Identifying biomarkers of response to treatment and long-term outcomes in lupus nephritis.

A thesis submitted to Imperial College London in candidature for the degree of Doctor of Philosophy by

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To Effie, for all the joy you bring. I hope these words are worthy.

ABSTRACT

Lupus nephritis (LN) is a common yet serious manifestation of systemic lupus erythematosus (SLE), carrying significant risk of morbidity and mortality. Treatments have, in real terms, progressed little in recent years and poor responses are associated with bad long-term outcomes. Clinically useful predictors of response to treatment and long-term outcomes are desperately needed for use both in clinic and in clinical trials as endpoints to aid the progression of new treatments.

Analysis of LN patients, staining and mass spectrometry (MS) were employed to examine the demographic, clinical, histopathological and proteomic profiles of LN patients.

470 patients and 803 biopsies were described in a database of LN patients treated over a 20-year period including the largest described cohort of patients treated with no oral glucocorticoids. Proteinuria level at biopsy, 'full house' pattern on immunohistochemistry and percentage of glomeruli with chronic damage predicted response to treatment. Proteinuria level and eGFR at one year predicted long-term outcome. Achieving both an eGFR >80mls/min/1.73m^2 and proteinuria <90mg/mmol predicted a good outcome at 7 years with odds ratio 9.922 (95% CI 4.979-19.774) $p<0.001$. LN was demonstrated to be treated successfully without oral glucocorticoids or cyclophosphamide. The membrane attack complex was shown to be strongly associated with LN, however not a useful marker of on-going activation of complement C5. For the first time, the proteome of LN renal biopsy tissue was explored using SWATH-MS. The pathways of mitochondrial processes, oxidative phosphorylation and metabolism were found to be downregulated in LN and even more so in non-responders.

Together, this body of work identifies predictors of response to treatment, parameters to target with treatments and possible pathogenic targets of new treatments. It indicates that rapid diagnosis of LN and flares followed by targeting of excellent renal function with early initiation of treatments is key to preserving renal function long-term.

STATEMENT OF CONTRIBUTION

Except where acknowledged below and referenced throughout, I declare that this thesis is entirely my own work and is based upon research carried out in the Department of Immunology and Inflammation (formally the Department of Medicine) at Imperial College London and the Stoller Biomarker Discovery Centre at the University of Manchester.

The basic protocol for protein extraction from tissue, including use of the pressure cycling technology, was kindly provided by Dr Tiannan Guo (Westlake University).

Mass spectrometry (MS), protein quantification and machine learning was performed by Bethany Geary in the Stoller Biomarker Discovery Centre at the University of Manchester as part of a collaboration. Artemis Papadaki produced the principal component analysis figures and heat maps on the resulting protein databases. Sample preparation for MS and the remainder of the proteomics analysis including differential expression, generation of volcano plots, correlation analysis and string analysis was performed by myself.

Plasma and urine samples were provided by Dr Iva Gunnarsson (Karolinska Institutet) for the C5b-9 enzyme linked immunosorbent assay (ELISA) analysis. Analysis performed by myself.

All staining was performed by myself except for the C9 and CD68 immunohistochemistry and C3c immunofluorescence which was performed by the Clinical Laboratory at Hammersmith Hospital according to their lab protocols.

Dr Anisur Rahman kindly provided patient data from the Rheumatology service at University College London Hospital for validation of the database work. Statistics analysis performed by myself.

The database was constructed by myself using mainly electronic notes and results software at Imperial College Healthcare NHS Trust as well as paper notes when required. Patients for the database were identified from the biopsy diagnosis database and a legacy database which was collected by Dr Tabitha Turner-Stokes. Visiting doctors assisted in the data collection of side effect data (Dr Neeru Sharma, Dr Sofia Correia and Dr Iara Karlla).

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LIST OF ABBREVIATIONS

A active

ACN acetonitrile

ACR American College of Rheumatology

ADAMTS13 ADAM metallopeptidase with thrombospondin type 1 motif 13

ALMS Aspreva Lupus Management Study

ANA antinuclear antibody

ANCA anti-neutrophil cytoplasmic antibody

APL anti-phospholipid syndrome

APOL1 apolipoprotein L1

ASS argininosuccinate synthetase

ATP adenosine triphosphate

AUC area under the curve

AURORA-1 Aurinia Renal Response in Active Lupus With Voclosporin

AZA Azathioprine

BAFF B-cell activating factor

BC Bordeaux University Hospital

BD Becton Dickenson

BLISS-LN Belimumab International Study in Lupus Nephritis

CARRA Childhood Arthritis and Rheumatology Research Alliance

C chronic

C complement

CC Hospital Universitario Cruces

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CKD chronic kidney disease

CKD-EPI CKD Epidemiology Collaboration

CNI calcineurin inhibitor

CR complete response

CTTNBP2NL CTTNBP2 N-terminal like protein

CyP cyclophosphamide

DDA data dependent acquisition

DDD dense deposit disease

DEXA dual-energy X-ray absorptiometry

DIA data independent acquisition

DN diabetic nephropathy

DNA deoxyribonucleic acid

dsDNA anti-double-stranded deoxyribonucleic acid

eGFR estimated glomerular filtration rate

ELISA enzyme linked immunosorbent assay

ELNT Euro-Lupus Nephritis Trial

EM electron microscopy

ER endoplasmic reticulum

ERA-EDTA European Renal Association-European Dialysis and Transplantation Association

ESKD end stage kidney disease

EULAR European Alliance of Associations for Rheumatology

FDR false discovery rate

FFPE formalin-fixation and paraffin-embedding

FHR-5 factor H related protein 5

G global

g grams

GEAS Systemic autoimmune disease group of the Spanish Society of Internal Medicine and Spanish Society of Nephrology

GWAS genome-wide association studies

HC healthy controls

HIF-1 hypoxia-inducible factor-1α

hnRNP heterogeneous nuclear ribonucleoprotein

HPF high powered field

HPLC high-performance liquid chromatography

HRQoL health-related quality of life

IAA iodoacetamide

IC immune complex

ICAT isotope coded affinity tag

ICHIS Integrated Computerised Hospital Information System

ICHT Imperial College Healthcare Trust

ICHTB Imperial College Healthcare NHS Trust Tissue Bank

IF immunofluorescence

IFTA interstitial fibrosis and tubular atrophy

Ig immunoglobulin

IHC immunohistochemistry

IL interleukin

IFN interferon

IP immunoperoxidase

IQR inter-quartile range

ISN International Society of Nephrology

iTRAQ isobaric tagging technology

IU international units

IV intravenous

KDIGO Kidney Disease Improving Global Outcomes

L litre

LLDAS Lupus Low Disease Activity State

LM light microscopy

LN lupus nephritis

LR logistic regression

MAC membrane attack complex

MALDI matrix-assisted laser desorption/ionisation

MAM mitochondria-associated ER membrane

MAR missing at random

MBL mannose binding lectin

MCAR missing completely at random

MCD minimal change disease

MERCs mitochondria and endoplasmic reticulum contact sites

mg milligram

MHC major histocompatibility complex

ml millilitre

MME metalloendopeptidase

MMF mycophenolate mofetil

MNAR missing not at random

MOM mitochondrial outer membrane

MP methylprednisolone

MPa megapascals

MPA mycophenolate acid

MRM multiple reaction monitoring

MS mass spectrometry

mTORC2 mammalian target of rapamycin complex 2

MYLUPUS efficacy and safety of enteric-coated mycophenolate sodium in combination with two glucocorticoid regimens for the treatment of active lupus nephritis

m/z mass-to-charge

n number of patients

NAD nicotinamide adenine dinucleotide

NADH nicotinamide adenine dinucleotide hydrogen

NADP nicotinamide adenine dinucleotide phosphate

NET neutrophil extracellular trap

NF-κB nuclear factor kappa B

NIH National Institute of Health

NIHR National Institute for Health Research

NOBILITY B-cell depletion with obinutuzumab for the treatment of proliferative lupus nephritis: a randomised, double-blind, placebo-controlled trial

NR non-response

NRES National Research Ethics Service

NT5E 5'-nucleotidase ecto

OP oral prednisolone

OR odds ratio

ORM1 alpha-1-acid glycoprotein

ORR overall response rate

PACS2 phosphofurin acidic cluster sorting protein 2

PBS phosphate buffered saline

PCA principal component analysis

PCT pressure cycling technology

PGD2 prostaglandin D2

PGDS prostaglandin H_2D-isomerase

PGH2 prostaglandin H2

PO oral treatments

Ppp4 serine/threonine-protein phosphatase 4 catalytic subunit

PPI protein-protein interaction

PPV positive predictive value

PR partial response

RAAS renin-angiotensin-aldosterone system

RCT randomised controlled trial

RITUXILUP Trial of Rituximab and Mycophenolate Mofetil Without Oral Steroids for Lupus Nephritis

ROC receiver operating characteristics

ROS reactive oxygen species

RPS Renal Pathology Society

RR relative risk

RRT renal replacement therapy

RTX rituximab

S segmental

SELUNE Study of Safety, Efficacy and Tolerability of Secukinumab Versus Placebo, in Combination With SoC Therapy, in Patients With Active Lupus Nephritis

SLICC Systemic Lupus International Collaborating Clinics

SKI severe acute kidney injury

SLE systemic lupus erythematosus

SNP single nucleotide polymorphism

SRM selected reaction monitoring

STAT4 signal transducer and activator of transcription 4

SWATH-MS sequential window acquisition of all theoretical fragment ion spectra mass spectrometry

TBM thin basement membrane

TCEP tris(2-carboxyethyl)phosphine hydrochloride

TGF-beta transforming growth factor-beta

TGFBR TGF-beta receptors

TGFBRAP1 transforming growth factor beta receptor associated protein 1

TIN tubulo-interstitial nephritis

TMB tetramethylbenzidine

TOF time-of-flight

Treg regulatory T cell

TRI tubuloreticular inclusion

TULIP-LN Treatment of Uncontrolled Lupus via the Interferon Pathway - Lupus Nephritis

TYK2 tyrosine kinase 2

u units

UCLH University College Hospital London

uPCR urine protein/creatinine ratio

VDAC voltage-dependent anion channel

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK

PUBLICATIONS

Glomerular membrane attack complex is not a reliable marker of ongoing C5 activation in lupus nephritis.

Hannah R. Wilson, Nicholas R. Medjeral-Thomas, Alyssa C. Gilmore, Pritesh Trivedi, Kathleen Seyb, Ramin Farzaneh-Far, Iva Gunnarsson, Agneta Zickert, Thomas D. Cairns, Liz Lightstone, H. Terence Cook, Matthew C. Pickering.

Kidney International March 2019; 95(3): 655-665

Manifestations of lupus in the kidney and how to manage them.

Hannah R. Wilson, Liz Lightstone.

Nephrology Dialysis Transplantation October 2017; 32(10): 1614–1616

Positive ANCA serology in patients with lupus nephritis is associated with distinct histopathological features on renal biopsy.

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Manuscript in preparation.

Using long term outcome data to redefine proteinuria and eGFR endpoints for lupus nephritis trials

Hannah R Wilson, Tabitha Turner-Stokes, Iara Santos, Hannah Beckwith, Neeru Agarwal, Sofia Correia, Camilla Pillay, Andrew Porter, Megan Griffith, Jeremy Levy, Thomas Cairns, H. Terence Cook, Liz Lightstone.

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PRESENTATIONS

Oral:

Using long term outcome data to redefine proteinuria and eGFR endpoints for lupus nephritis trials.

Hannah R. Wilson, Tabitha Turner-Stokes, Iara Santos, Megan Griffith, Jeremy Levy, Thomas D. Cairns, H. Terence Cook, Liz Lightstone.

American Society of Nephrology Kidney Week October 2018

20 years of lupus nephritis.

Hannah R. Wilson, Tabitha Turner-Stokes, Iara Santos, Megan Griffith, Jeremy Levy, Thomas D. Cairns, H. Terence Cook, Liz Lightstone.

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Complement in lupus nephritis.

Hannah R. Wilson, Nicholas R. Medjeral-Thomas, Alyssa C. Gilmore, Pritesh Trivedi, Kathleen Seyb, Ramin Farzaneh-Far, Iva Gunnarsson, Agneta Zickert, Thomas D. Cairns, Liz Lightstone, H. Terence Cook, Matthew C. Pickering.

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The role of the membrane attack complex in lupus nephritis.

Hannah R. Wilson, Nicholas R. Medjeral-Thomas, Alyssa C. Gilmore, Pritesh Trivedi, Kathleen Seyb, Ramin Farzaneh-Far, Iva Gunnarsson, Agneta Zickert, Thomas D. Cairns, Liz Lightstone, H. Terence Cook, Matthew C. Pickering.

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Poster:

A fingerprint of response to treatment in lupus nephritis: identification of a panel of 8 proteins from baseline renal biopsies that predict response.

Hannah R Wilson, Bethany Geary, Alyssa C Gilmore, Reyna Guitierrez, H Terence Cook, Anthony D Whetton, Liz Lightstone.

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Using long term outcome data to redefine proteinuria and eGFR endpoints for lupus nephritis trials: proteinuria targets need not be so stringent

Hannah R. Wilson, Tabitha Turner-Stokes, Iara Santos, Hannah Beckwith, Neeru Agarwal, Sofia Correia, Camilla Pillay, Andrew Porter, Megan Griffith, Jeremy Levy, Thomas Cairns, H. Terence Cook, Liz Lightstone.

Renal Association/British Renal Society UKKW 2019

Comparison of treatment in a large cohort of lupus nephritis: Excellent long-term outcome with no difference in response or flare between oral steroid and steroid-free regimens

Hannah R. Wilson, Camilla Pillay, Andrew Porter, Tabitha Turner-Stokes, Iara Santos, Hannah Beckwith, Neeru Agarwal, Sofia Correia, Megan Griffith, Jeremy Levy, Thomas Cairns, H. Terence Cook, Liz Lightstone.

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Comparison of treatment eras in a large cohort with lupus nephritis: Maintenance of long-term outcomes with significantly lower burden of immunosuppression & glucocorticoid use.

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Chronic changes in lupus nephritis biopsies – when to treat? Could complement C3c be an indicator for missed activity?

Hannah R. Wilson, Tabitha Turner-Stokes, Iara Santos, Megan Griffith, Jeremy Levy, Thomas D. Cairns, H. Terence Cook, Liz Lightstone.

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Twenty Years of Lupus Nephritis Management – Evaluation of a Multi-Ethnic Patient Cohort to Identify Factors Affecting Outcome.

Hannah R. Wilson, Tabitha Turner-Stokes, Megan Griffith, Jeremy Levy, Hannah Beckwith, Thomas D. Cairns, H. Terence Cook, Liz Lightstone.

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The spectrum of complement C9 staining in lupus nephritis.

Hannah R. Wilson, Nicholas R. Medjeral-Thomas, Alyssa C. Gilmore, Pritesh Trivedi, Kathleen Seyb, Ramin Farzaneh-Far, Iva Gunnarsson, Agneta Zickert, Thomas D. Cairns, Liz Lightstone, H. Terence Cook, Matthew C. Pickering.

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Factors associated with the development of ESRD in a recent large single centre cohort of patients with lupus nephritis

Iara Dasilva Santos, Hannah R. Wilson, Tabitha Turner-Stokes, Thomas D. Cairns, Liz Lightstone.

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1 CHAPTER ONE – INTRODUCTION AND BACKGROUND

Lupus nephritis (LN) is a common manifestation of systemic lupus erythematosus (SLE), a chronic autoimmune disease of complex aetiology. The gold standard for diagnosis is renal biopsy, current assessment of which however, provides little guidance as to optimal treatment protocols, response or prognosis. Response to treatment varies, is overall suboptimal and a significant proportion of patients have serious complications. With a varied and complex pathogenesis, mechanisms of injury in LN are multiple. These diverse mechanisms however produce a small range of histopathological appearances and clinical sequelae. Analysing the active pathways in LN tissue can provide further insight into pathogenesis, biomarkers for prognosis and targets for potential treatments. Here, the pathogenesis, diagnosis, and treatment of LN, will be reviewed, as well as the outcomes. The need for biomarkers and existing candidates will then be discussed followed by proteomics as a method of biomarker discovery.

1.1 SYSTEMIC LUPUS ERYTHEMATOSUS AND LUPUS NEPHRITIS

SLE is a chronic autoimmune disease which affects various tissues and organ systems in the body and typically follows a relapsing-remitting course. A significant proportion of patients, up to 2 in 3 of those with SLE, will develop lupus nephritis (LN), one of the most severe manifestations of SLE. Both the condition and its treatments are associated with considerable morbidity and mortality [1][2] with up to 25% of patients with LN developing end stage kidney disease (ESKD) [3], 33% of those with class IV disease at 10 years [4] and evidence of a 6-fold increase in mortality.[5] However, earlier diagnosis and initiation of treatment are associated with better outcomes.[6]

1.1.1 Epidemiology

The prevalence of SLE and LN varies worldwide. A recent systematic review identified the highest estimates of incidence and prevalence of SLE to be in North America at 23.2/100,000 person years (95% CI: 23.4, 24.0) and 241/100,000 people (95% CI: 130, 352) respectively.[7] The UK had an incidence of 4.9/100,000 person years (95% CI: 4.7, 5.1)[8] Females have a higher incidence of SLE as compared with males. This is the case for all age groups and ethnicities. The sex ratio ranged from 2:1 to 15:1 in the systematic review [7] with the higher incidence in females persisting for all age groups. However with smaller ratios at extremes of age, the peak incidence in men occurred later

than women; the fifth to seventh decade as opposed to the third to seventh for women.[7] In regards to ethnicity, incidence of SLE is highest in the Black population, followed by Asian and Hispanic populations and lowest in the Caucasian population.[7] For example, in a UK-wide study, incidence was 31.46/100,000 person years (95% CI 22.48, 44.03) in people of Black Caribbean ethnicity and 6.73/100,000 person years (95% CI 6.35, 7.14) in White people.[8]

1.1.2 Pathogenesis

The pathogenesis of LN is multifactorial and is characterised by immune complex (IC) mediated injury. The presence of ICs in glomeruli leads to a cascade of events which cause inflammation with the infiltration of lymphocytes and macrophages. Two key processes in this cascade are complement activation and the release of pro-inflammatory cytokines such as interferon alpha. Unchecked, this inflammation results in glomerular scarring which, along with tubular inflammation and subsequent scarring with interstitial fibrosis and tubular atrophy (IFTA), can lead to chronic kidney disease (CKD) and ESKD.[9][10]

The pathogenesis of glomerulonephritis involves four stages with two initial 'hits' [11]; genetic risk factors and environmental risk factors. Following these exposures, the innate immune system is activated involving complement and toll-like receptors, followed by the adaptive immune response. This response is antigen-specific, directed at auto-antigens and generates antigen-specific T and B cells which cause tissue damage through effector cells and complement.[11]

Over 60 genetic loci have been identified as risk factors for SLE using genome-wide association studies (GWAS).[12] These include single nucleotide polymorphisms (SNPs) involved in interferon (IFN) 1/ nuclear factor kappa B (NF-κB) signalling, B and T cell signalling, complement and apoptosis/debris clearance mechanisms which normally prevent large amounts of deoxyribonucleic acid (DNA) in extracellular compartments.[11][13] Most SNPs associated with SLE have been identified in non-coding regions of immune response related genes [14] which suggests a regulatory effect of these SNPs in the disease pathogenesis.[12]

There are many environmental factors which have been linked to SLE. These include viral infections including the Epstein-Barr virus, drugs and ultraviolet exposure.[11]

Following these 'hits', the key trigger of the immune system is self-antigens becoming exposed due to a deficiency in debris clearing mechanisms. These self-antigens become exposed to the immune system because of failures in apoptosis – apoptotic blebs have nuclear antigens on their surface and

increased apoptosis or defective clearance of apoptotic cells / blebs allows presentation to the adaptive immune system.[15][16] The autoimmune response provoked by exposure of these self-antigens leads to B cell production of antibodies which in turn form ICs in glomeruli. These ICs may form in situ,[17] be deposited or become trapped in the glomeruli.[11] Evidence points less towards a passive trapping.[18] Instead it is suggested that cross-reactivity with extracellular matrix and intracellular structures such as myosin cause the deposits to localise in the glomeruli.[15][19] B cells also activate autoreactive T cells and local inflammatory processes.

These ICs cause inflammation which can lead to permanent damage to the glomeruli and tubules. Involvement of all three pathways of complement have been implicated in this inflammation [20] with initiation by the classical pathway, amplification by the alternative pathway and involvement in deficiency of debris clearing by the lectin pathway.[20] Complement (C) components C3a and C5a act as chemoattractants which cause an influx of neutrophils and mononuclear cells, amplifying the inflammation.

Patients with active class III and IV disease have hypercellular glomeruli with leukocyte infiltration (commonly called a proliferative pattern of injury) and subendothelial deposits, usually with additional mesangial deposits. Subepithelial deposits occur in class V disease. Class V disease is distinguished from primary membranous disease by the presence of tubuloreticular inclusions (TRIs), seen in all classes of LN, as well as often being associated with mesangial and sometimes with subendothelial deposits. In addition, the deposits are composed of immunoglobulin (Ig)A, IgM, IgE and IgG1-3[11] rather than IgG4 seen in primary membranous disease.

1.1.3 Diagnosis and Classification

1.1.3.1 Diagnosis

Clinical indicators for suspicion of renal injury in SLE include:

- Proteinuria, usually the key presenting feature, which can present as new onset proteinuria: of >50 mg/mmol in spot urine protein:creatinine ratio (uPCR) or 24hr urine protein >500mg; or a doubling of “baseline” proteinuria to greater than 100mg/mmol;
- and / or a reduction in renal function which is present in half of all patients with LN[21,22];
- and / or an active urine sediment.

It is important to obtain baseline renal function if available and to interpret a creatinine reading at the upper limits of 'normal' with caution and in context of the patient. Abnormal values can be missed due to sitting within the normal range despite representing a significant change from the patient's baseline. Mean serum creatinine level is 60μmol/L in women of child-bearing age.[23] If a 24hr urine protein is difficult to obtain accurately as is often the case, a timed urine collection can be more accurate than a spot uPCR.

Hypertension can be an indicator of severe nephritis.

The kidney biopsy is the gold standard for the diagnosis of lupus nephritis and is used alongside clinical features and serological markers of SLE to guide treatment. The biopsy is also used to classify the lupus nephritis and provides evidence for prognosis such as an assessment of chronic damage. Technologies in use for the kidney biopsy have progressed from light microscopy alone to including immunohistochemistry and electron microscopy (EM).

1.1.3.2 Classification

Lupus nephritis is classified based on glomerular pathology with guidelines produced by the International Society of Nephrology (ISN) and the Renal Pathology Society (RPS) in 2004.[24] The abbreviated classification is reproduced below in Table 1.1.1.

Class	Description
Class I	Minimal mesangial lupus nephritis
Class II	Mesangial proliferative lupus nephritis
Class III	Focal lupus nephritis*
Class IV	Diffuse segmental (IV-S) or global (IV-G) lupus nephritis*
Class V	Membranous lupus nephritis
Class VI	Advanced sclerosing lupus nephritis

Table 1.1. Abbreviated International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification of lupus nephritis (2003). [24]

**A, active lesions only; A/C, active and chronic lesions; C, chronic lesions only.*

The guidelines also advise to indicate and grade (as mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.

A practical guide to the ISN/RPS classification of LN is displayed in figure 1.1[25]: it is class III or IV if there are active or sclerotic glomerular lesions with endocapillary hypercellularity, subendothelial deposits (wire-loops on light microscopy and seen on electron microscopy (EM)), intraluminal immune aggregates (hyaline thrombi) or crescents, amongst other features. Immunofluorescence typically shows "full house" deposition of immunoglobulins and complement. The nephritis is considered Class III where these changes are present in fewer than 50% of glomeruli or Class IV if in 50% or more. The activity of the glomerular lesions is also classified (all active (A), active/chronic (A/C) or all chronic(C)). For those with class IV the glomerular lesions are further described as predominantly segmental (S), if most glomeruli have <50% tuft involved, or global (G) where ≥50% of the tuft is involved.[26]

Class V LN involves membranous change, with "full house" granular capillary wall immunofluorescence and subepithelial immune deposits on EM. Class III/IV LN can coexist with Class V.[26] If the biopsy shows only mesangial hypercellularity then it is class II. These patients are likely to have minimal proteinuria and normal function. In Class I LN there are ICs detected by immunofluorescence or electron microscopy changes alone.[26]

It is worth noting that the classification of LN refers only to glomerular changes which are a consequence of glomerular IC deposition. Other manifestations can however be found including tubulointerstitial inflammation, vasculopathy and/or thrombotic microangiopathy (usually in association with antiphospholipid antibodies).[27] The degree of tubulo-interstitial scarring is also prognostic and should be documented.[26]

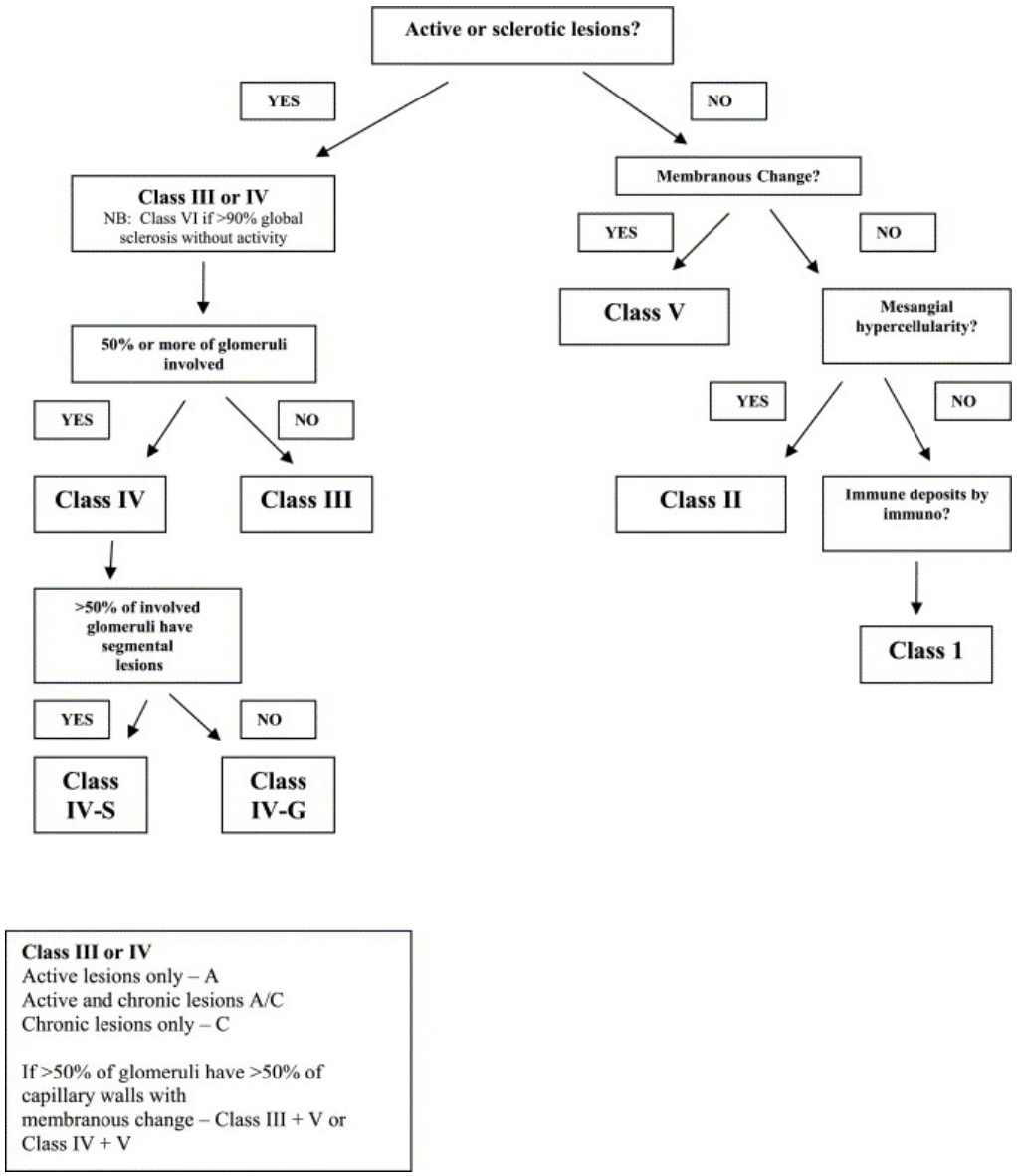


Figure 1.1 A practical guide to the ISN/RPS class selection demonstrated in a flowchart from Elliot et al, Am J Kidney Dis 2006 [25]

Image re-printed from 'Evolution of Lesions Over 10 Years in a Patient With SLE: Flowchart Approach to the New International Society of Nephrology (ISN)/Renal Pathology Society (RPS) Classification of Lupus Nephritis'. Elliot V et al. Am J Kidney Dis 2006.[25] with permission from Elsevier.

The ISN/RPS guidelines were reviewed in 2018 [28] with several alterations proposed including removal of the segmental and global allocations and the A, C and A/C grades with a replacement of the latter with the modified National Institute of Health (NIH) lupus nephritis activity and chronicity scoring indices.[29] The proposed modifications are to undergo a phase 2 'evidence-based

approach'[28] to assess the suitability before modifying the 2004 criteria. Specifically regarding the activity and chronicity indices, there is not as yet good evidence that they provide reliable prognostic information [24] and they are poorly reproducible.[24] The ISN/RPS classification recommends listing the proportion of glomeruli with features of active and chronic disease [24] and this is therefore the approach used in this thesis.

1.1.4 Treatment

The ESKD risk decreased significantly in the 1980's which corresponds to an increased use of immunosuppressant treatment. Treatment of lupus nephritis was initially, in the 1950s, solely with glucocorticoids – thought to be a game changer at the time but inadequate on their own and associated with many adverse side effects.[30] Treatment has however come a long way since this time with the proportion of patients treated with glucocorticoids alone decreasing from 54% in 1966 to 9% in 2010.[4] Despite evolution of treatments however, ESKD is still a relatively common outcome, with Hanly JG *et al.* recently identifying a rate of 10.1% at 10 years.[31][32]

Current treatments, in particular for proliferative LN, focus on induction and maintenance therapy phases.[33] Induction with intravenous (IV) cyclophosphamide (CyP) or mycophenolate mofetil (MMF) (target 2-3g daily) is recommended alongside glucocorticoids (IV then oral),[34] the dose and duration of the latter largely based on expert opinion rather than trial data. Wherever higher and lower doses of steroids have been compared, lower doses are associated with just as good or better outcomes and fewer side effects. At Imperial College Healthcare Trust (ICHT), IV methylprednisolone pulses of no greater than 500milligrammes (mg) each are used and for oral steroids, when used, a low starting dose (≤40mg/day, max 0.5mg/kg/day) with rapid taper is a strategy indicated to be effective and safer.[35] This strategy is also now endorsed by European Alliance of Associations for Rheumatology (EULAR) in their most recent guidelines.[36][26]

CyP (as per either the Euro-Lupus [37] or NIH [38] regimens) and MMF have similar efficacy[39]; however CyP may reduce fertility, though this is less likely with low dose regimens. Maintenance treatment is recommended with MMF (1-2g/day) or Azathioprine (AZA) (1.5-2.5mg/kg/day) alongside low-dose glucocorticoids for at least one (Kidney Disease Improving Global Outcomes (KDIGO)) to three (EULAR/European Renal Association-European Dialysis and Transplantation Association (ERA-EDTA)) years following complete remission.[34][26]

Treatment of pure class V is more controversial due to less robust evidence. It is worth noting that whilst prognosis is more benign, a significant proportion of patients reach ESKD by 10 years.[4] It is usually treated the same as class III or IV LN. Most guidelines agree that nephrotic range proteinuria (>3g/24hr or urine protein creatinine ratio (uPCR) >300mg/mmol) warrants immunosuppressive treatment.[34][26]

A significant proportion of patients do not respond to treatment. It is recommended with refractory disease to switch therapy from MMF to CyP or vice versa.[34] With relapsing disease it is advised to repeat the treatment which was effective at bringing about remission. In both scenarios a repeat renal biopsy should be considered as the pathogenic manifestations can change and may indicate alternative treatment.[26]

Multiple guidelines discussing the above recommendations were published in 2012, and compared by Wilhelmus *et al.* in 2016.[34] These were updated by EULAR in 2019 for SLE [40] and LN [36] and by KDIGO in 2021.[41] Recommendations for the use of MMF or low dose (Euro-Lupus [37] regimen) cyclophosphamide for induction treatment and MMF or AZA for maintenance treatment of LN are unchanged. Rituximab is still advised for use in refractory or relapsing disease only in the EULAR guidelines [36] however KDIGO add the recommendation for its use for corticosteroid minimisation.[41] In addition, KDIGO also include belimumab alongside MMF or CyP as an initial therapy. However, whilst B-lymphocyte biologics are included in the KDIGO guidelines for initial therapy, the key core first line recommendation continues to be glucocorticoids plus low-dose IV CyP or MMF. Calcineurin inhibitors tacrolimus and voclosporin are also listed as alternative treatments by KDIGO. EULAR guidelines also recommend consideration of use of high-dose cyclophosphamide for induction in severe disease.[36] In addition, there is advice to minimise prednisolone dose to start 0.3-0.5mg/kg/day (EULAR) or 0.6-1.0 mg/kg/day (KDIGO) and reduce to $\leq$7.5mg oral prednisolone/day (EULAR) or <2.5-5mg/day (KDIGO) by 3-6 months in maintenance treatment of SLE.[36][41] At the time of starting this body of work, almost none of the treatments described were licensed for use and there had been no new treatments licenced for lupus nephritis in a number of years with only one recent therapy, belimumab, gaining NICE approval for use for SLE in 2016. There have however since been some developments, including emerging roles for both biologics and multi-target therapy. The FDA have approved the use of voclosporin and belimumab specifically for LN and anifrolumab for moderate to severe SLE.[42] NICE have broadened their belimumab approval making it applicable to many more patients with LN. NICE guidance in relation to voclosporin is expected in January 2023 [43] and guidance on anifrolumab is awaiting

evidence.[44] Tacrolimus has shown promise as an alternative treatment for LN [45] and as noted, is now within both the EULAR and KDIGO guidelines although not highlighted as first line in either. Multi-target refers to the combination of low dose steroid, MMF and a calcineurin inhibitor (CNI); both the Aurinia Renal Response in Active Lupus With voclosporin (AURORA-1), which demonstrated a benefit of the Calcineurin inhibitor voclosporin in achieving complete remission (CR) [46] and a large phase III study from China using tacrolimus [47] have demonstrated superiority of such a combination as induction treatment in comparison to standard of care. The EULAR update is understandably cautious in its interpretation of these studies as only the phase 2 study of voclosporin, AURA-LV [48] was available at the time of the guidelines update. Disappointingly, the role of rituximab is yet to be defined by positive trial data though it is widely used and indeed recommended by LN guidelines (EULAR,[36] KDIGO [41] and American College of Rheumatology (ACR) [49]) and approved by NHSE for refractory LN whilst cohort data suggest benefit as primary therapy.[50] Clinical trials of rituximab have however thus-far failed to meet the primary endpoint [51] arguably for many reasons including high doses of concomitant steroids.[52] An interesting posthoc analysis of the LUNAR trial recently revealed however that patients who achieved B-cell depletion following treatment with rituximab had a higher chance of CR.[53] On the background of the failure of the LUNAR trial to demonstrate a benefit of rituximab in improving response rates in LN,[51] the NOBILITY (B-cell depletion with obinutuzumab for the treatment of proliferative lupus nephritis: a randomised, double-blind, placebo-controlled trial) trial was conducted, where the addition of obinutuzumab, an alternative anti-CD20 monoclonal antibody, to MMF and steroids was demonstrated to improve response rates to treatment vs placebo. As a proof-of-concept study significance was set at 0.2 for 83% Power. With this significance, overall response rate (ORR) (complete and partial response) and CR alone were significantly different from 52 weeks. At 104 weeks however, the difference was significant at $p<0.05$ (CR) and $p<0.01$ (ORR).[54] A Phase 3 study is underway.[26]

In clinical trials to date the rate of response to standard of care is 30-40% and standard of care PLUS the new treatment is, broadly speaking, approximately 50%,[55] indicating that there is significant room for improvement. Several further randomised trials in LN are ongoing or recently completed. These include: Treatment of Uncontrolled Lupus via the Interferon Pathway - Lupus Nephritis (TULIP-LN), investigating anifrolumab, a type I interferon receptor antibody, in addition to MMF and glucocorticoids, which unfortunately failed to reach its primary end point.[56] Anifrolumab has however received FDA approval for treatment of 'moderate-to-severe" SLE.[57] The Belimumab International Study in Lupus Nephritis (BLISS-LN) study demonstrated benefit of administering

belimumab alongside standard of care in achieving a renal response.[58] This study measured renal response at two years rather than the standard one year - however this time period has since been validated as predictive of long-term outcomes in a large cohort.[59] The addition of belimumab to rituximab and cyclophosphamide in refractory or recurrent LN was not shown to be of clinical benefit, despite prolonging the B cell depletion.[60] Promising results for deucravacitinib, a tyrosine kinase 2 (TYK2) inhibitor, in the treatment of SLE were recently described in a late breaking abstract at the European Alliance of Associations for Rheumatology (EULAR) 2022 Congress.[61] However a trial into its use in LN unfortunately had to be terminated due to lack of enrolment.[62] Secukinumab, an anti-interleukin (IL)-17A antibody which has shown promising case study results in LN[63] is currently recruiting in the Study of Safety, Efficacy and Tolerability of Secukinumab Versus Placebo, in Combination With SoC Therapy, in Patients With Active Lupus Nephritis (SELUNE).[64] Results of an anti-CD40L antibody (NCT00001789) have not yet been reported. Another potential target is complement C5, blockade of which has been shown to mitigate SLE in mice [65] with optimistic case study evidence in LN.[66] Ravulizumab, which, like Eculizumab, also inhibits the terminal complement pathway by C5 binding is currently undergoing Phase 2 study.[67]

There is very little evidence or agreement amongst guidelines in regard to treatment of class II LN except for the use of renin-angiotensin-aldosterone system (RAAS) inhibitors. Immunosuppression is largely directed towards extra renal manifestations. Class I LN requires no specific therapy as it has no clinical manifestations and is not associated with long-term renal impairment.[68] Patients with nephrotic range proteinuria who are found to have only class I or II LN on biopsy are likely to have lupus podocytopathy unless proliferative changes have been missed. Diagnosis can be confirmed with electron microscopy which demonstrates diffuse podocyte effacement. KDIGO recommends induction with glucocorticoid monotherapy and maintenance with an additional oral agent.[41][26]

Various adjunctive treatments are advised by the majority of guidelines particularly the use of hydroxychloroquine,[34] which is associated with sustained remission [69] and a reduced rate of relapse.[70] Screening with an ophthalmologist for retinopathy at baseline and yearly after 5 years is recommended.[34] RAAS inhibition for proteinuria and blood pressure control and statins for hyperlipidaemia are consistently recommended, as are consideration of calcium and vitamin D supplements, bisphosphonates, low-dose aspirin, anticoagulation in those with albumin <20g/L, ovarian protection with GnRH analogues if cumulative CyP dose >10g and avoidance of live vaccines.[34] In addition, low dose prophylactic antibiotics can be considered whilst complement

levels are low. Contraception is warranted for all patients on MMF or cyclophosphamide and pregnancy should be avoided until remission is sustained for at least 6 months.[26]

The side effect profile of current treatments is also significant with the relapsing remitting nature of the disease meaning that cumulative toxicity of long-term treatments is a major issue for patients. Steroids in particular are key culprits in a wide range of long-term complications, including osteoporosis, diabetes and hypertension.

1.1.4.1 Steroids

When first introduced, steroids were revolutionary as a treatment, changing lupus nephritis from a life-threatening condition into a treatable one.[71–73] In an early trial, five year survival of lupus nephritis was 73% in patients receiving steroid treatment compared to 41% in those receiving no steroid treatment.[74] However, from almost the earliest time they were used, there was a recognition that dosing and duration needed to be considered.[75] Furthermore, there was an early perception of the significant risks of high-dose steroid administration.[76] Despite these early concerns, most current treatment protocols still involve oral steroids, often at high doses and until recently there had been few systematic attempts to reduce their use and advise on the appropriate dose, duration of treatment, or weaning schedule. This is despite the fact that the consequences of chronic steroid use in terms of significant morbidity and mortality are widely recognised.[77] In addition, there is clear evidence that even small increases in dosing have a significant impact on toxicity. Al Salwah S. *et al.* showed that as little as 1mg/day increase in maintenance prednisolone dose is associated with a 2.8% increase in the risk of new organ damage.[78][32]

"Treat-to-target" [79] recommendations published in 2014 on the treatment of SLE advised to aim for the lowest glucocorticoid dose possible and aim to wean completely.[79] Recent EULAR recommendations for treatment published in 2019 advised a specific maximum dose of 7.5mg/day of prednisolone for maintenance treatment of SLE with the aim, if possible, to withdraw glucocorticoids, noting that use of steroid sparing treatments could assist in the ability to lower the doses of glucocorticoids and hopefully withdraw.[40] Specific guidance on doses during active treatment and duration are however still required as terms such as 'low dose' can be variably interpreted.

Undoubtedly, steroids are very effective in the short-term at rapidly dampening down inflammation, with a broad scope of influence. Their longer-term efficacy and use, is however, far less clear cut. On their own, they do not preserve renal function as shown very clearly by the long term data from the

NIH group.[80] Disease-modifying treatments are required to preserve renal-function in the long-term and the wealth of evidence of the detrimental effects of chronic use of steroids makes it increasingly difficult to argue that their benefits outweigh these.[81–84][32]

Interestingly, whilst the focus in LN has been to find alternative agents to cyclophosphamide, rather than to reduce steroids, the main driver to change in the world of kidney transplant was the priority given to minimising steroid exposure. The introduction of calcineurin inhibitors partly reduced steroid burden in transplantation but it was the advent of biologic treatments that allowed real steroid sparing.[85] Borrows *et al.* reported the use of a steroid-sparing regimen consisting of induction with methylprednisolone with just one week of oral steroids in addition to an anti-IL-2R antibody in high-risk patients, followed by maintenance treatment with tacrolimus and mycophenolate mofetil and no oral steroids. Results of this regimen showed greater than 50% reduction in rejection episodes, a reduction in new-onset diabetes from 16% to 6% at 3 years which was probably related to the low post-transplant weight gain of 3kg at 3 years.[86,87][32]

The consistent theme in transplant regimens using an induction agent followed by minimal steroids, does not solely reduce toxicity, but also leads to improvement in immunological outcomes – consistent with the idea of "less is more" when it comes to steroid dosing.[32]

There have been a few attempts in SLE and in LN to assess the impact of "lower" (often still quite high) doses of steroids (table 1.2). Consistently, where directly compared, lower doses of methylprednisolone or oral steroids were associated with at least as good efficacy outcomes and fewer adverse events, most notably serious infections and zoster episodes (table 1.2).[88–91] A good example is the small randomised controlled trial (RCT) MYLUPUS (Efficacy and safety of enteric-coated mycophenolate sodium in combination with two glucocorticoid regimens for the treatment of active lupus nephritis) which showed that combining myfortic with either standard (1mg/kg) or reduced (0.5mg/kg) dose prednisolone, led to equally good renal and extra renal outcomes at 24 weeks but fewer severe infections and no zoster (0/39) in the reduced dose group compared to 7 cases in 42 patients given 1mg/kg at baseline.[92][32]

Given this evidence, ICHT Lupus Centre undertook a cohort study in which all patients with biopsy-proven active lupus nephritis, not on steroids at baseline and with no rapidly progressive lupus nephritis or cerebral lupus, were treated with the Rituxilup regimen which included rituximab (1g) and methylprednisolone (500mg) on days 1 and 15, followed by maintenance treatment of mycophenolate mofetil (titrated up to mycophenolate acid (MPA) levels of 1.4-2.4mg/L) and no oral

steroids.[35] By one year, the majority of these patients achieved CR or partial response (PR).[35][32]

The AURORA-1 trial,[46] discussed previously, which compared voclosporin, a novel calcineurin inhibitor, with placebo in addition to standard of care with MMF and corticosteroids included a very rapid forced steroid taper, from a maximum of 1g methylprednisolone and 25mg oral prednisolone per day at the start down to 2.5mg by 16 weeks. In this study, voclosporin combined with MMF and low-dose steroids was superior to standard of care with significantly more patients achieving the primary endpoint of CR at 52 weeks than in the placebo group (41% vs 23%, OR 2.65; 95% CI 1.64-4.27; p<0.0001)[46]. Voclosporin was well tolerated with no difference in side effect rate when compared to placebo.[46][32]

Ruiz-Irastorza *et al.* published an observational comparative study of two cohorts: Hospital Universitario Cruces (CC) and Bordeaux University Hospital (BC).[93] Patients at both units were treated with cyclophosphamide or MMF in addition to both intravenous and oral steroids. Patients at CC were treated with repeated pulses of 125mg methylprednisolone and oral prednisolone ≤30mg/day with a taper over 12-14 weeks until 2.5-5mg/day. The BC protocol used fewer, higher doses of methylprednisolone (resulting in the same cumulative dose) and higher doses of prednisolone with a longer taper, the average 6-month dose being 21mg/day vs 8.3mg/day at CC ($p < 0.001$). CR rates were significantly higher at CC - 86% at one year, compared to 42% at BC ($p < 0.001$). Unsurprisingly, patients at CC had a lower risk of steroid-related side effects with an adjusted HR 0.19 (95% CI 0.04-0.89).[93][32]

These studies, along with a need for guidance regarding dose and duration of steroid use, led to the 2019 EULAR Guidelines update for LN to include some long-awaited information related to glucocorticoid use recommending lower starting doses (0.3-0.5mg/kg/day) reducing to ≤7.5mg/day by 3-6 months [36] and KDIGO to advise consideration of a reduced dose regimen starting at 0.5-0.6mg/kg/day tapering to <2.5mg by week 25 if response allows.[41] Current practice however still varies.

Studies with a single steroid dose					
Study	Steroid dose (n=number of patients) Mean actual dose over 24 weeks where available Maintenance dose	Total OP dose over 24 weeks for 60kg patient (grams (g))	Response rate, response as defined by the study.	Toxicity marker	Toxicity
ELNT, 2002 [37]	MP 3X 750mg OP Start dose 0.5-1mg/kg/day Maintenance dose 5-7.5mg until month 30	3.15	71% low dose CyP, 54% high dose CyP (over median 41 months follow-up)	Infections	44 events (27 patients) in patients.
ALMS, 2009 [94]	OP Start dose 60mg/day OP Mean dose 26mg/day (n=364)	4.3	56.2% MMF group, 53% CyP group (24 weeks)	Infections	130 events in 364 patients
RITUXILUP 2013 [35]	MP 2X 500mg	No OP	72% (11-58 weeks)	Adverse events	18% had serious adverse e with inpatient stay.
BLISS-LN 2020 [58]	MP 1-3X 500-1000mg OP Start dose 0.5-1mg/kg/day OP wean to 10mg by week 24	4.55	43% Belimumab group, 32% Placebo group at week 104.	Infections	141 patients in 448 patien
AURORA-1 2021 [46]	MP 2X 250-500mg OP start dose 20-25mg/day OP wean to 2.5mg/day by week 16	1.33	41% Voclosporin group, 23% Placebo group at week 52.	Infections	254 events in 356 patients
NOBILITY 2022 [54]	MP 1000-3320mg OP Start dose 0.5mg/kg/day OP wean to 7.5mg/day by week 12	2.45	35% Obinutuzumab, 23% Placebo group at week 52.	Infections	102 patients in 125 patien

Studies with comparison of different steroid doses								
Study	Steroid dose 'low dose' cohort (n=number of patients	Steroid dose 'high/standard dose' cohort (n=number of patients)	Response rate low dose	Response rate high /standard dose	p value	Toxicity marker	Toxicity low dose	Toxicity standard
Edwards, JC *et al.*[89]	300mg MP (n=10)	3000mg MP (n=11)	2/11 (18%) 'ideal' response	1/10 (10%) 'ideal' response	No data		No data	No data
Badsha H, *et al.*[88]	1-1.5g MP (n=26)	3-5g MP (n=29)	SLEDAI score reduced (6months) 19-> 2	SLEDAI score reduced (6 months) 20 to 0	No data	Serious infections	9 events in 7 patients	20 event 17patien
Fischer-Betz, R. *et al*.[90]	<20mg OP OD (n=21)	>20mg OP OD (n=19)	CR (24months) 71.4%	CR (24months) 52.6%	0.37	Infections	11 patients in 21 patients	12 patie 19 patie
MYLUPUS Zeher, M. *et al*.[92]	0.5mg/kg (n=39)	1mg/kg (n=42)	CR (24weeks) 20.5%	CR (24weeks) 19.0%	0.87	Infections	14 patients in 39 patients	24 patie 42 patie
Ruiz-Irastorza, G. *et al*.[91]	22mg/day mean (n=15)	49mg/day mean (n=30)	CR (12months) 47%	CR (12months) 30%	0.38	Toxicity due to glucocorticoids	1 patient in 15 patients	20 patie 30 patie

Table 1.2 Steroid dosing in key lupus nephritis studies [32]

Total OP dose over 24 weeks for 60kg patient is estimated based on protocol information and standard wean schedules. Table reprinted, amended and expanded from original publication 'Can we manage lupus nephritis without chronic corticosteroids administration? Lightstone L, Doria A, Wilson H, et al. Autoimmun Rev 2017[32]. ELNT – Euro Lupus Nephritis Trial; ALMS - Aspreva Lupus Management Study; RITUXILUP – Trial of Rituximab and Mycophenolate Mofetil Without Oral Steroids for Lupus Nephritis; BLISS-LN – Belimumab International Study in Lupus Nephritis; AURORA-1 – Aurinia Renal Response in Active Lupus With Voclosporin; NOBILITY - B-cell depletion with obinutuzumab for the treatment of proliferative lupus nephritis: a randomised, double-blind, placebo-controlled trial ; MYLUPUS - Efficacy and safety of enteric-coated mycophenolate sodium in combination with two glucocorticoid regimens for the treatment of active lupus nephritis. (MP=Methylprednisolone, OP=Oral Prednisolone.)

1.1.5 What are we trying to achieve with treatment?

In reviewing the management of lupus nephritis, it is important to consider exactly what we are trying to achieve with treatment. Certainly, preservation of both life and renal function. Hans-Joachim Anders displays clearly the nephron loss associated with a single episode of LN and with persisting LN in figure 1.2 (Image reprinted from 'Lupus Nephritis', Hans-Joachim Anders *et al.*, 2020)[95], demonstrating beautifully the need to achieve prompt renal response to LN flares to prevent ESKD.

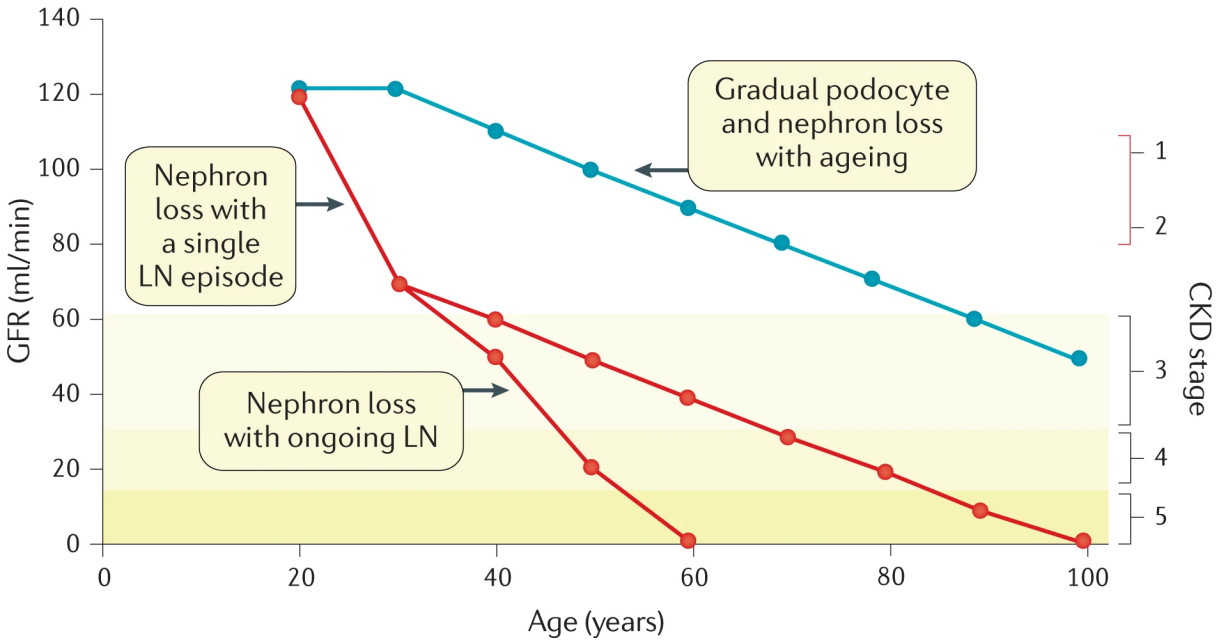


Figure 1.2 Nephron loss associated with a single episode of LN compared with persisting LN and usual ageing. Image reproduced with permission from Springer Nature from 'Lupus Nephritis', Hans-Joachim Anders et al., Nature Reviews Disease Primers, 2020.[95]**.**

Originally adapted from Anders, H.-J. & Rovin, B. A pathophysiology-based approach to the diagnosis and treatment of lupus nephritis. Kidney Int. 90, 493–501 (2016) [96].

However, as treatment has moved towards long term management of a more chronic condition and the rate of relapses remains relatively frequent, it is equally important to ensure a good quality of life and to minimise treatment-related toxicity, including the preservation of fertility. This requires rational therapy, which ideally would be targeted to modify specific pathophysiological dysfunctions. One key way to minimise treatment related toxicity is to minimise steroid exposure. Improving adherence to medication is also crucial, and again minimising steroids is very likely to assist this.[32]

Specific long term renal endpoints are preservation of renal function and avoiding end stage kidney disease. However, such endpoints are inconvenient in the practical timescale of the average controlled trial of treatments for LN and thus reliable proxies need to be determined.[32]

With current advances in personalised medicine, we may see a change in approach to treatment in the coming years with therapeutics tailored to specific pathogenic mechanisms of injury identified by 'omics analysis of biopsies, blood and/or urine.[26]

1.1.6 Response definitions and predictors

There is no single agreed definition of response to treatment. However, definitions do have some consensus.[34] Definitions all include reference to renal function and proteinuria with some also including urinary sediment. Guidelines generally agree that proteinuria of less than 0.5g/24hours or a urine protein creatinine ratio (uPCR) of less than 50mg/mmol as well as a near normal renal function constitute complete response. The 2021 KDIGO guidelines [41] update continues to recommend this definition however the EULAR guidelines update has relaxed the proteinuria target to <0.5-0.7g/24 hours.[36] Definitions discuss renal function in terms of 'normal', a 'return to baseline', a 'return to baseline ±15-25%' [35][34], 'stabilisation or improvement (…) ±10-15% of baseline' [41] or occasionally in terms of exact figures, for example, the Spanish Guidelines from the 'systemic autoimmune disease group of the Spanish Society of Internal Medicine and Spanish Society of Nephrology' (GEAS) [19] have the criteria of a serum creatinine <1.2mg/dL (106µmol/L). A serum albumin of >30g/dL is also required by GEAS as well as inactive urinary sediment (<5 red blood cells/high powered field (HPF), <5leucocytes/HPF and no casts), the latter also being required by the North American Childhood Arthritis and Rheumatology Research Alliance (CARRA).[97] In some cases, definitions of response in clinical trials have also included oral steroid dose limits.[98] Definitions of partial response (PR) also require a normalisation or 'stabilisation' of renal function, usually within 25% of baseline values. The renal function requirement was more lenient for PR than CR in previous KDIGO [99] and EULAR guidelines.[100] However updates from both bodies now require renal function to be within 10% [40] or 10-15% [41] of baseline estimated glomerular filtration rate (eGFR) for both CR and PR highlighting learning from research including that of this thesis [101,102] regarding the importance of preservation of excellent renal function for good long term outcomes. A decrease in proteinuria ≥50% and non-nephrotic is generally agreed upon. Non-response (NR) is generally understood to mean that the criteria for complete or partial response have not been met by 12 months following initiation of treatment.

Until recently, there was no clear definition of remission in SLE either, with studies using their own definitions and parameters.[103] However, the DORIS definition of remission in SLE was published in 2021 providing recommendations for a universal definition.[104] The DORIS taskforce crucially involved patient representatives, the need for which was particularly highlighted by a survey which showed that doctors can classify their patients as being 'in remission' despite significant continuing symptoms.[105]

Definitions of response used in guidelines, analysis of historical data and perhaps most importantly, clinical trials, are of critical importance as demonstrated by Wofsy *et al.* [106], when re-analysis of the abatacept in lupus nephritis trial (IM101075) identified that an alternative definition of complete response would lead to a different conclusion for the trial with the most striking difference giving a CR of 6% in the control group and 22-24% in the treatment groups in contrast to the initial study findings which suggested no difference.[106] In an era when many therapies which have promising results in phase 1 and 2 studies are failing to meet their primary endpoints in phase 3 clinical trials,[107–109] getting the definition of response (as well as ensuring the treatment is hitting its target) and therefore the endpoint right is critical. This has led to analyses of investigator and pharma-led clinical trials in SLE to identify the barriers to drug development.[110][111]

An international task force compiled 12 recommendations for "treating-to-target" in SLE published in 2014.[79] These included remission and prevention of damage accrual and interestingly also issues of health-related quality of life (HRQoL).[79] Achievement of a lupus low disease activity state (LLDAS) has also been assessed in a prospective cohort study where it was found to be 'associated with … protection against flare and damage accrual in SLE'.[112] It was therefore suggested that LLDAS would be a valuable endpoint for clinical studies.[112]

Response definitions used in this thesis are discussed in the methods.

1.1.6.1 Predictors of response

Data from the Aspreva Lupus Management Study (ALMS) trial (370 patients) was studied to identify predictors of response to treatment. Here, they identified that a higher proteinuria at baseline and longer duration of LN were associated with a lower chance of remission at six months. However, the area under the curve (AUC) for CR was 0.55, indicating relatively poor performance of the model as a predictor of response.[113] A further review of 212 patients treated between 1970 and 2011 identified that proteinuria at baseline and longer duration of LN were associated with less chance of recovery and longer time to recovery in those who did. Male sex and low serum complement were

also associated.[114] Using machine learning and a combination of clinical parameters and urine biomarkers, Ayoub *et al*. identified several candidate models for predictors of response. Commonly featured clinical predictors were race, sex, complement C3 and C4, dsDNA, eGFR at baseline and uPCR at baseline.[115]

1.1.7 Outcomes and predictors of outcomes

Current therapies induce complete response in approximately 30-50% of patients with lupus nephritis, identifying a clear need for improvements in our treatment of LN.[99] One result of the sub-optimal response to immunosuppressive treatments is increased risk of poor long-term outcomes. LN carries a significant risk of morbidity and mortality [2,116] [1][117] with up to 25% of patients with LN developing ESKD [3] and evidence of a 6-9-fold increase in mortality.[5] [118] Achieving even partial response has been shown to improve long term outcomes of ESKD and death compared to NR.[119] Tektonidou *et al.* identified those with class IV having the greatest risk of ESKD with a 15 year risk of 44% for those diagnosed in the 2000's whereas class V had a 20% 15 year risk when diagnosed during the same period.[4] Outcomes are worse in Black and Hispanic patients, including death and ESKD.[120] This could be due to more severe disease in Black patients including higher incidence of proliferative disease [121] which in turn could be due to a genetic preponderance for more severe disease. For example, the apolipoprotein L1 (*APOL1*) gene, 2 common variants of which only present in people of African ancestry, is responsible for much of the overall excess risk of chronic kidney disease (CKD) and ESKD in Black patients [122] and is also associated specifically with ESKD in LN.[123][124]

The effect of histological class on long term outcomes has previously been discussed. In a review of 499 patients diagnosed with LN between 1970 and 2016, Moroni *et al.* also identified male sex, arterial hypertension, no maintenance immunosuppression, renal function at baseline and activity and chronicity indices on the biopsy to each independently predict ESKD on multivariate analysis.[3]

Recognising and initiating treatment early in LN has been shown to improve management and response.[125][6] Delay ranging from only 3-5 months in treating LN has been shown to reduce chances of remission and increase flares as well as the risk of ESKD.[45]

Significant work is being undertaken to establish standardised outcomes in glomerular diseases for clinical trials involving both patients and healthcare professionals.[126] One cause of the poor response and outcome in some patients could simply be that the treatments are targeted at immune

mechanisms not as relevant to their specific key disease pathogenic pathways. Given the heterogeneous pathogenesis of LN, treatments targeted to the individual may well improve outcomes as well as reducing drug-related side effects.

1.1.7.1 Predictors of outcomes

Until recently, the key endpoint of complete renal remission was varyingly defined but most commonly determined by preservation of normal renal function (a difficult target given the likelihood that a 'baseline' renal function may well not be known in a previously healthy young patient) and proteinuria equivalent to less than 0.5g/day (urine PCR ≤ 50mg/mmol).[32] However, recent analyses of datasets from clinical trials where there is long term follow up have identified that this may well be too stringent to define those who do well from a renal perspective in the long term.[32] Dall'Era M *et al*., using the Euro-Lupus Nephritis cohort identified proteinuria of <0.8g/day at 12 months to be the best predictor of a good long-term renal outcome, as defined by creatinine <1.0mg/dl at 7 years.[127][32] Long term follow-up of the MAINTAIN trial found a similar value of 0.7g/day proteinuria at 12 months to best predict long-term renal outcome.[128][32] The FDA have yet to approve these as 1 year targets in new trials but many studies are now including these as secondary endpoints or in post hoc analysis[54] or even as the primary endpoint in the case of BLISS-LN [58] which used a threshold of uPCR <0.7.[32] The EULAR 2019 guidelines update for treatment of SLE noted the evidence that a proteinuria of ≤0.8g/day at 12 months is predictive of good long term outcomes from the Euro-Lupus data.[127] [40] The updated guidelines for LN have changed the definition of complete response to proteinuria <0.5-0.7 g/24 hours with '[near-]normal' eGFR.[36] The move to increasing the proteinuria target is as a result of a body of research both discussed here and inclusive of this thesis [101,102] as referenced as evidence of validation of the thresholds in a more diverse cohort by the team who validated the 2-year targets in the BLISS-LN trial.[59] This body of research demonstrates this higher threshold as better predictive of good long term outcomes. Ayoub *et al.* used the threshold of <0.8g/day proteinuria as a 'relaxed' definition of response in their study of predictors of response where it performed similarly to the more traditional threshold of <0.5g/d.[115]

Both proteinuria and renal function at 12 months were found by Mackay *et al.* to be predictive of CKD development at follow-up (12-48 months from initial presentation) using multivariable Cox proportional hazards regression.[129] In an effort to identify predictors of long-term outcomes: CKD; severe acute kidney injury (SKI); and the need for renal replacement therapy (RRT), Mackay *et al.* brought together data from 12 lupus cohorts and 3 RCTs (totalling 944 patients). Analysis was

performed on 550 patients after inclusion and exclusion criteria were applied. These patients were 51% White and all were treated with glucocorticoids.[129].

Individual features of chronic damage on the renal biopsy, as well as the chronicity index as a whole, have also been shown to be predictive of poor outcome in retrospective observational cohort studies including fibrous crescents and interstitial fibrosis and tubular atrophy.[130] The value of the chronicity index in predicting long-term outcomes was however not confirmed by the Lupus Nephritis Collaborative Study Group.[131] In this study, four renal pathologists independently scored biopsies showing proliferative LN for activity and chronicity scores and evaluated the results for predicting outcomes which were measured for a mean of just over 2 years. Neither in using receiver operating characteristics (ROC) curve or contingency analysis were activity or chronicity scores of value in predicting outcomes.[131] These data are however from 1989 and describe very severe disease. Subsequent studies have however been inconsistent in their findings of the utility of the indices [132] and therefore the ISN/RPS 2018 review of the classification suggests further evaluation.[28]

1.2 BIOMARKERS IN LUPUS NEPHRITIS

Although many of the known mediators of disease and inflammation have been quantitatively assessed in lupus nephritis, including anti dsDNA, anti-nucleosomes, anti-C1q, C3 and C5b-9, many of which are routinely measured, none have proven to correlate reliably with disease activity or prognosis and thus serve as a useful biomarker. Conventional markers such as renal function are also unable to differentiate activity of the LN from damage. Renal biopsy is the current gold standard for diagnosis and classification of lupus nephritis. Repeat biopsies are required in the event of a renal flare suspected from monitoring of clinical markers.[34] There is also mounting evidence that repeat renal biopsies are useful to monitor response to treatment, even when clinical remission has been achieved.[133] However, renal biopsy is an invasive procedure which carries a not inconsequential risk of bleeding [134][135][136] with one in 10 patients having some form of bleeding and one in 1500 requiring some form of investigation or intervention for bleeding.[137,138] It is therefore important that useful biomarkers, specific to SLE which can provide a quantitative indication of disease activity early in the process are identified in patient serum, plasma and urine as fluctuations in these can be much more easily and frequently monitored. Additionally, biomarkers better predictive of prognosis are required. This has proven difficult thus far in LN, possibly due to the heterogeneous nature of SLE and LN. As there are many routes to the manifestations of SLE, deficiencies or overactivation of one or other pathway will not be present in all patients. Because of this, it may be that a panel of biomarkers, perhaps coupled with a statistically derived score, is more useful than a single biomarker.

1.2.1 Existing candidates for biomarkers

Currently, in clinical practice, the key biomarkers used are: serum creatinine for renal function, spot urine PCR or 24hr urine protein for proteinuria, and in renal biopsies, immunoglobulin (Ig) deposition and complement markers C1q, C3 and C4 in renal tissue using immunohistochemistry.

Proteinuria is usually quantified using a spot urine protein-creatinine ratio (uPCR), however a uPCR from a 24-hour urine collection can provide a more accurate result but this is not practical to do on a frequent basis and is subject to collection errors. As well as errors in collection, proteinuria is also affected by coexistent infection, renal function, chronic glomerular scarring and comorbidities.

Additional regularly measured markers including C3, C4 and dsDNA in serum have not been proven to consistently correlate with disease activity or predict prognosis. A new biomarker needs to be

superior to those in current use at representing LN activity to the degree of the gold standard (renal biopsy) and predicting prognosis.

Several serum and urine biomarkers have been identified over recent years as candidate biomarkers.[139] None of these have as yet made it into mainstream practice, possibly due to the need for further validation, particularly in prospective studies where serial monitoring can be undertaken and correlation with activity can be investigated. Interesting candidates include pro-inflammatory cytokines, such as IL-6,[140] IL-16,[141] (urinary IL-16 correlating with response to treatment) and IL-17,[142] pro-inflammatory chemokines including urine MCP-1 [143,144] which has been shown to predict flares and correlate with disease activity [145,146] and CXCL11 (interferon-inducible T-cell alpha chemoattractant (I-TAC)),[147] adhesion molecules such as VCAM-1 [148] and growth factors such as transforming growth factor-beta (TGF-B), shown to be high in non-responders.[146][149][139] Urine soluble CD163, a protein released from macrophages has been identified in cross-sectional and longitudinal measurements to correlate with disease severity in LN as well as histological activity tracking with response to treatment.[150,151] Ayoub *et al*. recently reviewed the literature for urine biomarkers to use with clinical data in the production of models to predict response to treatment using machine learning. Their literature search revealed 283 original articles published over 10 years. The best performing biomarkers in the models were CXCL8, PTX3, CCL2, adiponectin and EGF.[115]

1.2.2 Complement in lupus nephritis

Complement levels and activity are common markers of disease activity in SLE and have also been shown to identify patients at risk of active class III and IV lupus nephritis.[152] 'Full house' staining on kidney biopsies, the co-localisation of IgG, IgA and IgM with C1q, C3 and C4, is the hallmark of LN.[153] Complement is widely accepted to play a significant role in the pathogenesis of LN, in particular through activation of the classical pathway by ICs,[20] but also through both lectin and alternative pathway activation.[154] The complement pathway, displayed in figure 1.3 is part of the innate immune system. The classical pathway is activated by C1 binding to IgM or IgG in ICs. The activated C1 cleaves C2 and C4, each into a and b components. In combination, C4b2a then acts as a C3 convertase which activates C3 through cleavage. The alternative pathway is activated by C3 binding of factor B. Through cleavage by factor D, a C3 convertase is formed which ultimately forms C3bBb, the alternative pathway C3 convertase. The lectin pathway is activated by the binding of mannose binding lectin (MBL) to carbohydrate groups on microbes or apoptotic material.[155]

Early complement components (C1q, C2 and C4) have roles in clearance of ICs and apoptotic cells. Consequently, deficiency in these proteins can be a risk factor for lupus nephritis.[155] When C1q is absent, ICs associate with plasmacytoid dendritic cells, generating IFN alpha. C1q also slows clearance of neutrophil extracellular traps (NETs) thus allowing them to promote type I IFN production.[156]

Conversely, the latter components of the complement cascade have been shown to be destructive in SLE and LN. Complement C3 is the central activation protein of the complement cascade and its activation results in the production of anaphylatoxin (C3a) and opsonin (C3b) and can trigger C5 activation. Complement C3c and C3d are formed by cleavage of C3b. C5 activation results in C5a, a chemoattractant recruiting inflammatory cells and an anaphylatoxin and membrane attack complex (MAC, C5b-9) which can damage cell membranes and induce erythrocyte lysis.

Factors involved in the alternative pathway have been shown to correlate with disease activity in LN.[154] Factor H, a major regulator of the alternative pathway is known to function by acting as a co-factor for Factor I mediated cleavage and inactivation of C3b and decay of C3-convertase, C3bBb. Deficiency in LN has been shown to be related to disease severity.[157] Deposition of factor H and properdin appear to be markers of activity in LN [154] and dysfunctions of factor H including clearance of apoptotic cells being identified in some patients with LN.[158] Factor H related protein 5 (FHR-5) works as an antagonist to factor H and high levels have been demonstrated in patients with LN.[159]

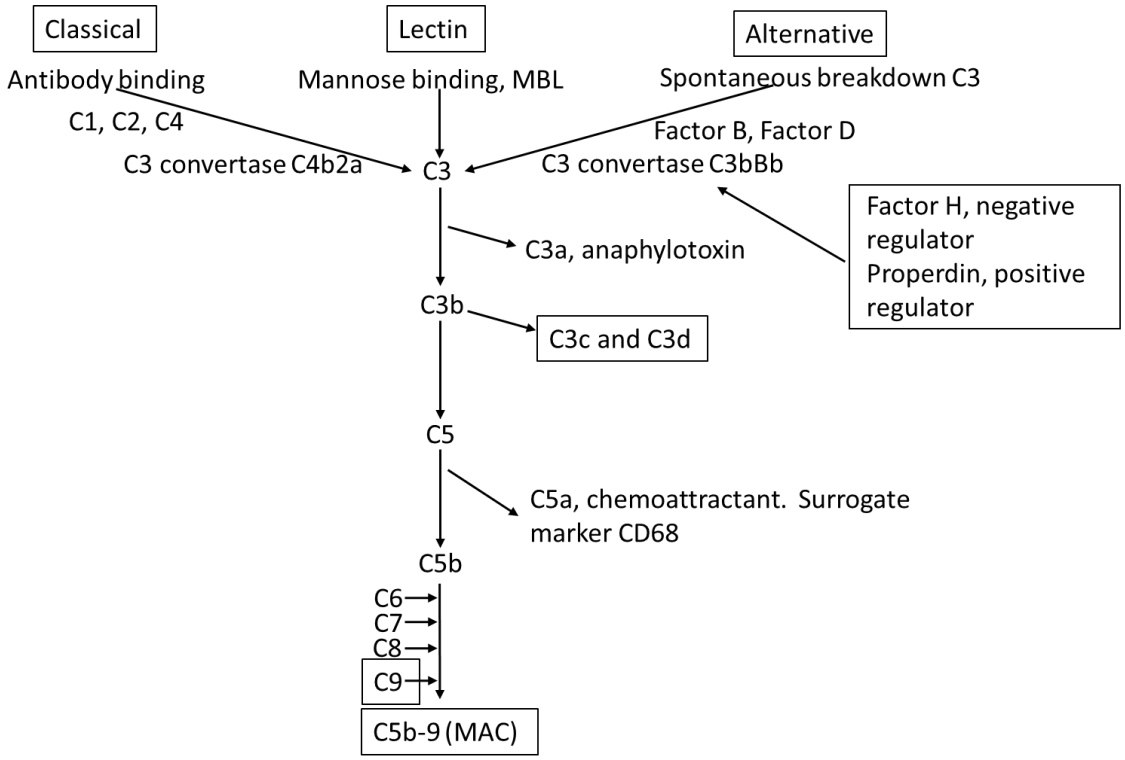


Figure 1.3 Figure displaying key elements of the complement cascade.

Targeting of components of the complement pathway including the alternative pathway by reducing factor B levels [160] and the terminal pathway by blocking C5 cleavage [65] have proven successful at treating murine SLE. Complement inhibitors are now being used to treat autoimmune disease, Avacopan, a C5a receptor inhibitor is approved by the FDA [161] and recommended by NICE [162] for treating ANCA associated vasculitis. Studies are also underway and recruiting, investigating targeting of the complement pathway in LN.[67][163] Thus highlighting the importance of understanding the role of complement in LN.

1.3 SUBVISUAL ANALYSIS OF THE RENAL BIOPSY

As previously noted, LN is diagnosed and classified on renal biopsy [164] which includes light microscopy, immunohistochemistry and electron microscopy. However, whilst the histological changes visible on microscopy are limited in number, the pathogenic mechanisms of injury which cause these changes are much more diverse.[165][166][167] [168] Thrombotic microangiopathy for example, is ultimately caused by damage to the endothelium which results in thrombosis of small vessels. This can be the result however of multiple different pathogenic pathways, including ADAM metallopeptidase with thrombospondin type 1 motif 13 (*ADAMTS13*) deficiency (genetic or associated with autoimmunity), drug-related damage and complement alternative pathway activation.[169] Other histopathological changes classic to LN can similarly be caused by combinations of activation or downregulation of multiple pathogenic pathways. This is in part because of the great heterogeneity in pathogenesis of lupus nephritis.[166] Specific pathogenic pathway disruption cannot therefore be determined from the biopsy alone. In addition, it cannot be determined from the biopsy, or indeed clinical presentation, the likelihood of response to treatment or prognosis, or specifically which treatments are most likely to be of benefit.

Molecular profiling of the kidney biopsy can identify and characterise pathogenic mechanisms of injury [165] allowing for prediction of response to therapy and individualisation of treatment, targeted to specific pathogenic mechanisms.

Biomarker discovery has largely focussed on identification of candidate biomarkers, selected for investigation due to identified roles in LN pathogenesis. An alternative approach, increasing in use over the past decade, is the exploratory approach where large arrays of, or all identifiable, molecular markers are reviewed without bias, using techniques such as transcriptomics or proteomics.

1.3.1 RNA analysis for identification of biomarkers

Molecular profiling using transcriptomics has been shown in LN to identify and characterise pathogenic mechanisms of injury. Gilmore *et al.* identified 52 genes common to all LN classes, differentially expressed compared to control, with enrichment for type 1 IFN, complement and Major Histocompatibility Complex (MHC) II pathways using the Nanostring ncounter platform.[170] Parikh *et al.*, using the same technology, demonstrated variation in transcript expression in baseline flare biopsies from patients with lupus nephritis who went on to be NR to standard treatment compared to those who went into CR.[168] Principal component analysis showed separation of CR

and NR. Their data suggested greater expression of interferon pathway genes in CR and complement genes in those who did not respond to treatment. Classical treatment approaches do not necessarily target complement pathways. The same group have published further data looking at changes in transcript expression between serial biopsies in treatment responders and non-responders. They identified that type I and II interferon pathways, the alternative complement pathway and T cell signalling pathways appear to be different in CR and NR.[171]

1.3.2 Proteomics

RNA profiles do not however provide the full picture [172] as the number of proteins far outweighs the number of coding genes due to alternative splicing and post-translational modifications.[173] mRNA abundance has been shown to be a poor predictor of protein abundance when protein was analysed using antibody microarrays,[174] two-dimensional electrophoresis [175] or mass spectrometry.[176] Post-translational modifications are regarded as of greater importance than the transcription and translation of the proteins themselves due to their influence on function.[177] Proteins, in comparison to RNA, also provide a more direct indication of the pathogenic pathways that are active. In addition, they are often the direct target of drug therapies. Tissue proteomics therefore can provide potentially the most reliable diagnostic and prognostic markers for lupus nephritis.

Protein identification and quantification can be achieved with antibody-based microassays, immunohistochemistry (IHC) panels and two-dimensional gel electrophoresis (2D-PAGE) which separates up to 10,000 proteins according to charge and mass. These techniques however can only look for a limited number of pre-determined proteins in the case of microarrays and IHC. 2D-PAGE requires mass spectrometry for protein identification in the absence of prior identification and annotation and is limited, missing low abundance proteins.[178]

1.3.3 Mass spectrometry

Mass spectrometry (MS) is the method of choice for protein identification due to the volume of data which can be obtained from relatively small biological samples. The technique has developed at a dramatic pace over recent years with the novel publication of single nephron proteomics.[179]

The most common process used in mass spectrometry involves proteolytic digestion of a substrate with trypsin which cleaves c-terminal arginine and lysine. This substrate can be a mixture of proteins

which can be excised from gel following separation and subjected to in-gel digest. Alternatively, whole tissue, dissected sections of tissue or other substrates such as serum, plasma or urine can be processed and digested with trypsin. Digestion forms peptides, which are ionised and the ions produced detected and sorted based on their mass-to-charge (m/z) ratio. The spectrometer selects precursor peptide ions for fragmentation. The mass spectra produced from this fragmentation are used to determine the amino acid sequence of the peptide. Bioinformatics software is then used to identify and quantify the proteins in the sample from the peptides detected. This process, where proteins are identified and quantified using specific peptide sequences, is known as 'bottom-up' proteomics. 'Top-down' proteomics conversely, involves the identification and quantification of specific intact proteins. Purified proteins are directly fragmented to obtain sequence data for identification. 'Bottom-up' proteomics involves inferring of proteins which could be inaccurate due to lack of peptide specificity. 'Top-down' proteomics measures both the intact and fragment ion masses resulting in a more reliable characterisation of proteoforms.[180] However, there are many technical challenges for 'top-down' proteomics including the poor solubility of proteins, the reduced sensitivity of MS to proteins and reduced throughput of MS with proteins.[181] This has limited 'top-down' proteomics to characterisation of single proteins or mixtures of a limited number of proteins.[182]

1.3.3.1 Discovery 'shotgun' and targeted proteomics

There are two major mass spectrometry techniques used for 'bottom-up' protein analysis, following the extraction of proteins from a sample, lysis of the proteins into peptides and their separation by gel or liquid chromatography and ionisation.[183] Shotgun proteomics is a technique where the mass spectrometer selects the most abundant peptide ions from a survey scan to fragment and then records their fragmented ion spectra at selected time points in a second mass analyser – tandem mass spectrometry. This technique can identify thousands of peptides per sample and is the method of choice for discovery proteomics, to discover the maximum number of proteins from a sample. Shotgun proteomics is however poorly reproducible, can miss peptides of low abundance and quantification of the peptides is of poor accuracy.[184]

An alternative technique is known as selected or multiple reaction monitoring (SRM/MRM). In this technique, specific peptides are identified in a sample over a continuous time period. This technique is highly reproducible and quantification of the abundance of the protein is much more accurate; however, the peptides for study need to be predetermined and the number which can be assessed is limited to approximately 100 per sample.[183]

1.3.4 Quantitative proteomics

Quantifying proteins using SRM is widely accepted to be highly accurate. Quantification of proteins in shotgun proteomics is however more difficult. The most common technique for quantification is spectral counting.[185][186] Spectral counting is label-free and is therefore easier to use than previously standard label-based quantification techniques which involve stable-isotope labelling with chemical modification of proteins, for example with isotope coded affinity tag (ICAT)[187], or peptides, with multiplexed isobaric tagging technology (iTRAQ) for example.[188][185] Labelling strategies are more accurate than label-free,[189] however are more expensive and require a high level of expertise.[190] Most importantly, when using proteomics for the purposes of biomarker discovery from human samples, labelling of tissues, especially human clinical samples, is not always possible.[189] Spectral counting essentially involves using a count of the number of fragmentation spectra for peptides of a specific protein to estimate abundance of that protein.[185] As a useful technique in human tissue, spectral counting has been used as a method of quantification in several studies involving human renal tissue including by Satoskar and colleagues when examining fresh frozen tissue to compare diabetic nephropathy (DN) with fibronectin glomerulopathy, LN and healthy controls (HC)[191] **a**nd by Sethi *et al*., when investigating C3 glomerulopathy.[192]

Spectral counting is however a semi-quantitative technique which is particularly problematic at measuring low abundance proteins. Additional issues involve quantification of short proteins, which is more variable and the range of detectable protein expression which is limited due to saturation effects at higher protein concentrations.[186] Larger proteins, producing more peptides will produce higher spectral counts than smaller proteins of equal abundance.[186] Methods can be employed to improve the accuracy, for example, in the study by Satoskar *et al.* spectral counts were normalised by linear scaling such that total spectral counts were equal,[191] however a large amount of quantitative information is still lost.

1.3.5 Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS)

As standard techniques do not reliably quantify unspecified peptides or proteins, especially those of low abundance, a technique called sequential window acquisition of all theoretical fragment ion spectra (SWATH)-MS has been developed which allows for more accurate quantification of all proteins in a sample. SWATH-MS is a relatively new technique for data acquisition described in

2012.[193] It combines the accuracy of SRM acquisition with the spread of shotgun or 'discovery' proteomics by recording fragment ion spectra of all precursor ions detectable throughout the entire mass range. It does this by fragmenting all precursor peptides generated from a sample which are within a certain mass-to-charge ratio (m/z) and retention time range. The machine cycles through the m/z range in segments of specified width or 'windows', producing a fragment ion spectrum which includes fragment ions of all precursor ions, a record which can be interrogated repeatedly as bioinformatics progresses.[191][194][183] This complete record can be produced from biopsy-level tissue samples.[195]

Shotgun proteomics is known as data dependent acquisition (DDA) as the instrument initially performs a short cycle where it runs a survey scan of parent ions identifying the m/z and intensity of these parent ions. Subsequent MS/MS scans involve fragmentation of these identified parent ions, generally selecting in order of abundance. SWATH-MS does not however require this initial survey scan and is therefore known as data independent acquisition (DIA). All parent ions within a certain m/z range or 'window' are fragmented and the fragments detected together.

1.3.6 Frozen versus formalin-fixed and paraffin-embedded tissue

Formalin-fixation and paraffin-embedding (FFPE) of tissue samples is a technique which allows the storage of biopsy samples for a number of years. The result of this is that many institutions, including our own, now house an incredibly informative, yet until recently, untapped resource for retrospective study using molecular profiling techniques. As recently as 2015 it was declared that proteins are not extractable from FFPE samples,[196] although studies pre-dating this review had explored the use of FFPE tissue. The particular difficulty is the extraction of proteins from FFPE samples, due to the formalin-induced cross-linking. Newer protein extraction techniques validated in several recent publications have however enabled the use of these samples.[197][198][199][200][201] In particular, heat-mediated retrieval techniques have tackled the issues with formalin-induced cross-linking of proteins. Fowler *et al.* demonstrated the increased protein yield using pressure in addition to heat in FFPE mouse tissue.[199] The pressure improves the hydration of the protein as well as improving the ability of the proteins to get into solution, thus allowing for hydrolysis of the formaldehyde cross-links.[202]

Proteomic analysis of FFPE tissue has in fact been described since 2005,[203] where Palmer-Toy *et al.* demonstrated similar peptide identification in frozen and FFPE tissue although with 20% proteins identified unique to frozen tissue and 40% unique to FFPE.[203] This was later studied in 2009 [198]

where Scicchitano *et al.* demonstrated that FFPE tissue identified the same number of proteins as frozen tissue despite protein yields from FFPE tissue being slightly smaller.[198] Further studies have shown comparative findings from frozen and FFPE tissue [198][204] and there are now a great number of studies which have used FFPE tissue to produce quantified protein data using MS [205][206][207][208][209][210] with validation using IHC.[211]

Preanalytical factors including tissue processing and fixation as well as storage time and conditions are known to have an impact on proteomic analysis of FFPE tissue.[201][212][206] In fact, in a comparison of murine FFPE and frozen tissue, a coefficient of variation of 43% was identified in FFPE tissue, 31% being related to pre-analytical factors and the remaining 12% related to the MS analysis.[213] In a study by Sprung *et al.* [214] looking at clear cell renal carcinoma, MRM analysis of 1982 FFPE and 2154 frozen samples found a coefficient of variation 18-20% in both tissue types, however, although the 104 of 114 targeted peptides were detectable in both FFPE and frozen tissue, signal intensities were reduced by 34% in the FFPE tissue.[214]

Pre-analytical techniques vary between studies and indeed, in long-stored samples, may not be known. In a study of diabetic nephropathy, tissue stored for three to seven days in formaldehyde prior to paraffin embedding and further processing did not yield sufficient protein for individual analysis, calculated to be 5µg.[215]

There is no current consensus on protein extraction technique for FFPE tissue [200] however the extraction buffer has been identified to play a role in variation in identity and quantity of proteins detected.[213] A recent review [216] attempted to develop a standardised extraction protocol from FFPE tissue by reviewing multiple steps of the protocol including buffers and heating conditions. It concluded that FFPE tissue is suitable for biomarker detection, even at very small amounts of laser dissected tissue. It also concluded that an aggressive disaggregation method was required for protein extraction. It did not however trial the use of elevated pressure.[216]

Thus FFPE tissue is currently used routinely in clinical studies investigating proteins using mass spectrometry such as in disease typing in amyloidosis,[217] where congo-red positive regions of FFPE renal samples are analysed, and in biomarker discovery.[192]

1.3.7 Pressure cycling technology (PCT)

Following on from the demonstration of pressure increasing protein yield in mouse FFPE liver tissue [199] and human FFPE aorta,[218] Guo *et al.* have demonstrated the benefit of PCT in increasing

protein yield in frozen tissue [195][219] and more recently, in FFPE prostate and lymphoma tissue.[220] The same team have also recently published an accelerated technique for FFPE PCT SWATH using hepatocellular carcinoma tissue [221] where they combine steps in the PCT protein extraction and in prostate tissue [222] where they demonstrate an accelerated SWATH technique. PCT essentially applies high pressure (up to 45,000 psi) to the tissue sample alternating with ambient pressure (14.7 psi). The changes in pressure applied are rapid: rise in 3 seconds and fall in milliseconds. It is designed to promote de-crosslinking, extraction and trypsin digestion of proteins from FFPE tissue.[220] The pressure helps the protein go into solution, therefore improving the denaturation and enzyme hydrolysis.[195,219] The cycling pressure is applied at multiple steps of the protocol: tissue lysis and protein extraction and enzymatic digestion. The length of time spent at high pressure per cycle and the number of cycles can be varied. Shao at al also demonstrated the increased protein yield using a 'MicroPestle' [219] which inserts into the micro-tube containing the sample in comparison to simply applying a cap to the tube around which the pressure is applied.

Uchida *et al.* have since used PCT in FFPE mouse liver along with SWATH-MS demonstrating that the technique can identify the same changes in protein expression related to disease as detected in fresh tissue.[220]

1.3.8 Proteomics in urine and serum in LN

The urine proteome, known as the 'liquid biopsy' of the kidney [223] has been studied extensively for biomarkers of renal disease and specifically LN.[224] In terms of biomarkers for LN, prostaglandin H_2D-isomerase (PGDS) has been shown to be elevated in active LN in comparison to inactive LN, disease controls (glomerular disease other than LN) and healthy controls [225] and in a panel of urinary proteins in paediatric patients with LN [226] and is of interest due to its involvement in the inflammatory response where it catalyses the isomerisation of prostaglandin H2 (PGH2) to produce prostaglandin D2 (PGD2) which is involved in the inflammatory response and acts as a chemoattractant.[225] It was also noted to have no correlation with eGFR.[225] Kwon *et al.* recently used SWATH-MS to examine urine of LN patients and identified several possible biomarkers, the most promising being alpha-1-acid glycoprotein (ORM1), of value in diagnosing early LN as it was identified in patients with non-significant proteinuria (uPCR <500mg/g / <50mg/mmol).[227]

Proteomics has also been employed in the serum in the effort to identify useful biomarkers in lupus nephritis.[139] Serum IGFBP2 has been found to differentiate SLE patients from healthy controls [228] as well as differentiating active LN from inactive LN and inactive SLE, performing better than

anti ds-DNA or C3 in identifying activity in regards to specificity.[229] It was then validated as a marker of disease activity and chronicity.[230] Furthermore, it has been shown in longitudinal studies to correlate with response.[231][139]

Nicolaou *et al.* [232] undertook a review of studies using MS-based proteomics in human samples to identify biomarkers of SLE. They identified 241 potential biomarkers, 11 of which had been validated and identified in more than one study. Many of these including annexin's A2 and A5, alpha-1-antitrypsin and haptoglobin, were identified for diagnosis of LN specifically.[232]

1.3.9 Proteomics in renal tissue using mass spectrometry

The use of proteomics in renal tissue has been touched upon, including the proof of concept study by Satoskar *et al.* [191] using frozen tissue where LN glomeruli were compared with disease and healthy controls and showed increased complement expression and a decrease in podocyte-associated proteins and those associated with cellular metabolism.[191] This study used fifteen 10µm sections from each case, laser dissecting an average of 75 glomerular cross-sections analysing with LC for separation of proteins and electrospray ionisation with tandem MS/MS for shotgun proteomics. The first study to use FFPE tissue to quantitatively assess isolated glomeruli in diabetic nephropathy required a large amount of tissue and pooling of samples to provide sufficient protein for LC-MS/MS with iTRAQ labelling.[215] Subsequently, MS has been performed on much smaller amounts of tissue such as by Sethi *et al.* in their study of C3 glomerulonephritis where 4 glomeruli were captured from 10µm FFPE sections.[192] This team, following LC MS/MS, use three algorithms to generate peptide and protein probability scores thereby generating a list of those proteins with >90% probability. A spectra value then indicates the number of mass spectra for quantification using spectral counting. The same method therefore both detects and quantifies the proteins. The same team have performed MS in dense deposit disease (DDD) to examine glomerular proteins in this disease, specifically those in the complement pathway.[233] Hobeika *et al.* recently published a proteomic analysis of the glomerular extracellular matrix using laser capture microdissection, again demonstrating the reproducibility of proteins extracted by frozen and FFPE tissue using liquid chromatography mass spectrometry.[197]

1.3.9.1 Proteomics in LN renal tissue using mass spectrometry

In addition to the proof of concept study by Satoskar *et al.* described above,[191] LN renal tissue has been investigated with MS by Alaiya *et al*.,[167] who used the technique of protein in gel-digestion

and matrix-assisted laser desorption/ionisation time-of-flight MS (MALDI-TOF-MS) to distinguish class IV LN from disease controls.[167] In this study, Alaiya *et al.* investigated class IV LN using fresh frozen tissue with 2-DE gel separation, identifying differentially expressed protein spots using MS. Differentially expressed proteins between LN IV-G, normal and anti-neutrophil cytoplasmic antibody (ANCA) vasculitis biopsies were validated using LC-MS/MS with new samples. 16 of the 25 identified proteins from 2-DE analysis were also identified using LC-MS/MS. Many of the identified proteins were noted to be linked to pro-inflammatory pathways or their regulators.[167] Many of the proteins identified were linked to metabolic processes such as glutamate dehydrogenase.[167] It is not specified if glutamate dehydrogenase was downregulated in LN; however glutathione peroxidase, an anti-oxidant, is noted to be downregulated in LN compared to healthy control.[167] iTRAQ labelling has previously been used to identify and quantify proteins from frozen tissue from LN subjects and healthy controls (renal biopsies taken prior to kidney donation).[234] 512 proteins were identified with significant up-regulation of 10 proteins including heterogeneous nuclear ribonucleoprotein (hnRNP-) and annexins and down-regulation of 8 proteins including arginosuccinate synthetase (ASS) and aldolase [234] again including those proteins involved in metabolic processes.

1.4 CONCLUSION

Current evidence available for predicting response and guiding treatment for LN is insufficient. Currently, prognosis is predicted using a combination of clinical parameters including creatinine,[235] proteinuria and plasma lupus biomarkers, histopathological features including class and markers of activity and chronicity [236] and monitoring of response to therapy.[237] However, clinical parameters often do not correlate with histology and neither can fully or reliably predict response rates or outcome or guide treatment. Additionally, response rates to standard immunosuppression protocols are varied and suboptimal. Identification of the proteomic profile of LN at the time of diagnosis could allow improved understanding of the pathogenesis of LN and provide evidence for potential new treatment targets. Comparison of the proteome of responders compared to non-responders could aid in prediction of response to therapy and prognosis. Exploration of the proteome could also aid individualisation of therapy with targeting of specific pathogenic mechanisms of disease, thus improving response to treatment and minimising the use of potentially toxic treatments with undesirable side effect profiles. In the longer term, such profiling and adaptation of treatment regimens could improve treatment response rates and, hence the degree of chronic kidney damage and renal prognosis.

1.5 EXPERIMENTAL QUESTIONS AND STUDY AIMS

1.5.1 Hypothesis

Profiling of the patient using demographic, clinical and histopathological data and the proteome of the kidney biopsy in lupus nephritis, using database exploration, staining and mass spectrometry, will identify variables which can be used to predict response and outcome in lupus nephritis. In addition, protein biomarkers identified can be used to predict pathogenic mechanisms of injury not yet targeted in lupus nephritis and those which vary in treatment responders and non-responders. This information could be used not only to predict response to treatment and long-term outcomes in lupus nephritis: a 'fingerprint of response' but also to generate hypotheses for new treatment targets.

1.5.2 Study Aims

- To construct a comprehensive database of lupus nephritis patients and fully characterise outcomes to identify demographic, clinical and histopathological predictors of treatment response and long-term outcomes.
- To characterise the protein expression profile in renal biopsies in lupus nephritis and identify active pathogenic pathways in lupus nephritis and then separately in treatment responders and non-responders in a retrospective cohort of patients.
- To use immunohistochemistry staining to characterise complement deposition to assess its variability in lupus nephritis and thereby provide insight into the complement pathway in lupus nephritis and the potential relationship to response to treatment.
- To identify the suitability of complement markers to identify patients with lupus nephritis in whom C5 blockade may be a useful treatment.
- To use immunohistochemistry staining to validate the key biomarkers identified by proteomic analysis which appear to differentiate between responders and non-responders.
- To use proteomic profiling of the kidney biopsy in lupus nephritis to generate hypotheses for new treatment targets.

1.5.3 Experimental questions

- Can a combination of demographic, clinical, histopathological and treatment data predict the response of a lupus nephritis patient to treatment, their risk of flare or their long-term outcome?
- How do complement markers vary in lupus nephritis and can any of them be used to predict response to treatment or long-term outcome in lupus nephritis?
- Can sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS) be performed in formalin fixed, paraffin embedded (FFPE) human renal tissue?
- Can SWATH-MS of human renal tissue differentiate treatment responders from non-responders and therefore enable prediction of response to treatment?
- Can SWATH-MS of human renal tissue provide information regarding pathogenic pathways in lupus nephritis and thereby generate hypotheses for new treatment targets?
- Can protein biomarkers identified in renal tissue using SWATH-MS also be identified by immunohistochemistry and be used to differentiate treatment responders from non-responders?

2 CHAPTER TWO-MATERIALS AND METHODS

2.1 GENERAL REAGENTS AND BUFFERS

Laboratory chemicals were purchased from Sigma-Aldrich (Dorset, UK), plastic consumables from Becton Dickenson (BD) Biosciences (Oxford, UK). Specific materials and their source are described in the relevant sections below. Table 2.1 describes the constituents of commonly used buffers and reagents. Milli-Q water is ultrapure water: distilled, purified water with additional removal of contaminants including particulates, bacteria, pyrogens, nucleases, proteases, VOCs, endocrine disruptors and organics (Milli-Q, Integral with Q-POD (Biopak Polisher CDUFBI001), Vent Filter MPK01 (TANKMPK01), Merck, Darmstadt, Germany)

Phosphate Buffered Saline (PBS) Tablets (Oxoid, BR00114G). Utilised in immunohistochemistry.	Sodium Chloride 8.0g Potassium Chloride 0.2g Di-sodium hydrogen phosphate 1.15g Potassium dihydrogen phosphate 0.2g 1L Milli-Q water pH 7.3 ± 0.2
Phosphate Buffered Saline (PBS) utilised in ELISA.	8g Sodium Chloride 1.16g Di-sodium hydrogen phosphate Potassium dihydrogen phosphate 0.2g Potassium Chloride 0.2g 1L Milli-Q water pH 7.0
Sodium Citrate	2.94g Sodium Citrate Tribasic 1L Milli-Q water

	Titrated to pH 6.0 with acetic acid
Tris EDTA	1.21g Tris Base 0.37g EDTA 1L Milli-Q water Titrated to pH 9.0 with HCL
Lysis Buffer	36.036g Urea (6M) 15.224g Thiourea (2M) 0.186g Ethylenediaminetetraacetic acid disodium salt dehydrate (5mM) 0.7906g Ammonium bicarbonate (100mM) 100millilitres (ml) Milli-Q water Titrated to pH 8.0
Coating Buffer (0.1M Sodium Carbonate)	7.13g Sodium Bicarbonate 1.59g Sodium Carbonate 1L Milli-Q water Titrated to pH 9.5 with NaOH

Table 2.1. Constituents of commonly used buffers.

2.2 DEMOGRAPHIC, CLINICAL AND HISTOPATHOLOGICAL DATA ACQUISITION

2.2.1 Formation of Database

A database was constructed of every patient with biopsy-proven LN between 01/01/1996 and 31/12/2016. Demographic, clinical, histopathological, treatment and outcome data were collected from clinical notes review and immunosuppression treatment documented for each patient over the course of their diagnosis with lupus nephritis.

Data was identified from patient electronic records including clinical notes review: Cerner (Cerner, North Kansas City, MO, USA), ICE (ICE Health Systems, Henderson, NV, USA) and the Integrated Computerised Hospital Information System (ICHIS), (IMS Maxims, Milton Keynes, UK) and clinic letters as well as existing databases.

A database of appropriate disease controls was identified including biopsies with a diagnosis of idiopathic membranous nephropathy, thin basement membrane disease and minimal change disease.

2.2.1.1 Ethics

This was a retrospective study. All patients gave their consent for treatment and received standard care according to the accepted unit protocols at Imperial College Healthcare Trust (ICHT) or University College Hospital London (UCLH) for the database validation cohort treated at UCLH. Data were stored within the hospital and anonymised when analysed on Imperial College London computers.

2.2.2 Baseline demographic data

Patients' clinical records were reviewed retrospectively to identify baseline demographic data including age, sex, ethnicity, reason for biopsy, duration of LN and features of American College of Rheumatology (ACR) criteria [238] and Systemic Lupus International Collaborating Clinics (SLICC) criteria.[239] Clinical data were also recorded from retrospective review of the clinical records. Serum creatinine and eGFR (CKD-EPI ml/min/1.73m^2) were recorded from the day of biopsy. Antinuclear antibody (ANA) was recorded as positive if ever positive. Serum dsDNA and complement C3 and C4 values were recorded and if ever abnormal. The highest urine protein:creatinine ratio (uPCR) (mg/mmol) from within four weeks of biopsy was recorded.

2.2.3 Class and active and chronic lesions

Renal biopsy reports were reviewed to determine ISN/RPS [240] features of activity, chronic lesions and class of LN and features of chronic tubulointerstitial damage as represented by percentage IFTA. Class and the definition of active and chronic disease/lesions were assigned according to the ISN/RPS classification. Active disease was defined by the presence of any of the following: endocapillary hypercellularity with or without leukocyte infiltration and with substantial luminal reduction, karyorrhexis, fibrinoid necrosis, rupture of glomerular basement membrane, crescents (cellular or fibrocellular), subendothelial deposits on light microscopy (wire loops) or intraluminal immune aggregates (hyaline thrombi). Chronic glomerular lesions were defined by the presence of any of the following: glomerular sclerosis (segmental or global), fibrous adhesions or fibrous crescents. If there were mixed active and chronic lesions, these biopsies were analysed in the active group. Biopsies in the chronic group had exclusively chronic features. Activity of the biopsy was quantified by the percentage of viable glomeruli showing any of the listed features of activity. The percentage of glomeruli with chronic lesions and the percentage containing crescents were also calculated.[241]

2.2.4 Response and Flare

Clinical data were analysed to define response to each treatment escalation following a renal biopsy. Response was defined by review of urine protein-creatinine ratio (uPCR) and eGFR using the CKD Epidemiology Collaboration (CKD-EPI) formula [242] calculated from creatinine. Baseline uPCR was defined as the highest uPCR within 4 weeks pre- or post- the diagnostic renal biopsy. Baseline creatinine was taken on the day of biopsy or the closest reading possible, within four weeks.

Complete response (CR) was defined as uPCR <50mg/mmol and eGFR ≥60ml/min/1.73m2, or if initial eGFR <60ml/min/1.73m2, with <20% reduction by one year post biopsy. Partial response (PR) was defined as uPCR <300mg/mmol with a ≥50% improvement from baseline and eGFR criteria the same as for CR. Non-response (NR) was defined as failing to achieve PR by one year. Time to remission was recorded. Remission was not applicable if treatment was not escalated following the biopsy. Flare from CR/PR was defined as a ≥50% increase in the uPCR, to >100mg/mmol, sustained over two consecutive readings +/- a fall in eGFR of ≥20% from baseline. In addition, a repeat biopsy as was standard practice at ICHT with a change in treatment or a change in treatment without repeat biopsy was required. Clinical outcomes of death, initiation of renal replacement therapy (dialysis or renal transplant), doubling of baseline creatinine and eGFR at 5, 7 and 10 years were identified. eGFR was

considered to have fallen below a threshold if the fall was sustained for a minimum of two consecutive readings with no sustained recovery.[241]

2.2.5 Treatment

Patients received the standard of care for treatment at our unit, according to their diagnosis, clinical features and contemporaneous accepted unit protocols. LN is currently treated at our centre with one of five protocols largely involving IV Rituximab or Cyclophosphamide. First line since 2005 is usually Rituximab (RTX) (2 doses of 1g, 2 weeks apart) and MMF (target 2-3g daily) (Rituxilup) with no oral glucocorticoids. If a patient is already on oral glucocorticoids, these are continued alongside RTX and MMF with avoidance of escalation if possible and active weaning of oral glucocorticoids as tolerated (Rituxirescue). In both of these regimens, MMF is continued as maintenance. Alternatively, IV CyP following the Euro-Lupus[243] protocol is given and in cases of severe disease, such as rapidly deteriorating renal function or cerebral lupus, RTX and CyP are given concurrently (RituxiCyP). Maintenance treatment standard is MMF for each of these regimens from 2001. Prior to this and in cases where MMF is being avoided, for example in preparation for pregnancy, maintenance therapy is AZA. If RTX and CyP are not suitable, oral treatments (PO) alone are given, most commonly MMF, occasionally calcineurin inhibition with Tacrolimus, either with or without oral glucocorticoids. Prior to the unit merger in 2005, standard treatment regimens included both NIH[244], followed by Euro-Lupus[243] CyP protocols and MMF with oral glucocorticoids.

2.2.6 Side Effects

Side effect and infection data was collected by reviewing C-reactive protein, microbiology results, clinic letters and discharge summaries from clinical records at ICHT. Positive microbiology or documented infection was recorded for one year following initiation of treatment. Dual-energy X-ray absorptiometry (DEXA) scan reports following at least one year of treatment were recorded as ‘normal’, ‘osteopenia’ or ‘osteoporosis’. Fractures, avascular necrosis and cancer events were recorded cumulatively during follow-up. The highest serum blood glucose level was recorded during the first year of treatment and HbA1c at one year of treatment. Diabetes mellitus documented as steroid-induced at any time point was recorded.

2.3 IMMUNOHISTOCHEMISTRY

At the time of biopsy, C1q, C3c, IgA, IgG and IgM staining is all performed by the clinical laboratory by immunofluorescence (IF) on frozen tissue. These are then scored on a 0 to 3+ scale, with a separate intensity score for mesangium and capillary wall. The recorded values were used in the database analysis. The recorded values for C3c were also used in the complement staining section of this thesis, except where no values were available and then C3c staining was performed in the research laboratory by immunoperoxidase (IP) on formalin-fixed, paraffin-embedded (FFPE) tissue.

Staining was performed in the clinical laboratory for C9 and CD68 by IP on FFPE tissue for research purposes and scored by myself.

All other staining was performed manually in the research laboratory by IP on formalin-fixed, paraffin-embedded (FFPE) tissue.

2.3.1 Tissue Sectioning

Sections were cut from tissue blocks for staining at 3μm for consistent staining results. Blocks were first trimmed with a scalpel to taper the edge of the block on the side from which the blade approaches to stabilise the block. The chill plate was set to 7°C and blocks laid tissue side up on the surface to chill for up to 15 minutes to allow for smoother cutting and prevent disassociation of the tissue from the wax. Blocks were turned tissue-side down for 1-2 minutes immediately prior to cutting. The microtome (Leica RM2255, Leica Biosystems, IL, USA), forceps (straight forceps 115mm, fine tip, 232-0034, VWR, PA, USA) and brushes (Zhuting, painter friend professional artist brush, Amazon, ASIN: B071K1XFBF) were cleaned with Milli-Q water followed by 100% Ethanol before a fresh blade (Feather microtome blades, S35) was inserted and angle of approach set to 5° before locking the block in the block holder and adjusting to neutral alignment. Excess wax was trimmed before switching to section mode and cutting tissue sections. Brushes and forceps were used to transfer each section in turn to the pre-heated water bath (Raymond A Lamb, Now Fisher Scientific E66SLD/230 Thermo Fisher Scientific, MA, USA) set at 37°C. The water bath was filled with distilled water to aid binding to the slides. Once sufficient sections were cut, samples were scooped onto labelled, charged polysine (631-0107, VWR, PA, USA) slides, one per slide. Slides were left standing upright to allow water to drain and prevent water being trapped under the wax. Slides were then transferred to a metal rack (Raymond A Lamb, now Fisher Scientific E1025-APD Thermo Fisher

Scientific, MA, USA Fisher Scientific) for baking in a pre-heated oven set to 37-40°C and were left to bake overnight to allow for optimal tissue adherence.

2.3.2 Pre-treatment of paraffin sections

Labelled sections were placed in a metal rack in two sequential baths of xylene (Xylene, >=97%, X/0200/17, Fisher, Thermo Fisher Scientific, MA, USA) for deparaffinisation followed by rehydration in a bath of 100% ethanol (Ethanol, absolute, >=99.8%, E/0650DF/C17, Fisher, Thermo Fisher Scientific, MA, USA), followed by 70% ethanol followed by running water, each for five minutes.

2.3.3 Antigen retrieval

Antigen retrieval was achieved with one of three buffers outlined in table 2.2. specified for each antibody in table 2.3. For each, either a water bath (Grant OLS200, Fisher Scientific, Thermo Fisher Scientific, MA, USA) or pressure cooker (Pressure King Pro, Argos, Milton Keynes, UK) was used to incubate the sections. In the water bath, 190ml was pre-warmed in a heat-proof box (VITLAB Universal Slide Staining Kit, BrandTech V99099, VWR, PA, USA). Slides were then added and incubated in a water bath for 30minutes. In the pressure-cooker, 1.5L was added to the pot, well above the minimum level, sufficient to cover slide racks. Loaded slide racks were added (up to two), the lid secured and cooker set to meat and 30 minutes. 30 minutes would begin following heating. Slides were washed in PBS (table 2.1) twice for five minutes each time (KCH-Vibrax-VXR, Kinematica AG, Switzerland).

Antigen Retrieval Method	Buffer	Temperature
Heat Sodium Citrate	Sodium Citrate	95°C, solution pre-warmed in microwave
Heat Tris-EDTA	Tris-EDTA	95°C, solution pre-warmed in microwave
Enzyme	Protease XXIV (P8038-1G, Sigma-Aldrich, Dorset, UK) 25mg reconstituted frozen powder to 1ml PBS.	37°C, solution pre-warmed in water bath.

Table 2.2. Antigen Retrieval Techniques

2.3.4 Blocking

Tissue sections were encircled using a paraffin pen (Imm Edge Pen H-4000 Vector Laboratories, CA, USA) to enclose solutions and blocked with Peroxidase block (0.03% hydrogen peroxide containing sodium azide, DAKO Envision Kit (K4007 or K4011, Dako, Agilent, CA, USA) for 30-60 minutes at room temperature to block for endogenous peroxidase activity. Slides were placed in a covered, humidified box (Stain Tray slide staining system, Z670146-1EA, Merck, Darmstadt, Germany) during incubations to minimise evaporation. Slides were then washed in PBS twice for five minutes each time. A second block of 30% normal goat serum (G9023-10ml, Sigma-Aldrich, Dorset, UK) was then applied for one hour at room temperature, incubating slides in the humidified incubation box to block non-specific binding of antibodies. It was tapped-off before application of the primary antibody.

2.3.5 Primary antibody

100μl of antibody was added to each slide before incubating overnight at 4°C.

Target Protein	Primary Antibody	Antibody Class	Primary Antibody Dilution	Antigen Retrieval	Peroxidase Time	Secondary Antibody	DAB time
C3c detects C3c and the C3c part of C3 and C3b	DAKO A0062	Anti-human Rabbit Polyclonal	1/100,000	Enzyme in water bath	60 minutes	Goat anti-rabbit	Seven minutes 30 seconds
C3d	Abcam ab136916	Anti-human Rabbit Monoclonal	1/1000	Sodium Citrate in water bath	30 minutes	Goat anti-rabbit	12 minutes
C5b9 detects solid-phase, membrane-bound and fluid-phase C5b-9 but not native C9	DAKO M0777	Anti-human Mouse Monoclonal	1/1000	Enzyme in water bath	30 minutes	Goat anti-mouse	Six minutes
Properdin	Bio-orbyt orb2097	Anti-human Rabbit Monoclonal	1/50	Tris EDTA in pressure cooker	30 minutes	Goat anti-rabbit	10 minutes
Factor H	Abcam ab118820	Anti-human Mouse Monoclonal	1/50	Sodium Citrate in water bath	60 minutes	Goat anti-mouse	10 minutes
FHR5	Abnova 81494-DO1P	Anti-human Rabbit Polyclonal	1/400	Enzyme in water bath	60 minutes	Goat anti-rabbit	Five minutes
C9	Leica NCL-CCC9	Anti-human Mouse Monoclonal	Pre-diluted	Enzyme in Leica Bond Autostainer	Five minutes	Anti-mouse	10 minutes
CD68	Dako, M0876	Anti-human Mouse Monoclonal	1/100	Enzyme in Leica Bond Autostainer	Five minutes	Anti-mouse	10 minutes
Cullin 2	Abcam ab226463	Anti-human Rabbit Polyclonal	1/100	Sodium Citrate in water bath	30 minutes	Goat anti-rabbit	4 minutes
Proteasome subunit alpha type 6	Abcam ab109377	Anti-human Rabbit Monoclonal	1/250	Sodium Citrate in water bath	30 minutes	Goat anti-rabbit	4 minutes

Table 2.3. Antibodies used for Immunohistochemistry

2.3.6 Secondary antibody

Following PBS wash twice, each for five minutes, a secondary antibody was applied (Dako Envision+system-HRP for use with rabbit primary antibody (K4011) or Envision+system-HRP for use with mouse primary antibody (K4007)) for detection of the primary antibody. The EnVision reagent contains a peroxidase labelled polymer conjugated to goat anti-mouse or anti-rabbit immunoglobulins: secondary antibodies directed against rabbit or mouse immunoglobulins, raised in a goat. The antibodies are contained in Tris-HCl buffer containing stabilising protein and an anti-microbial agent. This was applied and slides incubated for one hour in the humidified incubation box at room temperature. Slides were again washed twice in PBS, each for five minutes before staining.

2.3.7 Stain

DAB substrate buffer was made up from the Dako Envision kit, one millilitre of DAB+ substrate buffer solution, pH 7.5, containing hydrogen peroxide and a preservative to one drop of DAB+ Chromogen: 3,3'-diaminobenzidine chromogen solution. The DAB was applied to the sections for a pre-defined time period (determined in the antibody testing phase), optimised for colour reacting. A light tan colour with darker deposits was targeted. Once the reaction was complete, sections were transferred to PBS and washed twice, each for five minutes.

2.3.8 Counterstain and mount

Following filtering of the haematoxylin (Harris Haematoxylin Solution, Modified, HHS32-1L, Sigma-Aldrich,), slides were immersed for 60 seconds in haematoxylin followed by running water for two minutes. Slides were then immersed in 1% acid alcohol (990ml 70% ethanol with 10ml HCl) for one second only before being immersed in running water for five minutes. Slides were then passed through immersion in: 75% ethanol; 100% ethanol; 100% ethanol; xylene; xylene each for five minutes. Slides were then fixed and mounted one at a time using Pertex (SEA-0104-00A, CellPath, Powys) and a cover slip (631-0136, 631-0134, 631-0138, VWR, PA, USA) and left to dry for 24 hours in the fume hood before being reviewed on the microscope (Leica DM4B optical microscope, coupled with Leica DFC700T digital camera (Leica Microsystems, Wetzlar, Germany)).

2.3.9 Grading

C1q, C3c, IgA, IgG and IgM staining intensity performed at the time of biopsy was reported by one of two experienced renal histopathologists. Staining intensity on the remainder of the stains was assessed by one blinded observer after their grading was compared with the aforementioned histopathologists and found to correlate. Staining of C3c, C3d, C9, C5b9, Factor H, FHR5 and Properdin were graded using a standardised semi-quantitative scoring system as 0, +/- (trace, numerically represented as 0.5), 1, 2 or 3. Intensity was separately analysed in the glomerular capillary wall, mesangium and tubular basement membrane. For staining of the proteasome subunit alpha type 6 and cullin 2, intensity was also recorded using the same semi-quantitative grading system for the tubular cells, tubular brush border, glomerular capillary endothelium and peri-tubular capillaries as well as the glomerular capillary wall, mesangium and tubular basement membrane. Staining intensity was not evaluated in globally sclerosed glomeruli or regions of segmental sclerosis. For CD68 staining, the number of macrophages in each glomerulus was counted and recorded in each biopsy and the median and maximum glomerular count for each biopsy calculated. C3c values quoted for capillary wall and mesangial staining are from the time of biopsy and for tubular basement membrane staining are from manual staining.[241]

2.3.10 Automated processing

FFPE sections were processed in the clinical histopathological laboratory using the Leica Bond III automated IHC stainer. The automated stainer takes slides through dewax, peroxidase block of five minutes, primary antibody incubation for 15 minutes, post primary incubation for eight minutes, polymer incubation for eight minutes, mixed DAB refine for 10 minutes followed by haematoxylin for five minutes. Slides are then mounted using an automated slide mounter.[241]

2.3.11 Controls

Positive and negative controls were used with each batch of staining. For the complement staining, idiopathic membranous disease was used as a positive control. For the validation of the mass spectrometry, tissue suggested from the datasheet as positive control or tissue with known expression was used. For cullin 2, human breast cancer was used. For proteasome subunit alpha type 6 human colon was used. Thin basement membrane disease, minimal change disease and kidney biopsies reported as within normal parameters were used as a negative ‘disease’ control.

Omission of primary antibody was used as an additional negative control. Representative images of membranous nephropathy and 'normal' tissue are shown in the results chapter 4 in figure 4.1.[241]

2.4 Blood and Urine Sample Collection

Blood and urine samples were collected prospectively from patients with newly presenting or flaring lupus nephritis prior to intravenous treatment. If patients had received intravenous treatment for this flare of lupus nephritis, only DNA and RNA were collected. Blood and urine samples were collected under study number SALA1004 utilising the latest version of patient information sheets and consent form. Consent was taken by myself and consent form copies were stored in the patient's notes and a locked cabinet as well as being offered to the patient. Blood samples were collected by either myself or a registered nurse or phlebotomist at the time of clinically required blood tests or intravenous cannula insertion. Urine samples were collected when required clinically such as pregnancy testing at times of treatment or urine dip in clinic and surplus urine which would have otherwise been discarded was collected for research. If a urine sample was not required clinically patients were asked to provide one. Samples were labelled with a pre-printed label containing the research patient identifier (LNBB001, LNBB002 etc), sample number and sample type. Patients were approached for repeat samples between one and two years after the first sample collection if possible. Samples were collected as follows:

- 1 Blue Tempus tube (4342792 Fisher) containing 6ml stabilising reagent (3ml blood) for RNA. Tubes were shaken well until foamy before freezing at -80°C.
- 1 Purple 'lavender' large vacutainer tube (367525, BD). (EDTA, 10ml blood) for DNA. Tubes were frozen at -80°C straight away.
- 1 Red vacutainer tube (367837, BD) (6ml blood) for serum. Tubes were incubated for one hour at room temperature before centrifuging at 3000revs/min for 20minutes at 4°C. Serum was stored in microcaps.
- 2 Light purple 'lavender' vacutainer tube (367838, BD) (6ml blood) for plasma. Tubes were incubated for one hour at room temperature before centrifuging at 3000revs/min for 20minutes at 4°C. Plasma was stored in microcaps.
- 1 Specimen bottle for urine (SC306, Fisher, Thermo Fisher Scientific, MA, USA). The urine was collected on ice before centrifuging at 3000revs/min for 20minutes at 4°C and stored in microcaps.

2.5 ELISA

Quantification of C5b-9 in plasma and urine was assessed by direct enzyme linked immunosorbent assay (ELISA) using BD Biosciences Human C5b-9 ELISA Set Cat. No. 558315.[241]

The capture antibody (purified anti-human C5b-9) was diluted 1:250 in coating buffer (table 2.1) and microwells coated with 50µl of diluted capture antibody per well. The plate was then sealed and incubated overnight at 4°C. The following day, wells were aspirated and washed three times with ≥ 300µl per well of wash buffer (PBS (table 2.1) with 0.05% Tween-20 prepared on the day). After the final wash, the plate was inverted and blotted on absorbent paper to remove any residual buffer. Plates were then blocked using 150µl per well of assay diluent (PBS (table 2.1) with 10% fetal bovine serum (SH30088, BD) incubated at room temperature for one hour on a plate shaker (Labnet Orbit 1000, Labnet International, Edison, NJ, USA). Standards were prepared using reconstituted lyophilised standard at 30ng/ml which was allowed to equilibrate for 15 minutes and vortexed gently (Fisher Scientific FB15012 TopMix, 3000rpm, Fisher Scientific). 150µl of assay diluent was then added to 6 tubes, labelled 15ng/ml, 7.5ng/ml, 3.75ng/ml, 1.875ng/ml, 0.938ng/ml and 0.469ng/ml. Serial dilutions were then prepared by adding 150µl of the reconstituted standard to the first tube and then 150µl of each standard to the next tube, vortexing between each transfer. Assay diluent served as the zero standard (0ng/ml). Wells were again aspirated, washed three times with wash buffer and blotted. 50µl of each standard, sample and control (dilutions prepared in assay diluent) were pipetted into the wells, the plate sealed and incubated for two hours at room temperature on a plate shaker. Wells were again aspirated, washed three times with wash buffer and blotted. 50µl of working detector (250X detector antibody (Biotin anti-human C5b-9) and 250X Streptavidin-HRP in assay diluent) was then added to each well and the plate sealed and incubated for one hour at room temperature on a plate shaker. Wells were again aspirated, washed seven times with wash buffer and blotted. 50µl of substrate solution (Tetramethylbenzidine (TMB) and Hydrogen Peroxide (BD Pharmingen TMB Substrate Reagent Set (555214)) combined in equal volumes and vortexed) was then added to each well and the plate incubated without sealing for 30 minutes at room temperature in the dark. 25µl of stop solution (1M Sulphuric Acid) was then added to each well and the plate read straight away (within 30 minutes of stopping the reaction). Optical density was measured at 450nm using an ELx800 absorbance reader (Biotek). A standard curve was constructed of mean 450nm absorbance on the y-axis and log concentration of the x-axis and concentrations of the samples determined using a four parameter logistic curve fit using GraphPad Prism (version 7.02).

2.6 PROTEOMICS

2.6.1 Block cutting

Blocks were first trimmed with a scalpel to taper the edge of the block on the side from which the blade approaches to stabilise the block. The chill plate was set to 7°C and blocks laid tissue side up on the surface to chill for up to 15 minutes to allow for smoother cutting and prevent disassociation of the tissue from the wax. Blocks were turned tissue-side down for 1-2 minutes immediately prior to cutting. The microtome, forceps and brushes were cleaned with Milli-Q water followed by 100% Ethanol before a fresh blade was inserted and angle of approach set to 5° before locking the block in the block holder and adjusting to neutral alignment. Excess wax was trimmed before switching to section mode and cutting tissue sections. Appearance (stained or unstained) and rough estimation of size of the tissue was noted (number and length of cores). 3 to 6 sections were cut as previously described at 3µm depending on the quantity of tissue remaining. The microtome was then set to 150µm for one section to be cut for protein extraction. This section was placed in the appropriate microtube and weighed. Appearance and rough quantity of remaining tissue was noted.

1.5ml protein lo-bind Eppendorf tubes were prepped and weighed to 6 decimal places. The sample has been noted in method development to stay in the bottom of a 1.5ml tube and be thrown up out of a buffer in the 2ml round bottom tubes when being agitated in the Thermoshaker (Thermal Shake Lite, 460-0249, VWR, PA, USA.)

2.6.2 Dewax

Using fume hood, add 1ml of heptane to each Eppendorf tube. Then place Eppendorf tubes in ThermoMixer, set to 25°C and 800rpm for ten minutes. The heptane was then removed with gel loading tips and discarded. 1ml of heptane was again added to each Eppendorf tube which were again shaken for ten minutes at 25°C and 800rpm.

2.6.3 Rehydrate

Taking each Eppendorf tube in turn, the heptane was discarded using gel loading tips and the Eppendorf tube weighed. 1ml of 100% ethanol was then added before shaking at 800rpm for five minutes at 25°C. After discarding the ethanol, 200µl of 90% ethanol was added and shaken at

800rpm for five minutes at 25°C. Ethanol was again discarded before adding 200µl of 75% ethanol and shaking at 800rpm for five minutes at 25°C.

2.6.4 Acid Hydrolysis

After discarding the ethanol, 200µl of 0.1% formic acid was added and the Eppendorf tube shaken at 600rpm for 30 minutes at 30°C.

2.6.5 Basic Hydrolysis

Taking each Eppendorf tube in turn, the FFPE tissue was removed using forceps, dabbed on fresh tissue paper to remove residual acid and placed in a pre-weighed MicroTube (MT-96 Pressure BioSciences Inc., MA, USA). 15µl of 0.1M Tris-HCl (pH 10) was then added before closing with the 100µl cap (MC100-96, Pressure BioSciences Inc., MA, USA) and placing in a new Eppendorf tube and shaking at 500rpm for 30 minutes at 95°C. The samples were then immediately cooled on ice and 25µl lysis buffer (table 2.1) added to each microtube which was again closed with the 100µl cap before being transferred to -80°C freezer for storage prior to being transferred to Manchester for the next stage of processing.

2.6.6 Pressure Cycling Technology (PCT) -Assisted Lysis

Samples were removed from the -80°C freezer once the water bath (Haake AC 200 and S3, Thermo Scientific, Thermo Fisher Scientific, MA, USA) and barocycler (Barocycler NEP2320 PCT Sample Preparation System, Pressure BioSciences Inc.) had reached 30°C. The pump was turned on and set to 8 bar (115 psi) (Sil-Air 100-24 2 x 1/2 HP 4.5 CFM 6 Gallon Portable Oil Lubricated Silent Air Compressor, Silentaire Technologies). The 100µl cap was replaced with a micropestle (MTWS-MP-RK, Pressure BioSciences Inc.). Samples were then loaded, balanced, into the barocycler chamber which was dropped into the barocycler before the machine was pre-charged to remove all air bubbles from the system and then set for tissue cleavage. This applied 120 cycles of 45,000 psi for 50 seconds followed by ambient pressure for 10 seconds.

2.6.7 Protein Quantification

Samples were removed from the barocycler and stored in the fridge. Protein quantification was undertaken using a Qubit machine (Qubit 3 Fluorometer, Q33216, Invitrogen, Thermo Fisher

Scientific). 200µl total volume was used in the Qubit tubes. 10µl of standard (Qubit Protein Assay Kit, Q33211, Invitrogen, Thermo Fisher Scientific) was added to 190µl of working solution (1:200 dye : protein buffer (Qubit protein Reagent, Q33211 A, Invitrogen, Thermo Fisher Scientific and Qubit protein Buffer, Q33211 B, Invitrogen, Thermo Fisher Scientific)). 2µl of sample was added to 198µl of working solution. Samples and controls were incubated in the dark for 15 minutes and read on the Qubit machine at room temperature. The protein function was used, entering the original sample volume with output in µg/ml. Total protein in the 40µl sample could then be calculated.

2.6.8 Protein Reduction and Alkylation

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (C4706-10G powder ≥98% Sigma Aldrich) and iodoacetamide (IAA) (BioUltra 1149-25G, Sigma Aldrich) were simultaneously added to the solution at a final concentration of 10mM and 40mM respectively. 2µl of 100mM ammonium bicarbonate was also added to account for the 2µl removed for protein quantification. Samples were closed with the same micropestle and microtubes were transferred to black eppendorfs and incubated on a thermoshaker (Thermal Shake Touch 460-0205, VWR) at 25°C, shaking at 600rpm for 30 minutes.

2.6.9 Lys C digestion

20µg of lyophilised Lys-C (Lysyl Endopeptidase, Mass Spec Grade 121-05063, WAKO, Richmond, VA, USA) was reconstituted in 100mM ammonium bicarbonate to provide a ratio of 1:40 enzyme : protein when 52.5µl of enzyme was added. The highest protein concentration of the samples was used for this calculation. After 52.5µl of Lys-C was added, the samples were closed with a 100µl cap and returned to the barocycler. Lys-C digestion was then run, applying 20,000psi for 50 seconds and ambient pressure for 10 seconds for 45 cycles at 30°C.

2.6.10 Trypsin digestion

20µg of lyophilised Trypsin (Sequencing grade modified trypsin, Porcine, V511A, Promega Madison Wi, USA) was reconstituted in 100mM ammonium bicarbonate to provide a ratio of 1:20 enzyme : protein when 50µl of enzyme was added. The highest protein concentration of the samples was used for this calculation. After 50µl of Trypsin was added, the samples were closed with a 150µl cap (MTWS-MC150-RK, Pressure BioSciences Inc.) and returned to the barocycler. Trypsin digestion was then run, applying 20,000psi for 50 seconds and ambient pressure for 10 seconds for 90 cycles at

30°C. 15µl of 10% formic acid (56302-50ml-F, Mass Spec Grade, Eluent additive for LC-MS, Sigma) was added before samples were frozen at -80 overnight.

2.6.11 Peptide Clean Up

Peptide clean up was undertaken using Oasis HLB SPE µElution Plate 30µm, Oasis sample extraction products, as per manufacturers instructions (Waters, Part No 186001828BA). Clean-up ensured that the sample could be concentrated and buffer contaminants such as urea removed to increase signal to noise ratio.

2.6.11.1 Condition

200µl of 100% acetonitrile (ACN) (A955-1, OPTIMA LC/MS Grade, Fisher) was added to each column on the HLB µElution plate. The columns, metal tray and waste tray (Deepwell Plate 96/500µl, 0030501110, Eppendorf, Hamburg, Germany) were stacked in the Beckman Coulter Biomek NXp laboratory automation workstation with Amplius positive pressure extractor. Pressure was applied at 0.2 megapascals (MPa) until the ACN had passed through the columns into the waste tray. Waste was discarded.

2.6.11.2 Equilibrate

200µl of de-ionised water was added to the columns, pressure applied in the same manner and water discarded.

2.6.11.3 Load

Defrosted samples were then added to the columns, pressure applied and waste again discarded, the protein being stored in the columns.

2.6.11.4 Wash

200µl of 5% ACN was added to the columns, the waste tray replaced with a 'wash' tray (Deepwell Plate 96/500µl, 0030501110, Eppendorf) and the same pressure applied. The wash was transferred to 2ml round bottom tubes (Safe-Lock Tubes 2.0ml, 0030123344, Eppendorf). Non-lo-bind tubes were used as the lo-bind tubes do not allow for drying.

2.6.11.5 Elute

200µl 100% ACN was added to the columns, the wash tray replaced with an elute tray (Deepwell Plate 96/500µl, 0030501110, Eppendorf) and the same pressure applied. The elute was transferred to 2ml round bottom tubes.

2.6.11.6 Dry

Tubes containing wash and elute were then placed into the GeneVac miVac Quattro concentrator attached to the miVac Quattro Pump and dried for 4 hours at 20°C before being frozen at -80°C. The sample is expected to be in the elute, however the wash is saved in case any sample is taken up during this process.

Peptide is not measured in the samples to conserve the small volume of sample as the peptide measurement is not required for loading on the mass spectrometer.

2.6.12 Sequential window acquisition of all theoretical fragment ion spectra (SWATH) analysis

SWATH-MS analysis was performed on a 6600 TripleTOF mass spectrometer (Sciex, Warrington, UK) coupled to a Dionex Ultimate 3000 high-performance liquid chromatography (HPLC) (Dionex, Thermo, UK). Peptides were separated through an Acclaim PepMap 100 C18 column. Buffer A comprised 2% Acetonitrile, 0.1% Formic acid, 98% Water. Buffer B comprised 80% Acetonitrile, 0.1 % Formic acid and 20% Water. 3% v/v Acetonitrile and 0.1% v/v Formic acid was used as sample buffer. Peptides were loaded at 5 µl/min for 10 min prior to being eluted over a 120 minute gradient at 0.3 µl/min.

2.7 STATISTICAL ANALYSIS

Data analysis was performed using the GraphPad Prism (version 7.02) and IBM SPSS statistics software programmes. Categorical data are displayed as percentage and analysed with Fisher's exact tests. Multiple comparisons have been adjusted with a Bonferroni correction or Benjamini-Hochberg correction in the case of the proteomics data. Continuous data are displayed as median and range or inter-quartile range (IQR) as described in the text. Data were assessed for normality of distribution using the D'Agostino & Pearson normality test. Continuous parametric data were analysed using Student's t test (two-tailed) or one-way ANOVA with Tukey's multiple comparison's test depending on the number of categories. Non-parametric data were compared using Mann-Whitney test or Kruskal-Wallis with Dunn's multiple comparison's test depending on the number of categories. In calculations involving dsDNA, only data from patients who have ever had an elevated dsDNA were included unless otherwise stated.[241]

2.7.1 Specific to database analysis

Logistic regression analysis was performed to identify factors affecting response and long-term outcomes. Where necessary, median values were used to complete gaps in data to allow for inclusion in analysis. The response and outcomes of eGFR>80, RRT and death at 7 years were individually assessed using univariate binary analysis for each demographic, clinical and histopathological feature described followed by multivariate analysis including all features significant at univariate analysis.

Flare by different time-points included only those patients with the required amount of follow-up unless death occurred before the time point. Outcomes by different time-points included only those patients with the required amount of follow-up unless death occurred before the time-point, except for the logistic regression analysis for renal replacement therapy where all RRT initiation was included, even if patients did not have 7 years follow-up in total.

Correction for baseline characteristics was performed using binary or multinomial logistic regression analysis.

Kaplan Meier was performed for survival analysis with log-rank (Mantel-Cox) test of survival curve significance. To identify relevant cut-offs for eGFR and proteinuria at one year, predicting the

relevant outcome, receiver operating characteristics (ROC) curves were used. Median values were not used to complete gaps in data for ROC curve analysis.

For some of the analyses, patients were excluded if follow-up was <18 months in those who survived. Where this is the case, this is indicated in the text. In the treatment comparison analysis, patients were also excluded if treatment received was outside standard protocols. In this analysis, patients were categorised by the treatment they received for the first biopsy showing LN at our unit, in the time period stated, with the exception of three patients whose first biopsy was prior to the time period under review.

2.7.2 Specific to staining and complement studies

Comparisons between staining intensities and with markers of activity were performed using correlations. Parametric data were compared using the Pearson r correlation. Non-parametric data were compared using the Spearman r correlation. Mesangial staining was analysed separately to capillary wall staining.[241]

C5b-9 plasma concentrations were determined using interpolated values from the standard curve.[241]

2.7.3 Specific to proteomics

2.7.3.1 Power Calculation

Coefficient of variation has not been assessed in FFPE tissue using SWATH proteomic analysis. Coefficients of variation available in the literature are not applicable as the protocol and samples are significantly different. The coefficient described by Broeckx at al for example, using murine FFPE tissue for analysis by liquid chromatography tandem mass spectrometry, includes a large proportion (31%) related to pre-analytical factors.[245] Unfortunately, there was not sufficient protein extracted to attempt to make an assessment of coefficient of variation in this project. A sample size calculation [246] was therefore made using the planned effect (Cohen's d) size threshold of 2-fold difference in expression of proteins and a significance level of 0.05 for two-tailed t test and a power of 0.8. This provided a suggested sample size of 12, 6 samples in each group being compared. Sample size was limited on a practical basis by the available samples. Human samples will naturally have many variable factors, some of which are difficult to measure or control for, such as concordance with treatment or concurrent additional medical diagnoses. It also became apparent

that entire samples had to be removed from analysis and the data obtained from those processed had a relatively high rate of 'missingness', that is data missing at random (MAR) due to failures in reaching quality control thresholds. A slightly higher sample size was therefore used to overcome these issues. Finally, as discovery work, it was important to capture all available data and explore patterns. It may be that proteins which were not differentially expressed to the pre-determined level were nonetheless valuable to explore their role in differentiating between responders and non-responders.

2.7.3.2 Analysis of SWATH-MS data

Mass spectrometry data files were searched using openSWATH (Version: 2.0.0). Peptide matches were scored using pyProphet (version 0.18.3) and the search results aligned using the feature alignment script from MSproteomicstools. A false discovery rate (FDR) cut-off of 1% was applied before the decoys and non-proteotypic peptides were removed. A minimum of one proteotypic peptide was used for identification. Statistical tests and downstream analysis was performed using the R language (version 3.4.1). Throughout the analysis the random seed was set to 500. Resultant data was transformed and normalised using the Bioconductor (release 3.5) packages SWATH2Stats and MSstats. Normalisation was performed using MSstats' data process function using the median equalisation method, providing abundance values of normalised counts per second. Samples were removed from analysis if the minimum total ion intensity for the whole sample was below the acceptable threshold. Individual protein abundance values were reported as missing if the identification scoring was below the acceptable quality control thresholds. These could be due to interference in the signal, poorly shaped peaks and fluctuations in fragmentation, ionisation and ion flight amongst other reasons. All proteins were included in heat map and Principal component analysis (PCA) analysis as an overview of the data. Proteins were removed from downstream analysis if there were $\geq$80% unknown values.

1,000 iterations of machine learning testing was conducted using the randomForest R package (version 4.6-14). The dataset was split 70:30 at random into a training set and testing set. The training set was balanced to even out the classes using the SMOTE function of the DMwR R package. A model was created using randomForest and then tested on the testing set.

Heat maps and Principal component analyses were performed in R to visualise the data. Heatmaps were generated using Z scores of normalised counts and pheatmap in R. T-tests were performed to determine statistical significance between conditions and fold change calculated and transformed to

log2 fold change for visualisation. Volcano plots were produced on GraphPad Prism demonstrating differential expression of proteins plotting corrected p value (termed q value) (-log10 q) against log2 fold change. A threshold of log2 fold change = 1 or -1 was set for differential expression to identify proteins equivalent to a 2-fold change in linear space or a 0.5-fold change in terms of downregulation. P values were adjusted using the Benjamini-Hochberg correction to produce q values and a value of <0.05 taken as significant. Where descriptions of up-regulation or down-regulation are made, this is in reference to control. When case comparisons are made, differences are described as 'expressed at a higher/lower level in…'. Significantly differentially expressed genes were identified from the proteins.

Functional protein-association network visualisation was performed using the STRING database (https://string-db.org/), using the full STRING network. Network edges were defined by confidence, and interactions were set to medium confidence interaction score (≥0.4). Disconnected nodes within networks were displayed unless stated. Significantly differentially expressed genes were evaluated for enrichment of Gene Ontology (GO) terms (Biological Processes, Molecular Functions, Cellular Component), Reactome pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and STRING local network clusters. Visual interaction networks were generated with functional enrichments in GO terms (Biological Processes) and KEGG pathways. String provides an assessment of whether the number of interactions between the proteins is significantly more than expected (protein-protein interaction (PPI) enrichment value with an associated p value). A significant value indicates that the proteins are connected biologically in some way. Enriched pathways have a false discovery rate, a measure of the significance of the enrichment. These p values are corrected using the Benjamini-Hochberg correction.

Correlation calculations were performed using Spearman r correlation for non-parametric data. Box plots were created using Graphpad Prism to visualise individual protein abundance.

2.8 ETHICAL APPROVAL

This was a retrospective study. All patients gave their consent for treatment and received standard care according to the accepted unit protocols at Imperial College Healthcare Trust (ICHT) or University College Hospital London (UCLH) for the database validation cohort treated at UCLH.

2.8.1 Ethical approval for use of human tissue

Biopsies were performed for routine clinical indications and spare tissue was used for the research as approved by the Imperial College Tissue Bank. Tissue samples were provided by the Imperial College Healthcare NHS Trust Tissue Bank (ICHTB) (application number R16063). Other investigators may have received samples from these same tissues. The research was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. ICHTB is approved by National Research Ethics Service (NRES) to release human material for research (17/WA/0161).

2.8.2 Ethical approval for use of human plasma and urine

Blood and urine samples were collected from patients and healthy controls as part of the project "Analysis of Lymphocyte response to autoantigens in patients with glomerulonephritis", JRO reference number SALA 1004, Research Ethics Committee reference number 04/Q0406/25.

2.8.3 Ethical approval for use of human plasma and urine from Swedish group

Sampling of tissue and blood from the Karolinska Institutet was approved by the regional ethics committee in Stockholm, (number 2012/1550-31/3).

3 CHAPTER THREE – CLINICAL FACTORS AFFECTING RESPONSE

3.1 INTRODUCTION AND BACKGROUND

As reviewed in detail in the introduction, current rates of response to treatment are suboptimal with the result that LN carries a significant risk of morbidity and mortality.[2,116] What we are trying to achieve with treatment can be unclear as definitions of response vary [34] and historically have not been targeted to longer term outcomes. Current medications in use are largely unlicenced and whilst there is some promise from recent clinical trials, response to treatment is still arguably sub-optimal in these trials, with NOBILITY demonstrating a 41% CR rate [54] and BLISS-LN a 43% CR rate [58] at 104 weeks. Additionally, the follow-up is short-term and many new trial protocols still carry a significant glucocorticoid burden. Without clear targets for trials, potential treatments could be unfairly rejected.[106]

The side effect profile of current treatments is significant, especially that of glucocorticoids which carry risk of infection, weight gain and psychological disturbance when used short term and osteoporosis, diabetes and hypertension, among others when used for prolonged periods. Long term use of glucocorticoids often leads to difficulty in weaning off them completely due to perceived or genuine hypoadrenalism. With this in mind, when our lupus centre was established following the merger of two renal units in late 2005, a key aim for new protocols was to reduce dependence on glucocorticoids, achieved by increasing the use of CD20+ B cell depletion therapy.

Preliminary data regarding our 'Rituxilup' cohort treated with no oral steroids, demonstrated that over a follow up of 77 months, 30/39 (77%) patients had never had oral steroids. Whilst flares were not uncommon, the majority responded to re-treatment, again with no oral steroids and, importantly, flares did not predict poor renal outcome. This was instead predicted by a baseline creatinine >120μmol/l or a failure to achieve PR at 6 months.[247][32]

Preliminary data regarding our RituxiRescue cohort, on maintenance steroids prior to their diagnostic renal biopsy, have shown the majority have achieved CR or PR (76.3%). Importantly, just over 50% were off oral steroids completely at 5 years with mean time to stopping of 18.2 months. Of those remaining on steroids, mean dose at 5 years was 5.8mg. [Pillay, C,[248] and UK Kidney Week 2017, manuscript in preparation] There were slightly lower rates of first relapse in the RituxiRescue cohort, compared to the Rituxilup cohort. However, there were higher adverse event

rates related to the side effects of steroids, with the majority of the documented steroid toxicity complications being bone related.[32]

This review analyses the demographic, clinical, histopathological, response and outcome data of all LN patients treated at our centre over the last 20 years. It includes a review overall of our experience with LN and with patients characterised by treatment protocols to analyse the characteristics of our cohort and assess if and how baseline features and outcomes differ between the different treatment protocols. Including, as it does, the largest cohort of patients treated with an oral steroid free regimen, it specifically addresses the question of whether LN can be treated successfully with minimal glucocorticoids analysing a much greater patient number of the above cohorts as well as longer term data. It was also explored if there were any other factors, demographic, histopathological or clinical, identifiable at baseline, which could predict response to treatment. It also aims to understand how baseline features and short-term results can predict long-term outcomes as well as examine if a less stringent proteinuria cut-off, suggested by review of the Euro-Lupus Nephritis [36][127] and MAINTAIN [128][250] cohorts, can be validated. This is with the overall aim of predicting responses to treatment and identifying short term markers of efficacy which best represent long term outcomes. Besides the primary utility of predicting long term outcomes in the clinic, a key use for new thresholds is for clinical trials to accurately identify new treatment options which provide the best long-term outcomes.

3.2 CHAPTER AIMS

- To review the comprehensive database of demographic, clinical and histopathological information of all patients diagnosed with lupus nephritis at Imperial College Healthcare Trust (ICHT) between 01/01/1996 and 31/12/2016 to characterise and explore the cohort.
- To identify predictors of response to treatment from the database.
- To analyse the cohort by treatment protocol to explore patient selection for each treatment protocol in use at ICHT and compare short- and long-term outcomes between the treatment groups, in particular, exploring the largest documented cohort of patients treated with no oral glucocorticoids.
- To explore long term outcomes and identify short term variables which serve as predictors for long-term outcomes.
- To identify useful thresholds or targets for these short-term variables for use in the clinic and in clinical trials to predict good long-term outcomes.
- To validate these thresholds in a separate cohort.

3.3 RESULTS

3.3.1 Overview of Imperial Lupus Nephritis cohort

To evaluate baseline characteristics and their relationship to response and outcome, data were collected on 803 biopsies performed on 470 patients between 01/01/1996 and 31/12/2016. The cohort was 82% female with mixed ethnicity, largely split between south Asian, Black and White. Median age of diagnosis of SLE was 29 years (range 5-75 years, IQR 22-40 years) and LN 32 years (range 9-76 years, IQR 24-44 years). Patients had between one (60% of patients) and eight (1% of patients) biopsies recorded over the time period. Nearly half of the biopsies performed were for the first presentation of LN (45%). Median follow-up from the first biopsy performed at ICHT was 89.5 months (7 years), (range 0-25 years, IQR 3-11 years). Median follow-up from the diagnosis of SLE was 11 years, (range 0-58 years, IQR 6-17 years). Median follow-up from the diagnosis of LN was 8 years, (range 0-46 years, IQR 4-13 years). Baseline demographic and clinical data for all patients is shown in table 3.1. Baseline histopathological information is shown in table 3.2. Data on other histopathological features including the staining intensity of IgG, IgA, IgM, C3, C4 and C1q in the capillary wall and mesangium were also recorded; however these have not been included in the tables as individual stains were not identified as significant features in later analyses.

Baseline Data	**Value**
Sex	82% Female
Ethnicity	
• **South Asian**	32%
• **Black**	27%
• **White**	26%
• **East Asian**	3%
• **Mixed**	2%
• **Other/Unstated**	11%
Median biopsy number per patient	1 (range 1-8)
Indication for biopsy	
• **New presentation**	363 (45.2%)
• **Flare**	277 (34.5%)
• **Persistent proteinuria**	106 (13.2%)

• **Staging** • **Unknown**	55 (6.8%) 2 (0.2%)
ACR diagnostic criteria fulfilled	90.7%
SLICC diagnostic criteria fulfilled	95%
Clinical symptoms of SLE present (non-renal)	69.3%
dsDNA ever positive	76.6%
ANA ever positive	91.5%
Smith antibody ever positive	23.2%
APL ever positive	28.5%
Serum C3 (g/L) (normal range 0.7-1.7)	Median 0.85 Range 0.03-2.28 IQR 0.57-1.12
Serum C4 (g/L) (normal range 0.16-0.54)	Median 0.16 Range 0.00-0.96 IQR 0.08-0.27
Serum dsDNA (IU/mL) (normal range 0-30)	Median 102 Range 0-20171 IQR 31.5-384
Creatinine at the time of biopsy (µmol/L)	Median 86 Range 32-902 IQR 66-141
eGFR at the time of biopsy (ml/min/1.73m^2)	Median 78.89 Range 4.72-90 IQR 45.03-90
Proteinuria at the time of biopsy (mg/mmol)	Median 296 Range 0-4560 IQR 139.25-597.5
Hydroxychloroquine Prescribed	415 (60.3%) Total 688
Angiotensin Converting Enzyme or Angiotensin II Receptor Blocker Prescribed	475 (70.4%) Total 675

Table 3.1. Table displaying overall patient demographic and clinical data of the entire LN cohort.

ACR, American College of Rheumatology; ANA, antinuclear antibody; APL, Anti-phospholipid; C, complement; dsDNA, anti-double-stranded deoxyribonucleic acid; eGFR, estimated glomerular filtration rate; SLE, systemic lupus erythematosus; SLICC, Systemic Lupus International Collaborating Clinics.

Variable	Value
Class • **I** • **II** • **III** • **IV** • **V** • **III + V** • **IV + V** • **II + V** • **VI** • **Other**	 9 (1.1%) 49 (6.1%) 89 (11.1%) 250 (31.1%) 193 (24.0%) 74 (9.2%) 96 (12.0%) 5 (0.6%) 1 (0.1%) 37 (4.6%)
Percentage of glomeruli with active lesions (class III and IV disease only)	Median 33% Range 0%-100% IQR 0%-87%
Percentage of glomeruli with chronic lesions	Median 25% Range 0%-100% IQR 6%-54%
Percentage of glomeruli with crescents	Median 0% Range 0%-100% IQR 0%-0%
Necrosis	77 (9.9%) Total 776
Endocapillary proliferation • **None** • **Focal segmental** • **Diffuse segmental** • **Diffuse global** • **Focal global**	 433 (55.9%) 176 (22.7%) 83 (10.7%) 78 (10.1%) 5 (0.6%) Total 775
Mesangial proliferation • **None**	 218 (28.2%)

• **Focal segmental** • **Diffuse segmental** • **Diffuse global** • **Focal global**	59 (7.6%) 35 (4.5%) 448 (58%) 12 (1.6%) Total 772
Thrombotic Microangiopathy	49 (6.7%) Total 736
Subendothelial Deposits • **None** • **Small/Rare** • **Large/Conspicuous**	 268 (38.4%) 266 (38.1%) 164 (23.5%) Total 698
Subepithelial Deposits • **None** • **Small/Rare** • **Large/Conspicuous**	 153 (22.0%) 246 (35.4%) 296 (42.6%) Total 695
Mesangial Deposits	83 (12.1%) Total 684
Full house (IgA, IgG, IgM, C3, C1q all ≥1)	209 (28.5%) Total 734
Interstitial Fibrosis and Tubular Atrophy Percentage	Median 10% Range 0%-90% IQR 0% - 23.75%
Tubulointerstitial Inflammation • **None** • **Mild** • **Moderate** • **Severe (>50%)**	 481 (65.1%) 150 (20.3%) 84 (11.4%) 24 (3.2%) Total 739
Acute Tubular Injury	337 (47.7%) Total 707

Table 3.2 Histopathological data of all biopsies performed showing lupus nephritis.

Categorical data displayed as frequency and percentage, continuous data displayed as median, range and inter quartile range (IQR).

3.3.2 Chronic glomerular lesions, 'full house' staining and proteinuria level were predictive of response to treatment

On a review of all data, overall there was a rate of 45.7% complete response (CR) by one year (table 3.3). Response to treatment by one year was reviewed for all patients receiving an escalation of treatment with no exclusion criteria apart from requiring one year follow up unless CR was achieved, or death occurred before then.

Response to treatment	**Total number of biopsies**
Complete Response	274 (45.7%)
Partial Response	149 (24.9%)
Non-Response	176 (29.4%)
N/A No active disease on biopsy	148
	Total 747 (599 with CR/PR/NR)

Table 3.3 Overall response to treatment of the entire cohort in all biopsies (n=747) with an escalation of treatment and minimum one year follow-up (unless CR or death occurred before).

Data displayed as total number and percentage.

Multiple logistic regression analysis was performed on all biopsies which received an escalation in treatment following the biopsy and therefore also had a response calculated of CR, PR or NR. Variables included in the analysis were all listed parameters in tables 3.1 and 3.2 above as well as the histopathological features: intimal thickening; hyalinosis; vasculitis; foam cells; tubulointerstitial nephritis; and staining of IgA, IgG, IgM, C1q, C3, C4 in the mesangium and capillary wall. Treatment protocol followed, the inclusion of glucocorticoid treatment pre- and post-biopsy and the recording of an infection were also included. Table 3.4 lists the variables which were found to be significant in univariate analysis of influencing achievement of CR or PR and thus included in the multivariate analysis model. The significance in the multivariate model and odds ratios (ORs) are also listed showing that percentage of glomeruli with chronic lesions, having a 'full house' pattern on staining and level of proteinuria at biopsy were each predictive of response by one year. The higher the percentage of glomeruli with chronic lesions, the lower the chance of responding. The OR of response with 'full house' staining was 1.753 (1.097 – 2.800) indicating a higher chance of response with 'full house' staining. Proteinuria had an OR of 0.655 for each increase in the groups of proteinuria, meaning

that a higher proteinuria at baseline is associated with less chance of response for each increase between the groups 0-49, 50-299 and 300+ mg/mmol).

Variable	Significance in univariate model (p value)	Significance in multivariate model (p value)	Odds Ratio in multivariate model (95% CI)
Percentage of glomeruli with chronic lesions	0.000	0.004	**0.986 (0.976 – 0.996)**
'Full house' staining	0.002	0.019	**1.753 (1.097-2.800)**
Intimal thickening	0.014	0.528	0.873 (0.572-1.332)
Percentage interstitial fibrosis and tubular atrophy (analysed in deciles)	0.000	0.712	0.969 (0.819-1.146)
Serum C3 low	0.024	0.793	1.073 (0.635 – 1.811)
Serum C4 low	0.001	0.738	1.093 (0.647 – 1.846)
eGFR at biopsy (analysed in deciles)	0.02	0.943	1.003 (0.920 – 1.094)
Proteinuria at biopsy (analysed in three groups, 0-49, 50-299 and 300+)	0.002	0.027	**0.655 (0.450 – 0.952)**
SLICC criteria fulfilled	0.042	0.512	1.348 (0.553 – 3.287)
Oral steroids established pre biopsy	0.034	0.841	1.062 (0.588 – 1.918)
Oral steroids used post biopsy	0.006	0.198	1.478 (0.816 – 2.678)

Table 3.4 Variables identified in univariate analysis to be significant regarding achievement of CR or PR versus NR and thus included in the multivariate model.

Significance in univariate and multivariate models listed. Odds ratio in multivariate model listed. SLICC, Systemic Lupus International Collaborating Clinics.

The data were then analysed excluding PR, that is to assess the variables which were significant in achieving complete response alone. Table 3.5 lists the variables which were found to be significant in univariate analysis of influencing achievement of CR versus NR and were thus included in a second multivariate model. The significance in the multivariate model and ORs are also listed and show that the same three factors: percentage of glomeruli with chronic lesions, having a 'full house' staining pattern and proteinuria level at biopsy were predictive of achieving complete response by one year.

Variable	Significance in univariate model (p value)	Significance in multivariate model (p value)	Odds Ratio in multivariate model (95% CI)
Percentage of glomeruli with chronic lesions	0.000	0.000	**0.976 (0.963 – 0.989)**
Capillary Wall C3 Present	0.007	0.231	0.704 (0.396 – 1.251)
'Full house' staining	0.003	0.005	**2.437 (1.305 – 4.554)**
Intimal thickening	0.021	0.948	1.018 (0.594 – 1.744)
Percentage interstitial fibrosis and tubular atrophy (analysed in deciles)	0.000	0.669	0.954 (0.770 – 1.183)
Foam cells present	0.015	0.080	0.255 (0.055 – 1.178)
Serum C3 low	0.024	0.983	1.007 (0.529 – 1.915)
Serum C4 low	0.000	0.570	1.207 (0.631 – 2.308)
eGFR at biopsy (analysed in deciles)	0.009	0.625	1.028 (0.921 – 1.147)
Proteinuria at biopsy (analysed in three groups, 0-49, 50-299 and 300+)	0.000	0.000	**0.303 (0.184 – 0.499)**
ACR criteria fulfilled	0.029	0.281	0.280 (0.028 – 2.828)
SLICC criteria fulfilled	0.006	0.345	3.774 (0.240 – 59.293)
Oral steroids used post biopsy	0.005	0.147	1.761 (0.819 – 3.784)
Angiotensin Converting Enzyme or Angiotensin II Receptor Blocker Prescribed	0.016	0.170	0.662 (0.367 – 1.193)

Table 3.5 Variables identified in univariate analysis to be significant regarding achievement of complete response versus non-response and thus included in the multivariate model.

Significance in univariate and multivariate models listed. Odds ratio in multivariate model listed. ACR, American College of Rheumatology; SLICC, Systemic Lupus International Collaborating Clinics.

3.3.2.1 The relationship of baseline proteinuria to response has no clear useful threshold

ROC curve analysis was performed looking at proteinuria level at biopsy in patients with CR and PR versus those with NR. Given the identified significance of proteinuria level at biopsy in predicting response to treatment, the aim was to explore if a particular threshold could be identified which may be useful clinically to predict response to treatment. This analysis did not however indicate that a threshold would be useful in determining outcome. There was a poor AUC of 0.5678. p was 0.0093 and a threshold of 349.5mg/mmol was identified although this had only a sensitivity of 55.16% and specificity of 54.6% for predicting response (CR or PR).

3.3.3 Comparison of groups of patients receiving different treatments

3.3.3.1 Demographic characteristics

470 patients were treated for lupus nephritis (LN) between 01/01/1996 and 31/12/2016. 118 patients were excluded from analysis due to having <18 months follow-up or having received treatment outside of the standard protocols. 352 patients were therefore included in the analysis. The baseline characteristics of each group are described in Table 3.6 with baseline clinical parameters displayed in figure 3.1. There was no significant difference in sex, ethnicity or age at biopsy between each treatment group. Baseline eGFR and proteinuria were significantly different between the groups ($p<0.002$ and $p<0.001$ respectfully) (figure 3.1). Median follow-up of the entire cohort was 8 years (IQR 5-11), maximum 25 from time of the first biopsy performed.

75% of the biopsies represented a new presentation of LN. The proportion of flare as biopsy indication was significantly different between the groups overall ($p=0.011$); a higher proportion of biopsies were performed due to flare in the Rituxirescue, IV CyP and PO groups. Prior exposure to IV CyP was also recorded and analysed and was found to be higher in these same groups ($p=0.013$). It is likely therefore that these flaring patients were treated with IV CyP at another institution or prior to our study start date. There was no significant difference in the proportion of patients exposed to Rituximab previously between the groups.

3.3.3.2 Patients treated with Rituxilup had a lower eGFR at biopsy and higher proteinuria in comparison to those treated with Rituxirescue

Baseline proteinuria and eGFR were significantly different between the treatment groups (table 3.6 and figure 3.1) with baseline proteinuria higher in the Rituxilup group than the Rituxirescue group ($p=0.0006$) and highest in the RituxiCyP group. Baseline eGFR was highest in the Rituxirescue group and lowest in the IV CyP group and was significantly different between the Rituxilup group and the Rituxirescue group ($p=0.0388$). Baseline complement and dsDNA were not significantly different between the Rituxilup and Rituxirescue groups, however were so between all the treatment groups with most active serology seen in the group treated with RituxiCyP and least active seen in the oral treatment group (figure 3.1).

	Overall n=352	Rituxilup n=107	Rituxirescue n=104	IV CyP (NIH/ Euro-Lupus) n=46	RituxiCyP n=28	Oral n=67	p value
Demographic data							
Age at biopsy	34 (27-46)	36 (28-52)	33 (27-43)	33 (26-41)	34 (24-45)	38 (28-52)	ns
Sex (% female)	82	79	85	85	89	81	ns
Ethnicity (%)							ns
White	26	33	22	22	14	28	
Black	30	25	30	33	39	30	
South Asian	35	35	41	35	39	25	
East Asian	3	4	3	0	4	3	
Reason for biopsy (%)							p=0.011
First presentation LN	75	84	65	72	89	72	
Flare	23	14	33	28	7	25	
Failure to respond	2	2	2	0	4	3	
Length of follow-up (years)	8 (5-11)	8 (5-10)	7 (4-10)	13 (6-18)	5 (3-8)	11 (4-13)	p<0.0001
Baseline clinical data							
ANA (% ever positive)	92	83	95	100	100	91	P=0.001
C3 (g/L) (normal range 0.7-1.7)	0.76 (0.51-1.06)	0.89 (0.57-1.19)	0.75 (0.54-0.98)	0.58 (0.33-0.81)	0.54 (0.27-0.63)	0.96 (0.58-1.2)	p<0.0001 RL vs CyP p<0.01 RL vs RCyP p<0.0001 RR vs RCyP p<0.01 CyP vs PO p<0.001 RCyP vs PO p<0.0001

C4 (g/L) (normal range 0.16-0.54)	0.13 (0.07-0.2)	0.14 (0.08-0.26)	0.12 (0.07-0.19)	0.11 (0.04-0.19)	0.07 (0.04-0.13)	0.17 (0.09-0.26)	p<0.001 RL vs RCyP p<0.01 CyP vs PO p<0.05 RCyP vs PO p<0.01
dsDNA (% ever positive)	78	65	86	87	89	76	P=0.0032
dsDNA in those patients ever dsDNA positive (units/ml) (normal range 0-30)	165 (54-692)	189 (46-667)	222 (59-784)	115 (58-516)	843 (220-2158)	84 (37-200)	p<0.001 RL vs RCyP p<0.05 RCyP vs PO p<0.001
eGFR at biopsy (ml/min/1.73m^2)	86 (54-90)	77 (54-90)	90 (77-90)	63 (29-90)	72 (44-90)	86 (61-90)	P=0.0002 RL vs RR p=0.0388 RR vs CyP p<0.0001 CyP vs PO p=0.0364
Urine PCR at biopsy (mg/mmol)	330 (168-728)	438 (231-890)	239 (103-489)	330 (203-795)	598 (279-1007)	243 (131-525)	P<0.0001 RL vs RR p=0.0006 RL vs PO p=0.0125 RR vs RCyP p=0.0032 RCyP vs PO p=0.0135

Table 3.6 Demographic and clinical data for all patients receiving an escalation in treatment for lupus nephritis from the cohort comparing treatment protocols in use at Imperial College Healthcare Trust.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as number (percentage). LN, lupus nephritis; ANA, antinuclear antibody; C, complement; dsDNA, anti-double-stranded deoxyribonucleic acid; eGFR, estimated glomerular filtration rate (CKD-EPI); PCR, protein creatinine ratio; RL, Rituxilup; RR, Rituxirescue; RCyP, RituxiCyP; CyP, IV CyP; PO , Oral treatments.

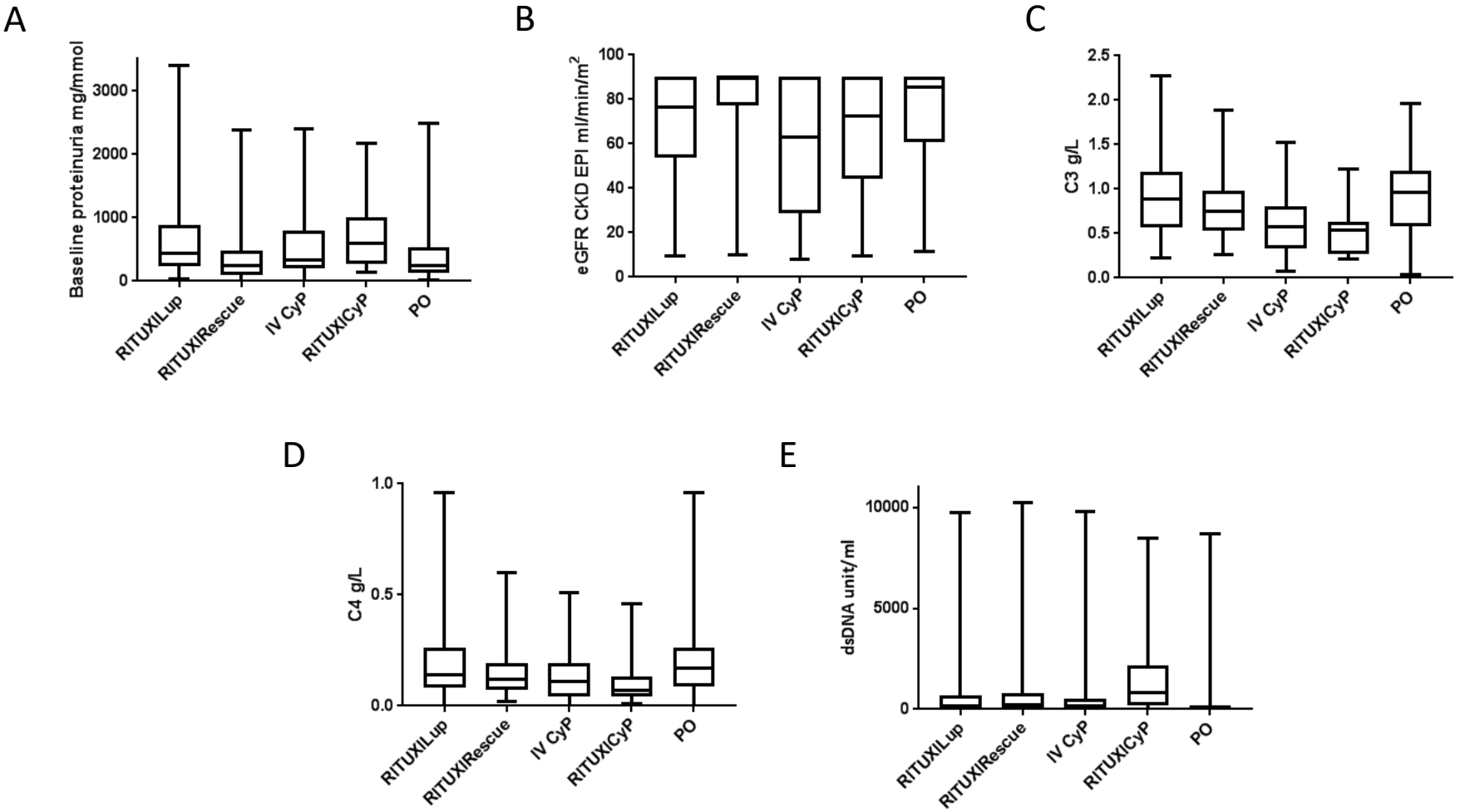


Figure 3.1 Box and whisker plots demonstrating baseline clinical parameters of the different treatment groups all of which were significantly different across the groups.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values. (A) Baseline proteinuria level (mg/mmol) ($p<0.0001$). (B) Baseline eGFR (CKD EPI ml/min/m^2) ($p=0.0002$). (C) Baseline complement C3 (g/L) ($p<0.0001$). (D) Baseline complement C4 (g/L) ($p<0.001$). (E) Baseline dsDNA (unit/ml) ($p<0.001$).

3.3.3.3 Pure class V disease is most commonly treated with Rituxilup or oral treatments whereas the most active proliferative disease is treated with IV CyP

26% of biopsies were pure class V. Class distribution was significantly different between the groups with a higher proportion of pure class V in the Rituxilup group and the oral group (table 3.7). Based on the percentage of glomeruli showing active disease, the most active proliferative disease was most likely to be treated with IV CyP or RituxiCyP. There was no difference in activity level of the biopsy between the Rituxilup and Rituxirescue groups. There was no difference in the amount of chronic damage in each group as measured by the percentage of glomeruli in the biopsy with chronic lesions and the percentage IFTA. Histological features are listed in table 3.7.

	Overall **n=352**	**Rituxilup** **n=107**	**Rituxirescue** **n=104**	**IV CyP (NIH/** **Euro-Lupus)** **n=46**	**RituxiCyP** **n=28**	**Oral** **n=67**	**p value**
Class							P<0.0001
I+II	**23 (7%)**	2 (2%)	5 (5%)	3 (7%)	0 (0%)	13 (19%)	
III/IV	**159 (45%)**	43 (40%)	54 (52%)	30 (65%)	16 (57%)	16 (24%)	
V	**90 (26%)**	36 (34%)	22 (21%)	7 (15%)	1 (4%)	24 (36%)	
III/IV+V	**75 (21%)**	26 (24%)	20 (19%)	6 (13%)	10 (36%)	13 (19%)	
Other	**5 (1%)**	0 (0%)	3 (3%)	0 (0%)	1 (4%)	1 (1%)	
Percentage glomeruli with active lesions in the biopsy (class III and IV and mixed)	47 (14-93)	36 (14-79)	39 (11-86)	100 (50-100)	63 (45-87)	32 (11-84)	p<0.001 RL vs CyP p<0.001 RR vs CyP p<0.001 CyP vs PO p<0.01
Percentage glomeruli with chronic lesions in the biopsy (class III and IV and mixed)	17 (4-39)	20 (5-42)	18 (4-39)	14 (0-48)	17 (0-31)	17 (1-58)	ns
Percent IFTA	5 (0-15)	10 (0-15)	5 (0-15)	5 (0-15)	5 (0-10)	10 (0-20)	ns

Table 3.7 Histopathological data for all patients receiving an escalation in treatment for lupus nephritis from the cohort.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as number (percentage). RL, Rituxilup; RR, Rituxirescue; RCyP, RituxiCyP; CyP, IV CyP; PO, Oral treatments. IFTA, interstitial fibrosis and tubular atrophy.

3.3.3.4 The majority (85%) of those treated with the oral-steroid free regimen Rituxilup, remained steroid-free at latest follow-up.

Glucocorticoids formed part of the treatment for every patient in the Rituxirescue and IV CyP group, the majority of the RituxiCyP and Oral groups (86% and 82% respectively) and none of the Rituxilup group. At one year of treatment, just nine patients (8%) of the Rituxilup group had started steroids, after a repeat biopsy and treatment regimen change in eight and due to uncontrolled skin and joint symptoms in one (table 3.8). At the patients latest follow-up, at 8 years (IQR 5-10 years), 85% of those in the Rituxilup group remained glucocorticoid-free. Median dose of those initiated on steroids was 10mg (IQR 5-10mg). In the Rituxirescue group 23 (22%) patients had had steroids successfully weaned by one year and at latest follow-up, 55% were glucocorticoid-free (table 3.8). The median dose of steroids at latest follow-up was 5mg (IQR 5-10mg). Overall, of all those where oral glucocorticoids formed part of the treatment (229 total), 33 (14%) were weaned at one year and 121 (53%) were steroid free at latest follow-up.

	Overall **n=352**	**Rituxilup** **n=107**	**Rituxirescue** **n=104**	**IV CyP (NIH/ Euro-Lupus)** **n=46**	**RituxiCyP** **n=28**	**Oral** **n=67**	**p value**
Glucocorticoids included in treatment	229 (65%)	0 (0%)	104 (100%)	46 (100%)	24 (86%)	55 (82%)	<0.0001
Glucocorticoids free at latest follow-up	237 (67%)	91 (85%)	57 (55%)	28 (61%)	20 (71%)	41 (62%)	<0.0001
Median dose at latest follow-up of those taking glucocorticoids (mg)	5	10	5	10	5	5	

Table 3.8 Glucocorticoid data for all patients with lupus nephritis receiving an escalation in treatment from the cohort.

First biopsy at Imperial College Healthcare Trust used. Categorical data displayed as number (percentage). RL, Rituxilup; RR, Rituxirescue; RCyP, RituxiCyP; CyP, IV CyP; PO, Oral treatments.

3.3.3.5 No difference in response to treatment by one year between the different treatment groups with a greater change in uPCR in the more proteinuric groups

There was no significant difference in response to treatment between the treatment groups (table 3.9). There was a significant difference in the baseline clinical features of the groups, however when these were corrected for with regression analysis, there remained no significant difference in response.

Tables 3.10 and 3.11 display the response, flare and outcome data when reviewing class III/IV and class V LN separately. In class III/IV LN, flare at 7 years is significantly different between the groups with more flares in the IV CyP group (table 3.10).

Analysing all classes together, the most similar treatment regimens, Rituxilup and Rituxirescue, had significantly different baseline clinical features: the Rituxilup group had a significantly higher baseline uPCR and lower eGFR than the Rituxirescue group. Despite this, there was no significant difference in rates of response to treatment by one year between these groups, even on sub-group analysis, with the Rituxilup group demonstrating a greater fall in uPCR, median (IQR) (-283.5 (-631 to -86), than the Rituxirescue group (-138 (-356 to -33) (table 3.12).

3.3.3.6 There was no difference in overall rates of flare between the treatment groups with those patients in the Rituxirescue and RituxiCyP groups flaring most quickly.

There was no significant difference in the overall rates of flare between each treatment group, however time to flare was significantly shorter (p=0.01) in Rituxirescue and RituxiCyP groups with a median time to flare of 5 months (IQR 2-18) in the Rituxirescue group and 7 months (1-35) in the RituxiCyP group. In those who had not flared by 5 years, subsequent flare rates were low (9% between 5-10 years) with no significant difference between the groups. Flare data is demonstrated in table 3.9.

3.3.3.7 There was no difference in long term outcomes between the groups with good renal function in the majority.

Median follow-up overall was 8 years (IQR 5-11). Long term outcomes of death, RRT, doubling of baseline creatinine and eGFR were reviewed at 7 years in those with 7 years follow-up or who had died before 7 years. The majority of patients in each group had an excellent outcome of eGFR $\geq$80 at 7 years except for the cyclophosphamide group where only 47% of patients had eGFR $\geq$80 at 7 years (table 3.9). When analysed as a composite outcome of death, RRT, doubling of baseline creatinine or eGFR<80, there were no significant differences between the treatment groups at 7 years(table 3.9). RRT rate at 7 years however differed between the groups with a p value of 0.045 when reviewed separately. On post-test comparison, only cyclophosphamide vs PO was significantly different with the Bonferroni correction with a p value of 0.025. Log-rank survival demonstrated p=ns for RRT in those patients with 7 years follow-up or who had died before 7 years (figure 3.2 C) when analysed by log-rank test (displayed by Kaplan Meier survival graph in figure 3.2). Figure 3.2 also displays survival graphs for survival, doubling of baseline creatinine and flare (p=ns).

	Overall **n=352**	**Rituxilup** **n=107**	**Rituxirescue** **n=104**	**IV CyP (NIH/ Euro-Lupus)** **n=46**	**RituxiCyP** **n=28**	**Oral** **n=67**	**p value**
Response by one year (%) **CR** **PR** **NR**	 **53** **24** **23**	 44 28 28	 59 22 19	 54 24 22	 54 21 25	 58 22 19	ns
Time to CR/PR (months)	**5 (2-9)**	6 (2-8)	5 (1-9)	8 (2-11)	5 (2-7)	5 (3-9)	ns
Flare by latest follow-up (%)	**20**	16	17	42	19	17	0.01
Time to flare (months (IQR))	**14 (5-48)**	13 (2-43)	5 (2-18)	24 (11-63)	7 (1-35)	63 (12-101)	0.0142 RR vs PO 0.0263
Flare by 1 year (from CR/PR) (%)	**8**	6	11	11	14	4	ns
Flare by 5 years (from CR/PR) (%)	**20**	19	21	31	27	10	ns
Flare by 7 years (from CR/PR) (%)	**24**	23	20	44	30	16	ns
Flare by 10 years (from CR/PR) (%)	**30**	21	28	55	50	22	ns
Flare between 1 and 5 years in those who haven't flared by 1 year (from CR/PR) (%)	**13**	14	11	25	11	5	ns
Flare between 5 and 10 years in those who haven't flared by 5 years (from CR/PR) (%)	**9**	0	0	23	0	14	ns
Outcomes at 5 years post biopsy **eGFR >60** **eGFR >80** **Doubling baseline creatinine** **RRT**	**n=279** **203 (73%)** **177 (63%)** **10 (4%)** **26 (9%)**	**n=**87 **65 (75%)** 54 (62%) 3 (3%) 8 (9%)	**n=**79 58(73%) 52 (66%) 2 (3%) 8 (10%)	**n=**41 24 (59%) 21 (51%) 3 (7%) 7 (17%)	**n=**20 14 (70%) 12 (60%) 1 (5%) 2 (10%)	**n=**52 42 (81%) 38 (73%) 1 (2%) 1 (2%)	ns

Death	**14 (5%)**	4 (4%)	5 (6%)	2 (5%)	0 (0%)	3 (6%)	
Outcomes at 7 years post biopsy	**n=232**	**n=**72	**n=**63	**n=**36	**n=**12	**n=**49	ns
eGFR >60	**154 (66%)**	45 (63%)	43 (68%)	19 (53%)	8 (67%)	39 (80%)	
eGFR >80	**134 (58%)**	37 (51%)	40 (63%)	17 (47%)	6 (50%)	34 (69%)	
Doubling baseline creatinine	**5 (2%)**	3 (4%)	0 (0%)	1 (3%)	1 (8%)	0 (0%)	
RRT	**32 (14%)**	11 (15%)	8 (13%)	8 (22%)	3 (25%)	2 (4%)	
Death	**17 (7%)**	5 (7%)	6 (9%)	3 (9%)	0 (0%)	3 (6%)	

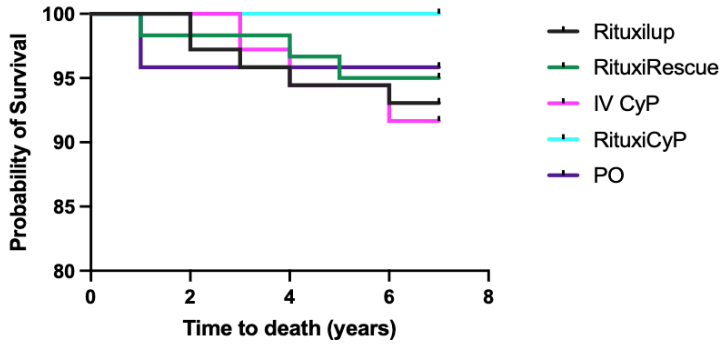
Table 3.9 Response and outcome data for all patients receiving an escalation in treatment for lupus nephritis from the cohort.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as number (percentage). CR, complete response; PR, partial response; NR, non-response; RRT, renal replacement therapy; RL, Rituxilup; RR, Rituxirescue; RCyP, RituxiCyP; CyP, IV CyP; PO , Oral treatments.

Figure 3.2 Kaplan-Meier Survival graphs for all patients with seven years follow-up data (unless died prior to seven years) demonstrating no significant difference between the treatment groups.

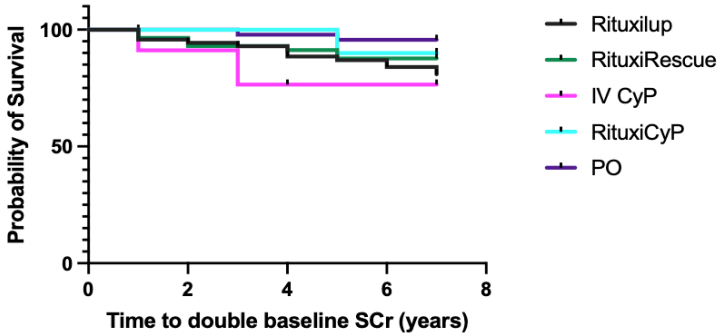
Kaplan-Meier survival graphs generated for all data displaying (A) Survival, (B) Doubling of baseline serum creatinine, (C) Renal Replacement Therapy and (D) Flare. p=ns for all analyses.

A



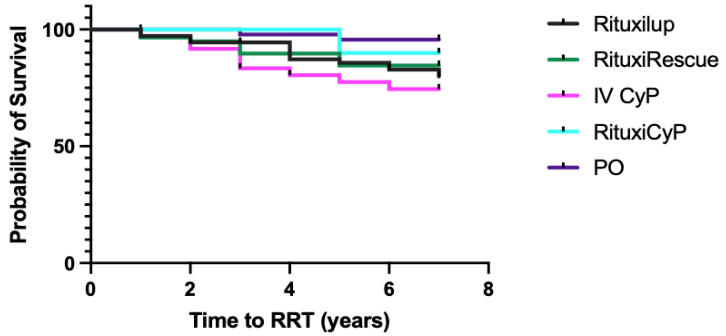
Time (yrs)	0	1	2	3	4	5	6	7
RITUXILup	72	72	72	70	69	69	68	67
RITUXIRescue	60	60	60	60	59	58	58	57
IV Cyp	36	36	36	36	35	35	34	33
RITUXICyp	12	12	12	12	12	12	12	12
Oral	48	48	48	48	48	48	48	46

B



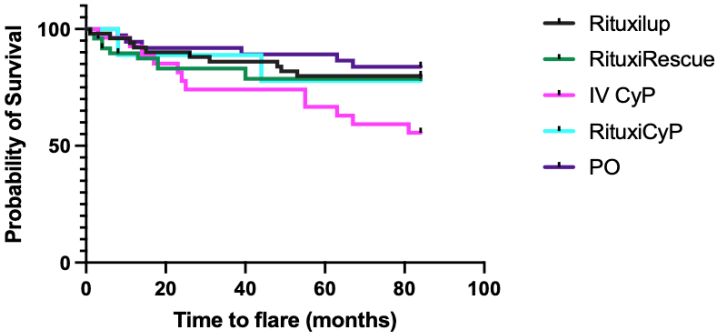
Time (yrs)	0	1	2	3	4	5	6	7
RITUXILup	71	71	68	65	63	59	58	55
RITUXIRescue	57	57	55	55	53	52	52	50
IV Cyp	34	34	34	31	26	26	26	25
RITUXICyp	10	10	10	10	10	10	10	9
Oral	48	48	48	46	46	45	45	44

C



Time (yrs)	0	1	2	3	4	5	6	7
RITUXILup	72	72	70	66	65	60	59	56
RITUXIRescue	59	59	56	55	55	52	52	49
IV Cyp	36	36	35	33	29	27	26	25
RITUXICyp	10	10	10	10	10	10	10	9
Oral	48	48	48	46	46	45	45	44

D



Time (months)	0	12	24	36	48	60	72	84
RITUXILup	51	47	46	43	42	40	40	39
RITUXIRescue	49	42	40	40	38	38	38	36
IV Cyp	27	26	22	21	21	20	17	15
RITUXICyp	9	9	9	9	8	8	8	7
Oral	37	36	35	35	34	34	32	31

	Overall n=234	Rituxilup n=69	Rituxirescue n=74	IV CyP (NIH/ Euro-Lupus) n=36	RituxiCyP n=26	Oral n=29	p value
eGFR at biopsy (ml/min/1.73m^2)		69 (52-90)	90 (74-90)	59 (31-59)	73 (50-90)	72 (50-90)	
Response by one year (%)							ns
CR	48	39	54	50	54	48	
PR	25	26	24	25	19	28	
NR	27	35	22	25	27	24	
Time to CR/PR (months)	6 (2-9)	6 (2-8)	5 (2-9)	8 (3-11)	4.5 (2-7)	7 (4-10)	ns
Flare by latest follow-up (%)	25	18	21	56	16	18	0.0021
Time to flare (months (IQR))	14.5 (6-45)	23 (2-49)	5.5 (3-17)	24 (11-63)	8 (6-44)	26.5 (10-81)	ns
Flare by 1 year (from CR/PR) (%)	11	7	14	15	11	5	ns
Flare by 5 years (from CR/PR) (%)	26	22	25	42	23	16	ns
Flare by 7 years (from CR/PR) (%)	29	24	24	57	22	18	0.04
Flare by 10 years (from CR/PR) (%)	36	22	35	63	33	20	ns
Flare between 1 and 5 years in those who haven't flared by 1 year (from CR/PR) (%)	18	1	12	35	11	12	ns
Flare between 5 and 10 years in those who haven't flared by 5 years (from CR/PR) (%)	10	0	0	33	0	9	ns
Outcomes at 5 years post biopsy	n=188	n=56	n=57	n=32	n=18	n=25	ns
eGFR >60	130 (69%)	41 (73%)	43 (75%)	16 (50%)	12 (67%)	18 (72%)	
eGFR >80	110 (59%)	34 (61%)	37 (65%)	13 (41%)	10 (56%)	16 (64%)	
Doubling baseline creatinine	8 (4%)	2 (4%)	1 (2%)	3 (9%)	1 (6%)	1 (4%)	
RRT	24 (13%)	7 (13%)	7 (12%)	7 (22%)	2 (11%)	1 (4%)	
Death	6 (3%)	1 (2%)	2 (4%)	1 (3%)	0 (0%)	2 (8%)	
Outcomes at 7 years post biopsy	n=154	n=47	n=44	n=29	n=11	n=23	ns
eGFR >60	99 (64%)	30 (64%)	31 (70%)	13 (45%)	8 (73%)	17 (74%)	
eGFR >80	83 (54%)	24(51%)	28(64%)	11 (38%)	6 (55%)	14 (61%)	
Doubling baseline creatinine	4 (3%)	2(4%)	0(0%)	1 (3%)	1 (9%)	0 (0%)	
RRT	29 (19%)	9(19%)	7 (16%)	9 (31%)	2 (18%)	2 (9%)	
Death	8 (5%)	1(2%)	3 (7%)	2 (7%)	0 (0%)	2 (9%)	

Table 3.10 Response and outcome data for all patients with lupus nephritis class III/IV +/- V receiving an escalation in treatment from the cohort.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as number (percentage). CR, complete response; PR, partial response; NR, non-response; RRT, renal replacement therapy; RL, Rituxilup; RR, Rituxirescue; RCyP, RituxiCyP; CyP, IV CyP; PO, Oral treatments.

	Overall n=90	Rituxilup n=36	Rituxirescue n=22	IV CyP (NIH/ Euro-Lupus) n=7	RituxiCyP n=1	Oral n=24	p value
eGFR at biopsy (ml/min/1.73m^2)		90 (61-90)	90 (86-90)	90 (90-90)	90 (90-90)	90 (85-90)	
Response by one year (%)							ns
CR	59	50	68	71	0	63	
PR	24	33	14	29	100	17	
NR	17	17	18	0	0	21	
Time to CR/PR (months)	6.5 (2-9)	7 (3-10)	5 (1-7)	8 (2-13)	5 (5-5)	5 (3-8)	ns
Flare by latest follow-up (%)	11	13	6	0	0	16	ns
Time to flare (months)	33 (11-66)	11.5 (3-23)	40 (40-40)	NA	NA	67 (63-107)	p=0.0214 RL vs PO 0.0485
Flare by 1 year (from CR/PR) (%)	3	7	0	0	0	0	ns
Flare by 5 years (from CR/PR) (%)	9	17	8	0	0	0	ns
Flare by 7 years (from CR/PR) (%)	15	22	9	0	NA	15	ns
Flare by 10 years (from CR/PR) (%)	20	20	14	0	NA	33	ns
Flare between 1 and 5 years	6	10	11	0	NA	0	ns
Flare between 5 and 10 years	10	0	0	0	NA	40	ns
Outcomes at 5 years post biopsy	n=70	n=29	n=16	n=6	n=1	n=18	ns
eGFR >60	56 (80%)	23 (79%)	10 (63%)	6 (100%)	1 (100%)	16 (89%)	
eGFR >80	50 (71%)	19 (66%)	10 (63%)	6 (100%)	1 (100%)	14 (78%)	
Doubling baseline creatinine	2 (3%)	1 (3%)	1 (6%)	0 (0%)	0 (0%)	0 (0%)	
RRT	4 (6%)	2 (7%)	2 (13%)	0 (0%)	0 (0%)	0 (0%)	
Death	5 (7%)	2 (7%)	3 (19%)	(0%)	(0%)	(0%)	
Outcomes at 7 years post biopsy	n=60	n=23	n=14	n=5	n=0	n=18	ns
eGFR >60	42 (70%)	14 (61%)	8 (57%)	5 (100%)	N/A	15 (83%)	
eGFR >80	(65%)	12 (52%)	8 (57%)	5 (100%)		14 (78%)	
Doubling baseline creatinine	(2%)	1 (4%)	0 (0%)	0 (0%)		0 (0%)	
RRT	(10%)	3 (13%)	3 (21%)	0 (0%)		0 (0%)	
Death	(10%)	3 (13%)	3 (21%)	0 (0%)		0 (0%)	

Table 3.11 Response and outcome data for all patients with lupus nephritis class V receiving an escalation in treatment from the cohort.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as number (percentage). CR, complete response; PR, partial response; NR, non-response; RRT, renal replacement therapy; RL, Rituxilup; RR, Rituxirescue; RCyP, RituxiCyP; CyP, IV CyP; PO, Oral treatments.

	Overall **n=352**	**Rituxilup** **n=107**	**Rituxirescue** **n=104**	**IV CyP (NIH/** **Euro-Lupus)** **n=46**	**RituxiCyP** **n=28**	**Oral** **n=67**	**p value**
Median (IQR) change in Proteinuria baseline to 1yr	-221 (-508 to -59)	-283.5 (-631 to -86)	-138 (-356 to -33)	-244.5 (-578 to -45)	-508 (-883 to -165)	-180 (-408 to -65)	P=0.0056 RL vs RR p=0.0266 RR vs RituxiCyP p=0.0320
Median (IQR) change in eGFR baseline to 1yr	0 (0-10)	0 (0 to 10)	0 (0 to 1)	2.75 (-1 to 23)	0 (0 to 27)	0 (-3 to 4)	P=0.0495 Ns between each group individually
Percent fall in proteinuria, median (IQR), baseline to 1yr	78 (39 to 92)	76 (37 to 90)	73 (40 to 90)	85 (27 to 95)	89 (47 to 96)	81 (37 to 91)	ns
Percent change in eGFR, median (IQR) baseline to 1yr	0 (0 to 12)	0 (0 to 16)	0 (0 to 0.3)	4 (-1.8 to 67)	0 (0 to 61)	0 (-4 to 6)	ns

Table 3.12 Change in baseline parameters of all patients with lupus nephritis receiving an escalation in treatment from the cohort.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as number (percentage). RL, Rituxilup; RR, Rituxirescue; RCyP, RituxiCyP; CyP, IV CyP; PO, Oral treatments.

3.3.3.8 There is a trend towards more infections in the Rituxirescue group however this did not reach statistical significance

27% patients had any infection recorded, either requiring inpatient or outpatient treatment, including 23% of the Rituxilup group and 30% of the Rituxirescue group. This conferred a 20% increase in the relative risk (RR) of infection for the Rituxirescue group which was not significant (RR 1.237 (95% CI 0.904-1.774)). Infection rates are reported in table 3.13.

Analysing the data by steroid treatment vs no steroid treatment revealed non-significant higher rates of infection in the steroid group compared to the non-steroid group (27% of the steroid group experienced at least one infection treated as an outpatient and 22% of the non-steroid group and 7% of the steroid group were hospitalised with infection compared to 3% of the non-steroid group).

3.3.3.9 No difference in rates of cancer or steroid-induced diabetes between the groups with the highest blood glucose in the Oral group.

There were two cases of recorded steroid induced diabetes within the year following treatment, one in the RituxiCyP group and one in the Oral group. There was no difference in blood glucose or HbA1c between the groups however the highest blood glucose was in the oral group. There were seven recorded cases of cancer with no difference in the distribution. There was no difference in the rates of osteopenia or osteoporosis between the groups.

	Overall	**Rituxilup n=107**	**Rituxirescue n=104**	**IV CyP (NIH/ Euro-Lupus) n=46**	**RituxiCyP n=28**	**Oral n=67**	**p value**
Urinary tract infection (%)							0.401
single	10	10	10	5	18	8	
multiple	8	7	13	10	0	0	
Chest Infection (%)							0.623
single	7	5	10	7	11	6	
multiple	1	1	2	0	4	0	
Skin infection (%)							0.545
single	3	3	3	7	4	2	
Multiple	0	0	0	0	0	2	

Viral infection (non shingles) (%)	2	1	2	2	7	0	0.156
Shingles (%)	1	0	0	0	4	3	0.039
Sepsis unknown origin (%)	2	1	2	2	4	3	0.652
Hospitalisation with infection (%)							0.113
single	5	1	7	7	11	6	
Multiple	0	1	0	0	0	0	
Outpatient infection (%)							0.766
single	13	14	14	10	14	11	
multiple	13	8	17	12	18	11	
Outpatient infection	No Steroids 22% Steroids 27%						0.303
Hospital infection	No steroids 3% Steroids 7%						0.087
Any infection hospital or outpatient (%)	27	23	31	27	32	25	0.702
Cancer (total number of cases)	7	3 (Neuroendocrine, lung& prostate, breast)	2 (Gallbladder, endometrial & bowel)	1 (B cell NHL)	1 (Kaposi Sarcoma)	0	0.493
Bone density (DEXA) (total number patients) • **Normal** • **Osteopenia** • **Osteoporosis**	n=47 20 22 5	n=5 2 1 2	n=19 6 11 2	n=9 5 4 0	n=3 1 1 1	n=11 6 5 0	0.165
Avascular necrosis (total number of cases)		1	2				0.901
Fracture (total cases)					1		0.083
HbA1c value closest to 1 year post treatment (median)	n=136 37	n=34 36	n=53 36	n=13 37	n=10 38.5	n=26 39	0.6301

Table 3.13 Side effect data for all patients with lupus nephritis receiving an escalation in treatment from the cohort.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as number (percentage). DEXA, dual-energy X-ray absorptiometry; RL, Rituxilup; RR, Rituxirescue; RCyP, RituxiCyP; CyP, IV CyP; PO, Oral treatments.

3.3.4 Predicting long term outcome and identification of thresholds

Of the 470 patients, 375 had disease on biopsy requiring escalation of immunosuppression and a minimum of 18 months follow-up from this biopsy and were therefore included in an analysis to assess predictors of long-term outcome.

Details of the biopsy and treatment are in reference to the first biopsy performed at ICHT following which there was an escalation in treatment. The median length of follow-up was 8 years (IQR 4-11). Median age at diagnosis of SLE was 29 (IQR 22-41) and at diagnosis of LN was 33 (IQR 25-45). Table 3.14 displays the demographic characteristics of the cohort.

3.3.4.1 Clinical characteristics

This was the first presentation of LN in 74.9% of patients. Median eGFR at biopsy was 84.9mls/min/1.73m^2 (IQR 53.9-90), 106 (28%) of patients had an eGFR <60 at the time of biopsy. Median proteinuria was 319mg/mmol (IQR 166-736), with 209 (56%) having uPCR >300 i.e. nephrotic range proteinuria, at the time of biopsy. Table 3.15 displays the clinical characteristics of the cohort.

Data	**Lupus Nephritis Cohort** **n=375**
Age at biopsy	34 (27-46)
Sex (% female)	81.6
Ethnicity (%) **White** **Black** **South Asian** **East Asian** **Mixed**	 25.6 28.8 34.9 3.2 3.7

Table 3.14 Demographic data for all patients receiving an escalation in treatment for lupus nephritis from the cohort on which predictors of long-term outcome were analysed.

Data	Lupus Nephritis Cohort n=375
Indication for biopsy • **New presentation** • **Flare** • **Persistent proteinuria**	 74.9% 22.9% 2.1%
C3 (g/L) (normal range 0.7-1.7)	0.76 (0.51-1.06)
C4 (g/L) (normal range 0.16-0.54)	0.13 (0.07-0.23)
dsDNA in those patients ever dsDNA positive (units/ml) (normal range 0-30)	158.5 (51.5-645.5)
dsDNA ever positive	78.4%
ANA ever positive	92%
Anti Smith ab ever positive	25.2%
Antiphospholipid ever positive	28%
Creatinine at biopsy (μmol/L)	83 (66-119.5)
eGFR at biopsy (ml/min/1.73m^2)	84.94 (53.88-90)
Urine PCR at biopsy (mg/mmol)	319 (166-736)
ACR criteria fulfilled	92%
SLICC criteria fulfilled	95.5%
Non-renal clinical symptoms of SLE	73.7%
Treatment group **Rituxilup** **Rituxirescue** **IV CyP NIH or Euro-Lupus** **RituxiCyP** **PO** **Other**	 28.5% 27.7% 12.5% 7.5% 17.9% 4%
Oral steroids	65% on steroids
Angiotensin Converting Enzyme or Angiotensin II Receptor Blocker Prescribed	66.3%
Hydroxychloroquine prescribed	59.9%

Table 3.15 Clinical data for all patients receiving an escalation in treatment for lupus nephritis from the cohort on which predictors of long-term outcome were analysed.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as percentage. ACR, American College of Rheumatology; SLICC, Systemic Lupus International Collaborating Clinics; ANA, antinuclear antibody; C, complement; dsDNA, anti-double-stranded deoxyribonucleic acid; eGFR, estimated glomerular filtration rate (CKD-EPI); PCR, protein creatinine ratio; PO, Oral treatments.

3.3.4.2 The majority of biopsies were proliferative with 71% of biopsies showing evidence of chronic lesions

65% of patients had a biopsy showing class III or IV lupus nephritis with or without class V disease. Active lesions were seen in a median of 46% (IQR 15-93%) of glomeruli of these biopsies (table 3.16). Chronic lesions were evident in 71% of these biopsies with a median of 13% (IQR 0-35%) of glomeruli in a biopsy showing chronic glomerular lesions. 25.6% of patients had pure class V disease. The majority (89%) of biopsies showed <30% interstitial fibrosis and tubular atrophy with only 1% containing more than 60%. Table 3.16 displays the histopathological features of the cohort.

Data	Lupus Nephritis Cohort n=375
Class (%)	
I/II	7.5
III/IV A	44.3
V	25.6
Mixed III/IV A +V	21.1
Other (podocytopathy in 3, APLS1, features V in 1, collapsing glomerulopathy 1)	1.6
Percentage active glomeruli per biopsy (class III/IVA +/- V)	46% (15%-93%)
Percentage chronic glomeruli per biopsy (class III/IVA, +/- V)	13% (0-35%)
Necrosis	12.7% (18.7% of III, IV and mixed)
Crescents	32.5% (49.6% of III, IV and mixed)
Percent IFTA	5% (0-15%)
IFTA	
<30%	89.2%
30-59%	9.7%
60-100%	1.1%
Sub-epithelial deposits	0 19.4%, 1+ 31.7%, 2+ 48.9%
Sub-endothelial deposits	0 31.9%, 1+ 38.3%, 2+ 29.8%
Full House immunofluorescence or immunoperoxidase (IgG, IgM, IgA, C3 and C1q all >/=1)	123, 34.6%
Immunoglobulin (at least 0.5 for one of IgM/A/G)	98%
Complement (at least 0.5 for one of C3, C4, C1q	95.2%

Table 3.16 Histopathological data for all patients receiving an escalation in treatment for lupus nephritis from the cohort on which predictors of long-term outcome were analysed.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as percentage. IFTA, interstitial fibrosis and tubular atrophy.

3.3.4.3 High levels of response to treatment

Using standard definitions of response to treatment, 76.8% of patients responded to treatment with 52.5% overall having complete response (table 3.17).

Data	**Lupus Nephritis Cohort** **n=375**
Creatinine at one year (μmol/L)	78 (65-104)
eGFR at one year (ml/min/1.73m^2)	90 (62.4175-90)
Urine PCR at one year (mg/mmol)	62 (23-192)
Response	CR 52.5% PR 24.3% NR 23.2%

Table 3.17 Response data for all patients receiving an escalation in treatment for lupus nephritis from the cohort on which predictors of long-term outcome were analysed.

First biopsy at Imperial College Healthcare Trust used. Continuous data displayed as median (inter-quartile range). Categorical data displayed as percentage. eGFR, estimated glomerular filtration rate (CKD-EPI); PCR, protein creatinine ratio.

3.3.4.5 Long term outcomes

At 7 years from the biopsy described, 57.1% of patients had an eGFR >80, 2.4% had doubled their baseline creatinine (median time 5years (IQR 4-9), 13.1% had started on RRT (median time 4years (IQR 1.5-7.5) and 9.3% had died (median time 4years (IQR 1-8.25). Table 3.18 shows outcomes at 1, 5, 7 and 10 years.

Data (outcomes mutually exclusive)	**Lupus Nephritis Cohort** **n=375**
Outcomes at one year	Total: 375
eGFR>80	266 (70.9%)
Doubling of baseline creatinine	5 (1.3%)
RRT	6 (1.6%)
Death	5 (1.3%)
Death on RRT	0 (0%)
eGFR 70-79	13 (3.5%)
eGFR 60-79	20 (5.3%)
eGFR <60	60 (16%)
Outcomes at 5 years	Total: 298
eGFR>80	187 (62.8%)
Doubling of baseline creatinine	11 (3.7%)
RRT	26 (8.7%)
Death	16 (5.4%)
Death on RRT	3 (1%)
eGFR 70-79	12 (4%)
eGFR 60-69	15 (5%)

eGFR <60	28 (9.4%)
Outcomes at 7 years	Total: 245
eGFR>80	140 (57.1%)
Doubling of baseline creatinine	6 (2.4%)
RRT	32 (13.1%)
Death	18 (7.3%)
Death on RRT	5 (2.0%)
eGFR 70-79	13 (5.3%)
eGFR 60-69	7 (2.9%)
eGFR <60	24 (9.8%)
Outcomes at 10 years	Total: 159
eGFR>80	76 (47.8%)
Doubling of baseline creatinine	4 (2.5%)
RRT	22 (13.8%)
Death	21 (13.2%)
Death on RRT	8 (5%)
eGFR 70-79	6 (3.8%)
eGFR 60-69	7 (4.4%)
eGFR <60	15 (9.4%)

Table 3.18. Outcome data at one, 5, 7 and 10 years from the first biopsy for all patients receiving an escalation in treatment for lupus nephritis from the cohort on which predictors of long-term outcome were analysed.

First biopsy at Imperial College Healthcare Trust used. Categorical data displayed as number (percentage). Data shown for patients with follow-up to the time point in question or who had died before. eGFR, estimated glomerular filtration rate (CKD-EPI); RRT, renal response therapy.

Univariate logistic regression analysis was performed on all database values including all demographic, histopathological and clinical features recorded, including but not limited to those features in the above tables describing the cohort. Logistic regression was performed to identify factors affecting achieving a good long term outcome at 7 years post biopsy, that is eGFR ≥80mls/min/1.73m^2 versus the composite outcome of doubling baseline creatinine, RRT, death and eGFR <80. Table 3.19 shows the variables revealed to be significant on univariate analysis for achieving an eGFR ≥80 at 7 years post first biopsy, which were included in the multivariate analysis model and their significance and Exp(B) values (equivalent to odds ratio) in the univariate and multivariate models.

Variable	Significance on univariate analysis p value	Odds Ratio on univariate analysis	Significance on multivariate analysis p value	Odds Ratio on multivariate analysis	95% CI on multivariate analysis	Significant on multivariate analysis	B on multivariate analysis
Age at Biopsy	0.000	0.966	0.798	0.996	0.969-1.025		-0.004
Sex	0.049	1.828	0.399	1.476	0.598-3.645		0.389
Percentage Chronic Glomeruli	0.000	0.963	0.457	0.993	0.974-1.012		-0.007
Percentage IFTA	0.000	0.901	0.059	0.956	0.912-1.002		-0.045
Full House	0.042	0.575	0.347	1.440	0.674-3.076		0.365
Clinical symptoms of SLE (Not Renal)	0.019	1.962	0.381	1.456	0.628-3.374		0.375
eGFR at biopsy (blocks 10)	0.000	1.580	0.089	1.183	0.975-1.436		0.168
uPCR at one year (3 groups)	0.000	0.350	0.002	0.424	0.247-0.734	***	-0.859
eGFR at one year (blocks 10)	0.000	2.224	0.000	1.672	1.265-2.211	***	0.514
Constant							-4.203

Table 3.19 Variables identified in univariate analysis to be significant regarding achievement of good long term outcome eGFR ≥80mls/min/1.73m² at 7 years versus the composite outcome of doubling of baseline creatinine, renal replacement therapy, death or eGFR <80 and thus included in the multivariate model.

Significance and odds ratio in univariate and multivariate models listed.

Multiple logistic regression (LR) analysis revealed both eGFR (p<0.001) and proteinuria (p=0.002) at one year post biopsy to significantly predict if eGFR at 7 years is >80 (table 3.19). eGFR at one year has an OR 1.67 (95% CI 1.27-2.21) for each decile of GFR to 90. Proteinuria at one year has an OR of 0.43 (95% CI 0.250-0.734) for change between each group of uPCR 0-50, 50-300, 300+.

Flare significantly affected outcome on univariate analysis. It was not included in the multivariate analysis model as to do so would exclude non-responders or correct for non-response thus affecting the reliability of the model. Flare ever has an OR of 0.341 (95% CI 0.184-0.635) for good outcome of eGFR >80 at 7 years (p=0.001).

Logistic regression (LR) analysis was also performed to identify factors affecting the outcomes of RRT and death at 7 years individually.

LR analysis revealed that ethnicity (Black/other), complement C3 level and eGFR and proteinuria at one year significantly affected risk of RRT at 7 years. Black patients had increased risk of RRT with OR 3.273 (95% CI 1.198-8.941) p=0.021. C3 had an OR of 0.212 (0.049-0.923) p=0.039 per g/L. eGFR has an OR 0.655 (95% CI 0.535-0.802) p<0.001 per decile up to 90 and proteinuria has an OR of 2.661 (95% CI 1.410-5.023) p=0.003 for change between each group of uPCR 0-50, 50-300, 300+.

LR analysis for risk of death at 7 years revealed that age at biopsy and proteinuria at one year post biopsy significantly affected the risk, with age at biopsy having an OR of 1.801 (95% CI 1.149-2.822) p=0.01 per decile and proteinuria having an OR of 2.577 (95% CI 1.128-5.887) p=0.011 for change between each group of uPCR 0-50, 50-300, 300+.

3.3.4.6 Identification of optimal cut-off point

In order to identify a cut off point for eGFR and proteinuria, ROC curves were generated (figure 3.3). These identified cut-offs for eGFR and uPCR predictive of eGFR ≥80 at 7 years after the first biopsy at ICHT with a diagnosis of lupus nephritis. The ROC curve for eGFR had an AUC of 0.8037 p<0.0001 and identified a cut-off of >80mls/min/1.73m^2 as predictive of good long-term outcome with eGFR ≥80 at 7 years with a sensitivity of 75.2% and specificity of 75.5% (figure 3.3 (A)). The positive predictive value (PPV) of this cut-off was 80% with a negative predictive value of 75% (table 3.20). The ROC curve for proteinuria had an AUC of 0.6811 p<0.0001 and identified a cut-off of <90mg/mmol as predictive of good long-term outcome with eGFR ≥80 at 7 years with a sensitivity of 64.1% and specificity of 61.3% (figure 3.3 (B)). PPV was 70% with NPV 60% (table 3.20).

Cut-offs of eGFR and uPCR predictive of eGFR ≥70 and ≥60 at 7 years were also investigated. The cut-off values identified were similar: predicting eGFR ≥70 at 7 years was eGFR at one year >80.39 and proteinuria <78, predicting eGFR ≥60 at 7 years was eGFR at one year >78.49 and proteinuria at one year <89.5.

Combining the cut-offs for eGFR and proteinuria did not improve the sensitivity of the measures, however it did improve the specificity: a combination of eGFR >80mls/min/1.73m2 and proteinuria <90mg/mmol at one year predicted good long-term outcome of eGFR ≥80 at 7 years with a sensitivity of 55.9% and specificity of 88.7%. PPV of this combination was 86.4% and NPV 61.0%. Using the new cut-off points, achieving both the eGFR and proteinuria thresholds predicts a good outcome (eGFR ≥80) at 7 years with an OR 9.922 (95% CI 4.979-19.774) p<0.001. Achieving only one or neither of the thresholds gives an OR 0.101 (95% CI 0.051-0.201) p<0.001 for a good outcome (eGFR≥80) at 7 years.

	Urine protein creatinine ratio <90
Sensitivity	64.1%
Specificity	61.1%
PPV	70%
NPV	60%
	eGFR >80
Sensitivity	75.2%
Specificity	75.5%
PPV	80%
NPV	60%
	Both urine protein creatinine ratio <90 and eGFR >80
Sensitivity	55.9%
Specificity	88.7%
PPV	86.4%
NPV	61.0%

Table 3.20 Features of the thresholds for urine PCR and eGFR as predictive of achieving a good long-term outcome.

Good long-term outcome defined by eGFR ≥80mls/min/1.73m^2 at 7 years post biopsy. eGFR, estimated glomerular filtration rate; PPV, positive predictive value; NPV, negative predictive value.

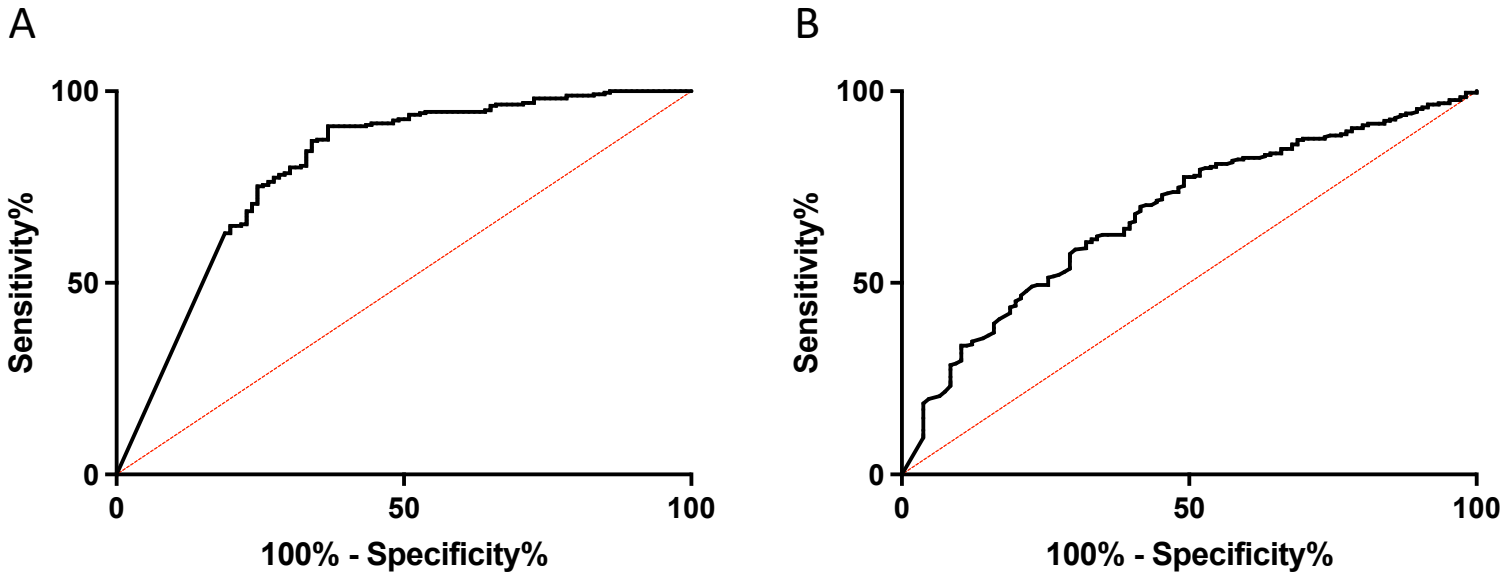


Figure 3.3 ROC curves of the sensitivity % and 100%-specificity % of (A) eGFR and (B) proteinuria level at one year post biopsy, predicting an excellent long-term outcome of eGFR ≥80ml/min/1.73m² at seven years post biopsy.

(A) eGFR area under curve 0.804 (95% CI 0.749 to 0.858) p<0.0001. (B) Proteinuria level are under curve 0.681 (95% CI 0.622 to 0.740) p<0.0001.

3.3.4.7 Validation of the cut-off points

To validate these identified targets for treatment, a dataset of patients with LN from the UCL Rheumatology Clinic comprising 188 patients was kindly shared with us for review. Taking those patients who were treated for their first episode of LN between 1996 and 2011 with known uPCR and eGFR at one year following treatment and known outcome at seven years following initiation of treatment, the cohort were compared to the ICHT cohort (table 3.21) taking just those patients with seven years follow-up (245 patients). The UCL cohort were younger with a higher percentage female and a greater proportion of White patients. The sex and ethnicity were however not significantly different. The UCL cohort had a higher eGFR at baseline and at one year and a lower baseline proteinuria (table 3.21). Outcomes were not significantly different.

Variable	ICHT (n=245)	UCL (n=53)	p value
Age at biopsy	34	26	0.0002
Sex	77.14% Female	88.68% Female	ns
Ethnicity **White** **Black** **South Asian**	 27.5% 29.6% 42.7%	 41.51% 32.08% 26.42%	ns
Baseline eGFR (ml/min/1.73m^2)	78.4 (50-90)	90 (72-90)	0.0037
Baseline proteinuria	316 (168-742)	224 (118-517)	0.0389
eGFR at one year (ml/min/1.73m^2)	87.24 (59-90)	90 (81-90)	0.003
Proteinuria at one year	63 (23-185)	47.5 (26-149)	ns
Outcomes at 7 years			ns
Death	9.39%	11.32%	ns
RRT	15.1%	15.09%	ns
Doubling baseline creatinine	2.45%	5.66%	ns
eGFR ≥80	57.14%	62.26%	ns
eGFR 70-79	5.306%	5.660%	
eGFR 60-69	2.857%	5.660%	
eGFR <60	9.796%	0%	

Table 3.21 Comparison of the ICHT and UCL cohorts. Demographic, clinical and outcome data.

Continuous data displayed as median (inter-quartile range), categorical data displayed as percentage. ICHT, Imperial College Healthcare Trust; eGFR, estimated glomerular filtration rate; RRT, renal replacement therapy.

Thresholds were evaluated individually and in combination (table 3.22). Individually, a uPCR of <90mg/mmol and an eGFR >80mls/min/1.73m^2 could identify those patients who would have a good outcome at seven years (eGFR ≥80mls/min/1.73m^2) with a high sensitivity (73% for uPCR and 94% for eGFR). However, the specificity of each was poor at 50% for uPCR and 55% for eGFR. The NPV of uPCR was also poor at 50%, however was much better for eGFR at 85%. Taking the two in combination resulted in a poorer sensitivity at 67% and NPV at 57% however the specificity and PPV were much improved and were comparable with the values for the ICHT dataset (table 3.22). Arguably, these are the values to optimise for clinical utility of the thresholds.

	Sensitivity	**Specificity**	**PPV**	**NPV**
uPCR <90 UCL	73%	50%	73%	50%
uPCR <90 ICHT	64%	61%	70%	60%
eGFR >80 UCL	94%	55%	78%	85%
eGFR >80 ICHT	75%	76%	80%	60%
Both uCPR <90 and eGFR >80 UCL	67%	81%	87%	57%
Both uCPR <90 and eGFR >80 ICHT	56%	89%	86%	61%

Table 3.22 Comparison of the utility of the uPCR and eGFR thresholds for prediction of a good long-term outcome in the ICHT and UCL cohorts.

ICHT, Imperial College Healthcare Trust; eGFR, estimated glomerular filtration rate; uPCR, urine protein creatinine ratio.

3.4 Discussion of results and future work

The database produced describes a large multi-ethnic cohort of LN patients treated at a central London hospital. Encompassing every patient treated for active lupus nephritis in the clinic over 20 years without exception, it represents the full range of histopathological appearances and treatment protocols in use at the clinic. Analysis of the features at baseline was able to give an insight into predictors of response to treatment. Delving deeper into the data, different treatment groups were compared, using 'real-world' data with a long follow-up period (median 8 years, range 0 to 25 years). It is the largest dataset describing an oral-steroid-free regimen. Importantly, it shows comparable response and outcomes to the same treatment regimen with pre-existing, continued oral steroids (Rituxirescue) and also to more standard protocols such as IV CyP and oral treatments such as MMF and glucocorticoids. It also provides longer follow-up outcome data for the original Rituxilup cohort[35] with the addition of further patients treated with this regimen. Whilst the outcomes are in line with other published cohorts and better than most trials, response rates to treatment remain sub-optimal overall and more effective treatment regimens for LN are required. That was the rationale for using the data to try to define the best predictors of long-term outcomes to help guide clinical trial protocols in order that investigation of new treatments is assessed by the most relevant outcomes. Further analysis identified the best performing predictors of long-term outcomes with clinically useful targets for treatment.

In this analysis, response to treatment, 'good' long term outcomes and flare are all discussed. Despite these being key outcomes managed daily in LN clinics and described in both retrospective database analyses and clinical trials, there are not strict definitions used worldwide. Moreover, the studies that have assessed predictors of long-term outcomes have all assessed different outcomes. The long-term follow-up of the Euro-Lupus cohort defined a good outcome in terms of renal function as measured by creatinine and used the value of ≤1.0mg/dl at 7 years.[127] The 7 year follow-up analysis of the MAINTAIN trial used the same value: creatinine <1mg/dL;[128] however the initial analysis of the long term follow up of the MAINTAIN trial investigated multiple different end points. Last serum creatinine ≤120% of baseline was used for their calculation of the predictive value of proteinuria <0.5g/d for long term outcomes.[250] In Mackay *et al.*'s study, the long-term outcomes selected were CKD, severe acute kidney injury (SKI) and the need for RRT.[129] CKD was defined as an increase in creatinine of ≥30% (±2%), that is the worst creatinine from the best creatinine sustained for 3 months and an eGFR <60ml/min/1.73m^2. The definition was derived from a National

Kidney Foundation and the US Food and Drug Administration workshop.[251] This demonstrates the variation in the literature and therefore the challenge of comparing the value of published predictors of outcomes.

The patient cohort described here is young and therefore a high target of eGFR ≥80ml/min/1.73m^2 at 7 years was deliberately chosen as the definition of a 'good' outcome in this analysis. A creatinine of 1.0mg/dl is equivalent to a CKD EPI of 76 ml/min/1.73m^2 in a 34-year-old (median age in this cohort) female. Rather than referring to CKD, eGFR thresholds were used, persisting over 2 readings. NICE guidelines advise that a reduction should be 'persistent'.[252] Outcomes were described at 7 years to be in alignment with the Euro-Lupus [127] and MAINTAIN long term analyses [128] and provide the longest follow-up possible whilst still containing a meaningful number of patients. Data were also reviewed at 10 years; however due to the change of treatment protocols over the years, some data had less follow-up than others making comparison at this time point less useful.

Whilst cyclophosphamide, MMF and glucocorticoids dominate the clinical guidelines for treatment of LN, Rituximab has been adopted by many centres over the last few years. Including patients treated with Rituximab at our centre from as early as 2005 allows this dataset to represent and thus be applicable to each common treatment in use today. Some treatment protocols have changed however, as would be expected over the years. Analysis therefore does include patients treated with NIH dosing of cyclophosphamide.

Whether response was considered to be CR or CR and PR, baseline proteinuria level, a 'full house' pattern on immunohistochemistry and the percentage of glomeruli with chronic lesions were each found to significantly affect response by one year. Baseline proteinuria was also identified by Ayoub *et al.* [115] to be predictive of response, featuring in most of their predictive models identified using machine learning. Other common clinical variables included in the models were baseline eGFR, race, age, sex, complement C3 and C4 and dsDNA. The clinical variables chosen for assessment in Ayoub's study were so due to their common use in the clinic as part of the assessment of a patient with LN. eGFR, race and proteinuria level were selected for many of the models to predict outcome, as were complement and dsDNA, however these latter variables had less impact. All of these listed clinical variables were analysed in this thesis for impact on response, using regression analysis, however were not found to be independently significant in predicting response to treatment in the multivariate model. The histopathological features which were significant in our cohort, 'full house' staining pattern and chronic glomerular lesions, were not evaluated by Ayoub *et al*..[115] Each of

the predictors in the ICHT data had a p value of <0.01 on multivariate analysis. The AUC for proteinuria was however only 0.5678. Study of the ALMS cohort identified AUC's of only 0.55 and 0.51 for CR and PR which included baseline UPCR as well as features of SLE activity and damage.[113] With this AUC, a satisfactory threshold could not be identified for proteinuria which was predictive of response by one year. A threshold of 349.5 mg/mmol could only predict response with slightly more than 50% sensitivity and specificity. This could be because the degree of proteinuria is dependent on both the amount of activity as well as the degree of chronic change in addition to other factors such as existing treatments including ACE or ARB use. Histological class was measured but not found to be predictive of response to treatment by Ayoub *et al.* or in these data. The presence of a 'full house' pattern could be a marker of activity of the disease. To quantify the degree of active disease and chronic damage related to LN, the percentage of glomeruli showing active and chronic lesions respectively was calculated and compared. Activity and chronicity indices were not used as they are not calculated at our institution. Whilst there has been some evidence of their use in predicting outcomes [29] the indices were not validated by the Lupus Nephritis Collaborative Study Group [131] and the ISN/RPS proposed guideline revisions suggest a phase 2 study using an evidence based approach is required to determine if a modification to the indices is valid. They do however still recommend use of a modified score in the meanwhile, albeit unvalidated.[28] The amount of interstitial fibrosis and tubular atrophy, which is associated with chronic damage, was not significant here in predicting response by one year in the multivariate model. This could be because the response definition requires only stability of renal function. It has previously been shown to be of great significance in predicting long term outcomes.[29]

The mixture of features of both active disease and chronic damage being associated with response to treatment is interesting. It could indicate that non-responders have more rapidly progressive disease, or present later in the disease process. Or it could indeed indicate that different disease processes are present in non-responders such as necrotic or fibrotic processes leading to an increase in chronic damage as well as the more active disease. Potentially relevant disease pathways can only be postulated from these data. It has been shown that early treatment in SLE increases the chances of remission [125] and prevents kidney damage.[6]

These predictors of response therefore provide potential insight into the mechanisms of disease in non-responders and better aid clinicians in predicting response to treatment at the stage of the biopsy.

In the comparison of treatment groups, patients in each group were identified to have different baseline characteristics which likely represent the characteristics on which their treatment decision was based. Patients with the most active disease histologically and lowest eGFR were generally treated with IV CyP which also conforms with our treatment protocols where those with rapidly deteriorating renal function are treated with IV CyP. In recent years, patients with the most active disease biochemically, as measured by uPCR, complement and dsDNA, were treated with RituxiCyP, which fits with our treatment protocols where patients with highly active disease or significant extra-renal features such as cerebral lupus are treated with RituxiCyP. Those with the least active disease by biochemical parameters were treated with oral therapies alone.

The patient groups who received the two most similar treatment regimens, Rituxilup and Rituxirescue, are different in that Rituxirescue patients had a higher eGFR and lower proteinuria at baseline than Rituxilup patients. This could be due to the higher proportion of patients with a biopsy indication of flare in the Rituxirescue group - the patients are known to have LN and are therefore under close monitoring, a change in the eGFR and proteinuria would be acted upon quickly, before deteriorating further as could happen in a new presentation where baseline parameters can deteriorate significantly before the patient becomes symptomatic enough to seek diagnosis and treatment. The higher proportion of biopsy indications for flare in the Rituxirescue group is to be expected as this treatment regimen is largely only chosen if patients are already on long-term glucocorticoids. Despite the worse baseline features of the Rituxilup group, response to treatment, flare rate and long-term outcomes were not significantly different to the Rituxirescue group, with a greater median change in eGFR and proteinuria in the Rituxilup group, arguably demonstrating that the oral steroids are not conferring any additional benefit in this treatment regimen. Indeed, only 9 patients (8%) of those on the Rituxilup regimen started oral steroids in the year following biopsy, due to a change in treatment for lupus nephritis in all but one and 85% remained steroid free at latest follow-up (median 8 years (IQR 5-10)).

Weaning of steroids was actively performed in the Rituxirescue group. Despite treating active disease and in a patient group on long-term steroids, steroids were able to be weaned in 22% of this group by 1 year and 55% by latest follow-up.

As described, the baseline characteristics in terms of biochemical and histological activity and reason for biopsy were significantly different between the treatment groups, consistent with our current protocols. Despite an arguably ‘worse’ baseline in some however, treatment response and long-term outcomes do not differ between the groups with the majority of patients in each group having

excellent renal function with eGFR >80 at 7 years except the cyclophosphamide group where this was 47%. This group were shown from baseline parameters to have the most 'severe' disease as fits with our treatment protocols where cyclophosphamide is used for rapidly progressive disease or rapidly deteriorating renal function. This review would suggest that lupus nephritis can be treated without oral steroids or cyclophosphamide when targeted appropriately, achieving the same response rate and long-term outcomes without the inherent side effects of these treatments.

There was no difference in the rate of flare overall or by 7 years, however flares occurred sooner in the Rituxirescue and RituxiCyP groups. This could be because the RituxiCyP were most active biochemically. It is unclear why the Rituxirescue group flare more quickly. Flare rate overall was 24% by 7 years which is slightly lower than published data which describes flare rates as between 27-67% patients.[253] Flare is a complex problem to untangle in a retrospective study – undoubtedly a proportion are associated with falls in concordance with medication.

There was no significant difference between mortality rates between the groups. There is a slightly higher number in the Rituxilup and Rituxirescue groups as compared to the RituxiCyP group. This could be due to an avoidance of IV CyP in patients who are particularly frail or at risk of infection but still requiring immunosuppression where IV CyP may be avoided.

There was no significant difference in the side effects between each group except for shingles rate and blood glucose which were both highest in the RituxiCyP group. The rate of side effects, infections in particular, is however low with an overall rate of 27% of patients having any recorded infection. Due to issues of data protection, it was only possible to record infections which required hospitalisation at ICHT, were recognised at a clinic appointment or were reported by patients and recorded in the clinic notes. This will have missed a large number of infections managed in the community. There was a larger rate of infections in those patients treated with steroids as would be expected, however this did not reach statistical significance, likely due to the small numbers.

What matters to patients is not outcomes at one or two years, currently measured in clinical trials. It is the long-term survival and preservation of renal function. It is important to identify therefore how 1-to-2-year endpoints from clinical trials translate to long-term outcomes of preservation of excellent kidney function. Long-term follow-up of clinical trials provides excellent datasets of very well characterised patients with uniform treatment protocols. The populations can, however, also be very uniform due to tight criteria for trial entry and the limited heterogeneity of trial patients. Trial protocols also do not necessarily translate to the clinic, such as frequency and standard of urine

microscopy or 24-hour urinary protein assessment and it is unclear if findings can translate to patients receiving different treatment protocols to that in the clinical trial.

Long term follow-up of the Euro-Lupus [127] and MAINTAIN [128] cohorts suggested a proteinuria cut off at 1 year of <0.8g/d and <0.7g/d respectively, better predict good renal outcomes at 7 years than usual CR criteria of <0.5g/d. The treatment protocols used are those recommended in current guidelines and thus highly applicable to patients currently treated for lupus nephritis, however each of these studies was undertaken in a mainly White (84% in Euro-Lupus [127] and 79% in MAINTAIN [128]) population in patients who all had proliferative renal disease.

Given the consistency of the findings from the Euro-Lupus and MAINTAIN cohorts however, it was investigated if the thresholds could be validated in a more-diverse, 'real-world' cohort. In identifying a value of <90mg/mmol as predictive of good long-term outcome, these data have indeed shown that the findings are translatable to the wider population. The sensitivity of the cut-off is however lower in the current dataset. This could possibly be due to the varied nature of the dataset, patients with co-morbidities and those unlikely to be concordant with treatment could be excluded from a clinical trial and these are patients who would be likely to do worse and potentially not in line with their results at one year. It could be that there are differences between ethnicities, classes of LN, treatment protocols or another variable. Tektonidou *et al.* identified a very different 10 year ESKD risk for patients with different classes of LN with those with class IV having the greatest risk with a 15 year risk of 44% for those diagnosed in the 2000's whereas class V had a 20% 15 year risk when diagnosed during the same period[4]. 47% of the patients described in the current cohort have more than one biopsy performed. As histopathological class and treatment protocols used can change with each biopsy, data was not sub-divided for this analysis. The proportion of patients having their first episode of nephritis was the same, 74% patients in Euro-Lupus [127], 87% in MAINTAIN [128] and 74.9% in this cohort.

This analysis adds weight to changing the targets in clinical trials of new treatments. Treatments may currently not advance to clinical use for LN if they fail to reduce proteinuria to less than 0.5g/day such as anifrolumab,[56] currently approved by the FDA for moderate SLE however not for severe LN and not currently recommended by NICE. There have however been some recent promising results and indeed in two recent clinical trials, the primary endpoint was changed to include a proteinuria target of uPCR <0.7 rather than <0.5 to align with the growing body of evidence, of which these data were a part,[102][101] of this threshold as predictive of long term outcomes.[58][56] The value of 0.8g/day was also recently used by Ayoub *et al.* as a 'relaxed'

definition of response in their study investigating variables affecting response in LN, as well as the more standard 0.5g/d. The performance of the models was similar for both the traditional and more lenient definitions of proteinuria and eGFR.[115] Analysis of the Hopkins Lupus Cohort using the BLISS-LN 2 year protocol also confirmed that proteinuria ≤0.7g/day and eGFR ≥60ml/min/1.73m^2 predicted good long term outcomes (survival with no ESRD).[59] Research from this thesis was cited here as evidence of a higher threshold in a more diverse cohort.[59] The threshold of 0.8g/day proteinuria at 12 months as a predictor of good long-term renal outcomes was noted in the recent EULAR 2019 SLE guidelines update [40] and in the EULAR LN guidelines update the definition of CR was modified to proteinuria <0.5-0.7g/day.[36]

In the Euro-lupus and MAINTAIN long-term analysis, addition of eGFR did not improve the predictive value of proteinuria with regards to good outcome. In contrast, in our cohort, eGFR at 12 months was identified alongside proteinuria as predictive of outcome. eGFR is considered to be the best measure of kidney function.[242] The CKD-EPI formula is accurate at both ends of the spectrum (up to 90mls/min/m^2) and over time.[254] Both proteinuria and creatinine at 12 months were found by Mackay *et al.* in their analysis of 550 patients (51% White) to be predictive of CKD at follow-up (12-48 months post presentation).[129] eGFR was actually, in our data, the best individual predictor of a good outcome with a PPV of 80% and an NPV of 75%. The combination of proteinuria and eGFR led to a reduction in the sensitivity to 56% however an increase in the PPV to 86% and specificity to 89%. It is arguably the specificity which is of greater importance, given that in the event of predicting a good outcome for a patient, in an effort to reduce treatment complications, treatment withdrawal may take place sooner or treatment may not be escalated further in the event that traditional CR criteria (proteinuria <50mg/mmol) had not been met, which would be usual practice. However, sacrificing sensitivity for specificity could lead to over treating of patients. In the long term follow-up of the MAINTAIN cohort, the NPV of proteinuria <0.7g/d was low (29%) [128] similar to their earlier analysis of proteinuria <0.5g/d predicting long term outcomes,[250] indicating that even when higher proteinuria cut-offs are used for this prediction and definition of response, eGFR also needs to be considered given that sub-optimal proteinuria is not a good predictor alone of a poor long-term outcome.

This study focussed on prediction of excellent eGFR. However, the eGFR and proteinuria predictive of outcomes of eGFR 60 and 70 at 7 years were also reviewed and was found to be the same as those values for eGFR 80, indicating that a very good eGFR at one year is required for eGFR at 7 years to be >60, let alone >80, highlighting the importance of eGFR at one year. The proteinuria is

also the same, meaning that a better proteinuria than 90 doesn't appear to predict or result in a better outcome. Interestingly in the initial analysis of long term follow-up of the MAINTAIN cohort, the fall in proteinuria was predictive of good long term outcome whether it was defined by eGFR ≤60ml/min/1.73m^2, creatinine ≤1mg/dL or <1.4 mg/dL or creatinine ≤120% of baseline as used in their 92% PPV at 12 months prediction of good outcome with early proteinuria decrease of ≤0.5g/d.[250]

The identified thresholds were validated using a multi-ethnic dataset of patients with LN from a rheumatology clinic in another part of central London. The dataset differed from that of the ICHT in that the patients were younger, with a higher proportion of female and White patients, although the sex and ethnic distributions were not significantly different. They had a lower baseline proteinuria and higher baseline eGFR. Despite these differences, the thresholds in combination performed comparably to the ICHT dataset with similar specificity and PPV. The specificity of each individual threshold was poor. The reasons for this could be multiple, such as flare or poor concordance with treatment. Of the three patients in the UCL cohort who were predicted to have a good outcome using both eGFR and UPCR but didn't, one died of an illness unrelated to the LN with renal disease in remission. The other two had flares which accumulated chronic damage. It would be impossible to identify a predictor of long-term outcomes with 100% sensitivity and specificity. Arguably, reducing treatment inappropriately would be of greater harm to a patient and therefore specificity and PPV of a threshold are the features to target. Using the thresholds in combination appears to achieve this. Future work will involve collecting prospective data to validate the thresholds and ideally would include repeat biopsies.

This analysis does not address the safety of treatment withdrawal in those with good outcome predicted but does add weight to the value of a trial addressing this question and provide evidence that the target for proteinuria in such a trial should be higher than 50mg/mmol.

There are limitations to this data analysis, in that it is a single-centre study and retrospective review. The patient groups for the treatment comparison are inherently different at baseline and therefore it is not a direct comparison of treatments: outcomes of one treatment may not be applicable to a cohort of patients with different baseline characteristics. Side effect data are incomplete and the patient population is 'real-world' including patients with co-morbidities and potential concordance issues. However, whilst data gathering is difficult in retrospective analyses, the ICHT Renal Unit was early to introduce computerised results and clinical notes storage, which improved the process for this dataset.

The data give valuable indications of factors which may predict response to treatment including the level of activity of the disease and the amount of chronic damage present. The data also demonstrate that with appropriate patient selection, equally good responses to treatment and outcomes can be achieved with no IV CyP or oral steroids and the side effects inherent to these treatments. When no oral steroids are used for treatment induction, the vast majority, 85% in this cohort, remain steroid free at the latest follow-up of median 8 years. The complete nature of the unselected, variably treated and multi-ethnic cohort meant that this analysis validates the findings from the long-term follow-up of the Euro-Lupus and MAINTAIN cohorts, with "real world" data, that proteinuria thresholds at one year are too stringent. These data also suggest that more attention needs to be paid to preservation of excellent renal function. In these data, a proteinuria threshold of <90mg/mmol and eGFR threshold of >80ml/min/1.73m^2 at 1 year, better predicted long term renal survival than current standard trial endpoints. As this is the key renal outcome that matters to patients, this is evidence that it is time to change the definition of success of treatments being trialled in LN.

3.5 SUMMARY OF KEY FINDINGS

The largest cohort of patients with an oral-steroid free treatment protocol has been described. In total 803 biopsies from 470 patients treated over a 20-year period with different standard protocols have been described.

Overall, there was a rate of 45.7% complete response by one year in the cohort.

Proteinuria level at biopsy, a 'full house' pattern on immunohistochemistry and the percentage of glomeruli with chronic lesions were each independently significant in predicting response to treatment by one year.

Proteinuria and eGFR at one year were independently significant for predicting excellent long-term outcome of eGFR >80ml/min/1.73m2.

No differences in response to treatment or long-term outcome were seen between treatment protocols in use indicating that LN can be successfully treated without the use of oral steroids or cyclophosphamide.

Use of oral steroid free treatments does require patient selection; however is not reserved for those with the 'best' baseline parameters.

The majority (85%) of those treated with the oral-steroid free regimen Rituxilup, remained steroid-free at latest follow-up indicating that those treated with an oral steroid free induction regimen can remain off oral steroids.

Steroid free treatment is not associated with an increased flare rate.

An eGFR of >80mls/min/1.73m^2 was predictive of good long-term outcome with eGFR >80 with a sensitivity of 75.2% and specificity of 75.5% (PPV 80%, NPV 75%). AUC 0.8037 p<0.0001.

A proteinuria of <90mg/mmol was predictive of good long-term outcome with eGFR >80 with a sensitivity of 64.1% and specificity of 61.3%. (PPV 70%, NPV 60%). AUC 0.6811 p<0.0001.

Achieving both the eGFR and proteinuria thresholds predicts a good outcome at 7 years with an OR 9.922 (95% CI 4.979-19.774) p<0.001.

eGFR and proteinuria targets were validated in a separate multi-ethnic cohort.

4 CHAPTER FOUR – COMPLEMENT STUDIES

4.1 INTRODUCTION AND BACKGROUND

Response rates to current treatment options are sub-optimal. Analysis of the demographic, clinical and histopathological features of patients with LN treated over the last 20 years at ICHT demonstrated that response to treatment is the key determinant of achieving good long-term outcomes. In chapter 3 it was reported that in the cohort of patients reviewed for this study, 45.7% achieved CR within one year of treatment and five years post biopsy, 63% had an eGFR >80m l/min/1.73m^2. In a recent clinical trial, only 23% achieved CR at one year in the standard of care group treated with low dose steroids and mycophenolate mofetil.[46]

Treatment options in LN are predominantly anti-inflammatory, rather than target-specific.[241] Side effects, especially from steroid use, are common.[241] New treatment approaches are required to improve response rates and consequently improve long term outcomes. Options for new treatment approaches include complement C5 inhibition.[241] Consequently, there is a need to identify which patients with LN are most likely to benefit from this approach.[241]

Complement is widely accepted to play a significant role in the pathogenesis of LN, in particular through activation of the classical pathway by ICs [20] but also through both lectin and alternative pathway activation.[154][241] Blockade of C5 is effective in murine SLE [65] and there are case reports of response to C5 inhibition using eculizumab.[66][241] Complement C3 is the central activation protein in the complement cascade and its activation results in the production of anaphylatoxin (C3a) and opsonin (C3b) and can trigger C5 activation.[241] Complement C3c and C3d are formed by cleavage of C3b.[241] C5 activation results in anaphylatoxin (C5a) and membrane attack complex (MAC, C5b-9) which can damage cell membranes and induce erythrocyte lysis.[241]

Previous studies have demonstrated: a high prevalence of MAC staining in LN including in the peritubular areas [255]; co-localisation of C3c and C5b-9 [256]; mesangial and mixed MAC staining distributions in class III and IV LN respectively [257]; and frequent presence of staining for both C5b-9 and mannose-binding lectin.[258][241] In contrast, C9 staining was demonstrable in only 43% of LN biopsies.[259][241] The kinetics of MAC staining in LN and its role in chronic LN are poorly characterised.[241] C5b-9 has additionally been studied in serum and urine and there is evidence to show that serum C5b-9 is a useful marker of activity.[259][241]

To identify which patients with LN might benefit from C5 blockade, MAC staining distribution in 57 LN biopsies was studied.[241] Alongside C5b-9, other elements of the complement pathway were also investigated to better understand their role in LN.[241]

4.2 CHAPTER AIMS

- To use immunohistochemistry staining to characterise complement deposition to assess its variability in lupus nephritis and thereby provide insight into the complement pathway in lupus nephritis.
- To explore if complement staining has an association with response to treatment in LN.
- To identify the suitability of complement markers to identify patients with lupus nephritis in whom C5 blockade may be a useful treatment.
- To explore the finding of positive C3c staining in LN biopsies showing chronic class III or IV LN and review the escalation of treatment in these patients.

4.3 RESULTS

4.3.1 Patient cohort characteristics and immunostaining of renal biopsies

Demographic, clinical and histopathological data from 57 biopsies (51 patients) with class III A or A/C (n=10), III C (n=10), IV A or A/C (n=12), IV C (n=12) and V (n=13) LN were obtained retrospectively (table 4.1). Median length of follow-up was 6.8 years (range 0.8-25.7). All patients fulfilled SLICC criteria for SLE.[241]

Immunostaining was performed on renal biopsy tissue for complement C3c, C3d, factor H, factor H related protein 5 (FHR5), properdin, C9 and C5b-9 and glomerular macrophage quantification using CD68 staining (table 4.2, figures 4.1 and 4.2). The staining intensities for glomerular capillary wall, mesangium and tubular basement membrane were scored and compared between LN subsets and controls (table 4.2).[241] Staining was performed by IP on FFPE tissue as discussed in the methods for the majority of stains. C3c performed at the time of biopsy in the clinical laboratory was performed in 35 cases by IF on frozen tissue and in 19 cases, IP on FFPE tissue.

Additionally, pre and post treatment biopsies from five further patients (ten biopsies) were collected from Karolinska University Hospital to provide a second cohort for serial C5b-9 staining and are included in the serial biopsies results. These biopsies are not included in the other analyses.[241]

Class LN	**III A or A/C** **n=10**	**III C** **n=10**	**IV A or A/C** **n=12**	**IV C** **n=12**	**V** **n=13**
ACR Criteria fulfilled (% yes)	90	90	100	92	100
SLICC Criteria fulfilled (% yes)	100	100	100	100	100
Age	35.5	37	30	31.5	50
Sex (% female)	80	80	91.67	83.33	76.92
Ethnicity (%)					
White	30	30	42	58	31
Black	30	30	50	33	31
South Asian	40	40	0	0	38
East Asian	0	0	8	8	0
Treatment in response to biopsy (%)	100	60	100	17	85
Treatment (%)					
Rituximab based	80	60	58	17	69
Cyclophosphamide based	10	0	25	0	0
Rituximab and Cyclophosphamide	0	0	17	0	8
Oral treatment	10	40	0	83	23
Reason for biopsy (%)					
First presentation LN	60	30	67	0	46
Flare	40	50	33	50	31
Partial response	0	0	0	33	8
Confirmation inactivity	0	20	0	17	15
C3 (g/L) (normal range 0.7-1.7)	0.62 (0.22-0.99)	0.97 (0.33-1.88)	0.60 (0.13-0.90)	1.11 (0.64-1.54)	0.9 (0.39-1.82)
C4 (g/L) (normal range 0.16-0.54)	0.1	0.18	0.06	0.26	0.15

	(<0.03-0.30)	(0.05-0.38)	(0.04-0.16)	(0.07-0.47)	(0.02-0.46)
dsDNA ab level in those patients ever dsDNA positive (units/ml) (normal range 0-30)	1377 (30-4937)	51.5 (<10-138)	809 (94-4036)	31 (6-218)	47 (9-274)
ANA (% ever positive)	90	90	92	83	100
Creatinine at biopsy (µmol/L)	64.5 (52-312)	74 (51-224)	75 (58-310)	100.5 (51-284)	66 (50-181)
eGFR at biopsy (ml/min/1.73m^2)	80.5 (15-90)	73 (26-90)	80 (14-89)	57 (18-90)	84 (36-90)
Urine PCR at biopsy (mg/mmol)	87 (0-404)	126 (28-2022)	252 (125-2371)	233 (35-666)	308 (27-3390)
Response at one year (%) **CR** **PR** **NR** **N/A**	 80 0 20 0	 10 0 50 40	 42 17 42 0	 0 8 8 83	 38 31 15 15
Time to CR/PR (months)	2 (0-11)	14 (14-14)	7 (1-11)	0 (0-0)	7 (4-9)
Duration LN (years)	0 (0-18)	5 (0-13)	0 (0-18)	3.5 (0.4-23)	0 (0-10)
RRT (%)	20	0	8	8	0
Death (%)	0	10	0	0	0
Doubling of creatinine which has not progressed to RRT in those alive (%)	0	0	33	8	0

Table 4.1. Demographic and clinical data of the staining cohort [241]***.***

LN, lupus nephritis; ACR, American College of Rheumatology; SLICC, Systemic Lupus International Collaborating Clinics; eGFR, estimated glomerular filtration rate; CR, complete response; PR, partial response; NR, non-response; RRT, renal replacement therapy.

Target	Diagnosis	Capillary Wall	Mesangium	Tubular Basement Membrane
C5b-9 % positive, median intensity (range)	LN III-A or A/C; n=10	60%, 0.75 (0-2)	80%, 1.5 (0-3)	60%, 0.75 (0-3)
	LN III-C; n=10	70%, 0.75 (0-2)	90%, 1 (0-2)	80%, 1 (0-2)
	LN IV-A or A/C; n=10	60%, 2 (0-3)	80%, 2 (0-3)	60%, 0.5 (0-3)
	LN IV-C; n=12	58%, 1.5 (0-2)	92%, 1 (0-2)	100%, 1 (0.5-2)
	LN V; n=13	100%, 3 (0.5-3)	46%, 0 (0-3)	69%, 0 (0-3)
	Membranous Nephropathy n=5	100% 3 (1-3)	0% 0 (0-0)	50% 0.5 (0-1)
	Thin Basement Membrane n=4	25% 0 (0-1)	100% 0.5 (0.5-0.5)	75% 0.5 (0-0.5)
	Minimal Change n=4	0% 0 (0-0)	100% 1 (0.5-1)	100% 2 (0.5-2)
	Normal n=6	0% 0 (0-0)	17% 0 (0-0.5)	17% 0 (0-2)
C3c[1,2]	LN III-A or A/C; n=10	30%, 0 (0-2)	100%, 2 (1-3)	70%, 0.5 (0-3)
	LN III-C; n=9	25%, 0 (0-2)	75%, 1 (0-2)	100%, 1 (0.5-3)
	LN IV-A or A/C; n=12	92%, 2.5 (0-3)	75%, 2 (0-3)	50%, 0.5 (0-1)
	LN IV-C; n=10	20%, 0 (0-1)	30%, 0 (0-1)	91%, 0.5 (0-2)
	LN V; n=13	100%, 2 (0.5-3)	23%, 0 (0-3)	85%, 1 (0-3)
	Membranous Nephropathy n=5	75% 2 (0-2)	25% 0 (0-1)	0% 0 (0-0)
	Thin Basement Membrane n=4	0% 0 (0-0)	0% 0 (0-0)	0% 0 (0-0)
	Minimal Change n=4	0% 0 (0-0)	0% 0 (0-0)	50% 0.5 (0-1)
	Normal n=6	0% 0 (0-0)	17% 0 (0-1)	N/A
C3d	LN III-A or A/C; n=10	100%, 1 (0.5-2)	60%, 1 (0-2)	50%, 0 (0-2)
	LN III-C; n=9	89%, 1 (0-2)	56%, 0.5 (0-2)	60%, 0 (0-3)
	LN IV-A or A/C; n=12	92%, 1 (0-3)	17%, 0 (0-0.5)	25%, 0 (0-3)
	LN IV-C; n=10	80%, 0.5 (0-3)	20%, 0 (0-1)	42%, 0 (0-3)
	LN V; n=13	100%, 2 (0.5-3)	38%, 0 (0-3)	54%, 0.5 (0-2)
	Membranous Nephropathy n=5	100%, 3 (2-3)	0%, 0 (0-0)	0%, 0 (0-0)
	Thin Basement Membrane n=4	0%, 0 (0-0)	0%, 0 (0-0)	0%, 0 (0-0)
Properdin	LN III-A or A/C; n=8	100%, 1 (0.5-3)	13%, 0 (0-0.5)	63%, 0.5 (0-0.5)
	LN III-C; n=9	67%, 1 (0-2)	0%, 0 (0-0)	22%, 0 (0-2)

	LN IV-A or A/C; n=12	100%, 2 (1-3)	8%, 0 (0-0.5)	83%, 1 (0-1)
	LN IV-C; n=11	100%, 2 (0.5-3)	0%, 0 (0-0)	91%, 1 (0-1)
	LN V; n=13	77%, 2 (0-3)	0%, 0 (0-0)	15%, 0 (0-2)
	Membranous Nephropathy n=3	100%, 2 (2-3)	0%, 0 (0-0)	33%, 0 (0-1)
	Thin Basement Membrane n=2	100%, 0.75, (0.5-1)	0%, 0 (0-0)	0%, 0 (0-0)
	Minimal Change n=1	100%, 1 (1-1)	0%, 0 (0-0)	0%, 0 (0-0)
	Normal n=4	100%, 1.5 (1-3)	50%, 0.5 (0-2)	50%, 0.5 (0-1)
Factor H	LN III-A or A/C; n=8	13%, 0 (0-0.5)	13%, 0 (0-0.5)	0%, 0 (0-0)
	LN III-C; n=9	25%, 0 (0-1)	0%, 0 (0-0)	0%, 0 (0-0)
	LN IV-A or A/C; n=12	8%, 0 (0-0.5)	0%, 0 (0-0)	0%, 0 (0-0)
	LN IV-C; n=9	11%, 0 (0-0.5)	11%, 0 (0-0.5)	0%, 0 (0-0)
	LN V; n=13	31%, 0 (0-0.5)	15%, 0 (0-0.5)	0%, 0 (0-0)
	Membranous Nephropathy n=2	100%, 1 (1-1)	0%, 0 (0-0)	0%, 0 (0-0)
	Thin Basement Membrane n=2	0%, 0 (0-0)	0%, 0 (0-0)	0%, 0 (0-0)
	Normal n=4	0%, 0 (0-0)	0%, 0 (0-0)	0%, 0 (0-0)
Factor H Related Protein 5	LN III-A or A/C; n=9	89%, 2 (0-3)	56%, 0.5 (0-3)	11%, 0 (0-1)
	LN III-C; n=10	80%, 1.5 (0-3)	40%, 0 (0-3)	10%, 0 (0-0.5)
	LN IV-A or A/C; n=12	83%, 1 (0-3)	67%, 1 (0-2)	0%, 0 (0-0)
	LN IV-C; n=10	70%, 1 (0-2)	60%, 0.5 (0-3)	0%, 0 (0-0)
	LN V; n=13	100%, 3 (0.5-3)	62%, 1 (0-2)	0%, 0 (0-0)
	Membranous Nephropathy n=2	100%, 2.5 (2-3)	0%, 0 (0-0)	0%, 0 (0-0)
	Thin Basement Membrane n=2	0%, 0 (0-0)	0%, 0 (0-0)	0%, 0 (0-0)
	Normal n=5	0%, 0 (0-0)	0%, 0 (0-0)	0%, 0 (0-0)
CD68[3] Maximum Glomerular Count Median (range)		**Maximum Glomerular Count Median (range)**		
	LN III-A or A/C; n=10	15 (5-34)		
	LN III-C; n=10	2 (0-8)		
	LN IV-A or A/C; n=10	20 (9-48)		

	LN IV-C; n=10	5 (1-14)		
	LN V; n=7	4 (2-11)		
	Membranous Nephropathy n=5	1 (0-4)		
	Thin Basement Membrane n=4	4 (2-7)		
	Minimal Change n=4	4 (0-8)		
	Normal n=6	2.5 (0-4)		

Table 4.2. Complement and CD68 immunostaining in LN and control cases [241]***.***

For complement staining, values represent percentage of biopsies with positive staining (grade 0.5 to 3 inclusive) and median staining intensity with range of values in parentheses; For CD68, values represent median maximum glomerular count value with range of values in parentheses; [1]p=0.024 capillary wall III and IV A or A/C vs III and IV C; [2]p=0.001 mesangial III and IV A or A/C vs III and IV C; [3]p<0.0001 III and IV A or A/C vs III and IV C[240].

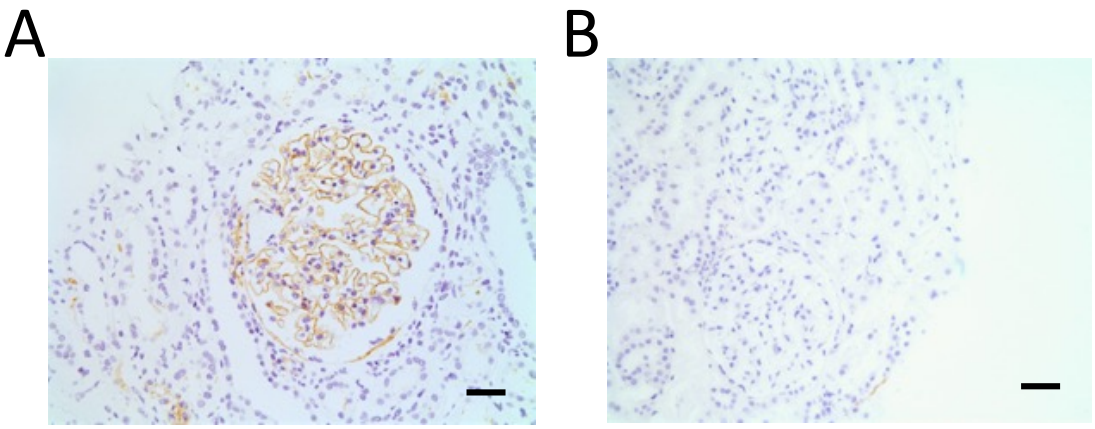
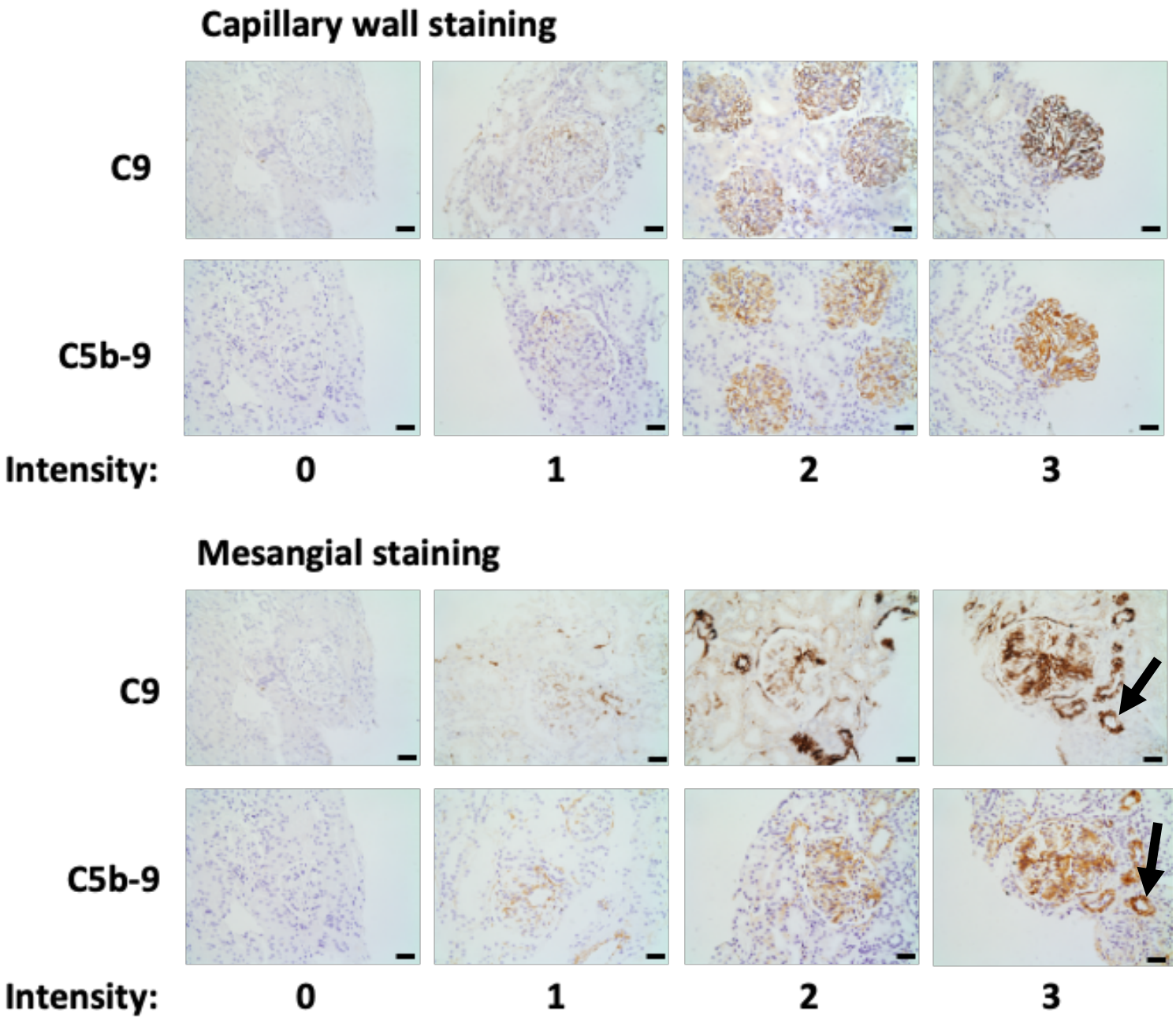


Figure 4.1 Glomerular immunostaining for complement C5b-9 in control biopsies.

Representative images of C5b9 staining in membranous nephropathy (A) and a normal renal biopsy (B). Grade 3 capillary wall staining is seen in membranous nephropathy. No staining is evident in the normal biopsy. Original magnification x20. Bar = 50 µm [240].

Figure 4.2 Glomerular immunostaining for complement C9 and C5b-9.

Representative images of capillary wall (upper panel) and mesangial (lower panel) staining using antibodies to either complement C9 or C5b-9. Serial sections from the same biopsy were used. Images depict grade 0, 1, 2 and 3 staining intensities. Staining intensity correlated for each antibody (capillary wall, r=0.92, p<0.0001 and mesangium, r=0.86, p<0.0001). Arrows indicate vascular staining. Original magnification x20. Bar = 50 µm [240].



4.3.2 Glomerular C5b-9 is near-ubiquitous in LN and does not differ between active disease and biopsies with chronic glomerular lesions only

To detect MAC, antibodies specific for C9 and C5b-9 were used. The staining pattern (mesangial vs. capillary wall) and intensities (graded 0 – 3) were equivalent for both antibodies (figure 4.2). There was vascular staining in LN (figure 4.2) and disease controls. C5b-9 was present in the glomeruli of all but one of the LN biopsies. In class III and IV LN the staining was typically mesangial with some granular capillary wall staining. In class V LN the staining was predominantly in a granular pattern along the capillary wall (table 4.2). It was hypothesised that active disease would have on-going complement consumption, whereas biopsies with chronic glomerular lesions only would not. This would predict that C5b-9 staining intensity would be stronger in active disease compared to chronic. However, the staining intensities did not differ (table 4.2). In view of this unexpected result, glomerular complement C3 staining was next examined.[241]

4.3.3 Glomerular C3c but not C3d differs between active and chronic class III and IV LN

Glomerular C3 was assessed using antibodies that detect C3c (recognising C3b, iC3b and C3c) or C3d. In contrast to C5b-9 staining, the C3c staining intensity was significantly different between active (III A, IV A) and chronic (III C, IV C) LN in both the capillary wall ($p=0.024$) and mesangium ($p=0.001$, Table 4.2). However, C3d staining intensity did not differ between active and chronic LN. Both capillary wall and mesangial C3c staining intensity correlated with glomerular macrophage count (figure 4.3), supporting the association between active LN and glomerular C3c. There was no correlation between glomerular macrophage count and either C3d or C5b-9. Of the biopsies with chronic LN only, of which all were positive for C5b-9; 44% were positive for C3c and C3d; 50% were positive for C3d only; and one biopsy (6%) was negative for both C3c and C3d, but remained positive for C5b-9. Notably, no biopsy showing chronic LN only was positive for C3c and negative for C3d. These data suggest C3c is cleared more rapidly than C3d and in turn, C3d is cleared more rapidly than C5b-9. Biopsies showing chronic LN only which were C3c-positive were also positive for immunoglobulin (Ig) with a positive correlation between mesangial C3c and mesangial IgG intensities ($r=0.677$, $p=0.002$) and capillary wall C3c and capillary wall IgA, IgG and IgM intensities ($r=0.753$, $p<0.001$).[241]

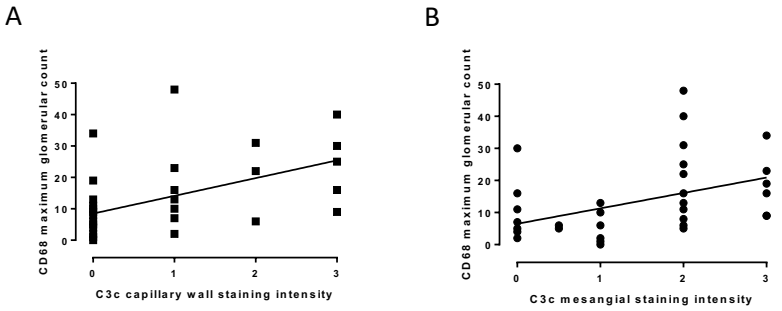


Figure 4.3 Glomerular complement C3c and CD68 maximum glomerular count correlate

C3c staining intensity in (A) capillary wall and (B) mesangium correlated significantly with the CD68 maximum glomerular count (r=0.57, p=0.0003 and r=0.51, p=0.002, respectively)[240].

4.3.4 Glomerular C3c and CD68, but not C3d or C5b-9, correlated with serum C3, C4 and anti-dsDNA titre

Capillary wall and mesangial C3c staining intensity and CD68 maximum glomerular count correlated negatively with serum C3 and C4 and positively with anti-dsDNA titre in class III and IV disease (figure 4.4, minimum r=0.43, p<0.02 in all instances for C3c and minimum r=0.60, p<0.0001 for CD68). In contrast, there was no correlation between serum C3, C4 and anti-dsDNA titre and C5b-9 staining. Capillary wall C3c (r=0.44, p=0.004) and C5b-9 (r=0.31, p=0.04) correlated with proteinuria in class III and IV disease. There was no association between capillary wall C5b-9 and proteinuria in class V LN, likely due to the lack of a large dynamic range of C5b-9 in class V disease. Serum C3 and C4 in the cases with serial biopsies increased between the first and second biopsy as displayed in Table 4.3. C3c staining intensity decreased and there was a significant negative correlation with serum C3 (r=-0.675, p=0.004) and C4 (r=-0.866, p=0.00003). There was no correlation between serum C3 or C4 and C3d or C5b-9.[241]

4.3.5 Glomerular C3c and CD68, but not C3d or C5b-9 correlated with histological markers of activity

Capillary wall C3c staining intensity and CD68 correlated positively with both active glomerular lesions and crescents and negatively with chronic glomerular lesions (figure 4.5, p≤0.01 in all cases). Interestingly, glomerular C3d and C5b-9 did not. Serum C3 and C4 levels also correlated significantly with histological parameters: negatively with active glomerular lesions and crescents and positively with chronic glomerular lesions (figure 4.5, minimum r=0.621 and p<0.000001 for active and chronic glomerular lesions and minimum r=0.379 and p<0.012 for crescents).[241]

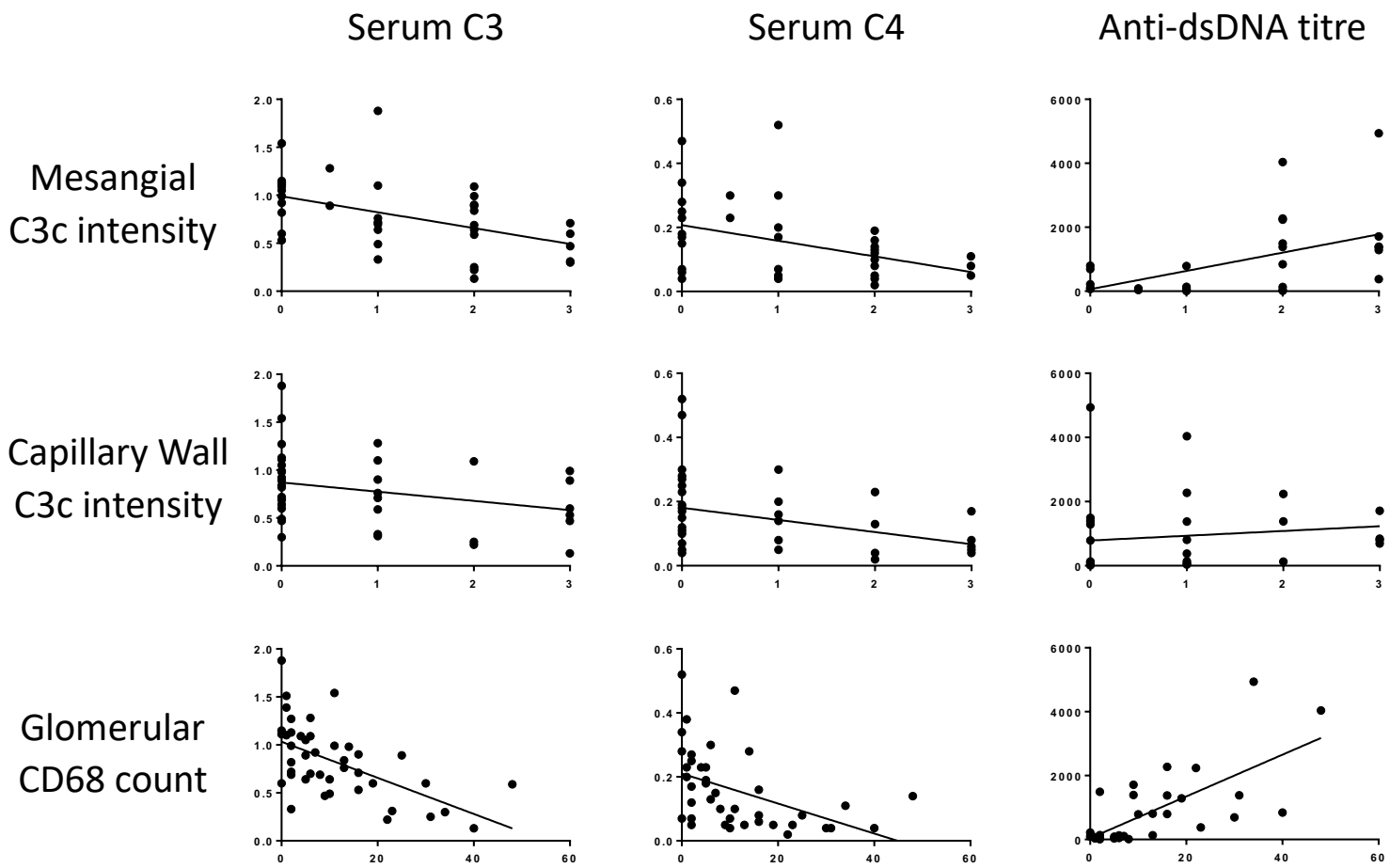


Figure 4.4 Glomerular complement C3c and CD68 maximum glomerular count correlate significantly with serum complement C3, C4 and anti-dsDNA titre.

Mesangial C3c staining intensity correlated significantly with serum C3 (r=-0.53, p<0.001), serum C4 (r=-0.44, p=0.004) and dsDNA (r=0.53, p=0.003). Capillary wall C3c staining intensity correlated significantly with serum C3 (r=-0.49, p=0.001), serum C4 (r=-0.56, p=0.0001) and dsDNA (r=0.43, p=0.017). CD68 maximum glomerular count correlated significantly with serum C3 (r=-0.64, p<0.00001), serum C4 (r=-0.61, p<0.0001) and dsDNA (r=0.78, p<0.000001) [240].

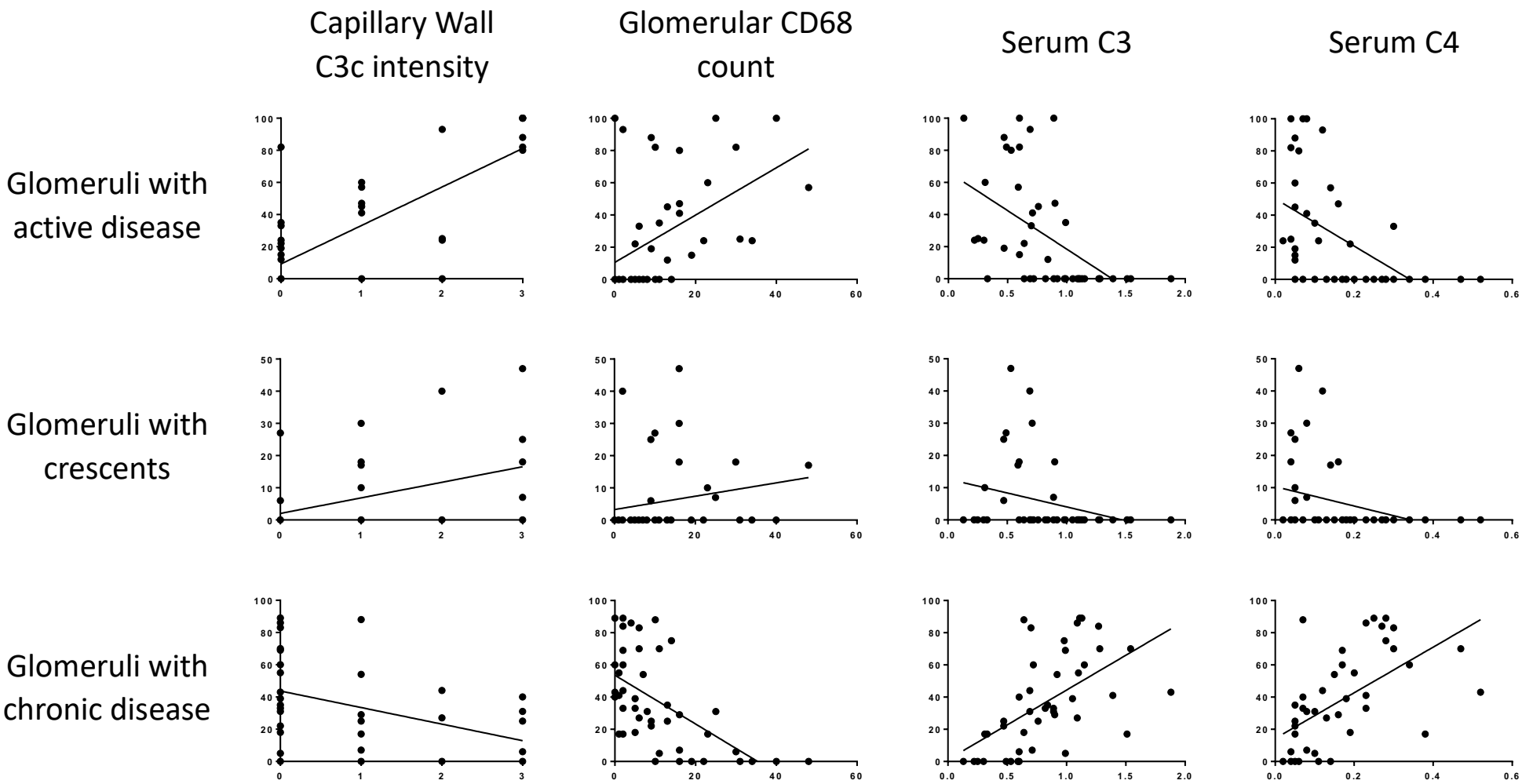


Figure 4.5 Glomerular complement C3c, CD68 maximum glomerular count and serum complement correlate significantly with histopathological features of the renal biopsy.

Percentage of glomeruli with active disease correlated significantly with capillary wall C3c staining intensity (r=0.66, p<0.00001), CD68 maximum glomerular count (r=0.75, p<0.0000001), serum C3 (r=-0.65, p<0.00001) and serum C4 (r=-0.62, p<0.00001). Percentage of glomeruli with crescents correlated significantly with capillary wall C3c staining intensity (r=0.48, p=0.002), CD68 maximum glomerular count (r=0.45, p=0.003), serum C3 (r=-0.38, p=0.01) and serum C4 (r=-0.38, p=0.01). Percentage of glomeruli with chronic disease correlated significantly with capillary wall C3c staining intensity (r=-0.41, p=0.01), CD68 maximum glomerular count (r=-0.61, p<0.0001), serum C3 (r=0.68, p<0.000001) and serum C4 (r=0.69, p<0.000001) [240].

4.3.6 More intense C5b-9 staining is associated with non-response to treatment

There was a significant difference in the intensity of C5b-9 capillary wall staining in class III and IV A or A/C disease between responses to treatment (p=0.01). Those patients with more intense MAC staining were more likely to be non-responders (figure 4.6 (A)). Conversely, patients with more intense mesangial C3c staining, in class III and IV A or A/C disease, were more likely to respond to treatment (figure 4.6 (B), p=0.04). Notably, C5b-9 staining intensity in the tubular basement membrane (figure 4.7) correlated with the percentage interstitial fibrosis and tubular atrophy (IFTA) (r 0.50, p=0.0001). The longevity of C5b-9 staining was next examined.[241]

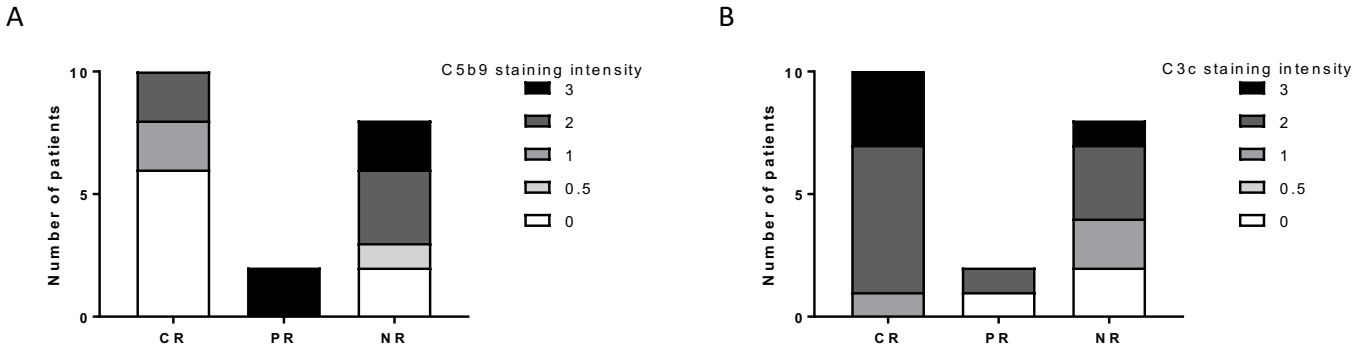


Figure 4.6 Capillary wall C5b-9 and mesangial C3c staining intensities varied with treatment response.

There was a significant difference in (A) C5b-9 capillary wall staining intensity between treatment response at one year. Patients with more intense C5b-9 staining were more likely to be non-responders (p=0.01). There was a significant difference in (B) C3c mesangial staining intensity between treatment response at one year. Patients with more intense C3c staining were more likely to be responders (p=0.04). CR, complete response; PR, partial response; NR, non-response [240].

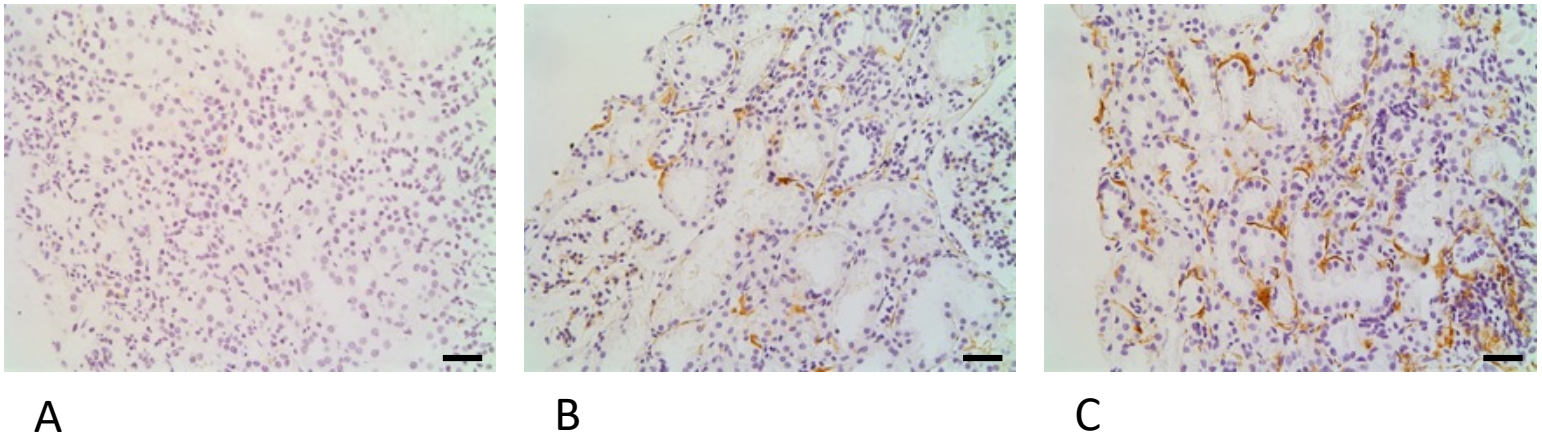


Figure 4.7 Representative images of tubular basement membrane C5b-9 staining

(A) Grade 0 staining in a case with 0% interstitial fibrosis and tubular atrophy (IFTA); (B) Grade 1 staining in a case with 15% IFTA; and (C) grade 2 staining in a case with 30% IFTA. Original magnification x20. Bar = 50 µm. The presence of IFTA positively correlated with the degree of C5b-9 staining (r=0.50, p=0.0001) [240].

4.3.7 C5b-9 staining can decrease over time but not in all cases

To further understand the kinetics of C5b-9 deposition and clearance, cases with serial biopsies taken pre- and post-treatment were reviewed: three patients from our centre and five from Karolinska University Hospital (table 4.3). C5b-9 staining was compared between pre- and post-treatment biopsies (figure 4.8). Staining was positive in the mesangium of seven and capillary wall of five of the pre-treatment biopsies. The C5b-9 staining in the mesangium decreased in five out of seven of the second biopsies, becoming negative in three, the shortest inter-biopsy interval being six months. In contrast, although capillary wall C5b-9 staining intensity decreased in the majority of the second biopsies, none became completely negative. The longest inter-biopsy interval was five years. It was therefore concluded that mesangial C5b-9 staining can resolve within six months whilst capillary wall staining is more persistent. To confirm that the C5b-9 detected in the post treatment biopsies was persisting rather than newly deposited, C5b-9 was measured in a very limited number of available samples, from serial plasma samples from the time of biopsy, the first showing active disease and the second, post treatment with histological remission. Figure 4.9 demonstrates the high level of C5b-9 in the plasma pre-treatment and lower level post-treatment.[241]

Case[1]	Time between biopsies	Biopsy number	Class	Response to treatment	C5b9 capillary wall	C5b9 mesangium	C3c capillary wall	C3c mesangium	C3d capillary wall	C3d mesangium	Maximum CD68	Serum C3	Serum C4
1	5 years	Biopsy 1	V	CR	2	1	0	0	1	0	5	1.22	0.19
		Biopsy 2	V (inactive)	Inactive	0.5	0	0	0	0.5	0.5	2	1.2	0.21
2	7 months	Biopsy 1	IV G A/C	PR	3	3	3	1	2	0	52	0.69	0.12
		Biopsy 2	IV C	Inactive	2	2	0.5	1	1	0	3	1.15	0.34
3	19 months	Biopsy 1	IV G A/C	NR	3	3	1	0.5	3	0	59	0.6	0.07
		Biopsy 2	IV S C	Inactive	1	0	0.5	0	3	0	3	1.11	0.28
4	13 months	Biopsy 1	II + V	CR	3	3	2		1	1	9	0.08	0.16
		Biopsy 2	II	Inactive	3	3	1		1	1	1	0.91	0.15
5	10 months	Biopsy 1	III	NR	2	2	3		0	2	0	0.58	0.09
		Biopsy 2	II	Inactive	3	3	2		1	1	4	0.64	0.10
6	6 months	Biopsy 1	IV	CR	0	2	1		0	1	22	0.30	0.11
		Biopsy 2	I	Inactive	0	0	1		2	2	2	1.15	0.17
7	8 months	Biopsy 1	III	CR	0	3	3		0	2	25	0.32	0.06
		Biopsy 2	II	Inactive	0	0.5	2		0	1	2	0.59	0.12
8	9 months	Biopsy 1	III	CR	0	0	2		0	1	13	0.2	0.10
		Biopsy 2	II	Inactive	0	0	1		0	0.5	4	0.4	0.10

Table 4.3. Staining intensity and serum complement markers in serial biopsies [241]***.***

[1]Cases 1-3 from our local hospital; cases 4-8 from Karolinska University Hospital. CR, complete response; PR, partial response; NR, non-response. Normal range for serum C3 is 0.7-1.7 and serum C4 is 0.16-0.54 [240].

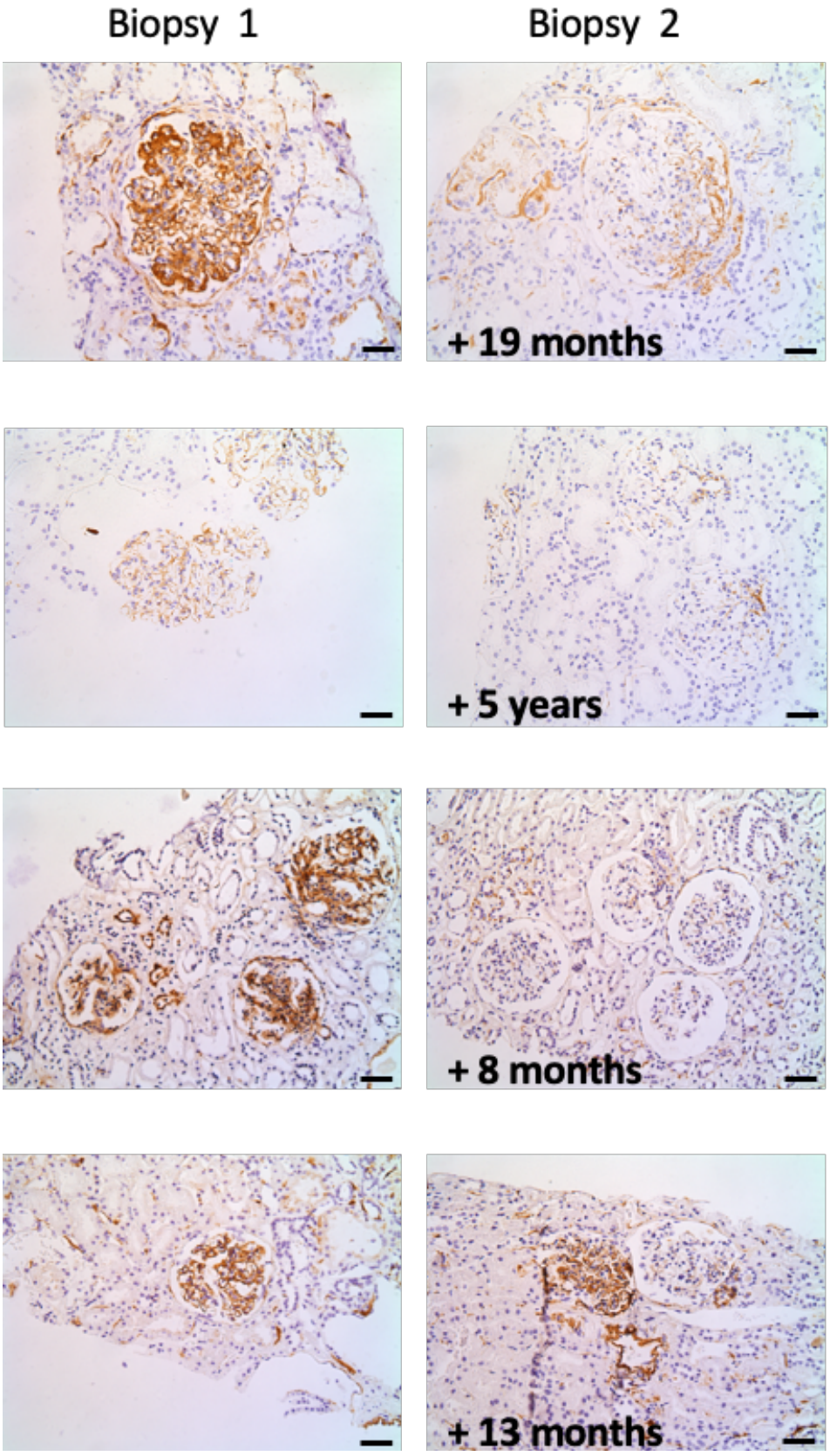


Figure 4.8 Glomerular C5b-9 in serial LN biopsies.

Representative C5b-9 staining from sequential LN renal biopsies: pre-treatment (biopsy 1) and post-treatment (biopsy 2), with varying inter-biopsy intervals. Original magnification x20. Bar = 50 µm [241].

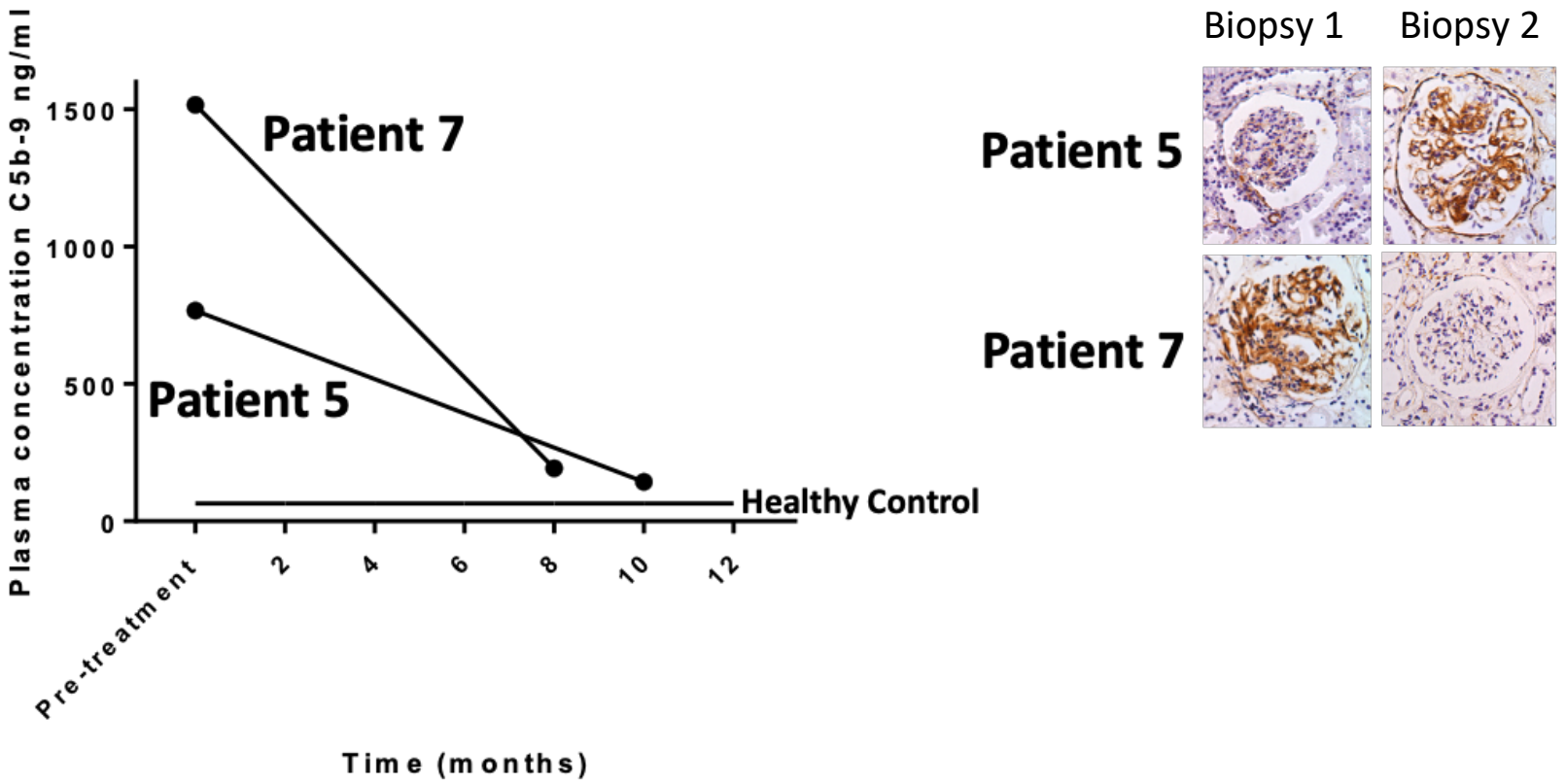


Figure 4.9 C5b-9 concentration in the plasma of patients with serial biopsies, pre and post treatment taken from the time of biopsy with C5b-9 staining from the time of biopsy for comparison.

C5b-9 plasma concentration was higher in both patients pre-treatment compared with post-treatment. In comparison to pre-treatment, C5b-9 staining increased in patient 5 in the post treatment biopsy and decreased in patient 7 in the post treatment biopsy. Original magnification x20. [241].

4.3.8 Factor H staining was rarely present and did not correlate with disease activity or response to treatment

Factor H staining was present only rarely in the lupus patients and always with weak intensity when present, either a trace alone or grade one staining (table 4.2). It was absent in the controls with no active disease or no complement mediated disease and present in the positive controls: membranous and human liver. It was more commonly present in the capillary wall when positive (table 4.2). There was no significant difference between factor H staining intensity in active and chronic LN. Factor H staining intensity did not significantly correlate with any of the other staining intensities. Factor H mesangial staining intensity correlated very weakly with proteinuria in class III and IV disease ($r=-0.347$, $p=0.035$) graph not shown. There was no significant relationship with response to treatment.

4.3.9 Factor H Related Protein 5 correlated weakly with C5b-9 staining in the capillary wall of LN patients

FHR5 staining was present in both the capillary wall and mesangium, usually both simultaneously and more commonly of greater intensity in the capillary wall (table 4.2). There was no significant difference between the intensity in active LN vs biopsies showing chronic LN only. It was largely absent from the tubular basement membrane. There was a weak correlation between FHR5 and C5b-9 and C9 in the capillary wall of class III and IV disease ($r=0.419$ ($p=0.008$), $r=0.475$ ($p=0.003$) respectively) and stronger correlations in class V disease ($r=0.758$($p=0.007$), $r=0.885$($p=0.0001$)). There was also a weak correlation between FHR5 and C3d in the capillary wall of class III and IV disease ($r=0.386$ ($p=0.018$)) and class V disease ($r=0.586$ ($p=0.0354$)). There was no correlation between C3c or CD68 and FHR5. There was also a correlation between C1q staining intensity in class V disease and FHR5 capillary wall staining ($r=0.739$ ($p=0.0039$)). FHR5 also correlated weakly with C5b-9 staining intensity in the mesangium ($r=0.327$ ($p=0.045$)) of class III and IV disease and negatively with proteinuria ($r=-0.396$($p=0.011$)). There was no significant relationship with response to treatment. Correlation graphs not shown.

4.3.10 Properdin staining was present in the capillary wall however did not correlate with disease activity or response to treatment

Properdin staining was present in the capillary wall in the majority of cases, however only a trace was present in two cases in the mesangium (table 4.2). There was no significant difference between the intensity in active LN vs biopsies showing chronic LN only. There was a weak correlation between properdin and C5b-9 and C9 in the capillary wall of class III and IV disease (r=0.364 (p=0.025), r=0.343 (p=0.041) respectively). There were also correlations in class III and IV disease between properdin in the mesangium and mesangial C3c (r=0.357, (p=0.028)) and C1q (r=0.371, (p=0.022)) as well as mesangial IgG (r=0.351, (p=0.036)) and the percentage of crescents (r=0.417, (p=0.007)). There was no significant relationship with response to treatment. Correlation graphs not shown.

4.3.11 C3c staining was present in some biopsies showing chronic LN alone and could be a surrogate for disease activity

In the complement staining discussed here, biopsies were analysed as active or chronic. It was noted that C3c staining correlated positively with the percentage of glomeruli showing active lesions and negatively with the percentage of glomeruli showing chronic lesions and a role has been identified as a potential marker of active disease. C3c staining was however not entirely absent from the biopsies showing only chronic LN. In the analysis of treatment response and outcomes described in the previous chapter, biopsies showing chronic changes alone (class III or IV C) were often excluded as many of these patients did not have an escalation of their immunosuppression treatment. Some patients did however have an escalation of treatment including eight (36%) of the patients with chronic LN changes only on biopsy in this staining cohort. Numerically, a greater percentage of those with positive C3c staining had escalation of their treatment (table 4.4). The difference however, using such small numbers, was not statistically significant. Of those who had their treatment escalated, the majority did not respond to this treatment by our stated definitions.

	C3c positive n=13	C3c negative n=9	p value
CD68 maximum glomerular count (median)	5	2	ns
Treatment escalated	6 (46%)	2 (22%)	ns
Response to treatment CR PR NR N/A	 1 0 5 7	 0 1 1 7	ns

Table 4.4 Biopsies from the complement staining cohort classified as class III/IV C demonstrating no significant difference between the treatment of those positive or negative for C3c staining.

On the basis of these observations, those biopsies from the whole database showing class III or IV chronic LN only were further examined to understand if there were particular characteristics which

prompted treatment for these patients. Outcomes were also reviewed to assess if positive C3c staining is a surrogate for disease activity on this wider cohort and if treatment of these patients is warranted.

129 biopsies were performed over the data collection period which showed class III C or IV C LN only. 5 biopsies were excluded due to lack of follow-up information. 53% of those remaining patients had escalation of treatment post biopsy. Table 4.5 shows the clinical and histopathological variables of these patients at the time of biopsy.

Baseline Variables	**Treatment escalated (n=66)**	**Treatment not escalated (n=58)**	**p value**
Indication for biopsy • **New presentation (16% biopsies)** • **Presumed renal flare (42% biopsies)** • **Persistent proteinuria (22% biopsies)** • **Staging (20% biopsies)**	 15 (23%) 37 (56%) 12 (18%) 2 (3%)	 3 (5%) 15 (26%) 16 (28%) 24 (41%)	 <0.0001
C3c positive staining	91%	56%	0.002
Subendothelial or subepithelial deposits on Electron Microscopy	90%	66%	0.015
Proteinuria uPCR (median, mg/mmol)	305 (163-533)	143 (80-361)	0.0002
eGFR (median)	77 (55-90)	58 (33-87)	0.008

Table 4.5 Clinical and histopathological variables of all patients with a biopsy showing class III/IV C LN at the time of biopsy showing significantly different features between those patients receiving an escalation in their treatment post biopsy and those not.

Continuous data displayed as median (range). Categorical data displayed as number (percentage) or percentage alone as indicated. uPCR, urine protein creatinine ratio; eGFR, estimated glomerular filtration rate; C, complement.

Clinical indication for biopsy was significantly different between the groups (table 4.5). As might have been suspected, where there was a clinical suspicion of new activity in the scenario of an initial presentation of LN or evidence of presumed renal flare, the decision was made to treat in a significantly higher proportion of patients, despite the lack of detected active disease on renal biopsy (table 4.5). Where the biopsy was being performed to rule out active disease, in the case of a staging biopsy, a significantly higher proportion of patients did not have escalation of treatment. Consistent with this finding, proteinuria was higher in the group where treatment was escalated (table 4.5). Interestingly, eGFR was also higher, indicating that treatment may have been initiated more commonly where it was felt there was more to gain or preserve. C3c staining was significantly higher in the treated group demonstrating that C3c positive staining is more common in the group with more active disease clinically and histopathologically in relation to deposits (table 4.5).

Short- and long-term outcomes were compared between the groups and are displayed in table 4.6. There was no longer any significant difference at one year between eGFR and proteinuria between the groups which was present at baseline, uPCR had improved in the group with escalated treatment however, despite the treatment, eGFR declined in this group. Long term survival with reasonable renal function was slightly higher in the treated group however there was no significant difference between the groups.

Outcome Variables	**Treatment escalated n=66**	**Treatment not escalated n=58**	**p value**
Median eGFR at one year	69	57	ns
Median uPCR at one year	177	130	ns
1–31-year survival with eGFR >60mls/min/1.73m2 (minimum 1yr follow-up)	44%	34%	ns

Table 4.6 Table displaying no significant difference in short- or long-term clinical outcomes between patients with treatment escalated and those without treatment escalated after a biopsy showing LN III/IV chronic disease only.

LN, lupus nephritis; uPCR, urine protein creatinine ratio; eGFR, estimated glomerular filtration rate

To better analyse if C3c positive staining is indicative of on-going activity, it was next examined whether chronic changes in the biopsy deteriorated in those patients who were C3c positive or negative, where a follow-up biopsy existed. Table 4.7 shows that whilst the changes were not significant, in those patients with C3c positive staining, chronic changes did appear to deteriorate with no deterioration in patients with C3c negative biopsies.

Histopathological variable	**C3c positive n=38**	**C3c negative n=8**	**p value**
Change in % of glomeruli in the biopsy showing chronic changes	Increase 8%	Decrease 8%	ns
Change in % interstitial fibrosis & tubular atrophy (IFTA)	Increase 7.5%	0%	ns

Table 4.7 Table comparing histopathological features of chronic changes between patients positive for C3c staining and those negative for C3c staining.

Analysis of treated versus untreated patients with chronic biopsies did not provide strong evidence that treatment was of benefit. However, the not treated group were significantly less active at baseline and were stable at one year and therefore are not a true comparator group. In order to assess if treatment could be of benefit in those patients with C3c positive staining, progression of chronic changes on the biopsy in patients who had a further follow-up biopsy was reviewed and analysed by the presence of C3c. Numbers were very small and there were no significant differences. However, it appears as though treatment of biopsies with C3c positive staining could possibly mitigate progressive scarring. This is displayed in table 4.8.

Histopathological variable	C3c positive with treatment escalated n=31	C3c positive with no treatment escalated n=7	p value
Change in % of glomeruli in the biopsy showing chronic changes	Increase 5%	Increase 21%	ns
Change in % interstitial fibrosis & tubular atrophy (IFTA)	Increase 7.5%	Increase 5%	ns

Table 4.8 Table comparing histopathological features of chronic changes between C3c positive patients with treatment escalated and those with no treatment escalated.

4.4 Discussion of results and future work

A retrospective study of complement deposition in 57 cases of LN was performed, with the initial focus being on MAC deposition. Location, staining intensity and kinetics were reviewed and relationship to complement C3c and C3d, properdin, factor H, FHR5, glomerular macrophage numbers, disease activity and treatment response studied. Both proliferative and membranous LN were studied as well as both active and chronic manifestations of disease, including pre- and post-treatment in three patients from my cohort and a further five patients from a separate cohort. C9 was found in the same distribution and intensity as C5b-9. Interestingly, C5b-9 was identified at a low level in the mesangium and tubular basement membrane of the negative disease controls: minimal change disease (MCD) and thin basement membrane disease. The reason for this is unclear. Previous studies have identified positive glomerular staining in MCD.[260] The mesangial staining could be related to intermittent passage of ICs through the mesangium, combined with the long half-life of C5b-9. The tubular basement membrane staining may relate to complement activation on the surface of tubular epithelial cells in the presence of proteinuria or secondary to acute tubular injury due to haematuria. MAC deposition was present in all but one LN case of the 57 studied, a class IV A biopsy, and in one patient from the second cohort. This indicated that evidence of C5 activation is almost always present in LN. This is consistent with previous studies of the terminal complement pathway in LN, which has included class II disease.[257] However, recently published work by Wang *et al*.,[259] found that the majority (57%) of biopsies were negative for C9. In this study activity and chronicity indices did not differ depending on the presence of C9, however patients negative for C9 were more likely to respond to treatment.[259][241]

Surprisingly, in this cohort, there was no significant difference in the intensity of MAC deposition between active and chronic LN. Significant MAC deposition was noted in some cases of inactive disease with scarring. When serial biopsies were reviewed, the C5b-9 staining did decrease in intensity in the majority of post-treatment biopsies which showed no active disease. However, the C5b-9 remained positive in the majority of the post-treatment biopsies and in two of the seven cases with a positive initial biopsy, staining intensity did not decrease. Assessment of C5b-9 in the very limited number of available plasma samples would suggest that there was not on-going activation of the terminal pathway at the time of the post-treatment biopsies. It can be concluded that C5b-9 staining can resolve, but that this appears to take months and it appears to clear more rapidly from the mesangium than the capillary wall.[241]

The presence of C5b-9 in biopsies with chronic LN only could be indicative of on-going activity. Ongoing C3 activation in some of the chronic cases was supported by positive C3c staining: 53% of

the class III and IV chronic LN cases had positive C3c, albeit of a much lower intensity than in active disease. Alternatively, persistent staining could be due to involvement of the MAC in the scarring process. Evidence from the serial biopsies however would suggest that persistence in chronic LN is most likely due to slow clearance of the MAC occurring over months rather than hours or days. This would lessen its utility as a marker of current or recent complement C5 activation.[241]

The persistence of the MAC in inactive LN biopsies could indicate a failure of our current treatments to target this pathway. In a reported case where eculizumab was used to treat refractory LN, C9 staining performed 18 months after eculizumab treatment was negative after being strongly positive pre-treatment.[261] However, in a series of patients with Dense Deposit Disease/C3 Glomerulopathy treated with eculizumab, C5b-9 was present in all of the post-treatment biopsies performed at one year.[262] Interestingly, C3 staining intensity also did not change in the post treatment biopsies in comparison to the pre-treatment.[262] The different findings could be due to the different time-points of the repeat biopsies or the nature of the different diseases. Timmermans *et al.* demonstrated how C5b-9 staining is predictive of complement mediated disease in TMA due to malignant hypertension.[263] In De Holanda *et al.*'s review of SLE with TMA, all were successfully treated with eculizumab.[264] C5b-9 staining was positive when performed. There were no data however on the longer-term appearance of this staining.[241]

In our cohort there was also a significant association between C5b-9 intensity and non-response to treatment. In this respect it is relevant that C5b-9 staining intensity in the tubular basement membrane correlated with IFTA. This could indicate a role for MAC in tubulointerstitial fibrosis and that C5b-9 could be a marker of chronic damage. Interestingly, PCA analysis of transcriptomics data has suggested there may be greater expression of complement genes in treatment non-responders.[168][241]

Under NHS England policy, eculizumab can only be approved for treatment for recurrent C3G in a transplant kidney if the biopsy shows the presence of MAC staining.[265] It seems logical to expect that a patient would not derive benefit from C5 inhibition if the biopsy is negative for MAC staining. However, our data show that when present, MAC staining does not always indicate current C5 activation. The combination of positive C3c, C5b-9 and CD68 (as a surrogate for C5a) staining would appear from these data to be good evidence of activation of both C3 and C5 pathways.[241]

Complement C3 staining was examined utilising an anti-C3c antibody (which recognises C3b, iC3b and C3c) and an anti-C3d antibody, which recognises C3d alone. Glomerular C3c staining intensity did not correlate with C3d staining. This likely reflects the different kinetics of clearance of C3c and C3d. Schulze *et al.* showed that glomerular C3c is cleared by 85% within 24 hours of cessation of C3

activity in experimental nephritis. However, glomerular C3d persisted beyond cessation of activation of C3, as measured by urinary excretion of C5b-9, in three rat models of complement-dependent glomerulonephritis.[266] The staining described here is consistent with these findings: C3d was identified both with and without C3c in the inactive biopsies whilst C3c was never present without C3d. In the serial biopsies, the difference in C3c intensity between the active and inactive biopsies was greater than the difference in C3d. The glomerular C3c staining intensity and CD68 count both correlated with markers of activity. The intensity of C3d and C5b-9 however did not correlate with disease activity.[241]

The presence of C3c in some of the biopsies showing chronic LN only was surprising as it could be expected that chronic disease would not be associated with on-going complement activation. It is possible that, due to sampling error, there was active disease which was not captured in these biopsies. There was a slightly higher CD68 count in the biopsies which were C3c positive, in concordance with the correlation of these two stains however it was not statistically significant. Notably, nine of the 13 chronic biopsies positive for C3c were performed due to either a new presentation of LN or a presumed flare. Six of these (46%) were then actively treated despite the lack of active disease seen on biopsy. Positive C3c in a chronic biopsy in our wider cohort was more likely to be associated with active management.[241]

Management of biopsies showing no active lesions (ISN RPS Class III C or IV C) is a clinical conundrum. "Treat-to-target" recommendations from an international task force advise against escalation of treatment targeting serological variables in clinically asymptomatic SLE patients.[79] It has been shown that on-going histological markers of activity on repeat biopsy are associated with increased chance of flare, even in those patients in clinical and serological remission.[133] However, management is still unclear in LN where a clinically indicated biopsy shows chronic lesions only. Based on the findings of the C3c staining in the cohort of 57 biopsies, all biopsies showing chronic changes alone in the full database were reviewed to further explore this question, as this staining is performed as part of the clinical routine at this unit.

If there is sufficient clinical evidence to indicate on-going renal activity, patients may still have treatment escalated to treat presumed activity missed due to sampling error, or indeed to treat concurrent extra-renal symptoms. Biopsies with this result may have been performed on a patient's first presentation to a renal unit or because of clinical evidence of activity either in the form or a flare or persistent proteinuria (partial or non-response). Alternatively, the biopsy could have been performed to rule out on-going activity, such as in the case of a pre-pregnancy or treatment withdrawal biopsy. The decision to treat is a clinical one and unsurprisingly, the data showed that a

much higher percentage of patients have treatment of the LN escalated in cases where there was high clinical suspicion of activity prior to the biopsy. However, significantly fewer cases have treatment escalated when the biopsy was being performed to rule out on-going activity. Patients in the group where treatment was escalated were significantly more likely to have C3c positive staining and deposits on EM. It is unclear if these factors helped guide the decision to treat or if they are associated with the other evidence of on-going activity such as proteinuria. Greater than half of the cases where treatment was not escalated have positive C3c staining and deposits on EM and so these do not appear to be key to the decision to escalate treatment. Proteinuria was higher in the group who received an escalation of treatment which is likely associated with activity. eGFR was also higher, suggesting that treatment may be tried in the absence of active disease on light microscopy (LM) if it is felt there is more to gain or preserve in terms of renal function by treating. There is evidence from analysis of these features that biopsies which show only chronic lesions on LM may indeed have on-going activity requiring escalation of treatment. This could be due to sampling error which raises the question of how much tissue is required for an adequate sample. The fact that subendothelial and subepithelial deposits were present in so many cases also raises the question of whether EM should be included in the criteria for active or chronic LN. There is no current requirement for this in either the current standard ISN/RPS classification criteria or the activity and chronicity indices.[29] Indeed, if it were to be included there would need to be an assessment of the age of the deposits. It also needs to be acknowledged that EM is not available for many centres worldwide and in many cases where it is available, reporting is unsuitably slow to be relied upon for urgent clinical treatment decisions. It appears from the data that more active cases clinically were selected for treatment escalation, despite the lack of LM evidence of activity. However, on review of the short and long-term outcomes, it is unclear if it is of benefit. The proteinuria does appear improved by one year post treatment, with the proteinuria in the untreated group unchanged, however the eGFR also falls in the treatment group, suggesting the treatment did not prevent loss of renal function. Long term survival on the basis of this single clinical decision is difficult to assess as these biopsies showing chronic lesions only are usually not the only biopsy performed for this patient. On review of the latest follow-up data, there was no significant difference between the patients who had treatment following a biopsy showing chronic LN or not.

The deterioration of chronic changes in subsequent biopsies in patients with positive C3c staining, albeit non-significant in comparison to C3c negative biopsies, would indicate that the positive C3c staining could well be associated with on-going activity. Treatment does appear to mitigate this deterioration, however the numbers in this analysis are again small. Alongside the evidence we

have for C3c being associated with activity, evidence from this analysis would imply that treatment could prevent further chronic changes from occurring.

Factor H, factor H related protein 5 and properdin deposition were also reviewed. Factor H, a negative regulator of the alternative pathway, was present in only 13/49 (26.5%) cases stained. When present in the capillary wall, there was always strong FHR5 staining, although the two did not correlate significantly. Perhaps given the auto-immune nature of SLE, it is unsurprising that factor H was generally absent or of a low intensity. Mouse models of SLE have demonstrated how factor H deficiency accelerates development of LN.[267] Interestingly, there was no significant difference in the intensity between biopsies showing active LN and those showing chronic LN only, indicating that it is not playing a significant role in regulating the disease where there is no active disease present.

Properdin staining was present in the capillary wall in the majority of cases, however only a trace was present in two cases in the mesangium. The correlations were somewhat contradictory and generally quite weak. It appeared that there were weak correlations with C5b-9 and C9 and capillary wall properdin in class III and IV disease, which have not been found to correlate with the amount of disease activity, whereas mesangial properdin correlated with C3c, C1q and IgG, markers of active disease.

Further exploration of the complement pathway, using staining, in particular to explore the lectin pathway on this cohort is planned. Biopsies with positive C3c staining showing evidence of chronic changes only, also warrant further exploration. Numbers were small and therefore staining is planned on chronic biopsies performed more recently including repeat biopsies. A cluster analysis approach to statistical analysis of the staining intensities of all the parts of the complement system in conjunction with demographic and clinical data is also planned to assess if there are possible categorisations of lupus nephritis biopsies which may provide more information as to likely response to treatment or longer-term outcomes than current categorisation methods such as histological class.

4.5 SUMMARY OF KEY FINDINGS

MAC deposition is strongly associated with LN, its clearance from glomeruli is slow, and it is not a reliable indicator of ongoing C5 activation.

More intense C5b-9 staining is associated with non-response to treatment (p=0.01)

More intense C3c staining in the mesangium is associated with response to treatment (p=0.04).

C5b-9 staining intensity in the tubular basement membrane correlated with the percentage interstitial fibrosis and tubular atrophy (IFTA) (r 0.50, p=0.0001)

C3c positive staining in class III/IV biopsies showing no active proliferative lesions is more common in patients with more active disease clinically and histopathologically in relation to deposits seen by electron microscope. It is also more common in patients who received an escalation of treatment despite a lack of active lesions.

5 CHAPTER FIVE – PROTEOMICS STUDIES

5.1 INTRODUCTION AND BACKGROUND

Achieving a good long-term outcome is the ultimate goal when treating lupus nephritis. On review of our cohort, 45.7% of patients respond satisfactorily to the current standard of care. Analysis of the demographic, clinical and histopathological features of patients and their biopsies revealed that mixed features of both activity and chronicity of LN: proteinuria level at biopsy; percentage of glomeruli showing chronic lesions and 'full house' pattern on staining, were predictive of response to treatment. Response to treatment, in turn, measured by the renal function and degree of proteinuria, is predictive of long-term outcomes. IHC staining identified that patients with more intense staining of the MAC, shown to be positive in active and chronic LN, were more likely to be non-responders to treatment while patients with more intense C3c staining, a feature related to disease activity, were more likely to respond to treatment. MAC staining in the tubular basement membrane correlated with the amount of IFTA, a marker of chronic damage.

Results thus far have therefore provided evidence for predictors of response and outcome. They have also provided parameters for assessment of new treatment options. However, mechanisms associated with response to treatment can only be speculated from these results. Mixed features of active disease and chronic lesions have been shown to be predictive of response to treatment. This could be due to: rapidly progressive disease producing early chronic features; delay in presentation allowing chronic changes to take place before diagnosis and treatment; or alternative pathways such as necrotic and fibrotic processes producing early chronic changes. Further exploration of the pathogenesis of LN, both to examine these hypotheses and to identify new treatment targets to improve response rates and thus long-term outcomes, is required. In pursuit of these aims, renal biopsy tissue was further interrogated by examining the renal biopsy proteome. This was made possible by the large repository of renal biopsy tissue surplus to clinical use, stored in the Imperial College Tissue Bank. Assessment of the transcriptomic profile has been performed in lupus nephritis demonstrating potential differences between treatment responders and non-responders.[168,171] Parikh *et al.*'s data suggested greater expression of interferon pathway genes in CR and complement genes in those who did not respond to treatment.[168] However, it is worth noting that these differences were largely identified from principal component analysis and the genes were not statistically differentially expressed between the groups. Following this, they identified that type I and II interferon pathways, the alternative complement pathway and T cell signalling pathways

appear to be differentially expressed in CR and NR when comparing each individually to control using the same technology.[171] Arguably however, a direct comparison of CR vs NR would more accurately describe any differences in expression of proteins between responders and non-responders. Gilmore *et al.* identified 52 significantly differentially expressed genes common to all LN classes with enrichment for type 1 IFN, complement and MHC II pathways.[170] RNA profiles do not however provide the whole story whereas the proteome can give a more direct view of the active pathways and processes.

The proteome of human renal biopsy tissue has been explored in various conditions [191][192][197] including LN where Alaiya *et al.* used MALDI-TOF MS to distinguish LN class IV from disease controls [167] and Satoskar *et al.* showed increased complement expression and decreased podocyte-associated proteins and those associated with cellular metabolism in frozen LN glomeruli.[191] Proteomics has not yet been used to compare LN treatment responders to non-responders. In the proteomics work performed in LN, shotgun proteomics was used which does not reliably quantify proteins, especially those of low abundance. Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS) however, allows for accurate quantification of all proteins in a sample. Thus allowing for the technique of discovery proteomics (no pre-defined proteins to identify) with the quantification accuracy of targeted proteomics.[268] It has been used in multiple tissues including frozen human kidney tissue,[219][195] mouse FFPE [269] and human FFPE tissue (hepatocellular carcinoma,[221] prostate [222] cancer and lymphoma tissue [220]). These data have shown good correlation between frozen and FFPE tissue.[220] In recent publications, even in an accelerated protocol, the quantitative assessment was shown to be accurate when compared with the targeted proteomic technique, multiple reaction monitoring (MRM) (gold standard for quantification).[222] SWATH-MS has also recently been utilised for the discovery of urine biomarkers in lupus nephritis by Kwon *et al.* who identified a promising biomarker (urine alpha-1-acid glycoprotein) for early LN detection.[227]

Given these described benefits of SWATH-MS, the ability to accurately quantify all proteins in a sample, it was therefore selected for use to analyse the proteome of FFPE LN renal biopsy tissue. Tissue left-over from a renal biopsy after clinical tests have been performed is often very small quantities, left as a strip of the core. Some of the samples used had been in storage for months or years. Clearly, repositories of this tissue are vast and an operational technique opens the door to great potential for study of these samples. The initial aim of this body of work was therefore to establish if proteomic analysis by SWATH-MS of baseline kidney biopsies was indeed possible in the quantities and quality of FFPE renal biopsy tissue remaining after clinical use and to optimise the method for such tissue. Following optimisation of the technique, two cohorts were processed

consisting of LN and thin basement membrane (TBM) as a control. From these cohorts, the pathogenesis of LN was investigated, to first identify if LN could be differentiated from control and to explore new potential treatment targets. Differences in class of LN were analysed to investigate if these could be distinguished from one another and if different disease processes could be identified. Finally, data were examined comparing responders to non-responders to assess if SWATH-MS could separate the groups and thus provide any insight as to if there is a pathogenic reason as to why some patients respond to immunosuppression whilst others do not. Finally, as a method of validation and with the aim of identifying a simpler technique for predicting response, staining was performed.

5.2 CHAPTER AIMS

- To identify if sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS) can be performed in formalin fixed, paraffin embedded (FFPE) human renal biopsy tissue including successful extraction of a sufficient quantity of protein and subsequent identification of proteins.
- To characterise the protein expression profile of the renal biopsy in a retrospective cohort of patients with lupus nephritis, using SWATH-MS and identify active pathogenic pathways in lupus nephritis.
- To analyse the protein expression profile of proliferative and membranous LN separately to identify similarities and differences.
- To explore if SWATH-MS of renal biopsy tissue can differentiate treatment responders from non-responders and therefore enable prediction of response to treatment.
- To use proteomic profiling of the kidney biopsy in lupus nephritis to generate hypotheses for new treatment targets.
- To validate the findings from SWATH-MS using immunohistochemistry and evaluate if staining could be used to differentiate treatment responders from non-responders.

5.3 RESULTS

5.3.1 Optimisation of the technique

A protocol was previously reported for extracting protein from punch biopsy samples using pressure cycling technology (PCT) [183][195] and later adapted for FFPE tissue.[220] However, these methods did not include the retrieval of protein from FFPE renal biopsy samples taken from storage with material surplus to clinical use which have very little tissue present in a long strip rather than a punch. The samples used had been in storage for at least 2 years (to reduce the likelihood of the need of the sample for clinical reasons) with some samples having been stored for much longer, including one from 22 years previously. The published protocol was therefore tested with these samples for feasibility and optimised to maximise protein extraction.

The final optimised methods for protein extraction and SWATH MS analysis are discussed in Chapter 2. Briefly, protein was extracted by altering the method of tissue retrieval as one large section from the FFPE block for pressure cycling technology (PCT) assisted lysis. Tissue cleavage time was extended to 120 cycles to maximise protein retrieval. To allow for protein quantification on such small protein yields, a Qubit 3 (Qubit 3 Fluorometer, Q33216, Invitrogen, Thermo Fisher Scientific) was used, which detects protein by fluorescence probes, a technique that only requires 1µl of sample to quantify even small amounts of protein and produces more specific data than the standard BCA assay (Thermo Scientific, Wilmington, DE; no. 23225 23227) used by Shao *et al*.[195]. When the BCA assay was tested and interactions investigated, it was found to be additionally unsuitable for these samples as urea, used in the published technique by Shao *et al*.[195] and with these samples, was found to interfere with the assay.

As a biobank of clinical samples was used for this research, some of the FFPE blocks had been previously stained by pathology. However, staining of the tissue was found to have no impact on protein yield.

Following optimisation of the technique of protein extraction as above, initial trials of data dependent acquisition (DDA) analysis were undertaken to assess if the amount of protein extracted was sufficient for MS to be performed and detect a valuable number of proteins. 2 LN samples were compared to 2 controls (tubulo-interstitial nephritis (TIN)). Using a single section of tissue from the block, 0.5-1.5mg of tissue was obtained per sample as weighed following de-wax and rehydration. Between 3.7-6.0µg of protein were extracted from each sample using the pressure-cycling technology with extended tissue cleavage time. Peptide yield was not measured in order to

conserve the sample, as it is not used to determine the amount of sample to be loaded onto the mass spectrometer. This makes the yield somewhat difficult to compare with the published data which more commonly use peptide mass and estimate the weight of the sample, calculated from the volume[195]. DDA fragments ions of greatest intensity only, identified in a survey scan, fragmenting a selection therefore, of all ions present. It was used for this experiment as a simpler technique as it was an initial screening experiment only. Using this technique, 1979 proteins were identified. As SWATH-MS fragments all ions over the whole m/z range, within selected m/z ranges (windows) it would be expected to identify more proteins. As it analyses each window in turn at several time points, it would also be expected to quantify the proteins with greater accuracy. As the intention of this experiment was simply to identify if sufficient protein could be extracted for MS to be performed, and only 4 samples were run with a sub-optimal control, differential expression (LN vs control) was not calculated. A PCA was however performed as a high-level analysis to assess if LN could be differentiated from control. LN was shown to separate from control on the PCA analysis as shown in figure 5.1 (A).

Following the identification of nearly 2000 proteins using DDA analysis, the SWATH technique was trialled on a small number of samples (8 class III or IV LN, 8 class V LN and 4 controls) to establish if more proteins were indeed detected using SWATH-MS compared to DDA analysis and then if LN could be clearly distinguished from control with this technique. This initial trial identified 4311 proteins and separated LN from control on PCA analysis (figure 5.1 (B)). A more suitable control of tissue from biopsies taken at the time of kidney transplant was used this time, more suitable due to less expected inflammatory processes in this tissue compared to TIN. Unfortunately, there was not sufficient of this kind of tissue to use as control for the forthcoming experiments. Again, due to the low numbers of samples, only high-level analysis of PCA was performed in this instance.

Given the large number of proteins identified and the clear separation of the LN from control on PCA, it was decided to proceed with the amended protocol and use of the SWATH technique on two larger cohorts of LN patients to include both proliferative and membranous LN and treatment responders and non-responders.

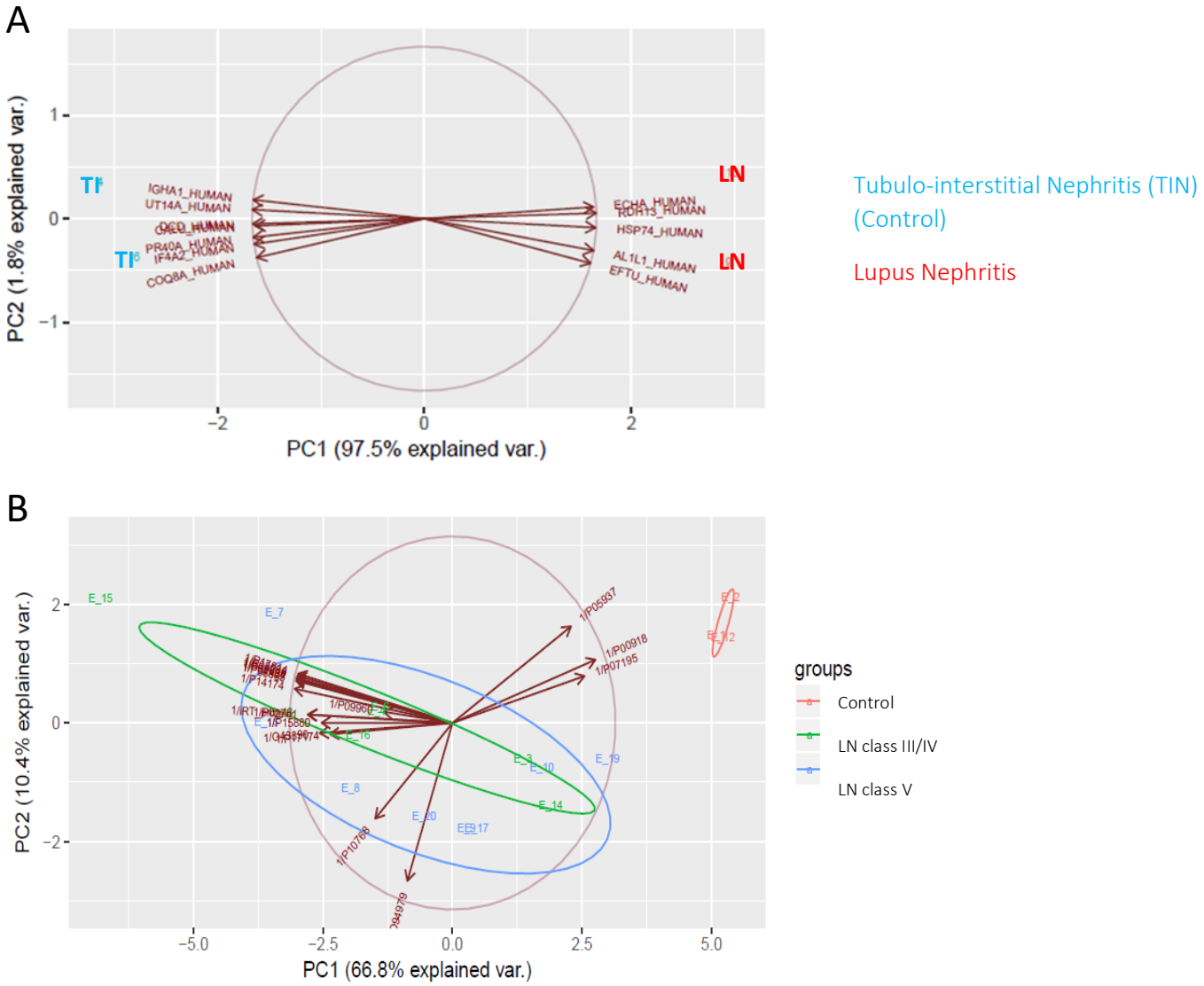


Figure 5.1 Principal component analysis (PCA) of preliminary experiments designed to validate techniques of protein extraction and mass spectrometry showing separation of LN from controls.

A) PCA of LN (n=2, red) compared to TIN (n=2, blue) from Data dependent acquisition (DDA) analysis. The four biopsies are indicated on the graph perimeter in representative colours. B) PCA of LN class III/IV (n=8, green), LN class V (n=8, blue) and control (n=4, red, time zero kidney transplant biopsies) using data generated by SWATH-MS. LN, lupus nephritis; TIN, tubulo-interstitial nephritis.

5.3.2 Cohort One

5.3.2.1 Overview and demographic data

Following optimisation of the methods required to conduct this analysis, we next selected our LN patient cohorts. First, an initial cohort of 32 FFPE tissue blocks from renal biopsies were analysed (cohort 1). These included 25 LN: 16 of which were class III/IV (8 CR and 8 NR), 9 class V (5 CR and 4 NR) and 7 controls (TBM). The demographics of the cohort are displayed in table 5.1. Though we had a heterogeneous cohort, there were no significant differences between groups in terms of ethnicity, reason for biopsy or treatment and so all groups were balanced for this analysis.

Demographic and phenotypic data for the cohort are displayed in table 5.1.

Diagnosis	Lupus Nephritis class IV CR n=8	Lupus Nephritis class IV NR n=8	Lupus Nephritis class V CR n=5	Lupus Nephritis class V NR n=4	Control (Thin Basement Membrane) n=7	p value (LN vs control and CR vs NR)
Median age (range)	34 (24-40)	38 (19-51)	53 (29-70)	48 (32-59)	38 (16-62)	ns
Sex (% female)	100%	75%	100%	100%	100%	ns
Ethnicity	White 0% Black 25% South Asian 50% Other 0%	White 25% Black 37.5% South Asian 25% Other 12.5%	White 60% Black 20% South Asian 20% Other 0%	White 25% Black 50% South Asian 25% Other 0%	White 28.5% Black 28.5% South Asian 14% Other 28.5%	ns
Reason for biopsy	First presentation 75% Flare 25% Persistent proteinuria 0% Staging 0%	First presentation 25% Flare 50% Persistent proteinuria 25% Staging 0%	First presentation 100% Flare 0% Persistent proteinuria 0% Staging 0%	First presentation 25% Flare 25% Persistent proteinuria 50% Staging 0%	N/A	ns
Treatment	Rituximab 75% CyP 0% RituxiCyP 25% Oral 0%	Rituximab 62.5% CyP 0% RituxiCyP 25% Oral 12.5%	Rituximab 100% CyP 0% RituxiCyP 0% Oral 0%	Rituximab 50% CyP 25% RituxiCyP 0% Oral 25%	N/A	ns

Table 5.1 Table displaying demographic and clinical information for the biopsies used in cohort one (n=32). There were no significant differences identified between the groups in any of the categories allowing for direct comparison of the groups.

LN lupus nephritis; CyP, cyclophosphamide; CR complete response; NR non-response.

5.3.2.2 Lupus Nephritis versus control

SWATH-MS analysis was conducted on our sample cohort 1, initially looking at the differences observed between LN and controls. From our total of 32 FFPE samples, 8 samples (4 IV CR, 3 IV NR and 1 V NR) were removed due to the minimum total ion intensity of the sample being below an acceptable threshold. Fortunately, the samples lost were from the categories with the largest initial numbers of samples. Of the remaining 24 samples, there were 7 controls (TBM) and 17 LN: 9 class III/IV (4 CR and 5 NR) and 8 class V (5 CR and 3 NR). SWATH-MS on these remaining 24 samples identified 5139 proteins. A quantity was reported for each of these proteins for each of the 24 samples. The method for identifying and quantifying the proteins is discussed in chapter 2. Briefly, the MS produces highly multiplexed fragment ion spectra. This output was searched using software (openSWATH) containing a comprehensive assay library to identify the proteins from the MS output. The same software quantifies the peptides and thus proteins from the sum of the fragment ion peaks. A single proteotypic peptide was required for identification. Several quality controls are in place to ensure accurate identification. If, for example, the peaks were not typically shaped for a particular peptide, identifying a protein in a particular sample, the abundance would not be reported, a zero, or blank is instead reported and the value for that protein abundance in that sample is 'missing' or unknown. 64% of values were missing in this cohort. Whilst this appears to be a large amount of data, this degree of 'missingness' is in keeping with other published works using the same technique[221,222] in which the accuracy of the protein identification and quantification is validated despite the amount of missing data. It is technically possible that any proteins reported as missing were so as they were below the lower limit of detection. Correlation analysis was therefore undertaken comparing the number of missing values for a protein across all the samples with the median abundance of the protein again across all the samples. This correlation was very poor (r=-0.035) indicating a very low chance that the missing data represent a true value below the lower limit of detection. Some of the missing values will likely represent true values below the lower limit of detection. However, due to the reporting of invalid data in the same way as low quantity proteins, a quantity of zero cannot be reported for a protein as zero cannot be distinguished from 'unknown'. The implications of this are discussed in the discussion. Given this large amount of missing data, some proteins had up to 92% of their values missing across all the samples. This would obviously affect the accuracy of further data analysis on these proteins including differential expression. The protein was therefore removed from analysis if there were $\geq$80% unknown values. 1694 proteins (33%) were removed leaving 3446 for further analysis. In doing so, nearly 50% of the missing values were removed from further analysis. This demonstrates that nearly half were concentrated in one third of the proteins.

Following from this, a hierarchical heatmap (figure 5.2) and PCA graphs (figure 5.3) were generated to visualise the data and any groupings. As noted, the heat map contains all protein abundance values from all samples. Missing values were input as a very high value and are demonstrated by a grey bar. This showed no clear separation of LN from control. It also showed no clear separation of the data by class, response to treatment, age or sex. The PCA analysis also showed no clear separation of LN and control. Briefly, PCA analysis identifies the axes on which there is the greatest separation of data, pictorially demonstrating clusters of samples (each biopsy in this case). It does this by plotting all possible combinations of each variable (here each protein abundance) for each biopsy, on a series of differently orientated X/Y axes (protein 1 vs protein 2, etc) with each biopsy a plot point on each X/Y axis. This results in a 3D image of multiple sets of axes. PCA analysis examines this 3D figure to identify the line cutting through all the axes on which there is most separation of the data, whereby combining all the original variables (protein abundances) will explain the most variance producing a new variable. It then identifies the next lines of greatest separation, with the effect of the first removed. These new variables are termed principal component 1 (PC1), PC2 etc and can then be plotted against one another on an X/Y axis, PC1 vs PC2, PC1 vs PC3 etc. Pictorially representing the biopsies by diagnosis, LN or control, we can then visualise if the data separates on our distinction of interest. Figure 5.3 shows principal components 1, 2 and 3 plotted in three configurations. None of the plots demonstrate clear separation or clustering of the groups. It had been expected that LN would separate from control on this analysis, given that separation had been identified in the same analysis applied to the preliminary exploratory trials of this experiment as discussed in the optimisation section of this results chapter.

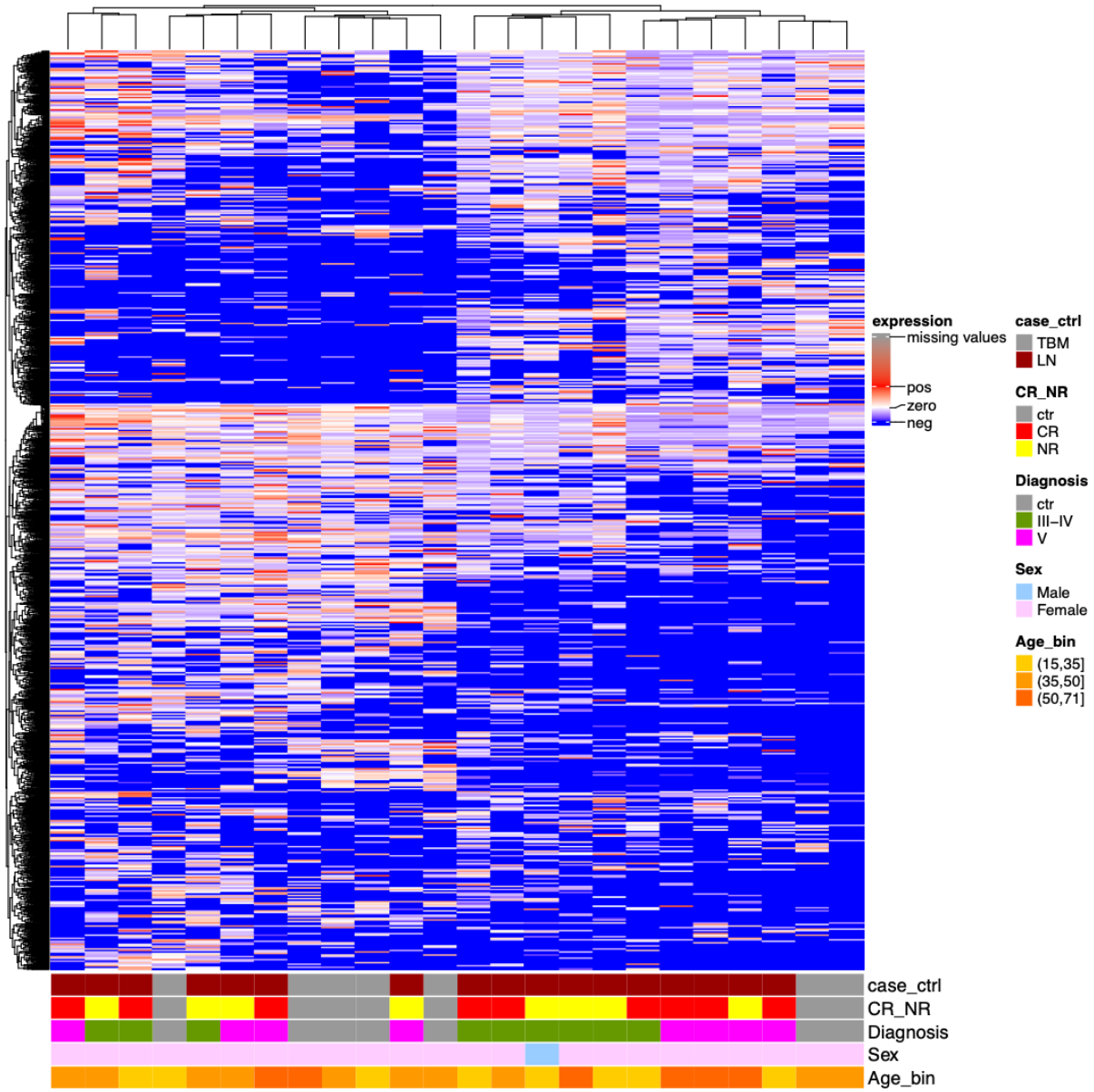


Figure 5.2 Hierarchical heat map of SWATH-MS data from LN and controls which shows no clear separation of LN from control or by class, response, age or sex.

LN (n=17) and TBM (n=7) samples were analysed by SWATH-MS for cohort one (n=24). The protein abundance values (n=5139) are displayed using z scores of the raw protein abundance values which are median normalised. The protein abundances are each therefore displayed on a pictorial relative scale of blue (negative) to red (positive). Missing protein abundance values are represented by a grey bar. Samples are annotated at the base of the heat map and identify each biopsy by: case (LN, maroon) or control (TBM, grey); response, (CR, red or NR, yellow); diagnosis (control (grey), class III/IV LN (green) or class V LN(purple)), sex (male, blue or female, pink) and age (arranged in groups (bins) 15-35, 35-50 and 50-71 displayed as graded yellow to orange. Ctr = control; LN, lupus nephritis; TBM, thin basement membrane; CR, complete response; NR, non-response.

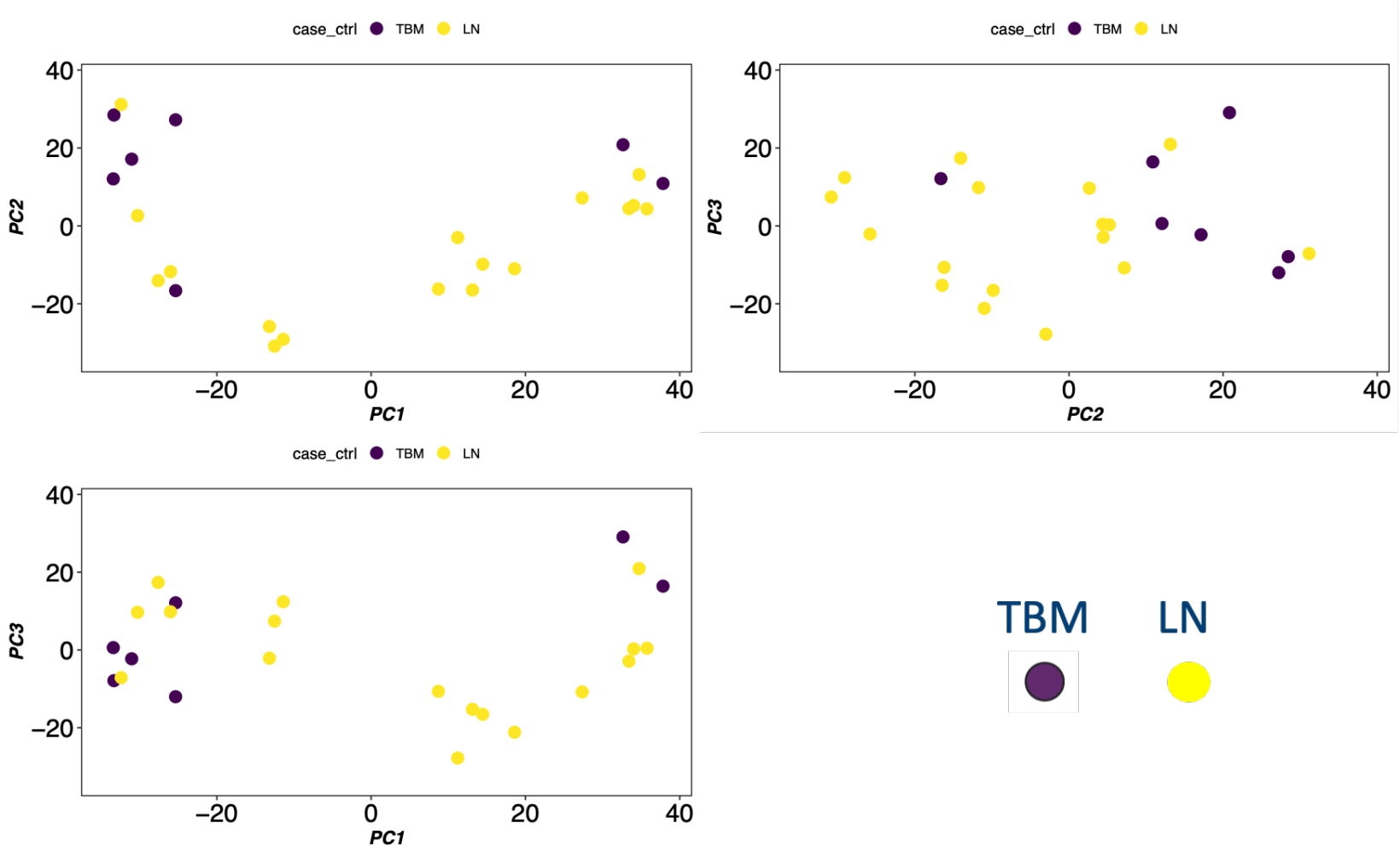


Figure 5.3 Principal component analysis of cohort one cannot clearly separate LN from controls.

PCA of SWATH-MS data (n=24 biopsies) in three configurations of principal components (PC) 1, 2 and 3. A shows PC1 vs PC2, B shows PC2 vs PC3 and C shows PC1 vs PC3. Samples are each represented by dots coloured yellow for LN (n=17) and purple for control, (TBM) (n=7). The first three principal components explain the majority of the variance of the data. LN, lupus nephritis; TBM, thin basement membrane; PC, principal component.

With no clear separation by heatmap or PCA, we next wanted to look more specifically at the proteins which were differentially expressed between LN and TBM to highlight pathways that may be involved in disease pathogenesis. This was undertaken using a volcano plot of relative expression represented by q value vs fold change with significantly different expression defined by q<0.05 and log2 fold change ≤-1 or ≥1 (figure 5.4). A log2 fold change ≥1 equates to a fold change ≥2 and a log2 fold change ≤-1 equates to a fold change ≤0.5 or abundance reduced by at least half. Analysis of all LN biopsies compared with TBM control identified only 4 differentially expressed proteins all downregulated in LN compared to control with a log2 fold change ≤-2. They were transforming growth factor beta receptor associated protein 1 (TGFBRAP1), Phosphofurin Acidic Cluster Sorting Protein 2 (PACS2), CTTNBP2 N-terminal like protein (CTTNBP2NL) and 5'-Nucleotidase Ecto (NT5E) (figure 5.5). Additionally, there were no links identified by String[270] between the proteins with regards to biological processes (p=1) (figure 5.6).

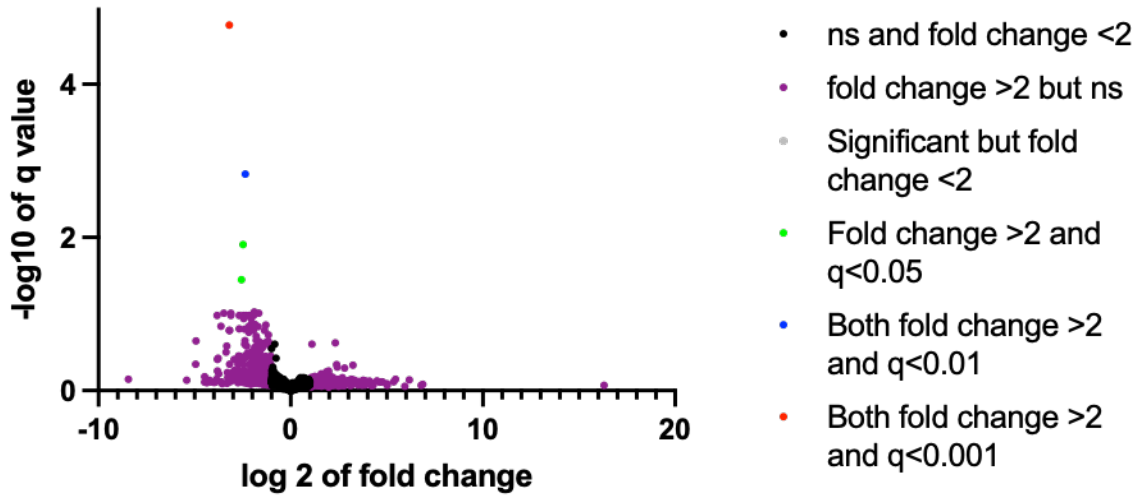


Figure 5.4 Volcano plot analysis identified 4 significantly downregulated proteins between LN and control.

A volcano plot was generated using SWATH-MS data from cohort one, displaying differentially expressed proteins between LN (n=17) and TBM (n=7) (control). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN and control with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN and control are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis; TBM, thin basement membrane.

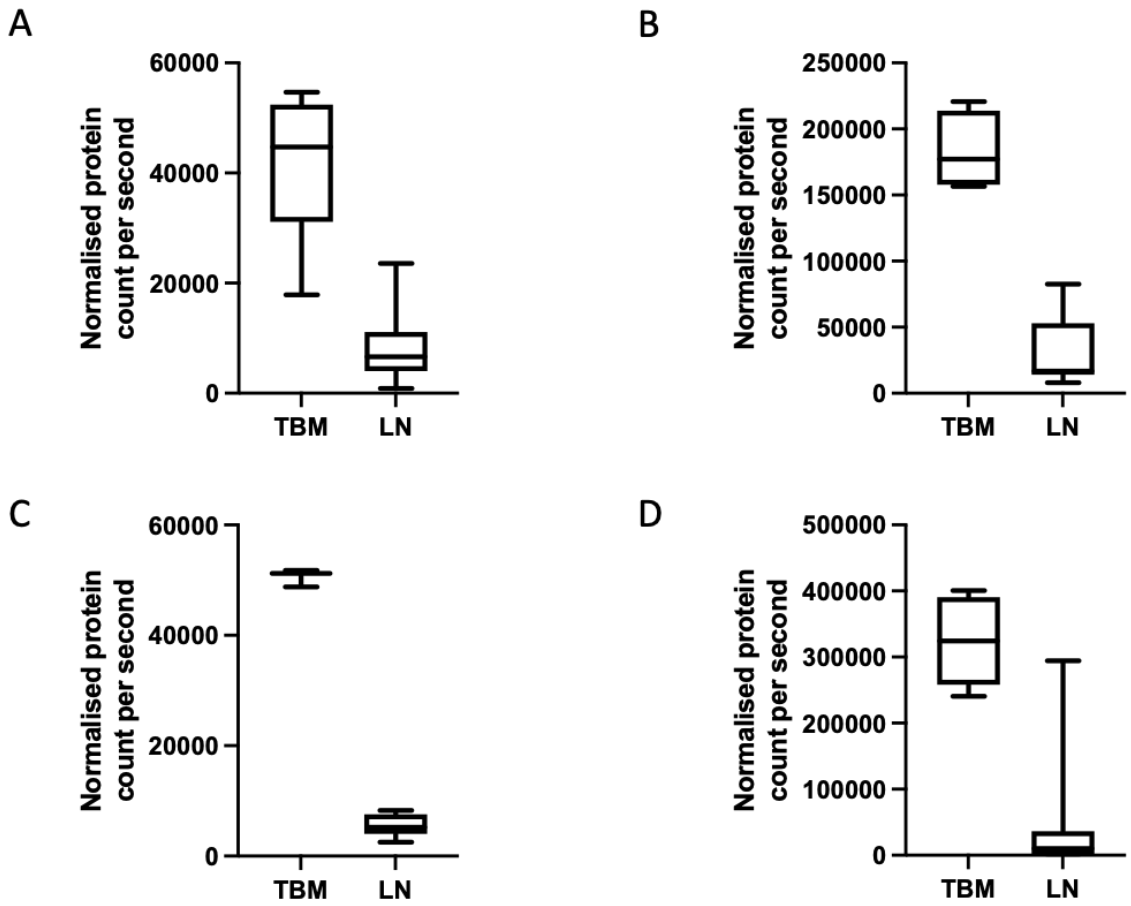


Figure 5.5 Box and whisker plots for listed proteins differentially expressed between LN and TBM in cohort one.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values. A – Transforming growth factor beta receptor associated protein 1 (TGFBRAP1). B – Phosphofurin Acidic Cluster Sorting Protein 2 (PACS 2). C – CTTNBP N-terminal like protein 2 (CTTNBP2NL). D – 5'Nucleotidase Ecto (NT5E).

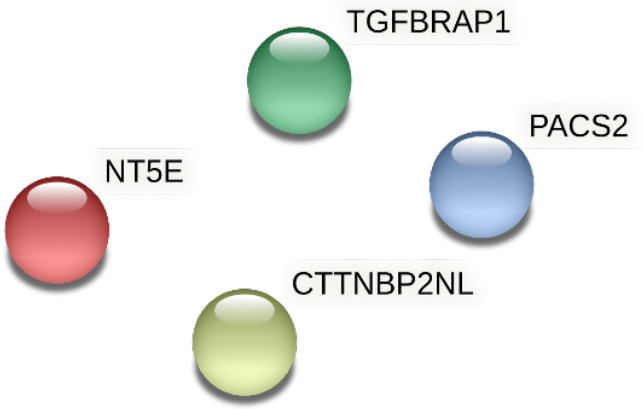


Figure 5.6 STRING interaction network identified no enriched pathways between the 4 significantly downregulated proteins between LN and control.

A STRING interaction network was produced for the 4 significant differentially expressed proteins downregulated between LN and control (n=17) and TBM (n=7) (medium confidence) in cohort one. PPI enrichment value, p=1. No enriched pathways. PPI, protein-protein interaction; LN, lupus nephritis; TBM, thin basement membrane.

5.3.2.3 Lupus Nephritis class analysis

The initial analysis of LN vs control, which identified no separation of the groups, contains both class III/IV and class V LN. As these represent quite different disease processes, respectfully proliferative and membranous, it was considered that by combining the two, some differences in protein expression may be being missed. The data were therefore next analysed looking at these disease processes separately. Initially, the PCA analysis was labelled by class. This showed no separation of the data by class (figure 5.7) indicating that this technique could not be used to identify the class of LN.

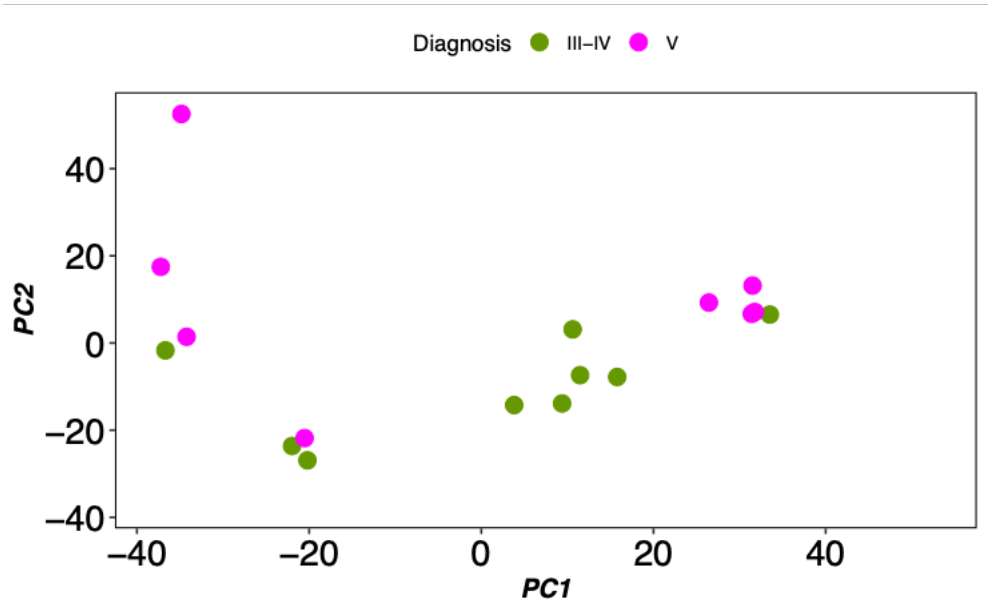


Figure 5.7 Principal component analysis of cohort one does not show clear separation of LN class III/IV from LN class V.

PCA of LN data (n=17 biopsies) displaying PC1 vs PC2 with biopsies coded by class. Samples are each represented by dots coloured green for LN class III/IV (n=9) and purple for LN class V (n=8). LN, lupus nephritis; PC, principal component.

The classes were therefore examined in further detail, again using a volcano plot of relative expression represented by q value vs fold change with significantly different expression defined by q<0.05 and log2 fold change ≤-1 or ≥1. When LN class III and IV were compared to control, 38 proteins were found to be significantly different, all downregulated with a log2 fold change ≤-1.5. The protein expression profile is shown in figure 5.8 and interactions between the proteins in figure 5.9. Calnexin was significantly downregulated in Class IV LN compared to TBM (figure 5.10). There were however no significant (p=0.738) links identified by String[270] between the significantly downregulated proteins (figure 5.9).

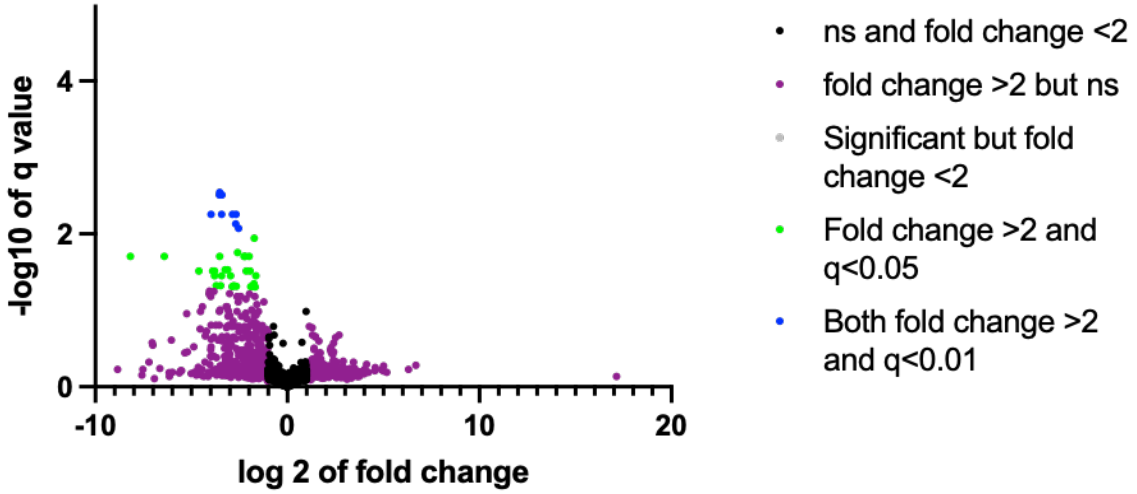


Figure 5.*8 Volcano plot analysis identified 38 significantly downregulated proteins between LN class III/IV and control.*

A volcano plot was generated using SWATH-MS data from cohort one, displaying differentially expressed proteins between LN class III/IV (n=9) and TBM (n=7)(control). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN class III/IV and control with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN class III/IV and control are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis; TBM, thin basement membrane.

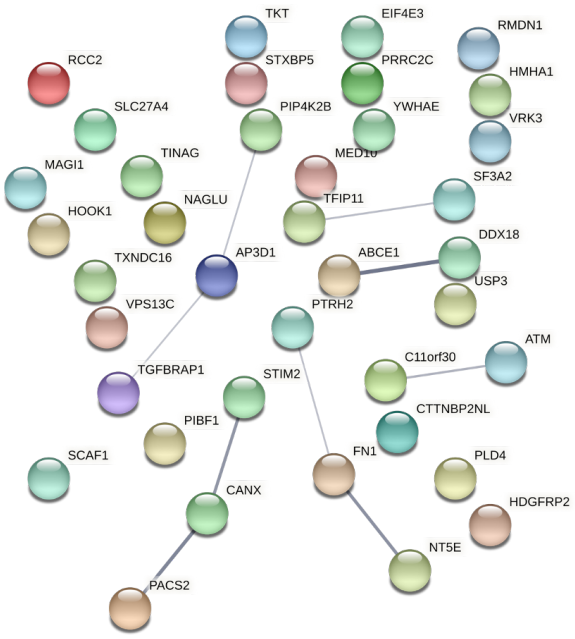


Figure 5.9 STRING interaction network identified no enriched pathways between the 38 significantly downregulated proteins between LN class III/IV and control.

A STRING interaction network was produced for the 38 significant differentially expressed proteins downregulated between LN class III/IV (n=9) and TBM (n=7) (medium confidence) in cohort one. PPI enrichment value, p=0.738. No enriched pathways. PPI, protein-protein interaction; LN, lupus nephritis; TBM, thin basement membrane.

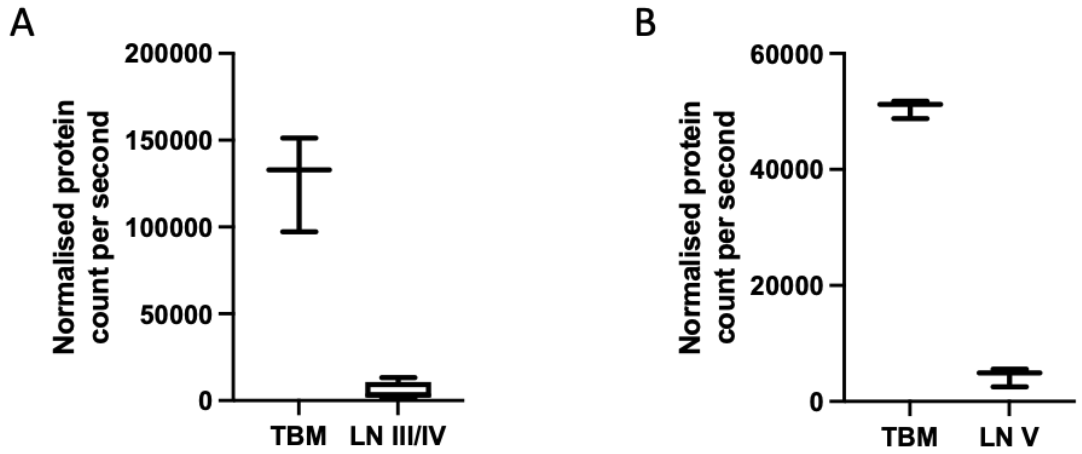


Figure 5.10 Box and Whisker plots for listed proteins differentially expressed between LN III/IV and TBM (A) and LN V and TBM (B) in cohort one.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values. (A) Calnexin. (B) CTTNBP2 N-terminal like protein (CTTNBP2NL).

Looking at membranous LN, again using a volcano plot of relative expression represented by q value vs fold change (figure 5.11), Class V LN had only one protein significantly downregulated compared to TBM: CTTNBP2NL (figure 5.10) and none upregulated.

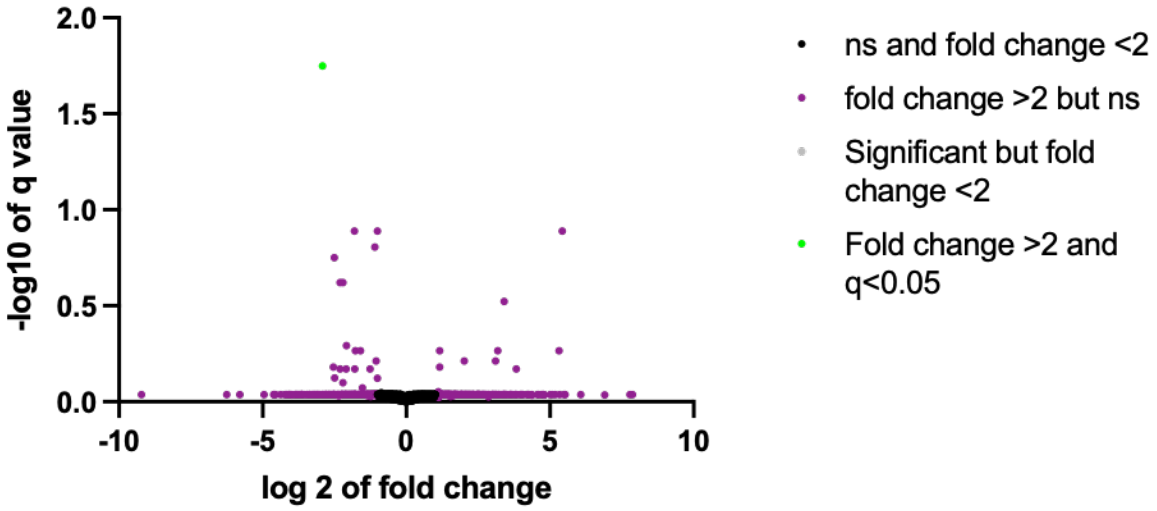


Figure 5.11 Volcano plot analysis identified 1 significantly downregulated protein between LN class V and control.

A volcano plot was generated using SWATH-MS data from cohort one, displaying differentially expressed proteins between LN class V (n=8) and TBM (n=7)(control). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN class V and control with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN class V and control are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis; TBM, thin basement membrane.

Thus, analysing the classes separately appeared to reveal significantly different proteins in proliferative LN compared to control, an effect which may have been dampened down by initial inclusion of the membranous disease. It was however only a small number of proteins. As a further analysis therefore, to investigate how different the classes of LN appear to be in regards to protein abundance, a correlation comparison was next made between the fold change difference in protein abundance in class III/IV LN vs TBM and class V LN vs TBM. Of the 3446 proteins identified in cohort one, 3125 proteins were identified in all LN class III/IV, LN class V and TBM (and thus had a fold change between each case and control comparison calculated). The correlation calculation identified a reasonable correlation coefficient of Spearman r = 0.5839 ($p<0.0001$). Figure 5.12 displays the correlation of the log2 fold change. This demonstrates that many of the proteins are expressed differentially to control in a similar fashion in class III/IV LN and class V LN within this cohort.

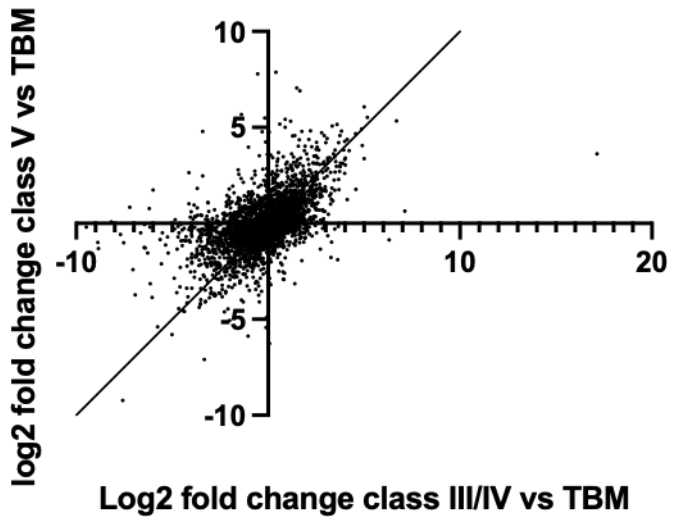


Figure 5.12 Proteins identified in LN class III/IV and class V relative to control correlate.

Correlation graph displaying the differential expression of proteins (n=3125) in LN class III/IV vs LN class V in cohort one. The log2 fold change of proteins identified in LN class III/IV (n=9) vs TBM (n=7) is displayed on the x axis compared to the log2 fold change of proteins identified in LN class V (n=8) vs TBM (n=7) displayed on the y axis. The graph displays a reasonable correlation coefficient of Spearman r = 0.5839 ($p<0.0001$). LN, lupus nephritis; TBM, thin basement membrane.

Class III/IV LN has been shown to have more significantly differentially expressed proteins compared to control than class V LN. Correlation analysis revealed that the differential expression of each class in relation to control is similar. In order to further elucidate differences between the classes of LN, a further direct comparison was made between them. This was again performed using a volcano plot of relative expression represented by q value vs fold change (figure 5.13), with significantly different expression defined by q<0.05 and log2 fold change ≤-1 or ≥1. Only three proteins were differentially expressed, all in higher levels in class V LN (figure 5.14). They were serine-protein kinase ATM which acts as a DNA damage sensor[271], E3 ubiquitin-protein ligase RNF114 which suppresses cellular senescence[272] and serine/threonine-protein phosphatase 4 catalytic subunit, a protein phosphatase involved in many cellular processes including apoptosis, DNA repair and checkpoint signalling of DNA damage as well as TNFα signalling[272] (figure 5.14). There were no significant (p=0.0957) links identified by String[270] between these proteins however.

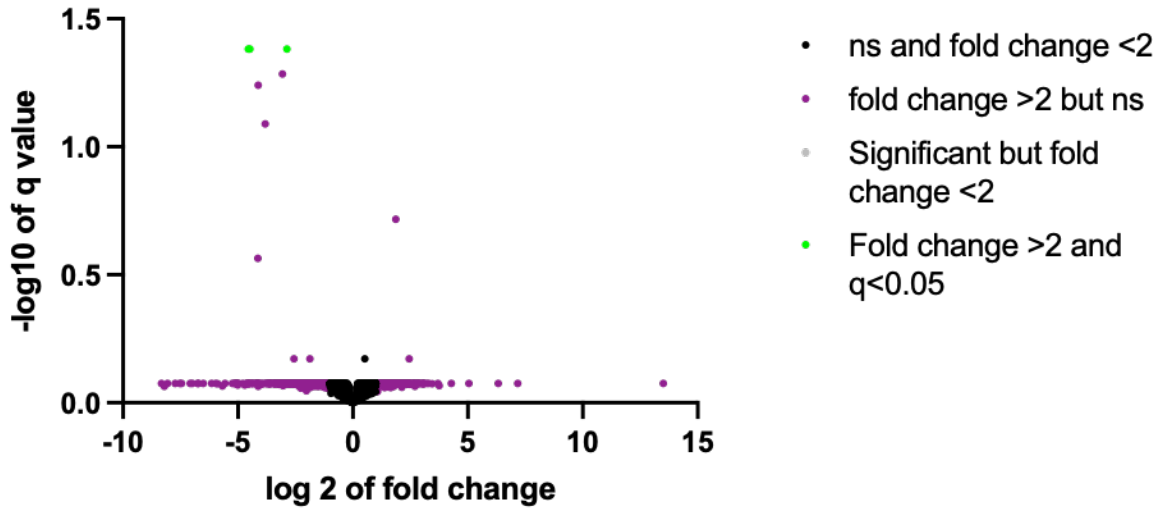


Figure 5.13 Volcano plot analysis identified 3 proteins significantly increased in LN class V compared to LN class III/IV.

A volcano plot was generated using SWATH-MS data from cohort one, displaying differentially expressed proteins between LN class III/IV (n=9) and LN class V (n=8). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN class III/IV and LN class V with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN class III/IV and LN class V are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis.

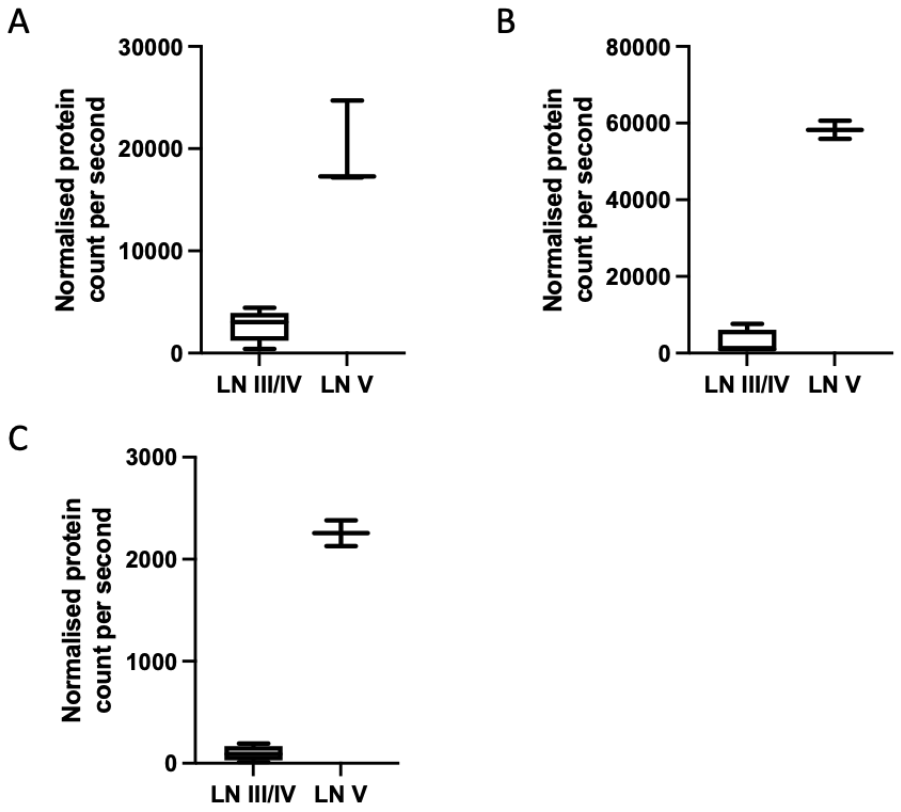


Figure 5.14 Box and whisker plots for listed proteins differentially expressed between LN III/IV and LN V in cohort one.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values. (A) Serine-protein kinase ATM. (B) E3 ubiquitin-protein ligase RNF114. (C) Serine/threonine-protein phosphatase 4 catalytic subunit.

5.3.2.4 Lupus nephritis responders versus non responders

Cohort one has thus far identified some proteins differentially expressed between LN and control. Following this, the LN cases were further examined to explore the hypothesis that there may be differentially expressed proteins in LN responders compared to non-responders.

Looking at our demographic data (table 5.1), there were no significant differences between CR and NR cases in regard to age, sex, ethnicity, reason for biopsy or treatment post biopsy thus allowing for a direct comparison between the groups without need for correction for these factors. There were numerically more patients with first presentation of LN (as opposed to flare or persistent proteinuria as biopsy indication) in the CR groups compared to NR, however this was not statistically significant (table 5.1). In the database analysis described in chapter 3, where all 599 biopsies which received treatment and thus had a recorded response to treatment were analysed, the reason for biopsy was not predictive of response. This numerical difference is therefore likely artefactual.

There were too few cases to examine the classes separately in this analysis and so protein abundances in all LN responders were directly compared to non-responders. This was again performed using the PCA analysis coding by response. This demonstrated no separation of the data (figure 5.15) making it unlikely that there would be significant differences in protein expression between responders and non-responders.

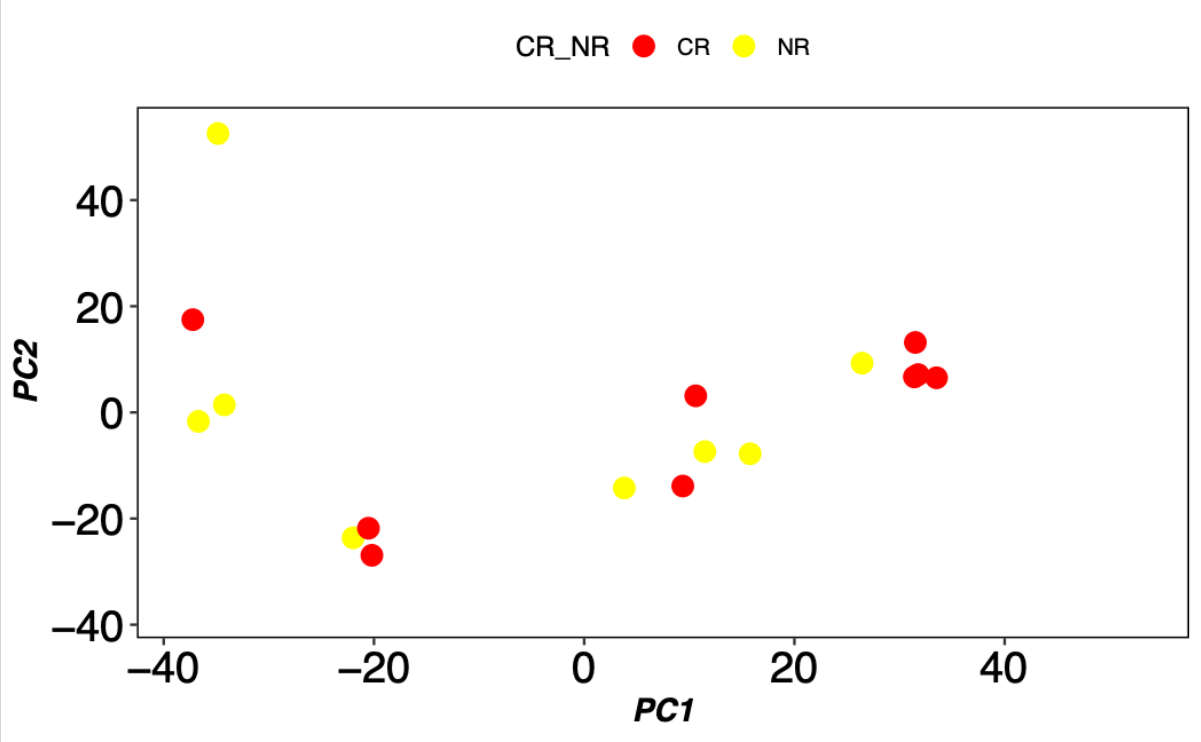


Figure 5.15 Principal component analysis of cohort one does not show clear separation of LN CR from LN NR.

PCA of LN data (n=17 biopsies) displaying PC1 vs PC2 with biopsies coded by response. Samples are each represented by dots coloured red for LN CR (n=9) and yellow for LN NR (n=8). LN, lupus nephritis; PC, principal component; CR, complete response; NR, non-response.

In order to look more closely for any differential expression, a volcano plot of relative expression represented by q value vs fold change (figure 5.16) was plotted, with significantly different expression defined by $q<0.05$ and log2 fold change $\leq$-1 or $\geq$1. There was no significant differential expression of proteins between CR and NR.

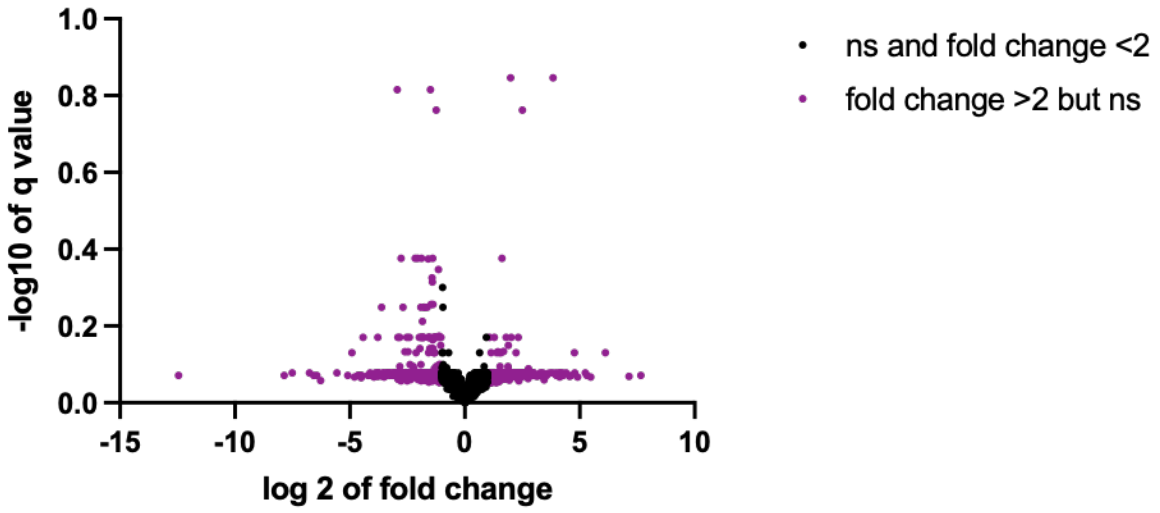


Figure 5.16 Volcano plot analysis identified no significantly differentially expressed proteins between LN CR and LN NR.

A volcano plot was generated using SWATH-MS data from cohort one, displaying differentially expressed proteins between LN CR (n=9) and LN NR (n=8). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN CR and LN NR with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN CR and LN NR are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis; CR, complete response; NR, non-response.

5.3.2.5 Machine learning analysis

The direct comparison of protein abundance between LN CR and NR using t tests with corrected p values and fold change for each protein revealed no significant differences. This kind of analysis, using a blunt correction of p values to represent significant differences in relative expression could potentially be missing patterns of different expression of proteins between the groups. An alternative method of analysis of this kind of 'big data' is to use machine learning to search for patterns in the data. In this instance, machine learning was employed to identify a panel of proteins which could separate CR from NR by way of a PCA using initially 70% of the data (randomly selected) to be a training set and the remaining 30% to be a test set. 1000 iterations (test panels of proteins) of the data were examined through machine learning.

This revealed that a panel of 8 proteins separated CR from NR when PCA was performed on the panel. These are listed in table 5.2 with box and whisker plots demonstrating the comparable quantities in figure 5.17. The PCA demonstrating the separation of CR from NR is shown in figure

5.18. Whether the proteins are expressed at higher or lower levels in CR compared to NR as measured by direct comparison of median protein abundance is also listed in table 5.2 however none of these differences were significant as assessed by t test with corrected p values and fold change as already noted in the previous CR vs NR analysis. Whilst this analysis was performed to identify a panel of proteins which could separate all LN CR from all LN NR, the proportion of classes in the training and test set were balanced. Figure 5.18 shows the PCA which demonstrates separation of CR from NR using the abundance of proteins listed in the panel in table 5.2. As each biopsy is plotted individually on the PCA, the points were labelled by both class and response (figure 5.18). The PCA shows that the classes grouped within the response groupings. This is represented by coloured circles on the graph. Whilst it shows CR separating from NR, III/IV CR separates from III/IV NR and the separation of V CR from V NR is greater with tighter groupings in this class. These tighter groupings in class V disease represent greater concordance in the protein abundances of these 8 proteins within the class V CR and NR than within the class III/IV CR and NR groups. The panel of proteins was analysed by String which revealed that there were not significantly more interactions between the proteins than expected (p=0.23). Therefore, there were no enriched pathways identified between the proteins.

Protein in Panel	Regulation in Response	Function
RuvB-like 1	Lower levels in CR	Cell proliferation.
Heterogeneous nuclear ribonucleoprotein F	Lower levels in CR	cRNA binding.
Cofilin-1	Lower levels in CR	Regulates actin skeleton dynamics, cell morphology and cytoskeletal organisation.
Transcription intermediary factor 1-beta	Lower levels in CR	Regulation DNA binding and repair.
Eukaryotic translation elongation factor 1 epsilon-1	Lower levels in CR	Negative regulator of cell proliferation and positive regulator of apoptosis.
60S ribosomal protein L9	Lower levels in CR	Structural constituent of ribosomes which catalyse protein synthesis.
Chloride intracellular channel protein 1	Lower levels in CR	Regulation of cell cycle. Protects intracellular environment against oxidation. Inflammation and apoptosis in Alzheimer's disease.
Moesin	Lower levels in CR	T and B cell homeostasis and self-tolerance.

Table 5.2 Panel of eight proteins from cohort one identified by machine learning to differentiate LN CR (n=9) from LN NR (n=8).

1,000 iterations of machine learning testing was conducted with the dataset split 70:30 at random into a training set and testing set with class balanced across the sets. PCA analysis was used to identify the separation of CR from NR. PCA, principal component analysis; LN, lupus nephritis; CR, complete response; NR, non-response.

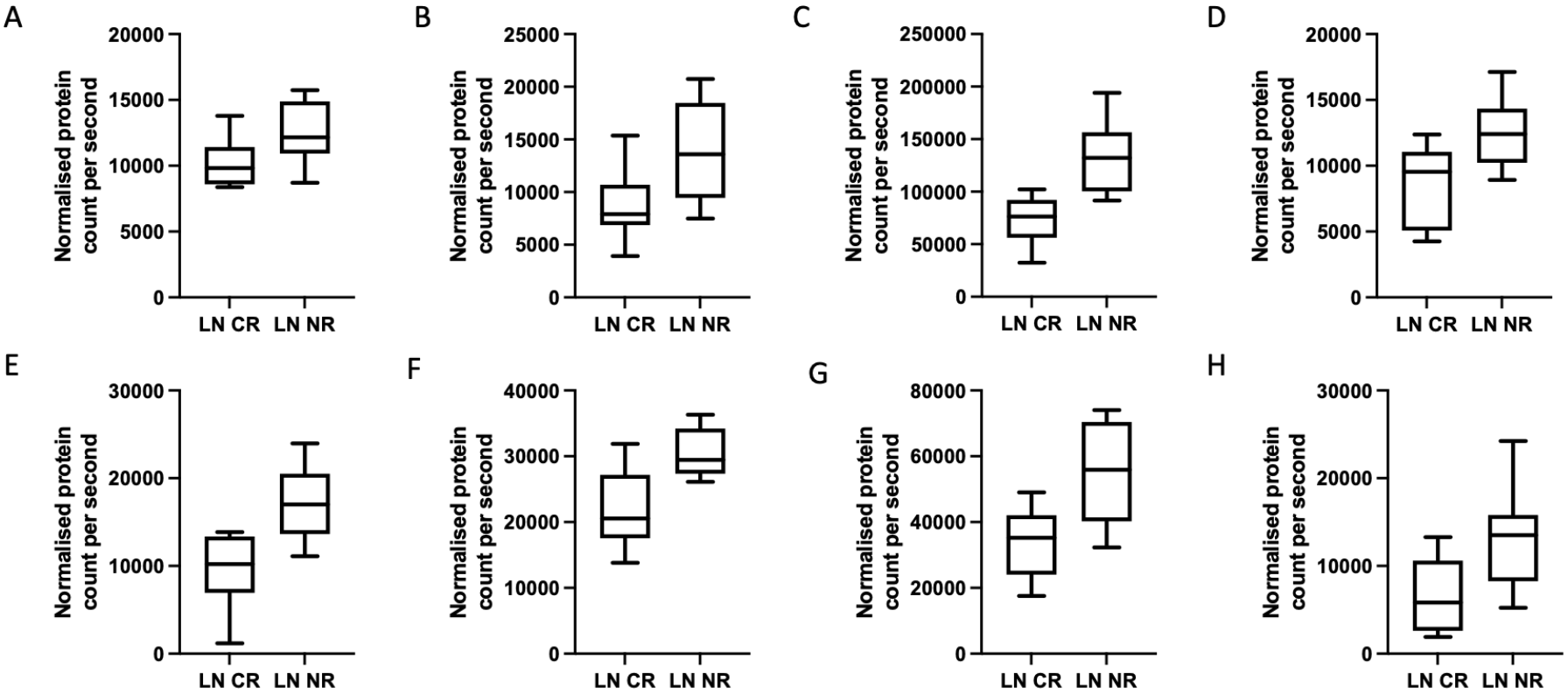


Figure 5.17 Box and whisker plots for listed proteins included in the panel identified by machine learning, separating LN CR and LN NR in cohort one.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values. A – RuvB like 1. B – Heterogeneous nuclear ribonucleoprotein F. C – Cofilin-1. D – Transcription intermediary factor 1-beta. E – Eukaryotic translation elongation factor 1 epsilon-1. F – 60S ribosomal protein L9. G – Chloride intracellular channel protein 1. H – Moesin.

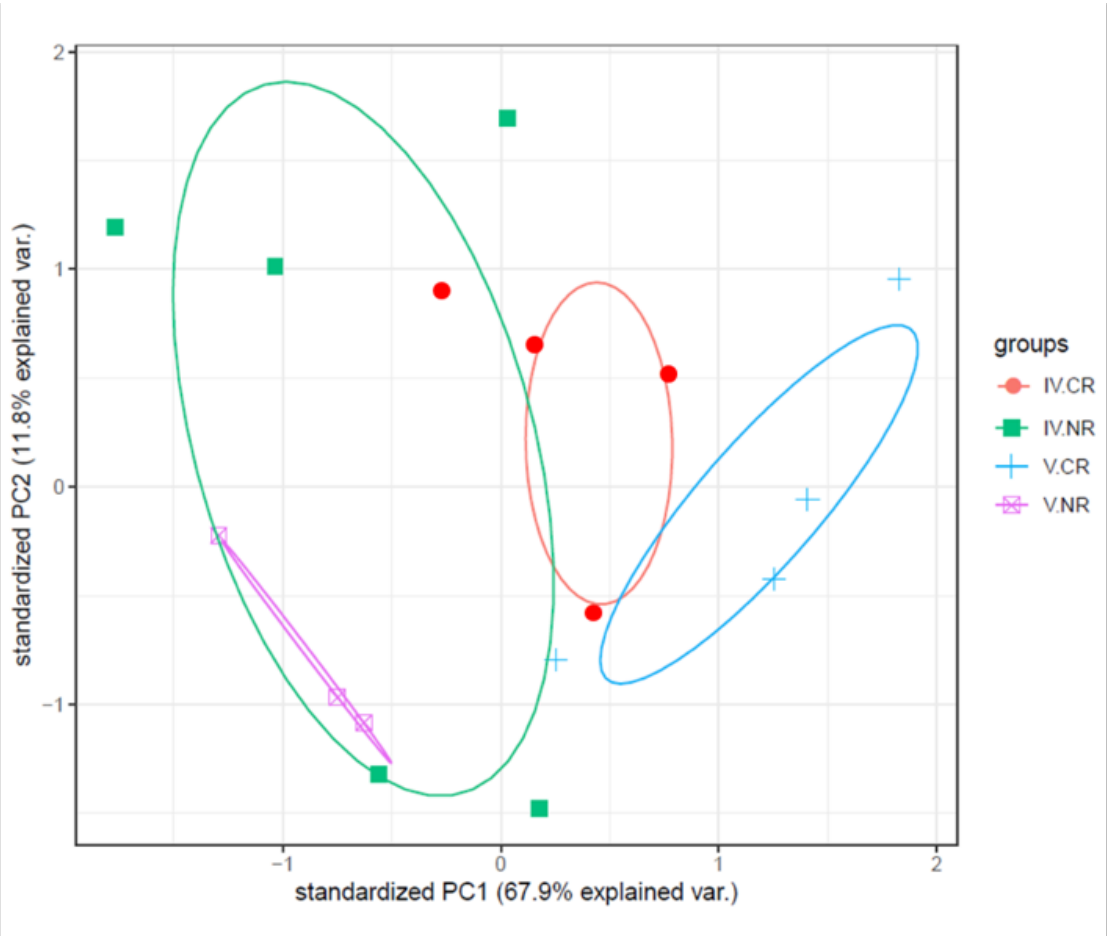


Figure 5.18 Principal component analysis of cohort one using the panel of 8 proteins identified in machine learning shows clear separation of the LN CR from LN NR which is more prominent in class V LN.

PCA of LN data (n=17 biopsies) using the panel of 8 proteins identified by machine learning. PC1 vs PC2 is displayed with biopsies coded by class and response. Samples are each represented by dots coloured red for LN class III/IV CR (n=4), green for LN class III/IV NR (n=5), blue for LN class V CR (n=5) and purple for LN class V NR (n=3). LN, lupus nephritis; PC, principal component; CR, complete response; NR, non-response.

5.3.3 Cohort Two

5.3.3.1 Overview and demographic data

The findings of cohort one were somewhat different to expected. Neither by heat map analysis, PCA nor differential expression of proteins using t test and fold change analysis, did LN biopsy tissue appear to show different expression of proteins to control. This was unexpected as obvious differences had been identified at all phases of preliminary experiments. This could have been for a number of technical reasons or simply be demonstrating the heterogeneity of LN as a disease with the occurrence by chance that this group of samples were not concordant enough in pathogenesis to display statistically significant differences in protein expression when compared to controls. That is, the experiment was not powered enough to show differences in protein abundance compared to control. It was therefore next planned to repeat the experiment on a larger number of samples to provide further information. For this new cohort two, 38 new FFPE renal biopsy tissue blocks were analysed. These included 32 LN: 17 of which were class III/IV (9 CR and 8 NR), 15 class V (8 CR and 7 NR) and 6 controls (TBM). The demographics of the cohort are displayed in table 5.3. Though we again had a heterogeneous cohort, there were no significant differences between groups in terms of ethnicity, reason for biopsy or treatment and so all groups were balanced for this analysis.

Demographic and phenotypic data for the cohort are displayed in table 5.3.

Diagnosis	Lupus Nephritis class III/IV CR n=9	Lupus Nephritis class IV NR n=9	Lupus Nephritis V CR n=8	Lupus Nephritis V NR n=6	Control (Thin Basement Membrane) n=6	p value (LN vs control and CR vs NR)
Median age (range)	30 (20-46)	38 (17-51)	36 (21-45)	33 (18-71)	43 (36-50)	ns
Sex (% female)	67%	67%	88%	67%	83%	ns
Ethnicity	White 33% Black 22% South Asian 44% Other 0%	White 22% Black 44% South Asian 33% Other 11%	White 13% Black 63% South Asian 13% Other 13%	White 17% Black 50% South Asian 33% Other 0%	White 17% Black 17% South Asian 67% Other 0%	ns
Reason for biopsy	First presentation 78% Flare 0% Persistent proteinuria 22% Staging 0%	First presentation 33% Flare 33% Persistent proteinuria 33% Staging 0%	First presentation 50% Flare 50% Persistent proteinuria 0% Staging 0%	First presentation 17% Flare 50% Persistent proteinuria 33% Staging 0%		ns
Treatment	Rituximab 66% CyP 11% RituxiCyP 22% Oral 0%	Rituximab 33% CyP 22% RituxiCyP 44% Oral 0%	Rituximab 50% CyP 25% RituxiCyP 13% Oral 13%	Rituximab 34% CyP 0% RituxiCyP 0% Oral 67%		ns

Table 5.3 Table displaying demographic and clinical information for the biopsies used in cohort two (n=38). There were no significant differences identified between the groups in any of the categories allowing for direct comparison of the groups.

5.3.3.2 Lupus Nephritis versus control

SWATH-MS analysis was conducted on sample cohort 2, initially looking at the differences observed between LN and controls. From our 38 FFPE samples, 9 samples (2 IV CR, 1 IV NR, 2 V CR and 4 V NR) were removed due to the minimum total ion intensity of the sample being below an acceptable threshold. Fortunately, there was again a spread over the different categories of class and response, however few class V NR samples remained. Remaining sample numbers were 29 which included 23 LN: 14 of which were class III/IV (7 CR and 7 NR), 9 class V (6 CR and 3 NR) and 6 controls (TBM). SWATH-MS on these remaining 29 samples identified 4561 proteins. Again, a quantity was reported for each of these proteins for each of the 29 samples. Again, some values were missing due to failures in quality control during the identification or quantification of that protein within that sample. 50% of the values were missing in this cohort. A lower value than cohort one but again in-keeping with published data using the same technique. Correlation was again performed comparing the number of missing values for a protein across all the samples and the median abundance of the protein again across all the samples to evaluate the chance that the missing values were reported as such due to the proteins being below the lower limit of detection in those particular samples. The correlation was found to be very poor (r=-0.032), indicating again, a very low chance that the missing values represent a true value below the lower limit of detection. Again, it is possible and even likely that some of the missing data will be so because it was below the lower limit of detection. However, it is not possible to separate those proteins with quantities close to zero from those essentially 'unknown'. Some proteins had up to 97% of their values missing across all the samples. This would again obviously affect the accuracy of further data analysis on these proteins including differential expression. The protein was therefore removed from analysis if there were $\geq$80% unknown values. More of the missing values were concentrated in fewer proteins in cohort two compared to cohort one, which allowed for removal of fewer proteins (1035, 23%) from analysis, leaving 3526 for further analysis. In doing so, again, nearly 50% of the missing values were removed from further analysis, demonstrating that in cohort 2, nearly half of the missing values were concentrated in 23% of the proteins. There were fewer proteins initially identified in cohort two compared to cohort one and so the resultant number of proteins for analysis was similar to cohort one.

Following from this, a hierarchical heatmap was generated (figure 5.19) and PCA analysis performed (figure 5.20) to visualise the data and observe any groupings. The heat map again contained all protein abundance values from all samples. Missing values were input as a very high value and are demonstrated by a grey bar. These data, in contrast to cohort one, do appear to show clear separation of LN from TBM control with the exception of one control sample which appears as an outlier. The heat map does not show convincing separation of cases by age or sex or by class of LN

or response. This would indicate that these factors do not require correcting for when exploring the differences in protein abundance between LN and control.

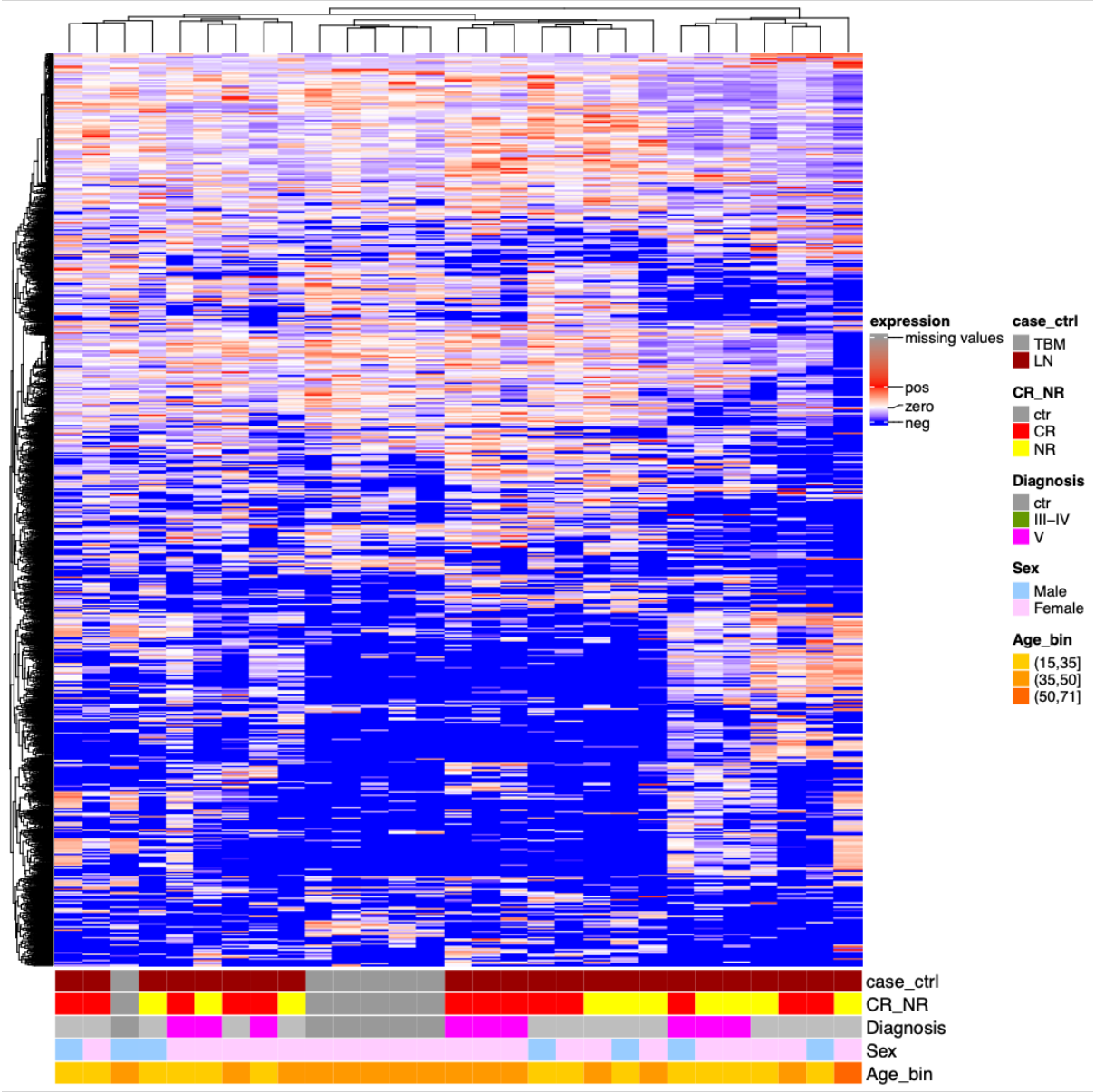


Figure 5.19 Hierarchical heat map of SWATH-MS data from LN and controls which shows clear separation of LN from control and no clear separation of the data by LN class, response to treatment, age or sex.

LN (n=23) and TBM (n=6) samples were analysed by SWATH-MS for cohort two (n=29). The protein abundance values (n=4561) are displayed using z scores of the raw protein abundance values which are median normalised. The protein abundances are each therefore displayed on a pictorial relative scale of blue (negative) to red (positive). Missing protein abundance values are represented by a grey bar. Samples are annotated at the base of the heat map and identify each biopsy by: case (LN, maroon) or control (TBM, grey); response (CR, red or NR, yellow); diagnosis (control (grey), class III/IV LN (green) or class V LN(purple)), sex (male, blue or female, pink) and age (arranged in groups (bins) 15-35, 35-50 and 50-71 displayed as graded yellow to orange. Ctr = control; LN, lupus nephritis; TBM, thin basement membrane; CR, complete response; NR, non-response.

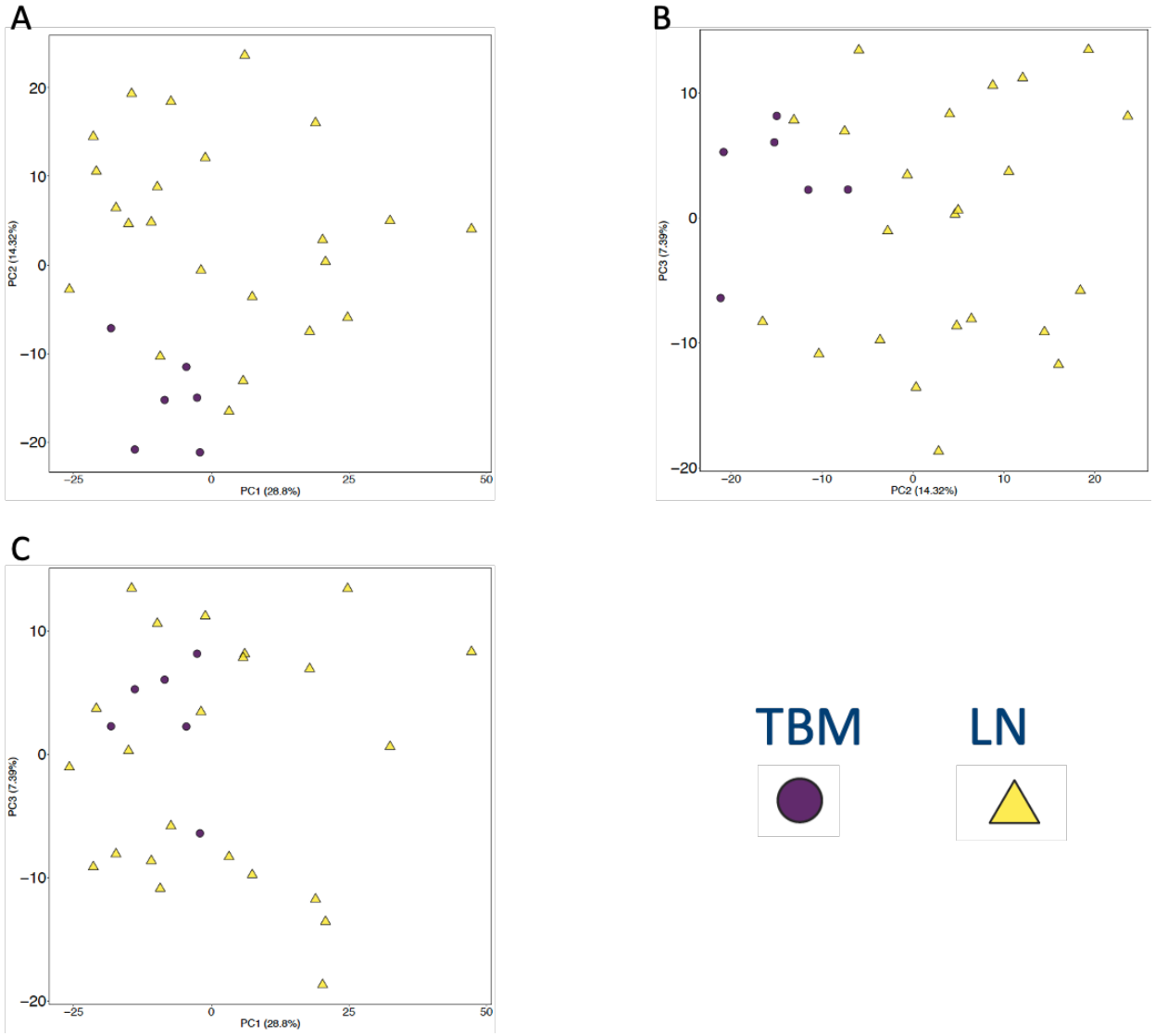


Figure 5.20 Principal component analysis of cohort two shows clear separation of LN from control.

PCA of SWATH-MS data (n=29 biopsies) in three configurations of principal components (PC) 1, 2 and 3. (A) shows PC1 vs PC2, (B) shows PC2 vs PC3 and (C) shows PC1 vs PC3. Samples are each represented by triangles coloured yellow for LN (n=23) and dots coloured purple for control, (TBM) (n=6). The first three principal components explain the majority of the variance of the data. LN, lupus nephritis; TBM, thin basement membrane; PC, principal component.

Figure 5.20 displays principal component analysis of the data overall. PCA draws out the biggest differences in protein expression across the whole dataset, identifying new variables which explain the most variance in the data. Again, in contrast to cohort one, the PCA for this cohort does show clear separation of the lupus nephritis and control. This can be seen clearest in figures 5.20 (A) and 5.20 (B) which show PC1 vs PC2 and PC2 vs PC3.

On finding the separation of LN and control on heat map analysis and PCA, we next wanted to identify if there were more proteins which were significantly differentially expressed between LN and control. We also wanted to examine specifically what these differentially expressed proteins were and if they congregated into pathways which might give insight into the disease pathogenesis of LN. In order to identify these differentially expressed proteins, a volcano plot of relative expression represented by q value vs fold change was constructed. Figure 5.21 displays the volcano plot showing the identified proteins and their relative expression represented by q value and fold change. Differentially expressed proteins as defined by q<0.05 and log2 fold change ≤-1 or ≥1 are represented by the green (q<0.05), blue (q<0.01) and red (q<0.001) dots. Analysis of all LN biopsies compared with TBM control identified 80 differentially expressed proteins with q<0.05 and log2 fold change ≤-1 or ≥1, 77 downregulated with log2 fold change ≤-1 and 3 upregulated with log2 fold change ≥1.

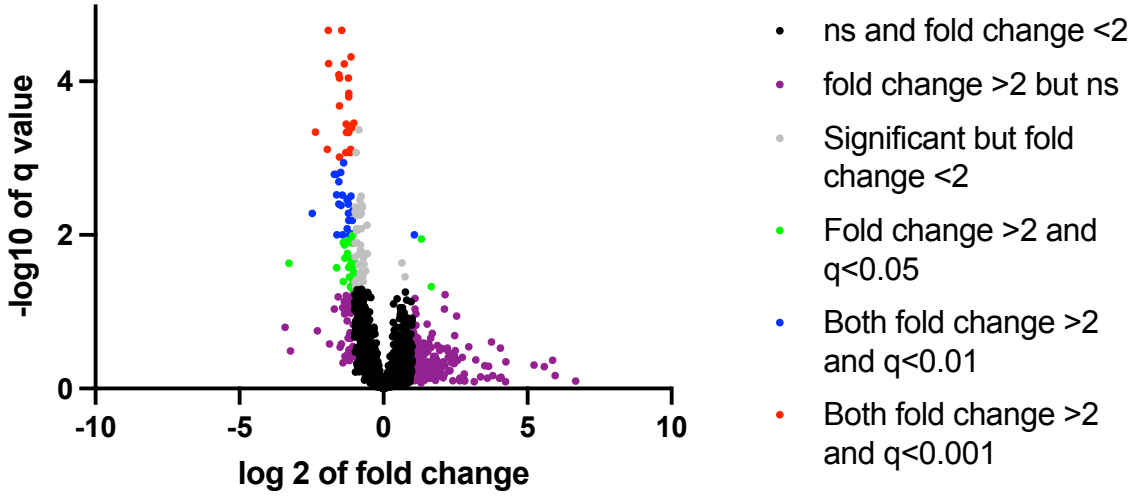


Figure 5.21 Volcano plot analysis identified 80 significantly differentially expressed (77 downregulated and 3 upregulated) proteins between LN and control.

A volcano plot was generated using SWATH-MS data from cohort two, displaying differentially expressed proteins between LN (n=23) and TBM (n=6) (control). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN and control with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN and control are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis; TBM, thin basement membrane.

These proteins were analysed by String[270] to identify if there were any links between the proteins in terms of interactions between the proteins or similarities in regards to participation in biological pathways or processes. String identified significantly more interactions than expected between the downregulated proteins ($p<1^{-16}$) however not between the upregulated proteins ($p=1$). These links and pathways are displayed in figure 5.22 (A)i) and ii) (downregulated) and (B) (upregulated). Pathways with some of the most significant enrichments are highlighted by colour of the protein dots. Proteins involved in more than one pathway have multiple colours. Significance of the enrichment is determined by the false discovery rate and p values are corrected for multiple testing with the Benjamini-Hochberg correction as was performed in differential expression analysis. In the downregulated proteins, this includes the biological processes of the oxidation-reduction process ($q=7.12^{-23}$), aerobic respiration ($q=8.92^{-16}$), mitochondrial electron transport, nicotinamide adenine dinucleotide (NAD) hydrogen (NADH) to ubiquinone ($q=0.0114$) and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) processes of oxidative phosphorylation ($q=6.57^{-9}$) and metabolic pathways ($q=2.88^{-18}$)(figure 5.22 (A)ii)). Many mitochondrial proteins were found to be downregulated in LN, including NADH dehydrogenase [ubiquinone] flavoprotein 1, the first enzyme of the mitochondrial electron transport chain. Lysosome-associated membrane glycoprotein 1 was also significantly downregulated. It plays an important role in NK cell cytotoxicity. Also downregulated were adenosine triphosphate (ATP) synthase F(0) complex subunit B1, involved in metabolic and catalytic activity as well as proteins involved in antioxidant functions (Thioredoxin-dependent peroxide reductase) and free-radical detoxification (NAD phosphate (NAD(P)) transhydrogenase) (figure 5.23).

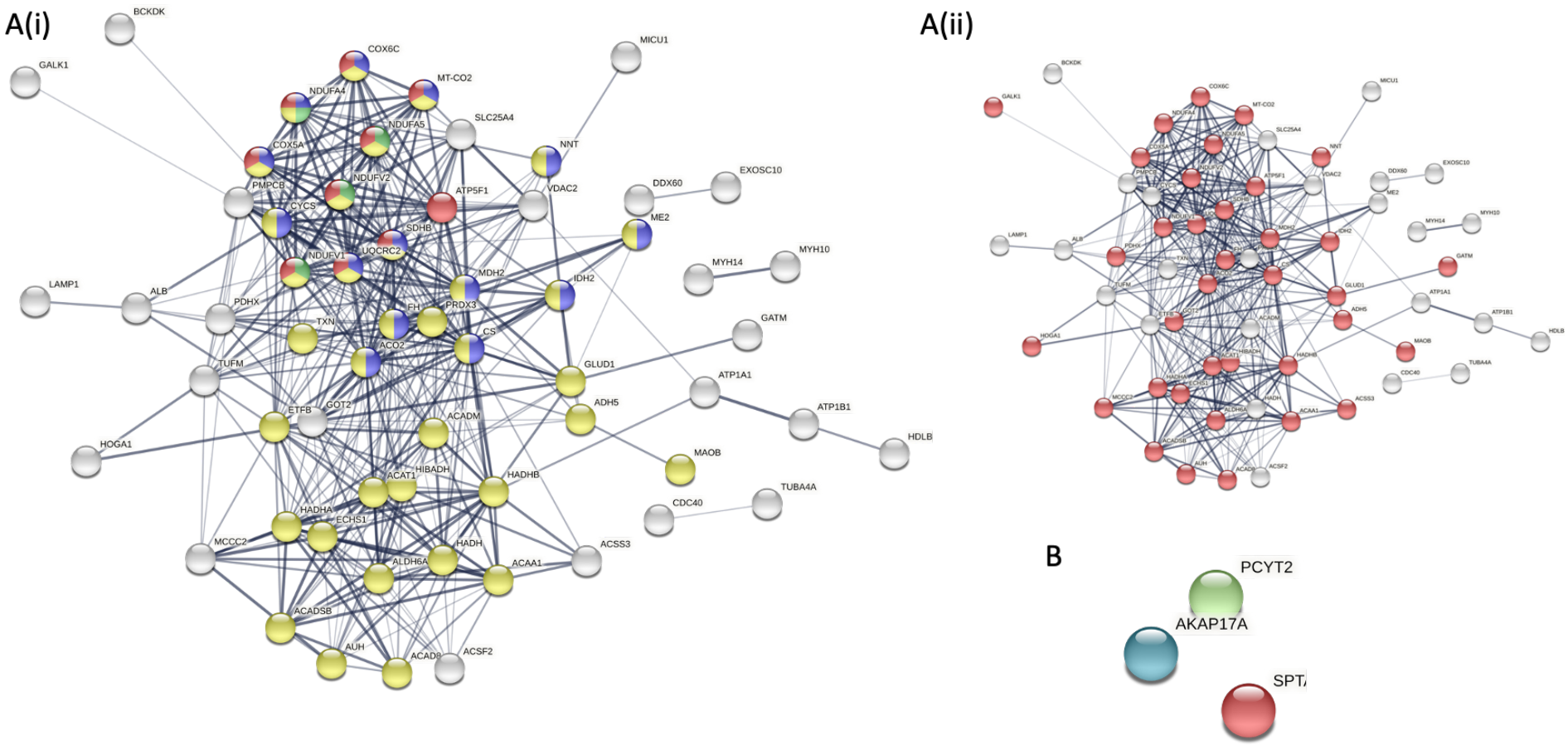


Figure 5.22 STRING interaction networks identified enriched pathways between the 77 downregulated proteins between LN and control.

STRING interaction networks were produced for the 80 significantly differentially expressed proteins between LN (n=23) and TBM (n=6) (medium confidence) in cohort two. (A) (i) and (ii) display the downregulated proteins (n=77) (PPI enrichment value $p<1\text{-}^{16}$). (A)(i) Red nodes – oxidative phosphorylation; blue nodes – aerobic respiration; yellow nodes – oxidation reduction process; green nodes – mitochondrial electron transport NADH to ubiquinone. Disconnected nodes hidden. (A)(ii) Red nodes – metabolic pathways. Disconnected nodes hidden. (B) displays the upregulated proteins (n=3) (PPI enrichment value p=1). No enriched pathways. PPI, protein-protein interaction; LN, lupus nephritis; TBM, thin basement membrane.

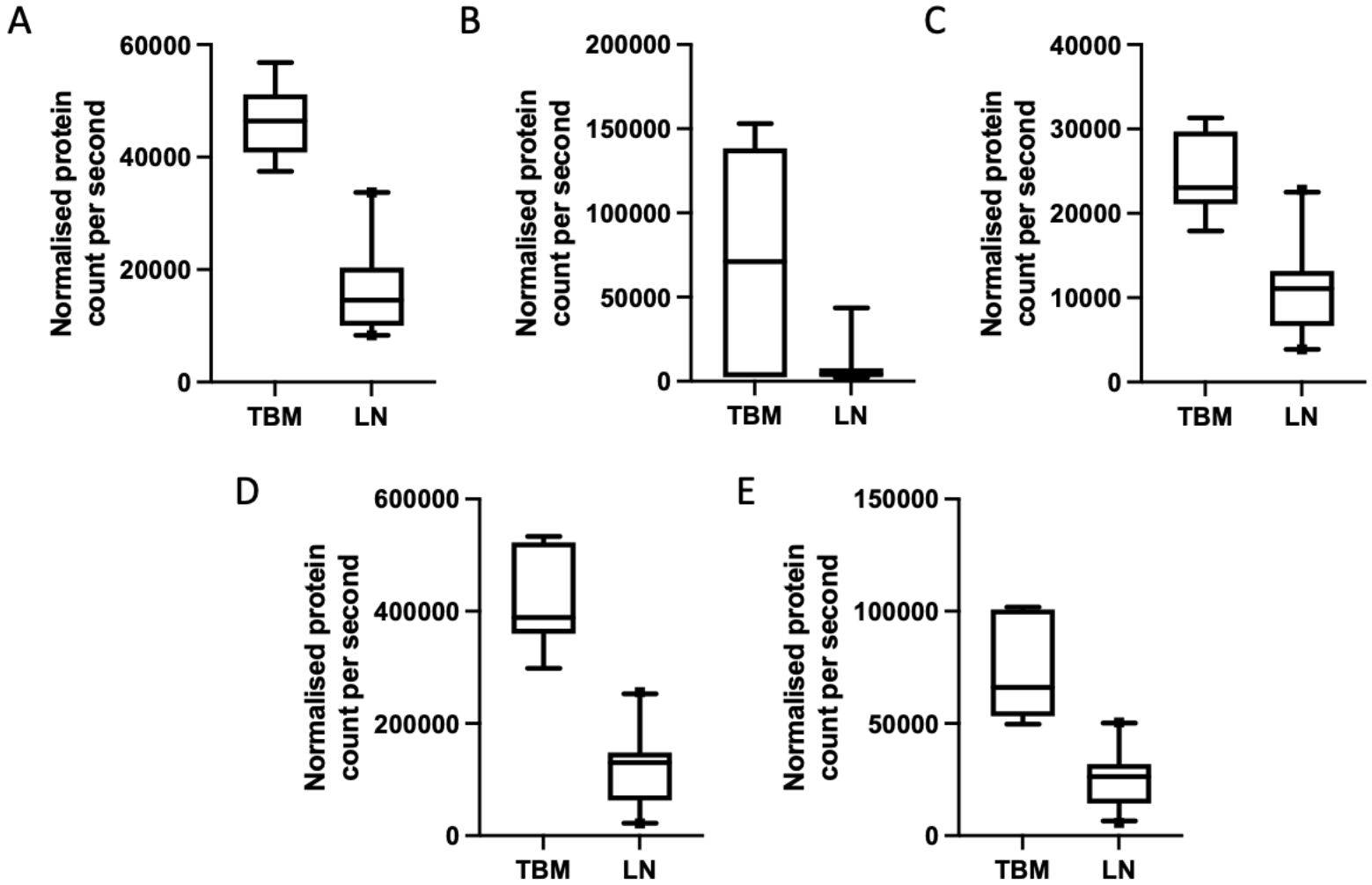


Figure 5.23 Box and whisker plots for listed proteins differentially expressed between LN and TBM in cohort two.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values. (A) NADH dehydrogenase [ubiquinone] flavoprotein 1. (B) Lysosome-associated membrane glycoprotein 1. (C) Adenosine triphosphate synthase F (0) complex subunit B mitochondrial. (D) Thioredoxin-dependent peroxide reductase mitochondrial. (E) NAD phosphate transhydrogenase mitochondrial.

5.3.3.3 Lupus Nephritis class analysis

In cohort one, it was demonstrated that more differentially expressed proteins were identified between LN and control when analysing proliferative and membranous LN separately indicating that mixing the disease processes could be hiding some of the differential expression of proteins. We therefore next went on to analyse proliferative and membranous LN separately. Initially the PCA analysis was labelled by class to identify if the classes of LN could be separated by this analysis. Interestingly, proliferative disease could be separated from membranous using PC2 vs PC3 and PC1 vs PC3 (figure 5.24 (B) and (C)). The control cases appear to cluster closer to the class III/IV disease.

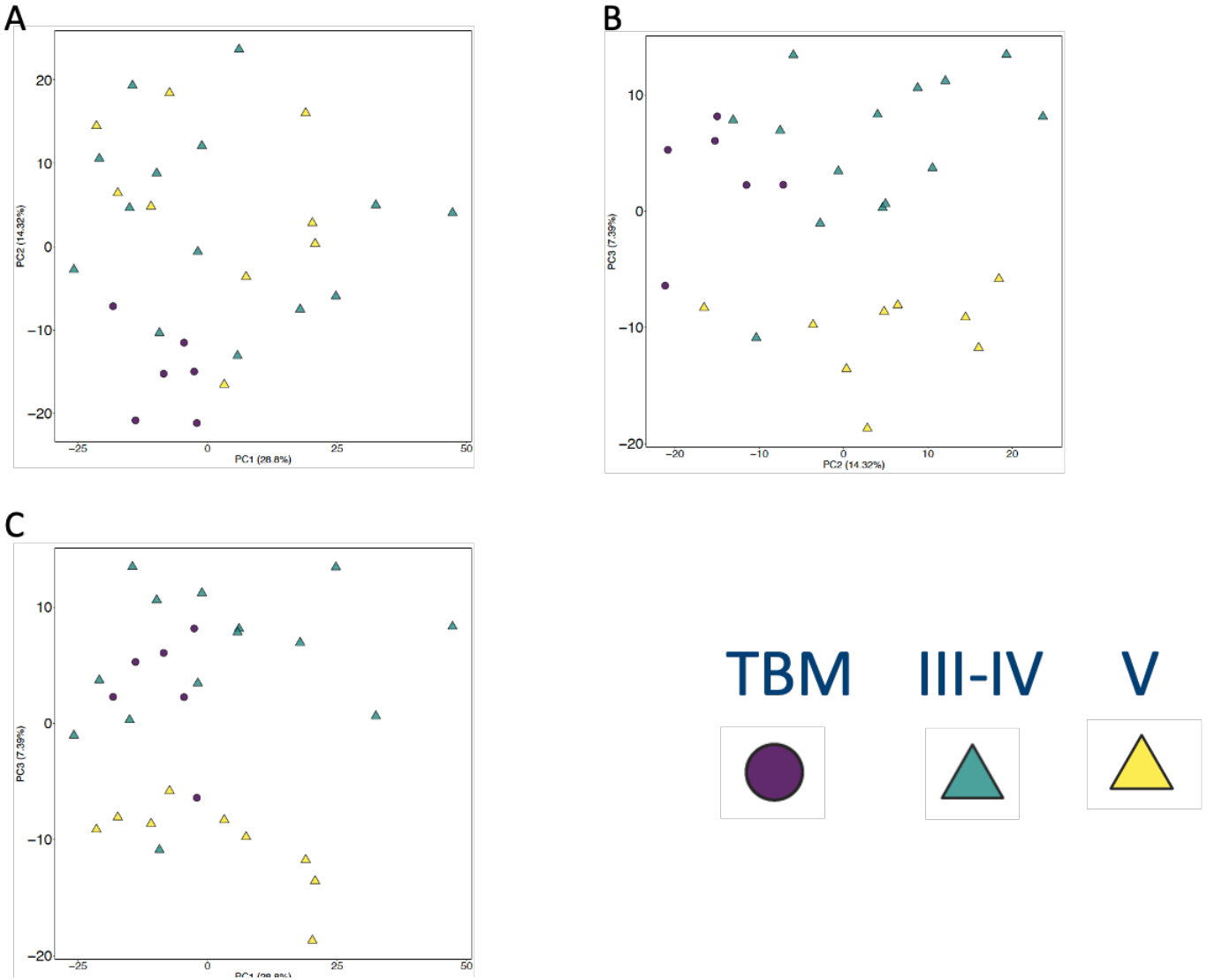


Figure 5.24 Principal component analysis of cohort two shows clear separation of the lupus nephritis and control samples as well as separation of the LN class III/IV from LN class V.

PCA of SWATH-MS data in cohort two data (n=29 biopsies) in three configurations of principal components (PC) 1, 2 and 3 with LN biopsies coded by class. (A) shows PC1 vs PC2, (B) shows PC2 vs PC3 and (C) shows PC1 vs PC3. Samples are each represented by triangles coloured green for LN class III/IV (n=14), triangles coloured yellow for LN class V (n=9) and dots coloured purple for control, (TBM) (n=6). The first three principal components explain the majority of the variance of the data. LN, lupus nephritis; TBM, thin basement membrane; PC, principal component.

To further explore the proteins and associated pathways contributing to this separation of data, a volcano plot was again employed plotting relative expression represented by q value vs fold change. When LN class III and IV were compared to control, 44 proteins were found to be significantly different with q<0.05 and log2 fold change ≤-1 or ≥1, 42 downregulated with a log2 fold change ≤-1 and 2 upregulated with a log2 fold change ≥1. The protein expression profile is shown in figure 5.25 and the links between the proteins demonstrated in figure 5.26. There were significantly more interactions than expected between the downregulated proteins ($p<1^{-16}$). Similar pathways were enriched to those identified in all LN vs TBM as shown in figure 5.22. This included the biological processes of the oxidation-reduction process ($q=6.68^{-16}$), aerobic respiration ($q=9.06^{-9}$), mitochondrial electron transport, NADH to ubiquinone (q=0.0016) and the KEGG processes of oxidative phosphorylation ($q=1.08^{-6}$) and metabolic pathways ($q=2.05^{-13}$)(figure 5.26 (A) and (B)).

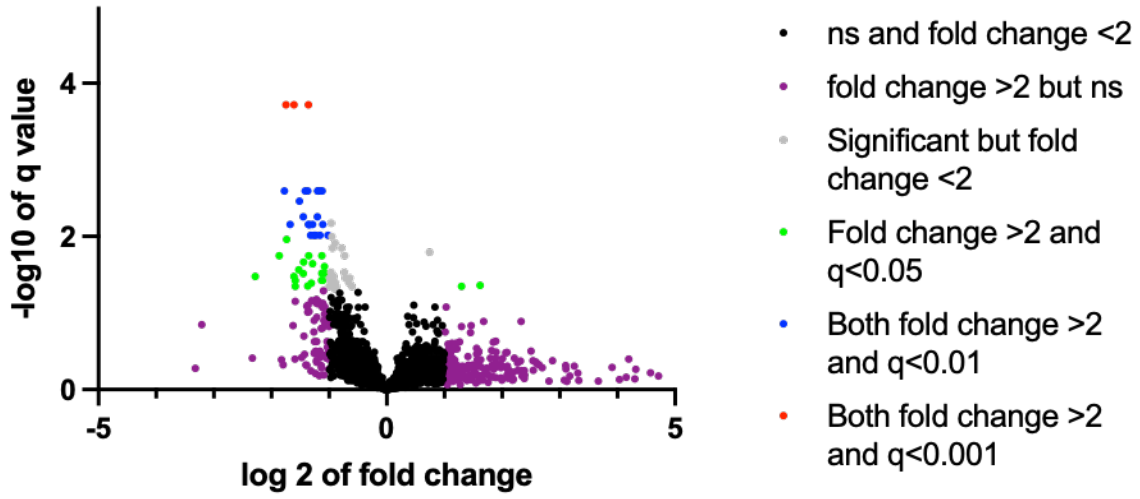


Figure 5.25 Volcano plot analysis identified 44 significantly differentially expressed proteins (42 downregulated and 2 upregulated) between LN class III/IV and control.

A volcano plot was generated using SWATH-MS data from cohort two, displaying differentially expressed proteins between LN class III/IV (n=14) and TBM (n=6) (control). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN class III/IV and control with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN class III/IV and control are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis; TBM, thin basement membrane.

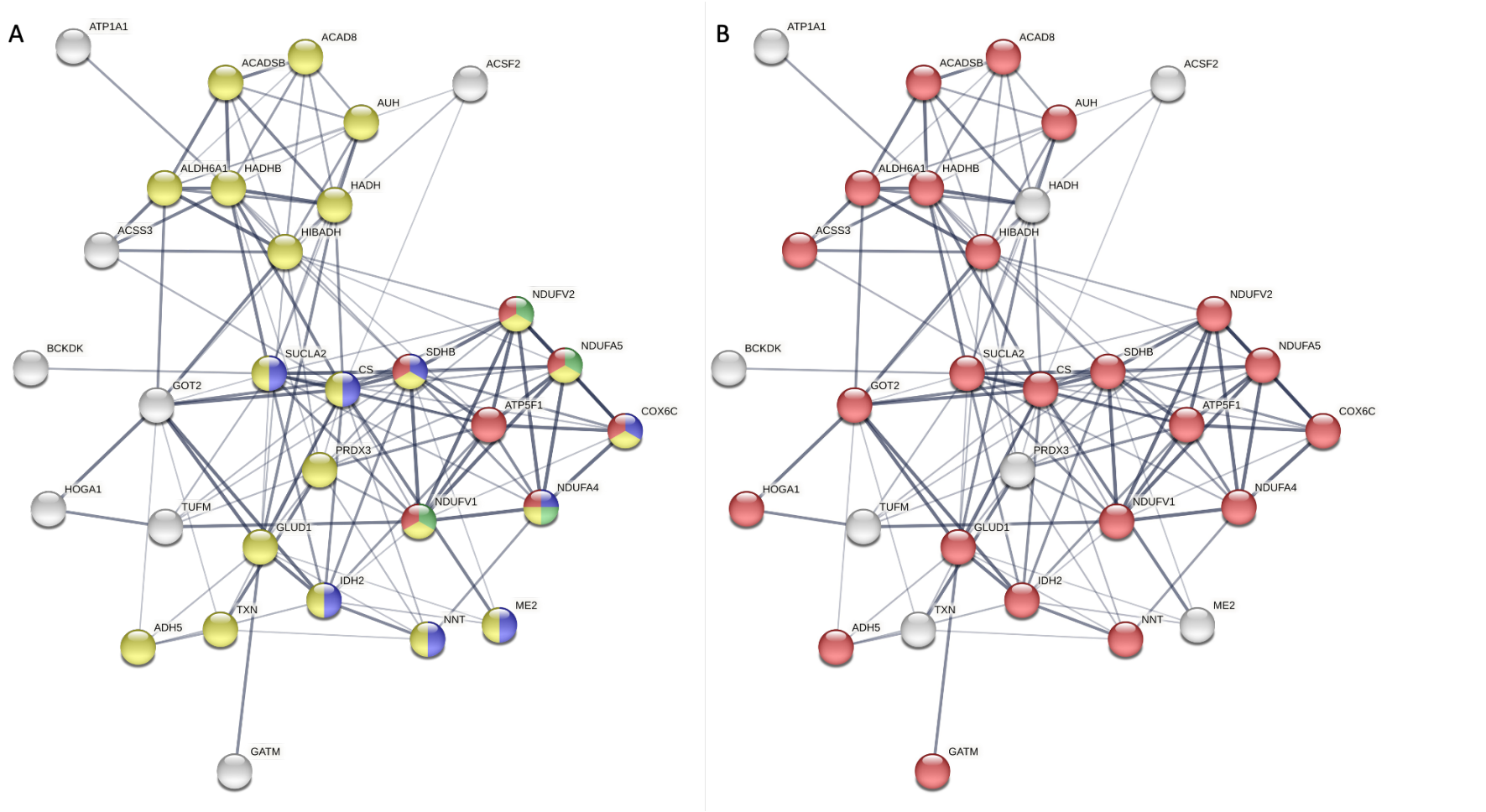


Figure 5.26 STRING interaction network identified enriched pathways between the 42 significantly downregulated proteins between LN class III/IV and control.

A STRING interaction network was produced for the 42 significant differentially expressed proteins between LN class III/IV (n=14) and TBM (n=6) (medium confidence) downregulated in cohort two. PPI enrichment value, $p<1^{-16}$. (A) Red nodes—oxidative phosphorylation; blue nodes—aerobic respiration; yellow nodes—oxidation-reduction process; green nodes—mitochondrial electron transport, NADH to ubiquinone. Disconnected nodes hidden. (B) Red nodes-metabolic pathways. Disconnected nodes hidden. PPI, protein-protein interaction; LN, lupus nephritis; TBM, thin basement membrane.

Looking at membranous LN, Class V disease had 142 significantly differentially expressed proteins compared to TBM, with q<0.05 and log2 fold change ≤-1 or ≥1. 115 downregulated with log2 fold change ≤1 and 27 upregulated with log2 fold change ≥1. The protein expression profile is shown in figure 5.27 and links between the proteins in figure 5.28. Interestingly, despite the number of proteins identified, the upregulated proteins did not show significantly more interactions than expected (p=0.549) and therefore nor significant enhancement of any particular pathways (no pathways with q<0.05). The downregulated proteins showed significantly more interactions than expected ($p<1^{-16}$) and again, a similar pattern of pathway enhancement to that identified overall in LN and in proliferative disease, however now with inclusion of enhancement of the ribosome KEGG pathway (q=0.0173). As previously, there was enrichment of the biological processes of the oxidation-reduction process ($q=1.69^{-20}$), aerobic respiration ($q=2.63^{-9}$), mitochondrial electron transport, NADH to ubiquinone (q=0.00021) and the KEGG processes of oxidative phosphorylation ($q=9.85^{-11}$) and metabolic pathways ($q=9.41^{-20}$)(figure 5.28)

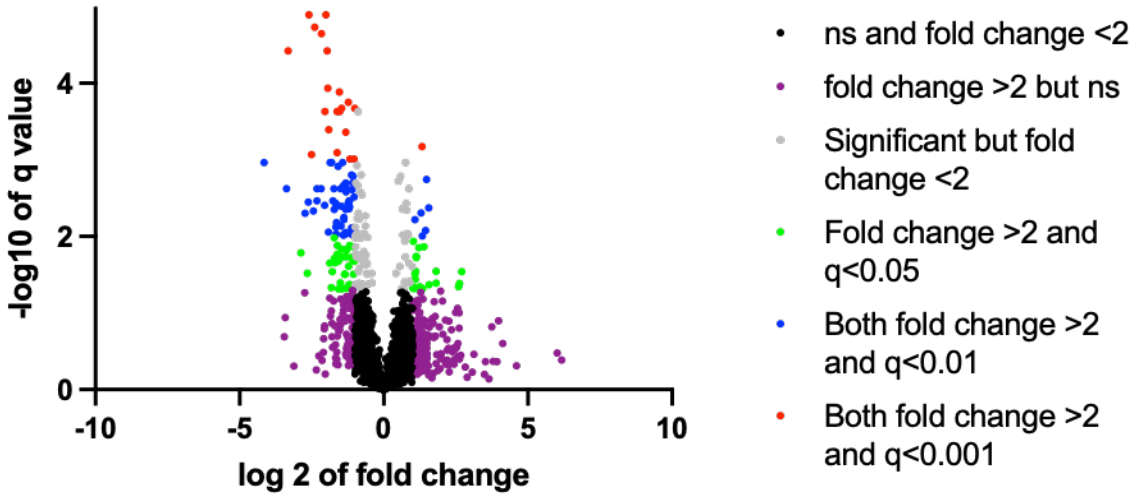


Figure 5.27 Volcano plot analysis identified 142 significantly differentially expressed proteins (115 downregulated and 27 upregulated) between LN class V and control.

A volcano plot was generated using SWATH-MS data from cohort two, displaying differentially expressed proteins between LN class V (n=9) and TBM (n=6) (control). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN class V and control with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN class V and control are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis; TBM, thin basement membrane.

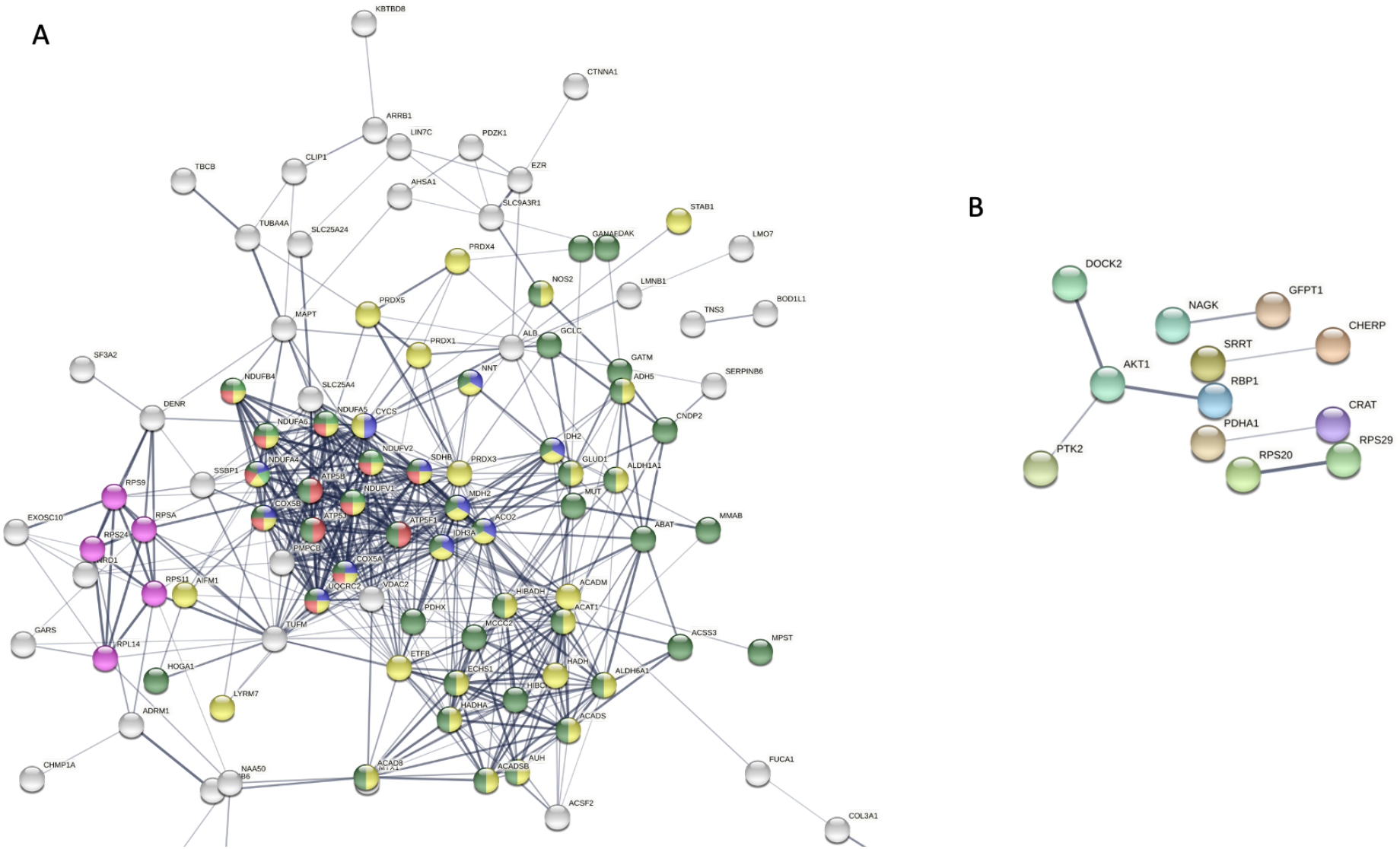


Figure 5.28 STRING interaction networks identified enriched pathways between the 115 significantly downregulated proteins between LN class V and control.

STRING interaction networks were produced for the 142 significant differentially expressed proteins between LN class V (n=9) and TBM (n=6) (medium confidence) (A) downregulated (n=115) and (B) upregulated (n=27) in cohort two. (A) Downregulated proteins. PPI enrichment value, $p<1^{-16}$. Red nodes—oxidative phosphorylation; blue nodes—aerobic respiration; yellow nodes—oxidation-reduction process; green nodes—mitochondrial electron transport, NADH to ubiquinone; pink nodes—ribosome pathway; olive nodes—metabolic pathways. Disconnected nodes hidden. (B) Upregulated proteins (PPI enrichment value, p=0.549). No enriched pathways. Disconnected nodes hidden. PPI, protein-protein interaction; LN, lupus nephritis; TBM, thin basement membrane.

Thus, analysing the classes separately has again appeared to identify more proteins significantly differentially expressed in LN compared to control. However, apart from the addition of the ribosome pathway in class V disease, the biological processes and pathways revealed by the differentially expressed proteins are the same, suggesting potentially a not so dissimilar disease process as expected in the classes.

To further evaluate the degree of similarity between the protein expression profile of each class of LN, a correlation comparison was made between the fold change difference in protein abundance in class III/IV LN vs TBM and class V LN vs TBM. Of the 3526 proteins identified in cohort two, 2942 proteins were identified in all LN class III/IV, LN class V and TBM (and thus had a fold change between each case and control comparison calculated). The correlation calculation identified a reasonable correlation coefficient of Spearman r = 0.6860 ($p<0.0001$). Figure 5.29 displays the correlation of the log2 fold change. This demonstrates that again many of the proteins are expressed differentially to control in a similar fashion in class III/IV LN and class V LN.

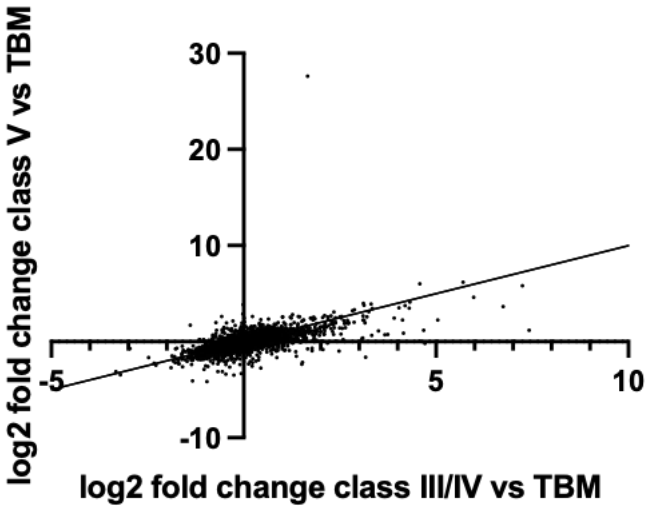


Figure 5.29 Proteins identified in LN class III/IV and class V relative to control correlate.

Correlation graph displaying the differential expression of proteins (n=2942) in LN class III/IV vs LN class V in cohort two. The log2 fold change of proteins identified in LN class III/IV (n=14) vs TBM (n=6) is displayed on the x axis compared to the log2 fold change of proteins identified in LN class V (n=9) vs TBM (n=6) displayed on the y axis. The graph displays a reasonable correlation coefficient of Spearman r = 0.6860 (p<0.0001). LN, lupus nephritis; TBM, thin basement membrane.

Finally, to further explore how the disease processes vary between Class III/IV LN and class V LN, a direct comparison was made between them (figure 5.30). This was again performed using a volcano plot of relative expression represented by q value vs fold change (figure 5.30), with significantly different expression defined by q<0.05 and log2 fold change ≤-1 or ≥1. Interestingly, only six proteins were differentially expressed with q<0.05 and log2 fold change ≥1 or ≤-1: five in higher levels in class V LN and one higher level in class III/IV. There were no significant (p=0.188 for those expressed at a higher level in class V) links between the differentially expressed proteins on String[270] analysis. Those expressed at higher levels in class V included tyrosine-protein kinase BTK (figure 5.31).

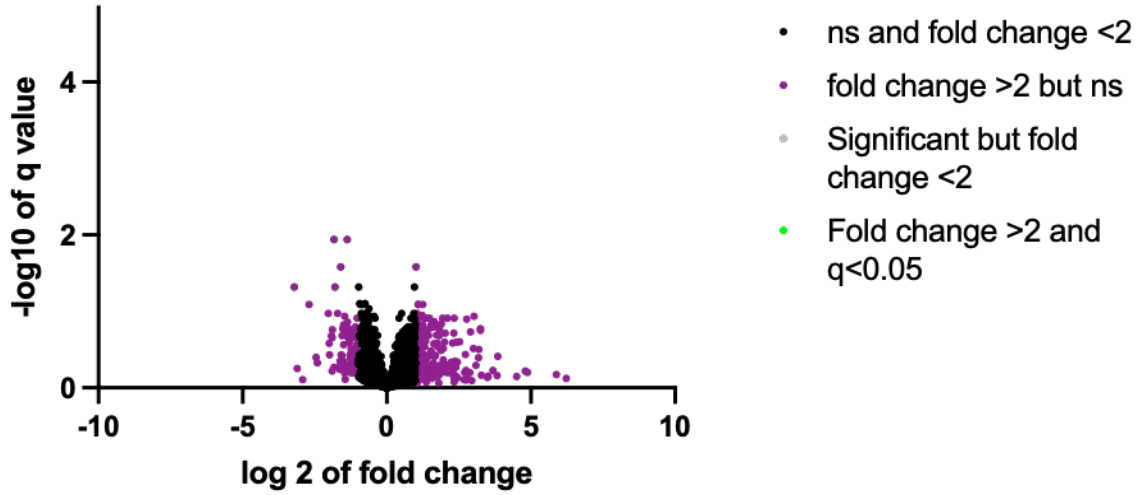


Figure 5.30 Volcano plot analysis identified 6 significantly differentially expressed proteins (5 expressed at a higher level in class V and 1 expressed at a higher level in class III/IV) between LN class III/IV and LN class V.

A volcano plot was generated using SWATH-MS data from cohort two, displaying differentially expressed proteins between LN class III/IV (n=14) and LN class V (n=9). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN class III/IV and LN class V with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN class III/IV and LN class V are coloured grey (q value significant but log2 fold change ≤1 and ≥-1), purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis.

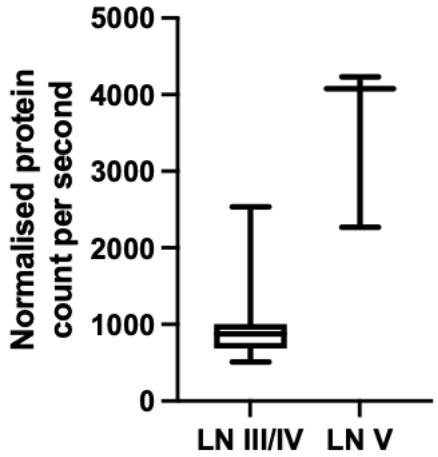


Figure 5.31 Box and whisker plot for tyrosine-protein kinase BTK, differentially expressed between LN III/IV and LN V in cohort two.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values.

5.3.3.5 Lupus nephritis responders versus non responders

Cohort two has identified several pathways enriched in LN compared to control. Class III/IV LN and class V LN indicated separation on PCA analysis and identified a greater number of significantly differentially expressed proteins between LN and control when analysed separately. However, on pathway analysis they were found to have similar disease processes responsible for the differential expression of proteins when each were compared to control. The small number of proteins differentially expressed on direct comparison had no significant links (p=0.188). It was next explored if differences in protein expression could be identified between LN responders and non-responders.

There was again no significant difference between the groups in regards to age, sex, ethnicity, reason for biopsy or treatment post biopsy. There were however, as previously, numerically more patients with first presentation of LN (as opposed to flare or persistent proteinuria) as biopsy indication in the CR groups compared to NR, however this was not statistically significant (table 5.3). Given the findings on the wider cohort of 599 biopsies described in chapter 3 that reason for biopsy was not predictive of response, this is likely again artefactual.

The original heat map analysis did not identify clear separation of the data by response to treatment (figure 5.19). Next therefore, the PCA analysis was visualised with biopsies categorised by response into CR and NR (figure 5.32). This also did not show separation of the data by these groups.

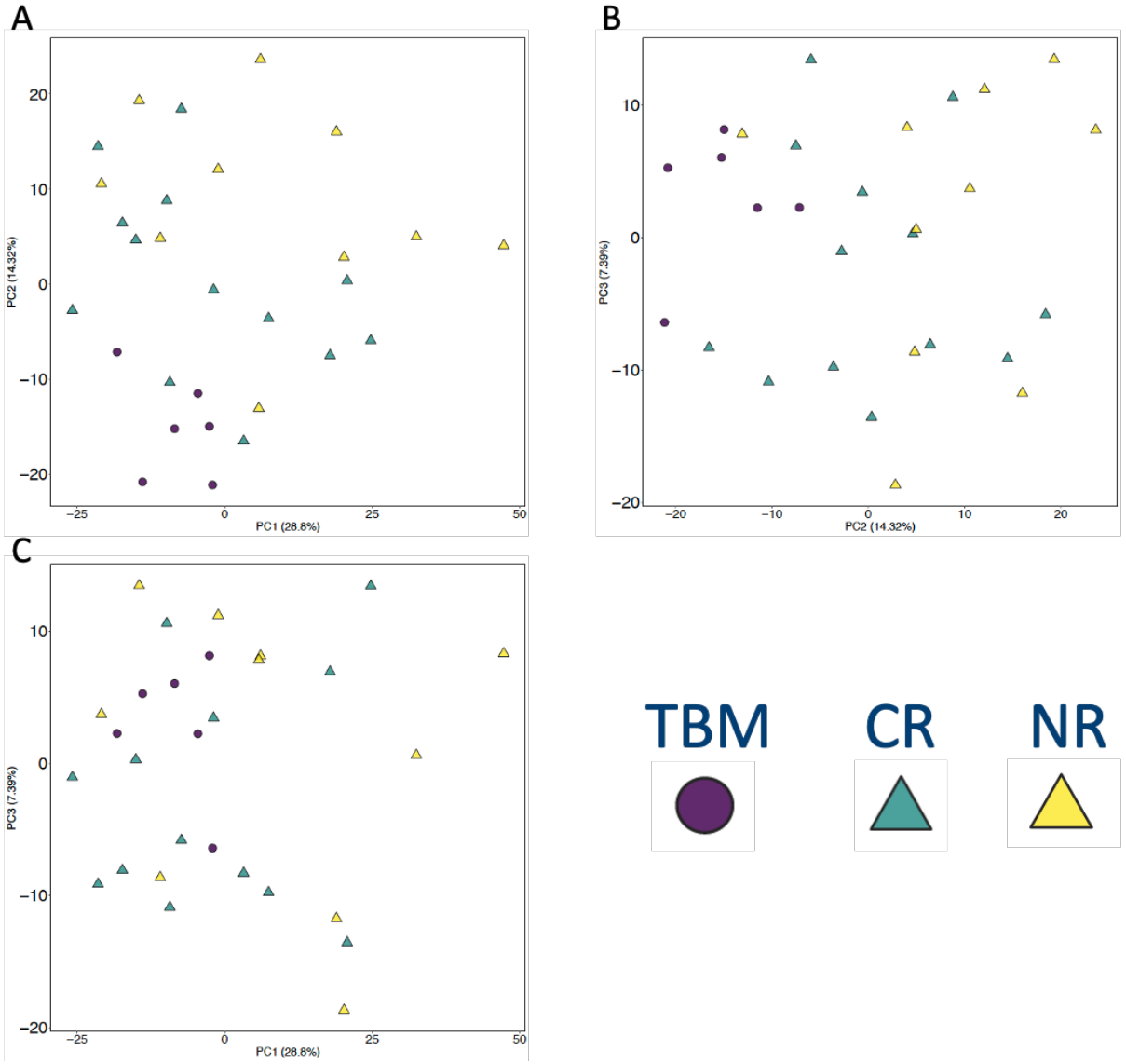


Figure 5.32 Principal component analysis of cohort two does not show clear separation of LN CR from LN NR.

PCA of cohort two data (n=29 biopsies) in three configurations of principal components (PC) 1, 2 and 3 with LN biopsies coded by response. (A) shows PC1 vs PC2, (B) shows PC2 vs PC3 and (C) shows PC1 vs PC3. Samples are each represented by triangles coloured green for LN CR (n=13), triangles coloured yellow for LN NR (n=10) and dots coloured purple for control, (TBM) (n=6). The first three principal components explain the majority of the variance of the data. LN, lupus nephritis; TBM, thin basement membrane; PC, principal component; CR, complete response; NR, non-response.

In order to examine more specifically any differences in protein abundance between CR and NR, a volcano plot was generated of relative expression represented by q value vs fold change (figure 5.33), with significantly different expression defined by q<0.05 and log2 fold change ≤-1 or ≥1. Due to the loss of so many class V NR cases in the initial stages of the SWATH-MS, there were again too few cases to examine each class of LN separately in the analysis of CR vs NR and so they were again examined altogether.

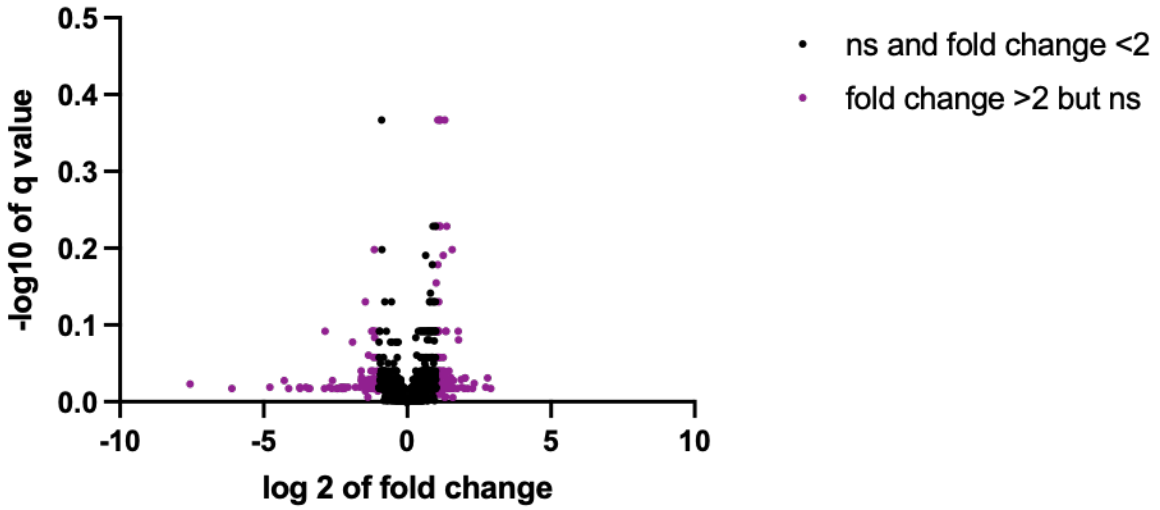


Figure 5.33 Volcano plot analysis identified no significantly differentially expressed proteins between LN CR and LN NR.

A volcano plot was generated using SWATH-MS data from cohort two, displaying differentially expressed proteins between LN CR (n=9) and LN NR (n=8). Relative expression is represented by log2 of fold change on the x axis and -log10 of q value on the y axis. q values are corrected p values using the Benjamini-Hochberg technique. Proteins were said to be significantly differentially expressed if log2 fold change was ≥1 or ≤-1 and q<0.05. Each protein is represented by a dot coloured according to the q value and fold change. Proteins which are significantly differentially expressed between LN class III/IV and control with log2 fold change ≥1 or ≤-1 are coloured green (q<0.05), blue (q<0.01) or red (q<0.001). Proteins not significantly differentially expressed between LN class III/IV and control are coloured purple (log2 fold change ≥1 or ≤-1 but q value not significant) or black (log2 fold change ≤1 and ≥-1 and q value not significant). LN, lupus nephritis; CR, complete response; NR, non-response.

There were again no statistically significant differences between responders and non-responders using the above criteria for significance (figure 5.33). With cohort one, at this point, machine learning was attempted as an alternative technique to identify if the proteome of the LN biopsy could be used to separate responders from non-responders. A panel of proteins was produced. However, the process of machine learning is a compromise between the size of the panel, the number of iterations the machine can attempt and the computer resources including time. Especially in the context of the heterogeneity of LN, the panel identified is likely to differ each time the programme is run. In the absence of specific proteins which could be used to predict response, clear demonstration of pathways could be very useful to indicate targets for new treatments. The size of the panel produced by machine learning did not allow for clear linkage of proteins into pathways. Therefore, with cohort two, this analysis was not repeated. Instead, with the acknowledgement that this is discovery analysis and therefore understood to be exploratory rather than conclusive, proteins which were differentially expressed between CR and NR with an uncorrected p value of <0.01 and log2 fold change $\leq$-1 or $\geq$1 were reviewed. This was again performed using t test and fold change analysis to calculate relative expression with significantly different expression defined by the aforementioned uncorrected p value and log2 fold change. It was accepted that this would likely include false positives. 19 proteins were identified to be differentially expressed p<0.01 and log2 fold change $\leq$-1 or $\geq$1 with 14 expressed at a higher level in CR compared to NR and 5 at a lower level in CR compared to NR.

Proteins differentially expressed with a p<0.01 (uncorrected) and their known links are displayed in Figure 5.34 in String interaction networks. This demonstrates that the proteins expressed at a higher level in CR compared to NR did have significantly more links than expected ($p=2.43^{-5}$) (figure 5.34 (A)). The proteins expressed at a higher level in NR compared to CR did not have significantly more links than expected (p=0.188) (figure 5.34 (B)). Enhanced pathways in proteins of a higher abundance in CR compared to NR are similar to those demonstrated in LN vs control. Pathways downregulated in LN are of greater abundance in responders compared to non-responders; the KEGG pathways of oxidative phosphorylation (q=0.0323) and metabolic pathways (q=0.033). The cellular component mitochondrion is also highlighted in figure 5.34 (A) (q=0.0023). Not shown on the figure but also enriched were the citric acid (TCA) cycle and respiratory electron transport (q=0.014) reactome pathway and the electron transport chain: OXPHOS system in mitochondria (q=0.0364) WikiPathway. This could be indicative of responders having a different metabolic state to non-responders.

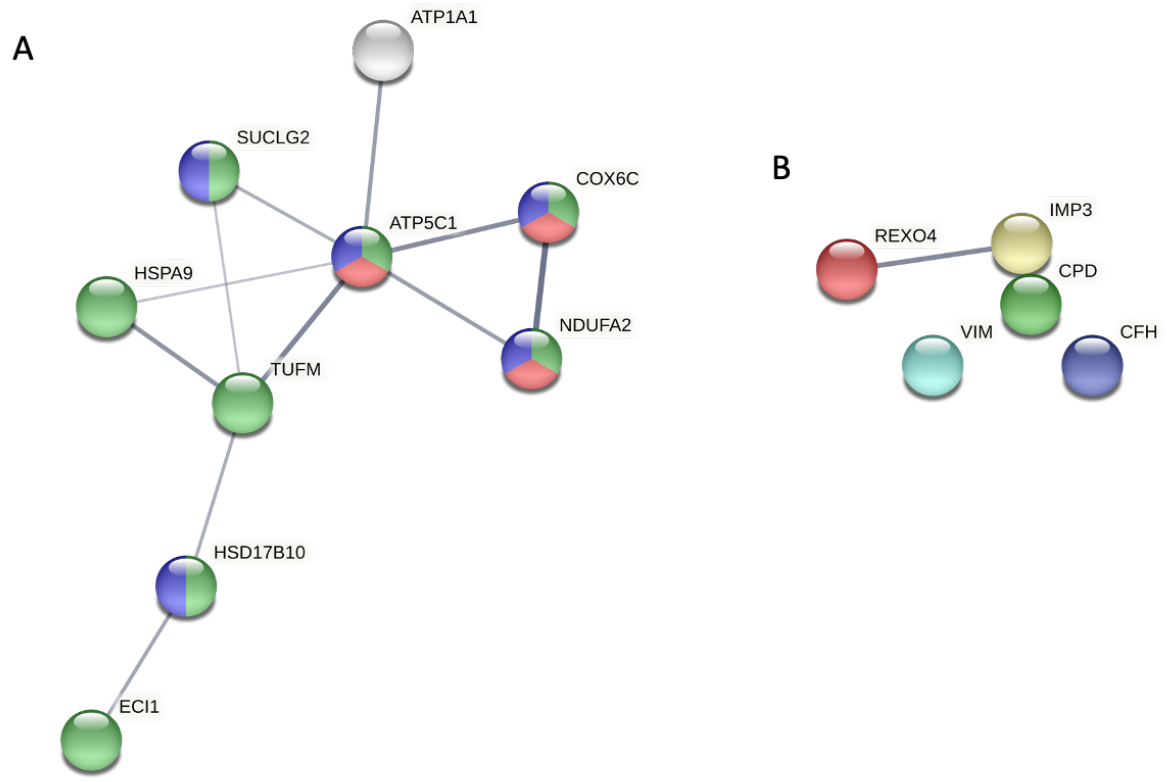


Figure 5.34 STRING interaction networks identified enriched pathways between the 14 proteins expressed at a higher level in LN CR compared to LN NR (uncorrected p<0.01).

STRING interaction networks were produced for the 19 differentially expressed proteins between LN CR (n=9) and LN NR (n=8) (medium confidence) with uncorrected p<0.01 and fold change ≤-1 or ≥1 (A) proteins expressed at a higher level in CR compared to NR (n=14) and (B) proteins expressed at a higher level in NR compared to CR (n=5) in cohort two. (A) Proteins expressed at a higher level in CR compared to NR. PPI enrichment value, $p=2.43^{-5}$. Red nodes—oxidative phosphorylation; blue nodes— metabolic pathways; green nodes—mitochondrial cellular components. Disconnected nodes hidden. (B) Proteins expressed at a higher level in NR compared to CR (PPI enrichment value, p=0.188). No enriched pathways. PPI, protein-protein interaction; LN, lupus nephritis; TBM, thin basement membrane; CR, complete response; NR, non-response.

5.3.3.6 Correlation of cohort one and two

Cohorts one and two have appeared to demonstrate quite different findings. Even on the initial overview of data where all proteins were displayed in a heat map for review, cohort one did not show separation of LN from control which had been expected following the preliminary data experiments. It was postulated that the cohorts could be different in protein identification or abundance or both. In order to further understand the degree of concordance in the findings of cohorts one and two they were directly compared. This was performed by directly comparing the overall cohorts in a correlation graph. 1931 proteins were identified in both datasets when analysing the proteins remaining after those with ≥80% zeros had been removed. This equates to 56% of the 3446 proteins identified in cohort one and 55% of the 3526 proteins identified in cohort two. Correlations were calculated between the mean abundance of proteins in the cases and controls and between the p values and fold change differences when comparing LN to TBM and LN CR to LN NR in each cohort. There were very poor correlations between protein abundance (figure 5.35 (A)i) and (A)ii)) in cohort one and two and between p values (figure 5.35 (B)i) and (B)ii)) and fold changes (figure 5.35 (C)i) and (C)ii)). Correlation coefficients (Spearman r) values ranged from -0.046 (CR vs NR uncorrected p value) to 0.255 (Mean abundance LN)(figure 5.35). This indicates that there was a difference in both identification of proteins and protein abundance between the cohorts and that the difference in abundance was not consistent within a cohort but varied between the proteins. Given that the data from cohort one was inconsistent with the remainder of the experiments, the cohorts were not combined for analysis.

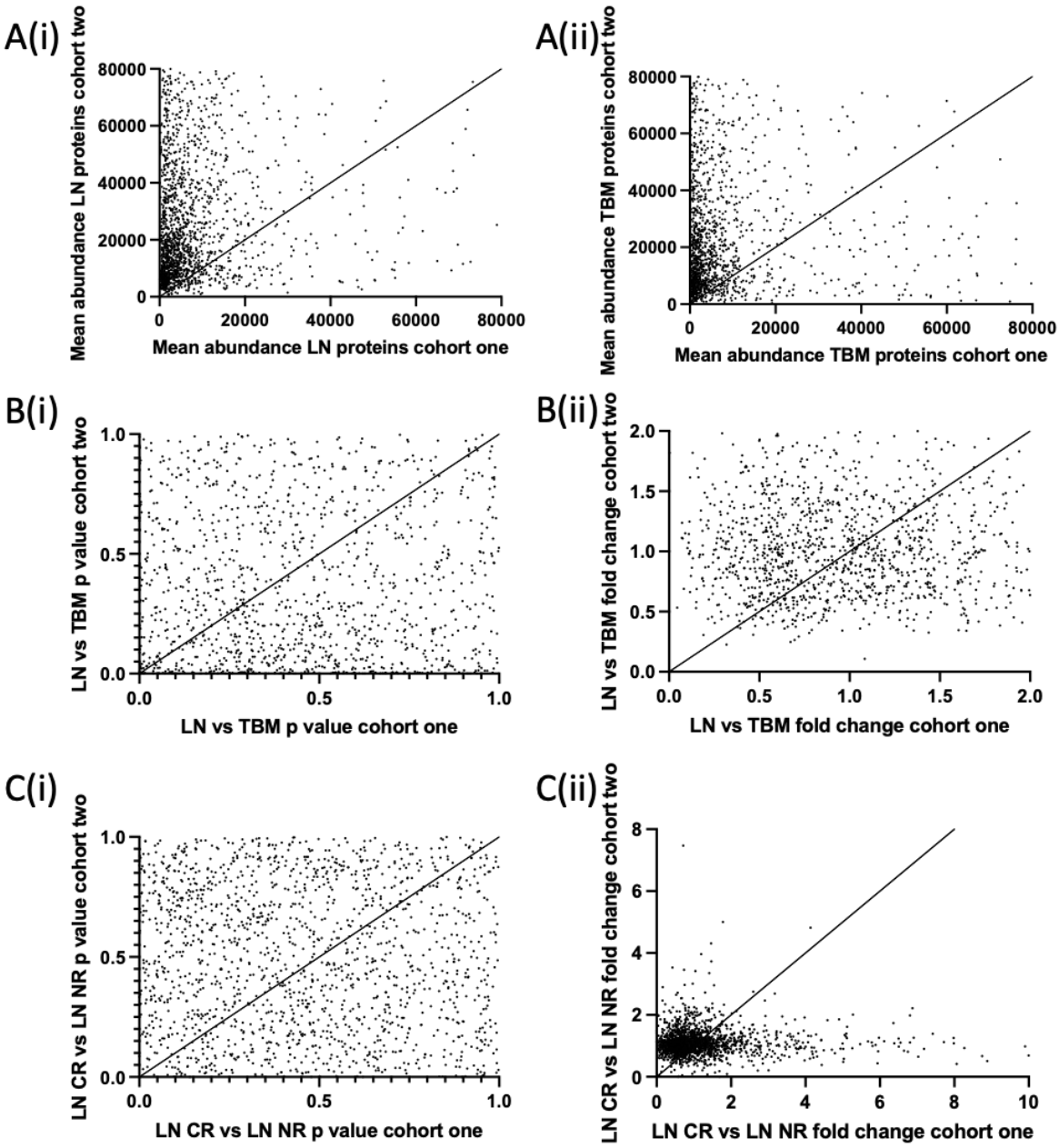


Figure 5.35 Proteins identified in cohort one and two did not correlate in regards to abundance or differential expression.

Correlation graphs comparing cohort one (n=24) and cohort two (n=29). 1931 proteins were identified in both datasets and included in these graphs. Correlation comparisons were made between (A)(i) Mean abundance of proteins in LN samples in cohort one (n=17) and cohort two (n=23), r=0.255; (A)(ii) Mean abundance of proteins in TBM samples in cohort one (n=7) and cohort two (n=6), r=0.199; (B)(i) LN (n=24) vs TBM (n=7) p value in cohort one compared with LN (n=29) vs TBM (n=6) p values in cohort two, r=0.022; (B)(ii) LN (n=24) vs TBM (n=7) fold change in cohort one compared with LN (n=29) vs TBM (n=6) fold change in cohort two, r=0.023; (C)(i) LN CR (n=9) vs LN NR (n=8) p value in cohort one compared with LN CR (n=13) vs LN NR (n=10) p value in cohort two, r=-0.046; (C)(ii) LN CR (n=9) vs LN NR (n=8) fold change in cohort one compared with LN CR (n=13) vs LN NR (n=10) fold change in cohort two, r=0.049. LN, lupus nephritis; TBM, thin basement membrane; CR, complete response; NR, non-response.

5.3.3.7 Immunohistochemistry

The results shown thus far demonstrate possible proteins and pathways which might be able to distinguish LN from control and suggests proteins and pathways for further exploration to establish if differences can be found in the baseline biopsy between future treatment responders and non-responders. The aims of this pursuit are threefold: firstly establishing if there is indeed a difference between responders and non-responders present at the time of the baseline biopsy; secondly, if so, developing a way to predict at the time of the biopsy if a patient is likely to respond to treatment; and thirdly, if differences were identified, using these to identify potential new treatment targets. In pursuit of the second aim, predicting likelihood of response at the individual level in clinic, immunohistochemistry was next explored as a possible technique which could realistically be employed at the time of biopsy, alongside routine stains. With the acknowledgement that no key single proteins were yet established as reliable or validated predictors of response, immunohistochemistry was performed to test the feasibility of the technique on possible predictive proteins and as a form of validation of some of the highlighted proteins. Sections cut at the time of block cutting of the first cohort were used following the analysis of this cohort. Proteins for staining were therefore selected from the first cohort which either had an uncorrected p value <0.05 when comparing CR to NR or were in the machine learning panel identified, distinguishing CR from NR where there was an available antibody. Suitable controls were identified from Protein Atlas.[273] Control specimens and active LN were stained with antibodies using the three previously described antigen retrieval methods, as per guidance from the manufacturer, to identify those with optimal staining which could be used on the LN samples to assess if the IHC would identify differences in staining intensity suggested by the proteomic findings.

MEK2/MAP2K2 (Anti-MEK2 antibody [OTI1A2] (ab140372)), MUM1 (Recombinant Anti-MUM1 antibody [EPR5653] (ab124691)) and Cofilin 1 (SAB2702206) were trialled with poor staining on the controls and therefore not pursued. BAT3/BAG-6 (ab88292), Moesin (ab52490) and PRKRIP1 (C134100) were trialled with suboptimal staining in LN. Cullin 2 (figure 5.36) and Proteosome subunit alpha type 6 (figure 5.38) provided reliable controls with staining in LN.

All biopsy cases with tissue remaining available for staining were included. Two cases were used for positive control in each stain. For Cullin 2, 27 samples were stained: 19 LN: 5 class III/IV CR, 5 class V CR, 5 class III/IV NR and 4 class V NR. 6 TBM and 2 human breast cancer were stained as controls. Median values of staining intensity and significant differences between CR and NR are listed in table 5.4. p values were calculated using unpaired t tests. For proteosome subunit alpha type 6, 26 samples were stained: 17 LN: 4 class III/IV CR, 5 class V CR, 4 class III/IV NR and 4 class V NR. 7 TBM

and 2 human colon were stained as controls. Median values of staining intensity and significant differences between CR and NR are listed in table 5.5. p values were calculated using unpaired t tests.

5.3.3.7.1 Cullin 2

Cullin 2 was present at a lower level in CR compared to NR (uncorrected p=0.013, q=0.676, log2 fold change = -4.428) in cohort one (figure 5.36). It demonstrated staining in the mesangium, capillary wall, tubules and tubule brush border. Median staining intensity in CR, NR and the TBM are listed in table 5.4. There was slightly more intense staining in the non-responders in the glomerular capillary wall and the mesangium. This reached statistical significance in the capillary wall staining intensity (p=0.047 unpaired t test). There was a similar intensity staining in the tubules and tubule brush border and overall. Figure 5.37 displays the staining in the capillary wall in complete responders and non-responders grouped into negative staining (score 0), mild staining (0.5-1) and intense staining (2-3), where the t test showed statistical significance. As displayed in figure 5.37, however, staining in the non-responders was very variable and it would not therefore be a reliable method of distinguishing CR from NR.

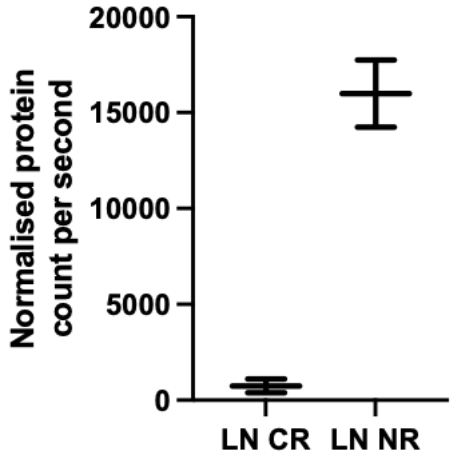


Figure 5.36 Box and whisker plot for cullin 2, expressed at a lower level in LN CR compared to LN NR in cohort one.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values.

Stain	Site of staining	LN CR	LN NR	TBM	CR vs NR p value
Cullin 2		n=10	n=9	n=6	
	Glomerular mesangium	1	2	1	0.167
	Glomerular capillary wall	0.75	1	0.75	**0.047***
	Tubules	2	2	2	0.825
	Tubule Brush Border	2	2	2	0.767
	Total	6.75	7.5	7.5	0.590

Table 5.4 Median staining intensity for Cullin 2 at different sites in the renal biopsy. LN CR (n=10), LN NR (n=9) and TBM (n=6) were compared.

T tests were used to compare staining intensities in the different parts of the renal biopsy. Staining was significantly different in the glomerular capillary wall where it was more intense in the non-responders (p=0.047). LN, lupus nephritis; TBM, thin basement membrane; CR, complete response; NR, non-response.

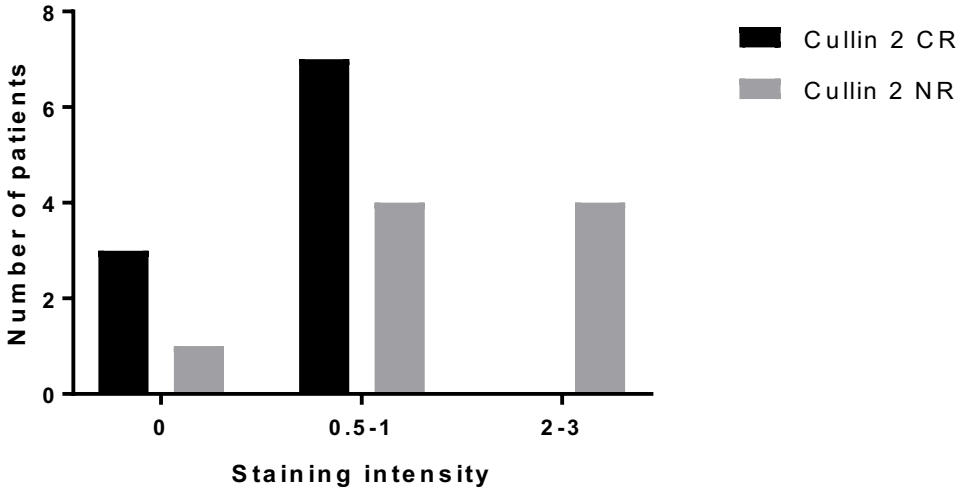


Figure 5.37 Bar chart demonstrating staining of cullin 2 in the glomerular capillary wall comparing LN CR to LN NR with variable staining intensity, especially in LN NR.

Bar chart demonstrating staining of cullin 2 in the glomerular capillary wall in cases from cohort one. LN CR (n=10) are shown with black bars and LN NR (n=9) are shown with grey bars. Staining intensity is grouped into negative (0), score 0.5-1 and score 2-3. LN, lupus nephritis; CR, complete response; NR, non-response.

5.3.3.7.2 Proteasome subunit alpha type 6

Proteasome subunit alpha type 6 was present at a lower level in CR vs NR (uncorrected p=0.005, q=0.564, log2 fold change = -1.746) in cohort one (figure 5.38). The antibody demonstrated staining in the glomerular mesangium and capillary wall as well as the tubules and tubule brush border. Median staining intensity in CR, NR and the TBM are listed in table 5.5. Staining was more intense in the non-responders in all areas. This was statistically significant using unpaired t tests in the mesangium (p=0.046), capillary wall (p=0.028) and overall total staining intensity (p=0.023) with all areas added together. This is displayed in figure 5.39 which shows the staining intensity, grouped into negative staining (score 0), score 0.5-1 and score 2-3, in the mesangium (A), capillary wall (B) and overall summative total (C), demonstrating the reduced staining in the CR and more intense staining in NR in all areas. Figure 5.40 shows an example of the staining in patients with CR (A)i) and ii) and NR (B)i) and ii).

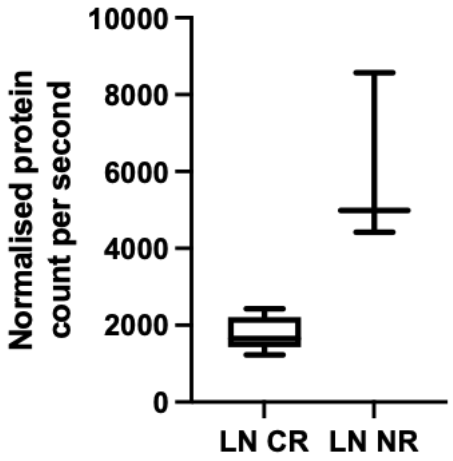


Figure 5.38 Box and whisker plot for proteosome subunit alpha type 6, expressed at a lower level in LN CR compared to LN NR in cohort one.

Line demonstrates median, box demonstrates inter-quartile range and whiskers represent minimum and maximum values.

Stain	Site of staining	LN CR	LN NR	TBM	CR vs NR p value
Proteasome subunit alpha type 6		n=9	n=8	n=7	
	Glomerular mesangium	0	0.5	0.25	**0.046***
	Glomerular capillary wall	1	1.5	0.5	**0.028***
	Tubules	0.75	1.5	0.5	0.122
	Tubule Brush Border	1	2	0.5	0.053
	Total	3	5.25	2	**0.023***

Table 5.5 Median staining intensity for Proteasome subunit alpha type 6 at different sites in the renal biopsy. LN CR (n=9), LN NR (n=8) and TBM (n=7) were compared.

T tests were used to compare staining intensities in the different parts of the renal biopsy. Staining was significantly different in the glomerular mesangium, (p=0.046); the glomerular capillary wall, (p=0.028) and overall total (p=0.023). Staining was more intense in the non-responders in these regions. LN, lupus nephritis; TBM, thin basement membrane; CR, complete response; NR, non-response.

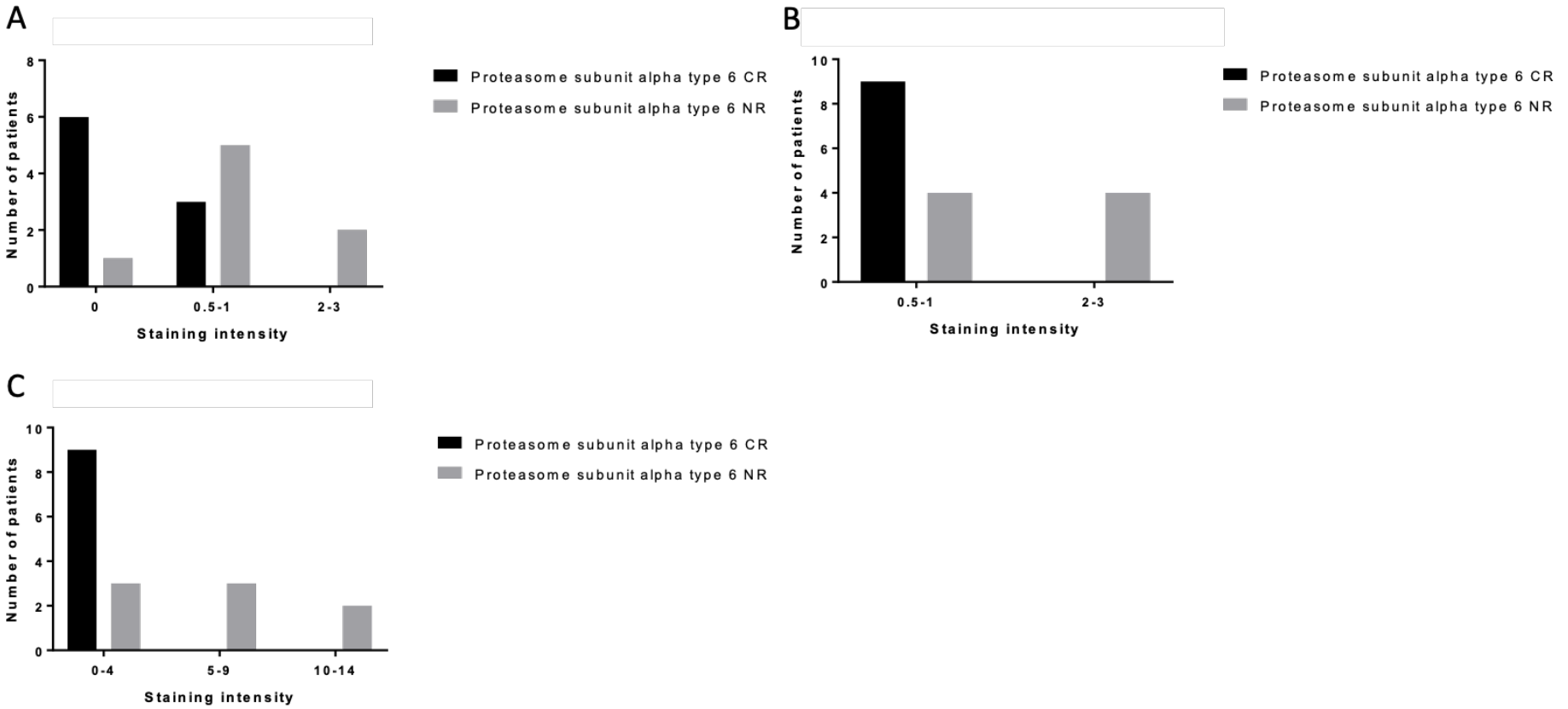


Figure 5.39 Bar charts demonstrating staining of Proteasome subunit alpha type 6 in LN CR compared to LN NR demonstrating more intense staining in LN NR.

Bar charts demonstrating staining of Proteasome subunit alpha type 6 in the (A) glomerular mesangium; (B) glomerular capillary wall and C) total summative staining in cases from cohort one. LN CR (n=9) are shown with black bars and LN NR (n=8) are shown with grey bars. Staining intensity is grouped into negative (0), score 0.5-1 and score 2-3. LN, lupus nephritis; CR, complete response; NR, non-response.

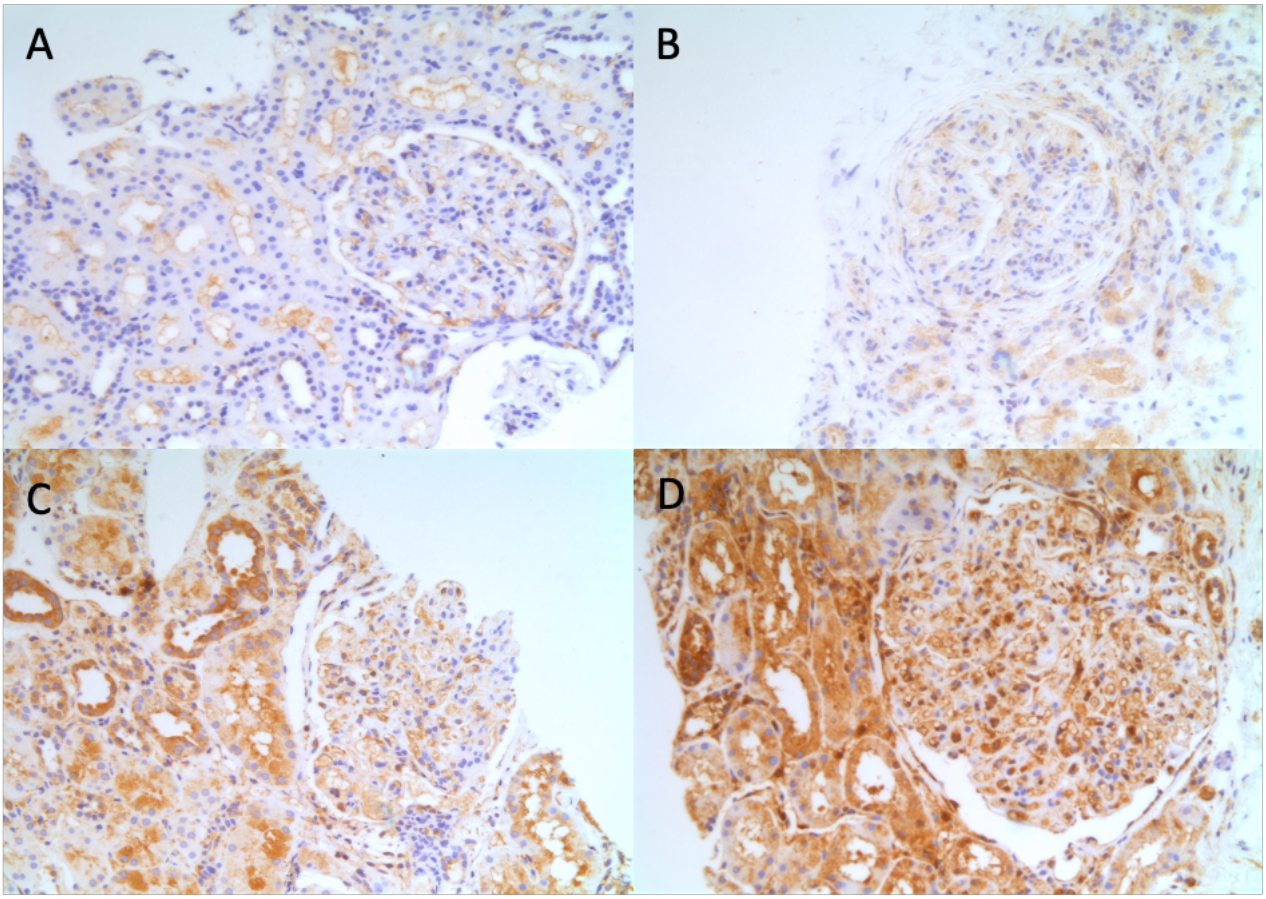


Figure 5.40 Renal biopsy immunostaining for proteasome subunit alpha type 6 demonstrating less intense staining in LN CR (A) and (B) compared to LN NR (C) and (D).

Representative images of proteasome subunit alpha type 6 staining in LN CR (A) and (B) and LN NR (C) and (D). Grade 0-1 staining is seen in LN CR and grade 2-3 staining is seen in LN NR. Original magnification x20. LN, lupus nephritis; CR, complete response; NR, non-response.

Figure 5.40 Renal biopsy immunostaining for proteasome subunit alpha type 6 demonstrating less intense staining in LN CR (A) and (B) compared to LN NR (C) and (D).

Representative images of proteasome subunit alpha type 6 staining in LN CR (A) and (B) and LN NR (C) and (D). Grade 0-1 staining is seen in LN CR and grade 2-3 staining is seen in LN NR. Original magnification x20. LN, lupus nephritis; CR, complete response; NR, non-response.

5.4 Discussion of results and future work

Protein yields from the FFPE blocks are extremely small (4-8µg protein). Despite this, it was demonstrated that this amount of protein can be successfully analysed by SWATH MS, identifying a large number of proteins, 5139 in cohort one and 4561 in cohort two. Using heat map analysis, PCA and differential expression as measured by t tests and fold change, LN was compared to control followed by the examination separately of proliferative and membranous LN. Finally, biopsies from patients who went on to respond to treatment were compared to biopsies of those who did not respond to treatment to explore if there were any differences in the protein expression profiles of these groups. LN was distinguished from control in cohort two with downregulation of metabolic pathways. Proliferative LN (class III and IV) and membranous LN (class V) were compared within each cohort and identified to have similar protein expression profiles. No significant differences were identified between responders and non-responders. There was however some evidence that may indicate that responders have a different metabolic activity to non-responders.

Accurate quantification of proteins was key to this work as it was specifically differential expression of proteins which was being assessed rather than the presence or absence of them. Recent work by Gao *et al.* [221] and Sun *et al.* [222] demonstrate the accuracy of quantification of proteins using SWATH-MS in FFPE tissue by comparing the technique with more established MS quantification techniques including MRM,[222] arguably the gold standard for pre-determined specific protein quantification in mass spectrometry. Protein identification and quantification underwent rigorous quality control. Preliminary data revealed separation of LN from control samples and the proteins identified by cohorts one and two were found to have some similarities to published data which will be reviewed. However, cohorts one and two did not provide similar results. The abundance of proteins, the fold change differences and p values did not correlate between cohorts. PCA analysis of cohort one could not separate LN from control which was unexpected. Both being human renal tissue, the most abundant proteins will be similarly expressed, in contrast to a comparison of cancer tissue to normal. However, PCA analysis is designed to draw out and demonstrate the largest differences that exist between the compared data and would therefore be expected to show these groupings. Indeed, in all the preliminary experiments and in cohort two, LN was distinguished from control by PCA analysis. Alaiya *et al*., despite analysing very small numbers of cases (5 class IV LN, 3HC, 4ANCA) with LC/MS/MS, reported that there was clear separation between the cases and controls with PCA.[167] It is interesting that more differences could be detected between class III/IV LN (arguably the more active disease process) vs TBM (38 differentially expressed proteins) in cohort

one, indicating perhaps that it is easier to see the differences when looking at the different disease processes separately. There were however no enriched pathways within the differentially expressed proteins as analysed by String software.[270] It is difficult therefore, for all these reasons, to draw any firm biological inferences from cohort one.

On initial review of the data, it was expected that certain proteins, such as C1q or TNF, which have previously been shown to be differentially expressed in LN vs TBM [171] would be present and show this differential expression. When cohort one did not show the separation expected between LN and control, these proteins were searched for individually. None were differentially expressed in LN vs TBM in either cohort. The validity of any research however is not based, of course, on its findings and the fact that the proteins expected to be differentially expressed were not found to be so, could be for many reasons. For some of the proteins, a missing value was reported for all controls and therefore a p value and fold change could not be determined. As has been discussed, this could be a true absence of the protein in the controls. Indeed, many of the inflammatory proteins could be expected to be of low abundance or absent in the controls. However, it is not possible to distinguish low abundance proteins, below the lower limit of detection, from values missing due to quality control issues. This issue of missing data will be discussed in greater depth, however it would introduce great bias to attempt to distinguish proteins of low abundance from unreported values on the grounds of what might be expected to be found. Indeed, in the complement staining described in chapter 4, C3c, CD68 and C5b-9 were unexpectedly identified in the negative disease controls. It was postulated that the presence of the complement in the mesangium and tubular basement membrane of the TBM controls could be related to complement activation on the surface of the tubular epithelial cells due to the proteinuria or related to acute tubular injury due to haematuria.

For some of the proteins where there might have been expected to be differential expression in LN compared to TBM, the proteins were identified in both the LN and control cases. It could have been that the study was not powered to find a significant difference. Additionally, the pathogenesis of LN is so heterogenous that it is difficult to say that the presence or absence of a specific protein is guaranteed in LN. There are known, for example, to be IFN high and IFN low groups of SLE patients [274] and low complement is not always seen in the serum of LN patients. In a review of biomarker studies using MS based proteomics in various human-derived specimens in LN, candidate biomarkers identified involved none of the 'expected' proteins searched for here.[232]

In the proteomics studies reported to date using LN renal tissue by Alaiya,[167] Sui [234] and Satoskar,[191] none of the listed proteins searched for here were identified except for complement which was identified (C1q, C3, C5, C9) in LN tissue by Satoskar alone.[191] There were similarities

identified between these data and that of these published studies. Satoskar identified downregulation of components of cellular metabolism in LN,[191] a key finding in the data presented here and also by Alaiya [167] and Sui.[234] Satoskar *et al*. did not specify which components of cellular metabolism were downregulated. However, they did specify that the anti-oxidant proteins, peroxiredoxins and glutathione S transferases were noted to be downregulated.[191] Treatment to counter glutathione depletion has been investigated as a possible treatment in SLE with mouse and human studies summarised by Perl.[275] In our data, mitochondrial glutamate dehydrogenase and a peroxidase were identified to be downregulated in LN compared to TBM in cohort two. Alaiya at al reported 28 differentially expressed proteins identified by MALDI-TOF MS distinguishing between HC, LN and ANCA tissue. They reported many to be "directly or indirectly associated with pro-inflammatory factors and regulators such as interlukin-1, TGF-β and NFkB".[167] The differentially expressed proteins identified by Alaiya *et al.* included moesin, which was also identified in our data in the machine learning panel differentiating CR from NR in cohort one. Alaiya's data also identified ATP synthase alpha and glutamate dehydrogenase, which, along with multiple ATP synthase subunits were differentially expressed in our data also, when LN was compared to TBM in cohort two. Sui *et al.* identified 18 differentially expressed proteins (uncorrected p value <0.05 and FC >1.5) between LN and HC in their proteomics study of renal tissue. Several proteins with oxidoreductase activity were identified to be downregulated in LN compared to TBM.[234] In a recent SWATH-MS study examining urine of HC, SLE and LN patients, the differentially expressed proteins (corrected p<0.05 and log2 fold change >1) were not all individually listed.[227] Of the listed proteins, none were differentially expressed in these data between TBM and LN although all were identified and the protein identified as the most promising biomarker for early LN detection by Kwon *et al*., alpha-1-acid glycoprotein (ORM1),[227] was identified in our data with a significant FC but not q value between LN and TBM. The difference identified in our data therefore did not reach statistical significance. In a review of biomarker studies in SLE identified using MS based proteomics in various human-derived specimens,[232] one of the most commonly postulated as a biomarker differentiating SLE from control was annexin A5, useful in detecting thrombophilia and LN. It was identified in our study however not differentially expressed between LN and control.

Transcriptomics work comparing LN to TBM has demonstrated 52 significantly differentially expressed genes common to all LN classes with enrichment for type 1 IFN, complement and MHC II pathways.[170] A separate group using the same NanoString nCounter platform comparing LN to normal kidney from pre-implantation biopsies of living donor kidneys identified some of the inflammatory genes such as C1QB and STAT1 expected to be upregulated in LN using uncorrected p

values in both their first study[168] and second,[171] however taking $p<0.01$ to be significant.[168,171] A Bonferroni correction was used in the first study for comparison of clinical features between the groups.[168] In our study, some genes related to inflammatory pathways were identified such as C1q and C5 however they were not differentially expressed between cases and controls. Transcriptomic and proteomic results are often poorly correlated.[276] Multiple studies have more recently integrated the results of both to further understand the biological mechanisms of study.[276][277] A poor overall correlation is common, as demonstrated by a recent study in the Human Protein Atlas project.[278] It is increasingly recognised however that transcriptomics and proteomics provide different, complementary information. Whilst transcriptomics represents gene expression, protein abundance is also affected by post translational regulation and modifications. The detection of more mRNA doesn't necessarily mean more protein production; equally the presence of a protein does not necessarily mean the protein is active, was produced recently or indicate what triggered the production of said protein.[279] Integrating the proteomics work with transcriptomics on FFPE renal biopsies, in particular using data where the proteomics and transcriptomics have been performed on the same samples will form a part of the future work of this project.

In our study, CR and NR did not separate on PCA analysis or heat map visualisation and there were no differentially expressed proteins between the groups in either cohort. Other groups have explored the comparison of CR vs NR in LN by looking at gene transcript expression using the NanoString ncounter platform.[168,171] In their first paper, Parikh *et al.*'s data suggested greater expression of interferon pathway genes in CR and complement genes in those who did not respond to treatment.[168] The same team went on to report that type I and II interferon pathways, the alternative complement pathway and T cell signalling pathways can discriminate CR from NR using the same technology.[171] These data however describe 4 NR samples compared to 5 CR (vs normal). These groups consisted of 3/5 of the CR class IV and all of the 4 NR class IV. Gilmore *et al*. using a similar panel on the same platform with much larger numbers did not produce the same findings (using TBM as a control).[170] Gilmore *et al.* did however see differences between the classes.[170] Given the split of classes between Parikh's CR and NR patients, the gene expression differences could be due to the weight of more class IV in one group than the other. Additionally, the differences described above were identified in the first study from principal component 1, that is the genes were not actually statistically differentially expressed between CR and NR but were key contributors to the separation seen on PC analysis. It is difficult to take biological significance from genes which were not identified to be significantly differentially expressed. Five transcripts were differentially expressed between CR and NR in this first study by Parikh *et al*.: Membrane

metalloendopeptidase (MME), FADD, CD274/PD-L1 (p=0.0002), Complement component C1S and integrin beta-2 (ITGB2) (p=0.0007). The latter two were also identified in cohort two; however, they were not significantly differentially expressed. In the second study by Parikh *et al*., a direct comparison of CR and NR was not performed, instead the team looked at CR vs control and NR vs control where differential expression of the pathways described was actually seen between LN and control. Arguably, a more appropriate comparison between CR and NR would be a direct comparison of the two rather than each to control as the latter which was performed does not inform if the differences reported are seen between CR and NR.

Whilst it is hard to make any biological inferences from the four proteins differentially expressed in LN vs control in cohort one, they do all have biological plausibility for being relevant to LN. Of note, none were differentially expressed between LN and control in cohort two. TGFBRAP1 is part of the transforming growth factor-beta (TGF-beta) 'superfamily' which binds to TGF-beta receptors (TGFBR), stimulating TGF-beta signalling.[280] A role in the pathogenesis of SLE has been postulated due to findings of reduced TGFBR1 in newly diagnosed SLE patients.[281] This study found that in vitro, Tofacitinib, the JAK inhibitor, upregulated TGFBR1 expression, thus reducing T cell activation and in MRL/lpr mice, decreased proteinuria. Thus providing evidence for a possible role for Tofactinib in treating SLE patients.[281] Phosphofurin Acidic Cluster Sorting Protein 2 (PACS2) is a protein involved in endoplasmic reticulum (ER) calcium ion homeostasis.[282] It is a mitochondria-associated ER membrane (MAM) resident protein and regulatory protein. The MAM controls mitochondrial physiology and apoptosis.[283] CTTNBP2 N-terminal like protein is a target of signal transducer and activator of transcription 4 (STAT4), a transcription factor known to be involved in IL12 responses and T helper cell differentiation.[284] This protein was the only one identified to be significantly differentially expressed in Class V LN compared to TBM in cohort one. The final protein identified to be differentially expressed in LN compared to control in cohort one was NT5E. This protein has been identified to produce adenosine by dephosphorylation of adenine nucleotides.[285] Adenosine acts as an immune modulator. Lack of NT5E in mice studies has been shown to be linked with autoimmune inflammation, proteinuria, reduction in renal function, glomerulitis and increased IgG and C3 deposition.[286]

All three proteins expressed in cohort one at a higher level in class V LN compared to class III/IV LN: serine-protein kinase ATM (a DNA damage sensor)[271], E3 ubiquitin-protein ligase RNF114 and serine/threonine-protein phosphatase 4 catalytic subunit (Ppp4), have been linked to processes involved in the pathogenesis of SLE. RNF114 acts as a negative regulator of NF-κB dependent transcription and modulates T cell activation and apoptosis and Ppp4 which regulates NF-κB and

mTOR pathways playing a role in the 'DNA damage response'[287]. It is difficult to infer from these proteins alone though, any different pathogenesis of Class V LN to Class III/IV LN.

A panel of 8 proteins was identified by machine learning which separated CR from NR as demonstrated on the PCA. As touched on in the results, machine learning is an alternative tool with which to examine big data. The output can differentiate groups based on a pattern of expression in a panel of proteins and explores all proteins for this pattern, not only those significantly differentially expressed between the groups. All proteins in the panel were present at lower levels in CR, many related to inflammation, apoptosis and tumour suppression or progression. None however were significantly differentially expressed and so little can be taken from the exact proteins in the panel.

Cohort two had 80 differentially expressed proteins between LN and TBM which could be separated on PCA analysis. 9 samples were lost to analysis due to technical issues; however, with a greater number of samples to begin with, power for comparisons was greater. More of the 'expected' proteins were detected however none of these were significantly different comparing LN to control. Reasons for this have already been postulated. It is therefore probably more feasible to attempt to draw biological reasonings from the cohort two data than from cohort one.

Of the 80 proteins differentially expressed between LN and TBM, the vast majority were downregulated in LN compared to TBM, likely in part due to the missing values, discussed later in this section. In brief, with fewer control samples than LN, it was more likely that all control samples would have a missing value than all LN samples. Differential expression cannot be calculated with a missing value for one category and thus proteins of an abundance below the lower limit of detection in control could not be compared to LN. Many of the downregulated proteins were mitochondrial proteins. Mitochondria and their dysfunction have been an interesting recent area of focus in study of the pathogenesis of SLE including as an area to target for new treatments.

The downregulation of mitochondrial proteins was in concordance with findings of a recent study which showed 'downregulation of mitochondria-derived genes and mitochondria-associated metabolic pathways' in transcriptomic analysis of CD4+ and CD8+ T cells from a group of SLE patients with LN who had enrichment of type-I IFN expression.[274] One of the downregulated proteins was NADH dehydrogenase [ubiquinone] flavoprotein 1, the first enzyme of the mitochondrial electron transport chain. Impaired function of this chain leads to increased oxidative stress.[288] NADH dehydrogenase oxidises NADH and reduces ubiquinone. Doherty *et al.* have previously reported increased NADH dehydrogenase activity in the electron transport chain at the internal mitochondrial membrane of peripheral blood lymphocytes from SLE patients.[289] Also downregulated were ATP synthase F(0) complex subunit B1, involved in metabolic and catalytic activity as well as proteins

involved in antioxidant functions (Thioredoxin-dependent peroxide reductase) and free-radical detoxification (NAD(P) transhydrogenase). It is possible that this represents a lack of anti-oxidant activity, which could leave the glomeruli vulnerable to oxidative stress and injury. Alternatively, it could represent overconsumption. Mitochondria of abnormal morphology and size, associated with impaired function by way of ATP synthesis, have been identified in SLE patients. The removal of free radicals has been shown to allow mitochondria which have developed abnormalities in their structure and size to return to normal.[288] Downregulation of ATP synthases were also identified in Alaiya's data and in a DNA microarray study of peripheral blood of SLE patients vs HC.[290] ATP synthases make ATP at the mitochondrial inner membrane.[291] As the key source of energy for metabolic pathways in cells, ATP and the electron transport chain are fundamental to intracellular processes. SLE patients show a reduction in ATP production in addition to abnormalities of mitochondria and membrane potential disruption.[288]

Oxidative stress has been shown to lead to the formation of macropores in the mitochondrial outer membrane (MOM) formed by voltage-dependent anion channel (VDAC) oligomers. mtDNA has been shown to be released through these macropores inducing type-1 IFNs. This mtDNA release has been shown to be reduced in MpJ-Fas^{lpr} "lupus"-prone mice by the use of a VDAC oligomerisation inhibitor. It was also shown to reduce lupus renal IC deposition and proteinuria in the mice.[292]

Glutamate dehydrogenase downregulation in LN was identified in both cohort two and the proteomics study by Alaiya *et al*..[167] It catalyses the conversion of glutamate to α-ketoglutarate. This is one reaction in the chain of glutaminolysis which is necessary for Th17 cell differentiation.[293] Th17 cells are involved in pathogenesis of autoimmunity via production of IL-17 [294] and the target of a drug, secukinumab, currently being evaluated in a phase 3 randomised control trial in LN.[64]

As noted, our data are consistent with those of the recently published study demonstrating downregulation of mitochondrial proteins and associated pathways in CD4+ and CD8+ T cells from patients with LN [274] and in a further study of peripheral blood leukocyte DNA in SLE patients.[295] Leucocytes taken from SLE patients were shown to have increased damage of mtDNA and decreased mtDNA and mtRNA expression with those with more damage also showing greater risk of LN.[295] It is interesting therefore that LN was associated with a downregulation of these pathways in our work. The downregulation of these pathways in LN could in theory be under-production or over-consumption or another form of removal. Paradoxically, other studies have shown increased mitochondrial oxidative metabolism in SLE, such as in SLE CD4+ T cells with an enhancement of mitochondrial respiration associated with the pathogenesis of SLE.[296] Additionally, increased

mitochondrial metabolism and glycolysis were found to correlate with CD4+ T cells from SLE patients and from lupus prone B6.Sle1.Sle2.Sle3 (TC) mice.[297] These paradoxical findings could therefore indicate that the finding of downregulation of the pathways in ours and others' studies might be most likely to reflect over-consumption rather than under-production.

It is furthermore interesting that in the analysis of CR vs NR in cohort two, using uncorrected p values <0.01, there was different expression of proteins involved in these same metabolic activity pathways identified in LN vs control, with higher expression in CR compared to NR. This could indicate that disease in NR is more metabolically active if the lower expression is indeed related to over-consumption. Combining transcriptomics with proteomics and metabolomics could help in understanding the processes leading to the downregulation.

Results from the database analysis and complement staining revealed a mixed pattern of features of activity and chronicity of disease being associated with non-response to treatment. It was postulated that this could be due to more rapidly progressive disease, a delay in presentation or different disease pathways in NRs which progress to chronic changes sooner such as necrotic or fibrotic disease pathways. The findings of the analysis of CR vs NR in cohort 2 using uncorrected p values <0.01 suggested that different pathways were not enhanced in responders compared to non-responders; however, the pathways downregulated in LN vs control were expressed at a lower level (non-significant) in NR compared to CR. This could indicate that more metabolically active or rapidly progressive disease is present in NR. If proven, this could explain why features of increased activity of disease and chronicity of disease are present in NR compared to CR. This may well be more evident on a repeat biopsy and is a focus of future planned work.

Given the growing body of evidence of the role of mitochondrial oxidative stress in the pathogenesis of LN, there has been exploration of targeting these pathways to treat disease. The antioxidant MitoQ has been shown to significantly reduce ROS production and NET formation in MRL-lpr mice.[298] It also reduced serum type 1 IFN levels and IC deposition in the kidneys of the mice.[298]

In our study, there were no proteins significantly differentially expressed in CR vs NR in either cohort one or two. Proteins were therefore explored for immunohistochemistry which were from the panel of proteins identified by machine learning or expressed at a different level with an uncorrected p value <0.05. As noted in the results, immunohistochemistry was explored with the aim of gently evaluating the technique as one to identify patients likely to respond to treatment on an individual basis, in the clinic. This was however undertaken with the clear acknowledgement that no individual proteins were identified which clearly differentiated CR from NR.

Cullin 2 was expressed at a lower level in CR compared to NR (uncorrected p=0.013 log2 fold change = -4.43) when measured by t test and fold change. However, as displayed in figure 5.37, staining in the non-responders was variable and it would not therefore be a reliable method of distinguishing CR from NR.

Proteosome subunit alpha type 6 was selected for staining as it was expressed at a lower level (not significant) in CR vs NR when assessed by t test and fold change (uncorrected p=0.005, log2 fold change= -1.75) and plays a key role in the maintenance of protein homeostasis by removing misfolded or damaged proteins that could impair cellular functions as part of ATP-dependent regulation. There was a significant difference in the staining in CR compared to NR in cohort one. It was however excluded from cohort two analysis as it was quantified in <20% of samples and there was not enough tissue to replicate the staining. Whilst the staining was unable to validate a significantly different protein expression between CR and NR, as this was not identified in the MS analysis, it does validate the MS findings of presence of the identified proteins in the tissue with a suggestion of a possible difference between CR and NR.

SWATH-MS was selected as a technique due to its ability to act as a discovery technique and accurately quantify all proteins detected, even low abundance ones. This is achievable as all precursors generated from a sample within a specific m/z ratio and retention-time range are fragmented and proteins accurately identified and quantified by continuously measuring the fragment ion spectra rather than only at specific time points as in shotgun proteomics which additionally only measures the most abundant proteins. However, it still has a lower limit of detection. Additionally, with such small quantities of protein extracted in this study, despite the advantages of using SWATH-MS in these size samples compared to other forms of MS, the quantity of protein extracted from the FFPE tissue was still a key limitation of these data. This limitation has multiple consequences leading to further limitations on protein detection and quantification. Firstly, there was not enough protein to perform technical replicates. Furthermore, most studies using SWATH-MS create sample-specific assay libraries, using fractionated or enriched samples, generated on the same mass spectrometer using DDA mode prior to the SWATH-MS acquisition. This generates a library specific to the dataset.[268] This had been the initial intention for these data; however, there was not enough protein extracted from the samples to do so. A generic library was therefore used which, whilst comprehensive, could have affected the accuracy of protein identification and quantification in these data.

It is difficult to compare the mass of protein extracted from the FFPE blocks with published data, and therefore identify to what degree this could be further optimised, as this information is often not

included in published works. The starting weight of the FFPE sample is also difficult to obtain accurately given the preservation techniques including dehydration and fixation in wax. It is also variably recorded when included, for example wet weight estimated by volume,[195] or in wax [220] which could be a punch or section, and is therefore difficult to compare to values recorded in this thesis after dewax. The published data using PCT for protein extraction describe peptide yield rather than protein yield and note yield of 20µg peptide per 0.5mg tissue [195]; however, the protein and peptide weights are not comparable and so this is difficult to compare to my findings. It certainly appears though, in this study, that it is likely that less protein was extracted per mg tissue (~5µg per 1mg tissue weighed post de-wax and rehydration) than the published data on using PCT for protein extraction in frozen tissue (~50µg peptide per 1mg wet tissue, ~80µg protein per 1mg wet tissue) , frozen mouse heart/liver/brain) [219] or FFPE tissue, (60µg peptide per mg dry weight in wax [220]). The group use BCA analysis for protein measurement which was unable to provide reliable results in this study as it is affected by the urea buffer which is also used in the above referenced studies. Peptide mass was not calculated as we were looking to conserve sample and this measurement was not used for loading onto the MS. Shao *et al.* note that 0.2-0.5mg wet human tissue reliably produced sufficient peptide for multiple SWATH-MS analyses at optimal sample loading. They went on to use 2-4mg tissue samples for the experiments due to the ability to more accurately weigh the starting weight of the sample at this range.[219] Protease inhibitors can be used in the lysis buffer and could potentially improve protein yield.[219] They were not used in this study due to the risk of interference with the trypsin-based digestion. The risk of sample loss in an attempt to remove the incompatible inhibitors was felt to be too great. This could however be tested as a potential modification to the protocol to improve protein yield.

Another limitation associated with the small quantity of protein extracted from the samples is that it limited the techniques available for validation of the data. Sections were taken at the time of sample preparation. This allowed for immunohistochemistry. However, only a few sections could be taken to preserve the small amount of tissue and these were insufficient for testing more than a few stains, the selection of which was obviously limited to those available. Other MS validation techniques including technical replicates being processed within the same run as well as batch replicates, processing the samples again at a separate time point, were not possible. It would also have been desirable to perform alternative MS quantification by MRM to validate the findings. It was also not possible to perform any other alternative techniques such as western blot, flow cytometry or transcriptomics. The SWATH technique using FFPE tissue has however been previously validated as discussed above.[222]

A large number of samples (8 in cohort one and 9 in cohort two) were lost due to the minimum total ion intensity of the sample being below an acceptable threshold. These were serendipitously spread across conditions but the power of the study was still weakened by this. Furthermore, there were a large number of missing values in the remaining samples which further reduced the power of each protein.

Missing data were reported for 64% of the protein values in cohort one and 50% in cohort two. As discussed briefly in the results, a value is missing in MS either because: a) it is below the lower limit of detection, 'missing not at random' (MNAR); b) there are random errors affecting the whole dataset, 'missing completely at random' (MCAR); or c) because of a factor which could be predicted by other variables, 'missing at random' (MAR).[299][300] Missing values due to processing problems leading to issues such as inaccurate peak detection would be classed as MAR.[299] However, MAR and MCAR can be difficult to distinguish and are treated in the same way. When values are MNAR, the data is 'left-censored.'[301] Graphically, this is represented by a lack of data on the left side of an otherwise standard, normal distribution bell curve. The missing data is concentrated at the lower end of the quantity data range. Neither cohort one nor two was left-censored. An assessment of correlation of the number of missing values for a protein and the mean value of the reported protein abundance was made for each cohort. The correlations were very poor indicating that the missing values were unlikely to largely represent values below the lower limit of detection. This was a strategy employed by McGurk *et al.* when exploring SWATH missingness. They concluded that it is not due to the limit of detection when they also found poor correlations.[301] It is likely that there are mixed reasons for the degree of missingness in these data but it could not be calculated if each individual missing value was MNAR or MAR. Some values may have been below the lower limit of detection. A more likely explanation for the majority however, given the lack of left-censoring of the data and the poor correlation of missingness rate with median quantity of the protein, is that they were missing due to quality control and the signal-to-noise ratio. Reasons for not passing the quality control thresholds can be interference in the signal, poorly shaped peaks and fluctuation in fragmentation, ionisation and ion flight. These all can lower the identification scoring below acceptable quality control thresholds. These are all MAR events and could therefore represent an absent or present protein at unknown quantity. For some proteins previously shown to be involved in lupus pathogenesis, there were missing data in the control samples and some positive values in the LN samples. It could be the case that the missing data in the control samples represent values close to the lower limit of detection. This was impossible to tell and would have been dangerous to assume, introducing significant bias to the analysis. This did mean however that proteins with all missing data in one category were removed

from analysis as t-test and fold change could not be performed. This more commonly occurred with proteins all reported as missing in controls. This could be why when significant differences were identified they were much more commonly downregulated in LN compared to control.

There is no standardised way of managing missing values in proteomics data.[302][300][299] Data MNAR could be replaced by the lower limit of detection of the mass spectrometer or be more reliably considered to be representing a value close to zero. These data could be replaced therefore with one number, a small value or zero.[301] Data MAR can be removed leaving a blank or imputation of the missing values can also be performed using the mean or other calculated values.[303] However, imputation techniques can introduce significant bias, overestimation of differences and lead to an overrepresentation of replicates.[301]

Apart from removal and imputation, other possible strategies for missing values are to rerun samples or remove the samples or identified substances with the most missing values. The first strategy was not possible with these samples as there was not enough protein for technical replicates. The second strategy does not have a standard application. One method used in metabolomics is the 80% rule [304][305] where only metabolites identified in 80% of samples are kept for analysis. A 'modified 80% rule' has also been suggested where data is kept when data are present for $\geq$80% of values among each biological group [306] to account for some values being MNAR and to prevent loss of these data. In this study, proteins with 80% or more missing values were removed from the analysis. To remove all the proteins with <80% values would remove a significant amount of the data and was not felt to be appropriate for discovery proteomics as true positives could be missed. It was applied here however across all samples rather than within biological groups as to do so could have biased the data to identify differences which were not true differences between the biological groups. It is acknowledged however that in doing this, true differences could be missed.

Another putative strategy for the missing data problem is measuring the same patient on several occasions over a set time period. If a protein quantity is recorded as zero consistently, it can be reliably said to be zero.[301] This strategy would be unsuitable for these data as kidney biopsies are performed infrequently and mostly only for significant clinical indications (rather than at protocolised time points for instance) due to the risks associated with the procedure. Additionally, the clinical condition of the patient would be different with each measurement.

The degree of 'missingness' was in line with recent studies using SWATH-MS. Gao *et al.* reported 54.62% missing values in their data on HCC murine tissue where 1975 proteins were quantified in at least 50% of samples and 4600 proteins quantified overall using the conventional PCT method. In

the HCC human cohort, missing value rate was 68.6%.[221] Sun *et al.*, performing SWATH-MS in prostate tissue identified a missing value rate of 74.79% using an accelerated SWATH-MS technique and 61.86% in the standard SWATH-MS technique. Despite this however, the panel of differentially expressed proteins identified was confirmed in two patient cohorts using three proteomics workflows including the standard and accelerated SWATH methods.[222]

The high rates of missingness described above are more recent work by the Guo team. Interestingly in the earlier work by Guo *et al.*, missingness across 1629 proteins in 12 samples was low at 12% and in heat map and t-test analysis the missing values were recorded as zero.[183] Later work by the same team using FFPE tissue acknowledged the presence of zeros but then did not discuss their frequency or how they were handled.[220] Hohne *et al.* removed samples with >1 missing quantification in their single nephron study[179]. The degree of missingness and how missing data are handled is often not discussed.[167][195][307][191][215][219][227]

Ultimately therefore it was felt that the strategy which would introduce the least bias would be to remove all the missing data. Although this would reduce power it was felt to be least likely to result in false positives.

MS outputs can be described by the number and identification of proteins, by the quantities and by the patterns. Differential expression in two conditions or case vs control is usually identified by t test p-value (<0.05) and fold change (>1.5-2). Correction of p values using the Benjamini-Hochberg technique to produce q values is a standard technique to prevent false positives. When performing 5000 t-tests, by chance some will show a difference. There is a risk however in discovery proteomics, that correction using the Benjamini-Hochberg technique could be too blunt a tool, a significant amount of data is lost with this strategy and false negatives could be identified.[308] It has been postulated that false positives are low risk and correction is unnecessary [308] especially when using bespoke libraries.[309] It was decided for this study to correct the p values, as, especially with the high proportion of missing values it was important to avoid false positives and overstating differences. As noted also, development of a bespoke library had not been possible in this study. By correcting the p values, accepting that an amount of false negatives would likely result, more biological inference could be taken from the identified q values. This was particularly felt to be important with these data as there was not enough protein to validate any findings. In discovery proteomics, when p values are left uncorrected, it can be accepted that there will be false positives and findings should be interpreted with the appropriate caution until validated.

Many studies analysing 'omics' data do not analyse using p values and fold changes. There is a school of thought that big data like this requires alternative methods of data analysis such as

machine learning.[310] It could be postulated that analysing each protein individually misses relationships between the proteins. Relationships between all upregulated or all downregulated proteins can be reviewed using various software, String [270,272] in this study. This analysis cannot however tease out relationships between proteins not known to have links or between these two different groups.

To this end, machine learning was performed to review the differences between responders and non-responders on cohort one and a panel of 8 proteins identified, essentially by trial and error, which differentiated CR from NR when PCA analysis was applied. There were no statistically significant differences found between these groups using q values. The combination of 8 proteins could however separate CR vs NR on PCA and predict response on a test set, an outcome interesting in itself. It may be that a panel identified by this kind of data analysis which takes into account how variations in protein quantity interact with one another, is of greater benefit in predicting response than a panel of the most differentially expressed proteins. The panel could not however be validated by cohort two. Published data also demonstrates that each run on MS provides slightly different results.[221] This could be in part due to the compromises made when generating the panel due to computational resources. Despite using standards we were unable to combine results between experiments as the quantities were not directly comparable. Given this and the heterogeneity of LN, the use of a different set of samples is further likely to produce a different panel of proteins again.

There was very little crossover of differentially expressed proteins in cohorts one and two. This could be for many reasons. Without being able to perform technical replicates or validation using MRM and with the variation in batch analysis we cannot rule out this being due to technique. However SWATH has been validated using this same protocol with small differences.[222] It could also be due to biological variation. This is the first time LN renal biopsy tissue has been studied with DIA MS. In a published study, it was demonstrated that a panel of 75 proteins identified using SWATH-MS could distinguish prostate Ca from benign disease in a separate cohort of samples.[222] LN arguably has potentially much greater heterogeneity between patients. In the previous discussed MS studies performed on LN renal biopsy tissue, the findings were not compared with a separate cohort for validation.[167][191][234] It is also entirely plausible that due to the heterogeneity of the disease, specific proteins may vary between patients to the degree that in a separate cohort, specific proteins will not be differentially expressed to the same degree.

When proteins were identified to be differentially expressed, their expression was not uniform among the samples. This was also reflected in the staining. LN is a disease with multiple and

variable pathogenic pathways. Viewing the uncorrected p value data for CR vs NR in cohort two identified that possibly the responders have a different level of metabolic activity with higher expression in CR of the pathways shown to be downregulated in LN. Clinically, knowing a patient is unlikely to respond to treatment would be of benefit, for example in deciding how much immunosuppression to give for LN if it is highly likely that they will be requiring a renal transplant and therefore significant lifelong immunosuppression. Arguably though, the greater value of this work, rather than identifying CR vs NR, is the identification of pathogenic pathways in LN not currently targeted by treatments thus providing options for future research and possible therapeutic avenues.

The key aim of this work was to assess if proteomic studies like this are feasible with small quantities of stored FFPE samples, followed by unbiased discovery. As the first study to perform PCT SWATH on FFPE renal tissue, this is valuable evidence that the technique can be used here. It is also a valuable addition to the small body of proteomic work in LN tissue. It aligns with a body of recent work describing the disruption of metabolic pathways and mitochondria in LN as well as identifying the same proteins differentially expressed in previous LN proteomics studies as well as additional proteins in the same pathways, validating these findings. A significantly greater number of cases have been used than before providing greater power. However, there is still a lack of power, in some part due to the degree of missingness. Although, despite being quite striking, it is interesting to see this is in keeping with recent SWATH studies, one of which was validated with MRM.

Much future work is planned. Remaining samples are being explored to assess if sufficient protein is available for MRM analysis in a limited number of samples investigating differentially expressed proteins from cohort two to validate quantities. Validation of the data using plasma and most excitingly, transcriptomics data from a matched cohort including some of the exact samples used in this study is planned. Additionally, plasma samples from different time periods in the course of disease have been collected to allow exploration of intra-patient variability of the markers pre and post treatment. Tissue samples are also being explored to assess if there are sufficient second biopsies available to power a study analysing the proteome of second biopsies in comparison to first to explore on-going activity of disease and progression of chronic changes in both responders and non-responders.

In summary, quantitative proteomics, a technique validated in human FFPE tissue, has been performed using very small quantities of protein remaining from clinical samples. To our knowledge, it is the first study using human FFPE LN renal biopsy tissue for PCT SWATH-MS. Additionally, it is the first proteomics study to investigate LN responders vs non-responders. Metabolic,

mitochondrial activity and anti-oxidant pathways have been shown to be downregulated in LN vs control, a finding aligning with an increasing body of research. There is also an indication that non-responders may have a different level of metabolic activity to responders. These data provide evidence for potential new treatment targets including a JAK inhibitor which has shown promising in vitro results and in a lupus mouse model[281]. Murine studies of a VDAC oligomerisation inhibitor[292] and the antioxidant mitoQ[298] have also shown promising results.

5.5 SUMMARY OF KEY FINDINGS

PCT-SWATH-MS is possible in human renal FFPE tissue remaining following clinical use. LN can be distinguished from control with 80 differentially expressed proteins. Metabolic, mitochondrial and anti-oxidant processes are down-regulated in LN compared to TBM. There were only 6 proteins differentially expressed between class III/IV LN and class V LN with no enrichment of specific pathways; however, when class V was compared to control there was enrichment of ribosomal pathways not seen when looking at class III/IV exclusively. There were no significantly differentially expressed proteins between treatment responders and non-responders. However, there was some evidence that the metabolic activity level of non-responders could be different to responders as proteins downregulated in LN compared to control were expressed at a lower level in non-responders compared to responders (uncorrected $p < 0.01$). This analysis did not suggest a different disease process in non-responders compared to responders. These data provide evidence for new targets for treatment, some of which have been investigated in murine studies with promising results.

6 CHAPTER SIX – DISCUSSION AND FUTURE WORK

6.1 DISCUSSION

Lupus nephritis (LN) is a common manifestation of systemic lupus erythematosus (SLE) for which response rates to treatment are often sub-optimal. This results in significant long-term renal complications including CKD and ESKD as well as increased mortality.[1][2][3] Current predictors of response and outcome such as histological class on renal biopsy are poorly validated and better performing measures are required to aid management in the clinic. Current standard of care treatments are anti-inflammatory or broadly immunosuppressive and provide relatively poor rates of response often with significant side effects. New targeted treatments are required to address these issues. In the years leading up to the beginning of this research, there had been some disappointing results from clinical trials, failing to achieve their treatment targets[56]. The past three years have however seen much more success with targeted therapies and indeed three new drugs licensed for treatment for LN – belimumab, voclosporin and anifrolumab.

ICHT has a large diverse cohort of patients with LN with computerised clinical data and renal biopsy samples dating back to 1996. This research therefore set out to examine the demographic, clinical, histopathological and proteomic profiles of these patients and their renal tissue with the aim of identifying clinically useful predictors of response to treatment and outcome as well as identification of potential new targets for treatment. Utilising database exploration, staining and mass spectrometry, biomarkers of response to treatment and outcome were identified and pathogenic mechanisms of disease in LN explored for both markers of and mechanisms of response and the identification of new treatment targets. Using the predictors of long-term outcome, new thresholds for clinical variables have been suggested for use in the clinic and in clinical trials.

A comprehensive database encompassing 20 years of treating LN at a central London hospital was compiled, consisting of 470 patients and 803 biopsies. It contains the largest cohort to date describing an oral-steroid free treatment protocol. Overall unselected rate of complete response by one year in the cohort was 45.7%. Proteinuria level at biopsy, a ‘full house’ pattern on immunohistochemistry and the percentage of glomeruli with chronic lesions were each independently significant in predicting response to treatment. This indicates that features of activity and chronicity of disease are key in influencing response to treatment. There were no significant differences in response to treatment or long-term outcomes seen between the different treatment protocols used indicating that LN can be successfully treated without the use of oral steroids or

cyclophosphamide. Whilst appropriate patient selection is undoubtedly important, the use of an oral steroid-free treatment regimen is not reserved for those with the 'best' baseline parameters. Additionally, steroid-free treatments were not associated with an increased flare rate.

To improve treatment response rates, new approaches are required. These will need robust clinical trials to prove their efficacy. There is not currently a unified definition of a good long-term outcome or clear prescribed short-term targets for clinical trials proven to be representative of long-term outcomes. This was therefore next addressed using the database to identify short-term clinical variables and their thresholds which are predictive of long-term outcomes. Trials in LN are not straight-forward, many recent trials had failed to meet their end-points and treatments in current use performing well in cohort studies had not demonstrated this efficacy in recent clinical trials[51]. We set out to identify more robust end-points using the data from our cohort. Both proteinuria and eGFR at 12 months were independently predictive of good long-term outcome defined as an eGFR $\geq$ 80mls/min/1.73m^2 at 7 years. An eGFR of >80mls/min/1.73m^2 at one year has a PPV 80% and proteinuria <90mg/mmol at one year has a PPV 70%. Achieving both the eGFR and proteinuria thresholds predicted a good outcome at 7 years with an OR 9.922 (95% CI 4.979-19.774) p<0.001. These thresholds were then validated in a separate multi-ethnic cohort from another central London hospital. These findings indicate that the current target for proteinuria (<50mg/mmol) is too stringent. The results are in line with analysis of long-term data from both the Euro-Lupus [127] and MAINTAIN [128] studies which identified thresholds of <0.8g/d and 0.7g/d respectively. The former was acknowledged in the EULAR 2019 guidelines update for SLE [40] and in the updated guidelines for LN the definition of CR was expanded to a target proteinuria of <0.5-0.7 g/24 hours.[36] Interestingly, a recent trial, BLISS-LN, changed the parameters of their primary end-point to a uPCR of <0.7 [58] due to the growing evidence, of which these data were a part,[102][101] of this threshold as a valuable predictor of long-term outcomes. Interestingly, the primary end-point for BLISS-LN was at two years – a time point not evaluated previously with respect to the proteinuria threshold of 0.7. However, a subsequent application of the BLISS-LN endpoints for proteinuria and eGFR at two years to the Hopkins Lupus cohort, confirmed association with both good long-term outcomes and reduction in flares.[59] The findings in this thesis for eGFR indicated that an excellent eGFR at one year should be a key aim in order to preserve nephrons and maintain reasonable renal function in the long term. In this analysis it achieved a superior AUC for predicting long term outcome and the combination of eGFR and proteinuria provided the best PPV of 86% and best specificity of 89%. It is arguably the specificity which is of most value in these targets as their value is in part the selection of patients in whom it is appropriate to rationalise treatments. These data provide evidence to support finessing targets for our treatments in the clinic and that endpoints in

clinical trials should be amended to a more lenient proteinuria target and perhaps more stringent eGFR targets at one year in order to best reflect likely preservation of kidney function long-term.

Exploration of complement deposition was undertaken as complement is well established to be involved in LN pathogenesis and a potential target for treatment with one drug targeting C5 currently in clinical use with case study evidence in LN [66] and two further drugs targeting the complement pathway being studied in clinical trials currently recruiting.[67][311] Complement deposition was explored to further understand its role in LN and identify if staining, a technique used routinely in LN biopsy analysis clinically, could be used to guide which patients may benefit from use of a complement C5 inhibitor. Staining revealed that deposition of the MAC is strongly associated with LN. However, it's clearance from the glomeruli is slow, meaning that it is not a reliable marker of on-going activation of complement C5.[241] Therefore, it is not a useful indicator on its own as to the likely benefit to an individual of a treatment targeting the MAC. More intense staining was associated with non-response to treatment. This could possibly be evidence of a more significant role of complement in the pathogenesis of LN in non-responders. Current standards of care do not specifically target the complement pathways. However, more intense C3c staining, a feature related to disease activity, was associated with a greater likelihood of response to treatment. It is also pertinent that MAC staining in the tubular basement membrane correlated with the percentage of interstitial fibrosis and tubular atrophy, a marker of chronicity. Markers of chronic LN were associated with non-response in the database studies. The staining results indicate that MAC may have a role in tubulointerstitial fibrosis and could be a marker of chronic damage. Presence of C5b-9 staining in chronic biopsies could however also potentially be a marker of on-going activity. C3c staining in the biopsies showing chronic LN alone indicated that there may be on-going LN activity in these patients not captured on the biopsy. Those with C3c positive staining were more likely to have more active disease clinically and more likely to receive an escalation in treatment despite a lack of active glomerular lesions on light microscopy. Thus, the combination of C3c, C5b-9 and CD68 (as a surrogate for C5a) positive staining would appear from these data to be good evidence of activation of both the C3 and C5 pathways, and could be used to guide if a C5 inhibitor might offer potential benefit in these patients.

Overall, analysis of the demographic, clinical and histopathological features of patients and their biopsies have revealed some predictors of response to treatment and long-term outcomes potentially related to activity and chronicity of the disease processes. Parameters for clinical trials of new treatments have been identified validating and expanding on existing data. Mechanisms of disease can however only be speculated from these results and the next step therefore was to explore the proteomic profile of LN in renal biopsies. SWATH-MS, a quantitative method of

discovery proteomics has recently been validated in human FFPE tissue.[220][221][222] It was performed here using very small quantities of protein and shown to be possible in human renal FFPE tissue, the first use, to our knowledge, of SWATH-MS in this tissue. Exploration of the proteome was undertaken to evaluate active pathogenic pathways in LN to identify new treatment targets and to identify if a difference could be seen between treatment responders and non-responders. The aim was to use the proteomic data to predict disease response at the time of the baseline biopsy and identifying if different pathogenic processes are responsible for disease in non-responders potentially allowing for a more targeted treatment approach. LN was shown to be discernible from control renal tissue using the technique. Metabolic pathways, mitochondrial processes and oxidative phosphorylation were downregulated as well as the biological processes of aerobic respiration and the oxidation-reduction process. Interestingly, it did not identify significant differences in the pathogenic processes of proliferative disease (class III/IV) compared to membranous (class V) although when class V disease alone was compared to control, there was additional downregulation of ribosomal pathways. There were no significantly differentially expressed proteins identified between responders and non-responders which also could not be separated on principal component analysis (PCA). This was the first study using proteomics to investigate the LN renal biopsy for differences between responders and non-responders. It added to a small body of work examining the proteome of LN renal biopsy tissue,[167][191][234] providing greater power using a significantly greater number of cases than previously. It validated and expanded on previous findings of the down-regulation of metabolic, mitochondrial and anti-oxidant processes in LN in these studies. It also aligned with a body of recent work describing the disruption of metabolic pathways and mitochondria in LN including downregulation of these pathways in CD4+ and CD8+ T cells.[274] This downregulation could be under-production, loss or, perhaps more likely, over-consumption. The downregulation could leave glomeruli vulnerable to oxidative stress and injury as well as possibly auto-immunity due to poor clearance and repair. Even though there were no significantly differentially expressed proteins between responders and non-responders, there was some evidence that proteins downregulated in LN compared to control were possibly expressed at a lower level in non-responders compared to responders (uncorrected $p<0.01$). This could possibly indicate that non-responders may have a different level of metabolic activity to responders. The non-significant differences between responders and non-responders seen did not however indicate a different disease process in non-responders; the same pathways demonstrated enhancement. The lower expression (using uncorrected $p<0.01$) of the same pathways in non-responders seen could possibly indicate more rapidly progressive disease explaining the dual findings in database and staining work of non-responders having both more active disease and more chronic damage. This

would support the data [6][125] suggesting rapid recognition and treatment of LN is key in achieving good long-term outcomes.

The proteomic data provide evidence for new treatment targets including a JAK inhibitor which has shown promising in vitro results and in a lupus mouse model.[281] Murine studies of a VDAC oligomerisation inhibitor and the antioxidant mitoQ have also shown promising results.[292][298] Data from the cohort analysis could provide appropriate clinical outcome targets in trials of these agents.

There are limitations to the data. As a retrospective study, the cohort database analysis will have gaps in the data. It is a 'real-world' cohort, which carries the strength of applicability and validation of findings from clinical trials. However, by its nature, the real-world cohort is varied, incomplete in places and carries with it the issue of adherence to treatments, for which there is no infallible or standard measure. Concordance with treatment is a true confounder to our central question of response as clearly, a lack of treatment will likely be associated with non-response. The majority of treatments did however contain an intravenous (supervised) portion and blood levels of medications were monitored in the clinic. Some features however could not be evaluated in full, in particular the side-effect data. As data is being collected retrospectively and patients seek treatment for side effects from multiple sources including their own GP or emergency department, clinic records with these details can be incomplete. Data protection prevented us from reviewing these alternate sources of care to obtain complete records.

The proteomics study was powered to identify a 2-fold difference in expression. Whole samples were however lost due to the minimum total ion intensity of the sample being below an acceptable threshold. Of the remaining samples, the data contained a relatively high proportion of missing values, although notably in line with other SWATH studies.[221][222] As a discovery study, the findings require validation. Ideally, a part of this validation would have been additional proteomics techniques on the same tissue such as MRM and ELISA. However, there was not enough protein obtained from the samples to achieve this. The very small quantities of protein also prevented the running of technical replicates and development of a bespoke library which would have improved the accuracy of identification.

This body of work, despite the limitations described, provides, as was its aim, valuable and novel insights into predictors of response to treatment of lupus nephritis and the long-term outcomes as well as validating previous findings within the body of LN research. It describes the largest cohort of patients with LN treated with an oral steroid-free protocol demonstrating that LN can be treated successfully without oral steroids or cyclophosphamide. New thresholds for targets of treatment in

the clinic and in future clinical trials have been provided. It describes the first use of exploration of the proteome of lupus nephritis renal tissue with mass spectrometry to compare treatment responders to non-responders. In doing so, insights into the pathogenesis of LN have been revealed, potential new treatment targets for lupus nephritis identified and possible insights into the metabolic activity in non-responders, worthy of future study.

6.2 Future Work

Much future work is planned in all spheres of study.

With regards to the database, further interrogation is planned including further investigation of the predictive value of percentage active and chronic glomeruli as compared to activity and chronicity indices, or indeed identification of new indices of activity and chronicity of value. Cluster analysis is also planned to identify if classification exists which is predictive of response to treatment or long-term outcomes or indeed provides better guidance than existing classifications in use such as histological class. It is also planned to explore the value of second biopsies performed on our cohort for reasons of partial response to treatment, non-response and pre-treatment change or withdrawal or pregnancy. This would be with the aim of gathering evidence on the use of repeat biopsies, a key aim of the EULAR 2019 guidelines for future research.

One limitation of this work is concordance to treatments. It is not currently officially recorded and therefore could not be integrated into data analysis. This provides a 'real-world' cohort, improving the utility and applicability of the findings. However, to include a measure of concordance, such as utilising the hydroxychloroquine levels taken at clinic visits as part of the standard of care at our unit, would be of benefit when analysing active disease pathways, especially in follow-up biopsies where this information could aid understanding as to pathways affected by current treatments.

With regards to the staining work, findings regarding C5b-9 are to be replicated and validated using the biopsies and plasma collected prospectively during the time of the research. This currently stands at 69 patients. The complement pathway is also to be further explored including the MBL pathway to provide a more complete picture of complement.

The findings regarding C3c staining in chronic biopsies warrants further investigation. It would be of value to explore this in more chronic cases and in the repeat biopsies and understand if C3c staining is acting as a marker of on-going inflammation. Further exploration of these biopsies showing no active disease but only chronic LN by proteomics would help understand the level of on-going activity and markers of active disease in LN.

Regarding the proteomics studies, as described, improvement of the technique and protein yield could be explored, including trial of protease inhibitors. Processed samples are being explored to assess if sufficient protein is available for MRM analysis in a limited number of samples to validate the findings of differentially expressed proteins from cohort two.

Data will be further explored using cluster analysis for cohort two, bringing clinical, demographic and histopathological data into this analysis to assess if a combination of these data can provide better predictors than one arm of the work alone.

Further validation of the proteomics is planned using plasma and most excitingly, transcriptomics data from a matched cohort including some of the exact samples used in this study. It is to be explored if integration of the proteomics and transcriptomics can provide more robust findings than each individually. Collaboration is planned here with a colleague who has performed Nanostring analysis on the ncounter platform on a cohort of FFPE renal biopsy samples. Additionally, plasma and urine samples from different time periods in the course of disease have been collected to allow exploration of intra-patient variability of the markers pre and post treatment and validation of the proteomics results using ELISA in these samples/media and a functional assessment of the pathways identified undertaken. Staining will next be explored with the aim of identifying a multiplex staining panel to predict response to treatment.

6.3 CONCLUSION

This body of work describes the demographic, clinical, histopathological and proteomic profiles of a large, diverse cohort of patients with lupus nephritis, treated at a specialist unit over a 20-year period. Examination has revealed clinically useful predictors of response to treatment and long-term outcomes. These include features of both the activity of disease and features of chronic damage including proteinuria level at biopsy, a 'full house' pattern on immunohistochemistry and the percentage of glomeruli with chronic lesions as well as elements of the complement pathway. Analysis of different treatments for LN including, crucially, an oral steroid-free regimen, demonstrated that LN can be treated successfully with no oral steroids or cyclophosphamide. Statistical analysis has revealed and validated targets and parameters for high-value evaluation of new treatments, with the target of optimal long-term outcomes. Analysis of the proteome, using a relatively new technique of SWATH-MS, has revealed insights into the pathogenesis of LN and thus suggestions of potential new treatment targets. There was also suggestion that non-responders may have a different metabolic activity level. This could align with the findings of more active disease with increased features of chronic damage identified in earlier work. Together, this body of work supports rapid recognition and treatment of LN, targeting excellent renal function as being key to preserving long term excellent outcomes.

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APPENDICES

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2. Lightstone L, Doria A, Wilson H, Ward FL, Larosa M, Bargman JM. Can we manage lupus nephritis without chronic corticosteroids administration? Autoimmun Rev 2017.
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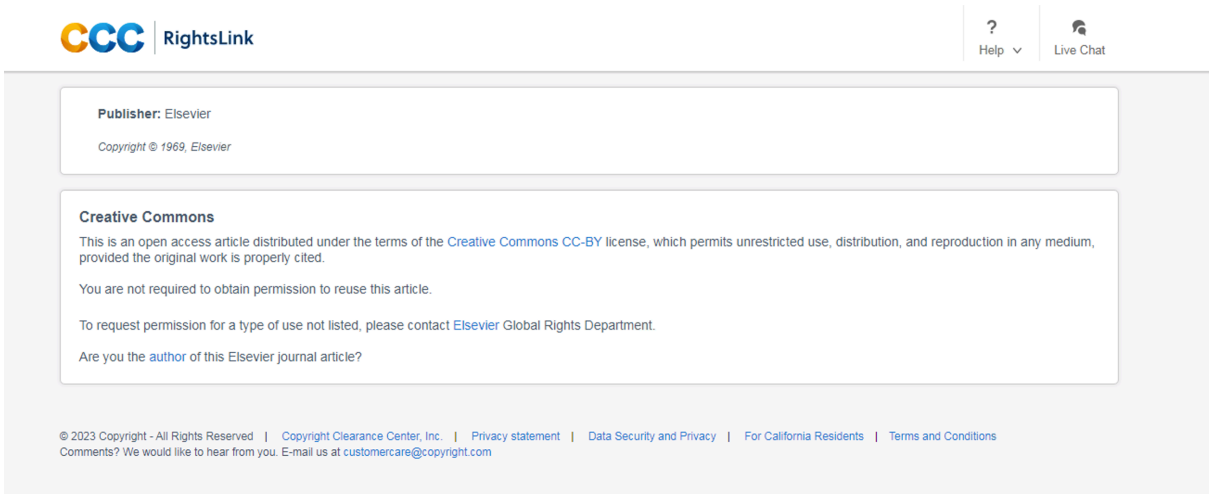
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