Developing a Lupus Nephritis urinary biomarker panel in children for use in a clinical trial

'Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Author's declaration

I declare that this thesis is the result of my own work. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree of qualification. The contribution of others is made explicit where this has taken place.

This research was carried out at the Institute in the Park, Alder Hey Children's NHS Foundation Trust, and the Department of Women and Children's Health, Institute of Translational Medicine, University of Liverpool.

Signature.....

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Abstract

Dr E Smith. Developing a Lupus Nephritis urinary biomarker panel in children for use in a clinical trial

Background: Juvenile-onset systemic lupus erythematosus (JSLE) is a rare, severe multisystem autoimmune disease affecting the kidney (Lupus Nephritis, LN) in up to 80% of children. Numerous individual urinary biomarkers have previously been investigated. No individual biomarker has achieved 'excellence' in differentiating active from inactive LN.

Aims: (1) To assess performance of traditional clinical biomarkers for LN monitoring and prediction of LN outcomes. (2) To select biomarkers warranting assessment in a 'LN biomarker panel'; to cross-sectionally assess if combining novel/traditional biomarkers improves active LN identification. (3) To validate the optimal UK LN biomarkers within independent, ethnically distinct JSLE cohorts. (4) To longitudinally assess if urine biomarkers predict LN flare/remission. (5) To streamline methods for LN urine biomarker panel quantification.

Methods: Clinical data and urine samples from UK, United States (US) and South African (SA) JSLE patients were utilised within cross-sectional and longitudinal studies assessing combinations of novel urine/traditional clinical biomarkers. A custom LN biomarker panel multiplex assay was developed/validated in collaboration with Merck Millipore.

Results: 37% of UK patients displayed active LN as an initial presenting feature, with a further 17% developing LN after a median of 2.04 years [IQR 0.8-3.7]. ACR score (>5) and C3 levels (<0.9g/L) at baseline were significant predictors of subsequent LN development. 39% of patients recovered from proteinuria following an LN flare during the study period, within a median of 17 months (IQR 4-49) with the remaining 61% continuing to have proteinuria after a median of 22 months (IQR 12-41). Younger patients (<14 yrs), those with a lower eGFR (<80mls/min) and haematological involvement at LN onset, displayed a longer time to proteinuria recovery. ESR, C3, WCC, neutrophils, lymphocytes and IgG contributed significantly to an optimal non-renal traditional biomarker model for active LN identification (AUC 0.72).

Novel urine biomarkers were selected for assessment by detailed literature review. Cross-sectional fitting a binary logistic regression models with data from 61 UK patients, the optimal biomarker combination included AGP+CP+LPGDS+TF (AUC 0.920). Inclusion of traditional biomarkers within the model did not improve the AUC further. The novel biomarker panel displayed equivalent ability for identifying active LN in 30 US and 23 SA patients (AUC of 0.991 and 1.00 respectively). Within the longitudinal study, including 244 observations from 80 UK/US/SA patients, a Markov Multi-State model identified AGP to be predictive flare, and CP of remission (model AIC =135). By entering individual AGP/CP patient values into the model, 3, 6, 9 and 12 month probabilities of disease state transition are provided.

The multiplex assay demonstrated acceptable cross reactivity between multiplexed antibodies, satisfactory spike recovery, intra/inter-assay precision. Linearity of dilution was unacceptable, therefore rigorous range finding in 106 UK/US/SA samples identified the optimal dilutions required for each biomarker. Combining multiplex biomarker values for AGP+CP+LPGDS+TF within a binary logistic regression model, equivalent ability for identification of active LN was seen (multiplex AUC = 0.998, ELISA AUC = 0.952).

Conclusions: This thesis has demonstrated and validated an 'excellent' urinary biomarker panel for active LN identification in three ethnically distinct JSLE cohorts. Different constituents of the biomarker panel are best at predicting LN flare/remission. A custom urine biomarker panel multiplex assay has been developed, demonstrating improved ability for active LN identification over existing ELISAs. Future clinical studies prospectively measuring the urine biomarker panel by multiplex are warranted, facilitating refinement of the Markov Multi-State Model and assessing if biomarker-led monitoring can actually improve renal outcomes for children with LN.

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Abbreviations

4-PL	four parameter logistic
A1mG	α-1-macroglobulin
Ab	antibody
ACEi	angiotensin converting enzyme inhibitor
ACR	American College of Rheumatology
AECOM	Albert Einstein College of Medicine
AGP	α1-acid-glycoprotein
AI	activity index
AIC	Akaike Information Criterion score
ANA	antinuclear antibodies
ANCA	antineutrophil cytoplasmic antibody
anti-dsDNA	anti-double stranded DNA
anti-RNP	anti-ribonucleoprotein
anti-Sm	anti-Smith
APLS	anti-phospholipid syndrome
AT2i	angiotensin 2 inhibitor
AUC	area under the curve
AUC ROC	area under the curve receiver operating curve analysis
AZA	azathioprine
BA	boric acid
BAFF	B-cell-activating factor
Bd	twice daily
BILAG	British Isles Lupus Assessment Group
BlyS	B-lymphocyte stimulator
C1q-CLR	collagenous region of C1q
C3/C4	complement 3/4
CARRA	Childhood Arthritis and Rheumatology Research Alliance
CCD	Charge Coupled Device
CD	cluster of differentiation
CD2AP	cluster of differentiation 2 associated protein
CHAQ	Childhood Health Assessment Questionnaire
CHQ	Childhood Health Questionnaire
CI	chronicity index
CICs	circulating immune complexes

CIs	confidence intervals
CMV	Cytomegalovirus
CO_2	carbon dioxide
CP	ceruloplasmin
Cr	creatinine
CrCl	creatinine clearance
CRP	c-reactive protein
CTU	clinical trials unit
CV	co-efficient of variation
CXCL16	chemokine (C-X-C motif) ligand 16
CXCR3	chemokine receptor 3
CYC	cyclophosphamide
Cyr61	cysteine rich protein 61
Cys-C	cystatin-C
DAT	direct antibody test
DCs	dendritic cells
DEXA	bone mineral density scan
DNA	deoxyribonucleic acid
EATC	Experimental Arthritis Treatment Centre for children
EBV	Epstein-Barr virus
ECG	electrocardiogram
ECLAM	European Community Lupus Activity Measure
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
eGFR	estimated glomerular filtration rate
ELC	Einstein Lupus Cohort
ELISA	enzyme linked immunoabsorbant assay
ENAs	extractable nuclear antigens
ESR	erythrocyte sedimentation rate.
EULAR	European League Against Rheumatism
EULAR/ERA-EDTA	European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association
FABP	fatty acid binding protein
FBC	full blood count
FCS	fetal calf serum
FGF-2	fibroblast growth factor-2

FH	family history
FLT-3L	FMS-related tyrosine kinase 3 ligand
Fn14	fibroblast growth factor-inducible 14
FSGS	focal segmental glomerulosclerosis
FT	freeze thaw
G-CSF	granulocyte colony stimulating factor
GBM	glomerular basement membrane
GM-CSF	granulocyte macrophage colony stimulating factor
GRO	chemokine (C-X-C motif) ligand 1
GST	glutathione-S-transferase
Hb	haemoglobin
HBA1c	glycosylated haemoglobin
HBSS	hanks balanced salt solution
HGF	hepatocyte growth factor
HIV	Human Immunodeficiency Virus
HKI	human kidney injury
hpf	high powered field
hr	hour
HRP	horseradish peroxidase
HRQOL	health related quality of life
HRs	hazard ratios
ICC	intra-class correlation coefficient
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL- 1RA	interleukin 1 receptor antagonist
in-act	inactive
incub	Incubation
IP-10	interferon-γ-inducible protein 10
IQR	interquartile ranges
ISN/RPS	International Society of Nephrology/Renal Pathology Society
ITS	insulin transferrin selenium liquid supplement
IVIG	intravenous immunoglobulin
IVMP	intravenous methylprednisolone
JIA	Juvenile Idiopathic Arthritis
JSLE	Juvenile-onset Systemic Lupus Erythematosus

KDIGO	Kidney Disease Improving Global Outcomes
KIM-1	kidney injury molecule 1
LPGDS	lipocalin-type prostaglandin-D synthetase
LEDs	light emitting diodes
LFTs	liver function tests
LN	lupus nephritis
LOD	linearity of dilution
M-CSF	macrophage colony stimulating factor
MALDI-TOF MS	surface-enhanced matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAS	macrophage activation syndrome
MCP-1	monocyte chemoattractant protein-1
MCS	microscopy / culture / sensitivities
MDC	macrophage derived chemokine
med	median
MFI	median fluorescent intensity
Min	minute
MIP-1α	Macrophage inflammatory protein-1α
MMF	mycophenolate mofetil
MRA	magnetic resonance angiogram
MRC CiC	Medical Research Council Confidence in Concept grant
MRI	magnetic resonance imaging
MSK	musculoskeletal
MTA	material transfer agreement
NA	not available or applicable
NAG	n-acetyl-b-d-glucosaminidase
NETosis	increased NET production
NETs	neutrophil extracellular traps
NGAL	neutrophil gelatinase associated lipoclain
NHE-3	N+ / H+ exchanger isoform 3
NICE	National Institute of Clinical Excellence
NIH	National Institute of Health
NIHR	National Institute of health Research
OD	optical density
OPG	osteoprotegerin
OPN	osteopontin

OR	adds ratio
p-value	probability value
pBILAG2004	paediatric British Isles Lupus Assessment Group 2004 assessment score
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pc	Bonferroni corrected probability value
PDGF-AA	platelet-derived growth factor-AA
Ped-SDI	paediatric version of the SDI
PGDS	prostaglandin-H2-D isomerase
PLUTO	Pediatric Lupus Trial of Belimumab Plus Background Standard Therapy
Pred	prednisolone
Pts	patients
QCs	quality controls
RA	rheumatoid arthritis
RANTES	regulated on activation, normal T cell expressed and secreted
RBP	retinol binding protein
RCTs	randomised controlled trials
RING	Rituximab for Lupus Nephritis With Remission as a Goal
Ritux	rituximab
RITUXILUP	Rituximab and Mycophenolate Mofetil Without Oral Steroids for Lupus Nephritis
RLU	relative light units
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	room temperture
SA	South Africa
SA Cohort	Paediatric Lupus Erythematosus in South Africa Cohort Study
sCD40L	soluble cluster of differentiation 40 ligand
SD	standard deviation
SDI	standardised damage index
SELDI-TOF	surface-enhanced laser desorption / ionization time of flight
SEM	standard error of the mean
Serum creat	serum creatinine
SF36	Short Form 36
SHARE	Single Hub and Access point for Paediatric Rheumatology in Europe
SLAM	Systemic Lupus Activity Measure

SLE	Systemic Lupus Erythematosus
SLEDAI	SLE disease activity index
SLICC	Systemic Lupus International Collaborating Clinics
SMILEY	Simple Measure of Impact of Lupus Erythematosus in Youngsters
SMR	standardised mortality rates
SOPs	standard operating procedures
SP-conjugate	streptavidin-peroxidase conjugate
SPSS	statistics programme for social sciences
STN	standard curve
Strep	streptavidin
strep-HRP	streptavidin horseradish peroxidase
TF	transferrin
TFF-3	trefoil Factor-3
TFTs	thyroid function tests
TI	tubulointerstitial
TIAI	tubulointerstitial activity index
TIMP-1	tissue inhibitor of metaloproteases-1
TLRs	toll like receptors
TMB	tetramethyl-benzidine
TNF-α	tumour necrosis factor- α
TNF-β	transforming growth factor- β
TWEAK	tumor necrosis factor-like weak inducer of apoptosis
U&Es	urea & electrolytes
UACR	urinary albumin creatinine ratio
UCL	University College London
UCLH	University College London Hospital
UK	United Kingdom
UoL	University of Liverpool
UPCR	urinary protein creatinine ratio
US	United States
US Cohort	Einstein Lupus Cohort
USA	United States of America
USS	ultrasound
UTI	urinary tract infection
VAS	visual analogue scale
VCAM-1	vascular cell adhesion molecule-1

VEGF	vascular endothelial growth factor
WCC	white cell count
WHO	World Health Organisation
WT-1	wils tumor-1
ZA2G	zinc α-2-glycoprotein
β2MG	beta-2-microglobulin
K-FLC	κ free light chain

1 General Introduction

1.1 Juvenile-onset Systemic Lupus Erythematosus

Juvenile-onset systemic lupus erythematosus (JSLE), also known as childhood-onset SLE, is a rare, severe multisystem autoimmune disease, occurring before the age of 17 years. It is often referred to as the archetypal systemic autoimmune disorder as it is characterised by widespread inflammation and damage, which can affect any organ. It is a very complex disease with marked heterogeneity between individual patients, causing anything from mild to life-threatening disease, following a relapsing and remitting course, and with an unpredictable natural history. JSLE is characterised by the production of autoantibodies directed against endogenous nuclear autoantigens, including anti-nuclear antibodies (ANA) and anti-double stranded DNA (anti-dsDNA).

1.1.1 Epidemiology of JSLE

Systemic Lupus Erythematosus (SLE) is rare in children, accounting for 15-20% of all SLE cases [1-6]. Onset is uncommon before the age of 5 years, but rises steadily during childhood, puberty, until mid-adulthood [3]. Very rare infantile forms of JSLE can be associated with a monogenic predisposition. Hyper-production of interferon-α (IFN-α) has been demonstrated in many types of monogenic lupus syndromes [7,8], following the identification of specific genes, responsible for Aicardi-Goutiers Syndrome, an autosomal recessive disease associated with high IFN-α production and early-onset of JSLE [9]. Defects in early components of the classical complement pathway have also been shown to predispose to early onset of JSLE [10]. Such forms of JSLE often have broader clinical phenotypic characteristics than other SLE patients, highlighting important differences between JSLE and adult onset SLE (see section 1.1.3).

The annual incidence of JSLE ranges between 0.36–2.5 per 100,000 children and young people [11-17]. This may be an underestimate as many cases go unrecognised over long periods, have mild disease, or do not initially fulfil criteria to enable a diagnosis to be made [18]. A recent UK epidemiological study using the General

Practice-based Clinical Practice Research Datalink has indicated that the incidence of SLE is 0.19 per 100,000 person-years in children aged 0-9 years, and 1.92 per 100,000 person-years in young people aged 10-19 years [17]. In other countries the incidence per 100,000 person-years has been shown to be 0.36 in Canada [19], 0.37 in Finland [15], 0.47 in Japan [16], 0.9 in Wisconsin USA [20] and 2.5 in Atlanta USA [21]. The female-to-male ratio differs between childhood and adult onset disease, from approximately 4:1 for pre-pubertal onset JSLE, increasing as puberty is approached (due to hormonal influences), and becoming approximately 9:1 during adulthood [17,19,22-25].

Similar to adult onset SLE, JSLE is more common amongst individuals of non-Caucasian ethnicity, especially African American, African, Hispanic, and Asian children [26-29]. Within the UK JSLE Cohort Study, the standardised incidence rate has been shown to be significantly higher in non-Caucasian patients as compared to Caucasian patients (incidence of 0.7-2.5 per 100,000 persons per UK ethnic population in non-Caucasians, as compared to 0.1 in Caucasians) [28]. In a study by Hiraki et al using International Classification of Disease-9 codes and the US Medicaid Analytic eXtract database (from 2000 to 2004), to identify JSLE patients from 47 US states and the District of Columbia, the incidence of JSLE was higher in all non-Caucasian ethnic groups (2.73, 2.45 and 4.16 per 100,000 in African Americans, Hispanics and Asians respectively, and 1.33 per 100,000 in Caucasians). Incidence of Lupus Nephritis (LN) was highest amongst Asian JSLE patients (2.08 per 100,000), followed by African American, Hispanic and Caucasians respectively (0.87, 0.85 and 0.3 per 100,000) [29]. Within an inception cohort of 265 patients followed at the Sick Kids Hospital in Toronto, non-Caucasian ethnicity was also significantly associated with increased JSLE prevalence, with non-Caucasian patients exhibiting a younger age at diagnosis with respect to Caucasian patients (12.6 vs. 14.6 yrs; $p=0.007$) and more LN (62% vs. 45%; $p=0.01$) than Caucasian patients [26].

Ethnicity is also known to influence disease severity in children. In a US study looking at outcomes of hospitalised JSLE patients, several differences were demonstrated according to ethnicity. Hispanic patients were shown to have longer hospital stays and higher in-hospital mortality. African Americans had more hospital admissions, and higher mortality. Increased end stage renal failure rates were also seen in African

American and Hispanic patients [30]. In a US adult SLE study, the standardised incidence ratio of end stage LN requiring renal replacement therapy was 11.1 for African American patients, 4.9 for 'other ethnicity' patients and 1.7 for Caucasian patients. In addition, amongst patients with end stage renal disease, African Americans displayed a higher mortality rate [31]. African American young adult SLE patients (18-30 years old) with end stage renal disease have also been shown to be at increased risk of death (hazard ratio (HR) 1.43) compared with non-African Americans [32]. Further details on ethnic differences in JSLE are detailed in section 5.1.1.

1.1.2 Clinical features and diagnosing JSLE

There is no single diagnostic test or validated diagnostic criteria for JSLE. Therefore, arriving at the diagnosis of JSLE relies upon an awareness that JSLE can occur in children and young people and considering it as part of a differential diagnosis. Comprehensive clinical assessment by a team experienced in the care of children with lupus and other connective tissue disorders is essential. This needs to be supported by a multi-disciplinary team, with judicious interpretation of investigations and careful stepwise exclusion of the broad and significant differential diagnoses. Diagnostic features can appear intermittently and cumulatively over many months (or even years) rather than in parallel, leading to potentially significant delays in making a diagnosis of JSLE [18]. The condition can present with non-specific symptoms, such as fatigue, arthralgia, mouth ulcers and headaches, incorrectly leading to the symptoms being attributed to 'being a teenager', exam stress, anorexia nervosa, or chronic fatigue syndrome. Conversely, the presentation can be acute with potentially life-threatening manifestations e.g. renal failure or seizures.

The American College of Rheumatology (ACR) revised criteria for SLE classification [33] were introduced to classify patients as having JSLE for the purpose of clinical trials, but are frequently used for diagnosing JSLE (see Table 1-1, and Appendix 1). A person is classified as having JSLE if they fulfil any 4 or more of the 11 criteria, serially or simultaneously, during any time interval (before the age of 17 years). The criteria alone are not a pre-requisite for commencing treatment as many individuals with two or three features will go on to fulfil further criteria. Patients may therefore be

treated pre-emptively where there is a high clinical index of suspicion for JSLE by a team experienced in the care of JSLE patients.

Criterion	Definition
Malar rash	Fixed erythema, flat or raised, over malar eminences, tending to spare nasolabial folds
Discoid lupus	Erythematous raised patches of adherent keratotic scaling and follicular plugging. Atrophic scarring in older lesions
Photosensitivity	Skin rash as a result of unusual reaction to sunlight
Oral or nasal ulceration	Oral or nasopharyngeal ulcers, usually painless, observed by a physician
Non-erosive arthritis	Non-erosive arthritis involving two or more peripheral joints, characterised by tenderness, swelling, or effusion
Serositis	Pleuritis—pleuritic pain, rub or pleural effusion. Pericarditis—documented by ECG or rub or pericardial effusion
Nephritis	Persistent proteinuria >0.5g/day or >3+, or Cellular casts: red cell, haemoglobin, granular, tubular or mixed
Neurological	Seizures or psychosis in the absence of offending drugs or metabolic derangement (e.g. uraemia, ketoacidosis, electrolyte imbalance)
Haematological	Haemolytic anaemia with reticulocytes, or Leucopenia <4000 per mm^3 on 2 or more occasions, or Lymphopenia <1500 per mm^3 on 2 or more occasions, or Thrombocytopenia <100,000 per mm^3 in absence of offending drugs
Immunological	Anti-DNA antibody, or Anti-Sm antibody, or Anti-phospholipid antibodies: Abnormal anticardiolipin antibody IgG or IgM Positive lupus anticoagulant False positive for syphilis for >6 months
Anti-nuclear antibody	Abnormal titre of ANA at any point in absence of drugs known to be associated with drug-induced lupus

Table 1-1: The revised ACR criteria for classification of SLE

ECG = electrocardiogram. Ig = immunoglobulin.

In 2012, the Systemic Lupus International Collaborating Clinics (SLICC) group proposed revised new SLICC classification criteria in order to improve clinical relevance [6]. Here, the criteria are divided into clinical and immunological. The patient must satisfy at least 4 new SLICC criteria, including at least one clinical criterion and one immunologic criterion, or the patient must have biopsy proven LN in the presence of ANA or anti–dsDNA antibodies (see Table 1-2 and Appendix 1). The SLICC criteria are again cumulative and have been validated in JSLE, performing better than the ACR criteria in terms of sensitivity and accuracy at the first visit, and within the first year of follow-up [34,35].

Clinical criteria			
Acute cutaneous lupus	• Lupus malar rash • Bullous lupus • Toxic epidermal necrolysis variant of SLE • Photosensitive lupus rash	• Maculopapular lupus rash • Subacute cutaneous lupus – non-indurated psoriaform and/or annular polycyclic lesions that resolve without scarring	
Chronic cutaneous lupus	• Classic discoid rash • Localized (above neck), generalized (below neck) • Hypertrophic (verrucous) lupus • Lupus panniculitis (profundus)	• Mucosal lupus • Lupus erythematosus tumidus • Chillblain lupus • Discoid lupus/lichen planus overlap	
Oral / nasal ulcers	• Palate, buccal, tongue (in absence of vasculitis, Behcet's, infection (e.g. herpes), inflammatory bowel, reactive arthritis)		
Non-scarring alopecia	• Diffuse thinning or hair fragility with visible broken hairs in the absence of other causes such as alopecia areata		
Arthritis	• Synovitis of ≥2 joints (with swelling or effusion) OR tenderness in ≥2 joints and ≥30 minutes of morning stiffness		
Serositis	• Typical pleurisy for more than 1 day OR pleural effusions OR pleural rub • Typical pericardial pain for >1 day OR pericardial effusion OR pericardial rub OR pericarditis by electrocardiography		
Renal	• Urine protein–to-creatinine ratio (or 24-hour protein) representing 500 mg protein/24 hours OR red blood cell casts		
Neurologic	• Seizures • Psychosis	• Mononeuritis multiplex • Myelitis	• Peripheral or cranial neuropathy • Acute confusional state
Haemolytic anemia	• No definition within SLICC criteria		
Leukopenia	• < 4,000/mm^3 at least once, in the absence of other known causes such as Felty's syndrome, drugs, and portal hypertension	**Lymphopenia** • <1,000/mm^3 at least once, in the absence of other known causes such as corticosteroids, drugs, and infection	
Thrombocytopenia	• <100,000/mm^3 at least once, in the absence of other causes (drugs, portal hypertension, and thrombotic thrombocytopenic)		
Immunological criteria			
ANA	• Level above laboratory reference range		
Anti-dsDNA antibody	• Level above laboratory reference range (or >2-fold the reference range if tested by ELISA)		
Anti-Sm	• Presence of antibody to Sm nuclear antigen		
Anti-phospholipid antibody	Positivity as determined by any of the following: • Positive test result for lupus anticoagulant • False-positive test result for rapid plasma reagin	• Medium or high-titer anti-cardiolipin antibody level (IgA, IgG, or IgM) • Positive test result for anti–β2-glycoprotein I	

Table 1-2: The SLICC criteria for classification of SLE [6]

1.1.3 Differences in the clinical features of JSLE and adult-onset SLE

Significant differences typically exist between JSLE and adult-onset SLE patients in relation to their disease severity and clinical phenotype. It has been demonstrated that JSLE patients have higher mean SLE disease activity index (SLEDAI) scores at the time of their initial presentation and when followed over time [1,2,36,37]. In JSLE there is a greater corticosteroid and immunosuppressive treatment burden than in adults [1,27,36-38], with more rapid accrual of disease related damage [1,36,39]. Previous studies have provided somewhat conflicting results regarding the differences in clinical phenotype across SLE patient age groups. The most consistent observation is that more LN and haematological disease is seen in children than adults [1,27,36-38,40-44]. Some studies have reported the frequency of neuropsychiatric involvement to be higher in JSLE [1,2,40] with others demonstrating no difference between JSLE and adult SLE [27,37,38]. Such differences may be attributed to differences in the case definitions used to define neuropsychiatric involvement, small patient numbers and retrospective data collection within most studies.

A recent study involving the UK JSLE Cohort Study and University College London Hospital (UCLH) SLE Cohort has described the effect of age at disease onset on SLE phenotype in 924 UK patients (413 JSLE and 511 adult-onset SLE). They found JSLE patients to have significantly more renal involvement, alopecia, apthous ulceration, ACR defined haematological involvement, thrombocytopenia, haemolytic anaemia, high anti-dsDNA, anti-Smith (anti-Sm) and anti-ribonucleoprotein (anti-RNP) positivity than adult SLE patients. Adult-onset SLE patients had significantly more arthritis, serositis and leucopenia. Standardised mortality rates (SMR) by age of onset declined over the decades, with an SMR of 18.3 in JSLE and 3.1 in adult onset SLE. The SMR was particularly elevated in the 0-9 age group at 87 [42]. A large meta-analysis, including 905 JSLE and 5993 adult SLE patients from 16 separate studies identified malar rash, ulcers, renal involvement, proteinuria, urinary cellular casts, seizures, thrombocytopenia, haemolytic anaemia, fever and lymphadenopathy to be more common in JSLE. Raynaud's, pleuritic pain and sicca syndrome were more common in adult SLE [43].

1.1.3.1 Differential diagnosis of JSLE

There is a wide differential diagnosis of JSLE, which needs to be systematically excluded. The main differential diagnoses to consider depend on the predominant organ affected at presentation, and include: other systemic autoimmune conditions, infections, immunodeficiency syndromes or malignancies. Many pharmacological agents have also been associated with 'lupus-like' syndromes, characterised by a transient increase in autoantibodies and cutaneous manifestations. Table 1-3 provides a summary of the differential diagnoses grouped according to the predominant organ affected at presentation.

Manifestations	Differential diagnoses	
Renal	Acute post-streptococcal glomerulonephritis Nephrotic syndrome	Henoch-Schonlein purpura Haemolytic uraemic syndrome Anti-GBM antibody disease
Haematological	Acute/chronic anaemia APL syndrome Evan's syndrome	Autoimmune & chronic benign neutropenia Recurrent fever syndromes Macrophage activation syndrome
Musculoskeletal	JIA Behçets syndrome Systemic sclerosis Vasculitis (Polyarteritis nodosa, Microscopic polyangitis, Wegners Granulomatosus)	Juvenile Dermatomyositis Mixed connective tissue disease Undifferentiated connective tissue disease Fibromyalgia Kawasaki's disease
Gastrointestinal	Autoimmune hepatitis	Autoimmune biliary disease
Neuropsychiatric	Epilepsy Generalised anxiety	Anorexia
Cardio-respiratory	Dilated cardiomyopathy Bacterial endocarditis	Rheumatic heart disease Myocarditis or pericarditis
Immunological	Chronic Granulomatous Disease Complement deficiency	Angioedema
Endocrine	Hypothyroidism	Hyperthyroidism
Malignancy	Lymphoma	Solid tumours
Toxic/ therapeutic	Definitely associated with chlorpromazine, hydralazine, isoniazid, methyldopa, procainamide, quinadine	Possibly associated a range of anti-hypertensives, anti-psychotics, anti-epileptics and antibiotics
Infectious	CMV, EBV Parvovirus B19	HIV, Hepatitis B & C Lyme disease

Table 1-3: Differential diagnosis of JSLE dependent upon the predominant organ/system involved at initial presentation.
GBM = glomerular basement membrane. JIA = Juvenile Idiopathic Arthritis. CMV = Cytomegalovirus. EBV = Epstein-Barr virus. HIV = Human Immunodeficiency Virus.

1.1.3.2 Holistic assessment of the child with JSLE

Optimisation of JSLE disease control relies upon careful multisystem assessment of the patient at baseline and at each clinical visit including history, examination and investigations. There is no single 'gold standard' biological marker that accurately reflects disease activity in JSLE. However, there are several JSLE disease assessment tools which can be used within the clinic, clinical studies and clinical trials to assess disease activity, response to treatment, disease-related damage, function and health related quality of life (HRQOL) (see sections 1.1.3.4 and 1.1.4).

At each clinical encounter, there needs to be a thorough search for symptoms and signs of JSLE activity within each organ system (mucocutaneous, musculoskeletal, renal, neuropsychiatric, cardiovascular, respiratory, gastrointestinal, renal and ophthalmic). Laboratory investigations are used to assess aspects of disease activity and organ damage, which may not be appreciable through history and examination alone. Routine investigations performed at each review may vary slightly between patients based on clinical presentation, but a standard, comprehensive approach is needed. As an example of this, the possible results of these investigations and their subsequent implications are summarised in Table 1-4.

Investigation	Possible results and implications
Hb	Decreased with anaemia of chronic disease or autoimmune anaemia
WCC	Decreased WCC due to lymphopaenia +/- neutropenia, lymphocytes may be decreased due to disease activity or immunosuppression Increased WCC suggests infection or response to corticosteroids decreased
Platelets	Decreased due to autoimmune or peripheral destruction, or as a complication of drug therapy**.** Note – need to consider MAS if pancytopenia
ESR	Increased in active disease Note - if sudden drop in ESR patients, consider MAS
U&Es	Increased creatinine is often a late sign of renal involvement suggesting chronic kidney disease. Use creatinine to calculate eGFR. Increased urea in dehydration and gastrointestinal bleeding. Monitoring for immunosuppressive drug toxicity.
LFTs	Increase in autoimmune hepatitis and with immunosuppressive drug toxicity
Bone profile	Increased alkaline phosphatase with chronic kidney disease, liver/bone disorders or vitamin D deficiency
Glucose/HBA1c	Increased in drug induced or disease related diabetes
Amylase/lipase	Consider in presence of abdominal pain to assess for pancreatitis
CRP	If increased consider infection. Other causes of raised CRP in SLE include serositis and polyarthritis
C3/C4	Levels correlate with disease activity, with decreased C3 and C4 seen in active disease
Anti-dsDNA	Serial titres are used as a measure of disease activity
Immunoglobulins	Increased in acute inflammation
Urinalysis, MCS, spot UACR or UPCR	Protein or blood on urinalysis in renal involvement. If protein present need quantitative measure such as spot UACR or UPCR. MC to exclude urine infection as the underlying cause

Table 1-4: Routine investigations for each clinical review

Investigation frequency approximately 3-monthly or more frequently if unwell. FBC = full blood count. Hb = haemoglobin. WCC = white cell count. MAS = macrophage activation syndrome. ESR = erythrocyte sedimentation rate. U&Es = urea & electrolytes. eGFR = estimated glomerular filtration rate. LFTs = Liver function tests. HBA1c = glycosylated haemoglobin. CRP = C-reactive protein. C3/C4 = complement ¾ fragments. MCS = microscopy/culture/sensitivities. UACR = urinary albumin creatinine ratio. UPCR = urinary protein creatinine ratio.

Table 1-5 shows additional investigations if clinically warranted which should be carried out at the time of the initial assessment and then annually.

Investigation	Possible results and implications
Clotting screen	Prolonged in APLS and MAS
Fibrinogen	Increased in acute inflammation, decreased in MAS
DAT	Positive in autoimmune haemolytic anaemia
Lupus anticoagulant and anti-cardiolipin antibodies	Increased in APLS. Associated with increased risk of pulmonary embolism, neuropsychiatric involvement, cardiovascular disease
TFTs and thyroid antibodies	JSLE associated with autoimmune thyroid disease. Can cause apparent worsening of JSLE symptoms (e.g. fatigue)
Fasting lipid profile	Dyslipidaemia increases risk of cardiovascular events; increased triglycerides in MAS
Vitamin D levels	Vitamin D deficiency common due to sun avoidance/sunscreen use, lack of exercise, treatments and can cause worsening of JSLE related symptoms (e.g. fatigue and muscle pain)
ANA	Positive in 95% of JSLE patients, may become positive after initially being negative at presentation
Anti-Ro, anti-La **Anti-Sm**	Most common ENAs seen. Ro - associated with photosensitivity. Both associated with Sjogren's syndrome, congenital heart block and neonatal lupus Associated with renal involvement
Anti-C1q	Marker of disease activity particularly renal disease but test not widely available
Immunity status	Measles and varicella IgG, prior to starting immunosuppressive treatment

Table 1-5: Investigations at time of the initial assessment and then annually (or as clinically indicated).

APLS = anti-phospholipid syndrome. MAS = macrophage activation syndrome. DAT = direct antibody test. TFTs = Thyroid function tests. ENAs = Extractable Nuclear Antigens.

Unexpected results from the above tests may relate to the time of sampling, treatment and patient related factors. The value of commonly available traditional biomarkers of JSLE activity for identifying active LN remains uncertain and is explored further in sections 1.2.3 and 3.1.3. Further investigations should be considered on an individual basis depending on the features observed or on previous organ involvement, balancing the risks of the procedure and the need for sedation and/or general anaesthesia in children.

Renal biopsy, renal ultrasound (USS), renal biopsy and measurement of GFR may be used to assess for LN. ECG, echocardiogram, visceral angiogram, and magnetic resonance angiogram (MRA) are useful for the assessment of potential cardiovascular involvement. When respiratory symptoms are present a chest x-ray, pulmonary function tests including transfer factor and CT chest should be considered. Magnetic resonance imaging or angiogram (MRI/MRA) of the brain, electroencephalogram, psychometric testing are appropriate when neuropsychiatric disease is suspected. Abdominal USS, upper and lower GI endoscopy is useful where persistent gastrointestinal symptoms seen. Bone mineral density scan (DEXA) is indicated annually for those at risk from osteoporosis.

A European initiative called SHARE (Single Hub and Access point for paediatric Rheumatology in Europe, launched in 2013) has derived evidence-based recommendations for diagnosis/monitoring and treatment of JSLE, providing consensus based guidance using the European League Against Rheumatism (EULAR) standard operating procedure, which are consistent with the approach to diagnosis detailed above [45].

1.1.3.3 The multi-disciplinary team and management of JSLE

In light of the complexity of JSLE, patient management often involves multiple specialists, including dermatologists, nephrologists, haematologists, immunologists, neurologists, cardiologists, gynaecologists, endocrinologists and gastroenterologists. Such specialists may assist with the initial diagnosis, and subsequent management of organ specific disease flares and long term complications. This process should be coordinated by a paediatric rheumatologist with experience of JSLE, who will have an overview of the patient, identify when additional expertise is required and ensure

continuity of care [46]. Nurse specialists play an essential role in providing education to the patient/family about the disease and its medications. They also assist in liaising with other specialists, allied healthcare professionals, school and wider social activity groups. It is important to ask the patient/family about the effect of their disease on social, educational and psychological functioning (see section 1.1.3.4). Psychologists are increasingly becoming integral to paediatric rheumatology teams, specifically assisting with management of debilitating neuropsychiatric manifestations, cognitive and behavioural difficulties, management of fatigue and development of self-management strategies for the patient/family. Physiotherapists, occupational therapists and play specialists are also extremely important, assessing function in the home and school, providing rehabilitation following disease flares, and helping patients to cope with procedures and limitations related to their diseases and its management. The general practitioner must also be kept fully up to date about medication regimens and monitoring, disease flares and vaccinations where applicable [46].

1.1.3.4 Assessment of psychosocial wellbeing in JSLE

JSLE most commonly develops during adolescence, which is a time of huge physical, psychological and social change for the young person. Effective multi-disciplinary care must therefore, not only consider medical concerns, but also HRQOL, the underlying priorities of adolescence, and the impact of JSLE on daily life/school. During adolescence, young people establish relationships with peers and partners, acquire independence and make career choices, which can be impacted upon by a multisystem illness which is unpredictable, relapsing and remitting. Therefore, measuring HRQOL and the psychosocial impact of disease is as important as measuring disease activity/damage. Validated scores include the Childhood Health Questionnaire (CHQ) and Short Form 36 (SF36) for assessing general health and well-being [47,48]. The CHAQ (Childhood Health Assessment Questionnaire) for measuring function, pain and providing a general evaluation of the disease using a visual analogue scale (VAS) [49]. These scoring systems are used as part of the UK JSLE Cohort Study and can be seen in Appendices 2-4. Specific adult SLE-related QOL tools have also been developed (e.g. LupusQoL [50]) but have not been validated in children. Since the establishment of the UK JSLE Cohort Study, the Simple Measure of Impact of Lupus Erythematosus in Youngsters (SMILEY) health related QOL tool has also been developed for children aged 5-18 years, with responses taking the form

of different facial expressions. The tool consists of four domains: effect on self, limitations, social, and burden of SLE. The US-English version of SMILEY has been shown to be valid, reliable and responsive to changes in disease activity [51]. SMILEY has been translated into over 30 languages, including a UK-English adaptation [52]. Future validation of this measure within the UK JSLE Cohort Study would be of interest.

1.1.4 Monitoring of JSLE disease activity

Disease activity, damage or HRQOL assessment measures in JSLE help to standardise serial disease monitoring, and are mainly used in clinical studies/trials to allow comparison of therapies and assess treatment response. They can be used in clinical practice where there are systems or expertise in place to accurately score the assessments. Within JSLE studies and clinical care, the British Isles Lupus Assessment Group (BILAG) [53,54] and SLEDAI [55] scores are most commonly used (see Appendices 5-8). Alternative measures, include the Systemic Lupus Activity Measure (SLAM) [56] and the European Community Lupus Activity Measure (ECLAM) [57-59]. The monitoring of JSLE related damage is detailed in section 1.1.9.1 below.

1.1.4.1 British Isles Lupus Assessment Group (BILAG) assessment tool

The BILAG score is used as an integral part of this thesis for quantifying disease activity and is therefore described in detail. In the 1980s, the newly formed UK BILAG group sought to develop a disease activity score which would be more comprehensive than contemporary global indices, facilitating accurate assessment of new activity, flare, remission in individual organs/systems. Using previous global scores, difficulties had been observed, for example with very sick patients with life threatening LN scoring the same as relatively well outpatients with low disease activity in a variety of organs [60]. The original/classic BILAG score was derived using a nominal consensus approach in 1993 [53], focusing on eight organs/systems (constitutional, mucocutaneous, neurological, musculoskeletal, cardiovascular/respiratory, vasculitis, renal and haematological). The score captured disease transitions, with the clinician being asked to grade clinical features as new, the same, worse or improving over the last 4 weeks and as compared to the preceding 4 weeks. The recorded items were converted into a corresponding alphabetical score (A-E) for each organ/system, based

on the principle of physician's intention to treat. **A** was for 'action', implying that the patient required treatment with at least 20mg of prednisolone daily or immunosuppressive therapy. **B** was for 'beware', with the patient requiring a lower level of immunosuppression. **C** implied 'contentment', meaning low disease activity requiring little treatment but still not fully recovered. **D** was for **'**discount' inferring that the patient had been active in the past but is no longer active. An **E** grade suggested that a patient had 'never ever' been active [53].

The original Classical BILAG was shown to be a comprehensive, reliable and valid tool which was sensitive to changes in SLE disease activity [61], and useful in observational adult and paediatric studies [62,63] and a drug trial [64]. There were, however, concerns that some of the items were capturing damage (e.g. avascular necrosis), that the renal domain required modification as it only captured worsening but not stable proteinuria, and did not include UPCR or UACR ratio measurements. It did not capture gastrointestinal or ophthalmic manifestations and the neuropsychiatric terminology was outdated. A new BILAG-2004 index was produced, including nine organ domains/systems; ophthalmic and gastrointestinal systems were added, the stand alone vasculitis section removed and the individual items placed alongside other more appropriate systems. A better BILAG glossary was also produced to improve standardisation of scoring, and the mucocutaneous and neurological system questions were re-arranged. The renal disease domain was improved and damage features removed from the score [54]. A numerical scoring system, A = 12, B = 8, C = 1, D/E = 0 was derived in order to convert the alphabetical score into a numerical total BILAG-2004 score [65]. Items on fatigue, migraine, cluster/tension headache, mood/anxiety disorders were also removed from the BILAG-2004 score due to poor inter-rater agreement [66]. The total BILAG score was shown to significantly associate with increasing or decreasing therapy, increasing ESR, anti-dsDNA levels and decreasing C3/C4 levels [67].

1.1.4.2 Organ (or system) specific BILAG scores, with a focus on the renal BILAG score

Yee et al, subsequently undertook a study examining whether the BILAG-2004 index was sensitive to change focusing on the principle of intention to treat, i.e. assessing whether an increase in therapy was associated with an increase in scores for all

systems, or if a decrease in score was associated with a decrease in treatment. This showed that system-specific scores were independently associated with treatment increases in all systems apart from the renal system. The score was sensitive to detection of new-onset LN and significant improvement of renal disease activity, but apparent fluctuation of the renal BILAG score between adjacent categories of Grades A and B or Grades B and C were noted, without changes in treatment. This was seen to be the case when assessments were close together or where intermittently active urine sediment (e.g. isolated sterile pyuria) or a brief rise in proteinuria were temporarily seen [68]. In clinical practice, treatment is not commonly increased on the basis of a single rise in proteinuria or a single failure of proteinuria to improve, accounting for such difficulties [68].

Use of the renal domain of the BILAG score is however of great importance within LN clinical studies, where the score is used to define LN activity rather than response to treatment alone. This approach is in keeping with the findings of Yee et al, specifically that the renal BILAG score is sensitive to detection of new-onset of LN activity and significant improvement of renal disease activity, despite not reflecting intention to treat [68]. It is not possible to undertake serial renal biopsies in order to define LN activity longitudinally, particularly in children, therefore, the renal BILAG score definition of active LN has been utilised within a very large number of studies looking at the ability of urine and serum biomarkers to identify and predict changes in LN disease activity in JSLE and adult SLE [69-78].

In a paediatric setting, total BILAG, SLEDAI and SLAM scores have been assessed in 35 JSLE patients on up to four occasions during the disease course: at the time of diagnosis, 6 months post diagnosis, at the time of disease flare, and 6 months post-flare. All three tools were found to be very sensitive to changes in disease activity, with no individual score showing overall superiority [79]. Two further studies have looked at the ability of the BILAG score for assessing LN disease activity in JSLE in particular. Zhou et al assessed the clinical data of 159 JSLE patients and showed the renal BILAG-2004 score to correlate better with the renal pathology activity index (see section 1.2.2) and 24-hour urinary protein levels than the SLEDAI-2000 score; area under the curve (AUC) of 0.93 for BILAG vs. 0.88 for SLEDAI [80]. Marks et al have also undertaken a prospective observational comparison study of the validity of the

BILAG score in 10 JSLE patients with biopsy proven LN and 11 JSLE patients without clinical, laboratory or biopsy proven evidence of LN. Renal biopsies were undertaken within 0.1-0.8 years of the BILAG assessment, and the numerical renal BILAG score (rather than alphabetical score) compared between groups. A significantly higher renal BILAG score was demonstrated in the LN group as compared to the non-nephritis group, demonstrating the ability of the renal BILAG score to identify active LN [63].

One of the main strengths of the BILAG score is that it has been designed from the outset to provide individual organ domain specific scores (including renal). Other measures use questions of relevance to assessing LN activity, but were not originally established in order to provide a renal specific score. The renal BILAG score is derived from more comprehensive composite renal data (including urine dipstick, 24-hour or spot proteinuria measurements, blood pressure, serum creatinine, GFR, urine sediment, histological evidence of LN and presence of nephrotic syndrome, see Appendices 5 and 6) than other scores such as the SLEDAI score (see Appendix 7), which is the most commonly used in the US, but only collects information on urinary casts, haematuria, proteinuria and pyuria [81]. The renal BILAG score has also been shown to associate better with the renal pathology activity index (see section 1.2.2) than the SLEDAI score within a paediatric study [80].

The main weaknesses of the renal BILAG score is that it has not undergone validation against treatment response in children, as described above by Yee et al for adult SLE [68]. As with other composite disease activity scores, by utilising traditional LN laboratory markers (e.g. proteinuria, GFR) to calculate the BILAG score, and then assessing urine biomarkers against the BILAG score, it becomes impossible to compare such traditional laboratory markers and novel biomarkers head to head. Some of the traditional biomarkers contributing to the renal BILAG score have been criticised individually as poor markers of active LN (see sections 1.2.3 and 3.1.3). However, the comprehensive nature of the BILAG ensures that a range of other factors are also considered alongside such markers.

The approach to use of the renal BILAG score in the present thesis has therefore been to draw on the strengths of the renal BILAG score for detection of new-onset LN, severe LN and significant improvement of disease activity [68], by defining active LN

as having a renal BILAG score of A or B in patients with current or previous histological confirmation of active LN, and inactive LN as a renal BILAG score D or E (without clinical, laboratory or biopsy proven evidence of LN). This approach is in keeping with the study of Marks et al assessing the validity of the renal BILAG score in children [63]. By grouping the alphabetical renal BILAG score categories together to differentiate active versus inactive LN, inaccuracies demonstrated with subtle fluctuations in the renal BILAG score during the study of Yee al al [68] are mitigated. Where renal BILAG C scoring episodes are included in a study (e.g. longitudinal studies), it has been considered best to group A, B and C grades together in view of the fluctuation between adjacent categories of B and C without changes in treatment as previously described [68].

1.1.4.3 Systemic Lupus Erythematosus Disease Activity Measure

The SLEDAI score is a global index which was initially derived in 1986 [55] and most recently updated by Gladman et al in 2002 (SLEDAI-2K [81]). Within the original SLEDAI score, rash, alopecia, mucous membrane lesions, and proteinuria were only scored only on their first occurrence or a recurrence, whereas in the SLEDAI-2K version these items are simply scored when present (regardless of whether new or recurrent). In contrast to the BILAG score, the SLEDAI score evaluates disease activity over the previous 10 days vs. the past month, with the BILAG score also recording partial improvement in features vs. present/absent. In view of this, Touma et al developed a response rate of 50% (SRI-50) to document a 50% improvement in SLEDAI, providing a measure of partial rather than complete improvement [82]. The SLEDAI-2K consists of 24 weighted items across 9 organ systems (central nervous system, vascular, renal, musculoskeletal, serosal, dermal, immunologic, constitutional, and hematologic), providing a maximum total score of 105, with all questions contained within a single page form, making it the shortest of the SLE disease activity tools.

The SLEDAI score has been shown to be sensitive to changes in disease activity within an international adult SLE validation study [83]. The majority of paediatric studies have utilised the original SLEDAI score. Brunner et al have assessed and compared whether the original SLEDAI, BILAG and SLAM scores are sensitive to clinical

change in disease activity in children. Assessing these scores at the time of diagnosis, six months post-diagnosis, at the time of a flare, and 6 months post-flare, all three measures were found to be highly sensitive to clinical change in children, with no measure displaying overall superiority [79]. The SLEDAI-2K score was used in a further study by Brunner at al looking at the minimal clinically important differences (MCID) of disease activity indices in JSLE. They found the MCID to be small (difference of 2 points) and similar to those reported for adults with SLE, but did not discriminate well between disease courses (detection rates for improvement or worsening were all <55%). Using a higher MCID (defined by a 70% predicted probability of improvement or worsening) a better ability to discriminate between changes in disease activity was seen, but the proportion of patients with disease activity change was underestimated. The authors therefore concluded that small changes in SLEDAI-2K disease activity scores can be clinically relevant, however, overall worsening, improvement and response to therapy, cannot be accurately captured by such measures of disease activity alone [84].

1.1.4.4 Systemic Lupus Activity Measure

The latest version of the SLAM is known as the SLAM-R [56]. Both versions feature more systemic features than the SLEDAI and record more subjective symptoms such as fatigue, arthralgia and myalgia. All SLAM items are weighted according to the severity of the item. In a paediatric study including 35 JSLE patients and comparing the SLAM score with the BILAG and SLEDAI scores, the SLAM was shown to be user friendly, although longer than the SLEDAI. The SLAM was also shown to demonstrate equivalent responsiveness to change in disease activity as compared to the BILAG and SLEDAI scores [79]. Similarly, in an international study that including 557 JSLE patients, the SLAM was found to be strongly associated with changes in disease activity [85].

1.1.5 Aetiological factors

The underlying cause of JSLE remains unknown, with JSLE pathogenesis continuing to be a very active area of translational research. Onset of SLE in the childhood years is associated with an increased number of susceptibility alleles, involving a range of

different genes which have provided important insights into JSLE pathogenesis [44]. The concordance rate for JSLE is 25% amongst monozygotic twins and 2% in dizygotic twins, highlighting that genetic contribution is important but not the sole cause of JSLE [86]. Variants in the genes associated with type 1 interferon (IFN) production and or signalling have been shown to play a major role in JSLE susceptibility [87], with higher serum IFN-α levels demonstrated in younger individuals with JSLE within family cohorts [88]. IFN-α hyper-production has been associated with a number of rare infantile forms of JSLE in addition to Aicardi-Goutiers Syndrome (see section 1.1.1), phenotypically differing from other JSLE patients. For example, TREX1 mutations have been connected with chilblain lupus and intracerebral calcifications [89], DNAse-IL3 mutations are related to early-onset SLE, ANAs, anti-dsDNA, ANCA [90], the AGS5 mutations are linked to chilblain lupus, intracerebral calcifications and mental retardation [91] and ACP5 mutations are associated with growth retardation, spondyloenchondrodysplasia, SLE, Sjögren, vitiligo, myositis, Raynaud, ANA and anti-dsDNA [92]. Defects in early components of the classical complement pathway have also been shown to cause apoptotic cell and immune complex clearance defects, predisposing to JSLE. For example, C1q deficiency is particularly associated with rash and LN [10], and C2, or C4 deficiency are strong risk factors for JSLE [93]. Several micro-RNAs have also been implicated in JSLE pathogenesis [94], associating with high disease activity [95].

Exposure to multiple environmental factors in the context of this genetic predisposition is thought to also trigger autoimmunity, leading to JSLE manifestations. Predisposing environmental factors associated with the onset of JSLE include certain medications, infections (see Table 1-3 above), and exposure to ultraviolet light [96]. Hormonal influences (especially oestrogens) are also thought to play a key contributory role in the development of JSLE as evidenced by the strong female predisposition seen in JSLE, occurrence of disease flares in pregnancy and increase in female to male ratio as puberty is approached [24,25].

1.1.6 JSLE pathogenesis

The complex pathogenesis of JSLE can be summarised and considered as four distinct yet interacting processes, where there is increased nuclear debris production and

exposure, loss of tolerance to self-antigen and generation of auto-antibodies, which in turn exert pathogenic effects due to the formation of immune complexes, resulting in inflammation and clinical disease manifestations [97]. It is increasingly being demonstrated that both the innate and adaptive immune systems play a role in JSLE pathogenesis, in contrast to previous beliefs that JSLE was a disease of the adaptive immune system. An overview of JSLE pathogenesis is presented, followed by a specific description of LN pathogenesis.

1.1.6.1 Increased nuclear debris exposure

JSLE serum contains increased IFN-α, which plays a prominent role in development of JSLE, as discussed in section 1.1.5. IFN-α is pro-apoptotic, leading to increased endogenous nuclear debris exposure [98]. Neutrophils are also known to play a key part in JSLE pathogenesis, with a neutrophil specific genes signature being the second most commonly expressed gene signature within JSLE peripheral blood mononuclear cells (PBMCs) [99]. This has been attributed to the presence of early neutrophil precursors within the blood, known as low density granulocytes [100]. JSLE neutrophils promptly undergo spontaneous apoptosis *in vitro*, which is accelerated in the presence of JSLE serum [98] and dysregulated [101]. Neutrophils are also known to release neutrophil extracellular traps (NETs), containing chromatin, neutrophil deoxyribonucleic acid (DNA) and histones into their local environment. These NETs usually bind pathogens and assist with their clearance under normal conditions [102]. Increased NET production (also known as NETosis) coupled with ineffective NET clearance can lead to prolonged exposure of nuclear debris to the immune system [103]. Cytokines such as IFN-α also promote NET formation by neutrophils [104]. Monocytes and macrophages are responsible for clearance of apoptotic cells and environmental debris. However, these cells exhibit impaired phagocytic abilities in the presence of JSLE serum [105-107]. A combination of these processes is thought to contribute to the increase in nuclear debris visible to the immune system in JSLE, stimulating production of nuclear autoantibodies [108].

1.1.6.2 Loss of tolerance to self-antigens and generation of autoantibodies

Dendritic cells (DCs) are the main antigen-presenting cells connecting the innate and adaptive immune systems. They are able to both induce activation of naïve T-cells and

stimulate B-cell growth and differentiation. DCs are normally responsible for generating T-cell tolerance to apoptotic cells through inactivation of autoreactive T-cells. JSLE serum and IFN-α can induce maturation of monocytes into myeloid dendritic cells which are able to function as antigen presenting cells [109]. In JSLE the magnitude of apoptotic material complexed with autoantibodies, and the activation of myeloid dendritic cells by IFN-α is thought to result in auto-antigens being presented to auto-reactive T-cells, which in turn stimulate auto-reactive B-cells. JSLE serum-induced monocyte-derived DCs have also been shown to promote B-cell responses directly, inducing differentiation of naive and memory B-cells into IgG and IgA secreting plasmablasts, producing auto-antibodies against self-derived nucleic acids [110]. An imbalance of T-cell subsets has also been demonstrated in SLE, with a reduction in regulatory T-cells (CD4 CD25+), correlating with an increase in JSLE disease activity [111]. In JSLE patients with active LN, a more pro-inflammatory T-cell profile has been demonstrated with high serum IL-17 and IL-23 levels, and reduced regulatory T-cells and transforming growth factor-β concentrations [112].

1.1.6.3 Generation of autoantibodies

Loss of B-cell tolerance has been the main focus of recent therapeutic agents in SLE (e.g. Rituximab, Belimumab, Blisimumab, Blisibimod, Atacicept, Epratuzumab, Ocrelizumab) [113]. B-cell derived antibodies stimulate DCs to produce IFN-α, amplifying the inflammatory cascade involving both the innate and adaptive immune systems. Loss of B-cell tolerance is thought to occur at an early stage of JSLE development, with ANAs demonstrable up to 9.4 years before clinical features of SLE become apparent [114]. There is often absolute B-cell lymphopenia, but an expansion of auto-reactive memory B-cell clones and increased levels of immature peripheral blood plasmablasts which are felt to be pathogenic, correlating with increased autoantibody production and SLE disease activity [115]. Serum B-cell-activating factor (BAFF, also known as B-lymphocyte stimulator, BlyS) levels are increased in SLE and have been linked to expansion of autoreactive B-cell clones, prolonging their survival. Defective DCs have been shown to stimulate BAFF production, contributing to the processes described above [116].

1.1.6.4 Immune complexes and tissue damage

Auto-antibodies (mainly against nuclear antigens e.g. anti-dsDNA and ENAs) associate with autoantigens and complement factors which are present within the bloodstream, forming circulating immune complexes (CICs). CICs can be deposited within target organs, activating complement, initiating an inflammatory response and tissue damage [117]. CICs play a key role in the pathogenesis of LN, and are associated with different LN histological sub-classes dependant on where they are deposited within the kidney (described in section 1.1.7 and Figure 1-3). There is murine evidence that anti-dsDNA and ANA antibodies cross-react with proteins within the kidney (e.g. α-actin), exerting direct pathogenic effects on renal cells [118]. Post mortem examinations have revealed auto-antibodies to the collagenous region of C1q to be deposited and concentrated in the renal glomeruli of SLE patients [119]. Within murine models, anti-C1q antibodies have been shown to be pathogenic in the glomeruli when in combination with other complement fixing autoantibodies and immune complexes [120]. A summary and overview of the processes involved in JSLE pathogenesis is shown in Figure 1-1.

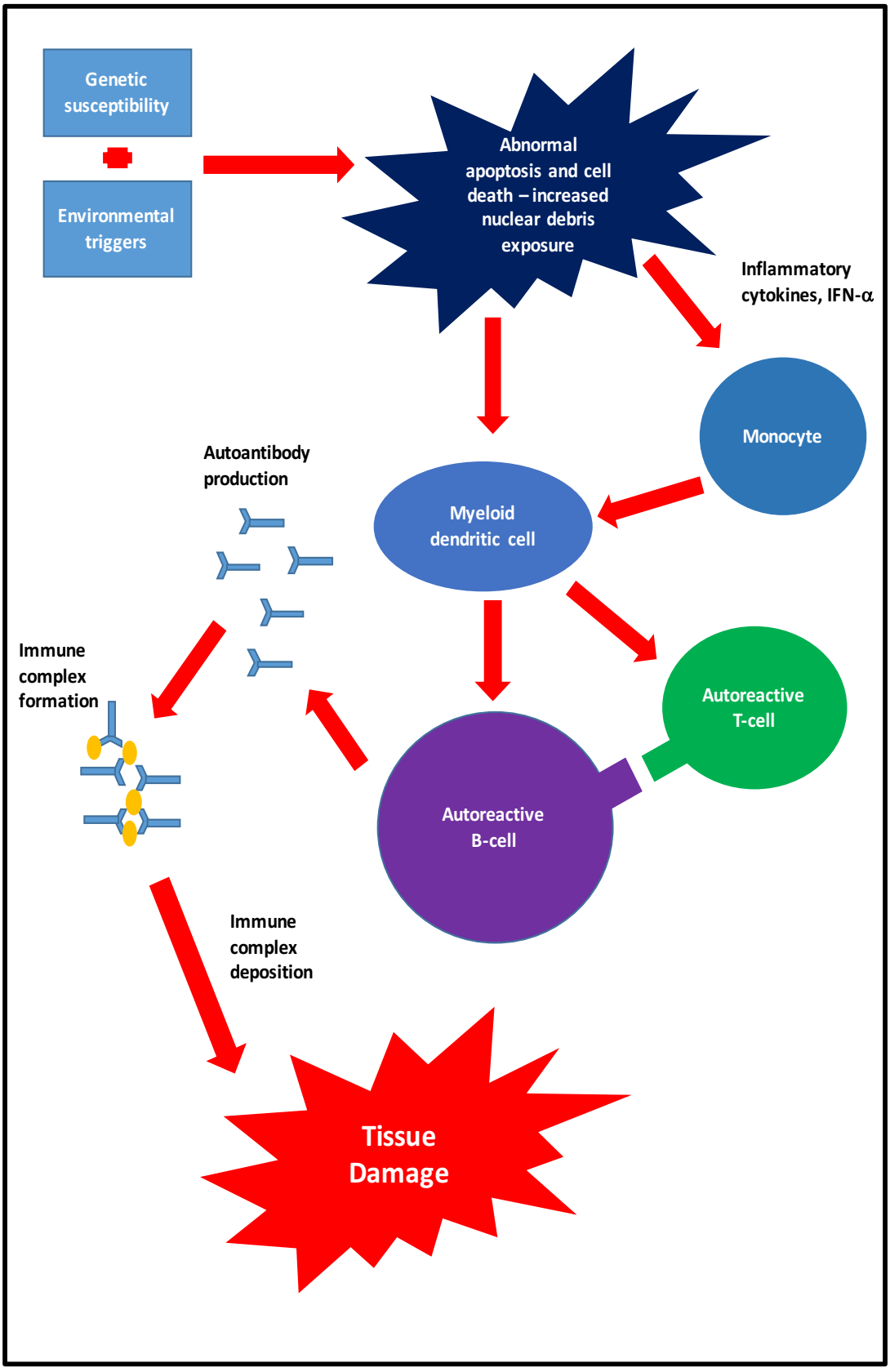


Figure 1-1: Overview of the processes involved in JSLE pathogenesis
Figure adapted from reference [113].

1.1.7 Pathogenesis of lupus nephritis

The kidney is made up of millions of nephrons, each containing a glomerular and a tubular region. The tubules are divided into separate components (proximal, distal, convoluted) and are largely responsible for water and electrolyte reabsorption/excretion. Each glomerulus consists of a ball of blood capillaries which enter the kidney from the afferent arteriole and leave as the efferent arteriole. Blood passing through the glomerulus is under high pressure, forcing waste substances to move through the glomerular filtration barrier and into the Bowman's capsule to form urinary ultra-filtrate. Under physiological conditions, proteins are held within the blood capillaries due to the highly efficient glomerular filtration barrier which consists of fenestrated endothelium, the collagenous glomerular basement membrane (GBM), and podocytes, with their interdigitating foot processes and slit diaphragms (see Figure 1-2). These three layers restrict movement of proteins into the urine on the basis of their molecular weight, size and electrical charge [121]. Molecules must be positively charged so they are not repelled by the negatively charged GBM. Specialised slit diaphragm proteins (e.g. nephrin, podocin, neph-1 [122]), are involved in intracellular signalling networks to maintain cellular polarity [123]. Mesangial cells sit amongst and support the glomerular capillaries, regulating blood flow by contracting. In glomerular diseases such as LN, this barrier is disrupted leading to proteinuria due to leakage of plasma/serum proteins (e.g. albumin, which is large and negatively charged) alongside secretion of specific proteins reflective of the inflammatory state and kidney damage [124].

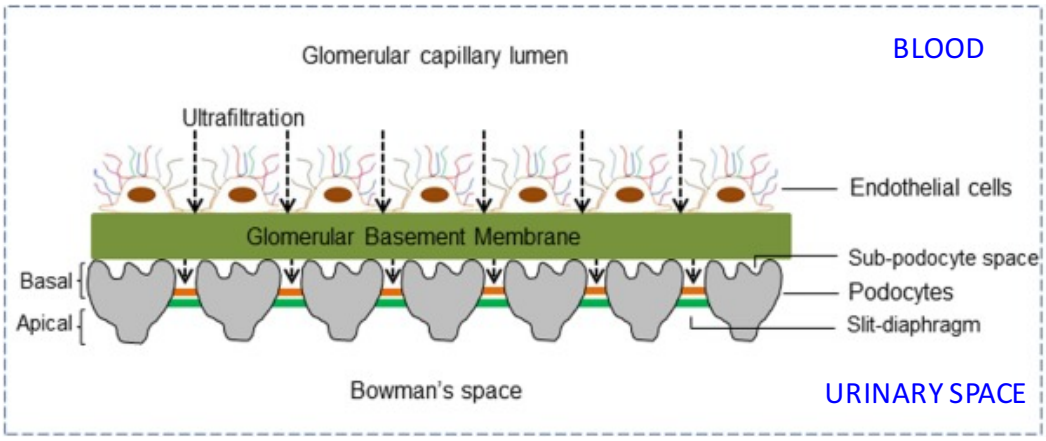


Figure 1-2: Architecture of the glomerular filtration barrier, displaying the three layers: endothelial, glomerular basement membrane, and podocytes.
Diagram adapted from [125].

The pathogenesis of LN is known to involve initiation of inflammation by CICs and subsequent development of immune mediated mechanisms of kidney tissue injury involving soluble inflammatory mediators, cellular infiltrates and non-immune mechanisms of tissue injury. These processes are summarised in Figure 1-3. As described above in section 1.1.6.4, immune complexes can be composed of ANA, anti-C1q and anti-glomerular antibodies, combined with opsonised apoptotic particles and NETs [126,127]. Sub-endothelial immune complexes (between the endothelial cells and GBM) are seen in class III and IV proliferative LN, causing injury to endothelial cells. They have access to the vascular space, allowing them to interact with and activate myeloid cells, promoting their recruitment into the kidney [128]. Sub-epithelial immune complexes (between the GBM and podocyte layers) lead to podocyte injury but less inflammation as the CICs only come into contact with the urinary space. The glomerular basement membrane can rupture, exposing sub-epithelial CICs to the whole glomerulus, and leading to a pro-inflammatory response with inflammatory cytokine/chemokine release (e.g. monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-α (TNF-α), IFN-γ) [129], complement activation, and activation of resident renal cells (e.g. podocytes) by CICs containing nucleic acids (through toll like receptors 7/9, TLRs) [130].

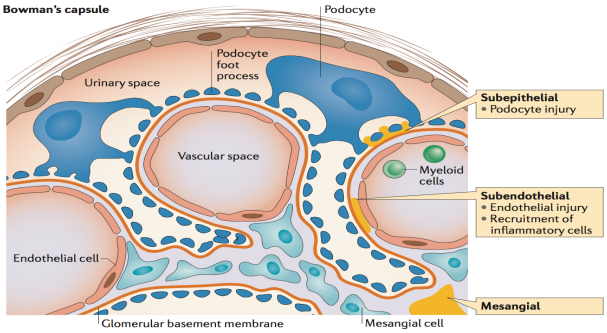
Inflammatory cells infiltrate the kidney through glomerular and interstitial blood vessels. Both the B and T-cells found within LN kidneys are clonally expanded, with evidence of a local adaptive immune response which could potentiate the effects of the systemic response. A proportion of B-cells derived from renal biopsies have been shown to be specific for vimentin, an intracellular protein which is extruded from apoptotic cells, with increasing vimentin levels correlating with reduced GFR [131]. Auto-antibodies to kidney proteins annexin 1 and α-enolase have also been detected in nephritic kidneys [132]. Macrophages recruited from the blood are initially pro-inflammatory but can switch to a reparative phenotype, assisting with tissue remodelling and resolution of inflammation [133,134]. DCs also infiltrate the kidneys during LN and may contribute to development of a local adaptive immune response [135].

Non-immune mechanisms of tissue injury also occur in parallel to those described above, potentiating renal damage. Maintenance of glomerular function relies on preservation of the nephrons complex structure including close mesangial to endothelial interaction, and podocyte to endothelial interaction, allowing diffusion of cytokines and growth factors through the GBM [136]. When nephrons are damaged and lost, compensatory mechanisms lead to an increase in intra-glomerular pressure/stress for the residual nephrons, with abnormalities of vascular function also leading to tissue hypoxia [137] (see Figure 1-3).

Initiation of inflammation by immune complexes composed of

- IgG antibodies
- ANA, anti-C1q, anti-glomerular
- Complement
- Apoptotic particles
- NETs

Immune complex deposition*



Sub-endothelial

- Injures endothelial cells
- Seen in class III & IV LN
- Access to vascular space
- Activates circulating myeloid cells and recruits them to renal tissue

Sub-epithelial

- Injures podocytes
- Seen in class V LN
- Less severe inflammatory response as immune complexes in contact with urinary space
- TLR mediated signals and inflammatory mediators

General effects

- Initiates complement cascade
- Activates glomerular cells
- Induces cytokine & chemokine release (e.g. MCP-1, TNF-α, IFN-α/γ)
- Activates TLR's enhancing the immune response

Cellular infiltrates

- Local adaptive immune response
  - B-cells – some specific to vimentin, annexin V, α-enolase
  - T-cells – produce IFN- γ, IL-21, IL-17 CD8+ cells with effector functions stimulated by local antigens
- DCs – propagate adaptive response
- Macrophages – pro-inflammatory infiltrating and resident macrophages

Parallel non-immune mechanisms of renal tissue injury

- Disruption of cell:cell interactions
  - mesangial:endothelial
  - podocyte:endothelial
- Rise in intra-glomerular pressure
- Glomerular stress
- Tissue hypoxia

Figure 1-3: Summary of processes involved in LN pathogenesis.
*Diagram displaying position of sub-endothelial/sub-epithelial deposits adapted from reference [138].

1.1.8 Disease management of JSLE

The management of JSLE must be undertaken by a multi-disciplinary team with experience of looking after children and young people with JSLE and similar complex connective tissue disorders. This is emphasised in the SHARE consensus evidence-based recommendations for management of JSLE [45]. Referral to an experienced specialist team is essential as soon as the diagnosis is suspected, as the longer the period of active disease, the higher the risk of long-term complications [139,140]. JSLE patients frequently require input from several specialist teams along with complex immunosuppressive treatment, co-ordinated by a paediatric rheumatologist. As survival rates from JSLE improve, there is increasing emphasis on preventing long-term complications and co-morbidities, such as premature atherosclerosis, osteoporosis, neurocognitive impairment, and (potentially) increased risk of malignancy, through optimal disease control and empowering patients to take responsibility for their own healthcare [141]. Immunosuppression is tailored to the individual patient, taking into account disease presentation and severity, patient preference, tolerance of drugs and the burden of the disease/treatment on QOL. Issues often arise in young people with complex chronic diseases such as JSLE in relation to treatment adherence, risk-taking behaviours, and poor clinic attendance; worsening clinical outcomes [142-148].

The sections to follow consider pharmacological management of mild-to-moderate JSLE manifestations, contrasted with severe disease. Mild to moderate disease is characterised by features affecting the following domains; constitutional, mucocutaneous and musculoskeletal, whereas severe disease is characterised by major organ involvement (e.g. LN, neurological). Management of LN is briefly discussed under management of severe disease (section 1.1.8.2) with a more detailed explanation of how LN treatment relates to LN histological sub-classes. The key clinical trials concerning LN induction and maintenance treatment, and published treatment recommendations relating to the management of LN in children are shown in section 1.2.4 below.

1.1.8.1 Management of mild to moderate disease

Glucocorticoids remain the established mainstay of JSLE management in the form of topical and/or low dose oral treatment for mild-to-moderate disease, or high dose oral

or intravenous treatment for severe disease. This is despite their well-recognised array of significant adverse effects. Within clinical care there is a strong emphasis on tapering to the smallest possible dose and use of corticosteroid sparing treatments to reduce side effects [113]. Corticosteroid free induction regimes are starting to emerge, showing promising results [149]. Clinical trials for example of Rituximab and mycophenolate mofetil (MMF) without oral corticosteroids for LN treatment compared to standard therapy have been attempted [150]. Defining the appropriate use of corticosteroids in the management of JSLE remains an important research question.

Hydroxychloroquine is recommended from diagnosis for all JSLE severities, either as monotherapy or in combination with other treatments. It is well recognised that hydroxychloroquine can improve symptoms [151], reduce the rate of flares and improve renal response to treatment in adult LN [152,153]. It has also been shown to reduce lipid abnormalities and vascular events in adult SLE [154].

Azathioprine is generally used for new mild to moderate disease and as maintenance treatment following severe manifestations [155,156], however, its efficacy has not been examined in JSLE clinical trials. MMF can be used as an alternative to azathioprine, depending on the type of manifestation, but is usually reserved for moderate to severe disease manifestations. Methotrexate is mainly used where prominent mucocutaneous or musculoskeletal symptoms are present [157], however the evidence for use as a monotherapy in JSLE is poor [158]. The mode of action, indications and side effects of the above medications are summarised in Table 1-6.

Medication	Mode of action	Indications	Side effects
Glucocorticoids	Acts on the innate and adaptive immune systems - ↓ cytokine expression, inhibiting access to sites of inflammation and interfering with cell function.	Induction and maintenance treatment. Different forms for mild/mod/severe disease.	Cataracts, adrenal suppression, striae, obesity, mood changes, growth failure, osteoporosis
Hydroxy-chloroquine	Positive effect likely to be due to inhibition of endosomal TLR's 3,7,9 inhibit the binding of lupus autoantigens and preventing IFN-α production.	All severities & manifestations. Continues indefinitely.	Safe in pregnancy or G6PD deficiency.
Azathioprine	Purine synthesis analogue. Immunosuppressive properties due to inhibition of T-cell mediated immunity and DNA synthesis.	New mild - mod disease or maintenance treatment. Safe in pregnancy.	Nausea, fatigue, hair loss. Genetic testing for TPMT activity prior to initiating therapy. Those with ↓ activity risk myelosuppression.
Methotrexate	The doses used in rheumatic diseases inhibits cell mediated immunity and cytokine production.	Arthritis and mucocutaneous disease.	Anticipatory nausea and vomiting. Myelosuppression and hepatotoxicity. Unsafe in pregnancy.
MMF	Inhibits the enzyme inosine monophosphate dehydrogenase, which is required for the proliferation of T and B cells.	Moderate to severe disease. LN induction and maintenance.	Lower dose initially, escalating to improve tolerance. Unsafe in pregnancy. Abdominal discomfort, diarrhoea, hepatotoxicity.
Cyclophosphamide	Potent broad-spectrum immunosuppressant which modulates the T cell response and B cell antibody production.	Induction therapy for severe disease at JSLE onset or during severe flares.	Infertility due to premature ovarian failure, Hair loss, risk of infection, nausea, vomiting, long-term ↑ risk of malignancy.
Plasma exchange	Removal of putative pathogenic autoantibodies and circulating immune complexes from the blood.	Adjuvant therapy in rapidly progressive life threatening disease.	Invasive, risk of anaphylaxis. Small risk of infection from blood products (mainly albumin, plasma) used in the procedure.
IVIG	Postulated to involve the Fc portion binding to the Fc receptors of macrophages & complement C3b and C4b, inhibiting binding of autoantibodies.	Adjuvant therapy in rapidly progressive life threatening disease. ITP.	Anaphylaxis, fever, urticaria, headache, abdominal pain, nausea, vomiting, small risk of viral contamination.
Rituximab	Chimeric anti-CD20 monoclonal antibody. Induces apoptosis upon binding to CD20 cell surface antigen expressed on immature, naive & memory B cells (not pro-B, early pre-B cells or plasma cells).	Severe, intractable disease or those patients experiencing unwanted adverse effects from alternative treatments.	Anaphylaxis, fever, hypotension, wheeze, urticarial rash, infections.
Belimumab	humanised monoclonal antibody, which binds to human soluble BAFF/BLyS protein preventing binding to its receptors. BAFF is vital for B cell survival and important for B cell maturation, immunoglobulin production and switching.	Adult SLE patients with active auto-antibody positive disease. The role of Belimumab in severe active LN / central nervous system involvement is unclear.	Nausea, diarrhoea, fever, infections, bronchitis, insomnia, pain in extremities, depression, and migraine

Table 1-6: Mechanisms of action, indications and side effects of commonly used medications in JSLE.

Mod = moderate. TLR's = toll like receptors. G6PD = Glucose-6-phosphate dehydrogenase deficiency. TPMT = enzyme thiopurine methyltransferase. C3/C4 = complement fragments 3/4. ITP = idiopathic thrombocytopenia purpura. CD20 = cluster of differentiation 20.

1.1.8.2 Management of severe disease in JSLE

Severe disease at JSLE onset or during a flare must be promptly recognised and treated to improve outcomes [139]. Along with use of high dose, systemic corticosteroids, cyclophosphamide is often used to treat major organ involvement or life threatening flares, when rapid disease control is required (e.g. neurological, systemic vasculitis, renal involvement) [159-163]. Recommendations vary between guidelines in relation to cyclophosphamide dosage and treatment duration [162,163]. Risks of cyclophosphamide side effects, including infertility, cancer and infections have increasingly led to MMF being used where possible as an alternative to cyclophosphamide, with MMF tending to be the commonest LN induction and maintenance treatment in recent years [164]. MMF has been shown to be equally effective, but less toxic than cyclophosphamide in the induction phase of LN management [165]. As a maintenance treatment, MMF has been shown to be significantly superior to azathioprine, regardless of whether MMF or cyclophosphamide was used as induction therapy (ALMS trial [166]). In the MAINTAIN trial, where all patients received Euro Lupus induction treatment [167] with low dose IV cyclophosphamide (6 doses) followed by either MMF or azathioprine, no difference was seen in relation to the time to renal flare between the two study arms [168]. Further details relating to cyclophosphamide, MMF and azathioprine use in LN management are provided in section 1.2.4, Table 1-9, Table 1-10 and Table 1-11.

Plasma exchange has been seen to be clinically useful in rapidly progressive life threatening disease, with a recent review summarising case series which highlight the utility of plasma exchange for severe therapy-resistant manifestations, refractory LN, diffuse alveolar haemorrhage, neuropsychiatric SLE, thrombotic thrombocytopenic purpura, catastrophicAPLS, hyperviscosity syndrome and cryoglobulinemia [169]. The mechanism of action is thought to be through removal of putative pathogenic auto-antibodies and CICs from the blood. Intravenous immunoglobulin (IVIG) may also be useful, and has been shown to be significantly associated with a reduction in SLE disease activity scores (e.g. SLEDAI, SLAM) and improvements in C3 levels [170].

Rituximab is currently the most commonly used biologic in JSLE, playing a role in treatment of disease which is refractory to first line induction treatment, or for those

patients experiencing unwanted adverse effects from alternative treatments (see Table 1-6). Three retrospective JSLE studies (including one from the UK JSLE Cohort Study) have demonstrated a reduction in steroid burden and disease activity following rituximab treatment [171-174]. Adult randomised control trials (RCTs) evaluating rituximab in non-renal SLE (as induction and maintenance treatment, EXPLORER trial [175]) and LN (as an adjuvant to MMF and steroids, LUNAR trial [176]) both failed to meet their primary or secondary outcome measures, however, suboptimal trial design was felt to contribute to these results. Two recent studies seek to examine the efficacy of rituximab in LN, including paediatric patients. The Rituximab and Mycophenolate Mofetil Without Oral Steroids for Lupus Nephritis (RITUXILUP) trial is an open-label, multicentre RCT assessing rituximab with MMF versus MMF and glucocorticoids only [150]. The Rituximab for Lupus Nephritis With Remission as a Goal (RING) trial is another phase III trial that will examine rituximab in refractory LN [177].

1.1.8.3 Emerging biologic agents

Belimumab is the only biologic agent which has been proven to be effective in adult-SLE RCTs alongside standard SLE therapy [178,179], demonstrating a favourable side-effect profile [180]. It is a fully humanised monoclonal antibody, which binds to human soluble BAFF/BLyS protein (see section 1.1.6.3), preventing it from binding to its receptors. Higher therapeutic benefit of belimumab was found in patients with autoantibody positive disease, greater disease activity, low complement and corticosteroid use at baseline. In early 2016, belimumab was approved by the National Institute of Clinical Excellence (NICE) for use in adult SLE patients with active auto-antibody positive disease [181]. Of note, patients with severe active LN or central nervous system involvement were excluded from the belimumab trials mentioned above [178,179]. However, numerous case reports have described successful use of belimumab in the context of proliferative LN [182], membranous LN [183], rituximab refractory LN [184] and sequential use of rituximab followed by belimumab [185,186], highlighting that further trials of belimumab in LN are required. A randomised, double-blind Pediatric Lupus Trial of belimumab Plus Background Standard Therapy (PLUTO) is currently assessing the pharmacokinetics, safety and efficacy of belimumab in 5-17 year olds with active, auto-antibody positive JSLE. Patients with severe active LN are again excluded from this trial [187].

Biologic agents that remain within the early phases of investigation in adult SLE have been summarised in a recent review [113] and include: blisibimod (an anti-BAFF peptibody), tabalumab (anti-BAFF humanised monoclonal antibody), atacicept (anti-BAFF and APRIL fusion protein), epratuzumab (an anti-CD22 human monoclonal antibody), ocrelizumab (a humanised anti-CD20 monoclonal antibody), abatacept (a CTLA-4 and immunoglobulin fusion protein), forigerimod (a 21-mer peptide targeting T-cells), sifalimumab (a humanised anti-IFN-α monoclonal antibody), rontalizumab (a humanised anti-IFN-α monoclonal antibody) and tocilizumab (a humanised anti-IL-6 monoclonal antibody) [113].

1.1.9 Prognosis in JSLE

1.1.9.1 Disease-associated damage

Patients developing JSLE during childhood now live longer and consequently have an increased risk of developing disease and treatment related damage. The SLICC Damage Index (SDI, see Appendix 1) has been developed to measure cumulative irreversible SLE related damage [188], and has been validated in a paediatric setting [189,190]. A questionnaire administered to paediatric rheumatologists scoring the SDI in the paediatric validation study [189] highlighted concerns that some of the SDI items would be more common amongst older populations, and that the score would benefit from the introduction of weightings, and some of the items being redefined. Within the UK JSLE Cohort Study, 28% of patients have been shown to have an SDI score of ≥1 within a relatively short follow-up period (median 4.5 years [IQR 2.7-6.9 years]), with neurological, renal, musculoskeletal damage and scarring alopecia displaying the highest prevalence. The SDI renal damage features include; eGFR<50%, persistent 24 hour proteinuria >=3.59g OR UACR of > 1000mg/mm OR > 10mg/mg, and end stage renal disease [28].

Seven JSLE studies counted within a meta-analysis including 1,559 JSLE patients have shown 39-65% of patients to demonstrate damage with mean SDI scores of 0.6-2.3 (after 4-10.8 years). Damage of the renal, musculoskeletal, neuropsychiatric and ocular systems were most commonly seen [26,39,190-192]. Cumulative disease activity over time and disease duration have been consistently reported as predictors

of damage [189,190,193,194], with JSLE patients accruing damage at a faster rate than adult onset patients [39,189,192-196]. Other predictors of damage in JSLE have been shown to include presence of major organ involvement at baseline and later in the disease course, use of immunosuppression, cumulative corticosteroid dose, male gender, and non-Caucasian ethnicity [1,12,26,39,190,191,194,197-200]. The largest of these seven studies included in the meta-analysis [39] devised a paediatric version of the SDI (Ped-SDI), which did not change any of the existing items to ensure harmonisation between the paediatric and adult scores, but included growth failure and delayed puberty as new damage domains. Further details on LN associated damage are provided in section 1.2.5.

1.1.9.2 Co-morbidities

Important co-morbid conditions may be linked to the JSLE disease process or treatment. Atherosclerosis is itself a chronic inflammatory condition influenced by JSLE related factors such as CICs, complement activation, anti-phospholipid antibodies, corticosteroid use, lipid abnormalities and endothelial dysfunction [201,202]. Irreversible renal failure is one of the most common long-term consequences of JSLE [203], affecting approximately 10% of children with proliferative LN within 5 years [199]. Renal failure relates to cardiovascular risk, with shared risk factors which can worsen both renal and cardiovascular outcomes (e.g. hypertension, atherosclerosis, glucose intolerance, lipid disorders and anaemia). Further information on renal co-morbidity is provided in section 1.2.5 below. Multiple factors may contribute to osteopenia and osteoporosis in JSLE, including inflammation, corticosteroid use, diseases activity, nutrition, physical inactivity, limited exposure to sunlight and delays in pubertal development, leading to significant risk of fracture [204,205]. JSLE patients show a significant reduction in parent-adjusted height z-scores, with males being most affected. Delays in pubertal onset has also been shown to occur in 15% of female and 24% of male JSLE patients [206]. Certain immunosuppressive treatments may be associated with longer-term co-morbidities, which must be mitigated where possible (e.g. long term side effects of cyclophosphamide including infertility, increased risk of malignancy, see Table 1-6). Prospective, long-term, collaborative follow-up studies are required to direct care of these co-morbid conditions and determine guidelines for their prevention.

1.1.9.3 Mortality

Life expectancy of SLE patients in developed countries has increased significantly over the last 20 years, with the 5-year survival rate reported to be 94-100%, disappointingly falling to 81-92% by 10 years [207,208], and 79% by 15 years [209,210]. Studies from developing countries have reported poorer survival rates, for example in Thailand the 5-year survival rate has been reported to be 77% [211], and in India, after a mean of 6.5 years follow-up the survival rate has been shown to be 63% [212]. Higher disease activity is associated with higher mortality in both JSLE and adult SLE [189,213]. Comparing JSLE and adult SLE, the overall survival rates are similar, however the baseline mortality rate of children is far lower, therefore the age adjusted mortality rate is far higher for children [1,2,43,214]. Studies in adult SLE have consistently shown low socioeconomic status, high disease activity and damage to be important predictors of poor survival [215-221]. In a study of 1,827 SLE patients within the multi-ethnic SLICC inception cohort, patients with LN had a significantly higher risk of death (HR = 2.98) than those without LN [222]. LN patients of African American origin [223] and with nephrotic range proteinuria at baseline [224] have been shown to have increased mortality rates. In JSLE, results are more conflicting between studies, but overall male gender, severe infections, LN, early damage and haematological involvement have all been associated with poor survival [207,221]. Within a Northern Indian JSLE study looking at LN outcomes over 24 years, end stage renal failure and infections were shown to be the two most common causes of death [225]. Large studies with prospective follow-up into adult care are required to determine accurate long-term survival rates and factors associated with reduced survival, in patients with JSLE, and LN in particular.

1.2 Lupus Nephritis

1.2.1 Clinical presentation of LN and its natural history

Up to 80% of JSLE patients develop LN [28], compared with 40-50% or adults [36,37]. LN may be part of the patients' initial presentation or occur later in the disease course [226]. The clinical presentation of LN is very heterogeneous, ranging from it being entirely asymptomatic to having overt features of nephrotic syndrome. Therefore, clinicians should proactively screen for symptoms and signs of LN at each clinical encounter, and undertake urinalysis routinely. Patients should be questioned about the

presence of foamy urine (suggesting proteinuria) or nocturia (an early sign of glomerular or tubular dysfunction). Microscopic haematuria may be identified by urinalysis, with macroscopic haematuria being a rare feature, which should prompt rapid review of the patient. Proteinuria mainly reflects the degree of glomerular involvement, with proteinuria of >3.5g per day leading to nephrotic syndrome, characterised by hypoalbuminaemia, hyperlipidaemia and peripheral oedema [227]. Timed (24-hour) urine collections represent the gold standard for proteinuria quantification but are seldom undertaken due to difficulties arising from collection accuracy, especially in children. Therefore, spot UPCR or UACR measurements are favoured, estimating the degree of proteinuria. Urinary abnormalities related to menstruation, infection, cross-contamination, orthostatic proteinuria and the timing of the urine sample should be considered and repeat urinalysis undertaken as necessary [228].

It is important to also assess for presence of active urine sediment in JSLE patients, with >10 red blood cells +/- >10 white cells per high powered field (hpf) being clinically significant. Misshapen or fragmented red cells suggest inflammatory glomerular or tubulointerstitial disease, whereas normal erythrocytes indicate bleeding in the lower urinary tract [227]. Glomerular abnormalities are more common than tubular dysfunction in LN, therefore it is appropriate to screen serum creatinine as a marker of glomerular function and to calculate the eGFR from serum creatinine and height [229]. True GFR is not measured very often as the test is labour intensive and involves radio-nucleotides. In severe LN, patients may present with rapidly progressive glomerulonephritis leading to acute renal failure and doubling of serum creatinine due to a loss of glomerular filtration function, usually in the context of proliferative glomerulonephritis, fibrinoid necrosis and cellular crescents in the renal biopsy. More frequently, renal function fluctuates with exacerbations/remission, with the concerning prospect of end-stage renal disease requiring dialysis or transplant, following progressive damage during LN flares.

1.2.2 Diagnosis and monitoring of LN

The ACR criteria for diagnosis of LN include a) persistent proteinuria of >0.5g per day or >3+ urine dipstick for albumin, or b) cellular casts, including red blood cell,

haemoglobin, granular, renal tubular cell, or mixed [230]. These laboratory criteria define the minimum measures for diagnosing kidney disease, and are commonly used as inclusion criteria when considering patients for clinical research studies. Renal biopsy is however the gold standard for diagnosing LN, characterising disease activity and providing insight into the degree of scarring and irreversible damage to the kidney [198,231]. At present, there is no international consensus as to the optimal timing of the initial renal biopsy in children. The invasive nature of renal biopsies, risk of complications such as bleeding or infection and need for anaesthetic in children, lead to a tendency for the procedure to be avoided until deemed absolutely necessary, with clinical concern that irreversible renal damage may occur during this period [232].

Renal biopsies were initially classified according to the 1974 World Health Organisation (WHO) LN classification criteria [233], but since 2003, following a consensus conference of nephrologists, pathologists, and rheumatologists, the International Society of Nephrology/Renal Pathology Society (ISN/RPS) LN criteria have been adopted, classifying specimens according to morphological patterns of glomerular injury and prognostic relevance [231,234]. LN is divided into five classes according to the ISN/RPS criteria, class II mesangial LN, class III focal proliferative LN, class IV diffuse proliferative LN, class V membranous LN and class VI advanced sclerosing LN. Active lesions are characterised by cellular crescent and fibrinoid necrosis, whereas chronic lesions consist of glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis (see Table 1-7 for further details) [234]. Inadequate treatment may lead to the conversion of active lesions to chronic irreversibly damaged lesions.

ISN/RPS LN class	Histological characteristics
Class II mesangial LN	Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits. May be a few isolated subepithelial or subendothelial deposits visible by immunofluorescence or electron microscopy, but not by light microscopy.
Class III focal proliferative LN	Active or inactive focal, segmental, or global endo- or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations.
Class IV diffuse proliferative LN	Active or inactive diffuse, segmental, or global endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental (IV-S) lupus nephritis when ≥50% of the involved glomeruli have segmental lesions, and diffuse global (IV-G) lupus nephritis when ≥50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation.
Class V membranous LN	Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations. Class V lupus nephritis may occur in combination with class III or IV in which case both will be diagnosed.
Class VI LN advanced sclerosis	≥90% of glomeruli globally sclerosed without residual activity.

Table 1-7: ISN/RPS 2003 classification of LN.

A number of studies have assessed inter-observer reproducibility of LN class scoring, comparing the WHO and ISN/RPS 2004 classification systems. Furness et al looked at 20 LN cases and demonstrated kappa values of 0.44 and 0.53 for inter-observer agreement between WHO and ISN/RPS scores respectively (0 = no agreement, 1 = perfect) [235]. Wilhelmus et al carried out a similar study where 360 members of the Renal Pathology Society were shown microphotographs of 30 cases and asked whether glomerular lesions were present and compatible with either class III or IV LN vs. other LN classes. The kappa value for presence of class III/IV LN was disappointingly low, at 0.39 [236]. Grootscholten et al assessed the intra-class correlation coefficient (ICC, >0.8 excellent; 0.6-0.8 good; 0.4-0.6 moderate; <0.4 poor) in 126 LN cases, demonstrating ICC scores of 0.182 and 0.414 for WHO and ISN/RPS criteria respectively [237]. The studies demonstrate higher consensus with the ISN/RPS 2004 classification system, but worryingly, even the ISN/RPS system does not perform perfectly.

In order to facilitate the application of the renal biopsy as a predictive tool, the National Institute of Health (NIH), developed composite activity and chronicity indices (AI and CI) for LN, focusing on the glomerular compartment of the kidney [238]. This was subsequently followed by the development of a tubulointerstitial activity index (TIAI) [239], recognising that changes in the tubulointerstitial (TI) compartment may be important for prognosis [240,241]. Table 1-8 shows a description of the histological characteristics of the AI, CI, TIAI.

	Activity Index	**Chronicity Index**
Glomerular abnormalities	1. Cellular proliferation 2. Fibrinoid necrosis karyorrhexis 3. Cellular crescents 4. Hyaline thrombi, wire loops 5. Leukocyte infiltration	1. Glomerular sclerosis 2. Fibrous crescents
Tubulo-interstitial abnormalities	1. Mononuclear-cell infiltration	1. Interstitial fibrosis 2. Tubular atrophy

Table 1-8: Components of the NIH LN AI, CI, TIAI scores
Each feature is graded on a scale of 0, 1, 2, or 3 (absent, mild, moderate, and severe, respectively). Fibrinoid necrosis and cellular crescents are weighted by a factor of 2. Maximum score of activity index is 24, and of chronicity index is 12 [238].

Specific histological features associated with poor renal prognosis include large subendothelial deposits, necrotising glomerular lesions, and ≥50% of glomeruli being affected by cellular crescents [242-244]. Patients with purely mesangial proliferative lesions/immune deposits restricted to the mesangial regions are less likely to develop progressive renal failure. However, the co-existence of ≥1 sub-endothelial deposit may suggest the presence of more advanced disease predictive of progressive renal failure [238,243,245-248].

LN sub-class, therefore, plays an important role in predicting outcome and guiding treatment pathways [249], with more aggressive treatment reserved for severe proliferative glomerular pathology (see section 1.2.4). Patients presenting with low-moderate levels of proteinuria without acute renal failure present a clinical dilemma, as they could have underlying mesangial lupus (class II), mild isolated membranous lupus (class V), or a proliferative lesion with either mild activity or be in the early stages of a more active lesion (class III, IV); therefore, significantly different treatment strategies would be indicated depending on which histological sub-class is present (see section 1.2.4).

The NIH AI and CI, has generated much controversy, with some investigators concluding that these indices are better predictors than WHO LN class, with high AI scores in the initial or repeat renal biopsy predicting the development of progressive renal disease and mortality [238,243]. However, others have questioned the reproducibility and validity of these scores, following inter-observer evaluation studies [250,251]. A further point of note is that LN is frequently focal, therefore, larger tissue samples provide more accurate assessment of glomerular involvement. This can be problematic in children as it has been suggested that in order to adequately rule out a focal lesion, a biopsy should contain a minimum of 10 glomeruli for light microscopic analysis [252].

1.2.3 Limitations of the current tests used in LN diagnosis and monitoring

In view of the risks and difficulties in undertaking repeated renal biopsies in children, clinicians rely heavily upon the less invasive, routine clinical biomarkers, when monitoring patients and making a decision as to whether to undertake a renal biopsy.

Such routine biomarkers include: proteinuria, urine microscopy, serum creatinine, eGFR, and also immunological, haematological and inflammatory biomarkers (see section 1.1.3.2). These investigations present limitations both diagnostically and prognostically during ongoing monitoring and follow up, deserving recognition. Following an LN flare, proteinuria has been shown to take a significant period of time to normalise in adult SLE patients, making it difficult to differentiate whether ongoing proteinuria reflects the recovery phase after a flare, is due to irreversible renal damage, or continued LN activity [253]. Changes in serum creatinine tend to lag behind changes in GFR and are also influenced by the child's age, gender and height [254]. Of concern, there have been reports of 'clinically silent LN' in patients with biopsy-defined LN but no proteinuria, normal urinalysis and normal renal function [255]. Repeat biopsy of patients who are in clinical remission has shown approximately one third to have ongoing evidence of LN activity, and approximately 60% to display CI features [256,257], suggesting that improved monitoring of sub-clinical disease is required to prevent under-treatment. Normal complement and anti-dsDNA antibodies may provide reassurance that active LN is improbable, however, studies looking at the accuracy of these tests for differentiating between patients with active and inactive LN have shown conflicting results [258-260].

1.2.4 Treatment of LN

The aim of LN treatment is to achieve remission as soon as possible in order to prevent irreversible kidney damage. In the initial induction phase, potent immunosuppressants are given with the aim of inducing LN remission. Subsequent long-term maintenance therapy involves lower doses of immunosuppressant medication, with the aim of preventing further renal flares whilst minimising treatment related adverse events. Long-term renal prognosis is better if a satisfactory response to initial induction therapy has been achieved [261,262]. As discussed in section 1.2.2 above, proliferative (class III/IV) LN carries the highest risk of progression to end stage renal disease, therefore intensive immunosuppressive treatment is warranted. The main studies influencing current clinical practice in relation to induction and maintenance treatment for class III/IV LN are shown in Table 1-9 and Table 1-10, and also discussed in section 1.1.8.2 above.

Study	Participants	Treatment groups	Follow-up	Key findings
Houssiau et al [167] **Euro Lupus trial**	90 pts (76 White, 6 Asian, 8 Afro-Caribbean or Black) with class III-V LN	Comparing high or low dose CYC 6 x monthly doses of CYC (high or low) + 2 x quarterly CYC doses IVMP for 3 days followed by oral pred	41 months	No difference between high-dose and low-dose CYC groups: • Treatment failure, 20% versus 16% • renal remission 54% versus 71% Long term follow-up study found no difference in death, doubling of serum creatinine and renal failure after 10 yrs [263]
Ginzler et al [264]	140 pts (79 Black, 28 Hispanic, 24 White and 8 Asian) with class III–V LN	Pred + MMF vs. Pred + CYC	24 weeks	Pred + MMF had a better safety profile and was more effective than pred + CYC: • Complete remission 22.5% vs. 5.8% • Partial remission 29.6% vs. 24.6%
Appel et al [165] **ALMS phase I**	370 pts (147 White, 123 Asian and 100 other race) with class III–V LN	Pred + MMF vs. Pred + CYC	24 weeks	Largest trial to date • Similar response rates: 56.2% versus 53% • 40.6% more adverse events in the CYC group N.B. response rate significantly higher in MMF group in Black, Hispanic, 'other race' patients

Table 1-9: Key trials of induction treatment in class III-V LN
Pts = patients. CYC = cyclophosphamide. MMF = mycoplenolate mofetil. Pred = prednisolone.

Study	Participants	Maintenance treatment	Follow up	Key findings
Contreras et al [265]	59 pts (29 Hispanic, 27 Black and 3 White) with LN class III or IV who had received 6 months of CYC induction therapy	• Pred + MMF vs. • Pred + AZA vs. • Pred + quarterly CYC	29, 30 and 25 months respectively	• Mortality and renal-failure-free survival inferior with pred + CYC • Relapse-free survival better with pred + MMF than with pred + CYC
Houssiau et al [168] **MAINTAIN trial**	105 pts (83 White, 13 Black and 9 Asian) with class III-V LN (WHO) who had received IVMP then pred plus low-dose CYC induction treatment	• Pred + MMF vs. • Pred + AZA	48 months	• Time to renal or systemic flare did not differ between groups • Similar proportions of renal flares: 19% with MMF vs. 25% with AZA
Dooley et al [166] **ALMS phase II trial**	227 pts (99 white, 76 Asian, 23 black and 29 'other' ethnicity) with class III–V LN who had received any induction therapy	• Pred + MMF vs. • Pred + AZA	36 months	• MMF was superior to AZA with regard to time to treatment failure, time to renal flare and time to rescue therapy • Treatment failure rates 16.4% vs. 32.4% • Renal flare rates 12.9% vs. 23.4%

Table 1-10: Key trials of maintenance treatment in class III-V LN

CYC = cyclophosphamide. MMF = mycoplenolate mofetil. Pred = prednisolone. IVMP = intravenous methylprednisolone. AZA = azathioprine.

Membranous, type V LN features often co-inside with proliferative class III/IV features, in which case their treatment is as per class the III/IV LN treatment described above. Pure class V LN is uncommon, displaying proteinuria as the principle manifestation and a better renal prognosis than class III/IV LN [244,266]. In class V LN cases where normal kidney function is present and proteinuria is within the sub-nephrotic range, treatment includes use of an angiotensin converting enzyme inhibitor (ACEi), with immunosuppressive therapy indicated as per the extra-renal manifestations. When proteinuria is within the nephrotic range, it is associated with increased risk or renal deterioration [267], therefore, immunosuppressive induction and maintenance treatment are indicated as per class III/IV LN treatment protocols [156,268,269] or alternatively including prednisolone and a calcineurin inhibitor (cyclosporin or tacrolimus). Class I or II LN (with proteinuria of <3g/day) should be treated as per extra-renal clinical manifestations. In Class II LN with >3g proteinuria per day, glucocorticoids ± a calcineurin inhibitor are utilised. In Class VI LN chronic lesions are present, therefore the risks of aggressive immunosuppression may outweigh the benefits, and treatment is as per the extra-renal manifestations [162,163,268,270].

Three different published recommendations for treatment of LN in JSLE exist, from the Childhood Arthritis and Rheumatology Research Alliance (CARRA) [163], European League Against Rheumatism and European Renal Association–European Dialysis and Transplant Association (EULAR/ERA-EDTA) [162] and Kidney Disease Improving Global Outcomes (KDIGO) CARRA SLE committee [268]. These recommendations are summarised in Table 1-11. The role of biologics in LN treatment remains largely inconclusive as discussed in section 1.1.8.3 above, with such treatments mainly being used in patients who do not respond adequately to conventional treatment or as an adjunct to conventional treatment.

	Protocol summary		
CARRA SLE sub-committee	• Glucocorticoids (oral, IV, or mixed oral/IV) AND • CYC: 6 x monthly intravenous doses (initial dose 500mg/m^2, subsequent doses increased, not to exceed 1500mg monthly). Dose should be adjusted if renal insufficiency or low WCC OR • MMF: 600mg/m^2/dose, bd, maximum dose of 1500mg bd. A lower dose can be used at initiation of treatment, but the dose should be escalated to the target dose within 4 weeks		
KDIGO clinical practice guidelines	Class I LN	Treat as dictated by extra-renal clinical manifestations	
	Class II LN	• Proteinuria >1g/day: treat as dictated by extra-renal clinical manifestations of SLE &ACE inhibitor. • Proteinuria >3g/day: treat with glucocorticoids +/- calcineurin inhibitor	
	Class III & IV LN	Induction therapy: • Glucocorticoids plus CYC or MMF Maintenance therapy: • Azathioprine (1.5–2.5mg/kg/day) or	Note for induction therapy dosage: • MMF (1–2 g/day in divided doses) • Low-dose oral glucocorticoids (10mg/day prednisone)
	Class V LN	• Normal kidney function and non-nephrotic range proteinuria - treat as per extra-renal manifestations • Persistent nephrotic proteinuria - glucocorticoids plus CYC or MMF or AZA or a calcineurin inhibitor	
	Class VI LN	• Immunosuppressants as dictated by extra-renal manifestations	
	Non-responders*	• Consider rituximab, IVIG or calcineurin inhibitor	
EULAR / ERA-EDTA	Class IIIA, IVA or IIIA/C, IVA/C ±V	• IVMP: 3 pulses (500–750mg) followed by oral prednisolone (0.5mg/kg/day for 4 weeks, reducing to <10mg/day by 4–6 months) • MMF: 3g/day for 6 months or CYC: cumulative dose: 3g over 3 months	
	Class V LN + nephrotic range proteinuria	• Oral prednisolone (0.5 mg/kg/day) + MMF (3g/day for 6 months) • Alternative for non-responders: CYC or calcineurin inhibitor or rituximab • Patients without adverse prognostic factors: AZA (2mg/kg/day) Subsequent treatment when improving • Prednisolone (5–7.5mg/day) + MMF (dose 2g/day) or AZA (2mg/kg/day) Continue for at least 3 years. Gradual drug withdrawal, glucocorticoids first, can then be attempted.	

Table 1-11: Published recommendations for treatment of LN in JSLE

IV = intravenous. CYC = cyclophosphamide. WCC = white cell count. Bd = twice daily. MMF = mycoplenolate mofetil. Pred = prednisolone. IVMP = intravenous methylprednisolone.*Non-responders = patients who have failed >1 recommended initial regime. IVIG = intravenous immunoglobulin. A = active. C = chronic. Table adapted from reference [109]. CARRA SLE sub-committee guidelines detailed in reference [159]. KDIGO clinical practice guidelines in reference [264] EULAR / ERA-EDTA guideline in reference [158].

1.2.5 Renal survival in JSLE

LN is one of the major causes of morbidity in JSLE, and has also been reported to be the most important predictor of mortality [271]. In the 1950s, the reported mortality rate was > 50% at 5-years for patients with LN [272]. Since the introduction of high-dose corticosteroids and immunosuppressive agents for LN in the late 1980s/early 1990s, the 5-year renal survival rate is now 84%–94% [198-200,211,212,273-276]. Longer term renal outcomes have been reported by some of these studies, reporting 10-15 year renal survival rates of 75–91% [198,199,211,275]. Renal survival has been shown to vary according to renal biopsy class, with poorer renal survival in proliferative (class III/IV) LN versus mesangial (class II) or membranous LN (class V) [198-200,273-275,277,278]. Adult SLE studies have demonstrated that Black and Hispanic patients have poorer renal survival than Caucasian patients [13], with marked variation in renal survival between developed and developing countries [13,222,279] (see section 5.1.1). In the United States (US), 1.9% of all adult and 3% of paediatric renal transplants are attributed to LN, emphasising the higher burden and severity of the disease in childhood [280]. In children commencing renal replacement therapy for LN, the 5-year mortality rate is 22% [281].

1.3 Urine biomarkers of LN

1.3.1 Pursuit of novel biomarkers

It is evident from the summary of data presented above that the performance of conventional blood, urine, biopsy tests for the diagnosis and monitoring of LN remain inadequate (sections 1.2.2 and 1.2.3). LN therapy could be more effective, treatment toxicity limited, and renal outcomes improved if LN onset, severity, and treatment responsiveness could be predicted. To this end, novel, non-invasive biomarkers of disease activity and prognosis are increasingly being investigated. To translate an experimental biomarker into clinical practice, Mischak et al [282] describe a six step process to be considered when developing and evaluating novel biomarkers studies. This process includes: (1) initial biomarker identification/verification, (2) evaluation of the results by independent experts, (3) evaluation in a suitable bio-bank of existing samples or newly collected samples, (4) evaluation in a clinical trial, (5) implementation in clinical practice, and (6) proving the cost-effectiveness of the

validated biomarker. The majority of LN urine biomarker studies to date have focused upon step one of this process.

1.3.2 Why urine?

Urine is increasingly becoming recognised as the most useful and desirable medium for biomarker discovery due to the non-invasive nature of sample collection and its ability to reflect kidney damage, given its close proximity to native renal cells. Plasma and serum are more complex sample types, which are in contact with multiple organs and therefore less likely to yield kidney specific biomarkers. The smaller number of core proteins in urine compared to plasma/serum (approximately 2000 vs. 10,000), also make urine a better medium for harvesting biomarkers [283,284]. Efforts to identify urinary biomarkers to date have related to specific candidate biomarkers implicated in the pathogenesis of LN or hypothesis free biomarker screens (e.g. using proteomic techniques).

1.3.3 Why assess novel biomarkers in children as well as adults?

JSLE and adult-onset SLE differ in many ways (see section 1.1.3), with more renal involvement [1,28,40,42,285,286], greater risk of renal failure and irreversible renal damage being seen in JSLE [203,287]. Such differences in LN severity may therefore bear influence on the level of biomarkers reflecting the degree of renal injury. Urine biomarkers derived from plasma proteins may also show age dependent differences in their levels. Childhood specific biomarker discovery and validation studies are therefore important to explore such issues. Children generally have fewer co-morbidities (e.g. diabetes, cardiovascular disease or hypertension), which could have confounding effects on biomarker discovery. Consequently, it may be that a biomarker is useful in a paediatric but not an adult-onset SLE population, or vice versa. This may account for some of the differences in proteins identified from paediatric and adult SLE urine proteomic studies [288-290].

In the next section, developments in identifying robust urinary biomarkers for LN will be reviewed. Biomarkers that are of particular relevance to children or have gone through validation in independent patient cohorts will be highlighted, as such markers

will be of key importance to this thesis, defining individual or combinations of biomarkers meriting advancement towards clinical practice.

1.3.4 Urine biomarkers relating to LN pathophysiology

1.3.4.1 Inflammatory cells as urine biomarkers

In a recent study of 19 adult SLE patients, urinary T-cells (CD8+ and CD4+), B-cells and CD14+ macrophages were shown to significantly distinguish between patients with active LN and non-LN SLE patients [291]. The same group has previously shown that high urinary CD4+ T-cells numbers are observed in proliferative LN, and that over time, normalisation of urinary CD4+ T-cell count is associated with lower disease activity and better renal function. Persistence or an increase in urinary T-cell numbers is associated with higher total SLEDAI scores, and poorer renal outcomes in terms of serum creatinine and proteinuria [292]. These results have been confirmed by a further group who demonstrated urinary CD4+ and CD8+ T-cell counts to significantly discriminate between active and inactive LN [293], decreasing in response to treatment [291]. The relatively large volume of urine required limits the use of urinary immune cells as biomarkers in children (approximately 100mls as opposed to 20-200µl in immune-assays), where it can be difficult to obtain such urine volumes. The urine also needs to be fresh and analysed within six hours of voiding [291].

1.3.4.2 Cytokines, chemokines and their receptors as urine biomarkers

Cytokines, chemokines and their receptors play an important role in the pathogenesis of LN. This has led to a variety of studies testing whether they may be used as urine biomarkers for LN.

1.3.4.2.1 Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 is one of the most extensively investigated urine biomarkers in LN. It is a leucocyte chemotactic factor, which acts to recruit inflammatory cells to the kidney [294]. Increased expression of MCP-1 has been demonstrated by immunohistochemistry and in situ hybridisation in renal endothelial cells, epithelial cells and infiltrating mononuclear cells in LN [295]. There is also a correlation between glomerular expression of MCP-1 and severe histological classes of LN (class III/IV), correlating with poor renal prognosis [296]. A large number of cross-sectional

studies have consistently demonstrated that urinary MCP-1 levels are elevated in patients with active LN, as compared to inactive LN and/or healthy controls (HCs) [70,72,297-302]. MCP-1 is also elevated in other types of glomerulonephritis [303].

Watson et al [74], examined urinary MCP-1 levels longitudinally in 64 patients from the UK JSLE Cohort Study, demonstrating MCP-1 to be an independent predictor for active renal disease at the time of the current review, and over time low urinary MCP-1 levels were a good predictor of LN improvement (AUC: 0.81; *p=0.013*). In an adult longitudinal study, urinary MCP-1 levels were demonstrated to be significantly higher during renal as opposed to non-renal flares, increasing 2-4 months pre-LN flare. Patients who responded to treatment showed a decrease in urinary MCP-1 levels over several months, whereas non-responders displayed persistently elevated levels. MCP-1 levels were also demonstrated to be higher in patients with proliferative LN versus membranous LN [299]. A further longitudinal study of 40 adult patients supports these results, with urinary MCP-1 levels falling in responders, remaining elevated in non-responders and correlating with histological severity [277]. Of note, an adult SLE study has shown urinary MCP-1 levels to be no better than anti-dsDNA antibody or C3 at differentiating renal versus non-renal SLE flares [304] with a further study finding that MCP-1 was unable to predict LN flare [305]. MCP-1 antagonists have been shown to ameliorate LN in MRL-(Fas)lpr autoimmune prone mice [306], and to permit a 75% reduction in the dose of cyclophosphamide required to control proliferative LN [307], but are not currently under investigation in humans.

In summary, the ten urinary MCP-1 studies described above across adult and paediatric cross-sectional and longitudinal studies [70,74,277,296,298,299,301,302,308,309] provide evidence to support the role of MCP-1 as an important biomarker involved in LN pathogenesis and monitoring. Further investigation of MCP-1 is indicated, either alone or in combination with other biomarkers, as part of this current thesis.

1.3.4.2.2 Neutrophil gelatinase associated lipoclain (NGAL)

Urinary NGAL has also been widely investigated in LN. It is a member of the lipocalin family of carrier proteins that is expressed by many cell types including neutrophils and renal tubular epithelial cells. It is responsible for cellular iron transport, apoptosis, bacteriostasis and tissue differentiation, and is implicated in the growth and

differentiation of epithelial cells [310]. NGAL is constitutively expressed at low levels in the kidneys [311], and is up-regulated in response to acute renal injury in response to inflammation, ischemia and infection [312-316]. Brunner at al carried out the first paediatric urinary NGAL study in a cohort of 35 JSLE patients, reporting higher urinary NGAL levels in JSLE compared to Juvenile Idiopathic Arthritis (JIA) patients. NGAL levels were strongly correlated with renal SLEDAI defined disease activity, but not with extra-renal disease activity. Subgroup analysis showed NGAL levels to be highly sensitive and specific for identifying JSLE patients with biopsy-proven nephritis [317]. In a subsequent study, they found the combination of NGAL, MCP-1 and creatinine clearance to be good at identifying LN chronicity (AUC 0.83) [318]. An independent Egyptian cohort including 35 JSLE patients, has demonstrated urinary NGAL to correlate with the renal SLEDAI score and be predictive of class III and IV LN, with 91% sensitivity and 70% specificity respectively [319]. Pitashny et al demonstrated urinary NGAL levels to be significantly higher in adult LN than non-LN patients, with NGAL levels correlating with renal but not with extra-renal SLEDAI scores [320]. 24-hour urinary NGAL excretion has been shown to be a biomarker of renal damage in adult SLE, correlating with serum creatinine and creatinine clearance [321].

In longitudinal studies, urinary NGAL has been demonstrated to be predictive of LN flare [74,315,322]. Suzuki et al studied 85 JSLE, 30 JIA patients, and 50 HCs, showing plasma and urinary NGAL levels to be increased in patients compared to controls [322]. A subsequent paper reporting ongoing longitudinal follow-up (≥ 3 visits) of this cohort demonstrated a 104% increase in urinary NGAL up to 3 months before worsening of LN [69]. Watson et al [74], demonstrated urinary NGAL to be a good predictor of worsening LN activity (AUC 0.76; $p=0.04$) in 64 UK JSLE Cohort Study patients. Similarly, in a study including an exploratory adult SLE cohort (Einstein Lupus Cohort) and a validation cohort (University College London), elevated urinary NGAL levels were found to rise 3-6 months before an LN flare in both cohorts, performing better than anti-dsDNA antibody for prediction of LN flare [71]. In contrast to the above, a longitudinal study involving 107 adult SLE patients was unable to identify an association between urinary NGAL and any measure of LN disease activity [308].

The overall evidence for urinary NGAL in LN monitoring, both in paediatric and adult and cross-sectional studies [69,71,74,315,317-322] suggests that further investigation, both alone or in combination with other biomarkers, is required as part of this current thesis with the aim of potentially moving urinary NGAL from being measured in the laboratory towards it being useful in clinical translation.

1.3.4.2.3 Vascular cell adhesion molecule-1 (VCAM-1)

Urinary VCAM-1 has not been investigated in JSLE patients to date. It is an adhesion molecule and member of the immunoglobulin superfamily, which is expressed and released by endothelial cells and glomerular parietal epithelial cells within the renal tubular system, and has been observed within the kidneys in murine and human lupus [323-325]. VCAM-1 interacts with integrins, supporting tethering and adhesion of leukocytes to endothelial cells, and therefore promoting their migration into organs such as the kidney [324]. Wu et al initially demonstrated urinary VCAM-1 to correlate with urinary protein levels and disease activity scores in three murine lupus nephritis models. They extended this work to 38 SLE patients, 15 HCs and 6 rheumatoid arthritis (RA) patients. SLE patients displayed significantly higher urinary VCAM-1 levels than HCs, with LN patients exhibiting the highest VCAM-1 levels. Urinary VCAM-1 levels also correlated with UPCR and renal SLEDAI score. Serum levels of VCAM-1 were also found to be increased in both murine and human LN, however, urine levels were higher, suggesting additional local VCAM-1 production within the kidney in active LN [326].

Molad et al subsequently looked at 24 SLE patients and HCs, showing urinary VCAM-1 to be significantly increased in SLE, correlating with SLEDAI, low C3, creatinine clearance and albuminuria [327]. Abd-Elkareem et al studied 50 SLE patients (30 active LN, 20 non-LN) and found elevated urinary VCAM-1 in class III, IV and V LN but not in class I/II, or non LN SLE patients [328]. In a larger adult study (121 SLE patients, 33 with active LN), urinary VCAM-1 was demonstrated to be significantly increased in active LN [329]. More recently, Singh et al showed VCAM-1 to discriminate between active and inactive LN (AUC 0.92), correlating with the presence of class IV LN [297]. In a longitudinal study of 107 SLE patients followed for up to eight clinic visits, urinary VCAM-1 levels were shown to correlate with renal SLICC score, UPCR and physicians' global assessment, but not renal histology [308].

The above indications for VCAM-1s potential role in LN immunopathogenesis, combined with the significant evidence for VCAM-1s role as a urine biomarker in adults [297,308,326-329], collectively supports the need for assessment of VCAM-1 in a paediatric setting as part of the current thesis, along with other promising urine biomarkers with strong evidence supporting their potential use in clinical translation.

1.3.4.2.4 Tumor necrosis factor-like weak inducer of apoptosis (TWEAK)

Urinary TWEAK is a soluble cytokine that is mainly produced by leukocytes, but also by resident renal cells, leading to elevated TWEAK expression within the kidney and urine of adults with LN [330,331]. Expression of Fibroblast Growth Factor-inducible 14 (Fn14), the TWEAK signalling receptor, is rapidly unregulated in context of tissue injury, autoimmune/inflammatory diseases, and has been shown to be present on podocytes, endothelial, mesangial and tubular cells [331]. Blocking TWEAK/Fn14 interactions in LN mouse models leads to improvement in disease [332], with Fn14 deficiency ameliorating diseases phenotype [333].

In a cross-sectional study of 83 adult SLE patients, urinary TWEAK levels were shown to be significantly higher in patients with active LN than non-LN SLE patients, with higher TWEAK levels during renal flares, correlating with the renal SLEDAI score [334]. Longitudinal follow-up of a subgroup of these patients within a larger multicenter cohort, identified urinary TWEAK levels to be significantly higher during a renal flare than 4-6 months before/after. There was no association between TWEAK and LN histological classes [330]. Two subsequent adult cross-sectional studies looking at urinary TWEAK have demonstrated significantly higher TWEAK levels in LN compared to non-LN patients [298,335]. All urinary TWEAK studies to date have utilised an ELISA assay which is produced by a pharmaceutical company, Biogen Indec, limiting the ability of independent investigators to directly validate their findings in distinct cohorts. A collaboration with Biogen or alternative assay would be required for TWEAK to be assessed as part of the current study. This was explored as part of the development work for this thesis, but was not possible within the timeframe of this thesis.

1.3.4.2.5 Interleukin-6 (IL-6)

Two small adult SLE studies have suggested a role for IL-6 as a urinary biomarker for LN. The first included 29 patients with active LN and showed IL-6 levels to be higher in those with WHO class IV LN on biopsy than other classes [336]. A second study of 27 SLE patient and 17 HCs found urinary IL-6 to be higher in active LN, compared to inactive LN and HCs [337]. However, in a study of 143 SLE patients and 73 HCs, IL-6 was higher in SLE patients, but no difference seen in those with/without LN [338]. On balance, the strength of the evidence for IL-6 is not sufficient to support investigation in JSLE, over other urine biomarkers.

1.3.4.2.6 Other chemokines and their receptors

Interferon-γ-inducible protein 10 (IP-10) is a chemokine produced by monocytes, endothelial cells and fibroblasts in response to IFN-γ. IP-10 promotes migration of T-cells expressing the IP-10 receptor (Chemokine receptor 3, CXCR3) into the kidney [339]. Tubulo-interstitial IP-10 expression has been shown to decrease in serial biopsies where an LN patient changes from having proliferative nephritis to membranous nephropathy [340]. Avihingsanon et al [341], evaluated urinary mRNA levels of IP-10 and CXCR3, demonstrating significantly higher levels in class IV LN compared with classes II, III and V. A significant reduction in IP-10 and CXCR3 mRNA levels was seen in patients responding to treatment, whereas levels remained elevated in treatment resistant patients. Abujam et al [304], assessed urinary IP-10 and MCP-1 levels in the urine of 138 adult SLE patients, demonstrating a significant difference in the levels of both biomarkers between active LN, active non-renal SLE patients and HCs. In ROC analysis, MCP-1 out performed IP-10 in its ability to identify active LN (AUC 0.78 vs. 0.68 respectively). No difference in urinary supernatant IP-10 levels could be detected between active LN, inactive LN patients or HCs within the UK JSLE Cohort Study [72]. RANTES (regulated on activation, normal T cell expressed and secreted) is a chemokine which is involved in T cell recruitment to inflamed sites. In adult patients with diffuse proliferative LN, urinary RANTES has been shown to be significantly higher during episodes of flare [305].

No evidence relating to urinary CXCR3, or RANTES is available in JSLE. These markers could therefore be considered for further investigation but the strength of evidence supporting them is far less than for MCP-1, NGAL and VCAM-1.

1.3.5 Proteomic based urine biomarker analysis

The normal urinary proteome contains a small but discrete number of proteins. In pathological situations the urinary proteome can provide information regarding damage to all segments of the nephron, underscoring the physiological and biological processes taking place. Advancements in proteomics have provided considerable insight into LN urine biomarkers, and the section to follow will review the implications from paediatric and adult urine proteomic studies to date.

1.3.5.1 Adult proteomic studies

One of the earliest LN urinary proteomic studies by Mosley et al used surface-enhanced laser desorption/ionization time of flight (SELDI-TOF), identifying two proteins with masses of 3340 and 3980 which distinguished active from inactive LN, each with 92% sensitivity and specificity. This study featured a limited prospective longitudinal cohort of six patients, showing these proteins to predict relapse and response to treatment earlier than traditional clinical markers [342]. Using 2D-electrophoresis in 16 patients with LN, focal segmental glomerulosclerosis (FSGS), diabetic and membranous nephropathy, Varghese et al used patterns of protein abundance to train an artificial neural network to create a prediction algorithm for identification of each disease. In an external validation set of 16 patients, the artificial neural network was found to be best for LN identification with a sensitivity of 86%, specificity of 89% and AUC of 0.84. Mass spectrometry identified the proteins to include α1-acid-glycoprotein (AGP), transferrin (TF), zinc α-2-glycoprotein (ZA2G), α-1-macroglobulin (A1mG), α-1-antitrypsin, retinol binding protein (RBP), haptoglobin, complement factor B, transthyretin, hemopexin and albumin [288]. The same group undertook a complementary study using the same methodology as described above to examine 20 urine samples from different ISN/RPS LN classes. With different combinations of proteins, the sensitivity and specificity for ISN/RPS class identification was class II 100%, 100% respectively; III 86%, 100%; IV 100%, 92%; and V 92%, 50%. The 50% class V LN specificity was due to inclusion of patients with mixed class III, IV, V disease. These patients were correctly identified as having class III or IV disease which is more clinically appropriate. The two proteins contributing most to the tests sensitivity were AGP and A1mG [343].

Zhang et al subsequently screened the low molecular weight proteome (<20kDa) in samples taken at baseline, pre-flare, flare, and post-flare by fractionating the urine to remove proteins larger than 30 kDa, and then spotting them onto weak cation exchanger protein chips for analysis by SELDI-TOF MS. 19 patients were investigated with class III (n=5), class IV (n=11), or class V LN (n=3). 27 proteins were found to be differentially expressed between flare intervals. A 20 amino acid isoform of Hepcidin and an albumin fragment (N-terminal region) were found to be increased 4 months pre-flare, returning to baseline at the time of renal flare, whereas a 25 amino acid isoform of Hepcidin decreased during a renal flare and returned to baseline 4 months post-flare (marker of treatment response). They also found α-1-antitrypsin to be increased at the time of flare [344].

Somparn et al undertook 2D electrophoresis on samples from 5 active and 5 inactive LN patients, revealing 16 protein spots whose levels differed significantly between the groups (serotransferrin, AGP, alpha-2-HS glycoprotein, haptoglobin, alpha-1-antitrypsin, albumin, ZA2G, immunoglobulin kappa chain (3 forms), RBP-4, beta-2-microglobulin (β2MG), transthyretin and prostaglandin-H2-D isomerase (PGDS)). They chose to validate ZA2G (due to the magnitude of change) and PGDS (only present in active LN) by ELISA, in 30 active LN, 26 inactive LN, 14 non-LN glomerular diseases and 8 HCs. Urinary ZA2G levels significantly differentiated patients with active and inactive LN but were also elevated in patients with non-LN glomerular diseases. Urinary PGDS was only significantly elevated in active LN [289].

1.3.5.2 Paediatric proteomic studies

Only two paediatric urinary proteomic studies have been carried out to date, both by Suzuki et al in US JSLE patients [290,345]. In the first, SELDI-TOF-MS was used in 32 JSLE and 11 JIA patients. A urinary proteomic signature was identified, consisting of eight proteins which displayed significantly higher peak intensities in patients with LN as compared to JIA/inactive LN patients. These peaks had mass-to-charge ratios of 2.76, 22, 23, 44, 56, 79, 100, and 133. There was no significant difference in biomarker peak intensities between distinct WHO LN classes. The 22, 23, 44, 79, and 100 kDa peaks were strongly correlated with SLEDAI-2K defined renal disease activity [345]. In the second study, surface-enhanced matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) identified TF, ceruloplasmin (CP), AGP, lipocalin-type prostaglandin-D synthetase (LPGDS), albumin and albumin-related fragments as the proteins contained within the above urinary proteomic signature. Immunonephelometry and ELISA were then used to assess the ability of this protein signature to monitor LN disease activity over time (98 JSLE, 30 JIA patients). All proteins were significantly higher in JSLE than JIA. Individual biomarkers displayed fair to good ability to identify SLEDAI or BILAG defined active LN (AUC values between 0.68-0.81), with the AUC improving to 0.85 when all biomarkers were considered together. Urinary TF, AGP and LPGDS were significantly increased 3 months before a SLEDAI based diagnosis of LN. However, significant increases in AGP and LPGDS over time were also demonstrated in patients with stable active LN, improved LN (AGP) and inactive LN (LPGDS) [290], highlighting the need for further prospective studies to improve understanding of the relationship of these biomarkers to disease activity over time. There is clearly overlap between some but not all biomarkers identified in adult/paediatric studies, highlighting the importance of undertaking biomarker studies in both patient groups rather than extrapolating between them.

1.3.5.3 Validation of proteomic results

The validity of biomarkers identified through proteomics must be re-assessed in separate patient groups to provide independent verification of the findings and evaluate biomarker sensitivity, specificity and predictive capabilities. This may either take the form of an independent proteomic study or through use of other protein binding techniques (e.g. ELISA or multiplex), to quantify the biomarkers in a larger group of patients. Such techniques are quicker, highly sensitive, specific for the marker of interest and readily available within many laboratories. Table 1-12 lists the proteins identified in LN proteomic studies that have undergone internal or external validation using additional methods for protein quantification ± further proteomic techniques.

Protein	Original proteomic study	Validation Studies	
		Proteomic	Other methods/techniques
Biomarkers identified in ≥2 proteomic studies and validated using other methods			
AGP	**Oates** [343]	**Varghese** [288] **Suzuki** [345]	**Suzuki [290]**, 98 JSLE, ELISAs, serially assessed AGP, TF, CP, LPGDS. AUC of 0.85 for active LN (SLEDAI/BILAG defined) identification when all biomarkers combined. **Brunner** [318], 76 SLE pts, ELISAs. AGP+MCP1+CP+UPCR = good for detecting LN histological activity (AUC 0.85). AGP+MCP-1+TF+CrCl+C4 = fair for membranous LN (AUC 0.75). **Watson** [72], 60 JSLE pts, ELISA. Urinary AGP significantly ↑ in active LN.
TF	**Varghese** [288]	**Suzuki** [345]	**Suzuki** [290] – as described above **Brunner** [318] – as described above
PGDS or LPGDS	**Suzuki** [345]	**Somparn** [289]	**Suzuki** [290] – as described above **Somparn** [289], ELISA, 30 active LN, 26 inactive LN, 14 non-LN kidney disease, 8 HCs, PDGS significantly ↑ in active LN. **Gupta** [346] 28 SLE with active LN, 6 inactive SLE, 12 active non-renal SLE, 19 HCs. ELISA. Significantly ↑ PGDS in active LN. Levels ↓ in those responding to treatment, remained ↑ in chronic kidney disease.
ZA2G	**Varghese** [288]	**Somparn** [289]	**Somparn** [289], ELISA, 30 active LN, 26 inactive, 14 non-LN kidney disease. ZA2G levels ↑ in active LN.
RBP-4	**Varghese** [288]	**Somparn** [289]	**Sesso** [77], 70 SLE pts, immunoenzymometric assay. RBP-4 significantly higher in active LN. **Marks** [347], 21 JSLE pts, 10 active LN, 11 inactive, ELISA. RBP significantly ↑ in active LN.
Biomarkers identified in a single proteomic study and validated using another technique			
Hemo-pexin	**Varghese** [288]	NA	**Brunner** [348], 47 active LN JSLE pts, ELISA. NIH AI predicted by NGAL, MCP-1, CP, adiponectin and KIM-1, Hemopexin in combination.
Hep-cidin	**Zhang** [344]	NA	**Mohammed** [349], 30 active LN, 30 non-LN SLE pts, 30 HCs, ELISA. Hepcidin ↑ in active LN.
K-FLC	**Somparn** [289]	NA	**Hanaoka** [350], 43 SLE pts, Nephrometric assay. Both κ/λ FLCs ↑ in class III/IV LN vs. I/II/IV.
β2MG	**Somparn** [289]	NA	**Tsai** [337], 15 active LN, 12 inactive LN, ELISA. Levels did not differ between groups. **Choe** [351], 64 SLE, ELISA, significant ↑ in active LN.
CP	**Suzuki [290]**	NA	**Suzuki** [290] - as described above. **Brunner** [318] - as described above. **Brunner** [348] - as described above

Table 1-12: Proteins identified in LN proteomic studies that have undergone validation using additional methods for protein quantification ± further proteomic techniques.

Pts = patient. AGP = α-1 acid glycoprotein. TF = transferrin. CP = ceruloplasmin, MCP-1 = monocyte chemoattractant protein. CrCl = Creatinine Clearance. ZA2G = Zinc α-2-glycoprotein. HCs = healthy controls. RBP= plasma retinol binding protein. NIH AI = National Institute for Health Activity Index. PGDS = Prostaglandin-H2-D isomerase, alternative name LPGDS = Lipocalin-type prostaglandin-D synthetase. Hepcidin = 20 & 25 amino acid isoforms. K-FLC= κ free light chain. β2MG = Beta-2-microglobulin

Figure 1-4 provides an overview of the evidence relating to protein urine biomarkers to date. It demonstrates how both urine biomarkers relating to LN pathophysiology (described in section 1.3.4) and those identified in proteomic studies (section 1.3.5) integrate and relate to the different clinical scenarios faced during the LN disease course.

Novel urinary biomarkers with the strongest evidence in a JSLE setting are emphasised within Figure 1-4 and include NGAL + TF as potential early predictors of flare and NGAL + AGP + TF + CP + LPGDS for identification of active LN. In the evaluation of flare severity (association with ISN/RPS class or NIH activity index), there is evidence for NGAL + AGP + MCP-1 + TF + CP + Adiponectin + KIM-1 and Hematopexin. MCP-1 use has been demonstrated for identification of non-responders and NGAL, MCP-1, NGAL for detection of chronicity.

This evidence synthesis highlights that by serially measuring a panel of the most promising biomarkers (AGP, TF, CP, LPGDS, MCP-1 and NGAL), it may be possible to identify distinct stages of the LN disease course depending on biomarker levels/combinations.

As mentioned in section 1.3.4.2.3 there is also strong evidence for urinary VCAM-1 in adult SLE, in relation to identification of an active LN flare, evaluation of flare severity and identification of chronicity, suggesting that this also warrants further evaluation.

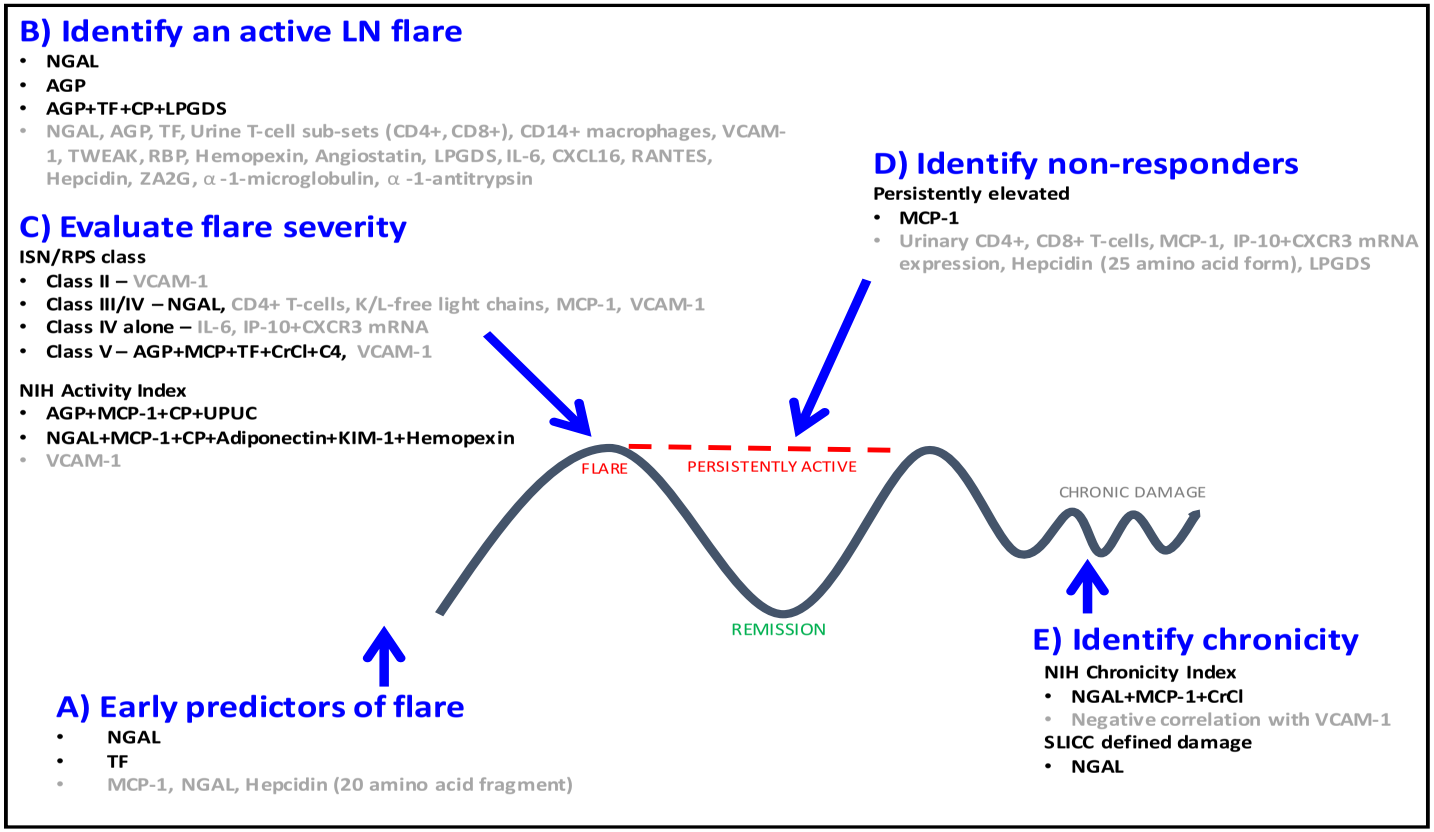


Figure 1-4: Overview of the evidence relating to urine biomarkers for LN monitoring to date.

Markers which are A) early predictors of flare, B) able to identify an active LN flare, C) used to evaluate flare severity, D) for identification of treatment non-responders, E) to identify chronic kidney damage. Urinary biomarkers shown in black are from paediatric studies, those in grey are from adult studies. NGAL = neutrophil gelatinase associated lipocalin. AGP = α-1 acid glycoprotein. TF = transferrin. CP = ceruloplasmin. LPGDS = Lipocalin- type prostaglandin-D synthetase. MCP1 = monocyte chemoattractant protein. VCAM-1 = vascular cell adhesion molecule 1. TWEAK = tumor necrosis like weak inducer of apoptosis. RBP= plasma retinol binding protein. Il-6 = interleukin 6. RANTES = Regulated on Activation, Normal T Expressed and Secreted, ZA2G = Zinc α-2-glycoprotein. ISN/RPS = International Society of Nephrology/Renal Pathology Society classification. CrCl = Creatinine Clearance. K/L-FLC= κappa/lamda free light chains. IP-10 = Interferon gamma-induced protein 10. CXCR3 = Chemokine (C-X-C Motif) Receptor 3. NIH = national institute for health. UPCR = urine protein/creatinine ratio. KIM-1 = kidney injury molecule 1.

1.4 Summary and key next steps for developing a panel of LN urine biomarkers for use in a future clinical trial

This extensive review of JSLE, LN and urine biomarkers for LN has demonstrated that JSLE is very complex, with an unpredictable disease course and potential for significant morbidity and mortality related to the disease itself and its treatment. JSLE is more severe than adult SLE with a greater burden relating to renal disease, which worryingly may translate into end stage renal disease requiring dialysis or transplant within childhood or early adult life. Much of the literature presented in this chapter relates to adult onset SLE. With 10 years of clinical data and an existing biobank of urine samples collected as part of the national UK JSLE Cohort Study, a unique opportunity is presented to explore clinical, demographic, and urinary biomarker related factors which either have not been explored in JSLE to date or require additional validation to elucidate or substantiate their role in the stratification of paediatric LN patients.

Previous work, both at the University of Liverpool (UoL) and within the literature has highlighted many promising urine biomarkers for LN in JSLE, providing insights into the pathophysiology and monitoring of LN. To date, no individual urine biomarker has achieved an 'excellent' predictive ability for identifying active LN on its own, but the most promising urine biomarkers in children have been shown to include MCP-1, NGAL, AGP, CP, LPGDS, TF, with strong adult SLE evidence for VCAM-1 (see section 1.3.4.2.3 and Figure 1-4). The more limited evidence for other cytokines, chemokines and their receptors (e.g. TWEAK, IL-6, IP-10, CXCL3) is presented above, but these markers do not stand out as having sufficient evidence for being advanced towards clinical translation.

Going forward, it is therefore important that the most promising urinary biomarkers are further validated to test the hypothesis that a combined urine biomarker panel will improve LN diagnostic accuracy and monitoring during the disease course. To enable any discoveries resulting from this work to move towards clinical translation, it is crucially important that adequate numbers of patients are available. Given the rarity of JSLE and the significant differences seen in disease phenotype and clinical outcomes

according to ethnic origin, it would appear prudent to seek international collaboration to ensure that adequate patient numbers are available, and to validate any promising results across different patient cohorts, in order to obtain robust conclusions deserving clinical translation.

Measurement of a biomarker panel in contrast to a single biomarker, does however introduce complexity into the methods required for biomarker quantification, also increasing the cost, sample volume, and time required for biomarker quantification per patient. Most existing point of care devices (e.g. pregnancy tests) are only able to quantify a single marker. It is therefore of key importance to balance accuracy with practicality and consider how such a test could be undertaken within a clinical trial setting, where a short turnaround time would be required for urine biomarker results. Such a trial is out with the scope of this thesis, but would represent the next step in determining whether urine biomarker-led monitoring actually improves renal outcome.

Having determined a range of key biomarkers which are found within the urine of patients with active LN, key questions present themselves regarding whether such biomarkers originate from the kidneys themselves, are passively filtered through the glomerulus, or are implicated in the pathogenesis of LN themselves. Working alongside cell biologists based within the UoL UK's Experimental Arthritis Treatment Centre (EATC) for Children (https://www.liverpool.ac.uk/translational-medicine/research/eatc/), this study also presents notable opportunities for back translation and investigation of the role of such biomarkers *in vitro*, as part of the current thesis, and in collaboration with other members of the EATC team.

1.5 Overarching hypothesis

As a result of the detailed review of evidence presented and summarised above, the overarching hypothesis of this thesis is that:

A combination of traditional clinical and promising non-invasive novel urinary biomarkers, as part of a 'LN biomarker panel', are better than traditional biomarkers alone at differentiating JSLE patients with active LN from those without, and in predicting fluctuations in LN disease activity over time, within ethnically distinct JSLE cohorts.

1.6 Overarching aims

1. To select biomarkers warranting further assessment as part of a potential 'LN biomarker panel' from both:
    a. A detailed review of the existing literature in the field (see above).
    b. Assessment of potential additional candidate biomarkers using commercially available kidney toxicity urine biomarker multiplex assays.

2. To use urine samples from UK JSLE Cohort Study patients to cross-sectionally assess if combining novel biomarkers can improve identification of active LN; and to assess whether addition of traditional JSLE disease activity data to a urine biomarker panel could help to improve active LN identification still further.

3. To assess if the developed UK 'optimal LN urinary biomarker panel' performs comparably within ethnically distinct JSLE patient cohorts from other countries.

4. To determine longitudinally, if the 'optimal LN urinary biomarker panel' as a whole, or individual constituent members of it, are able to predict LN flare and remission in advance.

5. To develop an appropriate assay platform for quantification of the validated 'LN urinary biomarker panel' in order to help streamline the process of urine biomarker panel quantification.

6. To investigate whether constituents of the ‘LN urinary biomarker panel’ are implicated in the pathogenesis on LN through investigation of an immortalised human podocytes cell line *in vitro*.

2 Methodology

2.1 Patient cohorts

2.1.1 The UK JSLE Cohort Study

In 2006, a multidisciplinary group of leading paediatric rheumatologists, nephrologists, adult rheumatologists, dermatologists and scientists from across the UK formed the 'UK JSLE Study Group'. Its members represented all major paediatric rheumatology centres across the UK, aiming to develop a comprehensive research programme to investigate the *'clinical characteristics and immunopathology of JSLE'*. The Study Group set up the national UK JSLE Cohort Study and Repository, running from the national coordinating centre within the Institute in the Park, UoL at Alder Hey Children's NHS Foundation Trust Hospital, with invited participation of all major paediatric rheumatology centres across the UK (see https://www.liverpool.ac.uk/translational-medicine/research/ukjsle/about/). The Study, which is ongoing, collects real world clinical data during clinic appointments and hospital admissions, as well as biological samples such as blood, urine and genetic material. The Study has national and local ethical approval and is supported by the National Institute for Health Research (NIHR) Clinical Research Network – Children (see https://www.crn.nihr.ac.uk/children/). The Study collects data from patients with definite or evolving/probable JSLE from 25 centres across the UK. The clinical data and bio-banked specimens collected as part of this study facilitate a wide range of research studies within Liverpool, nationally and internationally.

2.1.1.1 Inclusion and exclusion criteria

All children seen in UK paediatric rheumatology and nephrology centres across the UK are eligible for inclusion in the UK JSLE Cohort Study if they satisfy the following two criteria:

- They meet at least four of the twelve revised ACR diagnostic criteria for SLE [33] (see Appendix 1)
- Are aged <17 years at the time of presentation

Children with "probable" or "evolving" JSLE, defined as fulfilling two or three ACR criteria, and yet who in the opinion of the consultant paediatric rheumatologist may

well develop clinical features fulfilling ACR criteria of JSLE in time, may also be invited to participate in the study. Patients who have transferred to adult rheumatology care can continue to be followed in centres where the required study support is available. Patients are excluded from the study if they/their carer withhold consent or if they withdraw from the cohort study.

2.1.1.2 Patient recruitment

Parents, children and adolescents are provided with appropriate information sheets and the study is explained by their consultant or an appropriately trained professional such as research nurse. The family is given as much time as they need to decide whether or not they wish to participate, and reassured that they are free to withdraw at any time without giving an explanation, and that withdrawal will not in any way affect future management. Once the signed consent has been obtained, a study number is assigned. Written assent/consent is obtained from all parents, carers, patients as appropriate. An example of a participant information sheet and consent form is shown in Appendices 9 and 10. The study has full ethical approvals in place from the National Research Ethics Service North West, Liverpool East (REC reference 06/Q1502/77).

2.1.1.3 Clinical data and disease activity scores

The data collected for the purposes of the UK JSLE Cohort Study and Repository is the same as that which is collected routinely as part of routine good clinical practice. Standardised data collection forms have been developed to facilitate collection of these data across all units. Different data collection forms are collected at the time of initial presentation (baseline), at each clinic/hospital visit (approximately 3-4 monthly and during disease flares) and annually, as detailed below:

- *Baseline data collection*
    - Comprehensive demographic data (see Appendix 11)
    - ACR and new SLICC diagnostic criteria (see Appendix 1)
    - Paediatric British Isles Lupus Assessment Group (pBILAG2004) disease activity score form (see Appendix 5) – including additional information on medications, standard laboratory markers of JSLE disease activity and parental/physician's global assessment of disease activity. pBILAG2004 referred to as the BILAG throughout this thesis.

- Annual review form including a comprehensive review of blood results, potential ophthalmology reviews, DEXA scans, renal biopsies, documentation of significant infections, pubertal status and SLICC/ACR damage index review (see Appendix 12).
- CHAQ and CHQ/SF36 patient reported measures of disease status (see Appendices 2-4).

- *Clinic/hospital visits*
  - BILAG form
  - CHAQ and CHQ/SF36
- *Annual visit*
  - Update to ACR/SLICC diagnostic criteria
  - Annual review form including the SLICC/ACR damage index
  - CHAQ and CHQ/SF36

The pBILAG2004 disease activity score has been adapted from the original adult BILAG score [54] to include parameters of relevance to paediatric patients (e.g. normal blood pressure definitions), and has undergone preliminary validation in a UK paediatric cohort [63]. All BILAG data are collated anonymously at the study coordinating centre and stored in an electronic database, programmed to calculate BILAG disease activity scores [28]. For each patient visit organ specific BILAG scores are calculated (A–E) for eight organ domains (renal, constitutional, mucocutaneous, musculoskeletal, cardiorespiratory, gastrointestinal, neurological, haematological). Alphabetical scores correspond to the following degrees of JSLE disease activity; A - severe disease activity; B - moderate disease activity; C - stable mild disease activity; D - inactive disease, but previous organ involvement; E - no organ involvement ever (see section 1.1.4.1 and Appendix 6).

2.1.1.4 Urine sample collection and definition of active LN

A subset of the children participating in the UK JSLE Cohort Study [28] were recruited to the 'renal biomarkers in JSLE' sub-section of the UK JSLE Cohort Study, including all patients from Alder Hey Children's NHS Foundation Trust, Liverpool, and Great Ormond Street NHS Hospital for Children, London, UK. The urine samples included in the studies undertaken and presented in this thesis were collected during routine clinical care together with the clinical data and disease activity scores detailed above.

The UK JSLE Cohort Study has a material transfer agreement (MTA) to allow the collection and transfer of patient samples to the UoL (see Appendix 13).

The composite renal domain of the BILAG score is calculated from six specific items. These are: renal function (deterioration, based on serum creatinine and GFR); proteinuria (defined by urine dipstick, UPCR/UACR, or 24-hour protein levels); presence of nephrotic syndrome; active urinary sediment; severe hypertension; and histological evidence of active LN in the previous three months [54,63]. Different cut-offs for these clinical investigations correspond to the different renal BILAG disease activity scores (see Appendix 6) [54].

In light of the literature presented in section 1.1.4.2, LN activity was defined as follows for the purpose of the cross-sectional urine biomarker studies (in sections 3.5.5, 4.5 and 5.5.1):

- Active LN – if patients had a renal BILAG of A or B and previous histological confirmation of LN.
- Inactive LN - if they had a renal BILAG score of D or E.

Patient episodes where a renal BILAG of C was scored were not included in cross-sectional analyses. This was because these studies sought to identify biomarkers differentiating between the binary outcome of active versus inactive LN. Renal BILAG C patient episodes with mild or improving renal disease were therefore excluded.

2.1.2 US Einstein Lupus Cohort

The Einstein Lupus Cohort (referred to as the 'US Cohort' throughout this thesis) was established in 2002 by Professor Chaim Putterman at Montefiori Medical Centre, Bronx, New York, USA in association with the Albert Einstein College of Medicine (AECOM), initially collecting clinical data and urine samples from adult SLE patients only. Each sample was linked to an electronic database containing clinical information, disease activity measures (BILAG, SLICC, SLEDAI), laboratory parameters, demographic and medication information. In 2009, a paediatric sub-cohort was added collecting urine samples and clinical data from JSLE patients attending the Children's Hospital at Montefiore Medical Centre [334]. Samples were collected using comparable standard operating procedures (SOPs) to the UK JSLE Cohort Study, and

anonymised coded samples were stored at -80^{o}C without the addition of any additives (see section 2.2.5). Eligible patients were diagnosed with JSLE prior to 16 years of age and met ≥4 of the revised ACR SLE classification criteria [230]. Full ethical approvals were in place (Institutional Review Board at Einstein-Montefiore, IRB 2000-154).

A collaboration was established with Professor Chaim Putterman and Associate Professor Beatrice Goilav on behalf of the US Cohort as part of the current study. The development of this collaboration was led by Dr Eve Smith with support from Professor Michael Beresford. MTAs were negotiated with the support of the legal departments at AECOM and the UoL over a number of months in view of differences in US, UK and international laws/regulations and expectations regarding attribution of intellectual property. The agreed MTAs are shown in Appendix 14. A representative urine sample was selected from each patient recruited to the US Cohort. Where possible a sample was chosen during an active LN episode with a subsequent follow-up sample where possible for inclusion within the longitudinal study. For some patients only one urine sample was available. Urine microscopy and culture was not routinely carried out on US Cohort samples. However, samples showing urine dipstick signs of urinary tract infection (UTI) were excluded from the current study (presence of nitrate and leucocytes). Multiple aliquots of each urine sample were transferred on dry ice using the World Courier transfer service and arrived at the UoL frozen and in good condition. Anonymised, coded clinical data were also transferred.

2.1.3 University of Cape Town Lupus Cohort

The Paediatric Lupus Erythematosus in SA Cohort Study (referred to as the SA Cohort throughout this thesis) was established in 2013 by Professor Chris Scott (Department of Paediatric Rheumatology, University of Cape Town) and Dr Laura Lewandowski (Paediatric Rheumatology and International Child Health Fellow, Duke University, North Carolina, USA). It collects retrospective and prospective clinical data from JSLE patients attending the Red Cross Memorial and Groote Schuur Hospitals, Cape Town, South Africa. In 2015 Dr Smith and Professor Chris Scott submitted an ethics amendment, facilitating prospective collection of urine samples from JSLE patients during routine clinical care together with detailed demographic data, self-reported ethnicity data, clinical laboratory results and medication information (approval granted

HREC No 424-2013, see Appendix 15). Eligible patients were diagnosed with JSLE prior to 19 years of age and met ≥4 of the revised ACR SLE classification criteria. Patients were excluded if they had other diseases which might explain their ACR criteria (e.g. active tuberculosis, or HIV) or if their urine samples displayed urine dipstick signs of UTI (as above). Approval was also obtained to recruit HCs in the urine biomarker arm of the study. They were identified from elective theatre lists/out-patient clinics. These patients were also <19 years of age. A HC case report form (see Appendix 16) was developed to screen for the presence of inflammatory/connective tissue/kidney diseases.

A research nurse was employed for two days per week (between Feb 2015-16) to supplement the existing research support for the SA Cohort (funded through a MRC Confidence in Concept (CiC) grant awarded to Professor Michael Beresford and Dr Eve Smith, see section 6.1.3). The research nurse was responsible for consenting patients to the urine biomarker arm of the study, collecting the necessary clinical data to calculate the renal BILAG score, recruitment of HC patients, and transfer of samples from the clinic to the laboratory for processing and storage. Urine samples were processed using the same SOP as the UK samples, and stored at -80^{o}C without the addition of any additives (see section 2.2.5). Face to face meetings in Cape Town at the time of study set-up, and subsequent regular videoconferences between Dr Eve Smith, Professor Chris Scott and Research Sister Angel Putti were invaluable for troubleshooting, agreeing and monitoring study timelines.

The consent process differed for the SA Cohort as a large percentage of patients presented to clinic with a family member other than their parent or guardian. The parents of these patients were contacted by phone for consent, using a standardised script. A witness unrelated to the study team was present during the phone consent process and signed the consent form in the "witness" signature line. A copy of the consent forms "signed" via phone consent were either given to the caregiver to take home or a copy was provided at the next patient visit**.**

A MTA was negotiated with the support of the legal departments at the University of Cape Town and the UoL to facilitate transfer of the urine samples and anonymised coded clinical data. The agreed MTA is shown in Appendix 17. Multiple aliquots of

each urine sample were transferred using the same procedures as for the US samples. The number of samples per patient varied depending on when they were recruited to the study. Similar to the US Cohort, an active LN episode sample was chosen for inclusion in the cross-sectional analysis where possible, with the remaining samples being included in the longitudinal analysis.

2.2 Laboratory methods

A series of standard laboratory methods were used throughout this thesis, including the use of ELISA and multiplex bead assays, therefore the approach to these techniques and their optimisation is described here, with further details in relevant chapters. Techniques associated with urine/blood processing and storage, and podocyte culture are also detailed here, with individual experimental conditions discussed in the associated chapters.

2.2.1 Enzyme linked immunosorbent assay (ELISA) overview

ELISA is a common laboratory technique for quantifying the amount of analyte in a solution/sample. ELISA assays result in a coloured end product which correlates with the amount of analyte present in the original sample. The ELISA procedure begins with a coating step where the capture antibody is adsorbed onto a polystyrene 96 well plate. The liquid is removed and the plate washed. Several washes are performed (between 2-5 depending on the assay) to remove unbound materials and prevent them from interfering with the next step of the assay. During the wash process, it is important that excess wash buffer is removed to prevent dilution of the reagents included in the subsequent step. The plate should be blotted against clean paper towels to ensure complete removal of the liquid. Plate washers can be used to improve consistency across the plate. This is followed by a blocking step where an unrelated protein based solution is used to cover all unbound sites on the plate to prevent non-specific binding. The block is often left in place for 1 hour, and then removed and washed as above. When an 'ELISA kit' is used (e.g. for AGP, MCP-1, LPGDS, TF and CP in this thesis), these two steps are performed by the assay manufacturers.

The next step is the addition of the standards, samples and controls which are incubated at room temperature for 1-2 hours and then washed. The detection antibody is then

added (incubated from 30 minutes to 2 hours at room temperature depending on the assay), binding specifically to the target analyte. The detection antibody can either be enzyme conjugated (direct ELISA) or biotinylated, requiring the addition of an enzyme linked secondary antibody (e.g. with streptavidin horseradish peroxidase, strep-HRP) for 20-30 minutes (in-direct ELISA). The plate is washed after each of the steps described above. Both direct and in-direct ELISA assays then require a substrate solution to be added to the plate. This can come in a stabilized premixed form (e.g. Tetramethyl-benzidine and hydrogen peroxidase mixed together) or as two separate solutions which have to be mixed immediately prior to use (colour reagent A, stabilised hydrogen peroxidase + colour reagent B, stabilised chromogen, tetramethyl-benzidine). The plate is placed in the dark at room temperature, and the enzyme-substrate reaction leads to a colour change within each well (from clear to blue). Manufacturer's suggest an incubation time (usually between 10-30 minutes) but it is very important to monitor the plate during this step during the first run of the assay, as over development of the high standard points will affect the range of the standard curve. It is important that the same incubation time is used between assays where more than one plate is performed serially. An acidic stop solution is then added to the plate (e.g. sulphuric or hydrochloric acid) terminating the enzymatic reaction. The colour in the wells should change from blue to yellow. If the colour in the wells is green or does not appear uniform the plate was tapped gently to ensure thorough mixing with the acid. An overview of the ELISA assay procedure is shown in Figure 2-1.

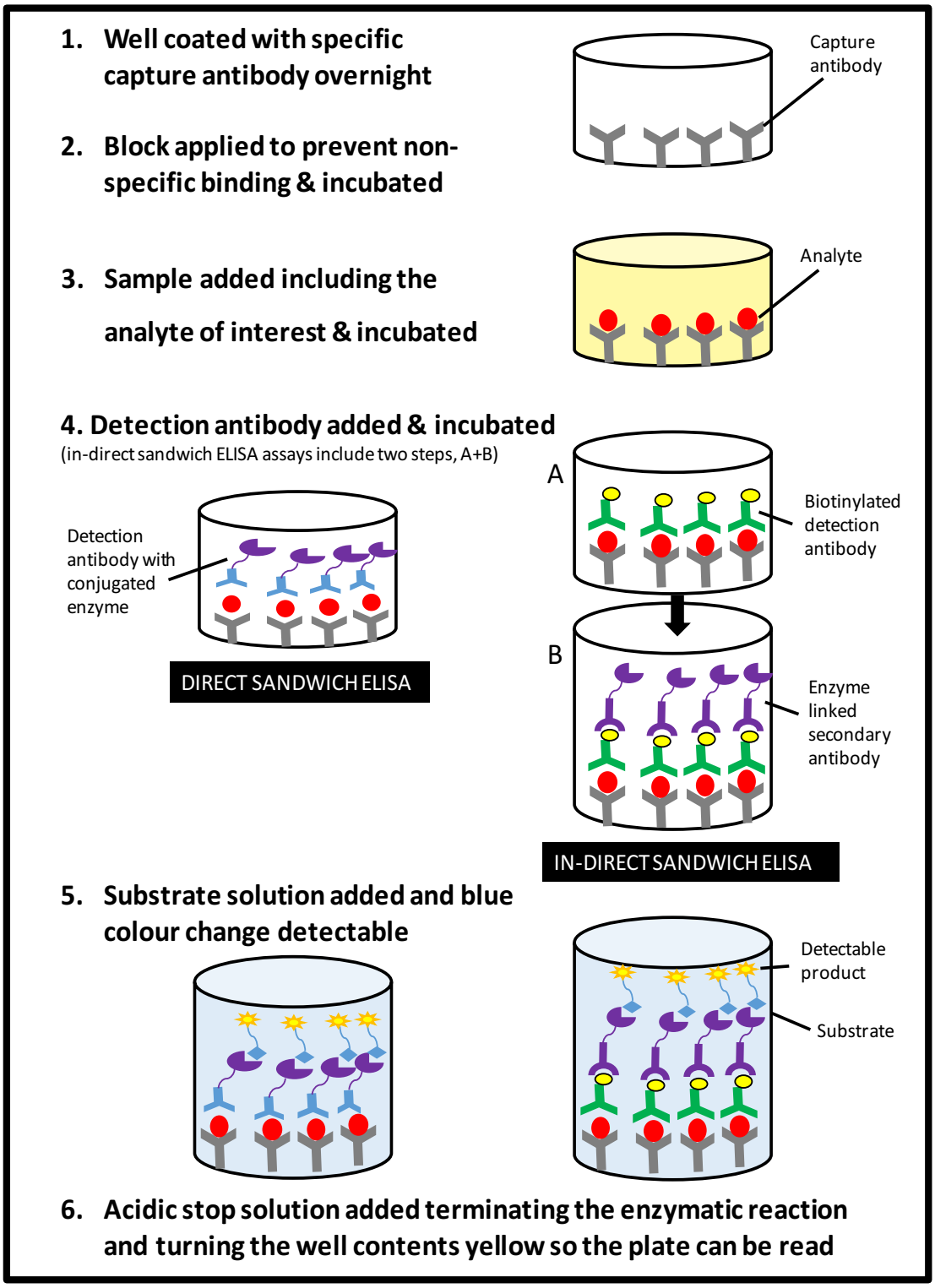


Figure 2-1: Direct and in-direct sandwich ELISA overview

The optical density (OD) of each well was then determined using a BioTek ELx808 microplate reader set to 450 nm. Duplicate readings for each standard and sample were averaged, and the average blank (negative control, assay diluent alone) OD reading was subtracted from all readings. The blank wells were expected to have an OD of <0.1, with a higher OD suggesting contamination of the assay diluent and invalidating the assay results. A four parameter logistic (4-PL) standard curve was fitted, plotting analyte concentration (x-axis) versus the OD (y-axis), using the KC Junior software which supports the BioTek ELx808 microplate reader. The curve fitting equation represents the relationship between the values and the optical density (R-square value) and should be as close to 1.0 as possible (see Figure 2-2). The standard curve was used to calculate the concentration of the analyte of interest within unknown samples, based upon their colour and thus their OD value.

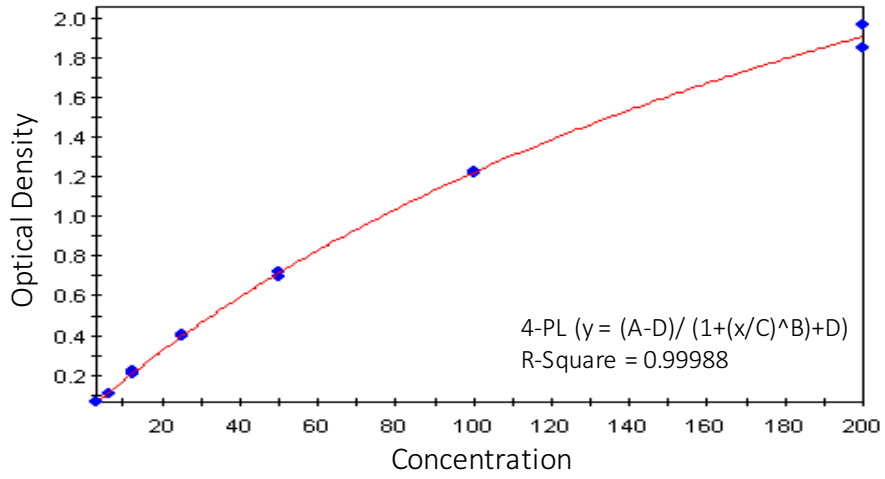


Figure 2-2: Typical 4-PL ELISA curve
The optical density of standard points (shown by blue diamonds) are plotted against their known concentrations and the line of best fit is placed using a 4-parameter logistic regression equation.

The details of the ELISA assays used in Chapters 4, 5 and 6 of this thesis are shown Table 2-1.

Analyte	Type	Capture Ab	Block	Sample incub	Type of detection Ab	Conjugate	Substrate solution	Stop solution	Read method
AGP (R&D Systems, DAGP00)	Direct quantative sandwich immuno-assay	Monoclonal capture Ab pre-coated	Yes, assay diluent RDI-73	2 hrs, RT	HRP conjugated polyclonal, 2 hr incub, RT	No	Colour reagent A (stabilised hydrogen peroxidase) + colour reagent B (stabilised chromogen TMB), 30 mins, RT, dark	2 N sulfuric acid	450 nm, 4-PL standard curve
CP (Assa Pro, EC4101-1)	In-direct quantative sandwich immuno-assay	Polyclonal capture Ab pre-coated	No	2 hrs, RT	Biotinylated polyclonal Ab, 1 hr incub, RT	SP conjugate 30 min incub, RT	Peroxidase chromogen substrate TMB, 10 mins, RT, dark	0.5 N hydro-chloric acid	450 nm, 4-PL standard curve
LPGDS (Bio Vendor, RD191113 100R)	Direct quantative sandwich immuno-assay	Polyclonal capture Ab pre-coated	No	1 hr, RT, orbital microplate shaker	HRP conjugated polyclonal Ab, 1 hr incub, RT, orbital microplate shaker	No	TMB added immediately (no wash step), 10 mins, RT, dark	Un-specified stop solution	450 nm, 4-PL standard curve
TF (GenwayG WB-36931E)	Direct quantative sandwich immuno-assay	Monoclonal capture Ab pre-coated	No	30 mins, RT	HRP conjugated polyclonal Ab, 30 min incub, RT, dark	No	TMB and hydrogen peroxidase, 5 mins, RT, dark	0.3 M sulfuric acid	450 nm, 4-PL standard curve
MCP-1 (R&D Systems, DCP00)	Direct quantative sandwich immuno-assay	Monoclonal capture Ab pre-coated	No	2 hrs, RT	HRP conjugated polyclonal, 1 hr incub, RT	No	Colour reagent A (stabilised hydrogen peroxidase) + colour reagent B (stabilised chromogen TMB), 30 mins, RT, dark	2 N sulfuric acid	450 nm, 4-PL standard curve
VCAM-1 (R&D Systems, DY809)	In-direct quantative sandwich immuno-assay	Monoclonal capture Ab added, incub overnight at RT	Yes, PBS + 10% FCS, 1 hr at RT	2 hrs, RT	Biotinylated polyclonal Ab, 2 hr incub, RT	SP conjugate 20 min incub, RT	Colour reagent A (stabilised hydrogen peroxidase) + colour reagent B (stabilised chromogen TMB), 20 mins, RT, dark	0.5 N hydro-chloric acid	450 nm, 4-PL standard curve

Table 2-1: Specific details of the ELISA assay protocols used within this study

Ab = antibody. Incubation = incub. RT = room temperature. HRP = horseradish peroxidase. TMB = tetramethyl-benzidine. 4-PL = 4 – parameter logistic. Streptavidin = Strep. SP-conjugate = Streptavidin-Peroxidase conjugate. Minute = min. Hour = hr.

2.2.2 ELISA assay validation

2.2.2.1 Co-efficient of variation between duplicate values

All individual patient samples were run in duplicate on the ELISA plate. The co-efficient of variation (CV) between the duplicate values was calculated, assessing for any differences between measurements which may have occurred due to pipetting errors, cross-contamination or temperature differences across the plate. It was calculated by determining the standard deviation (SD) of the duplicate values and dividing this by the mean of the two values. This was multiplied by 100 to obtain the % CV. The equation for CV calculation is shown below:

- CV (%) = ((value 1-value 2)/(mean of value 1+2)) x100

The larger the CV the greater the variance between the measurements. A CV of <15% was considered acceptable, otherwise the sample was re-run.

2.2.2.2 Spike and recovery

A spike recovery assay is another measurement of assay validity, assessing if substances present within the experimental environment (e.g. assay buffer, sample matrix components, complement, heterophilic antibodies) interfere with detection of the given analyte. Spike recovery experiments were carried out for assays which had not been validated for use with urine samples. This type of experiment involves adding a known amount of the standard substance to the urine sample (spiked sample), running the urine sample neat without any spike (un-spiked sample) and adding the same amount of standard to matched wells containing assay diluent only (expected spike value). The recovery is calculated using the equation below:

$$\textbf{\% Recovery} = \frac{\text{Observed - Neat}}{\text{Expected}} \times 100$$

Observed = spiked sample value
Neat = un-spiked sample value
Expected = amount spiked into sample

The % recovery should fall between 80-120%, demonstrating good retrieval of the spiked standard.

2.2.2.3 Linearity of dilution (LOD) approach

LOD experiments were carried out in-house for assays that were not validated for use in urine. If a sample type does not display LOD, this suggests that a sample component is interfering with the accurate detection of the analyte at a given dilution. Problems with poor LOD are most commonly seen at low dilution levels, where the concentration of interfering substance is highest. Practically LOD experiments indicate whether sample values generated from different dilutions can be directly compared, or whether the assay would have to be run at a single dilution to reduce error within the assay. LOD was considered acceptable if between 80-120%.

To test LOD, patient samples with a range of LN severities were serially diluted when spiked or un-spiked (as above). In general, a starting dilution was chosen that was less that the dilution suggested by the manufacturer, and a 1:2 dilution series prepared. The sample was vortexed briefly between each dilution. LOD was then calculated using the equation below where a neat sample was serially diluted as an example:

- % recovery (1:2) = ((observed concentration of 1:2 diluted sample) / (neat sample concentration/2)) x 100
- % recovery (1:4) = ((observed concentration of 1:4 diluted sample) / (neat sample concentration/4)) x 100
- % recovery (1:8) = ((observed concentration of 1:2 diluted sample) / (neat sample concentration/8)) x 100

2.2.2.4 Approach to range finding using patient samples

ELISA assays for AGP, CP, LPGDS, TF and MCP-1 had been commercially validated for use with urine, and therefore provided suggested starting dilutions. In the context of LN, there was clear potential for the samples to display high biomarker levels requiring further dilution, therefore range finding with 15 active LN, 15 inactive LN and 10 HC samples was carried out for each assay, assessing the percentage of samples detectable at a given dilution, the number of samples extrapolated and the number that were un-detectable for each analyte.

2.2.2.5 Sample dilutions to be used for each ELISA assay following in-house LOD and range finding experiments

A summary of the results from range finding and LOD experiments, along with suggested starting dilution is shown in Table 2-2. For TF, a starting dilution was not suggested by the manufacturers. 95% of samples were detectable when the assay was run at 1:100 dilution, with 16% of values extrapolated and 5% of samples off the high end of the curve. LOD was acceptable (102-106%) therefore less/more dilution would be possible as necessary. A starting dilution of 1:20 was suggested for CP by the assay manufacturers, however, the optimal dilution was identified to be 1:80. 21% of samples values were still extrapolated (high), suggesting that further dilution would be required for a proportion of samples (LOD 102-118%). Similarly, a starting dilution of 1:10 was suggested by the AGP assay manufacturers, however the optimal dilution was 1:80, and LOD was 103-115%, suggesting further/less dilution would be acceptable as necessary. 1:100 and 1:2 dilutions were suggested by the LPGDS and MCP-1 assay manufacturers, and found to perform optimally with LN samples (100% and 95% of samples detectable respectively), LOD was poor for both assays (106-169% and 95-135% respectively), therefore alternative dilutions were to be avoided (see Table 2-2).

	Dilution	Samples detectable	Samples extra-polated	Samples off STN curve	LOD	Optimal starting dilution
TF	neat	10/40 (25%)	5/10 (50%)	30/40 (75%, H)	na	**1:100**
	1:50	31/40 (78%)	10/31 (32%)	9/40 (22%, H)	106%	
	1:100	38/40 (95%)	6/38 (16%)	2/40 (5%, H)	104%	
	1:200	33/40 (83%)	6/33 (18%)	7/40 (17%, L)	102%	
CP	1:20	15/40 (38%)	3/15 (20%)	25/40 (62%, H)	na	**1:80**
	1:40	28/40 (70%)	5/28 (18%)	12/40 (30%, H)	102%	
	1:80	39/40 (98%)	8/39 (21%)	1/40 (2%, H)	114%	
	1:160	31/40 (78%)	15/31 (48%)	9/40 (22%, L)	118%	
AGP	1:10	12/40 (30%)	6/12 (50%)	28/40 (70%, H)	na	**1:80**
	1:40	28/40 (70%)	10/28 (36%)	12/40 (30%, H)	103%	
	1:80	38/40 (95%)	2/38 (5%)	2/40 (5%, H)	111%	
	1:160	36/40 (90%)	12/36 (33%)	4/40 (10%, L)	115%	
LPGDS	1:50	31/40 (78%)	5/31 (16%)	9/40 (22%, H)	na	**1:100**
	1:100	40/40 (100%)	2/40 (5%)	0/40 (0%)	106%	
	1:200	27/40 (68%)	7/27 (26%)	13/40 (32%, L)	143%	
	1:400	15/40 (38%)	11/15 (73%)	25/40 (62%, L)	169%	
MCP-1	neat	31/40 (78%)	5/31 (16%)	9/40 (22%, H)	na	**1:2**
	1:2	38/40 (95%)	2/38 (5%)	2/10 (5%, H)	95%	
	1:4	36/40 (90%)	9/36 (25%)	4/40 (10%, L)	119%	
	1:8	29/40 (73%)	11/29 (38%)	11/40 (27%, L)	135%	

Table 2-2: Summary of results from range finding and linearity of dilution experiments for TF, CP, AGP, LPGDS and MCP-1 with suggested starting dilution.

15 active LN, 15 inactive LN and 10 HCs samples included in the analysis. STN = standard curve. H = high. L = low.

2.2.2.6 Freeze thawing and biomarker stability

The effect of up to three freeze thaw cycles was assessed in two active LN, two inactive LN and two HC patients for each ELISA assay. Urine samples were always freeze thawed on ice. LPGDS displayed acceptable recovery for up to three freeze thaw cycles (95-98% recovery). TF could be freeze thawed for up to two cycles (96.5% recovery for one freeze thaw cycle, 92% for two). Both MCP-1 and AGP could only undergo one freeze thaw cycle (recovery of 94% and 91% respectively). CP levels fell by >15% after one freeze thaw cycle, therefore freeze thawing was avoided.

2.2.2.7 VCAM-1 R&D systems duo-kit ELISA assay

An R&D systems duo-kit (R&D Systems Ltd, Minneapolis, USA) was used to quantify urinary VCAM-1. This more basic type of ELISA assay had not been commercially validated for use with urine and therefore required extensive internal validation, including selection of an appropriate assay buffer, assessment of spike recovery, LOD, sample dilution, and biomarker stability following freeze thawing of samples. Three diluents (a) phosphate buffered saline (PBS) + 10% fetal calf serum (FCS, both Sigma Aldrich, UK)), (b) PBS + 20% FCS and (c) a commercially available reagent diluent concentrate (DY995, R&D Systems Ltd, Minneapolis, USA) were assessed within a spike recovery experiment to see if any difference in the recovery could be detected dependent on the assay diluent used. Very minor differences were seen between the three conditions when spike recovery experiments were carried out (average percentage recovery with buffer (a) = 99.8%, buffer (b) = 105.6% and buffer (c) = 104.1%), therefore the simplest buffer was chosen - PBS + 10% FCS. Samples were run neat, 1 in 50, 1 in 100 and 1 in 200 (in 15 active LN, 15 inactive LN and 10 HC patients) demonstrating LOD to be between 80-120%. 100% of samples were detected at 1 in 100 dilution, whereas with other dilutions, 11-33% of values were undetectable. Therefore, a 1 in 100 dilution was selected. Assessment of VCAM-1s stability with freeze thawing) showed VCAM-1 levels to fall on average by 22, 39 and 33% during freeze thaw cycles. Samples were not therefore used if they had previously been freeze thawed. A summary of the manufacturer and internal validation results for all ELISA assays is shown in Table 2-3.

Analyte	Type of assay	Spike recovery	Dilution required and LOD	Freeze thawing
VCAM-1[1]	R&D systems duo kit, DY809	100-106%	1:100 dilution, LOD 82-98%	No
MCP-1[2]	R & D systems quantakine, DCP00	96-120%	1:2 dilution LOD 95-135% Avoid dilution.	Yes – up to 1 FT cycle (94% recovery)
AGP[2]	R & D systems quantakine, DAGP00	86-111%	1:80, LOD 103-115%	Yes – up to 1 cycle (91% recovery)
Ceruloplasmin[2]	Assay Pro, EC4101-1	85-116%	1:80, LOD 111%	No
LPGDS[2]	BioVendor ELISA plate, RD191113100R	82-103%	1:100 dilution, LOD 106-169%. Avoid dilution.	Yes – up to 3 FT cycles (95-98% recovery)
Transferrin[2]	GenWay elisa plate, GWB-36931E	104-116%	1:100 dilution, LOD 102-106%	Yes – up to 2 FT cycles (92-96.5% recovery)
NGAL and Creatinine[3]	Abbott Architect	90-108%	1:10 dilution for NGAL and 1:50 for creatinine, automated 1:4 dilution if value high. LOD <10%	Up to 3 FT cycles

Table 2-3 Summary of validation of all assays used for biomarker quantification.
[1]All validation carried out in-house for use in urine. [2]Sample dilution and effect of freeze thawing determined in-house, whereas linearity of dilution and spike recovery determined by the manufacturer. [3]Commercially validated chemiluminescent microparticle immunoassays for use as a clinical test in urine and run in the Alder Hey Children's Hospital biochemistry laboratory on the Abbott Architect analyser. LOD = linearity of dilution. FT = freeze thaw.

2.2.3 Abbott Architect urine NGAL and Creatinine assays

Urinary NGAL and creatinine concentrations were measured using two separate commercially available clinical Abbott Architect chemiluminescent microparticle immunoassays (Abbott Laboratories, Texas, USA) in the Alder Hey Children's Hospital biochemistry laboratory. Monoclonal antibodies for the detection of NGAL or creatinine coat the microparticles. In the first step of the assays, sample and wash buffer were combined (1:10 dilution for NGAL and 1:50 for creatinine). An aliquot of the diluted sample, wash buffer and paramagnetic microparticles were combined and the reaction mixture washed. In the second step, anti-NGAL or creatinine acridinium-labelled conjugate was added. Following another wash cycle, pre-trigger and trigger solutions were added to the reaction mixture. The resulting chemiluminescent reaction was measured as relative light units (RLU), correlating with the amount of NGAL or

creatinine present in the samples. If the value was off the top of the standard curve, an automated 1:4 dilution was performed. The manufacturer reported the following assay performance characteristics: 89-108% recovery, LOD <10% when auto-dilution is required and up to three freeze thaw cycles acceptable. All urine biomarker results were standardised for urinary creatinine (Cr) quantified on the Abbott Architect analyser and presented in units per milligram creatinine (mgCr). See Table 2-3 above for a summary of the above information.

2.2.4 Multiplex protein assays

2.2.4.1 Multiplex protein assay – background

Traditional single analyte protein detection methods such as ELISA or western blotting are well established but can be very sample/time consuming and costly when used to measure numerous analytes. Multiplex (multi-analyte profiling technology) allows quantification of multiple proteins in a single well of a 96 well plate. The MAGPIX™ instrument used in this study uses super-paramagnetic 6.5-micron microsphere beads which are internally dyed with precise proportions of red and infra-red flurophores of differing intensities, giving each bead type a unique spectral signature. Using this approach, up to 500 distinct bead sets can be produced (MAGPIX™ instrument can only quantify up to 50 analytes simultaneously). Each bead has a unique number/bead region allowing differentiation between beads. Individual beads are then coated with a capture antibody which is specific for one analyte. In contrast to ELISA assays, the beads coated with the capture antibody are suspended rather than being attached to the wells. A biotinylated detection antibody mixture is then added and incubated, followed by streptavidin-PE. Within the MAGPIX™ analyser, a magnet captures and holds the magnetic beads in a monolyer while two light emitting diodes (LEDs) illuminate and excite the beads. The red LED interrogates the bead whilst the green one interrogates the bead label. The identity and quantity of beads corresponding to the concentration of the analyte is captured with a Charge Coupled Device (CCD) camera and fluorescent imager. This process is summarised in Figure 2-3.

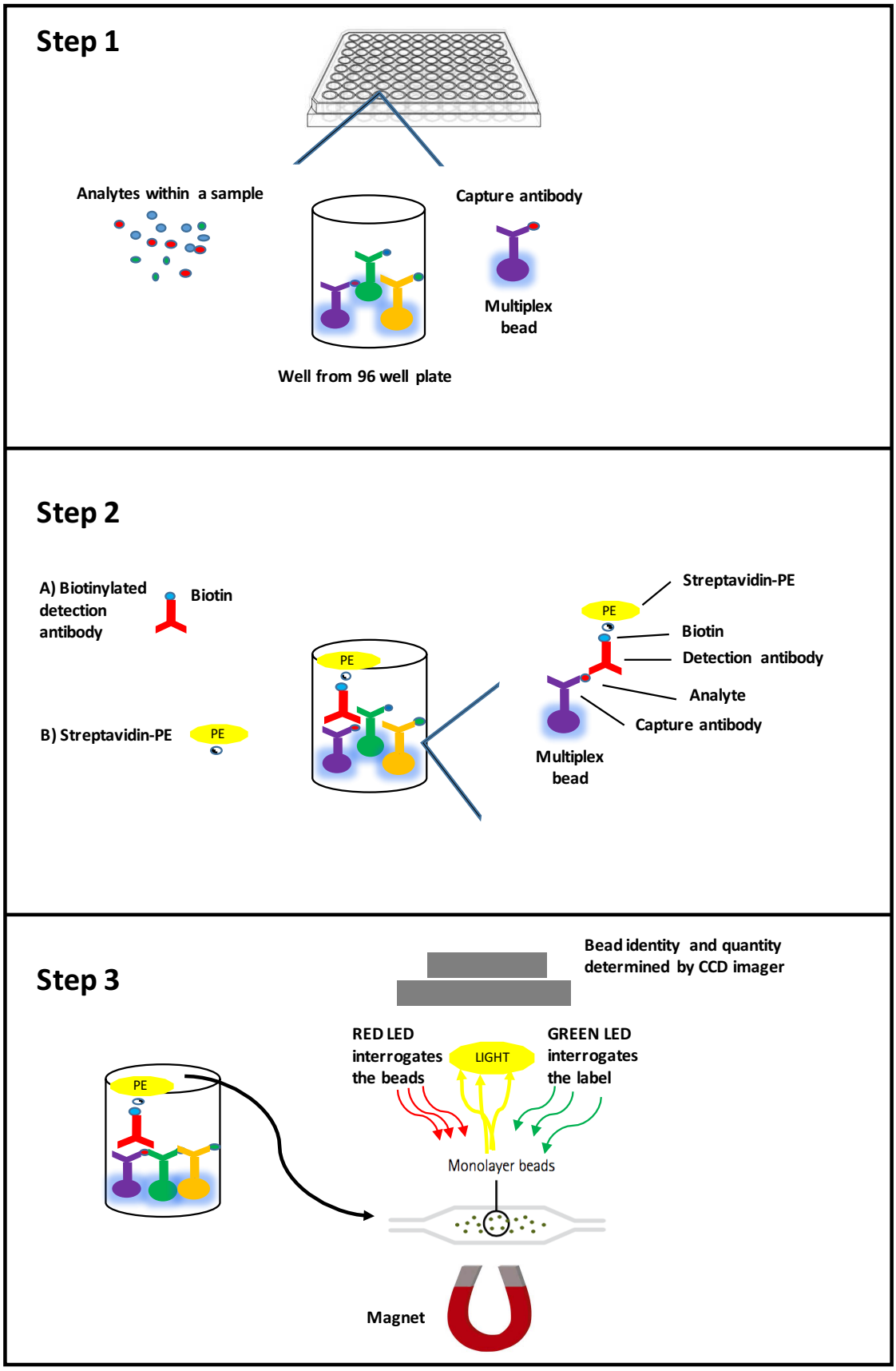


Figure 2-3: MAGPIX™ multiplex assay principle

In a multiplex assay different magnetic microsphere beads are found in suspension, coated with a capture antibody which is specific to one analyte. Step 1 - The beads and diluted samples are incubated. Step 2 – Biotinylated detection antibody is added and incubated, and then Streptavidin-PE is added and

incubated further. Step 3 – The assay is read in the MAGPIX™ instrument. A magnet is applied to the sample, holding the beads in a monolayer and two LED'S are used to identify the type and quantity of beads, corresponding to the concentration of the analyte present.

2.2.4.2 Multiplex protocol overview

Multiplex assays varied in terms of sample dilution, length of incubations and whether the beads are pre-mixed or require mixing, but generally followed the procedure detailed below. Before commencing an assay, the reagents were brought to room temperature. Urine samples were thawed, vortexed and then centrifuged to remove any debris which can interfere with the assay. For kits including individual bead vials, each antibody-bead vial was sonicated for 30 seconds and then vortexed for 1 minute. 60µL from each antibody bead vial was added to the mixing bottle, bringing the final volume to 3.0mL with bead diluent. Quality controls (QCs) and seven point working standards were reconstituted and prepared according to the manufacturer's instructions.

Prior to commencing the assay, 200µL of wash buffer was added to each well of the plate, the plate was sealed and mixed on a plate shaker for 10 minutes at room temperature. The wash buffer was removed from all wells by inverting the plate and tapping it onto absorbent towels several times. 25µL of each standard or control was added into the appropriate wells, with assay buffer used for the 0 pg/mL background standard. 25µL of appropriately diluted sample was added into the appropriate wells followed by 25µL of assay buffer to the all wells (sample, standard, control and background wells). 25µL of vortexed beads were then added to all wells (during addition of beads, the bead bottle was shaken intermittently to avoid bead settling). The plate was sealed, wrapped in foil and incubated with agitation on a plate shaker overnight at 4^{o}C, or for 2 hours at room temperature (20-25^{o}C) depending on the assay. Overnight incubation can improve assay sensitivity for some analytes.

The plate was then washed. During wash steps/prior to the well contents being decanted, a handheld magnet was applied to the plate for 60 seconds to settle the beads and prevent them from being lost. Any decanting or tapping on absorbent pads was undertaken gently to remove residual liquid. To wash the plate, it was removed from the magnet, 200µL of wash buffer added, the plate shaken for 30 seconds, reattached to magnet for 60 seconds, and the well contents removed as described above. The wash steps were repeated 2-3 times as recommended in the assay procedure.

25µL of detection antibody was added to each well, the plate sealed, covered with foil and incubated with agitation on a plate shaker for 1 hour at room temperature. 25µL of Streptavidin-Phycoerythrin was then added to each well containing the 25µL of detection antibody. The plate was sealed, covered with foil and incubated with agitation on a plate shaker for 30 minutes at room temperature. The well contents were gently removed, and the plate washed following the instructions above. 150µL of drive fluid was added to all wells and the beads re-suspended on a plate shaker for 5 minutes. The plate was run on a MAGPIX™ instrument with xPONENT® software. The raw data was exported and MILLIPLEX™ Analyst 5.1 software used to generate a 5-parameter logistic standard curve (5-pL) using the Median Fluorescent Intensity (MFI) data to calculate the analyte concentrations within samples.

2.2.4.3 Calculation of unknown values using multiple standard curves

Using the MILLIPLEX™ Analyst software, multiple standard curves are constructed plotting the known standard concentration on the x-axis and the MFI on the y-axis, to produce 5-pL standard curves for calculating analyte concentrations in samples, with the MFI for each sample being proportional to the amount of analyte present. An example of multiple multiplex standard curves is shown in Figure 2-4.

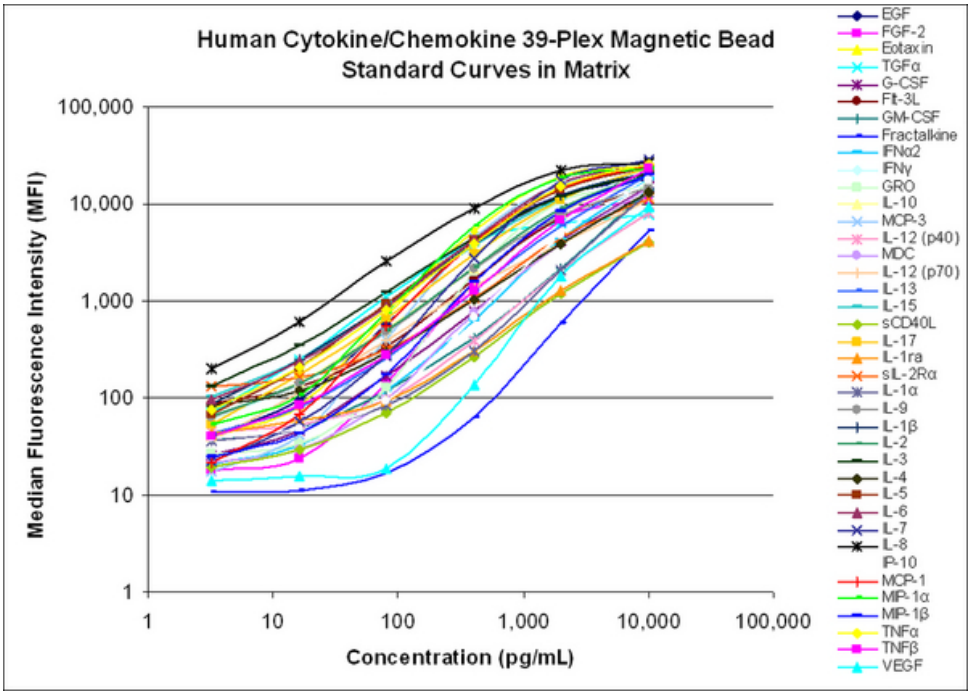


Figure 2-4: Multiplex assay multiple standard curves
Standards of a known concentration are plotted against the median fluorescent intensity for each analyte. The line of best fit is produced using MILLIPLEX™ analyst 5.1 software and 5-parameter logistic regression curves. The slope of the line can be used to calculate samples of unknown antigen concentration from their measured MFI.

2.2.4.4 Manufacturers validation for commercially available assays

All multiplex assays are assessed for cross-reactivity between the antibodies for each analyte within a given panel. This is carried out by spiking low, medium and high standards into the assay for a given analyte and assessing for increases in MFI for any of the other analytes. The percentage CV (described in section 2.2.2.1) of measurements made in duplicate or triplicate is quoted (considered acceptable by manufacturers when <10%). Inter-assay CV% (identical sample triplicates analysed using two different plates from the same lot number on the same platform, <15% difference considered acceptable) and intra-assay CV% (identical sample triplicates at different positions on the same plate, <10% difference considered acceptable). The manufacturers report spike and recovery values for low, medium and high concentration spikes, representing acceptable retrieval of the spiked standards when between 80-120%. Multiplex assays are also supplied with high and low QCs to be run during the assay, with the range for each analyte provided on a card insert within the kit, providing assurance of correct assay performance when the assay is run.

2.2.5 Collection, processing and storage of patient urine samples

In this study urine dipstick, microscopy and culture and sensitivities (MCS) excluded infection in UK patient samples. In US and SA patient samples, urine dipstick leucocytes/nitrites or the presence of clinical symptoms were assessed. The urine was processed as soon as possible or kept refrigerated until processing (up to 2 hours). The sample was centrifuged at 2000 rpm for 10 minutes. The urine supernatant was divided in to 1ml aliquots and stored in plastic Eppendorf's at -80^{o}C until required.

2.2.6 Collection, processing and storage of plasma/serum

2.2.6.1 Isolating monocytes and culturing them to become macrophages

10-20mls of blood was collected from HCs for isolation of white blood cells and subsequent monocyte isolation using CD14+ magnetic bead selection. Firstly, 5mls of blood was gently layered on top of 6mls of polymorph preparation solution (Axis-Shield PoC, Norway) in a sterile 12ml universal tube. This was centrifuged at 1000g for 30 minutes (without a break), allowing the cells to be separated according to their density. PBMCs formed the top layer of cells with neutrophils in the middle and red blood cells at the bottom of the sample (see Figure 2-5).

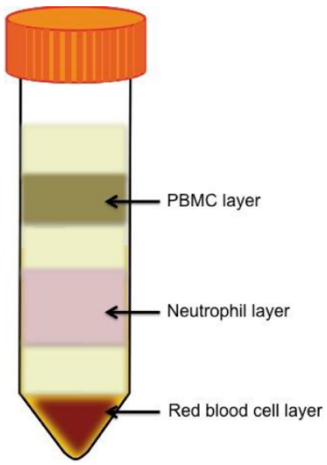


Figure 2-5: Depiction of the three cell layers formed after centrifugation of whole blood with polymorph preparation solution.

The band of PBMCs was carefully collected for these experiments and mixed in a sterile universal container with 10mls of Roswell Park Memorial Institute medium (RPMI) media (Lonza, UK). The cells were then re-centrifuged at 1000g for 10 minutes to become pelleted. Where the pellet appeared contaminated with red blood

cells, 1ml of RPMI and 9mls of 1% Ammonium Chloride solution were added for 3 minutes, and the cells re-pelleted (centrifuged at 1000g for 5 minutes). The cells were then re-suspended in 1ml of RPMI media, counted using a haemocytometer to identify the number of cells per ml (> 10 million PBMCs required), and then re-spun at 1000g for 5 minutes.

A CD14+ magnetic cell selection kit (EasySep^{TM}, Miltenyi Biotec Ltd, UK) was used to isolate monocytes from PBMCs. A buffer was made containing 50mls of cold PBS, 2.5mls FCS and 2Mm (0.037g) of filtered ethylenediamine tetra-acetic acid (EDTA, Sigma-Aldrich, UK), and chilled on ice. 80µl of buffer + 20µl of CD14+ micro-selection beads were added to pelleted cells, for every $1x10^7$ cells previously counted. The mixture was incubated for 15 minutes at 4^oC. 8mls of buffer was added to the solution, and then centrifuged for 10 minutes at 1000g. The cells were re-suspended in 0.5mls of the above buffer.

A magnetic selection column was then used to isolate the CD14+ monocytes. The column was washed with 0.5mls of EDTA buffer to moisten the column, and then the cells were passed through the column followed by 3 x 0.5ml buffer washes. CD14- cells passed through the magnetic column into a waste collection tube. The column was taken off the magnetic field and the CD14+ monocytes were washed out of the column using 1ml of sterile EDTA buffer. The cells were re-counted using a haemocytometer and re-suspended in macrophage media according to the number of cells present ($1x10^6$ cells/ml required in RPMI media, 10% FCS, 1% penicillin streptomycin and 10ng/ml of macrophage colony stimulating factor (M-CSF, R&D Systems Ltd, USA)) to promote differentiation of monocytes into macrophages. The cells were seeded into a 24 well plate and incubated for 6 days at 37^oC in 5% CO_2.These experimental conditions have previously shown within our research group to lead to macrophage differentiation. The purity of the isolated monocytes has also been confirmed within our group by staining and analysing for the percentage of cells bearing CD14 positivity immediately after the cell separation process. Using flow cytometry, the CD14+ cells have been gated and counted, demonstrating a very pure population of cells with a purity of >95% [352,353].

2.2.6.2 Activation of monocyte derived macrophages

Once differentiated into macrophages the cells were washed twice with PBS and then either exposed to 1ng/ml of IFN-γ or RPMI media alone for 48 hours, leading to an activated or inactivated state respectively. 1ng/ml of IFN-γ has previously been shown within our research group to be sufficient for macrophage activation using TNF- α as a marker of adequate macrophage activation. A dose of 1ng/ml IFN-γ was shown to achieve the maximum TNF-α concentration (1000 pg/ml), with cell viability maintained [353] and therefore this dose was also used for macrophage activation in the current study. After 48 hours incubation with the IFN-γ or media alone, the macrophage supernatant was removed, centrifuged and stored at -80^{o}C until required.

2.2.6.3 Isolation of neutrophils for use as positive controls in apoptosis experiments

Neutrophils are known to be short lived cells when cultured, with a high proportion undergoing apoptosis when cultured overnight. Neutrophils were isolated using a Hetacept/Histopaque (Sigma-Aldrich, UK) isolation method. 10mls of HC blood was aliquoted into two falcon tubes (5mls blood + 1ml Hetacept (Sigma-Aldrich, UK), mixed well and left at room temperature for 30 minutes in the hood until the plasma/PBMCs and red cells form two distinct layers (50:50 split). The PBMC/plasma layer was removed, PBS added (4-fold dilution) and centrifuged at 200g for 10 minutes. The liquid was poured off, the pellet re-suspended in 1ml of media, layered onto histopaque (Sigma-Aldrich, UK), and centrifuged at 2000rpm for 20 minutes.

The liquid (containing PBMCs) was removed and the resultant neutrophil pellet lysed to remove red cells (1ml of RPMI and 9mls of 1% Ammonium Chloride solution added to the cells for 3 minutes, cells centrifuged at 2000rpm for 5 minutes for the neutrophil pellet to re-form). The media was discarded and the cells re-suspended in 1ml of media. The neutrophils were counted using a haemocytometer and then plated [354].

The following morning, flow cytometry was used to confirm apoptosis using Annexin V staining (Sigma-Aldrich, UK) prior to running the Caspase 3/7 apoptosis assay to confirm the validity of the positive control. 100μl of Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich, UK) was added to 100microlitres of cells, and the mixture pipetted up and down. The plate was spun at 1500 rpm for 5 minutes, the fluid tipped

out and 99µl of HBSS and 1µl of Annexin added to each well. This was refrigerated for 15 minutes, a further 100µl of HBSS added and spun again for 5 minutes at 1500 rpm. The HBSS was tipped out, a further 200µl of HBSS added, the cells mixed up and down to re-suspend the pellet and then run through the flow cytometer to confirm apoptosis immediately prior to the Caspase 3/7 Assay (Promega Corporation, UK, see section 4.4.4.1). This showed Annexin V to be positive in 88.8% of neutrophils, confirming apoptosis in positive control cells (see Figure 2-6).

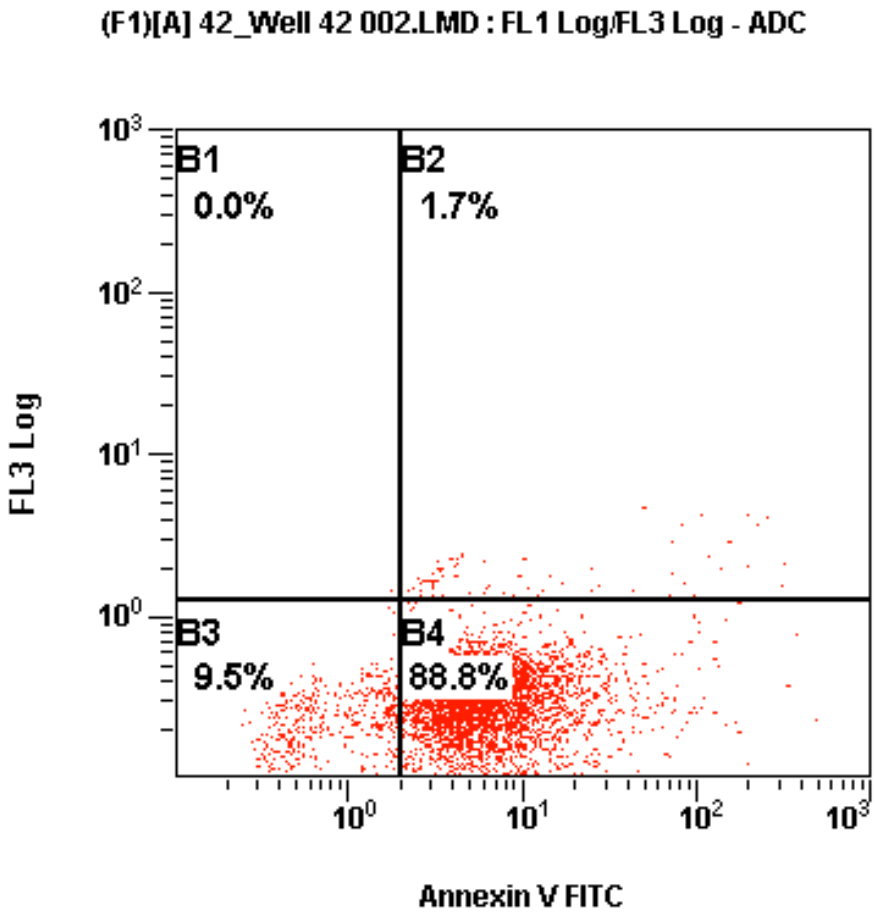


Figure 2-6: Flow cytometry dot plot confirming apoptosis in positive control cells (HC neutrophils cultured overnight).
88.8% of cells fell within the bottom right quadrant and were positive for Annexin V, therefore considered to be apoptotic. Cells in the bottom left hand quadrant were negative for Annexin V and therefore considered to still be viable.

2.2.7 Podocyte cell line culture

2.2.7.1 Background of the immortalized human podocyte cell line

Podocytes are highly specialised cells with cell bodies, major processes, and minor foot processes which are interlinked by slit diaphragms (see section 1.1.7). In the mature terminally differentiated state, podocytes lose their proliferative capacity, making them difficult to culture *in vitro*. In the past, only rather 'undifferentiated podocytes' of questionable cellular origin were available in culture, with de-differentiation often seen (e.g. loss of major processes) during the *in vitro* culture process [355,356]. A cell line was developed by Professor Moin Saleem and his research team at the University of Bristol Academic Renal Unit, to avoid such issues. They initially developed a conditionally immortalized podocyte cell line from the immortomouse, carrying a temperature sensitive T antigen as a transgene allowing the cells to proliferate at 33^{o}C and differentiate into mature podocytes at 37^{o}C. The differentiated cells display actin filaments, synaptopodin and microtubules forming processes which are similar to the podocyte processes *in vivo* [357]. On this basis they developed a human primary *in vitro* cell line, using cells which were obtained post nephrectomy from a 3-year old child's kidney transfected with a Simian vacuolating virus 40 (SV40) large T antigen gene using a retroviral vector. As above, at 33°C these cells proliferate and when thermoswitched to 37°C, they stop proliferating and terminally differentiate into mature podocyte cells which have been shown to express podocyte proteins including Wils tumor-1 (WT-1), nephrin, podocin, P-cadherin, CD2 associated protein (CD2AP) [358]. Clearly podocytes in culture differ from those *in vivo* in respect to the cell cycle, as the inability to replicate and regenerate is a key issue in the biology of podocytes playing a major role in the progression of renal injury. However, markers of cell proliferation (e.g. PCNA and Ki67) are downregulated when the cells are transferred to 37^{o}C, reflecting the pattern seen *in vivo* [358]. A pre-established collaboration between Professor Moin Saleem and Professor Michael Beresford has enabled use of this human podocyte cell line as part of this current work.

2.2.7.2 Culture techniques

The podocytes were grown in 25cm^2 flask in 5mls of 'podocyte cell media' containing RPMI-1640 media (Lonza, UK) with 10% FBS, 1% penicillin-streptomycin and 1% insulin transferrin selenium liquid supplement (ITS) (all Sigma-Aldrich, UK). Cells were removed from liquid nitrogen storage, hand thawed, and seeded at a 1x10^5

concentration in 5mls of podocyte cell media and placed at 33°C in 5% CO_2 until they reached 60% confluence. At this stage the cells could either be divided using trypsin EDTA (Sigma-Aldrich, UK) or thermoswitched to 37°C (with 5% CO_2) allowing the podocytes to terminally differentiate for experimental purposes.

To divide cells, the media was poured off, sterile cold PBS (Sigma-Aldrich, UK) used to wash the cells twice, and 1ml of trypsin (1x concentration; Sigma-Aldrich Ltd, USA) added to each flask. The cells were then placed at 37°C for a maximum of 5 minutes to allow cell detachment (monitored regularly, detachment visible under the microscope with cells moving across the flask). 10mls of podocyte media was added to the trypsinated cells, which were transferred into a sterile universal container and centrifuged at 1000*g* (10 minutes). The media was poured off and the pelleted cells re-suspended, counted and split into fresh flasks or plates for proliferation at 33°C. At each passage approximately 1/3 of cells were stored by placing them into a cryovial with 1ml of freezing media (1% Dimethyl Sulfoxide (Sigma-Aldrich, UK), 50% FCS, 50% podocyte cell media), slowly cooled in an iso-propanol container at -80°C for 24 hours, and then moved into long-term storage in liquid nitrogen.

For cytokine production and apoptosis experiments, the podocytes were seeded in 6 or 96-well plates, in 2mls or 100µl of podocyte cell media respectively. Once the cells reached 60% confluence at 33°C, they were thermoswitched to 37°C and allowed to terminally differentiate over 10-14 days, becoming the mature podocytes required for experimental purposes. Figure 2-7 shows podocytes when they are undifferentiated (at 33°C) and again when they are terminally differentiated (37°C). At both temperatures, podocyte media was replaced every 3-4 days.

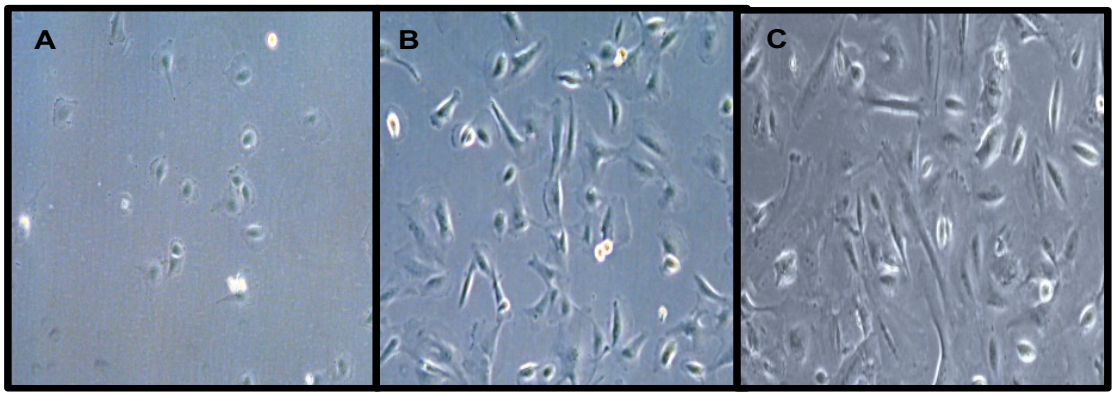


Figure 2-7: Morphology of podocytes at different cell culture stages.
A. Undifferentiated podocytes plated at 33°C. **B.** 60% confluent podocytes, day 1 at 37°C. **C.** Mature terminally differentiated podocytes after 10 days at 37°C.

Once terminally differentiated, the podocytes were washed twice with PBS prior to being exposed to the experimental test conditions (see section 4.4.4) for 48 hours at 37°C, in keeping with previous work within our laboratory [359]. Podocyte supernatant was then aspirated from the cells carefully, avoiding the podocyte cell layer, and stored at -80°C. All podocytes were within two passage of each other for experiments.

2.3 Statistical analysis

A full description of the statistical techniques employed is provided in the methods section of each chapter. In brief, descriptive statistics were undertaken using Statistics Programme for Social Sciences (SPSS, IBM Corporation, USA) or GraphPad Prism software (GraphPad software, USA). All results were assessed to see whether they represented normality using the Shapiro-Wilk test. In data that was normally distributed (parametric), the data was presented as mean values with the standard error of the mean (SEM). In non-normally distributed data (non-parametric) the results were represented as median values with interquartile ranges [IQR]. Statistical comparison between two non-related groups of non-parametric data used the Mann-Whitney U test and the student t-test for parametric data. Comparison of more than two groups used the Kruskal-Wallis (non-parametric) or the one-way ANOVA test (parametric) with a post-hoc test (e.g. Dunnet's test) to identify where the significance lay. For discrete variables with more than 2 categories, chi-squared tests were used. Bonferroni adjustment was made to account for multiple testing as appropriate, and the Bonferroni corrected *p-value,* p_c reported.

More complex statistical tests were undertaken using R version 3.1.1 [360] in collaboration with Dr Andrea Jorgenson (Senior Lecturer in Biostatistics, Institute of Translational Medicine, UoL) and Dr Peng Yin (Research Associate, Biostatistics, Institute of Translational Medicine, UoL). Tests included univariate logistic regression, Cox proportional hazards modelling (with Kaplan Meyer curves and risk tables to display the model outputs), multiple regression modelling (with HRs, 95% CIs), multiple imputation of missing clinical data using 'MICE' package in R [361], binary logistic regression modelling and application of the step Akaike Information Criterion score (AIC) function in R to select the final model, and area under the curve receiver operating curve analysis (AUC ROC). Analysis of longitudinal biomarker data was undertaken in collaboration with Dr Antonio Eleuteri (Computer Scientist, Department of Physics, UoL) and Professor Antony Fisher (Professor of Physics and Clinical Engineering, Department of Physics, UoL), and involved fitting of a Markov model of state transitions, quoting the corrected AIC in R. Urine biomarkers were explored as factors predicting state transitions. Bayesian multiple imputation of missing clinical variables was used. GraphPad Prism software or R version 3.1.1 [360] were used to produce all graphical illustrations.

2.4 Summary

This chapter demonstrates how this study is placed within the context of the UK JSLE Cohort Study and how international collaborations with ethnically distinct JSLE cohorts have been formed to advance work looking at urine biomarkers for LN.
The methods of patient recruitment, clinical data collection, sample processing and transfer have been described, highlighting the similarities and differences between different study sites.

The theory behind the experimental methods and optimisation of experimental protocols has been discussed.

The overview of the diverse range of statistical techniques employed in this work, made possible through collaborations within the UoL, has ensured that the results of this work are optimally explored and reported upon.

3 Clinical predictors of active LN and assessment of novel and traditional biomarkers for disease monitoring within the UK JSLE Cohort Study

3.1 Introduction

3.1.1 Clinical predictors of active LN development

Many patients will have LN as part of their initial presentation, but a proportion will go on to develop this manifestation later [362,363]. Early recognition and appropriate management of LN is important as early response to treatment is known to be associated with better renal outcome [261]. Long term survival of JSLE [198] and adult-onset SLE [364] patients with LN is reduced as compared to those without LN. Identifying those with or at risk of developing LN is important, so that clinicians can be extra vigilant in monitoring for LN. To date, investigation of clinical and demographic factors predicting future LN development in children has been limited to a single small North American study including 47 patients [362]. This study identified low serum albumin (odds ratio (OR): 4.8, 95% CI: 1.9–12.5) and positive dsDNA antibodies (OR: 3.2, 95% CI: 1.7–5.9) to be associated with development of LN within a median of 3.3±2 years. In longitudinal analyses, the same study identified isolated sterile pyuria (HR: 3, 95% CI: 1.1–6.4) and low serum albumin (HR: 3.4, 95% CI: 1.7–6.9) to be predictors of future LN [362]. This study included predominantly African American patients. Further exploration of such potential predictive factors is required in other ethnically distinct populations, to help the clinicians when monitoring patients and making treatment decisions.

3.1.2 Predictors of recovery from proteinuria following an LN flare

Following an LN flare, proteinuria has been shown to take a significant period of time to normalise in adults with LN, with 53% of patients requiring up to 2 years to recover and only 74% recovering by 5 years [253]. During this time, differentiating between proteinuria due to ongoing LN flare or chronic renal damage can be problematic. There is limited reliability of proteinuria measurements alone. This can at times result in repeated renal biopsies, despite there being no agreement as to the appropriate timing and indications for repeating a renal biopsy, particularly in children [232,365]. Several adult SLE studies have shown that an early reduction in proteinuria following initiation

of immunosuppressive therapy is associated with improved longer term renal outcomes [261,263,366,367]. Time to recovery from proteinuria in children with active LN receiving standard treatment has not been described to date. It is therefore of great interest to explore this within the national UK JSLE Cohort Study, to appreciate for how long proteinuria persists within a real world clinical setting (in contrast for example to a clinical trial setting). Identification of clinical and demographic prognostic factors at the onset of LN which are predictive of subsequent longer duration to resolution of proteinuria, could be useful for stratifying patients as high or low risk, helping to fine-tune the intensity and duration of early immunosuppressive therapy.

3.1.3 Traditional biomarkers and active LN identification

The predictive value of commonly available, so called 'traditional' biomarkers of JSLE activity for identifying active LN remains uncertain (see section 1.2.3) [259,368]. There have been reports highlighting 'clinically silent LN' patients who have no proteinuria, normal urinalysis and renal function, but biopsy defined active LN [255]. In clinical practice, paediatric rheumatologists and nephrologists rely heavily upon such non-invasive markers, as renal biopsies are invasive with significant potential associated complications, limiting their repeated use in children. Studies looking at the role of routinely measured immunological, haematological and inflammatory biomarkers in active LN identification in children are lacking. Therefore, assessment of the ability of these common, clinical biomarkers for differentiating the presence or absence of active LN, both individually and/or in combination, is of importance to patient care.

3.1.4 Novel urine biomarker identification

Screening for novel urine biomarkers using multiplex assays and profiling array kits (with capture and control antibodies for different biomarkers spotted onto nitrocellulose membranes) has become increasingly popular in the scientific literature for biomarker identification. This is in part due to the recognition that no single biomarker has emerged as pre-eminent, but also due to the increasing range of commercially available assays which can rapidly screen a large number of biomarkers [369]. Such assays have mainly been developed to detect drug-induced renal damage

and nephrotoxicity within the field of applied toxicology. The kidney is particularly susceptible to drug-induced injury as by the time the filtrate moves to the distal tubule and collecting ducts, its components can be concentrated more than 100 fold [370]. Monitoring of patients in clinical studies for signs of nephrotoxicity has previously been based upon serum creatinine and urea. However, both of these have limitations in their sensitivity and specificity, especially in individuals who still have significant renal reserve, where a large amount of kidney injury can occur without affecting GFR and serum creatinine. In patients who are ill with low muscle mass, there has to be an even greater decrease in GFR for serum creatinine to rise. Urea levels can increase with fluid depletion or if urea is increased due to protein supplementation, catabolic states, or blood within the gastrointestinal tract, impacting on ureas reliability as a marker of nephrotoxicity [370].

Several promising candidate novel urine biomarkers have emerged detecting injury to different, specific nephron segments (see Figure 3-1). Such sites are of key importance to diverse kidney pathologies, therefore, such markers have also been evaluated in acute kidney injury in very low birth weight infants [371], liver cirrhosis [372], renal transplantation [373], bladder cancer [374] and painful bladder syndrome [375]. In the context of LN, two studies have used multiplex to assess urine biomarkers. Kiani et al looked at osteoprotegerin (OPG) and MCP-1, showing both markers to be associated with the renal visual analogue scale and renal disease activity descriptors of the SLEDAI score [300]. The second study used multiplex technology to look at 27 cytokines, chemokines and cellular growth factors in the urine. MCP-1 was found to be the only immune mediator in urine, correlating with the SLEDAI score and demonstrating significantly higher levels in those with severe disease activity. The AUC value for detecting LN was 0.70 [376]. The utility of large commercially available kidney injury panels for identification of active LN has not been explored to date.

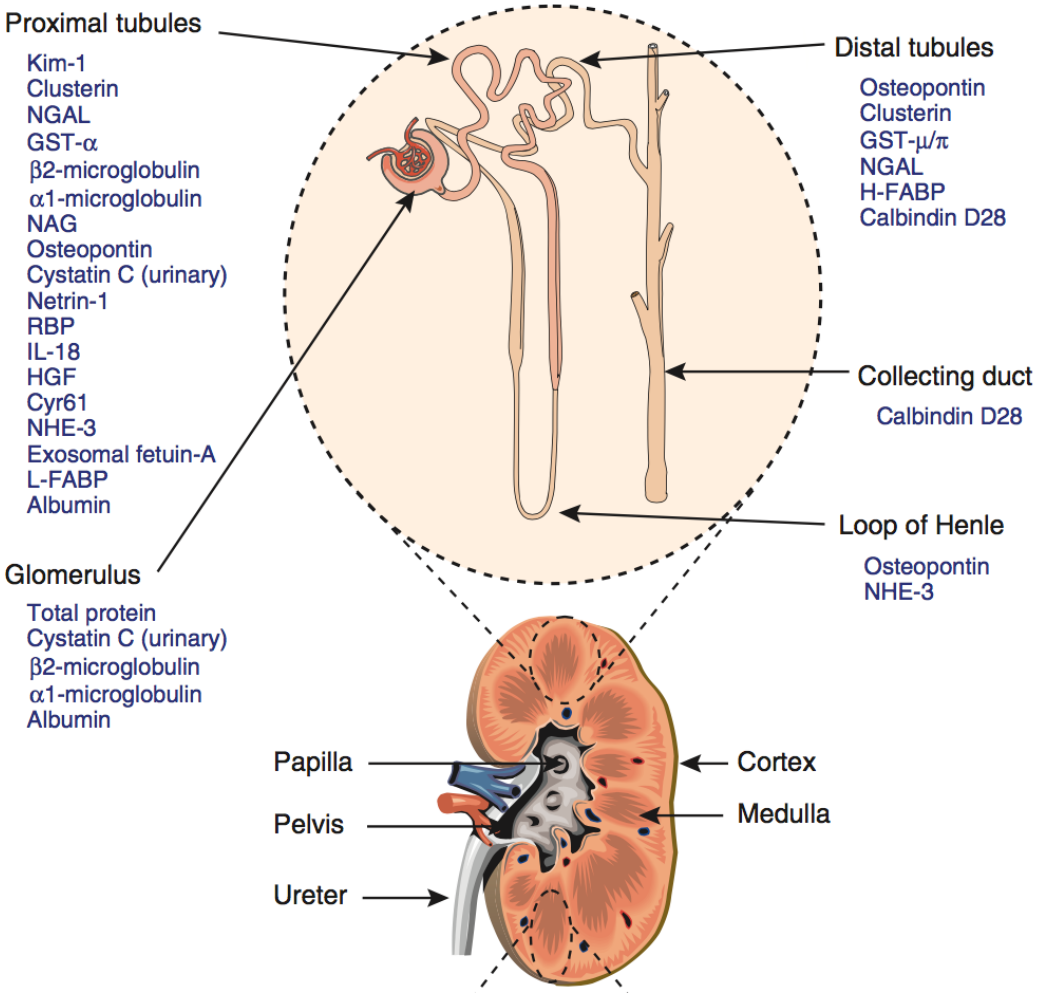


Figure 3-1: Nephron segment-specific kidney injury urine biomarkers .
KIM-1 = kidney injury molecule-1. NGAL = neutrophil gelatinase associated lipocalin. GST-α/μ/π = Glutathione-S-transferase-α. NAG = N-acetyl-b-d- glucosaminidase. RBP = Retinol-binding protein. HGF = hepatocyte growth factor. Cyr61 = cysteine rich protein 61. NHE-3 = N+ / H+ exchanger isoform 3. L-FABP = L-type fatty acid binding protein. H-FABP = H-type fatty acid binding protein. Figure adapted from [370].

3.2 Hypotheses

- Clinical and demographic factors can be used to differentiate patients with and without LN and to predict those who are likely to develop LN over their disease course.
- In patients with LN, such factors may also help to predict time to recovery from proteinuria following an active LN flare, and be used to monitor for changes in LN activity.
- Use of commercially available kidney toxicity assay will help to identify novel urine biomarkers for LN identification.

3.3 Aims

The primary aim of this chapter is to use existing data from within the UK JSLE Cohort Study to assess clinical and demographic factors differentiating patients with and without LN and whether such factors play a role in predicting the development of LN, subsequent time to recovery from proteinuria following an LN flare, and their potential use in monitoring LN disease activity. The secondary aim was to assess whether commercially available kidney toxicity assays can be used to identify new urine biomarkers for active LN identification.

The specific objectives of this study were:

1. To characterise patients with LN at baseline, and how they differ from patients without LN in terms of clinical and demographic factors.

2. For those without LN at baseline, who develop it at a later stage, to determine when they develop LN and whether there are clinical and demographic predictors at baseline which can predict subsequent development of LN.

3. To establish if clinical and demographic factors predict the occurrence and time to recovery from proteinuria following an active LN flare.

4. To ascertain the ability of traditional haematological, immunological and inflammatory biomarkers of JSLE disease activity to be able to identify and differentiate active LN from inactive LN, both individually and in combination.

5. To establish whether urine biomarkers quantifiable through a commercially available kidney toxicity panel assays can differentiate active from inactive LN patients.

3.4 Specific methods

3.4.1 Clinical features characterising patients with LN at baseline

Children participating in the UK JSLE Cohort Study (see section 2.1.1) between 2006-2016 were included in this study if they were:

- Managed in paediatric rheumatology care.
- Recruited to the UK JSLE Cohort study within 1 year of diagnosis.
- Identified as having active LN at the time of the initial presentation to paediatric rheumatology care (referred to as 'baseline' throughout).

Active LN was defined in the following ways:

- Biopsy defined LN documented on the baseline annual review study form (see Appendix 12).
- Nephritis on biopsy in the last 3 months documented on the baseline BILAG form (see Appendix 5).
- Renal BILAG defined LN. Characterised as having 'active LN' if they had a renal domain of BILAG of A or B, with complete renal BILAG data present, in contrast to 'inactive LN' if they had renal BILAG of D or E (see Appendix 6).

Patients with and without active LN at baseline were compared in terms of the clinical and demographic factors shown in Table 3-1 using univariate logistic regression. A Bonferroni correction was applied for multiple testing (35 tests) and the significance level adjusted to $p<0.00142$.

Demographic features	Haematological features at baseline	BILAG domain involvement[12]
• Gender • Age at JSLE diagnosis • Ethnicity[2] • Any FH of autoimmunity[1] **Renal features at baseline** • Proteinuria[3] • Severe hypertension[4] • Serum creatinine • Estimated GFR[5]	• Haemoglobin • White cell count • Neutrophils • Lymphocytes • Platelets **Immunological features at baseline** • C3 and C4[8] • Anti-dsDNA[9] • IgG, IgA, IgM[10] • ANA titre • Anti-Sm, RNP, Anti-Ro, Anti-La[11]	• Constitutional • Mucocutaneous • Neuropsychiatric • Musculoskeletal • Cardiorespiratory • Gastrointestinal • Opthalmological • Haematological • First ACR score • ESR[6] • CRP[7]

Table 3-1: Clinical and demographic factors investigated at baseline
[1]Any family history of autoimmunity (history of SLE, thyroid disease, rheumatoid arthritis, connective tissue disease, type-1 diabetes, 'other family history' documented). [2]Ethnicity (Caucasian/non-caucasian). [3]spot protein/creatinine or albumin/creatinine ratio. [4]blood pressure rising to > 170/110 mm Hg within 1 month with grade 3 or 4 Keith-Wagener-Barker retinal changes (flame-shaped haemorrhages or cotton-wool spots or papilloedema). [5]eGFR = estimated glomerular filtration rate. [6]ESR = erythrocyte sedimentation rate. [7]CRP = c-reactive protein. [8]C3 and C4 = complement factors 3 and 4. [9]Anti-dsDNA = anti-double stranded DNA antibodies. [10]Ig = immunoglobulin. [11]Antibodies to extractable nuclear antigens. [12]BILAG domain involvement = score of A or B for a given organ domain.

3.4.2 Predictors of developing LN over time

Those without LN at baseline were followed longitudinally to see if they subsequently developed LN during their disease course, and when this occurred. Those with only a single study visit were excluded from these analyses. The association between outcome (time to the development of LN) and each clinical and demographic variable at baseline (detailed in Table 3-1) was tested univariately using Cox Proportional hazard modelling. The endpoint for each patient was defined as the time from the baseline visit to the date of the first active LN episode (as defined in section 3.4.1). Patients who did not develop LN during follow-up were censored at the date of the last visit, therefore they did not change the cumulative probability of survival for the population (graphically depicted on Kaplan-Meier plots). Variables with missing data were tested with complete cases only univariately.

Covariates with a $p<0.2$ on univariate analysis were included in a multiple regression model. Where >10% of the data was missing for a given covariate, 'MICE' package in R version 3.2.0 was used to undertake multiple imputation [361]. This took place

for lymphocyte count, platelet count, C3 and C4. Covariates to be included in the final model were chosen using a backward stepwise model selection procedure (threshold *p<0.05*). HRs, 95% CIs and *p-values* were summarised for covariates present in the final model. The results were displayed graphically with Kaplan-Meier curves and risk tables. Within the Kaplan-Meier plots, the x-axis was time from zero (when observation began) to the last observed time point. The y-axis was the proportion of subjects with JSLE who survived without developing LN. A vertical drop indicates the occurrence of an event (i.e. a patient developing LN). Risk tables under each Kaplan-Meier plot provide information about the number of patients still at risk of the outcome at the specific time points. All analysis and preparation of figures was undertaken with R version 3.2.0 [360].

3.4.3 Predictors of recovery from proteinuria following an LN flare

All participants of the UK JSLE Cohort Study (see section 2.1.1) [28], managed within paediatric rheumatology care between 2006-2016, were included in the current study if they had:

- Active LN - defined in terms of either having renal biopsy defined active LN or renal BILAG defined LN (renal BILAG of A or B) **and**
- Proteinuria - defined as a UPCR or UACR ratio of > 50mg/mmol or a 24-hour urine protein of ≥0.5 g. Proteinuria cutoffs chosen on the basis of the renal BILAG score, where a UPCR or UACR ratio of > 50mg/mmol or a 24-hour urine protein of ≥0.5 g is required for a score of B to be achieved **and**
- At least 2 follow-up visits following the onset of proteinuria.

Those with <2 follow-up visits or without proteinuria were excluded. Patients were categorised as having **recovered** from proteinuria (if spot UPCR or UACR ratio was <25mg/mmol at two consecutive visits) or **not recovered** at the latest follow-up. This cut off was chosen on the basis of the renal BILAG score as a spot UPCR or UACR ratio of >25mg/mmol would lead to a renal BILAG score of C, indicating ongoing mild LN rather than recovery.

The study was undertaken on a cross-sectional basis. The groups were compared using two complementary approaches; firstly, a binary approach was used. The association between outcome (recovered versus not recovered) and clinico-demographic factors at

the time of LN onset were tested univariately using logistic regression modelling (see Table 3-2). Covariates with a *p-value <0.2* were then included in a multiple regression model. Where >10% of the data was missing for a given covariate 'MICE' package in R version 3.2.0 was used to undertake multiple imputation [361]. This took place for serum creatinine, anti-dsDNA antibody levels, ESR, C3 and C4. The following covariates were not included in the multiple regression model due to the high levels of missing data (>40%); urine sediment results, CRP, IgG, IgA and IgM. Covariates to be included in the final model were selected using a backward stepwise model selection approach (threshold *p<0.05*). AUC ROC analysis of the final model was undertaken, looking at the ability of the model to predict patient outcome.

Clinical features at time of LN onset	**Demographic features**	**Medication use (yes/no, at time of LN onset)**
• Proteinuria[1] • Severe hypertension[2] • Nephrotic syndrome[3] • Serum creatinine • Presence of active urine sediment • eGFR [4] • Haemoglobin • Total white cell count • Neutrophil count • Lymphocyte count • Platelets count • ESR[5] and CRP[6] • C3 and C4[7] • Anti-dsDNA[8] • IgG, IgA, IgM[9]	• Age at LN onset • Gender • Ethnicity • Length of disease **BILAG domain involvement** • Constitutional • Mucocutaneous • Neuropsychiatric • Musculoskeletal • Cardiorespiratory • Gastrointestinal • Opthalmological • Haematological **Total Numerical BILAG score**	• Hydroxychloroquine • Azathioprine • Mycophenelate Mofetil • Prednisolone • IVIG[10] • Rituximab (ever) • Cyclophosphamide (ever) • Angiotensin inhibitor or angiotensin receptor blocker **Other data** • Physician global assessment

Table 3-2: Clinical and demographic factors investigated at the time of LN with proteinuria onset

[1]Spot urine protein/creatinine or albumin/creatinine ratio. [2]Blood pressure rising to > 170/110 mm Hg within 1 month with grade 3 or 4 Keith-Wagener-Barker retinal changes (flame-shaped haemorrhages or cotton-wool spots or papilloedema). [3]Nephrotic syndrome criteria: heavy proteinuria (≥ 3.5 g/day or protein-creatinine ratio ≥ 350 mg/mmol or albumin-creatinine ratio ≥ 350 mg/mmol) and hypoalbuminaemia and oedema. [4]eGFR = estimated glomerular filtration rate. [5]ESR = erythrocyte sedimentation rate. [6]CRP = c-reactive protein. [7]C3 and C4 = complement factors 3 and 4. [8]Anti-dsDNA = anti-double stranded DNA antibodies. [9]Ig = immunoglobulin. [10]IVIG = intra-venous immunoglobulin.

The second approach looked at time to recovery from proteinuria following an active LN flare. Patients were censored when they had not recovered from proteinuria at the

final follow up visit. The endpoint for each patient was defined as the date of recovery from proteinuria, or the last visit date if censored. Cox proportional hazard regression modelling was used to univariately test the association between each variable of interest (see Table 3-2) and outcome. Variables with missing data were tested with complete cases only univariately. Covariates with $p<0.2$ on univariate analysis were included in a multiple regression model. As above, where >10% of the data was missing for a given covariate, 'MICE' package in R version 3.2.0 was used to undertake multiple imputation [361]. Covariates to be included in the final model were chosen by using a backward stepwise model selection procedure (threshold $p<0.05$). HRs, 95% CIs and *p-values* were summarised for covariates present in the final model. The results were displayed graphically with Kaplan-Meyer curves and risk tables. All analysis and preparation of figures was undertaken with R version 3.2.0 [360].

3.4.4 Traditional biomarkers and LN monitoring

Patients participating in the UK JSLE Cohort Study (see section 2.1.1) [28] between 2006-2016, followed up in paediatric or adult rheumatology care were cross-sectionally grouped according to the renal domain of BILAG disease activity score, as active LN or inactive LN. Individual patient visits were selected where the clinical data was most complete. So called 'traditional' JSLE immunological, haematological and inflammatory laboratory test results, routinely used in clinical practice, were compared between patients with active and inactive LN. These included: anti-dsDNA, C3, C4, ESR, CRP, Hb, WCC, neutrophils, lymphocytes, platelets IgG, IgA and IgM levels. As the definition of active LN was based on the composite renal BILAG score, that in turn is calculated from factors including: proteinuria, GFR, blood pressure, active urine sediment, plasma creatinine and recent biopsy findings, the performance of these traditional biomarkers could not be investigated.

Demographic and traditional biomarker data were expressed as median values and IQRs. A binary logistic multiple regression model was fitted to assess for association between a combination of traditional biomarkers and LN status. All traditional biomarkers (log-transformed) excluding those contributing to the renal BILAG score were included in an initial model and the 'stepAIC' function in R [360] applied to select a final model. This function compares different models based on all possible

combinations of biomarkers and chooses the model with the minimum AIC value. The AIC is a measure of the relative quality of a model relative to each of the other models, with a lower value representing better quality. The AUC for the final model was calculated. AUC values of 1.0–0.9, 0.9–0.8, 0.8–0.7, 0.7–0.6, 0.6–0.5 were considered "excellent, good, fair, poor and fail" respectively. Data analysis was undertaken using R version 3.1.1 [360]. Where Bonferroni adjustment was made to account for multiple testing, the Bonferroni corrected *p-value,* p_c is reported.

The number of patients included in the studies detailed in sections 3.4.1, 3.4.2, 3.4.3 and 3.4.4 above varied due to the timing of the analyses and inclusion criteria used for each study. The first study examining clinical features characterising patients with LN at baseline/predictors of LN over time (sections 3.4.1 and 3.4.2) included the lowest number of patients (n=331), due to exclusion of patients who were recruited to the UK JSLE Cohort Study >1 year after achieving a diagnosis of JSLE. Within the second study investigating predictors of recovery from proteinuria following an LN flare (section 3.4.3), patients had to have at least 2 follow-up visits with proteinuria measurements, leading to the exclusion of some patients (n=350). The greatest number of patients was seen in the study investigating traditional biomarkers and LN monitoring (n=370, section 3.4.4) as it included patients within the UK JSLE Cohort Study who were followed-up in paediatric or adult rheumatology, and there was no stipulation on the number of visits required per patient, therefore, even those with a single visit were eligible for inclusion.

3.4.5 Novel urine biomarker identification using Human Kidney Injury (HKI) Multiplex Assays

Three commercially available HKI Magnetic Bead panel multiplex assays (Merck Millipore, St Charles, USA) were undertaken looking at 21 different urinary biomarkers:

- HKI-1: Calbindin, Collagen IV, FABP-1, GST-α, GST-π, IP-10, KIM-1, Osteoactivin, Renin, Trefoil Factor-3 (TFF-3), and tissue inhibitor of metaloproteases-1 (TIMP-1).
- HKI-2: α-1-Microglobulin (A1mG), Albumin, Clusterin, Cystatin-C (Cys-C), Epidermal Growth Factor (EGF), NGAL, and osteopontin (OPN).
- HKI-3: Uromodulin, RBP-4, B2mG

The assays were undertaken as per manufacturer's instructions (see section 2.2.4.2, for further details on multiplex technology and procedures), running the urine samples at 1:2 dilution in assay buffer for HKI-1, 1:100 for HKI-2 and 1:500 for HKI-3. The analyses were carried out on an MAGPIX™ array reader (Merck Millipore, USA (see section 2.2.4.1).

A subset of the children participating in the UK JSLE Cohort Study [28] from Alder Hey Children's NHS Foundation Trust, Liverpool, and Great Ormond Street NHS Hospital for Children, London, UK, were included in this study. Urine samples were collected during routine clinical care together with detailed demographic and clinical data. Renal disease activity was defined as active LN or inactive LN (see section 2.1.1.4). At a later date, the three HKI multiplex assays were also carried out on patient samples from the US Cohort (see section 2.1.2) [334].

Biomarker concentrations were standardised to urinary creatinine and expressed as median values and IQRs. Mann Whitney U tests were used to compare biomarker concentrations between patient groups. A Bonferroni adjustment was applied to account for multiple testing according to the number of biomarkers tested per assay (11 for HKI assay 1, 7 for HKI 2 and 3 for HKI 3), and corrected p-values (p_c) reported.

3.5 Results

3.5.1 Clinical features of patients with active LN at baseline

The total study cohort consisted of 370 patients, of which 331 were eligible for the current study, due to exclusion of patients who were recruited to the UK JSLE Cohort Study >1 year after achieving a diagnosis of JSLE. A total of 121/331 (37%) patients had active LN as an initial presenting feature at baseline. Testing the association between clinical and demographic factors at baseline and outcome univariately, six factors differed significantly between active LN and non-LN patients after correction for multiple testing, including first ACR score ($p=3.6 \times 10^{-6}$), severe hypertension ($p=0.0006$), proteinuria ($p=5.7 \times 10^{-12}$), serum creatinine ($p=0.00024$), ESR ($p=0.00075$) and C3 ($p=1.3 \times 10^{-7}$) (see Table 3-3 for summary statistics of the two patient cohorts with and without active LN).

Clinical and demographic factors at baseline		Active LN (n=121)	No LN (n=210)	p_c[1]
Proteinuria[2] (mg/mmolCr, NA[3]=125)		63.1 [42, 153]	13 [8, 26]	***5.7x10^{-12}***
Serum creatinine (micromols/l, NA=56)		61 [45, 75]	53 [45, 62]	***0.00024***
First ACR Score of:	**4**	42/121 (35%)	112/210 (53%)	***3.6 x10^{-6}***
	5	32/121 (26%)	60/210 (29%)	
	6	22/121 (18%)	26/210 (12%)	
	7	17/121 (14%)	8/210 (4%)	
	8	7/121 (6%)	4/210 (2%)	
	9	2/121 (1%)	0	
Severe hypertension[4] (NA=15)		14/115 (12%)	2/201 (0.5%)	***0.00060***
ESR (mm/h, NA=66)		60 [22, 101]	32 [13, 67]	***0.00075***
C3 (mg/L, NA=60)		0.52 [0.35, 0.82]	0.91 [0.60, 1.21]	***1.3x10^{-7}***
Female gender (NA=2)		104/121 (86%)	167/208 (80%)	*0.20*
Caucasian ethnicity[5] (NA=3)		53/119 (45%)	118/209 (56%)	*0.0349*
Diagnosis age (years, NA=6)		13 [11.1, 14.5]	12.8 [10.5, 14.5]	*0.48*
FH of autoimmunity[6]		58/121 (48%)	113/210 (54%)	*0.30*
ANA titre (NA = 129)		640 [240, 1600]	640 [400, 1140]	*0.83*
Anti-Sm positive		26/121 (21%)	40/210 (19%)	*0.59*
Anti- RNP positive		27/121 (22%)	56/210 (27%)	*0.38*
Anti-Ro positive		36/121 (30%)	57/210 (27%)	*0.61*
Anti-La positive		17/121 (14%)	27/121 (22%)	*0.76*
eGFR (ml/min/m^2, NA=92)		102 [83, 135]	113 [101, 131]	*0.75*
Haemoglobin (g/dl, NA=23)		11.1 [9.0, 12.2]	11.3 [10.1, 12.5]	*0.086*
WCC (x10^9/L, NA=25)		5.8 [3.9, 9.6]	5.6 [4.0, 7.6]	*0.03*
Neutrophils (x10^9/L, NA=37)		3.6 [2.3, 7.4]	3.4 [2.2, 5.1]	*0.0021*
Lymphocytes (x10^9/L, NA = 34)		1.4 [0.8, 1.8]	1.3 [0.9, 1.9]	*0.56*
Platelets (x10^9/L, NA=28)		226 [152, 315]	272 [195, 337]	*0.024*
CRP (mg/L, NA=62)		5 [4, 7]	5 [4, 8]	*0.61*
C4 (g/L, NA = 63)		0.06 [0.03, 0.14]	0.11 [0.06, 0.18]	*0.046*
dsDNA Titre (IU/L, NA=60)		200 [34.5, 329]	53 [14.25, 200]	*0.16*
IgG (g/L, NA=117)		13.7 [9.8, 19.9]	16.2 [12.7, 22.7]	*0.45*
IgA (g/L, NA=117)		2.06 [1.33, 2.89]	1.97 [1.51, 2.57]	*0.55*
IgM (g/L, NA=119)		1.29 [0.77, 1.9]	1.3 [0.93, 1.9]	*0.88*
Constitutional involvement[7]		66/121 (55%)	94/210 (45%)	*0.087*
Mucocutaneous involvement		70/121 (58%)	143/210 (68%)	*0.062*
Neuropsychiatric involvement		15/121 (12%)	21/210 (10%)	*0.50*
Musculoskeletal involvement		66/121 (55%)	134/210 (64%)	*0.098*
Cardiorespiratory involvement		19/121 (16%)	23/210 (11%)	*0.21*
Gastrointestinal involvement		11/121 (9%)	13/210 (11%)	*0.33*
Ophthalmic involvement		5/121 (4%)	3/210 (1.4%)	*0.14*
Haematological involvement		82/121 (68%)	146/210 (70%)	*0.74*

Table 3-3: Clinical and demographic data at baseline in patients with and without active LN at baseline.

Summary statistics used for continuous variables (median, IQR), whereas number count and percentage detailed for discrete variables (for each category). [1]*p-values* are Bonferroni-corrected from univariate binary regression or Chi Squared tests appropriate, non-significant = *p>0.00142,* significant values shown in bold. [2]Baseline Proteinuria = spot UACR or UPCR measurements depending on hospital laboratory (mg/mmolCr). [3]Missing data shown in brackets with NA. [4]BILAG defined severe

hypertension = blood pressure rising above 95th centile for gender and age according to height centile within 1 month +/- grade 3 or 4 Keith-Wagener-Barker retinal changes (flame-shaped haemorrhages or cotton-wool spots or papilloedema). [5]Ethnicity classification simplified to Caucasian or non-Caucasian. [6]Any family history of autoimmunity including SLE, thyroid disease, rheumatoid arthritis, connective tissue disease, type-1 diabetes and 'other autoimmune disease' noted.[7]BILAG defined organ domain involvement (yes = BILAG score of A/B/C, no = D/E).

3.5.2 Predictors of time to developing LN over the disease course

Of the 210/331 patients without LN at baseline, 13 only had a single study visit and were therefore excluded from further analyses. A total of 197/210 patients without LN at baseline were therefore followed up longitudinally for a median of 3.1 years [IQR 1.5-5.0]. 34/197 (17%) patients developed LN during the study period after a median of 2.04 years [IQR 0.8-3.7]. Testing for association between each clinical and demographic factor at baseline and outcome (developed LN or did not develop LN) univariately, the following factors had *p-values* of *<0.2* and were included as covariates within a multiple regression model: any FH of autoimmunity, first ACR score, lymphocyte count, platelet count, C3, C4 and BILAG defined cardiorespiratory involvement (see Table 3-4).

Clinical and demographic factors			No LN(n=163)	Active LN(n=34)	p_c[1]
Factors considered in the multivariate model	Family history of autoimmunity[2]		85/163 (52%)	22/34 (65%)	*0.160*
	First ACR Score of:	4	90/163 (55%)	15/34 (43%)	*0.0038*
		5	50/163 (32%)	7/34 (21%)	
		6	17/163 (10%)	7/34 (21%)	
		7	4/163 (2%)	4/34 (12%)	
		8	2 (1%)	1/34 (3%)	
	Lymphocytes (x10^9/L, NA[3]=20)		1.4 [0.9, 1.9]	1.2 [0.8, 1.7]	*0.098*
	Platelet count (x10^9/L, NA=18)		283 [216, 345]	214 [149, 291]	*0.057*
	C3 (g/L, NA=30)		0.94 [0.67, 1.24]	0.63 [0.45, 0.83]	*0.0032*
	C4 (g/L, NA=32)		0.12 [0.08, 0.19]	0.06 [0.03, 0.12]	*0.067*
	Cardiorespiratory involvement		16/163 (10%)	5/34 (9%)	*0.17*
Demo-graphics	Female gender		127/163 [78%]	29/34 [85%]	*0.640*
	Caucasian ethnicity[4]		92/163 (56%)	17/34 (50%)	*0.400*
	Diagnosis age (years, NA=1)		12.8 [10.7, 14.6]	12.6[10.4,14.1]	*0.310*
Clinical features and laboratory investigations	ANA titre (NA=69)		640 [400, 1140]	640 [400,1600]	*0.898*
	Anti-Sm positive		30/163 (18%)	7/34 (21%)	*0.750*
	Anti- RNP positive		45/163	8/34	*0.40*
	Anti-Ro positive		45/163	8/34	*0.860*
	Anti-La positive		21/163	4/34	*0.840*
	Severe hypertension[5] (NA=8)		2/163	3/34	*0.990*
	Proteinuria[6] (NA=90)		13 [8, 26]	17 [10, 28]	*0.630*
	Ser creatinine (μmols/L,NA=30)		53 [45, 62]	56 [46, 62]	*0.821*
	eGFR (ml/min/m^2, NA=48)		114 [98.7, 130.5]	108 [101, 128]	*0.623*
	Haemoglobin (g/dl, NA=14)		11.3 [10.1, 12.4]	10.8 [9.4, 12.5]	*0.844*
	WCC (x10^9/L, NA=15)		5.8 [4.0, 7.9]	4.6 [3.9, 7.1]	*0.213*
	Neutrophils (x10^9/L, NA=19)		3.4 [2.2, 5.3]	3.4 [2.2, 4.4]	*0.462*
	ESR (mm/h, NA=31)		32.5 [14.5, 66.3]	40 [13.5, 83.3]	*0.903*
	CRP (mg/L, NA=35)		5 [4, 10]	4 [3.5, 5]	*0.788*
	dsDNA Titre (IU/L, NA=39)		44 [11.2, 200]	96 [20, 300]	*0.471*
	IgG (g/L,NA=61)		15.9 [12.1, 22.7]	17 [15.2, 22.2]	*0.737*
	IgA (g/L, NA=60)		2.0 [1.5, 2.5]	2.1 [1.7, 3.0]	*0.916*
	IgM (g/L, NA=63)		1.4 [0.9, 1.8]	1.3 [1.1, 2.3]	*0.647*
BILAG defined involvement	Constitutional involvement[7]		73/163 (45%)	18/34 (53%)	*0.44*
	Mucocutaneous involvement		108/163 (66%)	27/34 (79%)	*0.29*
	Neuropsychiatric involvement		17/163 (10%)	3/34 (9%)	*0.81*
	Musculoskeletal involvement		107/163 (66%)	23/34 (68%)	*0.96*
	Gastrointestinal involvement		9/163 (6%)	1/34 (3%)	*0.52*
	Ophthalmic involvement		2/163 (1.2%)	1/34 (3%)	*0.840*
	Haematological involvement		112/163 (69%)	26/34 (76%)	*0.48*

Table 3-4: Clinical and demographic factors at baseline for patients with/without LN longitudinally.

Summary statistics for continuous variables (median, IQR), number count and percentage detailed for discrete variables. [1]*p-values* are from univariate Cox Proportion hazard models. [2]Any FH of autoimmunity included SLE, thyroid disease, rheumatoid arthritis, connective tissue disease, type-1 diabetes and 'other autoimmune disease'.[3]Missing data shown in brackets. [4]Ethnicity simplified to Caucasian or non-Caucasian. [5]BILAG defined severe hypertension = blood pressure rising above 95th centile for gender and age according to height centile within 1 month +/- grade 3 or 4 Keith-Wagener-

Barker retinal changes. [6]Baseline Proteinuria = UPCR or UACR (mg/mmolCr). [7]BILAG defined extra-renal organ domain involvement (yes = BILAG score of A/B, no = D/E).

The multiple regression model identified the first ACR score (*p=0.014*) and C3 value (*p=0.0082*) as being significant covariate variables remaining after backward stepwise model selection. Patients with a higher ACR score and a lower C3 value at baseline were at greater risk of developing LN at any point in time (ACR score: HR 1.45, 95% CI 1.08-1.95 and C3: HR 0.27, 95% CI 0.10-0.68) (see Table 3-5). The concordance statistic was 0.710 for the final Cox proportional hazard model.

	p	**HR**	**(95% CI)**
First ACR score	*0.014*	1.45	1.08-1.95
C3	*0.0082*	0.27	0.10-0.68

Table 3-5: Multiple logistic regression model differentiating those who did/did not develop LN on the basis of their clinical and demographic data at baseline.

The Kaplan Meier plot for the first ACR score (see Figure 3-2) divides the patients into two sub-groups with an ACR score of <5 (n=161), and >5 (n=35). The median ACR score for those who developed LN was 5, therefore this was used as the cut-off for plotting the Kaplan-Meier curve. This plot demonstrates that at a given time, patients with a ACR score of >5 at diagnosis have an increased risk of developing LN (univariate analysis *p=0.0038*).

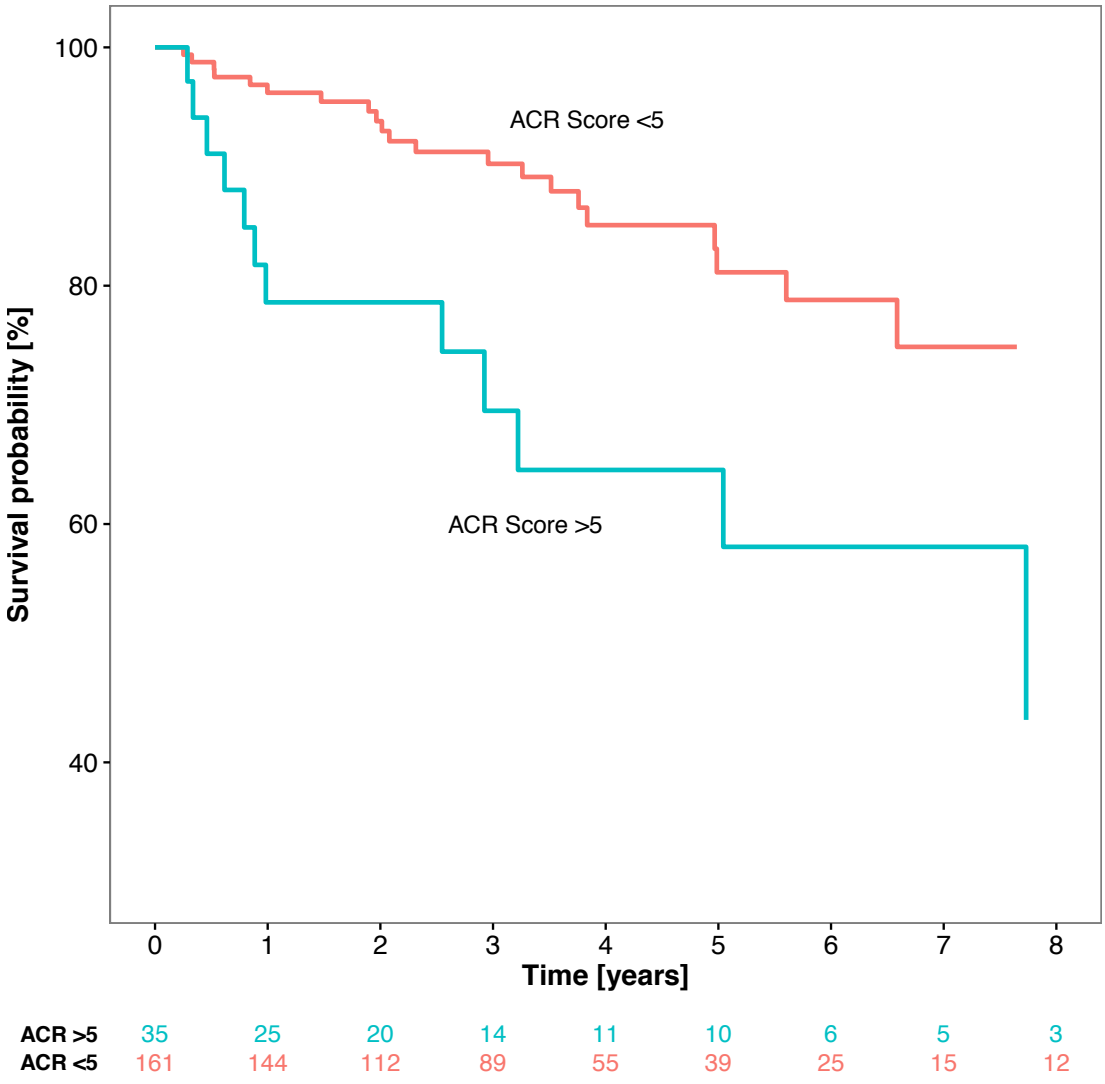


Figure 3-2: Kaplan-Meier plot for ACR score at baseline
Non-imputed data used for development of Kaplan-Meier plots, therefore n=156.

The Kaplan Meier plot for C3 (see Figure 3-3) divided the patients into two sub-groups with a C3 cut-off of >0.9g/L (in the normal range, n=77) or <0.9g/L (hypocomplementemia, n=78). This plot demonstrates that at a given time, patients with a C3 of <0.9g/L at diagnosis are at increased risk of developing LN (univariate analysis *p=0.0032*).

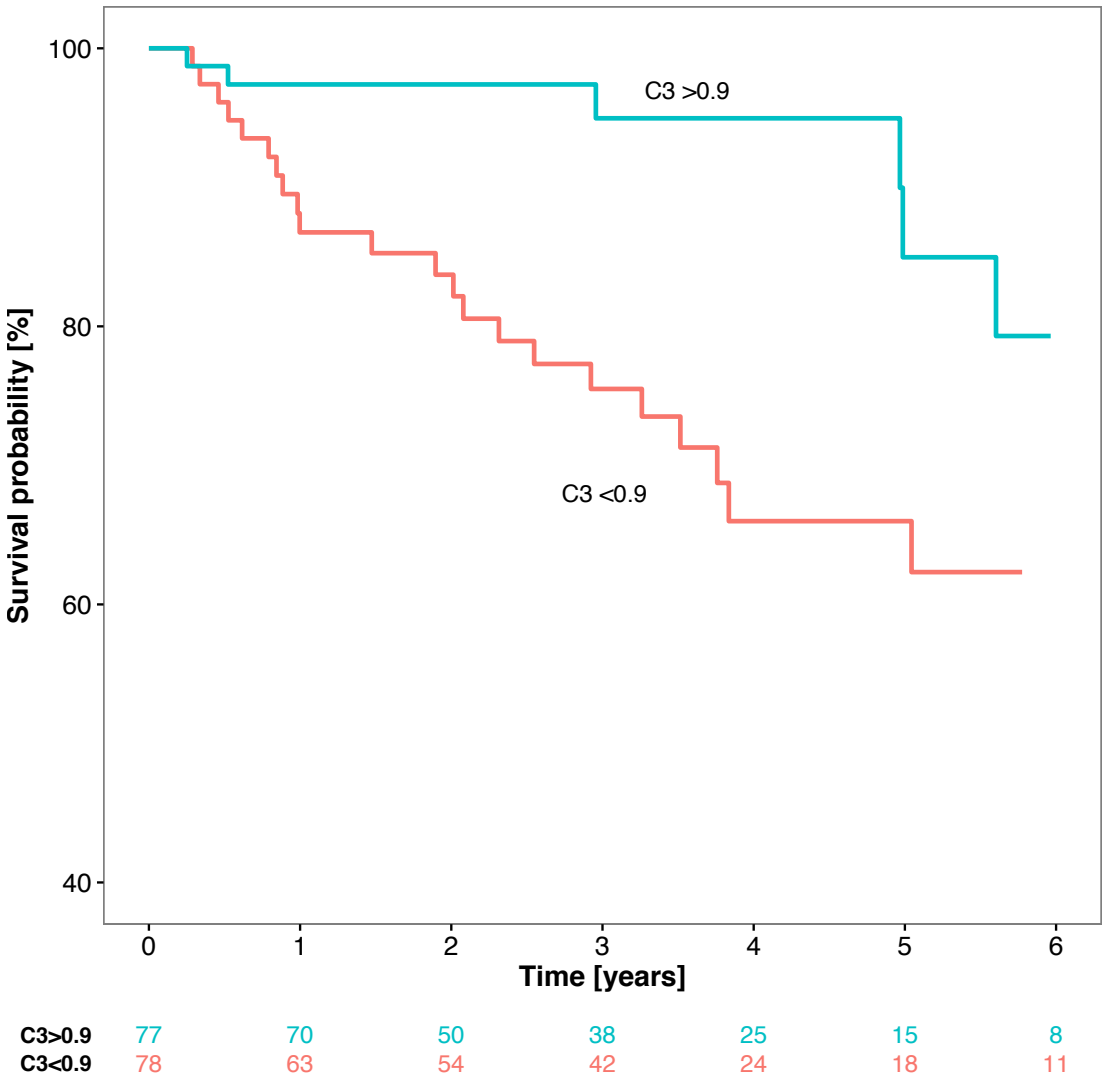


Figure 3-3: Kaplan-Meier plot for C3 at baseline (<0.9 vs. >0.9 g/L)
Non imputed data used for development of Kaplan-Meier plots, therefore n=156.

3.5.3 Predictors of recovery from proteinuria following an active LN flare

As detailed in section 3.5.1, the total study cohort consisted of 370 JSLE patients, 350 of which were considered in the current study due to the exclusion of patients with < 2 follow-up visits. Of these, a total of 64/350 (18%) JSLE patients were included in this specific analysis, having had active LN with proteinuria, and at least 2 consecutive follow-up visits following the development of proteinuria. Resolution of proteinuria was seen in 25/64 (39%) patients, within a median of 17 months (IQR 3.5-49.2). Proteinuria had not resolved in the remaining 39/64 (61%) after a median follow-up period of 22 months (IQR 12.1-41.1).

3.5.3.1 Do clinical and demographic factors at LN onset differentiate patients where proteinuria recovered or did not recover during the follow-up period?

In testing the association between each clinical and demographic factor (at the time of LN onset, detailed in Table 3-6) and outcome (proteinuria recovered or not-recovered) univariately, ethnicity (Caucasian or non-Caucasian), eGFR, Azathioprine use, physicians global assessment score, BILAG defined constitutional involvement, cardiorespiratory involvement and haematological involvement all had *p-values* of *<0.2* (see Table 3-6). These were therefore included as covariates within the multiple regression model.

	Clinical and demographic factors at LN onset	Not recovered (n=39)	Recovered (n=25)	*p*
Factors in the multivariate model	**Caucasian ethnicity[1]**	13/39 (33%)	13/25 (52%)	***0.141***
	eGFR (ml/min/m^2, NA = 2)	104 (29, 159)	121 (29,153)	***0.140***
	Azathioprine[2]	Y: 8, N: 31	Y: 2, N: 23	***0.194***
	Physician Global Assessment	23 (0, 75)	41 (1, 71)	***0.089***
	Constitutional involvement[3]	Y: 15, N: 24	Y: 15, N: 10	***0.095***
	Cardioresp involvement	Y: 3, N: 36	Y: 5, N: 20	***0.159***
	Haematological involvement	Y: 33, N: 6	Y: 13, N: 12	***0.006***
Demo-graphics	**Age at LN onset (days)**	5071 (2352, 6455)	4976 (2952, 6540)	*0.610*
	Female gender	32/39 (82%)	18/25 (72%)	*0.346*
	Length of disease (days)	225 (0, 4857)	27 (0, 2679)	*0.251*
Clinical features and laboratory investigations	**Baseline Proteinuria[4]**	149 (50, 2772)	252 (51, 1418)	*0.496*
	Severe hypertension[5](NA = 3)	Y: 6, N: 31	Y: 3, N: 21	*0.690*
	Nephrotic Syndrome[6](NA = 3)	Y: 7, N: 30	Y: 5, N: 19	*0.854*
	Serum creatinine (micromol/l,NA=8)	61 (34, 234)	50 (36, 177)	*0.305*
	Active urinary sed[7] (NA = 40)	Y: 5, N: 9	Y: 3, N: 7	*0.770*
	Haemoglobin (g/dl)	10.8 (5.6, 96)	11.3 (7.1, 14.9)	*0.595*
	WCC (x10^9/L)	4.8 (2.5, 22.4)	6.4 (0.5, 9.1)	*0.461*
	Neutrophils (x10^9/L, NA=2)	3.4 (1.1, 17.8)	3.44 (0.4, 12.33)	*0.577*
	Lymphocytes (x10^9/L, NA=2)	1.40 (0.1, 5.0)	1.53 (0.1, 5.42)	*0.515*
	Platelets (x10^9/L)	245 (77, 589)	225 (82, 522)	*0.635*
	ESR (mm/hr, NA=11)	40 (2, 170)	37.5 (4, 102)	*0.435*
	CRP (mg/l, NA=27)	5 (1, 19)	5 (1, 295)	*0.369*
	C3 (g/L, NA=7)	0.51 (0.18, 1.61)	0.71 (0.22, 1.31)	*0.546*
	C4 (g/L, NA=7)	0.06 (0.01, 0.90)	0.075 (0.02, 0.21)	*0.753*
	Anti-dsDNA (NA=22)	119 (0, 3503)	220 (42, 3770)	*0.841*
	IgG (g/L, NA=27)	14.6 (0.9, 70.2)	11.8 (2.8, 33.1)	*0.397*
	IgA (g/L, NA=28)	2.06 (0.8, 4.9)	2.36 (0.3, 3.7)	*0.907*
	IgM (g/L, NA=28)	1.11 (0.4, 9.6)	(0.07, 2.5)	*0.271*
Medications at baseline[3]	**Hydroxychloroquine**	Y: 21, N: 18	Y: 12, N: 13	*0.648*
	Mycophenalate Mofetil	Y: 10, N: 29	Y: 6, N: 19	*0.882*
	Prednisolone	Y: 24, N: 15	Y: 14, N: 11	*0.660*
	IV immunoglobulin (IVIG)	Y: 2, N: 37	Y: 2, N: 23	*0.646*
	Rituximab ever	Y: 2, N: 37	Y: 1, N: 14	*0.835*
	Cyclophosphamide ever	Y: 3, N: 36	Y: 2, N: 23	*0.964*
	ACEi or AT2i[8]	Y: 11, N: 28	Y: 4, N: 21	*0.266*
BILAG defined involvement	**Mucocutaneous involvement**	Y: 23, N: 16	Y: 15, N: 10	*0.935*
	Neuropsychiatric involvement	Y: 3, N:36	Y: 3, N: 22	*0.567*
	Musculoskeletal involvement	Y: 19, N: 20	Y: 14, N: 11	*0.570*
	Gastrointestinal involvement	Y: 0, N: 39	Y: 3, N: 22	*0.990*
	Opthalmological involvement	Y: 0, N: 39	Y: 2, N: 23	*0.992*
	Total numerical BILAG score	11 (3, 27)	11 (1, 53)	*0.333*

Table 3-6: Summary statistics and univariate logistic regression test results for all variables at the time of active LN with proteinuria onset, in those who did/did not recover from proteinuria.

Summary statistics used for continuous variables (median, min, max), whereas number count was detailed for discrete variables. Missing data shown in brackets with NA. [1]Ethnicity classification simplified to Caucasian or non-Caucasian. [2]Medication use (yes) or non-use (no) rather than absolute drug dose. [3]BILAG defined organ domain involvement (yes = BILAG score of A/B, no = D/E). [4]Baseline Proteinuria = UACR or UPCR measurements depending on hospital laboratory (mg/mmolCr). [5]BILAG defined severe hypertension = blood pressure rising above 95th centile for gender and age according to height centile within 1 month +/- grade 3 or 4 Keith-Wagener-Barker retinal changes. [6]Nephrotic syndrome = heavy proteinuria (> 50 mg/kg/day or > 3.5 g/day or UPCR> 350 mg/mmol or UACR > 350mg/mmol) + hypoalbuminaemia + oedema). [7]Active urine sediment = pyuria (> 5 white cells/hpf), haematuria (> 5red cells/hpf) or red cell casts in absence of other causes. [8]ACEi or AT2i = Angiotensin inhibitor or angiotensin receptor blocker.

Significant covariate variables remaining after backward stepwise model selection included ethnicity (Caucasian or non-Caucasian), eGFR, Azathioprine use, BILAG defined cardiorespiratory involvement and haematological involvement (all $p<0.05$, see Table 3-7).

Clinical and demographic factors at LN onset	**Odds ratio (95% CI)**	***p***
Ethnicity (Caucasian vs. non-Caucasian)	14.19 (2.52, 122.63)	*0.007*
eGFR	1.04 (1.015, 1.08)	*0.007*
Azathioprine use	0.093 (0.0070, 0.78)	*0.044*
Cardiorespiratory involvement	11.22 (1.57, 107.54)	*0.022*
Haematological involvement	0.13 (0.025, 0.53)	*0.007*

Table 3-7: Multivariate logistic regression model differentiating those who recovered and did not recover from proteinuria following an LN flare.
eGFR= estimated glomerular filtration rate, CI = confidence interval.

Caucasian patients, those with a higher eGFR and BILAG defined cardio-respiratory involvement at the time of LN onset were more likely to recover from proteinuria (following an LN flare) during the study period. Those receiving Azathioprine treatment and with BILAG-defined haematological involvement were less likely to be recovered. The ability of the multivariate model to predict the two patient outcomes (proteinuria recovered/not-recovered following an LN flare) was assessed using AUC ROC analysis, providing a good AUC value of 0.830 (see Figure 3-4).

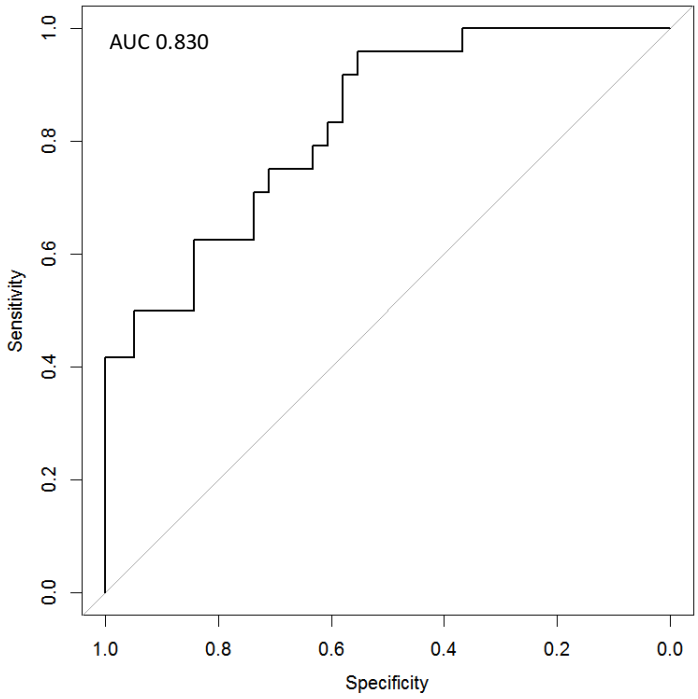


Figure 3-4: ROC curve of multivariate logistic model predicting the two patient outcomes (proteinuria recovered/not-recovered).

3.5.3.2 Do clinical and/or demographic factors at LN onset influence time to recovery from proteinuria following an LN flare?

The same clinical and demographic factors (at the time of LN onset, detailed in Table 3-2 above) were tested for association with the outcome (time to recovery from proteinuria following an LN flare) using Cox proportional hazard regression modelling. The following covariates, including age of LN onset, serum creatinine, eGFR, neutrophil count, physician global assessment and BILAG defined haematological involvement had p of <0.2 (see Table 3-8), and were therefore considered with a multiple regression model. All other co-variates had a p of >0.2 and were therefore not included within the multiple regression model (full data available in Appendix 18).

Clinical and demographic factors at LN onset	*p*
Age at LN onset	*0.013*
Serum creatinine	*0.184*
eGFR	*0.060*
Neutrophil count	*0.197*
Physicians global assessment	*0.107*
Haematological involvement	*0.038*

Table 3-8: Univariate association for each variable with outcome (time to recovery from proteinuria following LN flare) using Cox proportional hazard regression modelling.
Descriptive statistics for these variables are shown in Table 3-6, and therefore not repeated here.

The multivariate regression model identified younger patients, those with a lower eGFR and haematological involvement at the time of LN onset to have a longer time to recovery from proteinuria (see Table 3-9). For all three covariates, the 95% CI's for the HRs excluded one. The concordance statistic was 0.719 for the multivariate Cox proportional hazard model, which showed that the model accurately discriminated patients 71.9% of the time.

Clinical and demographic factors at LN onset	HR (95% CI)	*p*
Age at LN onset (years)	1.384 (1.0952, 1.7501)	*0.007*
eGFR	1.016 (1.0010, 1.0305)	*0.035*
Haematological involvement	0.324 (0.1294, 0.8115)	*0.016*

Table 3-9: Multivariate logistic regression model showing factors associated with time to recovery from proteinuria following an LN flare.
eGFR= estimated glomerular filtration rate, HR = hazard ration, CI = confidence interval.

The median age for those who recovered from proteinuria was 14 years, therefore the Kaplan Meyer plot for age (see Figure 3-5) divides the patients into two sub-groups aged <14 years (n=32), and >14 years (n=31). This plot demonstrates that at a given time, patients who are older at the time of LN onset are more likely to have recovered from proteinuria (univariate analysis *p=0.007*).

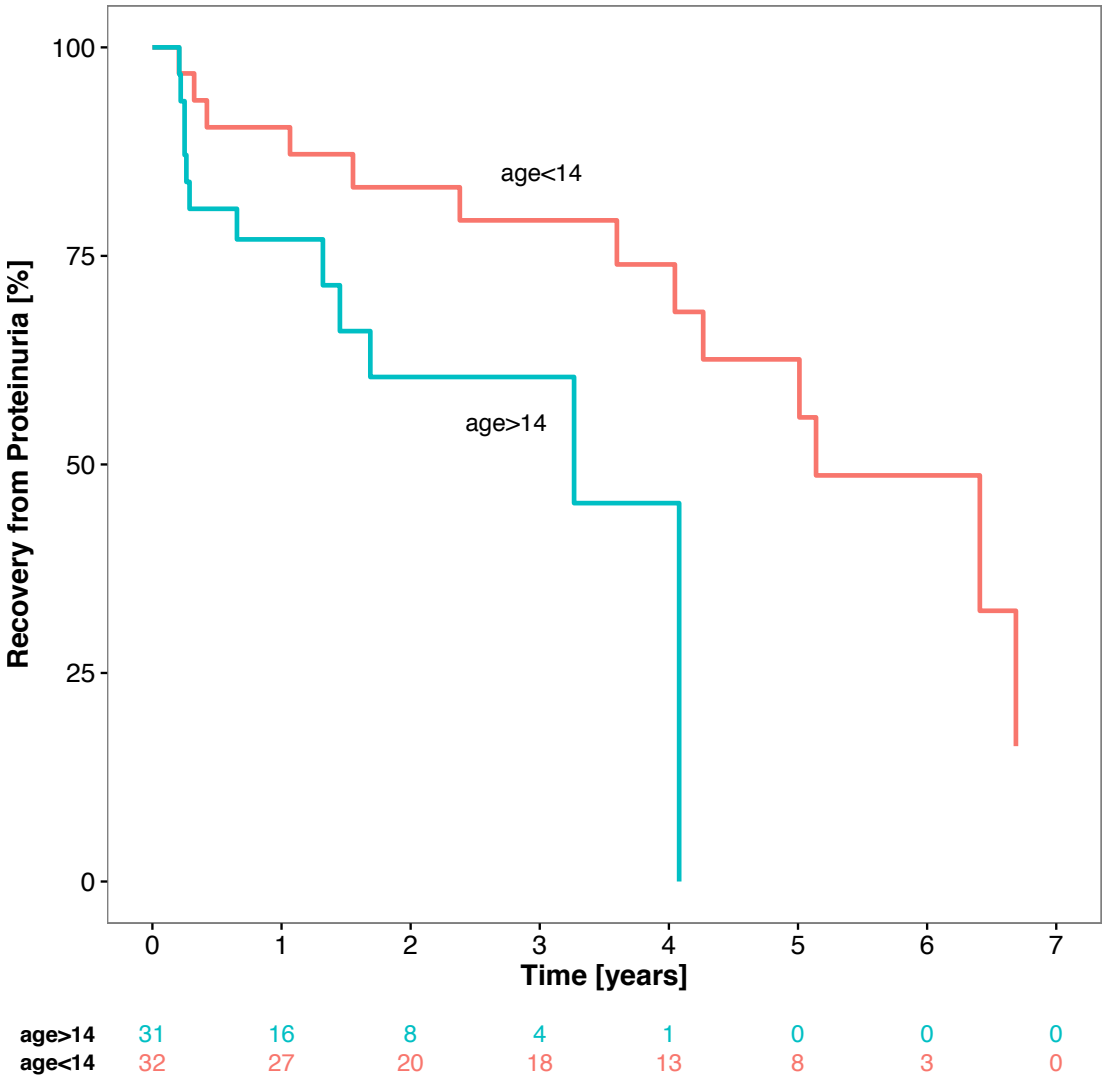


Figure 3-5: Kaplan-Meier plot for age (<14 vs. >14 years)

The Kaplan Meyer plot for eGFR (see Figure 3-6) divides the patients into two clinically relevant sub-groups with an eGFR of <80 mls/min (in the abnormal range, n=12) or >80 mls/min (in the normal range, n= 51). This plot demonstrates that at a given time, patients with a eGFR of >80 mls/min at LN onset are more likely to have recovered from proteinuria (univariate analysis *p=0.035*).

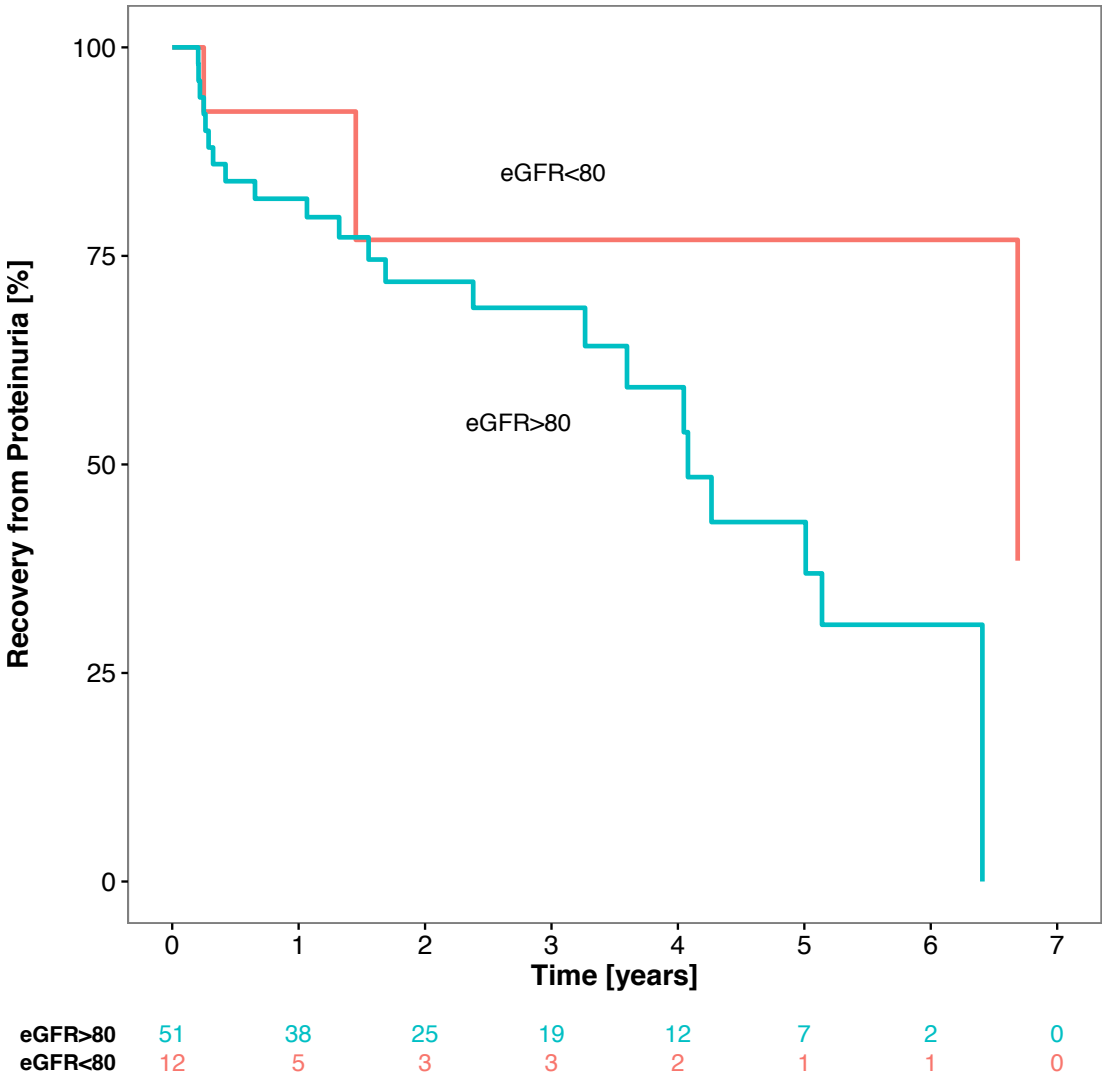


Figure 3-6: Kaplan-Meier plot for eGFR (<80 vs. >80 mls/min)

The Kaplan Meyer plot for haematological involvement (see Figure 3-7) divides the patients into those with and without haematological involvement (n=45 and 18 respectively). This plot illustrates that at a given time, patients without haematological involvement at LN onset are more likely to have recovered from proteinuria at each time point (univariate analysis *p=0.016*).

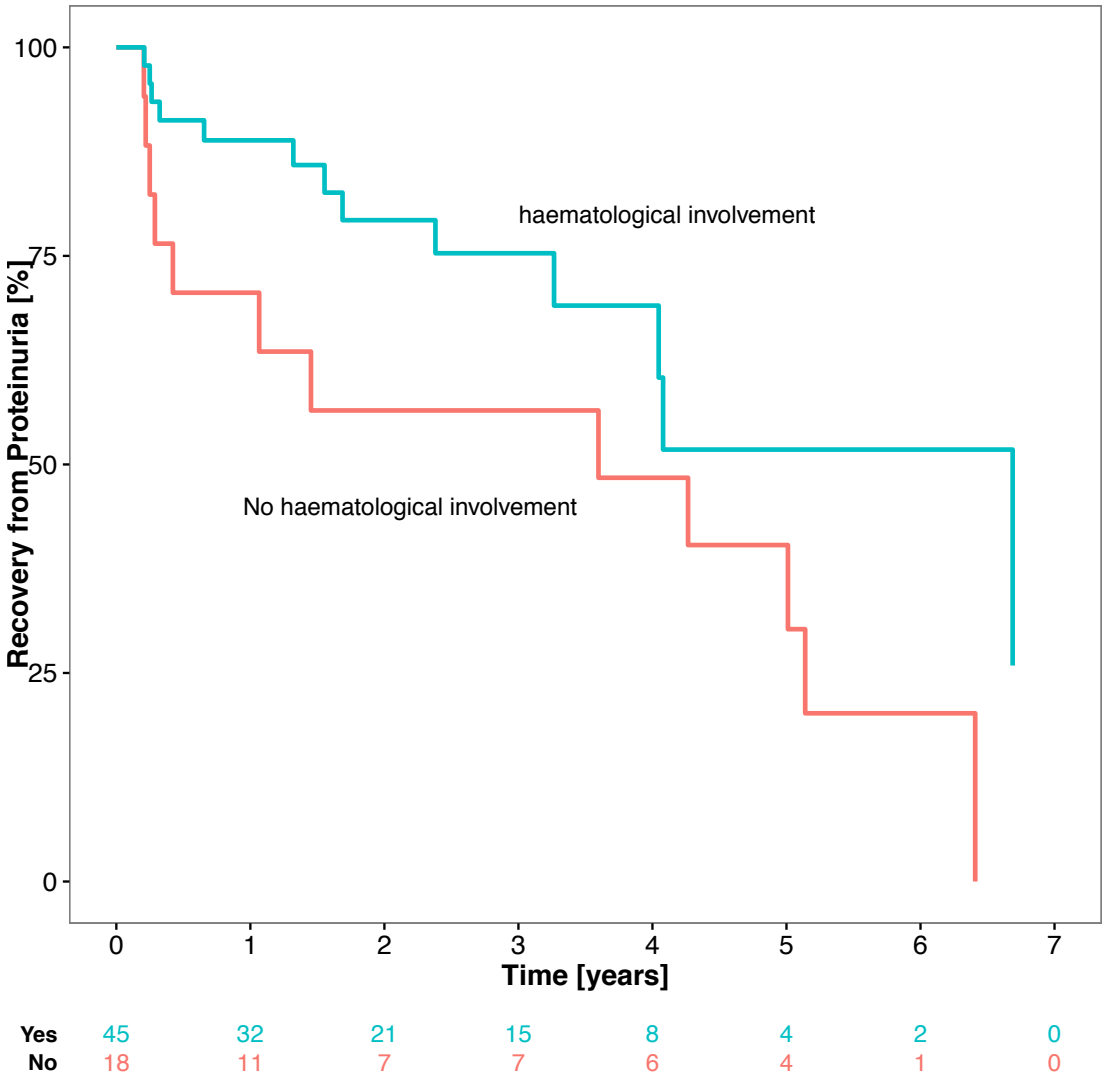


Figure 3-7: Kaplan-Meier plot for haematological involvement

3.5.4 Traditional biomarkers and identification of active LN

3.5.4.1 Patient demographics and traditional clinical biomarkers

A larger number of JSLE patients was seen in the current study (n=370), when compared to those presented in sections 3.5.1 and 3.5.3, due to inclusion of patients who were followed-up in paediatric or adult rheumatology, even if they only had a single study visit. 191 active LN and 179 inactive LN patients were investigated, with a median age of 12.7 and 12.8 years respectively at diagnosis. At the time of analysis, median disease duration for active and inactive LN patients was 1.5 [0-4.6] and 1.6 [0-5.7] years respectively. The non-renal traditional biomarker concentrations in the active and inactive LN patient groups are summarised in Table 3-10.

	Inactive LN (n=179)[1]	**Active LN (n=191)[1]**
Age at diagnosis (years)	12.7 [10.1-14.3]	12.8 [10.8-14.5]
Disease duration (years)	1.6 [0-5.7]	1.5 [0-4.6]
Female gender (n, %)	149 (83%)	159 (84%)
Caucasian ethnicity[2] (n, %)	95 (54%)	95 (51%)
Anti-dsDNA (IU/L, NA=62)	29 [7-154]	77 [13-261]
C3 (g/L, NA=31)	0.99 [0.75-1.23]	0.78 [0.43-1.08]
C4 (g/L, NA=32)	0.15 [0.08-0.20]	0.10 [0.05-0.19]
ESR (mm/h, NA=43)	18 [6-40]	40 [11-80]
CRP (mg/L, NA=64)	5 [3-7]	5 [3-7]
Haemoglobin (g/dl, NA=3)	12.1 [10.8-13.2]	11.5 [9.9-12.8]
White cells ($x10^9$/L, NA=5)	5.4 [4.2-7.1]	5.7 [4.0-8.8]
Neutrophils ($x10^9$/L, NA=5)	3.2 [2.1-4.3]	3.6 [2.4-6.4]
Lymphocytes ($x10^9$/L, NA=5)	1.3 [0.9-1.9]	1.3 [0.9-1.9]
Platelets ($x10^9$/L, NA=6)	270 [207-325]	261 [196-335]
IgG (g/L, NA=79)	13.7 [10.9-18.6]	12.0 [9.2-18.1]
IgA (g/L, NA=81)	1.8 [1.2-2.4]	1.9 [1.2-2.8]
IgM (g/L, NA=83)	1.2 [0.8-1.7]	1.0 [0.6-1.6]

Table 3-10: Key demographic data for the JSLE patients included in the traditional biomarkers study.
[1]Results expressed as median values and IQRs or absolute counts and percentages as appropriate.
[2]Ethnicity classification simplified to Caucasian or non-Caucasian.

3.5.4.2 Strength of traditional biomarkers for identifying active LN

A binary multiple logistic regression model was fitted including all non-renal traditional biomarkers, and then applying the 'StepAIC' function in R [360]. The final selected model included six biomarkers including ESR, C3, WCC, neutrophils, lymphocytes and IgG (see Table 3-11). The AUC corresponding to this final model was 0.724, demonstrating this optimal combination of traditional non-renal biomarkers to have 'fair' ability for active LN identification.

	Final model fitted by including all biomarkers in a multiple logistic regression[1]			
Biomarkers	**Co-efficient**	**Std. Error**	***p***	**AUC[2]**
ESR	0.019	0.007	*0.003*	0.724
C3	-1.035	0.488	*0.034*	
White cells	-0.699	0.423	*0.098*	
Neutrophils	0.795	0.427	*0.060*	
Lymphocytes	0.735	0.503	*0.144*	
IgG	-0.061	0.026	*0.017*	

Table 3-11: Binary logistic regression model for identification of active LN using traditional biomarkers.

[1]185 patients (88 active LN and 97 inactive LN) included in this model as patients excluded when traditional biomarker measurements were missing. [2]AUC: area under the curve.

3.5.5 Novel urine biomarker identification – HKI Multiplex Assays

3.5.5.1 Patient demographics

A total of 35 patients from the UK JSLE Cohort Study were included in HKI multiplex assay analysis, 13/35 (37%) were classed as active LN and 22/35 (63%) as inactive LN JSLE patients. Active and inactive LN patients had a median diagnosis age of 12 [8-13] and 10 [8-12] years respectively, with a median disease duration of 4.6 [2.9-6.2] and 4.5 [2.1-6.7] years at the time of biomarker analysis. All patients had a median of 5 [4-7] ACR criteria at diagnosis. 92% of the active LN and 82% of the inactive LN patients were female (see Table 3-12). Overall, 37% of patients were Caucasian, 26% Indian/Pakistani and 14% were of African origin. 9% were Caribbean or Chinese and 5% were mixed race

At a later date, 30 US Cohort patients were included; 16/30 (53%) were classed as having active LN, and 14/30 (47%) as inactive LN JSLE patients. Active and inactive LN patients had a median diagnosis age of 17 [15-19] and 15 [14-17] years

respectively, with a median disease duration of 1.7 [0.5-5.6] and 3.1 [1.2-4.8] years at the time of biomarker analysis. 71% of the active LN and 100% of the inactive LN patients were female (see Table 3-12). Overall, 55% of patients were African American, 41% Hispanic and 4% Asian.

	UK Cohort		US Cohort	
	Active LN (n=13)	**Inactive LN (n=22)**	**Active LN (n=16)**	**Inactive LN (n=14)**
Age at Diagnosis (years)	12 [8-13]	10 [8-12]	17 [15-19]	15 [14-17]
Disease duration (years)	4.6 [2.9-6.2]	4.5 [2.1-6.7]	1.7 [0.5-5.6]	3.1 [1.2-4.8]
Female	12 (92%)	18 (82%)	10 (71%)	16 (100%)
ACR criteria at diagnosis	5 [4-7]	5 [4-7]	5 [4.5-6.0]	5 [5.0-5.8]

Table 3-12: Demographic data for UK and US patients who contributed samples for HKI biomarker multiplex analysis.

3.5.5.2 Human Kidney Injury urine biomarker concentrations

Three different HKI assays were undertaken as detailed in section 3.4.5. For each assay, Table 3-13 shows the assay sensitivities in terms of the minimum detectable concentration for each biomarkers, low and high standard curve values (demonstrating the range of the assay), and the expected/actual QC values, whilst running each assay with UK and US Cohort samples. QC failures were reported when UK Cohort samples were run for GST-α , GST-π, TIMP-1 (QC2 values only, all higher than expected), however, the difference between the expected and actual QC values was minimal. A QC failure was also seen for Albumin (both QC1 and QC2) when UK Cohort samples were run. A large discrepancy was seen between the actual and expected QC1 levels (actual 10.26, minimum expected 24 ngmL), with a smaller difference seen for QC2s (actual 255.71, minimum expected 260 ngmL).

	Biomarker	Min DC[1]	Low STN[2] / High STN	Expected QC[3] range		UK Cohort actual values[4]		US Cohort actual values[4]	
				QC 1	QC 2	QC 1	QC 2	QC 1	QC 2
HKI assay 1	**Calbindin**	0.062	0.2 / 200	1.2-2.4	13-26	2.1	17.2	1.6	16.2
	GST-α[5]	0.043	0.05 / 50	0.38-0.78	3.4-7.1	0.7	**7.3**	0.6	7.1
	GST-π[6]	0.076	0.05 / 50	0.29-0.60	3.0-6.2	0.6	**6.9**	0.5	5.7
	Osteoact[7]	0.056	0.1 / 100	0.62-1.3	6.7-14	1.2	12.7	1.1	10.4
	TIMP-1[8]	0.078	0.1 / 100	0.61-1.3	6.9-14	1.3	**15.9**	1.2	10.3
	KIM-1[9]	0.014	0.05 / 50	0.30-0.63	3.3-6.8	0.6	6.5	0.6	5.0
	IP-10[10]	0.004	0.005 / 5	0.03-0.07	0.33-0.68	0.07	0.6	0.06	0.5
	Renin	0.016	0.02 / 25	0.15-0.31	1.5-3.2	0.3	2.7	0.3	2.4
	FABP-1[11]	3.061	6 / 6000	41-85	426-884	70.3	685	78.0	633
	Collagen-4	0.094	0.3 / 350	2.2-4.6	23-49	4.3	41.3	3.9	35.9
	TFF-3[12]	0.312	0.5 / 500	3.3-6.6	33-68	3.7	43.3	3.8	36.4
HKI assay 2	**EGF[13]**	0.02	0.01 / 10	0.06-0.13	0.64-1.3	0.08	0.9	0.08	0.8
	NGAL[14]	0.01	0.02 / 20	0.13-0.26	1.2-2.6	0.16	1.8	0.16	1.7
	Albumin	3.15	3.9 / 4000	24-49	260-540	**10**	**255**	24	300
	Clusterin	2.46	4.9 / 5000	29-60	306-636	33	40	33	370
	Cystatin C	0.16	0.02 / 20	0.12-0.24	1.2-2.5	0.2	1.8	0.2	1.6
	OPN[15]	0.40	0.6 / 600	4.0-8.4	38-78	4	47	5	43
	A1mG[16]	0.70	1.0 / 1000	6.1-133	66-138	6	83	6	78
HKI assay 3	**Uromod[17]**	0.06	0.1 / 100	0.60-1.25	6.4-13.2	1.2	9.4	0.7	11.4
	β2M[18]	0.02	0.1 / 100	0.30-0.63	3.0-6.2	0.5	4.3	0.4	4.9
	RBP-4[19]	0.08	0.05 / 50	0.63-1.31	6.3-13.1	1.0	9.3	0.7	9.0

Table 3-13: HKI assays 1-3: assay sensitivity, standard curve range and expected/actual quality control values.

All biomarker concentrations measured in ng/ml. [1]Min DC = minimum detectable concentration. [2]Low and high standard curve values demonstrate the standard curve range. [3]Expected quality control (QC) values provided by assay manufacturers. [4]Actual observed QC measurements from the UK and US cohorts, with those that are out of range highlighted in bold text. [5]GST-α = Glutathione-S-transferase-α. [6]GST-π = Glutathione-S-transferase-π. [7]Osteoact = Osteoactivin.[8]TIMP-1 = tissue inhibitor of

metaloproteases-1. [9]KIM-1 = kidney injury molecule-1. [10]IP-10 = Interferon gamma inducible protein 10. [11]FABP-1 = Fatty Acid Binding Protein 1. [12]TFF-3 = trefoil factor 3. [13]EGF = epidermal growth factor. [14]NGAL = neutrophil gelatinase associated lipoclain. [15]OPN = osteopontin. [16]A1mG = Alpha-1-microglobulin. [17]Uromod = Uromodulin. [12]β2M = beta-2-microglobulin. [13]RBP = Retinol-binding protein 4.

HKI-1 and 3 assay urine biomarker concentrations did not differ between active and inactive LN patients in either the UK or US Cohort (all pc=1.0, see Table 3-14). A 1:2 dilution was used when running the HKI-1 assay as per manufacturer's instructions. At this dilution FABP-1 levels fell below the lowest standard point and were extrapolated (see Table 3-13 and Table 3-14). For all other biomarkers, levels were detected in acceptable regions of the standard curve. With HKI-2, Albumin levels differed significantly between active LN and inactive LN patients (median of 522 ngmgCr [IQR 274-1631] and 46 ngmgCr [IQR 29-115] respectively, pc<0.001). No difference was seen in UK Cohort Albumin levels, although a QC failure was detected for Albumin when the UK samples were run (see Table 3-13) questioning the reliability of the results for this analyte. No other biomarkers displayed a significant difference between patient groups (see Table 3-14). For all HKI-2 assay biomarkers, patient values were detected in acceptable regions of the standard curve.

Biomarker		UK Cohort			US Cohort		
		Active LN (n=13)	Inactive LN (n=22)	p_c[1]	Active LN (n=16)	Inactive LN (n=14)	p_c[1]
HKI 1	**Calbin**[2]	3.4 [2.4-7.4]	5.4 [3.5-8.7]	1.0	0.4 [1.5-4.4]	0.2 [1.5-5.3]	1.0
	GST-α[3]	4.6 [19-99]	6.6 [19-83]	1.0	0.02 [2.4-18]	0.36 [0.9-4.0]	1.0
	GST-π[3]	2.4 [17-27]	7.4 [10-15]	1.0	1.6 [7.0-18]	1.2 [0.8-1.1]	1.0
	Osteoa[4]	0.63 [1.2-2.5]	0.89 [1.3-2.7]	1.0	0.6 [0.8-2.2]	0.60 [0.9-2.0]	1.0
	TIMP-1[5]	1.5 [3.7-16]	1.0 [1.7-3.3]	1.0	1.7 [3.9-7.4]	1.1 [1.6-3.1]	1.0
	KIM-1[6]	0.38 [0.9-3.0]	0.71 [0.9-1.5]	1.0	0.3 [0.7-1.2]	0.3 [0.4-0.7]	1.0
	IP-10[7]	0.02 [0.03-0.05]	0.02 [0.03-0.07]	1.0	0.01 [0.05-0.08]	0.0 [0.02-0.03]	1.0
	Renin	0.03 [0.06-0.16]	0.04 [0.06-0.10]	1.0	0.01 [0.03-0.03]	0.02 [0.03-0.06]	1.0
	FABP-1[8]	3.5 [12-17]	4.9 [15-32]	1.0	1.2 [5.8-16]	0.43 [1.6-6.3]	1.0
	Coll-4[9]	27 [44-63]	37 [41-54]	1.0	23 [32-47]	16 [24-32]	1.0
	TFF-3[10]	3.0 [7.8-20]	3.4 [6.5-9.0]	1.0	3.5 [6.3-21]	1.4 [2.6-5.4]	1.0
HKI 2	**EGF**[11]	42 [5-60]	53 [30-87]	1.0	18 [12-23]	21 [14-29]	1.0
	NGAL[12]	48 [16-146]	46 [19-120]	1.0	35 [15-196]	28 [12-105]	1.0
	Alb[13]	125 [58-391]	60 [51-254]	1.0	522 [274-1631]	46 [29-115]	**<0.001**
	Clust[14]	1442 [800-4124]	1581[1073-4238]	1.0	1586 [1257-4055]	2188 [845-5854]	1.0
	Cys C[15]	30 [5-54]	35 [26-54]	1.0	28 [19-32]	21 [18-36]	1.0
	OPN[16]	1506 [499-3517]	2739[1668-4419]	1.0	1246 [911-2065]	999 [792-1431]	1.0
	A1mG[17]	373 [313-3324]	836 [377-1572]	1.0	741 [228-1565]	339 [190-855]	1.0
HKI 3	**Urom**[18]	1954 [358-3701]	2976[1161-8992]	**0.22**	812 [502-1565]	796 [578-2669]	1.0
	β2M[19]	258 [28-724]	151 [118-219]	1.0	1012 [563-2032]	1362 [660-2032]	1.0
	RBP4[20]	651 [457-2611]	813 [616-1340]	1.0	187 [47-497]	401 [101-976]	1.0

Table 3-14: HKI Multiplex panel 1, 2, 3 urine biomarker concentrations in UK and US Cohort patients.

Biomarker concentrations standardised to urinary creatinine (ngmgCr) and expressed as median values and IQRs. [1]Mann Whitney U tests used to compare biomarker concentrations between active and inactive LN patients, and Bonferroni adjustment applied to account for multiple testing within each assay (HKI 1 = 11 tests, HKI 2 = 7 tests, HKI 3 = 3 tests), and corrected *p-values* (p_c) reported. Significant differences highlighted with bold text. [2]Calbin = calbindin. [3]GST-α or π = Glutathione-S-transferase-α or π. [4]Osteoa = osteoactivin. [5]TIMP-1 = tissue inhibitor of metaloproteases-1. [6]KIM-1 = kidney injury molecule-1. [7]IP-10 = Interferon gamma inducible protein 10. [8]FABP1 = Fatty Acid Binding Protein 1. [9]Coll-4 = collagen 4. [10]TFF-3 = trefoil factor 3. [11]EGF = epidermal growth factor. [12]NGAL = neutrophil gelatinase associated lipoclain. [13]Alb = albumin. [14]Clust = clusterin. [15]Cys C = cystatin C. [16]OPN = osteopontin. [17]A1mG = Alpha-1-microglobulin. [18]Urom = uromodulin. [19]β2M = beta-2-microglobulin. [20]RBP = Retinol-binding protein 4.

3.6 Discussion

Using existing clinical data from the UK JSLE Cohort Study, the main aim of this chapter was to explore the ability of clinical and demographic factors to differentiate patients with and without LN. In addition, it sought to determine whether such factors play a role in predicting the development of LN, the time to recovery from proteinuria following an LN flare, and their utility in identifying active LN. These analyses will in turn potentially help inform clinicians to be aware of patients who have an increased risk of future LN development, or slow proteinuria recovery from active LN.

The role of traditional biomarkers in ongoing LN monitoring was found to be limited. The use of bio-banked urine samples was therefore adopted to screen for new urine biomarkers of LN activity using commercially available HKI assays.

Clinical features characterising patients with LN at baseline. A total of 37% of UK JSLE Cohort Study patients were found to have active LN at baseline, with a further 17% of patients developing LN after a median of 2.04 years [IQR 0.8-3.7]. These data highlight the 'high risk' period for LN development. Overall, 54% of patients were identified as ever having had LN. Other studies have reported 50-80% of JSLE patients to be affected by LN [28,29,37,108]. Differences in LN prevalence between cohorts may be due to variations in patient ethnicity and the method used for active LN identification. Watson et al looked at LN prevalence within the UK JSLE Cohort between 2006 and 2011, demonstrating 36% of patients to have ACR defined LN and 80% to have LN if defined using disease activity assessed by the renal BILAG score [28]. Within the current study, LN patients were defined on the basis of biopsy and/or a renal BILAG score of A or B, whereas in the previous study renal BILAG C patients (mild renal disease) were also included in the renal BILAG defined LN group [28]. Hiraki et al demonstrated an LN prevalence of 37% in children, using the US Medicaid Analytic database for case identification [29], whereas Brunner at al reported a 78% LN prevalence in the Cincinnati JSLE cohort when LN was defined as a renal SLEDAI domain score of >0 [36]. Sule et al studied another US JSLE cohort from Philadelphia, demonstrating 30% of patients to have developed LN during one month of diagnosis (defined by biopsy or meeting the ACR criteria for LN), with a further 30% developing LN within a median of 3.2±2 years [362].

Within the current study, six clinical and demographic factors were found to differ significantly between those with and those without LN at baseline. These were: the patient's first ACR score, presence of severe hypertension and levels of proteinuria, serum creatinine, ESR and C3. Both ACR score and C3 levels at baseline were also identified as significant predictors for subsequent LN development. Such factors were also investigated by Sule et al [362], identifying low serum albumin and positive anti-dsDNA as differentiating LN and non-LN patients. They also identified isolated sterile pyuria and low serum albumin as predictors of future LN [362]. Their study included predominantly African American patients and a smaller samples size than the UK study detailed within this chapter.

Predictors of recovery from proteinuria following an LN flare – A total of 39% of patients were shown to recover from proteinuria following an LN flare during the study period, within a median of 17 months (IQR 3.5-49.2) with the remaining 61% continuing to have proteinuria after a median of 22 months (IQR 12.1-41.1). This observation provides useful information on the length of time necessary for proteinuria to resolve following an LN flare in clinical practice, as opposed to a clinical trial setting. This study included more patients than the previous study discussed above (characterising patients with LN at baseline and identifying factors predicative of subsequent LN development), as patients did not have to be recruited to the UK JSLE Cohort Study within one year of JSLE diagnosis to be eligible. The spot UPCR or UPCR ratio cut off chosen for inclusion in this study was >50ng/mmol (for either quantification method), corresponding with the minimum level required to score a renal BILAG score of B. The spot UPCR or UACR ratio cut off for recovery was chosen to be <25mg/mmol, on the basis that such a proteinuria level would not be adequate to even score a renal BILAG C score.

The frequency and severity of LN is known to be increased in patients of African, Hispanic and Asian origin [30,32,222,281]. It is therefore not surprising that ethnicity differentiated patients who did and did not recover from proteinuria following an LN flare (*p=0.007*). Patients of non-Caucasian ethnicity were grouped due to the small numbers seen in individual ethnic minority groups, however, further assessment of such groups could reveal additional differences. Reductions in eGFR usually occur

following significant renal damage and may be preceded by a period of hyper-filtration [377-379]. It is therefore also not unexpected that low eGFR was found to differentiate those who did/did not recover from proteinuria following LN flare, and bear influence on time to recovery. Patient use of azathioprine at the time of LN onset differentiated patients who did from those who did not recover from proteinuria during follow-up. Azathioprine has been shown to display equivalence with MMF as maintenance treatment for LN [168]. However, it is not usually used as an induction treatment, having been shown to be inferior to MMF and cyclophosphamide when used for induction [380]. As this study collects historic data it may be that some patients received Azathioprine induction treatment. Younger patient age at the time of LN onset was shown to influence time to recovery from proteinuria, likely reflecting the more severe disease phenotype and potential genetic predisposition to JSLE/LN seen in younger patients [28,36,40,381-384]. Haematological involvement may affect the ability to intensify immunosuppressant treatment due to concerns about treatment toxicity, thereby differentiating patients who did/did not recover from proteinuria. There is no clear evidence as to why cardio-respiratory involvement should differentiate those who did/did not recover, however it can be speculated that associated factors like hypertension or poor renal perfusion could play a role. Awareness of these factors is important for stratification of patients at the time of LN onset, and considering the intensity and duration of early immunosuppressive therapy.

Traditional biomarkers and LN monitoring – This study included a larger number of active LN patients (191/370) compared to the previous studies looking at clinical predictors for LN (155/331) and recovery of proteinuria following an LN flare (64/350). The results from the current study included data from a single patient visit whereas the first two studies featured more stringent inclusion criteria, e.g. requirement for recruitment to the UK JSLE Cohort within 1 year of diagnosis, >1 follow-up visit, serial proteinuria data, patients followed up within paediatric practice only, thus reducing the number of available patients.

ESR, C3, WCC, neutrophils, lymphocytes and IgG were found to contribute significantly to the optimal model for active LN identification, displaying ‘fair’ test accuracy (AUC 0.724). These results complement those of an adult SLE study which showed decreases in C3 to be associated with renal disease activity [368]. In a

prospective study serially monitoring C3/C4 levels in adult SLE patients, C4 was demonstrated to deteriorate before C3, starting 25 and 20 weeks respectively before the LN flare becoming clinically detectable [385]. Together with anti-dsDNA antibody levels, C3/C4 levels have been shown to have a good negative predictive value for active LN in a 6-year prospective study of 228 LN patients [386]. Our findings relating to ESR are also in keeping with those of a recent study that showed ESR to be correlated with renal involvement according to the Lupus Activity Index VAS and the SLEDAI score. ESR was also correlated with haematuria and proteinuria. Over time, a change in ESR between two visits was highly correlated with a concurrent change in the renal VAS [387].

Anti-dsDNA antibody levels did not feature in the final multiple logistic regression model. Similar results have previously been reported in a study assessing anti-dsDNA antibody and C3/C4 levels, in 53 adult SLE patients 3-9 months preceding a flare. For all three tests, sensitivity and specificity for predicting renal and non-renal flares was in the region of 50% and 75% respectively, with positive and negative likelihood ratios being close to 1.0, suggesting little clinical value as a routine test [259]. In contrast, high titres of anti-dsDNA have been shown to differentiate proliferative from non-proliferative LN at the time of renal biopsy [258], with some studies concluding that increased anti-dsDNA antibody levels should prompt pre-emptive treatment due to anti-dsDNA's strong ability to predict SLE flares [388]. These conflicting results may be due to differences in sample size, disease activity measures and the frequency of biomarker testing.

Overall, the ability of traditional non-renal immunological, haematological and inflammatory biomarkers for identifying active LN flares was rather disappointing. It is recognised however, that in clinical practice such markers would be considered alongside the information gained from traditional renal biomarkers (e.g. proteinuria, blood pressure, serum creatinine, GFR, urine sediment, and recent biopsy findings). As the definition of active LN was based on the composite renal BILAG score (calculated from the above renal biomarkers) it was not possible to add such traditional renal biomarkers as covariates within the regression model analysis. This would be necessary to provide the overall picture of the diagnostic tests currently available to the clinician. Further large, prospective longitudinal studies are required to look more

closely at the relationship between all traditional biomarkers and changes in LN activity using different outcome measures over time.

Novel urine biomarker identification using HKI multiplex assays – A total of 21 urine biomarkers previously implicated in drug induced HKI were assessed in 40 UK JSLE Cohort Study and 30 US Cohort patients, using commercially available multiplex assays. The only significant difference in biomarker levels between patient groups was for Albumin in the US Cohort ($p_c<0.001$). No difference was seen in UK Cohort Albumin levels, although a QC failure was detected for Albumin when these samples were run (see Table 3-13). Other QC failures were reported when UK Cohort samples were run for GST-α , GST-π, TIMP-1 (QC2 values only). Ideally these assays would be repeated but given the cost, that urinary Albumin is already known to be elevated in LN [344,345] and that differences in GST-α , GST-π, TIMP-1 were not seen between US active and inactive LN patient groups, therefore repeating these assays was not considered worthwhile.

Of the 21 markers explored by the HKI assays, only IP-10, NGAL, KIM-1, A1mG and uromodulin have previously been investigated in LN urine. In keeping with the results of this study, previous work using UK JSLE Cohort urine samples and ELISA techniques also failed to demonstrate a significant difference in urinary IP-10 levels between active and inactive LN patients [72]. NGAL has previously been shown to predict LN flare within the UK JSLE Cohort Study [74], but, like the current study, was unable to differentiate active and inactive patients on a cross-sectional basis [389]. Further investigation of its role is warranted in larger patient numbers/using distinct techniques for NGAL measurement.

KIM-1, a marker of proximal tubular damage has been assessed in 37 active LN and 20 inactive LN patients. 24 hour KIM-1 levels (ng/24h) were identified to be significantly higher in active LN than inactive LN urine samples [390]. This study differed from the current multiplex study where spot/random urine samples were included. Urinary KIM-1 has also been demonstrated to be part of a urine biomarker panel predictive of renal biopsy NIH Activity Index in 47 JSLE patients [348], however, its ability to differentiate active and inactive LN in isolation was again not investigated. A1mG, a glomerular marker, is one of a number of proteins associating

with ISN/RPS LN class and chronicity within an adult proteomic study [343]. In contrast to the current study, Uromodulin (also known as Tamm-Horsfall Glycoprotein) has been shown to be lower in patients with active LN than inactive LN patients in a small adult study including 15 active and 12 inactive LN patients [337]. From these HKI assays, it is clear that a more targeted approach to novel biomarker analysis is required, validating markers with good existing evidence in adult or paediatric SLE (see section 1.3), with the aim of optimising such tests.

3.7 Summary

Using existing clinical data from the UK JSLE Cohort Study, this chapter has explored the ability of basic clinical and demographic factors to stratify JSLE patients as high or low risk for LN at the time of their initial presentation and during the disease course. It has demonstrated that proteinuria can be persistent following an LN flare in children within a real world clinical setting. The characteristics of patients who are at increased risk of having prolonged proteinuria following an LN flare are described. Early reduction in proteinuria following initiation of immunosuppressive therapy has been shown to be associated with improved longer term renal outcomes in adult SLE, therefore, appreciation of those at risk of prolonged proteinuria may help the clinician to change, or fine-tune the intensity and duration of early immunosuppressive therapy. Paediatric rheumatologists and nephrologists rely heavily upon non-invasive blood and urine markers during ongoing LN monitoring. This study highlights that at best, haematological, immunological and inflammatory markers only display 'fair' ability for differentiating active from inactive LN. This significantly limits their use in this regard.

Urine biomarkers that have been developed for identifying drug-induced renal damage and nephrotoxicity have not proven in this present study to be useful in an LN setting. This highlights that a more targeted approach to novel biomarker analysis is required in LN, with the aim of accelerating the translation of such tests into clinical practice.

3.8 Conclusions

- Active LN was demonstrated at baseline in 37% of UK JSLE Cohort Study patients, with a further 17% developing LN after a median of 2.04 years [IQR 0.8-3.7], highlighting the 'high risk' LN period.
- First ACR score, presence of severe hypertension, levels of proteinuria, serum creatinine, ESR and C3, differed significantly between those with and without LN at baseline. ACR score (>5) and C3 (<0.9g/L) at baseline were identified as significant risk factors for subsequent LN.
- 39% of patients were shown to recover from proteinuria following an LN flare during the study period, within a median of 17 months (IQR 3.5-49.2). At a given time, patients who were older (>14 years), had a normal eGFR (>80 mls/min) and no haematological involvement at the time of LN onset, were more likely to have recovered from proteinuria following an LN flare.
- At best, the optimal haematological, immunological and inflammatory markers (namely: ESR, C3, WCC, neutrophils, lymphocytes and IgG) displayed only a 'fair' ability for identifying active LN, limiting their use in this regard.
- Urine biomarkers of drug-induced renal damage/nephrotoxicity have not proven useful in an LN setting.
- A more targeted approach, validating the most promising biomarkers to date is required, with the aim of accelerating their translation into clinical practice.

4 Targeted analysis of urine biomarkers and development of a urine biomarker panel for identifying active LN

4.1 Introduction

In Chapters 1 and 3, evidence from the existing literature and UK JSLE Cohort Study demonstrated that conventional markers of JSLE disease activity such as anti-dsDNA, C3/C4, ESR and full blood count parameters are poor at adequately identifying the occurrence of LN flares.

In Chapter 3, screening of urinary biomarkers previously implicated in kidney injury models and/or drug toxicity studies was undertaken in urine samples from patients in the UK JSLE Cohort Study, showing that such markers are not informative in an LN setting.

Building on this, Chapter 4 includes targeted analysis of promising urine biomarkers identified from the existing literature (see section 1.3), assessing whether combinations of such markers can improve active LN identification, over and above individual markers.

4.1.1 Promising individual urine biomarkers in LN

The existing evidence for urinary biomarkers in LN has been reviewed extensively in Chapter 1. From this, the most promising biomarkers to date warranting further evaluation appear to be MCP-1, NGAL, VCAM-1, AGP, CP, LPGDS and TF (see section 1.3). Urinary MCP-1, NGAL and VCAM-1 have been shown to individually outperform both traditional and novel serum biomarkers for identification of LN, with extensive evidence for MCP-1 and NGAL within paediatric studies (see sections 1.3.4.2.1 and 1.3.4.2.2) [74,297,304,328,329]. There is strong evidence for VCAM-1 in adult SLE but this had not been investigated in JSLE and warrants investigation (see section 1.3.4.2.3) [297,308,326-329]. AGP, TF and CP have been identified within two JSLE proteomic studies [290,345] and validated with alternative forms of protein binding techniques (ELISA and immunonephelometry), adding strength to the initial proteomic findings (see section 1.3.5.2 and Table 1-12) [318,348]. Within the UK

JSLE Cohort Study, urinary MCP-1 and AGP have been shown to differentiate patients with active LN, inactive LN and HCs [72]. Longitudinally, MCP-1 was found to be a good predictor of improving disease (AUC = 0.81), and NGAL was a fair biomarker of worsening disease [74]. Adult-onset SLE studies have indicated similar results [299,300].

4.1.2 Biomarker panels

To date, no individual urine biomarker has achieved an 'excellent' predictive ability (AUC>0.9) for identifying active LN on its own. The individual constituents of the JSLE LN proteomic signature described by Suzuki et al, including TF, CP, AGP, LPGDS, albumin and albumin-related fragments [290,345], displayed fair to good ability to identify SLEDAI or BILAG-defined active LN (AUC values between 0.68-0.81), with the AUC improving to 0.85 when all of the biomarkers were considered together [290]. This observation highlighted the potential value of combining urinary biomarkers to improve diagnostic accuracy. A subsequent study demonstrated that combinations of urine biomarkers improve prediction of renal function loss in JSLE and SLE [391].

The findings of proteomic biomarker studies are often criticised due to poor reproducibility. It is therefore important that the validity of biomarkers identified using these methods is assessed on distinct groups of patient cohorts, providing independent verification (see Table 1-12) [282,284]. Certain proteomic technology platforms may preferentially detect specific types of protein (e.g. high or low molecular weight), restricting their ability to actually undertake completely hypothesis free biomarker screening [392]. In the above proteomic studies by Suzuki et al [290,345], it is interesting that previously identified promising biomarkers such as MCP-1, VCAM-1 and NGAL were not actually detected. This may in part be due to limitations of the proteomic techniques used. LN phenotype and severity can also vary with age, ethnicity and race [1,32,281], therefore it is also important to cross-validate previous results in ethnically distinct childhood SLE cohorts.

4.1.3 Effect of biomarker exposure on human podocytes

Having determined a range of key biomarkers which are present in the urine of patients with active LN, a key question was whether these biomarkers originated from the kidneys themselves, reflecting local injury, or were being filtered through the glomerulus. This is currently under investigation by cell biologists within the University of Liverpool EATC for children. To explore this further, their impact upon a conditionally immortalised human podocyte cell line (see section 2.2.7) [358] was investigated as part of the current study, to gain an insight into whether such biomarkers may also be implicated in the pathogenesis of LN.

In LN there is known to be increased urinary excretion of podocytes (identified in urine sediment), coupled with decreased expression of podocyte markers within kidney biopsies from LN patients [393]. Podocytes (see sections 1.1.7 and 2.2.7) are highly differentiated cells that are in direct contact with the urinary space and glomerular basement membrane, overlying the glomerular capillaries. They are formed of a body with extending major processes that branch further into intricately interdigitated foot processes, separated by a slit diaphragm. Although podocytes form the outer part of the glomerular filter, they are the cells that are most commonly affected during nephrotic diseases [394]. Loss of these cells due to apoptosis (or necrosis) would lead to their appearance in the urine, gross structural damage to the glomerulus and significant defects in the filtration barrier. It is therefore of relevance to assess in the first place, the effect of active synthetic or recombinant forms of these biomarkers in relation to podocyte apoptosis.

A variety of cytokines, growth factors and their receptors have been shown to be produced by podocytes as part of LN and other kidney diseases [395,396]. Within our research group, podocytes have been shown to produce the chemokine MCP-1 in response to IFN-γ activated macrophage media [359]. Similarly, in diabetic nephropathy in-vitro models, high glucose levels have been shown to induce MCP-1 production [396]. Podocytes have also been shown to produce TWEAK [397], TNF-α [396], IL-8 [398], IL-6 and IL-16 [399]. Following exposure of podocytes to active synthetic or recombinant forms of biomarkers, it would therefore be important to

undertake cytokine multiplex analysis, to examine whether a range of cytokines, chemokines and their receptors are produced.

4.2 Hypotheses

- That a combination of novel biomarkers, identified through proteomic and candidate urine biomarker studies will improve the identification of active LN compared to those without active LN in children on a cross-sectional basis when compared to traditional biomarkers, as part of developing a potential 'LN urinary biomarker panel'.
- That such urinary biomarkers are implicated in the pathogenesis of LN, inducing apoptosis and cytokine production in human podocytes.

4.3 Aims and objectives

The primary aim of this study was to assess if combining novel biomarkers can improve active LN identification. The secondary aim was to assess if addition of traditional JSLE disease activity data to a urine biomarker panel could help to further improve identification of active LN. The final aim was to assess whether the most promising urinary biomarkers for active LN identification induced apoptosis and cytokine production by human podocytes.

The specific objectives of this chapter are:

1. To assess the performance of individual promising urine biomarkers for the identification of active LN in UK JSLE Cohort Study patients.

2. To assess if combining novel urine biomarkers in a biomarker panel, can improve identification of active LN, over and above individual biomarkers.

3. To evaluate the effect of adding traditional biomarkers data to such a urine biomarker panel, to see if this further improves identification of active LN.

4. To assess urine biomarker levels in patients with and without active extra-renal JSLE disease.

5. To assess podocyte apoptosis using a Caspase-Glo® 3/7 assay following exposure to different concentrations of the most promising urine biomarkers for active LN identification.

6. To simultaneously quantify 41 different cytokines, growth factors and their receptors in podocyte supernatant following exposure to the most promising biomarkers previously identified in LN urine, using a magnetic bead panel multiplex assay.

4.4 Specific methods

4.4.1 Patients

A subset of the children participating in the UK JSLE Cohort Study (see section 2.1.1) [28] were recruited, comprising all JSLE patients contributing to the UK JSLE Cohort Study recruited from Alder Hey Children's NHS Foundation Trust, Liverpool, and Great Ormond Street NHS Hospital for Children, London, UK between 2010 and 2014, in whom urine samples were collected. Exclusion criteria included patients with UTI or patients in whom no urine samples were collected. Patients were categorised as active LN and inactive LN according to the renal domain of BILAG disease activity score (see section 2.1.1.4).

4.4.2 Extra-renal disease activity classification

Within the inactive renal group, patients were subdivided further as having 'any active extra-renal involvement' (BILAG A/B in the following BILAG domains: constitutional, mucocutaneous, neuropsychiatric, musculoskeletal, cardiorespiratory, gastrointestinal, ophthalmic or haematological, see Appendix 6) or 'no extra-renal involvement' (BILAG score of D or E in all extra-renal domains) to allow assessment of biomarker levels according to whether extra-renal JSLE disease activity is present.

4.4.3 Laboratory techniques

4.4.3.1 Biomarker quantification

Urine samples were collected and stored as per section 2.2.5. Pre-coated ELISA kits were used for quantification of CP (St Charles, Assay Pro, USA), TF (GenWay, San Diego, USA), LPGDS (BioVendor, Brno, Czech Republic), AGP and MCP-1 (R&D Systems Ltd, Minneapolis, USA) as per the manufacturer's instructions. These ELISA kits were already commercially validated for use with urine samples, therefore assessment of different assay buffers and spike recovery was not undertaken in-house. The required sample dilutions, LOD and ability to freeze thaw samples were however determined in-house. Further details on standard ELISA protocols and the in-house validation of these assays is provided in sections 2.2.2 and Table 2-2 and Table 2-3). Urinary NGAL and creatinine concentrations were measured using commercially available chemiluminescent microparticle Abbott Architect clinical immunoassays (Abbott Laboratories, Texas, USA) in Alder Hey Children's Hospital lab (see section 2.2.3). All biomarker results were standardised for urinary Cr concentration and presented in units per milligram creatinine (mgCr).

4.4.4 Effect of biomarker exposure on human podocytes

Full details of the podocyte culture techniques employed are detailed in section 2.2.7.2. In both the apoptosis and cytokine production experiments, podocytes were cultured until they were terminally differentiated at 37^{o}C, washed twice with sterile PBS and then exposed to either 'high' or 'low' concentrations of biomarker for 48 hours, in keeping with previous work within our laboratory [359]. The cells were then either assessed for apoptosis using a Caspase-Glo® 3/7 assay (Promega corporation, UK, see below) or the podocyte supernatant was removed, centrifuged and stored at -70^{o}C until required for cytokine analysis. The 'high' biomarker concentration corresponded to the median biomarker level detected in the urine of active LN patients from the UK JSLE Cohort Study (not corrected for urinary creatinine), and the 'low' concentration matched that of inactive LN patients (see Table 4-1 and section 4.5.2). The impact of all biomarkers together at 'high' +/- 'low' levels was also assessed. The most promising biomarkers from the UK JSLE Cohort study were chosen for inclusion in these experiments, namely AGP, CP, LPGDS and TF. Each experiment was repeated three times.

Biomarker	High concentration	Low concentration	Details of biomarker used in experiments
AGP	20,600 ng/ml	300 ng/ml	Active native purified human AGP, full length protein, ABCAM, UK
Cerulopasmin	4700 ng/ml	700 ng/ml	Active native purified CP protein, Antibodies-Online, UK
Transferrin	4900 ng/ml	1200 ng/ml	Active recombinant TF protein, Antibodies-Online, UK
LPGDS	1200 ng/ml	300 ng/ml	Active recombinant LPGDS Cayman Chemical, USA

Table 4-1: Concentrations and manufacturers of biomarkers applied to podocytes in the in-vitro experiments

4.4.4.1 Podocyte apoptosis and biomarker exposure

The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase-3 and 7 activities in cultures of adherent cells, and is designed for use with multi-well plate culture systems. Caspase 3/7 are members of the cysteine aspartic acid-specific protease (caspase) family which have key effector roles in apoptosis. The assay provides a luminogenic caspase-3/7 substrate which when added to the podocytes, causes cell lysis and is subsequently cleaved in the presence of caspase 3/7, resulting in the production of a luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase 3/7 activity present. The caspase and luciferase enzyme activities reach steady state so that the luminescent signal peaks after one hour (see Figure 4-1 for an overview of the assay). The Caspase-Glo® 3/7 Assay has been used in previous studies looking at apoptosis in the podocyte cell line included in this study [400,401]. Podocytes were cultured in white walled tissue culture plates for the luminescence assay and concurrently in a normal clear tissue culture plate, in order to be able to monitor the cells and assess the degree of differentiation by light microscopy. The full range of in-vitro test conditions for each Caspase-Glo® 3/7 Apoptosis Assay experiment were as follows:

1. Caspase 3/7 Glo® reagent alone (blank)
2. Untreated podocytes (negative control)
3. Podocytes + high AGP levels
4. Podocytes + low AGP levels

5. Podocytes + high CP levels
6. Podocytes + low CP levels
7. Podocytes + high TF levels
8. Podocytes + low TF levels
9. Podocytes + high LPGDS levels
10. Podocytes + low LPGDS levels
11. Podocytes + high CP + AGP + LPGDS + TF
12. Positive control (apoptotic neutrophils extracted previous day, see section 2.2.6.3)

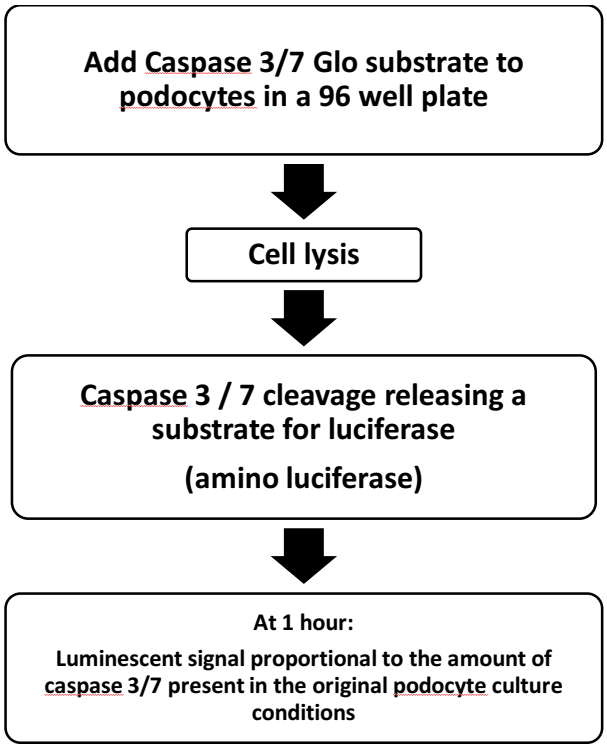


Figure 4-1: Caspase-Glo® 3/7 Apoptosis assay overview

4.4.4.2 Podocyte cytokine production and biomarker exposure

Using an HCYTOMAG-60K human cytokine/chemokine magnetic bead panel multiplex assay (Merck Millipore, USA), 41 different cytokines, growth factors and their receptors were simultaneously quantified in podocyte supernatant following exposure to high and low biomarker concentrations as detailed above. The markers quantified by the HCYTOMAG-60K multiplex assay included; Eotaxin, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 1 receptor antagonist (IL- 1RA), Macrophage inflammatory protein (MIP-1α), transforming growth factor β (TNF β), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), FMS-related tyrosine kinase 3 ligand (FLT-3L), Fractalkine, chemokine (C-X-C motif) ligand 1 (GRO), MCP-3, macrophage derived chemokine (MDC), Platelet-derived growth factor-AA (PDGF-AA), soluble cluster of differentiation 40 ligand (sCD40L), MCP-1, EGF, IFN-α, IFN-γ, IL-10, IL-12P40, IL-12P70, IL-13, IL-15, IL-17A, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MIP-1β, RANTES, TNFα, TGF-α, PDGF-AB/BB, and IL-9. The analysis was carried out an a MAGPIX™ array reader (Merck Millipore, USA) as per the manufacturer's instructions (see section 2.2.4 for further details on multiplex techniques and procedures). The full list of experimental conditions is detailed below:

1. Podocytes alone (negative control)
2. AGP high
3. AGP low
4. Ceruloplasmin high
5. Ceruloplasmin low
6. LPGDS high
7. LPGDS low
8. Transferrin high
9. Transferrin low
10. All biomarkers together – LOW
11. All biomarkers together – HIGH
12. Positive control – IFN-γ activated macrophage media (see section 2.2.6.2)

4.4.4.3 Positive controls in apoptosis / cytokine production experiments

The positive controls varied according to the experiment. For the apoptosis experiments, neutrophils were isolated using a Hetacept/Histopaque (Sigma-Aldrich, UK) isolation method and cultured overnight (see section 2.2.6.3). Neutrophils are known to be short lived when cultured, with a high proportion undergoing apoptosis which can be confirmed by flow cytometry using Annexin V staining (see Figure 2-6). For cytokine production experiments, the positive control comprised of podocytes exposed to supernatant from IFN-γ activated human macrophages (see section 2.2.6.2), as such conditions have previously been shown to cause MCP-1 production by the podocyte cell line [359].

4.4.5 Statistical analysis

4.4.5.1 Cross-sectional urine biomarker analysis

Summary statistics for demographics, baseline clinical data and biomarker data were provided in terms of median values and IQRs. Pearson's chi-square test (binary data) and univariate logistic regression (quantitative data) were used to check for differences in demographic and clinical factors between different patient groups. Due to the number of factors explored, a Bonferroni adjustment was applied to account for multiple testing (32 comparisons).

Mann Whitney U tests were used to compare biomarker concentrations between active and inactive LN patients. A Bonferroni adjustment was again applied to account for multiple testing (7 comparisons). A binary logistic regression model was fitted to assess for association between a combination of biomarkers and LN status (outcome: LN active=1; LN inactive=0). All novel biomarkers (log-transformed) were included in an initial model and the 'stepAIC' function in R [360] applied to select a final model. The AUC for the final model was calculated. Each of the remaining novel biomarkers were then added into the final model in turn, in order of statistical significance according to the original model including all novel biomarkers, and the AUC calculated. This allowed exploration of the effect of each biomarker on the model's AUC. Traditional biomarkers found to be significantly associated with active/inactive LN status in univariate analysis were then added to the model and the AUC again calculated. AUC values of 1.0–0.9, 0.9–0.8, 0.8–0.7, 0.7–0.6, 0.6–0.5 were considered "excellent, good, fair, poor and fail" respectively [402].

To assess biomarker levels according to whether extra-renal JSLE disease activity was present, log biomarker levels from inactive LN patients with 'any active extra-renal involvement' were compared with inactive LN patients with 'no extra-renal involvement' using a Mann Whitney U test. Due to the six biomarkers explored, a Bonferroni adjustment was applied to account for multiple testing, and the Bonferroni corrected *p-value,* p_c reported.

4.4.5.2 Effect of biomarker exposure on human podocytes analysis

Descriptive statistics (mean and SEM) quoted for Caspase 3/7 luminescence readings. Distribution of the data assessed using Shapiro-Wilks tests. ANOVA analysis with Dunnets post hoc test used to assess for significant differences in apoptosis between podocytes alone and all experimental conditions. For the HCYTOMAG assay, median values and IQRs were quoted. Multiple t-tests with 5% false discovery rate used to identify cytokine levels differing significantly between podocytes alone and when exposed to individual conditions.

All data analysis and generation of graphical illustrations was undertaken using SPSS Ltd version 21.0, R version 3.1.1 [360] and GraphPad Prism version 6.0.

4.5 Results

4.5.1 Clinical and demographic data

The study cohort consisted of 61 JSLE patients, 15 (25%) were classed as JSLE active LN (2/15 renal BILAG score=A, 13/15=B) and 46 (75%) as JSLE inactive LN (27/46 renal BILAG score=D, 19/46=E). JSLE patients with active and inactive LN had a median age of 15.8 [14.8-17.1] and 15.4 [13.8-17.5] years respectively, with a disease duration of 2.8 [0.7-3.9] and 2.4 [0.8-4.8] respectively at the time of biomarker analysis. 86.7% of the active LN and 62.5% of the inactive LN patients were female. There was no difference in patient ethnicity between patient groups ($p_c = 1.0$). All JSLE patients had a median of 5 ACR classification criteria at diagnosis [IQR 4-7] (see Table 4-2).

		Active LN (n=15)[1]	Inactive LN(n=46)[1]	p_c[2]
Current age (years)		15.8 [14.8-17.1]	15.4 [13.8-17.5]	1.0
Disease duration (years)		2.8 [0.7-3.9]	2.4 [0.8-4.8]	1.0
Gender (Female n, %)[3]		13 (86.7%)	35 (62.5%)	1.0
ACR criteria at diagnosis		5 [4-7]	5 [4-7]	1.0
Patient ethnicity (n, %)				
Caucasian		2 (13.3%)	23 (50.0%)	
African		3 (20.0%)	5 (10.8%)	
Caribbean		2 (13.3%)	2 (4.4%)	1.0
Mixed race		3 (20.0%)	0 (0.0%)	
Indian / Pakistani		3 (20.0%)	11 (23.9%)	
Chinese		2 (13.3%)	5 (11.0%)	
Medications (n, %)[4]				
Prednisolone		12/15(80%)	21/46 (46%)	1.0
Hydroxychloroquine		13/15 (87%)	31/46 (67%)	1.0
Azathioprine		2/15 (13%)	13/46 (28%)	1.0
Mycophenolate mofetil		11/15 (73%)	19/46 (41%)	0.96
Cycolphosphamide ever		3/15 (20%)	2/46 (4%)	1.0
Rituximab ever		5/15 (33%)	0/46 (0%)	**0.03**
ACEi[5] or AT2[6] blocker		4/15 (27%)	6/46 (13%)	1.0
Laboratory measures	**(n)[7]**			
eGFR[8] (ml/min/m^2)	**53**	100 [70-112]	116 [105-127]	0.96
UACR (mg/mmolCr)	**52**	92.4 [22.7-153.4]	0.90 [0.7-1.6]	**<0.001**
ESR (mm/h)	**60**	55.0 [20-90]	9.0 [3.0-23.0]	**<0.001**
CRP[9] (mg/L)	**40**	5.0 [4.0-5.0]	5.0 [4.0-5.0]	1.0
Anti-dsDNA[10] (IU/L)	**54**	48.0 [15.0-263.0]	1.9 [0.0-52.0]	0.29
C3[11] (g/L)	**40**	0.96 [0.5-1.2]	1.09 [0.9-1.2]	1.0
C4[12] (g/L)	**41**	0.14 [0.03-0.25]	0.17 [0.10-0.25]	1.0
WCC[13] (x10^9/L)	**60**	5.6 [4.9-7.0]	5.8 [4.4-7.6]	1.0
Lymph[14] (x10^9/L)	**60**	1.66 [1.35-1.9]	1.78 [1.16-2.5]	1.0
Neutrophils (x10^9/L)	**45**	4.3 [1.8-5.0]	3.3 [2.4-4.8]	1.0
Haemoglobin (g/dl)	**45**	10.8 [8.9-13.3]	13.1 [11.9-14.3]	1.0
Platelets (x10^9/L)	**45**	315.5 [237.3-411.3]	275.0 [232.8-375.8]	1.0
IgG[15] (g/L)	**31**	10.3 [7.5-18.5]	13.3 [9.1-13.7]	1.0
IgA[16] (g/L)	**31**	1.8 [0.67-4.20]	1.2 [0.99-1.86]	1.0
IgM[17] (g/L)	**31**	0.84 [0.24-1.22]	0.85 [0.49-1.29]	1.0
Extra-renal involvement[18]				
Constitutional		4 (27%)	5 (11%)	1.0
Mucocutaneous		4 (27%)	14 (30%)	1.0
Neuropsychiatric		2 (13%)	0 (0%)	1.0
Musculoskeletal		6 (40%)	10 (22%)	1.0
Cardiorespiratory		0 (0%)	0 (0%)	NA
Gastrointestinal		1 (7%)	1 (2%)	1.0
Opthalmic		0 (0%)	0 (0%)	NA
Haematological		8 (53%)	20 (44%)	1.0

Table 4-2: Key demographic and clinical data for the UK JSLE patients studied.
[1]Expressed as median values and IQRs or numbers and percentages. [2] p-values are Bonferroni-corrected p-values from Chi Squared tests or univariate binary regression as appropriate. Abbreviations: [3]gender data missing on 1 patient. [4]Current medication use described for regular medications, those taken in courses/intermittently are described as having been used 'ever'. [5]ACEi = angiotensin converting enzyme inhibitor, [6]AT2 = angiotensin 2 blocker. [7]Number of patients contributing to analysis for each laboratory parameter. [8]GFR = glomerular filtration rate, [9]CRP = C-reactive protein, [10]anti-dsDNA = anti-double stranded DNA antibody, [11]C3 = complement factor 3, [12]C4 = complement factor 4, [13]WCC = total white cell count, [14]Lymph = lymphocyte count, [15]IgG = immunoglobulin G, [16]IgA = immunoglobulin A, [17]IgM = immunoglobulin M, [18]BILAG defined extra-renal involvement.

The number of patients receiving Rituximab treatment was significantly higher in the active LN group ($p_c<0.05$), reflecting severity of disease, but use of other medications did not differ significantly between the patient groups. Of the laboratory parameters investigated, UACR and ESR were significantly higher in the active LN patients (all $p_c<0.05$). No significant difference was seen between patient groups in the occurrence of extra-renal BILAG defined organ involvement (see Table 4-2).

4.5.2 Individual novel urine biomarkers

Figure 4-2 depicts the distribution of novel urinary biomarker concentrations standardised to urinary creatinine in the patient groups (raw data shown in Appendix 19). Patients with active LN had significantly higher urinary concentrations of AGP, CP, VCAM-1, MCP-1, and LPGDS than inactive LN patients (all $p_c<0.05$). Urinary TF and NGAL concentrations did not differ significantly between the patient groups (see Figure 4-2 and Table 4-3) ($p_c = 0.06$ and 1.0 respectively).

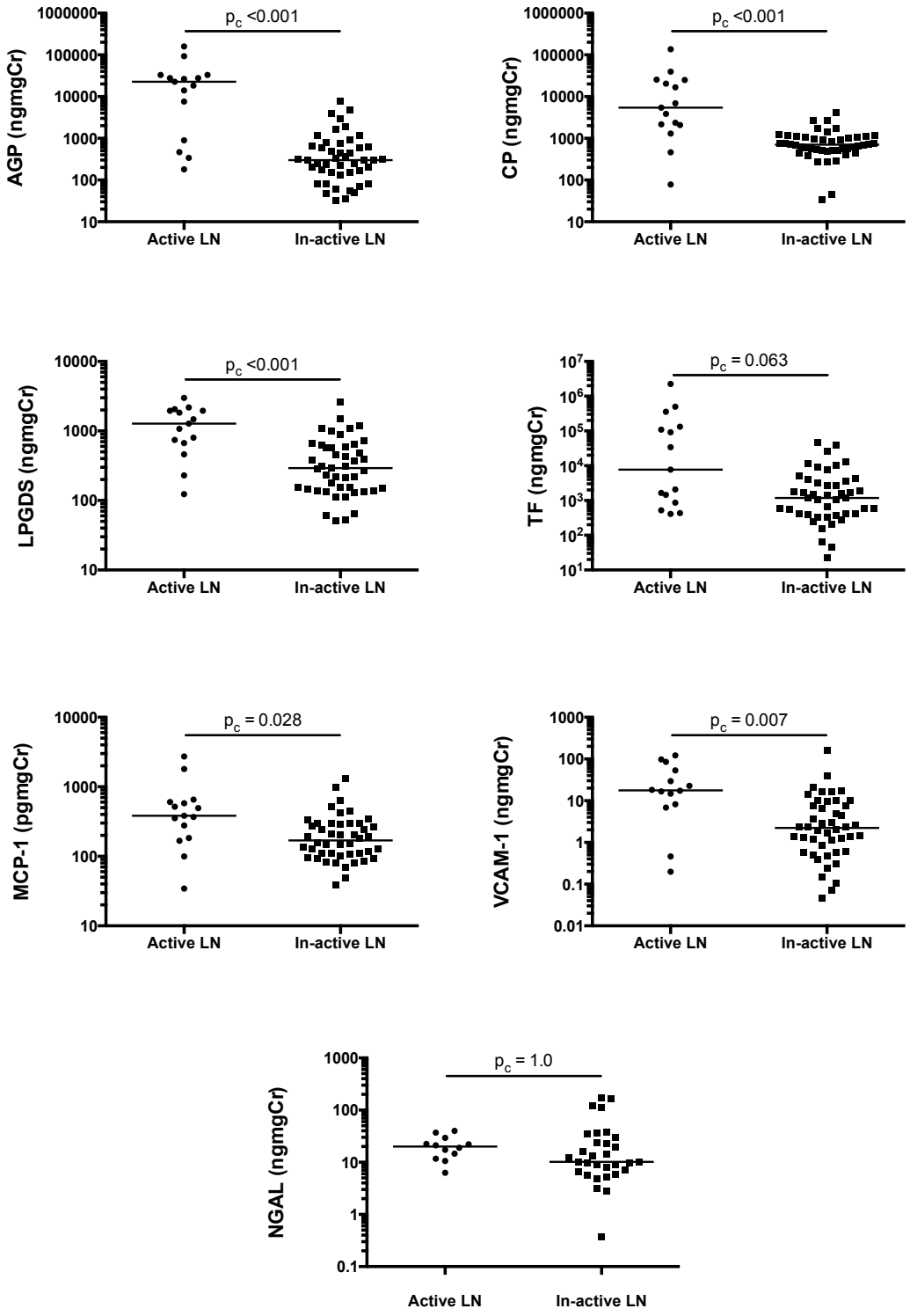


Figure 4-2: Distribution of urinary biomarker concentrations in patient groups.
Median value shown by horizontal line for each group. Mann Whitney U tests used to compare distribution of biomarker concentrations between patient groups (active LN versus inactive LN). P-values quoted are correct p-values, p_c. The raw data relating to this figure are shown in Appendix 19.

Biomarkers	Patient groups		Active vs. inactive LN[1] (p_c)
	Active LN	Inactive LN	
AGP (ngmgCr)	20,559 [788-29,144]	304 [144-708]	*<0.001*
CP (ngmgCr)	4,638 [1891-25,062]	701 [505-1,010]	*<0.001*
VCAM-1[2] (ngmgCr)	18 [8-62]	2 [1-8]	*0.007*
LPGDS (ngmgCr)	1175 [617-1,984]	288 [143-601]	*<0.001*
MCP-1 (pgmgCr)	376 [180-599]	157 [105-295]	*0.028*
TF (ngmgCr)	4,916 [781-188,415]	1,188 [402-3,822]	*0.063*
NGAL (ngmgCr)	20 [12-22]	10 [4-30]	*1.000*

Table 4-3: Urine biomarker concentrations standardised to urinary creatinine in active and inactive LN.

Biomarker concentrations standardised to urinary creatinine and expressed as median values and IQRs. Mann Whitney U tests used to compare biomarker concentrations between patient groups. [1]A Bonferroni adjustment was applied to account for multiple testing (7 tests), and corrected *p-values* reported (p_c). [2]VCAM-1 measurement missing from 1 patient. The raw data relating to this table are shown in Appendix 19.

4.5.3 Combining novel urine biomarkers

On fitting a binary logistic regression model including all novel biomarkers, and applying the 'stepAIC' function in R [360], the final model included both AGP and CP (see Table 4-4). AUC for this final model was 0.88.

Model including all biomarkers[1]			
Biomarker	**Co-efficient**	**Std. Error**	***p-value***
AGP	0.692	0.35	*0.047*
CP	0.551	0.36	*0.127*
VCAM-1	-0.228	0.38	*0.553*
LPGDS	0.870	0.76	*0.254*
MCP-1	-0.046	0.86	*0.957*
TF	0.256	0.23	*0.275*
Model after variable selection[2]			
AGP	0.782	2.84	*0.004*
CP	0.602	0.34	*0.080*

Table 4-4: Binary logistic regression models including all biomarkers and after variable selection

[1]59 patients included in novel biomarker models including VCAM-1 due to a missing measurement.
[2]Model selected after applying the 'stepAIC' function in R.

On addition of LPGDS, the AUC increased to 0.90, increasing further to 0.92 on addition of TF. Addition of VCAM-1 and MCP-1 into the model however, did not increase the AUC any further (see Table 4-5). The receiver-operating curve (ROC) generated by the model including AGP, CP, LPGDS and TF is shown in Figure 4-3.

Biomarkers included in binary logistic regression models		**AUC ROC**
Novel biomarker combinations	AGP + CP	0.88
	AGP + CP + LPGDS	0.90
	AGP + CP + LPGDS + TF	0.92
	AGP + CP + LPGDS + TF + VCAM-1[1]	0.92
	AGP + CP + LPGDS + TF + VCAM-1 + MCP-1	0.92
Novel/traditional biomarker combination	AGP + CP + LPGDS + TF + ESR[2]	0.91

Table 4-5: Effect on AUC by adding novel and traditional biomarkers to the logistic regression model

[1]59 patients included in novel biomarker models including VCAM-1 due to missing biomarker measurements. [2]60 patients included in novel and traditional biomarker model, due to missing ESR data for one patient.

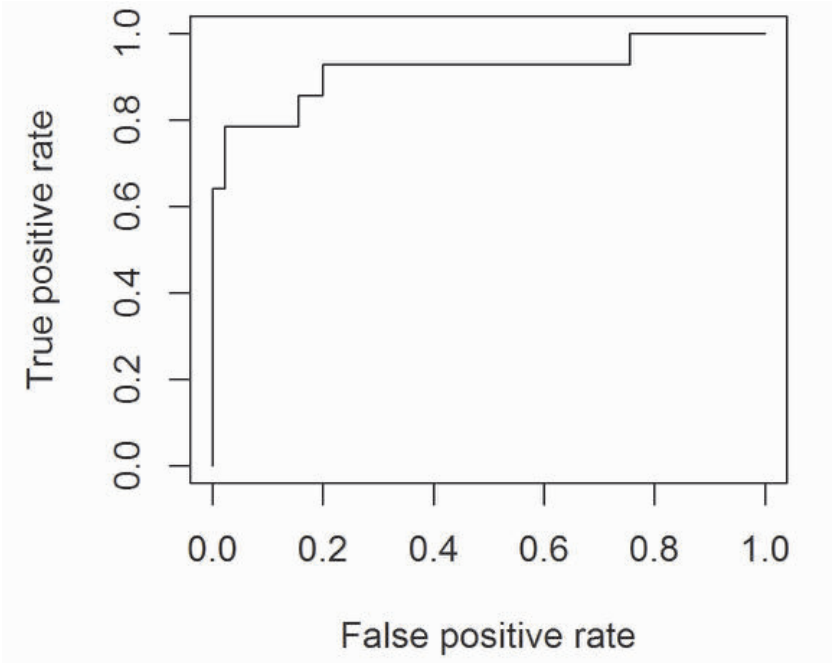


Figure 4-3: Receiver operating curve for the optimal novel biomarker combination - AGP, CP, LPGDS and Transferrin (AUC 0.92).

4.5.4 Urine biomarkers and extra-renal involvement

There was no significant difference in urinary biomarker levels in inactive LN patients with active extra-renal involvement and no extra-renal involvement (see Table 4-6). Within the inactive LN group, urinary biomarker levels did not differ between renal-BILAG score D (inactive renal disease but previous involvement) and renal-BILAG score E patients (no previous renal involvement) (all $p_c>0.05$). Urine biomarker concentrations uncorrected for creatinine also did not alter the above findings.

Novel biomarkers	**Inactive LN patients**		**Comparison between patients with / without extra-renal involvement (p_c)[1]**
	With extra-renal involvement	**Without extra-renal involvement**	
AGP (ngmgCr)	511 [285-1149]	299 [165-748]	*1.0*
CP (ngmgCr)	835 [557-1365]	1027 [627-1320]	*1.0*
VCAM-1[2] (ngmgCr)	5 [1.5-20]	3 [0.6-10]	*0.28*
LPGDS (ngmgCr)	342 [169-856]	365 [204-584]	*1.0*
MCP-1 (pgmgCr)	143 [101-298]	152 [95-240]	*1.0*
TF (ngmgCr)	789 [372-4133]	1188 [359-3511]	*1.0*
NGAL (ngmgCr)	13 [8-52]	9 [5-26]	*1.0*

Table 4-6: Urine biomarker concentrations in inactive LN patients with/without extra-renal involvement.

Biomarker concentrations standardised to urinary creatinine and expressed as median values and IQRs. Mann Whitney U tests used to compare biomarker concentrations between patient groups. [1]A Bonferroni adjustment was applied to account for multiple testing, corrected *p-values* (p_c) reported. [2]VCAM-1 measurement missing from 1 patient.

4.5.5 Comparing and combining novel and traditional biomarkers

Traditional biomarkers which do not contribute to the composite renal BILAG score were assessed for their ability to identify active LN in the patients specifically included in this study, with Table 4-7 showing the AUC for each traditional biomarker. ESR was the best traditional biomarker with a fair AUC of 0.796. C3 and dsDNA showed a poor ability to identify active LN (AUC's of 0.645 and 0.617 respectively). C4 performed worst with an AUC of 0.593. Inclusion of all traditional biomarkers together in a regression model did not improve the AUC (see Table 4-7). Of the traditional biomarkers assessed univariately in Table 4-2, ESR was significantly different between active and inactive LN patients ($p_c<0.05$). Addition of this traditional biomarker to the optimal novel biomarker logistic regression model including AGP, LPGDS, TF and CP, did not improve the AUC further (AUC 0.91, see Table 4-5).

Traditional biomarkers	AUC ROC
dsDNA[1]	0.617
C3[2]	0.645
C4[3]	0.593
ESR[4]	0.796
All biomarkers	0.783

Table 4-7: AUC values corresponding to ability of traditional biomarkers to identify active LN.

AUC values obtained from logistic regression model probabilities for each traditional biomarker and all biomarkers together. [1]dsDNA = anti-double stranded DNA antibody, [2]C3 = complement factor 3, [3]C4 = complement factor 4, [4]ESR – erythrocyte sedimentation rate.

4.5.6 Effect of biomarker exposure on human podocytes *in vitro*

4.5.6.1 Podocyte apoptosis

Using a Caspase-Glo® 3/7 Assay, luminescence in RLU corresponding to Caspase 3/7 activity was determined following exposure of podocytes to the conditions listed in section 4.4.4.1. All results were normally distributed (Shapiro-Wilk test, $p>0.05$ for all experimental conditions). Podocytes alone displayed a mean luminescence of 1509 (±100) RLU. In response to the experimental conditions applied to the podocytes, the mean luminesence varied between 1219-1669 RLU. ANOVA analysis with Dunnet's multiple comparison tests compared the luminescence obtained with each experimental condition, to podocytes alone. No significant difference were seen in response to any of the experimental conditions (all $p_c > 0.05$) except from the positive control wells (apoptotic neutrophils, RLU 6075 ± 354 RLU, $p_c<0.0001$, see Table 4-8 and Figure 4-4).

Experimental condition	Luminescence (RLU)	Comparison between pod alone & pods + conditions (p_c-*value*)
Caspase 3/7 Glo reagent alone (blank)	0	*NA*
Podocytes alone	1509 ± 100	*NA*
Podocytes & High AGP	1291 ± 173	*0.995*
Podocytes & Low AGP	1429 ± 207	*0.999*
Podocytes & High CP	1608 ± 278	*0.999*
Podocytes & Low CP	1268 ± 106	*0.994*
Podocytes & High TF	1455 ± 48	*0.999*
Podocytes & Low TF	1219 ± 84	*0.983*
Podocytes & High LPGDS	1607 ± 70	*0.999*
Podocytes & Low LPGDS	1501 ± 122	*1.000*
Podocytes & High AGP+CP+TF+LPGDS	1669 ± 27	*0.999*
Positive control (apoptotic neutrophils)	6075 ± 354	*< 0.0001*

Table 4-8: Luminescence corresponding to Caspase 3/7 activity following exposure of podocytes to AGP, CP, TF and LPGDS.
Values quoted are the mean ± standard error of the mean. All comparison between experimental conditions and podocytes alone were corrected for multiple testing (p_c-*value*). RLU = reactive light units. Pods = podocytes.

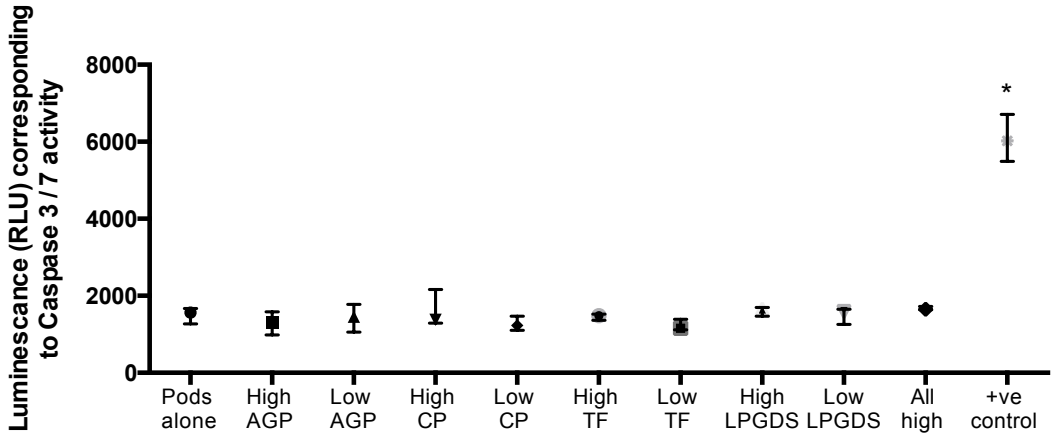


Figure 4-4: Luminescence corresponding to Caspase 3/7 activity in podocytes exposed to varying experimental conditions.
All experimental conditions include podocytes except from the assay positive control which is comprised of apoptotic neutrophils. Abbreviations: RLU = reactive light units, Pods = podocytes, +ve control = positive control. *significant result for comparison of podocytes alone vs. positive control (p_c *<0.0001)*.

Apoptosis was confirmed in additional positive control wells using flow cytometry and Annexin V staining immediately prior to the Caspase Glo® 3/7 Assay. This showed Annexin V to be positive in 88.8% of neutrophils, suggesting that the Caspase 3/7 Glo® assay results were reliable (see section 2.2.6.3 and the flow cytometry dot plot shown in Figure 2-6).

4.5.6.2 Podocyte cytokine production

Table 4-9 shows the median/IQR levels of each cytokine in podocyte supernatant following exposure of the podocytes to high/low concentrations of LN urinary biomarker. Multiple t-tests with a 5% false discovery rate were applied to log transformed data to assess the impact of the 10 experimental conditions on 41 markers, identifying markers differing significantly between podocytes alone and podocytes exposed to individual experimental conditions. Treatment with 'high CP' and 'all biomarkers high' was associated with a significant reduction in TNF-α secretion compared to podocytes alone; median of 19 and 20 pg/ml TNF-α respectively, as opposed to 26 pg/ml with podocytes alone (both *p<0.001*). No other differences in cytokine level were demonstrated according to the urine biomarker treatment conditions applied.

MCP-1 production was significantly increased in the presence of the positive control; 15182 pg/ml [9919-16613] compared to the podocyte alone levels, 9289 pg/ml [8182-9748] ($p<0.001$). The assays high/low QCs were also within range for all analytes. All podocyte supernatant samples were run undiluted, with the dynamic range of the assay being very wide (3.2–10,000 pg/ml). However, despite this IL-9, IL-1β, IL-3 and MIP-1β were undetectable in the podocyte supernatant suggesting that these markers are not presently secreted by podocytes.

Marker (pg/ml)	Treatment conditions									
	Pods alone	**AGP high**	**AGP low**	**CP high**	**CP low**	**LPGDS high**	**LPGDS low**	**TF high**	**TF low**	**ALL high**
TNF-α	26 [25-26]	21 [20-22]	24 [22-25]	**19 [17-20]***	22 [19-24]	23 [23-27]	22 [19-22]	20 [19-30]	26 [26-27]	**20 [19-21]****
EGF	14 [12-13]	13 [12-13]	13 [0.1-14]	12 [12-13]	13 [11-13]	12 [11-15]	13 [11-13]	12 [10-16]	13 [13-14]	13 [12-15]
FGF	88 [81-102]	80 [73-80]	75 [0-76]	76 [73-98]	76 [73-79]	76 [74-92]	73 [73-75]	78 [57-94]	81 [79-87]	83 [83-87]
VEGF	2112 [2002-2126]	2267 [1926-2465]	2130 [2095-2163]	1901 [1781-1979]	2200 [2149-2298]	2325 [2067-2455]	1961 [1885-2112]	2158 [2092-2710]	1968 [1910-2185]	2407 [2074-2577]
MCP-1	9289 [8182-9748]	7962 [7768-8715]	8908 [8573-9243]	6944 [6192-8744]	7678 [6364-8907]	8937 [7978-9070]	9205 [8336-9629]	7298 [7093-11035]	8818 [8315-8906]	7856 [7168-8267]
IL-6	12838 [11712-13729]	11608 [11442-12150]	12826 [12493-13159]	11836 [11395-13833]	12521 [11734-14851]	12298 [11953-14115]	12176 [11872-12441]	15251 [12090-15514]	13434 [12533-14279]	11790 [11635-13702]
IL-4	10 [8-14]	9 [9-10]	10 [10-11]	9 [7-13]	10 [8-10]	8 [6-13]	8 [6-8]	9 [5-17]	12 [10-13]	10 [9-14]
GM-CSF	287 [276-292]	247 [214-264]	304 [286-323]	212 [202-275]	273 [214-294]	264 [257-329]	261 [247-276]	270 [241-359]	293 [267-313]	281 [271-281]
G-CSF	26 [25-27]	23 [22-25]	22 [21-23]	25 [18-25]	22 [21-32]	25 [20-34]	19 [19-21]	21 [12-37]	32 [23-45]	23 [22-23]
IL-1α	3 [2-3]	3 [2-3]	3 [2-3]	3 [2-3]	3 [2-4]	3 [1-4]	2 [2-3]	2 [1-3]	3 [3-4]	2 [2-3]
FLT-3L	29 [28-29]	29 [26-30]	31 [27-35]	29 [22-31]	28 [25-32]	28 [23-34]	25 [24-27]	29 [24-44]	34 [26-35]	30[29-33]
IL-8	1621 [1552-1701]	1348 [1328-1746]	1717 [1664-1771]	1308 [1243-1424]	1575 [1282-1592]	1851 [1598-2069]	1524 [1485-1679]	1518 [1375-2188]	1616 [1561-1925]	1554 [1417-1706]
PDGF-AA	283 [272-358]	309 [258-371]	305 [299-310]	245 [226-289]	279 [278-308]	310 [283-331]	291 [272-301]	507 [494-711]	343 [322-373]	493 [490-498]
IL-12 P70	5 [3-5]	5 [5-5]	5 [2-8]	7 [3-8]	6 [5-7]	7 [5-9]	4 [4-5]	12 [5-16]	8 [4-8]	8 [3-8]
Fractaline	119 [112-126]	114 [101-114]	112 [108-116]	114 [91-131]	114 [97-118]	111 [94-136]	91 [88-104]	98 [73-174]	134 [116-142]	121 [103-123]
Eotaxin	264 [250-286]	223 [201-254]	279 [264-294]	199 [197-216]	230 [205-242]	254 [235-279]	262 [251-273]	249 [226-331]	254 [235-280]	191 [181-202]
TNF-β	5 [4-5]	4 [3-4]	3 [3-4]	4 [3-7]	4 [3-6]	4 [3-7]	2 [2-5]	3 [0.1-7]	5 [4-5]	4 [4-5]
RANTES	13 [11-19]	12 [8-16]	10 [10-11]	13 [10-17]	11 [7-15]	13 [11-14]	10 [10-11]	15 [2-20]	17 [15-19]	12 [11-14]
MIP-1α	15 [15-16]	14 [14-15]	14 [14-15]	14 [13-15]	14 [13-14]	14 [13-14]	14 [13-14]	14 [13-15]	15 [14-16]	14 [14-15]
IP-10	24 [16-30]	22 [20-24]	25 [22-27]	26 [24-28]	18 [14-25]	19 [14-24]	18 [17-26]	35 [13-48]	28 [25-29]	21 [16-25]
IL-7	15 [10-15]	10 [10-11]	13 [12-13]	12 [10-15]	12 [11-16]	12 [12-15]	9 [8-10]	10 [5-19]	15 [14-15]	13[11-13]
IL-5	2 [2-3]	2 [2-2]	2 [2-2]	2.0 [2-2]	2 [2-2]	2 [2-2]	2 [2-2]	2 [2-3]	2 [2-3]	2 [2-2]
IL-2	3.2 [3-4]	3.1 [2.1-3.7]	3.3 [2.5-4.2]	3.1 [2.1-3.7]	3.2 [2.8-4.0]	3.3 [2.2-4.5]	2.5 [2.2-2.5]	2.5 [1.4-3.6]	3.4 [3.0-4.7]	3.4 [2.9-3.6]
IL-1RA	8.6 [7.0-9.9]	7.4 [6.5-8.0]	8.0 [7.0-9.1]	7.2 [4.8-11]	7.2 [5.3-9.8]	7.8 [5.8-11]	5.7 [4.8-6.8]	7.0 [2.0-13]	12 [9.7-12]	7.6 [7.4-9.1]
Il-17A	2 [2-2]	1 [0.1-1]	1 [1-1]	2 [1-2]	0.6 [0.6-3]	2 [1-4]	0.7 [0.5-1]	2 [1-3]	3 [1-3]	1 [1-2]
sCD-40L	6 [5-6]	4 [3-5]	5 [5-6]	5 [4-6]	4 [4-6]	5 [3-7]	3 [3-6]	5 [2-7]	7 [5-7]	5 [4-6]
IL-15	6 [6-6]	5 [4-6]	5 [5-6]	6 [4-7]	5 [4-6]	6 [5-7]	5 [4-5]	5 [3-11]	7 [6-7]	6 [6-6]

PDGF-AB/BB	1312 [1162-1337]	1027 [996-1029]	1376 [1329-1423]	1013 [960-1031]	1188 [1080-1353]	1396 [1388-1581]	1300 [1275-1360]	1235 [1174-1687]	1276 [1275-1435]	1120 [894-1189]
IL-13	4 [3-5]	3 [3-3]	2 [1-2]	5 [4-7]	3 [3-4]	3 [2-5]	4 [4-4]	5 [4-7]	4 [2-6]	3 [3-4]
MDC	11 [11-13]	11 [11-12]	11 [11-11]	11 [10-13]	11 [10-12]	11 [9-13]	10 [9-12]	12 [8-14]	11 [11-13]	12 [11-13]
IL-12 P40	20 [19-23]	17 [12-20]	17 [14-20]	16 [12-23]	17 [15-20]	15 [14-22]	14 [12-21]	14 [7-23]	17 [17-20]	17 [15-21]
MCP-3	28 [27-29]	28 [22-29]	26 [22-29]	27 [22-33]	29 [22-32]	28 [24-30]	21 [19-28]	28 [18-35]	29 [26-31]	28 [28-30]
IL-10	4 [4-5]	3 [3-4]	4 [2-5]	3 [3-6]	4 [2-5]	4 [2-6]	2 [2-4]	3 [1-6]	5 [4-6]	4 [4-4]
GRO	4126 [3694-4461]	3635 [3313-5190]	4435 [4007-4864]	3532 [3285-3723]	4776 [3410-4955]	5235 [4396-5490]	4050 [3957-4154]	3748 [3337-5914]	4356 [3372-5317]	4258 [3978-4594]
IFNγ	5 [5-7]	5 [5-6]	6 [5-7]	6 [2-8]	8 [3-9]	4 [3-8]	4 [3-6]	5 [5-7]	6 [5-7]	6 [5-8]
IFNα	32 [30-33]	30 [30-31]	31 [25-38]	29 [22-35]	25 [22-34]	27 [23-32]	25 [24-33]	27 [23-43]	40 [40-42]	29 [28-30]
TGFβ	106 [100-110]	96 [89-115]	106 [105-107]	90 [82-93]	97 [88-100]	103 [91-114]	97 [92-99]	94 [90-137]	97 [92-112]	99 [93-99]
IL-9	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
IL-1β	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
IL-3	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
MIP-1β	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 4-9: Cytokine/chemokine levels in podocyte supernatant in response to different concentrations of urine biomarkers.
*$p=0.002$, **$p=0.001$, Median and IQR levels shown for each analyte. Multiple t-test applied with a 5% false discovery rate applied, and significant differences in cytokine/chemokine level highlighted in bold with *p-value* shown in the corresponding column. Abbreviations: pods alone = untreated podocytes. high = level of biomarker seen in active LN urine. low = level of biomarker seen in inactive LN urine. EGF = epidermal growth factor. FGF = fibroblast growth factor. VEGF = vascular endothelial growth factor. TNF-α = tumor necrosis factor α. MCP = monocyte chemoattractant protein. IL = interleukin. GM-CSF = granulocyte macrophage colony stimulating factor. G-CSF = granulocyte colony stimulating factor. FLT-3L = FMS-related tyrosine kinase 3 ligand. PDGF-AA = Platelet-derived growth factor-AA. TNF-β = tumor necrosis factor-β. RANTES = regulated on activation, normal T cell expressed and secreted. MIP = Macrophage inflammatory protein. IP-10 = Interferon gamma-induced protein 10. IL-1RA = interleukin 1 receptor antagonist. sCD40L = soluble cluster of differentiation 40 ligand. PDGF-AB/BB = Platelet-derived growth factor-AB/BB. MCD = macrophage derived chemokine. GRO = chemokine (C-X-C motif) ligand 1. IFN = interferon. TGFβ = transforming growth factor β. N=3 for all conditions.

4.6 Discussion

Using a cross-sectional cohort of patients recruited to the UK JSLE Cohort Study, the aim of this study was to determine if urinary AGP, CP, VCAM-1, TF, LPGDS, MCP-1 and NGAL are able to differentiate between JSLE patients with active and inactive LN. By simultaneously measuring each of these urinary biomarkers at a single patient visit, the association between different novel urinary and traditional serum biomarkers, both individually and in combination has been assessed. A 'LN urinary biomarker panel' demonstrated that it was able to improve significantly the identification of active LN, over and above individual biomarkers.

The identification and validation of LN biomarkers in children is important as they generally have fewer co-morbidities and more frequent renal involvement than adult counterparts. Some of the biomarkers included in this study have been largely studied individually within adult SLE studies. Overall, an ideal biomarker must be non-invasive, accurate, demonstrate good sensitivity and specificity for the condition in question, and sustain cross-validation within ethnically distinct patient cohorts (see section 1.3.1). In a condition such as JSLE, a biomarker must be organ domain specific as patients may concurrently display involvement of multiple organs. This study has shown that extra-renal JSLE disease activity does influence urine biomarker levels.

This work builds upon that originally performed by Suzuki et al who showed AGP, CP, LPGDS and TF to be higher in active than inactive LN in a US JSLE cohort. When included in a model with albumin, and albumin-related fragments, their combined AUC for active LN identification (renal BILAG/SLEDAI defined) was 0.85 [290]. Within samples from the UK JSLE Cohort Study, additional improvement with a stronger AUC value (0.92) has been demonstrated when AGP, CP, LPGDS and TF were combined in a regression model. Of the additional biomarkers assessed in the current study, VCAM-1 and MCP-1 levels also differed significantly between active and inactive LN when tested individually. However, these biomarkers did not further improve the AUC when added into the model already including AGP, CP, LPGDS and TF. Both of these Cohorts include comparable patients in terms of age, gender and length of disease. However, there were marked differences in the terms of ethnicity; only 17% were Caucasian within the US Cohort and 41% in the UK Cohort. Notably,

African, African American and Asian patients often have more severe disease and more kidney involvement in SLE [32,281,403]. This transatlantic comparison of a similar biomarker panel provides considerable strength to the hypothesis that a panel approach involving some or all of these urinary biomarkers may be an important way of differentiating active from inactive LN.

It is important to consider the origin and renal-specificity of these novel biomarkers. AGP (also called Orosomucoid) belongs to the immunocalin family, a group of binding proteins which bind and transport small hydrophobic molecules and have immunomodulatory functions. AGP is also one of the major acute phase proteins, mainly secreted by hepatocytes as part of the systemic response to inflammatory mediators (IL-1, IL-6, IL-8) or stressful stimuli such as physical trauma or bacterial infections. AGP production has also been reported in several tissues outside the liver including alveolar stimulated macrophages [404], human endothelial cells [405], cultured monocytes [406,407], resting and activated polymorphs [408,409]. In active LN, increased production as part of the acute phase response, coupled with production by cells infiltrating the kidney, may be responsible for the high urinary levels demonstrated.

CP is involved in the metabolism of iron and carries the vast amount of circulating copper in the plasma [410]. TF is a metal binding protein with high affinity for iron, binding and moving it into cells and tissues. Iron is important to normal immune function, contributing to cell differentiation and growth. Alterations in iron homeostasis have been associated with several rheumatological and autoimmune diseases [411]. Both CP and TF differ from albumin in terms of their molecular radii and isoelectric points, and have been shown to predict the onset of microalbuminuria at an early stage in type 2 diabetes [412,413].

LPGDS is secretory protein of the lipocalin superfamily that acts as an enzyme responsible for the production of prostaglandin D2. It is produced in the brain by the choroid plexus or leptomeninges and is continuously secreted through cerebrospinal fluid into blood. LPGDS is similar to albumin in chemical properties but is much smaller, allowing it to pass more readily through the glomerular capillary walls [414]. In type-2 diabetes, urinary LPGDS has been shown to increase in the early stages of

kidney injury [415]. Hypertensive patients also excrete more urinary LPGDS than normotensive patients [416].

This study has shown for the first time in JSLE that urinary VCAM-1 levels differ significantly between active and inactive LN (good biomarker, AUC 0.80). Previous studies looking at VCAM-1 in adults have found VCAM-1 to be associated with physician's global estimate of disease, UPCR, SLICC renal activity score [308] and the revised SLAM-R [329], class III, IV and V LN [328], and the renal pathology NIH AI [297]. Urinary levels are higher than blood levels, suggesting that the inflamed kidney may represent an important source of urinary VCAM-1 [326]. Although VCAM-1 is not contained within the optimal biomarker panel, it warrants further evaluation longitudinally to assess its performance in predicting LN flares.

Exposure of podocytes to the biomarkers AGP, CP, TF and LPGDS at 'high'/'low' concentrations (corresponding to the median levels detected in the urine of active/inactive LN patients) did not appear to induce podocyte apoptosis. The Caspase-Glo® 3/7 Assay is an established method for detecting apoptosis in adherent cells, and has been used by others working with this conditionally immortalised podocyte cell line [400,401]. Further investigation could include a time series, where the biomarker exposure occurs for a shorter/longer period. Although exposure to the biomarkers did not induce podocyte apoptosis, biomarker exposure could lead to structural damage to the glomerulus and defects in the filtration barrier due to conformational changes within the podocyte, or podocyte detachment [417,418]. Future experiments could therefore assess podocyte detachment by trypsinizing the cells until they are suspended and subsequently allowing them to settle in culture medium containing the experimental conditions. After a set period, cells in suspension and adherent cells could be collected and counted to assess for differences in cell attachment according to the experimental conditions [400,419]. The podocyte slit diaphragm is most commonly affected by glomerular disease [394] and assessment of slit diaphragm proteins such as nephrin, podocin and CD2-associated protein by immunofluorescence [420] could also provide insights into slit diaphragm structure and function in response to urine biomarker exposure.

Regarding cytokine production in response to 'high'/'low' biomarker concentrations, TNF-α levels were significantly decreased in the presence of 'high' CP or 'all biomarkers high'. In both conditions, 'high' CP may have led to the decrease in TNF-α as it was a common factor across both conditions. CP is the major copper-carrying protein in the blood, but also assists TF in iron transport [410]. CP is mainly produced by hepatocytes but also activated macrophages as part of the acute-phase response [421,422]. Copper plays a role in stress-induced release of cytokines such as TNF-α, IL-1 α/β and TGF-β by immune cells [423]. TNF-α is a pro-inflammatory cytokine involved in systemic inflammation and is mainly produced by activated macrophages, although it can be produced by many other cell types, including podocytes [396]. In Wilson's disease, where low CP and high copper levels are seen, serum TNF-α is known to be increased as compared to controls [424]. In addition, serum copper has been shown to correlate positively with TNF-α levels in RA patients [425], therefore CP (the copper scavenger) may correlate negatively with TNF-α. In the current study, when CP levels are high TNF-α is decreased, therefore it is hypothesised that a negative feedback loop may be in operation. Soluble TNF receptor levels may also influence TNF-α levels under theses cpnditions [426], requiring further investigation.

Certain limitations to this study warrant recognition and should be addressed in future work. The definition of active LN was based on the composite renal BILAG score, calculated from proteinuria, GFR, blood pressure, active urine sediment, plasma creatinine and recent biopsy findings, therefore one could not directly compare such traditional markers with the novel urinary biomarkers studied. Patients were considered to have an active LN episode if they had a renal BILAG score of A or B **and** previous histological confirmation of LN at some stage during their disease. This influences the ability to directly compare the results of the current study with others which do not include this stipulation. This condition was felt to be important in ensuring the active LN patient group were truly representative of patients with active LN. Inspection of the clinical data of JSLE patients followed longitudinally revealed that at times, patients may score a renal BILAG score of B (e.g. on the basis of having 2+ urine dipstick at a single visit) but have no change to their steroid or immunosuppressive treatment suggesting that the clinician had not been convinced that the patient had renal involvement. At subsequently visits, such patients do not

demonstrate further renal BILAG A or B scores, suggesting that this has been an isolated incident and that the patient may not have had true active LN. In patients with a renal BILAG score of A or B **and** previous histological confirmation of LN at some stage during their disease, changes in renal BILAG score were more persistent and reflective of changes in treatment, suggesting that this approach to patient stratification is more appropriate.

The cross-sectional nature of this study also limits the ability to comment on the relationship of such biomarkers with other stages of the fluctuating LN disease course (e.g. prediction of flare/remission). Although only a quarter of patients had 'active' disease, the numbers included are comparable or in excess of many previous studies [72,74,297,299,328,329]. These promising findings require validation in a larger, international, longitudinal, prospectively collected study including children and young people with the full range of active (severe or mild) and inactive disease. Concurrent investigation of the role of such biomarkers in LN mouse models or more sophisticated *in vitro* LN models, will also help to improve understanding of LN pathophysiology.

4.7 Summary

This study has demonstrated that combining novel urine biomarkers improves LN identification in JSLE patients. Further validation of this 'excellent' novel urine biomarker panel is required in independent international JSLE cohorts, both cross-sectionally and longitudinally, to define biomarker profiles that predict LN flare and remission. It is anticipated that a future urinary biomarker point of care testing device will help to improve the renal outcome for JSLE patients through biomarker-led renal monitoring in routine clinical practice.

4.8 Conclusions

- The optimal excellent 'LN urinary biomarker panel' identified within the UK JSLE Cohort Study for cross-sectionally differentiating active from inactive LN patients includes: AGP, CP, LPGDS and TF (AUC 0.920).
- Combining traditional biomarkers with the optimal novel biomarker panel did not improve the AUC further.

- Exposure of podocytes to the biomarkers AGP, CP, TF and LPGDS at ‘high’/‘low’ concentrations reduced TNF-α production only, and did not induce podocyte apoptosis.
- Next steps should therefore include:
    - Investigation of whether the above biomarker panel performs comparably within ethnically distinct JSLE patient cohorts.
    - Assessment of whether constituents of the LN urinary biomarker panel are able to longitudinally predict LN flare or remission in advance.

5 International validation of the urine biomarker panel and longitudinal analysis

5.1 Introduction

5.1.1 Ethnic differences in SLE and LN

Further investigation of the excellent novel 'LN urinary biomarker panel' described in Chapter 4 for identification of active LN is required in independent JSLE cohorts, both cross-sectionally and longitudinally, to cross-validate previous findings and define biomarker profiles that predict LN flare and remission. Significant differences in disease phenotype and clinical outcome have been shown to occur in JSLE patients of different ethnicities (see section 1.1.1). Hispanic JSLE patients have been shown to have significantly longer lengths of hospital stay, more re-admissions and higher in-hospital mortality than their non-Hispanic counterparts, including White, African American, Asian and American Indian patients. African American JSLE patients have been shown to be significantly more likely to be admitted to intensive care than the other patient groups mentioned above. Both African American and Hispanic patients are also more likely to have end-stage renal disease and higher mortality rate than White, and American Indian JSLE patients [30]. The crude death rate of adult SLE patients of African descent has also been shown to be three times higher than Caucasians [427]. In a further study conducted across 47 US states, the risk of death was significantly higher among Native American and Black adult SLE patients compared with Caucasian patients. Whilst, Hispanic and Asian patients had lower mortality risks compared with Caucasians [428].

Survival rates of adult SLE patients in developing countries are substantially lower than in the developed world. In an adult SLE study including mainly Black patients seen at a tertiary institution in Soweto, South Africa, the 5-year survival rate was 78% [429], compared with the reported > 90% 5-year survival rates in many industrialised countries including Sweden [430], the United Kingdom [431], Italy [432] and in a multicentre study across seven European countries [433]. Within a South African adult SLE Cohort, multivariate analysis showed LN to be the sole predictor of patient mortality [429]. In an American adult SLE study, African Americans with end stage renal failure were at significantly increased risk of death compared with their non-

African contemporaries [32]. In Cape Town, South African adults with proliferative LN have also been shown to have lower renal specific survival rates than in non-African countries with 5- and 10- year survival rates of 63% and 52% respectively [279]. The SLICC Inception Cohort [434], the largest multi-ethnic/racial SLE inception cohort of patients from across Europe, North America and Canada has recently reported the cumulative renal survival at 5 and 10 years to be 96.7% and 95.7% at 5 and 10 years respectively [222], highlighting the marked differences in renal survival between developed and developing countries.

The above examples of differences between SLE disease phenotype according to ethnicity emphasise the importance of cross-validating our experimental findings in different SLE populations rather than automatically generalising between them, to see if urine biomarkers can still differentiate active from inactive disease activity irrespective of the ethnic origin of the cohort, and provide new evidence for an internationally applicable urine biomarker panel for LN.

In the current chapter, two international collaborations with the US Einstein Lupus Cohort (referred to as the 'US Cohort' [334], described in section 2.1.2) and the Paediatric Lupus Erythematosus in South Africa Cohort (referred to as the 'SA Cohort' [435], described in section 2.1.3) were developed to investigate the urine biomarker panel in ethnically distinct cohorts. Within the SA cohort, Lewandowski et al have recently demonstrated that 61% of patients initially presented with LN (documented by renal biopsy), with 63% of these patients displaying severe ISN/RPS class III or IV LN. The increased LN severity within this cohort as compared to the well described North American CARRA registry cohort [436] is highlighted within their recent paper. 15% of patients within the SA Cohort required dialysis vs. 1% in the CARRA cohort ($p<0.001$), with strikingly higher transplantation rates seen in the SA cohort; 8% vs. <1% in their North American peers ($p<0.001$) [435].

Validation of the UK JSLE Cohort 'biomarker panel' in such ethnically distinct and 'severe' LN cohorts will provide important insight into these proposed urinary biomarkers on an international level.

5.1.2 Longitudinal assessment of urine biomarkers

Renal biopsy remains the gold standard for diagnosing and monitoring LN. In children, biopsy is associated with significant risks of bleeding (in up to 25% of patients), infections (0.2% risk), adverse reactions to sedatives, and poses a significant psychological burden to the child and family [437-440]. At diagnosis, renal biopsy provides prognostic information and guides treatment so its benefits outweigh these risks; however, it has a limited role in ongoing monitoring. Currently, children with long-standing LN could potentially require multiple biopsies throughout their disease course due to the limitations of traditional blood and urine tests in LN monitoring (see sections 1.2.2, 1.2.3 and 1.2.4). Urine biomarkers have been shown to be better than serum biomarkers in differentiating renal disease from other organ manifestations (see section 4.5.4 and [73,322]). There is therefore an urgent need to develop a non-invasive urine biomarker panel test that allows the clinician to quickly and accurately determine an individual child's LN activity and predict fluctuations in disease activity over time. Such a test could prompt treatment intensification, alleviating or preventing disease flare and modifying the course of disease to improve renal outcome. Similarly, identifying patients who will go into remission over subsequent months could help to limit their time on potentially toxic treatment, personalising their care.

Constituents of the biomarker panel including AGP, TF and LPGDS have been shown to be elevated at least 3 months before an LN flare becomes diagnosed clinically. However, significant increases in AGP and LPGDS were also demonstrated in patients with stable active LN, improving LN (AGP) and inactive LN (LPGDS) [290], highlighting the need for further prospective studies to improve understanding of the relationship of these biomarkers to disease activity over time. The effect of combining such markers longitudinally also warrants assessment, to see if this improves the ability to predict LN flare or other stages of the LN disease process. MCP-1 did not emerge as part of the urine biomarker panel demonstrated in Chapter 4, but a paediatric longitudinal biomarker study has shown low urinary MCP-1 levels to be a good predictor of future renal disease improvement [74], therefore further longitudinal evaluation is required. In an adult SLE longitudinal study, urinary MCP-1 has been shown to increase 2-4 months before an LN flare, decreasing in patients who respond to treatment, and remaining persistently elevated in non-responders [299]. Similarly, urinary VCAM-1 was not part of the optimal urinary biomarker panel but in an adult

SLE longitudinal study it was shown to correlate with renal SLICC score, UPCR and physicians global assessment over time [308]. The UK, US and SA JSLE Cohorts collect serial samples during routine clinical care, presenting the opportunity to assess the above markers in a 'real world' longitudinal setting.

5.2 Hypotheses

- The 'optimal urinary biomarker panel' identified within the UK JSLE Cohort will perform to a comparable level within the distinct, and ethnically diverse US and SA JSLE Cohorts.
- Individually or collectively, constituents of the 'optimal urinary biomarker panel' will play a role in the prediction of LN flare and remission over time.

5.3 Aims and objectives

The primary aim of this study was to assess if the 'optimal urinary biomarker panel' for identification of active LN identified in the UK JSLE Cohort Study performed in a comparable way within two international, ethnically distinct JSLE cohorts from the US and SA. The secondary aim was to determine longitudinally, using data and samples from all three cohorts, if the urinary biomarker panel itself, or its constituent members, are able to predict LN flare and remission in advance.

The specific objectives of this chapter were:

1. To evaluate the performance of the UK optimal urinary biomarker panel cross-sectionally within the US and SA Cohorts, to see if the same 'excellent' ability for identification of active LN is demonstrated.

2. To compare the urine biomarker concentrations in HC patients (from within the SA Cohort) to those within the SA JSLE patient groups.

3. To assess urine biomarker levels in patients with proven renal biopsy versus renal BILAG-defined LN, to assess performance of the renal BILAG score as a proxy measure of LN disease activity.

4. Using data from all three 'real world' JSLE cohorts from three different countries, to develop a Markov Multi-State model of urine biomarker dynamics in LN, assessing their ability to predict future LN flare and remission.

5.4 Specific methods

5.4.1 Patient cohorts

5.4.1.1 The US and SA Cohorts and renal disease activity classification

The US Cohort included patients followed at the Children's Hospital at Montefiore, Bronx, NY, USA [334]. SA Cohort patients attend Red Cross Memorial and Groote Schuur Hospital Hospitals, Cape Town, South Africa (see sections 2.1.2 and 2.1.3 for more details). HCs with non-inflammatory non-infective diagnoses, normal urinalysis and no symptoms of UTI were also recruited within the SA Cohort. In both cohorts, urine samples were collected during routine clinical care together with detailed demographic data, self-reported ethnicity data, clinical laboratory results and medication information. Renal disease activity was defined as per UK JSLE Cohort patients (see section 2.1.1.4).

5.4.2 Laboratory techniques

US and SA urine samples were processed following the same SOP as UK samples and stored at -80^{o}C prior to transfer to the University of Liverpool on dry ice (see section 2.2.5). The same ELISA kits, techniques and conditions were used for biomarker quantification as with the UK samples previously (see section 2.2.2 and Table 2-3). All biomarker results were standardised for urinary creatinine (Cr) concentration and presented in units per milligram creatinine (mgCr).

5.4.3 Statistical analysis

5.4.3.1 International validation of the urine biomarker panel – cross sectional

Summary statistics for demographics, baseline clinical data and biomarker data are presented as median values and IQRs. Univariate logistic regression (quantitative data) and Pearson's chi-square test (binary data) were used to assess for differences in demographic and clinical factors between different patient groups. Due to the number of factors explored, a Bonferroni adjustment was applied to account for multiple testing (15 comparisons per cohort).

Mann Whitney U tests with Bonferroni adjustments were used to compare biomarker concentrations between active and inactive LN patients (7 comparisons for US patients and 6 for SA patients), as well as inactive and HC SA patients (6 comparisons). When comparing urinary biomarker levels in patients where a diagnosis of LN was made on the basis of recent renal biopsy results versus BILAG defined nephritis alone, Bonferroni adjusted Mann Whitney U tests were also used.

The US data was analysed following the same methodological approach as the UK data (see section 4.4.5.1). On using a standard logistic regression approach to analyse the SA data, problems were encountered due to a phenomenon known within the statistical literature as 'separation', whereby a linear function of the covariates within a regression model can generate perfect predictions of the outcome variable. This phenomenon is discussed in detail in reference [441]. A consequence is that maximum likelihood estimates of the parameters suffer from bias when using standard logistic regression, and the standard errors of parameters are over-inflated. One solution to the problem is to use Firth's Penalised Likelihood Logistic Regression [41,442,443], which removes the bias from the maximum likelihood estimators, and is the approach used to analyse the SA data. Again, the baseline model included biomarkers AGP + CP for consistency with the baseline model identified in the UK dataset, and each of the remaining novel biomarkers were then added to the baseline model in turn in the same way as was done in the UK dataset, and the AUC for each updated model calculated. The data from all three cohorts were then pooled, and analysed in the same way as the US and UK data given the larger sample size (see section 4.4.5.1). AUC values of 1.0–0.9, 0.9–0.8, 0.8–0.7, 0.7–0.6, 0.6–0.5 were considered "excellent, good, fair, poor and fail" respectively [402].

5.4.3.2 Longitudinal analyses of urinary biomarkers

A subset of children included in the UK, US and SA JSLE cohorts had >1 urine sample available for novel urine biomarkers measurement over time. As the samples were collected during routine clinical care, the time between samples and the number of samples varied between patients. Six novel urinary biomarkers (AGP, CP, LPGDS, TF, MCP-1 and VCAM-1) were quantified by ELISA (see section 2.2.2 and Table 2-3). Patients were seen to transition between active LN (renal BILAG A/B), mildly active LN (renal BILAG C) and inactive LN (renal BILAG D/E). A binary outcome of 'any LN activity'/inactive LN was required for longitudinal analysis. Inspection of urine biomarker values for renal BILAG C patient episodes showed them to cluster with renal BILAG A/B episodes (see Figure 5-6). This grouping is also in keeping with the previous BILAG validation study [68] by Yee et al (assessing the BILAG intention to treat principle), which showed fluctuation between adjacent renal BILAG categories of A and B or B and C to occur without changes in LN treatment (see section 1.1.4.2). The longitudinal 'any LN activity group' therefore included patients with a renal domain BILAG score of A, B or C & previous histological confirmation of LN (State 2), or inactive LN (renal domain BILAG score D or E, State 1).

Biomarker and clinical-demographic data including age, sex, duration of disease, ethnicity, C3/C4, and anti-dsDNA antibodies were considered within the models. Some predictors contained missing (at random) data which would reduce the sample size available for the model, multiple imputation based on the Bayesian Alternating Conditional Expectation algorithm [444], was therefore used to avoid this. Heterogeneity was found in the distributions of some of the prognostic factors and states (statistical significance assessed via Kolmogorov-Smirnov and Fisher tests, for continuous and binary data, respectively). To address this, Huber's robust "sandwich" estimate [444] of the co-variance matrix of model parameters was utilised, taking into account the intra-cluster correlation of the data in each cohort. The effect of such a correction of the covariance matrix using a Huber's estimate is to increase the standard errors of the parameter estimates, thus reducing the chances of false rejections of statistical tests, and increasing the width of the confidence intervals.

A baseline homogeneous Markov Multi-State model of disease state transitions was fitted by Dr Antonio Eleuteri, Department of Physics and Clinical Engineering, UoL. The model was fitted assuming that individuals independently transition among the two states of active and inactive LN according to a continuous-time Markov process as depicted in Figure 5-1 below. At a given time-point, the aim of the model would be to provide the instantaneous probability of a patient staying in the same state (active or inactive LN) or transitioning (active to inactive, of vice versa).

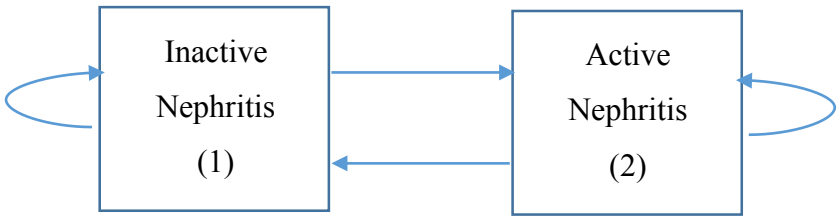


Figure 5-1: State transitions assumed by the Markov Multi-State model.

A 2 x 2 transition intensity matrix was derived, and then extended so that the transition intensities could depend upon prognostic factors. Within the present data set there were 13 potential predictors (novel biomarkers and clinico-demographic factors), which would require a model with 28 parameters. While no formal definition of an *effective sample size* for Markov Multi-State models exists in literature to date [444], it was assumed the somewhat restrictive definition of *the smallest number of observed state transitions*. In these data it was observed that only n=10 transitions from the inactive to the active state, so this was our effective sample size when developing the model. Using the empirical assessment of model complexity in regression analysis [444], it was estimated that no more than two predictors can be used in the model without the risk of overfitting.

To select candidate predictors, the expected relative Kullback-Leibler information [445] estimated by the AIC score was used. The AIC is essentially a measure of information loss induced by approximating the data generating process with a model, the best model is therefore one with the lowest AIC score. All data analysis was undertaken using R version 3.1.1 [360]. Graphical illustrations were generated in R or using GraphPad Prism version 6.0. Where Bonferroni adjustment was made to account for multiple testing, the Bonferroni corrected p-value, p_c is reported.

5.5 Results

5.5.1 Cross-sectional biomarker panel validation - US and SA cohorts

5.5.1.1 US Cohort

The US Cohort consisted of 30 JSLE patients, 16 (53%) were classed as active LN (11/16 renal BILAG score=A, 5/16=B) and 14 (47%) as inactive LN JSLE patients (6/16 renal BILAG score=D, 8/16=E). Active and inactive LN JSLE patients had a median age of 15 [14-17] and 17 [15-19] years respectively, with a disease duration of 3.1 and 1.7 years at the time of biomarker analysis. One hundred percent of the active LN and 71% of the inactive LN patients were female. US Cohort patients were largely African/African American (53%) and Hispanic (43%), whereas the UK JSLE Cohort patients assessed in Chapter 4 were predominantly Caucasian (41%) and Indian (23%). All active LN patients had biopsy proven LN during their disease course, with the ISN/RPS 2003 classes as follows; class III = 19%, class IV = 19%, class V = 31%, mixed class III/V = 31%, and a median of 5 ACR classification criteria at diagnosis. Active LN and inactive LN patients differed significantly in terms of their UACR and use of ACEi/AT2 blockers (both $p<0.05$, see Table 5-1).

5.5.1.2 SA Cohort

The SA JSLE Cohort consisted of 23 JSLE patients and 18 HCs, 9 JSLE patients (39%) were classed as having active LN (8/9 renal BILAG score=A, 1/9=B) and 14 (61%) as inactive LN (5/14 renal BILAG score=D, 9/14=E). Active LN, inactive LN patients and HCs had a median age of 13 [11-15], 14 [13-15] and 11 [10-12] years respectively, with a disease duration of 2.8 and 2.6 years for the JSLE patients at the time of biomarker analysis. 89% of the active LN, 86% of the inactive LN patients and 78% of the HCs were female. SA JSLE patients were mainly Coloured[1] (55% of active LN and 57% on inactive) and Black African (34% of active LN and 29% of inactive). 11% of active LN and 7% of inactive LN patients were Indian/Asian. 45% of HCs were Coloured[1] and 55% were Black African. All active LN patients had biopsy proven LN

[1] Coloured is an ethnic label for people of mixed ethnic origin who possess ancestry from Europe, Asia, and various Khoisan and Bantu ethnic groups of Southern Africa.

during their disease course, with the ISN/RPS 2003 classes as follows; class II = 44%, class IV = 34%, class V = 22%, and a median of 6 ACR classification criteria at diagnosis. Active LN and inactive LN patients differed significantly in terms of their UACR ($p<0.01$, see Table 5-1).

	US Cohort			SA Cohort		
	Active LN	**Inactive LN**	p_c	**Active LN**	**Inactive LN**	p_c
Age[1]	15 [14-17]	17 [15-19]	*ns*	13 [11-15]	14 [13-15]	*ns*
Duration[2]	3.1 [1.2-4.8]	1.7 [0.5-5.6]	*ns*	2.8 [0.7-4.9]	2.6 [1.8-4.0]	*ns*
Female	16 (100)	10 (71)	*ns*	8 (89)	12 (86)	*ns*
ACR[3]	5 [5.0-5.8]	5 [4.5-6.0]	*ns*	6 [4-8]	5 [4-6]	*ns*
Prednisolone[4]	14 (88)	12 (86)	*ns*	6 (67)	8 (57)	
MMF[5]	7 (44)	3 (21)	*ns*	3 (33)	7 (50)	
CYC[6] **ever**	9 (56)	4 (29)	*ns*	1 (11)	0	*ns*
Ritux[7]**ever**	6 (38)	5 (36)	*ns*	1 (11)	0	
ACEi/AT2[8]	10 (63)	1 (7)	***0.03***	5 (56)	1 (7)	
GFR[9]	126 [90-160]	110 [100-123]	*ns*	136 [72-156]	150 [118-160]	*ns*
UACR[10]	555 [137-2059]	9 [3-19]	***0.03***	210 [90-415]	15 [10-20]	***<0.01***
Serum creat[11]	53 [44-71]	66 [62-73]	*ns*	39 [36-78]	40 [35-51]	*ns*
dsDNA[12]	156 [96-179]	87 [23-178]	*ns*	48 [12-244]	17 [9-42]	*ns*
C3[13]	0.8 [0.7-1.0]	1.0 [0.8-1.2]	*ns*	1.0 [0.7-1.1]	1.2 [0.9-1.3]	*ns*
ESR[14]	-	-	-	61 [25-83]	50 [20-77]	*ns*

Table 5-1: Clinico-demographic data of US and SA Cohort patients at the time of urinary biomarker quantification.

Data expressed as median values and IQRs in square brackets, or numbers and percentages in curved brackets. *p-values* are Bonferroni-corrected (p_c) from Chi Squared tests or univariate binary regression as appropriate, ns = non-significant, $p_c>0.05$. [1]Age in years at time of analysis. [2]Duration = disease duration in years. [3]ACR = number of American College of Rheumatology criteria for SLE fulfilled at diagnosis. [4]Current medication use described for regular medications, those taken in courses/intermittently are described as having been used 'ever'. [5]MMF= mycophenolate mofetil. [6]CYC = cyclophosphamide. [7]Ritux = rituximab. [8]ACEi/AT2= angiotensin converting enzyme inhibitor or angiotensin 2 blocker. [9]GFR = glomerular filtration rate, [10]UACR = urinary albumin creatinine ratio (mg/mmolCr), [11]Serum creat = serum creatinine (μmol/L). [12]dsDNA = anti-double stranded DNA antibody (IU/L), [13]C3 = complement factor 3 (g/L), [14]ESR = erythrocyte sedimentation rate (mm/h), ESR not routinely measured in the US Cohort.

5.5.1.3 Individual novel urine biomarkers

Figure 5-2 shows the distribution of novel urinary biomarker concentrations in US and SA Cohort patients, compared to UK JSLE Cohort patients. Patients with active LN from both US and SA validation cohorts had significantly higher urinary concentrations of AGP, CP, LPGDS, TF, MCP-1 and VCAM-1 than inactive LN patients (all $p_c<0.05$). Whilst similar for AGP, CP, LPGDS, MCP-1 and VCAM-1, this contrasts to the UK JSLE Cohort results where a significant difference in TF levels was not seen between active and inactive LN ($p_c = 0.06$). Some subtle variation was seen in the strength of the corrected *p-values* assessing the biomarkers' ability to differentiate active and inactive LN biomarker concentrations between cohorts for LPGDS, MCP-1 and VCAM-1. NGAL levels did not differ between US Cohort patient groups ($p_c=1.0$) and were not quantified in the SA Cohort (see Table 5-2). In the SA Cohort, none of the urine biomarker levels differed significantly between inactive LN and HC patients (all p_c *values 1.0*, see Table 5-3).

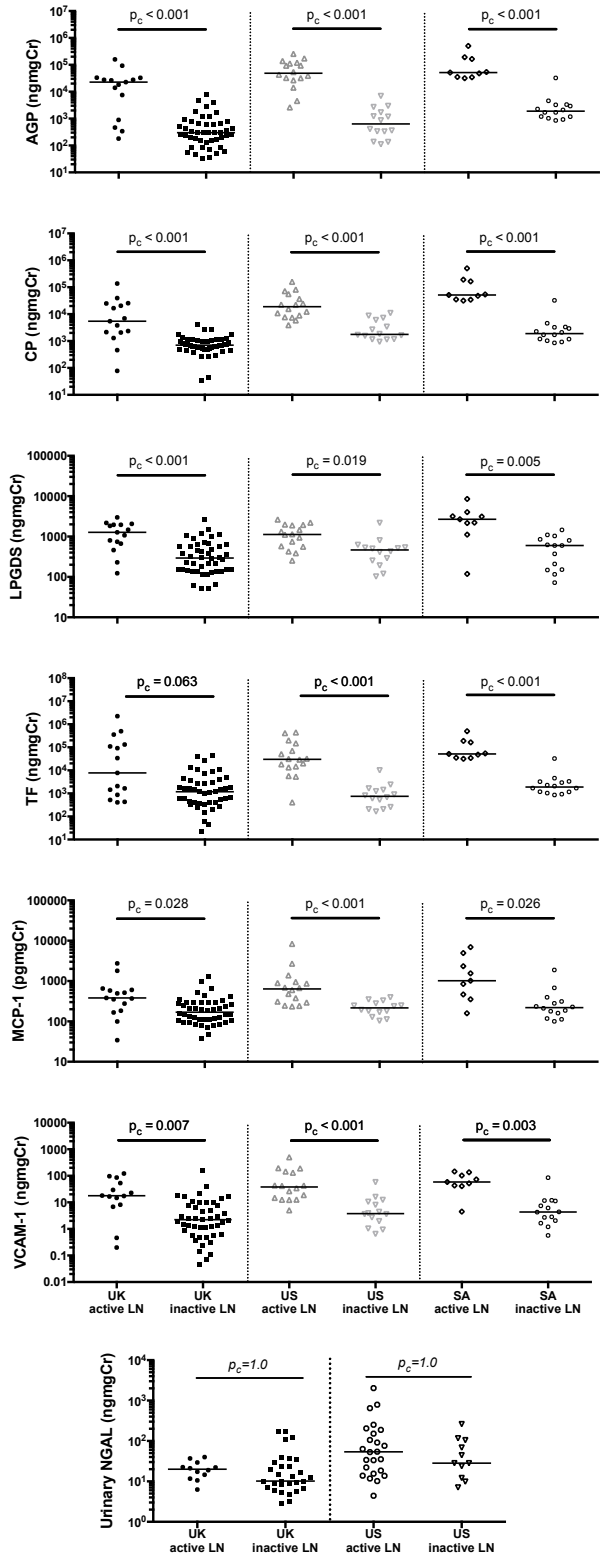


Figure 5-2: Distribution of biomarker concentrations in active/inactive LN patients from the UK, US and SA JSLE cohorts.

Median biomarker values shown by horizontal line. Mann Whitney-U tests used to compare distribution of biomarker concentrations between patient groups. Bonferroni corrected *p-values* (p_c). The raw data relating this figure are shown in Appendix 19.

	UK			US			SA		
	Active LN	**Inactive LN**	p_c	**Active LN**	**Inactive LN**	p_c	**Active LN**	**Inactive LN**	p_c
LPGDS	1175 [617-1,984]	288 [143-601]	*<0.001*	1324 [562-1939]	467 [238-561]	*0.0192*	2683 [1640-3602]	601 [151-900]	*0.0054*
TF	4,916 [781-188,415]	1,188 [402-3,822]	*0.063*	31334 [18278-148865]	748 [243-1495]	*<0.001*	63630 [38071-156026]	433 [221-1020]	*<0.001*
CP	4,638 [1891-25,062]	701 [505-1,010]	*<0.001*	22354 [10903-56080]	1765 [1181-6393]	*<0.001*	51714 [34861-177503]	1901 [1140-3276]	*<0.001*
AGP	20,559 [788-29,144]	304 [144-708]	*<0.001*	59173 [32491-119917]	635 [283-1965]	*<0.001*	145435 [54746-250367]	680 [394-2985]	*<0.001*
MCP-1	376 [180-599]	157 [105-295]	*0.028*	668 [378-950]	216 [159-291]	*<0.001*	1020 [410-3642]	219 [150-334]	*0.026*
VCAM-1	18 [8-62]	2 [1-8]	*0.007*	34.4 [14.8-130.9]	3.8 [1.8-10.9]	*<0.001*	58.8 [41.9-119]	4.3 [1.9-11.4]	*0.003*
NGAL	20 [12-22]	10 [4-30]	*1.0*	53 [19-179]	28 [7-108]	*1.0*	NA	NA	*NA*

Table 5-2: Urine biomarker concentrations standardised to urinary creatinine in active and inactive LN patients from the UK, US and SA JSLE Cohorts.

P-values quoted are corrected, p_c. Median values and interquartile ranges provided. MCP-1 concentration in pgmgCr, all other biomarkers in ngmgCr. NGAL levels not measured in SA JSLE patients.

Biomarker	Inactive LN [med, IQR], n=14	HC [med, IQR], n=18	Inactive vs. HC (p_c)
VCAM-1	4 [2-11]	4 [2-6]	*1.0*
MCP-1	219 [150-334]	296 [186-448]	*1.0*
LPGDS	601 [151-900]	577 [314-765]	*1.0*
AGP	680 [394-2985]	605 [408-1458]	*1.0*
CP	1901 [1140-3276]	1700 [1324-2999]	*1.0*
TF	433 [221-1020]	425 [234-928]	*1.0*

Table 5-3: Urine biomarker concentrations standardised to urinary creatinine in inactive LN and HC patients from the SA Cohort.
P-values quoted are corrected, p_c. Med = median. Median values and IQRs provided. MCP-1 concentration in pgmgCr, all other biomarkers in ngmgCr. NGAL levels not measured.

5.5.1.4 Combinations of urine biomarkers

In the US and SA cohorts, binary logistic regression models including AGP, CP, LPGDS and TF again produced the optimal AUCs (0.991 and 1.0 respectively). As a combination of biomarkers led to excellent identification of active LN in both validation cohorts, AUCs were also calculated for all three cohorts combined (see Table 5-4).

Biomarker combinations	UK Cohort[1]	US Cohort[2]	SA Cohort[3]	All cohorts together
AGP + CP	0.881	0.982	0.992	0.937
AGP + CP + LPGDS	0.900	0.982	0.992	0.942
AGP + CP + LPGDS + TF	0.920	0.991	1	0.951
AGP + CP + LPGDS + TF + VCAM-1	0.920	0.987	NA[4]	0.953
AGP + CP + LPGDS + TF + VCAM-1 + MCP-1	0.920	NA[4]	NA[4]	0.952

Table 5-4: Effect on AUC of adding biomarkers to the regression models in individual cohorts or together.
[1]59 UK patients included in novel biomarker models including VCAM-1 due to missing biomarker measurements. [2]30 US patients included in novel biomarker models. [3]23 SA patients included in novel biomarker models. [4]Patient number precludes fitting of a model.

The combined model for all three cohorts, including AGP, CP, LPGDS and Transferrin again gave an excellent AUC (0.951). However, adding VCAM-1 slightly improved the AUC still further (0.953). The receiver-operating curve (ROC) generated by the optimal three cohort model is shown in Figure 5-3.

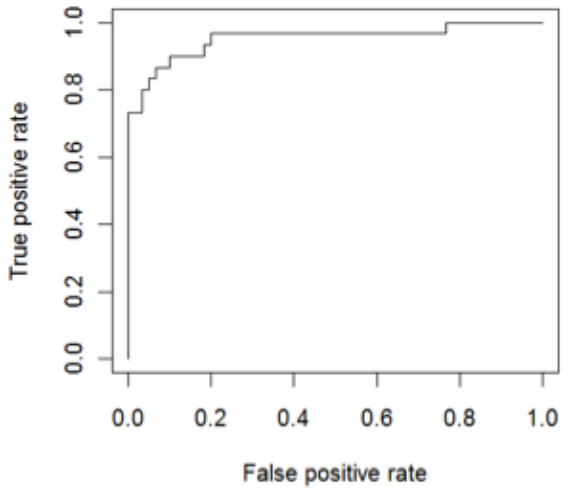


Figure 5-3: ROC generated from the optimal binary logistic regression model when data from all three cohorts were combined.
Optimal model includes AGP, CP, LPGDS, TF and VCAM-1 (AUC = 0.953).

5.5.1.5 Urine biomarker concentrations in biopsy versus renal BILAG defined LN

Urine biomarker levels from 12 samples from the US Cohort patients which were taken at the time of, or within six weeks of renal biopsy (8 at the time or biopsy, 2 up to 3 weeks before biopsy and 2 up to 6 weeks post biopsy), were compared with 11 patient samples who had had biopsy proven LN at some other point during their disease course but currently had a composite renal BILAG score-based diagnosis of active LN. Urinary AGP, CP, LPGDS, TF, MCP-1 and VCAM-1 levels did not differ significantly between the two groups of active LN patients (all $p_c = 1.0$; see Figure 5-4). Urine samples from the UK and SA Cohorts were not available close enough to the time of renal biopsy, therefore comparable groups were not available for inclusion in these analyses. Within the US Cohort samples taken at the time of, or within six weeks of renal biopsy, urinary biomarker levels appeared to be lower in class II ISN/RPS 2003 LN sub-class as compared to all other classes, however the numbers available were limited and therefore statistical tests were not possible (see Figure 5-5).

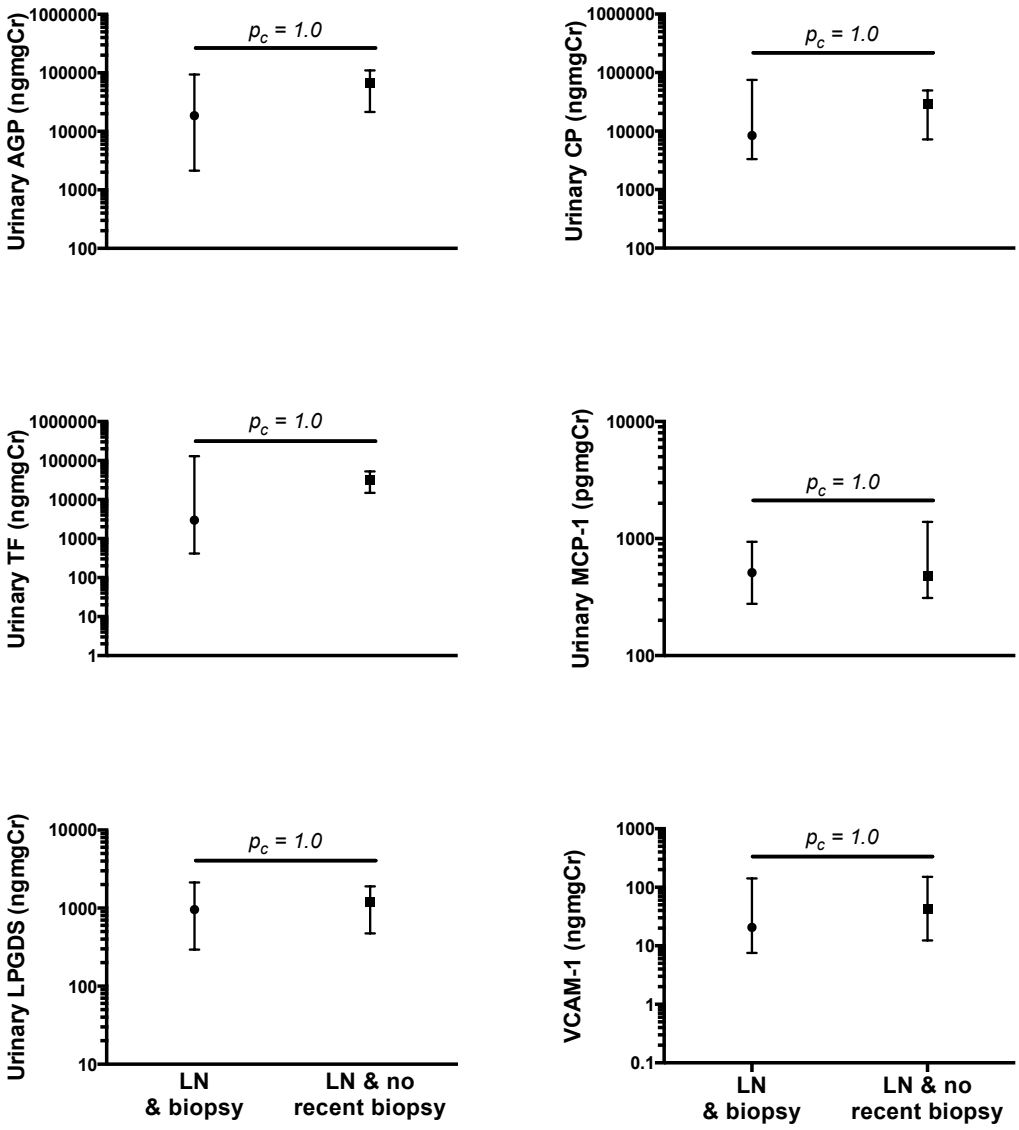


Figure 5-4: Urine biomarker concentrations in US Cohort patients with biopsy vs. BILAG defined active LN.

LN & biopsy patients, n=12. LN no biopsy, n=11. *P-values* quoted are corrected, p_c.

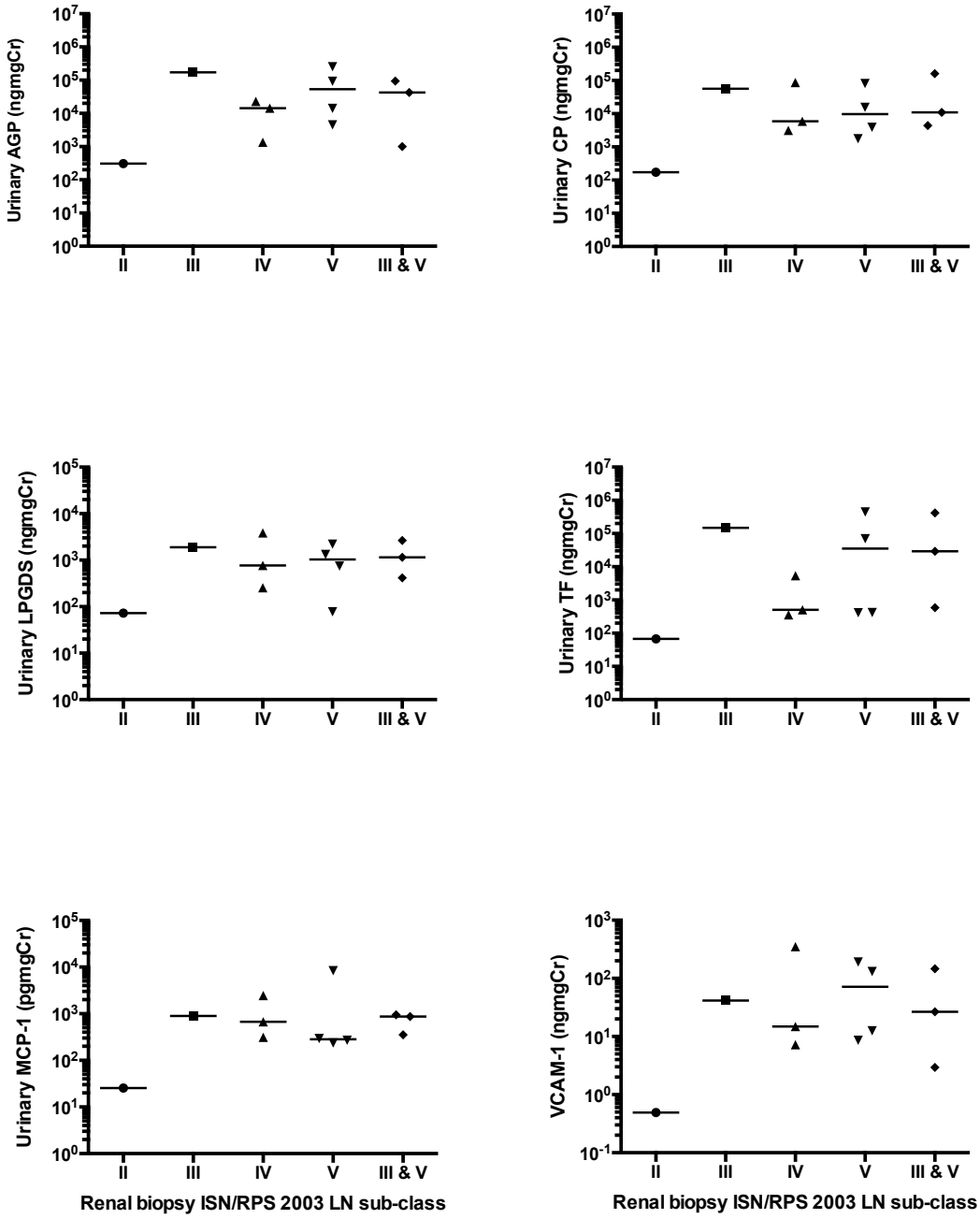


Figure 5-5: Urine biomarker concentrations according to renal biopsy ISN/RPS 2003 LN subclass.

Urine samples taken at the time of or within 6 weeks of renal biopsy. Individual patient values shown in the figure and median biomarker concentrations shown by the horizontal line. Total n =12 with 1 class II patient, 1 class III patient, 3 class IV, 4 class V and 3 mixed class III and V.

5.5.2 Longitudinal analysis within three 'real world' JSLE Cohorts

5.5.2.1 The longitudinal data

This study included 184 observations from 57 UK patients, 27 from 13 US patients, and 33 from 10 SA patients. Across the data set there were 10 transitions from the inactive to active LN disease state (1→2 transition), 18 from active to inactive LN (2→1 transition), with 93 and 43 remaining inactive and active respectively between time points (see section 5.4.3.2). The median follow-up period was 10.85 months [IQR 6.07-23.0]. Patients included within the original cross-sectional studies who only had one visit were excluded from these analyses. Inspection of the longitudinal urine biomarker values for renal BILAG C patient episodes showed them to cluster with renal BILAG A/B episodes (see Figure 5-6) rather than D/E episodes. On the basis of this observation and the findings of Yee et al (see [68] and section 5.4.3.2), the longitudinal active LN group therefore included patients with a renal domain BILAG score of A, B or C & previous histological confirmation of LN (State 2), or inactive LN where the renal domain BILAG score was D or E (State 1).

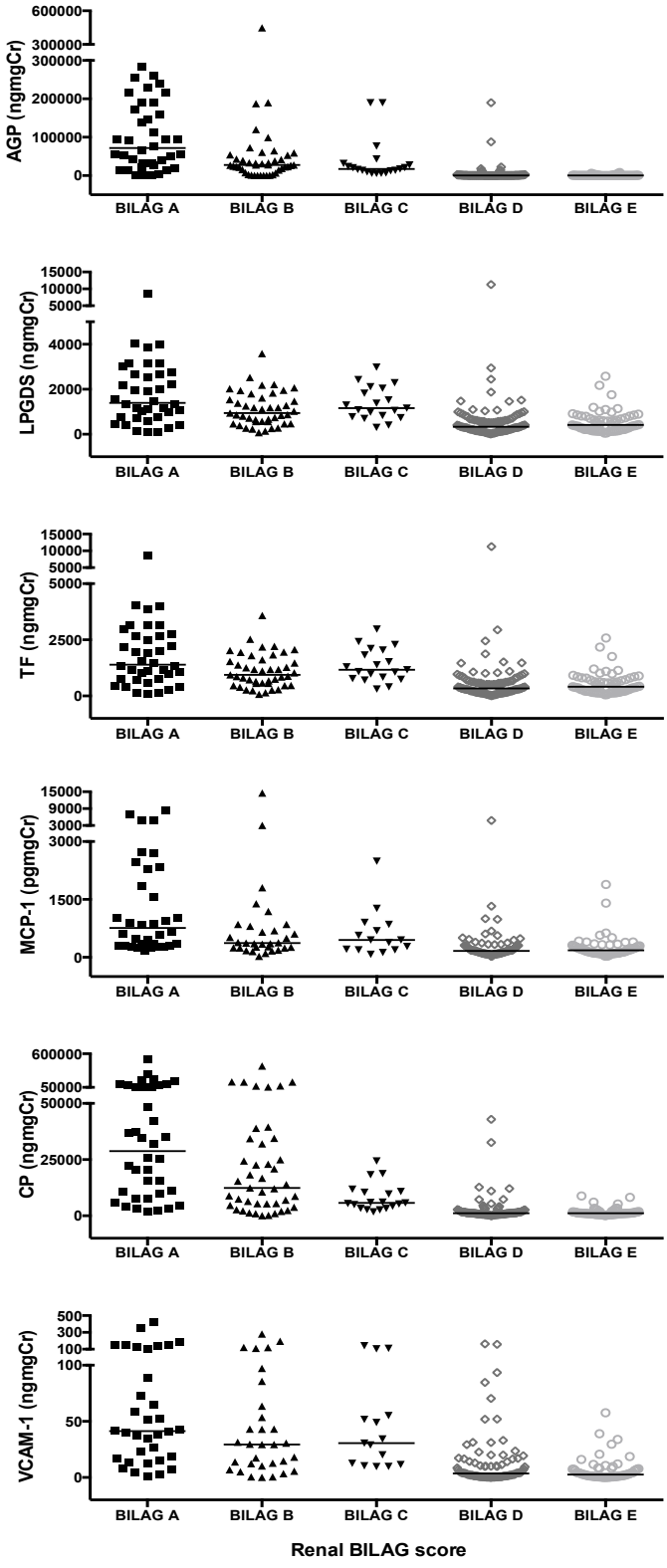


Figure 5-6: Urine biomarker values for UK, US, SA Cohort patients included in the longitudinal analysis according to renal BILAG score.

Horizontal line represents the median biomarker concentration. Each data point represents a biomarker value at a given time.

5.5.2.2 Developing a Markov Multi-State model of LN urine biomarker dynamics

A series of time-homogeneous Markov Multi-State models were fitted to the data using routines from the R library ***msm*** [446]. The AIC values for each model are shown in Table 5-5. The baseline model, including no factors had a AIC of 147.85, therefore models with a lower AIC than this had better evidence than the baseline model. The two models with the highest evidence are therefore ones including AGP (AIC 139.81) and CP (AIC 141.40) as predictors.

No factor	**LPGDS**	**MCP-1**	**CP**	**AGP**	**VCAM-1**	**TF**
147.85	152.14	154.74	141.40	139.81	142.59	151.64
Ethnicity	**Sex**	**Age**	**Disease duration**	**dsDNA-antibody**	**C3**	**C4**
151.41	154.35	154.57	153.89	147.35	155.88	157.12

Table 5-5: AIC values obtained by fitting a series of time-homogeneous Markov Multi-State models to each of the 13 potential predictors (novel biomarkers and clinico-demographic factors).

Combining both AGP and CP in a model (no more than two predictors allowed, due to the small effective sample size), the AIC was 157.19, so the increased complexity of the model did not improve the evidence for the model. The hazard ratios for this model (with 95% CIs) are shown in Table 5-6. Inspection of the confidence intervals associated with the prognostic factors added to the model revealed that they include the value 1 in the case of CP for state 1-2 transitions (inactive to active), and in the case of AGP for state 2-1 transitions (active to inactive). This suggested that the data is not informative enough to reliably assess the impact of CP/AGP on both of these transitions.

State transitions	**Baseline model**	**AGP**	**CP**
1-2	0.25 (0.03, 1.89)	1.80 (1.08, 3.01)	0.46 (0.17,1.28)
2-1	0.19 (0.03, 1.41)	1.22 (0.78, 1.91)	0.33 (0.18, 0.62)

Table 5-6: Hazard ratios for state transition dependent upon each prognostic factor in the full model containing both AGP and CP.
The baseline model was fitted using mean AGP and CP values. Hazard ratios and 95% confidence intervals shown. State 1 = inactive LN, State 2 = active LN.

A simplified model was then fitted with each biomarker only impacting on one transition, i.e. AGP for the 1-2 transition (inactive to active, predicting flare) and CP for the 2-1 transition (active to inactive, predicting remission). The new simplified model's AIC was 127.53, which is the lowest among both the single factor models, and the previous full model. Table 5-7 shows the HRs with 95% Cis for the simplified model.

State transitions	Baseline model	AGP	CP
1-2	0.59 (0.24, 1.45)	1.49 (1.10, 2.02)	-
2-1	2.11 (0.90, 4.94)	-	0.60 (0.39, 0.93)

Table 5-7: Transition intensities with hazard ratios for each prognostic factor in the simplified model containing AGP and CP.
The baseline model was fitted using mean AGP and CP values. State 1 = inactive LN, State 2 = active LN. Hazard ratios with 95% confidence intervals shown.

5.5.2.3 Assessing the performance of the optimal Markov Multi-State model

Table 5-8 shows the optimal model's performance in predicting state prevalence up to one year, in three-months intervals, when AGP was seen to impact on 1-2 transitions and CP on 2-1 transitions. This is also represented graphically in Figure 5-7.

	State 1 (BILAG D, E)		State 2 (BILAG A, B, C)	
Time	**Observed**	**Expected**	**Observed**	**Expected**
3 months	50	51	23	22
6 months	41	42	18	17
9 months	33	35	16	14
1 year	21	22	11	10

Table 5-8: Observed and expected state prevalence's of state 1 and 2 over a 1-year period using the optimal Markov Multi-State model.
State 1 = inactive LN, State 2 = active LN.

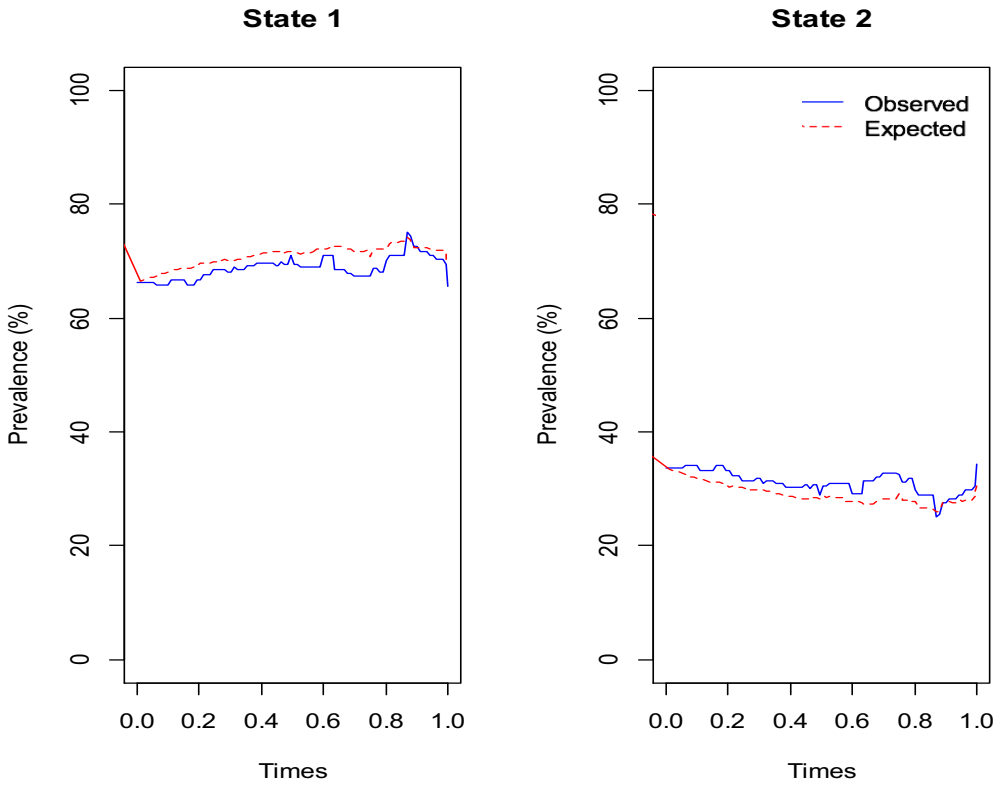


Figure 5-7: Observed and expected state prevalence over a 1-year period.
The blue line represents the observed state prevalence, whereas the red dotted line shows the state prevalence expected by the model. State 1 = inactive LN, State 2 = active LN. Times are shown in fractions of a year.

5.5.2.4 Using the Multi-State Markov model of LN urine biomarker dynamics - a worked example

Using a test subject where the AGP and CP values are known (patient 10045, who had active LN at the time of biomarker quantification), predictions of LN disease state over 3, 6 and 12 months can be demonstrated. For example, with a:

- CP of 2090 ngmgCr (log CP 7.64)
- AGP of 14043 ngmgCr (log AGP 9.55)

The Markov Multi-State model predicts that the mean time that they will remain in state 2 (active LN) = 0.41 years [0.16, 1.03]. Over the course of the 12 months the probabilities of state transition for this patient are shown in Table 5-9:

3 months	**To**	
From	**State 1**	**State 2**
State 1	0.79	0.21
State 2	0.40	0.60

6 months	**To**	
From	**State 1**	**State 2**
State 1	0.71	0.29
State 2	0.55	0.45

12 months	**To**	
From	**State 1**	**State 2**
State 1	0.66	0.34
State 2	0.64	0.36

Table 5-9: Test subjects probabilities of state transition at 3, 6 and 12 months following urine biomarker quantification.
State 1 = inactive LN, State 2 = active LN.

Therefore, the probability of this patient (currently with active LN) of remaining active at 3 months is moderate (HR 0.60), however by 6 and 12 months the probability of remaining active falls (HR 0.45 at 6 months and 0.36 at 12 months). Table 5-10 shows the actual LN disease course of this patient during the subsequent follow-up visits, showing that at 3 months the patient had a renal BILAG B score, becoming inactive (renal BILAG D) by 6 months.

Time	renal BILAG	SBP	DBP	Sev Hyper	U Dip	UACR	Serum creat	eGFR	Act Sed	Biopsy
0	A	98	60	N	4+	628	39	152	NA	Y
3/12	B	102	50	N	1+	9.6	49	120	Y	N
6/12	D	98	59	N	1+	3.2	49	118	N	N
12/12	D	100	60	N	1+	0.9	51	116	N	N

Table 5-10: Clinical LN disease course of the patient during follow up visits after urine biomarker quantification.

Time = time following biomarker quantification. SBP = systolic blood pressure. DBP = diastolic blood pressure. Sev Hypert = severe hypertension. Y/N = yes or no. U Dip = urine dipstick. UACR = urine albumin creatinine ratio. Serum creat = serum creatinine. eGFR = estimated GFR. Act Sed = active sediment. Biopsy = biopsy defined LN within the last 3 months.

5.6 Discussion

By simultaneously measuring urinary AGP, CP, VCAM-1, TF, LPGDS and MCP-1 in two distinct, ethnically diverse validation cohorts of JSLE patients, the primary aim of this study was to evaluate the performance of the UK optimal urine biomarker panel, to see if the same 'excellent' ability for LN identification could be demonstrated. In all three cohorts (UK, US, SA) the optimal urinary biomarker combination for identification of active LN was shown to include AGP, CP, LPGDS and TF with excellent an AUC values of 0.920 in the UK, 0.991 in the US anda perfect AUC of 1.0 in the SA Cohort. This is the first LN urine biomarker panel study to include exploratory and validation cohorts.

The secondary aim was to use longitudinal data from all three 'real world' cohorts to see if constituents of the biomarker panel are able to predict LN flare and remission in advance, using Markov Multi-State modelling. During the longitudinal study period, a relatively small number of patients transitioned from inactive to active LN (or vice versa), so the model was restricted to containing two biomarkers (to avoid overfitting). The two biomarkers with the highest evidence for predicting changes in LN activity were found to be AGP and CP. The optimal model including both AGP and CP suggested that AGP was best at predicting flare, and CP was best for predicting remission. Using this model, it is possible to develop individual patient predictions of LN disease state over 3, 6, 9 and 12 months.

As mentioned in Chapter 4, previous studies complementing our work have focused on identification of biomarker combinations reflective of LN histological subtypes in patients with biopsy proven LN. Brunner et al's study of 28 childhood onset and 48 adult SLE patients assessed biomarker combinations differentiating biopsy defined activity, chronicity or membranous LN in samples taken within 2 months of biopsy. The best predictive ability was seen for LN activity, when MCP-1, AGP, CP and UPCR were considered together (AUC 0.850) [318]. Within the UK, US and SA cohorts, we have demonstrated stronger AUC values (0.920, 0.991 and 1.0 respectively) for identification of active LN with urinary AGP, CP, LPGDS and TF in combination. When the results from the UK, US and SA Cohorts are pooled, VCAM-1 also adds to the diagnostic ability of the above biomarker panel.

The improved performance of the urine biomarker panel in the validation cohorts (US and SA) as compared to the original exploratory UK Cohort is interesting, and may be partially explained by differences in the ethnicity and severity of LN between the patient cohorts. The UK JSLE Cohort comprised of predominately Caucasian and Indian patients, the US Cohort of African American and Hispanic patients, and the SA Cohort mostly included Coloured and Black African patients. Notably, African and African American patients are known to often have more severe kidney involvement in SLE [32,281,403]. In the US and SA Cohorts there was also a greater proportion of active LN patients relative to the total patient numbers (53 and 39% in US/SA respectively, vs. 25% in UK). Although standardized SOPs were used throughout the course of the study, there could be operator-related improvements in lab techniques which could reduce variability in the assays and bear influence on these results.

Using samples from the US Cohort we were able to assess for potential differences in urine biomarker levels dependent on how the diagnosis of active LN was derived. In the UK and SA Cohorts all patients with 'active LN' had biopsy proven LN at some point during their disease course, but at the time of urine sampling they were defined as having active LN based upon their composite renal BILAG score (A or B). Within the US Cohort twelve samples were available which were taken at the time of or within 6 weeks of renal biopsy. Biomarker levels in these samples could therefore be compared to those in a further eleven patient samples who had a composite renal BILAG score-based diagnosis of active LN (but biopsy proven LN at some point during their disease course) as per the UK and SA patients. Urinary AGP, CP, LPGDS, TF, MCP-1 and VCAM-1 levels did not differ significantly between the two groups of 'active LN' patients (all $p_c = 1.0$), supporting the grouping of these patients together.

In the current study, all urinary biomarker levels appeared to be lower in class II ISN/RPS 2003 LN sub-class as compared to all other classes, however the number of patients available was limited and therefore statistical analysis was not possible. Some members of the urinary biomarker panel have been shown to correlate with histological subclasses in the literature. Abd-Elkareem et al studied 50 adult SLE patients and found elevated urinary VCAM-1 in class III, IV and V LN but not in class I/II LN [328]. Singh et al found VCAM-1 to have a positive correlation with the presence of

class IV LN [297]. In an adult SLE longitudinal study, Rovin et al demonstrated MCP-1 levels to be higher in patients with proliferative LN (WHO class III/IV) as opposed to membranous (class V) nephritis [299].

A recent study by Brunner et al assessing how urine biomarkers relate to composite NIH histological AI/CI indices (rather than distinct histological sub-classes), has looked at samples taken at the time of biopsy from 47 children. They demonstrated NGAL, MCP-1, CP, adiponectin, hematopexin and KIM-1 to be the best predictors of NIH LN AI, proposing a biomarker based Renal Activity Index for Lupus (RAIL) [348]. The RAIL includes additional biomarkers to those assessed in the current study, also within a biomarker panel. These results require further validation in larger international cohorts, assessing the measurement properties of the RAIL, cutoff scores in different populations and their ability to reflect ISN/RPS LN classes. In contrast to the markers validated in the current study, it remains unclear whether these biomarkers would be able to differentiate patients with active LN versus inactive LN as all patients in the above study had definite biopsy-defined active LN at the time of biomarker quantification.

In this study it was not possible to demonstrate a significant difference in urinary NGAL levels between those with active LN/inactive LN in either the UK or US cohorts on a cross-sectional basis. This is in contrast with previous work which has shown NGAL to be highly sensitive/specific for identification of biopsy proven LN in children [74]. These results may be explained by differences in the timing of the sample and the outcome measures used. Urinary NGAL has previously been shown to be a useful predictor of impending flare in the UK JSLE Cohort [74], and the adult arm of the Einstein Lupus Cohort [71]. Kiani et al were also unable to detect an association between urinary NGAL and LN in a prospective study including 107 adult SLE patients [308]. These observations may be due to urinary NGAL levels peaking before flares, and receding before it becomes clinically detectable [320]. Urinary NGAL has also been demonstrated as a marker of renal damage in LN [321], which may also explain why patients with a history of biopsy proven LN have higher urinary NGAL levels.

Longitudinal LN urine biomarker studies to date have featured individual biomarkers and small cohorts of patients from single countries [74,290,299,308]. The clear benefits of the current study deriving a Markov Multi-State model of LN urine biomarker disease dynamics, are the relatively large number of patients (n=97, with 184 individual visits), the international representation within the study population, and the rigorous statistical methods used. Of the six novel urine biomarkers, and seven clinico-demographic factors evaluated in the model, AGP and CP were found to be most informative. Fitting of the time homogeneous Markov Multi-State model showed AGP to be predictive of active LN flare, and CP to be predictive of remission. Looking at the AIC for each candidate predictor, VCAM-1 for example had a similar AIC value to AGP and CP (VCAM-1 = 142.59, AGP = 139.81, CP = 141.40), so if more data were available, including more LN disease state transitions, it would also be interesting to look at the impact of adding VCAM-1 to the model.

In contrast to the cross-sectional analysis where renal BILAG C patient episodes were excluded, the longitudinal active LN group included patients with a renal BILAG score of A, B or C. Inspection of the urine biomarker values for renal BILAG C patient episodes showed them to cluster with renal BILAG A/B episodes (see Figure 5-6) rather than D/E episodes. As discussed in sections 1.1.4.2 and 5.4.3.2, this approach was in keeping with the previous BILAG validation study [68] by Yee et al, which showed fluctuation between adjacent renal BILAG categories of A and B or B and C to occur without changes in LN treatment. Clinically it was also felt most appropriate to group patients with 'any LN activity' together in this way. Exclusion of these patient episodes within the longitudinal analysis would have led to long gaps between urine sampling episodes, and inclusion of a third category would increase the complexity of the modelling process given the sample size.

Despite these limitations, this study has demonstrated significant data regarding a panel of urine biomarkers which are able to predict changes in LN disease activity in ethnically diverse cohorts. The Markov Multi-State model therefore warrants further rigorous testing in a larger, prospectively conducted clinical trial of biomarker-led LN monitoring. These data support the argument eluded to in the current Chapters introduction, that very few tests are ideal biomarkers at every time point, therefore a longitudinal biomarker panel-based approach is required (see section 5.1.2), with AGP

and CP demonstrating predictive ability for different stages of the disease process. Validating these results in the context of a clinical trial (rather than a prospective observational study) would ensure that samples and clinical data are collected on a comprehensive, very regular and systematic basis. This would allow for more thorough assessment of the predictive abilities of the Markov Multi-State model. Such a study could be adequately powered to facilitate assessment of other markers which may improve the model further (e.g. VCAM-1) and ultimately could assess whether urine biomarker-led monitoring can actually improve renal outcome for patients.

5.7 Summary

This study has demonstrated and validated, a renal-specific 'excellent' novel urinary biomarker panel for recognition of active LN in three ethnically diverse JSLE populations. This adds considerable strength to the original proposed panel findings. Development of a Markov Multi-State model of LN urine biomarker disease dynamics demonstrated that AGP is predictive of active LN flare, and CP is predictive of remission in three independent cohorts from three continents, representing a significant step in the advancement of developing a urinary biomarker panel for LN. This is the first urine biomarker panel study to include both cross-sectional and longitudinal exploratory and validation cohorts, providing a firm foundation for development of a longitudinal clinical trial of urine biomarker-led monitoring in LN. To this end, efforts must be made to improve the method of urine biomarker panel quantification as current ELISA techniques are very time consuming and costly, prohibiting their use in a real-time study of biomarker-led monitoring, where a short turnaround time for results would be required.

5.8 Conclusions

- The optimal 'LN urinary biomarker panel' derived within UK JSLE Cohort Study samples displayed an equivalent ability for active LN identification in both the US and SA JSLE cohorts.
- Within a Markov Multi-State model of LN urine biomarker dynamics, AGP was found to be best at predicting LN flare and CP was best for predicting LN remission.

- To enable development of a future clinical trial of urine biomarker-led monitoring, urine biomarker panel quantification must be streamlined to allow the biomarker panel to be quantified quickly and conveniently.

6 Developing a urine biomarker panel test for use in a clinical trial in children

6.1 Introduction

6.1.1 Making steps towards a prospective clinical trial of urine biomarker-led monitoring in LN

As noted in Chapter 1, a six step process should be considered when translating an experimental biomarker into clinical practice, including: (1) initial biomarker identification, (2) evaluation of the results by independent experts, (3) further evaluation in a suitable biobank of existing samples or newly collected samples, (4) evaluation in a clinical trial or prospective study, (5) implementation in clinical practice, and (6) proving the cost-effectiveness of the validated biomarker [282].

The majority of LN urine biomarker studies to date have focused upon step one and two of this process. Through chapters 1-5 of this present thesis, step three has been reached by (1) identifying a panel of biomarkers which accurately identify LN in a UK JSLE population; (2) undertaking independent confirmatory analyses of the results by statistician's out with the laboratory research team; (3) subsequently evaluating the biomarker panel in two independent, international cohorts (US biobank of existing samples and SA prospectively collected samples). The next step necessary would therefore be to evaluate this biomarker panel within a prospective clinical trial. However, improving the method by which these multiple biomarkers are quantified is needed to make such a trial realistic.

6.1.2 Need for streamlined biomarker panel analysis to facilitate a future prospective clinical trial

Individual ELISA assays were used for urine biomarker quantification in Chapters 4 and 5. However a need to streamline biomarker panel analysis through a single combined biomarker assay was recognised, allowing the biomarker panel to be quantified quickly and conveniently as part of a future clinical trial. Such an assay would be expected to use a smaller sample volume than previous individual ELISA assays, whilst providing comparable results, and would then be a key enabler of a future larger prospective clinical trial of biomarker-led monitoring in clinical care.

After undertaking a series of introductory meetings with several industry partners, the multiplex assay emerged as the most suitable method to combine these biomarkers into a single test. External commercial small-to-medium sized enterprise (SME) companies specialising in developing small point of care devices (e.g. similar to a pregnancy test) were only able to offer devices which would measure 1 or 2 biomarkers in combination. In 2014, Dr Smith was selected from >150 international entrants as the winner of the Merck Millipore 2014 MAGPIX™ Grant program. She was awarded a MAGPIX™ Luminex Multiplex Instrument for accurate magnetic bead-based biomarker quantification using multi-analyte panels (worth £30,000), MILIPLEX Analyst 5.1 software, technical support (worth £5000) and £10,000 of assays (see Appendix 20). Using this technology, it is possible to simultaneously measure up to 50 analytes in a 25µl patient sample.

Dr Smith was awarded this prize on the basis of her proposal for using this platform for assessing the urinary biomarker panel, recognising that the *'MAGPIX™ instrument could really make a difference to your research and potentially benefit human health'.* As part of this, Millipore were keen to showcase how its products can help *'real world academics'* in the translation of biomarkers. The off-the-shelf multiplex kits developed by Merck Millipore (and other multiplex assay manufacturers) did not however measure our candidate biomarkers together, nor had they been validated for use in urine. To take full advantage of this technology and get the most out of Merck Millipore's prize, funding was sought to develop an LN urinary biomarker panel multiplex assay with Merck Millipore.

6.1.3 MRC Confidence in Concept Award

In March 2015, Dr Smith led a UoL MRC Confidence in Concept (CiC) scheme grant application, along with Professor Beresford and co-applicants; Professor Matthew Peak (Director of Research, Alder Hey Children's NHS Foundation Trust) and Dr Louise Oni (NIHR Academic Clinical Lecturer, Women and Children's Health, University of Liverpool). Collaborators included Professor Chris Scott (University of Cape Town, SA), Assistant Professor Beatrice Goilav (Albert Einstein College of Medicine, New York, USA) and Mr Lawrence Rentoul (Merck Millipore, Nottingham,

UK). This scheme aimed to accelerate the transition from discovery science to the early stages of therapeutic/diagnostic development and was therefore ideally suited to our work (see Appendix 21).

Applications had to be for projects that would cost no more than £50,000 and run between 6-12 months. Applications had to show how MRC CiC funding would provide a firm foundation for subsequent competitive funding or partnership with industry. The MRC was keen to promote academic-industry interactions and so applicants were strongly encouraged to explore how an award could be used to develop these interactions, which could include in-kind contributions from an industry partner. The application was successful and focused upon completing the assessment of urine biomarker panels within the international JSLE Cohorts (US/SA), development and validation of a custom multiplex assay for use in JSLE patient urine samples with Merck Millipore (using the multiplex platform gifted by them), and optimisation of the assay to replicate the results previously measured by ELISA.

It was anticipated that working with Merck Millipore to simplify methods for biomarker quantification, through development of a proof-of-concept custom single multiplex assay could position this work in a strong position to apply for a future substantive grant (e.g. MRC, NIHR Invention for Innovation, Wellcome Trust Translation Award, Arthritis Research IUK) to undertake a larger prospective multi-centre study of urine biomarker-led monitoring in clinical care, with clear potential for commercial and healthcare benefits. The outputs from the MRC CiC award will be detailed in the current chapter.

6.1.4 Considering the design of a prospective clinical trial of urine biomarker-led monitoring in LN - can patients send their urine samples through the post?

In a future clinical study or trial of urine biomarker-led monitoring, frequent, fixed time point serial urine samples would need to be collected to accurately assess the ability of the biomarker panel to predict events such as LN flare, remission, treatment response and prognosis. To reduce the impact of such a study on patients' lives, these samples would ideally be sent to hospital through the post so that an increased frequency of hospital attendance is not required. Protease inhibitors are used by some

investigators for long-term storage of urine samples to prevent degradation of proteins over time [447]. Certain analysis techniques (e.g. high-performance liquid chromatography or urinary exosome analysis) have been shown to be particularly susceptible to the effects of prolonged storage, and therefore protease inhibitors are used routinely to prevent such problems [448,449]. Evidence for routine use of protease inhibitors for the analytes seen within our urinary biomarker panel is lacking, therefore our standard procedure has been to centrifuge samples, aliquot the urine supernatant and store at -80^{o}C as soon as possible after the patient voiding.

It is anticipated that ice packs +/- protease inhibitors may prove beneficial with samples undergoing delayed processing and being transported through the post. In a study looking at NGAL and KIM-1 levels in samples which were stored at 4°C and −80°C with or without protease inhibitors, urinary NGAL and KIM-1 concentrations were stable for up to 48 hours when stored at 4°C and up to 6 months when stored at −80°C, independent of the addition of protease inhibitors [450]. With an ice pack sent through the post, it may not be possible to maintain a consistent temperature of 4^{o}C, and therefore protease inhibitors may confer additional benefit. Boric acid (BA) is a preservative which can be used in the clinical setting where sample transport delays are expected before bacterial culture, to inhibit non-specific (mixed) bacterial growth and contamination [451]. BA is known to interfere with routine biochemical urine UPCR ratio measurement, so although it may have a beneficial effect on reducing bacterial contamination it may affect the integrity of the biomarkers and therefore warrants further investigation.

It would therefore be necessary to undertake a series of experiments to determine whether home urine collection (in the appropriate manner) would provide an opportunity for a clinical study of frequent monitoring of urine biomarker levels, ensuring prospective validation of the utility of the LN urine biomarker panel is practical, and has minimal impact of the patients' day to day life and activities.

6.2 Hypothesis

- A multiplex assay, specifically developed for use in JSLE patient samples, will rapidly and accurately quantify AGP, CP, LPGDS, TF, VCAM-1 and MCP-1 in a

single assay when compared to the six individual ELISA assays which are currently in use.

6.3 Aims and objectives

The primary aim of the study presented in this chapter was to develop a multiplex assay for quantification of the internationally validated LN urinary biomarker panel in collaboration with industry partner Merck Millipore, in order to streamline the process of biomarker quantification.

The secondary aim was to assess if urine samples can undergo delayed processing (48 hours after sample collection) and still maintain urine biomarker stability by transporting samples in the presence of additives and/or a cool environment, allowing patients to send in samples from home as part of a future prospective clinical study or trial. Comparison of urine biomarker levels in patients with UTI, mixed growth or no growth samples is also important, as samples sent from home are more likely to display such issues.

The specific objectives of this chapter were:

1. To identify antibody pairs capable of detecting each biomarker within urine samples and assess for cross reactivity between them to determine if they were appropriate for use in a multiplex assay format.

2. To assess for JSLE urine/sample related matrix effects that could impact on the accuracy of the multiplex results through spike/recovery and LOD experiments.

3. To undertake range finding experiments on a large number of JSLE patient samples to be clear on the sample dilution/dilutions required for a future prospective clinical trial.

4. To assess the agreement between ELISA and multiplex biomarker results, comparing the ability of both assays for detection of active LN disease state.

5. To assess the stability of urine biomarkers in samples which have been processed using accepted standard techniques compared with those that undergo delayed processing but are maintained in the presence of certain experimental conditions (protease inhibitors +/- BA +/- an ice pack).

6. To compare urine biomarker levels in patients with a UTI, mixed growth or no growth in their urine sample.

6.4 Specific methods

6.4.1 Custom multiplex assay development

The assay was developed by the Merck Millipore Multiplex Research and Development Team, St Charles, US, with close collaboration, project management and discussion with the team at the UoL, led by Dr Smith. Regular e-mail discussions, exchange of update reports, teleconferences and a face to face meeting, led by Dr Smith, were important in ensuring that the optimal assay was developed, and tailored to the disease state (LN). Following development of a prototype assay, further rigorous in-house beta testing was carried out at the UoL by Dr Smith to ensure that the results obtained by Merck Millipore could be replicated in a 'real world' clinical-academic research laboratory, that optimal dilutions were chosen based on a wide range of patient samples, and that this new assay provided equivalent results to existing ELISA techniques. Close communication was maintained with the Merck Millipore team during the beta-testing process by Dr Smith.

Multiplex assay principles and the specifics of the MAGPIX™ instrument used in this study are described fully in section 2.2.4 and Figure 2-3. Multiplex assay development is an iterative process, involving stages of optimisation due to the potential for complex interactions between assay components. The ability to test for multiple analytes simultaneously provides many benefits over traditional single analyte testing but can lead to problems with cross-reactivity between analytes and high background levels in the target matrix due to non-specific binding. A variety of potential capture antibodies (for a particular biomarker) can be coupled to different microsphere sets and then

tested with the individual candidate detection antibodies, allowing for rapid identification of the best-performing capture and detection antibody pairs. Polyclonal and monoclonal antibodies can be used as detection antibodies. If a monoclonal detection antibody is used it should be specific for a different epitope than the capture antibody. Detection antibodies are typically biotinylated with streptavidin-PE as the reporter, eliminating the need for a separate reporter labelling step.

Assay conditions which may require optimisation, including sample volume/dilution, buffers/blocking agents, reaction volume, number of microsphere beads per reaction (2000–5000 per region per well), concentration of capture reagent for coupling, detection antibody/reporter concentration, and incubation times. The final step is to undertake the fully multiplexed assay to determine sensitivity and interference when all analytes and reagents are present in the reaction [452]. These specific stages of the development process were undertaken by Merck Millipore using their existing protocols, and not shared or presented here to protect their existing IP relating to multiplex assay development. The performance of the assay was however re-evaluated with JSLE urine samples at the UoL. 5p log standard curves were plotted for all analytes and unknown values determined using MILLIPLEX™ Analyst 5.1 software [453].

6.4.1.1 Selection of antibody pairs and assessment of cross-reactivity

Merck Millipore used existing antibody pairs from their commercially available assays for the following analytes; VCAM-1 and MCP-1 (already present in their human cytokine/chemokine panel 1 assay), AGP and CP (present in their Human Neurological Disorders Panel 2). They did not have existing antibody pairs for TF or LPGDS and therefore were required to firstly undertake microsphere coupling (primary amines on antibodies covalently linked to the carboxyl groups on the surface of the microspheres using a standard two-step carbodiimide coupling procedure [452]), and secondly check a range of antibody pairs for suitability. Generally, reagents used for other singleplex immunoassays will perform well in multiplex assays, provided they have sufficient sensitivity and specificity.

For TF, Merck Millipore tested all possible configurations using one standard, three monoclonal and one polyclonal TF antibody. Only one combination of antibodies

(including a monoclonal capture antibody and a polyclonal detection antibody conjugated to streptavidin-R-phycoerythrin) were able to detect the standard and TF in urine samples (antibody suppliers not shared by Merck Millipore).

LPGDS, and in particular the "Lipocalin" specific version of Prostaglandin D Synthase, proved more difficult to detect in urine using commercially available antibodies. Merck Millipore tested all possible antibody combinations using one standard, one monoclonal and two polyclonal antibodies, but could not get any significant signals. We suggested they contact BioVendor the manufacturers of the ELISA kits (https://www.biovendor.com/country26/product/immunoassays/prostaglandin-d-synthase-lipocalin-type-human-elisa), who were willing to share their reagents. Using the BioVendor antibodies, the monoclonal capture antibody was coupled to the microspheres and the standard and native LPGDS could be detected by a polyclonal detection antibody (conjugated to streptavidin-R-phycoerythrin) in all samples.

Once antibody pairs were selected, cross-reactivity to non-target proteins was assessed by adding a single standard in turn to multiplexed beads and detection antibodies (all six analyte beads and detection antibodies together). The MFI for each analyte was assessed in the presence of the individual spiked standard. One should expect to see a high MFI corresponding to the given standard which has been spiked in, with background MFI levels for all other analytes. If cross-reactivity is present, one would see MFI levels above background for analytes other than the one specifically spiked in, with dose dependent variations in the MFI depending on the size of the spike. In such a situation, it may be necessary to replace or alter the concentration of the cross-reacting capture or detection antibody [452].

6.4.1.2 Range finding

Preliminary range finding and determination of the optimal sample dilution for use with the assay was carried out by Merck Millipore using UK JSLE patient and HC samples which were transferred from the UoL. Multiple aliquots of eight inactive LN, nine active LN and nine HC samples were transferred for use in the assay development process. In a multiplex assay, there needs to be careful assessment of a range of representative samples to ensure that values are detected for each analyte at a given

dilution. It may be that a single dilution is not achievable, and that each sample needs to be run at two or three dilutions. The range finding process continued as part of the beta testing process at the UoL using a larger number of samples from UK JSLE Cohort study, US and SA cohorts. During this process we assessed the percentage of samples detectable at a given dilution, the number of samples extrapolated and the number of samples that were un-detectable for each analyte. The optimal dilution was chosen and standard curves plotted showing where the patient samples are detected on the standard curve. Ideally most samples should be on the linear portion of the standard curve.

6.4.1.3 Spike recovery and linearity of dilution

Assay buffer components, multiplexed antibodies, urine sample matrix and JSLE related factors such as complement, heterophilic antibodies or rheumatoid factor all have potential to impact on the accuracy of multiplex results [452]. It was therefore important to validate the assay with urine from JSLE patients with varying disease activity through spike/recovery and LOD experiments. These techniques are explained fully in sections 2.2.2.2 and 2.2.2.3, but in brief, a known amount of standard is 'spiked' into the sample type of interest (observed spiked concentration) and the assay buffer alone (expected amount of spike). The level of the biomarker in the neat 'un-spiked' sample is also quantified. The recovery is calculated from the difference between the observed spiked concentration minus the amount of spike in the neat un-spiked samples, divided by the expected amount of spike. This is multiplied by 100 to get the percentage recovery.

Samples were also serially diluted to test for LOD and assess whether sample values generated from different dilutions are comparable, or whether a single dilution is required to reduce error within the assay. If a sample does not exhibit linear dilution, this suggests that a component within the sample is interfering with accurate detection of a specific analyte at a given dilution and that a single dilution should be used for the assay to obtain comparable results. Both spike/recovery and LOD should be in the range of 80-120%.

6.4.1.4 Intra and inter-assay precision

In order to define the precision or repeatability of immunoassays it is important to report the inter-assay (between plate) and intra-assay (within a plate) CV. The CV is calculated by dividing the SD of a set of measurements by the mean of the set. An inter-assay CV of <15% is generally acceptable and intra-assay CV should be <10% [452].

- Inter-assay overall % CV = (SD of plate means ÷ mean of plate means) x 100
- Intra-assay % CV for each sample = (SD 1 and 2 ÷ duplicate mean) x 100

6.4.1.5 Comparison of multiplex performance with existing ELISA techniques

In clinical laboratory medicine it is common to assess agreement between two quantitative methods of measurement (e.g. two methods for measuring haemoglobin in the blood), ensuring that the values obtained are within a similar range and that any differences between the tests are minimal. Bland Altman plots are frequently used to quantify agreement between two measurements [454]. Acceptable limits of agreement must be defined *a priori*, based upon the clinical utility of the test. A graph is then produced plotting the difference in the two paired measurements (y axis) against the mean of these two measurements (x axis). The 95% Cis for the difference is calculated and marked on the plot, and this interval is compared to the acceptable limits of agreement to assess whether the interval is within those limits. This approach requires the difference in measurements between the two assays to be normally distributed (or assume a normal distribution when log transformed) [455]. Otherwise a non-parametric Bland Altman approach may be used which involves estimating the 2.5th and 97.5th quantiles of the distribution of differences and superimposing these centiles on the scatter diagram, representing the 95% confidence interval of the difference between the two measurement methods. This non-parametric approach is less reliable than the parametric methods described, but is a useful alternative depending upon the dataset [454].

For novel biomarkers which are experimental, the level of agreement between different methods of biomarker measurement can be considered secondary to whether the two tests show the same ability to identify the disease state of the patient [452] (i.e. the presence of active LN). Descriptive statistics (median values and IQRs) were used to inspect the distribution of biomarker measurements using the two techniques. Mann

Whitney U tests and Firth's penalised-likelihood binary logistic regression with AUC ROC analysis using the 'logistf' R package [360] were used to assess and compare the ability of the ELISA and multiplex assays to detect disease state, both univariately and in combination, allowing comparison between techniques.

6.4.2 Stability of biomarkers with delayed sample processing

6.4.2.1 Urine sample processing protocols

This series of experiments was carried out in two stages. During stage 1, 13 different urine processing conditions were compared using urine samples from two HCs. On the basis of these results, a smaller number of conditions were compared in six JSLE patients during stage 2. In clinical practice, patient urine samples are usually subdivided, with the majority of the sample going for clinical tests and the remainder being used for research. This approach was therefore more practical and minimised the amount of urine required per patient during stage 2 experiments.

During stage 1 experiments, the protocol below was followed:

1. 5mls of urine was aliquoted into a tube containing Boric Acid, producing a concentration of 10g/L (SIGMA, UK).
2. 0.5mls of urine (from the original sample) were aliquoted into tubes labelled one to seven using a Pasteur pipette (the amount of urine was important due to the presence of protease inhibitors in some tubes).
    a. **Tube 1 -** spun at 2000rpm for five minutes and then frozen straight away at -80°C as per the usual urine processing SOP (see section 2.2.5).
    b. **Tube 2 -** kept at room temperature.
    c. **Tube 3 -** placed between ice packs.
    d. **Tube 4 -** mixed using the Pasteur pipette (contained protease inhibitor 1, Protease/Phosphatase Inhibitor Cocktail (100X), Cell Signalling Technologies, USA) and kept at room temperature.
    e. **Tube 5 -** mixed using the Pasteur pipette (contained protease inhibitor 2, Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail (100X), ThermoFisher, USA) and kept at room temperature.
    f. **Tube 6 -** mixed using the Pasteur pipette (contained protease inhibitor 1) and placed between the ice packs.

g. **Tube 7 -** mixed using the Pasteur pipette (contained protease inhibitor 2) and placed in between the ice packs.

3. Using the urine containing boric acid - 0.5ml of urine was aliquoted into tubes labelled eight to thirteen using the Pasteur pipette.
    a. **Tube 8 –** kept at room temperature.
    b. **Tube 9 –** placed between the ice packs.
    c. **Tube 10 -** mixed using the Pasteur pipette (contained protease inhibitor 1) and kept at room temperature.
    d. **Tube 11 –** mixed using the Pasteur pipette (contained protease inhibitor 2) and kept at room temperature.
    e. **Tube 12 –** mixed using the Pasteur pipette (contained protease inhibitor 1) and placed between the ice packs.
    f. **Tube 13 -** mixed using the Pasteur pipette (contained protease inhibitors 2) and placed in between the ice packs.
4. After 48 hours, samples 2-13 were centrifuged at 2000 rpm for five minutes and frozen at -80 °C.

During stage 2, the same protocol was used but a limited number of conditions included.

6.4.2.2 Biomarker quantification and analysis

These experiments pre-dated the completion of the multiplex assay, therefore ELISA techniques were used for quantification of AGP, CP, LPGDS, TF, MCP-1 and VCAM-1, as detailed in Table 2-3. Biomarker concentrations in samples exposed to the treatment conditions were compared to those processed in the standard fashion, and expressed as a percentage of the standard processing concentration.

6.4.3 Urinary tract infection, contamination of urine with commensals and urine biomarker levels

Urine samples collected during the UK JSLE Cohort Study undergo microscopy, culture and antibiotic sensitivity testing within the respective hospital microbiology department, to assess for UTIs. Samples are reported as displaying A) UTI, pure growth of a single organism with bacterial counts of $\geq 10^8$ cfu/L, B) mixed growth

(urine containing more than one organism, usually due to difficulty with sample collection technique resulting in contamination with faecal or skin flora) or C) no-growth, in accordance with the UK standards for microbiology investigations, issued by Public Health England [456]. ELISA techniques were used to quantify urinary biomarker levels (see Table 2-3) in samples displaying UTI or mixed growth. Descriptive statistics (median value and IQR) were used to describe the biomarker levels in UTI or mixed growth urine in relation to samples displaying no-growth (samples included in previous analyses, see section 4.5.2). Mann Whitney U tests were used to compare biomarker levels in UTI versus no-growth urine samples, and mixed growth versus no-growth urines.

6.5 Results

6.5.1 Cross-reactivity between antibodies for different biomarkers

Cross-reactivity assessment was carried out by Merck Millipore, spiking in standards 5, 6 and 7 (for each biomarker) in turn. The MFI was assessed for each biomarker to identify potential antibody cross-reactivity. When LPGDS standard was spiked into the assay, the MFI for TF, CP, AGP, MCP-1 and VCAM-1 remained the same as the background MFI when no standard was spiked in (average of 33 RFU). Similar results were demonstrated when MCP-1 and VCAM-1 standards were spiked in (see Table 6-1). When the TF standard was spiked in the MFI for CP was 249 RFU (close to the TF background levels, range 236-300 RFU), whereas the MFI for all other analytes was well below background level. The inverse was seen when CP standards were spiked in (TF MFI levels equivalent to CP background levels). When AGP standard was spiked into the assay the CP and TF MFIs were increased above the background level (range 416-498 RFU) but were still less than the lowest AGP standard (from MFI of 1636 RFU). For all three analytes, the MFI did not serially increase with the different concentrations of spiked standard suggesting that there was not significant cross-reactivity.

Marker	Std spiked	Average MFI from duplicate readings					
		LPGDS	TF	CP	AGP	MCP-1	VCAM-1
LPGDS	**0**	33	137	30	38	28	18
	5	3987	143	32	40	28	18
	6	7846	145	32	43	28	18
	7	9468	146	33	47	28	19
TF	**0**	31	254	285	446	31	16
	5	29	5239	271	442	30	16
	6	30	9322	275	430	30	16
	7	31	12155	284	460	31	17
CP	**0**	29	238	275	445	30	17
	5	29	239	5377	421	28	16
	6	31	248	8051	439	29	17
	7	33	295	11042	475	32	18
AGP	**0**	33	145	32	39	29	18
	5	32	141	32	1644	28	18
	6	33	149	33	2326	29	18
	7	34	150	40	2743	28	18
MCP-1	**0**	31	136	30	38	27	18
	5	33	137	30	40	2459	26
	6	35	141	32	41	9118	66
	7	35	145	33	44	17741	18
VCAM-1	**0**	33	128	28	39	26	17
	5	35	143	32	42	29	5007
	6	33	136	31	42	28	9428
	7	36	150	38	46	28	11287

Table 6-1: Assessment of cross-reactivity between different antibodies within the six plex custom multiplex assay.

Std = standards spiked in; 0 = blank/media spiked in, 5 = standard 5, 6 = standard 6, 7 = standard 7. The grey shading highlights the average MFI readings for the standards that have been spiked in. The MFI's obtained for all other analytes should be compared to these readings to assess for cross-reactivity.

6.5.2 Range finding in UK JSLE Cohort Study patients and determination of the optimal dilution

6.5.2.1 Merck Millipore range finding results

Having established antibody pairs able to detect all biomarkers (except for LPGDS initially) with minimal cross reactivity, range finding was initiated. The antibodies for LPGDS were identified at a later stage and therefore range finding was undertaken separately. Samples from JSLE patients with active LN (n=8), inactive LN (n=4) and HCs (n=8) were run at 1 in 10 dilution to get an indication of the dilution that may be required (see Table 6-2). 11/20 (55%) of TF values were off the scale (high). 1/20 (5%) of CP values were high. No AGP values were off the scale, but 4/12 (20%) were extrapolated (>400000 pg/ml). 1/20 (5%) MCP-1 values were extrapolated (low, <4pg/ml). 4/20 (20%) VCAM-1 levels were off the scale (low) and a further 7 values (35%) were extrapolated (low, <122pg/ml).

Sample	TF (pg/ml)	CP (pg/ml)	AGP (pg/ml)	MCP-1 (pg/ml)	VCAM-1 (pg/ml)
A 1	>199299	>4539495	461809	69.4	454
A 2	>199299	490029	326811	55.3	2544
A 3	>199299	185119	465316	5.4	202
A 4	>199299	996411	503111	7.3	715
A 5	>199299	603039	307432	56.2	623
A 6	>199299	58406	383882	194.3	76
A 7	>199299	187368	478064	355.9	845
A 8	>199299	354828	240772	39.4	2102
IA 1	22746	2107	108086	25.3	124
IA 2	9709	2399	11570	11.5	<57.52
IA 3	>199299	22499	285050	29.4	95
IA 4	51832	8928	170713	6.9	61
HC 1	52071	2412	44147	19.3	<57.52
HC 2	22023	2747	17683	3.2	<57.52
HC 3	42992	2742	73336	44.0	85
HC 4	59997	10550	304849	19.6	157
HC 5	5501	2286	13737	4.1	68
HC 6	32499	999	48794	40.5	<57.52
HC 7	>199299	36989	114256	21.7	101
HC 8	>199299	15970	132015	16.9	67
Number off the scale	11/20 (55%) H	1/20 (5%) H	0	0	4/20 (20%) L
Number extrapolated	0	0	4/20 (20%) H	1/20 (5%) L	7/20 (35%) L

Table 6-2: First multiplex range finding experiment at 1 in 10 dilution.
A = active, IA = inactive, HC = healthy control. H = high. L = low. Dark grey shaded cells were off the scale. Light grey shaded cells were high but extrapolated.

The standard curves could not be adjusted to go higher/lower due to the curve already flattening in these areas. It was concluded that TF, CP and AGP required more than 1 in 10 dilution and MCP-1/VCAM-1 required less. TF, CP, AGP and LPGDS were therefore re-run at 1 in 100, 1 in 200, 1 in 400 and 1 in 800 and MCP-1/VCAM-1 were run neat (see Table 6-3). This experiment suggested that LPGDS could be run at any dilution, TF and AGP should be run at ≥1 in 200 dilution, and CP at 1 in 100 or less. 100% of MCP-1 and VCAM-1 values were detected when the assay was run neat. Initial expectation was that the assay could be run at a single (or at most two) dilutions to save space on costly multiplex assay plates. Given the small samples size it was concluded that more range finding experiments would be required during beta testing.

Analyte	Dilution	Samples detectable at given dilution	Number of samples extrapolated	Number of samples off the curve	Optimal dilution
TF	**1 in 100**	5/7 (71%)	0	2/7 (29% H)	1 in 200 appears best
	1 in 200	6/7 (86%)	0	1/7 (14% H)	
	1 in 400	6/7 (86%)	1/6 (17%)	1/7 (14% H)	
	1 in 800	6/7 (86%)	1/6 (17%)	1/7 (14% L)	
CP	**1 in 100**	7/7 (100%)	3/7 (43%)	0	1 in 100 or less appears best
	1 in 200	4/7 (57%)	0	3/7 (43% L)	
	1 in 400	4/7 (57%)	0	3/7 (43% L)	
	1 in 800	4/7 (57%)	0	3/7 (43% L)	
AGP	**1 in 100**	7/7 (100%)	2/7 (29%)	0	>1 in 200 best
	1 in 200	7/7 (100%)	0	0	
	1 in 400	7/7 (100%)	0	0	
	1 in 800	7/7 (100%)	0	0	
LPGDS	**1 in 100**	7/7 (100%)	0	0	Any dilution possible
	1 in 200	7/7 (100%)	0	0	
	1 in 400	7/7 (100%)	0	0	
	1 in 800	7/7 (100%)	0	0	
MCP-1	**Neat**	7/7 (100%)	0	0	Neat
VCAM-1	**Neat**	7/7 (100%)	0	0	Neat

Table 6-3: Second Merck Millipore multiplex range finding experiment
Samples run at 1 in 100, 1 in 200, 1 in 400 and 1 in 800 dilutions for TF, CP, AGP, LPGDS and neat for MCP-1 and VCAM-1. 3 active LN, 2 inactive LN and 2 HCs samples included in the analysis. H = high. L = low.

6.5.2.2 Beta testing – range finding in a greater number of samples – UK JSLE Cohort patients

A rigorous approach to range finding was undertaken during the beta testing process. Initially 10 active and 11 inactive UK JSLE patient samples were run neat, at 1 in 10, 1 in 100, 1 in 400 and 1 in 800 dilutions. The assay was run as a six plex for all analytes. Once two target dilutions were chosen the assay was run in a larger number of samples from the UK JSLE Cohort, US and SA cohorts to assess the dilutions required on an international level. Table 6-4 shows the results obtained when the initial UK JSLE Cohort samples were run neat, at 1 in 10, 1 in 100, 1 in 400 and 1 in 800 dilutions.

Analyte	Dilution	% of samples detectable at the given dilution	Number of samples extrapolated	Number of samples off the curve
LPGDS	Neat	20/20 (100%)	0/20 (0%)	0/20 (0%)
	1 in 10	21/21 (100%)	0/21 (0%)	0/21 (0%)
	1 in 100	20/20 (100%)	0/20 (0%)	0/20 (0%)
	1 in 400	**20/20 (100%)**	**0/20 (0%)**	**0/20 (0%)**
	1 in 800	20/20 (100%)	0/20 (0%)	0/20 (0%)
TF	Neat	15/20 (75%)	5/15 (33%)	5/20 (25%)
	1 in 10	16/21 (76%)	4/16 (25%)	5/21 (24%)
	1 in 100	**19/21 (90%)**	**3/19 (16%)**	**1/21 (5%, L)**
	1 in 400	**19/20 (95%)**	**0/19 (0%)**	**1/20 (5%, L)**
	1 in 800	17/21 (81%)	1/21 (5%)	4/21 - 19%
CP	**Neat**	**19/20 (95%)**	**3/19 (16%)**	**1/20 (5%, L)**
	1 in 10	**19/20 (95%)**	**1/19 (5%)**	**1/20 (5%, L)**
	1 in 100	17/20 (85%)	2/17 (12%)	3/20 (15%)
	1 in 400	15/20 (75%)	1/15 (7%)	5/20 (25%)
	1 in 800	13/21 (62%)	2/13 (15%)	8/21 (38%)
AGP	Neat	15/20 (75%)	4/20 (20%)	5/15 (33%)
	1 in 10	14/21 (66%)	3/14 (21%)	7/21 (33%)
	1 in 100	18/20 (90%)	4/18 (22%)	2/21 (10%)
	1 in 400	**19/20 (95%)**	**0/19 (0%)**	**1/20 (5%, H)**
	1 in 800	**21/21 (100%)**	**2/21 (9.5%)**	**0/21 (0%)**
MCP-1	**Neat**	**20/20 (100%)**	**0/20 (0%)**	**0/20 (0%)**
	1 in 10	**20/20 (100%)**	**0/20 (0%)**	**0/20 (0%)**
	1 in 100	20/20 (100%)	0/20 (0%)	0/20 (0%)
	1 in 400	18/19 (95%)	6/18 (33%)	1/19 (5%)
	1 in 800	18/21 (86%)	8/18 (44%)	3/21 (14%)
VCAM-1	**Neat**	**20/20 (100%)**	**1/20 (5%)**	**0/20 (0%)**
	1 in 10	17/21 (81%)	6/17 (35%)	4/21 (19%)
	1 in 100	8/20 (40%)	4/8 (50%)	12/20 (60%)
	1 in 400	4/20 (20%)	4/4 (100%)	16/20 (80%)
	1 in 800	3/21 (14%)	3/3 (100%)	18/21 (86%)

Table 6-4: First UK JSLE Cohort beta testing range finding experiment in 10 active and 11 inactive UK JSLE patient samples.
Samples run neat, at 1 in 10, 1 in 100, 1 in 400 and 1 in 800 dilutions. N=20 for some analytes/dilutions due to a value being excluded for technical reasons (inadequate bead count). The best dilutions for each analyte are highlighted in bold text and grey background. H = high. L = low.

LPGDS levels were detectable at all dilutions in all patients, with no values requiring extrapolation. Plotting the patient sample values on their standard curve, it was clear that the values fell on the linear portion of the curve when a dilution of 1 in 400 was used (see Figure 6-1). On balance, a dilution of 1 in 400 also proved best for TF and AGP. At this dilution, sample values were detectable for 95% of patients, with no values requiring extrapolation. One sample (5%) had a low TF level and one had a high AGP level at 1 in 400 dilution. MCP-1 and VCAM-1 values were both detectable in 100% of patients when run neat, with one VCAM-1 sample (5%) being extrapolated. 95% of CP values were detectable when samples were run neat or at 1 in 10 dilution, with less samples values being extrapolated at 1 in 10 dilution (16% high vs. 5% high). At both dilutions, one sample was undetectable due to being low. In view of the plan to ultimately run the assay at two dilutions CP was run neat in future assays (see Table 6-4 and Figure 6-1).

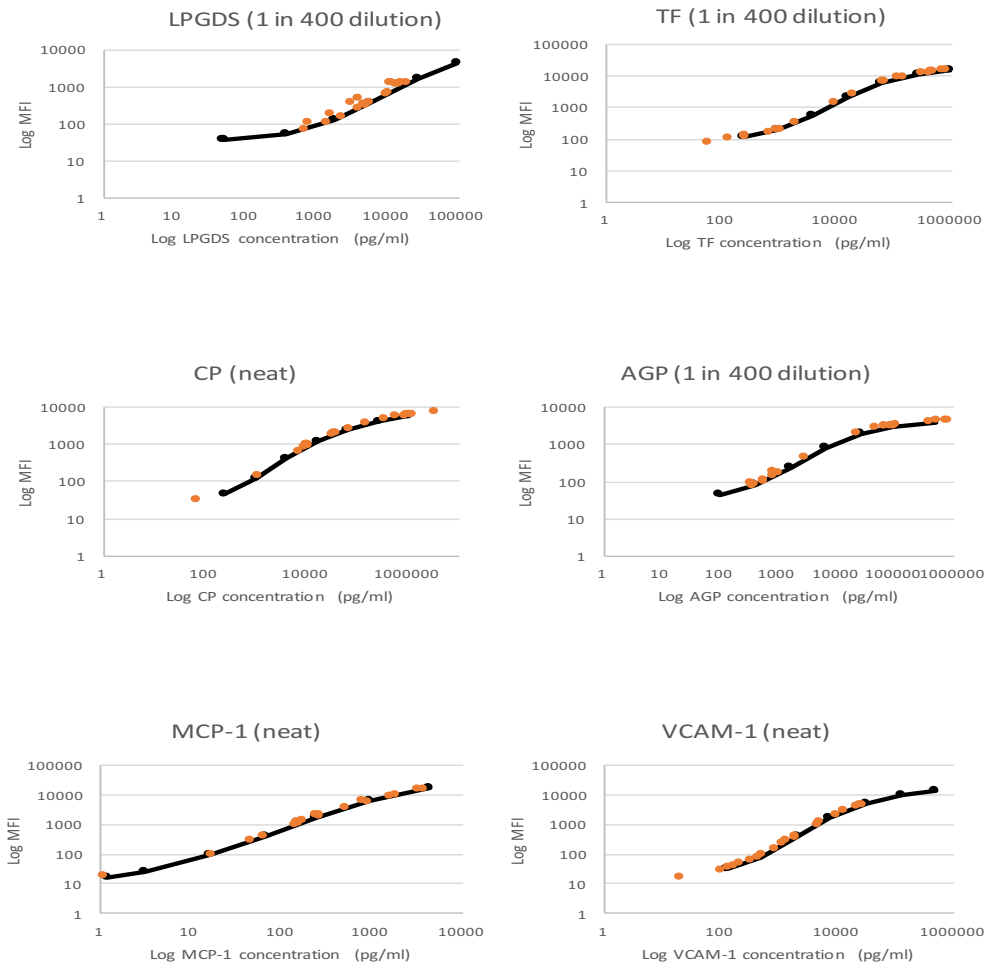


Figure 6-1: Beta testing standard curves showing the position of UK JSLE Cohort patient samples on the standard curves.
CP, MCP-1, VCAM1 = neat. LPGDS, TF, AGP = 1:400. Each red dot represents a patient sample value. MFI = median fluorescence intensity.

On the basis of the above results, the multiplex assay was run on a further 35 UK JSLE Cohort patients (28 inactive and 7 active LN) at the two chosen dilutions (neat and 1 in 400) to verify that biomarker values could be obtained using these dilutions. A similar percentage of sample values was detected for LPGDS, CP, AGP, MCP-1 and VCAM-1 as compared to the previous beta testing experiment. Less TF values were detectable (86% vs. 95%) but given the need to run the assay at two dilutions it was decided that TF should continue being run at 1 in 400 dilution (see Table 6-5).

Analyte and dilution	% of samples detectable at a given dilution	Number of samples extrapolated	Number of samples off the curve
LPGDS (1 in 400)	35/35 (100%)	0	0
TF (1 in 400)	30/35 (86%)	0	5 (15%, L)
CP (neat)	31/35 (91%)	3/31 (9.7%)	1 (3%, H)
AGP (1 in 400)	35/35 (100%)	2/25 (6%)	0
MCP-1 (neat)	35/35 (100%)	0	0
VCAM-1 (neat)	35/35 (100%)	2/35 (5.7%)	0

Table 6-5: Second UK JSLE Cohort beta testing range finding experiment

Run in a further 7 active and 28 inactive UK JSLE patient samples run neat and at 1 in 400 dilution. H = high. L = low.

6.5.2.3 Range finding in international samples – US and SA Cohort patients

The range finding was extended to include samples from US and SA JSLE patients, to assess if the assay could be used in the same way across different countries. Samples from 28 US (14 inactive and 14 active LN) and 23 SA patients (14 inactive and 9 active LN) were run neat and at 1 in 400 dilutions as per the UK samples. The percentage of sample values detectable for LPGDS, TF, AGP, MCP-1 and VCAM-1 were similar across all three international cohorts, using the dilutions derived from UK JSLE Cohort beta testing. CP levels were found to be higher in the US and SA patients, with only 72 and 77% of samples respectively being detectable when the samples were run neat. At 1 in 400 dilution this improved to 96% detectable across both cohorts, therefore this dilution is preferred to obtain CP levels within the US and SA cohorts (see Table 6-6). This highlights a potential need for country/ethnic group specific validation of the assay prior to use.

	% of samples detectable at a given dilution	Number of samples extrapolated	Number of samples off the curve
US Cohort			
LPGDS (1 in 400)	28/28 (100%)	0	0
TF (1 in 400)	28/28 (100%)	2/28 (7%)	0
CP (neat)	21/29 (72%)	4/21 (19%)	8/29 (28%), H
CP (1 in 400)*	27/28 (96%)	3/27 (11%)	1/28 (4%), L
AGP (1 in 400)	24/28 (86%)	0	4/18 (14%), H
MCP-1 (neat)	29/29 (100%)	1/29 (3%)	0
VCAM-1 (neat)	29/29 (100%)	1/29 (3%)	0
SA Cohort			
LPGDS (1 in 400)	26/26 (100%)	0	0
TF (1 in 400)	25/26 (96%)	0	1/26 (4%), H
CP (neat)	20/26 (77%)	2/20 (10%)	6/26 (23%), H
CP (1 in 400)*	25/26 (96%)	1/25 (4%)	1/26 (4%), L
AGP (1 in 400)	23/26 (89%)	0	3/26 (11%), H
MCP-1 (neat)	25/26 (96%)	0	1/26 (4%), H
VCAM-1 (neat)	26/26 (100%)	0	0

Table 6-6: Third beta testing range finding in US and SA JSLE cohort patients

Samples run neat or at 1 in 400 dilution as indicated in the table. *Optimal dilution for CP in US and SA cohort patients.

6.5.3 Spike recovery results

6.5.3.1 Merck Millipore spike recovery results

Standards 2, 3 and 4 (low, medium and high) were spiked into four active LN samples, two inactive LN and two HC samples. During this experiment TF, CP, AGP and LPGDS were run together as part of a four plex at 1 in 100 dilution. MCP-1 and VCAM were run together as a two plex, neat. Average spike recovery results were between 80-120% for all analytes when the standards were spiked into the assays (see Table 6-7). Further spike recovery experiments would therefore be required during the beta testing phase, to run the assay as a six plex at the optimal dilutions determined during beta testing experiments.

Sample	STN spiked	% recovery					
		4 plex assay (1 in 100 dilution)				2 plex assay (neat)	
		LPGDS	TF	CP	AGP-1	MCP-1	VCAM-1
A 1	**2**	93	95	103	99	108	101
	3	87	98	102	78	102	101
	4	75	96	106	90	95	94
A 2	**2**	94	100	107	109	108	105
	3	87	103	97	108	104	108
	4	82	100	99	105	112	111
A 3	**2**	93	94	98	84	102	101
	3	93	95	104	88	101	93
	4	79	98	109	90	90	81
A 4	**2**	93	95	102	98	110	99
	3	91	95	99	100	117	99
	4	82	99	101	106	118	94
IA 1	**2**	92	High	100	115	94	92
	3	86		109	81	97	83
	4	75		110	70	79	72
IA 2	**2**	100	104	106	103	96	91
	3	88	108	98	101	105	87
	4	80	111	103	110	109	81
HC 1	**2**	88	High	91	76	106	69
	3	92		104	106	100	60
	4	76		109	109	95	64
HC 2	**2**	94	102	108	96	99	88
	3	87	103	99	100	105	80
	4	81	105	97	99	87	73
Average % recovery		**87**	**100**	**102**	**97**	**102**	**89**

Table 6-7: Merck Millipore spike recovery results

TF, CP, AGP and LPGDS were run together as part of a four plex (1 in 100 dilution) and MCP-1 and VCAM-1 were as a two plex (neat). Percentage recovery shown for each patient when standards 2, 3, 4 were spiked in, along with the overall average percentage recovery for each analyte. STN = standard, A = active, IA = inactive, HC = healthy control.

6.5.3.2 Beta testing – spike recovery in UK samples

Standard 3 (medium sized spike) was added to three inactive, three active and three HC samples. The assay was run as a six plex at the two chosen optimal dilutions for each analyte (LPGDS, TF, AGP at 1 in 400, CP, MCP-1 and VCAM-1 neat). Percentage recovery was calculated for each patient along with the overall average percentage recovery for each analyte. Average spike recovery results were acceptable (between 80-120%) for all analytes at the chosen dilutions (see Table 6-8). Three individual patient VCAM-1 percentage recoveries were <80%, however the overall average recovery was 82%.

	% recovery					
Sample	**LPGDS (1 in 400)**	**TF (1 in 400)**	**CP (neat)**	**AGP (1 in 400)**	**MCP-1 (neat)**	**VCAM-1 (neat)**
IA 1	92	99	114	94	59	81
IA 2	93	97	81	102	104	80
IA 3	106	102	101	102	106	91
A 1	98	116	96	74	102	69
A 2	95	81	102	102	87	91
A 3	94	98	98	95	85	82
HC 1	108	104	104	104	96	73
HC 2	97	99	89	88	101	90
HC 3	96	96	85	92	93	77
Ave % recovery	**98**	**99**	**97**	**95**	**93**	**82**

Table 6-8: Beta testing spike recovery results when the assay was run as a six-plex at the two chosen optimal dilutions.
Percentage recovery shown for each patient along with the overall average percentage recovery for each analyte. A = active, IA = inactive, HC = healthy control.

6.5.4 Linearity of dilution

6.5.4.1 Merck Millipore linearity of dilution

LOD was determined in four active LN samples, two inactive LN and two HC samples. Similar to the spike recovery experiments, TF, CP, AGP and LPGDS were run together as part of a four plex with a 1 in 100 starting dilution. MCP-1 and VCAM were run together as a two plex, starting with neat samples. LOD was tested by diluting the initial samples 1 in 2, 1 in 4 and 1 in 8, and the average LOD for each dilution is presented. On average TF, CP, MCP-1 and VCAM-1 displayed acceptable LOD (80-120%), whereas LPGDS and AGP did not (LPGDS 192%, AGP 133%). Although the

average LOD was acceptable for TF, some individual samples displayed a LOD below 80%. Similarly, for CP, MCP-1 and VCAM-1, some individual % LOD's were above 120%, with the average LOD remaining within the acceptable range (107, 99, 98% respectively). For some samples, dilution resulted in biomarker levels going below the limits of detection of the assay, therefore the number of patients included in determination of the average LOD percentage was less than 7 in these instances (TF n = 6, CP n=4, VCAM-1 n=5, see Table 6-9). Further LOD experiments were required during the beta testing, running the assay as a six plex in a greater number of samples.

Sample	Dilution	4 plex assay, starting dilution 1 in 100				2 plex assay, starting dilution neat	
		LPGDS	TF	CP	AGP	MCP-1	VCAM-1
A1	1 in 2	173	114	98	84	96	88
	1 in 4	269	117	100	141	86	75
	1 in 8	362	111	104	178	81	64
A2	1 in 2	128	83	Low	97	97	73
	1 in 4	144	72		95	96	62
	1 in 8	155	57		99	99	57
A3	1 in 2	122	99	101	112	99	101
	1 in 4	153	102	104	152	102	97
	1 in 8	166	96	108	210	96	88
A4	1 in 2	115	Low	Low	101	105	93
	1 in 4	129			128	88	Low
	1 in 8	134			90	81	
IA 1	1 in 2	176	106	101	138	129	159
	1 in 4	295	110	102	144	164	180
	1 in 8	477	108	96	130	145	160
IA 2	1 in 2	106	85	Low	114	91	Low
	1 in 4	129	121		153	84	
	1 in 8	107	63		131	Low	
HC 1	1 in 2	148	Low	117	147	90	Low
	1 in 4	254		125	199	91	
	1 in 8	426		133	253	Low	
HC 2	1 in 2	124	70	Low	96	84	80
	1 in 4	153	54		103	77	58
	1 in 8	153	Low		88	Low	133
Ave % LOD at each dilution	**1 in 2**	**137**	**90**	**104**	**111**	**99**	**99**
	1 in 4	**191**	**93**	**106**	**139**	**99**	**94**
	1 in 8	**248**	**82**	**109**	**147**	**100**	**100**
	Overall	**192**	**91**	**107**	**133**	**99**	**98**

Table 6-9: Merck Millipore linearity of dilution results

TF, CP, AGP and LPGDS run together as part of a four plex (1 in 100 starting dilution). MCP-1 and VCAM run together as a two plex (neat starting dilution). Samples diluted 1 in 2, 1 in 4 and 1 in 8, and the average linearity for each dilution presented. A = active, IA = inactive, HC = healthy control, Ave = average, LOD = linearity of dilution. Low = analytes that were un-detectable at the given dilution.

6.5.4.2 Beta testing – linearity of dilution results

LOD was assessed using the six plex assay in 10 active and 11 inactive LN patient samples which were run neat, at 1 in 10, 1 in 100, 1 in 400 and 1 in 800 dilutions. LOD was found to be problematic for all analytes, improving with dilution for TF, CP and AGP. LPGDS and VCAM-1 did not display acceptable LOD for any dilutions. MCP-1 displayed acceptable LOD when going from neat to 1 in 10 dilution. At dilutions over 1 in 100, TF, CP and AGP displayed acceptable LOD (80-120%), suggesting that if a sample value was unobtainable due to being high and off the standard curve, then the assay could be repeated and the sample diluted further. See Table 6-10 for full results.

	Average LOD for each analyte (%)					
	LPGDS	**TF**	**CP**	**AGP**	**MCP-1**	**VCAM-1**
1 in 10	850	373	310	265	**113**	168
1 in 100	5423	1165	431	4138	155	157
1 in 400	202	**99**	**86**	**100**	181	64
1 in 800	126	**102**	**95**	**118**	142	128

Table 6-10: Beta testing average LOD results for each analyte when the assay was run as a six plex.
Acceptable LOD results shown in bold.

6.5.5 Merck Millipore - intra/inter assay precision

Intra-assay precision was assessed by running the two quality control's (QC1 & 2) 16 times within a single assay at different positions on the plate, assessing the average CVs. All CVs were <10%, ranging between 4.1-8.5% (see Table 6-11). To assess inter-assay variability, the QC's were run on three different plates/on three different days. Average inter-assay CV's ranged between 3.6-7.8% (see Table 6-11).

	CV (%)					
	LPGDS	**TF**	**CP**	**AGP**	**MCP-1**	**VCAM-1**
Average intra-assay precision	5.1	4.0	6.3	8.5	4.8	4.5
Average inter-assay precision	7.8	7.2	3.6	5.3	5.4	6.6

Table 6-11: Merck Millipore intra and inter-assay CVs using QCs 1 and 2

6.5.6 Beta testing – intra and inter assay precision

Intra-plate precision was assessed using patient samples run neat and at 1 in 400 dilution from 10 active and 11 inactive UK JSLE patients. Average CVs for the different biomarkers varied between 3.6-7.6%. Inter-assay precision was assessed using six UK JSLE patients across six different assays. LPGDS, CP, AGP, MCP-1 and VCAM-1 displayed acceptable inter-assay CV's (<15%), with TF showing a slightly high CV of 16.4% (Table 6-12).

	CVs (%)					
	LPGDS	**TF**	**CP**	**AGP**	**MCP-1**	**VCAM-1**
Average intra-assay precision	5.1	3.6	4.9	6.3	7.6	3.9
Average inter-assay precision	4.4	16.4	5.4	14.3	2.8	15.0

Table 6-12: Beta testing intra and inter-assay CVs.

6.5.7 Assessment of the multiplex assay in three international JSLE cohorts

6.5.7.1 Patient demographics

Biomarker analysis was undertaken on a total of 106 JSLE patients across the three cohorts; 54 from the UK, 29 US and 23 SA. Samples were not available from all UK/US patients included in previous work, therefore the demographic details of the patients included in the multiplex analysis are shown below. The proportion of female patients in the UK/US/SA cohorts was 74%, 86%, 91% respectively. Age at time of biomarker analysis was similar in the UK and SA cohorts (median of 13.6 and 13.5 years respectively) and higher in the US Cohort (median of 16 years). The length of disease at the time of biomarker analysis was similar across the three cohorts (median of 2.3-2.6 years). See Table 6-13 for further details.

	UK	US	SA
Patients	54	29	23
Gender	40 F (74%)	25 F (86%)	21 F (91%)
Age	13.6 [11.8-15.6]	16 [14-18]	13.5 [12.0-15.3]
Length of disease	2.3 [1.3-4.1]	2.5 [0.7-4.7]	2.6 [0.9-3.5]
Ethnicity[1]			
Caucasian	19 (35%)	**Caucasian** 0	**Caucasian** 1 (4%)
African/ Caribbean	15 (28%)	**African American** 16 (55%)	**Black** 7 (31%)
Indian/ Pakistani/ Bangladeshi	13 (24%)	**Hispanic** 12 (41%)	**Coloured** 13 (56%)
Asian/ Chinese	7 (13%)	**Asian** 1 (4%)	**Indian/ Asian** 2 (9%)

Table 6-13: Demographics of the patients included in the multiplex assay biomarker analysis from the UK, US and SA JSLE cohorts.
F = female. Age and length of disease given in years with median value and IQR in square brackets. [1]Self-reported ethnicity classification categories vary between the patient cohorts.

6.5.8 Head to head assessment of multiplex vs. ELISA assay performance

6.5.8.1 Comparing multiplex and ELISA absolute values

Figure 6-2 and Table 6-14 show the biomarker levels for all three cohorts combined when run by multiplex vs. ELISA. They highlight that differences in the absolute biomarker values can be seen depending on the type of assay used. For some analytes the levels measured by multiplex are consistently higher than those measured by ELISA, or vice-versa. With LPGDS, higher values are seen with multiplex than ELISA, whereas with CP, VCAM-1 and AGP, lower levels are seen with multiplex than ELISA. Similar levels were seen between the assays for TF and MCP-1. The *p-values* obtained for individual biomarkers when comparing active and inactive LN biomarker values are the same, regardless of whether multiplex or ELISA has been used to quantify the marker (see Table 6-14).

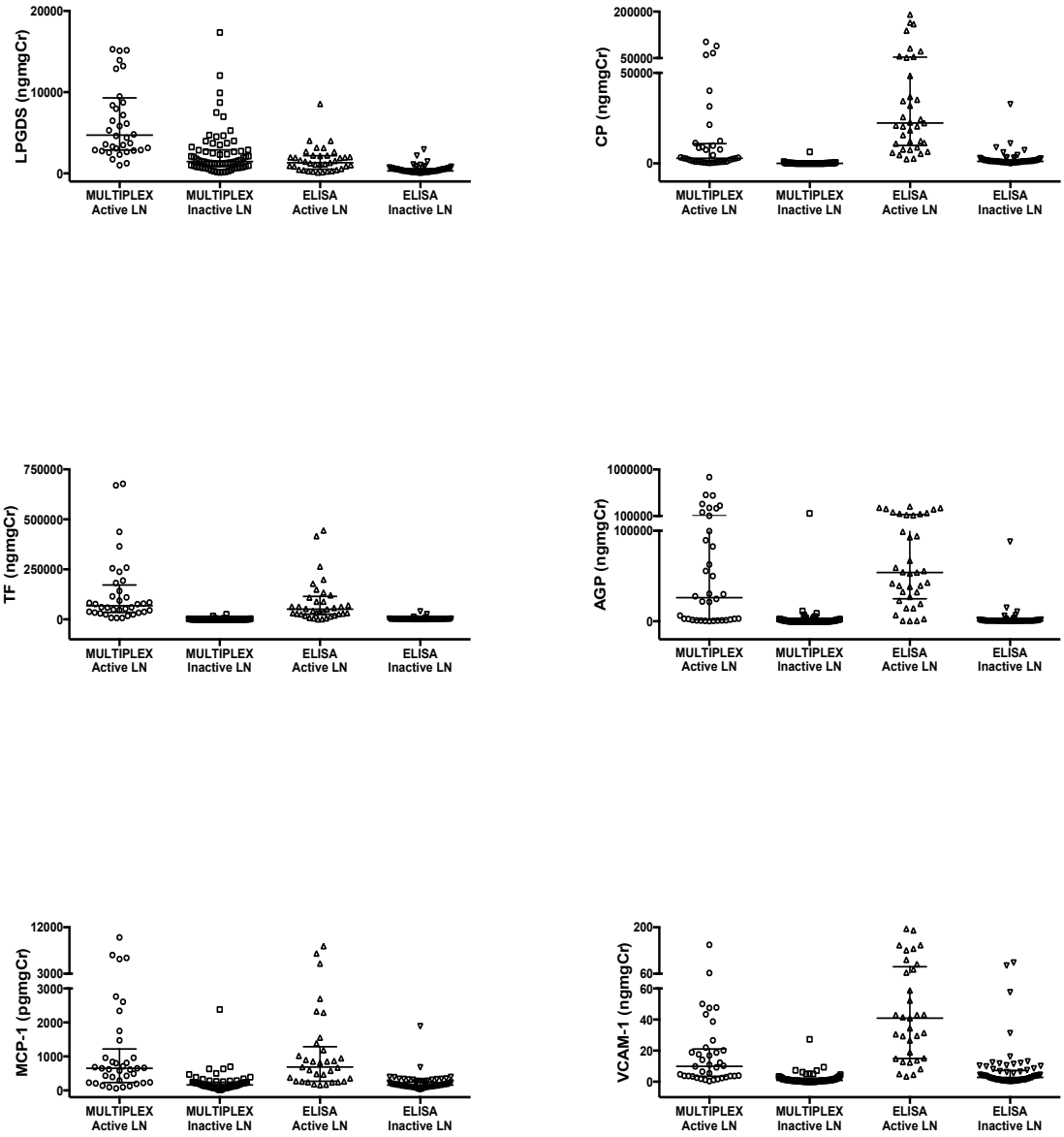


Figure 6-2: Distribution of urine biomarker values in active and inactive LN patients quantified by multiplex assay or ELISA in patients from all three JSLE cohorts.

	MULTIPLEX			ELISA		
	Active LN	**In active LN**	***p_c value***	**Active LN**	**In active LN**	***p_c value***
LPGDS	4714 [2872-9291]	1454 [840-2889]	*<0.0001*	1324 [515-2184]	312 [157-592]	*<0.0001*
CP	2836 [957-10903]	40 [14-158]	*<0.0001*	22354 [9910-52954]	1137 [632-1773]	*<0.0001*
TF	68191 [34734-171614]	407 [162-843]	*<0.0001*	51198 [25889-115025]	789 [256-1658]	*<0.0001*
AGP	26175 [1544-103177]	66 [14-1386]	*<0.0001*	53911 [24899-129315]	338 [153-841]	*<0.0001*
MCP-1	652 [233-1219]	163 [104-242]	*<0.0001*	689 [270-1287]	159 [108-248]	*<0.001*
VCAM1	10 [4-21]	1 [0-2]	*<0.0001*	41 [15-81]	3 [1-7]	*<0.0001*

Table 6-14: Absolute urine biomarker values in active and inactive LN patients quantified by multiplex assay or ELISA in patients from all three cohorts.
All biomarker concentrations expressed as ng per mg creatinine (ngmgCr) except MCP-1 (pgmgCr). Median values and IQRs shown. P_c = Bonferroni correction adjusted *p-values* (adjusted for the 6 Mann Whitney U tests comparing active and inactive LN for each biomarker quantification technique).

Initial assessment of the differences in measurements between the two assays identified that these were not normally distributed (all Shapiro-Wilkinson test *p-values* <0.05). Attempts to achieve normality through log transformation were unsuccessful. Given that these novel biomarkers are experimental, and that existing clinically relevant cut-offs do not exist, it was difficult to determine clinically meaningful acceptable limits of agreement between the two tests *a priori.* We therefore constructed non-parametric Bland Altman plots, showing the 95% CIs to gauge where the majority of differences in measurement between ELISA and multiplex techniques lay (see Figure 6-3).

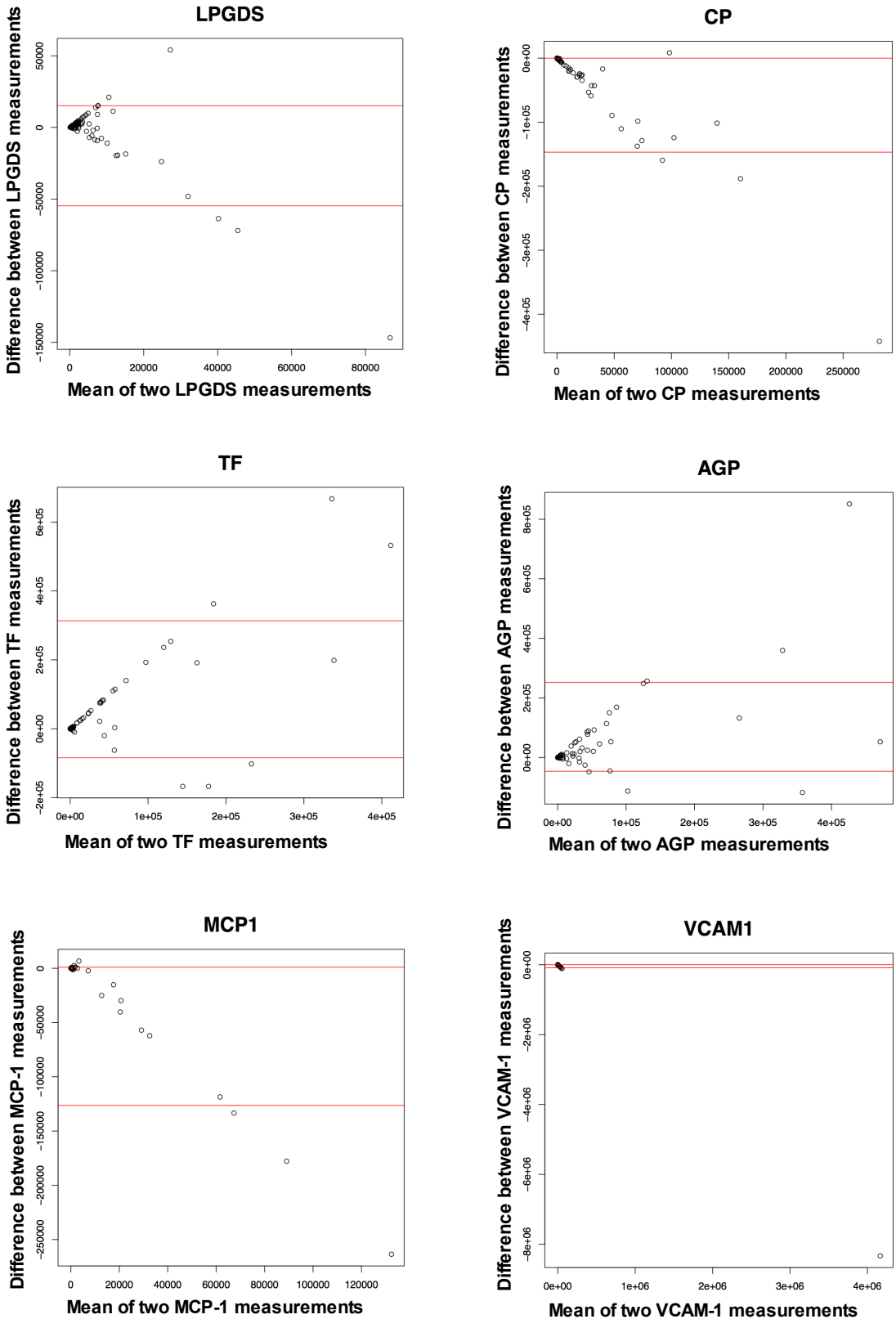


Figure 6-3: Non-parametric Bland Altman plots

The non-parametric Bland Altman plots show the difference between individual biomarker values quantified by ELISA or multiplex, against the mean biomarker value for the two techniques.

The narrowest 95% CIs were seen for VCAM-1 (-84242 to 313577) suggesting best agreement between the ELISA and multiplex assays for this biomarker. For all other analytes, as the mean biomarker level increased (along the x-axis) the points became more divergent leading to widening of the 95% CIs for LPGDS (-54731 to 15161), CP (-146869 to -36), TF (-84242 to 313577), AGP (-46190 to 252122) and MCP-1 (126368 to 966). For TF and AGP, the higher the mean TF or AGP value, the higher the mean difference became. Conversely, for LPGDS, CP and MCP-1, the higher the mean value, the more negative the mean difference became. Using either assay, high biomarker levels are more likely to be extrapolated and therefore 'best guess' values are generated by the assay software. Each assay will therefore have its own inherent error which will contribute to the divergence between the points [452]. In keeping with this, VCAM-1 levels were not frequently extrapolated, therefore the CIs were narrow.

6.5.9 Comparison of multiplex and ELISA techniques for identification of active LN disease state

Firth's penalised binary regression with AUC ROC analysis was used to compare the ability of multiplex and ELISA assays to detect disease state, both univariately and in combination. Table 6-15 shows the AUC values for active LN identification generated from running Firths's penalised binary regression models looking at each biomarker univariately with ELISA or multiplex. Similar AUC values are obtained regardless of the biomarker quantification technique, e.g. for LPGDS the ELISA AUC value is 0.826 and the multiplex AUC value is 0.829. For all biomarkers the multiplex AUC is higher than the ELISA AUC, suggesting that the multiplex technique is better at correctly identifying active LN disease state.

Biomarkers	Univariate penalised binary regression with AUC ROC analysis	
	ELISA	Multiplex
LPGDS	0.826	0.829
TF	0.829	0.996
CP	0.901	0.983
AGP	0.934	0.979
MCP-1	0.812	0.818
VCAM-1	0.863	0.899

Table 6-15: AUC values generated from Firth's penalised binary regression models looking at each biomarker univariately with ELISA or multiplex.

Previous analysis of the ELISA data from all three international cohorts combined identified the best panel of biomarkers for active LN identification to combine AGP, CP, LPGDS, TF and VCAM-1, with an AUC of 0.953 (see Table 5-4). Combining the multiplex biomarker data in the same order as was previously used for the ELISA data led to a progressive increase in AUC as biomarkers were added to the model. The optimal multiplex model contained all six biomarkers whereas the ELISA model contained five. The increase in AUC associated with addition of extra biomarkers after the model included AGP, CP, LPGDS and TF was however very modest (see Table 6-16).

Biomarker combinations included in the Firth's penalised binary regression models	ELISA AUC	Multiplex AUC
AGP	0.880	0.979
AGP + CP	0.937	0.986
AGP + CP + LGPDS	0.942	0.985
AGP + CP + LGPDS + TF	0.951	0.995
AGP + CP + LGPDS + TF + VCAM-1	0.953	0.997
AGP + CP + LGPDS + TF + VCAM-1 + MCP-1	0.952	0.998

Table 6-16: Comparison of biomarker combination AUC ROC values when ELISA or multiplex assays used.

The combined three cohort multiplex dataset was subsequently considered in isolation, developing a Firth's penalised regression model from scratch. All biomarkers were forced into the initial model and the 'backward' function used to select variables for the final model. The final model contained TF only, and the AUC for this was evaluated showing TF in isolation to produce an AUC of 0.996 (see Table 6-17).

	Model including all biomarkers		
	Co-efficient	**Std. Error**	***p***
TF	2.62	0.98	*0.003*
AGP	-1.88	1.24	*0.203*
MCP-1	0.72	0.66	*0.385*
VCAM-1	0.94	0.84	*0.452*
CP	0.28	0.68	*0.784*
LPGDS	0.17	0.98	*0.888*
	Final model after variable selection		
TF	1.97	0.48	*<0.001*

Table 6-17: Penalised regression models for multiplex dataset, initially including all biomarkers, and after variable selection.

As per previous ELISA work, the change in AUC was then evaluated after incorporating each of the other biomarkers in turn. The order used for adding them to the model was dependent on the order of statistical significance in the original model including all biomarkers (see Table 6-17). Again, combining biomarkers led to a progressive increase in AUC with the optimal model including TF + AGP + MCP-1 + VCAM-1 (AUC 0.999, see Table 6-18). When building this model, the addition of AGP to TF did not increase the AUC (remaining at 0.996), therefore the increase in AUC noted above is likely to be due to the combination of TF, MCP-1 and VCAM-1. With a starting AUC of 0.996 for TF alone, further increases in AUC were smaller than those seen when combinations of biomarkers were considered in previous ELISA models (see Table 5-4). Comparing the ELISA and multiplex model combinations, it is evident that a more accurate identification of disease state (active LN) can be obtained with the multiplex assay, using a smaller number of biomarkers.

Biomarker combinations included in the penalised binary regression models	AUC
TF	0.996
TF + AGP	0.996
TF + AGP + MCP-1	0.997
TF + AGP + MCP-1 + VCAM-1	0.999
TF + AGP + MCP-1 + VCAM-1 + CP	0.998
TF + AGP + MCP-1 + VCAM-1 + CP + LPGDS	0.998

Table 6-18: Effect on AUC of adding further biomarkers to the multiplex penalised regression model.

6.5.10 Stability of biomarkers during sample postage

6.5.10.1 Stage 1 – Healthy control samples

Two adult female HCs aged 34 and 33 years were included in stage 1. Urine dipstick analysis was normal, with no signs of infection. Conditions 5, 6, 11 and 12 proved best, maintaining biomarker levels within 20% of the standard processing levels. These four conditions included an ice pack and a protease inhibitor +/- BA. With the simpler processing conditions (e.g. 48 hours at room temperature, 48 hours on ice), biomarker levels were often > or < 20% of the standard processing levels, and therefore considered unacceptable (see Table 6-19).

Urine processing conditions	% of Standard Processing (average)					
	VCAM-1	AGP	CP	TF	LPGDS	MCP-1
1. 48 hours at RT	75	71	81	65	90	83
2. 48 hours on ice	92	80	157	72	75	105
3. Prot inhibitor 1 (RT)	90	65	221	105	80	114
4. Prot inhibitor 2 (RT)	117	130	141	133	90	111
5. Prot inhibitor 1 + ice	80	81	113	80	104	104
6. Prot inhibitor 2 + ice	86	102	99	90	108	98
7. BA + room temp	86	75	108	130	77	102
8. BA + ice	79	92	98	89	117	80
9. BA + prot inhibitor 1 (RT)	78	79	145	91	75	100
10. BA + prot inhibitor 2 (RT)	85	80	97	79	70	75
11. BA + prot inhibitor 1 + ice	81	100	104	90	119	100
12. BA + prot inhibitor 2 + ice	90	119	92	98	120	104

Table 6-19: Stability of urine biomarkers in HC samples following exposure to different urine processing conditions.
Biomarker values presented as an average % of standard processing value. RT = room temperature. Ice = ice-pack. Prot = protease. Green font highlights biomarker levels falling within 10% of standard processing levels. Blue font highlights those falling within 20% of standard processing levels. Black font indicates values which are > or < 20% of the standard processing levels and considered unacceptable. RT = room temperature, BA = boric acid.

In view of these results, the following conditions were included in stage 2 of the experiment:

1. Standard processing
2. Protease inhibitor 1 + ice
3. Protease inhibitor 2 + ice
4. BA + protease inhibitor 1 + ice
5. BA + protease inhibitor 2 + ice

6.5.10.2 Stage 2 results – JSLE patient samples

Six JSLE patients were included in stage 2 (5 female:1 male), with a median age of 15.6 years (IQR 12.1-16.6) and length of disease of 1.8 years (IQR 13.2-16.2). The testing conditions were re-numbered as outlined in Table 6-20. Condition 1, including protease inhibitor 1 and an ice pack, proved to be better than other conditions for

maintaining biomarker levels between 80-120% of the standard processing biomarker levels. Compared with standard processing, biomarkers levels in the presence of condition 1 were as follows; VCAM-1 111%, AGP 87%, CP 120%, TF 99%, LPGDS 108%, MCP-1 106%. The second best condition was condition 3 (including protease inhibitor 1, BA and an ice pack), however, MCP-1 measurements were 78% of the standard processing values and therefore considered unacceptable (see Table 6-20). In the presence of conditions 2 and 4, between 2-3 markers differed by >20% of standard processing levels, therefore these conditions were favoured least.

Urine processing conditions	% of Standard Processing (average)					
	VCAM-1	AGP	CP	TF	LPGDS	MCP-1
1. Prot inhibitor 1 + ice	111	87	120	99	108	106
2. Prot inhibitor 2 + ice	114	95	122	85	116	62
3. BA + prot inhibitor 1 + ice	114	104	116	106	106	78
4. BA + prot inhibitor 2 + ice	116	121	113	171	117	426

Table 6-20: Stability of urine biomarkers in JSLE patient samples following exposure to different urine processing conditions.
Average % of standard processing quoted. Prot = protease. BA = boric acid. Green font highlights biomarker levels falling within 10% of standard processing levels. Blue font highlights those falling within 20% of standard processing levels. Black font indicates values which are > or < 20% of the standard processing levels, and considered unacceptable.

6.5.11 Urinary tract infection, contamination of urine with commensals and urine biomarker levels

Ten samples from JSLE patients with mixed growth and seven JSLE patients with UTI were included in these analyses. All patients with UTI had a pure growth of E-coli, whereas those with mixed growth had different combinations of the following organisms; e-coli, coliforms, proteus, klesiella, enterococcus and staphylococcus. These patients were all female and had inactive LN, with a renal BILAG score of D or E. Urine biomarker levels from these patients were compared to those of the 46 UK patients with inactive LN included in precious analyses, who had no growth on microscopy, culture and antibiotic sensitivity testing (see
Table **4-3**).

Urine biomarker levels in the above patient groups are shown in Table 6-21. CP, LPGDS and TF levels were higher in patients with UTI than the other groups, although this difference did not achieve significance (all $p>0.05$). Urine biomarker levels were comparable between mixed growth and no growth samples, and again no significant difference was seen (all $p>0.05$). These results therefore suggest that the presence of UTI or a mixed growth within the urine does not influence biomarker levels.

	Urine culture result			***p*-values**	
Biomarker	**UTI (n=3)**	**Mixed growth (n=10)**	**No-growth (n=46)**	**UTI vs. No-growth**	**Mixed growth vs. No-growth**
AGP	324 [233-556]	892 [162-5300]	304 [144-708]	*0.825*	*0.384*
CP	1093 [699-1382]	814 [438-2249]	701 [505-1010]	*0.871*	*0.528*
LPGDS	325 [233-532]	199 [143-874]	288 [143-601]	*0.937*	*0.389*
MCP-1	277 [111-330]	132 [106-568]	157 [105-295]	*0.221*	*0.908*
VCAM-1	2 [2-5]	2 [0.4-23]	2 [1-8]	*0.996*	*0.844*
TF	523 [284-600]	696 [356-1162]	1,188 [402-3822]	*0.192*	*0.222*

Table 6-21: Urine biomarker levels in samples with proven UTI, mixed growth or no-growth.
All biomarker values in ngmgCr except for MCP-1 which is in pgmgCr. Median values and IQRs presented. Mann-Whitney U tests used to compare biomarker levels between groups. Abbreviations: UTI = urinary tract infection.

6.6 Discussion

The main aim of the study presented in this chapter was to assess feasibility of a custom multiplex assay for quantification of the internationally validated LN urinary biomarker panel. This was done in collaboration with an industry partner, Merck Millipore, and undertaken in order to streamline the process of urine biomarker quantification. In this chapter the steps involved in the development and validation of this multiplex assay have been presented. They highlight the process undertaken to identify antibody pairs capable of detecting each marker within the urine, assessment for cross reactivity between antibodies, exploration for JSLE urine sample matrix effects, rigorous range finding in three international JSLE cohorts, and the comparison of multiplex and ELISA assays for their ability to identify LN disease state. The major advantage to this approach is that it reduces cost, processing time and the volume of sample required, as compared to ELISA techniques. Notably, the multiplex assay format increased diagnostic accuracy over and above existing ELISA assays. It will therefore maximise the amount and value of information collected from very small sample volumes.

The secondary aim of this work was to assess if urine samples could undergo delayed processing (e.g. 48 hours after sample collection) and still maintain urine biomarker stability by transporting samples in the presence of additives or a cool environment, allowing patients to send in samples from home as part of a future prospective clinical trial. Initial testing of 13 separate combinations of conditions (with/without different protease inhibitors, ice packs, BA) took place in samples from HCs. A smaller number of conditions were then assessed in JSLE patient urine samples. Use of a protease inhibitor and an ice-pack was, on balance, best at maintaining biomarker stability within 87-120% of the standard processing concentrations.

The associated secondary aim was to compare urine biomarker levels in JSLE patients who had a concomitant UTI, mixed growth or no-growth in their urine. This is of importance as it will be difficult to assess samples sent from home for infection given the delayed sample processing time. No significant difference was seen in urine biomarker levels between UTI, mixed growth and no-growth samples. This

information will be of importance when planning a future study of urine biomarker-led monitoring in LN.

During the multiplex assay development process, when LPGDS, MCP-1 and VCAM-1 standards were individually spiked into an assay containing the multiplexed beads and detection antibodies, no cross-reactivity was demonstrated. However, when TF, CP and AGP standards were spiked in the MFIs for other analytes did display subtle increases (see section 6.5.1). Non-specific interaction between different antibodies and/or unrelated analytes in the reaction mix must be assessed for when using a multiplex assay format, as many proteins have closely related structures with highly conserved epitopes which can lead to cross-reactivity. Of crucial importance, there was not a dose response relationship (i.e. increasing the concentration of the spike did not increase the MFI detected for the non-specific analytes) suggesting that this apparent constant low level of cross reactivity will occur across all samples and not vary according to the concentration of endogenous biomarker within a sample. This is a recognised problem in multiplex assay development and is often accepted as long as a dose response relationship is not seen [452]. Merck Millipore did not feel that these observations represented the presence of significant cross-reactivity and highlighted that the MFIs seen for these analytes were always lower than that of the lowest standards.

The accuracy of the assay was assessed through spike recovery and LOD experiments. During alpha testing, Merck Millipore added low, medium and high concentration spikes into samples from patients with a range of different disease activities. During the beta testing stage a medium sized spike was used to minimise the contributory effect of the endogenous biomarker component whilst avoiding the assay's upper limit of detection. The average percentage recovery for each analyte was between 80-120% across both experiments, meeting existing acceptable criteria for spike recovery experiments [452,457,458], and suggesting that JSLE urine as a matrix did not interfere with analyte recovery.

Dilution is a useful additional check on assay accuracy and provides practical information as to whether an individual sample can be re-run more/less diluted at a later stage if the initial value is out with the assay's limit of detection. Several samples

were assessed from patients with a range of LN disease activity to ensure that the analyte values fell across the working range of the assay. During the alpha testing stage, Merck Millipore ran their LOD experiments in a four plex (for LPGDS, TF, CP and AGP) and two plex assay (MCP-1 and VCAM-1) whereas during the beta testing stage, the full six plex assay was used. They diluted eight patient samples 1 in 100, 1 in 200, 1 in 400 and 1 in 800 and demonstrated acceptable LOD on average for TF, CP, MCP-1 and VCAM-1 (between 80-120%), but not for LPGDS and AGP (LPGDS 192%, AGP 133%). During beta testing the six plex assay was used with a larger number of samples (10 active, 11 inactive LN) run neat, 1 in 10, 1 in 100, 1 in 400 and 1 in 800. LOD was found to be problematic for all analytes. This may be a result of the assay being run as a six plex vs. a four/two plex, pipetting errors or differences in antibody affinity dependent on the amount of diluent present. Samples containing high affinity antibody can show over-recovery on dilution, whereas low affinity antibodies may show lower recovery on dilution [452].

Under these circumstances, to avoid the introduction of errors associated with poor LOD, significant effort was made to assess a large number of patient samples to identify a single dilution factor for each analyte that would permit the measurement of the biomarkers in the majority of patients [452,457]. After identification of the optimal single dilution for each analyte in UK patients, these dilutions were re-tested in US and SA patient samples. Minor country-specific differences were seen in the optimal dilutions required for each analyte. In all countries, AGP, LPGDS and TF could be run at 1 in 400 dilution and MCP-1/VCAM-1 could be run neat. However, in the UK cohort CP should be run neat, whereas in US/SA patients, a 1 in 400 dilution was required. This extensive evaluation of individual biomarker concentration ranges in a total of 106 JSLE patients has provided strong evidence for the individual dilutions required, mitigating the demonstrated effects of poor LOD.

The head-to-head assessment of multiplex assay performance as compared to existing ELISA techniques showed that a more accurate identification of disease state can be obtained with the multiplex assay as compared with ELISA techniques, using a smaller number of biomarkers. Combining the multiplex biomarker data in the same order as was previously used for the ELISA data, a similar progressive increase in AUC was seen with addition of biomarkers to the model. Due to the increased accuracy of each

marker individually, the increases in AUC associated with the addition of each extra biomarker were more modest than previously seen with the ELISA analysis. However, overall the multiplex provided a better AUC for identification of disease state than ELISA analysis (multiplex AUC = 0.998, ELISA AUC = 0.952). Repeating the penalised binary regression modelling process from the beginning using a hypothesis free approach based on the multiplex data alone, TF was identified as the best individual marker within the final model (AUC 0.996) and although addition of AGP, MCP-1 and VCAM-1 led to the highest AUC, the improvement was again marginal (AUC of 0.999).

Other investigators have similarly found multiplex assays to outperform ELISA's for accuracy and identification of disease state [459,460]. This is thought to be based on a range of factors. Firstly, multiplex assays are based on detection of fluorescence as opposed to colorimetric detection, leading to better sensitivity. Secondly, with multiplex assays there is higher avidity of the capture antibodies to beads due to covalent coupling, leading to a higher density of capture antibodies per surface area. In contrast, ELISA plates are passively coated leading to increased risk of being washed off and higher background signals due to non-specific binding of the detection antibody [452].

The non-parametric Bland Altman plot for VCAM-1 suggested a high level of agreement between the ELISA and multiplex assays for this marker. For all other analytes, as the mean biomarker level increased (beyond low-medium biomarker values) the points became more divergent, broadening the 95% CIs. This is likely to relate to the relative precision of both assays at the high end of their standard curves. Using either assay, high biomarker levels are more likely to be extrapolated and imprecise, therefore a new method of biomarker quantification could not be expected to demonstrate agreement. Even if the original assay was re-run multiple times using the same high biomarker concentration sample, poor agreement is likely to be obtained between measurements due to the samples position on the non-linear portion of the standard curve [452]. Clearly use of data obtained beyond the lowest/upper quantifiable limit of the calibration curve should be undertaken with caution as the variability of these values is high. However, reporting of these numeric results can be justified on the basis that it provides actual numerical estimates rather than the

assignment of ‘high’ or ‘low’, as such values cannot be considered in analyses and may have a major impact on the study conclusions if an individual study group yields many values in these regions [457]. It is thought that these factors may be contributing to the wide CIs demonstrated on the non-parametric Bland Altman plots.

Looking towards a clinical trial of urine biomarker-led monitoring in LN, this study has shown that use of a protease inhibitor and ice pack for 48 hours before sample processing helps to maintains urine biomarker stability within 87-120% of its standard immediate processing concentration. Urine biomarker levels were also shown to be comparable between UTI, mixed growth and no-growth JSLE urine samples. To date, only urinary MCP-1 has been looked at in the context of UTI and shown not to be increased in elderly patients with acute cystitis or asyptomatic bacteruria [461]. These interesting results suggest that patient samples could be sent to hospital directly by patients and that the presence of UTI or a mixed growth does not influence biomarker levels. These analyses have been undertaken using the ELISA assays and should be repeated in the future using multiplex in a greater number of JSLE patients with a wider range of LN disease activity.

Drawing on the data from Chapters 3-6, the next chapter will bring these data together and discuss the design and implementation of a clinical trial of urine biomarker-led monitoring in LN, building on the firm foundations developed during this PhD.

6.7 Summary

The study included in this chapter has demonstrated feasibility of a custom multiplex assay for quantification of a urine biomarker panel in LN patients in collaboration with industry partner Merck Millipore. Use of this assay will streamline the process of urine biomarker quantification by reducing the cost, processing time and the volume of sample required for biomarker measurement. The multiplex assay has also shown an improved capacity for active LN disease state identification as compared to existing ELISA techniques.

6.8 Conclusions

- The multiplex 'LN urinary biomarker panel' assay has proven to be better than existing ELISA techniques for identification of active LN disease state.
- 'LN urinary biomarker panel' stability is maintained despite delayed urine sample processing, when in the presence of an ice pack and protease inhibitor.
- Presence of UTI or mixed bacterial growth do not influence 'LN urinary biomarker panel' levels.
- The results from Chapters 3-6 evidently lay the foundations for a future clinical trial of urine biomarker-led monitoring.

7 Discussion

The overarching hypothesis for this thesis was that:

'***A combination of traditional clinical and promising non-invasive novel urinary biomarkers, as part of a 'LN biomarker panel', are better than traditional biomarkers alone at differentiating JSLE patients with active LN from those without, and in predicting fluctuations in LN disease activity over time, within ethnically distinct JSLE cohorts'.***

The following steps were undertaken to investigate this hypothesis, with the clinical and research implications of these results discussed below.

- In Chapter 3, prospective clinical data collected over 10 years from the UK JSLE Cohort Study were interrogated, demonstrating the number of UK JSLE patients developing LN, their clinical and demographic characteristics, and the 'high risk' period for LN development. Clinical and demographic factors were shown to differentiate those who did/did not recover from proteinuria following an LN flare (during the follow-up period). Recognition of such factors predictive of a longer time to recovery could have important implications for treatment choices. Non-renal specific, haematological, inflammatory and immunological traditional biomarkers which are routinely measured in clinical practice were shown to only display a 'fair' test accuracy for active LN identification.
- Novel urine biomarkers warranting further assessment as part of an 'LN biomarker panel' were identified through detailed literature review (published in Clinical Immunology as a review, see Appendix 22). Multiplex screening of urine biomarkers previously implicated in drug induced human kidney injury was undertaken, but did not demonstrate any biomarkers worth pursuing (Chapter 3). The ultimate biomarkers selected for further investigation included AGP, CP, VCAM-1, TF, LPGDS, MCP-1 and NGAL, based upon the strength of evidence on how they relate to the LN disease course in JSLE, and the likelihood that further assessment as part of the current study could facilitate translation towards clinical practice.

- In Chapter 4, urine samples from UK JSLE Cohort study patients were used to cross-sectionally assess if individual biomarkers differed significantly between active and inactive LN patients. Novel and traditional markers of JSLE disease activity were then combined to assess whether a 'LN biomarker panel' could improve active LN identification. An optimal cross-sectional urine biomarker panel for active LN identification was identified, demonstrating an 'excellent' ability for active LN identification (AUC 0.920). Combining traditional biomarkers with the optimal novel urine biomarker panel did not improve the AUC further.
- At the end of Chapter 4, immortalised human podocytes were exposed to AGP, CP, LPGDS and TF (the optimal 'LN urinary biomarker panel' constituents) *in vitro*, to investigate whether these biomarkers could influence processes which are implicated in LN pathogenesis (podocyte apoptosis and cytokine production). No significant differences were seen with respect to these outcomes *in vitro*, and further investigation is required (discussed in section 7.2.4 below).
- In Chapter 5, to robustly validate the above biomarker panel and determine whether it performed comparably within ethnically distinct JSLE patient cohorts, collaborations were developed with the US Einstein Lupus Cohort [334] and the SA Paediatric Lupus Erythematosus in South Africa Cohort [435]. The optimal 'LN urinary biomarker panel' derived within UK JSLE Cohort samples displayed equivalent ability for active LN identification in both the US and SA JSLE cohorts (AUC values of 0.991 and 1.0 respectively).
- To determine longitudinally, if constituents of the LN urinary biomarker panel were able to predict LN flare or remission in advance, data from all three cohorts were combined within a Markov Multi State model of LN urine biomarker dynamics (Chapter 5). AGP was found to be best at predicting LN flare and CP was best for predicting LN remission. Using this model, individual patient predictions of LN disease state over the subsequent three, six, nine and twelve months can be determined.
- In Chapter 6, a multiplex 'LN urinary biomarker panel' assay was developed and validated in collaboration with industry partner Merck Millipore, supported by the award of an MRC CiC grant, in order to streamline the process of urine biomarker panel quantification, and enable development of a future clinical trial of urine

biomarker-led monitoring. The multiplex assay proved to be better for identification of active LN disease state than existing ELISA techniques.

- At the end of Chapter 6, as an aid to the proposed clinical trial, 'LN urinary biomarker panel' stability was investigated following delayed urine sample processing (after 48 hours), demonstrating a protease inhibitor and ice pack to maintain urine biomarker stability within 87-120% of standard immediate processing concentrations.

The overarching hypothesis of this thesis has therefore been thoroughly examined, identifying important insights from clinical and demographic data, deriving/cross-validating an 'LN urinary biomarker panel' to differentiate JSLE patients with and without LN, and identifying biomarkers which predict LN flare/remission, within three ethnically distinct JSLE cohorts. Through development and validation of a multiplex assay capable of LN biomarker panel quantification, we are now in a strong position to undertake a future paediatric clinical trial to prospectively validate this panel, and in due course to determine whether urine biomarker-led monitoring improves renal outcome in JSLE.

7.1 Clinical and research implications of this study

7.1.1 Clinico-demographic factors and LN

Early recognition of LN is important as early treatment and adequate response to treatment is known to be associated with better renal outcome [261]. Identification of those at risk of a prolonged LN course will help the clinician to start, modify, and fine-tune the intensity and duration of immunosuppressive therapy. In this present study, 36% of UK JSLE Cohort Study patients were found to have LN at the time of their initial presentation. A further 17% developed LN during a median follow up of 2.04 years [IQR 0.8-3.7], highlighting a 'high risk' period for development of LN in this cohort. This observation is important when considering the study design, sample size and inclusion criteria for a future clinical trial of urine biomarker-led monitoring, as such a trial will need to capture patients who undergo LN disease state transitions (active to inactive LN transitions, and vice versa), to test further the urine biomarker Markov Multi State model derived in Chapter 5.

Six clinical features were univariately found to differentiate patients with active LN at baseline from those without LN. These were: first ACR score, presence of severe hypertension, level of proteinuria, serum creatinine, ESR and C3. By highlighting clinical features which differentiate those with active LN from those without LN at baseline, these features may serve as a prompt for renal biopsy at an early stage. Both an ACR score of >5, and C3 levels of <0.9 mg/L at baseline, were also identified as significant independent risk factors for subsequent LN development within a multivariate model, over a median of 3.1 years [IQR 1.5-5.0]. Within a future clinical trial of urine biomarker-led monitoring, recruitment of patients with a high ACR score and low C3 at baseline would therefore also be anticipated to improve the yield of recruits who develop active LN.

Within the UK JSLE Cohort Study, 39% of patients were shown to recover from proteinuria following an LN flare within a median of 17 months (min 2.4, max 78), with the remaining 61% continuing to have proteinuria despite a median of 22 months follow-up (min 2.3, max 132). In an adult SLE study looking at time to recovery from proteinuria in patients with LN receiving standard treatment, proteinuria was shown to recover in 53% of patients by 2 years [253], suggesting that with current treatment regimens, children may take longer to go into remission than adults, likely relating to their more severe LN phenotype. Clinico-demographic factors univariately differentiating patients who did recover during follow-up compared to those who did not, included ethnicity, younger age at LN onset, the presence of reduced GFR, azathioprine use and concomitant BILAG defined haematological/cardio-respiratory involvement at the time of active LN with proteinuria development. Within a multivariate model, normal eGFR (>80mls/min), older age (>14 years), and concomitant haematological involvement at the time of active LN with proteinuria development, were all independently associated with increased rate of recovery from proteinuria at a given time-point. Recognition of such clinico-demographic factors predictive of longer time to recovery could also have important implications for treatment choices. For example, helping to modify the intensity and duration of immunosuppressive therapy. They may also impact on the design and conduct of the prospective clinical trial of urine biomarker-led monitoring in LN, whereby patients displaying the above features could either have more frequent biomarker monitoring, or more rapid treatment escalation in response to biomarker changes.

Clinicians currently rely heavily upon non-invasive blood and urine biomarkers as part of ongoing LN monitoring. This study highlights that although the non-renal-specific traditional biomarkers including ESR, C3, WCC, neutrophils, lymphocytes and IgG, can assist with identification of active LN, they rather disappointingly only display a 'fair' test accuracy (AUC 0.724) for active LN identification. In actual routine clinical practice, the information gained from these traditional biomarkers would be naturally supplemented with the information gained routinely from renal markers (e.g. proteinuria, BP, serum creatinine, eGFR). This will go some way to further improving the clinician's ability to accurately identify active disease. However, as our definition of active LN was based on the composite renal BILAG score (calculated directly from these traditional renal markers) we were unable to add such factors as covariates within the regression model analysis. At the same time, reports of 'clinically silent LN' in patients with no proteinuria, normal urinalysis/renal function, but biopsy defined LN [255], raise concerns about the role of such renal markers in LN monitoring, reinforcing the need for novel biomarkers for LN monitoring. A manuscript has been submitted on the basis of this work examining traditional non-renal biomarkers in LN monitoring (see Appendix 23).

7.1.2 Novel urine biomarkers within the UK JSLE Cohort Study

Notably, multiplex screening of urine biomarkers previously implicated in drug induced human kidney injury did not identify any specific biomarkers for LN within samples from the UK JSLE Cohort Study. This may relate to the fact that the majority of the biomarkers screened for were tubular injury markers, as tubules are more commonly affected by drug toxicity [370], whereas LN predominantly affects the glomerulus (see sections 1.1.7 and 3.5.5). JSLE urine specific factors may also have interfered with these assays, despite them being commercially validated for use with urine. Rather than searching *de novo* for further novel urinary biomarkers using for example, a urinary proteomic technique, an evidence-based, targeted approach was taken. This was undertaken initially nationally, and then internationally, validating biomarkers with a strong existing evidence in JSLE and/or adult SLE.

Within the UK JSLE Cohort Study, AGP, CP, LPGDS and TF were shown to constitute an optimal cross-sectional urine biomarker panel for active LN identification (AUC 0.920). This provided significant strength to the hypothesis that a urinary biomarker panel approach is better for differentiating active from inactive LN patients compared to any specific individual biomarker. These urinary biomarkers have previously been shown to be higher in patients with biopsy defined active LN compared to those with inactive LN in a US JSLE cohort. In the US study, a slightly lower AUC (0.85) was demonstrated for their ability to identify active LN, despite combination with further markers (albumin, and albumin-related fragments) additional to AGP, CP, LPGDS and TF [290].

In the current study, extra-renal JSLE disease activity was also shown not to influence urine biomarker levels, suggesting that such markers are renal specific. VCAM-1 and MCP-1 levels also differed significantly between UK patients with active compared to inactive LN. However, their addition to the above optimal urine biomarker panel model did not improve the AUC further. Both of these biomarkers have previously been shown to play a significant role in predicting changes in LN activity [74,308], therefore, they continued to be investigated longitudinally within Chapter 5 and were incorporated within the custom multiplex assay. Combining non-renal traditional biomarkers with the optimal novel biomarker panel did not significantly improve the AUC further, highlighting the improved performance of novel over traditional biomarkers.

7.1.3 International validation and longitudinal analyses of the novel LN urine biomarker panel

Significant differences in disease phenotype, clinical outcomes, complications, mortality and renal-specific survival rates have been shown to occur in JSLE patients of different ethnicities [30,32,222,428-433]. The UK optimal LN urine biomarker panel combination displayed the same ‘excellent’ ability for active LN identification in both a US [334] and SA [462] cohort; with AUC values of 0.991 and 1.0 respectively (see Chapter 5). Previously published urine biomarker panel studies in JSLE have failed to undertake such cross-cultural validation. The improved performance of the urine biomarker panel within these cohorts may be explained by differences seen in ethnicity and LN disease severity between the cohorts. Within the US Cohort, patients

were predominantly African American and Hispanic, whereas the SA Cohort mainly included Coloured and Black African patients. African and African American patients have been shown to have a more severe LN phenotype [32,281,403,435], and both validation cohorts featured a greater proportion of active LN patients than in the original UK JSLE Cohort (25% active LN in UK cohort, 53% in US and 39% in SA). Overall, in all three JSLE patient cohorts, the LN urine biomarker panel (AGP, CP, LPGDS and TF) performed extremely well, despite ethnic diversity, a wide range of disease severity, and assessment within severe LN cohorts. A manuscript has been accepted for publication based upon the UK/US biomarker panel results (see Appendix 24).

Combining data from all three cohorts, it was possible to assess constituents of the biomarker panel within a Markov Multi-State model of LN urine biomarker dynamics, to see if the urine biomarkers were able to predict LN flare and remission in advance (see Chapter 5). Only two biomarkers could be included within this model to avoid overfitting (model complexity limited by the number of patients undergoing disease state transitions, active to inactive LN or vice versa). Within the optimal longitudinal model, AGP was found to be best at predicting LN flare and CP was best for predicting LN remission. These data therefore support the hypothesis that a biomarker panel can also improve prediction of different stages of the LN disease process.

Figure 7-1 summarises the evidence stemming from this study as described in sections 7.1.1–7.1.3 above. It outlines when clinico-demographic factors could also be of use when monitoring patients for active LN. It also shows how both clinico-demographic factors and novel urine biomarkers can integrate as part of such monitoring, forming the basis for further longitudinal validation and a clinical trial assessing the impact of urine biomarker-led monitoring in LN.

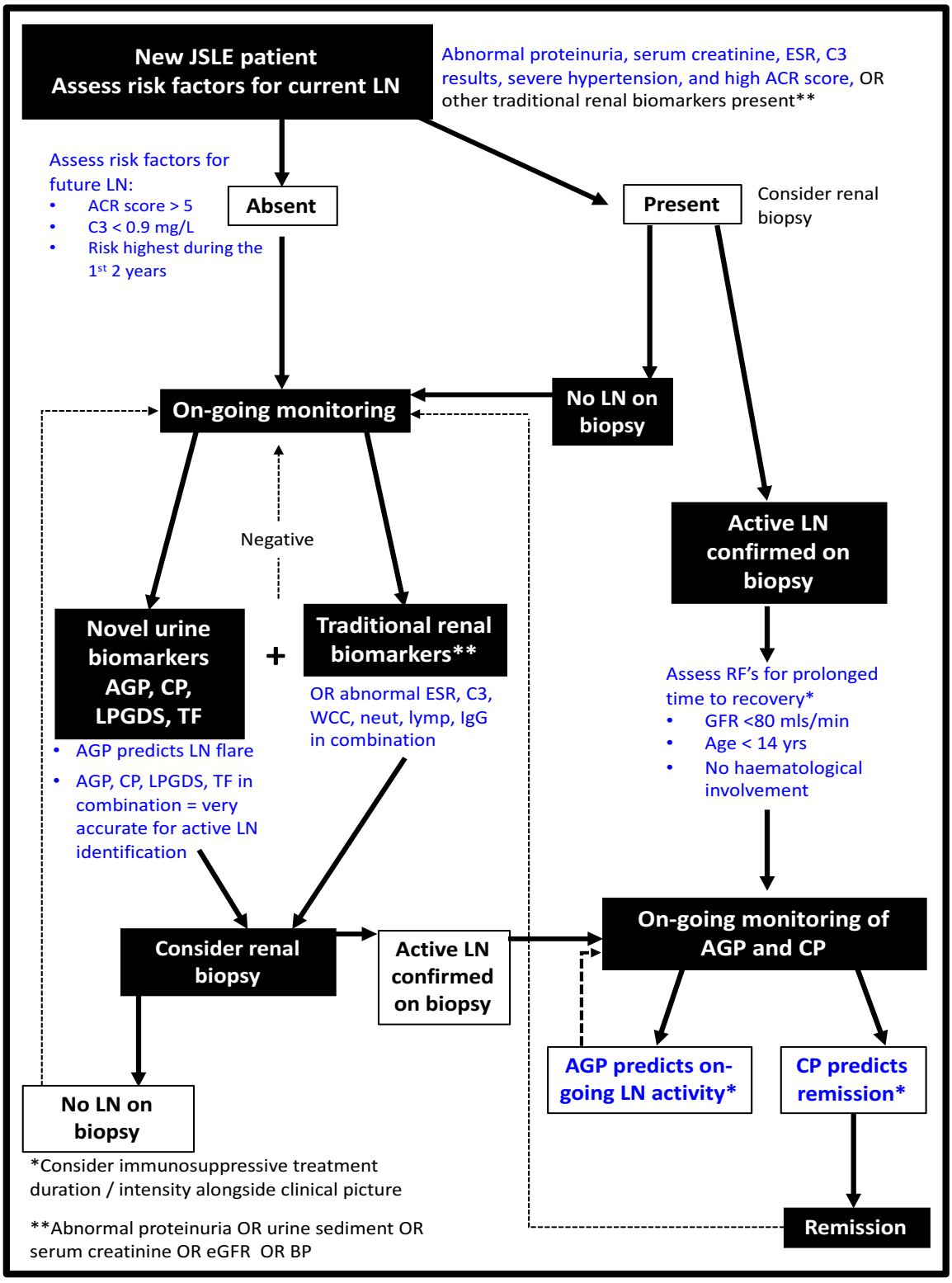


Figure 7-1: Summary of the evidence stemming from this PhD, outlining when clinico-demographic factors and novel biomarkers should be considered during the monitoring of JSLE patients.

Blue text is used to highlight evidence derived from the current study.

7.1.4 Advancing towards a prospective clinical trial of urinary biomarker panel-led monitoring of LN

7.1.4.1 Multiplex assay development

The need for streamlined biomarker panel quantification was recognised following cross-validation and longitudinal assessment of the biomarker panel. This was regarded as crucial for enabling development of a future clinical trial of urine biomarker-led monitoring, where rapid assessment of the urine biomarker panel would be required. A custom, multiplex LN urine biomarker panel assay was therefore developed in collaboration with industry partner Merck Millipore, undergoing beta testing in 107 cross-sectional UK/US/SA cohort samples, allowing head-to-head assessment of the multiplex assay's performance as compared to existing ELISA techniques. Combining the multiplex biomarker data in a binary logistic regression model, in the same order as with ELISA analysis, the optimal urine biomarker model again included AGP, CP, LPGDS, and TF (AUC = 0.998). The multiplex assay also provided a better AUC for identification of active LN disease state than previous ELISA techniques (AUC = 0.952). The multiplex approach reduces processing time, cost and the volume of precious patient sample required. Having developed a custom multiplex assay and demonstrated feasibility for its use, we are now in a strong position to apply for competitive funding for a future clinical trial.

7.1.4.2 Biomarker stability - delayed sample processing or urinary tract infection

In a future clinical study, frequent, fixed time point serial urine samples need to be collected to accurately assess the ability of the biomarker panel to predict events such as LN flare, remission, treatment response and prognosis. This study has shown that use of a protease inhibitor and ice pack for 48 hours before sample processing helps to maintain urine biomarker stability within 87-120% of its standard immediate processing concentration. Urine biomarker levels were also shown to be comparable between UTI, mixed growth and no growth JSLE urine samples. These results suggest that patients could send urine samples straight to hospital through the post, reducing the impact of the proposed study on patients' lives.

7.2 Limitations

7.2.1 The inherent difficulties of cohort studies

The clinical data and samples included in this study have been collected as part of the 'real world' UK JSLE Cohort Study. As with many cohort studies, data is prospectively collected alongside routine clinical care with the addition of retrospective data where necessary. Compared for example to the study design of an RCT, the follow-up visit interval is extremely variable and the amount of missing data is likely to be far higher than if the data was collected during a shorter, tightly regulated RCT. This is evident within this thesis when assessing patients with and without LN at diagnosis, and during follow up. Patients recruited during the early years of this study had more retrospectively collected baseline data, leading to multiple imputation of some clinical variables or exclusion of certain patients.

One of the main advantages of observational cohort studies is the ability to recruit larger numbers of patients than would be possible in a standard RCT design (given also the cost per patient), increasing the generalisability and the external validity of the results, length of follow-up, and reducing the clinical trial/placebo related effects that can be seen in RCTs [463]. The UK JSLE Cohort Study is the largest JSLE cohort in Europe, with representation from across the UK, mitigating some of the difficulties detailed above and providing strength to the results obtained. Similar sized cohorts are seen in the US, differing in terms of patient ethnicity, and influencing the ability to make direct inferences between populations. The UK JSLE Cohort Study case report forms are very comprehensive, collecting a broad range of disease activity, damage and outcome data, but clearly the possibility remains that other uncollected characteristics may also affect outcome.

7.2.2 Definitions of renal disease activity

Within the current study, patients were considered to have active LN on the basis of their renal BILAG score and having a previous renal biopsy demonstrating active LN (for the reasons outlined in section 1.1.4.2). The BILAG score is the most comprehensive composite scoring system, providing assessment of new activity, flare, remission in individual organs/systems [53,54,65,66], whereas other indices have been designed to provide a global score [56,57,59,464]. The wide range of outcome

measures used to define active LN influences the ability to directly compare results between different studies. In section 5.5.1.5, urine biomarker concentrations were shown to be the same between US Cohort patient samples which were taken at the time (or within 6 weeks) of renal biopsy, as compared to patients who had biopsy proven LN at some other point during their disease course but currently had a composite renal BILAG score-based diagnosis of active LN. This suggests that the definition of active LN utilised within this study is a reasonable proxy for biopsy defined active LN.

It was not possible to determine the relationship between constituents of the urinary biomarker panel and different ISN/RPS LN biopsy classes due to the small number of samples collected at the time of renal biopsy. Novel urine biomarkers and traditional renal biomarkers could also not be directly compared due to the definition of active LN being calculated from proteinuria, GFR, blood pressure, active urine sediment, plasma creatinine and recent biopsy findings (components of the composite renal BILAG score). The renal BILAG score is also unable to differentiate renal activity and damage. To address the above limitations, future work, including urine samples collected at the time of renal biopsy, is required.

7.2.3 Patient numbers and length of follow-up

The SA Cohort included the smallest number of patients, requiring a different statistical approach (Firth's Penalised Likelihood Logistic Regression) to that which was used with the UK and US cohorts. The longitudinal Markov Multi-State model was also limited by the number of transitions in disease state observed. Further urine biomarkers or clinical factors could have been included within a more complex model if a greater number of state transitions were seen. The nature of the study, being 'real world' without rigid follow-up intervals means that some episodes of flare may be missed and that there will be differences in the timing of the sample in relation to LN flare between patients. These problems are inherent to undertaking research in rare paediatric rheumatic diseases, and through inclusion of three cohorts the samples size has been optimised. In future studies it would be of interest to look at additional Asian, Chinese and South American cohorts to increase ethnic diversity of the study population.

The analyses looking at clinico-demographic features and how they relate to a variety of renal outcomes could be developed further by undertaking more complex longitudinal analysis, looking at the impact of treatment regimens and other clinico-demographic factors over time. Given the multitude of treatment strategies, different steroid regimens, and varying lengths of treatment, such analysis would be challenging. Within the current study patients with different lengths of follow-up are included, leading to censoring before the study outcome is reached for some patients. Repeating these analyses in 5-10 years would therefore be of interest to increase the length of follow-up.

7.2.4 *In vitro* podocyte cell line experiments

No difference was seen in podocyte apoptosis or cytokine production following exposure to urine biomarkers *in vitro*. The biomarkers used in these experiments were 'active' native or recombinant proteins (see Table 4-1). However, it is not possible to be certain whether these proteins were sufficiently biologically similar to the biomarkers present within urine. Other co-factors present within the urine could also modulate the effect of such biomarkers, but be lacking within the experimental conditions. The biomarker concentrations used in the experiments were chosen on the basis of the concentrations detected within LN urine, however in the future it would be interesting to look for a dose response relationship. It may also be that other native renal cells (e.g. endothelial cells) react to these biomarkers, and this also requires further investigation. It is also possible that the conditionally immortalized podocytes used in these experiments may be inherently resistant to apoptosis and an alternative podocyte specific positive control should therefore have been included to investigate for this (e.g. puromycin aminonucleoside treatment, [465]). Of key importance, cell biologists within the UoL, as part of the UK EATC for Children, are assessing whether these biomarkers originate from the kidney itself, or are passively filtered through the glomerulus.

7.2.5 Urine biomarker panel multiplex assay development

LOD was shown to be unacceptable during the beta testing phase of assay development. To avoid the introduction of errors associated with non-linearity, rigorous range finding was undertaken in order to determine the optimal sample dilutions in 106 UK, US, SA patient samples. In all countries, it was demonstrated that AGP, LPGDS and TF should be run at 1 in 400 dilution and MCP-1/VCAM-1 should be run neat. There was a difference demonstrated between cohorts for CP (UK cohort run neat, US/SA run at 1 in 400 dilution). This extensive evaluation has therefore overcome the difficulties associated with poor LOD.

Comparing ELISA and multiplex biomarker values head to head, differences could be seen between values, especially for samples measured at the high end of the standard curves where a greater number of extrapolated values are seen. However, for novel biomarkers which are experimental, the level of agreement between different methods of biomarker measurement can be considered secondary to whether the two tests show the same ability to identify the disease state of the patient [452]. A more accurate identification of active LN disease state could be obtained by multiplex as compared with ELISA techniques; AUC for identification of active LN was 0.998 and 0.952 respectively.

A summary of the limitations detailed above, along with potential solutions and suggestions for future research, are detailed in Table 7-1 and considered within the following section on next steps.

Limitations	Potential solutions and plans for future research
Cohort study approach	• Future prospective study with strict, regular/more frequent follow-up visits, run like a tightly regulated clinical trial/RCT. • Clinico-demographic factors to be re-assessed to see if the results of the current study can be validated. • To capture more transitions in disease state: o Patients send their urine samples directly to hospital at regular intervals, increasing urine sampling frequency. o Promote patient recruitment at/of: ▪ Initial JSLE diagnosis (highest LN risk from diagnosis-2 years). ▪ Those with an ACR score of >5 or C3<0.9mg/l at baseline (independent risk factors for LN development).
Definition of LN activity based upon the renal BILAG score	• Obtain urine samples and clinical data at the time of renal biopsy to assess the association between urinary biomarkers and LN sub-classes. o Recent UK JSLE Cohort Study ethics amendment approved to facilitate use of excess renal biopsy tissue for research purposes. This study will raise awareness of the need for urine, tissue samples and clinical data at the time of renal biopsy. o International collaboration likely to be required to obtain sufficient samples in a timely manner. • Use of a biopsy based LN (ISN/RPS class, AI or CI) outcome measure will enable head to head comparison of urine/traditional renal biomarkers.
Ethnic diversity	• Despite this being the 1st JSLE biomarker study to include a validation cohort, further ethnic diversity required (e.g. Chinese, South American).
Origin and role of urine biomarkers in LN pathogenesis	• Under investigation by cell biologists within the UK EATC for children. • Development of a complex *in vitro* LN model including podocytes ± co-culture with other renal cells (e.g. endothelial, mesangial) ± immune cells (e.g. macrophages, neutrophils, DC's) or supernatant from such cells ± cytokines and other factors implicated in LN pathogenesis (e.g. IFN-α, NETs). o Enable assessment of the origin of these biomarkers. o Re-investigate the impact of urine biomarkers on apoptosis and cytokine production.

Table 7-1: Limitations and suggestions for future research.

7.3 Next steps

Leading on from these findings, two clinical studies are required to advance the urine biomarker panel towards translation into clinical practice. Further input is required from a paediatric clinical trials unit (CTU) and patients before proceeding further, but a preliminary overview for these studies is discussed below using a PICO format (P: patient or population, I: intervention or indicator, C: comparison or control and O: outcome) [466]. A preliminary outline of these two studies, based on the findings of the thesis, are now summarised.

7.3.1 'LUPUS MODEL' Pilot study (LUPUS Multiplex Outcomes During Evaluation Longitudinally)

Rationale: The prototype longitudinal Markov Multi-State Model requires further validation, measuring the urine biomarkers by multiplex (as opposed to ELISA) in prospectively collected JSLE urine samples which have been collected at frequent/regular time points. Increasing the number of disease state transitions observed will facilitate assessment of further urine biomarkers or clinical factors within a more complex Markov Multi-State Model.

Prospective urine sampling at regular intervals (4-6 weekly) will increase understanding of biomarkers which predict flare, persistently active LN, remission, and help to decide upon the sampling frequency required in clinical study 2 (see section 7.3.2). UK centres with the highest rates of LN will be targeted for this study to capture the maximum number of disease state transitions (e.g. London, Birmingham, Liverpool, Manchester, Scotland). Through such a validation study, the pilot/feasibility data underpinning a larger and more costly clinical trial of urine biomarker-led monitoring would be strengthened. A PICO for the Lupus MODEL study is shown below (also see Figure 7-2).

P: JSLE patients within the UK JSLE Cohort, who prospectively send urine samples to the hospital every 4-6 weeks by post (capturing the period before, during and after an LN flare). At the time of routine clinic appointments (3-4 monthly) half of the urine sample would be processed immediately and the other half sent to hospital through the post.

I: Urine biomarker quantification by bespoke LN urinary biomarker multiplex assay.

C: Ability of urine biomarker panel constituents (namely: AGP, CP, LPGDS, TF, MCP-1 and VCAM-1), and the most promising clinico-demographic factors to predict flare, persistently active LN, and remission.

O: Primary - Robust assessment and refinement of the Markov Multi-State Model, identifying whether constituents of the urine biomarker panel can individually or in combination, predict flare, persistently active LN, remission, in order to finalise the longitudinal biomarker panel warranting assessment during the subsequent clinical trial of urine biomarker-led monitoring. Secondary – A) Assessment of when biomarker values change in advance of subsequent LN flare or remission. B) Identification of the frequency of biomarker quantification required within a clinical trial of urine biomarker-led monitoring.

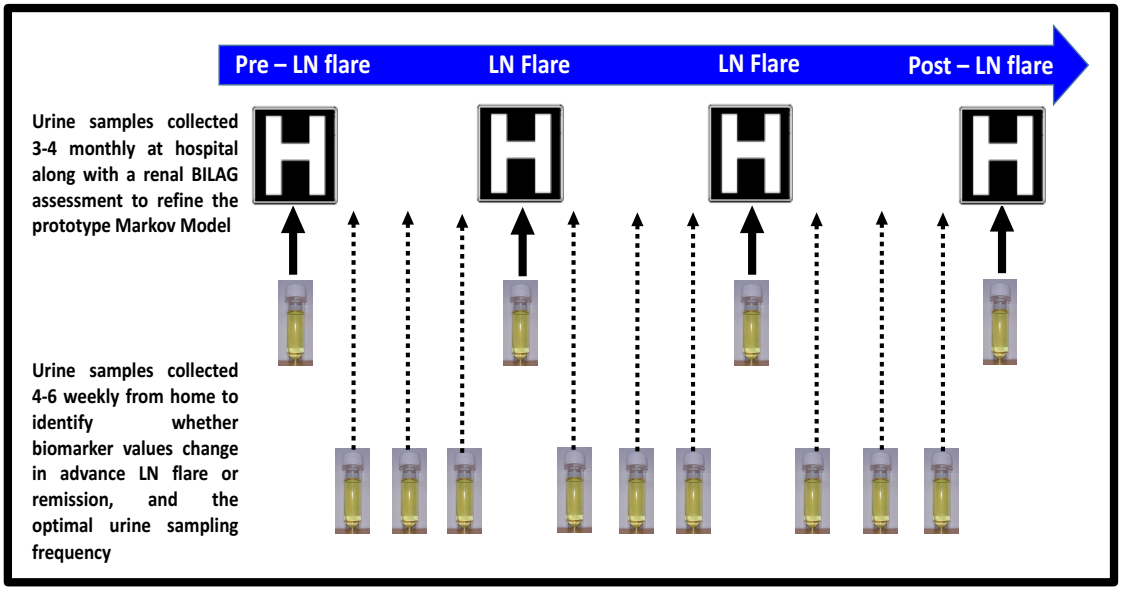


Figure 7-2: Overview of proposed 'LUPUS MODEL' pilot study
Samples would be collected 4-6 weekly (by post) to identify whether biomarker values change in advance of LN flare or remission, predicting such events. Assessment of these frequent samples will also help to identify the optimal urine sampling frequency. At clinic visits, a urine sample will be obtained and a renal BILAG assessment undertaken. Half of the urine sample will be processed immediately (standard urine processing SOP) and the other half sent through the post (postal SOP) in order to assess biomarker stability in samples which have been analysed by multiplex.

Further pre-study work required:

- Use custom multiplex assays to assess existing longitudinal JSLE samples from UK/US/SA cohorts to undertake preliminary Markov Multistate Modelling using multiplex data (validation the work which has already been undertaken in Chapter 5).
- Multiplex and urine biomarker stability assessment.
- Multiplex and biomarker levels in the presence of UTI and mixed growth samples.
- Consultation with patients, parents and key funding stakeholders (e.g. Association of Medical Research Charity members including Arthritis Research UK, Lupus UK, and the National Institute of Health Research Evaluation, Trials and Studies (NETS; http://www.nets.nihr.ac.uk/mis/about funding programmes), along with a paediatric CTU and the MRC North West Coast Hub for Trials Methodology Research (https://www.liverpool.ac.uk/translational-medicine/departmentsandgroups/nwhtmr/) on study design to optimise study acceptability and recruitment in view of the intensive follow-up schedule of the prospective study group.

7.3.2 Clinical trial: 'Can urine biomarker-led monitoring improve clinical outcomes for children with LN?'

Rationale: To justify translation of urine biomarkers into routine clinical care, the ultimate question is whether urine biomarker-led monitoring can improve renal outcomes. Specifically, this study would managed LN patients in accordance with a pre-defined biomarker based treatment algorithm, which uses the Markov Multi-State Model predictions to guide treatment. Such a study would be informed by the results of the LUPUS MODEL pilot/feasibility study (see section 7.3.1). This study would indicate which constituents of the biomarker panel merit quantification, the urine sampling frequency and the mode of sample transport (post or standard processing). Given the unique opportunities that this study would present, an up-to-date review of the literature on urine biomarkers, and those predictive of treatment response (see section 7.3.3) would also be undertaken at that time to assess whether further urine biomarkers merit parallel assessment. Treatment algorithms and study outcomes would be defined through an expert consensus process at the start of the study (e.g. using Delphi and Nominal Group techniques). Given the cost and long follow up

period required in such a study, discussions would be held with a CTU and the MRC North West Hub for Trials Methodology Research, University of Liverpool [467], to consider whether an adaptive trial design could be used.

P: Prospectively recruited JSLE patients (<18 years at the time of SLE onset) who are within 2 years of diagnosis (high risk period for LN development).

I: Urine biomarker quantification and adjustment of LN treatment in accordance with a pre-defined algorithm which uses biomarker based Markov Multi-State Model predictions to guide treatment.

C: Control JSLE patients (<18 years at the time of SLE onset) who are monitored and treated in accordance with standard clinical care, without urine biomarker quantification.

O: To be determined through consensus process.

Potential primary outcomes could include:

A) Time to achievement of renal BILAG defined remission (score of D) within time 'x' OR
B) Complete renal response within time 'x', defined by UPCR OR UACR of ≤20mg/mmol (=<0.2mg/mg) in a spot urine AND eGFR ≥60ml/min, or if <60ml/min at screening, not fallen by >20% compared to screening AND inactive urinary sediment defined as ≤5 red blood cells and ≤5 white blood cells per hpf and no cellular casts [468].

Potential secondary outcomes could include:

A) Low disease activity (D/E) in extra-renal organs/systems with no more than 7.5mg prednisolone (or equivalent) daily for at least 14 days before assessment.

B) SLE Responder Index (SRI) score at time points 'x' and 'y' [469].

C) BILAG-based Combined Lupus Assessment (BICLA) score at time points ‘x’ and ‘y’ [470].

D) Change in SLEDAI [81] or numeric BILAG [65] at time points ‘x’ and ‘y’.

E) Change in physician’s VAS at time points ‘x’ and ‘y’.

F) Change in daily oral prednisolone requirement at time points ‘x’ and ‘y’.

G) Change in patient-reported outcomes (e.g. VAS, pain, and fatigue, LupusQoL [50], SF-36 [47], CHAQ [49] at time points ‘x’ and ‘y’.

H) Patient questionnaire on perceived acceptability and practicality of urine biomarker based monitoring and treatment.

I) Standard questionnaire reporting healthcare utilisation during the study period.

J) Longer term outcomes could include number of LN flares at 2 and 5 years or renal survival (absence of end-stage renal failure, dialysis or transplant). This would require significant follow-up and funding.

Pre-study work required:

- LUPUS MODEL pilot study.
- Consultation within UK paediatric rheumatology and nephrology communities to estimate the number of eligible patients per month and consider of international recruitment through existing collaborations and the Paediatric Rheumatology European Society (PReS) JSLE Working Group.
- Comprehensive review of JSLE disease activity and outcome measures.
- Comprehensive review of adaptive trial design methodologies and consultation of a paediatric CTU.
- Consultation with patients, parents and key stakeholders (e.g. Lupus UK) on study design to optimise study acceptability, recruitment and retention.

7.3.3 Performance of urine biomarkers as predictors of response to treatment

Adult clinical studies looking at response to treatment [150] and clinical trials [187] are increasingly including JSLE patients as part of their study protocols. Both the US FDA paediatric exclusivity program [471] and European Union Paediatric Drug Regulations [472] provide financial incentives for drugs studied in paediatric populations, with the aim of increasing research and drug development for children. Establishing links with industry and chief investigators undertaking investigator-led trials could enable inclusion of the LN urinary biomarker panel as secondary outcomes within such study protocols, to assess whether constituents of the urine biomarker panel are able to predict response to treatment. MASTERPLANS: 'An open label observational study to identify predictors of response to rituximab and MMF in patients with SLE including cutaneous or renal manifestations' [473] is currently being set-up and due to recruit JSLE patients over the age of 12 years. This study will collect urine samples alongside other biological specimens, enabling assessment of whether urine biomarkers can predict treatment response to Rituximab or MMF in JSLE and adult SLE patients with LN. Such opportunistic investigation within well conducted phase II and III trials is an important step towards gathering robust evidence for urine biomarker-led monitoring in LN.

7.4 Final conclusions

Evidence presented within this thesis has demonstrated and validated an excellent urine biomarker panel for identification of active LN in ethnically distinct UK/US and SA JSLE cohorts. It has shown that different constituents of the biomarker panel are best suited at predicting the occurrence of LN flare and remission longitudinally, utilising a prototype longitudinal Markov Multi-State model of urine biomarker dynamics. To advance these laboratory observations towards clinical translation, a custom multiplex assay has been developed in collaboration with industry partner Merck Millipore and validated at the UoL in JSLE urine samples from UK/US/SA cohorts. The multiplex assay has been shown to be better at identifying active LN than existing ELISA techniques, and substantially reduces the time, cost and quantity of patient sample required for urine biomarker panel quantification. This work has laid

the foundations for clinical studies longitudinally measuring the urine biomarker panel by multiplex, allowing refinement and inclusion of further biomarkers within the Markov Multi-State Model. The second clinical study would examine whether urine biomarker-led monitoring can actually improve outcomes for children with LN, and is anticipated to yield pivotal evidence for the translation of urine biomarkers into routine clinical practice, producing significant progress in the field of urine biomarker monitoring for the benefit of children with JSLE.

Appendix contents

Appendix 1: UK JSLE Cohort Study ACR and SLICC form

SLICC CLASSIFICATION CRITERIA AT ANNUAL REVIEW

JSLE – ACR CLASSIFICATION CRITERIA AT ANNUAL REVIEW

Study No ☐☐☐☐☐☐ Date ☐☐/☐☐/☐☐ Retrospective ☐ Prospective ☐

These are cumulative. Please add **new** criteria only or **tick the box if no new criteria since last review** ☐

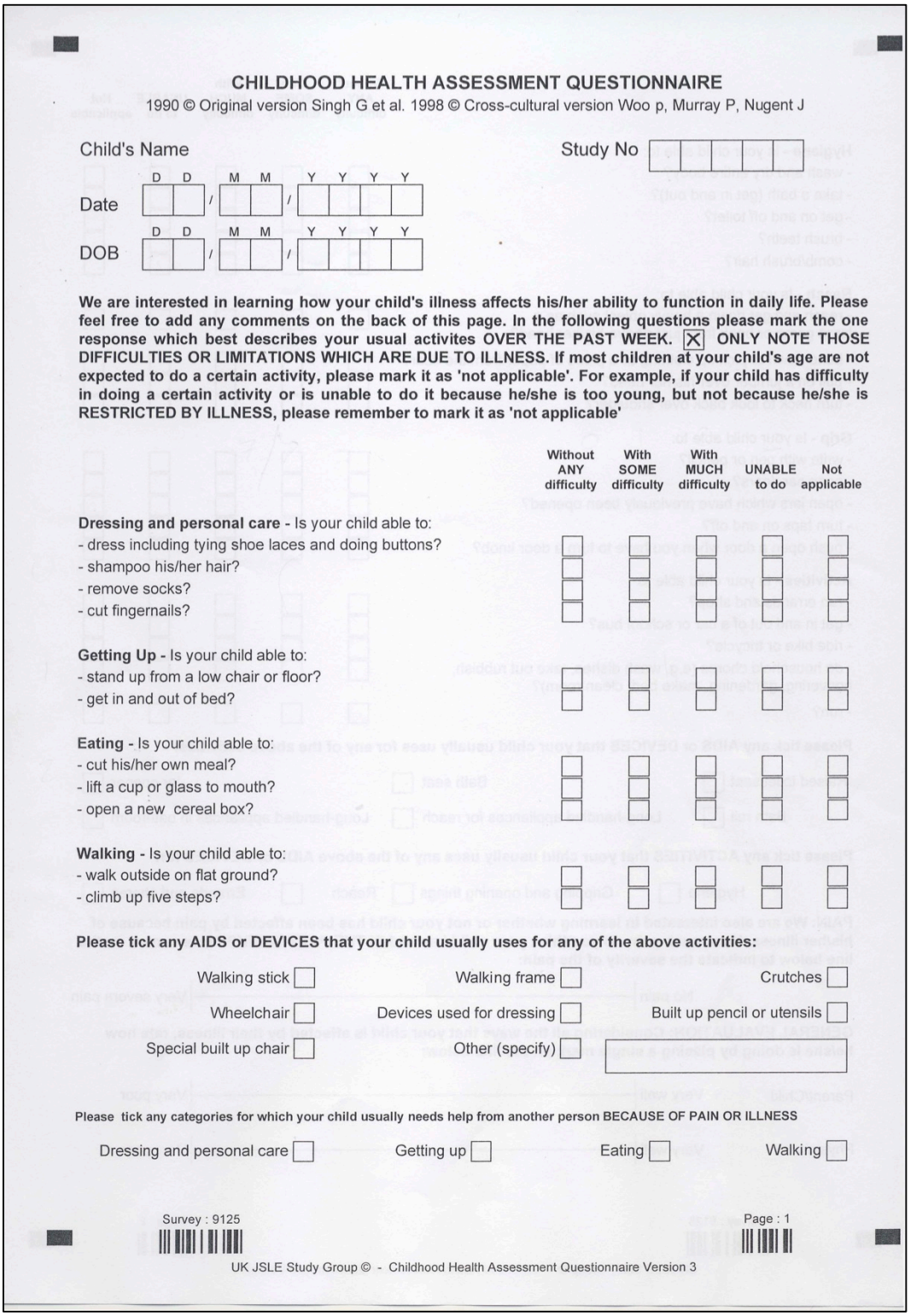
Criterion		Present	Subtype		Definition
1	Malar Rash	☐			Fixed erythema, flat or raised, over malar eminences, tending to spare the nasolabial folds
2	Discoid Lupus	☐			Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3	Photosensitivity	☐			Skin rash as a result of the unusual reaction to sunlight, by patient history or physician observation
4	Oral/nasal ulcerations	☐			Oral or nasopharyngeal ulceration, usually painless, observed by physician
5	Non-erosive Arthritis	☐			Non-erosive arthritis involving 2 or more peripheral joints , characterised by tenderness, swelling or effusion
6	Serositis	☐	A	☐	Pleuritis-convincing history of pleuritic pain or rub heard, or evidence of pleural effusion
		☐	B	☐	Pericarditis–documented ECG or rub, or evidence of pericardial effusion
7	Nephritis	☐	A	☐	Persistent proteinuria > 0.5g/day or > +++ (on protein dipstix) if quantification not performed
			B	☐	Cellular casts – may be red cell, haemoglobin, granular, tubular or mixed
8	Neurologic	☐	A	☐	Seizures in the absence of offending drugs or metabolic derangements (e.g. uraemia, ketoacidosis, electrolyte imbalance)
			B	☐	Psychosis in the absence of offending drugs or metabolic derangements (e.g. uraemia, ketoacidosis, electrolyte imbalance)
9	Haematological Disorder	☐	A	☐	Haemolytic anaemia with reticulocytes
			B	☐	Leucopenia < 4,000/mm³ total on 2 or more occasions
			C	☐	Lymphopenia < 1,500/mm³ on 2 or more occasions
			D	☐	Thrombocytopenia < 100,000/mm³ in absence of offending drugs
10	Immunological Disorder	☐	A	☐	Anti-DNA: antibody to native DNA in abnormal titre
			B	☐	Anti-Sm: presence of antibody to Sm nuclear antigen
			C	☐	+ve finding of anti-phospholipid antibodies based on:
			C1	☐	Abnormal level of IgG or IgM anti-cardiolipiantibody
			C2	☐	Positive test result for lupus anticoagulant (standard method)
			C3	☐	False +ve serologic result for syphilis > 6 mths
11	ANA	☐			Abnormal titre ANA at any time point in absence of drugs known to be associated with "drug-induced lupus"

If less than 4 criteria why evolving Lupus?	

Study No								Date			/			/		
These are cumulative. Please add **new** criteria only or **tick the box if no new criteria since last review**																

		Criterion	Present	Subtype	Definition
Clinical Criteria	1	**Acute cutaneous lupus** *in the absence of dermatomyositis*	☐	A ☐	Lupus malar rash (do not count if malar discoid)
				B ☐	Bullous lupus
				C ☐	Toxic epidermal necrolysis variant of SKE
				D ☐	Maculopapular lupus rash
				E ☐	Photosensitive lupus rash
				☐	Subacute cutaneous lupus (Non-indurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with postinflammatory dyspigmentation or telangiectasias)
	2	**Chronic cutaneous lupus**	☐	A ☐	Classic discoid rash localised (above the neck)
				B ☐	Classic discoid rash generalised (above and below the neck)
				C ☐	Hypertrophic (verrucous lupus)
				D ☐	Lupus panniculitis (profundus)
				E ☐	Mucosal lupus
				F ☐	Lupus erythematosus tumidus
				G ☐	Chilblains lupus
				H ☐	Discoid lupus / lichen planus overlap
	3	**Ulcers**	☐	A ☐	Oral ulcers (Palate/Buccal/Tongue) *in absence of other causes, such as vasculitis, Behcet's disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis and acidic foods*
				B ☐	Nasal ulcers - *in the absence of other causes, such as vasculitis, Behcet's disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis and acidic foods*
	4	**Nonscarring alopecia**	☐		Diffuse thinning or hair fragility with visible broken hairs *in the absence of other causes such as alopecia areata, drugs, iron deficiency and androgenic alopecia*
	5	**Synovitis**	☐		Involving 2 or more joints, characterised by swelling or effusion OR tenderness in 2 or more joints and at least 30 minutes of morning stiffness
	6	**Serositis**	☐	A ☐	Typical pleurisy for more than 1 day OR pleural effusions OR pleural rub
				B ☐	Typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day OR pericardial effusion OR pericardial rub OR pericarditis by ECG *in the absence of other causes such as infection, uraemia, and Dressler's pericarditis*
	7	**Renal**	☐	A ☐	Urine protein-to-creatinine ratio (or 24-hour urine protein) representing 500mg protein/24 hours
				B ☐	Red blood cell casts
	8	**Neurological**	☐	A ☐	Seizures
				B ☐	Psychosis
				C ☐	Mononeuritis multiplex *in the absence other known causes such as primary vasculitis*
				D ☐	Myelitis
				E ☐	Peripheral or cranial neuropathy *in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus*
				F ☐	Acute confusional state *in the absence of other causes, including toxic/metabolic, uraemia, drugs*
	9	**Haemolytic anaemia**	☐		
	10	**Leukopenia or lymphopenia**	☐	A ☐	Leukopenia (<4,000/mm^3 at least once) *in the absence of other known causes such as Felty's syndrome, drugs and portal hypertension*
				B ☐	Lymphopenia (<1,000/mm^3 at least once) *in the absence of other known causes such as corticosteroids, drugs and infection*
	11	**Thrombocytopenia**	☐		Thrombocytopenia <100,000/mm^3 at least once *in the absence of other known causes such as drugs, portal hypertension , and thrombotic thrombocytopenic purpura*
	1	**ANA**	☐		ANA level above laboratory reference range
	2	**Anti-dsDNA**	☐		Anti-dsDNA antibody level above laboratory reference range (or >2-fold the reference range if tested by ELISA)
	3	**Anti-Sm**	☐		Presence of antibody to Sm nuclear antigen
	4	**Antiphospholipid antibody positivity**	☐	A ☐	Positive test result for lupus anticoagulant
				B ☐	False-positive test result for rapid plasma reagin
				C ☐	Medium- or high-titer anticardiolipin antibody level (IgA, IgG, or IgM)
				D ☐	Positive test result for anti-β_2-glycoprotein I (IgA, IgG, or IgM)
	5	**Low complement**	☐	A ☐	Low C3
				B ☐	Low C4
				C ☐	Low CH50
	6	**Direct Coombs' test**	☐		*In the absence of haemolytic anaemia*

Appendix 2: UK JSLE Cohort Study CHAQ



CHILDHOOD HEALTH ASSESSMENT QUESTIONNAIRE

1990 © Original version Singh G et al. 1998 © Cross-cultural version Woo p, Murray P, Nugent J

Child's Name

Study No

Date D D / M M / Y Y Y Y

DOB D D / M M / Y Y Y Y

We are interested in learning how your child's illness affects his/her ability to function in daily life. Please feel free to add any comments on the back of this page. In the following questions please mark the one response which best describes your usual activites OVER THE PAST WEEK. ☒ ONLY NOTE THOSE DIFFICULTIES OR LIMITATIONS WHICH ARE DUE TO ILLNESS. If most children at your child's age are not expected to do a certain activity, please mark it as 'not applicable'. For example, if your child has difficulty in doing a certain activity or is unable to do it because he/she is too young, but not because he/she is RESTRICTED BY ILLNESS, please remember to mark it as 'not applicable'

	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	UNABLE to do	Not applicable
Dressing and personal care - Is your child able to:					
- dress including tying shoe laces and doing buttons?	☐	☐	☐	☐	☐
- shampoo his/her hair?	☐	☐	☐	☐	☐
- remove socks?	☐	☐	☐	☐	☐
- cut fingernails?	☐	☐	☐	☐	☐
Getting Up - Is your child able to:					
- stand up from a low chair or floor?	☐	☐	☐	☐	☐
- get in and out of bed?	☐	☐	☐	☐	☐
Eating - Is your child able to:					
- cut his/her own meal?	☐	☐	☐	☐	☐
- lift a cup or glass to mouth?	☐	☐	☐	☐	☐
- open a new cereal box?	☐	☐	☐	☐	☐
Walking - Is your child able to:					
- walk outside on flat ground?	☐	☐	☐	☐	☐
- climb up five steps?	☐	☐	☐	☐	☐

Please tick any AIDS or DEVICES that your child usually uses for any of the above activities:

Walking stick ☐ Walking frame ☐ Crutches ☐

Wheelchair ☐ Devices used for dressing ☐ Built up pencil or utensils ☐

Special built up chair ☐ Other (specify) ☐

Please tick any categories for which your child usually needs help from another person BECAUSE OF PAIN OR ILLNESS

Dressing and personal care ☐ Getting up ☐ Eating ☐ Walking ☐

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	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	UNABLE to do	Not applicable
Hygiene - Is your child able to:					
- wash and dry entire body?	☐	☐	☐	☐	☐
- take a bath (get in and out)?	☐	☐	☐	☐	☐
- get on and off toilet?	☐	☐	☐	☐	☐
- brush teeth?	☐	☐	☐	☐	☐
- comb/brush hair?	☐	☐	☐	☐	☐
Reach - Is your child able to:					
- reach and get down a heavy object such as a large game or book from just above his/her head?	☐	☐	☐	☐	☐
- bend down to pick up clothing or a piece of paper from the floor?	☐	☐	☐	☐	☐
- pull on a jumper over his/her head?	☐	☐	☐	☐	☐
- turn neck to look back over shoulder?	☐	☐	☐	☐	☐
Grip - Is your child able to:					
- write with pen or pencil?	☐	☐	☐	☐	☐
- open car doors?	☐	☐	☐	☐	☐
- open jars which have previously been opened?	☐	☐	☐	☐	☐
- turn taps on and off?	☐	☐	☐	☐	☐
- push open a door when you have to turn a door knob?	☐	☐	☐	☐	☐
Activities - Is your child able to:					
- run errands and shop?	☐	☐	☐	☐	☐
- get in and out of a car or school bus?	☐	☐	☐	☐	☐
- ride bike or tricycle?	☐	☐	☐	☐	☐
- do household chores (e.g. wash dishes, take out rubbish, hoovering, gardening, make bed, clean room)?	☐	☐	☐	☐	☐
- run?	☐	☐	☐	☐	☐

Please tick any AIDS or DEVICES that your child usually uses for any of the above activities:

Raised toilet seat ☐ Bath seat ☐ Jar opener ☐

Bath rail ☐ Long-handled appliances for reach ☐ Long-handled appliances in bathroom ☐

Please tick any ACTIVITIES that your child usually uses any of the above AIDS or DEVICES for:

Hygiene ☐ Gripping and opening things ☐ Reach ☐ Errands and chores ☐

PAIN: We are also interested in learning whether or not your child has been affected by pain because of his/her illness. How much pain do you think your child has had IN THE PAST WEEK? Place a mark on the line below to indicate the severity of the pain:

No pain |——————————————————| Very severe pain

GENERAL EVALUATION: Considering all the ways that your child is affected by their illness, rate how he/she is doing by placing a single mark on the line below:

Parent/Child Very well |——————————————————| Very poor

Physician Very well |——————————————————| Very poor

Survey : 9125

Appendix 3: UK JSLE Cohort Study CHQ

CHILD HEALTH QUESTIONNAIRE (CHQ) (For children five years or older)

Child's Name

Study No

Date D D / M M / Y Y Y Y DOB D D / M M / Y Y Y Y

This booklet asks about your child's health and well-being. Your individual answers will not be shared with anyone. If you choose not to participate it will not affect the care your child receives.

Answer the questions by marking one of the appropriate boxes with a check mark as shown. ☒

Certain questions may look alike, but each one is different. Some questions ask about problems your child may not have, but it is important for us to know that too.

Please answer every question. There are no right or wrong answers, just choose the response that you think fits your situation best.

If you are unsure how to answer a question, please give the best answer you can and make a comment in the margin. All comments will be read, so please fell free to make as many as you wish.

At the end, please go back and check once again that every item has been answered.

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SECTION 1: YOUR CHILD'S GENERAL HEALTH

1.1 In general, would you say your child's health is

Excellent ☐ Very good ☐ Good ☐ Fair ☐ Poor ☐

SECTION 2: YOUR CHILD'S PHYSICAL ACTIVITIES

The following questions ask about physical activities your child might do during a day.
2.1 During the past 4 weeks has your child been limited in any of the following activities due to health problems?

	Yes, limited a lot	Yes, limited somewhat	Yes, limited a little	No, not limited
a. Doing things that take a lot of energy, such as playing football or netball, running?	☐	☐	☐	☐
b. Doing things that take some energy, such as riding a bike or roller skating?	☐	☐	☐	☐
c. Ability (physically) to get around the neighbourhood, playground or school?	☐	☐	☐	☐
d. Walking 100 metres or climbing one flight of stairs?	☐	☐	☐	☐
e. Bending, lifting or stooping?	☐	☐	☐	☐
f. Taking care of themselves, that is, eating, drinking, dressing, bathing or going to the toilet?	☐	☐	☐	☐

SECTION 3: YOUR CHILD'S EVERYDAY ACTIVITIES

3.1 During the past 4 weeks has your child's schoolwork or activities with friends been limitied in any of the following ways due to EMOTIONAL difficulties or problems with his/her behaviour?

	Yes, limited a lot	Yes, limited somewhat	Yes, limited a little	No, not limited
a. Limited in the KIND of schoolwork or activities with friends he/she could do?	☐	☐	☐	☐
b. Limited in the AMOUNT of time he/she could spend on schoolwork or activities with friends?	☐	☐	☐	☐
c. Limited in PERFORMING schoolwork or activites with friends (it took extra effort)?	☐	☐	☐	☐

3.2 During the past 4 weeks has your child's schoolwork or activities with friends been limitied in any of the following ways due to problems with his/her PHYSICAL health?

	Yes, limited a lot	Yes, limited somewhat	Yes, limited a little	No, not limited
a. Limited in the KIND of schoolwork or activities with friends he/she could do?	☐	☐	☐	☐
b. Limited in the AMOUNT of time he/she could spend on schoolwork or activities with friends?	☐	☐	☐	☐

SECTION 4: PAIN

4.1 During the past 4 weeks how much bodily pain or discomfort has your child had?

None ☐ Very mild ☐ Mild ☐ Moderate ☐ Severe ☐ Very severe ☐

4.2 During the past 4 weeks how often has your child had bodily pain or discomfort?

None of the time ☐ Once or twice ☐ A few times ☐ Fairly often ☐ Very often ☐ Every/almost every day ☐

Study No _____ Date _____

SECTION 5: BEHAVIOUR

Below is a list of items that describe children's behaviour or problems they sometimes have.
5.1 How often during the past 4 weeks did each of the following statements describe your child?

	Very often	Fairly often	Sometimes	Almost never	Never
a. Argued a lot?	☐	☐	☐	☐	☐
b. Had difficulty in concentrating or paying attention?	☐	☐	☐	☐	☐
c. Not told the truth?	☐	☐	☐	☐	☐
d. Taken things which didn't belong to them?	☐	☐	☐	☐	☐
e. Had tantrums or a hot temper?	☐	☐	☐	☐	☐

5.2 Compared to other children your child's age, in general would you say his/her behaviour is

Exellent ☐ Very good ☐ Good ☐ Fair ☐ Poor ☐

SECTION 6: WELL-BEING

The following are about children's moods
6.1 During the past 4 weeks how much time do you think your child:

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Felt like crying?	☐	☐	☐	☐	☐
b. Felt lonely?	☐	☐	☐	☐	☐
c. Acted nervous?	☐	☐	☐	☐	☐
d. Acted bothered or upset?	☐	☐	☐	☐	☐
e. Acted cheerful?	☐	☐	☐	☐	☐

SECTION 7: SELF ESTEEM

The following ask about your child's satisfaction with self, school and others. It may be helpful if you keep in mind how other children your child's age might feel about these areas.
7.1 During the past 4 weeks how satisfied do you think your child has felt about:

	Very satisified	Somewhat satisified	Neither satisfied nor dissatisfied	Somewhat dissatisfied	Very dissatisfied
a. His/her school ability?	☐	☐	☐	☐	☐
b. His/her athletic ability?	☐	☐	☐	☐	☐
c. His/her friendships?	☐	☐	☐	☐	☐
d. His/her looks/appearance?	☐	☐	☐	☐	☐
e. His/her family relationships?	☐	☐	☐	☐	☐
f. His/her life overall?	☐	☐	☐	☐	☐

SECTION 8: YOUR CHILD'S HEALTH

The following statements are about health in general.
8.1 How true or false is each of these statements for your child?

	Definetly true	Mostly true	Don't know	Mostly false	Definetely false
a. My child seems to be less healthy than other children I know	☐	☐	☐	☐	☐
b. My child has never been seriously ill	☐	☐	☐	☐	☐
c. When there is something going around my child usually catches it	☐	☐	☐	☐	☐
d. I expect my child will have a very healthy life	☐	☐	☐	☐	☐
e. I worry more about my child's health than other people worry about their children's health	☐	☐	☐	☐	☐

8.2 Compared to one year ago, how would you rate your child's health now?

Much better now than one year ago ☐ Somewhat better now than one year ago ☐ About the same now as one year ago ☐ Somewhat worse now than one year ago ☐ Much worse now than one year ago ☐

SECTION 9: YOU AND YOUR FAMILY

9.1 During the <u>past 4 weeks</u> how MUCH emotional worry or concern did each of the following cause YOU?

	None at all	A little bit	Somewhat	A lot	A great deal
a. Your child's physical health?	☐	☐	☐	☐	☐
b. Your child's emotional well-being or behaviour?	☐	☐	☐	☐	☐
c. Your child's attention or learning difficulties?	☐	☐	☐	☐	☐

9.2 During the <u>past 4 weeks</u> were you limited in the amount of time YOU had for your own needs because of:

	Yes, limited a lot	Yes, limited somewhat	Yes, limited a little	No, not limited
a. Your child's physical health?	☐	☐	☐	☐
b. Your child's emotional well-being or behaviour?	☐	☐	☐	☐
c. Your child's attention or learning difficulties?	☐	☐	☐	☐

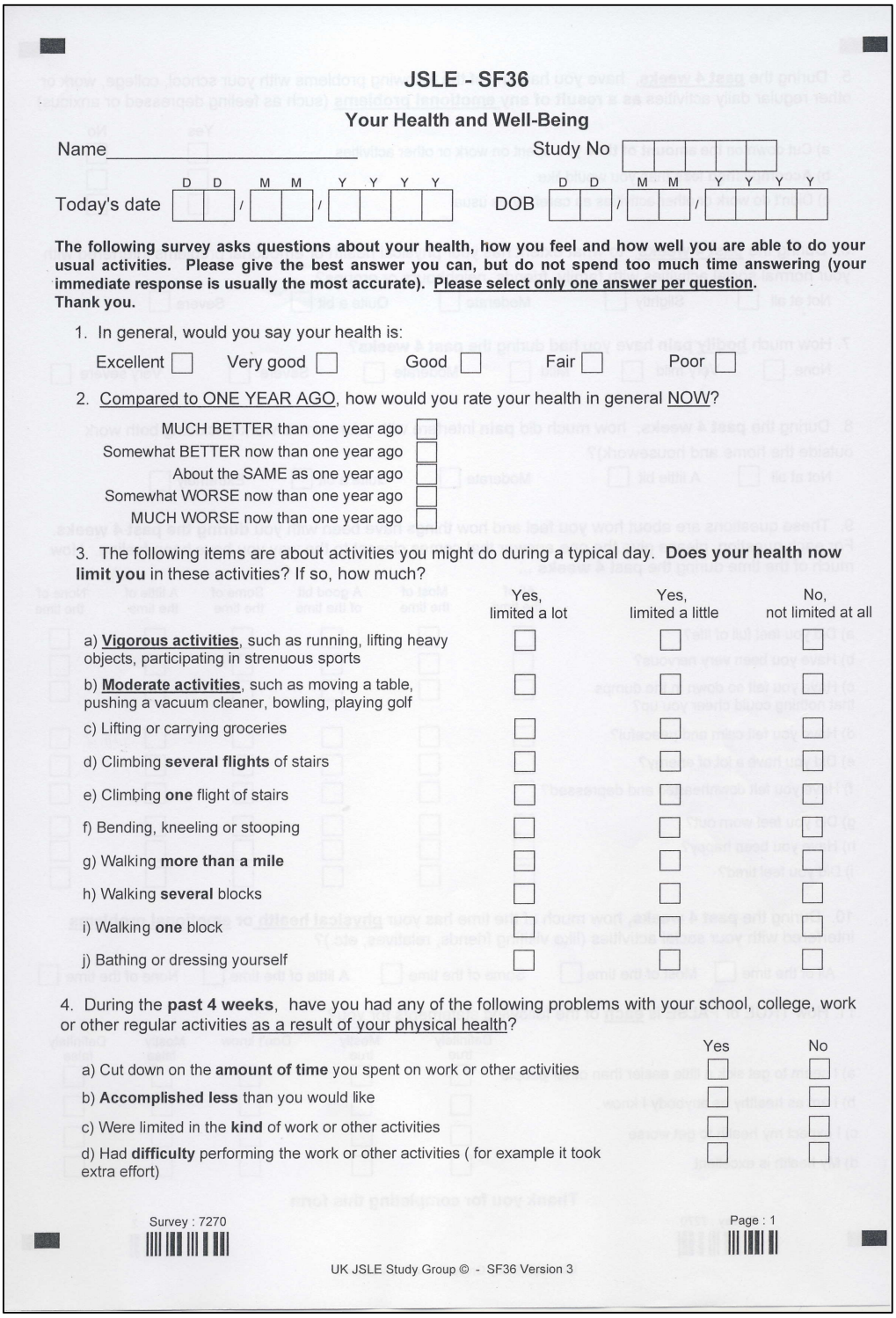
9.3 During the past 4 weeks <u>how often</u> has your child's health or behaviour:

	Very often	Fairly often	Sometimes	Almost never	Never
a. Limited the types of activities you could do as a family?	☐	☐	☐	☐	☐
b. Limited various everyday family activities (eating meals, watching TV)?	☐	☐	☐	☐	☐
c. Limited your ability as a family to 'get up and go' on a moment's notice?	☐	☐	☐	☐	☐
d. Caused tension or conflict in your home?	☐	☐	☐	☐	☐
e. Been a source of disagreements or arguments in your family?	☐	☐	☐	☐	☐
f. Caused you to cancel or change plans (personal or work) at the last minute?	☐	☐	☐	☐	☐

9.4 Sometimes families may have difficluty in getting along with one another. They do not always agree and they get angry. In general, how would you rate your family's ability to get along with one another?

Exellent ☐ Very good ☐ Good ☐ Fair ☐ Poor ☐

Appendix 4: UK JSLE Cohort Study SF36



JSLE - SF36

Your Health and Well-Being

Name_____ Study No

Today's date D D / M M / Y Y Y Y DOB D D / M M / Y Y Y Y

The following survey asks questions about your health, how you feel and how well you are able to do your usual activities. Please give the best answer you can, but do not spend too much time answering (your immediate response is usually the most accurate). Please select only one answer per question. Thank you.

1. In general, would you say your health is:

Excellent ☐ Very good ☐ Good ☐ Fair ☐ Poor ☐

2. Compared to ONE YEAR AGO, how would you rate your health in general NOW?

MUCH BETTER than one year ago ☐
Somewhat BETTER now than one year ago ☐
About the SAME as one year ago ☐
Somewhat WORSE now than one year ago ☐
MUCH WORSE now than one year ago ☐

3. The following items are about activities you might do during a typical day. **Does your health now limit you** in these activities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No, not limited at all
a) **Vigorous activities**, such as running, lifting heavy objects, participating in strenuous sports	☐	☐	☐
b) **Moderate activities**, such as moving a table, pushing a vacuum cleaner, bowling, playing golf	☐	☐	☐
c) Lifting or carrying groceries	☐	☐	☐
d) Climbing **several flights** of stairs	☐	☐	☐
e) Climbing **one** flight of stairs	☐	☐	☐
f) Bending, kneeling or stooping	☐	☐	☐
g) Walking **more than a mile**	☐	☐	☐
h) Walking **several** blocks	☐	☐	☐
i) Walking **one** block	☐	☐	☐
j) Bathing or dressing yourself	☐	☐	☐

4. During the **past 4 weeks**, have you had any of the following problems with your school, college, work or other regular activities as a result of your physical health?

	Yes	No
a) Cut down on the **amount of time** you spent on work or other activities	☐	☐
b) **Accomplished less** than you would like	☐	☐
c) Were limited in the **kind** of work or other activities	☐	☐
d) Had **difficulty** performing the work or other activities (for example it took extra effort)	☐	☐

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5. During the **past 4 weeks**, have you had any of the following problems with your school, college, work or other regular daily activities **as a result of any emotional problems** (such as feeling depressed or anxious)

	Yes	No
a) Cut down on the **amount of time** you spent on work or other activities	☐	☐
b) **Accomplished less** than you would like	☐	☐
c) Didn't do work or other activities as carefully as usual	☐	☐

6. During the **past 4 weeks**, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours or groups?

Not at all ☐ Slightly ☐ Moderate ☐ Quite a bit ☐ Severe ☐

7. How much **bodily pain** have you had during the **past 4 weeks**?

None ☐ Very mild ☐ Mild ☐ Moderate ☐ Severe ☐ Very severe ☐

8. During the **past 4 weeks**, how much did **pain** interfere with your normal work (including both work outside the home and housework)?

Not at all ☐ A little bit ☐ Moderate ☐ Quite a bit ☐ Extremely ☐

9. These questions are about how you feel and how things have been with you **during the past 4 weeks**. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past **4 weeks ...**

	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
a) Did you feel full of life?	☐	☐	☐	☐	☐	☐
b) Have you been very nervous?	☐	☐	☐	☐	☐	☐
c) Have you felt so down in the dumps that nothing could cheer you up?	☐	☐	☐	☐	☐	☐
d) Have you felt calm and peaceful?	☐	☐	☐	☐	☐	☐
e) Did you have a lot of energy?	☐	☐	☐	☐	☐	☐
f) Have you felt downhearted and depressed?	☐	☐	☐	☐	☐	☐
g) Did you feel worn out?	☐	☐	☐	☐	☐	☐
h) Have you been happy?	☐	☐	☐	☐	☐	☐
i) Did you feel tired?	☐	☐	☐	☐	☐	☐

10. During the **past 4 weeks,** how much of the time has your **physical health or emotional problems** interfered with your social activities (like visiting friends, relatives, etc.)?

All of the time ☐ Most of the time ☐ Some of the time ☐ A little of the time ☐ None of the time ☐

11. How TRUE or FALSE is **each** of the following statements for you?

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
a) I seem to get sick a little easier than other people	☐	☐	☐	☐	☐
b) I am as healthy as anybody I know	☐	☐	☐	☐	☐
c) I expect my health to get worse	☐	☐	☐	☐	☐
d) My health is excellent	☐	☐	☐	☐	☐

Thank you for completing this form

Survey : 7270

Appendix 5: UK JSLE Cohort Study BILAG form

JSLE – BILAG Form

Routine ☐ Flare ☐ Retrospective ☐ Prospective ☐

Study No | Date / /

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Height cm | Weight kg | Systolic BP mmHg | Diastolic BP mmHg

Urinalysis (score 0, trace, 1+, 2+, 3+). Not Done ☐
If abnormal urinalysis send urine for Microscopy, culture, sensitivities (MCS)

Proteinuria ☐ Haematuria ☐ Leucocytes ☐ Nitrites ☐ Menstruating Y / N

NB. The following bloods should be sent as part of BILAG: FBC, ESR, CRP, C3, C4, dsDNA, total Igs, Creatinine, urinary protein/Cr or Alb/Cr ratio. **Please tick to confirm done** ☐ Yes ☐ No

To be used with Glossary: * = Definition in Glossary
Only features attributable to JSLE to be recorded and refer only to last 4 week compared with previous 4 weeks

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions). **A blank box will be assumed to = O or No. Please tick to confirm** ☐ Yes ☐ No

General		
* 1	Pyrexia (documented >37.5°C)	
* 2	Weight Loss – unintentional; >5%	
* 3	Lymphadenopathy/splenomegaly	
4	Fatigue/malaise/lethargy	
5	Anorexia	
Mucocutaneous		
* 6	Skin eruption – severe active (not discoid/bullous/panniculitis)	
* 7	Skin eruption – mild	
* 8	Active discoid lesions: generalised/extensive	
* 9	Active discoid lesions: localised include lupus profundus	
* 10	Alopecia (severe, active)	
* 11	Alopecia (mild)	
* 12	Panniculitis/bullous lupus (severe)	
*13	Panniculitis/bullous lupus (mild)	
* 14a	Angio-oedema (severe)	
* 14b	Angio-oedema (mild)	
* 15	Mucosal ulceration (severe)	
*16	Mucosal ulcers (mild)	
* 17	Malar erythema	

18	Subcutaneous nodules					
19	Pernionic skin lesions					
*20	Peri-ungual erythema / chilblains					
21	Swollen fingers	Y				N
22	Sclerodactyly	Y				N
23	Calcinosis	Y				N
24	Telangiectasia	Y				N
25	Splinter haemorrhages	Y				N
Neurological						
26	Impaired level of consciousness					
* 27	Cognitive dysfunction					
* 28	Acute psychosis or delirium or confusional state					
* 29	Psychosis					
* 30	Seizure disorder					
* 31	Status epilepticus					
* 32	Cerebral vascular disease (not due to vasculitis)					
* 33	Cerebral vasculitis					
* 34	Aseptic meningitis					
* 35	Mononeuropathy (single/multiplex)					
36	Ascending or transverse myelitis					

JSLE – BILAG Form

Study No | | | | | | |

Date | | | / | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

* 37	Demyelinating syndrome	
* 38	Myelopathy	
Neurological continued		
* 39	Acute inflammatory demyelinating polyradiculoneuropathy	
40	Peripheral neuropathy	
* 41	Cranial neuropathy	
* 42	Plexopathy	
* 43	Polyneuropathy	
* 44	Autonomic disorder	
* 45	Disc swelling	
* 46	Chorea	
* 47	Cerebellar ataxia (isolated)	
* 48	Movement disorder	
* 49	Lupus headache (severe, unremitting)	
* 50	Episodic migrainous headaches	
* 51	Tension headache	
* 52	Cluster headache	
* 53	Headache from IC hypertension	
* 54	Organic depressive illness	
* 55	Mood disorder (depression/mania)	
* 56	Anxiety disorder	
* 57	Organic brain syndrome	
Musculoskeletal		
* 58	Definite myositis (severe)	
* 59	Myositis with incomplete criteria	
* 60	Myositis (mild)	
* 61	Myalgia	
* 62	Severe polyarthritis – with loss of function	
* 63	Moderate arthritis	
* 64	Arthralgia	
* 65	Tendonitis/tenosynovitis	

66	Tendon contractures and fixed deformity	Y			N
67	Aseptic necrosis	Y			N
Cardiovascular & Respiratory					
* 68	Pleuropericardial pain				
* 69	Dyspnoea				
* 70	Cardiac failure				
* 71	Friction rub				
* 72	Effusion (pericardial or pleural)				
* 73	Mild or intermittent chest pain				
74	Progressive CXR changes – lung fields	Y			N
75	Progressive CXR changes – heart size	Y			N
76	ECG evidence of pericarditis/ myocarditis/endocarditis	Y			N
* 77	Cardiac arrhythmia including tachycardia (>100 no fever)	Y			N
* 78	Pulmonary function fall by >20%	Y			N
79	Cytohistological evidence of inflammatory lung disease	Y			N
* 80	Myocarditis - mild				
* 81	New valvular dysfunction				
* 82	Cardiac tamponade				
* 83	Pleural effusion with dyspnoea				
* 84	Pulmonary haemorrhage/vasculitis				
* 85	Interstitial alveolitis/pneumonitis				
* 86	Shrinking lung syndrome				
* 87	Aortitis				
* 88	Coronary vasculitis				
Vasculitis					
* 89	Major cutaneous vasculitis including ulcers				
* 90	Major abdominal crisis due to vasculitis				
91	Recurrent thromboembolism (excluding strokes)				
92	Raynaud's				

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

93	Livido reticularis					
Vasculitis continued						
94	Superficial phlebitis					
* 95	Minor cutaneous vasculitis (nailfold, digital, purpura, urticaria)					
96	Thromboembolism (excl stroke) 1st episode	Y				N
Renal						
* 99	Severe hypertension	Y				N
103	Newly documented proteinuria (level 4)	Y				N
* 104	Nephrotic syndrome	Y				N
* 108	Histological evidence of active nephritis within 3 months	Y				N
Gastrointestinal						
* 109	Lupus peritonitis					
* 110	Abdominal serositis or ascites					
* 111	Lupus enteritis/colitis					
* 112	Malabsorption					
* 113	Protein losing enteropathy					
* 114	Intestinal pseudo-obstruction					

* 115	Lupus hepatitis	
* 116	Acute lupus cholecystitis	
* 117	Acute lupus pancreatitis	
Ophthalmic		
* 118	Orbital inflammation with myositis and/or proptosis	
* 119	Keratitis – severe	
* 120	Keratitis – mild	
121	Anterior uveitis	
* 122	Posterior uveitis/retinal vasculitis – severe	
* 123	Posterior uveitis/retinal vasculitis – mild	
124	Episcleritis	
* 125	Scleritis – severe	
* 126	Scleritis – mild	
* 127	Retinal / choroidal vaso-occlusive disease	
* 128	Isolated cotton-wool spots (cytoid bodies)	
* 129	Optic neuritis	
* 130	Anterior ischaemic optic neuropathy	

Function

Patient CHAQ ☐ • ☐☐

Global assessment on CHAQ (0: Very well – 100: Very ill ☐☐☐

Physicians clinical global score — Very well 0 ——— 100 Very ill

Important assessment of current clinical status

In relation to the patients LUPUS ACTIVITY at this time point, please tick the box that best describes your opinion as to their current flare status:

1. Clinical improvement – no clinical flare ☐
2. No change in Lupus activity ☐
3. Minor flare (may not require specific therapy) ☐
4. Moderate flare ☐
5. Major flare ☐

A flare of JSLE has been defined as "a measurable worsening of SLE disease activity in at least one organ system, involving new or worse signs of disease that may be accompanied by new or worse JSLE symptoms; depending on the severity of the flare, more intensive therapy may be required" [1]

1. Brunner HI, Klein-Gitelman MS, Higgins GC, et al. Arthritis Care Res. 2010;62(6):811-20.

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Treatment	Current dose	Revised dose
Hydroxychloroquine (mg/day)		
Azathioprine (mg/day)		
Mycophenolate (mg/day)		
Cyclosporin (mg/day)		
Prednisolone (mg/day)		
Methotrexate (mg/week)		
Oral		
Subcut		

	Current dose	Total number of pulses since last visit
IVIG (g/pulse)		

Rituximab:		Cyclophosphamide:	IV	Oral
Total dose per cycle (mg)		Total dose since last visit (mg)		
Number of infusions per cycle		Number of infusions since last visit		
Number of cycles since last visit		Cumulative dose		

IV methyl-prednisolone in last 3 months	<5 pulses		>=5 pulses	

Other Drugs

Aspirin		Bisphosphonate		Oral contraceptive pill	
Angiotensin receptor blocker		Ca** blockers		Statins	
Other biological DMARDS		ACEi		Diuretic		Anticoagulant	

Clinicians Intention re Medication

Please tick which of the following best describes your intention to change treatment, and explain why:

1) Decrease in treatment
- Disease improvement
- Side effects of treatment
- Compliance problems
- Weaning regimen

2) Increase in treatment
- Disease worsening
- Standard dose increment
- Dose increment due to weight

3) Change in DMARD
- Concerns over efficacy
- Planned maintenance treatment
- Side effects of treatment
- Compliance problems

4) No change in treatment
- Active disease (induction phase)
- Stable, not yet decrease
- Patients choice
- Not on Medication

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Results Page

Microscopy & culture results

MCS results	1. Proven UTI (>1x10^5 growth (cfu) of a single organism)	Y / N	2. Mixed growth / contamination	Y / N
	3. Microscopy results: a) White cell count	(per hpf)	b) Red cell count	(per hpf)
	c) Red cell casts	Y / N	d) White cell casts	Y / N

Renal function

* 102a	Urinary Alb/Cr ratio (mg/mmol Cr)		105	Creatinine (plasma/serum)	
* 102b	Urinary Protein/Cr Ratio (mg/mmol Cr)		106a	GFR-EDTA clearance (exact) (mls/min. 1.73m^2)	
102c	24hr/Urinary Protein(g)		106b	GFR: bt/creat ratio (estimate)	
	Not done		107	Active urinary sediment	Y N

Haematology

* 131	Haemoglobin g/dl		* 135	Platelets x 10^9/l	
* 132	Total white cell count x 10^9/l		* 136	Evidence of active haemolysis	Y N
* 133	Neutrophils x 10^9/l		137	Coomb's test positive	Y N
134	Lymphocytes x 10^9/l		* 138	TTP	Y N

Other measures of disease activity

ESR (mm/hr)		dsDNA	
CRP		IgG	
C3 (g/l)		IgA	
C4 (g/l)		IgM	
Viscosity (mPa.s) If no ESR		Ferritin (µg/L)	

Appendix 6: BILAG scoring guidance

BILAG2004 INDEX SCORING

- scoring based on the principle of physician's intention to treat

Category	Definition
A	**Severe disease activity requiring any of the following treatment:** **1. systemic high dose oral glucocorticoids (equivalent to prednisolone > 20 mg/day)** **2. intravenous pulse glucocorticoids (equivalent to pulse methylprednisolone ≥ 500 mg)** **3. systemic immunomodulators (include biologicals, immunoglobulins and plasmapheresis)** **4. therapeutic high dose anticoagulation in the presence of high dose steroids or immunomodulators** **eg: warfarin with target INR 3 - 4**
B	**Moderate disease activity requiring any of the following treatment:** **1. systemic low dose oral glucocorticoids (equivalent to prednisolone ≤ 20 mg/day)** **2. intramuscular or intra-articular or soft tissue glucocorticoids injection (equivalent to methylprednisolone < 500mg)** **3. topical glucocorticoids** **4. topical immunomodulators** **5. antimalarials or thalidomide or prasterone or acitretin** **6. symptomatic therapy** **eg: NSAIDs for inflammatory arthritis**
C	**Mild disease**
D	**Inactive disease but previously affected**
E	**System never involved**

CONSTITUTIONAL

Category A
Pyrexia recorded as 2 (same), 3 (worse) or 4 (new) **AND**

Any 2 or more of the following recorded as 2 (same), 3 (worse) or 4 (new):

Weight loss
Lymphadenopathy/splenomegaly
Anorexia

Category B
Pyrexia recorded as 2 (same), 3 (worse) or 4 (new) **OR**

Any 2 or more of the following recorded as 2 (same), 3 (worse) or 4 (new):

Weight loss
Lymphadenopathy/splenomegaly
Anorexia

BUT do not fulfil criteria for Category A

Category C
Pyrexia recorded as 1 (improving) **OR**

One or more of the following recorded as > 0:

Weight loss
Lymphadenopathy/Splenomegaly
Anorexia

BUT does not fulfil criteria for category A or B

Category D
Previous involvement

Category E
No previous involvement

MUCOCUTANEOUS

Category A
Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Skin eruption - severe
Angio-oedema - severe
Mucosal ulceration - severe
Panniculitis/Bullous lupus - severe
Major cutaneous vasculitis/thrombosis

Category B
Any Category A features recorded as 1 (improving) **OR**

Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Skin eruption - mild
Panniculitis/Bullous lupus - mild
Digital infarcts or nodular vasculitis
Alopecia - severe

Category C
Any Category B features recorded as 1 (improving) **OR**

Any of the following recorded as > 0:

Angio-oedema - mild
Mucosal ulceration - mild
Alopecia - mild
Periungual erythema/chilblains
Splinter haemorrhages

Category D
Previous involvement

Category E
No previous involvement

NEUROPSYCHIATRIC

Category A
Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Aseptic meningitis
Cerebral vasculitis
Demyelinating syndrome
Myelopathy
Acute confusional state
Psychosis
Acute inflammatory demyelinating polyradiculoneuropathy
Mononeuropathy (single/multiplex)
Cranial neuropathy
Plexopathy
Polyneuropathy
Status epilepticus
Cerebellar ataxia

Category B
Any Category A features recorded as 1 (improving) OR

Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Seizure disorder
Cerebrovascular disease (not due to vasculitis)
Cognitive dysfunction
Movement disorder
Autonomic disorder
Lupus headache - severe unremitting
Headache due to raised intracranial hypertension

Category C
Any Category B features recorded as 1 (improving)

Category D
Previous involvement

Category E
No previous involvement

MUSCULOSKELETAL

Category A
Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Severe Myositis
Severe Arthritis

Category B
Any Category A features recorded as 1 (improving) OR

Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Mild Myositis
Moderate Arthritis/Tendonitis/Tenosynovitis

Category C
Any Category B features recorded as 1 (improving) OR

Any of the following recorded as > 0:

Mild Arthritis/Arthralgia/Myalgia

Category D
Previous involvement

Category E
No previous involvement

CARDIORESPIRATORY

Category A

Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Myocarditis/Endocarditis + Cardiac failure
Arrhythmia
New valvular dysfunction
Cardiac tamponade
Pleural effusion with dyspnoea

Pulmonary haemorrhage/vasculitis
Interstitial alveolitis/pneumonitis
Shrinking lung syndrome
Aortitis
Coronary vasculitis

Category B
Any Category A features recorded as 1 (improving) **OR**

Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Pleurisy/Pericarditis
Myocarditis - mild

Category C
Any Category B features recorded as 1 (improving)

Category D
Previous involvement

Category E
No previous involvement

GASTROINTESTINAL
Category A
Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Peritonitis
Lupus enteritis/colitis
Intestinal pseudo-obstruction
Acute lupus cholecystitis
Acute lupus pancreatitis

Category B
Any Category A feature recorded as 1 (improving) OR

Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Abdominal serositis and/or ascites
Malabsorption
Protein losing enteropathy
Lupus hepatitis

Category C
Any Category B features recorded as 1 (improving)

Category D
Previous involvement

Category E
No previous involvement

OPHTHALMIC

Category A
Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Orbital inflammation/myositis/proptosis
Keratitis - severe
Posterior uveitis/retinal vasculitis - severe
Scleritis - severe
Retinal/choroidal vaso-occlusive disease
Optic neuritis
Anterior ischaemic optic neuropathy

Category B
Any Category A features recorded as 1 (improving) OR

Any of the following recorded as 2 (same), 3 (worse) or 4 (new):

Keratitis - mild
Anterior uveitis
Posterior uveitis/retinal vasculitis - mild
Scleritis - mild

Category C
Any Category B features recorded as 1 (improving) OR

Any of the following recorded as > 0:

Episcleritis
Isolated cotton-wool spots (cytoid bodies)

Category D
Previous involvement

Category E
No previous involvement

RENAL

Category A

Two or more of the following providing 1, 4 or 5 is included:

1. Deteriorating proteinuria (severe) defined as

(a) urine dipstick increased by ≥ 2 levels (used only if other methods of urine protein estimation not available); or

(b) 24 hour urine protein > 1 g that has not decreased (improved) by 25%; or
(c) urine protein-creatinine ratio > 100 mg/mmol that has not decreased (improved) by 25%; or
(d) urine albumin-creatinine ratio > 100 mg/mmol that has not decreased (improved) by 25%

2. Accelerated hypertension
3. Deteriorating renal function (severe) defined as

(a) plasma creatinine > 130 mol/l and having risen to > 130% of previous value; or
(b) GFR < 80 ml/min per 1.73 m2 and having fallen to < 67% of previous value; or
(c) GFR < 50 ml/min per 1.73 m2, and last time was > 50 ml/min per 1.73 m2 or was not measured.

4. Active urinary sediment
5. Histological evidence of active nephritis within last 3 months
6. Nephrotic syndrome

Category B
One of the following:

1. One of the Category A feature

2. Proteinuria (that has not fulfilled Category A criteria)
(a) urine dipstick which has risen by 1 level to at least 2+ (used only if other methods of urine protein estimation not available); or

(b) 24 hour urine protein ≥ 0.5 g that has not decreased (improved) by 25%; or
(c) urine protein-creatinine ratio ≥ 50 mg/mmol that has not decreased (improved) by 25%; or
(d) urine albumin-creatinine ratio ≥ 50 mg/mmol that has not decreased (improved) by 25%

3. Plasma creatinine > 130 mol/l and having risen to ≥ 115% but ≤ 130% of previous value

Category C
One of the following:

1. Mild/Stable proteinuria defined as

(a) urine dipstick ≥ 1+ but has not fulfilled criteria for Category A & B (used only if other methods
of urine protein estimation not available); or
(b) 24 hour urine protein > 0.25 g but has not fulfilled criteria for Category A & B ; or
(c) urine protein-creatinine ratio > 25 mg/mmol but has not fulfilled criteria for Category A & B; or
(d) urine albumin-creatinine ratio > 25 mg/mmol but has not fulfilled criteria for Category A & B

2. Rising blood pressure (providing the recorded values are > 140/90 mm Hg) which has not fulfilled criteria for Category A & B, defined as

(a) systolic rise of ≥ 30 mm Hg; and
(b) diastolic rise of ≥ 15mm Hg

Category D
Previous involvement

Category E
No previous involvement

Note: although albumin-creatinine ratio and protein-creatinine ratio are different, we use the same cut-off values for this index

HAEMATOLOGICAL

Category A
TTP recorded as 2 (same), 3 (worse) or 4 (new) OR

Any of the following:

Haemoglobin	< 8 g/dl
White cell count	< 1.0 x 109/l
Neutrophil count	< 0.5 x 109/l
Platelet count	< 25 x 109/l

Category B
TTP recorded as 1 (improving) OR

Any of the following:

Haemoglobin	8 - 8.9 g/dl
White cell count	1 - 1.9 x 109/l
Neutrophil count	0.5 - 0.9 x 109/l
Platelet count	25 - 49 x 109/l

Evidence of active haemolysis

Category C
Any of the following:

Haemoglobin	9 - 10.9 g/dl
White cell count	2 - 3.9 x 109/l
Neutrophil count	1 - 1.9 x 109/l
Lymphocyte count	< 1.0 x 109/L
Platelet count	50 - 149 x 109/l

Isolated Coombs' test positive

Category D
Previous involvement

Category E
No previous involvement

Appendix 7: SLEDAI score

Weight	Tick	Descriptor	Definition
8	☐	Seizure	Recent onset, exclude metabolic, infectious or drug causes
8	☐	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behaviour. Exclude uraemia and drug causes
8		Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function, with rapid onset and fluctuating clinical features, inability to sustain attention to environment, plus at least 2 of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, increased or decreased psychomotor activity. Exclude metabolic, infectious, or drug causes
8	☐	Visual disturbance	Retinal changes of SLE. Include cytoid bodies, retinal haemorrhages, serous exudates or haemorrhages in the choroid, or optic neuritis. Exclude hypertension, infection, or drug causes
8	☐	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves
8	☐	Lupus headache	Severe, persistent headache, may be migrainous, but must be non-responsive to narcotic analgesia
8	☐	CVA	New onset of cerebrovascular accident(s). Exclude arteriosderosis
8	☐	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter haemorrhages, or biopsy or angiogram proof of vasculitis
4	☐	Arthritis	≥2 joints with pain and signs of inflammation (i.e. tenderness, swelling or effusion)
4		Myositis	Proximal muscle aching weakness, associated with elevated creatinine phosphokinase/aldolase or electromyogram changes or a biopsy showing myositis
4	☐	Urinary casts	Haem-granular or red blood cell casts
4	☐	Hematuria	>5 red blood cells/high power field, Exclude stone, infection or other cause
4	☐	Proteinuria	>0.5g/24h
4	☐	Pyuria	>5 white blood cells/high power field. Exclude infection
2	☐	Rash	Inflammatory type rash
2	☐	Alopecia	Abnormal, patchy or diffuse loss of hair
2	☐	Mucosal ulcers	Oral or nasal ulcerations
2	☐	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening
2	☐	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation
2	☐	Low complement	decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory
2	☐	Increased DNA binding	increased DNA binding by Farr assay above normal range for testing laboratory
1	☐	Fever	>38°C. Exclude infectious cause
1	☐	Thrombocytopenia	<100,000 platelets/× 10^9/L, exclude drug causes
1	☐	Leucopenia	<3000 white blood cells/× 10^9/L, exclude drug causes
	☐	TOTAL SCORE (Sum of weights next to descriptors marked present)	

Appendix 8: BILAG Glossary

Extended Paediatric BILAG 2004 Definitions

Guidance on filling out BILAG forms

- It is implicit in this scoring system that all features scored must ONLY be **attributable to SLE and not due to damage, infection, thrombosis (in absence of inflammatory process) or other conditions**
- Assessment refers to manifestations occurring in the **last 4 weeks compared with the previous 4 weeks**
- In some manifestations, it may be difficult to differentiate SLE from other causes as there may not be any specific test and the decision would then lies with the **physician's judgement on the balance of probabilities**

Guidance for scoring

The questionnaire asks whether features are improving, the same, worse or new.

- **NEW (4):** manifestations are recorded as new when it is a new episode occurring in the last 4 weeks (compared to the previous 4 weeks) that has not improved and this includes new episodes (recurrence) of old manifestations
  - Note - a new episode occurring in the last 4 weeks but also satisfying the criteria for improvement (below) would be classified as improving (scoring a 1) instead of new (4)

- **WORSE (3):** this refers to manifestations that have deteriorated in the last 4 weeks compared to the previous 4 weeks

- **SAME (2):** This refers to manifestations that have been present for the last 4 weeks and the previous 4 weeks without significant improvement or deterioration
- this also applies to manifestations that have improved over the last 4 weeks compared to the previous 4 weeks but do not meet the criteria for improvement

- **IMPROVING (1):** Definition of improvement:
  - (a) the amount of improvement is sufficient for consideration **of reduction in therapy** and would not justify escalation in therapy, **AND:**
  - (b) improvement must be **present currently and for at least 2 weeks** out of the last 4 weeks, **OR:** manifestation that has **completely resolved and remained absent** over the **whole of last 1 week**

- **NOT PRESENT (0)**

- **NOT DONE:** it is important to indicate if a test has not been performed (particularly laboratory investigations) so that this will be recorded as such in the database & not as normal or absent (which is the default)

- Most are self-explanatory but definitions are available for most descriptors (see below)
- Ophthalmic manifestations need to be assessed by ophthalmologist
- For descriptors that are based on measurements (in renal and haematology systems), it is important to indicate if these are not due to lupus (for consideration of scoring) as they are usually recorded routinely into a database (eg drug side effects).

BILAG score calculation:

- This intends to reflect and be based upon the physician's intention to treat.
- Patients score as A-E for each of the 9 BILAG domains (general, mucocutaneous, neurological, musculoskeltal, cardiovascular/respiratory, vasculitis, renal, gastrointestinal, ophthalmic).

Category	Definition
A	**Severe disease activity requiring any of the following treatment:** 1. systemic high dose oral glucocorticoids (equivalent to prednisolone > 20 mg/day) 2. intravenous pulse glucocorticoids (equivalent to pulse methylprednisolone ≥ 500 mg) 3. systemic immunomodulators (include biologicals, immunoglobulins and plasmapheresis) 4. therapeutic high dose anticoagulation in the presence of high dose steroids or immunomodulators e.g: warfarin with target INR 3 - 4
B	**Moderate disease activity requiring any of the following treatment:** 1. systemic low dose oral glucocorticoids (equivalent to prednisolone ≤ 20 mg/day) 2. intramuscular or intra-articular or soft tissue glucocorticoids injection (equivalent to methylprednisolone < 500mg) 3. topical glucocorticoids 4. topical immunomodulators 5. antimalarials or thalidomide or prasterone or acitretin 6. symptomatic therapy e.g: NSAIDs for inflammatory arthritis
C	**Mild disease**
D	**Inactive disease but previously affected**
E	**System never involved**

BILAG - Glossary of terms

CONSTITUTIONAL		
No	**Parameter**	**Definition (as required)**
1.	Pyrexia	Temperature > 37.5°C documented
2.	Weight loss	Unintentional weight loss > 5%
3.	Lymphadenopathy	palpable lymph node more than 1 cm diameter; having excluded infection
4.	Fatigue or malaise or lethargy	
5.	Anorexia	
MUCOCUTANEOUS		
6.	Skin eruption - Severe active	• >18% (2/9) body surface area • Any lupus rash except panniculitis, bullous lesion & angio-oedema • Body surface area (BSA) is defined using the rules of nines (used to assess extent of burns) as follows: Palm (excluding fingers) = 1% BSA each lower limb = 18% BSA, each upper limb = 9% BSA, torso (front) = 18% BSA, torso (back) = 18% BSA, head = 9% BSA, genital (male) = 1% BSA
7.	Skin eruption - Mild	• ≤ 18% body surface area • Any lupus rash except panniculitis, bullous lesion & angio-oedema
8.	Active discoid lesions	Generalised / extensive
9.	Active discoid lesions: localized include lupus profundus	Lupus profundus: Erythematous elevated plaques with an overlying discoid skin lesion
10.	Alopecia (severe, active)	Clinically detectable (diffuse or patchy) hair loss with scalp inflammation (redness over scalp)
11.	Alopecia (mild)	Diffuse or patchy hair loss without scalp inflammation (clinically detectable or by history)
12.	Panniculitis / bullous (severe)	Severe if any one of: • > 9% body surface area; • facial panniculitis; • pannicultis that threatens to ulcerate; • pannicultitis that threatens integrity of subcutaneous tissue (beginning to cause surface depression) on >9% body surface area. Panniculitis presents as palpable and tender subcutaneous induration / nodule. Established surface depression and atrophy associated with fat necrosis alone is likely to be due to damage
13.	Panniculitis (mild)	As for severe but ≤ 9% body surface area and does not fulfill any criteria for severe panniculitis

14.	a. Angio-oedema (severe) b. Angio-oedema (mild)	• Potentially life-threatening eg: stridor, affecting tongue or lips. • Angio-oedema is a variant form of urticaria which affects the subcutaneous, submucosal and deep dermal tissues • Not life threatening
15.	Mucosal ulceration (severe)	• Disabling (significantly interfering with oral intake) • Extensive & deep ulceration (must have been observed by a physician)
16.	Mucosal ulceration (mild)	Localised and / or non-disabling ulceration
17.	Malar erythema	Malar rash must have been observed by a physician and has to be present continuously (persistent) for at least 1 week to be considered significant (to be recorded)
18.	Subcutaneous nodules	
19.	Perniotic skin lesions	
20.	Peri-ungal erythema / chillblains	Chilblains are localised inflammatory lesions (may ulcerate) which are precipitated by exposure to cold
21.	Swollen fingers	
22.	Sclerodactyly	Localized thickening and tightness of the skin of the fingers or toes.
23.	Calcinosis	Calcium salt depositis in the skin or subcutaneous tissues
24.	Telangiectasia	Dilatation of the capillaries causing them to appear as small red or purple clusters, often spidery in appearance on the skin.
25.	Splinter haemorrhages	Small areas of bleeding (**hemorrhage**) under the fingernails or toenails
NEUROPSYCHIATRIC		
26.	Impaired level of consciousness	
27.	Cognitive dysfunction	Significant deficits in any cognitive functions: • simple attention (ability to register and maintain information) • complex attention • memory (ability to register, recall, and recognize information e.g. learning, recall) • visual-spatial processing (ability to analyze, synthesize and manipulate visual-spatial information) • language (ability to comprehend, repeat and produce oral / written material e.g. verbal fluency, naming) • reasoning/problem solving (ability to reason and sbstract) • psychomotor speed • executive functions (e.g. planning, organizing, sequencing) in absence of disturbance of consciousness or level of arousal o sufficiently severe to interfere with daily living neuropsychological testing should be done if possible or corroborating history from third party if possible exclude substance abuse

28.	Acute psychosis or delirium or confusional state	Severe disturbance in the perception of reality characterised by: delusions, hallucinations, incoherence, marked illogical thinking or catatonic behaviour. Acute confusional state: acute disturbance of consciousness or level of arousal with reduced ability to focus, maintain or shift attention includes hypo- and hyperaroused states and encompasses the spectrum from delirium to coma
29.	Psychosis	• Delusion or hallucinations does not occur exclusively during course of a delirium • exclude drugs, substance abuse, primary psychotic disorder
30.	Seizure disorder	Independent description of seizure by reliable witness
31.	Status epilepticus	Seizure or series of seizures lasting ≥ 30 minutes without full recovery to baseline
32.	Cerebrovascular disease (not due to vasculitis)	Anyone with supporting imaging: (not due to vasculitis) stroke syndrome; transient ischaemic attack; intracranial haemorrhage; • exclude hypoglycaemia, cerebral sinus thrombosis, vascular malformation, tumour, abscess • Cerebral sinus thrombosis not included as definite thrombosis not considered part of lupus activity
33.	Cerebral vasculitis	Should be present with features of vasculitis in another system and supportive imaging &/or biopsy findings
34.	Aseptic meningitis	• Criteria (all): acute/subacute onset headache, fever, abnormal CSF (raised protein and/or lymphocyte predominance) but negative cultures • Exclude CNS/meningeal infection, intracranial haemorrhage • Preferably photophobia, neck stiffness, signs of meningeal irritation should be present but not essential
35.	Mononeuropathy (single/multiplex)	Supportive electrophysiology study preferred
36.	Ascending or transverse myelitis	
37.	Demyelinating syndrome	• Discrete white matter lesion with associated neurological deficit not recorded elsewhere • Ideally there should have been at least one previously recorded event • Supportive imaging required • Exclude multiple sclerosis
38.	Myelopathy	• Acute onset of rapidly evolving paraparesis or quadriparesis and/or sensory level • Exclude intramedullary and extramedullary space occupying lesion
39.	Acute inflammatory demyelinating polyradiculoneuropathy	Criteria: • progressive polyradiculoneuropathy • loss of reflexes • symmetrical involvement • increased CSF protein without pleocytosis • supportive electrophysiology study
40.	Peripheral or cranial neuropathy	
41.	Cranial neuropathy	Except optic neuropathy which is classified under ophthalmic

42.	Plexopathy	• Disorder of brachial or lumbosacral plexus resulting in neurological deficit not corresponding to territory of single root or nerve • Supportive electrophysiology study required
43.	Polyneuropathy	• Acute symmetrical distal sensory and/or motor deficit • Supportive electrophysiology study required
44.	Autonomic disorder	Any one: • fall in blood pressure to standing > 30/15 mmHg (systolic/diastolic) • increase in heart rate to standing ≥ 30bpm • loss of heart rate variation with respiration (max – min < 15 bpm, expiration:inspiration ratio < 1.2, Valsalva ratio < 1.4) • loss of sweating over body and limbs (anhidrosis) by sweat test exclude drugs and diabetes mellitus
45.	Disc swelling / cytoid swellings	Isolated cotton-wool spots. Also known as cytoid bodies (see ophthalmology – scored here for original Paed BILAG)
46.	Chorea	Jerky involuntary movements affecting especially the shoulders, hips, and face.
47.	Cerebellar ataxia	Cerebellar ataxia in isolation of other CNS features usually subacute presentation
48.	Movement disorder	Exclude drugs
49.	Severe headache (unremitting)	Disabling headache unresponsive to narcotic analgesia & lasting ≥ 3days exclude intracranical space occupying lesion and CNS infection
50.	Episodic migrainous headaches	With/without aura recurrent attacks of headache lasting 4 – 72hours may be preceded by neurological aura (lasting up to 1 hour)
51.	Tension headache	Recurrent episodes of headaches lasting minutes to days
52.	Cluster headache	Attacks of severe unilateral headache lasting 15 - 180 minutes attacks at least once every other day and up to 8 times a day attacks occur in clusters (series of weeks or months) separated by remissions of usually months or years
53.	Headache from IC hypertension	Exclude cerebral sinus thrombosis
54.	Organic depressive illness	Associated with somatic symptoms and severe enough to merit treatment with anti-depressive medication
55.	Mood disorder (depression/mania)	Prominent & persistent disturbance in mood characterised by depressed mood or markedly diminished interest or pleasure in almost all activities or elevated, expansive or irritable mood should result in significant distress or impaired functioning
56.	Anxiety disorder	Prominent anxiety, panic disorder, panic attacks or obsessions or compulsions resulting in clinically significant distress or impaired functioning

57.	Organic Brain disorder	Organic brain syndrome: impaired orientation, memory or other intellectual function in the absence of metabolic, psychiatric or pharmacological causes. Clinical features develop over a short period (usually hours to days) and tend to fluctuate over the course of the day. a) clouding of consciousness with reduced capacity to focus and sustain attention to environment b) i perceptual disturbance: misinterpretations, illusions or hallucinations ii incoherent speech iii insomnia or daytime drowsiness iv increased or decreased psychomotor activity c) disorientation and recent memory impairment
MUSCULOSKELETAL		
58.	Definite myositis (severe)	≥ 3 criteria: proximal muscle weakness; elevated muscle enzymes; positive muscle biopsy; abnormal EMG; positive MRI Exclude endocrine and drug-induced myopathy
59.	Incomplete myositis	2 above criteria
60.	Myositis (mild)	Raised muscle enzymes with myalgia but without significant weakness Asymptomatic raised muscle enzymes not included Exclude endocrine and drug-induced myopathy
61.	Myalgia	Inflammatory muscle pain which does not fulfill the criteria for arthritis or myositis
62.	Severe polyarthritis – with loss of function	Observed active synovitis ≥ 2 joints with marked loss of functional range of movement and significant impairment of activities of daily living and has been present on several days (cumulatively) over the last 4 weeks
63.	Arthritis (Moderate)	Active synovitis ≥ 1 joint (observed or through history) with some impairment of function, which has been present on several days over the last 4 weeks
64.	Arthralgia	Inflammatory joint pain (worse in morning with stiffness, usually improves with activity and not brought on by activity) which does not fulfill the above criteria for arthritis
65.	Tendonitis / tenosynovitis	Tendonitis / tenosynovitis with some impairment of function, which has been present on several days over the last 4 weeks
66.	Tendon contractures & fixed deformity	Fixed deformity caused by permanent shortening of muscles, tendons, and/or ligaments
67.	Aseptic necrosis	Generally avascular necrosis of bone associated with lupus.
CARDIOVASCULAR & RESPIRATORY		

68.	Pleuropericardial pain	Localized sharp or dull pain in chest aggravated by respiration • convincing history &/or physical findings that you would consider treating • in absence of cardiac tamponade or pleural effusion with dyspnoea do not score if you are unsure whether or not it is pleurisy/pericarditis
69.	Dyspnoea	On exertion
70.	Cardiac failure	Cardiac failure due to myocarditis or non-infective inflammation of endocardium or cardiac valves (endocarditis) • cardiac failure due to myocarditis is defined by left ventricular ejection fraction ≤ 40% & pulmonary oedema or peripheral oedema • cardiac failure due to acute valvular regurgitation (from endocarditis) can be associated with normal left ventricular ejection fraction diastolic heart failure is not included
71.	Friction rub	Audible on auscultation
72.	Effusion (pericardial or pleural)	On Echo / CXR
73.	Mild / intermittent chest pain	Non-specific (not clearly pleuritic, pericardial, musculoskeletal or angina)
74.	Progressive CXR changes – lung fields	
75.	Progressive CXR changes – heart size	
76.	ECG evidence of pericarditis / myocarditis	
77.	Cardiac arrhythmia	No fever (except sinus tachycardia) due to myocarditis or non-infective inflammation of endocardium or cardiac valves (endocarditis) Confirmation by ECG required (history palpitations insufficient)
78.	Pulmonary function fall by >20%	>20% less than expected (for height, weight, sex, age), or >20% fall in total lung capacity (FVC) and / or DLCO expected less >20%
79.	Histological evidence of inflammatory lung disease	
80.	Mild myocarditis	Inflammation of myocardium with raised cardiac enzymes &/or ECG changes and without resulting cardiac failure, arrhythmia or valvular dysfunction
81.	New valvular dysfunction	New cardiac valvular dysfunction due to myocarditis or non-infective inflammation of endocardium or cardiac valves (endocarditis)
82.	Cardiac tamponade	Supporting imaging required
83.	Pleural effusion with dyspnoea	Supporting imaging required
84.	Pulmonary haemorrhage/vasculitis	Inflammation of pulmonary vasculature with haemoptysis &/or dyspnoea &/or pulmonary hypertension supporting imaging &/or histological diagnosis

85.	Interstitial alveolitis/pneumonitis	Radiological features of alveolar infiltration not due to infection or haemorrhage required corrected gas transfer Kco (< 70% normal) or fall of >20% if previously normal on going activity would be determined by clinical findings and lung function tests, and repeated imaging may be required in those with deterioration (clinically or lung function tests) or failure to respond to therapy
86.	Shrinking lung syndrome	Acute reduction (> 20% if previous measurement available) in lung volumes (to < 70% predicted) in the presence of normal corrected gas transfer (Kco) & dysfunctional diaphragmatic movements
87.	Aortitis	Inflammation of aorta with or without dissection with supporting imaging abnormalities accompanied by > 10 mm Hg difference in BP between arms &/or claudication of extremities &/or vascular bruits repeated imaging would be required to determine on-going activity in those with clinical deterioration or failure to respond to therapy
88.	Coronary vasculitis	Inflammation of coronary vessels with radiographic evidence of non-atheromatous narrowing, obstruction or aneurismal changes
VASCULITIS		
89.	Major cutaneous vasculitis including ulcers	Accompanied by infarction in past month, extensive gangrene and / or ulceration or skin infarction
90.	Major abdominal crisis due to vasculitis	See also Gastrointestinal (scored here in original Paed BILAG)
91.	Recurrent thromboembolism (excluding strokes)	e.g. Recurrent pulmonary embolism or deep vein thrombosis
92.	Raynaud's	Usually fingers or toes change in colour to white, then blue and then red, as the bloodflow returns. It may associated with pain, numbness, swelling, tingling, and a painful "pins and needles" sensation. Can affect other areas e.g. nose, earlobes.
93.	Livido reticularis	Mottled reticulated vascular pattern that appears as a lace-like purplish discoloration of the skin.
94.	Superficial phlebitis	Inflammation of a superficial vein under the skin.
95.	Minor cutaneous vasculitis	Localised single or multiple infarct(s) over digit(s) or tender erythematous nodule(s) E.g. digital vasculitis, nailfold infarcts, purpura, urticaria
96.	Thromboembolism 1st episode	First pulmonary embolism or deep vein thrombosis

RENAL		
97.	Systolic blood pressure	BP measurement should be confirmed on repeat. Percentile detailed in table 2 below.
98.	Diastolic blood pressure	BP measurement should be confirmed on repeat. In <13 years, diastolic phase 4; >13 years phase 5. Percentiles detailed in table 2 below.
99.	Severe hypertension blood pressure	Severe BP by Task Force criteria (see table 2 of percentiles below) rising to that level within 1 month +/- accompanied by Grade III or IV retinal changes (haemorrhages, exudates, papilloedema)
100.	Proteinuria on dipstick	- = 0; + = 1; ++ = 2; +++ = 3
102 a	Urine albumin-creatinine ratio	On freshly voided urine sample – First early Morning Urine (EMU) preferable
102 b	Urine protein-creatinine ratio	On freshly voided urine sample – First early Morning Urine (EMU) preferable
104	Nephrotic syndrome	Criteria: heavy proteinuria (> 50 mg/kg/day or > 3.5 g/day or protein-creatinine ratio > 350 mg/mmol or albumin-creatinine ratio > 350mg/mmol) hypoalbuminaemia oedema – First early Morning Urine (EMU) preferable
105	Creatinine (plasma/serum)	
106 a	GFR measured	Using Cr EDTA clearance (mLs/min/1.73m^2). Not performed routinely but please provide measurement if undertaken.
106 b	GFR estimated	The Pbilag database will calculate this directly when height and serum creatinine measurements are provided (eGFR = 40 x height (cm) / creatinine (μmol/L)
107	Active urinary sediment	Uncentrifuged specimen, definition is: pyuria (> 5 WCC/hpf), haematuria (> 5 RBC/hpf) or red cell casts in absence of other causes. Therefore mark as having active sediment if any of the above are present.
108	Histology of active nephritis	WHO Class II, III, IV or V within last 3 months or since previous assessments if seen less than 3 months ago. Glomerular sclerosis without inflammation not regarded as evidence of active nephritis
GASTROINTESTINAL		
109	Lupus Peritonitis	Serositis presenting as acute abdomen with rebound/guarding
110	Abdominal serositis or ascites	Not presenting as acute abdomen
111	Lupus enteritis or colitis	Vasculitis or inflammation of small or large bowel with supportive imaging &/or biopsy findings

112	Malabsorption	With abnormal D- xylose absorption test or increased faecal fat excretion after exclusion of coeliac's disease (poor response to gluten-free diet) and gut vasculitis
113	Protein-losing enteropathy	Diarrhoea with hypoalbuminaemia or increased fecal excretion of iv radiolabeled albumin after exclusion of gut vasculitis
114	Intestinal pseudo-obstruction	Subacute intestinal obstruction due to intestinal hypomotility
115	Lupus Hepatitis	Raised transaminases in absence of autoantibodies specific to autoimmune hepatitis (eg: anti-smooth muscle, anti-liver cytosol 1) &/or biopsy appearance of chronic active hepatitis
116	Acute lupus cholecystitis	After exclusion of gallstones and infection
117	Acute lupus pancreatitis	Usually associated multisystem involvement
OPHTHALMIC		
118	Orbital inflammation	orbital inflammation with myositis &/or extra-ocular muscle swelling &/or proptosis supportive imaging required
119	Severe keratitis	Sight threatening includes: corneal melt peripheral ulcerative keratitis
120	Mild keratitis	Not sight threatening
121	Anterior uveitis	
122	Severe posterior uveitis &/or retinal vasculitis	sight-threatening &/or retinal vasculitis not due to vaso-occlusive disease
123	Mild posterior uveitis &/or retinal vasculitis	not sight-threatening not due to vaso-occlusive disease
124	Episcleritis	
125	Severe scleritis	Necrotising anterior scleritis Anterior &/or posterior scleritis requiring systemic steroids/immunosuppression &/or not responding to NSAIDs
126	Mild scleritis	Anterior &/or posterior scleritis not requiring systemic steroids excludes necrotising anterior scleritis
127	Retinal/choroidal vaso-occlusive	Includes: retinal arterial & venous occlusion disease serous retinal &/or retinal ligment epithelial detachments secondary to choroidal vasculopathy
128	Isolated cotton-wool spots	Also known as cytoid bodies
129	Optic neuritis	Excludes anterior ischaemic optic neuropathy

130	Anterior ischaemic optic neuropathy	Visual loss with pale swollen optic disc due to occlusion of posterior ciliary arteries
HAEMATOLOGY		
131	Haemoglobin	Exclude dietary deficiency & GI blood loss. Measurement in g/dl.
132	White cell count	Exclude drug-induced cause of abnormality. Measurement in x 10^9/l
133	Neutrophil count	Exclude drug-induced cause of abnormality. Measurement in x 10^9/l
134	Lymphocyte count	Exclude drug-induced cause of abnormality. Measurement in x 10^9/l
135	Platelet count	Exclude thrombocytopenia of antiphospholipid Syndrome & drug-induced cause
136	Active haemolysis	Positive Coomb's test & evidence of haemolysis (raised bilirubin or raised reticulocyte count or reduced haptoglobulins)
137	Isolated positive Coomb's test	Without features of active haemolysis described under 136 above.
138	TTP	• thrombotic thrombocytopaenic purpura • clinical syndrome of micro-angiopathic haemolytic anaemia and thrombocytopenia in absence of any other identifiable cause
TREATMENT		
• Provide the current does that they have been taking and the revised dose if changed during clinic/admission. • ACEi = angiotensin converting enzyme inhibitor • Ca** blocker = calcium channel blocker • Rituximab and cyclophosphamide are usually given as a number of infusions in a cycle of treatment. o For Rituximab - a cycle may be two infusions, given two weeks apart, although some centres may do otherwise. Document the **number infusions per cycle**, the total dose from **all infusions given within the cycle** (mg) and **the total number of cycles since last visit**. For example, if giving two infusions of 600mg, the total dose per cycle will be 1,200mg. Please ensure height and weight are recorded on the form as usual. o For cyclophosphamide – a number of infusions may be given over a certain period (e.g. 6 months) representing a course of cyclophosphamide treatment. Document the number of individual infusions given since the last visit, the total dose given since the last visit (mg) and the total cumulative dose to date. o		
CLINICIANS INTENTION REGARDING MEDICATIONS		
• These data will assist with validation of the pBILAG score assessing whether it reflects the clinicians intention to treat. Tick what best describes your treatment plan and the rationale for treatment changes.		

TABLE 1: 95TH PERCENTILE OF BLOOD PRESSURE IN BOYS AND GIRLS 3 TO 16 YEARS OF AGE, ACCORDING TO HEIGHT

Blood Pressure	Age	Height Percentage for Boys				Height Percentage for Girls			
		5th	25th	75th	95th	5th	25th	75th	95th
	yr	mmHg				mmHg			
Systolic	3	104	107	111	113	104	105	108	110
	6	109	112	115	117	108	110	112	114
	10	114	117	121	123	116	117	120	122
	13	121	124	128	130	121	123	126	128
	16	129	132	136	138	125	127	130	132
Diastolic	3	63	64	66	67	65	65	67	68
	6	72	73	75	76	71	72	73	75
	10	77	79	80	82	77	77	79	80
	13	79	81	83	84	80	81	82	84
	16	83	84	86	87	83	83	85	86

*** The height percentiles were determined with standard growth curves.**

Data are adapted from those of the Task Force on High Blood Pressure in Children and Adolescents

TABLE 2: CLASSIFICATION OF HYPERTENSION BY AGE GROUP

Age Group	Significant Hypertension (mmHg)	Severe Hypertension (mmHg)
Newborn 7 d 8-30 d	Systolic BP > 96 Systolic BP > 104	Systolic BP > 106 Systolic BP > 110
Infant (< 2yrs)	Systolic BP > 112 Diastolic BP > 74	Systolic BP > 118 Diastolic BP > 82
Children (3-5 yrs)	Systolic BP > 116 Diastolic BP > 76	Systolic BP > 124 Diastolic BP > 84
Children (6-9 yrs)	Systolic BP > 122 Diastolic BP > 78	Systolic BP > 130 Diastolic BP > 86
Children (10-12 yrs)	Systolic BP > 126 Diastolic BP > 82	Systolic BP > 134 Diastolic BP > 90
Adolescents (13-15 yrs)	Systolic BP > 136 Diastolic BP > 86	Systolic BP > 144 Diastolic BP > 92
Adolescents (16-18 yrs)	Systolic BP > 142 Diastolic BP > 92	Systolic BP > 150 Diastolic BP > 98

Appendix 9: UK JSLE Cohort Study Participant Information Sheet example (16-18 years)

UK Children's Lupus Study

Information sheet for patients aged 16 years and older

(JSLE patients – All centres except Liverpool & GOSH)

Why is this study being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells of children with and without Lupus, to see why Lupus cells are reacting against themselves. We are also looking at urine to better understand the kidney problems seen in Lupus.

Why am I being asked to take part?

Because you have Lupus.

Do I have to take part?

It's up to you. You can change your mind at any point without giving a reason. This will not affect your hospital care.

What will happen if I say yes?

- Your doctor will collect information about your age, gender and how you are when you visit the hospital.
- When you are having your usual blood tests we will collect a little extra blood. We will use this to:
  - Measure your autoantibodies and white cells (these usually fight infection)
  - Study the genetics of Lupus
  - Study the kidney involvement
- We will also ask if you can give us a urine sample.
- We will tell your GP you are in the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect information on all people in the UK e.g. if someone dies or develops cancer. If such an event happened, they would let us know about it.
- We will ask for your email address.
- We will be in touch about any future studies in Lupus to see if you are interested in taking part.

Will it do some good if I say yes?

By doing experiments on your samples you will help us to improve understanding of Lupus and create better treatments.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented in scientific meetings and published in medical journals. This may take several years. Individual test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group". It is run from Alder Hey Children's Hospital.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department, Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved.

Has the study been checked?

The Liverpool Research Ethics Committee has approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised.

All your study forms will be kept in your hospitals research office, stored in a locked filing cabinet. Your name and hospital details will be kept on a list in the research office so we know you are in the study. This office is locked when not-attended, for confidentiality.

All information kept on study computers (at the University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

UK JSLE Cohort Study & Repository
Info Sheets Version 1.4 February 2015

Appendix 10: UK JSLE Cohort Study consent form example (16-18 years)

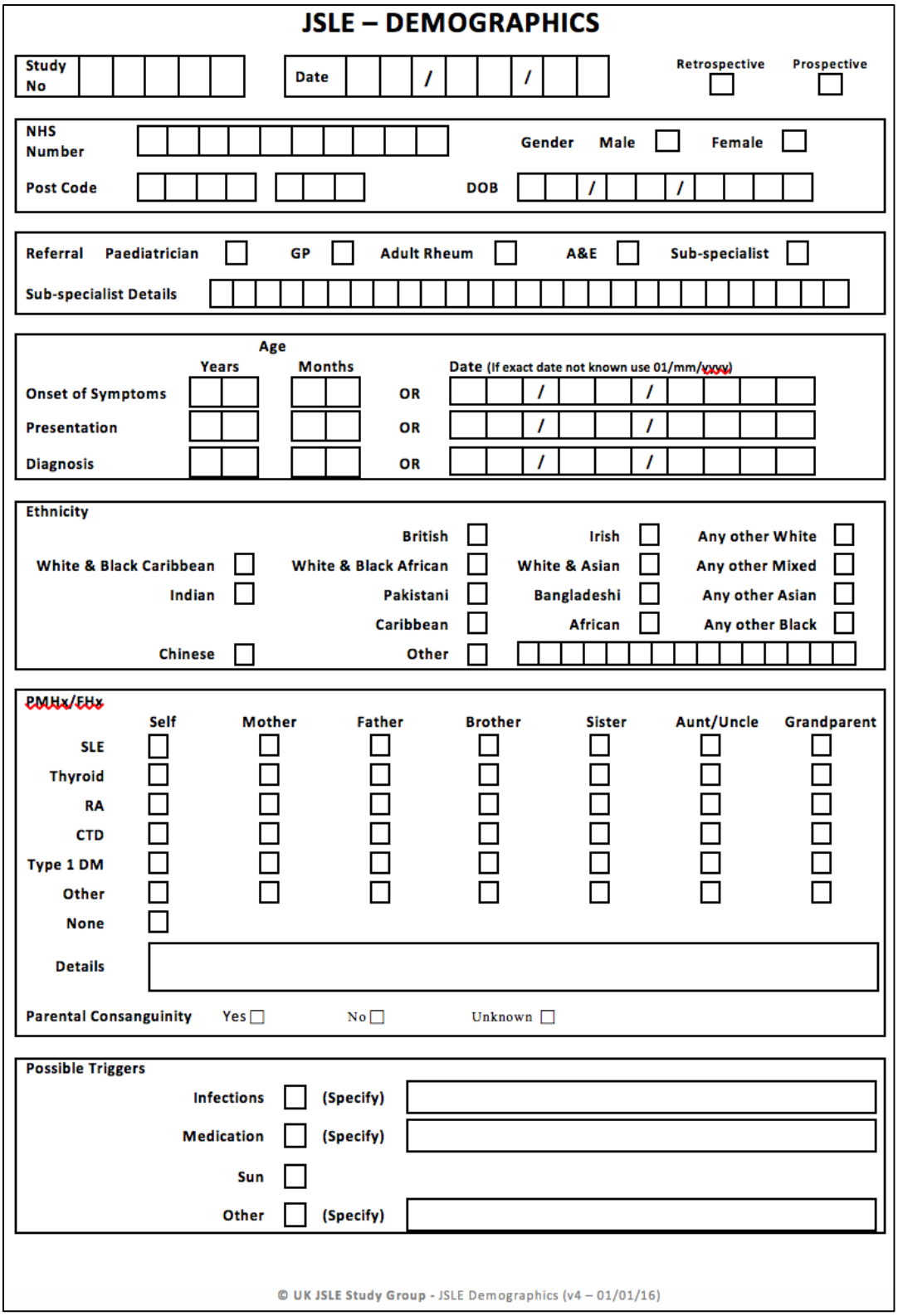
Patient's Consent Form (on local centre headed paper)
(Liverpool and GOSH only)
UK Juvenile SLE Cohort Study and Repository

		Please INITIAL box
1.	I have read and understand the information sheet (Version 1.4 -2nd February 2015) for the above study and have had the chance to ask questions	
2.	I understand taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records	
4.	I agree that a small amount of my blood may be used to investigate the immune system	
5.	I agree that a small amount of my blood may be collected and gifted to the "UK JSLE Study Group." It will be stored for future genetic studies. I understand that no result on my genes will be fed back to me or anyone else.	
6.	I agree that a small amount of my blood and urine samples may be used to look into ways of detecting kidney damage in lupus.	
7.	I agree that if I was to need a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital, to look at how the kidney is affected by Lupus.	
8.	**To be completed by patients who have had a kidney biopsy in the past:** I agree that a small amount of left over kidney tissue from my previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
9.	I agree that DNA can be extracted and stored from any kidney biopsy samples that I provide I understand that no result on my genes will be fed back to me or anyone else.	
10.	I agree that I will take part in the above study	
11.	I agree to allow researchers to make contact with me about other studies or a follow-up of this study through my doctors and my NHS number	
12.	I give permission for my GP to be informed that information about me is to be held on the study database	
13.	I agree to being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
14.	I agree to provide my email address. I understand that this will be stored securely. I understand it will only be used to provide me with information about the study such as the newsletter and to provide a way for the study group to contact me for follow up in the future.	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

Appendix 11: UK JSLE Cohort Study Baseline demographics form



JSLE – DEMOGRAPHICS

Study No | Date / / | Retrospective ☐ | Prospective ☐

NHS Number | Gender Male ☐ Female ☐

Post Code | DOB / /

Referral Paediatrician ☐ GP ☐ Adult Rheum ☐ A&E ☐ Sub-specialist ☐

Sub-specialist Details

	Age Years	Age Months	Date (If exact date not known use 01/mm/yyyy)
Onset of Symptoms			OR / /
Presentation			OR / /
Diagnosis			OR / /

Ethnicity

	British ☐	Irish ☐	Any other White ☐
White & Black Caribbean ☐	White & Black African ☐	White & Asian ☐	Any other Mixed ☐
Indian ☐	Pakistani ☐	Bangladeshi ☐	Any other Asian ☐
	Caribbean ☐	African ☐	Any other Black ☐
Chinese ☐	Other ☐		

~~PMHx/FHx~~

	Self	Mother	Father	Brother	Sister	Aunt/Uncle	Grandparent
SLE	☐	☐	☐	☐	☐	☐	☐
Thyroid	☐	☐	☐	☐	☐	☐	☐
RA	☐	☐	☐	☐	☐	☐	☐
CTD	☐	☐	☐	☐	☐	☐	☐
Type 1 DM	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐
None	☐						

Details

Parental Consanguinity Yes ☐ No ☐ Unknown ☐

Possible Triggers

Infections ☐ (Specify)

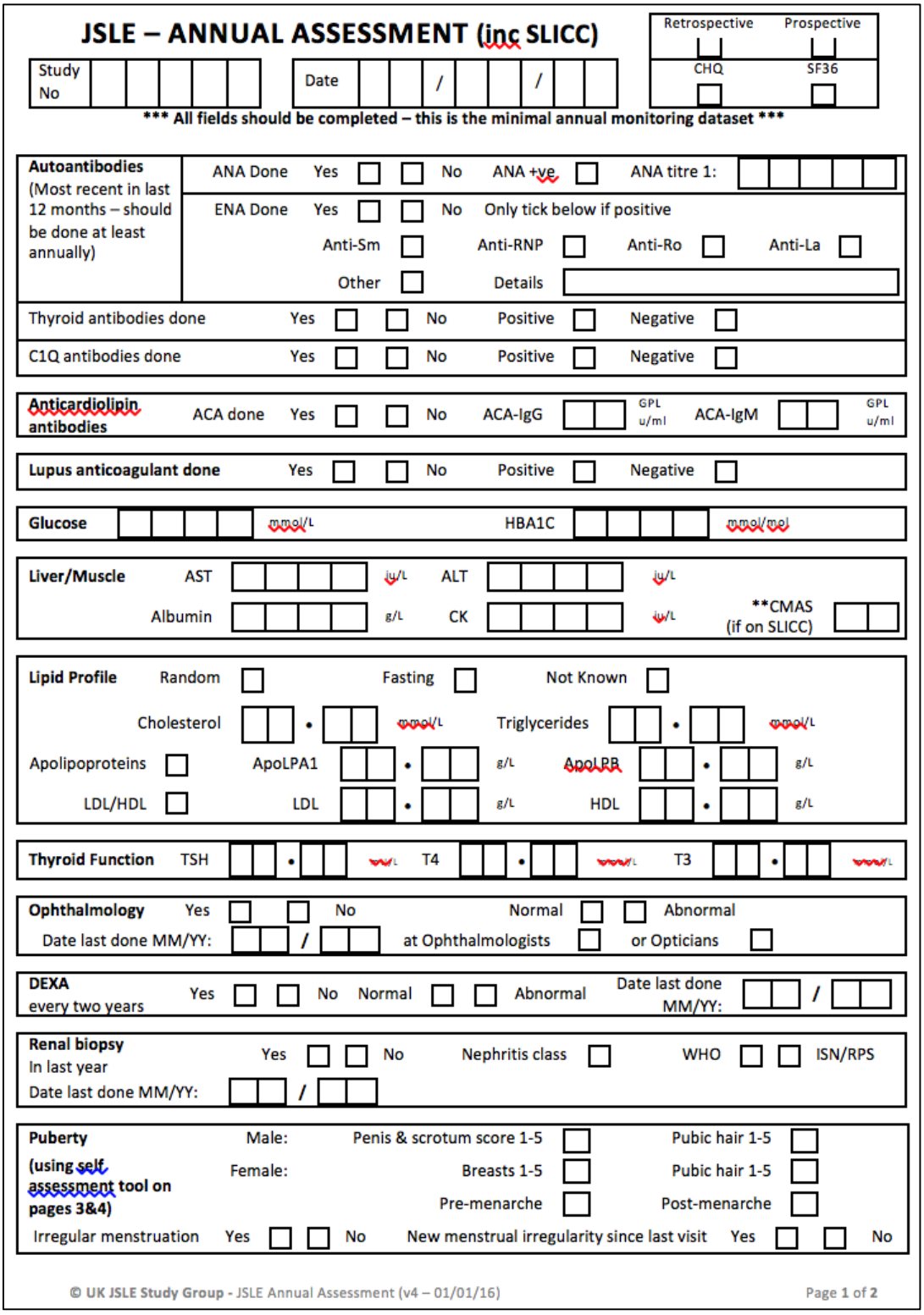
Medication ☐ (Specify)

Sun ☐

Other ☐ (Specify)

© UK JSLE Study Group - JSLE Demographics (v4 – 01/01/16)

Appendix 12: UK JSLE Cohort Study Annual Assessment form



JSLE – ANNUAL ASSESSMENT (inc SLICC)

Retrospective ☐ Prospective ☐

Study No ☐☐☐☐☐ Date ☐☐/☐☐/☐☐ CHQ ☐ SF36 ☐

*** All fields should be completed – this is the minimal annual monitoring dataset ***

Autoantibodies (Most recent in last 12 months – should be done at least annually)

ANA Done Yes ☐ ☐ No ANA +ve ☐ ANA titre 1: ☐☐☐☐☐

ENA Done Yes ☐ ☐ No Only tick below if positive

Anti-Sm ☐ Anti-RNP ☐ Anti-Ro ☐ Anti-La ☐

Other ☐ Details ☐

Thyroid antibodies done Yes ☐ ☐ No Positive ☐ Negative ☐

C1Q antibodies done Yes ☐ ☐ No Positive ☐ Negative ☐

Anticardiolipin antibodies ACA done Yes ☐ ☐ No ACA-IgG ☐☐ GPL u/ml ACA-IgM ☐☐ GPL u/ml

Lupus anticoagulant done Yes ☐ ☐ No Positive ☐ Negative ☐

Glucose ☐☐☐☐ mmol/L HBA1C ☐☐☐☐ mmol/mol

Liver/Muscle AST ☐☐☐☐ iu/L ALT ☐☐☐☐ iu/L

Albumin ☐☐☐☐ g/L CK ☐☐☐☐ iu/L **CMAS (if on SLICC) ☐☐

Lipid Profile Random ☐ Fasting ☐ Not Known ☐

Cholesterol ☐☐.☐☐ mmol/L Triglycerides ☐☐.☐☐ mmol/L

Apolipoproteins ☐ ApoLPA1 ☐☐.☐☐ g/L ApoLPB ☐☐.☐☐ g/L

LDL/HDL ☐ LDL ☐☐.☐☐ g/L HDL ☐☐.☐☐ g/L

Thyroid Function TSH ☐☐.☐☐ T4 ☐☐.☐☐ T3 ☐☐.☐☐

Ophthalmology Yes ☐ ☐ No Normal ☐ ☐ Abnormal

Date last done MM/YY: ☐☐/☐☐ at Ophthalmologists ☐ or Opticians ☐

DEXA every two years Yes ☐ ☐ No Normal ☐ ☐ Abnormal Date last done MM/YY: ☐☐/☐☐

Renal biopsy In last year Yes ☐ ☐ No Nephritis class ☐ WHO ☐ ☐ ISN/RPS

Date last done MM/YY: ☐☐/☐☐

Puberty (using self assessment tool on pages 3&4)

Male: Penis & scrotum score 1-5 ☐ Pubic hair 1-5 ☐

Female: Breasts 1-5 ☐ Pubic hair 1-5 ☐

Pre-menarche ☐ Post-menarche ☐

Irregular menstruation Yes ☐ ☐ No New menstrual irregularity since last visit Yes ☐ ☐ No

© UK JSLE Study Group - JSLE Annual Assessment (v4 – 01/01/16) Page 1 of 2

JSLE – ANNUAL ASSESMENT (Page 2 of 2)

To be used with Glossary A blank box will be assumed to = 0. Please tick to confirm	☐ Yes ☐ No

Parameters	SLICC Damage Criteria	0	1	2	3
Ocular (either eye by clinic assessment)	Ocular cataract EVER				
	Retinal change OR optic atrophy				
Neuropsychiatric	Cognitive impairment OR major psychosis				
	Seizures requiring therapy for 6 months				
	Cerebral vascular accident ever (Score 2 if >1), or resection not for malignancy				
	Cranial or peripheral neuropathy (excluding optic)				
	Transverse myelitis				
Renal	Estimated or measured GFR <50%				
	Proteinuria 24h, >=3.5g OR ACR > 1000mg/mm OR > 10mg/mm				
	End stage renal disease (regardless of dialysis or transplantation)				
Pulmonary	Pulmonary hypertension (right ventricular prominence, or loud P2)				
	Pulmonary fibrosis (physical and x-ray)				
	Shrinking lung (x-ray)				
	Pleural fibrosis (x-ray)				
	Pulmonary infarction (x-ray) OR resection not for malignancy				
Cardiovascular	Angina OR coronary artery bypass				
	Myocardial infarction ever (score 2 if >1)				
	Cardiomyopathy (ventricular dysfunction)				
	Valvular disease (diastolic murmur, or a systolic murmur > 3/6)				
	Pericarditis x 6 months or pericardectomy				
Peripheral Vascular	Claudication x 6 months				
	Minor tissue loss (pulp space)				
	Significant tissue loss ever (e.g. loss of digit or limb, resection) (Score 2 if > 1)				
	Venous thrombosis with swelling, ulceration, OR venous stasis				
Gastrointestinal	Infarction or resection of bowel (below duodenum), spleen, liver or gall bladder (Score 2 if > 1)				
	Mesenteric insufficiency				
	Chronic peritonitis				
	Stricture OR upper gastrointestinal tract surgery ever				
	Pancreatic insufficiency requiring enzyme replacement or pseudocyst				
Musculoskeletal	Atrophy or weakness (**If yes please record CMAS on page 1 of this form)				
	Deforming or erosive arthritis (including reducible deformities, excluding avascular necrosis)				
	Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis)				
	Avascular necrosis (Score 2 if > 1)				
	Osteomyelitis				
	Ruptured tendons				
Skin	Alopecia				
	Extensive scarring of panniculum other than scalp and pulp space				
	Skin ulceration (not due to thrombosis) > 6 months				
Other	**Diabetes** (regardless of treatment)				
	Malignancy (excluding dysplasia) (Score 2 if > 1)				
	Premature gonadal failure / secondary amenorrhoea				

Appendix 13: Great Ormond Street Hospital MTA

JSLE MATERIAL TRANSFER AGREEMENT

BETWEEN: The University of Liverpool, The Foundation Building, 765 Brownlow Hill, Liverpool, L69 7ZX (**"Liverpool"**); and

Central Manchester University Hospitals NHS Foundation Trust, Saint ~~Mary's Hospital~~, Oxford Road, Manchester, M13 9WL (**"Manchester"**). Cobbett House

together the "**Recipients**"

AND: Great Ormond Street Hospital for Children NHS Trust, Great Ormond Street, London WC1N 3JH (the **"Provider"**);

Whereas: (A) Liverpool Paediatric Research Ethics Committee has granted approval (REC 06/Q1502/77) for the collection and repository of data and materials in pursuance of a research study entitled "UK Juvenile Systemic Lupus Erythematosus Registry and Repository: Clinical characteristics and immunopathology of juvenile-onset systemic lupus erythematosus".

(B) The Provider has been granted Site Specific Assessment approval through its local research and development committee to participate as a contributing centre in the above mentioned study.

Upon: Professor Michael Beresford (the "**Chief Investigator**") an employee of Liverpool and Professor Yanick Crow an employee of Manchester wish to obtain cells, DNA, RNA, serum and plasma (the "**Material**") for the above mentioned study (the "**Purpose**"). The Provider confirms it is willing to supply the Material subject to the following terms.

It is Agreed by the parties as follows:

1. DEFINITIONS AND INTERPRETATIONS

1.1. **"Confidential Information"** means all information including without limitation all ideas, techniques, processes, know-how, routines, specifications, formulae, drawings, methods and other knowledge concerning the Material and the use of the Material in any Replicates or Derivates.

1.2. **"Derivative"** means any material created from the Material that is substantially modified but still represents a non-severable improvement to or amendment of the Material.

1.3. **"Intellectual Property"** shall mean patent applications, patents, trademarks, service marks, registered designs, domain names, applications for any of the foregoing, trade and business names, unregistered trademarks and service marks, know-how, copyrights, rights in designs, rights in databases, rights in inventions, rights in improvements and rights of the same or similar effect or nature, in any part of the world.

1.4. **"Replicate"** means any biological or chemical material representing substantially unmodified copy of all or part of the Material.

1.5. **"UK JSLE Study Group"** means the UK Juvenile Systemic Lupus Erythematosus Study Group which is a multi disciplinary multi centre collaborative group of paediatric rheumatologists, nephrologists and dermatologists and other specialists, adult rheumatologists and nurse specialists, lay representative and basic scientists. Its members represent almost all of the major paediatric centres in the UK. Its aims are to develop a comprehensive research program to investigate the clinical characteristics and immunopathology of JSLE.

6.5. If any provision of this Agreement is declared void or unenforceable by a Court of competent jurisdiction it shall be severed from the Agreement and the remaining provisions shall continue to the fullest extent permitted by law.

6.6. Nothing in this Agreement creates a relationship of employment, agency or partnership between the parties.

6.7. The Provider shall not be entitled to assign this Agreement without the express written permission of the Recipients.

6.8. This Agreement contains the entire agreement between the parties. No amendments or modifications to this agreement will be of any effect unless in writing signed by authorised representatives of all parties.

6.9. This Agreement is subject to the laws of England and both parties hereby submit to the exclusive jurisdiction of the English Courts.

Signed by and on behalf of **Liverpool** acting by a duly authorised signatory:

Name: **Stephanie Laidlaw**

Date: **Research Contracts Officer**
Legal, Risk and Compliance
University of Liverpool 17/11/11

Signed by and on behalf of **Manchester** acting by a duly authorised signatory:

Name: L WEBSTER

Date: 9/1/2012

Signed by and on behalf of **Provider** acting by a duly authorised signatory:

Name:

Date: 25/10/11

Appendix 14: Einstein Lupus Cohort MTA

A-00001788

Albert Einstein College of Medicine
of Yeshiva University
Jack and Pearl Resnick Campus
1300 Morris Park Avenue, Bronx, NY 10461

JOHN L. HARB
Office of Biotechnology

Phone: (718) 430-3357
Fax: (718) 430-8938
E-mail: mta@einstein.yu.edu

MATERIAL TRANSFER AGREEMENT
FOR TRANSFER OF MATERIAL
TO ACADEMIC, NON-PROFIT ORGANIZATIONS

In response to the RECIPIENT's request for the MATERIAL [insert description]

Urine from Juvenile Systemic Lupus Erythematosus patients and healthy controls

the PROVIDER asks that the RECIPIENT and the RECIPIENT SCIENTIST agree to the following before the RECIPIENT receives the MATERIAL:

1. The above MATERIAL is the property of the PROVIDER and is made available as a service to the research community.

2. THIS MATERIAL IS NOT FOR USE IN HUMAN SUBJECTS.

3. The MATERIAL will be used for teaching or not-for-profit research purposes only.

4. The MATERIAL will not be further distributed to others without the PROVIDER's written consent. The RECIPIENT shall refer any request for the MATERIAL to the PROVIDER. To the extent supplies are available, the PROVIDER or the PROVIDER SCIENTIST agrees to make the MATERIAL available under a separate Simple Letter Agreement to other scientists for teaching or not-for-profit research purposes only.

5. The RECIPIENT agrees to acknowledge the source of the MATERIAL in any publications reporting use of it.

6. Any MATERIAL delivered pursuant to this Agreement is understood to be experimental in nature and may have hazardous properties. THE PROVIDER MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED. THERE ARE NO EXPRESS OR IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE MATERIAL WILL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS. Unless prohibited by law, RECIPIENT assumes all liability for claims for damages against it by third parties which may arise from the use, storage or disposal of the MATERIAL except that, to the extent permitted by law, the PROVIDER shall be liable to the RECIPIENT when the damage is caused by the gross negligence or willful misconduct of the PROVIDER.

7. The RECIPIENT agrees to use the MATERIAL in compliance with all applicable statutes and regulations.

8. The MATERIAL is provided at no cost, or with an optional transmittal fee solely to reimburse the PROVIDER for its preparation and distribution costs. If a fee is requested, the amount will be indicated here: No fee.

The PROVIDER, RECIPIENT and RECIPIENT SCIENTIST must sign both copies of this letter and return one signed copy to the PROVIDER. The PROVIDER will then send the MATERIAL.

PROVIDER INFORMATION and AUTHORIZED SIGNATURE

Provider Scientist: Dr Chaim Putterman

Albert Einstein College of Medicine of Yeshiva University
1300 Morris Park Avenue, Bronx, NY 10461

Name of Authorized Official: John L. Harb

Title of Authorized Official: Assistant Dean of Scientific Operations

Certification of Authorized Official: This Simple Letter Agreement has / __has not [check one] been modified. If modified, the modifications are attached.

Signature of Authorized Official — Date: 3/31/15

RECIPIENT INFORMATION and AUTHORIZED SIGNATURE

Recipient Scientist: Dr Eve Smith

Recipient Organization: The University of Liverpool

Address: The Foundation Building, 765 Brownlow Hill, Liverpool, L69 7ZX

Name of Authorized Official: Sharon Grant

Title of Authorized Official: Institute of Translational Medicine, Institute Manager

Signature of Authorized Official: SC Grant

Date: 25 March 2015

Certification of Recipient Scientist: I have read and understood the conditions outlined in this Agreement and I agree to abide by them in the receipt and use of the MATERIAL.

Recipient Scientist — Date: 25/3/15

Appendix 15: Paediatric Lupus Erythematosus in SA Cohort Study ethical approval February 2015

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

HUMAN RESEARCH ETHICS COMMITTEE
- 3 FEB 2015
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

Form FHS006: Protocol Amendment

HREC office use only (FWA00001637; IRB00001938)		
☐ Approved	✔ Type of review: Expedited	☐ Full committee
This serves as notification that all changes and documentation described below are approved.		
Signature Chairperson of the HREC		Date 3/2/2015

Note: All major amendments should include a **PI Synopsis** justifying the changes for the amendment (please see notice dated 23 April 2012)

5/2/2015

Principal Investigator to complete the following: (Please ensure the Consent docs are labelled Correctly)

1. Protocol information

Date (when submitting this form)	31.1.15
HREC REF Number	424-2013
Protocol title	Paediatric Lupus Erythematosus in South Africa
Protocol number (if applicable)	IRB00001938
Principal Investigator	Associate Professor Christiaan Scott
Department / Office Internal Mail Address	Red Cross Memorial Hospital, Klipfontein Road, Rondebosch, 7700, Cape Town, South Africa

1.1 Is this a major or a minor amendment? (see FHS006hlp) Major (tick box) Minor (tick box)	☐ Major	✔ Minor
1.2 Does this protocol receive US Federal funding?	☐ Yes	✔ No
1.3 If the amendment is a major amendment and receives US Federal Funding, does the amendment require full committee approval?	☐ Yes	✔ No

2. List of Proposed Amendments with Revised Version Numbers and Dates

Please itemise on the page below, all amendments with revised version numbers and dates, which need approval.
This page will be detached, signed and returned to the PI as notification of approval. Please add extra pages if necessary.

1. **Addition of urine sample collection and storage for biomarker analysis**
   a. JSLE patients within the existing Red Cross Memorial Hospital JSLE Cohort will be approached at their routine visits and re-consented for collection and storage or urine samples alongside clinical data that is already collected as part of the Cohort study (see revised study protocol, version 2, dated 31.1.15).
   b. All urine samples will undergo dipstick testing as part of routine clinical care. Samples from JSLE patients with abnormalities on dipstick will be sent for

Appendix 16: Paediatric Lupus Erythematosus in SA Cohort Study Healthy Control Case Report form

Paediatric Control Case Report Form

Demographic data

Date	Age	Ethnicity	Allocated study ID

Clinical data

Current reason for hospital visit (please specify)	

Current urine symptoms	**YES**	**NO**
• Pain on passing urine		
• Increased urinary frequency		
• Menstruating		

Past history of the following conditions	**YES**	**NO**
• Juvenile Systemic Lupus Erythematosus		
• Juvenile Idiopathic Arthritis		
• Juvenile Dermatomyositis		
• Inflammatory bowel disease (ulcerative colitis / crohn's)		
• Kidney problems (please specify)		
• Any other illness of note (please specify)		

Urinalysis results (score 0, trace, 1+, 2+, 3+)

Proteinuria		**Haematuria**	

Leucocytes		**Nitrites**	

Appendix 17: Paediatric Lupus Erythematosus in SA Cohort Study MTA

MATERIAL TRANSFER AGREEMENT

Made and entered into by and between

UNIVERSITY OF CAPE TOWN

A university incorporated in terms of the Higher Education Act, 1997, and the statute of the University of Cape Town, promulgated under Government Notice No. 1199 of 20 September 2002, whose administrative offices are located at Bremner Building, Lower Campus, Lovers' Walk, Rondebosch, 7700, South Africa herein represented by Julie Nadler-Visser, in her capacity as Contracts Manager of the University of Cape Town and she being duly authorised thereto

(hereinafter referred to as "**Provider**")

and

THE UNIVERSITY OF LIVERPOOL

The Foundation Building, 765 Brownlow Hill, Liverpool, L69 7ZX, United Kingdom

(hereinafter referred to as "**Recipient**")

(collectively hereinafter referred to as the "**Parties**" and individually as the "**Party**")

1

AGREED TO FOR:

PROVIDER

JULIANNE NADLER-VISSER
Contracts Manager
Contracts & Intellectual Property Services
Dept of Research & Innovation
University of Cape Town

Signature

Name (duly authorised)
Julie Nadler-Visser

Position: Contracts Manager

17 July 2015
Date

RECIPIENT

Signature

Sharon Grant
Name (duly authorised)

Institute Manager, Institute of Translational Medicine

16 July 2015
Date

INVESTIGATOR

Signature

Name: Professor Michael Beresford

Position: Professor of Child Health

8

Appendix 18: Univariate association of each clinico-demographic variable with outcome in analysis looking at time to recovery from proteinuria following active LN

Clinical and demographic factors	*p-value*
Age at LN onset	***0.013***
Serum creatinine	***0.184***
eGFR (ml/min/m^2)	***0.0602***
Neutrophil count (x10^9/L)	***0.197***
Physicians global assessment (0-100 scale)	***0.107***
Haematological involvement[1]	***0.0377***
Gender	*0.322*
Ethnicity (Caucasian or non-Caucasian)	*0.421*
Length of disease	*0.934*
Baseline Proteinuria (urine protein or albumin:creatinine ratio, mg/mmolCr)[2]	*0.989*
Severe hypertension[3]	*0.253*
Nephrotic syndrome[4]	*0.765*
Active urinary sediment[5]	*0.466*
Haemoglobin (g/dl)	*0.979*
WCC (x10^9/L)	*0.426*
Lymphocytes (x10^9/L)	*0.879*
Platelets (x10^9/L)	*0.342*
ESR (mm/h)	*0.353*
CRP (mg/L)	*0.601*
C3 (g/L)	*0.986*
C4 (g/L)	*0.537*
Anti-dsDNA antibody titres (IU/L)	*0.577*
IgG (g/L)	*0.296*
IgA (g/L)	*0.636*
IgM (g/L)	*0.252*
Hydroxychloroquine[6]	*0.328*
Azathioprine	*0.377*
Mycophenolate Mofetil	*0.475*
Prednisolone	*0.581*
Intravenous immunoglobulin (IVIG)	*0.762*
Rituximab ever	*0.351*
Cyclophosphamide ever	*0.463*
ACEi or AT2i[7]	*0.607*
Constitutional involvement	*0.411*

Mucocutaneous involvement	*0.936*
Neuropsychiatric involvement	*0.901*
Musculoskeletal involvement	*0.272*
Cardiorespiratory involvement	*0.663*
Gastrointestinal involvement	*0.680*
Opthalmological involvement	*0.919*
Total numerical BILAG score	*0.424*

Univariate association of each clinicao-demographic variable with outcome, in analysis looking at time to recovery from proteinuria following active LN.

Cox proportional hazard regression modelling used. [1]BILAG defined organ domain involvement. [2]Baseline Proteinuria = UPAC or UAUC measurements depending on hospital laboratory). [3]BILAG defined severe hypertension. [4]Nephrotic syndrome = heavy proteinuria (> 50 mg/kg/day or > 3.5 g/day or protein-creatinine ratio > 350 mg/mmol or albumin-creatinine ratio > 350mg/mmol) + hypoalbuminaemia + oedema. [5]Active urine sediment = pyuria (> 5 WCC/hpf), haematuria (> 5 RBC/hpf) or red cell casts in absence of other causes. [6]Medication use (yes) or non-use (no) considered rather than absolute drug dose. [7]ACEi or AT2i = Angiotensin inhibitor or angiotensin receptor blocker. Note – descriptive statistics for these variables are shown in Table 3-6 and therefore not repeated.

Appendix 19: ELISA urine biomarker raw data from UK/US/SA JSLE patients

Cohort	STUDY ID	DATE	BILAG	LPGDS	MCP-1	CP	AGP	VCAM-1	TF
UK	10003	02/11/2011	D	830	80	712	299	11	406
UK	10003	30/05/2012	D	621	81	618	217	70	168
UK	10003	13/03/2013	D	584	137	1197	166	3	59
UK	10004	07/04/2010	D	534	95	2596	1746	0	556
UK	10004	14/04/2011	D	503	179	1128	102	0	352
UK	10004	19/12/2012	D	581	195	728	440	1	252
UK	10017	14/07/2010	C	1825	125	3351	27697	49	14180
UK	10017	07/10/2010	D	52	48	282	59	1	593
UK	10017	12/01/2011	D	941	98	1168	13179	163	35008
UK	10021	20/10/2010	D	204	236	2771	324	7	5972
UK	10021	09/02/2011	D	312	337	529	774	6	1423
UK	10030	19/07/2011	E	705	572	1757	285	2	355
UK	10030	17/10/2011	E	210	45	755	110	0	257
UK	10030	31/01/2013	E	179	149	445	250	1	1051
UK	10034	18/11/2009	D	327	110	1066	224	2	813
UK	10034	13/01/2010	D	302	53	301	343	2	789
UK	10034	06/10/2010	D	52	86	117	108	0	53
UK	10035	19/12/2009	E	413	271	554	231	8	1405
UK	10035	10/03/2010	E	631	630	795	515	8	770
UK	10035	03/11/2010	E	50	39	45	50	2	46
UK	10036	22/09/2010	D	112	84	277	83	1	3110
UK	10040	10/02/2010	D	132	606	NaN	1553	1	1876
UK	10040	26/01/2011	D	288	86	505	154	0	336
UK	10041	02/12/2009	D	123	34	462	181	0	522
UK	10041	30/06/2010	D	271	85	826	647	8	37
UK	10041	20/10/2010	D	345	92	680	653	3	1156
UK	10042	03/11/2010	D	1100	1000	1001	1199	158	23
UK	10042	25/08/2011	D	575	465	1197	910	0	1840
UK	10043	15/12/2010	E	135	111	266	205	7	590
UK	10043	09/02/2011	E	57	131	430	211	2	539
UK	10043	24/08/2011	E	342	204	606	263	4	1847
UK	10045	11/09/2009	A	1073	277	2089	14043	17	2078
UK	10045	08/06/2010	D	168	153	832	355	20	226
UK	10045	06/10/2010	D	41	26	65	362	0	76
UK	10045	09/03/2011	D	219	NaN	527	925	NaN	402
UK	10045	19/10/2011	D	330	NaN	643	1092	NaN	299
UK	10045	17/10/2012	D	473	NaN	1328	1741	NaN	1114
UK	10045	06/03/2013	B	718	NaN	997	2510	NaN	889
UK	10049	04/05/2011	E	287	219	984	268	1	1734
UK	10049	23/11/2011	E	131	341	604	83	0	155
UK	10049	10/06/2013	D	481	137	729	622	52	3921
UK	10050	22/08/2012	E	352	157	1115	21	1	154
UK	10050	17/10/2012	E	387	510	1079	33	0	63
UK	10050	16/10/2013	E	287	134	1284	88	0	69
UK	10051	06/12/2010	D	42	92	1376	183	1	184

UK	10051	02/09/2011	D	64	103	817	134	0	360
UK	10052	06/10/2011	E	584	94	1390	445	1	415
UK	10052	13/03/2013	E	1141	143	1042	3397	34	373
UK	10057	26/08/2011	E	218	298	701	595	3	659
UK	10057	15/05/2012	E	411	137	1026	409	0	3622
UK	10057	22/08/2012	E	1015	283	834	858	30	3690
UK	10060	10/07/2012	E	856	1402	1871	1162	4	579
UK	10060	03/10/2012	E	365	96	969	591	3	1646
UK	10061	09/01/2013	E	985	33	1318	2668	4	2160
UK	10061	06/02/2013	E	916	31	1365	3710	11	519
UK	10061	15/05/2013	E	729	94	511	938	2	210
UK	10062	02/10/2013	B	669	369	1298	896	0	867
UK	10062	13/11/2013	B	574	274	964	1018	6	1239
UK	10062	21/01/2014	D	334	150	474	151	3	190
UK	10062	09/07/2014	D	52	NaN	773	131	NaN	478
UK	10062	01/10/2014	D	134	NaN	1288	145	NaN	302
UK	10064	18/09/2013	E	2578	422	1725	1149	39	4344
UK	10069	20/08/2014	A	3164	NaN	99720	214944	NaN	243086
UK	10069	29/10/2014	A	468	NaN	11350	39416	NaN	31826
UK	15001	30/12/2010	E	477	319	910	309	7	2535
UK	15001	29/09/2011	E	267	246	557	296	5	1579
UK	15002	06/05/2010	D	129	NaN	1718	1709	NaN	3442
UK	15002	31/03/2011	D	180	NaN	660	529	NaN	3067
UK	15004	05/08/2010	B	142	NaN	1934	15686	NaN	27842
UK	15004	23/05/2011	D	124	71	361	360	24	255
UK	15004	16/08/2012	C	406	280	6046	13510	10	83439
UK	15004	20/12/2012	B	230	184	2364	22787	29	109114
UK	15004	31/07/2014	D	305	NaN	1223	7096	NaN	7855
UK	15005	19/07/2012	D	484	80	401	166	10	413
UK	15005	17/01/2013	D	586	94	663	255	2	319
UK	15015	18/08/2011	D	112	302	1121	178	2	589
UK	15015	15/08/2013	D	104	165	2146	86	1	3041
UK	15024	04/10/2012	D	217	178	1320	365	4	903
UK	15024	04/04/2013	D	569	188	1765	277	23	3149
UK	15024	03/10/2013	D	462	240	1069	304	4	322
UK	15032	06/10/2011	D	893	135	938	754	16	1188
UK	15032	15/03/2012	D	283	107	1357	7	5	1728
UK	15032	29/09/2012	D	524	77	1052	748	29	116
UK	15047	15/12/2011	A	983	1838	37507	49407	65	43815
UK	15047	15/03/2012	B	278	1188	2700	52798	3	53043
UK	15047	19/04/2012	A	1958	580	20521	158901	8	1457
UK	15048	05/08/2010	B	1180	NaN	73900	23426	NaN	185367
UK	15048	03/05/2012	A	4009	2289	20417	501	89	286
UK	15048	30/08/2012	A	2994	2733	25419	93109	23	499890
UK	15053	29/11/2012	D	132	125	902	282	1	306
UK	15053	16/05/2013	D	151	1322	389	231	2	425
UK	15054	15/12/2011	D	1477	183	1611	2564	5	725
UK	15054	15/11/2012	D	523	160	1711	274	4	1436

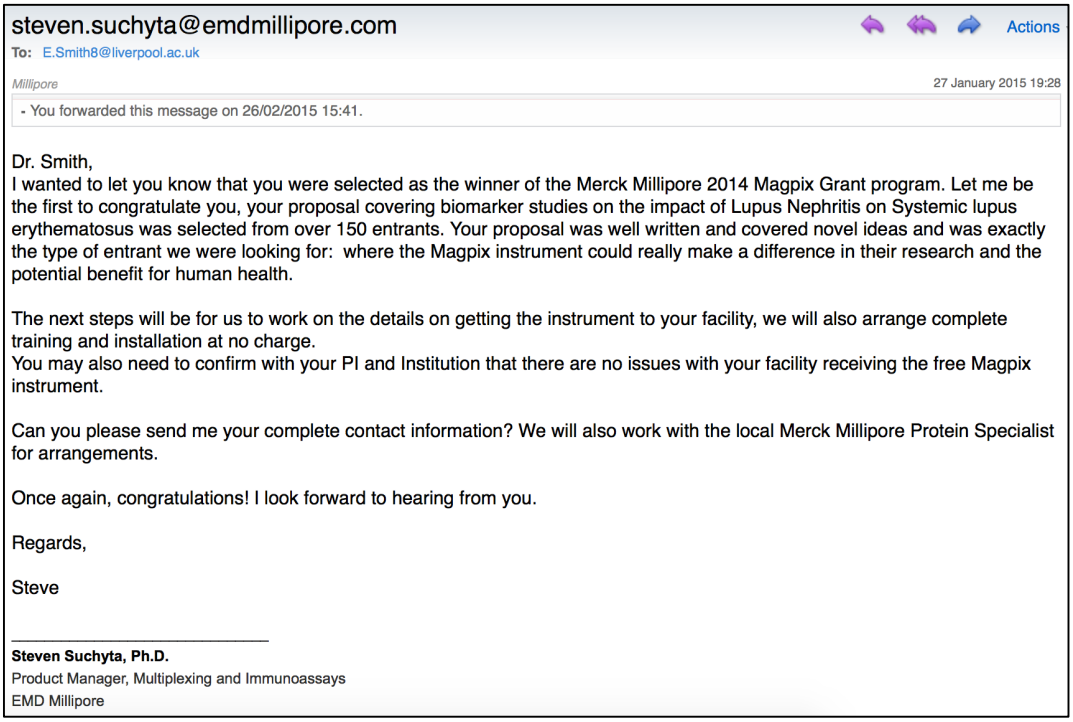
UK	15054	07/11/2013	D	573	182	766	230	8	7
UK	15056	16/02/2012	E	134	70	478	206	2	1040
UK	15058	21/07/2011	D	220	116	34	154	0	1763
UK	15060	05/04/2012	D	234	164	1025	260	1	9749
UK	15060	05/07/2012	D	164	28	1025	229	1	2286
UK	15060	04/04/2013	D	520	86	1598	221	10	5893
UK	15062	24/04/2011	D	161	135	379	3901	13	851
UK	15062	29/09/2011	D	340	137	1608	1315	2	779
UK	15062	19/01/2012	D	147	157	689	492	0	1908
UK	15063	02/02/2012	D	617	126	748	333	10	1182
UK	15063	21/06/2012	D	875	91	1144	432	4	74
UK	15063	20/12/2012	D	1005	175	1100	449	1	430
UK	15066	04/10/2011	D	407	484	2531	401	1	450
UK	15066	18/04/2013	D	233	174	2154	193	0	206
UK	15066	17/10/2013	D	152	290	709	48	14	10419
UK	15068	06/09/2012	B	1257	261	6443	14451	64	63018
UK	15068	07/02/2013	B	1835	383	78	464	18	356368
UK	15068	18/07/2013	D	192	114	812	2851	4	2735
UK	15068	06/03/2014	D	964	NaN	5428	5043	NaN	6846
UK	15068	03/07/2014	D	792	NaN	1369	6307	NaN	2376
UK	15070	04/08/2011	C	775	NaN	2657	190000	NaN	50576
UK	15070	20/10/2011	D	674	NaN	12765	190000	NaN	8159
UK	15070	03/01/2013	**A**	1572	1015	3118	55405	52	1781
UK	15070	07/02/2013	C	1382	854	24418	5954	29	15585
UK	15070	04/07/2013	B	2063	1802	6941	26527	97	1647
UK	15070	02/01/2014	B	2024	NaN	4639	41772	NaN	42177
UK	15070	06/02/2014	B	1382	NaN	10638	30770	NaN	24376
UK	15070	29/05/2014	C	1296	NaN	2741	16108	NaN	21366
UK	15070	31/07/2014	C	835	NaN	1728	23684	NaN	21010
UK	15073	16/06/2011	B	1278	353	3836	27821	15	7754
UK	15073	20/10/2011	B	862	NaN	5354	6858	NaN	7568
UK	15073	16/01/2014	D	99	NaN	1459	732	NaN	2069
UK	15074	18/10/2012	D	298	274	648	298	1	2746
UK	15078	26/02/2012	**D**	2947	187	1284	14939	93	175
UK	15078	21/06/2012	B	2181	167	2161	33115	122	411
UK	15079	06/12/2012	C	2046	209	5267	43378	104	38823
UK	15079	21/11/2013	B	1958	605	16711	33034	0	34234
UK	15079	30/01/2014	C	1164	690	11825	77102	55	4811
UK	15079	03/07/2014	B	1183	NaN	13919	42905	NaN	23518
UK	15079	16/10/2014	B	1610	NaN	18213	98974	NaN	28614
UK	15080	02/02/2012	E	1198	296	911	637	21	4965
UK	15080	17/05/2012	E	1756	101	1591	560	19	2148
UK	15080	04/07/2013	E	680	92	1284	225	10	12420
UK	15082	17/05/2012	E	131	64	487	117	0	17004
UK	15082	02/08/2012	E	139	110	441	83	0	279
UK	15082	17/01/2013	E	162	104	2199	511	1	31807
UK	15083	05/07/2012	A	148	275	25648	19379	14	63000
UK	15083	07/02/2013	D	385	81	1732	318	0	40192

UK	15083	16/01/2014	D	24	NaN	2075	535	NaN	4034
UK	15083	20/03/2014	D	42	NaN	848	236	NaN	1745
UK	15083	03/07/2014	D	7	NaN	642	50	NaN	1725
UK	15085	21/03/2013	D	225	72	3036	289	1	1238
UK	15086	06/12/2012	D	521	97	970	639	19	29465
UK	15086	06/06/2013	D	291	156	1176	183	4	968
UK	15086	09/12/2013	D	661	147	569	371	10	9159
UK	15087	20/03/2013	B	268	265	138977	38177	31	88443
UK	15087	19/12/2013	B	1471	494	39475	18331	7	132431
UK	15087	04/09/2014	**C**	984	NaN	10453	190000	NaN	84102
UK	15088	06/06/2013	D	806	338	2500	245	4	850
UK	15088	01/08/2013	D	996	444	2625	312	10	1514
UK	15089	04/04/2013	E	139	107	616	36	1	4133
UK	15089	18/07/2013	E	442	185	2809	96	0	2631
UK	15090	05/09/2013	E	213	387	1107	408	1	1179
UK	15090	21/11/2013	E	156	195	1122	55	1	11382
UK	15092	19/12/2013	E	215	152	627	70	0	561
UK	15102	02/02/2012	D	470	289	2009	22356	33	18221
UK	15102	20/09/2012	C	305	78	3249	20664	10	9839
UK	15106	07/04/2011	D	2450	503	7216	1474	14	50227
UK	15106	17/11/2011	D	1072	266	4130	3909	17	45936
UK	15106	19/04/2012	D	1871	564	12113	12276	17	11402
UK	15118	07/02/2013	D	309	294	1150	1638	5	25531
UK	15118	18/07/2013	D	906	311	1328	1521	4	926
UK	15119	17/11/2011	D	383	NaN	1159	198	NaN	690
UK	15119	17/05/2012	D	NaN	NaN	1314	72	NaN	771
UK	15119	16/08/2012	D	479	NaN	604	182	NaN	216
UK	15119	29/11/2012	D	385	NaN	1256	157	NaN	324
UK	15119	03/01/2013	D	116	NaN	395	126	NaN	203
UK	15119	18/07/2013	D	280	153	524	209	1	3282
UK	15119	17/10/2013	B	745	654	24943	7581	54	92001
UK	15119	02/01/2014	D	457	982	1195	1270	3	7919
UK	15120	06/05/2010	A	1317	NaN	10002	190000	NaN	161713
UK	15120	20/10/2011	B	841	NaN	38930	27933	NaN	72390
UK	15120	03/05/2012	B	257	NaN	31991	24655	NaN	48415
UK	15120	18/10/2012	**B**	1179	339	8676	22867	43	64861
UK	15120	20/12/2012	B	462	100	5440	27756	86	2255697
UK	15120	07/02/2013	B	1027	172	20908	300	31	8331176
UK	15120	29/05/2014	**A**	704	NaN	15530	190000	NaN	138306
UK	15120	04/09/2014	B	1796	NaN	137442	187564	NaN	256747
UK	15122	07/03/2013	D	249	371	1674	329	17	9287
UK	15122	04/07/2013	D	154	206	556	248	1	13137
UK	15122	03/10/2013	D	83	141	548	474	0	1142
UK	15123	01/03/2012	D	401	104	1290	1190	31	39806
UK	15123	19/04/2012	D	11260	4782	42931	18147	52	100628
UK	15123	30/08/2012	D	424	210	1178	662	1	2731
UK	15124	19/04/2012	B	876	806	7482	432	30	88357
UK	15124	29/11/2012	B	461	240	5406	21489	10	62124

UK	15124	07/03/2013	B	803	516	136641	340	17	434
UK	15124	05/06/2014	B	564	NaN	34355	190000	NaN	207767
US	AA5173	28/02/2012	A	254	672	5885	14258	15	5356
US	AD5	26/07/2010	E	120	278	3395	112	2	907
US	AD5	21/09/2010	D	71	182	3298	155	1	978
US	AF5156	11/04/2011	B	1939	1386	72057	119917	195	199170
US	AV5	27/10/2009	D	504	182	10997	6988	4	10181
US	AV5	18/11/2009	E	193	396	8808	862	3	812
US	BD5082	03/06/2010	E	2172	351	6085	2984	16	1246
US	BP5191	07/11/2013	A	1981	2701	36746	138713	34	51198
US	BS5180	11/04/2013	A	747	295	3917	4521	13	412
US	CD5059	11/01/2010	E	531	129	1188	360	1	683
US	DC5	18/09/2009	E	811	193	1696	1219	58	527
US	DC5	14/04/2010	D	707	129	3888	535	10	390
US	DF5315	13/02/2015	A	427	482	22354	32491	19	20046
US	DH5	31/03/2010	A	769	311	3133	1343	7	355
US	DH5	15/09/2010	D	293	249	1834	136	1	208
US	EA5	11/08/2010	A	2645	950	160039	94067	146	416412
US	EA5	14/03/2014	B	1539	853	12046	59173	110	26539
US	JC1387	31/07/2009	A	578	311	7484	26691	40	13566
US	JS5063	20/01/2010	D	413	169	1628	338	4	164
US	JV5	18/07/2013	A	1148	352	42255	93792	1	52695
US	JV5	09/05/2014	D	102	112	1554	1178	2	1665
US	KB5148	14/02/2011	B	388	247	22633	39354	12	32288
US	MA1385	29/07/2009	A	1460	600	7701	111382	43	31334
US	MG5	12/01/2012	A	416	352	4423	1004	3	584
US	MG5	13/02/2015	D	253	104	1159	137	1	604
US	MS5177	07/02/2013	A	2206	8353	81430	254412	191	444887
US	PB5309	15/01/2015	A	1324	236	15621	92812	131	69862
US	RA5	11/11/2009	A	1901	898	56080	171898	42	148865
US	RA5	26/02/2014	B	466	149	22932	64695	14	17263
US	RR5	15/12/2010	B	941	689	24371	32221	13	14856
US	RR5	28/10/2011	C	703	449	5909	12317	10	4843
US	RR5	08/01/2015	B	644	853	15383	60421	29	65493
US	RS5	08/12/2009	E	541	329	1137	410	8	255
US	RS5	28/02/2012	B	73	25	172	307	0	68
US	RT5	26/08/2009	E	515	240	938	332	4	207
US	RT5	12/08/2014	B	2522	3015	56044	72504	119	95764
US	SB5	06/12/2010	E	431	182	2684	2711	9	1438
US	SB5	23/08/2012	A	77	271	1767	14188	37	420
US	SL5	10/08/2009	A	3862	2456	87003	22869	349	503
US	SL5	28/12/2009	D	624	247	7317	1717	3	2416
US	TE5302	06/01/2015	B	562	379	8917	2601	5	5715
US	TO5189	17/10/2013	A	1147	868	10903	42765	27	29279
US	TP5312	20/01/2015	B	1113	243	12383	54293	43	18278
SA	PID001	07/07/2015	E	621	113	2090	410	12	659
SA	PID001	12/01/2016	E	275	139	604	41	2	210
SA	PID003	19/01/2016	E	602	100	1700	572	7	228

SA	PID005	05/05/2015	E	599	160	1190	406	4	123
SA	PID008	14/01/2016	A	3171	6931	54193	53911	59	25239
SA	PID010	25/08/2015	E	209	1889	3339	790	3	1245
SA	PID012	26/02/2015	E	855	235	1175	359	3	201
SA	PID012	05/05/2015	E	894	271	1973	8108	6	776
SA	PID012	25/08/2015	E	796	114	738	111	4	113
SA	PID012	20/10/2015	E	380	191	1867	156	1	209
SA	PID012	02/02/2016	E	452	98	1244	39	0	545
SA	PID013	04/05/2015	A	8561	2329	164366	283704	146	120935
SA	PID013	21/07/2015	A	2508	245	34657	31313	38	20863
SA	PID013	20/10/2015	A	2771	4982	271804	216754	419	211558
SA	PID013	14/01/2016	A	1040	362	106411	76937	145	69716
SA	PID013	02/02/2016	B	3582	14509	399096	447270	282	231025
SA	PID016	10/03/2015	C	1060	192	10809	6808	13	7657
SA	PID016	20/10/2015	C	1524	192	18745	17046	34	25224
SA	PID016	19/01/2016	B	2207	355	34493	27012	43	40502
SA	PID017	06/03/2015	A	3131	160	48369	66981	73	177987
SA	PID018	13/01/2016	E	116	118	928	38	2	352
SA	PID019	18/03/2015	D	72	178	857	821	1	422
SA	PID019	04/08/2015	D	183	305	3376	3540	2	3759
SA	PID020	25/11/2015	A	1117	850	502873	145435	136	134065
SA	PID022	20/10/2015	A	2162	466	190640	239383	41	264122
SA	PID023	22/01/2016	D	373	281	1711	412	2	142
SA	PID026	31/03/2015	E	148	211	1034	135	1	401
SA	PID027	25/08/2015	A	118	1020	51714	55581	4	57666
SA	PID036	13/04/2015	D	152	188	4585	1985	4	1651
SA	PID080	28/07/2015	E	804	397	2935	1317	6	444
SA	PID080	25/08/2015	E	368	83	5240	665	0	2406
SA	PID080	14/01/2016	E	149	230	990	228	1	887
SA	PID082	31/08/2015	D	1035	228	32647	87919	85	25274
SA	PID083	15/04/2015	E	1098	313	3255	5985	12	944
SA	PID083	22/01/2016	E	626	227	8210	5793	7	9192
SA	PID084	20/10/2015	C	2114	449	18266	20289	31	26929
SA	PID085	08/01/2015	A	2683	1555	31926	261352	102	63630
SA	PID085	18/03/2015	C	2980	903	5468	9459	52	994
SA	PID085	04/05/2015	C	1087	1267	5729	5424	20	2110
SA	PID085	07/07/2015	D	627	307	1860	1381	20	146
SA	PID085	28/07/2015	D	1514	328	2942	6671	31	239
SA	PID085	25/08/2015	C	737	384	5003	31796	110	1773
SA	PID085	02/02/2016	D	672	370	1656	602	3	546
SA	PID086	25/03/2015	C	2289	568	4320	13519	12	1974
SA	PID086	04/05/2015	D	1463	679	2288	10558	11	714
SA	PID092	22/06/2015	A	4034	4956	35230	228195	52	35640
SA	PID092	18/01/2016	D	215	394	3791	957	1	294
SA	PID095	13/10/2015	C	2425	2490	9727	6137	142	2347

Appendix 20: Merck Millipore 2014 Magpix Grant award program confirmation



steven.suchyta@emdmillipore.com

Actions

To: E.Smith8@liverpool.ac.uk

Millipore 27 January 2015 19:28

- You forwarded this message on 26/02/2015 15:41.

Dr. Smith,
I wanted to let you know that you were selected as the winner of the Merck Millipore 2014 Magpix Grant program. Let me be the first to congratulate you, your proposal covering biomarker studies on the impact of Lupus Nephritis on Systemic lupus erythematosus was selected from over 150 entrants. Your proposal was well written and covered novel ideas and was exactly the type of entrant we were looking for: where the Magpix instrument could really make a difference in their research and the potential benefit for human health.

The next steps will be for us to work on the details on getting the instrument to your facility, we will also arrange complete training and installation at no charge.
You may also need to confirm with your PI and Institution that there are no issues with your facility receiving the free Magpix instrument.

Can you please send me your complete contact information? We will also work with the local Merck Millipore Protein Specialist for arrangements.

Once again, congratulations! I look forward to hearing from you.

Regards,

Steve

_____

Steven Suchyta, Ph.D.
Product Manager, Multiplexing and Immunoassays
EMD Millipore

Appendix 21: Successful MRC Confidence in Concept (CiC) scheme grant application

Date: Friday, 10 April 2015 14:09

To: "Beresford, Michael" <mikega1@liverpool.ac.uk>

Cc: "Keenlyside, Stephanie [keenly]" <keenly@liverpool.ac.uk>

Subject: Confidence in Concept - Beresford

Dear Michael

Thank you for your application to the University of Liverpool Confidence in Concept scheme.

The scheme was once again extremely popular with 26 applications across a broad range of topics, many of which were multi-disciplinary in nature.

Your application was reviewed by the evaluation committee and discussed at the panel meeting held on 8th April. All projects were evaluated by Dr Richard Armer (Head of Drug Discovery, RedX Oncology), Dr Phil Bentley (Distinguished Scientist, Novartis), Professor Chris Chamberlain (VP and Head Experimental Medicine and Diagnostics, UCB), Dr Geoff Davison (CEO, Bionow), Dr David Pardoe (Head of Growth Projects, MRC Technology) and Dr Neil Warburton (COO, Mast Group). The panel meeting was Chaired by Professor Bob Burgoyne.

I am pleased to inform you that the panel scored your application strongly and agreed, in principle, that it should be funded through the CiC scheme. However, because the total budget of all of the successful projects exceeds the money available from MRC, I will have to review individual budgets and ask for a small reduction from each PI. I will be back with more details on this soon, together with any specific comments from the panel.

Regards

Neil

Dr Neil French
Senior Scientific Operations Manager
MRC Centre for Drug Safety Science
Department of Molecular and Clinical Pharmacology
Sherrington Building
Ashton Street
The University of Liverpool

Appendix 22: Published manuscript - Urinary biomarkers in childhood lupus nephritis (review)

This text box is where the unabridged thesis included the following third party copyright material:

Smith EMD, Beresford MWB. Urinary biomarkers in childhood lupus nephritis. Clinical Immunology 2016 (published on-line first, June 29th, http://dx.doi.org/10.1016/j.clim.2016.06.010).

Appendix 23: Manuscript under consideration - Do classical blood biomarkers of JSLE identify active Lupus Nephritis? Evidence from the UK JSLE Cohort Study

This text box is where the unabridged thesis included the following third party copyright material:

Smith EMD, Jorgensen AL, Beresford MWB. Do classical blood biomarkers of JSLE identify active Lupus Nephritis? Evidence from the UK JSLE Cohort Study. Lupus journal (re-submitted September 2016).

Appendix 24: Accepted manuscript confirmation - International validation of a urinary biomarker panel for identification of active lupus nephritis in children

This text box is where the unabridged thesis included the following third party copyright material:

Smith EMD, Jorgensen AL, Midgley A, Oni L, Goilav B, Putterman C, Wahezi D, Rubinstein T, Ekdawy D, Corkhill R, Jones CA, Marks SD, Newland P, Pilkington C, Tullus K, Beresford MW. International validation of a urinary biomarker panel for identification of active lupus nephritis in children. Pediatric Nephrology 2016 (Sept 3rd, Epub ahead of print]; DOI: 10.1007/s00467-016-3485-3.

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