>
<td>0.024</td>
</tr>
</tbody>
</table>

Table 7-8: Traditional cardiovascular risk factors in SLE patients at baseline
Patients with vitamin D deficiency had significantly lower systolic blood pressure than those that had replete levels of vitamin D. The table shows the median (IQR) or n(%) values for traditional cardiovascular risk factors between the groups.
BP = blood pressure, PVD = peripheral vascular disease, MI=myocardial infarction, TIA=transient ischaemic attack, HDL=high density lipoprotein, LDL=low density lipoprotein, TGs=triglycerides.

7.5.2 Aortic pulse-wave velocity
Vitamin D deficient patients had lower mean arterial BP, estimated central systolic BP, and pulse pressure than replete patients. Similarly, the augmentation indices were significantly lower in the vitamin D deficient group indicating more compliant arteries (table 7-9).
### Table 7-9: Vascular stiffness and vitamin D status in SLE patients

The table shows the median (IQR) for the parameters. *aPWV is normally distributed and so the mean (SD) is presented. Comparisons were by Mann-Whitney U test (and two-tailed t-test for aPWV).

<table>
<thead>
<tr>
<th>Arteriograph Parameter</th>
<th>Deficient (n=22)</th>
<th>Replete (n=18)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>45.0 (39.0, 51.0)</td>
<td>58.0 (48.0, 64.0)</td>
<td>0.003</td>
</tr>
<tr>
<td>Mean arterial BP (mmHg)</td>
<td>93.0 (89, 99)</td>
<td>102 (95.1, 109)</td>
<td>0.040</td>
</tr>
<tr>
<td>Central systolic BP (mmHg)</td>
<td>129.2 (118, 144)</td>
<td>149.1 (140, 158)</td>
<td>0.016</td>
</tr>
<tr>
<td>Central pulse pressure (mmHg)</td>
<td>52 (41, 57)</td>
<td>65 (54, 73)</td>
<td>0.031</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>66 (61, 74)</td>
<td>62 (58, 66)</td>
<td>0.073</td>
</tr>
<tr>
<td>Brachial augmentation index (%)</td>
<td>15.4 (5.55, 32.9)</td>
<td>41.9 (27.6, 51.4)</td>
<td>0.013</td>
</tr>
<tr>
<td>Aortic augmentation index (%)</td>
<td>45.2 (38.2, 53.5)</td>
<td>58.4 (50.3, 61.2)</td>
<td>0.032</td>
</tr>
<tr>
<td>aPWV (m/s)*</td>
<td>9.80 (2.66)</td>
<td>11.2 (2.99)</td>
<td>0.135</td>
</tr>
<tr>
<td>Diastolic Reflection Area (DRA)</td>
<td>37 (32, 45)</td>
<td>42 (38, 51)</td>
<td>0.172</td>
</tr>
</tbody>
</table>

Serum vitamin D was associated with MAP, and brachial and aortic augmentation indices in unadjusted linear and ordered logistic regression models. In age-adjusted models these associations were no longer significant (table 7-10). In contrast, the diastolic reflection area (DRA) was not associated with vitamin D status in an unadjusted model, but after adjustment for age and mean arterial blood pressure, was significantly associated with vitamin D status such that vitamin D deficient patients had a lower DRA (and thus less optimal coronary arterial filling).
### Table 7-10: Association between Arteriograph parameters and vitamin D status at baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted</th>
<th>Age-adjusted</th>
<th>Adjusted (age, mean arterial BP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>p</td>
</tr>
<tr>
<td>Brachial Augmentation Index (%)</td>
<td>5.25</td>
<td>1.39, 19.9</td>
<td>0.015</td>
</tr>
<tr>
<td>Aortic Augmentation Index (%)</td>
<td>4.07</td>
<td>1.15, 14.4</td>
<td>0.030</td>
</tr>
<tr>
<td>Diastolic Reflection Area</td>
<td>2.26</td>
<td>0.669, 7.61</td>
<td>0.190</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>95% CI</td>
<td>p</td>
</tr>
<tr>
<td>aPWV (m/s)</td>
<td>1.44</td>
<td>-0.47, 3.36</td>
<td>0.135</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>0.271</td>
<td>0.007, 0.53</td>
<td>0.044</td>
</tr>
</tbody>
</table>

The association between measures of vascular function and vitamin D status was determined using ordered logistic regression models (linear regression models for aPWV). The table shows the unadjusted, age-adjusted and age- and MAP-adjusted models.

#### 7.5.3 Flow-mediated dilatation

It should be noted that 4 FMD scans were not technically adequate for interpretation. All 4 of these patients belonged to the deficient group and were the first 4 patients recruited into the study. Furthermore, 1 additional patient in the deficient group was not able to tolerate GTN (thus for %GTN-dilatation and ED/EI dilatation there were results available for 17 subjects).

Baseline diameter was lower in vitamin D deficient patients and this association persisted after adjustment for age in a linear regression analysis ($\beta=0.001$, [0.0001, 0.018], $p=0.047$). The %FMD and ED/EI ratio did not differ between the 2 groups (table 7-11). In a multivariable linear regression model the baseline arterial diameter remained associated with 25(OH)D level after adjustment for age, systolic blood pressure, anti-hypertensive use and smoking status (table 7-12).
Table 7-11: Flow-mediated dilatation (FMD) in vitamin D deficient and replete SLE patients
The table shows the median (IQR) values for the parameters of FMD (except * which shows mean [sd]). Comparisons were made by Mann-Whitney U test (except * which were normally distributed and compared using 2-tailed t-test).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Deficient SLE (n=18)</th>
<th>Replete SLE (n=18)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline diameter (mm)*</td>
<td>3.25 (0.300)</td>
<td>3.54 (0.250)</td>
<td>0.004</td>
</tr>
<tr>
<td>Post Deflation diameter (mm)*</td>
<td>3.44 (0.322)</td>
<td>3.69 (0.321)</td>
<td>0.032</td>
</tr>
<tr>
<td>Absolute change in diameter (mm)</td>
<td>0.182 (0.116, 0.334)</td>
<td>0.100 (0.034, 0.239)</td>
<td>0.173</td>
</tr>
<tr>
<td>% FMD</td>
<td>5.47 (3.21, 9.58)</td>
<td>2.85 (0.981, 6.35)</td>
<td>0.114</td>
</tr>
<tr>
<td>% GTN dilatation</td>
<td>11.3 (10.7, 18.0)</td>
<td>10.4 (5.73, 13.3)</td>
<td>0.113</td>
</tr>
<tr>
<td>ED/EI</td>
<td>47.0 (18.6, 57.6)</td>
<td>47.3 (9.59, 85.2)</td>
<td>0.564</td>
</tr>
</tbody>
</table>

Table 7-12: Multivariable regression analysis of factors associated with brachial artery diameter at baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>B Coefficient</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D</td>
<td>0.010</td>
<td>0.003, 0.017</td>
<td>0.004</td>
</tr>
<tr>
<td>Age</td>
<td>0.010</td>
<td>0.001, 0.018</td>
<td>0.031</td>
</tr>
<tr>
<td>Mean arterial BP</td>
<td>-0.005</td>
<td>-0.012, 0.003</td>
<td>0.214</td>
</tr>
<tr>
<td>Antihypertensive use</td>
<td>0.168</td>
<td>0.008, 0.327</td>
<td>0.040</td>
</tr>
<tr>
<td>Current smoker</td>
<td>-0.134</td>
<td>-0.391, 0.123</td>
<td>0.294</td>
</tr>
</tbody>
</table>

7.5.4 Endothelial microparticles
The number of endothelial microparticles in the plasma was measured as described and quantified in terms of the number of AnnV+/CD31+/CD42b- events per millilitre of plasma. There was no significant difference between the number of EMPs in healthy controls compared to the SLE patients (median [IQR] 23,844 [10,704, 47,673] vs. 16,147 [4883, 47,259] EMPs/ml (p=0.569)) (figure 7-8). In comparison the number of EMPS was significantly higher than both of these groups in a population of SLE patients with active disease. This data was kindly supplied by Dr B Parker and the EMPs were measured using the same protocol (110;477). In this population the median SLEDAI score was 6 (5,13) the median number of EMPs was 147,170 (50,944, 278,755). The median [IQR] EMP numbers did not differ between vitamin D deficient and replete patients (11,946 [4178, 46,052] vs. 23,744 [6013, 59,010], p=0.228).
Figure 7-8: Endothelial microparticles in patients with SLE and healthy control subjects
The number of EMPs was not different between the SLE patients in this study and HCs (a). A separate cohort with highly active disease (*) had significantly higher numbers of EMPS compared to either HC, or to the clinically stable SLE patients in this study. Within this study population there was no difference in EMP numbers between vitamin D deficient and replete patients (b). The points show individual patient results and the bars show median values for the group. The Mann-Whitney U test was used to compare the groups.

Univariate linear regression models of log-transformed EMP number were used to investigate the association between number of EMPs and traditional- and SLE-related factors. In an unadjusted univariate analysis, there was no association seen between EMPs and serum 25(OH)D (β=0.009 [-0.033, 0.051], p=0.663), FMD (-0.063 [-0.185, 0.060], p=0.305) or ED/EI dilatation (-0.006 [-0.022, 0.011], p=0.487). There was however an association between EMPs and use of antihypertensive drugs, personal history of CVD, aPWV, lupus disease duration and lupus damage all of which remained significantly associated after adjustment for age (table 7-13).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted</th>
<th>Age Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>95% CI</td>
</tr>
<tr>
<td>aPWV</td>
<td>0.239</td>
<td>0.042, 0.435</td>
</tr>
<tr>
<td>Anti-hypertensive drugs</td>
<td>1.16</td>
<td>0.053, 2.26</td>
</tr>
<tr>
<td>History of CVD</td>
<td>1.66</td>
<td>0.175, 3.14</td>
</tr>
<tr>
<td>Disease Duration</td>
<td>0.055</td>
<td>0.009, 0.101</td>
</tr>
<tr>
<td>SLICC-DI</td>
<td>0.597</td>
<td>0.182, 1.01</td>
</tr>
</tbody>
</table>

Table 7-13: Univariate analysis of factors association with endothelial microparticles
aPWV = aortic pulse wave velocity, SLICC-DI = SLICC Damage Index

In a multivariable model including the 5 items above plus age only aPWV remained independently associated with EMPs (β=0.252 [0.025, 0.480], p=0.031).
7.6 Longitudinal analysis of SLE patients receiving vitamin D supplementation

As described above, 2 patients in the replete group were lost to follow-up, leaving 22 vitamin D deficient patients and 16 replete patients to be studied at each time point. Follow-up visits were timed at 3 months after the patient started vitamin D replacement or 3 months after the last study visit in patients who were vitamin D replete. The median (IQR) follow-up time was 112 (92, 140) days, but was longer in the deficient group due to delays in some patients starting treatment (table 7-14).

<table>
<thead>
<tr>
<th></th>
<th>Deficient Group (n=22)</th>
<th>Replete Group (n=16)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up time (days)</td>
<td>130 (111, 154)</td>
<td>97 (91, 107)</td>
<td>0.0032</td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>28.6 (18.3, 35.0)</td>
<td>-0.62 (-3.99, 2.63)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>-8.5 (-14.5, 0.5)</td>
<td>2.5 (-6.0, 7.0)</td>
<td>0.0389</td>
</tr>
<tr>
<td>Corrected calcium (mmol/l)</td>
<td>0.060 (-0.05, 0.15)</td>
<td>-0.03 (-0.10, 0.025)</td>
<td>0.0526</td>
</tr>
</tbody>
</table>

Table 7-14: Changes in vitamin D, PTH and calcium over the follow-up period

The median 25(OH)D in the deficient group at baseline and follow-up was 13.1 (10.3, 16.5) and 40.3 (32.2, 45.2), and in the replete group was 34.5 (30.8, 40.2) and 36.7 (31.7, 43.1) ng/ml (figure 7-9). Furthermore, the change in 25(OH)D was strongly inversely correlated with the baseline 25(OH)D, suggesting that there was a ceiling effect. The range for the increase in 25(OH)D in the treated patients was 11.2-59.8ng/ml. The change in 25(OH)D was associated with a reduction in serum PTH in the deficient group (47 [38, 66] to 40 [30, 47]) but not in the replete group (37 [29, 60] to 39 [33, 48] pg/ml). There was a trend towards a difference in changes in calcium levels between the 2 groups but the absolute increase in serum calcium with vitamin D treatment was only small (2.34 [2.24, 2.38] to 2.38 [2.27, 2.45] nmol/l).
Figure 7-9: Differences in 25(OH)D in the 2 groups between baseline and follow-up visits

There was an increase in vitamin D level in the deficient group but no change in the replete group (a). The columns in show the median 25(OH)D and the error bars show the IQR. In (b) the fold change in 25(OH)D in the treated group is plotted against the baseline 25(OH)D. The fold change is strongly inversely correlated with the baseline 25(OH)D. The Spearman’s ρ correlation coefficient is shown.

7.6.1 Changes in disease activity over time

The study cohort had low disease activity at baseline. Over the follow-up period there was no significant change in SLEDAI or global BILAG scores in either group. In the deficient group 8 patients (36%) had an increase in SLEDAI score at follow-up (p=0.067), however this was not matched by an increase in global BILAG score (table 7-15). The increase in SLEDAI was due predominantly to mucocutaneous disease. The only parameter in which there was a difference was in ds-DNA titre which did not change in the deficient group but decreased by a median (IQR) of 1 (-1.0, 0) iU/ml in the replete group (p=0.009). Whilst this change was statistically significant, a change of 1 iU/ml is not of clinical significance. There was no change in any of the cytokines measured.
## Change over Time

<table>
<thead>
<tr>
<th>Lupus Disease Activity</th>
<th>Deficient (n=22)</th>
<th>Replete (n=16)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLEDAI</td>
<td>0 (0, 2)</td>
<td>0 (0, 0)</td>
<td>0.336</td>
</tr>
<tr>
<td>Increase in SLEDAI score</td>
<td>8 (36.4%)</td>
<td>2 (12.5%)</td>
<td>0.067</td>
</tr>
<tr>
<td>BILAG (New A or B)</td>
<td>3 (13.6%)</td>
<td>1 (6.25%)</td>
<td>0.429</td>
</tr>
<tr>
<td>Global BILAG*</td>
<td>0 (0, 1)</td>
<td>0 (0, 1)</td>
<td>0.428</td>
</tr>
<tr>
<td>Increase in global BILAG</td>
<td>8 (36.4%)</td>
<td>7 (43.8%)</td>
<td>0.870</td>
</tr>
<tr>
<td>Ds-DNA (iu/ml)</td>
<td>0 (0, 1.0)</td>
<td>-1.0 (-1.0, 0)</td>
<td>0.0085</td>
</tr>
<tr>
<td>C3 (g/l)</td>
<td>0.01 (-0.09, 0.06)</td>
<td>0 (-0.12, 0.06)</td>
<td>0.794</td>
</tr>
<tr>
<td>C4 (g/l)</td>
<td>0 (-0.02, 0.01)</td>
<td>0.005 (-0.015, 0.025)</td>
<td>0.379</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>0.006 (0.983)</td>
<td>-0.098 (1.02)</td>
<td>0.554</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>0 (-4.0, 6.0)</td>
<td>-2.00 (-5.0, 2.0)</td>
<td>0.480</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>0 (-2.5, 6.5)</td>
<td>-3.50 (-6.5, 4.0)</td>
<td>0.170</td>
</tr>
<tr>
<td>Cytokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL6 (pg/ml)</td>
<td>0 (0, 0.94)</td>
<td>0 (-0.38, 0.66)</td>
<td>0.526</td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>0 (-1.1, 0)</td>
<td>0 (-10.2, 5.3)</td>
<td>0.742</td>
</tr>
<tr>
<td>IP10 (pg/ml)</td>
<td>-1.15 (-15.2, 22.6)</td>
<td>1.65 (-16.7, 28.5)</td>
<td>0.734</td>
</tr>
<tr>
<td>BAFF (pg/ml)</td>
<td>-5.30 (-20.5, 32.9)</td>
<td>-6.45 (-12.3, 9.85)</td>
<td>0.941</td>
</tr>
</tbody>
</table>

**Table 7-15: Changes in markers of disease activity at baseline and follow-up**

The table shows the median (IQR) or n (%) values. The Mann-Whitney U test was used for continuous variables and Chi-squared test for categorical variables.

### 7.6.2 Changes in traditional cardiovascular risk factors and aPWV over time

There were no differences in the change in traditional cardiovascular risk factors, aPWV or other vascular parameters, or EMPs over time between the 2 groups (table 7-16).
<table>
<thead>
<tr>
<th>Modifiable Risk Factors</th>
<th>Deficient Group (n=22)</th>
<th>Replete Group (n=16)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>-0.100 (-0.50, 0.60)</td>
<td>-0.100 (-0.15, 0.20)</td>
<td>0.618</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>0.01 (-0.24, 0.22)</td>
<td>0.04 (-0.05, 0.16)</td>
<td>0.619</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>-0.20 (-0.37, 0.28)</td>
<td>-0.14 (-0.23, 0.14)</td>
<td>0.667</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.100 (0, 0.4)</td>
<td>0.100 (0, 0.1)</td>
<td>0.425</td>
</tr>
<tr>
<td>Creatinine(µmol/l)</td>
<td>0.00 (-0.40, 0.30)</td>
<td>0.10 (-0.10, 0.70)</td>
<td>0.099</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>0.006 (-0.31, 0.67)</td>
<td>-0.098 (-0.82, 0.20)</td>
<td>0.554</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.00 (-1.0, 2.2)</td>
<td>1.10 (-0.1, 1.8)</td>
<td>0.4201</td>
</tr>
<tr>
<td>Arteriograph</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>-1.00 (-8.0, 9.0)</td>
<td>-4.00 (-6.0, 3.0)</td>
<td>0.676</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>-3.00 (-6.0, 4.0)</td>
<td>1.00 (-7.0, 4.0)</td>
<td>0.923</td>
</tr>
<tr>
<td>Mean arterial BP (mmHg)</td>
<td>-2.00 (-7.0, 6.0)</td>
<td>-1.00 (-6.0, 4.0)</td>
<td>0.836</td>
</tr>
<tr>
<td>Aortic PWV (m/s)</td>
<td>0.150 (-0.60, 1.05)</td>
<td>-0.300 (-1.90, -0.10)</td>
<td>0.285</td>
</tr>
<tr>
<td>Brachial augmentation index</td>
<td>0.650 (-6.40, 11.0)</td>
<td>0.850 (-25.5, 5.50)</td>
<td>0.248</td>
</tr>
<tr>
<td>Diastolic Reflection Area (DRA)</td>
<td>3.00 (-5.00, 5.00)</td>
<td>-3.50 (-6.00, 1.00)</td>
<td>0.157</td>
</tr>
<tr>
<td>Markers of Endothelial Damage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AnnV+/CD31+/CD42b- EMPs (events/ml)</td>
<td>4876 (-22373, 17089)</td>
<td>-2856 (-10366, 47402)</td>
<td>0.783</td>
</tr>
</tbody>
</table>

Table 7-16: Changes in cardiovascular risk factors, vascular stiffness measures and markers of endothelial damage over time

The change in each variable from baseline to the follow-up visit is shown in the table. The p value was obtained from a Mann-Whitney U test to look for differences in the median change between the 2 groups.

EMP = endothelial microparticles

### 7.6.3 Changes in endothelial function over time

The primary outcome for the study was the change in endothelial function (FMD) relative to the change in vitamin D. Given the associations between FMD and both age and baseline arterial diameter in this study, ED/EI dilatation was also included as an outcome. Furthermore, brachial artery diameter was associated with vitamin D at baseline. The median change over time in brachial diameter was -0.014mm in the deficient group and 0.087mm in the replete group (p=0.836).

When the changes in FMD and ED/EI were considered in terms of the patient groups there was a trend towards an increase in the ED/EI in the treated patients, but not in the replete patients. There was no significant change in FMD in either group (figure 7-10). Of note, the FMD at baseline was lower in the vitamin D replete group compared to the deficient group.
This may be due to increased age and increased baseline arterial diameter within the replete group given that the ED/EI dilatation was similar.

Figure 7-10: Changes in FMD and ED/EI in between baseline and follow-up
There was a non-significant increase in both FMD and ED/EI dilatation in the treated group but a less clear trend in the non-treated group. Panel (a) shows the results for FMD and panel (b) the results for ED/EI. The columns show the median values and the error bar shows the IQR. The baseline (pre) and follow-up (post) visit values were compared using the Wilcoxon matched-pairs signed rank test for paired non-parametric data. Healthy controls are shown for comparison.

There was wide variation in the change in 25(OH)D in the treated group. Consideration of the group as a whole may therefore mask any effects of vitamin D on FMD or ED/EI dilatation. The relationship between the change in vitamin D and change in FMD or ED/EI was therefore investigated by looking for a correlation between these two parameters. There was no correlation between change in vitamin D and change in FMD. In contrast, however, there was a significant correlation between the change in vitamin D and change in ED/EI dilatation in the treated group (but not the untreated replete group) (p=0.006) (figure 7-11). In this analysis the absolute change in vitamin D was used, but the relationship was also seen when the relative change in vitamin D (i.e fold change) is also used (r=0.509, p=0.0386).
Figure 7-11: Changes in FMD and ED/EI relative to final 25(OH)D and change in 25(OH)D
Scatter plots to show the correlation between the change in 25(OH)D over the follow-up period and the change in FMD (a) and change in ED/EI (b). The Spearman’s r correlation coefficient is shown.

As the distribution of ED/EI dilatation was not normal and contained negative values, ED/EI values were split into quartiles to explore factors associated with change in ED/EI. In univariate analysis the change in vitamin D was significantly associated with change in ED/EI dilatation (table 7-17). This associations remain significant after adjustment for age (OR 1.12, [1.02, 1.24], p=0.017). Adjustment for other variables was not performed as the number of subjects was low (n=17).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unadjusted</th>
<th>Age-adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age at baseline (years)</td>
<td>1.04</td>
<td>0.951, 1.13</td>
</tr>
<tr>
<td>25(OH)D at baseline (ng/ml)</td>
<td>0.985</td>
<td>0.807, 1.20</td>
</tr>
<tr>
<td>Change in 25(OH)D (ng/ml)</td>
<td>1.13</td>
<td>1.03, 1.24</td>
</tr>
<tr>
<td>Change in PTH (pg/ml)</td>
<td>0.974</td>
<td>0.933, 1.02</td>
</tr>
<tr>
<td>Change in Systolic BP (mmHg)</td>
<td>0.996</td>
<td>0.931, 1.07</td>
</tr>
<tr>
<td>Change in brachial diameter (mm)</td>
<td>3.19</td>
<td>0.07, 146</td>
</tr>
<tr>
<td>Change in SLEDAI score</td>
<td>0.899</td>
<td>0.513, 1.58</td>
</tr>
</tbody>
</table>

Table 7-17: Univariate analysis of parameters associated with change in ED/EI dilatation in treated patients
Ordered logistic regression was used in a univariate model to identify predictors of the change in ED/EI over the follow-up period. The OR describes the odds of moving to a higher quartile of ED/EI for each unit increase in the variable.
7.6.4 Changes in myeloid angiogenic cell number
The number of MACs in random fields was enumerated as described in Chapter 3 at the baseline visit and at follow-up. The number of MACs was significantly higher in the deficient group at the follow-up visit (mean [sd] 158.9 [70.0] vs. 206.6 [74.3], p=0.0152). There was no difference in numbers in the replete group (220.3 [67.0] vs. 209.8 [51.1], p=0.474 (figure 7-12). There was no correlation between the change in MAC number and the change in %FMD (r=0.418, p=0.157) or ED/EI dilatation (r=0.112, p=0.733).

Figure 7-12: Changes in the number of MACs between baseline and follow-up in SLE patients
There was a significant increase in the number of MACs in the deficient group over the follow-up period. The columns show mean number of cells per random field and the error bars show standard error. In the deficient group there were n=17 paired samples and n=5 in the replete group. Comparisons were made using paired t tests, *p<0.05.

7.7 Discussion
Our hypothesis was that endothelial dysfunction in SLE was driven, in part, by vitamin D deficiency and that vitamin D replacement could restore this abnormality. We found that vitamin D improved endothelial function (measured in terms of ED/EI dilatation) in patients with clinically stable SLE.

Our screening study aimed to identify vitamin D deficient and replete SLE patients and also healthy control subjects for the validation studies. The median 25(OH)D was similar in the 2 groups although approximately 50% of SLE patients were taking either low or high dose vitamin D supplements. Across the study population there was significant seasonal variation in 25(OH)D with higher levels in summer and autumn. Seasonal variations in vitamin D levels are well reported in the general population. In a prospective cohort of 125 adults in Greater Manchester with monthly 25(OH)D measurement, there was a difference of 10ng/ml between February and September (299). In lupus patients a significant seasonal difference was seen in some (410;424;431), but not all studies (422). This is interesting given that the majority of SLE
patients are advised to wear high factor sun block and to cover the skin where possible, and that these behaviours can attenuate seasonal variation in 25(OH)D levels in patients with skin disease (432). This suggests that even small amounts of sunlight exposure are sufficient to influence 25(OH)D levels. More detailed questioning of SLE patients in the main study also showed that sunlight avoidance behaviour influenced vitamin D levels. Vitamin D deficient SLE patients were significantly more likely to regularly cover arms and legs and tended to be more likely to report photosensitivity or fulfil the photosensitivity criterion in the 1997 Updated ACR Classification criteria.

The only other factor which significantly influenced 25(OH)D levels in this patient group was the use of high dose (but now low dose) vitamin D supplements. It is difficult to predict how much these supplements increase 25(OH)D levels in the patient population. In other groups, 800IU/day (the dosage in 2 Calcichew D₃ Forte tablets) increased serum 25(OH)D by approximately 8ng/ml (573) whilst higher dose supplements (i.e. 20,000IU/week) increased vitamin D levels by around 25ng/ml (574). It is assumed that the response to vitamin D is the same in SLE patients compared to healthy controls. There are no studies which have reported the change in vitamin D levels in response to treatment regimens similar to those used in this study or those described above. A high-dose strategy of 1400IU/day for 40 days, followed by 400IU/day resulted in a mean increase in 25(OH)D of 13ng/ml at 12 months in patients with SLE (575). This was around 50% of the increase seen in this study in which the recommended regional protocol comprised a bolus of 400,000IU over 10 days and a maintenance dose of 20,000IU a week. This strongly suggests that higher dose long term therapy is required in SLE patients with lower levels of 25(OH)D.

At the screening visit all subjects underwent aPWV and associated measures of vascular function using the TensioMed Arteriograph™. A direct comparison between the healthy control group and SLE group is limited as the SLE patients were significantly older than the HC group and this was not the primary purpose of this study. Differences in blood pressure, augmentation index, aortic pulse-wave velocity and estimates of coronary vessel filling between HCs and SLE patients were no longer seen after the analyses were adjusted for age. In the lupus patient group, arterial stiffness was strongly associated with both age and blood pressure but not with 25(OH)D. This is in contrast to a previous study in which aPWV was independently associated with 25(OH)D in SLE patients after adjustment for both of these parameters (470). This difference may be due to the patients in the previous study having more active disease which is strongly associated with both aPWV and 25(OH)D levels. Interestingly, there was an inverse association between aPWV and SLEDAI score in the
screening study. This should be interpreted with some caution as the population had predominantly inactive disease (median SLEDAI was 0).

In the main study of 22 deficient and 18 replete patients the two groups there was a significant difference in age, with the replete population a median of 11 years older. The observed differences in blood pressure and augmentation index were all attenuated after adjustment for age suggesting that age was a confounding factor. The exception to this was the diastolic reflection area. The odds of being in a higher tertile of DRA increased by 5-fold for each 1ng/ml increase in 25(OH)D, after adjustment for age and blood pressure. Interestingly, this association is not seen in the unadjusted analysis, suggesting that differences in age and blood pressure between the groups were masking the difference. The DRA is an arbitrary value calculated by the Arteriograph™ based on the principle that a normally timed reflected wave returns to the aorta during diastole and thus increases the coronary perfusion pressure. This result should be interpreted with some caution, however, as it was the only Arteriograph parameter to be associated with 25(OH)D after age/blood pressure adjustment. Furthermore, the methodology for calculating this parameter is unknown and so blood pressure adjustment may be inappropriate. The relevance of DRA is also uncertain as it has not been validated in clinical studies.

In this clinically stable SLE population there was no increase in the number of circulating EMPs compared to healthy controls. Within the SLE population, however, EMPs were associated with both disease duration and the accumulation of organ damage. The same protocol for EMP isolation was used, allowing a direct comparison to be made with a cohort of SLE patients with active disease. These patients had much higher levels of EMPs which fell when the inflammation was controlled with immunosuppressive therapies (110). Parker et. al. (2012) found that in active lupus patients, there was a correlation between EMPs and FMD, although over time there was not an association between change in EMPs and change in FMD (477). In this study there was no association between EMPs and FMD, ED/EI dilatation or serum 25(OH)D. Despite levels being similar between patients and HCs there was an independent association between EMPs and arterial stiffness which is consistent with studies in other subject groups (576-578).

The primary outcome for the observational study was a change in FMD over 3 months in patients receiving high dose vitamin D supplements. The baseline brachial artery diameter at baseline was independently associated with vitamin D levels but did not significantly change over the follow-up period in the vitamin D treated group. In patients with SLE, it has been
reported that baseline artery diameter is significantly smaller in patients with active disease (579). In a study by Holewijn et. al. (2009) in the general population, increased baseline diameter was associated with carotid IMT and prevalent CVD whilst FMD was not (580).

There was no difference in FMD at baseline between deficient and replete lupus patients. Whilst FMD is a well-validated technique for the assessment of endothelial function, considerable care needs to be taken to avoid the introduction of variability. The assessment of FMD in this study was based on guidelines published by the International Brachial Artery Reactivity Task Force which were later updated by Thijssen et. al. (2011) (85;475). Of note, the recording periods were slightly shorter than recommended due to file storage limitations, but as the peak diameter was observed between 45 and 75 seconds post deflation further recording beyond 120 seconds was deemed unnecessary. The coefficient of variation in this study was similar to that previously reported by our group (477). Despite the care taken, in this study FMD was strongly influenced by both age and baseline arterial diameter. This is of significance given that the vitamin D replete patients were older with increased brachial artery diameter. In order to allow for these differences the %FMD was normalised to the %GTN dilatation (to give the ED/EI ratio). This approach has been successfully used before both in patients with SLE and other patient groups (581;582).

This is the first study to demonstrate an association between serum vitamin D and endothelial function in patients with SLE. There was a significant association between the change in 25(OH)D and the change in ED/EI dilatation over time in the treated group. In the replete group there was a minimal change in the vitamin D levels and no change in ED/EI dilatation. In the regression analyses, only the change in 25(OH)D was associated with a change in endothelial function. These remained after adjustment for age, but other confounding variables cannot be excluded. The question remains whether the change in 25(OH)D or the final 25(OH)D concentration has the greatest effect on ED/EI dilatation. There were insufficient numbers of subjects in the study to allow the investigation of threshold effects. A further study is needed to identify the serum level of vitamin D which would result in a significant increase in endothelial function. Furthermore, in this study endothelial dysfunction was used as a surrogate marker for the development of CVD and for clinical cardiovascular events. An interventional trial with cardiovascular outcomes is desirable but is likely to be unachievable in a rare condition such as lupus. Given that vitamin D changes surrogate makers in SLE, if a reduction in CVD events were found in the general population or other patient groups it might be reasonable to extrapolate these results to patients with lupus.
Importantly the change in ED/EI dilatation was not associated either with changes in PTH or serum calcium or disease activity. The only other significant change over the follow-up period was an increase in the number of MACs in the deficient (but not the replete) group. Mechanistically it could be proposed that an increase in endothelial repair mechanisms could results in an improvement in endothelial function but this study does not provide sufficient evidence to support this. Interestingly, there was no correlation between the change in ED/EI and the change in the number of MACs. As described previously, however, the number of MACs is not associated with their overall angiogenic capacity.

This study has some limitations. Recruitment to the study occurred more slowly than predicted and as such the study remains under powered. The aim was to recruit 42 patients to the vitamin D deficient arm. The power calculation was based on a mean change in vitamin D of 6.12ng/ml. In this study however the mean change in the treated group was 28.6ng/ml. Although an association with ED/EI dilatation was seen, the study may still not have sufficient power to identify an association with %FMD. As the numbers are small it is not possible to give full consideration to other confounding parameters which may have impacted on the endothelial function.

In addition there was significant variability in the change in 25(OH)D levels seen in the treated group. As this was an observational study the patients may have received different preparations or dosages of cholecalciferol as any treatment was at the discretion of their GP. Similarly, there may have also been difficulties with patient compliance with the treatment. The patients all had low disease activity at recruitment. This was advantageous as any changes in endothelial function were unlikely to be due to changes in disease activity. Conversely, however, it is not possible to determine whether high dose vitamin D has additional benefit on disease activity in SLE. Modest vitamin D dosages have been shown to be associated with a small decrease in active disease (455).

This study has suggested a network of associations between SLE and markers of endothelial repair, function and damage. The associations in the context of those reported by other groups are shown in figure 7-13.
In summary, the results from this chapter show that in patients with stable disease, treatment of vitamin D deficiency results in improved endothelial function and an increase in MACs similar to that seen \textit{in vitro}. Although the mechanism by which this improvement occurs requires further study, it can be proposed that endothelial repair mechanisms are targeted by vitamin D. Further studies are also needed to determine the minimum change in vitamin D needed to result in a clinically significant improvement in endothelial function and to determine whether this results in a reduction in cardiovascular events.

\section*{7.8 Summary}

- Vitamin D levels in SLE patients were influenced by season and prior vitamin D therapy but were not significantly different from levels seen in healthy controls.

- Vitamin D deficient patients were more likely to be younger, non-white, take sun avoidance measures and have a history of renal disease and to be taking prednisolone and immunosuppressant drugs.

- Arterial stiffness in SLE patients was independent of 25(OH)D but strongly driven by age and blood pressure.

- Change in serum 25(OH)D was significantly associated with an improvement in endothelial function (as measured by endothelium-dependent:endothelium-independent dilation) and an increase in the number of MACs in clinically stable vitamin D deficient SLE patients.
Chapter 8: Final discussion and future directions
8 Final discussion and future directions

The studies presented in this thesis aimed to determine the effects of vitamin D on endothelial function and repair mechanisms in patients with SLE. Vitamin D deficiency is prevalent in the lupus population and associated with both vascular dysfunction and lupus disease activity in cross-sectional and a small number of longitudinal studies. There is however, little evidence that vitamin D replacement can improve vascular function in this high risk population. Vitamin D offers an attractive novel therapy as it is relatively inexpensive, well-tolerated and has low toxicity. The studies in this thesis were designed to determine whether vitamin D can modify endothelial function in SLE patients, and to determine the mechanism by which this may occur.

The first part of this thesis addressed the role of vitamin D in endothelial repair mechanisms. A significant amount of time was spent characterising MACs from HCs and SLE patients, demonstrating that they have the ability to migrate towards endothelial cells and have a pro-angiogenic capacity in vitro. The comparison of MAC function between the 2 patients groups, and vitamin D deficient and replete SLE patients is novel and supports previous observations that endothelial repair mechanism are impaired in SLE.

The role of IFN in the development of endothelial function and vascular damage in SLE is unclear. Our studies of the effects of IFN on healthy aortic endothelial cells in vitro strongly suggest that IFN has only an indirect effect on the endothelium. Other important target cells for IFN within the vasculature include macrophages, T cells, platelets and MACs. This study focussed on the detrimental effects of IFN on MACs to develop a model of failed endothelial repair, as might be seen in SLE. Interestingly, whilst MACs were sensitive to IFNα, there were differences in function between IFN-treated healthy MACs and MACs isolated from SLE patients, suggesting that other factors are important.

It is important to recognise that the role of MACs in endothelial repair in vivo is not completely understood. Whilst the injection of MACs augments angiogenesis in animal models of hind-limb ischaemia, the ability of MACs either to recognise sites of vascular damage and/or stimulate endothelial cells to migrate and proliferate has not yet been shown in human studies. The in vitro models were therefore developed to measure what might be considered important functions of MACs in the process of endothelial repair.

Vitamin D had beneficial effects on MAC number and function, in both the IFN model and MACs from SLE patients. Vitamin D increased the number of lupus MACs and restored their phenotype toward that of healthy controls. This reduced activation status was associated with reduction in expression of interferon-regulated protein IP10, whilst the production of pro-
angiogenic factors was not affected. This effect led to an increased angiogenic capacity in an
*in vitro* model of angiogenesis. Recently, Wong et. al. (2014) demonstrated that 1,25(OH)₂D₃
promoted vascular regeneration in a diabetic hind-limb ischaemia model. Importantly, tissue-
specific VDR knock-out models identified that myeloid cells mediate the restorative effect
of vitamin D (583). This observation strongly supports the *in vitro* data in our study and validates
the use of MACs to model the effects of vitamin D on vascular repair.

Vitamin D also augmented the ability of MACs to interfere with TNF-mediated down-regulation
of eNOS in aortic endothelial cells. Down-regulation of eNOS is an important process in the
development of endothelial dysfunction. The changes in eNOS expression were small but
apparent at both the transcript and protein level. The model used proved to be unsuitable for
the detection of nitric oxide, due to its lack of sensitivity, and thus changes in NO
bioavailability could not be demonstrated. It is also important to note that eNOS undergoes
phosphorylation which modulates its activity and ultimately NO production. Further studies
are needed to identify which secreted factor (or factors) modulates TNF signalling in
endothelial cells or directly regulate eNOS expression.

The second part of this thesis aimed to translate the *in vitro* findings into the lupus patient
population by determining the effect of vitamin D on endothelial function. In this
experimental study, clinically stable deficient patients treated with high doses of vitamin D
showed an improvement in endothelial function over the follow-up period which correlated
with the change in serum 25(OH)D. Changes in serum PTH calcium did not correlate with
change in endothelial function suggesting that the effect is due to vitamin D itself rather than
secondary effects on calcium homeostasis. The changes in ED/EI support the changes in eNOS
expression observed *in vitro*.

This study was not designed to address the question of whether there is a threshold effect of
vitamin D on endothelial function or which treatment regimens had the most beneficial effect.
Further, larger studies are needed to address this question, ideally in the context of a
randomised controlled trial. The magnitude of the changes in endothelial function observed
and the association with 25(OH)D could be used to calculate sample size for such a study. In
addition, the persistence of the changes on endothelial function should be demonstrated over
a longer period of time to confirm the durability of this effect.

The improvement in endothelial function was not associated with either change in arterial
stiffness or in markers of endothelial damage that were measured, namely EMPs. The number
of EMPs in this stable cohort was low and did not differ from the healthy control population.
Despite this, the number of EMPs was strongly associated with aortic stiffness and with longer
disease duration. Whilst studies have shown rapid changes in endothelial function in SLE patients in response to a variety of agents, there have been no interventional studies showing a change in vascular stiffness. It is however possible that aPWV might change in SLE with the correct intervention, as patients with rheumatoid arthritis show an improvement in aPWV after 8 weeks of treatment with TNF-inhibitors (584). In addition, all indices of vascular stiffness do not change together and some interventions may change the augmentation index but not the aPWV (585).

The use of a stable cohort allowed the changes in vessel function to be considered independently from any effect on lupus disease activity. As might be expected, therefore, there were no significant changes in any of the clinical measures of disease activity or in levels of serum cytokines. It would be interesting to further explore the immunomodulatory effects of vitamin D in the lupus population. The in vitro studies above show a significant effect of vitamin D on macrophage function, and others have identified anti-inflammatory effects on other cell types including T and B cells. Whilst it is unlikely that vitamin D alone would be sufficient to treat active SLE, it may have an additive, or even synergetic action with immunosuppressant therapy. Whilst the reduction in disease activity in the Hopkins Lupus Cohort was modest, this observation should encourage further clinical trials of vitamin D in active SLE.

A significant limitation of this study is that both clinical observations and in vitro experiments were primarily conducted in vitamin D deficient patients. It is therefore not possible to extrapolate these results to vitamin D replete patients. The beneficial effects of vitamin D on endothelial function may not be confined to deficient patients, particularly given that the deficient and replete groups did not significantly differ in terms of endothelial function at baseline. This suggests that rather than simply correcting abnormal endothelial function caused by vitamin D deficiency, high dose vitamin D therapy may have benefit regardless of vitamin D status. In order to address this, in vitro studies of MAC function could be carried out using PBMCs from replete patients. The effects on replete SLE patients could be addressed as part of a clinical trial as described above, providing that safety concerns around the potential development of hypercalcaemia could be addressed. Similarly this study cannot determine whether these effects are confined to SLE or also occur in the general population. Given the observations of Tarcin et al. (2009), that vitamin D can improve endothelial function in vitamin D deficient healthy controls, this seems a reasonable conclusion to draw (339).

In the angiogenesis assay, the effect of vitamin D on lupus MACs appeared to be down-regulation of IP10 which restored the angiogenic capacity towards that of healthy controls. It
is difficult to predict the effects of vitamin D on HC MACs. It could be argued that as IP10 levels were similar between SLE patients and HC, that vitamin D may have the same beneficial effect on angiogenesis in the control population via reduction in IP10 expression by MACs. Conversely, the increase in angiogenesis seen by IP10 blockade in SLE only increased network formation to the same level induced by HC MACs. This may mean that the HC MACs are already functioning optimally in terms of angiogenesis or that the in vitro assay has a threshold effect. Further studies are needed to determine the implications of changes in IP10 secretion in vivo. The receptor for IP10 (CXCR3) is differentially expressed in the vasculature and is increased in the presence of inflammation (586). The anti-angiogenic (and thus potentially anti-reparative) effect of IP10 may therefore be augmented in the presence of inflammation. Blockade of CXCR3 may therefore offer a novel approach to vasculoprotection in patients with SLE.

Taken together, these results support the hypothesis that vitamin D can improve endothelial function in SLE, an effect which is mediated, at least in part, by the augmentation of endothelial repair mechanisms. Further clinical trials are needed to determine whether vitamin D can offer a safe and effective means of reducing the cardiovascular disease burden in patients with SLE.
Appendices
## 9 Appendices

### Appendix 1: Laboratory materials used

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Appendix 2: Cell culture media and buffers used

**EGM-MV2**
Endothelial Cell Basal Media MV2 (Promocell, Germany) supplemented with 5% fetal bovine serum (Sigma-Aldrich, UK), 100 U/ml penicillin and 100μg/ml streptomycin (Sigma-Aldrich, UK) and EGM-MV2 supplement pack; (final concentration given below)
- Epidermal growth factor (EGM) 5ng/ml
- Basic fibroblast growth factor (bFGF) 10ng/ml
- Insulin-like growth factor (IGF) 20ng/ml
- Vascular endothelial growth factor-165 (VEGF-165) 0.5ng/ml
- Ascorbic acid 1μg/ml
- Hydrocortisone 0.2μg/ml

**ECGM**
Endothelial Cell Basal Medium (Promocell, Germany) supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100μg/ml streptomycin and EGM supplement pack; (final concentration given below)
- Endothelial cell growth supplement (ECGS, from bovine hypothalamus) 0.004ml/ml
- Epidermal growth factor (EGF) 0.1ng/ml
- Basic fibroblast growth factor (bFGF) 1ng/ml
- Heparin 90μg/ml
- Hydrocortisone 1μg/ml

**EGM**
Endothelial Basal Medium (Lonza, Switzerland) supplemented with 20% fetal bovine serum, 100 U/ml penicillin and 100μg/ml streptomycin and EGM supplement pack; final concentrations not available from supplier
- Bovine brain extract (BBE) with heparin
- Epidermal growth factor (EGF)
- Hydrocortisone
- Ascorbic acid
- Gentamicin and amphotericin B (GA-1000)

**EBM**
Endothelial Basal Medium (Lonza, Switzerland) supplemented with 1% (w/v) bovine serum albumin (Sigma-Aldrich), 100 U/ml penicillin and 100μg/ml streptomycin, gentamicin-amphotericin B (GA-1000) and ascorbic acid.

**RPMI-1640**
RPMI-1640 (Sigma-Aldrich, UK) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin and 100μg/ml streptomycin.

**Phosphate Buffered Saline (10x)**
- Sodium chloride (NaCl) 1.37M
- Potassium chloride (KCl) 27mM
- Disodium phosphate (Na₂HPO₄) 0.1M
- Potassium phosphate (KH₂PO₄) 18mM
PBS+ (1x)
Sodium chloride (NaCl) 137mM
Potassium chloride (KCl) 2.7mM
Disodium phosphate (Na2HPO4) 10mM
Potassium phosphate (KH2PO4) 1.8mM
Calcium chloride (CaCl2) 1mM
Magnesium chloride (MgCl2) 0.5mM

3D Collagen Matrix
For triplicate wells solution 1 and solution 2 were mixed thoroughly on ice.

Solution 1:
9.75µl M199 medium Modified (10x)
102.2µl M199 medium Modified (1x)
0.525µl 5N NaOH

Solution 2:
15.25 µl 0.1% acetic acid
72.2µl type 1 rat tail collagen (Becton Dickson, UK)

Tris-Borate-EDTA (TBE) Buffer (10x): 1l volume
Tris-Base 108g
Boric Acid 55g
EDTA (0.5M, pH 8.0) 40ml

RIPA Buffer
Sodium chloride (NaCl) 105mM
Tris (pH 7.2) 50mM
Sodium deoxycholate 1% (w/v)
Triton X-100 1% (v/v)
Sodium dodecyl sulphate (SDS) 1% (w/v)
Glycerol 5% (v/v)
DTT 1mM
Sodium orthovanadate (Na3VO4) 50µM
Sodium pyrophosphate 200µM
Made to 10ml with dH2O, pH7.2, stored at -20°C

5x Laemmli sample buffer
Tris (pH 6.8) 1M
SDS 5% (w/v)
Glycerol 25% (v/v)
β-mercaptoethanol 5% (v/v)
Made to 10ml with dH2O

SDS-Page Running Buffer
Tris 25mM
Glycine 190mM
SDS 0.1% (w/v)

SDS-Page Transfer Buffer
Tris 25mM
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Appendix 3: Expression and function of 1α-hydroxylase in MACs

Rationale
The enzyme 1α-hydroxylase (CYP27B1) is present in renal tissue and is essential for the conversion of 25(OH)D to 1,25(OH)₂D₃ and therefore the activation of vitamin D. Expression is not limited to the kidney and the 1α-hydroxylase transcript can be detected in a multitude of other tissue types including macrophages (255). The macrophage is, however, one of the few tissue types in which this enzyme has been shown to be functional, notably in the context of patients with hypercalcaemia sarcoidosis who demonstrated dysregulated activation of vitamin D. Given that MACs have a myeloid phenotype, the expression and activity of 1α-hydroxylase in MACs was investigated. If 1,25(OH)₂D₃ could be reliably generated within MACs, then this would support an autocrine/paracrine effect of vitamin D in endothelial repair (rather than an endocrine effect of circulating 1,25(OH)₂D₃).

Expression of 1α-hydroxylase
Expression of 1α-hydroxylase was determined by RT-PCR. The primer sequence for 1α-hydroxylase was 5’-ACTCAGGAAGCCAGGTGAGA-3’ (forward) and 5’-GACGAAGGACCAACCAGGTA-3’ (reverse). β-actin was used as a reference gene as described previously. Reverse transcription was conducted as described and RT-controls were produced using the same master mix with the omission of reverse transcriptase. PCR was carried out with denaturation at 95°C for 1 minutes followed by 30 cycles of 95°C for 30 seconds, 59°C for 30 seconds and 72°C for 30 seconds, with a final annealing step at 72°C for 7 minutes. The PCR products were run on a 1% (w/v) agarose gel with 0.005% (v/v) ethidium bromide and visualised under UV light (figure 9-1).

The expression of 1α-hydroxylase was identified in all 3 cell types tested (HAoECs, PBMCs and Day 8 MACs). PBMCs and MACs were derived from healthy control subjects.
Figure 9-1: Expression of 1α-hydroxylase in 3 cell types
The PCR product was 239 base pairs in size and present in all 3 cell types: HAoECs, PBMCs, and MACs. No bands were seen in the RT negative controls, thus excluding genomic DNA contamination.

Conversion of 25(OH)D to 1,25(OH)₂D
To measure the 1α-hydroxylation of 25(OH)D by MACs, the concentration of 1,25(OH)₂D₃ in the cell culture media was measured by HPLC in the Specialist Assay Laboratory at Central Manchester University Hospitals (Dr J Berry). In some experiments, TNFα (10ng/ml) was added to activate 1α-hydroxylase. To validate this approach, the production of 1,25(OH)₂D₃ was also measured in THP-1-derived macrophages. THP-1 cells were cultured in RPMI-1640 supplemented with 2mM Glutamine and 10% FBS. Prior to the experiment, THP-1 cells (50,000 cells per 24-well plate) were differentiated into macrophages by the addition of 50nM phorbol myristate acetate (PMA) for 72 hours. Complete differentiation was determined by the presence of adherent cells on light microscopy. At t=0, 25(OH)D (±10ng/ml TNFα) was added to the cells in fresh, warm culture media. The cell culture media was removed at the designated time point for 1,25(OH)₂D₃ analysis.

THP-1 macrophages synthesised 1,25(OH)₂D₃ from 25(OH)D over a 48 hour time period. Negligible concentrations of 1,25(OH)₂D₃ were seen the culture media alone (not shown). The production of 1,25(OH)₂D₃ was associated with a decrease in the concentration of 25(OH)D over the same time period. In contrast in MACs there was no change in 1,25(OH)₂D₃ after 24 hours over a range of concentrations of 25(OH)D (1nM-1µM). The addition of 10ng/ml TNFα did not change the production of 1,25(OH)₂D₃ in either cell type (measured over 48 hours in THP-1 macrophages and at 24 hours in MACs) (figure 9-2).
The production of 1,25(OH)₂D₃ was clearly seen in THP-1 macrophages over 48 hours. This was accompanied by a decrease in the concentration of 25(OH)D. No difference was seen between concentrations in the presence or absence of 10ng/ml TNFα (a). The graph shows concentration of 25(OH)D in nmol/l on the left y axis and concentration of 1,25(OH)₂D₃ in pmol/l on the right y axis. In MACs there was no change in the concentration of 1,25(OH)₂D₃ at 24 hours with increasing concentrations of 25(OH)D (b). The concentration of 1,25(OH)₂D₃ in pmol/l is shown on the right y axis and 25(OH)D at 24 hours on the left y axis as a comparator. The addition of TNFα to MACs did not result in an increase in 1α-hydroxylase activity (c).

The expression of 1α-hydroxylase was observed by RT-PCR in MACs, PBMCs and HAoECs. In endothelial cells, expression has been reported previously (396). In the experiments above, measurement of 1α-hydroxylase activity was confined to myeloid cells. Macrophages have been shown to express high levels of CYP27B1 compared to monocytes (587). Whilst 1,25(OH)₂D₃ was generated from 25(OH)D in THP-1 macrophages, this was not seen in MACs. The principle differences may be due to the effects of PMA, which can induce 1α hydroxylase activity (588). Interestingly TNFα, which has been reported to activate CYP27B1 in HUVEC, had no effect in either cell type (THP-1 macrophages or MACs) (397). The experiments above demonstrate that in the culture system used, MACs did not activate vitamin D. This justifies the use of 1,25(OH)₂D₃ in the experimental models of MAC function.
Appendix 4: Validation of non-invasive measures of vascular function

A reliability study was conducted using healthy control subjects in order to demonstrate that the measures of aPWV and FMD are robust. This is of particular importance given that FMD is a highly user-dependent technique. Current guidelines recommend that reproducibility data is calculated and presented by each group using FMD as a study outcome (475). Intra-observer variability was measured for both PVW and FMD and is reported in terms of correlation (Pearson’s correlation coefficient r), agreement (Bland-Altman method) and variance relative to the mean value (coefficient of variation, CV). Pearson’s correlation co-efficient was deemed appropriate as all data were normally distributed (D’Agostine and Pearson normality test). In addition, the repeatability of aPWV measurement was determined.

**Pulse-wave velocity: repeatability**

Aortic pulse-wave velocity was measured in healthy subjects are previously described. In order to assess repeatability, aPWV was measured in a single subject on n=10 occasions. The subject was fasted and avoided alcohol, nicotine and caffeine as per the Arteriograph guidelines. The measurement of JSD was carried out in duplicate on the first visit and this value used for the remaining 9 measurements. The Arteriograph measurement was carried out by different individuals in order to mimic how real-life assessments would be carried out using an automated device.

The coefficient of variation (CV) describes the variance of the measure relative to the mean value obtained. The CV was determined using the following formula:

\[
CV = \left( \frac{SD}{mean} \right) \times 100
\]

The coefficients of variation for BP, BAix and aPWV within a single subject are shown in table A1.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPWV (m/s)</td>
<td>7.30</td>
<td>0.228</td>
<td>3.09</td>
</tr>
<tr>
<td>Brachial Augmentation Index (%)</td>
<td>-66.72</td>
<td>3.61</td>
<td>5.40</td>
</tr>
<tr>
<td>Aortic Systolic BP</td>
<td>111</td>
<td>4.69</td>
<td>4.23</td>
</tr>
</tbody>
</table>

Table 9-1: Repeatability of Arteriograph measures in a single subject

* aPWV aortic pulse-wave velocity; BP blood pressure; SD standard deviation; CV coefficient of variation

**Pulse-wave velocity: intra-observer reliability**

Intra-observer reliability was determined by measuring aPWV on 2 occasions, 1-2 weeks apart (n=10 individual subjects). All measurements were conducted as described above. The
The correlation coefficient for aPWV was 0.779 (figure 9-3). This is lower than previous large validation studies which demonstrate that the Arteriograph method has a correlation coefficient of \( r = 0.97 \) for aPWV (589).

**Figure 9-3: Correlation between measures of aortic pulse-wave velocity at 2 time points**

The agreement between 2 measures of aPWV 1-2 weeks apart was assessed using the Bland-Altman method (590). The plot shows the difference between the mean of the 2 measures and the difference between them. Figure 9-4 shows good agreement between the measures conducted 1-2 weeks apart. Table 9-2 summarizes the 3 different approaches used to determine reliability of aPWV measurement.

**Figure 9-4: Bland-Altman plot of aortic pulse-wave velocity at 2 time points**
**Table 9-2: Variability in measurement of aortic pulse-wave velocity**

<table>
<thead>
<tr>
<th>Measurement of Intra-observer Variability</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman’s r correlation coefficient</td>
<td>0.779, p=0.008</td>
</tr>
<tr>
<td>Bland-Altman 95% limits of agreement</td>
<td>-0.590 to 1.25</td>
</tr>
<tr>
<td>CV (%)</td>
<td>6.12</td>
</tr>
</tbody>
</table>

**Flow-mediated dilatation: intra-observer reliability**

The published guidelines for the measurement of brachial artery FMD recommend that intra-observer reliability data is obtained for the measurement of the brachial artery diameter and both endothelial-dependent (flow-mediated) and endothelial-independent (GTN-mediated) dilatation (475). Flow-mediated dilatation was measured in n=10 healthy subjects and GTN response in n=9 subjects 1-2 weeks apart as described above. A single healthy control was not able to tolerate GTN due to severe headache. Small differences between absolute diameters can result in large differences in both FMD and GTN response as both of these are expressed as a %change in diameter.

The Spearman r correlation coefficients for baseline arterial diameter, FMD and GTN-mediated dilatation and ED/EI dilatation were r=0.936, r=0.849, r=0.504 and r=0.288 respectively (figure 9-5). In terms of measurement of ED/EI there was a single outlier who had a very low FMD response at the first visit, with a normal GTN response. If this outlier is excluded, then the correlation coefficients improve to r=0.917, p=0.001 for FMD and r=0.691, p=0.069 for ED/EI dilatation. Whilst the Corretti guidelines for the measurement of FMD discuss the importance of reproducibility testing, there is no consensus on the value of an acceptable CV (475). The CVs for both baseline arterial diameter and %FMD were similar to that reported by Parker (2012) (477).
Figure 9-5: Correlation coefficients for measurements of endothelial function in healthy controls

The graphs show the scatter plots of test and re-test values for baseline arterial diameter (a), FMD (b), GTN-mediated dilatation (c), and ED/EI dilatation (d). The lines show r=1. The Spearman’s r correlation coefficient is presented. The numbers of pairs are n=10 in (a) and (b), and n=9 in (c) and (d).

Bland-Altman plots were used to describe the difference between the measures at the 2 visits against the mean of the measure. Baseline diameter showed good agreement, whilst %FMD, %GTN-response and ED/EI dilatation showed less agreement (figure 9-6). The 3 validation measures used for the 4 different parameters of endothelial function are summarised in table 9-3.
Figure 9-6: Bland-Altman plots of the measures of endothelial function
The graphs show the Bland-Altman plots for baseline arterial diameter (a), FMD (b), GTN-mediated dilatation (c), and ED/EI dilatation (d). The horizontal lines show the mean and 2 standard deviations of the mean.

<table>
<thead>
<tr>
<th>Measurement of Intra-observer Variability</th>
<th>Baseline Diameter (mm) (n=10 pairs)</th>
<th>FMD (%) (n=10 pairs)</th>
<th>GTN Response (%) (n=9 pairs)</th>
<th>ED/EI Dilatation (%) (n=9 pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman’s correlation coefficient</td>
<td>0.936 p&lt;0.0001</td>
<td>0.849, p=0.002</td>
<td>0.504, p=0.166</td>
<td>0.317, p=0.410</td>
</tr>
<tr>
<td>Bland-Altman 95% limits of agreement</td>
<td>-0.221 to 0.402</td>
<td>-6.526 to 8.201</td>
<td>-15.9 to 13.2</td>
<td>-69.4 to 79.5</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.03</td>
<td>32.3</td>
<td>28.6</td>
<td>51.3</td>
</tr>
<tr>
<td>CV (%) in study by Parker (2012) (477)</td>
<td>3.50</td>
<td>45.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 9-3: Variability in the measurements of arterial diameter, FMD and GTN response in healthy subjects

**Effect of age and baseline arterial diameter on FMD**
It is well recognised that the baseline arterial diameter is associated with both the absolute post-deflation diameter and the %FMD. This was examined in all subjects at both visits. There
was a strong correlation between the baseline diameter and post-deflation maximal diameter, and thus the %FMD (figure 9-7).

![Figure 9-7: Correlation between baseline arterial diameter and %FMD](image)

Scatter plots to show the correlation between the baseline diameter and maximum post-dilatation diameter (a) and %FMD (b). The graph shows all measurements of FMD in both HCs and SLE patients. The Spearman r correlation coefficient is shown.

Similarly, there was a correlation between a subject’s age and the %FMD and the %GTN-response. This has biological plausibility as both endothelial health and smooth muscle function are expected to deteriorate with age (figure 9-8). This was measured in HCs and SLE patients at baseline.

![Figure 9-8: Correlation of %FMD and %GTN-mediated dilatation with age](image)

Scatter plots to show the correlation between the age of the subject and %FMD (a) and %GTN-mediated dilatation (b). The graphs show measurements of HCs and SLE patients, each subject only contributing once. The Spearman r correlation coefficient is shown.

If the %FMD is normalised against the %GTN response, to give the ED/EI dilatation, there is no longer any correlation with either age or baseline diameter (figure 9-9). Therefore this removes these 2 confounding factors from the analysis. This is of particular importance given the vitamin D deficient and replete populations differed significantly in terms of age.
Figure 9-9: Correlation of ED/EI dilatation with age
Scatter plots to show the correlation between the ED/EI dilatation and baseline diameter (a) and age (b). The Spearman r correlation coefficient is shown.
Appendix 5: Comparison of measurements of vascular function between SLE patients and healthy controls

During the screening phase of the clinical study, data on vascular function were collected on all patients and healthy controls. As described in Chapter 7, the SLE and control populations differed significantly in terms of a number of parameters including age, vitamin D use, season of recruitment and ethnicity. Due to these differences, it is difficult to compare the vascular function of the 2 groups. An exploratory analysis was carried out to identify differences in vascular function between patients and controls and to identify if any differences were due to disparities between the groups.

aPWV measured using the Tensiomed Arteriograph™ and other vascular parameters (e.g. blood pressure, brachial augmentation index) were compared using the Mann-Whitney U test. Many of the parameters were significantly higher (or trended towards being so) in SLE patients compared to controls. The DRA was significantly lower in SLE patients. Only heart rate and pulse pressure did not show any clear differences (table 9-4)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy (median, IQR)</th>
<th>SLE (median, IQR)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>19</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>120 (113, 126)</td>
<td>124 (117, 139)</td>
<td>0.0949</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>74.0 (72.0, 78.0)</td>
<td>79.0 (73.0, 86.5)</td>
<td>0.0304</td>
</tr>
<tr>
<td>Pulse Pressure (mmHg)</td>
<td>44.0 (41.0, 55.0)</td>
<td>46.0 (41.0, 55.0)</td>
<td>0.583</td>
</tr>
<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>89.0 (86.0, 93.0)</td>
<td>96.5 (87.0, 102)</td>
<td>0.0299</td>
</tr>
<tr>
<td>Heart Rate (beats/min)</td>
<td>61.0 (54.0, 67.0)</td>
<td>65.5 (59.5, 74.0)</td>
<td>0.101</td>
</tr>
<tr>
<td>Brachial Augmentation Index (%)</td>
<td>-26.6 (-46.8, -4.2)</td>
<td>9.00 (-6.4, 34.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Aortic Augmentation Index (%)</td>
<td>24.2 (13.9, 35.5)</td>
<td>41.4 (33.6, 53.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aPWV (m/s)</td>
<td>6.20 (5.8, 7.0)</td>
<td>9.10 (7.0, 11.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Central Systolic BP</td>
<td>112 (106, 125)</td>
<td>126 (118, 146)</td>
<td>0.0045</td>
</tr>
<tr>
<td>Central Pulse Pressure</td>
<td>39.0 (34.0, 53.0)</td>
<td>48.0 (42.0, 63.0)</td>
<td>0.0032</td>
</tr>
<tr>
<td>Diastolic Reflection Area*</td>
<td>53.0 (45.0, 64.0)</td>
<td>41.5 (37.0, 52.0)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Table 9-4: Vascular function measured using the Arteriograph in SLE patients and healthy controls
Arterial function was significantly impaired in SLE patients compared to controls in terms of most of the parameters measured. The table shows the median (IQR) values and the Mann-Whitney U test was used to identify significant differences between the groups.

Selected parameters were investigated in univariate ordered logistic regression models and subsequently adjusted for age. Mean arterial blood pressure was used as a marker of blood pressure. The models used the vascular parameter as the independent variable and the
patient group (patient or control) as the dependent variable. An OR<1 means that the value is higher in patients than controls (table 9-5).

<table>
<thead>
<tr>
<th>Unadjusted</th>
<th>Age-adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>aPWV (m/s)</td>
<td>0.108</td>
</tr>
<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>0.340</td>
</tr>
<tr>
<td>Brachial Augmentation Index (%)</td>
<td>0.119</td>
</tr>
<tr>
<td>Diastolic Reflection Area*</td>
<td>4.57</td>
</tr>
</tbody>
</table>

Table 9-5: Regression models of vascular parameters between SLE patients and healthy controls with adjustment for age
Vascular variables were split into tertiles and ordered logistic regression used to determine whether being a patient/control predicted being in a higher tertile for that parameter. In an unadjusted analysis, all 4 parameters were significantly associated with the patient/control status. None of these associations remained significant after adjustment for age. OR = odds ratio, CI = confidence interval

A comparison of measurements of endothelial function between SLE patients and the healthy controls recruited for the validation studies shows that the baseline arterial diameter and the maximal post deflation diameter were also significantly higher in lupus compared to HCs (table 9-6 and figure 9-10).

<table>
<thead>
<tr>
<th>Healthy (n=10)</th>
<th>All SLE (n=36)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline Diameter (mm)</strong>*</td>
<td><strong>3.04 (0.322)</strong></td>
<td>3.40 (0.310)</td>
</tr>
<tr>
<td><strong>Post Deflation Diameter (mm)</strong>*</td>
<td><strong>3.25 (0.324)</strong></td>
<td>3.57 (0.340)</td>
</tr>
<tr>
<td>Absolute Diameter change (mm)</td>
<td>0.222 (0.184)</td>
<td>0.165 (0.157)</td>
</tr>
<tr>
<td>% FMD</td>
<td>7.60 (6.89)</td>
<td>4.94 (4.92)</td>
</tr>
<tr>
<td>% GTN dilatation</td>
<td>15.4 (5.45)</td>
<td>11.9 (5.99)</td>
</tr>
<tr>
<td>ED/El</td>
<td>47.4 (34.2)</td>
<td>40.0 (40.4)</td>
</tr>
</tbody>
</table>

Table 9-6: Baseline endothelial function measurements in SLE Patients and healthy controls
The table shows the mean (sd) values which were compared using a two-tailed t-tests (all variables were normally distributed across the combined HC/SLE population).
There was a trend towards lower %FMD (a) and lower %ED/EI dilatation (b) in patients with SLE compared to healthy controls.

Linear regression models were again used to adjust for the differences in age between the 2 groups. The associations between baseline diameter and post deflation diameter and the status of the subject (i.e. patient or control) were no longer seen once the values were adjusted for age (table 9-7).

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th>Adjusted for age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>95% CI</td>
</tr>
<tr>
<td>Baseline Diameter</td>
<td>-0.36</td>
<td>-0.59, -0.14</td>
</tr>
<tr>
<td>Post Deflation Diameter (mm)</td>
<td>-0.33</td>
<td>-0.59, -0.08</td>
</tr>
<tr>
<td>Absolute Diameter change (mm)</td>
<td>0.02</td>
<td>-0.09, 0.14</td>
</tr>
<tr>
<td>% FMD</td>
<td>2.66</td>
<td>-1.21, 6.54</td>
</tr>
<tr>
<td>% GTN dilatation</td>
<td>3.52</td>
<td>-1.81, 8.84</td>
</tr>
<tr>
<td>ED/EI</td>
<td>7.32</td>
<td>-22.5, 37.2</td>
</tr>
</tbody>
</table>

Table 9-7: Linear regression models of endothelial function between patients and healthy controls with adjustment for age

In summary, the patient and healthy control populations differed significantly in terms of blood pressure, vascular stiffness and brachial artery diameter. These differences were all confounded by the older age within the SLE group compared to the HC group and were no longer significant when age was taken into account in regression models. No differences in vascular function were therefore detected between the HCs and clinically stable SLE patients.
10. Reference list


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